Jagged–Notch-mediated divergence of immune cell crosstalk maintains the anti-inflammatory response in visceral leishmaniasis

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ABSTRACT
Notch signaling governs crucial aspects of intercellular communication spanning antigen-presenting cells and T-cells. In this study, we investigate how Leishmania donovani takes advantage of this pathway to quell host immune responses. We report induction of the Notch ligand Jagged1 in L. donovani-infected bone marrow macrophages (BMMs) and subsequent activation of RBPJκ (also known as RBPJ) in T cells, which in turn upregulates the transcription factor GATA3. Activated RBPJκ also associates with the histone acetyltransferase p300 (also known as EP300), which binds with the Bcl2L12 promoter and enhances its expression. Interaction of Bcl2L12 with GATA3 in CD4+ T cells facilitates its binding to the interleukin (IL)-10 and IL-4 promoters, thereby increasing the secretion of these cytokines. Silencing Jagged1 hindered these events in a BMMs–T cell co-culture system. Upon further scrutiny, we found that parasite lipophosphoglycan (LPG) induces the host phosphoinositide 3-kinase (PI3K)/Akt pathway, which activates β-catenin and Egr1, the two transcription factors responsible for driving Jagged1 expression. In vivo morpholino-silencing of Jagged1 suppresses anti-inflammatory cytokine responses and reduces organ parasite burden in L. donovani-infected Balb/c mice, suggesting that L. donovani-infused host Jagged1–Notch signaling skew macrophage–T cell crosstalk into disease-promoting Th2 mode in experimental visceral leishmaniasis.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Leishmania donovani, Bone marrow macrophages, CD4+ T cells, Jagged1–Notch signaling, Anti-inflammatory cytokines

INTRODUCTION
Resolution of leishmanial infection relies on coordinated interactions between components of innate and adaptive immunity (Tripathi et al., 2007). Host defense against leishmanial infection is critically dependent upon the presence of functional crosstalk between macrophages and T cells. T cell polarization toward a Th1 or Th2 mode plays a key role in driving disease outcome towards either disease progression or resolution of leishmaniasis (Rosas et al., 2005; Tripathi et al., 2007). Notch signaling is one of the many immune signaling pathways known to skew T cell polarization, depending upon the availability of the ligand. The most well-studied functions of Notch signaling linked with immunity is the regulation of B and T cell lineage differentiation (Tangigaki and Honjo, 2007) and T cell activation (Eagar et al., 2004). Canonical Notch-dependent signaling begins with the binding of extracellular domain of one of the Notch receptors [for instance, exposure of CD4+ cells to Dll1 induces T cell differentiation toward Th1 (Maekawa et al., 2003) mode. On the other hand, stimulation with Jagged1 induces interleukin (IL)-4 secretion, thereby directing Th2 cell differentiation (Amsen et al., 2004). Interplay between these ligands not only determines the host T cell immune response to either defense or susceptibility towards infection but is also crucial for determining the fate of T cells. Several pathogens are known to exploit Notch signaling by up regulating different Notch ligands for their survival. For instance, DL4, the principal Notch ligand, is preferentially overexpressed in dendritic cells (DCs) during mycobacterial infection. Functional blocking of Dl4 in vivo during mycobacteria-induced pulmonary granuloma formation leads to a significant inhibition in Th17 cellular responses, and observation of larger granulomas (Ito et al., 2009). Moreover, treatment with anti-Dll4 antibody seems to block IL-17 production by CD4+ T cells while overexpression of Dll4 enhances IL-17 secretion, implying that Dll4 has a significant role in inducing Th17 activity during a mycobacterial challenge (Ito et al., 2009). A recent study showed that macrophages, although not DCs, upregulated expression of Notch ligand Dll1 in response to stimulation with influenza virus (Ito et al., 2011). Furthermore, targeted neutralization of Dll1 in response to influenza virus challenge resulted in increased viral clearance. Similar to this, CD4+ T cells stimulation with Dll1-Fc seemed to induce Th1 differentiation during Leishmania major infection (Maekawa et al., 2003). It was also seen that redundant Notch1 and Notch2 signaling is capable of activating the Th1 response toward L. major infection (Auderset et al., 2012). Progressive visceral leishmaniasis (VL) infection is typically associated with skewing towards a disease-conducive Th2 phenotype that suppresses the host-defensive Th1 phenotype (Auderset et al., 2012; Maekawa et al., 2003). However, whether Leishmania donovani exploits Jagged–Notch signaling to direct macrophage–T cell crosstalk towards induction of Th2 responses is completely unknown. Moreover the intracellular
mechanisms that facilitate the induction of Jagged–Notch receptor–ligand expression, interaction as well as effector cytokine responses during experimental VL are yet to be explored. Therefore, in the current study, we investigate whether \textit{L. donovani} modulates host Jagged–Notch signaling in order to promote Th2 responses during macrophage–T cell crosstalk in experimental VL.

**RESULTS**

\textit{L. donovani} induces an anti-inflammatory cytokine response in T cells through Jagged1-ligand mediated GATA3 expression

Disease resolution in leishmaniasis relies upon dynamic interactions between various members of the innate and adaptive immunity; activation of targeted macrophages and T cell populations are very important for appropriate cytokine production and thereby killing of the parasite. Recent evidence suggests that Notch signaling, which is activated upon interaction of ligand–receptor between immune cells, serves to modulate T cell-mediated immunity (Ito et al., 2011; Okamoto et al., 2009). Since Notch ligands are present on antigen-presenting cells, which bind to appropriate receptors featured on neighboring T cells to initiate signaling, we initially hypothesized that these ligands may be exploited by \textit{L. donovani} to modulate macrophage–T cell interaction towards Th2 mode for its own survival. To confirm these, we first carried out expression profiling of all the Notch ligands (Jagged and Delta isoforms) in isolated bone marrow macrophages (BMM\textsubscript{s}) following \textit{L. donovani} infection. Real-time PCR analysis revealed increased expression of Jagged1 ligand, among the isoforms, during \textit{L. donovani} infection in

![Fig. 1. \textit{L. donovani} induces Jagged1–Notch signaling during BMM\textsubscript{s}–CD4\textsuperscript{+} T cell co-culture.](image-url)

(A) BMM\textsubscript{s} were infected with \textit{L. donovani} (\textit{L. d}) promastigotes for 0–24 h and real-time PCR was performed with the isolated mRNA to check expression of Jagged1, Jagged2, Delta1 (Dll1), Delta3 (Dll3), and Delta4 (Dll4). The expression changes were calculated relative to uninfected macrophages after normalizing with GAPDH used as endogenous control. (B) BMM\textsubscript{s} were infected with stationary phase \textit{L. donovani} promastigotes for indicated time intervals and expression of Jagged1 was analyzed by immunoblotting. Equal protein loading was ensured through β-actin staining. (B1) Densitometric analyses for the immunoblots are represented as bar graph adjacent to the respective panels. The error bar represents mean±s.d. for biological replicates (\textit{n}=3). **P<0.001, ns, not significant (one-way ANOVA test). (C,D) CD4\textsuperscript{+} T cells were incubated with \textit{L. donovani}-infected (24 h) and uninfected BMM\textsubscript{s} (M\textsubscript{a}s) for respective time periods as indicated in the Materials and Methods. Nuclear and cytosolic extracts were prepared from isolated T cells and translocation of the ICD was analyzed by immunoblotting. (C1,D1) Densitometric analyses for the immunoblots are represented as bar graph adjacent to the respective panels. The error bar represents mean±s.d. for biological replicates (\textit{n}=3). *P<0.05, **P<0.005, ***P<0.0001; ns, not significant (one-way ANOVA test). (E,F) BMM\textsubscript{s} were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with \textit{L. donovani} for 24 h. CD4\textsuperscript{+} T cells were incubated with these infected macrophages for 24 h. Nuclear and cytosolic extracts were prepared from isolated T cells and translocation of ICD was analyzed by immunoblotting. (G) BMM\textsubscript{s} were transfected (24 h) with either control or Jagged1 siRNA followed by infection with \textit{L. donovani} promastigotes for 24 h. The efficacy of Jagged1 siRNA was evaluated by immunoblot analysis. (G1) Densitometric analysis for the immunoblots is represented as bar graph adjacent to the respective panels. The error bar represents mean±s.d. for biological replicates (\textit{n}=3). **P<0.0001 (unpaired two-tailed t-test). Significance is indicated for the control siRNA versus Jagged1 siRNA, (H) BMM\textsubscript{s} were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with \textit{L. donovani} for 24 h. T cells were incubated with these infected macrophages for 24 h. RBPJ\textsubscript{K} was immunoprecipitated from whole-cell lysate and co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with ICD and anti-RBPJ\textsubscript{K} antibody. (I) BMM\textsubscript{s} were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with \textit{L. donovani} for 24 h. T cells were incubated with these infected macrophages for 24 h. RBPJ\textsubscript{K} was immunoprecipitated from whole-cell lysate and co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with anti-ICD and anti-RBPJ\textsubscript{K} antibody. (J,K) Macrophages were transfected with Jagged1 or scrambled control siRNA for 24 h and were infected with \textit{L. donovani} promastigotes (24 h). In a separate set of experiments, T cells were pre-incubated with isotype control or Notch1 antibody (20 µg/ml) for 1 h. T cells were then incubated with infected BMM\textsubscript{s} for 48 h. Level of (J) IL-4 and (K) IL-10 in the culture supernatants were measured using the sandwich ELISA kit. Error bar represents the mean±s.d. of at least two independent experiments performed in triplicate. **P<0.005, ***P<0.0001 (one-way ANOVA test). Significance is indicated for the control versus infected groups. (L) Macrophages were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by \textit{L. donovani} infection for 24 h. Infected macrophages were co-cultured with CD4\textsuperscript{+} T cells for 24 h. Numbers of surviving intra-macrophagic amastigotes were counted by Giemsa staining. Error bar represents the mean±s.d. of at least two independent experiments performed in triplicate. **P<0.0005 (unpaired two-tailed t-test). Significance is indicated for the control siRNA versus Jagged1 siRNA,
BMMds, with highest level of induction noted at 12 h (6.1-fold) (Fig. 1A). Similar Jagged1 induction was also noted for protein levels (Fig. 1B) as studied by immunoblot analysis. Densitometric analysis revealed a clear and robust induction of Jagged1 in infected macrophages at 8- and 16-h post infection (Fig. 1B1).

Amongst the four types of Notch receptors, N1 and N2 are selectively expressed in T cells upon activation, with N1 playing the major role in effector T cells. Upon ligand binding, the intracellular domain (ICD) of N1 gets proteolytically cleaved and migrates to the nucleus, where it serves as a transcriptional activator (Amsen et al., 2004). Therefore, we next assessed the nuclear migration of N1 ICD domain in T cells incubated with L. donovani-infected macrophages. Immunoblot analysis suggested a gradual disappearance of N1 ICD from the cytosol with its significant enrichment in nuclear fractions of T cells incubated with infected macrophages whereas no nuclear enrichment of N1 ICD was observed in T cells incubated with control macrophages (Fig. 1C,D). This was also confirmed by densitometric analysis of the respective blot, where a significant reduction in N1 ICD was observed in the cytosol of T cells incubated with L. donovani-infected macrophages at 24 h (Fig. 1C1) with a concomitant increase in the nucleus (Fig. 1D1). We next assessed whether silencing of Jagged1 in L. donovani-infected-BMMds could inhibit the nuclear migration of N1 ICD in T cells. For this, we transfected the macrophages with siRNA against Jagged1 for 24 h and then infected them with L. donovani parasite. These siRNA-treated infected macrophages were then co-cultured with CD4+ T cells and infected them with L. donovani-infected macrophages (Fig. 1E,F). The specificity and efficacy of Jagged1 siRNA were determined by immunoblotting (Fig. 1G). Jagged1 expression was found to be significantly reduced (>70%) in infected macrophages upon treatment with Jagged1 siRNA when compared with control siRNA (Fig. 1G1).

The N1 ICD translocates to the nucleus and activates several different regulatory proteins, including DNA-binding protein RBPJκ (also known as RBPJ). RBPJκ serves as a transcriptional repressor by physically interacting with corepressor SMRT (also known as NCOR2), and the assembly of SMRT–RBPJκ complex is inhibited by the Notch ICD, which replaces SMRT and binds to RBPJκ, leading to its activation. RBPJκ activation can modulate expression of different genes (Jung et al., 2013). Therefore, we first determined whether L. donovani infection can induce activation of RBPJκ in co-cultured T cells by evaluating the association of RBPJκ with the N1 ICD by performing a co-immunoprecipitation assay. Our results showed a time-dependent increase in association of N1 ICD with RBPJκ in T cells incubated with L. donovani-infected BMMds, suggesting its activation (Fig. 1H). To this end, we also assessed whether Jagged1 silencing in macrophages could reduce the association of migrating N1 ICD and RBPJκ in T cells incubated with L. donovani-infected BMMds. We observed that silencing of Jagged1 in macrophages reduced association of N1 ICD and RBPJκ in co-cultured T cells, which was intact in T cells incubated with control siRNA treated-infected macrophages (Fig. 1I). Next, to identify the functional role of Jagged1–Notch1 interaction in promoting Th2 response during macrophage–T cell interaction, we performed knockdown of Jagged1 by siRNA in BMMds and functional blocking of Notch1 in T cells with a respective antibody. We observed that Jagged1 siRNA-treated L. donovani-infected macrophages had a significantly lower level of the IL-10 and IL-4 cytokines (63.1% and 61.7% inhibition, respectively, compared with control siRNA-treated cells) in the co-cultured T cells. Similarly, functional blocking of Notch1 in T cells markedly attenuated both IL-10 and IL-4 cytokines level in the T cells co-cultured with infected BMMds (62.5 and 58.2% inhibition, respectively, compared with control IgG-treated cells, Fig. 1J,K). These results suggest the role of upregulated Jagged1 in L. donovani-infected macrophages is in governing the T cell specific anti-inflammatory immune response. Inhibitions of CD4+ T cell differentiation to Th2 mode populations are associated with effective clearance of parasite from infected macrophages. As knockdown of Jagged1 markedly reduced disease progressive Th2 cytokine responses, we next assessed parasite multiplication in Jagged1-silenced L. donovani-infected macrophages co-cultured with T cell by Giemsa staining. Our results demonstrated that amastigote multiplication was significantly attenuated (64.3% decrease) in Jagged1-knockdown infected macrophages during the co-culture experiment with T cells (Fig. 1L). These results suggest the possible role of Jagged1 in providing a favorable environment for the parasite by inducing a Th2 response, which may help in successful propagation of the parasite.

**L. donovani induces GATA3 and Bcl2L12 expression in T cells incubated with infected BMMds**

The binding of classical transcription factor GATA3 to the promoter regions of Th2 cytokines (IL-10 and IL-4) is enhanced by Bcl2L12, which is crucial for Th2 commitment during CD4+ T cell differentiation (Yagi et al., 2011). This indicates that L. donovani might exploit these two transcriptional regulators in CD4+ T cells to switch immune response towards Th2 mode and to verify that, we assessed the functional significance of GATA3 and Bcl2L12 in IL-10 and IL-4 cytokine synthesis in T cells during infection. For that, siRNA-mediated silencing of GATA3 and Bcl2L12 in T cells was performed followed by incubation with L. donovani-infected BMMds. Silencing of GATA3 and Bcl2L12 reduced the secretion of IL-10 and IL-4 cytokine in T cells when incubated with infected macrophages; however, the secretion was drastically reduced when both the genes were silenced together (Fig. 2A,B). So, we next determined the expression level of GATA3 and Bcl2L12 in T cells co-cultured with L. donovani-infected macrophages. We used the in vitro co-culture system as described in Fig. 1 to determine GATA3 and Bcl2L12 expression in T cells, and observed that both GATA3 and Bcl2L12 mRNA expression was significantly induced in a time-dependent manner with maximum induction observed at 36 h post co-incubation (Fig. 2C). Similar induction of GATA3 and Bcl2L12 was reflected at the protein level, as observed by immunoblot analysis (Fig. 2D).

Next, we assessed the involvement of Bcl2L12 in promoting the binding of GATA3 on the IL-10 and IL-4 promoters. However, before proceeding, we analyzed time-dependent binding pattern of GATA3 on the IL-10 and IL-4 promoter regions of Th2 cytokines (IL-10 and IL-4) is enhanced with GATA3 and Bcl2L12 in IL-10 and IL-4 cytokine synthesis in T cells during infection. For that, siRNA-mediated silencing of GATA3 and Bcl2L12 in T cells was performed followed by incubation with L. donovani-infected BMMds. Silencing of GATA3 and Bcl2L12 reduced the secretion of IL-10 and IL-4 cytokine in T cells when incubated with infected macrophages; however, the secretion was drastically reduced when both the genes were silenced together (Fig. 2A,B). So, we next determined the expression level of GATA3 and Bcl2L12 in T cells co-cultured with L. donovani-infected macrophages. We used the in vitro co-culture system as described in Fig. 1 to determine GATA3 and Bcl2L12 expression in T cells, and observed that both GATA3 and Bcl2L12 mRNA expression was significantly induced in a time-dependent manner with maximum induction observed at 36 h post co-incubation (Fig. 2C). Similar induction of GATA3 and Bcl2L12 was reflected at the protein level, as observed by immunoblot analysis (Fig. 2D).

We next, we assessed the involvement of Bcl2L12 in promoting the binding of GATA3 on the IL-10 and IL-4 promoters. However, before proceeding, we analyzed time-dependent binding pattern of GATA3 on the IL-10 and IL-4 promoters with a chromatin immunoprecipitation (ChIP) assay. ChIP analysis showed that association of GATA3 with the IL-10 and IL-4 promoter increases in the T cells in a time-dependent fashion when we co-cultured T cells with L. donovani-infected macrophages, and the maximum recruitment was observed at 24 and 36 h post incubation (Fig. 2E,F). Upon further validation by PCR analysis of the positive control (input sample), it was found that soluble chromatin samples collected for every time point contained equal quantity of chromatin fragments bearing the IL-10 and IL-4 promoter. Furthermore, to validate the role of Bcl2L12 in promoting the binding of GATA3 on the IL-10 and IL-4 promoters, Bcl2L12 was silenced in T cells by
siRNA treatment and these cells were then incubated with L. donovani-infected BMMFs, and binding of GATA3 was assessed by ChIP assay. We observed that silencing of Bcl2L12 significantly reduced binding of GATA3 on the IL-10 and IL-4 promoters in the CD4+ T cells during co-culture experiment, whereas in the chromatin fractions isolated from control siRNA-transfected T cells, strong binding was observed between GATA3 and the IL-10 and IL-4 promoter (Fig. 2G,H).

L. donovani induces the Jagged1–Notch pathway to redirect the immune response toward Th2 mode

We next proceeded to evaluate the cellular events behind the GATA3- and Bcl2L12-mediated activation of the Th2 response during infection and check the possible involvement of the Jagged1–Notch pathway in inducing these transcriptional regulators in CD4+ T cells. For this, Notch signaling was blocked by means of Notch1 neutralizing antibody in CD4+ T cells followed by co-culture with L. donovani-infected BMMFs, and expression of GATA3 and Bcl2L12 were assessed at the protein level in isolated T cells. We observed significantly reduced GATA3 and Bcl2L12 expression in anti-Notch1 antibody-treated CD4+ T cells when compared to IgG-treated T cells in the co-culture system, which confirms the role of Notch signaling in GATA3 and Bcl2L12 expression (Fig. 3A). It has been demonstrated that Notch1 induces the Bcl-2 family members in T cells (MacKenzie et al., 2004) and RBPJκ-dependent Notch signaling regulates Bcl-2 family members (He et al., 2018). RBPJκ is also known to indirectly modulate the expression of Bcl2 family gene as it forms a complex with the Notch1 ICD and p300 (also known as EP300) acetyl transferase. Given that, following Notch signaling activation, the Notch1 ICD replaces the corepressor SMRT from SMRT–RBPJκ complex, leading to Notch1 ICD binding to p300 which then potentiates...
Notch1 ICD-mediated transcriptional activation of target genes. Hence, we next assessed whether *L. donovani* induces the expression of Bcl2L12 by inducing the recruitment of p300 by ChIP analysis. We observed a time-dependent increase in p300 binding with Bcl2L12 promoters in T cells incubated with *L. donovani*-infected (24 h) and control macrophages for different time periods as described in the Materials and Methods. PCR was carried out using primers specific to the Bcl2L12 promoter region after immunoprecipitation with anti-p300 or control IgG antibody. (C) T cells were pre-incubated with isotype control or Notch1 antibody for 1 h. BMMFs were infected with *L. donovani* promastigotes for 24 h. T cells were incubated with the infected macrophages for 12 h. ChIP analysis was performed in harvested CD4+ T cells to assess the binding of p300 to the Bcl2L12 promoter. (D) ChIP assays were performed to observe the binding of RBPJκ to the GATA3 promoter region after immunoprecipitation with anti-RBPJκ or control IgG antibody. (E) T cells were transfected with RBPJκ or scrambled control siRNA for 24 h and incubated with infected or uninfected macrophages for 36 h and expression of GATA3 was analyzed by western blotting. Equal protein loading was ensured through β-actin staining. (F,G) BMMFs were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with *L. donovani* (24 h) and incubated with T cells to assess Bcl2L12 and GATA3 promoter occupancy. Chromatin was prepared from harvested CD4+ T cells and ChIP analysis was performed as described in the Materials and Methods section. PCR was carried out using primers specific to GATA3 and Bcl2L12 promoter region after immunoprecipitation with anti-RBPJκ or anti-p300, respectively. Images shown are representative of three repeats.

Notch1 ICD-mediated transcriptional activation of target genes. Hence, we next assessed whether *L. donovani* induces the expression of Bcl2L12 by inducing the recruitment of p300 by ChIP analysis. We observed a time-dependent increase in p300 binding with Bcl2L12 promoters in T cells incubated with *L. donovani*-infected BMMFs (Fig. 3B). Interestingly, functional blocking of Notch signaling with Notch1-neutralizing antibody markedly impaired the binding of p300 to Bcl2L12 in the CD4+ T cells during the co-culture experiment, with this binding remaining in IgG antibody-treated T cells (Fig. 3C).

Notch signaling activates RBPJκ, which binds to the upstream promoter of GATA3, a transcription factor critical for inducing Th2 differentiation and limiting Th1 differentiation (Amsen et al., 2007, 2004). Hence, we next assessed the association of RBPJκ with the GATA3 promoter in T cells co-cultured with *L. donovani*-infected macrophages. For this, CD4+ T cells were incubated with *L. donovani*-infected macrophages for the respective time points and the total chromatin pool was extracted and analyzed with a ChIP assay. ChIP analysis showed that association of RBPJκ with the GATA3 promoter increases in the T cells in a time-dependent fashion in co-culture experiments and maximal recruitment was observed at 24 and 36 h post incubation (Fig. 3D). Upon further validation of results via PCR, it was found that soluble chromatin samples collected from every time point contained equal quantity of chromatin fragments containing the GATA3 promoter. We also determined the expression of GATA3 in absence of RBPJκ. For this, RBPJκ siRNA-treated T cells were incubated with *L. donovani*-infected BMMFs and expression of GATA3 was assessed by immunoblot analysis. We observed reduced GATA3 expression in RBPJκ siRNA-treated cells with respect to control siRNA-transfected T cells in the co-culture...
system, which confirms the role of RBPJκ in GATA3 expression (Fig. 3E).

Next, we investigated the effect of Jagged1–Notch interaction in modulating RBPJκ and p300-mediated activation of GATA3 and Bcl2L12, respectively, in T cells. To this end, Jagged1 silencing was carried out in L. donovani-infected BMMφs, and its effect on the RBPJκ binding to GATA3 and p300 binding to Bcl2L12 in CD4+ T cells was assessed with a ChIP assay during the co-culture experiment. We observed that silencing of Jagged1 in macrophage reduced binding of RBPJκ to the GATA3 promoter and also significantly reduced the binding of p300 to Bcl2L12 promoter in the CD4+ T cells in the co-culture experiment, whereas in the chromatin fractions isolated from control siRNA-transfected cells T cells, strong binding was observed between RBPJκ and the GATA3 promoter, and p300 and the Bcl2L12 promoter (Fig. 3F,G). These results suggest that L. donovani upregulates the GATA3 and Bcl2L12 expression in T cells via the Jagged1/Notch/Notch ICD/RBPJκ pathway.

**Signaling events involved behind Jagged1 induction in L. donovani-infected BMMφs**

We next proceeded to identify the transcriptional machinery and pathways behind Jagged1 induction in L. donovani-infected BMMφs. In order to identify the transcription factor-binding sites on Jagged1 promoter, we went through published articles and found possible involvement of Egr1, β-catenin and HIF-1α (Ishiguro et al., 2017; Kim et al., 2013; Wang et al., 2018), so we first assessed binding of these transcription factors to the endogenous Jagged1 promoter in infected macrophages by ChIP analysis. Time kinetic analysis revealed that L. donovani induced binding of Egr1 and β-catenin on Jagged1 promoter as early as after 4 h of infection, with this binding becoming much higher at 12 h and 16 h post infection (Fig. 4A,B). On the contrary, chromatin fractions derived from uninfected macrophages showed an absence of any interaction of Egr1 and β-catenin with the Jagged1 promoter, implying that basal expression level of Jagged1 was not under the control of these transcription factors. Upon subsequent validation of positive control (input sample) through PCR, it was further confirmed that soluble chromatin samples harvested for every time point contained equal quantity of chromatin fragments bearing Jagged1 promoter. We did not observe any binding of transcription factor HIF-1α to the Jagged1 promoter in infected macrophages as observed up to 24 h post infection (data not shown). Since binding of Egr1 and β-catenin on Jagged1 promoters requires their nuclear translocation, we next performed immunoblot analysis to assess the subcellular distribution of these transcription factors in the nuclear and cytosolic fractions.

**Fig. 4. Egr1 and β-catenin-mediated induction of Jagged1 in L. donovani-infected BMMφs.** (A,B) ChIP assays were performed to assess the binding of Egr1 and β-catenin to the Jagged1 promoter in L. donovani (L.d)-infected BMMφs for respective time periods. PCR was carried out using primers specific to Jagged1 promoter region after immunoprecipitation with anti-Egr1, anti-β-catenin or control IgG antibody. (C,D) Macrophages were infected with L. donovani promastigotes for respective time periods, nuclear and cytosolic extract were prepared and translocation of Egr1 and β-catenin was analyzed by immunoblotting. (E) Macrophages were transfected (24 h) with either control or Egr1 or β-catenin or both Egr1 and β-catenin siRNA followed by infection with L. donovani promastigotes for 16 h. Expression of Jagged1 was evaluated by immunoblot analysis. (F-H) Macrophages were pre-treated with wortmannin (1 µM) or LY294002 (10 µM) for 1 h followed by infection with L. donovani promastigotes for 16 h. Cytosolic (F) and nuclear (G) translocation of Egr1 and β-catenin, as well as expression of Jagged1 (H), were evaluated by immunoblot analysis. Images shown are representative of three repeats.
cytosolic fraction of *L. donovani*-infected BMMds. For this, BMMds were infected with *L. donovani* for 16 h and analyzed for nuclear translocation of Egr1 and β-catenin. Immunoblot analysis showed increased nuclear enrichment of Egr1 and β-catenin in *L. donovani*-infected macrophages with their gradual disappearance from the cytosol (Fig. 4C,D), whereas no nuclear enrichment was observed in uninfected macrophages. To more directly evaluate the functional role of Egr1 and β-catenin in regulating expression of Jagged1 in *L. donovani*-infected macrophages, siRNA-mediated knockdown was performed. For this, BMMds were transfected with respective siRNA for 24 h and then infected with *L. donovani* promastigotes for 16 h. We observed that knockdown of Egr1 and β-catenin drastically reduced the expression of Jagged1 in infected macrophages as compared to infected macrophages treated with control siRNA, with the maximum reduction in Jagged1 protein level observed upon treatment of macrophages with both Egr1 siRNA and β-catenin siRNA (Fig. 4E). Next, we sought to identify the upstream pathways responsible for Jagged1 induction during infection. Earlier reports suggested that *Leishmania* activates the phosphoinositide 3-kinase (PI3K)/Akt pathway, as well as Egr1 and β-catenin, separately in infected macrophages (Ruhland and Kima, 2009; Gupta et al., 2016; Srivastav et al., 2014). So to determine whether this pathway regulates Egr1 and β-catenin-mediated upregulation of Jagged1, we assessed whether transcriptional activation of both Egr1 and β-catenin was under the control of the PI3K/Akt pathway. For this PI3K/Akt pathway was blocked in BMMds by employing selective inhibitors (i.e. wortmannin or LY294002) for 1 h prior to *L. donovani* infection and then checked for nuclear translocation of Egr1 and β-catenin. In the same experimental set up, we also checked for Jagged1 expression in infected macrophages. We observed reduced nuclear translocation of both β-catenin and Egr1 in *L. donovani*-infected cells pre-treated with pharmacologic inhibitors of PI3K/Akt pathway, with retention of these proteins detected in cytosolic extract (Fig. 4F,G). Furthermore, inhibitor treatment also reduced Jagged1 expression in infected BMMds (Fig. 4H). These results suggest that *L. donovani* induces the expression of Jagged1 in macrophages through PI3K/AKT-mediated Egr1 and β-catenin activation.

*L. donovani* LPG is responsible for PI3K/Akt-mediated activation of Egr1 and β-catenin in infected BMMds

Lipophosphoglycan (LPG) is a prominent surface glycoconjugate molecule found on *Leishmania* promastigotes and plays a crucial role in immune evasion and also impairs the activation of pro-inflammatory pathways in host immune cells (Lima et al., 2017; Olivier et al., 2005; Rojas-Bernabe et al., 2014). To determine the role of LPG (a predominant *L. donovani* virulent factor) in the activation of PI3K/Akt pathway, BMMds were infected with either wild-type *L. donovani* (WT *L. donovani* Dd8 or WT *L. donovani* 1S) or isogenic LPG-defective *L. donovani* 1S (lpg1-KO promastigotes) parasites, and the activation of Akt proteins (assessing all isoforms of Akt) and nuclear translocation of Egr1 and β-catenin were assessed. Our results show that lpg1-KO promastigotes failed to induce phosphorylation of Akt in BMMds whereas both WT promastigotes significantly induced Akt phosphorylation in macrophages (Fig. 5A). Similarly, the nuclear translocation of Egr1 and β-catenin was significantly impaired when the BMMds were infected with lpg1-KO parasites, as observed by reduced nuclear accumulation and increased cytosolic abundance of both Egr1 and β-catenin (Fig. 5B,C). As a result, it was worthwhile to assess the binding pattern of these transcription factors to Jagged1 promoter in BMMds infected with WT and lpg1-KO parasites. We observed reduced binding of both Egr1 and β-catenin when we infected the BMMds with the lpg1-KO promastigotes, which was not affected in macrophage infected with the WT parasites (Fig. 5D,E). As the binding of both Egr1 and β-catenin was important for Jagged1 expression, we next investigated the role of parasite LPG in modulating the expression of Jagged1 at the protein level. We observed that the induction of Jagged1 was much higher in BMMds infected with WT promastigotes but was less pronounced in the BMMds infected with the lpg1-KO mutant (Fig. 5F). Next, to identify the role of the parasite LPG in modulating the Notch signaling-mediated Th2 response, we assessed binding of RBPJκ to GATA3 promoter and p300 to the Bc2L12 promoter by incubating the CD4+ T cells with either the WT or lpg1-KO parasite-infected BMMds. As expected, we observed reduced association of RBPJκ to the GATA3 promoter as well as p300 to the Bc2L12 promoter when the macrophages were infected with lpg1-KO parasites (Fig. 5G,H) whereas the association was intact when the BMMds were incubated with the WT parasites. These suggest that parasite virulence factor LPG plays a major role in modulating the immune response towards Th2 direction by inducing Jagged1 in BMMds, which, in turn, activates N1 ICD/RBPJκ pathway in CD 4+ T cells.

**Role of Jagged1 in in vivo parasite survival and pro- and anti-inflammatory cytokine balance**

Because our *ex vivo* experiments established the fact that induction of macrophage Jagged1 by *L. donovani* is essential for GATA3-mediated IL-4 and IL-10 synthesis in T cells, we next evaluated the role of Jagged1 in disease progression of VL in *L. donovani*-infected Balb/c mice. To this end, we first detected the expression of Jagged1 at both mRNA and protein level in splenocytes isolated from control and *L. donovani*-infected Balb/c mice at various time points. Similar to the data obtained *ex vivo*, the splenocytes of mice showed induction of Jagged1 at both mRNA and protein levels at all time points, with maximum induction observed at 4 weeks post infection (Fig. 6B,C). Furthermore, in order to evaluate the role of Jagged1 in *in vivo* disease progression, morpholino (antisense oligonucleotides that can block translation, Fig. 6A) mediated knockdown was performed. The efficacy and specificity of the morpholino on Jagged1 expression was further assessed by western blot analysis in splenocytes of infected mice at different time points of infection. Treatment of mice with Jagged1 morpholino led to considerable reduction in the Jagged1 protein level (≈70% decrease) when compared with infected mice administered control morpholino (Fig. 6D). Both liver and spleen parasite burdens were found to be reduced in infected Balb/c mice treated with Jagged1 morpholino (64.5 and 67.08% reduction, respectively) compared with control morpholino-treated infected mice at 6 weeks post-infection, further establishing the role of Jagged1 in parasite survival (Fig. 6E,F). We then proceeded to determine the consequences of Jagged1 inhibition on the *in vivo* anti-inflammatory cytokine response. *In vivo* silencing of Jagged1 in infected Balb/c mice resulted in decreased IL-10 (55.4% decrease as compared to the control morpholino-treated infected mice, Fig. 6G) and IL-4 synthesis (67.5% decrease as compared to the control morpholino-treated infected mice, Fig. 6H) at 4 weeks post infection. These results suggest that activation of Jagged1–Notch signaling *in vivo* might be associated with induction of anti-inflammatory cytokine response, which, in turns facilitates parasite survival in the infected Balb/c mice.
DISCUSSION

Notch signaling is activated upon ligand–receptor interactions between neighboring cells and was recently found to play a crucial role in regulating cell mediated immunity during antigen presenting cell–T cell crosstalk (Amsen et al., 2004). Because of its central role in shaping immunity, many pathogens exploit Notch pathway to escape immune host defense. For instance, *E. chaffeensis*, a nontagulate intracellular bacterium activates the Notch pathway to inhibit the ERK1/2 and p38 mitogen-activated protein kinase (MAPK) pathways, which are required for subsequent TLR2 and TLR4 expression and evasion of host immunity (Lina et al., 2016). *Schistosoma japonica* upregulates Notch1–Jagged1 signaling-dependent M2 polarization of macrophages, which might play an important role in liver granuloma and fibrosis in schistosomiasis (Zheng et al., 2016). During experimental encephalomyelitis, Dll1 expression is upregulated on dendritic cells, which seem to be involved in the differentiation of pathogenic Th1 and Th17 cells (Elyaman et al., 2007). However, the biological significance of Notch pathway in modulating immune response during macrophage–T cell crosstalk had not yet been explored in the case of experimental VL. Moreover, the mechanistic details involving the pathway responsible for upregulation of Notch ligands were also not known. In this study, we observed that *L. donovani* infection in macrophages specifically upregulates Jagged1 expression out of all Notch ligands. Upregulated Jagged1 then activates the canonical Notch pathway in T cells, which was verified by nuclear translocation of ICD. It is known that the Notch ICD is translocated to the nucleus but, as it does not have an intrinsic DNA-binding activity, it assembles with transcription factor recombination signal binding protein RBPJκ, in order to serve as a transcriptional activator. ICD binding converts RBPJκ from being a transcriptional repressor into being an activator (Amsen et al., 2004).

Another coactivator capable of forming physical interactions with Notch is MAML1, which facilitates Notch signaling from all four Notch receptors by binding with the ankyrin repeat motif of the Notch ICD. MAML1 is believed to stabilize the interaction between the Notch ICD and RBPJκ, as well recruiting the histone acetyltransferase p300 (Wallberg et al., 2002), which plays a major role in facilitating Notch ICD-mediated transcriptional activation of the target genes. Various studies have revealed that acetylation of chromatin templates by p300 strictly depends on RBPJκ, the Notch1 ICD and MAML1 (Oswald et al., 2001; Wallberg et al., 2002). In our case, we observed that *L. donovani* mediates recruitment of Notch1–RBPJκ to the Bcl2L12 promoter region, where p300 binds to the Bcl2L12 promoter and induces its transcriptional activation. Activation of this complex is also associated with different pathological situations; for instance, in many cancers the protooncogene c-Myc (Myc) is also acetylated by p300, which is recruited by Notch transcription complexes, and this correlates with Myc expression (Weng et al., 2006; Yashiro-Ohtani et al., 2014).
The Notch-ICD–RBPJκ complex trans-activates promoters containing RBPJκ-binding sites. Thus, this association is important for determining the fate of T cell-mediated immune response. However, many pathogens are known to modulate this association for their own survival and benefits. For instance, the Kaposi’s sarcoma-associated herpesvirus (KSHV) gene product, virally encoded G protein-coupled receptor (vGPCR), has been proposed to play important roles in KSHV-induced angiogenesis and vGPCR gene expression is upregulated by binding of RBPJκ on its sites in the vGPCR promoter. These results suggest a potential role for Notch signaling in the induction of vGPCR gene expression and KSHV-induced angiogenesis (Liang and Ganem, 2004).

Furthermore, B. anthracis edema toxin is known to activate the Notch signaling pathway in murine macrophages and human monocytes by increasing levels of RBPJκ (Larabee et al., 2013). In our case, we observed that L. donovani infection increased the expression of GATA3 in T cells co-cultured with infected macrophages by facilitating the association of RBPJκ on the GATA3 promoter, which contains putative binding site for RBPJκ. This is achieved by upregulation of Jagged1 in infected macrophages followed by its association with the Notch receptor N1, which initiates translocation of its ICD into the nucleus of T cells where it binds to RBPJκ and activates it. Hence, our results are in accordance with the existing studies that suggest that, unlike differentiation into the Th1 cell, differentiation into Th2 cells is RBPJκ dependent (Amsen et al., 2004; Betcher and Burnham, 1992; Tindemans et al., 2017).

Leishmania promastigotes activates PI3K/Akt signaling in infected macrophages in order to accomplish immunosuppression. For instance, activation of PI3K/Akt signaling during Leishmania amazonensis infection abrogates the production of IL-12 p70 by inhibiting the transcription of its p40 subunit in BMMΦs (Ruhland and Kima, 2009). Similarly, Leishmania promastigotes engage PI3K/Akt signaling, which confers resistance to the infected BMMΦs cell from activators of apoptosis (Ruhland and Kima, 2009).
et al., 2007). L. donovani is also known to activate Egr1 and β-catenin transcription factors; however, the pathway that initiates their activation is not clear (Gupta et al., 2016; Srivastav et al., 2014). Our findings also provide evidence that the increase in expression of Jagged1 in L. donovani-infected macrophages is accomplished by PI3K/Akt-mediated activation of transcription factor Egr1 and β-catenin. We and others have found that LPG can induce the PI3K/Akt pathway in macrophages. Induction of this pathway results in SRF-mediated nuclear translocation of Egr2 and, consequently, increased expression of SOCS1, which, in turn, inhibits Jak1/Stat1-mediated IL-12 cytokine secretion (Chandrakar et al., 2020). Recent findings also reported that Leishmanial LPG induced the autophagy of neutrophils by activating the PI3K/Akt signaling pathway (Pitale et al., 2019). In line with these results, we observed that L. donovani LPG could also induce the expression of Jagged1 in infected BMMDs, possibly by activating PI3K/Akt signaling axis.

Altogether, our study provides substantial evidences of how L. donovani modulates the existing communication between macrophages and T cells by exploiting Notch1–Jagged1 signaling for its own survival. These findings imply new therapeutic strategies that might be developed to inhibit Notch1–Jagged1 signaling, thereby reversing the Th2 polarization of the T cell, which might subsequently attenuate progression of VL.

**MATERIALS AND METHODS**

**Reagents, antibodies and kits**

Medium RPMI 1640 and Medium 199 were commercially purchased from Sigma-Aldrich (MO, USA). Heat-inactivated fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific (MA, USA). Antibodies against Jagged1 (28H8), ICD (cleaved Notch1) (D3B8), GATA3 (D13C9), Egr1 (15F7), β-catenin (D10A8), p-Akt, Akt (pan), Histone H3, GAPDH (14C10) and β-actin were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-RBPJκ (ab25949) antibody was purchased from Abcam (Cambridge, UK). The Simple ChIP Enzymatic Chromatin IP Kit was purchased from Cell Signaling Technology (Massachusetts, USA). ChIP grade antibodies for GATA3 (D13C9), Egr1 (15F7), β-catenin (D10A8) were purchased from Cell Signaling Technology (Massachusetts, USA). ChIP grade anti-RBPJκ (ABE384) antibody was purchased from Millipore (MA, USA). Bcl2L12 (BS-5797R), p300 (RW105) antibody was purchased from Thermo Fisher Scientific (MA, USA). Neutralizing antibody for Notch1 Monoclonal (A6) was purchased from Thermo Fisher Scientific. IgG antibody (IgG2B) was purchased from R&D Systems (MN, USA). Mouse-specific, small interfering RNA (siRNA) for Jagged1, GATA3, Bcl2L12, RBPJκ, Egr1, β-catenin and scrambled siRNA, transfection medium and transfection reagent were purchased from Santa Cruz Biotechnology (CA, USA). IL-4 and IL-10 cytokine BD OptEIA kits were purchased from BD Biosciences (NJ, USA). Wortmanin and C8156 (Thermo Fisher Scientific, USA) were confirmed and purity was assessed with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). RNA (1 μg) from each experimental group was subjected to cDNA synthesis by using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR was then performed in Step One plus real-time PCR system (Thermo Scientific) using SYBR green-based detection primers. The PCR amplification conditions were: 40 cycles of 95°C for 15 s and optimized annealing temperature for 30 s, extension at 72°C for 30 s. The housekeeping gene, GAPDH, was selected as internal standard to check the variability in amplification. Relative quantitation was undertaken by using the comparative ΔΔCt method, and the normalization of data were performed relative to GAPDH mRNA levels and expressed as fold change compared with uninfected controls. All samples were run in triplicate. The primer sequences were as follows: Jagged 1 forward, 5′-AGAAGTCAGA- GTTCAGAGGCGTCC-3′ and reverse, 5′-TGTTTCCAGAGGCGTCC-3′; Dll4 forward, 5′-AGTAGAAGGCGTGTCACTAC- GCAC-3′; Jagged 2 forward, 5′-TGCTGTTGAGGTCGGCTATGCT-3′ and reverse, 5′-TGGTCCACCTGAGCACTCCTCG-3′; Dll1 forward, 5′-GG- ACCTCAGTGAGGCACTGG-3′ and reverse, 5′-GGCAATTGCGTA- GGTTGTTCTAG-3′; Dll3 forward, 5′-AGTTGCACTTCTCCACCGC-3′ and reverse, 5′-ACGGCACTTACACGCTTCC-3′; Dll4 forward, 5′-
GTGAACTGCACATCGGATTG-3' and reverse, 5′-GTGGCAAGACGA-AGTGTGTTGGG-3'; BeL2L2 forward, 5′-TTCGCCAGTCTATGGCCCTGG-3' and reverse, 5′-CCAGGTTCAGATGACAGACC-3'; GATA3 forward, 5′-CTTGGGCTGTACATAAAGCTCT-3' and reverse, 5′-ACCATCTGGGAACCTGATC-3'; and GAPDH forward, 5′-TGTCAGTGGCAAGATTGGAGA-3' and reverse, 5′-GGTGCTCGCTCCTGGAAAGAT-3'.

**Immunoblot analysis and co-immunoprecipitation**

Adhered BM-Mφs were co-cultured with T cells for different time points, harvested and washed with chilled 1× PBS. For the preparation of whole-cell lysates, RIPA buffer (Sigma), supplemented with 1 mM PMSF and 1× protease inhibitor cocktail (P8340, Sigma) was used. The lysates were kept on ice for 10 min and centrifuged at 5000 g for 10 min at 4°C to obtain clear supernatants. Further, samples were separated by SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose membrane. Nitrocellulose membranes were incubated in blocking buffer (Tris-buffered saline (TBS) containing 4% fraction V bovine serum albumin (Sigma)) for 1 h at room temperature and further incubated with primary antibody at 4°C overnight. After washing three times with TBS with 0.1% Tween 20, membranes were incubated with appropriate HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were then washed with TBS containing 0.1% Tween 20 three times for 5 min each time. For detection, bands on the membrane were visualized using SuperSignalTM West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) according to the manufacturer’s guidelines. Densitometric analyses for the immunoblots were performed using ImageJ software. Band intensities were densitometrically quantified, and values were normalized to input and expressed in arbitrary units. The ratios of ODs of particular bands/input are indicated as bar graphs adjacent to figures.

For immunoprecipitation, whole-cell lysates were prepared using the RIPA cell lysis buffer. Immunoprecipitation was carried out by incubating cell lysate (500 µg proteins) with 5 µg corresponding antibodies (anti-RBPJκ) and kept at rocking conditions overnight at 4°C. Protein G plus-agarose (40 µl/sample; Santa Cruz Biotechnology) was added, and samples were further kept rocking for another 1 h. The immunoprecipitates were washed with lysis buffer twice and once with 1× PBS, and dissociation was performed by boiling in electrophoresis sample buffer. The specific association of ICD with RBPJκ was measured by western blotting using anti-N1 ICD antibody.

**Preparation of nuclear and cytoplasmic extracts**

Control BM-Mφs, infected BM-Mφs or infected BM-Mφs co-cultured with CD4+ T cells were taken, washed with PBS (pH 7.4) and homogenized in chilled lysis buffer containing 10 mM HEPEs pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM PMSF, 0.5 mM diethiothitol and leupeptin, 1 µg/ml of aprotinin and pepstatin A, with the help of a Dounce homogenizer. Homogenates were kept on ice for 45 min and treated with 0.5% NP-40 and the mixture was then centrifuged at 5000 g for 10 min at 4°C. The supernatants were kept aside for analysis of cytosolic proteins and pellets were extracted in nuclear extraction buffer (20 mM HEPES pH 7.8, 1.5 mM MgCl2, 400 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT and 1 µg/ml of aprotinin, leupeptin and pepstatin A) for 30 min on ice and were centrifuged at 15,000 g for 15 min. The amount of protein in each sample was measured and extracts were stored at −80°C.

**Chromatin immunoprecipitation assay**

The ChIP assay was carried out using the Simple ChIP Enzymatic Chromatin IP Kit from Cell Signaling Technology. Briefly, 4×106 cells (control BM-Mφs, infected BM-Mφs or infected BM-Mφs co-cultured with T cells) were subjected to cross-linking with 1% formaldehyde for 10 min at 37°C, followed by treatment with glycine solution for 5 min at room temperature to stop reaction. The cells were further processed as described previously (Parnar et al., 2018). Briefly, cells were washed with chilled PBS, pooled, pelleted down and further incubated for 10 min in lysis buffer enriched with 1 mM PMSF, diethiothitol (DTT), and protease cocktail inhibitor mix. The pelleted nuclei were collected and resuspended in buffer containing DTT, subjected to digestion by micrococcal nuclease, and further homogenized on ice. Following centrifugation, sheared chromatin was incubated overnight at 4°C with anti-RBPJκ, or Egr1 or β-catenin antibody and anti-IgG, as a negative control and precipitation was performed using ChIP-grade Protein G-agarose beads. An aliquot of the chromatin fraction incubated without antibody was taken as the input control sample (2%). Samples containing immunoprecipitated chromatin complexes were then washed sequentially in low-salt and high-salt wash buffers at 4°C and protein–DNA cross-links were reversed in presence of Proteinase K at 65°C for 2 h. Further purification of DNA fragments was performed using the spin columns. Then, 2 µl DNA from this was used as a template for PCR amplification. PCR was carried out in conditions of denaturation at 94°C for 30 s, standardized annealing temperature (RBPJκ, 48°C; Egr1, 48°C; β-catenin, 48°C; p300, 51.6°C; GATA3 on IL-4, 55.6°C; GATA3 on IL-10, 51.9°C) and extension at 72°C for 30 s for 40 cycles followed by 10 min at 72°C. For the following primers designed to amplify specific regions involving putative transcription factor-binding sites: RBPJκ forward, 5′-CACAGGAGGGTCACTACATC-3′ and reverse, 5′-AACCTGGCCTGAATCC-3′; Egr1 forward, 5′-GGGCTTCAAATGTTAGCT-3′ and reverse, 5′-ACCTATACGGGCTGCTCTC-3′; β-catenin forward, 5′-TAGAAGGGTAAAGGCTGCTA-3′ and reverse, 5′-CTTTCCCGGAGTAATTG-3′; GAGGAGAGCTTGT-3′ and reverse, 5′-ATGGGCTACATTGCTGCTT-3′; GATA3 on IL-4 forward, 5′-AGGAGAGCCAGTTGGCAACCTA-3′ and reverse, 5′-CAATGCTGCGAGAGGCCTC-3′; GATA3 on IL-10 forward, 5′-CCTACTGAAAGGGAAGTCCG-3′ and reverse, 5′-GACCTATGAAAGCCCTTGTT-3′.

**sirRNA-mediated knockdown in macrophages and T cells**

sRNA targeted against Jagged1, RBPJκ, GATA3, Egr1 and β-catenin (Santa Cruz Biotechnology) were transfected into BM-Mφs or T cells according to the manufacturer’s protocol. Briefly, 20 nM of control or target specific siRNA (dissolved in siRNA transfection medium and incubated with siRNA transfection reagent) were added to BM-Mφs or T cells and incubated for 5 h. After transfection, macrophages or T cells were incubated for additional 24 h in antibiotic-free complete medium followed by infection with L. donovani and co-incubation with T cells and processed for cytokine estimation by ELISA, lysate preparation for immunoblots or co-immunoprecipitation analysis.

**Cytokine estimation by ELISA**

Cytokine levels from harvested supernatants of infected macrophage-T cell co-culture were measured using a selective kit as per the manufacturer’s instructions (BD Biosciences, USA). Briefly, ELISA plates (Nunc Maxisorp) were coated with specific capture antibodies at 4°C, followed by blocking with assay diluents (PBS containing FBS) and were then incubated with supernatants for 2 h at room temperature, washed and incubated with the specific biotinylated detection antibody for 2 h, and incubated with streptavidin–horseradish peroxidase. After appropriate washes, signal was developed by addition of 3,3′,5,5′-tetramethylbenzidine (TMB) and optical density was measured at 450 nm after addition of 1 M H3PO4 as stop solution.

**Vivo-morpholino-mediated knockdown of Jagged1 in Balb/c mice**

To inhibit Jagged1 translation in vivo, translation blocking Vivo-morpholino oligonucleotides targeting Jagged1 (5′-CGTCCGGGCTCCATGATACTCCTCGGTG-3′) or control morpholino (5′-CCTCTATCCCTCAGTTACAATTTATA-3′) were synthesized and purchased from Gene Tools, USA. Vivo-morpholinos were solubilized in sterile PBS at 0.5 mM concentration and injected into Balb/c mice (n=5/group) via tail vein twice per week at 10 nmol (5 mg/kg body weight) dose, starting 1 week before infection and until 6 weeks post infection. The efficiency of knockdown was assessed on weeks 2, 4 and 6 post infection in the splenocytes of control and Jagged1 morpholino-treated animals by immunoblotting. Organ parasite burdens in spleen and liver were assessed on weeks 2, 4 and 6 post infection and expressed as Leishman Donovan units (LDUs; Parnar et al., 2018).

Splenocytes from all experimental groups of Balb/c mice were similarly isolated on weeks 2, 4 and 6 post infection and processed for cytokine estimation.
Statistical analysis
The experimental data were analyzed using GraphPad Prism 5.00 software and are presented as mean±s.d. of at least two independent experiments done in triplicate. Significance between experimental groups were derived using two-tailed Student’s-t test (for two groups), either paired or unpaired as appropriate, or one-way ANOVA followed by Tukey’s post-test for multiple groups. P<0.05 was considered to be statistically significant and P>0.05 was considered not significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
Supplementary information available online at https://jcs.biologists.org/lookup/doi/10.1242/jcs.252494.supplemental

References

Judicious use of two-tailed Student t-test as appropriate, or one-way ANOVA followed by Tukey and promote intracellular survival. TRP120 activates canonical notch signaling to downregulate TLR2/4 expression during infection of naive CD4+ T cells. Immunity 19, 549-559. doi:10.1016/S1074-7613(03)00270-X


Table S1. Antibodies for immunoblotting

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Table S2. Antibodies for ChIP assays

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<td>Rabbit Polyclonal Antibody</td>
<td>ABE384</td>
<td>Millipore, Germany</td>
<td>1:50</td>
</tr>
<tr>
<td>5</td>
<td>p300 (RW105)</td>
<td>Mouse monoclonal Antibody</td>
<td>MA1-1-6622</td>
<td>Thermo Fisher Scientific, USA</td>
<td>1:50</td>
</tr>
</tbody>
</table>
Table S3. Antibodies for functional blocking/neutralization

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Name of Antibody</th>
<th>Type of Antibody</th>
<th>Catalog No.</th>
<th>Manufacturer</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Notch1 (A6)</td>
<td>Mouse monoclonal Antibody</td>
<td>MA5-11961</td>
<td>Thermo Fisher Scientific, USA</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>IgG (IgG2B)</td>
<td>Mouse monoclonal Antibody</td>
<td>MAB004</td>
<td>R&amp;D Systems, USA</td>
<td>10µg/ml</td>
</tr>
</tbody>
</table>

Table S4. siRNAs for transfection

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Name of Protein</th>
<th>Catalog No.</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Jagged1</td>
<td>sc-44336</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>2</td>
<td>GATA3</td>
<td>sc-35453</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>3</td>
<td>Bcl2L12</td>
<td>sc-141674</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>4</td>
<td>RBP-Jκ</td>
<td>sc-38215</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>5</td>
<td>Egr1</td>
<td>sc-35267</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>6</td>
<td>β-catenin</td>
<td>sc-29210</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>7</td>
<td>scrambled siRNA</td>
<td>sc-142627</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
</tbody>
</table>