Role of IL-21 in host pathogenesis in experimental visceral leishmaniasis

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Abstract Visceral leishmaniasis (VL) is a major global health problem but still remains one of the neglected tropical diseases. Currently available chemotherapeutics are associated with severe toxicity and increased drug resistance. There is a need to explore for novel therapeutic strategies that could modulate host immune responses or work in synergy with chemotherapy of VL. Therefore, understanding the host immunological changes that play a vital role in disease pathology is a prerequisite for designing any interventions. We have investigated the role of IL-21 during the course of *Leishmania donovani* infection and after drug treatment. BALB/c mice were used to investigate the mRNA levels of IL-21 during active *Leishmania donovani* infection and after treatment using real time polymerase chain reaction (RT-PCR). Mice were divided in four groups i.e. Control (Group A), Infected (Group B), Uninfected treated (Group C) and Infected treated (Group D). Animals of Group C and D were treated with Amphotericin B. IL-21 mRNA levels in the spleen were estimated on days 1, 3, 7, 14, 17, 21, 28, 35, 45 and 60 post infection and also during course of treatment. We found that IL-21 mRNA levels was significantly up-regulated in the infected group with a fourfold increase at D60 p.i. ($p < 0.001$) and it was decreased significantly after the treatment. Our results suggest that IL-21 mRNA is associated with pathogenesis of *Leishmania donovani* infection

and that therapeutics designed to suppress IL-21 could provide promising antileishmanial activity.

Keywords Amphotericin B · Interleukin-21 · Pathogenesis · mRNA expression · Visceral leishmaniasis

Introduction

Visceral leishmaniasis (VL) is one of the neglected tropical diseases targeted for elimination. It is caused by the intracellular protozoan parasite *Leishmania donovani*. Amphotericin B alone or its lyposomised forms remains the mainstay for treatment of VL (Sundar and Chatterjee 2006). Moreover, Miltefosine the only oral drug for VL which is of recent introduction for VL treatment possess limitations like high cost and contraindication during pregnancies. Although Amphotericin B is an effective drug, it causes severe side effects such as anaphylaxis, thrombocytopenia, convulsions, nephrotoxicity and hypokalemia including fever and chills related to infusion. Chemotherapy works more efficaciously in synergy with the host immune responses particularly the cell mediated immune response (Croft et al. 2006). Therefore, it imperative to understand host immune responses and to identify lead molecules of the immune system that can be modulated for better efficacy of chemotherapy (Singh and Sundar 2014). IL-21 is a novel pleiotropic cytokine discovered in the year 2000 and has shown significant role in disease pathology of various diseases. Numerous studies have shown the therapeutic potential of this cytokine both in mice as well as in humans and clinical trials are underway to consider it as a target of immunotherapy (Spolski and Leonard 2014; Niu and Chen 2013) However, the role of IL-21 in the context of VL is not yet properly understood. Keeping this in view, we

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undertook the present study to investigate the role of IL-21 during the course of infection in BALB/c mouse at various intervals of infection and after treatment.

Materials and methods

Animals

BALB/c mouse (*Mus musculus*) aged 4–8 weeks old of either sex, bred and maintained in the animal facility of ICMR-Regional Medical Research Centre, NE Region Dibrugarh were used in the study. All experiments were conducted as per the guidelines of CPCSEA, ICMR New Delhi and SOP of the Institutional Animal Ethics Committee of ICMR-RMRC, Dibrugarh. This study was approved by the Institutional Animal Ethics Committee of ICMR-RMRC, Dibrugarh.

Parasites

Leishmania donovani strain (MHOM/IN/80/DD8) was obtained from Central Drug Research Institute, Lucknow and promastigote form is maintained in M199 medium (Sigma-Aldrich, USA) supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich, USA) and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin).

Animal infection

Metacyclic promastigotes were isolated from 5 to 6 day old stationary culture as per the methods of Melby et al. (2001). An inoculum containing 1×10^6 metacyclic promastigotes in 100 µl of phosphate-buffered saline (PBS) were used to infect BALB/c mice via the intra-cardiac route. Control mice received same volume of PBS only. Mice were divided into four groups Control (Group A), Infected (Group B), Uninfected treated (Group C) and Infected treated (Group D) and in each group three mice were taken.

Chemotherapy

Group C (Uninfected) was inoculated with Amphotericin B (1 mg/kg of body weight) in 100 µl inoculum at alternate days for a period of 15 days. Similarly, Group D (Infected) was treated with same volume of Amphotericin B (1 mg/kg of body weight) starting from day 14 of infection alternately for a period of 15 days.

Animal sacrifice

Animals of the Group A and B were sacrificed on day 1, 3, 7, 14, 17, 21, 28, 35, 45 and 60 for harvesting of spleen

tissues. Similarly, animals of Group C (Uninfected treated) and D (Infected treated) were sacrificed on day 17 onwards (days 17, 21, 28, 35, 45 and 60). Time points for the study of IL-21 mRNA expression were chosen as per earlier studies with minor modifications. Briefly day 0, 3 and 7 were chosen to assess the immunological changes during acute phase of infection; day 14, 17, 21 and 28 splenic proliferation stage and day 45 and day 60 stationary or chronic phase (Rousseau et al. 1997; Stager et al. 2003; Mukherjee et al. 2003; Osorio et al. 2012).

Determination of parasite load

Parasite load in the spleens of mice of Group B (Infected) and Group D (Infected treated) were determined at D7, D14, D21, D28, D35 and D60 time intervals by qPCR using the standard curve method. For determination of parasite load DNA was extracted using the Dneasy Blood and Tissue kit according to the manufacturer's instructions. Standard curve was constructed using serially diluted DNA from *L. donovani* parasites. Amplification was done using primers specific for kinetoplast DNA. The sequences of the primer are: forward primer: 5'-CCTATTTTACACCAA CCCCCAGT-3' and reverse primer: 5'-GGGTAGGGGCG TTCTGCGAAA-3' (Nicolas et al. 2002). Real time PCR was performed in a total reaction volume of 20 µl containing 10 µl of 2X SYBR Green PCR Master Mix, 10 pmol of each forward and reverse primer using 2 µl of template DNA. qPCR was performed under the following cycling conditions: Initial denaturation at 95 °C for 30 min followed by 30 cycles each consisting of annealing and extension at 94 °C for 30 s and 60 °C for 10 s. A non template control (NTC) consisting of 2 µl Nuclease free water was included in the reaction for validation of any possible contaminations/nonspecific reactions. Quantification of the parasites in the unknown samples was determined by interpolating the CT values obtained from the unknown samples on the standard curve.

Real time PCR for gene expression

Total RNA was extracted from 10 mg of fresh spleen tissue samples using the TRIzol reagent (Invitrogen, Life Technologies) and treated using DNase I (Sigma) according to the manufacturer's instructions and the remaining portion stored in RNA-later. Reverse transcription for cDNA synthesis was performed using 1 µg of total RNA and random primers in a total reaction volume of 50 µl. Real-time PCR was performed in ABI 7500 StepOne PLUS (Applied Biosystem) using the primers IL-21 forward: 5'-GGCT CCTTCAAAAGATGATTC-3' and IL-21 reverse: 5'-CAAGATGTACTCCTGC ATTC-3' and IL-10 Forward: 5'-CAGGACTTTAAGGGTTACTTG-3' and reverse: 5'-ATTT

TCACAGGGGAGAAATC-3'. The thermal conditions for PCR is as follows: 95 °C for 30 min followed by 30 cycles of annealing and extension at 94 °C for 30 s and 60 °C for 10 s. A no template control and No RT control was included in all the tests and all assays were performed in duplicates. The relative quantification of cytokines was normalized to housekeeping gene β -actin by the delta–delta Ct method and results were represented as fold increase over control mice.

Statistical analysis

Data analysis was done using GraphPad Prism version 5.01. by ANOVA with Bonferroni post tests for multiple comparisons. Experiments were done twice. The results are expressed as mean $\pm$ SD. Spearman analysis was performed to analyze the correlation between the cytokines and parasite load. p value of < 0.05 was considered as significant.

Results

We analysed the parasite load in the spleen of Infected and Infected Amphotericin B treated groups of mice. We found that parasite burden in the Infected group was slightly increased at D7 p.i. A greater increase in parasitemia was observed on D14 p.i. which continued to increase till D35 p.i and the peak was observed on D60 p.i. Splenic parasite burden in Infected Amphotericin B treated group decreased gradually after treatment (Fig. 1).

Estimation of IL-21 mRNA levels in mice infected with *Leishmania donovani* was done to assess the role of this cytokine during active *L. donovani* infection. We noted that IL-21 was up-regulated during the infection, however,

Fig. 1 Parasite load in the spleen of BALB/c mice of Infected and Infected Amphotericin B treated mice at various time intervals post infection and treatment. Results are representative of two independent experiments

significant up regulation was observed at D7, D14, D17, D21, D28, D35 and D60. The highest up-regulation was observed on D60 p.i. ($p < 0.001$) which has shown four-fold increase in expression in infected group compared to controls. The effect of Amphotericin B on the level of IL-21 mRNA in the Uninfected Amphotericin B treated group was found significantly down regulated compared to controls ($p < 0.001$) at all intervals except for D35 and D60 p.t. where marginal down regulation were recorded. In Infected Amphotericin B treated group, low level of IL-21 mRNA was detected at D17 p.t. ($p < 0.01$) which decreased further significantly at D21 and D28 p.t. ($p < 0.001$) (Fig. 2). We assessed the mRNA expression of IL-10 at different time intervals. We found that at the initial phase of infection at D1 p.i., IL-10 levels were almost equal to the control and the highest peak was observed on D21 p.i. which have shown 7.8-fold increase over control ($p < 0.001$). After treatment with Amphotericin B, mRNA levels of IL-10 were initially high at D17 (4.4-fold) after which it decreased significantly till D60 (last observation period). There was a direct correlation between parasite load and IL-21 expression ($r = 0.92$, $p = 0.007$) in the Infected group.

Discussion

Our findings suggest that IL-21 is associated with the pathogenesis of *L. donovani* infection as evident from increased levels of IL-21 during active infection. However, IL-21 levels gradually decreased after treatment. Our results suggest that Amphotericin B has the ability to downregulate the anti-inflammatory cytokines IL-21 and

Fig. 2 Relative mRNA expression of IL-21 in Control, Infected, Uninfected Amphotericin B treated and Infected Amphotericin B treated mice ($n = 3$) at various time intervals post infection and treatment determined by real time-polymerase chain reaction. At each time point, IL-21 expression was measured as fold change over control. Each bar represents mean of three mice and error bars show $\pm$ SD. Results are representative of two independent experiments. * p value < 0.05 ; *** p value < 0.001

IL-10 and this resulted in the decrease in parasite growth after treatment. Although parasite proliferation was arrested; however complete clearance of parasites was not achieved even after chemotherapy. This could be because of the high parasite load during infection and therefore it can be assumed that neither chemotherapy alone nor an efficient T cell response could eliminate all tissue parasites (Mukherjee et al. 2003; Murray 2005; van Griensven et al. 2010).

Our results are concordant with the findings of Ansari et al. (2011) where they have observed elevated mRNA levels of IL-21 in splenic aspirate cells from patients with active VL. Their findings suggest that IL-21 is a disease promoting cytokine which along with IL-27 regulates the differentiation and expansion of pathogenic IL-10. However, Pitta et al. (2009) could not detect IL-21 in PBMC cultures from kala-azar infected human subjects. Another study on cutaneous leishmaniasis caused by *Leishmania braziliensis* suggests active production of this cytokine along with other anti-inflammatory cytokines in lesions suggesting active role of this cytokine in disease pathogenesis (Costa et al. 2015). Our results are in agreement with other infectious diseases like TB where elevated IL-21 levels was associated with pediatric TB and assumed to play a role in disease pathogenesis along with TGF- β and IL-23 levels (Pavan Kumar et al. 2013). IL-10 is the main disease promoting cytokine in visceral leishmaniasis. Therefore we predicted that IL-10 could participate in disease pathogenesis together with IL-21. Concordant with other studies we observed elevated levels of IL-10 along with IL-21 which suggest that both the cytokines are associated with pathogenesis of *Leishmania donovani* infection (Ansari et al. 2011). There are many evidences suggesting elevated levels of IL-10 during active VL infection in patients and its role in pathogenesis (Caldas et al. 2005; Ansari et al. 2006; Peruhype-Magalhães et al. 2006).

Recently, researchers have exploited the therapeutic potential of IL-21 as an immunotherapeutic molecule to enhance immune functions in some infectious diseases and also in cancer (Korn et al. 2007; Niu and Chen 2013). Although, there is paucity of evidence on the role of IL-21 in the case of Leishmaniasis; recent studies targeting IL-21 as a therapy in various diseases suggest that this molecule might have potential to develop new treatment agent for VL.

Researchers around the globe are in search of such molecules that could provide a lead for development of therapeutics that possess reduced toxic effects and highly effective to combat dreaded parasitic diseases like VL. The elevated expression of IL-21 and its subsequent down regulation after treatment provides valuable insights to explore the intrinsic role of IL-21 as a target of

immunotherapy. Further studies are needed for generating more evidences to substantiate the role of IL-21 in chemotherapy and its importance in the treatment of VL infection.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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