Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing \textit{Leishmania} infection

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ABSTRACT

Intracellular survival of \textit{Leishmania donovani} demands rapid production of host ATP for its sustenance. However, a gradual decrease in intracellular ATP in spite of increased glycolysis suggests ATP efflux during infection. Accordingly, upon infection, we show here that ATP is exported and the major exporter was pannexin-1, leading to raised extracellular ATP levels. Extracellular ATP shows a gradual decrease after the initial increase, and analysis of cell surface ATP-degrading enzymes revealed induction of the ectonucleotidases CD39 and CD73. Ectonucleotidase-mediated ATP degradation leads to increased extracellular adenosine (eADO), and inhibition of CD39 and CD73 in infected cells decreased adenosine concentration and parasite survival, documenting the importance of adenosine in infection. Inhibiting adenosine uptake by cells did not affect parasite survival, suggesting that eADO exerts its effect through receptor-mediated signalling. We also show that \textit{Leishmania} induces the expression of adenosine receptors A\textsubscript{2A}R and A\textsubscript{2B}R, both of which are important for anti-inflammatory responses. Treating infected BALB/c mice with CD39 and CD73 inhibitors resulted in decreased parasite burden and increased host-favourable cytokine production. Collectively, these observations indicate that infection-induced ATP is exported, and after conversion into adenosine, propagates infection via receptor-mediated signalling.

KEY WORDS: ATP, Ectonucleotidase, Adenosine receptor, \textit{Leishmania donovani}, Macrophage

INTRODUCTION

\textit{Leishmania donovani}, the causative agent of fatal visceral leishmaniasis, is an obligate intracellular parasite inoculated into mammals by the bite of its vector, the sand fly. Once transmitted to the host, the infective promastigote forms are rapidly phagocytosed by macrophages (de Menezes et al., 2016) and the parasite, along with combating the antimicrobial defence arsenals of the macrophages, has to survive within the harsh environment of the phagolysosomes (Cunningham, 2002). In order to succeed, \textit{Leishmania} requires high energy levels; thus, the energy needs of both the parasite for its survival and of the host cell to resist infection, have drawn attention (Schaible and Kaufmann, 2007). As the intracellular parasite and the host cell utilise the same host energy sources, knowledge about the source of cellular energy and the pathway in which it is utilised within infected macrophage seems extremely important to address the molecular signalling of infection.

Adenosine 5′-triphosphate (ATP), the universal energy currency of a cell, fuels various vital processes necessary for maintenance of a cell and also acts as an intracellular and extracellular messenger (Corriden and Insel, 2010; Trautmann, 2009). The main source of ATP is the catabolic part of carbohydrate metabolism where glycolysis is followed by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (O’Neill and Pearce, 2016). However, during certain infections or inflammatory conditions, the pyruvate generated via glycolysis gets converted into lactate and leads to lesser but faster ATP production (Kelly and O’Neill, 2015). ATP generated within a cell, apart from acting as a source of energy, may act as a signalling molecule both intracellularly and even extracellularly (Corriden and Insel, 2010; Trautmann, 2009). Various stimuli such as stress, hypoxia, inflammation and reactive oxygen species (ROS) are found to induce ATP release from cells with the help of various ATP release channels such as the maxi-anion channel, the gap junction hemichannel, the P2X7 receptor and even via vesicular exocytosis in a non-lytic mechanism (Kojima et al., 2017). Released extracellular ATP (eATP) often acts as a signalling molecule and regulates various cellular processes (Corriden and Insel, 2010). Initially, it was found to play an important role in modulating the nervous and vascular systems (Housley et al., 2009; North and Verkhratsky, 2006; Schwiebert et al., 2002). Recent studies have shown that eATP may inhibit infection by intracellular bacterial pathogens (Coutinho-Silva et al., 2003; Lammas et al., 1997) and also regulate chemotaxis of immune cells (Chen et al., 2006; Kronlage et al., 2010). However, the concentration of eATP is critically maintained as a high eATP level acts as a ‘danger signal’ and can promote inflammation through the P2X7-receptor-dependent activation of the NLRP3 inflammasome, leading to production of pro-inflammatory cytokine IL-1β (Cassel and Sutterwala, 2010). The ATP level in the extracellular environment is usually maintained with the help of various ATP hydrolysing enzymes such as ecto-apyrase, pyrophosphatas, phosphodiesterases and ecto-adenosine triphosphatases (eATPase), amongst which the prevalent one is CD39 (also known as ENTPD1), a member of the ectonucleotide diphosphohydrolase (e-NTPase) family, CD39 dephosphorylates ATP to ADP and AMP (Antonioli et al., 2013). AMP thus generated is further converted into adenosine 5′-monophosphate (ADO) by the ecto-5′-nucleotidase CD73 (also known as NT5E). These enzymes are found to be expressed on the surface of macrophages, endothelial cells and various subsets of lymphocytes (Antonioli et al., 2013). Therefore, these enzymes are not only important for maintaining intermediate metabolite concentration but also play a significant role in modulating cellular immune responses. CD73 expression on tissues and T helper cells was found to promote...
colonisation and thus prolonged murine salmonellosis infection (Alam et al., 2014). Infection of dendritic cells with different species of Leishmania has also been shown to upregulate the expression and activity of CD39 and CD73 (Figueiredo et al., 2012), although the consequences have not been studied in detail.

Extracellular adenosine (eADO) generated by CD39 and CD73 is further acted upon by adenosine deaminase (ADA), which catalyses the deamination of adenosine to inosine. This is the only enzyme known to regulate the adenosine concentration, and absence of ADA has been associated with increased eADO level, leading to anti-inflammatory responses (Cronstein, 1994; Haskó and Cronstein, 2013). Adenosine is an immunomodulator generated at sites during tissue injury, inflammation and ischemia, and mainly elicits its action on cells through one of its four G-protein-coupled receptors: A_1, A_2A, A_2B and A_3 (Fredholm et al., 2001; Olah and Stiles, 1995). Adenosine was found to inhibit superoxide production, adhesion of cells to the endothelial barrier (Bouma et al., 1996), and proliferation and expansion of T-cells through the A_2A receptor (Hoskin et al., 2008; Huang et al., 1997). A_1R on the other hand, has been found to transduce pro-inflammatory signalling of adenosine (Haskó and Cronstein, 2004). Thus, the effect of adenosine on a given cell depends on the adenosine receptor subtype present and the signal it transduces.

The present study was initiated to address how the infective parasite and the infected macrophage cope with the demand for rapid requirement of ATP. Characterisation of infected host cell metabolic profiling and possible ATP efflux encouraged us to determine the fate of the extracellular ATP produced. Further investigation revealed that ATP is exported in the extracellular environment via a specific channel and gets hydrolysed to adenosine by cell surface ectonucleotidases. This not only enables the parasite to escape macrophage immune activation, but creates an anti-inflammatory cellular environment necessary for intra-macrophage survival of Leishmania through receptor-mediated signalling.

RESULTS

Host ATP generation during L. donovani infection
Successful survival and replication of intracellular pathogens require substantial amount of energy, i.e. ATP production. To quantify the energy status of an infected cell, we measured the intracellular ATP (iATP) concentration, which is expected to be increased following L. donovani infection. However, the iATP level showed a decreasing pattern up to 8 h post-infection in RAW macrophages (Fig. 1A). This contradictory observation led us to check the level of ATP in peritoneal macrophages, which also depicted a similar profile (Fig. 1B). Therefore, to investigate the metabolic status of RAW 264.7 macrophage cells during the early stages of L. donovani infection (0–8 h), the extracellular acidification rate (ECAR) and the mitochondrial oxygen consumption rate (OCR) were measured using live-cell extracellular flux analysis. ECAR values were found to be higher in infected cells (3.6-fold increase compared with uninfected cells at 3 h post-infection, P<0.001) indicating higher glycolytic activity (Fig. 1C). In contrast, the OCR values at 3 h post-infection showed a significant reduction (29.5% decrease compared with uninfected control, P<0.001), suggesting a lower mitochondrial respiration rate (Fig. 1C). A similar profile was obtained for peritoneal macrophages (Fig. 1D). Expression of the three enzymes involved in the irreversible steps of glycolysis, namely hexokinase 1 (HK1), phosphofructokinase (PFk) and pyruvate kinase M2 (Pkm2) also showed significant transcriptional upregulation during infection (3.3-, 4.2- and 5.1-fold, respectively, compared with uninfected control at 4 h post-infection, P<0.001) (Fig. 1E). In order to determine whether this upregulation of glycolysis is induced by live L. donovani, the transcript level of the enzymes was also assessed with paraformaldehyde fixed (PFA Fx) L. donovani, which showed almost unaltered levels of all three enzymes 4 h post-infection (Fig. 1F). Reduced mitochondrial respiration (Fig. 1C,D), led us to check whether pyruvate generated in infected macrophage cells from increased glycolysis is converted to lactate instead of entering the TCA cycle. Extracellular lactate concentration was found to be gradually increased with a maximum (3.5-fold increase, P<0.001) at 8 h post-infection (Fig. 1G), which corroborated well with our earlier observations. However, PFA-fixed L. donovani did not show any significant change in lactate level (Fig. 1G). To find out whether this early upregulation of glycolysis has an effect on intra-macrophage survival of L. donovani, macrophages were pre-treated with a graded concentration of 2-deoxyglucose (2-DG), an inhibitor of glycolysis, for 4 h followed by infection with promastigotes for 24 h. Treatment with 2-DG did not affect the viability of RAW 264.7 cells but the number of intracellular parasites was found to be significantly reduced at 10 mM concentration of 2-DG (59.9% decrease compared with untreated infected cells, P<0.01) (Fig. 1H). However, treatment of both promastigotes and axenic amastigotes with 10 mM 2-DG did not substantially affect parasite viability (Fig. S1B). Taken together, these results indicate that L. donovani infection causes a metabolic shift towards glycolysis during the initial hours of infection, which is essential for parasite survival within macrophages. However, the ATP generated via glycolysis is either utilised by the cell or effluxes to the extracellular environment.

ATP efflux during infection promotes parasite survival
Since ATP concentration showed a decreasing pattern (Fig. 1A,B) in spite of a higher glycolytic rate in infected macrophages, to understand the fate of glycolysis generated ATP, we measured the extracellular ATP concentration of infected cells, as macrophages are capable of releasing ATP (Dosch et al., 2018). Accordingly, it was found that at 2 h post-infection, the ATP concentration was significantly higher in the cell culture supernatant in comparison to uninfected cells (2.9- and 2.6-fold increase in RAW and peritoneal macrophages, respectively, P<0.001), indicating release of extracellular ATP during infection (Fig. 2A). Pre-treatment of infected macrophages with 2-DG, reduced the eATP concentration implying that the infection-induced ATP release was glycolysis dependent (Fig. 2A). Next, to evaluate the mechanism of ATP release, infected RAW cells were pre-treated with 100 µM amounts of inhibitors of various ATP release channels {10Panx for pannexin-1 (also known as PANX1; Pelegrin and Surprenant, 2006), carbonoxolone (CBX) for pannexin and connexin-related channels (Chekeni et al., 2010), flufenamic acid (FFA) for anion transporter (Guinamard et al., 2013) and gadolinium chloride (GdCl₃) for maxi-anion channel (Zhao et al., 2017)}, then the extracellular ATP level was measured at 2 h post-infection. Of all the inhibitors, 10Panx and CBX treatment led to significant reduction of extracellular ATP level (Fig. 2B). However, the extent of inhibition was much less (36.2% decrease, P<0.01) in case of CBX in comparison to 10Panx, which showed a 56.3% decrease (P<0.001) (Fig. 2B). Higher doses of 10Panx and CBX did not lead to any further significant decrease in the eATP concentration, as revealed by a dose titration experiment (Fig. 2C). Maximum significant inhibition was obtained at a dose of 100 µM for both 10Panx and CBX (Fig. 2C), which agreed with the administered dose used in our inhibition study (Fig. 2B). Since inhibition of 10Panx exerted maximum effect on ATP release, we next assessed the expression of pannexin-1 in infected macrophages at both the mRNA and protein level. Expression of pannexin-1 was found to be significantly higher in both cases (4.3- and 6.0-fold
induction for mRNA and protein level at 2 h post-infection, respectively, compared with uninfected control, $P < 0.001$) up to 6 h post-infection (Fig. 2D,E). Inhibition of export of ATP from infected macrophages using $10^{-9}$M 10Panx further led to a significant reduction in the number of intracellular parasites (56.9% decrease over infected control, $P < 0.001$) (Fig. 2F). Infected cells when supplemented with 100 µM ATP showed a marked reversal in the inhibition of intracellular parasite count even upon treatment with $10^{-9}$M 10Panx (1.6-fold increase over infected cells pre-treated with $10^{-9}$M 10Panx, $P < 0.01$) (Fig. 2F) thereby suggesting the relevance of ATP export during infection. The cell viability of the promastigotes and axenic amastigotes in the presence of 100 µM 10Panx was found to be almost unaltered (Fig. S1B). These observations suggest that the ATP generated by the increased rate of glycolysis during L. donovani infection gets exported outside cell, mostly through pannexin-1, and this export seems to be beneficial for intracellular parasite survival.

**Role of macrophage ectonucleotidases in the hydrolysis of eATP**

Excess eATP is also detrimental for cell viability as it may lead to inflammatory signals and even to apoptosis (Cauwels et al., 2014). Therefore, the time kinetics of eATP level was investigated in...
infected peritoneal macrophages during the early hours of infection (Fig. 3A). The eATP level started decreasing from 2 h post-infection and was markedly reduced at 8 h post-infection when infected with live promastigotes (Fig. 3A). Unlike live *Leishmania*, PFA-fixed *L. donovani* significantly increased the extracellular ATP concentration even during the later stages of infection (6 and 8 h) (Fig. 3A). In order to counter the detrimental effect of eATP, cells normally utilise various ATP hydrolysing enzymes to control the levels of ATP (Antonioli et al., 2013). We therefore evaluated expression of the main ATP hydrolysing enzymes of macrophage cells: CD39, CD73 and adenosine deaminase (ADA). Time kinetics revealed increased expression of CD39 and CD73 in both RAW and peritoneal macrophages as observed up to 24 h post-infection (2.8- and 3.9-fold in RAW and 2.6- and 3.8-fold in peritoneal macrophages, respectively, compared with uninfected control at 6 h post-infection, *P*<0.001) (Fig. 3B,C). However, expression of ADA was found to be very low throughout the time course in both cell types (Fig. 3B,C). The expression kinetics of macrophage-associated ectonucleotidases were also assessed following infection with PFA-fixed *L. donovani*, which showed almost undetectable expression of all the three enzymes CD39, CD73 and ADA (Fig. 3D). Increased expression of CD39 and CD73 prompted us to evaluate their ectonucleotidase activity. To this end, infected peritoneal macrophages were incubated in the presence of ATP (to evaluate CD39 activity) or AMP (to evaluate CD73 activity) and the ectonucleotidase activity was assessed by measuring inorganic phosphate (Pi) release (Fig. 3E,F). A time-dependent increase in hydrolysis was found for both ATP and AMP, and pre-treatment of cells with specific inhibitors of CD39 (POM-1) and CD73 (APCP) reversed this hydrolysis, thereby documenting specific involvement of CD39 and CD73 in hydrolysing extracellular ATP. Low expression of ADA indicated that extracellular adenosine produced during nucleotide hydrolysis might not be processed further and extracellular adenosine level indeed showed an increasing pattern with maximum level 8 h post infection (3.4-fold increase in comparison to uninfected cells, *P*<0.001) (Fig. 3G). On the other hand, infection with PFA-fixed *L. donovani* failed to increase the eADO level as opposed to infection with live parasites.
(Fig. 3G), thereby suggesting specific contribution of live parasites in converting host ATP to eADO. In order to determine whether generated adenosine aids in parasite survival, infected macrophages were pre-incubated with increasing concentrations of POM-1 and APCP. It was found that there was a significant decrease in the number of intracellular parasites upon treatment with either POM-1 (100 µM) or APCP (100 µM) (54.5% and 58.6% reduction, respectively, compared with infected control, \( P < 0.001 \)) (Fig. 3H). However, neither POM-1 nor APCP had any effect on the viability of the macrophages or promastigotes or axenic amastigotes at the above concentrations (Fig. S1A,B). Infected cells were also treated with POM-1 and APCP in the presence or absence of adenosine

Fig. 3. See next page for legend.
in comparison to uninfected cells at mRNA level and 2.9- and 4.5-fold, respectively, at protein level, $P<0.001$ (Fig. 4C,D). The observation was further verified by fluorescence microscopy using Texas Red-conjugated anti-A$_{2A}$R antibody and FITC labelled anti-A$_{2B}$R antibody, which showed significantly enhanced expression of both A$_{2A}$R and A$_{2B}$R (Fig. 4E). Next, to determine the role of adenosine receptor-mediated signalling, infected macrophages were pre-treated with specific inhibitors of A$_{2A}$R and A$_{2B}$R, ZM 241385 and MRS 1754, respectively, and parasite survival was determined by measuring intracellular parasite count. Individual treatment with either ZM 241385 (1 $\mu$M) (Kreckler et al., 2006) or MRS 1754 (1 $\mu$M) (Kreckler et al., 2006) led to moderate decrease in intracellular parasite count (48.1% and 54.2%, respectively, compared with the control, $P<0.001$) (Fig. 4F). However, a marked reduction in intracellular parasite count was obtained when both the inhibitors were used in combination (61.5% reduction compared with control infected, $P<0.001$) suggesting synergistic inhibition (Fig. 4F). None of the inhibitors showed any significant effect on macrophage or parasite survival in promastigote or axenic amastigote culture (Fig. S1A,B). Furthermore, this inhibition was obtained despite the presence of adequate concentration of adenosine in extracellular environment (Fig. 4F, inset) confirming that adenosine-mediated effects were exerted by specific receptors. To investigate whether inhibitor treatment modulates the expression profile of A$_{2A}$R and A$_{2B}$R, control and infected macrophages were pre-treated with either ZM 241385 or MRS 1754, and expression of A$_{2A}$R and A$_{2B}$R was determined by western blot analysis. The expression profile of both receptors remained unaltered upon treatment with specific inhibitors (Fig. 4G), confirming that inhibitors modulate only the receptor-mediated signalling, not their expression level. Since adenosine is an endogenous regulator of inflammation (Haskó and Cronstein, 2013), we investigated whether this upregulated expression of A$_{2A}$R and A$_{2B}$R following infection had any role in modulation of cytokine profile, by measuring levels of cytokines and chemokines in infected cells pre-treated with ZM 241385 and MRS 1754. LPS-induced production of IL-12 and TNF-$\alpha$, which was significantly decreased in L. donovani-infected cells, was found to be increased upon treatment with ZM241385 (1 $\mu$M) (4.1- and 3.0-fold increase for TNF-$\alpha$ and IL-12, respectively, compared with infected cells, $P<0.001$ and $P<0.01$, respectively) (Fig. 4H). However, pre-treatment with the A$_{2B}$R-specific antagonist MRS1754 (1 $\mu$M) did not affect pro-inflammatory cytokine production (Fig. 4H). On the other hand, the infection-induced increased IL-10 and TGF-$\beta$ production was found to be reduced by treatment with MRS 1754 (71.8% and 44.1% reduction in comparison to infected cells, $P<0.001$) and did not show any significant change when infected cells were pre-treated with ZM 241385 (Fig. 4I). Pre-treatment of infected cells with adenosine (100 $\mu$M) marginally increased the levels of IL-10 and TGF-$\beta$ (Fig. 4I). Like cytokines, inflammatory chemokines CCL3 and CCL5 production were also upregulated over infected control on a very small scale upon treatment with ZM (Fig. 4I). However, MRS1754 did not seem to have any effect on either CCL3 or CCL5 modulation. All these observations suggest that extracellular adenosine produced during infection exerts its effect via the receptors A$_{2A}$R and A$_{2B}$R and maintains an intracellular anti-inflammatory milieu necessary for parasite survival.

**Effect of the ectonucleotidase inhibitors POM-1 and APCP on infection in BALB/c mice**

To evaluate the importance of extracellular ATP degradation on parasite survival in visceral leishmaniasis, L. donovani-infected cells were infected with live or PFA-fixed L. donovani promastigotes for the indicated time periods and the eATP concentration in the culture supernatant was measured. Results are shown as bar graphs. (A) Peritoneal macrophages (PM)$\phi$s were infected with live L. donovani promastigotes for indicated time periods (0–24 h) and the protein level expression of CD39, CD73 and ADA measured by immunoblotting in RAW (B) and PM)$\gamma$s (C) cells. (D) Peritoneal macrophages were infected with PFA-fixed L. donovani promastigotes for the indicated time periods and the protein level expression of CD39, CD73 and ADA measured by immunoblotting. $\beta$-actin was used as an endogenous control. (E) PMFs were infected with L. donovani promastigotes alone or in the presence of increasing concentrations of POM-1 (50, 100 and 200 $\mu$M). Ectonucleotidase activity was then measured for CD39 by the amount of P$_{i}$ released using ATP as substrate. (F) PMFs were infected with L. donovani promastigotes for the indicated time periods and extracellular adenosine concentration (eADO) was measured in culture supernatant. (H) PMFs were infected with L. donovani for 24 h in the presence of increasing concentrations of POM-1 (50, 100 and 200 $\mu$M) or APCP (50, 100 and 200 $\mu$M) and the number of parasites per 100 macrophages were analysed by DAPI staining. (I) Number of parasites per 100 macrophages were measured in 24-h-infected PMFs in the presence of POM-1 (100 $\mu$M) and APCP (100 $\mu$M) alone or in combination of POM-1+APCP+ADO (100 $\mu$M) or ATP-$\gamma$S (100 $\mu$M). Bands were quantified by densitometry and are shown as bar graphs. Results are representative of three independent experiments and error bars are expressed as means $\pm$ s.d. $n=3$; ns, not significant, **$P<0.01$, ***$P<0.001$ (Student's t-test).

(ADO) or non-hydrolysable ATP analogue (ATP-$\gamma$S) (Fig. 3I). The extent of inhibition in the presence of POM-1 and APCP in combination was found to be 62.4%, which is not a very significant extent of inhibition in the presence of POM-1 and APCP in APCP-treated cells was found to be markedly reversed upon treatment with adenine (100 $\mu$M) (2.2-fold increase in comparison to parasite count in PMF-1- and APCP-treated cells, $P<0.001$) (Fig. 3I). All these observations suggest that extracellular ATP generated during infection is degraded by the cell surface ectonucleotidases to produce adenosine to propagate infection. Extracellular adenosine exerts its effect via A$_{2A}$ and A$_{2B}$ receptors

The fact that increased extracellular adenosine concentration facilitated parasite survival prompted us to determine its role on infection. Adenosine generated by the action of ectonucleotidases may either be taken up by the host cell through nucleoside transporters (Khan et al., 2007) or may exert its effect through signalling via specific cell surface receptors (Hasko and Cronstein, 2004). However, pre-treatment of cells with various concentrations of the adenosine uptake blocker dipyridamole (Di) did not have any effect on the intracellular parasite count (Fig. 4A) suggesting thereby that adenosine exerts its effect via specific adenosine receptor-mediated signalling. Adenosine primarily acts through one of the subtypes of four G-protein-coupled receptors A1, A$_{2A}$, A$_{2B}$ and A3 to establish an anti-inflammatory environment (Fredholm et al., 2001; Olah and Stiles, 1995). Infection increased expression of both A$_{2A}$R receptor (A$_{2A}$R) and A$_{2B}$ receptor (A$_{2B}$R) (2.2- and 3.0-fold, respectively, compared with control, $P<0.001$), whereas almost undetectable expression was obtained for A1R and A3R (Fig. 4B). Time kinetics studies further showed persistence of enhanced expression of A$_{2A}$R and A$_{2B}$R at both the mRNA and protein level (2.9- and 3.4-fold, respectively, at 8 h post-infection...
mice were administered intra-peritoneally (i.p.) with either the CD39-specific inhibitor POM-1 or the CD73-specific inhibitor APCP. Dose-titration experiments were carried out to assess the efficacy of the inhibitors with a dose range of 2.5–20 mg/kg body weight/day given up to 4 weeks at 3 day intervals starting at 11 days post-infection. The infection was allowed to proceed for 4 weeks, after which the animals were sacrificed, and the anti-leishmanial potency was assessed in terms of parasite burden in liver and spleen. During the experimental period, all the animals remained healthy. Maximum suppression of organ parasite burden was obtained...
at a dose of 10 mg/kg/day (Fig. 5A,B). Next, to find out the effectiveness of POM-1 and APCP on the progression of visceral leishmaniasis, infected BALB/c mice were injected with POM-1 and APCP at a dose of 10 mg/kg body weight/day up to 4 weeks at 3 day intervals after 11 days of infection (Fig. 5C) and the liver and spleen parasite burden (LDU) was determined. In both POM-1- and APCP-treated mice, the cells were treated with adenosine (100 µM) for measurement of the cytokines (I,J). Bands were quantified by densitometry and are shown as bar graphs. Results are representative of three independent experiments and error bars are expressed as mean±s.d. *n=5; ns, not significant, **P<0.01, ***P<0.001 (Student’s t-test).
APCP-treated mice, the parasite burden was found to be markedly lowered (65.1% and 71.4% in liver and 68.6% and 74.8%, respectively, in spleen at 4 weeks post-infection compared with infected control, P<0.001) (Fig. 5D,E). Haematoxylin and Eosin (H&E) staining of sections of liver from both POM-1- and APCP-treated infected mice at 4 weeks post-infection revealed well-defined zones of granuloma formation, a hallmark of macrophage infiltration, compared with untreated infected mice (Fig. 5F). Although POM-1 or APCP treatment did not cause any visible health issues, the tissue architecture was found to be a little disarranged even when administered without infection (Fig. S1C). The number of hepatic mature granulomas was found to be significantly increased in sections from both POM-1- and APCP-treated infected mice at 4 weeks post-infection in comparison to untreated infected mice (Fig. 5G). The protein expression of CD39 and CD73 was also determined in splenocytes isolated from control, infected and POM-1 and APCP administered mice after 4 weeks of infection (Fig. 5H). Although expression of CD39 and CD73 was significantly increased in splenocytes of infected mice at 4 weeks, treatment with the inhibitors did not show any change in the expression of CD39 and CD73. This is not surprising as POM-1 and APCP inhibit the ectonucleotidase activity rather than the expression level of CD39 and CD73. The levels of pro-inflammatory cytokines TNF-α and IL-12 were next analysed in the supernatant of splenocytes isolated from control, infected and POM-1- and APCP-treated infected mice at 4 weeks post-infection. In the case of POM-1 and APCP treatment, a marked increase in the level of TNF-α and IL-12 was observed at 4 weeks post-infection compared with infected controls (774.6±39.8 pg/ml TNF-α and 974.4±22.9 pg/ml IL-12 for POM-1 and 769.4±22.1 pg/ml TNF-α and 990.4±30.9 pg/ml IL-12 for APCP compared with 102.6±21 pg/ml TNF-α and 101.4±24.8 pg/ml IL-12 for infected control, respectively, P<0.001) (Fig. 5I). In contrast, both POM-1 and APCP treatment led to marked decrease in the level of IL-10 and TGF-β evaluated at 4 weeks post-infection (403.2±35.3 pg/ml IL-10 and 439.1±52.1 pg/ml TGF-β for POM-1 and 408.4±22.8 pg/ml IL-10 and 434.4±50.7 pg/ml TGF-β for APCP compared with 1333.2±48.2 pg/ml IL-10 and 748.2±56.8 pg/ml TGF-β for infected control, respectively, P<0.001) (Fig. 5J). Apart from regulating pro- and anti-inflammatory cytokines, POM-1 and APCP significantly modulated the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. Both POM-1- and APCP-treated infected mice showed increased levels of IFN-γ (679.3±20.0 pg/ml for POM-1 and 597.7±27.5 pg/ml for APCP compared with 99.7±24.3 pg/ml for infected control, P<0.001) and IL-4, a decrease in concentration was observed in inhibitor-treated infected mice (167.0±25.9 pg/ml for POM-1 and 178.3±30.5 pg/ml for APCP compared with 406.7±27.1 pg/ml for infected control, P<0.001) (Fig. 5J). While measuring the cytokines in splenocytes isolated from infected inhibitor-treated mice, in one set of experiments, adenosine was added in the culture medium. The presence of adenosine partially reversed the inhibitory effect of POM-1 and APCP on cytokine production, thereby suggesting the importance of adenosine-receptor mediated signalling in infection (Fig. 5J). All these results validate our in vitro observations and further suggest the potential of CD39 and CD73 as chemotherapeutic targets in visceral leishmaniasis.

**DISCUSSION**

Immediately upon entry inside the macrophages, *Leishmania* not only has to fight against the antimicrobial defence system of the host, but also has to adjust to the harsh environment inside phagolysosomes (Cunningham, 2002; Liu and Uzonna, 2012). This process calls for a rapid supply of energy, i.e. ATP, within the infected host cell. The present study was performed to delineate the source and fate of ATP of infected macrophages; which apart from being an energy currency, may also act as an important intracellular and extracellular signalling molecule (Corrêda and Insel, 2010; Trautmann, 2009). In the study, we documented that upon *L. donovani* infection, there is a diminishing pattern of intracellular ATP during early hours of infection even though there was an increased rate of host cell glycolysis (Warburg effect) suggesting that there is an upregulated ATP concentration in infected cells. It was found that the parasite effluxes excess ATP to the extracellular environment via ion exporter pannexin-1. An increase in extracellular ATP may lead to inflammation-mediated host cell immune activation (Casale and Sutterwala, 2010) and to escape this, the parasite activates the host macrophage’s ATP-degrading enzymes CD39 and CD73 for converting extracellular ATP (eATP) to adenosine. Extracellular adenosine signals through adenosine receptors A2A R and A2B R and provides an anti-inflammatory milieu essential for parasite survival (Fig. 6). To our knowledge, this is the first comprehensive report to demonstrate that ATP produced through altered host cell metabolism not only serves as an energy provider, but it also leads to generation of extracellular adenosine as an immune signalling molecule to facilitate infection (Fig. 6).

ATP is the primary energy currency of a cell, and according to our hypothesis, the iATP level of infected macrophages should increase. However, quantification of the iATP level significantly declined over time and this compelled us to evaluate the source of ATP, i.e. the metabolic status of *Leishmania*-infected macrophages during early infection. Contradictory to the iATP level, infection resulted in a heightened rate of glycolysis followed by lactate production. Intracellular pathogens are at times known to exploit host metabolic resources for their own benefit (Abu Kwaik and Bumann, 2013). Alteration of carbohydrate metabolism, the major ATP-generating pathway of the cell has also been reported (Bravo-Santano et al., 2018; Czyz et al., 2017). Pyruvate produced via glycolysis usually enters into the TCA cycle, but under certain circumstances gets converted into lactate. Although this is energetically less efficient compared with oxidative phosphorylation, it provides energy at a much faster rate. Intracellular infection by various pathogens like *Plasmodium berghei*, *Mycobacterium tuberculosis* and *Toxoplasma gondii* were also found to induce glycolysis and favour the intracellular persistence of the parasites (Li et al., 2008; Nelson et al., 2008; Shi et al., 2015). Metabolic profiling data of dengue virus-infected human cells have also shown that upregulation of glycolysis favours virus replication (Fontaine et al., 2015). Transcriptomic analysis of *L. major*-infected BMDM cells and extracellular flux analysis of *L. infantum*-infected cells have also shown an early upregulation of glycolysis following infection (Moreira et al., 2015; Rabhi et al., 2012).

However, the significant decrease in iATP level despite a higher glycolytic rate needed an explanation. Although ATP is a chemical source of cellular energy, it also plays an important role as a signalling molecule. An increase in cellular ATP concentration may lead to induction of apoptosis, and inhibition of macrophage apoptosis has been considered to be a survival strategy of the parasite (Saha et al., 2018). One way out for *Leishmania* to get rid of excess ATP is via its efflux from the host cell. Extracellular ATP level in infected macrophages indeed showed an increase over control. Inhibition studies in infected macrophages documented that amongst ATP release channel proteins (Kojima et al., 2017), pannexin-1 was shown to be of interest (Saha et al., 2018). One way out for *Leishmania* to get rid of excess ATP is via its efflux from the host cell. Extracellular ATP level in infected macrophages indeed showed an increase over control. Inhibition studies in infected macrophages documented that amongst ATP release channel proteins (Kojima et al., 2017), pannexin-1 was shown to be of interest (Saha et al., 2018).
with *P. falciparum* infection of human erythrocytes, where ATP is released via pannexin-1 channels (Alvarez et al., 2014). A significant increase of ATP in the cell supernatant was also found in vesicular stomatitis virus-infected cells and mice, where the increase took place in an exocytosis- and pannexin channel-dependent manner, leading to a reduction in virus replication (Zhang et al., 2017).

ATP in the extracellular environment (eATP) is kept under tight regulation as this molecule primarily acts as a ‘signal’ leading to activation of an inflammatory state. eATP is known to induce IL-12 production (Langston et al., 2003; Schurr et al., 2000) and even lead to elimination of intracellular pathogens (Junger, 2011; Langston et al., 2003; Piccini et al., 2008). *L. amazonensis* has been shown to be sensitive in response to eATP possibly because of induction of host cell apoptosis (Chaves et al., 2009). However, in the present study, eATP showed a gradual decrease over time and coincided well with increased expression of two major ATP degrading enzymes, CD39 and CD73. Infection with *L. braziliensis*, *L. amazonensis* and *L. major* has also been shown to increase the expression and activity of CD39 and CD73 in dendritic cells (Figueiredo et al., 2012). However, a study involving PBMCs isolated from patients of visceral leishmaniasis has shown decreased frequency of CD73+ T-cells in comparison to healthy controls (Rai et al., 2011). This could be because similar stimulus is capable of differential induction of the same enzymes in different cell types but more investigation is needed to be carried out to confirm this. Although *Leishmania* upregulated the expression of both CD39 and CD73, the expression of ADA, which irreversibly deaminates adenosine, the final product of the reaction catalysed by CD73, remained almost undetectable. An increased level of extracellular adenosine during infection further validated the observation. Moreover, decreased parasite survival in the CD39 and CD73 inhibited system was found to be reversed by adenosine added externally, which suggests that the parasite needs adenosine and deliberately keeps the expression of ADA at a low level. A similar finding was reported in *L. braziliensis* infection, where the presence of adenosine increased the severity of the disease (de Almeida Marques-da-Silva et al., 2008). It is worth mentioning that expression of CD26, which helps ADA to anchor on the surface of host cells, has been found to be impaired in visceral leishmaniasis (Rai et al., 2012). The parasite *Leishmania* itself is also found to express surface ectonucleotidases to convert tri-nucleotides like ATP to adenosine (Berrêdo-Pinho et al., 2001; Meyer-Fernandes et al., 1997; Vasconcellos Rde et al., 2014). It has been shown that increased ectonucleotidase activity on the surface of these parasites is directly correlated with increased infectivity and virulence levels of the parasite (de Almeida Marques-da-Silva et al., 2008; Maioi et al., 2004; Peres et al., 2017). *L. amazonensis* has been found to have the highest activity of ectonucleotidases, leading to a higher concentration of adenosine (de Souza et al., 2010; Leite et al., 2012). *Leishmania* promastigotes having higher ectonucleotidase activity were also found to have greater inhibitory effect on immune activation of dendritic and Th cells (de Almeida Marques-da-Silva et al., 2008; Ribeiro et al., 2017). However, following engulfment by macrophages, *Leishmania* will be present within the phagolysosomes (Cunningham, 2002) and *Leishmania* ectonucleotidases probably will not have any effect on the macrophage’s extracellular ATP concentration. Adenosine is a well-known anti-inflammatory molecule and has been found to favour the intracellular growth of many pathogens. Pathogenesis of *Toxoplasma*, *Salmonella* and *Helicobacter* has also been found to be dependent on the presence of host adenosine (Alam et al., 2009; Costales et al., 2011).
2018; Mahamed et al., 2015). Adenosine generated outside a cell may exert its effect either by entering the cell through nucleoside transporter channels or through adenosine-receptor-mediated signalling pathway (Haskó and Cronstein, 2004; Khan et al., 2007). Both instances were found to be associated with creating an anti-inflammatory milieu inside the cell (Haskó and Cronstein, 2004), and justified the action of *Leishmania* to maintain a high adenosine level in the extracellular environment. Treatment with the adenosine uptake blocker dipyridamole, did not have any effect on the intracellular survival of parasites and indicated the involvement of adenosine receptors in *L. donovani* infection. Accordingly, we found that among the four adenosine receptor subtypes A₁, A₂A, A₂B and A₃, infection led to specific upregulation of A₂A and A₂B. Inhibition of signal transduction via these receptors showed upregulation of pro-inflammatory cytokines and downregulation of anti-inflammatory cytokines, ultimately suppressing the intracellular persistence of *Leishmania*. Few studies have shown an individual contribution of adenosine receptors in infection (Figueiredo et al., 2017; Lima et al., 2017; Vijayamahantesh et al., 2016); however, the present study is perhaps the first to report the crucial role played by both A₂₅R and A₂BR in the pathogenesis caused by *L. donovani*.

In summary, the present study highlights how a parasite channel host ATP to the extracellular milieu for favouring its survival. Adenosine generated as a result of the process is crucial, as it inhibits inflammasome activation and activates parasite-favourable cytokine production. The importance of adenosine was also verified in *vivo*, where infected mice were treated with either the CD39 inhibitor, POM-1 or the CD73 inhibitor, ACPCP. Treatment with these inhibitors resulted in a significantly decreased liver and spleen parasite burden associated with a host-favourable pro-inflammatory environment. This study therefore not only unveiled the role of ATP in infection but identified ectonucleotidases as a potential therapeutic target in visceral leishmaniasis.

**MATERIALS AND METHODS**

**Cells and parasites**

*L. donovani* promastigotes of strain MHOM/IN/1983/AG83 were maintained in Medium 199 (M199, Invitrogen) supplemented with Hanks salt containing HEPES (12 mM), L-glutamine (20 mM), 10% heat inactivated FBS, 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen) at 22°C. *L. donovani* axenic amastigotes were cultured as described previously (Ghosh et al., 2013). The murine macrophage cell line RAW 264.7 (National Repository for Cell lines/Hybridomas, India) was maintained at 37°C, 5% CO₂ in DMEM (Invitrogen) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Peritoneal macrophages (PMφ) were isolated from peritoneum of 6- to 8-week-old female BALB/c mice as described earlier (Gross et al., 2003) and cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin and 100 U/ml penicillin. All the cells were checked for contamination before starting experiments.

**Reagents and antibodies**

Adenosine (A9251), adenosine 5′-monophosphate sodium salt (AMP, A1752), adenosine 5′-(α, β-methylene) diphasphate (APCP, M3763), adenosine 5′-triphosphate disodium salt hydrate (ATP, A6419), Adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATP-γs, A1388), carbonoxolone disodium salt (C4790), dipyridamole (D9766), D- (+)-glucose solution (G8769), 2-deoxyxylucose (2-DG, D6134), gadolinium(III) chloride hexahydrate (G7532), L-glutamine (G8540), oligomycin (O4876) and sodium pyruvate solution (S8636) were obtained from Sigma-Aldrich (St Louis, MO, USA). Reagents such as ZM 241385 (ab12018) and MRS 1754 (ab120397) were purchased from Abcam (Cambridge, UK). POM-1 (21160) and 10Panx (3348) were obtained from Cayman Chemical (Ann Arbor, Michigan, USA) and Tocris (Bristol, UK), respectively.

The antibodies against pannexin-1 (Chen et al., 2019) (ab139715, 1:1000; Lot# GR3241457-3), CD39 (Shah et al., 2014) [ab108248, EPR3678(2), 1:1000; Lot# GR60408-12], CD73 (Xu et al., 2018a) (ab175396, 1:1000; Lot# GR318955-11) and ADA (ab175310, 1:1000; Lot# GR3181658-9) were purchased from Abcam (Cambridge, UK). The antibodies against adenosine A₂₅ receptor (Haschemi et al., 2007) (NBP1-39474, 7F6-G5-A2, 1:1000 for WB, 1:100 for microscopy; Lot# B-1) and Adenosine A₂BR receptor (Xu et al., 2018b) (NB2P-41312, 1:2000 for WB, 1:100 for microscopy; Lot# 811-1802) were purchased from Novus Biologicals (Centennial, Colorado, USA). Mouse monoclonal β-actin antibody (A2228, 1:5000; Lot# 066M4860V), alkaline phosphatase– conjugated anti-mouse (A3562, 1:10,000; Lot # SLBV4379) and anti-rabbit (A3687, 1:10,000; Lot# SLBV1976) secondary antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Goat polyclonal secondary antibody to Mouse IgG - H&L (TR) (ab6787, 1:200; Lot# GR3207552-3) and goat anti-Rabbit IgG H&L (FITC) (ab6717, 1:200; Lot# 3237804-2) were obtained from Abcam (Cambridge, UK).

**Glycolysis and oxidative phosphorylation assay**

Cellular glycolytic rate and oxidative phosphorylation levels were assessed using the Seahorse Bioscience Extracellular Flux Analyzer (XF24, Agilent Technologies, Santa Clara, California, USA) by measuring the extracellular acidification rate (ECAR) (indicative of glycolysis) and oxygen consumption rate (OCR) (indicative of respiration). RAW 264.7 cells (8×10⁴) and peritoneal macrophages (2×10⁴) were seeded in wells of XF24-cell culture microplates and incubated overnight. The monolayer of cells were infected with *L. donovani* parasites in a 1:10 ratio for the indicated time points. Before assessment, the cells were washed with XF assay medium, i.e. XF base medium supplemented with 4 mM glutamine for glycolysis stress test kit assay and for the mito stress test kit assay, the base medium was supplemented with 4 mM glutamine, 1 mM pyruvate and 5.5 mM glucose. The cells were then incubated with 525 µl of the respective media for 1 h at 37°C in non-CO₂ incubator. ECAR and OCR were then measured as recommended by the manufacturer. After completion of measurements, the cells were lysed in the plates using 25 µl RIPA lysis buffer (Sigma; St Louis, MO, USA) supplemented with 3 mM PMSF and 3 mM protease inhibitor mixture (Calbiochem; San Diego, CA, USA). Protein concentrations in the clear supernatant were determined using the Bradford assay and used to normalise the data which were analysed by Wave Desktop software (Wave 2.6.0) which is freely available for download.

**Lactate measurement**

Lactate in the cell culture medium was assessed using a colorimetric L-Lactate Assay Kit (ab65331, Abcam) according to the manufacturer’s instructions. After the indicated time points, the cell culture supernatant was collected and deproteinised using the perchloric acid/KOH method. 50 µl of the deproteinised samples were mixed with 50 µl of reaction mixture and incubated at room temperature for 30 min in the dark. The samples were analysed by measuring absorbance at 450 nm.

**ATP measurement**

Intracellular and extracellular ATP levels were quantified using the luminescence ATP Determination Kit (A22066, Molecular Probes; Eugene, Oregon, USA). For intracellular ATP, cells (3×10⁴) were infected with *L. donovani* promastigotes for varying periods of time. Cells were then washed with PBS and lysed in 200 µl Glo-lysis buffer (Promega; Madison, Wisconsin, USA). Samples were boiled; centrifuged and 500 ng protein was used to measure ATP content using the kit. For extracellular ATP, cells (5×10⁴/well) in 500 µl of fresh serum-free DMEM in 24-well plates were infected with live or PFA-fixed *L. donovani* promastigotes for varying time periods. At specific time points, supernatants were collected and used to quantify ATP.

**Adenosine measurement**

Adenosine in the cell culture supernatant after the indicated time points was quantified using the Adenosine Assay Kit (MET-5090, Cell Biolabs; Centennial, Colorado, USA). Mouse monoclonal β-actin antibody (A2228, 1:5000; Lot# 066M4860V), alkaline phosphatase– conjugated anti-mouse (A3562, 1:10,000; Lot # SLBV4379) and anti-rabbit (A3687, 1:10,000; Lot# SLBV1976) secondary antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Goat polyclonal secondary antibody to Mouse IgG - H&L (TR) (ab6787, 1:200; Lot# GR3207552-3) and goat anti-Rabbit IgG H&L (FITC) (ab6717, 1:200; Lot# 3237804-2) were obtained from Abcam (Cambridge, UK).
The macrophage cells were incubated in serum-free DMEM and infected with live or PFA-fixed *L. donovani* for the specified time periods and then the cell culture medium was collected and centrifuged to obtain the clear supernatant. 50 μl of the sample was used to quantify adenosine.

### Ectonucleotidase activity

Ectonucleotidase activity of *L. donovani*-infected macrophages was determined using the Malachite Green colorimetric assay, which measures the amount of liberated inorganic phosphate (P_i) (Lanzetta et al., 1979). 2×10^5 macrophage cells (post-infection and treatment) were washed with reaction buffer (1 mM CaCl_2, 145 mM NaCl, 5 mM KCl, 10 mM HEPES and 1 mM MgCl_2, pH 7.4). The reaction was initiated by adding 1 mM ATP for CD39 or 1 mM AMP for CD73 to the culture dish and stopped by addition of the cell supernatant to a vial containing 1 ml of cold 25% activated charcoal in 0.1 M HCl. The vials were then centrifuged at 1500 g for 10 min and 50 μl of each of the centrifuged samples were quantified for released inorganic P_i using different concentration of P_i as standard. Nonspecific P_i release was determined by incubating cells in the absence of substrate ATP or AMP, which were added after stopping the reaction.

### Immunoblotting

Cells (RAW 264.7 and PMEs) were harvested and lysed using ice-cold lysis buffer (Cell Signaling Technology; Danvers, Massachusetts, USA) supplemented with 3 mM PMSF (Sigma) and 3 mM protease inhibitor mixture (Calbiochem; San Diego, CA, USA). Protein concentration in the clear supernatant was determined using the Bradford assay. From each sample, an equal amount of protein (50 μg or 20 μg) was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane, followed by blocking the membrane with 5% BSA and overnight incubation with specific primary antibody in the desired dilution. After washing with TBS, membranes were incubated with alkaline phosphatase-conjugated secondary antibody and detected by hydrolysis of 5-bromo-4-chloro-3-indolylphosphate (BCIP) chromogenic substrate. β-actin was used as a loading control. Band intensities were quantified by using ImageJ.

### Cytotoxicity assay

Cytotoxic or cytostatic effects were observed for the administered doses of the reagents by examination of cellular morphology and cell viability of RAW 264.7, peritoneal macrophages, promastigotes and axenic amastigotes by MTT assay kit (Roche Applied Science; Penzberg, Germany).

### Assessment of intracellular infection

In *in vitro* infection experiments, RAW 264.7 cells and peritoneal macrophage cells were infected with stationary phase *L. donovani* promastigotes at a 10:1 parasite/macrophage ratio (Kar et al., 2010). Infection was allowed to proceed for 4 h, the non-internalised parasites were removed by washing the plates with PBS and cells were cultured. Determination of the number of intracellular parasites was done by first fixing the cells in methanol and then staining with the nuclear stain DAPI (4′,6-diamidino-2-phenylindole; 1 μg/ml) along with RNase A (10 μg/ml) for 15 min (Gupta et al., 2016). Images were analysed using Olympus Fluoview software (version 3.1a, Tokyo, Japan).

### Cytochrome and chemokine analysis by ELISA

ELISA was performed using a sandwich ELISA kit (Quantikine M; R&D Systems, Minneapolis, MN, USA) for the various cytokines from PMEs and splenocytes. The detection limits of these assays were >5.1, >2.5, >2.4, >4.6 and >2 pg/ml for the cytokines TNF-α, IL12p70, IFN-γ, IL-10, TGF-β and IL-4, respectively and >4.7 and >7.8 pg/ml for the chemokines CCL3 and CCL5, respectively.

### Fluorescence microscopy

RAW 264.7 macrophages (2×10^5) were plated onto 18 mm² coverslips in DMEM medium and infected with *L. donovani* promastigotes (cell:parasite ratio 1:10). The cells were then washed twice in PBS and fixed with methanol at room temperature. The cells were permeabilised with 0.1% Triton X-100 and incubated with blocking solution (1% BSA) for 1 h at room temperature followed by overnight incubation with primary antibody against A2BR and Aβ2R at 4°C. After washing, coverslips were incubated with both FITC- and Texas Red-conjugated secondary antibody for 1 h at room temperature. Cells were stained with DAPI (4′,6-diamidino-2-phenylindole, 1 μg/ml) plus 10 μg/ml RNase A in PBS to label the nucleus, mounted on slides and visualised using Olympus IX81 microscope equipped with a FV1000 confocal system using a 100×/60× oil immersion Plan Apo (N.A. 1.45) objectives. The images thus captured were analysed by Olympus Fluoview (version 3.1a; Tokyo, Japan) and mounted using Adobe Photoshop software.

### In vivo infection

Animal maintenance and experiments were performed in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (New Delhi, India). The protocol was approved by the Institutional Animal Ethics Committee (IAEC). For *in vivo* infection, 6- to 8-week-old female BALB/c mice (~20 g) were injected via the tail vein with 1×10^7 stationary phase *L. donovani* promastigotes. Infection was assessed by removing the liver and spleen from infected mice up to 4 weeks after infection. Parasite burden was determined from Giemsa-stained impression smears (Srivastav et al., 2012). Liver and spleen parasite burdens, expressed as Leishman–Donovan units (LDU), were calculated as the number of amastigotes/1000 nucleated cells/organ mass (in grams) (Murray et al., 1993). Splenocytes from BALB/c mice were isolated and cultured as described earlier (Arsenescu et al., 2005). Splenocytes were stimulated with soluble leishmanial antigen (20 mg/ml) and with adenosine (100 μM) where applicable for 48 h, followed by measurement of pro-inflammatory cytokines by ELISA (Das et al., 2001).

### Histopathology

Livets isolated from BALB/c mice were fixed in 10% formalin (Merck) and embedded in paraffin wax. Tissue sections (5 mm) were made with a microtome (Leica Biosystems) and stained with H&E to study the microarchitecture using light microscope (Nikon Microscope, Singapore). To quantify density of granuloma formation in the liver tissue, the total number of granulomas (immature and mature) were counted in 50 consecutive microscopic fields of sections derived from mice of the test groups.

### Densitometric analysis

Densitometric analysis for all experiments was carried out using ImageJ software. Band intensities were quantitated densitometrically and the values obtained were normalised to endogenous control and expressed in arbitrary densitometric units. The ratios of OD of particular bands to endogenous control are represented as bar graphs adjacent to figures.

### Statistical analysis

Most of the experiments were performed at least three times. All the data are shown as means±s.d. of three independent experiments unless otherwise stated. *p* values are given in the legends of figure. Student’s *t*-test was used to assess the statistical significance of intergroup comparisons using GraphPad Prism 7.0 and *P*<0.05 was considered to be significant.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

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