

Co-factor-independent phosphoglycerate mutase of *Leishmania donovani* modulates macrophage signalling and promotes T-cell repertoires bearing epitopes for both MHC-I and MHC-II

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SUMMARY

Immunoactivation depends upon the antigen potential to modulate T-cell repertoires. The present study has enumerated the effect of 61 kDa recombinant *Leishmania donovani* co-factor-independent phosphoglycerate mutase (rLd-iPGAM) on mononuclear cells of healthy and treated visceral leishmaniasis subjects as well as on THP-1 cell line. rLd-iPGAM stimulation induced higher expression of interleukin-1 β (IL-1 β) in the phagocytic cell, its receptor and CD69 on T-cell subsets. These cellular activations resulted in upregulation of host-protective cytokines IL-2, IL-12, IL-17, tumour necrosis factor- α and interferon- γ , and downregulation of IL-4, IL-10 and tumour growth factor- β . This immune polarization was also evidenced by upregulation of nuclear factor- κ light-chain enhancer of activated B cells p50 and regulated expression of suppressor of mother against decapentaplegic protein-4. rLd-iPGAM stimulation also promoted lymphocyte proliferation and boosted the leishmaniacidal activity of macrophages by upregulating reactive oxygen species. It also induced 1.8-fold higher release of nitric oxide (NO) by promoting the transcription of inducible nitric oxide synthase gene. Besides, *in silico* analysis suggested the presence of major histocompatibility complex class I and II restricted epitopes, which can proficiently trigger CD8⁺ and CD4⁺ cells, respectively. This study reports rLd-iPGAM as an effective immunoprophylactic agent, which can be used in future vaccine design.

Key words: *Leishmania donovani*, co-factor-independent phosphoglycerate mutase, innate immunity, adaptive immunity, visceral leishmaniasis.

INTRODUCTION

Even after serious efforts of various national and international agencies, the deadline to control *Leishmania donovani* (Ld)-induced visceral leishmaniasis (VL) has been rescheduled again and again in the Indian subcontinent. This is due to the increasing rate of resistance to anti-leishmanial drugs, limited therapeutic options, lack of sterile cure, appearance of post-kala-azar dermal leishmaniasis, the existence of asymptomatic infection and HIV-VL co-infection, etc. Presently, there is no vaccine in use. Though few vaccine candidates are under clinical trial (Chakravarty *et al.* 2011; Coler *et al.* 2015), above situation warrants the introduction of potent immunomodulators, which can be used in future vaccine modules. Most of the earlier proposed vaccines failed to ascertain the high degree of antigenicity and persistency, which are

essential for a suitable vaccine candidate. Major hurdles on the way of anti-leishmania vaccine are variation in immunogenicity due to human lymphocyte antigen expression, gene variation as well as parasite-induced pathogenic pressure over vaccine candidate (Kumar and Engwerda, 2014). Many of the earlier subunits or protein vaccines (Skeiky *et al.* 1998; Stager *et al.* 2000; Agallou *et al.* 2011; Ghosh *et al.* 2001; Rafati *et al.* 2006; Goto *et al.* 2007; Carrillo *et al.* 2008) failed due to the fact that they were either tested in murine model or with cell lines, which provided good data inputs but may not mimic human cells. The present study has been carried out on peripheral blood of various healthy individuals from different endemic and non-endemic zones and treated cases of VL to decipher above problem. In this study, the presence of T-cell epitopes restricted to CD4⁺ or CD8⁺ cells has been also examined on phosphoglycerate mutase (PGAM) of *Ld*.

PGAM is a cytosolic enzyme, involved in glycolysis as well as gluconeogenesis pathway. It catalyses the

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reversible conversion of 3-phosphoglyceric acid (3-PGA) to 2-phosphoglyceric acid (2-PGA). Unlike co-factor-dependent PGAM (dPGAM) of human subjects, *Leishmania* contains co-factor-independent PGAM (iPGAM). iPGAM exhibits no sequence similarity with human dPGAM (Chevalier *et al.* 2000; Nowicki *et al.* 2009), which suggest its vaccine potential as well as the drug target. Basic Local Alignment Search Tool protein (BLASTP) analysis of the *Ld*-iPGAM and whole human protein shows only 30% identity and 9% query cover particularly with neural cell adhesion molecule L1 isoform 3 precursors. iPGAM of *L. donovani* exist in both the promastigotes and amastigotes forms, which is also an additional advantage regarding its vaccine potential. It becomes critical enzyme in the amastigotes form for its existence in human host because gluconeogenesis but not glycolysis is an essential pathway for its survival during amastigote form (Naederer *et al.* 2006). Recombinant iPGAM of *Brugia malayi* was also explored for its vaccine utility (Singh *et al.* 2014) and have been suggested as a possible target for drug designing. In the present study, recombinant iPGAM of *Ld* (*rLd*-iPGAM) has been reported with its potential to upregulate host-protective immune response augmenting antigen-presenting cell (APC) function, activation of effector cells and required anti-leishmanial activity. *In silico* study has suggested that *Ld*-iPGAM bears major histocompatibility complex (MHC) class I and II restricted epitopes (6 HLA A0201 and 5 DRB104010). Bearing these epitopes, *rLd*-iPGAM induced upregulation of interleukin-1 β (IL-1 β) on APCs as well as IL-1 β receptor and activation marker CD69 on CD4⁺ or CD8⁺ cells. This early activation was resulted in the dominance of host-protective cytokines, *viz.* IL-2, interferon- γ (IFN- γ), IL-17, IL-12 and tumour necrosis factor- α (TNF- α). Besides, it upregulated the expression of reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) in target cells in the presence of meager suppressor of mother against decapentaplegic protein-4 (SMAD-4) expression and upregulated nuclear factor- κ light-chain enhancer of activated B cells p50 (NF- κ B p50). Present study ensures *rLd*-iPGAM as a good immunoprophylactic agent, which can be explored in future vaccine designing.

MATERIALS AND METHODS

Leishmania donovani culture and isolation of genomic DNA

A reference strain of *Ld* (MHOM/IN/83/AG83) was cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS). Promastigotes culture was maintained as per protocol being used elsewhere (Singh *et al.* 2011).

Late log phase parasite was harvested by centrifugation at 900 *g* at 4 °C for 20 min (Hermle, Germany). The pellets (10⁸ promastigotes) were washed thrice with phosphate-buffered saline (PBS) and spun at 900 *g* for 20 min at 4 °C. The washed pellet was used to isolate DNA using DNA Purification Kit (Qiagen, Germany). Isolated DNA was electrophoresed on 0.8% agarose gel, and DNA concentration was evaluated and stored for further use.

Amplification and cloning of Ld-iPGAM

Ld-iPGAM gene-specific primers were designed manually and checked with NEB Cutter and Oligocalc tools. Primers were as following: forward 5' TTT TGG ATC CAT GTC GAA TCT CCT CTT GAC 3' (*Bam*HI restriction site is underlined) and reverse 5' TTT TAA GTC TTC AGT TCT CGA CAT AGA TC 3' (*Hind* III restriction site is underlined). These primers were synthesized commercially (IDT, Gurgaon, India). The *Ld*-iPGAM gene (NCBI Accession no. CBZ39131.1) was amplified using above primers in Thermal cycler (Bio-Rad, USA). The PCR condition was set as the first cycle at 95 °C for 2 min and 30 cycles at 94 °C for 1 min, 58.1 °C for 1 min, 72 °C for 1 min and last cycle at 72 °C for 10 min. The PCR product was electrophoresed on 1% agarose gel along with 1 kb ladder (BR Biochem. Life Sciences, India). The amplified product was eluted using gel extraction kit (Qiagen, Germany). Vector (pET-28a) was isolated from the fresh culture using plasmid isolation kit (Qiagen, Germany). Isolated pET-28a and eluted amplicon were digested separately with *Bam*HI and *Hind* III (Promega, USA) and ligated using T4 DNA ligase (Fermentas, Thermo Fisher Scientific, USA). Competent *Escherichia coli* DH5- α cell was transfected with the recombinant plasmid. Next day, the transformation was confirmed by colony PCR as well as by restriction double digestion using *Bam*HI and *Hind* III.

Expression and purification of iPGAM

The pET-28a-*Ld*-iPGAM construct was isolated from DH5- α and re-transformed in competent *E. coli* BL-21 (DE3, an expression host for recombinant construct). This transformation was again confirmed by colony PCR and restriction double digestion. A 50 μ L fresh inoculum of transformed BL-21 was inoculated in 5 mL Luria broth supplemented 1 μ g mL⁻¹ kanamycin. The culture was incubated at 37 °C in a shaker incubator at 220 rpm till the O.D₆₀₀ reached to 0.5–0.6. Further, 0.8 mM isopropyl β -D-1-thiogalactopyranoside was added to the culture and incubated at 25 °C for overnight. A 1 mL of the recombinant cells culture was pelleted and lysed with 2 \times sample loading buffer [100 mM Tris base (pH 6.8), 4% sodium dodecyl

sulphate (SDS), 20% glycerol, 0.2% bromophenol blue, 200 mM β -mercaptoethanol] and electrophoresed in SDS-12% polyacrylamide gel electrophoresis (PAGE) to confirm the protein expression. The uninduced culture was run in parallel as a control. The result was analysed in comparison to protein marker (Puregene).

For the expression of recombinant protein, transformed BL-21 (with pET28a-r*Ld*-iPGAM) was inoculated in 200 mL LB medium supplemented with $1 \mu\text{g mL}^{-1}$ kanamycin. The culture was extended as described earlier in this manuscript. The culture was harvested at 6000 g for 15 min and resuspended in 5 mL lysis buffer (50 mM Tris base pH 8, 300 mM NaCl, 0.1% Triton X-100) containing 50 μL of 1 mM phenylmethylsulfonyl fluoride, 50 μL of 10 mg mL^{-1} lysozyme and incubated for 2 h at 25 °C. The suspension was sonicated for 8×60 s (with 60 s intervals between each pulse) at 70 amplitude on the ice. Sonicated cells were centrifuged at 14 000 g for 15 min and the supernatant containing protein of interest was mixed with 600 μL Ni^{2+} nitrilotriacetic acid bead (Qiagen, Germany) in a column and incubated at 4 °C for 1 h in a shaker incubator. The column content was washed with buffer (50 mM NaH_2PO_4 , 20 mM imidazole, 300 mM NaCl, pH 8) for 7–8 times and r*Ld*-iPGAM was eluted using elution buffer (50 mM NaH_2PO_4 , 250 mM imidazole, 300 mM NaCl, pH 8). The purity of eluted r*Ld*-iPGAM was analysed by SDS-12% PAGE (Laemmli, 1970). The concentration of purified recombinant protein was estimated by Bradford method using bovine serum albumin (BSA) as standard.

Preparation of soluble leishmanial antigen

For the preparation of soluble leishmania antigen (SLA), the late log phase parasites were centrifuged in 15 mL centrifuge tubes (Tarson, India) at 900 g for 20 min in a cooling centrifuge (Hermle, Germany). The pellet was washed twice with PBS by centrifuging at 900 g at 4 °C for 20 min. The washed *Ld* pellet was subjected to six freezes and thaw cycle. The lysate was centrifuged at 30 000 g for 30 min, and the supernatant was collected in aliquots and stored at -80 °C for further use.

Confirmation of purified protein by Western-blot and lipopolysaccharide (LPS) test

Western blotting was performed to confirm the presence of recombinant protein using anti-hexahistidine antibody (Towbin *et al.* 1979). Briefly, the purified iPGAM was electrophoresed in SDS-12% PAGE and transferred to 0.22 μm nitrocellulose membrane (Sigma-Aldrich, USA) in the presence of transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH 8.2) using semi-dry blotter (Bio-rad, USA) at 15 V for 30 min. The transferred

nitrocellulose paper was treated with blocking buffer (5% BSA, PBS) for overnight and washed with Tris buffer saline-tween 20 [TBS-T (Tris base, NaCl, 0.1% tween-20 and 0.2% BSA, pH 7.5)] for three times. The membrane was incubated with anti-hexahistidine antibodies (1 : 2500 for 1 h, Santa-Cruz Biotech, USA) followed by three washing with TBS-T. The membrane was further incubated with horseradish peroxidase conjugated anti-rabbit antibody (1 : 1000, MERCK Biosciences, Bangalore, India). The blotted membranes were introduced with 3-3'-diaminobenzidine (0.06% H_2O_2 , Ameresco, Solon, USA) solution till the band appeared without background. The membrane was rinsed with plenty of distilled water to stop the reaction and analysed. The concentration of bacterial LPS contamination was determined in the r*Ld*-iPGAM using Limulus amoebocyte lysate test (Thermo-Scientific, USA) as per manufacturer's instructions. A 0.12 $\mu\text{g mg}^{-1}$ of LPS contamination was detected in the r*Ld*-iPGAM. The contamination was removed using a polymyxin B-agarose column (Sigma, USA) according to the manufacturer's instructions. The purified r*Ld*-iPGAM was then stored at -80 °C for further use.

Enzymatic activity of r*Ld*-iPGAM

The enzymatic activity of r*Ld*-iPGAM was carried out spectrophotometrically by measuring the decrease of UV absorbance at 340 nm due to oxidation of nicotinamide adenine dinucleotide hydrogen (NADH). As it is known that the amount of product produced in the reaction corresponds to the amount of NADH oxidized to nicotine adenine dinucleotide in the conversion of 3-PGA to 2-PGA. The assay was performed using 1 mL mixture containing 0.1 M HCl pH 7.6, 1 mM MgCl_2 , 1 mM adenosine diphosphate, 0.56 mM NADH, 0.1 mM CoCl_2 , 5 mM 3-PGA, 1 U mL^{-1} pyruvate kinase (Sigma), 1 U mL^{-1} enolase (Sigma), 1 U mL^{-1} pyruvate dehydrogenase (Sigma) and 1 $\mu\text{g mL}^{-1}$ r*Ld*-iPGAM at 25 °C.

Fluorescence staining of early activation marker CD69 on lymphocytes

Whole blood from the healthy subject ($n = 13$, observation of clinical symptoms and haematological changes expressed in a different study group has been attached in online Supplementary Table S1) was used to study the expression of surface markers lies on innate immune cells. A 100 μL whole blood was taken in culture plate (Nunc, Denmark), stimulated with or without r*Ld*-iPGAM and incubated in the CO_2 incubator at 37 °C for 24 h (for that blood was mixed with RPMI medium 1640+10% FBS in 1 : 1 ratio). Anti-CD69 fluorescein isothiocyanate (FITC) antibody (BD Biosciences) was added and further incubated for 20 min at room temperature

in dark condition. A 1 mL fluorescence-activated cell sorting (FACS) lysis buffer™ (1×) was added, vortexed and incubated for 10 min. Cells were centrifuged at 200 *g* for 5 min. The pellet was washed with 2 mL PBS, and finally, samples were suspended in 500 μ L PBS and acquired to study the fluorescence on FACS Caliber™ (BD). In order to explore the role of r*Ld*-iPGAM on CD4⁺ and CD8⁺ cells, expression of CD69 was also evaluated on peripheral blood mononuclear cells (PMNCs). For this, PMNCs were isolated from peripheral blood of healthy subjects (*n* = 13) by density gradient centrifugation over Histopaque1077 (Sigma-Aldrich). The PMNCs were washed with PBS and counted on 0.1 mm Naubauer chamber (Fein Optia, JENA, Germany). Cells were suspended at 1×10^6 PMNCs mL⁻¹ in RPMI-1640 complete media (RPMI + FBS). The cells were stimulated with or without r*Ld*-iPGAM and incubated in the CO₂ incubator at 37 °C for 24 h. The cultured cells were washed and stained with anti-CD4 Phycoerythrin Cy5 (PECy5) or anti-CD8 PECy5 and anti-CD69 FITC by following protocols discussed above.

Fluorescence staining of T cells and macrophages for qualitative evaluation of rLd-iPGAM-induced intracellular cytokines

PMNCs (1×10^6 cells mL⁻¹) from healthy and treated VL subjects (*n* = 13, online Supplementary Table S1) were stimulated with or without *Ld* or r*Ld*-iPGAM or phorbol 12-myristat 13-acetate (PMA) and ionomycin and incubated in the CO₂ incubator at 37 °C for 96 h. The culture was blocked using GolgiPlug (1 μ g mL⁻¹, BD) before 4 h of harvesting. Non-adherent cells were collected from culture supernatant and harvested to study intracellular cytokine of innate and adaptive immune cell. Centrifuged supernatant was used for enzyme-linked immunosorbent assays (ELISA). Adherent cells (macrophages) were scraped out by keeping the plate on ice using ice-chilled PBS and used for onward macrophage study. Cells were washed with PBS and surface antibody, like anti-CD4 PECy5, anti-CD8 PECy5, anti-CD14 PerCP and anti-IL-R PE, was added in respective falcon tubes and incubated for 20 min at 4 °C. A cell sample was again washed with PBS. Cytofix (BD Biosciences) was added and incubated for 30 min at 4 °C. After incubation, cells were permeabilized by adding 1 mL permwash buffer (1×) (BD Biosciences) and kept at 4 °C for 5 min. Samples were washed and intracellular antibodies, like anti-IL-1 β PE, anti-IFN- γ FITC, anti-IL-10 PE, anti-IL-2 FITC, anti-IL-4 FITC, anti-IL-12 FITC, anti-IL-17A PE, anti-transforming growth factor (TGF)- β FITC, anti-NF- κ β P50 PE, anti-SMAD PE, were added in respective tube followed by incubation for 30 min at 4 °C. Samples were gently

mixed with 1 mL permwash buffer (1×) and washed thoroughly. Tubes were further centrifuged and washed with 2 mL stain solution (PBS with 0.09% NaN₃ and 1% FBS). Finally, cell pellets were suspended in 500 μ L stain buffer and acquired on FACS Caliber (BD).

Quantitative evaluation of rLd-iPGAM-induced cytokine expression by PMNCs

The cytokines, like IL-10, IFN- γ , TNF- α and IL-12, were evaluated in culture supernatants of PMNCs stimulated with or without r*Ld*-iPGAM or *Ld* for 96 h. Phytohaemagglutinin (PHA) stimulation was used as positive control. Quantitative evaluations of cytokines were performed using kit contents and user manual of BD OptEIA kit. Absorbance was measured at 540 nm in ELISA reader (Bio-Rad).

Assessment of lymphocyte proliferation responses after rLd-iPGAM stimulation

PMNCs from healthy and treated VL subjects were cultured in 96-well flat-bottom tissue culture plates (Nunc, Denmark) with or without SLA or r*Ld*-iPGAM or PHA (1 μ g mL⁻¹, Sigma) as positive control. The LTT (lymphocyte transformation test) assay was carried out following protocol by Garg *et al.* (2005) but using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Roche diagnostics) instead of ³H thymidine. The culture was incubated at 37 °C in 5% CO₂ for 3 days in case PHA and 5 days for unstimulated blank, SLA, and r*Ld*-iPGAM. A 50 μ L of XTT was added to 100 μ L of the culture of each well. Absorbance was measured at 480 nm with 650 nm as reference wave length.

Evaluation of the effect of rLd-iPGAM on induction of ROS

PMNCs from the healthy subjects were taken in 12-well plate and incubated at 37 °C in CO₂ incubator with 5% CO₂ for overnight. Next days, adherent macrophages were collected from the cultured plate by following the same protocol as mentioned earlier. A 100 μ L of cultured and washed macrophages from healthy subjects were taken into four different 12 \times 75 mm², Falcon™ tubes. First tube without any stimulation was taken as negative control, second tube was challenged with *Ld*. Similarly, third tube was stimulated with r*Ld*-iPGAM and lastly, the LPS was added in a fourth tube as a positive control. All tubes were incubated in a water bath for 15 min. All tubes were taken out from water bath and added with 200 μ M dihydrorhodamine 123. A 4 μ M n-formylmethionyl-leucyl-phenylalanine was added in LPS as well as r*Ld*-iPGAM

containing tubes. Further tubes were incubated at 37 °C for 10 min. The samples were treated with 1 mL 1× FACS lysis buffer™ (BD) and centrifuged at 200 g for 5 min. The supernatant was discarded and resuspended in 500 µL stain buffer (PBS+1% FBS+0.09% NaN₃). Samples were acquired on FACS Caliber™ using Cell Quest software (BD). The ROS produced by stimulated cells were measured based on mean fluorescence intensity (MFI) detected by the flow cytometry.

Evaluation of rLd-iPGAM-induced nitric oxide (NO) synthesis in PMNCs culture supernatant

PMNCs from healthy donor were cultured (1×10^6 cells mL⁻¹) with or without rLd-iPGAM or Ld or PHA in 5% CO₂ incubator at 37 °C for 96 h. Nitrate and nitrite were quantified to evaluate NO synthesis in the culture supernatant of PMNCs in the presence or absence of rLd-iPGAM, Ld and PHA. The assay was performed using the contents and kit manual of NO Assay Kit (Thermo Fisher). Absorbance was recorded at 540 nm wavelength.

Evaluation of iNOS by semi-quantitative RT-PCR

Transcription of iNOS was evaluated by isolating the total RNA from PMNCs culture stimulated with or without rLd-iPGAM using Trizol. Reverse transcription was performed using an anchored oligo dT (H-dT11 M, where M represents adenine, cytosine, or guanine; GenHunter). The synthesized cDNAs were amplified by PCR for iNOS gene. The condition for RT-PCR included an initial denaturation at 94 °C for 5 min, 94 °C for 45 s, 56 °C (iNOS) or 58 °C glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 45 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. RT-PCR was performed for 25 cycles, which was within the linear range of amplification of the corresponding mRNA species. The sequences of primers-iNOS: forward primer- 5' AGCCAG AAGCGCTATCACGAAGAT-3' reverse primer 5-AATG CAGAGCTGGCTCCATCCTTA-30 GAPDH Primer Fwd 5'TCAT CATCTCTGC CCCCTCTG3', Rev 50-CGCCTGCTTCACCA CCTTCTT-3. The RT-PCR end product was also run on 1.5% agarose gel, stained with ethidium bromide, and finally documented and quantified using the Bio-Rad gel documentation system and associated Quantity One software. PCR products were compared with respect to the GAPDH.

Evaluation of NO, TNF-α, IL-12 and IL-10 by rLd-iPGAM stimulated THP-1 cells

The human monocytic cell line, THP-1, was maintained in RPMI-1640 medium supplemented with 10% FBS at 37 °C in a humidified atmospheric

incubator with 5% CO₂. The 1×10^6 THP-1 cells mL⁻¹ were cultured with 5 nM phorbol 12-myristate 13-acetate in 24-well plate for 48 h in order to get differentiation and maturation into macrophages. After incubation, cells were washed and cultured in fresh medium in the presence or absence of rLd-iPGAM or LPS. The culture supernatant was collected for evaluation of NO production using a kit (Thermoscientific) and cytokine ELISA for TNF-α, IL-12 and IL-10, using the BD OptEIA kit.

MHC class I and II epitopes prediction

For the screening of promising MHC class I and II epitopes, we mainly relied on HLA-A*0201 and HLA DRB1 0401 population. The amino acid sequence of 2,3-bisphosphoglycerate-independent PGAM (ACC No: CBZ39131.1) was retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>). The retrieved sequence was screened for 9 mer HLA-A*0201 restricted epitope using SYFPEITHI (which predicts the antigen-specific cytotoxic T-cell epitopes using matrix-based algorithm; Schuler *et al.* 2007), RANKPEP (Reche *et al.* 2004) and IEDB (<http://tools.iedb.org>) according to our previous methodology with certain alteration (Dikhit *et al.* 2015; Amit *et al.* 2016). As it is known that RANKPEP is a position-specific scoring matrices-based bioinformatics tool, which is used to predict peptide binders to MHC-I molecules from protein sequences or sequences alignment. Besides, it also predicts the MHC-I ligands having C-terminal end, which is likely to be resultant of proteasomal cleavage. IEDB is the library of an experimentally measured immune epitopes. The database includes the tools that predict the MHC class I and II binding epitopes.

For 15 mer HLA DRB1 0401 restricted epitopes, SYFPEITHI, IEDB and NETMHC II (Nielsen and Lund, 2009) servers were used. The consensus epitopes were selected based on Troat *et al.* theory as described previously (Dikhit *et al.* 2015; Amit *et al.* 2016). BLASTP search against *Homo sapiens* was performed (Altschul *et al.* 1997; Kar *et al.* 2013), and the peptides with 100% identity were excluded from the study.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6. One-way analysis of variance was performed to evaluate the statistical significance of obtained data.

RESULTS

Ld-iPGAM was successfully cloned, expressed, purified and confirmed

Ld-iPGAM gene was amplified by PCR using genomic DNA of a reference Ld strain MHOM/IN/83/AG83.

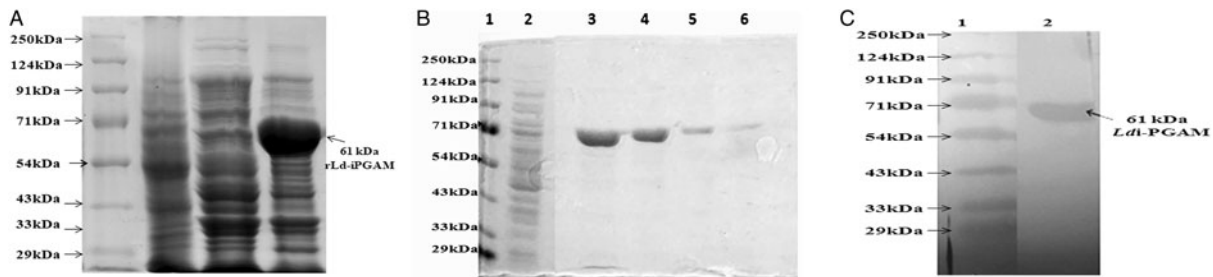


Fig. 1. The expression and purification of rLd-iPGAM. (A) SDS-12% PAGE of expressed protein (rLd-iPGAM) stained with coomassie brilliant blue R 250 (CBB R 250). Lane 1: protein molecular weight marker, lane 2: SLA, lane 3: uninduced transformed BL-21 and lane 4: expressed ~61 kDa rLd-iPGAM. (B) SDS-12% PAGE of purified rLd-iPGAM. Lane 1: protein molecular weight marker, lane 2: SLA and lanes 3, 4, 5 and 6: first, second, third and fourth elutions of purified iPGAM. (C) Western blotting of rLd-iPGAM protein showing reactivity with anti-hexahistidine antibody. Lane 1: protein marker, lane 2: purified rLd-iPGAM stained with anti-hexahistidine antibody.

The amplified product was cloned, expressed and examined by SDS-PAGE. Overexpressed ~61 kDa band of Ld-iPGAM was recognized in comparison to SLA and uninduced BL-21 (Fig. 1A). The purified recombinant protein, as shown in Fig. 1B, was confirmed by Western blotting as an anti-hexahistidine monoclonal antibody reactive 61 kDa band (Fig. 1C). The specific activity of rLd-iPGAM measured after performing enzymatic assays was 32 U mg^{-1} .

rLd-iPGAM-induced dose-dependent expression of CD69 on lymphocytes

rLd-iPGAM-induced expression of CD69 was examined on human lymphocytes to evaluate the antigenicity as well as the optimal concentration of the antigen. Flow-cytometry data showed maximum (>10%) upregulation of CD69 on lymphocytes at $5 \mu\text{g mL}^{-1}$ rLd-iPGAM. The expression of CD69 was dose-dependent with further dilutions of rLd-iPGAM (Fig. 2A). However, some diminishing effect was also noticed on higher concentration of rLd-iPGAM. This experiment was also carried out on CD4⁺ and CD8⁺ cells (Fig. 2B and C). There was 2.3- and 2.06-fold ($P \leq 0.001$) higher expression of CD69 on CD4⁺ and CD8⁺ cells, respectively, in response to $5 \mu\text{g mL}^{-1}$ rLd-iPGAM (Fig. 2D and E). PMA and ionomycin induced 9.8- and 7.5-fold higher expression of CD69 on CD4⁺ and CD8⁺ cells, respectively. Therefore, the $5 \mu\text{g mL}^{-1}$ concentration of rLd-iPGAM was taken into account for further experiments.

rLd-iPGAM stimulation induced higher expression of IL-1 β and IL-1R

After finalizing the concentration of rLd-iPGAM for *in vitro* studies, its effect on phagosome was evaluated. For this, effect of rLd-iPGAM on a leucocyte endogenous mediator and mononuclear cell factor was evaluated. rLd-iPGAM stimulation induced

1.90-fold higher IL-1 β expression in macrophages in comparison to unstimulated control (Fig. 3A). rLd-iPGAM stimulation also induced higher expression of IL-1R on CD4⁺ and CD8⁺ cells in comparison to unstimulated control (Fig. 3B and C).

rLd-iPGAM-induced host-protective immune activation in healthy as well as in treated VL subject

Early cytokine IL-2 released from T lymphocytes for its activation towards effector function was also evaluated in response to rLd-iPGAM. Percentage of IL-2⁺CD4⁺ cell was increased by 1.90- and 2.16-fold in healthy and treated VL subjects, respectively, after stimulation with rLd-iPGAM in comparison to unstimulated control (Fig. 4A). Depriving and generous effect was also noticed after Ld or PMA and ionomycin stimulation, respectively.

As it is well known that cellular activation either proceed towards helper cell type 1 (Th1) or type 2 (Th2). Evaluation of a signature cytokine IL-4 for Th2 was essential to decipher the pathogenic response of rLd-iPGAM. Upon stimulation with the recombinant protein, expression of the IL-4⁺CD4 cell was reduced by 0.27- and 0.40-fold in healthy and treated VL subjects, respectively (Fig. 4B). However, an increasing trend of IL-4⁺CD4 cells were observed in samples stimulated with Ld or PMA and ionomycin.

On the other hand, a prompt effect of rLd-iPGAM was observed on IFN- γ , which is pre-requisite for protection against leishmaniasis. rLd-iPGAM stimulation induced 1.72-fold ($P < 0.05$) and 1.35-fold ($P < 0.05$) higher expression of IFN- γ in CD4⁺ cells from healthy and treated VL donor, respectively (Fig. 5A). As expected, in samples of treated VL donor, but not in healthy donor, IFN- γ expression was higher after Ld stimulation. PMA and ionomycin treatment, as positive control increased IFN- γ by 3.22- and 1.56-fold in healthy and treated subjects, respectively. At this point,

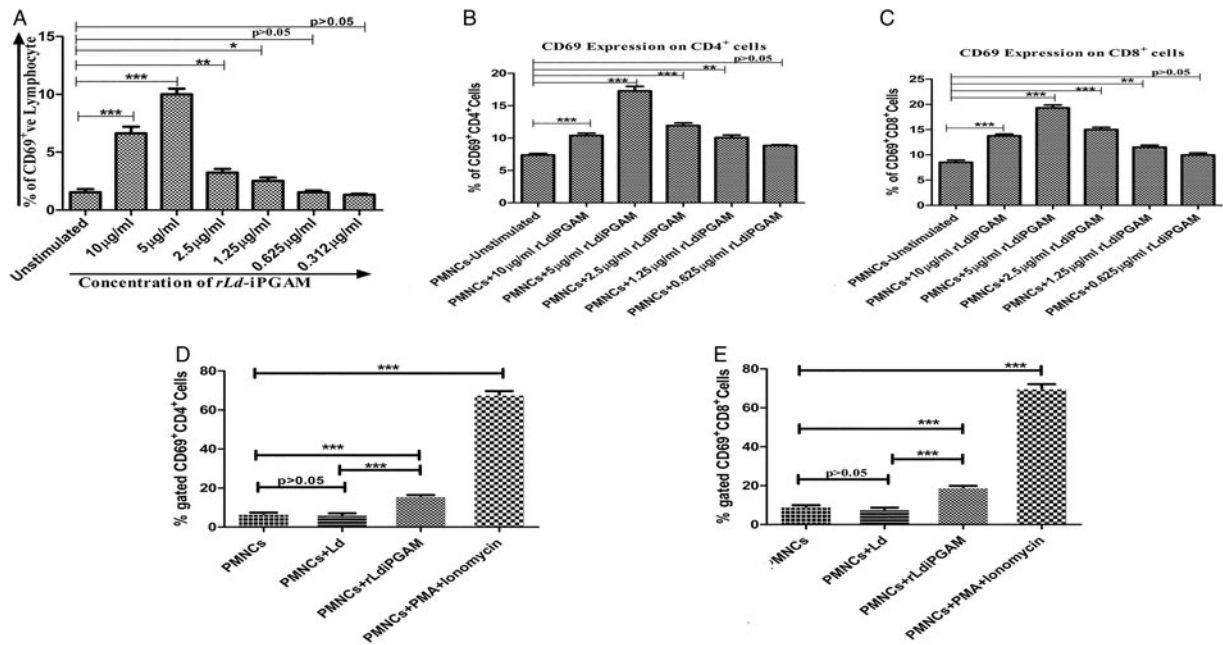


Fig. 2. The expression of CD69 on whole lymphocytes, CD4⁺ as well as CD8⁺ cells. (A) Expression of CD69 on whole lymphocytes (gated population of whole blood cells) upon stimulation with or without rLd-iPGAM in a series of 2-fold serial dilution. (B) Expression of CD69 on CD4⁺ cells (gated population from PMNCs) upon stimulation with or without rLd-iPGAM in a series of 2-fold serial dilution. (C) Expression of CD69 on CD8⁺ cells (gated population from PMNCs) upon stimulation with or without rLd-iPGAM in a series of 2-fold serial dilution. (D) Expression of CD69 on CD4⁺ T cells (gated population from PMNCs) after stimulation with or without *Leshmania donovani* (*Ld*) or rLd-iPGAM or PMA and ionomycin. (E) Expression of CD69 on CD8⁺ T cells (gated population from PMNCs) after stimulation with or without *Ld* or rLd-iPGAM or PMA and ionomycin. Bar represents the mean ± s.d. of five different experiments. Asterisk (*) is significant of ($P < 0.05$), double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

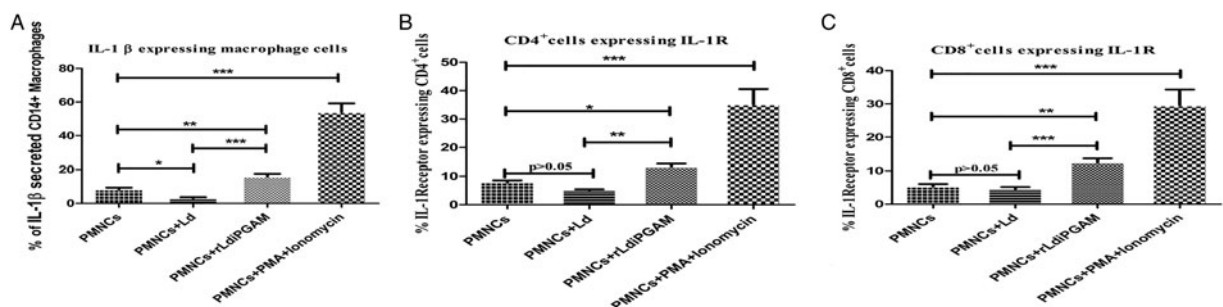


Fig. 3. The secretion of IL-1β by macrophages and expression of its receptor IL-1R on CD4⁺ and CD8⁺ cells after stimulation with or without *Leshmania donovani* (*Ld*) or rLd-iPGAM or PMA and ionomycin. (A) Secretion of IL-1β by CD14⁺ macrophages. (B) Expression of IL-1R on CD4⁺ cells. (C) Expression of IL-1R on CD8⁺ cells. Bar represents the mean ± s.d. of three different experiments. Asterisk (*) is significant of ($P < 0.05$), double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

evaluation of a signature regulatory cytokine IL-10 was crucial for rLd-iPGAM. rLd-iPGAM stimulation reduced the expression of IL-10 by 0.33- and 0.48-fold in both healthy as well as treated VL donors (Fig. 5C). Stimulation with *Ld* as well as PMA and ionomycin increased IL-10 expression in healthy samples. However, PMA and ionomycin but not *Ld* increased IL-10 expression in treated subjects. Like percentage of CD4⁺ IFN-γ⁺ cells, CD8⁺ IFN-γ⁺ cells were also upregulated in samples from treated VL and healthy human subjects in

response to rLd-iPGAM (Fig. 5B). However, it was reduced by 0.48-fold in healthy samples and increased by 1.36-fold in treated VL samples in response to *Ld*. On the other hand, rLd-iPGAM stimulation reduced the percentage of IL-10 expressing CD8⁺ cells in samples of treated VL and healthy subjects (Fig. 5D). However, *Ld* stimulation upregulated IL-10 expressing CD8⁺ cells in samples from healthy, but not treated VL subjects. The PMA and ionomycin treatment showed positive response as expected.

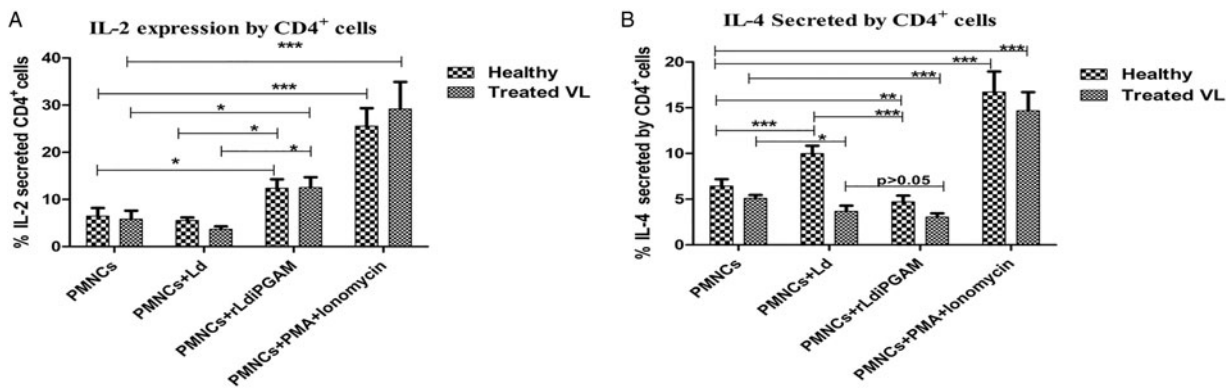


Fig. 4. The secretion of IL-2 and IL-4 by CD4⁺ cells after stimulation with or without *Leishmania donovani* (*Ld*) or r*Ld*-iPGAM or PMA and ionomycin. (A) IL-2 secreted by CD4⁺ cells. (B) IL-4 secreted by CD4⁺ cells. Bar represents the mean ± s.d. of two different experiments. Asterisk (*) is significant of ($P < 0.05$), double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

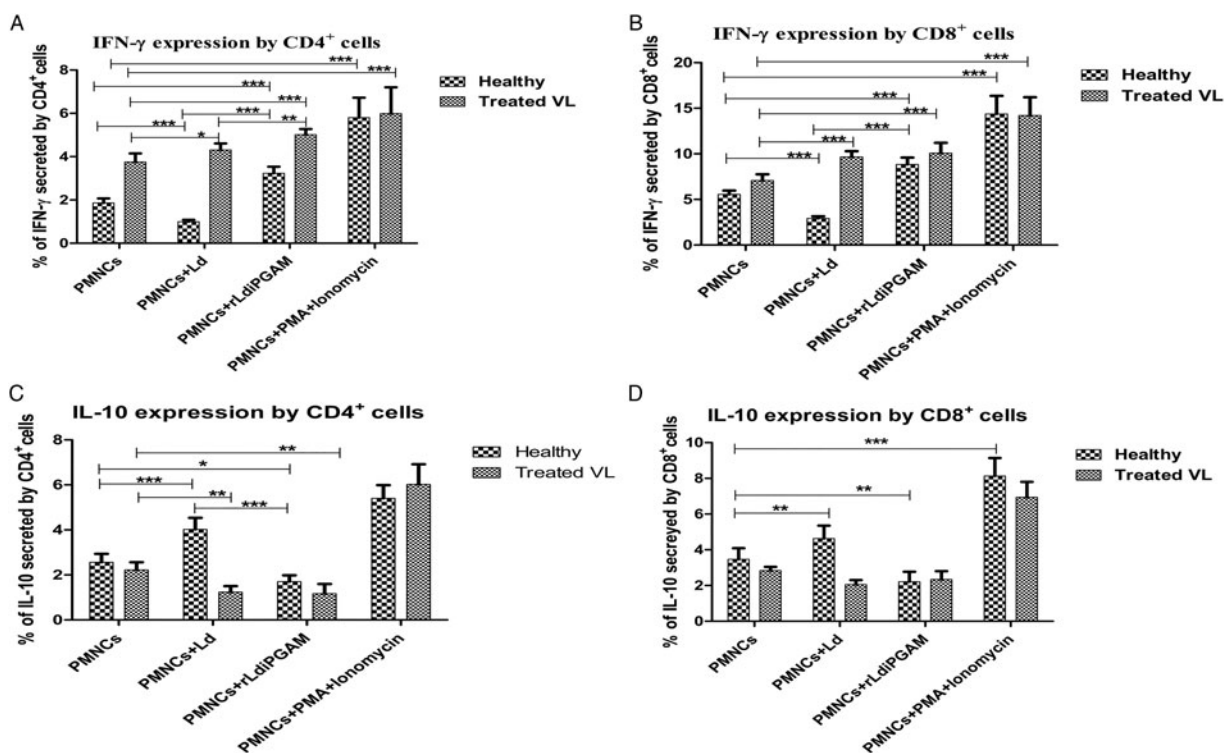


Fig. 5. The secretion of intracellular cytokines IFN- γ and IL-10 by CD4⁺ and CD8⁺ cells after stimulation with or without *Leishmania donovani* (*Ld*) or r*Ld*-iPGAM or PMA and ionomycin. (A) IFN- γ secreted by CD4⁺ cells. (B) IFN- γ secreted by CD8⁺ cells. (C) IL-10 secreted by CD4⁺ cells. (D) IL-10 secreted by CD8⁺ cells. Bar represents the mean ± s. d. of four different experiments. Asterisk (*) is significant of ($P < 0.05$), double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

Effects of r*Ld*-iPGAM was also evaluated in the expression of another pro-inflammatory cytokine IL-17, induced by IL-1 and other inflammasomes, expressed by a set of T-helper and works synergistically with other inflammatory cytokines to protect from infection. r*Ld*-iPGAM induced upregulation of CD4⁺ IL-17 cells as well as CD8⁺IL-17 in samples from healthy and treated VL donors (Fig. 6A and B). In comparison, *Ld* downregulated IL-17-positive CD4⁺ or CD8⁺ cells in healthy donors, whereas upregulated in samples from

treated VL. Upregulated expression of IL17⁺ cells was observed after PMA and ionomycin stimulation.

rLd-iPGAM charging boosts host-protective signalling of macrophages

Macrophage signalling plays a key role in providing immunity against the intracellular parasite *Ld*. Therefore, r*Ld*-iPGAM-induced responses of macrophages were evaluated. IL-12 expressing CD14⁺ macrophages were increased significantly after

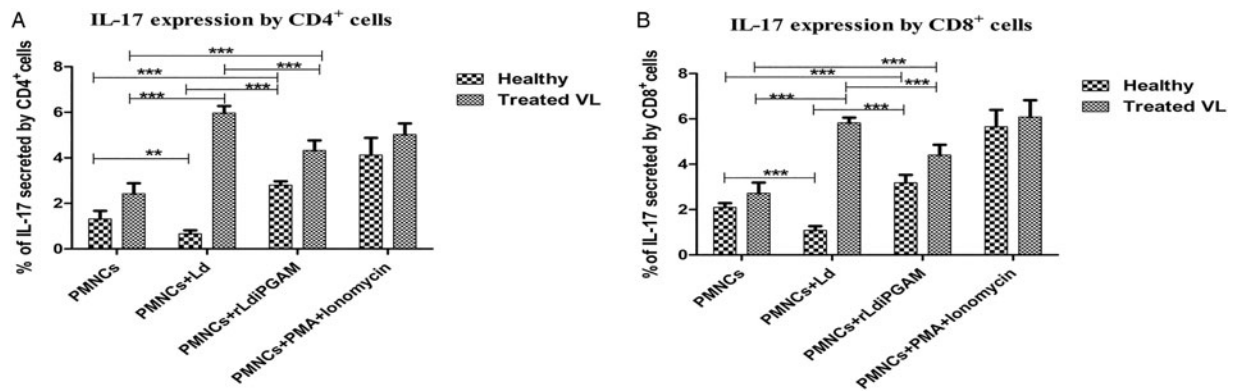


Fig. 6. The secretion of intracellular cytokines IL-17 by CD4⁺ and CD8⁺ cells after stimulation with or without *Leishmania donovani* (*Ld*) or r*Ld*-iPGAM or PMA and ionomycin. (A) IL-17 secreted by CD4⁺ cells. (B) IL-17 secreted by CD8⁺ cells. Bar represents the mean \pm s.d. of two different experiments. Double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

stimulation with r*Ld*-iPGAM or PMA and ionomycin in healthy and treated VL samples, respectively (Fig. 7A). *Leishmania donovani* stimulation, however, reduced IL-12 expressing macrophages in healthy donors, but increased in treated VL subjects.

Leishmania donovani is known to upregulate TGF- β , a negative regulator of immunity, under its survival strategy. *Leishmania donovani* as well as PMA and ionomycin were observed upregulating TGF- β expressing macrophages, which also resulted in higher expression of SMAD-4. But r*Ld*-iPGAM stimulation, showing its immunogenic strength, reduced the expression of TGF- β ⁺CD14⁺ macrophages (Fig. 7B) as well as SMAD-4 to half (Fig. 7C).

r*Ld*-iPGAM-induced immune activation was expected to translate in higher expression of NF- κ B p50. There was 2.68-fold upregulation of NF- κ B p50 expression in macrophages after r*Ld*-iPGAM stimulation (Fig. 7D). Whereas, pathogenic responses of *Ld* stimulation was resulted in reduced expression of NF- κ B p50. The PMA and ionomycin upregulated the NF- κ B p50.

Qualitative data of r*Ld*-iPGAM-induced cytokines was supported by quantitative assay by ELISA

Few of the key cytokines were also evaluated quantitatively to confirm flow data. For this, sandwich ELISA was performed with culture supernatants of PMNCs stimulated with or without r*Ld*-iPGAM. Secretion of IFN- γ was increased by 2.46-fold, IL-10 was reduced by 0.32-fold, TNF- α and IL-12 were also significantly increased by 1.27- and 2.51-fold after stimulation with r*Ld*-iPGAM in comparison to unstimulated control (Fig. 8A–D). In response to *Ld* stimulation, as predicted, IL-10 was raised by 1.2-fold. Unlike IL-10, the level of IFN- γ was decreased by 0.25-fold, TNF- α concentration was reduced to half, and the level of IL-12 decreased by 0.30-fold in comparison to unstimulated healthy control sample. Stimulation with PHA, as a positive

control, increased the secretion of all these cytokines. All these cytokines except IL-10 and TNF- α were also increased in treated VL samples in response to r*Ld*-iPGAM.

r*Ld*-iPGAM stimulation induced strong lymphoproliferative responses (LTT assay)

PMNCs cultured in the presence or absence of SLA, r*Ld*-iPGAM and PHA were assessed by XTT. The results of the proliferative response of lymphocytes against r*Ld*-iPGAM showed significantly higher stimulation in healthy and treated VL (mean OD 1.90 ± 0.186 and 2.386 ± 0.243) than SLA (mean OD 0.772 ± 0.105 and 1.146 ± 0.198) (Fig. 9A).

r*Ld*-iPGAM stimulation charged macrophages to enhance ROS

To ascertain whether the immunoprotective response of r*Ld*-iPGAM translates into parasiticidal activities of macrophages, ROS activity was evaluated. Macrophages showed increased ROS production upon r*Ld*-iPGAM stimulation in comparison to unstimulated control. The stimulated macrophages exhibited 2.1-fold higher ROS activity (MFI = 344.2) in comparison to unstimulated macrophage cells (MFI = 158.2). However, *Ld* challenge decreased the ROS activity (MFI = 78.2) by half and LPS stimulation increased it significantly (MFI = 566.6) (Fig. 9B).

r*Ld*-iPGAM stimulation activated iNOS and induced NO synthesis

NO play a crucial role in the elimination of *Leishmania* amastigotes during the healing process. *Leishmania* is well known, and as also evidenced by the present result, for reducing NO synthesis; but r*Ld*-iPGAM stimulation induced 1.8-fold higher production of NO ($P < 0.05$; Fig. 10A). The expression of iNOS transcripts also increased by 1.733-fold after r*Ld*-iPGAM stimulation (Fig. 10B and C).

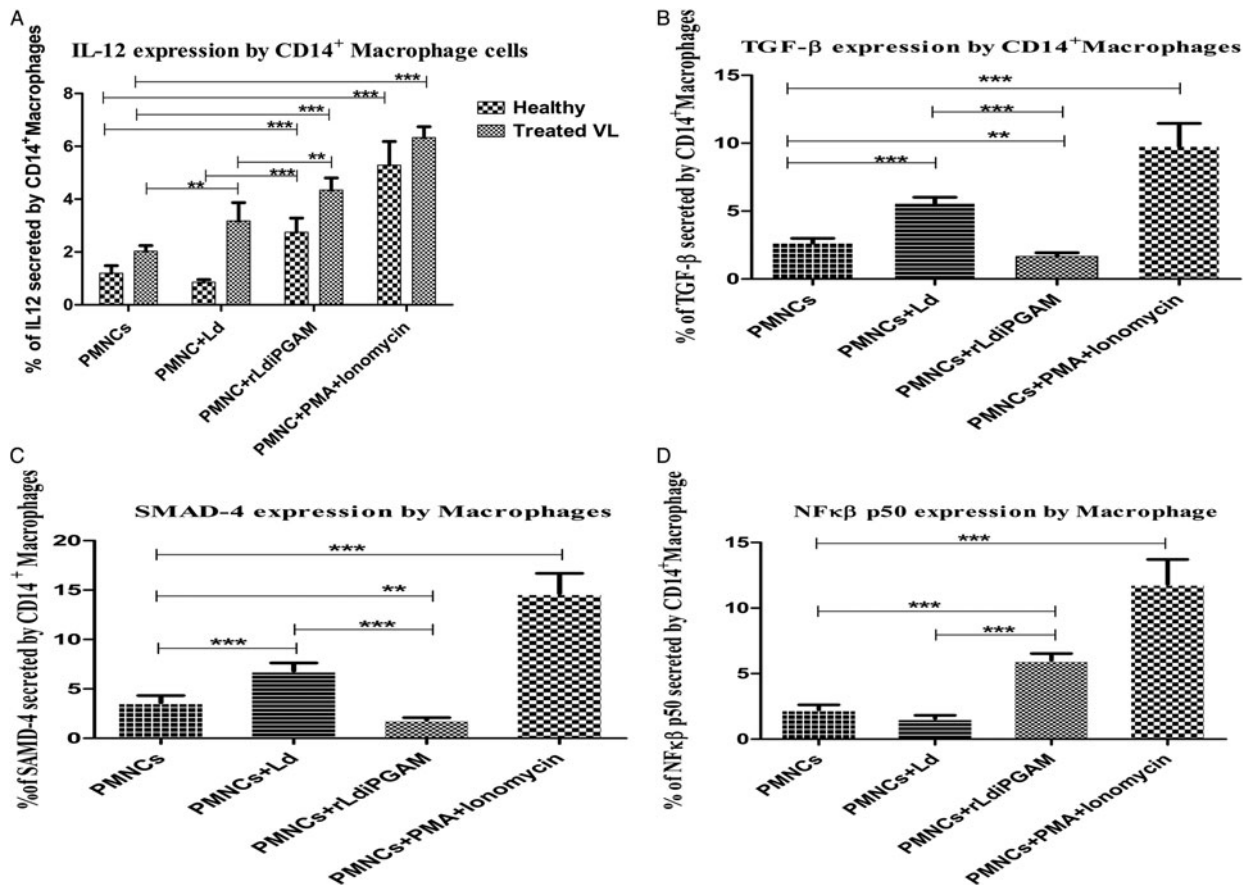


Fig. 7. The secretion of intracellular cytokines and transcription factors in macrophages, stimulated with or without *Leishmania donovani* (*Ld*) or *rLd-iPGAM* or PMA and ionomycin, as positive control. (A) IL-12 secreted by CD14⁺ macrophages. (B) TGF- β secreted by CD14⁺ macrophages. (C) SMAD-4 secreted by CD14⁺ macrophages. (D) NF- κ B p50 secreted by CD14⁺ macrophages. Bar represents the mean \pm s.d. of four different experiments. Double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

rLd-iPGAM charging directs the THP-1 cells towards host-protective responses

In support of the above experiments, immunomodulatory capacity of *rLd-iPGAM* was also evaluated on a monocyte cell line THP-1. NO generation increased by 2.16-fold in the THP-1 cells stimulated with *rLd-iPGAM* in comparison to unstimulated THP-1 cells (Fig. 11A). The NO production was 3-fold higher in response to LPS. Level of TNF- α and IL-12 was also increased by 2.28 and 2.69-fold (Fig. 11B and C). Unlike TNF- α and IL-12, secretion of IL-10 was decreased by more than half-fold as compared with unstimulated THP-1 cells (Fig. 11D). However, LPS stimulation significantly increased the level of TNF- α , IL-12 and IL-10.

Immunoactivation potential of *iPGAM* was supported by in silico study

In the present study, possible MHC class I/II restricted epitopes were predicted based on Trost *et al.* theory by combining the predictions from different algorithms. The epitopes that are highly conserved will be tolerated in the host. BLASTP

analysis revealed the peptide (SLAKLKDAV) with 100% query coverage and 88% identity against zinc finger homeobox 2, isoform CRA_d of *H. sapiens* and excluded from the study. The peptide ALKSGMYDV was found to be a consensus high binding affinity with MHC class I allele (online Supplementary Table S2) and shared only 67% identity with *H. sapiens*. Though, Syfpeithi score did not reflect the best binding affinity for the HLA DR1 restricted epitope (LGAFITKEGSTLHLIG), but IEDB and NETMHC II analysis revealed it as best possible epitope (online Supplementary Table S3).

DISCUSSION

Immunity to *Ld* infection depends largely upon cell-mediated immunity, particularly the upregulation of Th1 cells (Howard and Liew, 1984; Liew, 1991a, b). Besides CD8⁺ cells are also considered as a good contributor in the adaptive immune response during VL (Nateghi *et al.* 2010). Therefore, T-cell stimulating antigens are being considered as a good vaccine target for intracellular pathogens (Kamhawi *et al.* 2014).

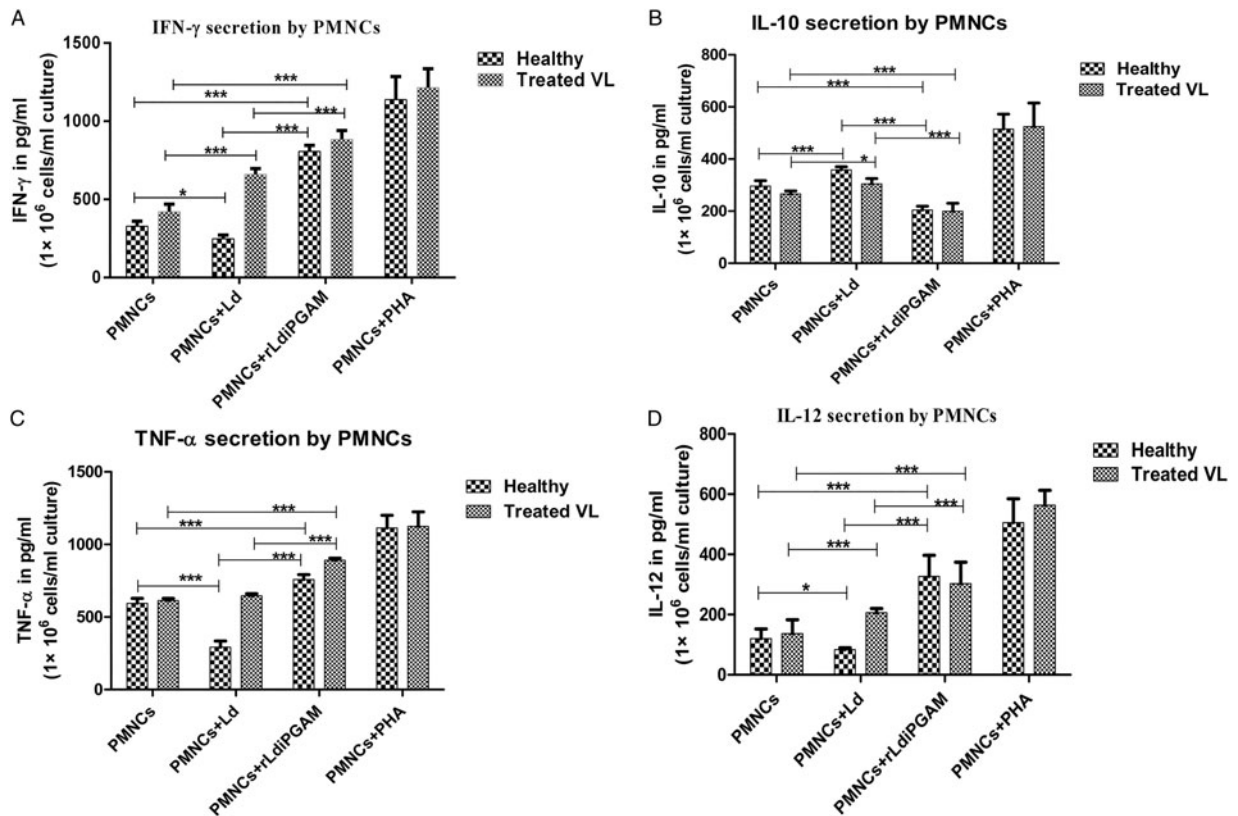


Fig. 8. The quantitative data of IFN- γ , IL-10, TNF- α and IL-12 evaluated by ELISA using culture supernatant of 1×10^6 PMNCs mL^{-1} incubated with or without *Leshmania donovani* (*Ld*) or *rLd*-iPGAM and PHA, as positive control. (A) IFN- γ in pg mL^{-1} . (B) IL-10 in pg mL^{-1} . (C) TNF- α in pg mL^{-1} . (D) IL-12 in pg mL^{-1} . Bar represents the mean \pm s.d. of four different experiments. Asterisk (*) is significant of ($P < 0.05$), double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

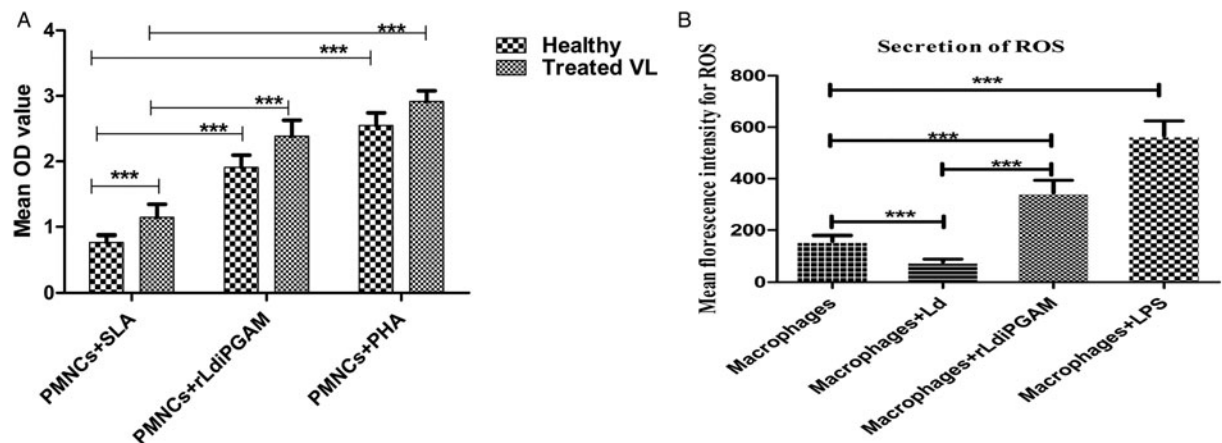


Fig. 9. Lymphocyte proliferation response shown by PMNCs and the ROS release by active macrophage. (A) LTT response of PMNCs from healthy donors against SLA, *rLd*-iPGAM and PHA. Proliferation was represented as the mean OD of stimulated culture–(minus) mean OD of unstimulated culture, as negative control. (B) The ROS produced by macrophages stimulated with or without *Ld* or *rLd*-iPGAM or LPS, as positive control. The data of ROS were measured *via* mean fluorescence intensity (MFI). Bar represents the mean \pm s.d. of two different experiments. Triple asterisk (***) is significant of ($P < 0.001$).

rLd-iPGAM having immunogenic potential induced earlier inducible cell surface glycoprotein CD69 on lymphocytes. Though, the aim of this experiment was to find a suitable dose ($5 \mu\text{g mL}^{-1}$)

of *rLd*-iPGAM for further experiments, higher expression of CD69 on CD4⁺ and CD8⁺ cells was an early assurance of lymphocyte activation, phosphorylation and proliferation.

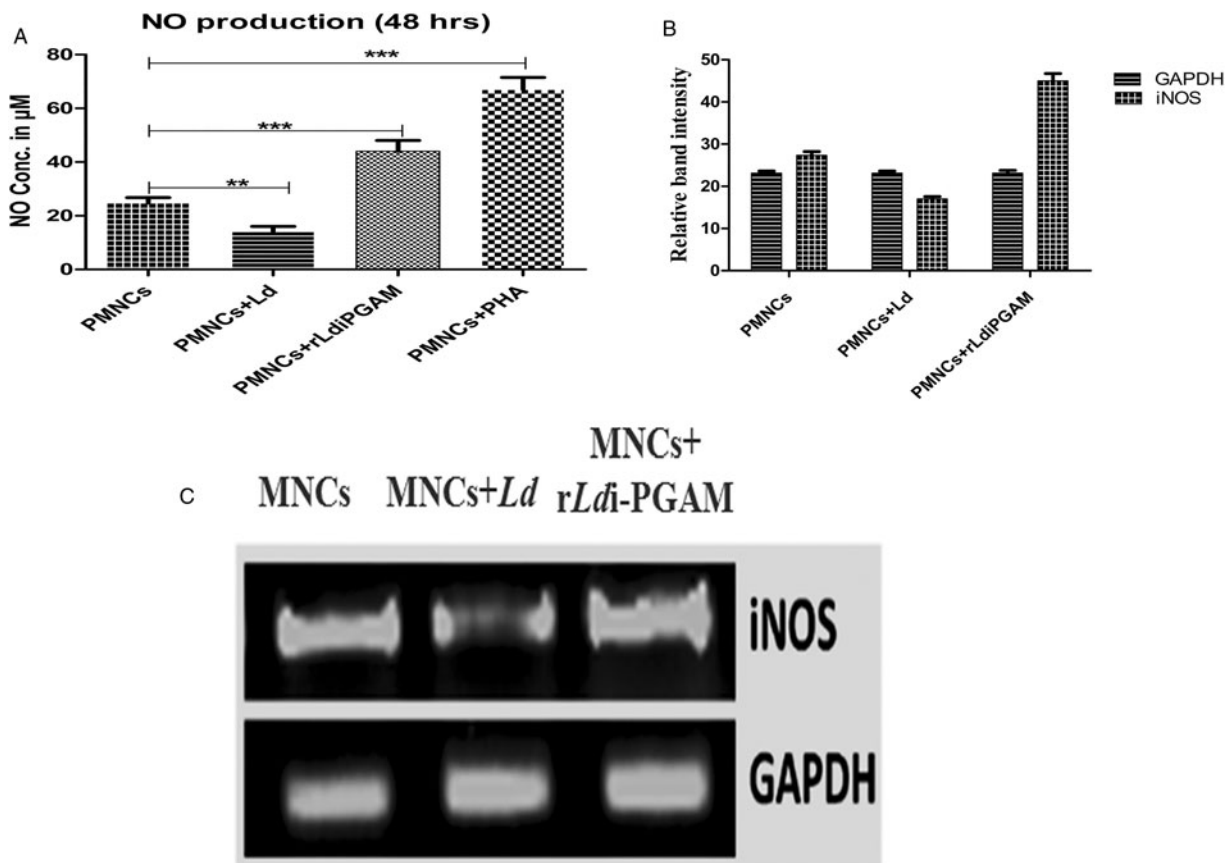


Fig. 10. Secretion of NO and activation of corresponding iNOS in PBMCs from healthy donor treated with or without *Leishmania donovani* (*Ld*) or *rLd*-iPGAM or PHA. (A) Amount of NO secreted. (B) Relative amplification of iNOS by real-time PCR (C) Amplification of iNOS in end product by RT-PCR in agarose gel. Bar represent the mean \pm s.d. of single independent experiments. Double asterisk (**) is significant of ($P < 0.01$) and triple asterisk (***) is significant of ($P < 0.001$).

IL-1 β is the member of IL-1 family and mediates the inflammatory responses. It is involved in cell proliferation, differentiation, apoptosis and facilitates host resistance to infection (Djalma *et al.* 2013). *rLd*-iPGAM induced upregulation of IL-1 β , which promote T-cell activation. Its counter-receptor on lymphocyte binds with a group of 11 cytokines and helps in inflammatory responses to infections. *rLd*-iPGAM stimulation was able to promote interaction of IL-1 β with IL-1R to help in transendothelial migration of immunocompetent cells.

It is evidenced that IL-2 treatment induces antimicrobial activity by promoting IFN- γ secretion (Christopher *et al.* 1993) and helps in resistance to infection (Murray *et al.* 1993). CD8 $^+$ also supports Th1 response of CD4 $^+$ cells being prone to IL-2 expression (Coler *et al.* 2007; Qila *et al.* 2013). *rLd*-iPGAM stimulation reverted the *Ld*-induced downregulation of IL-2 due to better expression through APCs and IL-1 β interaction with IL-1 β receptor upon CD4 $^+$ cells.

IL-4, a well-known Th2 cytokine in the *Leishmania* pathogenesis (Aseffa *et al.* 2002) was also regulated by *rLd*-iPGAM in both healthy as well as in treated samples. Though some of the authors do not recognize IL-4 as a pathogenic

cytokine, its higher expression serves as a signature cytokine during VL (Swain *et al.* 1990).

The immunoprophylactic effect of *rLd*-iPGAM was strengthened by the stimulatory response in upregulation of IFN- γ ⁺CD4 $^+$ and IFN- γ ⁺CD8 $^+$ cells and downregulation of IL-10 $^+$ CD4 $^+$ and IL-10 $^+$ CD8 $^+$ cells from healthy and treated VL subjects. Higher expression of IFN- γ ⁺CD4 $^+$ and IFN- γ ⁺CD8 $^+$ in *rLd*-iPGAM stimulated cells from a treated VL donor is probably due to the presence of memory cells against *rLd*-iPGAM. This possibility was also strengthened by the pathogenic effect of *Ld* stimulation on cells of the healthy donor, which was reversed by a higher Th1/Th2 ratio in the sample of treated VL cases; but we could not examine the *rLd*-iPGAM-induced dynamics of memory cell in the present study design.

IL-17 helps in protecting against human VL (Pitta *et al.* 2009). It induces host-protective signalling against *Ld* (Nascimento *et al.* 2011; Huber *et al.* 2013). Similar to IFN- γ , IL-17 $^+$ CD4 $^+$ cells (Th-17) and IL-17 $^+$ CD8 $^+$ cells (Tc-17) expression was raised upon stimulation with *rLd*-iPGAM in cells of treated VL. Cells of treated VL subject recognized *Ld* and responded well to express IL-17, which was also observed in the case of *rLd*-iPGAM stimulation.

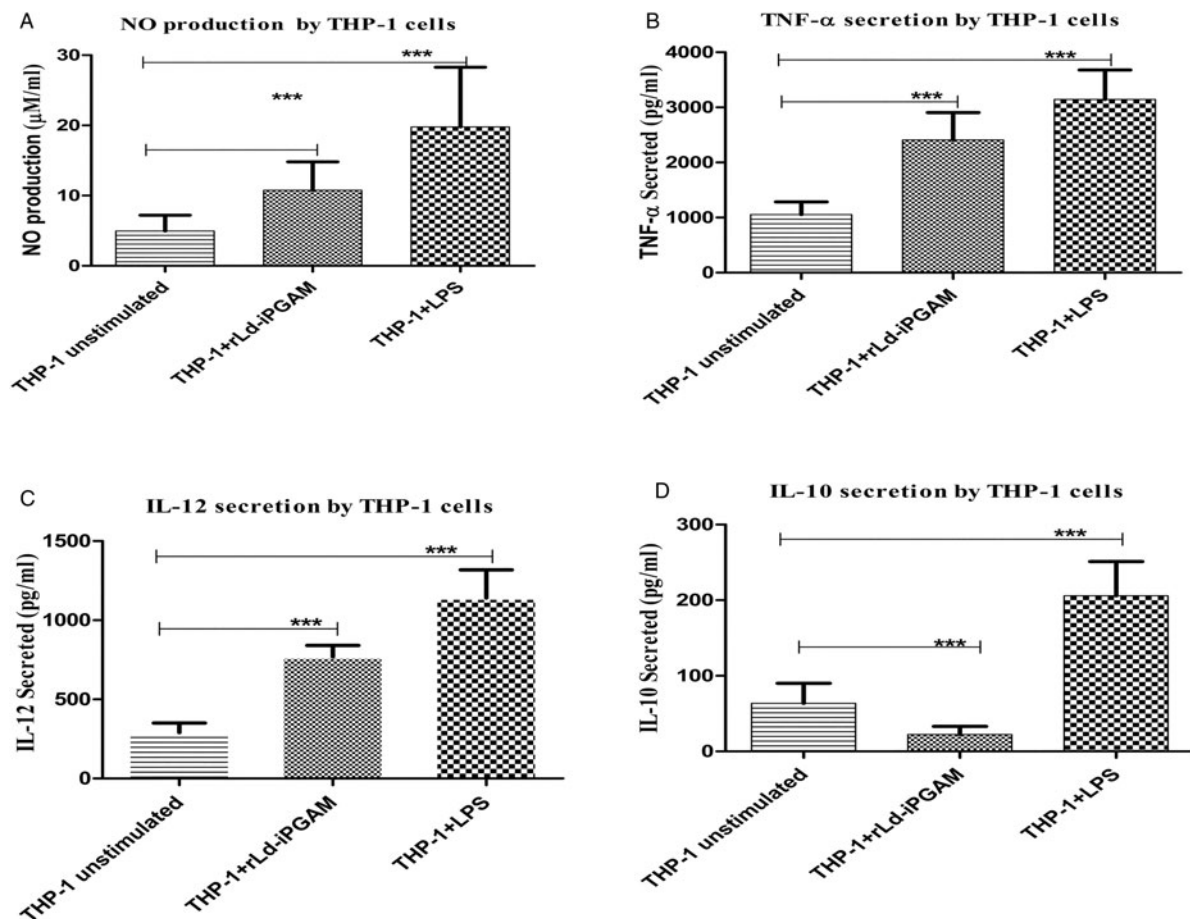


Fig. 11. The responses shown by THP-1 cell line stimulated with or without *rLd*-iPGAM or LPS. (A) NO production by THP-1 cell line. (B) TNF- α (in pg mL^{-1}) secreted by THP-1 cell line. (C) IL-12 (in pg mL^{-1}) secreted by THP-1 cell line. (D) IL-10 (in pg mL^{-1}) secreted by THP-1 cell line. Bar represents the mean \pm s.d. of four different experiments. Triple asterisk (***) is significant of ($P < 0.001$).

rLd-iPGAM induced higher expression of Th17 in the healthy sample as well, being more immunogenic in comparison to *Ld* parasite as a whole.

IL-12 interacts with T cells and helps the initiation and maintenance of Th1 responses (Ghalib *et al.* 1995; Murray *et al.* 2006). Thus, it mediates essential signalling between macrophages and T cells for the production of IFN- γ . *Leishmania donovani* downmodulate the IL-12 expression, which was reverted by *rLd*-iPGAM. Macrophages from treated VL responded both *Ld* and *rLd*-iPGAM producing IL-12. However, the response against *rLd*-iPGAM was better. Thus, *rLd*-iPGAM not only activated the T cells, but also modulated the macrophages for protective secondary signalling. IL-12 binding with IL-12 receptor induces expression of IFN- γ and IL-17. *Leishmania donovani* stimulation, as predicted, enhanced the secretion of TGF- β due to the presence of parasite-derived factor cathepsin-B. TGF- β enhances parasite survival in macrophages (Kira *et al.* 2003) by downmodulating NO production (Rebecca *et al.* 2014). *rLd*-iPGAM downmodulated TGF- β expression most probably due to lack of cathepsin-B or due to host immune pressure. This *rLd*-iPGAM-induced downregulation of

TGF- β was resulted in decreased accumulation of SMAD-4 in the nucleus (Roelen *et al.* 2003). It is known that downregulated SMAD-4 fails to promote IL-1 receptor-associated kinase-M, which ultimately leads a concomitant upregulation of downstream NF- κ B p50 (Srivastav *et al.* 2015).

The qualitative assay of cytokines evaluated by flow cytometry was supported by quantitative evaluation of few cytokines, like IFN- γ , IL-10, TNF- α and IL-12. IFN- γ is known to act in synergy with another macrophage-derived cytokine TNF- α . Therefore, along with IFN- γ , higher expression of TNF- α activates macrophages to express iNOS (Anselmo *et al.* 2009). Therefore, upregulation of IFN- γ and TNF- α upon *rLd*-iPGAM stimulation increased the iNOS expression, which in turn enhanced the production of NO and promoting the clearance of *Ld*. Pro-inflammatory cytokines TNF- α and IL-12 play a critical role in the induction of NO and ROS. Thus, upregulation of TNF- α and IL-12 upon *rLd*-iPGAM stimulation boosting the leishmanicidal activity of macrophages. Unlike IFN- γ , IL-10 was reduced in samples from healthy and treated VL subjects upon *rLd*-iPGAM stimulation. This extracellular cytokine data collected through ELISA

finally confirmed the host-protective immune response established by recombinant protein.

Development of strong cell-mediated immunological response is the key of protective signalling against VL. The significant lymphoproliferative response shown by PMNCs against r*Ld*-iPGAM stimulation ensures that recombinant protein having the potential to activate and develop strong T-cell-mediated immunity against pathogenesis.

ROS production through an oxidative burst is responsible for the leishmanicidal activity (Murray, 1981; Gannt *et al.* 2001; Bisti *et al.* 2006), but it has been reported that *Ld* inhibits the production of O₂⁻ inside the host, so the level of O₂⁻ and H₂O₂ is significantly lower in monocytes from VL patient as compared with healthy subjects (Kumar *et al.* 2001, 2002). *Leishmania donovani* survive inside the host by using the antioxidant system or by suppressing macrophages ROS production (Murray, 1981). We observed that charging of r*Ld*-iPGAM significantly increased the ROS generation by activating macrophages, which ensures that recombinant protein is able to defend the host from parasitic infection.

Binding of IFN- γ and TNF- α with macrophages activates iNOS, which in turn produces NO by breaking L-arginine to L-citrulline. NO plays a significant role in controlling parasite and overcome VL. It has direct parasiticidal activity in addition to its role in signalling to evoke innate macrophage responses (Pandya *et al.* 2016). This result was also supported by the confirmation of upregulation of iNOS in the presence of r*Ld*-iPGAM.

Th1 stimulatory nature of r*Ld*-iPGAM was validated and supported by directing the THP-1 cells through increasing the level of NO, TNF- α , IL-12 and simultaneously decreasing IL-10.

The efficacy of r*Ld*-iPGAM was supported by *in silico* study, which suggested that this antigen has highly conserve MHC class I and II restricted epitopes that could trigger the desired immune response in the host. As previously reported of the 10% top scored peptides predicted by SYFPEITHI and BIMAS, 85% peptides have the ability to trigger a desired immune response (Rammensee *et al.* 1999; Parker *et al.* 1994). Beside, highly conserved epitopes will be tolerated in the host. Therefore, this study recommends r*Ld*-iPGAM as a potential immunomodulating agent, which can be used in future vaccine module.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182017001494>.

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