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Lack of galectin-3 increases Jagged1/Notch activation in bone marrow-derived dendritic cells and promotes dysregulation of T helper cell polarization

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ABSTRACT

Galectin-3, an endogenous glycan-binding protein, is abundantly expressed at sites of inflammation and immune cell activation. Although this lectin has been implicated in the control of T helper (Th) polarization, the mechanisms underlying this effect are not well understood. Here, we investigated the role of endogenous galectin-3 during the course of experimental *Leishmania major* infection using galectin-3-deficient (*Lgals3*$^{-/-}$) mice in a BALB/c background and the involvement of Notch signaling pathway in this process. *Lgals3*$^{-/-}$ mice displayed an augmented, although mixed Th1/Th2 responses compared with wild-type (WT) mice. Concomitantly, lymph node and footpad lesion cells from infected *Lgals3*$^{-/-}$ mice showed enhanced levels of Notch signaling components (Notch-1, Jagged1, Jagged2 and Notch target gene Hes-1). Bone marrow-derived dendritic cells (BMDCs) from uninfected *Lgals3*$^{-/-}$ mice also displayed increased expression of the Notch ligands Delta-like-4 and Jagged1 and pro-inflammatory cytokines. In addition, activation of Notch signaling in BMDCs upon stimulation with Jagged1 was more pronounced in *Lgals3*$^{-/-}$ BMDCs compared to WT BMDCs; this condition resulted in increased production of IL-6 by *Lgals3*$^{-/-}$ BMDCs. Finally, addition of exogenous galectin-3 to *Lgals3*$^{-/-}$ BMDCs partially reverted the increased sensitivity to Jagged1 stimulation. Our results suggest that endogenous galectin-3 regulates Notch signaling activation in BMDCs and influences polarization of T helper responses, thus increasing susceptibility to *L. major* infection.

1. Introduction

Protein-glycan interactions have recently attracted the attention of immunologists as novel regulators of immune cell homeostasis and host–pathogen interactions (Kooyk and Rabinovich, 2008). Galectin-3 is a member of a family of endogenous lectins that bind β-galactoside-containing glycoconjugates and share structural homology in their carbohydrate recognition domains (CRDs) (Rabinovich and Toscano, 2009; Sato et al., 2009; Norling et al., 2009; Dumic et al., 2006). Besides its C-terminal CRD, galectin-3 presents an N-terminal domain, which enables its self-oligomerization leading to the formation of multimeric galectin-3

Abbreviations: Th, T helper; WT, wild-type; *Lgals3*$^{-/-}$, galectin-3-deficient mice; BMDC, bone marrow-derived dendritic cell; DCs, dendritic cells; NICD, Notch intracellular domain; APC, antigen presenting cell; Treg, T regulatory cell; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; LPS, lipopolysaccharide; DLL4, Delta-like-4; JAG1/JAG2, Jagged1/Jagged2.

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molecules (Hsu and Liu, 2004; Sato et al., 2009). Interestingly, galectin-3 is an ubiquitously expressed lectin, occurring in both intracellular and extracellular compartments, despite the lack of a signal peptide required for classical secretion (Hughes, 1997). This lectin is produced by stromal and immune cells, and is widely expressed at sites of tissue inflammation (Chen et al., 2005; Acosta-Rodríguez et al., 2004; Sundblad et al., 2011), where it plays essential roles in host responses to pathogens by modulating glycan-mediated pathogen recognition and subsequent development of innate and adaptive immune responses. In fact, galectin-3 can modulate immune cell activation and differentiation and control acute and chronic inflammatory responses (Esteban et al., 2011; Argüeso et al., 2009; Ferraz et al., 2008; Kohatsu et al., 2006; Silva-Monteiro et al., 2007; Bernardes et al., 2006; Nieminen et al., 2008; Farnworth et al., 2008; Breuilh et al., 2007; Jiang et al., 2009; Demetriou et al., 2001; Chen et al., 2009).

In previous studies, we used different experimental infection models in galectin-3-deficient (*Lgals3*$^{-/-}$) mice to investigate the immunoregulatory properties of galectin-3. We have demonstrated that galectin-3 suppresses the production of IL-12 by dendritic cells (DCs), limits the secretion of IL-6 and IL-1β by macrophages and negatively regulates the number and function of regulatory T cells (Tregs) (Bernardes et al., 2006; Ferraz et al., 2008; Fermino et al., 2013). Additionally, galectin-3 interferes with mice susceptibility to *Paracoccidioides brasiliensis* infection by increasing the inflammatory responses and favoring development of Th2-polarized immune responses (Ruas et al., 2009). However, in spite of considerable progress in elucidating galectin-3 functions, the mechanisms underlying the immune regulatory roles of this lectin during T helper cell differentiation still remain to be elucidated.

Notch signaling pathway is highly conserved from Drosophila to mammals and was originally identified in differentiation and developmental processes (Bray, 2006). In mammals, the signaling is triggered when one of the five ligands (Delta-1, -3, -4 and Jagged1 and 2) binds to one of the four Notch receptors (Notch-1-4) present in a neighboring cell, thus inducing Notch receptor proteolysis and release of the Notch intracellular domain (NICD). Next, NICD translocates to the nucleus where it initiates a signaling cascade culminating in the transcriptional regulation of Notch target genes (*Hes* and *Hey* family) (Ito et al., 2012). In the past years, Notch signaling has also emerged as a critical component of immune system regulation (Shang et al., 2016; Ito et al., 2012), controlling multiple steps of T and B cell development in both central and peripheral lymphoid organs (Shang et al., 2016). The expression of Jagged or Delta-like ligands in antigen presenting cells (APCs) has been shown to regulate the differentiation of naïve T helper cells into Th1 or Th2 effector subsets. While APCs expressing Delta-like ligands induced Th1 differentiation, expression of Jagged ligands was associated with Th2-skewed responses (Radtke et al., 2013).

Recently, interactions of galectin-3 with the Notch-1 receptor and Notch signaling activation was shown to inhibit osteoblast differentiation (Nakajima et al., 2014). Moreover, our group has shown that Tregs isolated from *Lgals3*$^{-/-}$ mice exhibit altered expression of Notch signaling components during *L. major* infection (Fermino et al., 2013). In the present study, we show that DCs from *Lgals3*$^{-/-}$ mice display an altered expression of Notch ligands and receptors which contribute to augment Th2 responses in the BALB/c model of *L. major* infection. Our results suggest that a cross-talk between galectin-3 and the Jagged1/Notch signaling pathways may contribute, at least in part, to the control of T helper polarization programs.

2. Material and methods

2.1. Mice

Galectin-3-deficient (*Lgals3*$^{-/-}$) mice were generated and backcrossed to BALB/c background for nine generations as previously described (Hsu et al., 2000). Age-matched wild-type (WT) mice in BALB/c background were used as control in all the experiments. Mice were housed and cared under approved conditions at the Animal Research Facilities of Faculdade de Medicina de Ribeirão Preto-USP and the London School of Hygiene and Tropical Medicine (London, UK). All of the animals used in the experiments were 6- to 8-week-old males.

2.2. L. major *infection*

Experiments were performed with *L. major* strain LV 39. The strain was maintained *in vivo* in BALB/c mice to keep its infectivity. For experimental infection, parasites were obtained from lymph nodes of infected mice and grown *in vitro* as described (Launois et al., 1997; Zimmermann et al., 1998). Promastigote forms were washed twice in PBS before infection. Mice were infected subcutaneously 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 with a vernier caliper and lesion size expressed as the difference in thickness between the infected footpad and the non-infected contralateral footpad.

2.3. Real time quantitative PCR analysis

For quantification of mRNA relative expression we utilized the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, 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, Madison, WI, USA). The reaction mixture contained 4 μg of total RNA, 20 pmol of oligodT primer (Invitrogen Life Technologies, Carlsbad, CA, USA), 40 U of RNAsin, 1 mM of dNTP mix, and 1 U of reverse transcriptase buffer. It was then immediately used or stored at −20 °C. PCR amplification and analysis were achieved using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA). All reactions were performed with SYBR Green PCR Master Mix (Applied Biosystems) using a 10 μL final volume in each reaction, which contained 1 μL of template cDNA, 2.5 pmol of each primer and 5 μL of SYBR Green. The cycles were processed according to the manufacturer's instructions. Each sample was tested in duplicate and all quantifications were normalized using β-actin as endogenous control. The primers used for all PCR amplification are described in Table 1.

2.4. In vitro *restimulation*

For *in vitro* restimulation after infection with *L. major*, total lymph node cells (5×10^6) were collected 14 days post-infection and stimulated with 20 μg/mL of *L. major* antigen for 72 h at 37 °C/5% CO_2/95% humidity in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 0.1% 2-ME (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM l-glutamine, and 5 mM HEPES in a final volume of 1 mL. Culture supernatants were collected after 72 h of stimulation and stored at −20 °C for cytokine measurement.

Table 1
Primer sequences for Real Time PCR.

Gene	Sequence of primer pair (5′-3′)	Accession number
β-actin	Forward: AGCTGCGTTTTACACCCTTT Reverse: AAGCCATGCCAATGTTGTCT	NM_007393.3
DLL4	Forward: CAGTTGCCCTTCAATTTCACC Reverse: TTTCCTGGCGAAGTCTCTGG	NM_019454.3
IFN-γ	Forward: TGCCATCGGCTGACCTAGAG Reverse: TCTCAGAGCTAGGCCGCAG	NM_008337.3
GATA-3	Forward: AAGAAAGGCATGAAGGACGC Reverse: GTGTGCCCATTTGGACATCA	NM_008091.3
Hes-1	Forward: CTCTGGGGACTGAGAAGAAAAA Reverse: GCATCCAAAATCAGTGTTTTCA	NM_008235.2
Hey-1	Forward: CACGCCACTATGCTCAATGT Reverse: TCTCCCTTCACCTCACTGCT	NM_010423.2
IL-1β	Forward: AAATACCTGTGGCCTTGGGC Reverse: CTTGGGATCCACACTCTCCAG	NM_008361.3
IL-4	Forward: GTCTCTCGTCACTGACGGCA Reverse: CGTGGATATGGCTCCTGGTAC	NM_021283.2
IL-6	Forward: TCAATTCCAGAAACCGCTATGA Reverse: GAAGTAGGGAAGGCCGTGGT	NM_031168.1
IL-10	Forward: TGACTGGCATGAGGATCAGC Reverse: AGTCCGCAGCTCTAGGAGCA	NM_010548.2
IL-12p40	Forward: AACCATCTCCTGGTTTGCCA Reverse: CGGGAGTCCAGTCCACCTC	NM_001303244.1
Jagged1	Forward: CCAGAACCCTTGTCACTACGG Reverse: CACGGGAATGGCAAGTCTTT	NM_013822.5
Jagged2	Forward: TATACCTGCGACCAGTACGGC Reverse: CCGTGGAGCAAATTACATCCTT	NM_010588.2
Notch-1	Forward: CTTGGCTGCCCGATACTCTC Reverse: CATGTAACGGAGTACGGCCC	NM_008714.3
T-bet	Forward: CACTAAGCAAGGACGGCGAA Reverse: CCACCAAGACCACATCCACA	NM_019507.2

2.5. Cytokines detection

IL-12p40, IL-10 and IFN-γ were quantified by ELISA with a commercially available kit, according to the manufacturer's instructions (OptEIA set; PharMingen). The sensitivity limits of the assays were 15 pg/mL for IL-12p40 and 30 pg/mL for IFN-γ and IL-10.

2.6. Generation of BMDCs

BMDCs were prepared by a modification (Jiang et al., 2003) of the procedure described by Inaba et al. (Inaba et al., 1992). A single-cell suspension of bone marrow cells from femurs and tibias of normal WT and *Lgals3*$^{-/-}$ mice (BALB/c background) were seeded into 24-well plates (1×10^6 cells/well) and cultured for 9 days at 37 °C/5% CO_2 in RPMI-1640 complete medium supplemented with 20 ng/mL GM-CSF (R&D Systems, Minneapolis, MN, USA). On Day 9, BM cell cultures were stimulated with 1 μg/mL LPS (Sigma-Aldrich) to generate mature BMDCs. The percentage of differentiated cells was confirmed to be higher than 80% by flow cytometry.

2.7. Stimulation of BMDCs with immobilized Jagged1 and Delta-like-4

To activate Notch signaling, 96-well plates (for cytokine analysis) and 6-well plates (for protein and RNA analysis) were previously coated with 0.25, 0.5, 1 or 2 μg/mL of rhJagged1 or rhDelta-like 4 (R&D system) diluted in PBS, and incubated overnight at 4 °C. After 24 h, plates were washed twice with PBS. After this step, BMDCs suspension from *Lgals3*$^{-/-}$ and WT mice were seeded on Jagged1- or Delta-like-4-coated plates for an additional 24 h. When indicated, BMDCs were pre-incubated with human recombinant galectin-3 (30 μg/mL) for 15 min before being added to Jagged1- or Delta-like-4-coated wells. The supernatants were then removed and cells were collected for subsequent RNA or protein extraction. Human recombinant galectin-3 was prepared based on procedures previously described (Stowell et al., 2008).

2.8. Flow cytometry

BMDCs from *Lgals3*$^{-/-}$ and WT mice were stimulated or not with 1 μg/mL of LPS for 24 h. 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 CD11c mAb (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 goat anti-Jagged1 or anti-Delta-like-4 (Santa Cruz Biotechnology) followed by staining with specific Alexa 488-labeled secondary Ab (BD Bioscience). Cells were analyzed on a FACScan flow cytometer (BD Biosciences, MD, USA). Lymph node cells isolated from *L. major*-infected *Lgals3*$^{-/-}$ and WT mice were stained (intracellular) with a PE-labeled rabbit-anti-Notch-1 Ab (Santa Cruz Biotechnology).

2.9. Western blotting

Cells were lysed in RIPA buffer and 50 μg of proteins were separated by NovexNuPAGE SDS-PAGE gel system (Invitrogen) and then transferred overnight to a PVDF membrane (Invitrogen). The membrane was incubated with anti-cleaved Val1744 (NICD1) (1:500) and anti-galectin-3 (M3/38) Ab. Anti-β-actin-peroxidase Ab (1:20,000) was used as a loading control. Horseradish peroxidase (HRP)-conjugated secondary Ab were detected using the enhanced chemiluminescence (ECL) reagent (GE Healthcare).

3. Results

3.1. Lgals3$^{-/-}$ mice on BALB/c background display mixed and exacerbated T cell responses during L. major infection

Genetic background influences the outcome of *Leishmania major* infection. Susceptibility of BALB/c mice to *L. major* infection is associated with the development of a *L. major*-specific Th2 response, while C57BL/6 mice resistance is due to their ability to mount a *L.*

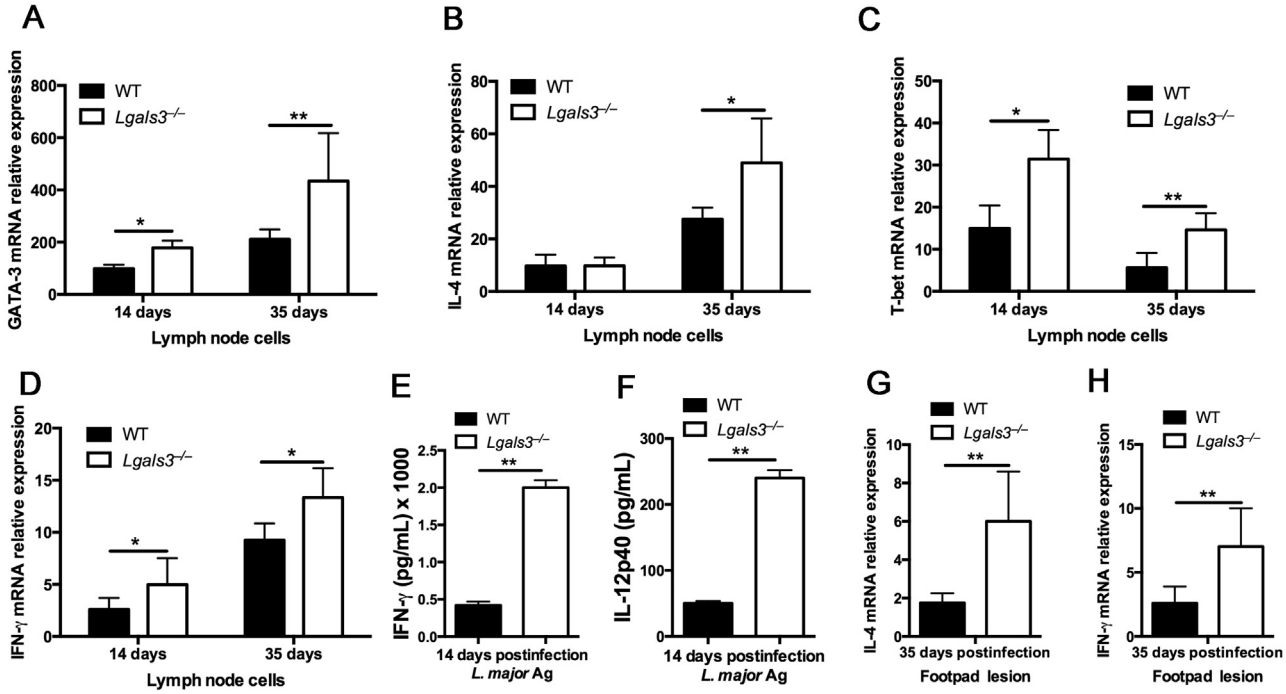

Fig. 1. Th1/Th2 bias during early and chronic *L. major* infection in *Lgals3*$^{-/-}$ BALB/c mice. (A–D) Relative quantification of GATA-3 and T-bet mRNA levels and cytokines in lymph nodes from *L. major*-infected WT and *Lgals3*$^{-/-}$ mice at 14 and 35 days post-infection. Total RNA from lymph node cells (5 mice/group) was isolated and analyzed by Real Time PCR for expression of GATA-3 (A), IL-4 (B), T-bet (C) and IFN-γ (D). (E–F) To quantify cytokine production, lymph node popliteal cells from 5 mice/group (14 days post-infection) were collected and re-stimulated *in vitro* with 20 μg/mL of *L. major* antigen. IFN-γ (E) and IL-12p40 (F) concentrations were determined by ELISA. (G-H) After 35 days post-infection, total RNA from footpad lesion was isolated and analyzed by Real Time PCR for expression of IL-4 (5 mice/group) (G) and IFN-γ (H). These results are representative of three independent experiments. *$p < 0.05$;**$p < 0.001$.

major-specific Th1 response (Sacks and Noben-Trauth, 2002). Since *Lgals3*$^{-/-}$ mice on a BALB/c background are more susceptible to *L. major* infection (Fermino et al., 2013), we investigated whether the Th1/Th2 cytokine balance is altered in the absence of galectin-3. *Lgals3*$^{-/-}$ and WT BALB/c mice were inoculated with 1×10^7 metacyclic promastigotes into one hind footpad, and the development of the lesion was monitored weekly. At 14 and 35 days post-infection, popliteal lymph node cells were collected and analyzed for expression of cytokines and transcription factors by real-time PCR. At the early phase of infection (14 days), lymph node cells from *Lgals3*$^{-/-}$ mice expressed higher mRNA levels of GATA-3 (Fig. 1A), a Th2-specific transcription factor, in comparison to WT mice. However, the mRNA levels of the hallmark Th2 cytokine, IL-4, were similar between WT and *Lgals3*$^{-/-}$ cells in this period (Fig. 1B). At a later phase of infection (35 days) lymph node cells from *L. major*-infected *Lgals3*$^{-/-}$ displayed increased mRNA levels of GATA-3 and IL-4 (Fig. 1A and B), when compared to *L. major*-infected WT mice. On the other hand, mRNA levels of the transcription factor T-bet and the Th1 cytokine IFN-γ, both characteristic of a Th1 cytokine profile, were both increased in the lymph nodes of *Lgals3*$^{-/-}$ mice at 14 and 35 days post-infection, compared with WT mice (Figs. 1C and D), suggesting a non-selective increase of Th1 and Th2 profiles in the context of galectin-3 deficiency. Supporting these findings, we found that lymph nodes cells isolated from infected *Lgals3*$^{-/-}$ mice had increased levels of the Th1-type cytokines IFN-γ and IL12p40 as compared with lymph node cells derived from WT mice following *in vitro* restimulation with *L. major* antigens (Fig. 1E and F). Similar to the results obtained in lymph nodes at 35 days after infection, the footpad lesions of *Lgals3*$^{-/-}$ mice displayed increased IL-4 and IFN-γ mRNA levels when compared to the footpad lesions of WT mice (Fig. 1G and H). Interestingly, the course of *L. major* infection in *Lgals3*$^{-/-}$ mice on C57BL/6 background did not differ statistically from WT mice with regards to footpad swelling, although *Lgals3*$^{-/-}$ mice developed a heightened Th1 polarized immune response and displayed a lower parasite load in their footpad at 14 days post-infection (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.molimm.2016.06.005). Altogether, these results suggest that galectin-3 deficiency favors non-selective up-regulation of Th1 and Th2 profiles in response to *L. major* infection.

3.2. L. major-*infected Lgals3*$^{-/-}$ *mice display enhanced expression of Notch signaling components*

Previous studies showed that the Notch signaling pathway plays an important role in T-cell activation and Th1/Th2 polarization (Amsen et al., 2009a,b). It is well documented that individual Notch receptors may induce Th2 polarization by directly regulating GATA-3 expression and influencing IL-4 secretion (Amsen et al., 2009a,b, 2007; Fang et al., 2007). Therefore, we next investigated whether Notch signaling deregulation could be responsible for the mixed Th1/Th2 response observed in *L. major*-infected *Lgals3*$^{-/-}$mice. As shown in Fig. 2, *L. major*-infected *Lgals3*$^{-/-}$ mice expressed higher Notch-1 receptor mRNA levels both in lymph nodes (Fig. 2A) and footpad lesion (Fig. 2B) at 35 days post-infection. We also detected heightened expression of Notch-1 cell surface protein on purified $CD4^+$ T cells from *L. major*-infected *Lgals3*$^{-/-}$ mice (Fig. 2C), and increased mRNA levels of the Notch-target gene Hes-1 in lymph node cells (Fig. 2D). These results suggest that galectin-3 deficiency results in an increased Notch signaling activity. At later stages of the *L. major* infection, we found that lymph node cells from *Lgals3*$^{-/-}$ mice have higher Jagged1 and Jagged2 mRNA expression than cells from WT mice (Fig. 2E and F). No differences were observed in Delta-like-4 mRNA levels (Fig. 2G). When analyzing footpad lesions we found that *L. major*-infected *Lgals3*$^{-/-}$ mice expressed significant higher mRNA levels of Jagged1 and Delta-like-4 (Fig. 2H and I, respectively) in comparison with WT mice. These results show

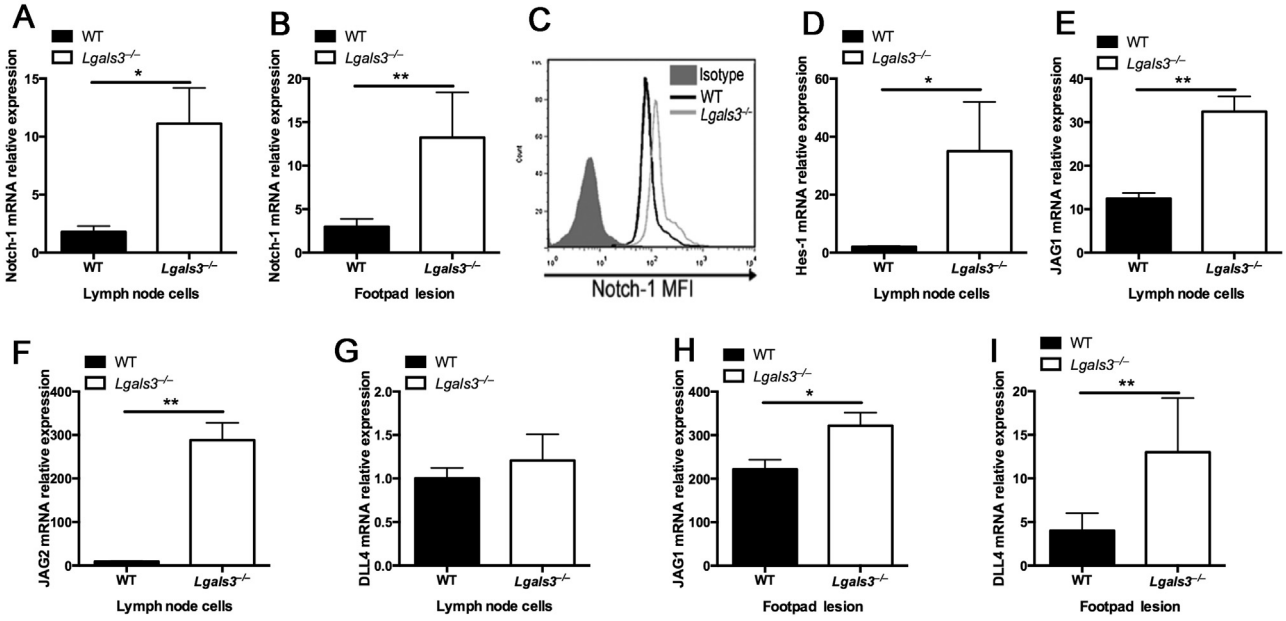

Fig. 2. Enhanced expression of Notch signaling components in *L. major*-infected Lgals3$^{-/-}$ mice. After 35 days post-infection, total RNA from lymph node cells or footpad lesion cells from WT and Lgals3$^{-/-}$ mice were converted into cDNA and analyzed by Real time PCR for Notch-1 (A and B), Hes-1 (D), JAG1 (E and H), JAG2 (F) and DLL4 (G and I) expression (5 mice/group). Alternatively, CD4^{+} T cells were purified from pooled lymph nodes of both genotypes (5 mice/group), labeled intracellularly with and anti-Notch-1 mAb and analyzed by flow cytometry (C). Data are representative of 3 independent experiments $^{*}p < 0.01$, $^{**}p < 0.01$.

that Notch-1 receptor and its ligands Jagged1 and Delta-like-4 are up-regulated during *L. major* infection in *Lgals3*$^{-/-}$ mice, suggesting that an activated Notch signaling pathway accompanies and could be responsible for the mixed Th1/Th2 phenotype observed during *L. major* infection.

3.3. Dendritic cells from Lgals3$^{-/-}$ mice display increased expression of Notch ligands Delta-like-4 and Jagged1

In mice, several Th1-promoting stimuli have been shown to induce Delta-like 4 expression in DCs, whereas Th2-promoting stimuli induce Jagged1 expression on these cells (Amsen et al., 2009a,b). Since the expression of Notch ligands by DCs plays a key role in the generation of Th1 and Th2 responses and we found a higher expression of Jagged1 in CD11c^{+} cells purified from lymph node cells of *Lgals3*$^{-/-}$ mice 35 days post-infection with *L. major* (Supplementary Fig. S2 in the online version at DOI: 10.1016/j.molimm.2016.06.005), we examined the expression of Jagged1 and Delta-like-4 in resting and LPS-stimulated BMDCs from *Lgals3*$^{-/-}$ and WT mice. LPS-treated WT BMDCs presented increased Delta-like-4 mRNA levels in comparison to control (Fig. 3A), whereas no difference was found in Jagged1 mRNA levels (Fig. 3B). However, when analyzing *Lgals3*$^{-/-}$ BMDCs we found that LPS stimulation strongly up-regulated both Jagged1 and Delta-like-4 mRNA expression (Fig. 3A and B). Interestingly, the expression levels of Jagged1 and Delta-like-4 in LPS-treated *Lgals3*$^{-/-}$ BMDCs were higher than in LPS-treated WT BMDCs (Fig. 3A and B), supporting a non-selective activation of Th1 and Th2 responses in *Lgals3*$^{-/-}$-infected mice. We further analyzed the cell surface expression of Jagged1 and Delta-like-4 ligands by flow cytometry in *Lgals3*$^{-/-}$ and WT BMDCs after LPS treatment and, in the presence or absence of exogenously added galectin-3. Similar to our qPCR results, we found that LPS-treated *Lgals3*$^{-/-}$ BMDCs presented a higher cell surface expression of Jagged1 and Delta-like-4 as compared to WT cells (Fig. 3C and D). Interestingly, incubation with recombinant galectin-3 before LPS stimulation, prevented enhanced expression of Jagged1 and Delta-like-4 observed in *Lgals3*$^{-/-}$ DCs (Fig. 3C and D), suggesting that exogenous galectin-3 counteracts the effects of galectin-3 deficiency. Moreover, in WT BMDCs, LPS induced an up-regulation of Delta-like-4 and galectin-3 pretreatment was able to prevent this effect (Fig. 3D). Since the expression pattern of Notch ligands correlates with Th1/Th2 cell polarization, our data suggest that the unbalanced expression of Notch ligands Jagged1 and Delta-like-4 found in *Lgals3*$^{-/-}$ DCs may contribute at least in part to the lack of clear polarization profiles during *L. major* infection.

3.4. BMDCs from Lgals3$^{-/-}$ mice show increased Notch signaling activation and enhanced cytokine production

Since differences in the expression levels of Jagged1 and Delta-like-4 ligands may influence the ability of DCs to induce polarized Th1/Th2 responses, we sought to investigate the status of Notch signaling activation in bone marrow DCs (BMDCs) from *Lgals3*$^{-/-}$ and WT mice and whether it could affect the production of cytokines by these cells. We then evaluated the mRNA expression levels of the Notch-target gene *Hes-1* in *Lgals3*$^{-/-}$ and WT BMDCs stimulated with or without LPS. We found that basal *Hes-1* expression was higher in WT BMDC compared to *Lgals3*$^{-/-}$ BMDC (Figs. 4 A, 5 A). However, treatment with LPS up regulated Hes-1 expression to a greater extent in *Lgals3*$^{-/-}$ BMDCs than in WT DCs (Fig. 4A). In addition, we evaluated the mRNA expression of several cytokines including IL-12p40, IL-6, IL-1ß and IL-10 and the secretion of IL-12p40 and IL-10, in *Lgals3*$^{-/-}$ or WT BMDCs. We found that LPS up regulated IL-12p40, IL-6, IL-1ß and IL-10 mRNA levels both in *Lgals3*$^{-/-}$ and WT BMDCs and, similar to the Notch target gene *Hes-1*, mRNA expression levels of all studied cytokines were higher in *Lgals3*$^{-/-}$ BMDCs as compared to WT BMDCs (Fig. 4B–E). Likewise, we found that LPS increased the secretion of IL-12p40 and IL-10 in BMDCs in comparison to resting cells. Once again, *Lgals3*$^{-/-}$ BMDCs produced higher amounts of IL-12 and IL-10 than their WT counterpart (Fig. 4F and G). These findings suggest that galectin-3 deficiency leads to increased activation of the Notch signaling pathway, and enhanced production of both pro- and anti-inflammatory cytokines.

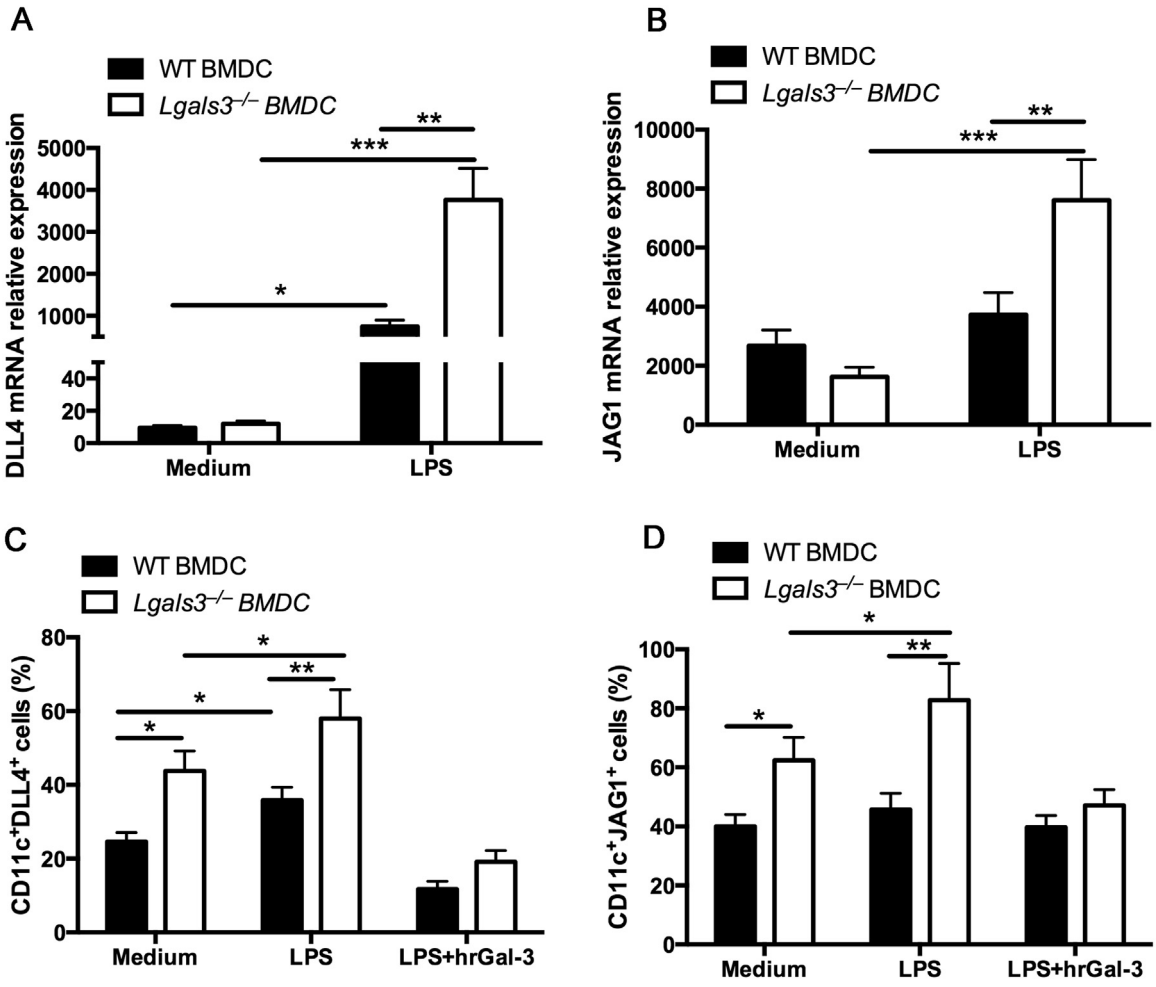

Fig. 3. Increased expression of Notch ligands Delta-like-4 and Jagged1 in BMDCs from *Lgals3*$^{-/-}$ mice. (A–D) Bone marrow-derived DCs were obtained from uninfected WT and *Lgals3*$^{-/-}$ mice. The total bone marrow cells (pooled from 3 mice/group) were cultured with GM-CSF plus IL-4 and stimulated with LPS, in presence or absence of hrGal-3 (30 μg/μL), as indicated. Relative quantification of DLL4 (A) and JAG1 (B) mRNAs was performed by Real Time PCR. (C–D) CD11c^{+} BMDCs from WT and *Lgals3*$^{-/-}$ mice were analyzed for DLL4 (C) or JAG1 (D) ligand expression by flow cytometry, and the percentage of double positive cells (CD11c^{+}DLL4^{+} or CD11c^{+}JAG1^{+}) is shown. Data are representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

3.5. *BMDCs from Lgals3*$^{-/-}$ *mice are more sensitive to Jagged1 stimulation*

To verify the functional activity of Notch receptors in BMDCs, we initially evaluated the expression of Notch-responsive genes such as *Hes-1* and *Hey-1* in *Lgals3*$^{-/-}$ or WT BMDCs cultured for 20 h on Delta-like-4 or Jagged1-coated culture plates. As seen in Fig. 5A, WT BMDCs in control conditions expressed higher mRNA levels of Hes-1 than *Lgals3*$^{-/-}$ BMDCs. Delta-like-4 ligand induced an increase of *Hes-1* mRNA expression in WT BMDCs, whereas no significant change was observed in *Lgals3*$^{-/-}$ BMDCs when compared to control conditions. In both situations (medium and Delta-like-4 stimulated), WT BMDCs presented higher levels of *Hes-1* mRNA compared to *Lgals3*$^{-/-}$ BMDCs. Surprisingly, in the presence of Jagged1, *Hes-1* was significantly up-regulated in *Lgals3*$^{-/-}$ BMDCs while no changes were observed in WT BMDCs in comparison to control conditions. When analyzing *Hey-1* expression we found that no changes on its mRNA levels upon stimulation with Delta-like-4 or Jagged1 ligands in WT BMDCs (Fig. 5B). On the contrary, the expression of Hey-1 in *Lgals3*$^{-/-}$ BMDCs was down-regulated upon stimulation with Delta-like-4 and significantly up-regulated after Jagged1 stimulus. On the other hand, after Jagged1 stimulus, *Lgals3*$^{-/-}$ BMDCs showed significant increased levels of *Hey-1* mRNA than WT BMDCs. These results indicate that *Lgals3*$^{-/-}$ and WT BMDCs exhibit a differential susceptibility to each Notch ligands being *Lgals3*$^{-/-}$ cells highly sensitive to Jagged1-triggered signals. We then cultured *Lgals3*$^{-/-}$ or WT derived-BMDCs for 6 h in the presence of immobilized Delta-like-4 or Jagged1 proteins and measured the protein levels of Notch-1 intracellular domain (NICD1) by Western blot. We found that Delta-like-4 was more prone to activate Notch signaling in WT BMDCs than Jagged1 ligand in WT BMDCs (Fig. 5C and D). On the other hand, in *Lgals3*$^{-/-}$ BMDCs we observed that both Delta-like-4 and Jagged1 were able to induce Notch-1 cleavage at the same extent. Interestingly, upon Jagged1 stimulation, NICD1 levels were increased in *Lgals3*$^{-/-}$ BMDCs in comparison to WT BMDCs (Fig. 5C and D). These data indicate that in the absence of galectin-3, stimulation by Jagged1/Notch signaling activation is preferred relative to Delta-like-4/Notch.

3.6. *Ligand-induced Notch signaling in Lgals3*$^{-/-}$ *BMDCS upregulates Jagged1, Delta-like-4 and IL-6 mRNA levels*

It has been previously reported that Notch signaling positively regulates IL-6 expression in macrophages and Notch-1 interacts with the IL-6 promoter in RAW264.7 cells activated by LPS/IFN-γ (Wongchana and Palaga, 2012). Therefore, we analyzed the mRNA levels of the Notch target gene IL-6 in the presence of different concentrations of the ligands Jagged1 and Delta-like-4. We

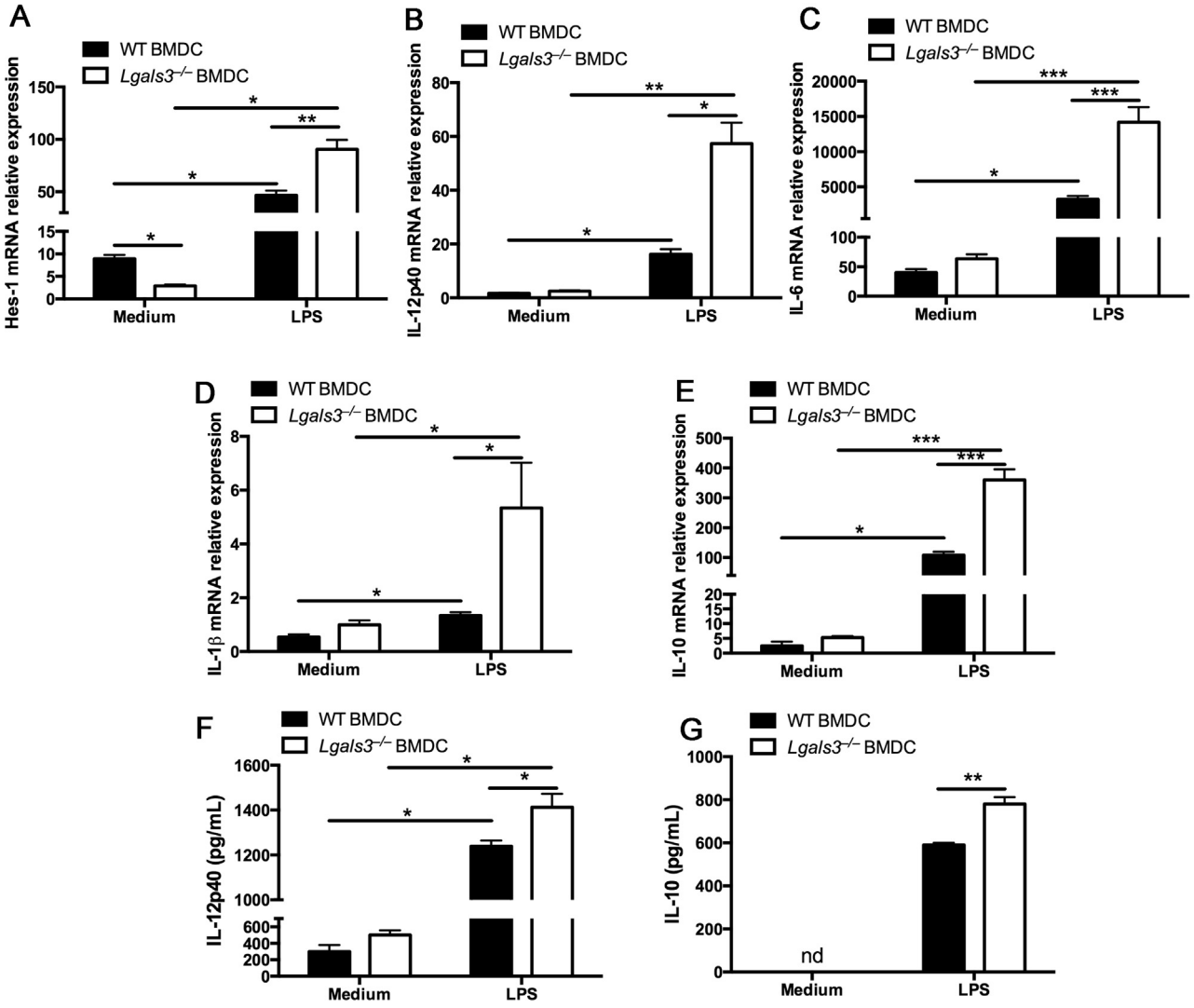

Fig. 4. BMDCs from Lgals3$^{-/-}$ mice show increased Notch signaling activation and cytokine production. Bone marrow-derived dendritic cells were obtained from uninfected WT and *Lgals3*$^{-/-}$ mice as described in Material and Methods section and stimulated with 1 μg/mL *E. coli* LPS for 24 h. Cells were then collected and the total mRNA was converted into cDNA. The relative mRNA levels of Hes-1 (A), IL-12p40 (B), IL-6 (C), IL-1β (D) and IL-10 (E) were determined by Real time PCR, using β-actin as endogenous control. Data represent the average of 3 experiments performed in triplicates each. Alternatively, the supernatants of BMDCs stimulated with LPS or cultured with medium alone were harvested and the levels of IL-12p40 (F) and IL-10 (G) were measured by ELISA. nd = non-detectable levels. $^{*}p < 0.01$, $^{**}p < 0.01$, $^{***}p < 0.001$.

observed that Delta-like-4 (0.25, 0.5 and 2 μg/mL) stimulation up-regulated IL-6 mRNA levels in both *Lgals3*$^{-/-}$ and WT BMDCs in comparison to the control medium, showing a 'bell-shaped' dose-response curve (Fig. 6A). However, *Lgals3*$^{-/-}$ BMDCs were much more sensitive to Delta-like-4 stimulation than WT BMDCs (Fig. 6A). Interestingly, IL-6 mRNA levels were also up-regulated in a concentration-dependent manner with 0.25, 0.5 and 2 μg/mL of immobilized Jagged1 in *Lgals3*$^{-/-}$ BMDCs as compared to control medium (Fig. 6B). On the other hand, WT BMDCs were only able to up-regulate IL-6 with the highest concentration of immobilized Jagged1 (2 μg/mL). Thus, galectin-3 deficiency lowers the threshold of Notch signaling.

Since Delta-like-4/Notch signaling induces the expression of Delta-like 4 itself (Sainson et al., 2005), we also checked the mRNA levels of Jagged1 and Delta-like-4 after ligands stimulation. We observed that in the presence of the immobilized Jagged1 the mRNA levels of Jagged1 (Fig. 6C) and Delta-like-4 (Fig. 6D) were increased in *Lgals3*$^{-/-}$ BMDCs, although no significant changes were found in WT BMDCs in comparison to control medium. However, after Delta-like-4 stimulation, we found increased levels of Jagged1 and Delta-like-4 mRNA in *Lgals3*$^{-/-}$ BMDCs. On the other hand, a decrease in Jagged1 and no changes in Delta-like-4 mRNA levels were observed in WT BMDCs as compared to control medium (Fig. 6C and D). With both Jagged1 and Delta-like-4, the levels of Jagged1 and Delta-like-4 mRNA were always higher in *Lgals3*$^{-/-}$ BMDCs than in WT BMDCs. These results support our previous observations indicating that in the absence of galectin-3, DCs are more prone to be activated by Notch ligands than WT DCs, being particularly susceptible to Jagged1.

3.7. Galectin-3 controls Notch activation in BMDCs

Galectin-3 is secreted by macrophages (Sato and Hughes, 1994), dendritic cells (Van Stijn et al., 2009) and is highly expressed in footpad tissues at the infection sites of *Leishmania major* (Fermino et al., 2013). Therefore, we aimed to study whether extracellular galectin-3 found at the infection site could be able to modulate Notch signaling activation and cytokine production in BMDCs. For this purpose, *Lgals3*$^{-/-}$ and WT BMDCs were treated with 30 μg of recombinant| human galectin-3 in the presence of immobilized

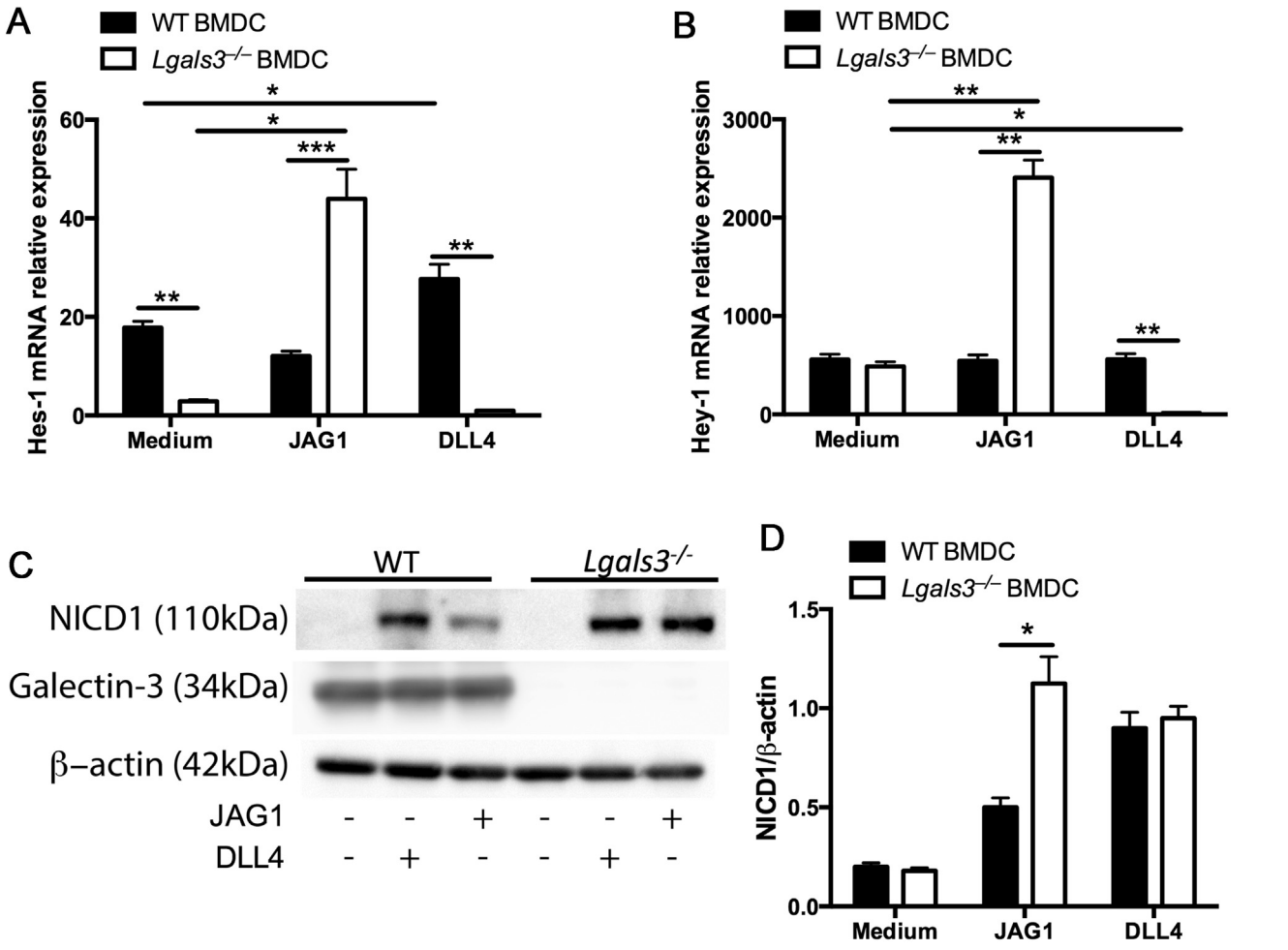

Fig. 5. BMDCs from Lgals3$^{-/-}$ mice are more sensitive to Jagged1 stimulation. BMDCs were generated from WT and *Lgals3*$^{-/-}$ cells and stimulated with immobilized JAG1 (1 μg/mL), DLL4 (1 μg/mL) or medium alone for 24 h. Following incubation with the corresponding stimuli, cells were collected and analyzed for Hes-1 (A) and Hey-1 (B) relative mRNA expression, using the Real time PCR method. Data are representative of three experiments performed in triplicates. (C) Alternatively, the levels of Notch-1 intracellular domain (NICD1) in JAG1 and DLL4-stimulated BMDCs (6 h of stimulus) were assessed by Western blot and normalized against β-actin. Galectin-3 levels are shown. The relative density of NICD1 was determined by ImageJ software (D). *$p < 0.01$, **$p < 0.01$, ***$p < 0.001$.

Delta-like-4 or Jagged1 ligands (0.5 μg/mL) and the mRNA levels of *Hes-1*, *Hey-1*, IL-10 and IL-6 were evaluated. We found that the addition of exogenous galectin-3 was sufficient to significantly decrease *Hes-1* mRNA levels in WT cells. Moreover, in Delta-like-4-stimulated WT BMDCs galectin-3 also reduced the expression of *Hes-1* mRNA levels (Fig. 7A). Also, the addition of exogenous galectin-3 to *Lgals3*$^{-/-}$ BMDCs led to down-regulation of *Hes-1* mRNA levels induced by Jagged1 stimulation (Fig. 7A).

Regarding the Notch target gene *Hey-1*, the addition of galectin-3 down-regulated *Hey-1* in WT BMDCs when compared to the controls (Fig. 7B). We also observed down-regulation of *Hey-1* mRNA expression in *Lgals3*$^{-/-}$ BMDCs stimulated with Jagged1 in the presence of exogenous galectin-3 (Fig. 7B).

When IL-6 was analyzed, we found that WT BMDCs stimulated with galectin-3 showed increased mRNA expression of this pro-inflammatory cytokine in comparison with control medium. However, WT BMDCs did not show up-regulation of IL-6 mRNA levels after stimulation with Notch ligands (Fig. 7C). Interestingly, *Lgals3*$^{-/-}$ BMDCs up-regulated IL-6 mRNA expression following treatments with galectin-3 and Notch ligands and administration of galectin-3 and Notch ligands together had a synergistic effect on IL-6 mRNA expression (Fig. 7C). Finally, we found that galectin-3 treatment induced down-regulation of IL-10 mRNA in *Lgals3*$^{-/-}$ BMDC stimulated with Delta-like-4 or Jagged1 ligands (Fig. 7D). Addition of exogenous galectin-3 partially rescued dysregulated expression of Notch target genes observed in *Lgals3*$^{-/-}$ BMDCs. Overall our data suggest that galectin-3 released into inflammatory sites during *L. major infection* might play a key role in modulating Notch signaling and downstream events, leading to regulation of T helper cell differentiation.

4. Discussion

More than 20 years ago, immunologists have found that resistance and susceptibility to experimental infection with the intracellular protozoan *L. major* was associated with the selective development of Th1 and Th2-dominated immune responses. Since then, this infectious disease has been widely used as a prototypic model to identify key regulatory mediators, lineage-specific transcription factors, and immunoregulatory cytokines (Mougneau et al., 2011).

Here we demonstrated that in the BALB/c susceptible model of *L. major* infection, *Lgals3*$^{-/-}$ mice display a dysregulated Th1/Th2 cytokine balance and exhibit increased expression of Notch-1 and the ligands Jagged1, Jagged2 and Delta-like-4 ligands. We also showed that BMDCs from *Lgals3*$^{-/-}$ mice are more sensitive to Jagged1 stimulation in terms of Notch signaling activation and

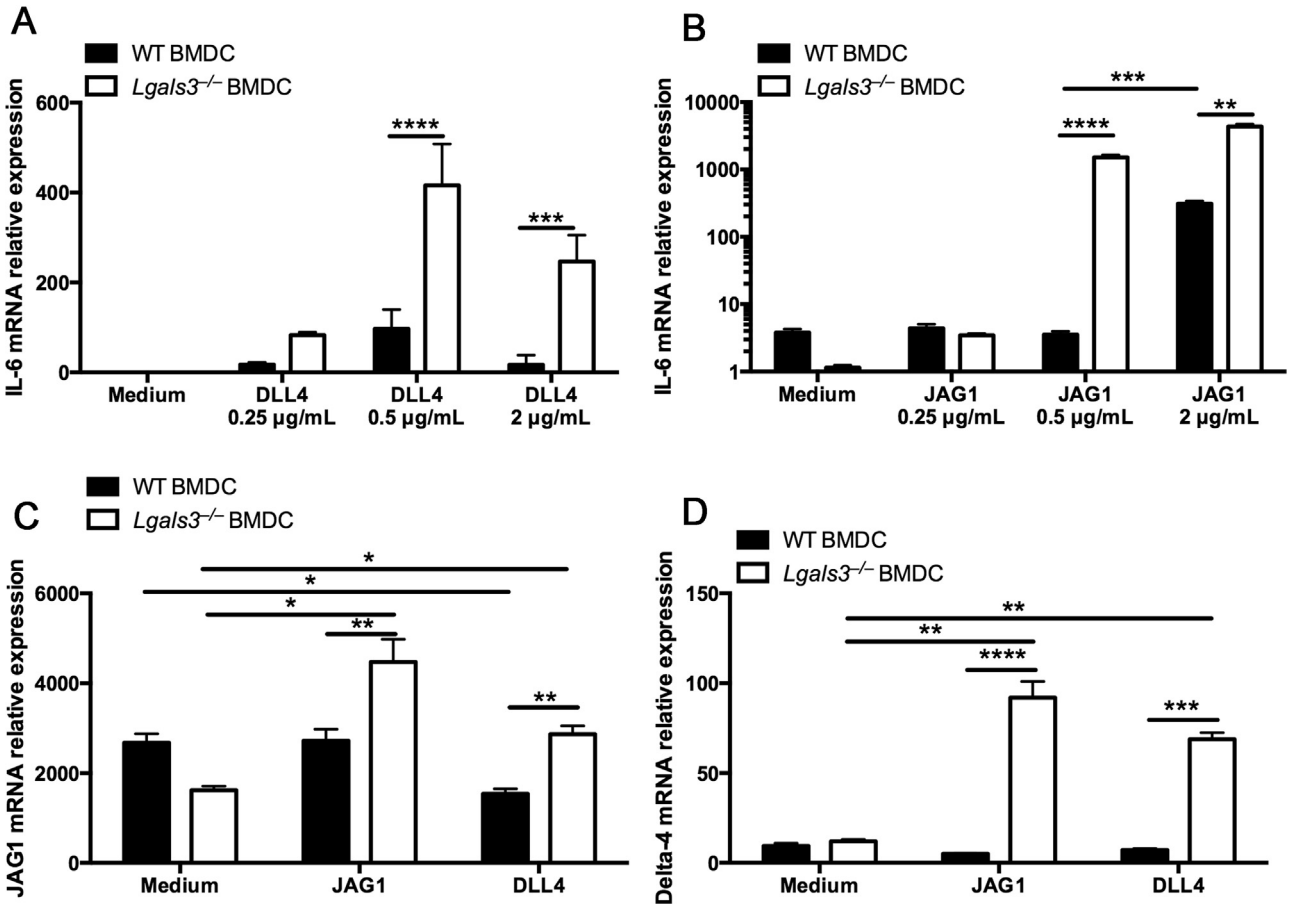

Fig. 6. Up-regulation of Jagged1, Delta-like-4 and IL-6 mRNA levels in Lgals3$^{-/-}$ BMDCS induced by Notch ligands. (A–B) BMDCs from WT and *Lgals3*$^{-/-}$ mice were seeded in 96-well plates previously coated with DLL4 or JAG1 or only medium, at the indicated concentrations. After 24 h, BMDCs stimulated with DLL4 (A) and JAG1 (B) were harvested and analyzed for IL-6 relative expression by Real time PCR. (C–D) BMDCs from *Lgals3*$^{-/-}$ and WT mice were stimulated with immobilized 1 μg/mL JAG1 or DLL4 and the relative expression of JAG1 (C) and DLL4 (D) mRNAs was determined by Real time PCR. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0005$, ****$p < 0.0001$.

cytokine production, a condition that was prevented when exogenous galectin-3 was added prior to stimulation.

Galectin-3 has been described as an important regulator of immune and inflammatory responses (Henderson and Sethi, 2009; Liu and Rabinovich, 2010). However, given its multiple and partially overlapping functions, it has proven challenging to demonstrate the precise mechanism underlying the pro- or anti-inflammatory roles of galectin-3 *in vivo*. Previous studies in galectin-3-deficient mice have revealed a central role for galectin-3 as a negative regulator of adaptive immune responses. It has been suggested that galectin-3 regulates both Th1 and Th2 response as *Lgals3*$^{-/-}$ mice developed lower Th2 responses and higher Th1 responses in a murine model of asthma (Zuberi et al., 2004). Moreover, and in contrast to galectin-1, galectin-3 binds equally to both Th1- and Th2-differentiated subsets, thus substantiating the lack of preferences of this endogenous lectin for polarized T helper cell populations (Toscano et al., 2007). This effect could be related to glycan-binding preferences of galectin-3 over galectin-1 with respects to recognition of α2,6-linked terminal sialic acid (Hirabayashi et al., 2002).

In previous studies, we have reported that galectin-3 influences the interface of innate and adaptive immunity by suppressing IL-12 production by DCs, driving the development of Th1-type responses upon *Toxoplasma gondii* infection (Bernardes et al., 2006). In addition, during the early course of *R. equi* infection *Lgals3*$^{-/-}$ mice (C57BL/6 background) are much more resistant to *R. equi* infection and this resistance was associated with higher production of cytokines by *Lgals3*$^{-/-}$ macrophages. These results suggested that galectin-3 may regulate innate immune responses by diminishing IL-1β production by macrophages (Ferraz et al., 2008). Moreover, in a *P. brasiliensis* murine model of infection, we found that *Lgals3*$^{-/-}$ mice (C57BL/6 background) displayed increased susceptibility to fungal infection, associated with the inability of these mice to mount an adequate inflammatory response, impaired DTH responses, high serum levels of specific antibodies, and development of Th2-polarized immune responses (Ruas et al., 2009).

In the present study we found an increased mixed Th1/Th2 response in *Lgals3*$^{-/-}$ mice upon *L. major* infection and identified galectin-3 as a molecular regulator of the Jagged1/Notch-1 signaling pathway. The Notch signaling pathway is well known for its role in binary cell fate decisions (Bray, 2006). In some settings, Notch regulates such decisions by a lateral inhibition mechanism, in which adoption of a primary fate is inhibited by Notch signaling, allowing cells to differentiate into a secondary fate by default. In other settings, Notch acts by actively promoting expression of lineage-differentiation genes (Bray, 2006). Its role in driving T-cell differentiation has emerged and it is well established that the Notch signaling pathway plays an important role in Th1/Th2 polarization (Amsen et al., 2009a,b). T-bet and GATA-3 are transcription factors that regulate the differentiation of naïve CD4^{+} T cells into Th1 and Th2 lineage, respectively (Mullen et al., 2001; Zheng and Flavell, 1997), and Notch can drive Th2 differentiation through direct transactivation of the GATA-3 gene (Amsen et al., 2009a,b). Although Notch signaling has been extensively implicated in cell-fate selection through the development of the immune system, less

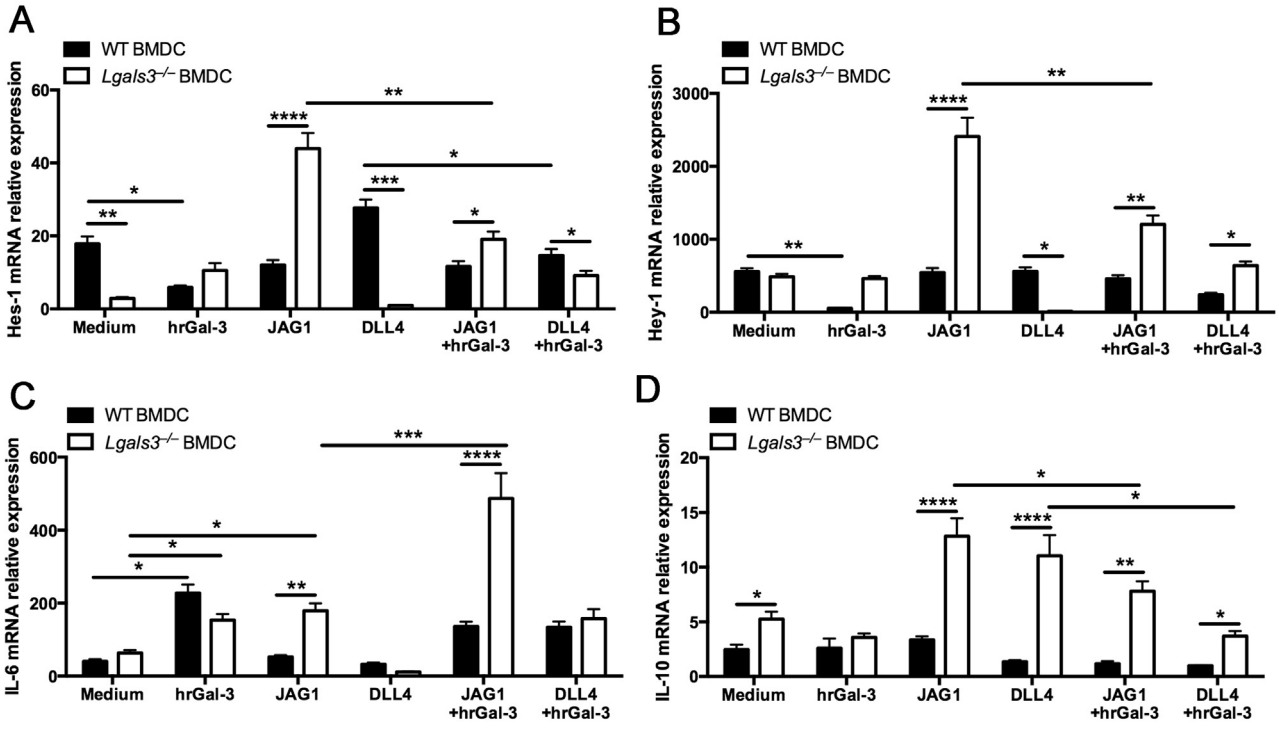

Fig. 7. Human recombinant Galectin-3 down regulates ligand-induced Notch signaling in BMDCs. Bone marrow-derived DCs were obtained from uninfected WT and *Lgals3*$^{-/-}$ mice and stimulated with immobilized 1 μg/mL of JAG1 or DLL4, in presence or absence of soluble hrGal-3 (30 μg/mL), for 24 h. Then, cells were harvested and analyzed for mRNA expression of Hes-1 (A), Hey-1 (B), IL-6 (C) and IL-10 (D) by Real time PCR. Results are representative of 3 independent assays performed in triplicates. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0005$, ****$p < 0.0001$.

attention has been paid to the function of Notch receptors in mature immune cells expressing high levels of those proteins. Skewing of CD4$^+$ T cells into different lineages depends on signals endorsed by antigen-presenting cells (APCs), which translate information about pathogen or inflammatory threats to T cells. Notch ligands act as signaling molecules responsible of conveying this information into T helper cytokine responses. In this regard, Notch ligands function as instructive signals that promote divergent effects during T helper cell differentiation. While Delta-like ligand promotes Th1 responses, Jagged ligand instructs naïve CD4$^+$ T cells to differentiate into the Th2 lineage (Amsen et al., 2004). We found, in the *L. major* experimental model, that expression of Notch-1 as well as Delta-like 4 and Jagged ligands in lymph node and/or foodpad lesion cells is increased in the absence of galectin-3, up-regulating both Th1 and Th2 mediators and transcription factors. Therefore, galectin-3 deficiency favors non-selective up-regulation of Th1 and Th2 cytokines in response to *L. major* infection, suggesting that this endogenous lectin may contribute to limit exacerbated T cell responses irrespective of their cytokine profile. Moreover, dysregulation of immune response exhibited by *Lgals3*$^{-/-}$ mice may partially explain the increased susceptibility of *Lgals3*$^{-/-}$ mice to *L. major* infection (Fermino et al., 2013).

The crosstalk between galectin-3 and the Notch signaling pathway on Th1/Th2 responses is also evidenced by its influence in BMDC biology. We showed that BMDCs from naïve *Lgals3*$^{-/-}$ mice displayed a mixed and enhanced expression of Jagged1 and Delta-like-4 mRNAs, and upon activation with LPS, an up-regulation of the Notch target gene Hes-1 was also observed in *Lgals3*$^{-/-}$ BMDCs. This phenomenon was accompanied by heightened expression of proinflammatory cytokines, which together corroborate the hypothesis that galectin-3 down-modulates expression of Notch ligands as well as Th1-inducing cytokines on DCs. These effects may contribute to T helper cell polarization during infection and could probably affect the clearance of the intracellular parasites.

In our study, we also found that activation of Notch signaling pathway in BMDCs with immobilized Jagged1 ligand is more prominent in *Lgals3*$^{-/-}$ cells compared to WT cells, and the expression of IL-6 was also augmented in Gal-3-deficient cells. It is well known that Notch activation requires that Jagged1/DLL4 ligands expressed on a 'signal-provider' cell could interact with Notch receptor on neighboring 'signal-receiving' cells (trans-interaction) (Cordle et al., 2008). The EGF repeats of mammalian Notch1 carry not only O-fucose and O-glucose glycans but also present complex N-glycans that could serve as ligands for galectin-3 (Stanley and Okajima, 2010). On the other hand, both Jagged1 and DLL4 ligands can be modified by O- and N-linked glycans (Panin et al., 2002; D'Souza et al., 2008). Therefore, we hypothesize that galectin-3 may contribute to bridge Notch receptor with specific ligands, thereby modulating Notch signaling. In fact, unpublished data from our laboratory demonstrate that galectin-3 preferentially binds to Jagged1 (Santos et. al., unpublished data). These observations led us to propose that soluble galectin-3 binds to Jagged1 ligands present on DCs (and possibly other APCs) and restrict Notch signaling activation in those cells, thus inhibiting the production of Th2-driving cytokines. More than fifteen years ago, Demetriou et al. (2001) demonstrated that galectin-3 directly restrains initiation of TCR signaling and T cell activation by forming multivalent complexes with glycans present on the TCR. Similar to interactions between galectin-3 and the TCR, it is possible that this endogenous lectin may form "lattices" through binding to specific glycans on Notch receptors/ligands to attenuate or deactivate Notch signaling activation. The hypothesis of interaction between galectin-3 and Notch signaling components is illustrated in Fig. 8. It should be emphasized that soluble galectin-3 is found at high concentrations in infection/inflammation sites and is secreted by different immune

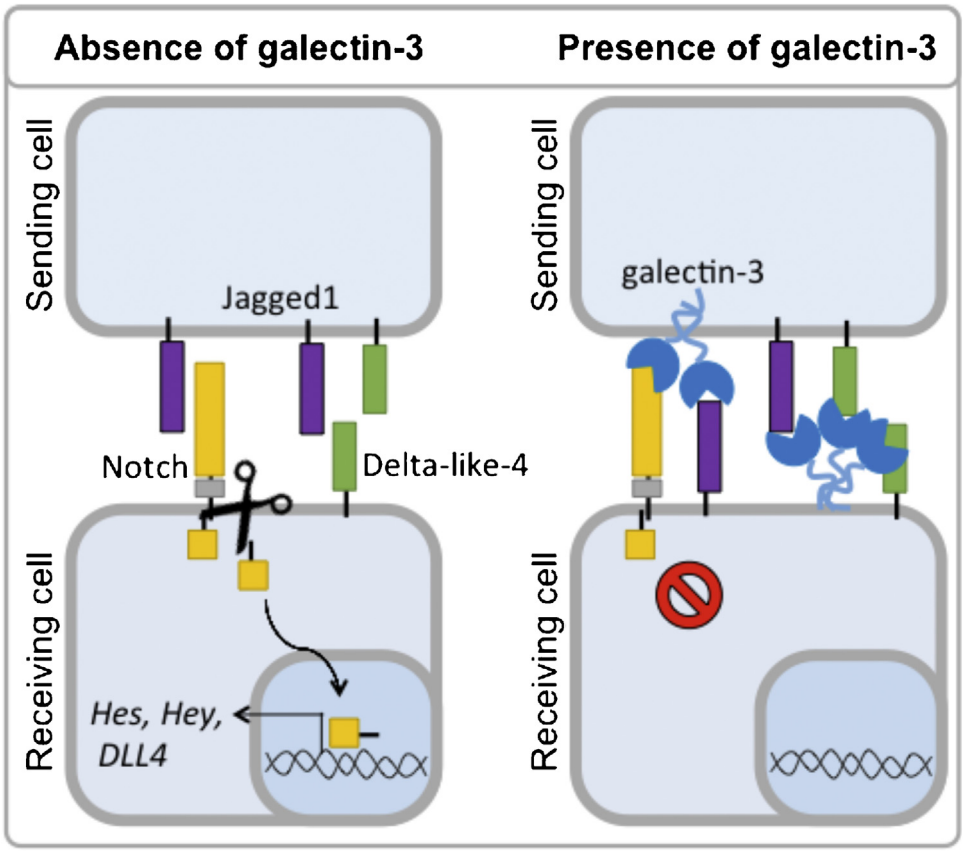

Fig. 8. Hypothesis model of Notch signaling modulation by galectin-3. (A) Notch-1 expressed in target cells is activated by Jagged1 or Delta-like-4 ligands present in the transmiting (effector) cell. NCDI is cleaved and translocated to the nucleus, where it triggers the transcription of Notch target genes, ultimately leading to Th1/Th2 polarization. (B) Because Notch-1 and Delta-like-4/Jagged1 ligands are glycoproteins, extracellular galectin-3 may interact with the Notch machinery. When present at high concentration (infection sites), galectin-3 creates a rigid lattice that could restrain the movement of glycoproteins, thus impairing ligand-receptor interaction and Notch signaling activation. As a result, Th1/Th2 responses are suppressed.

cells (Liu and Rabinovich, 2005). In fact, in our study, pre-treatment of naïve *Lgals3*$^{-/-}$ BMDCs with soluble recombinant galectin-3, followed by Jagged1 stimulus, prevented increased expression of IL-6.

On the other hand, *Lgals3*$^{-/-}$ BMDCs showed to be less efficient in responding to Delta-like-4 activation than WT BMDCs. Since Delta-like-4/Notch activation in APCs is associated with Th1 responses, it is possible that binding of galectin-3 to Delta-like-4 potentiates the activation of Notch signaling pathway, a situation that could be critical during Th1-related infections. It is also possible that the selective binding of galectin-3 to Notch ligands, depending on the infection context, represents a universal mechanism by which this lectin fine tunes adaptive immune responses, setting the threshold of Notch signaling activation and tailoring anti-microbial immunity.

The BALB/c mice model used in this study, which is particularly sensitive to *Leishmania major* infection, may reflect a particular set of patients with higher disease severity. Indeed, while pharmacological therapy can increase the therapeutic response to Leishmaniasis, host immunity is essential to resolve the infection. In fact, the host ability to mount a *L. major*-specific Th1 response is necessary to control parasite multiplication and dissemination. It is known that Notch receptor and ligands are increased in the cutaneous and mucosal lesions of patients with Leishmaniasis and, are associated with poor response to therapy (Rodrigues et al., 2001). Moreover, Notch signaling is required for a proper Th1 immune response following *L. major* infection (Utsunomiya et al., 2015; Auderset et al., 2012). Therefore, understanding the mechanisms by which galectin-3 regulates Notch signaling activation may contribute to the development of new immunotherapeutic targets in *L. major* infection.

5. Conclusions

In summary, our findings provide the first evidence of a role for endogenous galectin-3 in modulation of T helper responses during *L. major* infection and shed light on potential downstream mechanisms, such as Notch signaling, underlying the immunoregulatory activity of this glycan-binding protein *in vivo*.

Conflict of interests

Authors declare that they have no conflict of interest.

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