

## RESEARCH ARTICLE

## SPECIAL ISSUE: CELL BIOLOGY OF HOST–PATHOGEN INTERACTIONS

# 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 (BMMφs) and subsequent activation of RBPJ $\kappa$  (also known as RBPJ) in T cells, which in turn upregulates the transcription factor GATA3. Activated RBPJ $\kappa$  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<sup>+</sup> 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 BMMφ–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  $\beta$ -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*-induced host Jagged1–Notch signaling skews 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<sup>+</sup> 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 (Tanigaki 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 [of which there are four forms in mammals; Notch1–Notch4 (N1–N4)] with its cognate ligands on neighboring cells. In mammals, there are five Notch ligands designated as Delta-like 1 (Dll1), Dll3 and Dll4, Jagged1 and Jagged2. Regulation of Notch signaling is not only specific to the cell type but also depends upon the spatial expression of Notch ligands and receptors (Radtke et al., 2010). Several studies have outlined the role of Notch receptors in Th1/Th2 differentiation; for instance, exposure of CD4<sup>+</sup> T 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, Dll4, the principal Notch ligand, is preferentially overexpressed in dendritic cells (DCs) during mycobacterial infection. Functional blocking of Dll4 *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<sup>+</sup> 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 mortality, and decrease in IFN- $\gamma$  secretion, thereby leading to reduced viral clearance. Similar to this, CD4<sup>+</sup> 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

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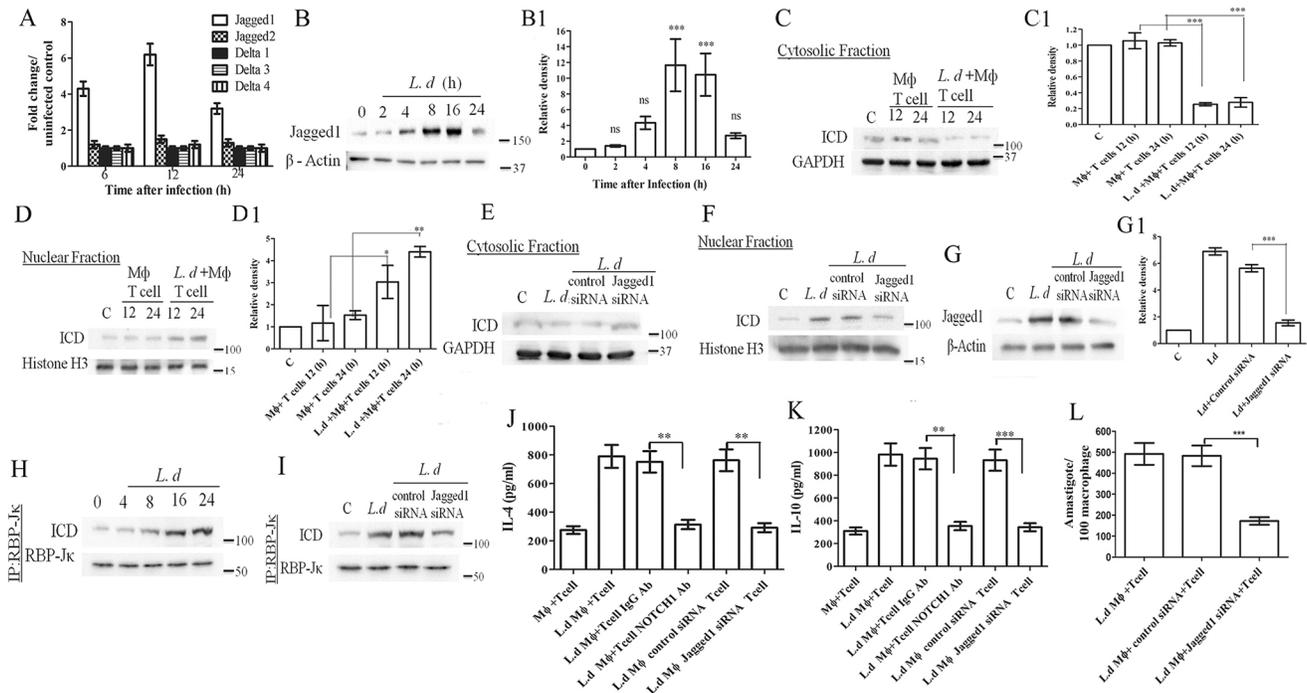
mechanisms that facilitate the induction of Jagged–Notch receptor–ligand expression, interaction as well as effector cytokine responses during experimental VL are yet to be explored. Therefore, in the current study, we investigate whether *L. donovani* modulates host Jagged–Notch signaling in order to promote Th2 responses during macrophage–T cell crosstalk in experimental VL.

## RESULTS

### *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 *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 (BMMφs) following *L. donovani* infection. Real-time PCR analysis revealed increased expression of Jagged1 ligand, among the isoforms, during *L. donovani* infection in



**Fig. 1. *L. donovani* induces Jagged1–Notch signaling during BMMφ–CD4<sup>+</sup> T cell co-culture.** (A) BMMφs were infected with *L. donovani* (*L.d*) promastigotes for 0–24 h and real-time PCR was performed with the isolated mRNA to check expression of Jagged1, Jagged2, Delta1 (DII1), Delta3 (DII3) and Delta4 (DII4). The expression changes were calculated relative to uninfected macrophages after normalizing with GAPDH used as endogenous control. (B) BMMφs were infected with stationary phase *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 ( $n=3$ ). \*\*\* $P<0.0001$ ; ns, not significant (one-way ANOVA test). (C, D) CD4<sup>+</sup> T cells were incubated with *L. donovani*-infected (24 h) and uninfected BMMφs (Mφ) 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 ( $n=3$ ). \* $P<0.05$ , \*\* $P<0.005$ , \*\*\* $P<0.0001$ ; ns, not significant (one-way ANOVA test). (E, F) BMMφs were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with *L. donovani* for 24 h. CD4<sup>+</sup> 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) BMMφs were transfected (24 h) with either control or Jagged1 siRNA followed by infection with *L. donovani* promastigotes for 24 h. The efficacy of Jagged1 siRNA was evaluated by immunoblot analysis. (G1) Densitometric analysis for the immunoblots are represented as bar graph adjacent to the respective panels. The error bar represents mean±s.d. for biological replicates ( $n=3$ ). \*\*\* $P<0.0001$  (unpaired two-tailed  $t$ -test). Significance is indicated for the control siRNA versus Jagged1 siRNA. (H) RBPJ $\kappa$  was immunoprecipitated from CD4<sup>+</sup> T cells incubated with BMMφs infected with *L. donovani* for different time points. Co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with ICD and anti-RBPJ $\kappa$  antibody. (I) BMMφs were transfected with Jagged1 or scrambled control siRNA for 24 h, followed by infection with *L. donovani* for 24 h. T cells were incubated with these infected macrophages for 24 h. RBPJ $\kappa$  was immunoprecipitated from whole-cell lysate and co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with anti-ICD and anti-RBPJ $\kappa$  antibody. (J, K) Macrophages were transfected with Jagged1 or scrambled control siRNA for 24 h and were infected with *L. donovani* promastigote (24 h). In a separate set of experiments, T cells were pre-incubated with isotype control or Notch1 antibody (20  $\mu$ g/ml) for 1 h. T cells were then incubated with infected BMMφs 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 *L. donovani* infection for 24 h. Infected macrophages were co-cultured with CD4<sup>+</sup> 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.

BMMφs, 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-BMMφs 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<sup>+</sup> T cells, and nuclear migration of the ICD was observed by immunoblot analysis. *In vitro* knockdown of Jagged1 ligand in macrophages has profound effect on nuclear migration of N1 ICD in T cells, as we observed a significant reduction in N1 ICD in the nuclear fraction of T cells co-cultured with Jagged1 silenced *L. donovani*-infected BMMφ (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).

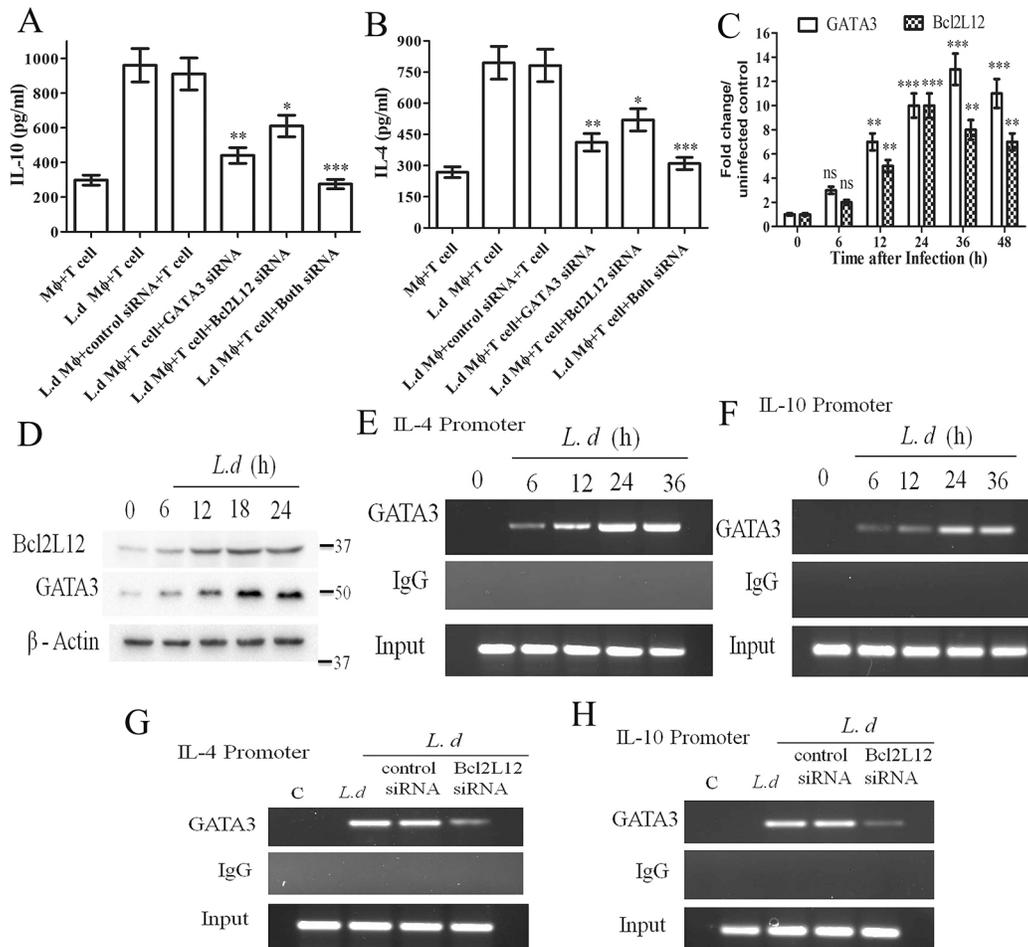
The N1 ICD translocates to the nucleus and activates several different regulatory proteins, including DNA-binding protein RBPJκ (also known as RBPJ). RBPJκ serves as a transcriptional repressor by physically interacting with corepressor SMRT (also known as NCOR2), and the assembly of SMRT–RBPJκ complex 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 BMMφs, suggesting its activation (Fig. 1H). To this end, we also assessed whether Jagged1 silencing in macrophages could reduce the association of migrated N1 ICD and RBPJκ in T cells incubated with *L. donovani*-infected BMMφs. 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 Jagged 1 by siRNA in BMMφs 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 BMMφs (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<sup>+</sup> 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 BMMφs**

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<sup>+</sup> T cell differentiation (Yagi et al., 2011). This indicates that *L. donovani* might exploit these two transcriptional regulators in CD4<sup>+</sup> 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 BMMφs. 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 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



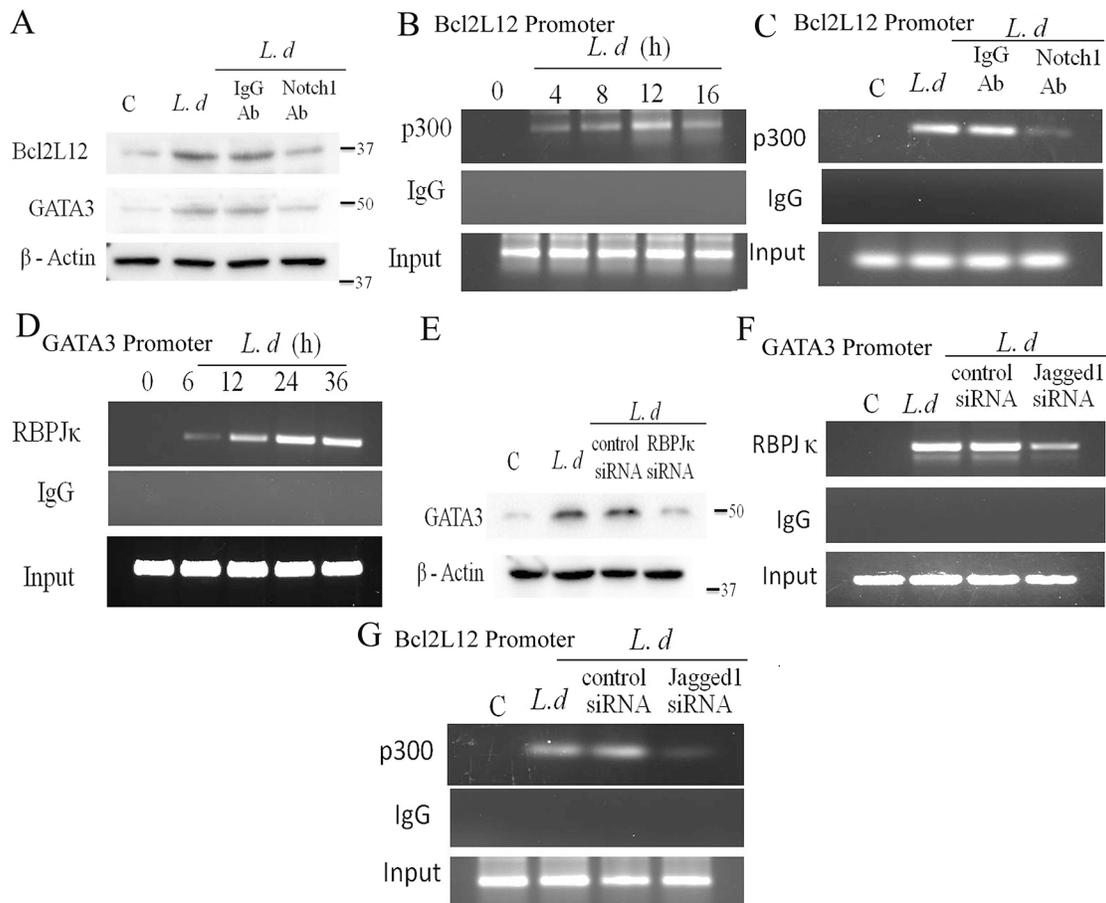
**Fig. 2. GATA3 and Bcl2L12 induces IL-4 and IL-10 in T cell co-cultured with infected BMMφs.** (A,B) BMMφs (Mφ) were transfected with siRNA against GATA3 or Bcl2L12, both (GATA3 and Bcl2L12) siRNAs or scrambled control siRNA for 24 h followed by infection with *L. donovani* (*L.d*) promastigotes (24 h). T cells were incubated with the infected macrophages for 48 h. Levels of (A) IL-10 and (B) IL-4 in the culture supernatants were measured by ELISA assay. (C) Real-time quantitative PCR was performed to check mRNA expression of GATA3 and Bcl2L12 in CD4<sup>+</sup> T cells incubated with *L. donovani*-infected macrophage for different time periods, the expression changes were calculated relative to control macrophages after normalizing to GAPDH, used as endogenous control. (D) Expression of GATA3 and Bcl2L12 in T cells in the same experimental systems as mentioned above was analyzed by immunoblotting. Equal protein loading is ensured through β-actin staining. (E,F) ChIP assays were performed to observe the binding of GATA3 to the IL-4 and IL-10 promoter in T cells incubated with *L. donovani*-infected and control macrophages for different time periods as described in the Materials and Methods section. PCR was carried out using primers specific to the IL-4 and IL-10 promoter regions after immunoprecipitation with anti-GATA3 or control IgG antibody. (G,H) CD4<sup>+</sup> T cells were transfected with Bcl2L12 or scrambled control siRNA for 24 h and incubated with infected (24 h) or uninfected macrophages for 36 h. ChIP analysis was performed as described in the Materials and Methods section. PCR was carried out using primers specific to IL-4 and IL-10 promoter region after immunoprecipitation with anti-GATA3 or control IgG antibody. Error bar in all panels represents the mean±s.d. of at least two independent experiments performed in triplicate. \**P*<0.05, \*\**P*<0.005, \*\*\**P*<0.0001, ns, not significant (one-way ANOVA test). Significance was determined for infected versus uninfected groups.

siRNA treatment and these cells were then incubated with *L. donovani*-infected BMMφs, 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<sup>+</sup> 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<sup>+</sup> T cells. For this, Notch signaling was blocked

by means of Notch1 neutralizing antibody in CD4<sup>+</sup> T cells followed by co-culture with *L. donovani*-infected BMMφs, 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<sup>+</sup> 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



**Fig. 3. Notch signaling switches the immune response towards Th2 direction in T cells co-cultured with infected BMMφs.** (A) CD4<sup>+</sup> T cells were pre-incubated with isotype control or Notch1 antibody (20 μg/ml) for 1 h. BMMφs were infected with *L. donovani* (*L.d*) promastigotes for 24 h. T cells were incubated with the infected BMMφs for 48 h. Expression of GATA3 and Bcl2L12 in T cells were analyzed by immunoblotting. GAPDH was used as an endogenous control. (B) ChIP assays were performed to observe the binding of p300 to the Bcl2L12 promoter 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. BMMφs 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<sup>+</sup> 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 in T cells incubated with *L. donovani*-infected and control macrophages for different time periods. PCR was carried out using primers specific to 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) BMMφs 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<sup>+</sup> 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 BMMφs (Fig. 3B). Interestingly, functional blocking of Notch signaling with Notch1-neutralizing antibody markedly impaired the binding of p300 to Bcl2L12 in the CD4<sup>+</sup> 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<sup>+</sup> 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. Chromatin fractions from uninfected macrophages exhibited negligible binding of RBPJκ to the GATA3 promoter (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 BMMφs 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

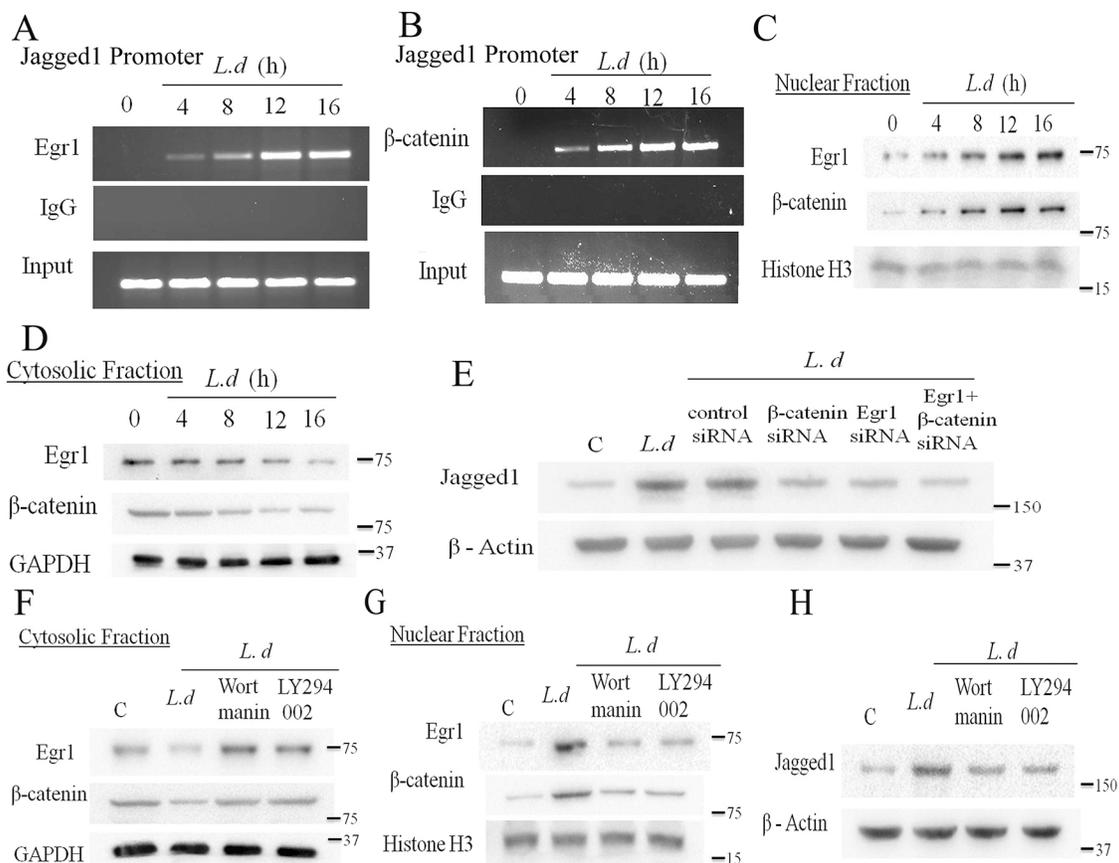
system, which confirms the role of RBPJ $\kappa$  in GATA3 expression (Fig. 3E).

Next, we investigated the effect of Jagged1–Notch interaction in modulating RBPJ $\kappa$ - and p300-mediated activation of GATA3 and Bcl2L12, respectively, in T cells. To this end, Jagged1 silencing was carried out in *L. donovani*-infected BMM $\phi$ s, and its effect on the RBPJ $\kappa$  binding to GATA3 and p300 binding to Bcl2L12 in CD4<sup>+</sup> T cells was assessed with a ChIP assay during the co-culture experiment. We observed that silencing of Jagged1 in macrophage reduced binding of RBPJ $\kappa$  to the GATA3 promoter and also significantly reduced the binding of p300 to Bcl2L12 promoter in the CD4<sup>+</sup> 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 RBPJ $\kappa$  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/RBPJ $\kappa$  pathway.

### Signaling events involved behind Jagged1 induction in *L. donovani*-infected BMM $\phi$ s

We next proceeded to identify the transcriptional machinery and pathways behind Jagged1 induction in *L. donovani*-infected

BMM $\phi$ s. In order to identify the transcription factor-binding sites on Jagged1 promoter, we went through published articles and found possible involvement of Egr1,  $\beta$ -catenin and HIF-1 $\alpha$  (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  $\beta$ -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  $\beta$ -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-1 $\alpha$  to the Jagged1 promoter in infected macrophages as observed up to 24 h post infection (data not shown). Since binding of Egr1 and  $\beta$ -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



**Fig. 4. Egr1 and  $\beta$ -catenin-mediated induction of Jagged1 in *L. donovani*-infected BMM $\phi$ s.** (A,B) ChIP assays were performed to assess the binding of Egr1 and  $\beta$ -catenin to the Jagged1 promoter in *L. donovani* (L.d)-infected BMM $\phi$ s for respective time periods. PCR was carried out using primers specific to Jagged1 promoter region after immunoprecipitation with anti-Egr1, anti- $\beta$ -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  $\beta$ -catenin was analyzed by immunoblotting. (E) Macrophages were transfected (24 h) with either control or Egr1 or  $\beta$ -catenin or both Egr1 and  $\beta$ -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 wortmanin (1  $\mu$ M) or LY294002 (10  $\mu$ M) for 1 h followed by infection with *L. donovani* promastigotes for 16 h. Cytosolic (F) and nuclear (G) translocation of Egr1 and  $\beta$ -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 BMMφs. For this, BMMφs 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, BMMφs 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 BMMφs 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 BMMφs (Fig. 4H). These results suggest that *L. donovani* induces the expression of Jagged1 in macrophages through PI3K/AKT-mediated Egr1 and β-catenin activation.

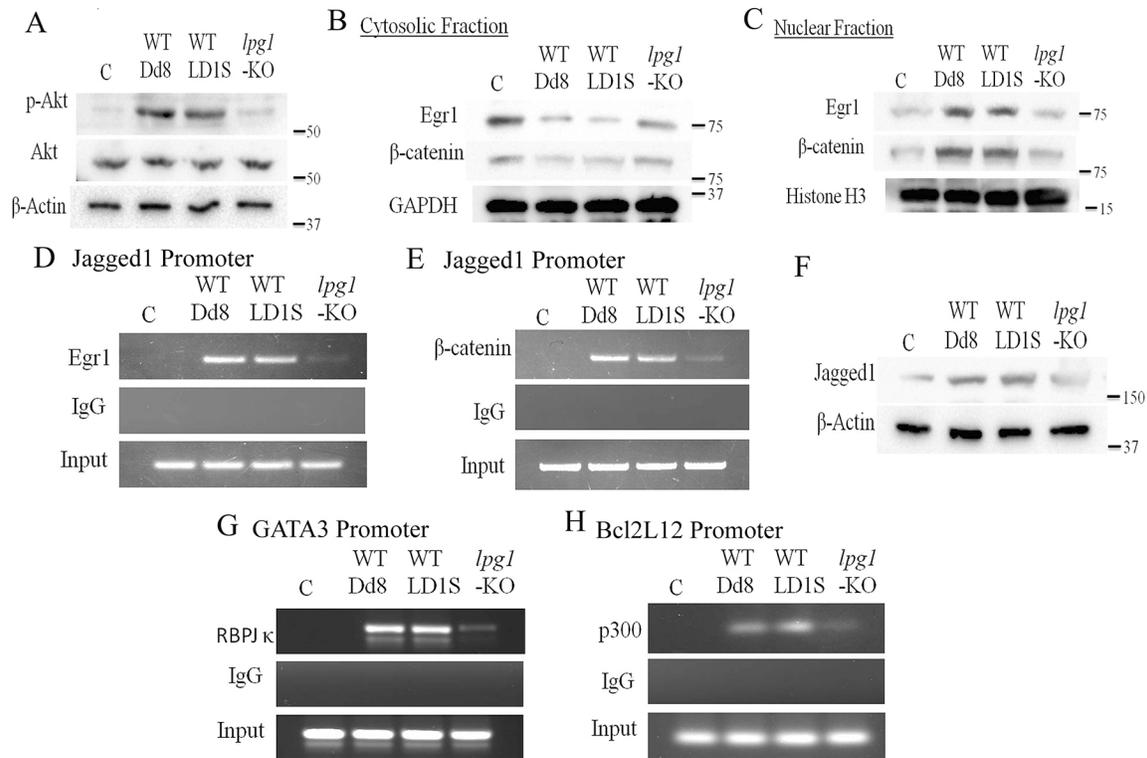
#### ***L. donovani* LPG is responsible for PI3K/Akt-mediated activation of Egr1 and β-catenin in infected BMMφs**

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, BMMφs 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 BMMφs 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 BMMφs 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 BMMφs infected with WT and *lpg1*-KO parasites. We observed reduced binding of both Egr1 and β-catenin when we infected the BMMφs 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 BMMφs infected with WT promastigotes but was less pronounced in the BMMφs 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 Bcl2L12 promoter by incubating the CD4<sup>+</sup> T cells with either the WT or *lpg1*-KO parasite-infected BMMφs. As expected, we observed reduced association of RBPJκ to the GATA3 promoter as well as p300 to the Bcl2L12 promoter when the macrophages were infected with *lpg1*-KO parasites (Fig. 5G,H) whereas the association was intact when the BMMφs 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 BMMφs, which, in turn, activates N1 ICD/RBPJκ pathway in CD4<sup>+</sup> 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 infected 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.



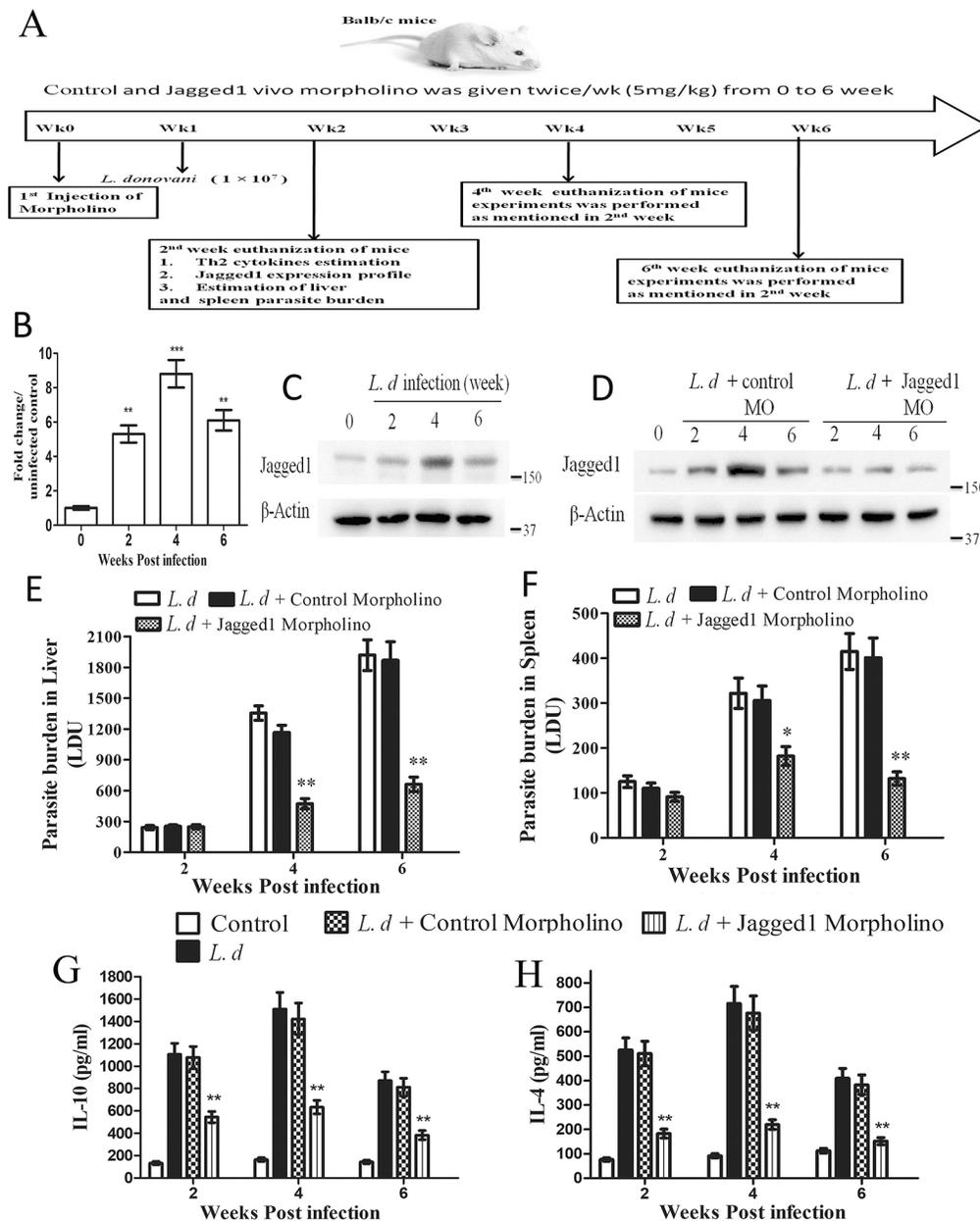
**Fig. 5. Leishmanial LPG induces PI3K/Akt-mediated Jagged1 induction in infected BMMφs.** (A) BMMφs were infected with either *L. donovani* (Dd8) promastigotes or WT or *lpg1*-KO promastigotes (*L. donovani* 1S strain) for 6 h and phosphorylation of Akt (p-Akt) was observed by immunoblot analysis. (B,C) Macrophages were infected with *L. donovani* (Dd8), *lpg1*-KO parasites or WT promastigotes for 12 h, nuclear and cytosolic extract were prepared and translocation of Egr1 and β-catenin was analyzed by immunoblotting. (D,E) BMMφs were infected with *L. donovani* (Dd8) promastigotes, WT or *lpg1*-KO promastigotes for 12 h and ChIP assays were performed to observe the binding of Egr1 and β-catenin to the Jagged1 promoter. PCR was carried out using primers specific to Jagged1 promoter region after immunoprecipitation with anti-Egr1, anti-β-catenin or control IgG antibody. (F) Macrophages were infected with WT or *lpg1*-KO promastigotes for 16 h and expression of Jagged1 was evaluated by immunoblot analysis. (G,H) Macrophages were infected with WT or *lpg1*-KO promastigotes for 24 h and then incubated with CD4<sup>+</sup> T cells. Chromatin was prepared from harvested CD4<sup>+</sup> 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.

## 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*, an obligate 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, 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).



**Fig. 6. Effect of *in vivo* knockdown of Jagged1 on parasite survival and Th2 cytokine.** (A) Schematic representation of the experimental protocol for Vivo-morpholino-mediated blocking of Jagged1 in Balb/c mice to determine parasite burden and cellular immune response. (B–D) Infected Balb/c mice were treated with control and Jagged1 Vivo-morpholino as indicated in the schematic diagram in A. Balb/c mice were infected with  $1 \times 10^7$  *L. donovani* promastigotes via the tail vein. Splenocytes were isolated from infected mice of different group and expression of Jagged 1 was evaluated in week 2, 4 and 6 by (B) isolating mRNA and checking by real time PCR and (C) by immunoblotting. (D) To verify the efficacy of Jagged1 morpholino, immunoblotting was performed in splenocytes isolated from *L. donovani*-infected mice treated either with control Vivo-morpholino or Jagged1-specific Vivo-morpholino at different time intervals as indicated. (E,F) Hepatic and splenic parasitemia was determined at week 2, 4 and 6 post infection from different groups of infected mice and expressed as the mean  $\pm$  s.d. LDU (G) IL-10 and (H) IL-4 in culture supernatants isolated from splenocytes of control, *L. donovani*-infected, *L. donovani*+control morpholino-treated mice, and *L. donovani*+Jagged1 morpholino-treated mice at week 2, 4 and 6 post infection were determined by ELISA. Results in B, E–H represent the mean  $\pm$  s.d. of two independent experiments performed in triplicate. \* $P < 0.04$ ; \*\* $P < 0.001$ , \*\*\* $P < 0.0006$  (one-way ANOVA test). Significance is indicated for the control morpholino versus Jagged1 morpholino-treated groups in E–H and between the uninfected group (0 weeks) and infected groups (2, 4 and 6 weeks) for B.

The Notch-ICD–RBPJ $\kappa$  complex trans-activates promoters containing RBPJ $\kappa$ -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 RBPJ $\kappa$  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 RBPJ $\kappa$  (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 RBPJ $\kappa$  on the GATA3 promoter, which contains putative binding site for RBPJ $\kappa$ . 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 RBPJ $\kappa$  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 RBPJ $\kappa$  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 BMM $\phi$ s (Ruhland and Kima, 2009). Similarly, *Leishmania* promastigotes engage PI3K/Akt signaling, which confers resistance to the infected BMM $\phi$ s cell from activators of apoptosis (Ruhland

et al., 2007). *L. donovani* is also known to activate Egr1 and  $\beta$ -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  $\beta$ -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 BMM $\phi$ s, 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),  $\beta$ -catenin (D10A8), p-Akt, Akt (pan), Histone H3, GAPDH (14C10) and  $\beta$ -actin were purchased from Cell Signaling Technology (Massachusetts, USA). Anti-RBPJ $\kappa$  (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),  $\beta$ -catenin (D10A8) were purchased from Cell Signaling Technology (Massachusetts, USA). ChIP grade RBPJ $\kappa$  (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, RBPJ $\kappa$ , Egr1,  $\beta$ -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 LY294002 were purchased from Sigma-Aldrich (MO, USA). Jagged1 and control Vivo-Morpholinos were purchased from Gene Tools (Oregon, USA). Further details of antibodies used for immunoblotting, ChIP assays and neutralization are shown in Tables S1, S2 and S3, respectively. Further details of siRNAs used are shown in Table S4.

### Animal ethics statement

The animal study was conducted in strict compliance with guidelines laid out by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animal experimental protocol was affirmed by the Institutional Animal Ethics Committee (registration no. 34/GO/ReBiBt-S/Re-L/99/CPCSEA) of the Council for Scientific and Industrial Research–Central Drug Research Institute (Lucknow, India) [IAEC approval no: IAEC/2020/03/Renew0 dated 03.01.2020]. For experimental studies, female Balb/c mice (20–25 gm, 6–8 week) were housed in climate-controlled and photo period controlled (12-h light/dark cycles) animal house and fed with standard rodent pellets and drinking water. Euthanasia of mice was performed by CO<sub>2</sub> inhalation at the end of the experiments.

### Isolation of murine bone marrow macrophages

For the isolation of BMDMs, femur and tibia bones from Balb/c mice were dissected, and leftover tissue on the bones was discarded. Ends of each bone were excised, and bone marrow cells were flushed out using sterile PBS. The cells were washed with PBS twice and then grown in Petri dishes in 10% FBS-containing Dulbecco's modified Eagle's medium containing L-glutamine and high glucose (11320033; Thermo Fisher Scientific, MA, USA) and supplemented with 20 ng/ml macrophage colony-stimulating factor (M-CSF, also known as CSF1), 1% glutamine and 50  $\mu$ M 2-mercaptoethanol for 3 days in a 5% CO<sub>2</sub> atmosphere at 37°C. Medium was changed on the third day and cells were washed with PBS to remove non-adherent ones. Adherent BMDMs were harvested on day 6 to carry out experiments.

### Purification of CD4<sup>+</sup> T cells from splenocytes of Balb/c mice

Balb/c mice were euthanized and spleens were gently cut and removed for preparation of single-cell suspensions. Thereafter, spleens were gently sheared and passed through a 40- $\mu$ m cell strainer to disrupt clumps. Following that, the cell suspension was spun at 200 g for 10 min at 4°C, and the contaminating erythrocytes in the splenocyte fraction was lysed with RBC lysis buffer. Cells were then centrifuged again and re-suspended in chilled MACS buffer (1 $\times$  PBS pH 7.2, 2 mM EDTA and 0.5% bovine serum albumin), and CD4<sup>+</sup> T cells were purified using CD4<sup>+</sup> magnetic beads using a CD4<sup>+</sup> T cells magnetic cell separation kit (Miltenyi Biotec). The cells were subsequently given a brief wash, centrifuged, and treated with CD4<sup>+</sup> T cells beads (1  $\mu$ l beads/10<sup>6</sup> cells) at 4°C for 20 min. After subsequent washes, the cells were passed through a MACS MS column that was washed with MACS buffer to finally obtain CD4<sup>+</sup> T cells. The elutes containing CD4<sup>+</sup> positive T cells were tested for sample homogeneity by flow cytometry (BD FACS Aria) and found to possess ~90% CD4<sup>+</sup> positive T cells.

### Macrophage infection, and co-culture with T cells

*Leishmania donovani* strain MHOM/IN/80/Dd8, *L. donovani* 1S Sudan strain and the isogenic *lpg1*-knockout (KO) mutant (Prive and Descoteaux, 2000) were cultured as promastigotes in M-199 medium (Sigma) supplemented with 10% heat-inactivated FBS (Invitrogen) at 24 $\pm$ 2°C. BMM $\phi$ s were infected with *L. donovani* promastigotes for 4 h at 10 parasites per macrophage. After infection, cells were washed at least six times to remove ungested parasites followed by incubation in complete medium for 20 h. The isolated CD4<sup>+</sup> T cells were activated (cultured with platebound anti-CD3, 2 mg/ml) and were incubated with *L. donovani*-infected BMM $\phi$ s at a ratio of 1 BMM $\phi$  for 5 CD4<sup>+</sup> T cells, and later on T cells or the existing supernatant were removed and analyzed.

### Real time PCR

In brief, total RNA from *L. donovani*-infected BMM $\phi$ s or from co-cultured CD4<sup>+</sup> T cells was isolated using the RNeasy Minikit (Qiagen). RNA integrity was confirmed and purity was assessed with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). RNA (1  $\mu$ g) from each experimental group was subjected to cDNA synthesis by using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCRs 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  $\Delta\Delta$ Ct 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-GTTTCAGAGGCGTCC-3' and reverse, 5'-AGTAGAAGGCTGTACCAA-GCAAC-3'; Jagged 2 forward, 5'-TGCTGTGGAGGTGGCTATGTCT-3' and reverse, 5'-TGTTTCCACCTTGACCTCGGT-3'; Dll1 forward, 5'-GG-ACCTCAGTGAGAGGCATATGG-3' and reverse, 5'-GGCAATTGGCTA-GGTTGTTTCATG-3'; Dll3 forward, 5'-AGTTGCACCTTCTCTACCGCG-3' and reverse, 5'-ACGGCATTATCAGGCTCTTC-3'; Dll4 forward, 5'-

GTGAAGTGCACATCAGCGATTG-3' and reverse, 5'-GTTGCAGACGA-AGTTGTTTGGG-3'; Bcl2L12 forward, 5'-TTCCGAGTTCTATGCCCTG-3' and reverse, 5'-CCAGTTTACGATGCAGAGCC-3'; GATA3 forward, 5'-CTGTGGGCTGTACTACAAGCTTCA-3' and reverse, 5'-ACCCATGG-CGGTGACCATGC-3'; and GAPDH forward, 5'-TTGCAGTGGCAAAGT-GGAGA-3' and reverse, 5'-GGGTCTCGCTCTGGAAGAT-3'.

### Immunoblot analysis and co-immunoprecipitation

Adhered BMMφs 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 5500 *g* for 10 min at 4°C to obtain clear supernatant. 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 SuperSignal™ 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 BMMφs, infected BMMφs or infected BMMφs co-cultured with CD4<sup>+</sup> 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 MgCl<sub>2</sub>, 0.5 mM PMSF, 0.5 mM dithiothreitol 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 MgCl<sub>2</sub>, 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, 4×10<sup>6</sup> cells (control BMMφ, infected BMMφs or BMMφs 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 (Parmar 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, dithiothreitol (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 with the following primers designed to amplify specific regions involving putative transcription factor-binding sites: RBPJκ forward, 5'-CAGGAGCAGGTCACATTTCA-3' and reverse, 5'-AACCTGGCTGATGTGAATCC-3'; Egr1 forward, 5'-GGCGTCCAAGCTTTAGTCTG-3' and reverse, 5'-ACTTATAGCGGGCTGCCTCT-3'; β-catenin forward, 5'-TAGAAGGGTTGAGGCGCTAA-3' and reverse, 5'-CTTTCCCGGAGT-CAGACTTG-3'; p300 forward, 5'-CAACTGGCTTTCAGGATTC-3' and reverse, 5'-ATGGCTGACTTTGGCATCT-3'; GATA3 on IL-4 forward, 5'-AGGAGAGCCAGTGGCAACCCTA-3' and reverse, 5'-CAATGCTGGC-AGAGGTCTCTCT-3'; GATA3 on IL-10 forward, 5'-CCTACTGAAGGG-AAGGTCCAG-3' and reverse, 5'-GACCTTACCATGGCCCTGTT-3'.

### siRNA-mediated knockdown in macrophages and T cells

siRNA targeted against Jagged1, RBPJκ, GATA3, Egr1 and β-catenin (Santa Cruz Biotechnology) were transfected into BMMφs 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 BMMφs 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 H<sub>3</sub>PO<sub>4</sub> 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'-CGTCCCGGCTCTA-ATATACTCCGTG-3') or control morpholino (5'-CCTCTACCTCAGT-TACAATTTATA-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; Parmar et al., 2018).

Splenocytes from all experimental groups of Balb/c mice were similarly isolated at 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

Conceptualization: P.C., S.K.; Methodology: P.C., A.S., A.R., M.D., N.P., A.D., S.K.; Validation: P.C., S.K.; Formal analysis: P.C., A.S., S.K.; Investigation: P.C., S.K.; Resources: S.K., A.D.; Data curation: P.C., A.S., A.R., M.D.; Writing - original draft: P.C., S.K.; Writing - review & editing: P.C., A.S., N.P., A.D., S.K.; Visualization: S.K.; Supervision: S.K.; Project administration: S.K.; Funding acquisition: S.K.

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### Supplementary information

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