 DOI: 10.1002/eji.201343381 Eur. J. Immunol. 2013. 43: 1806–1817

European Journal of Immunology

Galectin-3 negatively regulates the frequency and function of $CD4^+CD25^+Foxp3^+$ regulatory T cells and influences the course of *Leishmania major* infection

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Galectin-3, an endogenous glycan-binding protein, plays essential roles during microbial infection by modulating innate and adaptive immunity. However, the role of galectin-3 within the $CD4^+CD25^+Foxp3^+$ T regulatory (T_{REG}) cell compartment has not yet been explored. Here, we found, in a model of *Leishmania major* infection, that galectin-3 deficiency increases the frequency of peripheral T_{REG} cells both in draining lymph nodes (LNs) and sites of infection. These observations correlated with an increased severity of the disease, as shown by increased footpad swelling and parasite burden. Galectin-3-deficient (*Lgals3*$^{-/-}$) T_{REG} cells displayed higher CD103 expression, showed greater suppressive capacity, and synthesized higher amounts of IL-10 compared with their wild-type (WT) counterpart. Furthermore, both T_{REG} cells and T effector (T_{EFF}) cells from *Lgals3*$^{-/-}$ mice showed higher expression of Notch1 and the Notch target gene Hes-1. Interestingly, Notch signaling components were also altered in both T_{REG} and T_{EFF} cells from uninfected *Lgals3*$^{-/-}$ mice. Thus, endogenous galectin-3 regulates the frequency and function of $CD4^+CD25^+Foxp3^+$ T_{REG} cells and alters the course of *L. major* infection.

Keywords: Galectin-3 · IL-10 · *Leishmania major* · T regulatory (Treg) cells · Notch signaling

Introduction

Galectins are a family of glycan-binding proteins composed of 15 members that are conserved throughout animal evolution and share sequence similarities in their carbohydrate-recognition domain [1–3]. Galectin-3, a widely distributed member of the family, plays pleiotropic roles in innate and adaptive immunity by regulating cytokine production, phagocytosis, chemotaxis,

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signaling, and survival [4–7]. Through these mechanisms, galectin-3 has been proposed to control host immunity against several infectious agents [1, 6–8]. Yet, despite considerable evidence on the role of galectin-3 in the control of immune responses, its contribution to T regulatory (T_{REG}) cell function during microbial attack has not yet been explored.

T_{REG} cells, either inducible or naturally occurring, suppress effector T (T_{EFF})-cell responses through different mechanisms including cell–cell contact and secretion of immunosuppressive cytokines such as IL-10, TGF-β, and/or IL-35 [9]. Interestingly, galectin-1 and -10 have been proposed to mediate the immunosuppressive activity of Foxp3$^+$ T_{REG} cells [10, 11] and galectin-3 has been postulated as a potential marker for human T_{REG} cells [12]. In addition, galectin-3 increases the severity of autoimmune neuroinflammation by decreasing the frequency of T_{REG} cells [13], suggesting that this lectin might also influence the T_{REG} cell compartment during microbial infection.

We took advantage of the availability of galectin-3-deficient (*Lgals3*$^{-/-}$) mice on a BALB/c background in order to investigate the function of T_{REG} cells during the course of *Leishmania major* infection. This experimental model has provided extensive information on the factors that regulate the development of CD4$^+$ T helper (Th) cells in vivo [14] and has contributed to dissect the role of T_{REG} cells during intracellular infections [15–18].

Here, we show that *Lgals3*$^{-/-}$ mice display higher frequency of T_{REG} cells both in draining lymph nodes (LNs) and infection sites during *L. major* infection. Moreover, *Lgals3*$^{-/-}$ T_{REG} cells produce higher amounts of IL-10, have enhanced suppressive capacity, and show altered Notch expression compared with wild-type (WT) mice. Thus, endogenous galectin-3 influences T_{REG} cell number and function during parasitic protozoa infection.

Results

Leishmania major infected *Lgals3*$^{-/-}$ mice show increased frequency of CD4$^+$CD25$^+$Foxp3$^+$ T_{REG} cells

To investigate the role of galectin-3 within the T_{REG} cell compartment, we first compared the outcome of *L. major* infection in *Lgals3*$^{-/-}$ and WT mice on BALB/c background. Mice were inoculated with 1×10^7 metacyclic promastigotes into one hind footpad, and the development of the lesions was monitored weekly. *Lgals3*$^{-/-}$ mice developed more pronounced footpad swelling starting from 35 days postinfection and exhibited an increased parasite burden (at day 35) compared with WT mice (Fig. 1A). To examine the possible mechanisms underlying the increased susceptibility to *L. major* infection, we examined the impact of galectin-3 deficiency in different immune cell types. We found no significant differences in the frequency of F4/80$^+$ macrophages, CD11c$^+$ dendritic cells (DCs), and CD4$^+$ and CD8$^+$ T cells in draining LNs from *Lgals3*$^{-/-}$- and WT-infected mice at day 35 postinfection (Fig. 1B). However, we found a higher percentage of CD4$^+$CD25$^+$ T_{REG} cells in *L. major* infected *Lgals3*$^{-/-}$ versus WT mice (Fig. 1C). To further characterize this CD4$^+$CD25$^+$ T cell population, we isolated CD4$^+$ T cells from *Lgals3*$^{-/-}$- or WT-infected mice and analyzed the frequency of Foxp3$^+$ cells within the CD4$^+$CD25$^+$ gate. The percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells was higher in draining LNs from *Lgals3*$^{-/-}$ compared with WT mice (Fig. 1D). To determine whether the number of T_{REG} cells was increased at sites of infection in *Lgals3*$^{-/-}$ mice, footpad lesions were assessed for Foxp3 by immunohistochemistry. The frequency of Foxp3$^+$ cells in the footpad tissue from *Lgals3*$^{-/-}$mice was considerably higher when compared with WT mice (Fig. 2A and B). In addition, real-time RT-PCR analysis showed increased Foxp3 mRNA expression in footpad tissue from *Lgals3*$^{-/-}$-infected animals as compared with their WT counterpart (Fig. 2C). Of note, galectin-3 protein was detected at high levels in footpad tissue from WT mice (Fig. 2A; panel a).

As CD103 facilitates the homing and retention of T_{REG} cells at sites of *L. major* infection [17], we examined whether expression of this molecule was altered in the absence of galectin-3. CD4$^+$CD25$^+$ T cells from *L. major* infected *Lgals3*$^{-/-}$ mice displayed higher CD103 expression compared with their WT counterpart. However, we found similar CD62L expression in CD4$^+$CD25$^+$ T cells from *Lgals3*$^{-/-}$ and WT mice (Fig. 2D), showing selectivity in galectin-3-mediated control of T_{REG} cell specific markers. Taken together, these data suggest that endogenous galectin-3 controls the frequency of Foxp3$^+$ T_{REG} cells and modulates CD103 expression on these cells during the course of *L. major* infection.

T_{REG} cells from *Lgals3*$^{-/-}$ mice display higher suppressive activity

Because T_{REG} cells were found at higher numbers both in draining LNs and footpad lesions of *L. major* infected *Lgals3*$^{-/-}$ mice, we investigated the contribution of endogenous galectin-3 to the suppressive function of these cells. CD4$^+$CD25$^-$ T cells (T_{EFF}) were purified from LNs of WT-infected mice (Fig. 3A) and were restimulated in vitro with *L. major* antigen in the presence of CD4$^+$CD25$^+$ T_{REG} cells from either *Lgals3*$^{-/-}$ or WT mice at various T_{EFF}:T_{REG} ratios (Fig. 3B). Analysis of T-cell proliferation in co-cultures of T_{EFF}:T_{REG} cells (ratios of 1:1 and 1:0.5) indicated that CD4$^+$CD25$^+$ T_{REG} cells from *Lgals3*$^{-/-}$ mice had greater suppressive activity than T_{REG} cells isolated from WT mice. Moreover, while T_{REG} cells from either *Lgals3*$^{-/-}$ or WT mice suppressed IFN-γ and IL-4 production by CD4$^+$CD25$^-$ T cells (T_{EFF}), inhibition of cytokine production was much more pronounced when T_{EFF} cells were co-cultured with *Lgals3*$^{-/-}$ T_{REG} cells (Fig. 3C and D). Because the immunosuppressive activity of T_{REG} cells is in part mediated by IL-10 and TGF-β, we examined production of these cytokines in draining LNs from WT- and *Lgals3*$^{-/-}$-infected mice. Nonpurified LN cells (Fig. 3E) or purified T_{REG} cells (Fig. 3F) from *L. major* infected *Lgals3*$^{-/-}$ mice restimulated ex vivo with *L. major* antigen showing enhanced IL-10 mRNA expression as compared with cells obtained from WT mice. Furthermore, increased amounts of TGF-β transcripts were also detected in purified T_{REG} cells from *Lgals3*$^{-/-}$ compared with WT mice (Fig. 3G). Thus, endogenous galectin-3 not only controls T_{REG}-cell frequency in LN and

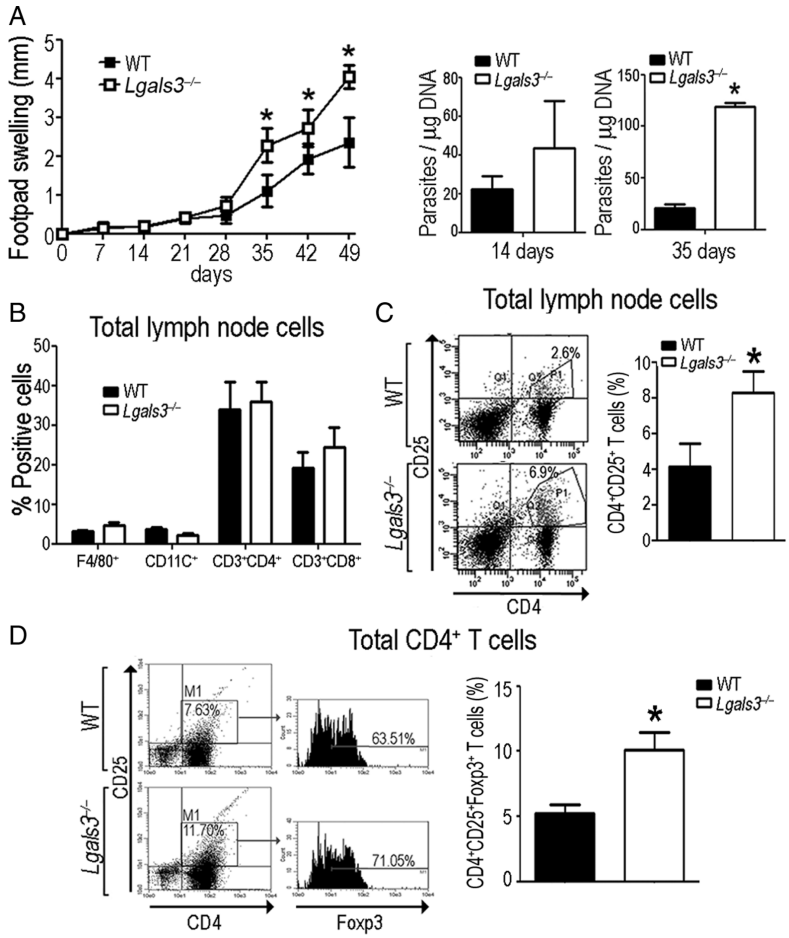

Figure 1. *Leishmania major* infected *Lgals3*$^{-/-}$ mice display higher frequency of T_{REG} cells in draining LNs. (A–D) *Lgals3*$^{-/-}$ and WT mice were infected with 1×10^7 stationary phase *L. major* LV39 promastigotes in the footpads. (A) Changes in footpad lesions between *Lgals3*$^{-/-}$ and WT mice (left) and parasite burden measured by real-time PCR at day 35 postinfection (right). Data are representative of three similar experiments and show the mean ± SD from ten mice per group. (B, C). At 35 days postinfection, cell phenotype on popliteal LN was assessed by immunostaining for F4/80, CD11c, CD3, CD4, and CD8 (B) or for CD4 and CD25 (C) and analyzed by flow cytometry. Data represent the mean + SD of positive cells from five mice per group. (D) Foxp3 expression within the gated $CD4^+CD25^+$ T cell population of total $CD4^+$ T cells purified from LNs of WT or *Lgals3*$^{-/-}$ mice at day 35 postinfection. Bar graph shows the mean + SD of three experiments performed with $CD4^+$ T cells purified from total LN cells pooled from three mice per group. $^*p < 0.05$; Student's t-test.

infection sites, but also limits the immunosuppressive function of these cells during the course of parasitic protozoa infection.

Galectin-3 controls IL-10 production by T_{REG} cells even in the absence of infection

To better characterize T_{REG} cells from *Lgals3*$^{-/-}$ mice, we next evaluated the expression of CD25, CTLA4, CD103, and CD62L in $CD4^+Foxp3^+$ T cells from uninfected WT and *Lgals3*$^{-/-}$ mice. Despite the higher percentage of $CD4^+Foxp3^+CD25^+$ T_{REG} cells found in uninfected *Lgals3*$^{-/-}$ mice, the expression of CD62L, CD103, and CTLA4 did not differ significantly between WT and *Lgals3*$^{-/-}$ animals (Fig. 4A). However, in vitro stimulated T_{REG} cells purified from *Lgals3*$^{-/-}$ mice synthesized considerably higher amounts of IL-10 compared with in vitro stimulated WT T_{REG} cells (Fig. 4B). Thus, endogenous galectin-3 controls IL-10 production by T_{REG} cells either in the absence or presence of *L. major* infection.

Lgals3$^{-/-}$ mice T_{REG} cells display higher expression of Jagged-1 and the Notch target gene Hes-1

Previous studies showed that T_{REG} cells preferentially express the Notch ligand Jagged-1, which confers an immunosuppressive phenotype to these cells [19–21]. We analyzed expression of Jagged-1 on T_{REG} and T_{EFF} cells purified from uninfected WT and *Lgals3*$^{-/-}$ mice. Remarkably, T_{REG} cells from *Lgals3*$^{-/-}$ mice showed higher Jagged-1 expression even in the absence of stimulation when compared with WT T_{REG} cells (mean fluorescence intensity 139.50 ± 3.21 versus 96.68 ± 0.84, respectively; Fig. 5A). In contrast, T_{EFF} from *Lgals3*$^{-/-}$ mice display higher Jagged-1 expression only after in vitro stimulation, in comparison with T_{EFF} cells isolated from WT mice (mean fluorescence intensity 115.48 ± 4.87 versus 81.31 ± 2.05, respectively; Fig. 5A).

It has been reported that Notch signaling plays an important role during development, expansion, and function of both T_{EFF} and T_{REG} cells [22]. We analyzed the expression of Notch receptors on T_{EFF} and T_{REG} cells isolated from uninfected WT and *Lgals3*$^{-/-}$ mice. We found that resting T_{EFF} cells from *Lgals3*$^{-/-}$ mice displayed enhanced expression of Notch-1, Notch-3, and the Notch target gene Hes-1 (Fig. 5B). Unlike T_{EFF} cells, T_{REG} cells from *Lgals3*$^{-/-}$ and WT mice exhibited similar mRNA expression levels for Notch-1 and Notch-3 in the absence of stimulation (Fig. 5B). Notch-3 mRNA expression on *Lgals3*$^{-/-}$ T_{REG} cells did not change after stimulation and was lower than that synthesized by WT cells (Fig. 5B). However, after stimulation with anti-CD3 and anti-CD28 mAb, *Lgals3*$^{-/-}$ T_{REG} cells displayed increased Hes-1 mRNA expression (Fig. 5B). Interestingly, expression of galectin-3

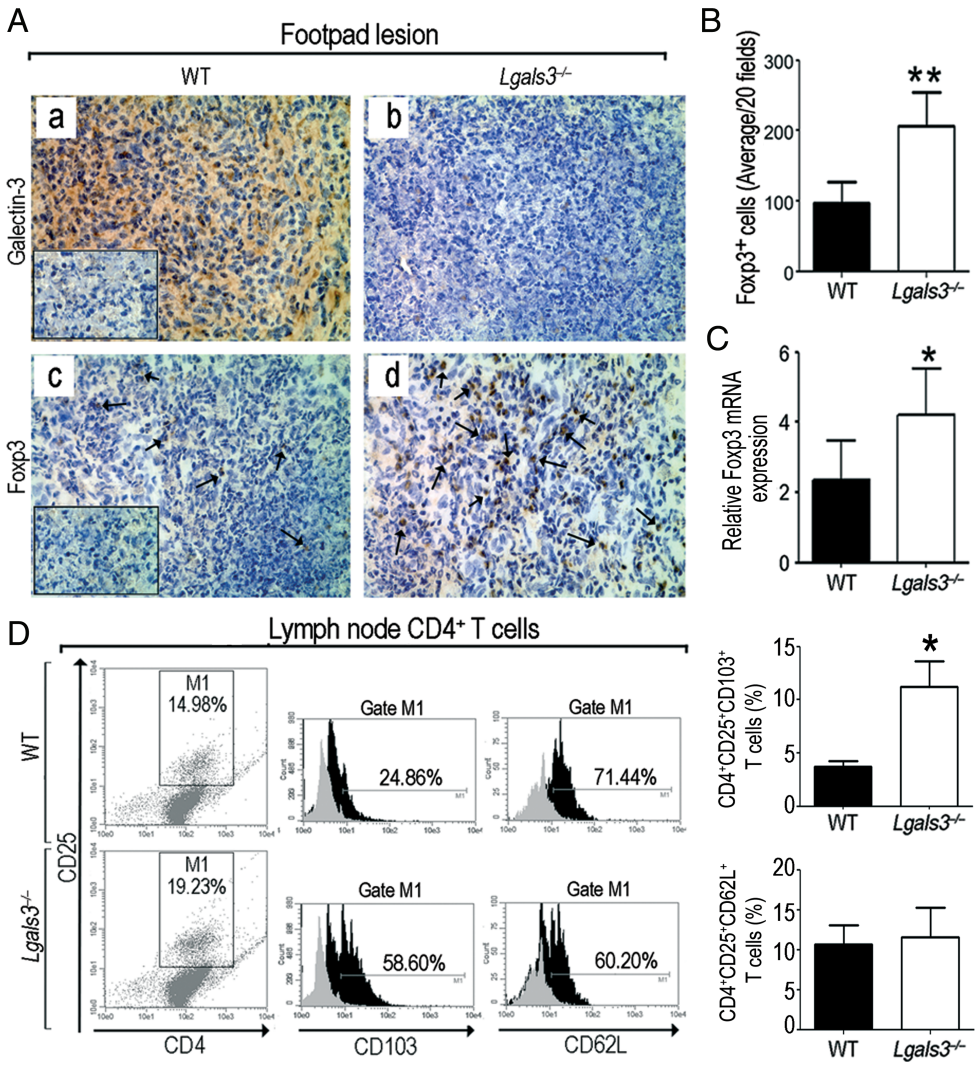

Figure 2. *Leishmania major* infected *Lgals3*$^{-/-}$ mice display higher frequency of T_{REG} cells at sites of infection. (A) Immunohistochemical staining of galectin-3 (panels a and b) and Foxp3 (panels c and d) in the footpad lesions of WT and *Lgals3*$^{-/-}$ mice at day 35 postinfection. Photomicrographs depict galectin-3 staining (brown color) and hematoxylin counterstaining (magnification: 40×). As expected, *Lgals3*$^{-/-}$ mice lack galectin-3 expression (panel b). Data are representative of two experiments using three mice per group. Black arrows indicate Foxp3^{+} cells. Insets depict controls omitting primary antibodies. (B) Number of Foxp3^{+} cells in footpad lesions of *Lgals3*$^{-/-}$ and WT mice 35 days after infection. Immunostained cells were counted in 20 different fields under phase contrast objective microscope (40× magnification) and data represent the mean + SD of two experiments with three mice per group. (C) Real-time RT-PCR. mRNA was isolated from footpad tissue 35 days after infection with *L. major*. cDNA was prepared from each group and quantitative real-time PCR was performed to analyze Foxp3 mRNA expression. Data show the mean + SD of three experiments with five mice per group. (D) Flow cytometry of CD103 and CD62L surface expression shown on double positive (CD4^{+}CD25^{+}) T cells purified from popliteal LNs from WT or *Lgals3*$^{-/-}$ mice at day 35 postinfection. Numbers on histograms represent average percentages of positive cells and bar graphs represent the mean percentage of triple-positive cells + SD of three independent experiments performed with cells pooled from three mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$; Student's t-test.

mRNA was substantially upregulated after stimulation with anti-CD3 and anti-CD28 antibodies in both T_{EFF} and T_{REG} WT cells (Fig. 5C).

To further dissect the role of galectin-3 within the T_{REG}-cell compartment during infection, we isolated T_{EFF} and T_{REG} cells from draining LNs of *L. major* infected *Lgals3*$^{-/-}$ and WT mice and analyzed Notch-1 and Notch-3 mRNA expression by real-time PCR and flow cytometry. T_{EFF} cells from *Lgals3*$^{-/-}$ mice showed increased mRNA expression for Notch-1 and Notch-3 (Fig. 6A) and enhanced Notch-1 protein expression (Fig. 6B), when compared with their WT counterpart. However, despite expressing high amounts of Notch-1 receptor (Fig. 6C), T_{REG} cells from *Lgals3*$^{-/-}$ mice displayed lower mRNA and protein levels of Notch-3 receptor (Fig. 6D), similar to T_{REG} cells from uninfected *Lgals3*$^{-/-}$ mice (Fig. 5B). Notably, galectin-3 expression was upregulated in T_{EFF} and T_{REG} cells from WT-infected mice (Fig. 6E); however, we could find no significant change in Jagged-1 expression between T_{EFF} and T_{REG} cells from WT- and *Lgals3*$^{-/-}$-infected mice (Fig. 6F). Thus, selected components of the Notch signaling pathway are altered in the absence of galectin-3 and

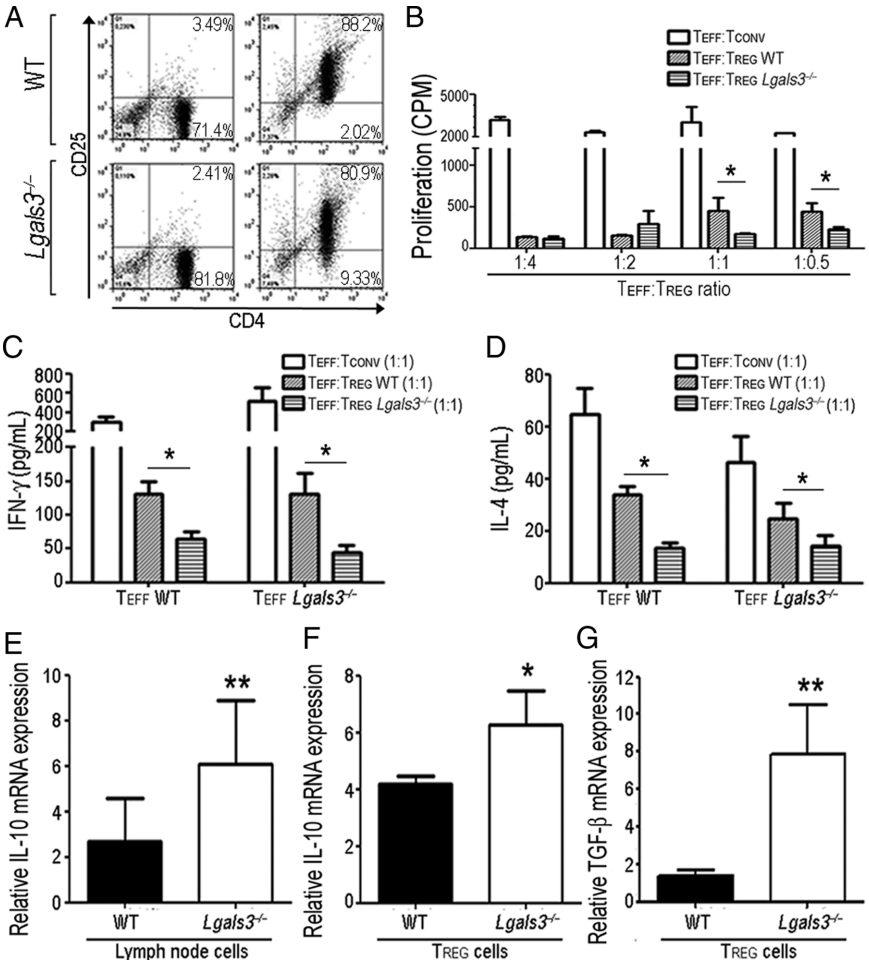

Figure 3. T_{REG} cells from *L. major* infected *Lgals3*$^{-/-}$ mice show increased suppressive capacity. (A–G) T_{EFF} and T_{REG} cells were isolated from popliteal LNs from *Lgals3*$^{-/-}$ or WT mice chronically infected with 1×10^7 stationary phase *L. major* LV39 promastigotes. T_{EFF} and T_{REG} cells were isolated using a mouse T_{REG} cell isolation kit (Miltenyi). All data are representative of three experiments, each one performed with purified cells pooled from four mice per group. (A) Purity of T_{EFF} and T_{REG} cells fluorescently stained with CD4 and CD25 antibodies. (B–D) In vitro suppressive activity of T_{REG} cells isolated from popliteal LNs of *L. major* infected mice. (B) T_{EFF} cells from WT mice were restimulated with *L. major* antigen and co-cultured for 72 h with different ratios of either WT or *Lgals3*$^{-/-}$ T_{REG} cells as indicated. Proliferation was measured by (^{3}H)-thymidine incorporation. Data represent the mean + SD of three independent experiments. (C, D) T_{EFF} cells from *L. major* infected WT and *Lgals3*$^{-/-}$ mice were co-cultured with T_{REG} cells from WT or *Lgals3*$^{-/-}$ mice at a 1:1 ratio for 72 h. Supernatants were collected for measuring IFN-γ and IL-4 by ELISA (mean + SD of three independent experiments). (E–G) Total LN cells (E) or purified T_{REG} cells (F, G) from *L. major* infected WT and *Lgals3*$^{-/-}$ mice were assessed for IL-10 and TGF-β_1 mRNA expression by real-time PCR. Data show mean + SD of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t*-test.

might contribute to the intrinsic immunoregulatory activity of this endogenous lectin within the T_{REG}-cell compartment.

Enhanced Notch activation accounts for the higher production of IL-10 in T_{REG} cells from *Lgals3*$^{-/-}$ mice

To further examine the possibility that endogenous galectin-3 could interfere with Notch activation in T_{REG} cells, we then isolated naïve CD4$^+$CD25$^-$ T cells from the spleens of noninfected WT or *Lgals3*$^{-/-}$ mice and activated these cells with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence of IL-2 and TGF-β. After 5 days, cells were harvested and analyzed for CD25 and Foxp3 expression. The differentiation rate was comparable in cells isolated from either *Lgals3*$^{-/-}$ or WT animals. About 60% of stimulated CD4$^+$CD25$^-$ T cells became CD4$^+$CD25$^+$ double positive cells and among them, 50% were also positive for Foxp3 (Fig. 7A and B). When CD4$^+$CD25$^-$ T cells were cultured in the presence of the γ-secretase inhibitor *N*-((3,5-difluorophenyl)acetyl)-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) (10 μM), T_{REG}-cell differentiation was completely abolished in both KO and WT groups (Fig. 7B). However, in vitro induced T_{REG} cells from *Lgals3*$^{-/-}$ mice synthesized higher amounts of IL-10 (Fig. 7C) compared with WT mice, similar to conventional T_{REG} cells isolated from infected and noninfected *Lgals3*$^{-/-}$ mice (Figs. 3F and 4B, respectively). Moreover, in vitro induced T_{REG} cells from *Lgals3*$^{-/-}$ mice were much more resistant to blockade of Notch activation and produced greater amounts of IL-10 even after treatment with DAPT (Fig. 7D). These results suggest that galectin-3 might not directly affect the in vitro differentiation of T_{REG} cells, but reinforces a critical role for this lectin in the control of IL-10 production and modulation of Notch activation.

Discussion

In the present study, we identified a role for endogenous galectin-3 as a negative regulator of T_{REG} cell frequency and function during *L. major* infection. Moreover, our results show that endogenous galectin-3 selectively influences downstream molecular targets including IL-10 and Notch signaling.

Galectin-3 is an immunoregulatory lectin widely distributed in different tissues including sites of inflammation and infection [1, 23] and modulates the fate and function of different cell types [5, 24, 25]. With regard to T cells, galectin-3 is expressed by activated but not resting CD4$^+$ and CD8$^+$ T cells [25]. Although

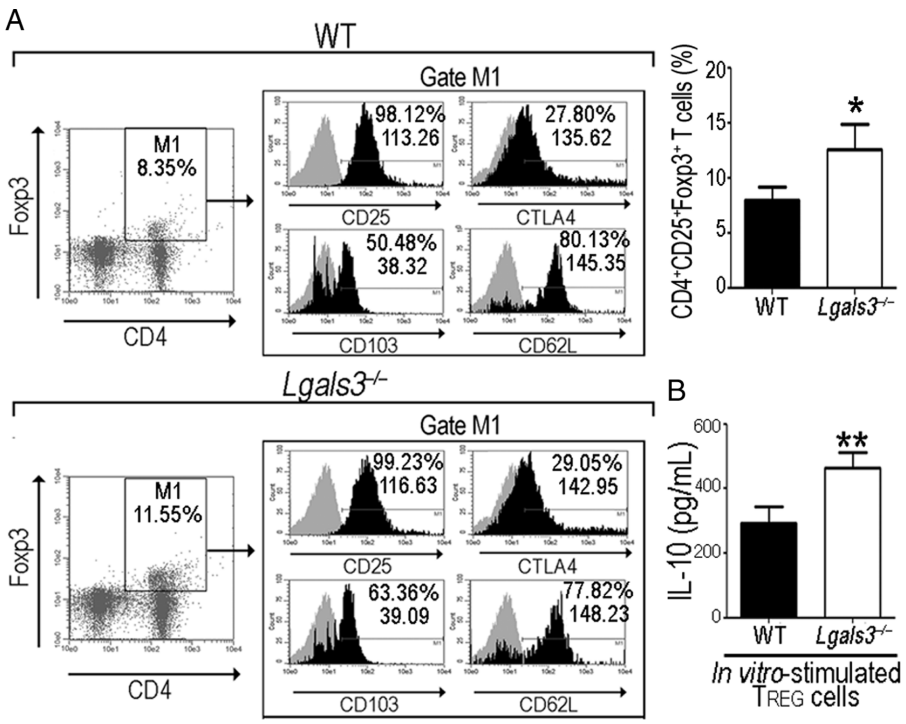

Figure 4. In vitro stimulated T_{REG} cells from noninfected *Lgals3*$^{-/-}$ mice produce higher amounts of IL-10. (A) Flow cytometry analysis of CD25, CTLA4, CD103, and CD62L expression in double-positive (FoxP3$^+$CD4$^+$) T cells isolated from spleen of noninfected WT and *Lgals3*$^{-/-}$ mice. Three independent experiments were performed, using purified cells pooled from three mice per group. Upper numbers represent the average percentages and lower numbers represent the mean fluorescence intensity (MFI) of cells expressing the indicated surface markers. Bar graph shows the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells (mean + SD of three experiments). (B) T_{REG} cells were purified from noninfected animals and stimulated with 0.5 μg/mL of anti-CD3 and anti-CD28 mAbs for 72 h. After this period, supernatants were collected and IL-10 was determined by ELISA (mean + SD of three experiments). $^*p < 0.05$, $^{**}p < 0.01$; Student's *t*-test.

different groups have reported several roles for exogenous and endogenous galectin-3 in T-cell activation, differentiation, and apoptosis [26, 27], the function of this lectin within the T_{REG}-cell compartment is largely unknown. We found increased percentage of peripheral T_{REG} cells in noninfected *Lgals3*$^{-/-}$ compared with WT mice. Remarkably, the frequency of T_{REG} cells at infection sites and draining LN was significantly increased during chronic leishmaniasis in *Lgals3*$^{-/-}$ mice compared with WT mice. Several possibilities may explain this phenomenon, including selective attraction of T_{REG} cells by tolerogenic DCs present in secondary lymphoid organs and infected tissues [28] and/or active proliferation of T_{REG} cells in vivo following antigenic stimulation [29]. Given our previous observations that galectin-3 has inhibitory effects on IL-12 production by DCs [5], the increased activation of DCs from *Lgals3*$^{-/-}$ mice could lead to enhanced migration of T_{REG} cells to sites of infection. In addition, T_{REG} cell homing is dictated by the expression of cell adhesion molecules, including CD103 [17] and CD62L [30], which regulate their tissue-specific trafficking, recruitment, and function. Our findings show that draining LNs from *Lgals3*$^{-/-}$-infected mice contains higher frequency of T_{REG} cells, which display increased expression of CD103. Whether endogenous galectin-3 could affect T_{REG}-cell recruitment via CD103-mediated mechanisms remains to be elucidated. Alternatively, as expression of CD103 is upregulated by TGF-β [31], the higher production of TGF-β by *Lgals3*$^{-/-}$ T_{REG} cells could also account for the upregulated expression of this molecule.

In the past few years, new findings have challenged the classical Th1/Th2 paradigm in mice "resistant" and "susceptible" to *L. major* infection. These findings revealed that IL-10 is one of the crucial factors responsible for the susceptibility to *L. major* infection, besides the traditional IL-4R pathway [32–34]. In *L. major* infection, T_{REG} cells have been reported as an important source of IL-10 [33–35], which contributes to susceptibility to infection [15, 16, 18, 35, 36]. Although we found that *Lgals3*$^{-/-}$ T_{REG} cells produce higher amounts of IL-10 than WT T_{REG} cells that could influence susceptibility to *L. major* infection, we cannot rule out the possibility that this endogenous lectin could also influence IL-10 production by other immune cells, including macrophages or B cells. This effect is important given recent studies showing the role of IL-10-producing B cells in controlling susceptibility to *L. major* infection [37]. Moreover, we previously found that macrophages from *Lgals3*$^{-/-}$ mice produce higher amounts of IL-10 in comparison with WT mice [7], suggesting that IL-10 may serve as a general effector target of the immunoregulatory activity of galectin-3. These results raise the question of whether galectin-3 could play a pivotal role in controlling IL-10 gene transcription and ultimately limiting T_{REG} cell functionality. Our findings add to the recently documented role of galectin-3 in modulating the severity of *L. major* infection by facilitating neutrophil recruitment to sites of infection [38]. Thus, distinct galectin-3-regulated mechanisms may dictate susceptibility to *L. major* infection.

Notch receptors and their ligands are important factors that contribute to the generation, expansion, and function of T_{REG} cells [22]. Notch-3 expression is a hallmark of T_{REG} cells and Notch-3-mediated signaling positively regulates the expansion of T_{REG} cells [39]. We found that Notch-1 and Notch-3 receptors are differentially expressed on T_{REG} cells from WT versus *Lgals3*$^{-/-}$ mice. Surprisingly, in our model, Notch-3 expression was found to be downregulated in T_{REG} cells from infected *Lgals3*$^{-/-}$ mice. Despite this fact, we detected high levels of Hes-1 transcripts in *Lgals3*$^{-/-}$ mice, suggesting a more pronounced activation of this pathway. In fact, Anastasi et al. [39] showed that transgenic

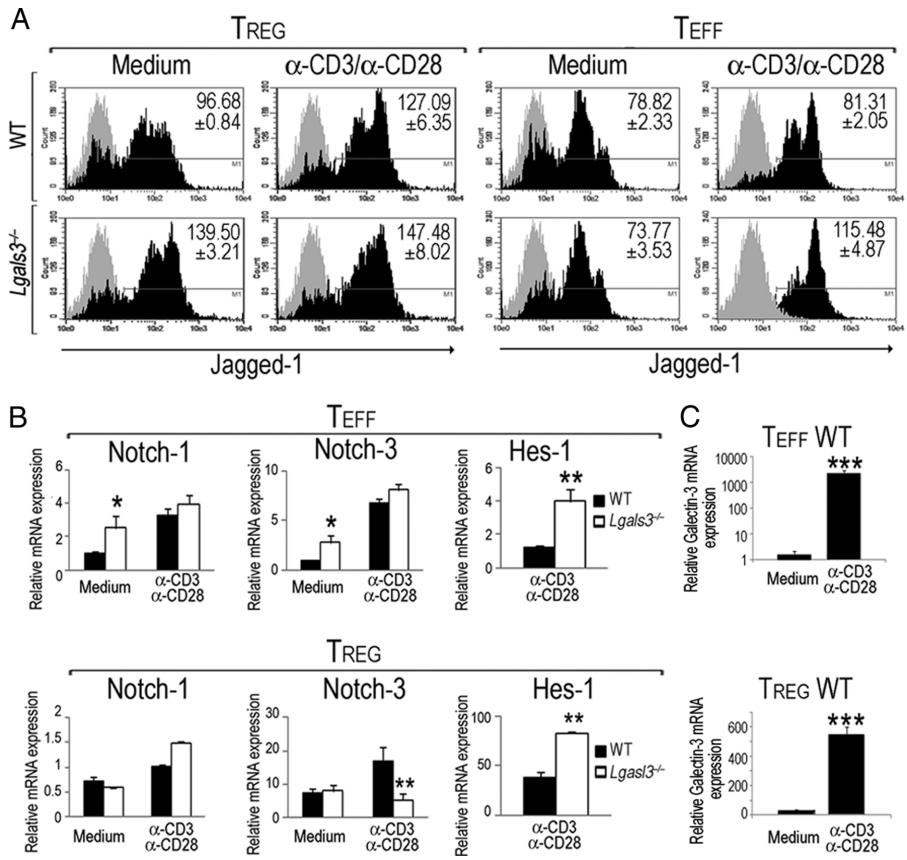

Figure 5. Lack of galectin-3 induces increased expression of Jagged-1, Notch-1, and the target gene Hes-1 in in vitro stimulated T_{EFF} and T_{REG} cells. (A–C). T_{REG} cells and T_{EFF} cells were purified from the spleen of noninfected WT and *Lgals3*$^{-/-}$ mice and stimulated with plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for 24 h. Three independent experiments were performed, using purified cells pooled from three mice per group. (A) Cells were stained intracellularly with an anti-Jagged-1 mAb and analyzed by flow cytometry. Numbers represent the average mean ± SD from three independent experiments. (B–C) T_{EFF} and T_{REG} cells were analyzed for mRNA expression of Notch signaling components (B) and galectin-3 (C) by real-time PCR. Data show mean + SD from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test.

mice overexpressing the active intracellular domain of Notch-3 display increased accumulation of T_{REG} cells in lymphoid organs and increased expression of IL-10. Activation of Notch signaling directly affects T_{REG}-cell function by regulating Foxp3 expression through RBP-J- and Hes1-dependent mechanisms [40, 41]. In addition, recent reports show that Notch signaling regulates IL-10 production by Th1 cells through a STAT4-dependent mechanism that converts pro-inflammatory Th1 cells into T cells with regulatory activity [42]. These observations led us to propose that increased IL-10 production in *Lgals3*$^{-/-}$ mice during infection was, at least in part, associated with higher activation of Notch signaling in these cells. This hypothesis has been confirmed by the fact that in vitro differentiated T_{REG} cells from *Lgals3*$^{-/-}$ mice produced more IL-10 and were more resistant to inhibition of the Notch pathway.

A few reports have already demonstrated a direct correlation between increased expression of Jagged-1 by antigen presenting cells and the induction of T_{REG} cells [20, 21]. Interestingly, Asano and colleagues [19] reported that Jagged-1 is highly expressed by T_{REG} cells and that blockade of this ligand inhibits T_{REG} cell suppressive function in vitro. In our study, the higher expression of Jagged-1 by T_{REG} cells from uninfected *Lgals3*$^{-/-}$ mice may account, at least in part, for their enhanced suppressive capacity. Interestingly, T_{EFF} cells activated by Jagged-1 are considerably more sensitive to T_{REG}-cell-mediated suppressive activity [43]. Taken together, these findings suggest that galectin-3 may negatively control the number and suppressive function of T_{REG} cells by modulating components of the Notch pathway. Interestingly, mice lacking c-Rel, a member of the NF-κB family of transcription factors implicated in T_{REG}-cell differentiation, IL-10 production, and Th skewing, also showed exacerbated leishmaniasis [44]. Whether c-Rel regulates galectin-3 expression remains to be established.

Finally, as galectin-1 and galectin-10 positively regulate T_{REG}-cell function [10, 11] and galectin-3 negatively regulates T_{REG}-cell expansion in the context of autoimmune [13] or infectious diseases (our results), we postulate that a balance among different members of the galectin family may play a homeostatic role in the modulation of T_{REG} cells. Our data provide an alternative mechanism to explain alterations in T_{REG}-cell function during *Leishmania* infection with broad implications in immunopathology.

Materials and methods

Mice

Galectin-3-deficient (*Lgals3*$^{-/-}$) mice were generated as described [45] and backcrossed to BALB/c mice for nine generations. Age-matched WT mice on BALB/c background were used as controls. The Ethics Committee on Animal Research of the University of São Paulo approved all the procedures described. Mouse experiments were approved (Protocol 097/2005) by the Faculdade de

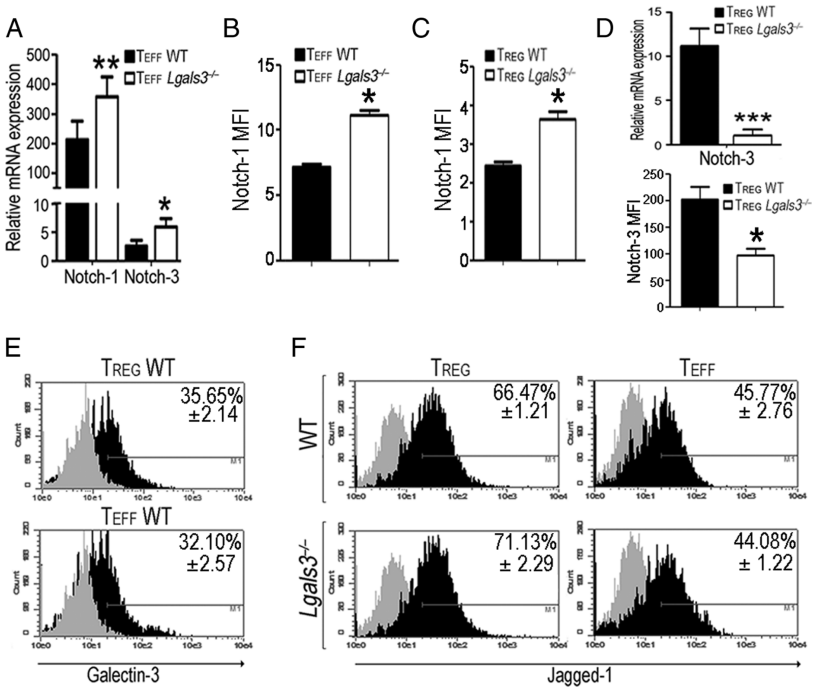

Figure 6. Lack of galectin-3 induces increased expression of Notch-1 receptor during *L. major* infection. T_{EFF} cells (A, B) and T_{REG} cells (C, D) were purified from popliteal LNs from *L. major* infected WT and *Lgals3*$^{-/-}$ mice 35 days postinfection and expression of Notch-1 and Notch-3 was assessed by real-time PCR (A, D, upper panel) or by flow cytometry using anti-Notch-1 or anti-Notch-3 mAbs (B–D, lower panel). (E, F) Flow cytometry analysis showing the percentage of positive cells expressing galectin-3 and Jagged-1. All data represent mean ± SD of three experiments performed with cells pooled from four mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's *t*-test.

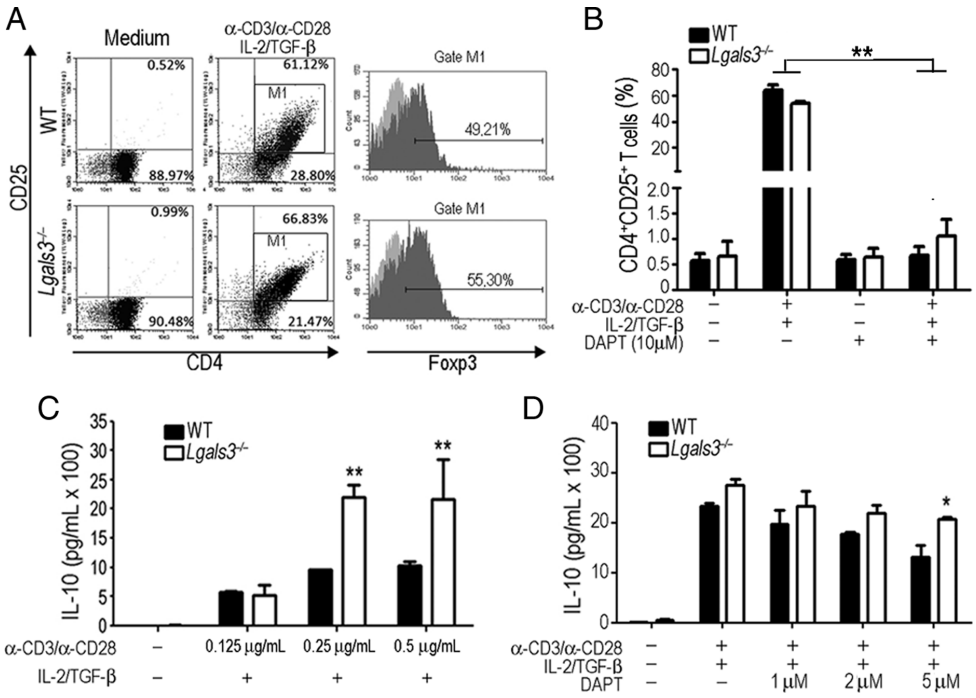

Figure 7. In vitro differentiated T_{REG} cells from *Lgals3*$^{-/-}$ mice show enhanced IL-10 production in the absence or presence of Notch signaling. (A) Naïve $CD4^{+}CD25^{-}$ T cells were isolated from spleen of noninfected WT or *Lgals3*$^{-/-}$ mice and differentiated toward a T_{REG} cell profile by stimulation with plate-bound anti-CD3 (0.5 μg/mL) and soluble anti-CD28 (0.5 μg/mL) mAbs, IL-2 (20 ng/mL), and TGF-β_1 (3 ng/mL). After 5 days, cells were harvested and analyzed by flow cytometry for expression of CD4, CD25, and Foxp3. Data show average percentage of positive cells, representative of three experiments each performed with cells pooled from four mice per group. (B) Naïve $CD4^{+}CD25^{-}$ T cells were differentiated toward a T_{REG}-cell phenotype as described in (A), in the presence or absence of 10 μM of the Notch signaling inhibitor DAPT. The percentage of cells positive for CD25 is shown (mean + SD of three experiments). (C, D) Supernatants were collected from in vitro differentiated T_{REG} cells cultured with increasing concentrations of anti-CD3 (0.125–0.5 μg/mL) and anti-CD28 (0.125–0.5 μg/mL) antibodies (C) or with fixed concentrations of anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL) mAbs and increasing concentrations of DAPT (D) to assess IL-10 by ELISA. Data show the mean + SD of three experiments performed with cells pooled from three mice per group. $^{*}p < 0.05$; $^{**}p < 0.01$; Student's *t*-test.

Table 1. Primers used in real-time PCR.

Gene	Sequence of primer pair (5′–3′)	PCR product size (bp)
Actb (β-actin)	Forward: AGCTGCGTTTTACACCCTTT Reverse: AAGCCATGCCAATGTTGTCT	100
Foxp3	Forward: ATTGAGGGTGGGTGTCAGGA Reverse: TCCAAGTCTCGTCTGAAGGCA	100
Lgals3	Forward: GACAGTCAGCCTTCCCCTTTGAGAG Reverse: AGGCACACAGGGCCGGTTTCGG	100
Hes1	Forward: CTCTGGGGACTGAGAAGAAAAA Reverse: GCATCCAAAATCAGTGTTTTCA	100
Il10	Forward: TGACTGGCATGAGGATCAGC Reverse: AGTCCGCAGCTCTAGGAGCA	100
Notch1	Forward: CTTGGCTGCCCGATACTCTC Reverse: CATGTAACGGAGTACGGCCC	100
Notch3	Forward: GGTTCCCTGAGGGTTTTGC Reverse: GGCCATGTTCTTCATTCCCA	100
Tgfb1	Forward: GACTCTCCACCTGCAAGACCA Reverse: GGGACTGGCGAGCCTTAGTT	100

Medicina de Ribeirão Preto-USP Institutional Animal Care and User Committee approved protocols. All animals used were 6- to 8-week-old males.

Leishmania major infection

Experiments were performed with *L. major* strain LV39 maintained in BALB/c mice by serial s.c. passages. For experimental infection, parasites were grown in vitro as described [46]. Promastigote forms were washed twice in PBS before infection. Mice were infected s.c. in one hind footpad with 1×10^7 stationary phase *L. major* promastigotes in a final volume of 50 μL. Lesion development was monitored weekly, and the noninfected contralateral footpad was used as control. Parasite burden was determined by real-time PCR [47].

Flow cytometry

Cells were obtained from draining LNs or spleen of noninfected mice as indicated. Cells were incubated for 30 min with CD16/CD32 mAb (Fc blocking, clone 2.4G2, BD Bioscience, MD, USA), followed by surface staining with PE-conjugated anti-mouse F4/80 (R&D Systems, MN, USA), anti-mouse CD11c, anti-CD4, anti-CD8, anti-CTLA4, anti-CD62L, anti-CD103, or anti-CD25 antibodies, and/or with FITC-conjugated anti-CD3, anti-CD8, and anti-CD25 antibodies (all from eBioscience, CA, USA). For intracellular staining, cells were permeabilized using the Fix & Perm Buffer kit (eBioscience) according to the manufacturer's protocol, and stained with PECy5-labeled Foxp3 antibody (eBioscience), FITC-Notch-1 or FITC-Notch-3 antibodies (all from Biolegend, CA, USA), or with anti-Jagged-1 or rat anti-galectin-3 M3/38 mAbs [48] followed by staining with specific Alexa 488-labeled antibodies (BD Bioscience). Cells were analyzed on a FACScan flow cytometer (BD Biosciences).

Cytokine determination

Cytokines (IL-4, IL-10, and IFN-γ) were determined by ELISA using commercially available kits, according to manufacturer's instructions (BD Biosciences). The sensitivity limits of the assays were 7 pg/mL for IL-4 and 30 pg/mL for IL-10 and IFN-γ.

T_{REG} cell isolation and in vitro suppression assay

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were isolated from pooled draining LN cells of *L. major* infected mice or from spleens of normal mice ($n = 4$) using a mouse T_{REG}-cell isolation kit (Miltenyi Biotec, Bergish Gladcach, Germany) according to the manufacturer's instructions. The suppressive capacity of T_{REG} cells was studied in co-culture suppression assays, which were set up in 96-well plates in RPMI 1640 (Gibco, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum Gibco). Proliferation was assessed by (^{3}H)-thymidine incorporation. Briefly, $CD4^+CD25^-$ (T_{EFF}) cells isolated from draining LNs of infected WT mice (or *Lgals3*$^{-/-}$ mice, when indicated) were seeded at 5×10^4 cells per well and restimulated with 20 μg/mL of *L. major* antigen. Then, $CD4^+CD25^+$ T_{REG} cells or $CD4^+CD25^-$ T (T_{EFF}) cells from either WT- or *Lgals3*$^{-/-}$-infected mice were incorporated to cultures at different ratios. At day 5, proliferation was measured by adding 0.5 μCi (^{3}H)-thymidine (Amersham Biosciences, Piscataway, NJ, USA) to each well. After 12 h, radioactivity was measured using a β-plate counter (Packard, Canberra, Australia). Culture supernatants were collected for cytokine measurement by ELISA. Tests were set up in triplicate.

In vitro generation of T_{REG} cells

For differentiation of naïve $CD4^+CD25^-$ T cells into a T_{REG}-cell phenotype, $CD4^+CD25^-$ T cells were enriched from total spleen

cells of WT or *Lgals3*$^{-/-}$ mice by negative selection. CD4$^+$CD25$^-$ T cells were resuspended at 1×10^5 cells per well in RPMI 1640 medium plus 5% fetal bovine serum, seeded in a 96-well plate coated with anti-CD3 mAb (BD Biosciences) at the indicated concentrations, and stimulated with soluble TGF-β_1 (3 ng/mL), IL-2 (20 ng/mL), and anti-CD28 mAb (at the indicated concentrations) (all from BD Biosciences). In some experiments, cells were cultured in the presence of different concentrations of DAPT (1–10 μM, Sigma-Aldrich). After 5 days of culture, cells were harvested and analyzed for CD25 and Foxp3 by flow cytometry as described above. Cytokines were measured in culture supernatants by ELISA.

Immunohistochemistry

Footpad tissue from infected WT and *Lgals3*$^{-/-}$ mice was frozen in Tissue Tek (Qiagen, CA, USA) medium and cut into 8–10 μm sections. Immunohistochemistry for galectin-3 and Foxp3 was performed as described [49], using the polyclonal rat anti-Foxp3 antibody (eBioscience) or monoclonal rat anti-galectin-3 M3/38 antibody [48], and the biotinylated anti-rat secondary antibody (R&D Systems). Slides were analyzed using a Nikon Eclipse E800 microscope (Nikon USA, Melville, NY, USA) equipped with a digital camera Nikon DXM1200.

Real-time quantitative PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, CA, USA), following the manufacturer's instructions. cDNA synthesis was performed in a final volume of 20 μL using ImProm-II Reverse Transcriptase (Promega Corporation, WI, USA). PCR amplification was performed with SYBR Green Master Mix (Applied Biosystems, CA, USA) and analyzed with an ABI Prism 7500 sequence detector (Applied Biosystems), using the $2^{-\Delta\Delta CT}$ method [50]. The primers used for PCR amplification are listed in Table 1.

Statistical analysis

Results are expressed as the mean ± SD of the indicated number of experiments. Statistical analysis of control and experimental groups was performed by Student's *t*-test using Prism 5 GraphPad (La Jolla, CA, USA) software. Differences were considered statistically significant when $p \leq 0.05$.

Acknowledgements: We thank Marcelo Dias Baruffi for helpful discussion, Julio Siqueira and Domingos Soares de Souza Filho for expert animal care, Vani MA Correa for excellent technical assistance, and João Santana da Silva for the CD103 antibody. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq) to E.S.B. and M.C.R.B. and grants from Fundación Sales and Agencia Nacional de Promoción Científica y Tecnológica (Argentina) to G.A.R.

Conflict of interest: The authors declare no commercial conflict of interest.

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Abbreviations: **DAPT**: N-((3,5-difluorophenyl)acetyl)-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester · **T_{EFF} cell**: T effector cell

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Received: 25/1/2013
Revised: 18/3/2013
Accepted: 11/4/2013
Accepted article online: 16/4/2013