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Up regulation of A_{2B} adenosine receptor on monocytes are crucially required for immune pathogenicity in Indian patients exposed to *Leishmania donovani*

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ABSTRACT

Adenosine, an endogenous purine nucleoside is one such extracellular signalling molecule whose role in regulation of anti-inflammatory cytokines and immune pathogenicity in visceral leishmaniasis is not fully understood. Here, we investigated the relationship between *Leishmania donovani* infection and expression of A_{2B} receptor on monocytes in VL patients in their pre and post treatment stage. We also investigated the molecular mechanisms influencing the interaction between immunopathogenicity and infection by exposing *Leishmania donovani* pulsed macrophages to Adenosine. A direct correlation of up-regulated A_{2B} expression on monocytes with increased parasite load was also observed. Our results also suggested that A_{2B} receptor activation is critically required for the stimulatory effect of adenosine on IL-10 production and suppression of nitric oxide release. The stimulatory effect of adenosine on *Leishmania donovani* induced IL-10 production required ERK1/2 activation and is p-38 MAPK independent.

1. Introduction

Among the various forms of leishmaniasis, visceral leishmaniasis (VL) caused by *Leishmania donovani* (*L. donovani*), is the most severe in the Indian subcontinent and is fatal if untreated. This has further been compounded by the wide spread development of unresponsiveness to the sodium antimony gluconate (SAG). Exploration of alternative drugs, including oral miltefosine and parenteral amphotericin-B as first line treatments are tried but newer drugs are expensive and have side effects [1–3].

Critically, absence of an effective Th1 response has been claimed as a major cause for disease pathology and development of drug unresponsiveness among patients with VL [4]. Interferon-gamma (IFN-γ) is the pre-dominant cytokine in anti-leishmanial defence however, a strong parasite induced immunosupression ensues following *L. donovani* infection by over expression of transforming growth factor β (TGF-β) in $CD4^+$ which obstruct in release of free radical generation and IL-12 production in macrophages of the infected hosts [5,6]. As shown in a previous study, interferon-γ treatment used in combination with sodium stibogluconate produced unexpectedly discouraging results in Indian Kala-azar patients [1]. If immunotherapy with IFN-γ is not able to trigger a protective Th1 response, it suggests another level of barrier on the Th1 components, which could be an anti-inflammatory substance like adenosine.

As known, ATP and other nucleotides are released from cells at sites of inflammation of different tissues are known to bind to specific purinergic (P2) receptors and this activity induces anti-parasite effects [7–9]. Unlike the effect of P2 receptor, adenosine receptors were observed with anti-inflammatory effects in infection by *Leishmania* [10,11]. As previously shown, *Leishmania* expresses e-NTPDase/nucleotidase on its surface, which degrades ATP into adenosine and may have implication in growth of parasite and pathology [12]. One study from Indian subcontinent also reported elevated serum adenosine level in human VL cases despite increased serum adenosine deaminase level [13]. Adenosine interacts with one or more of four of G-protein-coupled

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receptors (A_1, A_{2A}, A_{2B} and A_3) through which it exerts immunosuppressive effects [14]. Although most potent anti-inflammatory and immune suppressive effects of adenosine were reported due to A_{2B} receptor expressed pre-dominantly on APC, these are less studied in infection like VL. Here, our study is an attempt to investigate the role played by A_{2B} receptor during VL in human patients.

2. Methods

2.1. Clinical samples and diagnosis

10 healthy and 15 active VL cases from endemic and non-endemic areas of Bihar were selected for these studies. All the active VL patients presented characteristic signs and symptoms of the disease and diagnosis was confirmed by the presence of *L. donovani* in Giemsa stained bone marrow/splenic aspirate and positive serology (direct agglutination test, DAT). At the time of study, the duration of illness ranged from 1 to 6 months and patients were untreated. Successfully treated cases were without hepatospleenomegaly and fever after completion of Amphotericin B treatment (15 injections of 1 mg/kg body weight applied with very slow infusion of 5% dextrose on alternate days). Each of the healthy control subjects had no apparent history of VL and was not a resident of VL endemic areas. Recommendations outlined in the Helsinki Declaration were followed and Ethical approval was obtained from the Ethical Review Committee of RMRIMS, Patna, India for the study. Approaching subjects unwilling to give their informed consent or were positive for tuberculosis, malaria, kidney, heart and liver diseases, HIV or with symptoms of asthma or rheumatic arthritis were excluded from this study.

2.2. Expression level of A_{2B} adenosine receptors in VL patients before start of treatment and after completion of antileishmanial therapy

PBMCs were isolated from 10 of each group, VL confirmed and healthy donors. 1×10^5 cells were suspended in 100 μl phosphate buffer saline (PBS) in a FACS Tube and 5 μg rabbit ant-human A_{2B} antibody (Santacruz biotechnology) was added and incubated at 4 °C in dark for 30 min. Cells were washed twice with 1 ml PBS and pellet was resuspended 100 μl PBS and 5 μg goat-antirabbit-PE conjugated antibody (Santacruz biotechnology) was added and incubated at 4 °C for 30 min in dark. Cells were washed twice with 1 ml PBS and the pellet was resuspended in 100 μl PBS and stained with mouse anti-human CD14-FITC conjugated (BD bioscience San Diego, CA, USA) incubated at 4 °C in dark for 30 min. Cells were washed in 1 ml PBS and resuspended in 450 μl stain buffer for examination of fluorescence on monocytes ($CD14^+$) for A_{2B} adenosine receptor by FACS-Calibur Flow-Cytometer (Becton Dickinson, San Diego, USA) using Cell Quest Pro software.

2.3. In vitro A_{2B} receptor expression

PBMCs were isolated from ten healthy donors and 2×10^6 cells per well were seeded on 6 well plate and incubated overnight at 37 °C CO_2 incubator. Next day, non-adherent cells were washed twice with sterile PBS and adherent monocytes were pulsed with 1:10 monocytes to metacyclic *L. donovani* promastigotes for 12–16 h. Cells were scraped and washed twice with PBS at 500 g for 5 min at room temperature. 1×10^5 cells were suspended in 100 μl phosphate buffer saline (PBS) in a FACS tube and 5 μg rabbit ant-human A_{2B} antibody (Santacruz biotechnology) was added and incubated at 4 °C in dark for 30 min. Cells were washed twice with 1 ml PBS and pellet was resuspended 100 μl PBS and 5 μg goat-antirabbit-PE conjugated antibody (Santacruz biotechnology) was added and incubated at 4 °C for 30 min in dark. Cells were washed twice with 1 ml PBS and the pellet was resuspended in 100 μl PBS and stained with mouse anti-human CD14-FITC (BD bioscience San Diego, CA, USA) incubated at 4 °C in dark for 30 min. Cells were washed in 1 ml PBS and resuspended in 450 μl stain buffer for examination of fluorescence on monocytes ($CD14^+$) for A_{2B} adenosine receptor by FACS-Calibur Flow-Cytometer (Becton Dickinson, San Diego, USA) using Cell Quest Pro software.

2.4. IL-10 and nitric oxide measurement

PBMCs were isolated from VL confirmed patients and healthy donors by Histopaque (Sigma–Aldrich) method. 2×10^6 cells/well were seeded on 6 well plate (Nunc) and incubated over night at CO_2 incubator. After 12 h, non-adherent cells were washed twice with sterile PBS and fresh media was added to all the wells and cultured in the presence or absence of 100 μM adenosine. In antagonistic experiments, non-adherent cells were washed and adherent cells were pre-treated with 25 and 50 μM adenosine receptor antagonist CGS 15943 (Tocris bioscience UK) and 25 and 50 μM A_{2B} receptor antagonist MRS1574 (Tocris bioscience UK) for 1 h before stimulating with 100 μM adenosine or *L. donovani* or both. Plates were incubated in CO_2 incubator for 24 h and culture supernatant was collected and centrifuged at 1000 g for 10 min at 4 °C. The secreted IL-10 was estimated using BD OptiEIA (BD bioscience San Diego, CA, USA) according to manufacturer's protocol. The colour intensity was measured at 450 nm by ELISA plate reader (Biorad i MARK).The same sample is used for the estimation of nitric oxide by nitrite/nitrate assay kit (Sigma–Aldrich) according to manufacturer's protocol. The colour intensity was measured at 570 nm by ELISA plate reader (Biorad i MARK).

2.5. Cell signalling analysis

200 μl bloods stimulated in the presence or absence of 100 μM adenosine (Sigma–Aldrich) or *L. donovani* or both at intervels of times (5, 10, 15 and 30 min) at 37 °C water bath and mean fluorescence intensity for ERK1/2 and p38 MAPK were analysed in VL patients and control. For STAT3 evaluation, cells were stimulated in the presence or absence of 100 μM adenosine or *L. donovani* or both in the presence or absence of 5 μg/ml neutralizing IL-10 at different time intervals as above at 37 °C. The RBCs were lysed in 2 ml Phosflow Lyse/Fix buffer (BD biosciences, USA) for 10 min were permeabilized by 1 ml chilled Phosflow buffer III (BD bioscience) for 30 min on ice. The washed cells were consecutively co-incubated with PE-conjugated anti-CD14 antibodies (BD bioscience USA) and with Phosflow-p38 MAPK-PerCP, Phosflow-ERK1/2-PE and Phosflow-SAT3-Alexa fluoro 647 (BD bioscience USA) at 4 °C in dark for 30 min. Stained cells were washed with 3 ml stain buffer (600 g for 5 min.). Supernatant was discarded and sample was re-suspended in 500 μl stain buffer. Samples were analysed using the Flow-Cytometer and software described above. All these experiments were performed in duplicate at different time interval (5, 10, 15 and 30 min) and ten [10] VL patients as well as ten [10] healthy control were used for *in vitro* experiments.

2.6. Statistical analysis

Student's two-tailed paired *t* test or unpaired *t* test was used to determine the difference between the groups studied. Statistical analysis was performed using GraphPad Prism 5, USA software