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Visceral leishmaniasis: A novel nuclear envelope protein 'nucleoporins-93 (NUP-93)' from *Leishmania donovani* prompts macrophage signaling for T-cell activation towards host protective immune response

Manish K. Singh[a,1], Fauzia Jamal[a,1], Amit K. Dubey[a,b], Pushkar Shivam[a], Sarita Kumari[a], Pushpanjali[a], Chayanika Bordoloi[b], S. Narayan[a], V.N.R. Das[a], K. Pandey[a], P. Das[a], Shubhankar K. Singh[a,*]

[a] Rajendra Memorial Research Institute of Medical Sciences, Patna 800007, India
[b] National Institute of Pharmaceutical Education and Research, Hajipur 844102, India

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ABSTRACT

The shift of macrophage and T-cell repertoires towards *proinflammatory* cytokine signalling ensures the generation of host-protective machinery that is otherwise compromised in cases of the intracellular *Leishmania* parasite. Different groups have attempted to restore host protective immunity. These vaccine candidates showed good responses and protective effects in murine models, but they generally failed during human trials. In the present study, we evaluated the effect of 97 kDa recombinant nucleoporin-93 of *Leishmania donovani* (r*Ld*-NUP93) on mononuclear cells in healthy and treated visceral leishmaniasis (VL) patients and on THP-1 cell lines. r*Ld*-NUP93 stimulation increased the expression of the early lymphocyte activation marker CD69 on $CD4^+$ and $CD8^+$ T cells. The expression of the host protective pro-inflammatory cytokines IFN-γ, IL-12 and TNF-α was increased, with a corresponding down-regulation of IL-10 and TGF-β upon r*Ld*-NUP93 stimulation. This immune polarization resulted in the up-regulation of NF-κB p50 with scant expression of SMAD-4. Augmenting lymphocyte proliferation upon priming with r*Ld*-NUP93 ensured its potential for activation and generation of strong T-cell mediated immune responses. This stimulation extended the leishmanicidal activity of macrophages by releasing high amounts of reactive oxygen species (ROS). Further, the leishmanicidal activity of macrophages was intensified by the elevated production of nitric oxide (NO). The fact that this antigen was earlier reported in circulating immune complexes of VL patients highlights its antigenic importance. In addition, *in silico* analysis suggested the presence of MHC class I and II-restricted epitopes that proficiently trigger $CD8^+$ and $CD4^+$ T-cells, respectively. This study reported that r*Ld*-NUP93 was an effective immunoprophylactic agent that can be explored in future vaccine design.

1. Introduction

Even after serious efforts made by different agencies, cases of visceral leishmaniasis (VL) have been cited continuously in India, particularly in the Bihar provinces. An increasing rate of resistance to anti-leishmanial drugs, limited therapeutic options, a lack of sterile cure, the appearance of post kala-azar dermal leishmaniasis and the existence of asymptomatic infection and HIV-VL co-infection are among the major hurdles in achieving the goal of Kala-azar elimination programme. These all fortify the existence of a reservoir for the continuation of parasite transmission. Although a few vaccine candidates are under clinical trial [1,2], there is still not a single effective commercial vaccine against human leishmaniasis. Most of the proposed vaccines failed to achieve high degrees of antigenicity and persistency, which are the key determinants of an effective vaccine candidate. Variation in immunogenicity due to human lymphocyte antigen expression, drug susceptibility and parasite-induced pathogenic pressure in host and over-immune responses to vaccine candidates were the major hurdles in the way of anti-*Leishmania* vaccines [3–5]. In addition, many of the earlier proposed subunit or protein vaccines [6–12] failed to generate protective immunity against pathogen because they were tested in either murine model or in cell lines, providing good data but not mimicking

* Corresponding author at: Microbiology Division, RMRIMS (ICMR), Agamkuan, Patna 800007, India.
E-mail address: shubhankark.singh@icmr.gov.in (S.K. Singh).
[1] Both authors contributed equally to this work.

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human cells. Considering the above situation, the present study was carried out on peripheral blood of healthy individuals from various VL endemic and non-endemic zones and treated cases of VL to decipher this problem.

The nuclear pore complex (NPC) is a large macromolecular assembly that acts as a gatekeeper in the pores of the nuclear envelope. It facilitates transportation of proteins and other biological molecules between the cytoplasm and the nucleoplasm [13,14]. NPCs are composed of approximately 30 different proteins known as nucleoporins (NUPs) [15,13,16]. Amongst the NUPs, NUP93 is essential for the structural assembly of the nuclear pore complexes [17]. It is known that the NUP93 of *L. donovani* exists in both the promastigote and amatigote forms, an additional advantage regarding its vaccine potential. *Leishmania* species caused cleavage of NUP proteins after infection, and cleavage sites in NUP93 were also identified for metalloprotease GP63 [18]. Jamal et al. [38] showed its presence in circulating immune complexes (CICs) from VL patients. The presence of nucleoporin in CICs was evidence of the activation of B-cells against NUP93, which may alternatively activate T-cells, as the former also act as efficient antigen presenting cells. Therefore, considering its immunogenicity, this antigen was selected to explore the immunoprophylactic activity.

For the first time, the immunoprophylactic potential of r*Ld*-NUP93 has been evaluated in peripheral blood mononuclear cell (PMNCs) from healthy and treated VL patients. However, PMNCs from active VL cases were not included in this study, as they may not respond to leishmanial antigen [19]. Additionally, PMNCs from VL patients typically do not proliferate or produce IFN-γ in response to stimulation with recombinant protein of *Leishmania* species [20–22]. Similar findings were observed during our study with insignificant data, so they were removed from the text. r*Ld*-NUP93 significantly up-regulated the early activation marker CD69 on $CD4^+$ or $CD8^+$ T cells. This early activation resulted in the dominance of the host protective cytokines IFN-γ, IL-12 and TNF-α and the regulation of IL-10 and TGF-β. In addition, it up-regulated the expression of reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) in target cells in the presence of meagre SMAD-4 expression and up-regulated NF-κB p50. A systematic effect of r*Ld*-NUP93 in a protective cascade of immune molecules ensures its capability as a promising immune prophylactic agent to be used in vaccines.

2. Materials and methods

2.1. L. donovani culture and isolation of genomic DNA

A reference strain of *L. donovani* (MHOM/IN/83/AG83) was cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS). Promastigote culture was maintained as per a protocol used elsewhere [23]. Late log phase parasites were harvested by centrifugation at 900*g* at 4 °C for 20 min (Hermle, Germany). The pellets were washed with PBS three times and spun at 900*g* for 20 min at 4 °C. The washed pellets (10^8 promastigotes) were used to isolate DNA using the DNA Purification Kit (Qiagen, Germany). Isolated DNA was electrophoresed using 0.8% agarose gels, and DNA concentration was evaluated using Nanodrop (Thermo Fisher Scientific) and stored for further use.

2.2. Amplification and cloning of L. donovani NUP93

Ld-NUP93 gene specific primers were designed manually and checked with NEB Cutter and Oligocalc tools. Primers were as follows: Forward, 5′-TTTTGGATCCATGTTTAGCTCGACT-3′ (*Bam*HI restriction site is underlined) and reverse, 5′-TTTTGCGGCCGCACTCAGCACATATAAAC-3′ (*Not*I restriction site is underlined). These primers were synthesized commercially (IDT, India). The *Ld*-NUP93 gene (NCBI Reference Sequence: XM_003865331.1) was amplified using the primers above in a thermal cycler (Bio-Rad, USA). The PCR condition was set as the first cycle at 95 °C for 5 min and 30 cycles at 94 °C for 1 min, 56.7 °C for 1 min, 72 °C for 3 min and the last cycle at 72 °C for 10 min. The PCR product was electrophoresed using 1% agarose gel along with a 1 kb ladder (BR Biochem. Life Sciences, India). The amplified product was eluted using a gel extraction kit (Qiagen, Germany). A vector (pET-28a) was isolated the fresh culture using a plasmid isolation kit (Qiagen, Germany). Isolated pET-28a and eluted amplicons were digested separately with *Bam*HI and *Not*I (Promega, USA) and ligated using T4 DNA ligase (Fermentas, Thermo Fisher Scientific, USA). Freshly prepared competent *Escherichia coli* DH5α cells were transfected with the recombinant plasmid. On a subsequent day, the transformation was confirmed by colony PCR and by restriction double digestion using *Bam*HI and *Not*I.

2.3. DNA sequence analysis and alignment

Gene inserts of the pET-28a-*Ld*-NUP93 construct were sequenced by Sanger sequencing methods. The cloned genes insert were isolated from vector pET28a with the help of restriction enzymes *Bam*HI and *Not*I. To verify the gene inserts cloned in the vector, specific forward and reverse primers for *Ld*-NUP93 gene were used. DNA sequencing was performed commercially by Applied Biosystems. Chromatogram analysis was performed using the Finch TV 1.4.0 software. Homology searches and sequence alignment were performed using the nucleotide BLAST program http://www.ncbi.nlm.nih.gov/BLAST.

2.4. Expression and purification of L. donovani NUP93

The pET-28a-*Ld*-NUP93 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 µl fresh inoculum of transformed BL-21 was inoculated in 5 ml Luria broth supplemented with 1 µg/ml kanamycin. The culture was incubated at 37 °C in a shaker incubator at 220 rpm until the OD_{600} reached 0.5–0.6. Then 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and incubated at 37 °C overnight (14–16 hrs). One ml of the recombinant cells culture was pelleted and lysed with 2× SLB [100 mM Tris base (pH-6.8), 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.2% bromophenol blue, 200 mM β-mercaptoethanol] and electrophoresed by SDS-12% p*olyacrylamide gel electrophoresis* (PAGE*)* to confirm protein expression. The uninduced culture was run in parallel as a control. The result was analysed in comparison to protein marker (Puregene).

Recombinant *Ld*-NUP93 was purified by following a previously described protocol [24]. The concentration of purified recombinant protein was estimated by the Bradford method using bovine serum albumin (BSA) as the standard.

2.5. Preparation of soluble Leishmania 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 *L. donovani* pellet was subjected to six cycle of freeze and thaw. 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.

2.6. Confirmation of purified protein by western blot and LPS test

Western blotting was performed to confirm the presence of recombinant protein using anti-hexahistidine antibody following a protocol described earlier [25]. Briefly, purified r*Ld*-NUP93 was subjected to SDS-12% PAGE and transferred to 0.22 µm nitrocellulose membranes

(Sigma-Aldrich USA) in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH-8.2) using a semi-dry blotter (Bio-Rad, USA) at 15 V for 30 min. The transferred nitrocellulose paper (NCP) was treated with blocking buffer (5% BSA, PBS) overnight and was washed with Tris-buffered saline-Tween 20 [TBS-T (Tris base, NaCl, 0.1% Tween-20 and 0.2% BSA, pH-7.5)] 3 times. The membranes were incubated with anti-hexahistidine antibodies (1:2500 for 1 hr, Santa-Cruz Biotech, USA), followed by three washes with TBS-T. The membranes were further incubated with *horseradish peroxidase* (HRP)-conjugated anti-rabbit antibodies (1:1000, MERCK Biosciences). The blotted membranes were exposed to DAB (3-3′-diaminobenzidine, 0.06% H_2O_2, Ameresco, USA) solution until the bands appeared without background. The membranes were rinsed with plenty of distilled water to stop the reaction and analysed. The concentration of bacterial lipopolysaccharide (LPS) contamination was determined in the r*Ld*-NUP93 using the Limulus amoebocyte lysate (LAL) test (Thermo-Scientific, USA) as per manufacturer's instructions. LPS contamination of 0.12 μg/mg was detected in the r*Ld*-NUP93. The contamination was removed using a polymyxin B-agarose column (Sigma, USA) according to the manufacturer's instructions. After the removal steps, very little residual LPS was detected in r*Ld*-NUP93 (1.25 pg/μg of r*Ld*-NUP93). The purified r*Ld*-NUP93 was stored at −80 °C in aliquots for further use.

2.7. Generation of polyclonal antibody

Generation of polyclonal antibody against r*Ld*-NUP93 was performed in a rabbit after obtaining approval from the animal ethical committee of Rajendra Memorial Research Institute of Medical Sciences. Prior to immunization, pre-immune serum was collected. Subsequently, the rabbit was immunized using 150 μg r*Ld*-NUP93 with complete Freunds adjuvant. After 15 days, the rabbit was again immunized using 150 μg of r*Ld*-NUP93 with incomplete Freunds adjuvant as a first booster. Subsequently, only antigen was administered as a second booster, three days prior to serum collection. For immunoblotting experiments, purified r*Ld*-NUP93 was electrophoresed in 12% SDS-PAGE and transferred to 0.22 μm nitrocellulose membranes (Sigma, USA) in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH-8.2) with a semi-dry blotter (Bio-Rad) at 15 V for 30 min. Blocking was done in blocking buffer (5% BSA, PBS) overnight, and membranes were washed with Tris-buffered saline-Tween 20 [TBS-T (Tris base, NaCl, 0.1% Tween-20 and 0.2% BSA, pH-7.5] 3 times. The membranes were incubated for 2 hrs in rabbit generated polyclonal antibody (1:65 ratio) followed by three washes in TBS-T. HRP-conjugated anti-rabbit antibodies were used as secondary antibodies. The membranes were further incubated with DAB (3-3′-diaminobenzidine, 0.06% H_2O_2) solution until the band appeared without background. The membranes were rinsed with plenty of distilled water.

2.8. Sample selection

Peripheral blood was collected from healthy and VL patients after obtaining written informed consent following the guidelines of the institutional ethical committee. Those who were apparently healthy and free from any health related complains or recognizable clinical symptoms, coming from non-VL endemic areas, were selected for the healthy blood samples. Residual blood samples collected from VL patients for routine investigations (before and after treatment) were used for the immunological investigations in this study. VL patients were diagnosed by rK39 strip test (InBios, India) and confirmed by microscopic examination for *L. donovani* amastigotes in Giemsa-stained spleen or bone marrow smears. Treated VL samples were collected from the patients receiving complete courses of amphotericin B treatment and being clinically cured. Details of the clinical, haematological and biochemical investigations are given in Table 1.

2.9. Fluorescence staining of early activation marker CD69 on lymphocytes.

Whole blood from healthy subjects (n = 13) was used to study the expression of surface markers on lymphocytes. To explore the role of r*Ld*-NUP93 on T-cell activation and proliferation, expression of the early activation lymphocyte marker CD69 was evaluated in PMNCs [24,26]. PMNCs were isolated from peripheral blood of healthy subjects (n = 13) by density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, USA). The PMNCs were washed with PBS and counted in a 0.1 mm Neubauer chamber (Fein Optia, JENA, Germany). Cells were suspended at 1×10^6 PMNCs/ml in RPMI-1640 complete media (RPMI + FBS). The cells were stimulated with or without r*Ld*-NUP93 and incubated in the CO_2 incubator at 37 °C for 24 hrs. The cultured cells were washed and stained with anti-CD4 PECy5 or anti-CD8 PECy5 and anti-CD69 FITC and further incubated for 20 min at room temperature in the dark. Cells were centrifuged at 200*g* for 5 min. The pellets were washed with 2 ml PBS, and the samples were suspended in 500 μl PBS and acquired to measure fluorescence on a FACSCaliber™ (BD).

Table 1
Clinical symptoms, hematological as well as biochemical parameters of healthy subject and VL patients before and after anti-leishmanial therapy. n: number of sample, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase.

Study groups	Healthy (n = 13)		Visceral leishmaniasis (VL) patients (n = 13)			
			Before treatment		After treatment	
	Male (n = 8)	Female (n = 5)	Male (n = 9)	Female (n = 4)	Male (n = 9)	Female (n = 4)
Age	22–45	25–45	21–45	23–45	21–45	23–45
Weight (kg)	55–75	45–65	52–69	42.5–62	53.1–72.6	42.6–63
Body mass index (kg/m^2)	23.92 ± 2.91	18.9 ± 1.75	14.65 ± 2.27	12.98 ± 2.02	19.80 ± 1.96	15.57 ± 1.77
Body temperature (in ^{0}F)	97.5 ± 0.5	97.5 ± 0.45	100.59 ± 1.892	100.86 ± 1.831	97.24 ± 0.375	97.45 ± 0.35
Hepatomegaly (in cm)	0	0	3.28 ± 2.76	2.8 ± 1.70	0.7 ± 0.928	0.59 ± 0.813
Splenomegaly (in cm)	0	0	7.8 ± 3.69	6.9 ± 3.01	0.8 ± 1.141	0.71 ± 1.040
Haemoglobins (g/dl)	12.96 ± 0.955	10.98 ± 0.895	7 ± 1.64	5.8 ± 1.64	9.31 ± 0.856	8.1 ± 0.792
WBC (white blood cells) (per mm^2)	6820 ± 1581.50	6998 ± 1248.66	2550 ± 949.10	2615 ± 1045.10	5509 ± 486.75	5676 ± 687.15
Lymphocytes (%)	32.86 ± 5.73	33.61 ± 6.9	43.71 ± 8.195	45.78 ± 6.5	28.7 ± 2.547	29.6 ± 2.74
SGOT (U/L)	7.97 ± 4.858	7.743 ± 3.855	35.7 ± 16.213	31.4 ± 13.935	28.47 ± 8.60	21.5 ± 7.122
SGPT (U/L)	8 ± 1.581	7.4 ± 1.437	31 ± 13.730	29.96 ± 14.533	23 ± 9.281	21.7 ± 7.836
Blood urea (mg/dl)	20.51 ± 4.725	20 ± 5.591	24.1 ± 6.749	23.5 ± 7.581	20.4 ± 11.834	21 ± 10.761
Serum Creatinine (mg/dl)	0.7 ± 0.168	0.56 ± 0.142	0.469 ± 0.163	0.447 ± 0.144	0.499 ± 0.144	0.504 ± 0.143
Sodium (mEq/L)	137 ± 7.592	142 ± 5.352	138.8 ± 3.049	142.3 ± 4.836	136.6 ± 5.253	139 ± 3.789
Potassium (mEq/L)	4.456 ± 0.660	4.353 ± 0.576	4.36 ± 0.377	3.87 ± 0.474	4.71 ± 0.954	5 ± 1.422

2.10. Fluorescence staining of T cells and macrophages for qualitative evaluation of rLd-NUP93-induced intracellular cytokines

PMNCs (1×10^6 cells/ml) from healthy and treated VL patients (n = 13, Table 1) were stimulated with or without *L. donovani* (*Ld*) or r*Ld*-NUP93 or PMA and ionomycin and were incubated in the CO_2 incubator at 37 °C for 96 hrs by providing feed pulsing after 48 hrs. The cultures were blocked using GolgiPlug™ (1 μg/ml, BD) for the final 2 hrs. Non-adherent cells were collected from culture supernatants and were harvested to study intracellular cytokines of innate and adaptive immune cells. Centrifuged supernatants were 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 subsequent macrophage studies. Cells were washed with PBS, and the surface antibodies anti-CD4 phycoerythrin Cy5 (PECy5), anti-CD8 PECy5 and anti-CD14 PerCP were added in their own Falcon tubes and incubated for 20 min at 4 °C. The cell samples were 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 maintained at 4 °C for 5 min, Samples were washed, and the intracellular antibodies anti-IFN-γ FITC, anti-IL-10 PE, anti-IL-12 FITC, anti TGF-β FITC, anti NF-κβ P50 PE and anti-SMAD PE were added in their respective tubes 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% NaN_3 and 1% FBS). Finally, cell pellets were suspended in 500 μl stain buffer and sorted by FACSCaliber (BD).

2.11. Quantitative evaluation of rLd-NUP93-induced cytokine expression by PMNCs

The cytokines IL-10 (Cat. No. 555157, BD), IFN-γ (Cat. No. 555142, BD), TNF-α (Cat. No. 555212, BD) and IL-12 (Cat. No. 555183, BD) were evaluated in culture supernatants of PMNCs stimulated with or without r*Ld*-NUP93 or *Ld* for 96 hrs. Phytohaemagglutinin (PHA) stimulation was used as a positive control. Quantitative evaluations of cytokines were performed using kit contents according the user manual of the BD OptEIA kit. Absorbance was measured at 450 nm in an ELISA reader (Bio-Rad).

2.12. Assessment of lymphocyte proliferation responses after rLd-NUP93 stimulation

PMNCs from healthy and treated VL patients were cultured in 96-well flat-bottom tissue culture plates (Nunc, Denmark) with or without SLA or r*Ld*-NUP93 or PHA (1 μg/ml, Sigma) as positive control. The purpose of using SLA in place of *Ld* was to avoid the unwanted response generated by the dehydrogenase activity of the parasite. The LTT (lymphocyte transformation test) assay was carried out following the protocol of Garg et al. [27], except 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% CO_2 for 3 days in case of PHA and 5 days for the unstimulated blank, SLA and r*Ld*-NUP93. Fifty microlitres of XTT was added to 100 μl of the culture of each well. Absorbance was measured at 480 nm, with 650 nm as the reference wavelength.

2.13. Evaluation of the effect of rLd-NUP93 on induction of reactive oxygen species (ROS)

PMNCs from healthy subjects were placed in 12-well plates and incubated at 37 °C in CO_2 incubator with 5% CO_2 overnight. After 14–16 h of incubation, the adherent macrophages were collected from the culture plates by following the protocol mentioned earlier. One hundred microlitres of cultured and washed macrophages from healthy subjects were placed into four different 12 × 75 mm, Falcon™ tubes. In the first tube, cells without stimulation were used as the negative control. Cells in the second tube were challenged with *Ld*. Similarly, cells in the third tube were stimulated with r*Ld*-NUP93, and finally, the cells in the fourth tube were stimulated with LPS as a positive control. All tubes were incubated in a water bath for 15 min. The samples were removed from the water bath and 200 μM dihydrorhodamine 123 (DHR) was added. N-formylmethionyl-leucyl-phenylalanine (FMLP, 4 μM) was added to samples stimulated with LPS and r*Ld*-NUP93. The samples were further incubated at 37 °C in a water bath for 10 min. The samples were treated with 1 ml 1× FACS lysis buffer™ (BD) and centrifuged at 200*g* for 5 min. The supernatants were discarded, and cell pellets were resuspended in 500 μl stain buffer (PBS + 1% FBS + 0.09%NaN_3). Samples were acquired on FACSCalibur™ using Cell Quest software (BD). The ROS produced by stimulated cells were measured based on their mean fluorescence intensity.

2.14. Evaluation of rLd-NUP93-induced NO (nitric oxide) synthesis in PMNC culture supernatants

PMNCs from healthy donors were cultured (1×10^6 cells/ml) with or without r*Ld*-NUP93 or *L. donovani* or PHA in a 5% CO_2 incubator at 37 °C for 96 hrs. Nitrate and nitrite were quantified to evaluate NO synthesis in the culture supernatants of PMNCs in the presence or absence of r*Ld*-NUP93, *Ld* and PHA. The assay was performed according to the contents and kit manual of nitric oxide Assay Kit (Thermo Fisher). Absorption was recorded at 540 nm.

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

The human monocytic cell line, THP-1 was maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum at 37 °C in a humidified atmospheric incubator with 5% CO_2. THP-1 cells (1×10^6 cells/ml) were cultured with 5 nM phorbol 12-myristate 13-acetate in 24 -well plates for 48 hrs to achieve differentiation and maturation into macrophages. Cells were then washed and cultured in fresh medium in the presence or absence of r*Ld*-NUP93 or LPS. The culture supernatants were collected to evaluate NO production using a kit (Thermo Fisher Scientific) and cytokine ELISA to measure TNF-α, IL-12 and IL-10 using the BD OptEIA kit.

2.16. MHC class I and MHC class II epitope prediction

To screen the availability of promising MHC class I and MHC class II epitopes in *Ld*-NUP93, we relied on HLA-A*0201, 0207, 0205, A1101 and HLA DRB1 0401 populations. The amino acid sequence of NUP93 (XP_003865379.1) was retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). The retrieved sequence was screened for 9-mer HLA-A*0201, 0207, 0205, and A1101 restricted epitope using SYFPEITHI (predicting the antigen-specific cytotoxic T-cell epitopes using a matrix-based algorithm; [28], RANKPEP [29] and IEDB (http://tools.iedb.org) according to our previous methodology [24]). RANKPEP is a Position Specific Scoring Matrices (PSSMs)-based bioinformatics tool that is used to predict peptide binders to MHC-I molecules from protein sequences or sequences alignment. It also predicts the MHC-I ligands having C-terminal ends that are likely to be the results of proteasomal cleavage. IEDB is the library of experimentally measured immune epitopes. The database includes a tool that predicts the MHC class I and class II binding epitopes.

For 15-mer HLA DRB1 0401 restricted epitopes, SYFPEITHI, IEDB and NETMHC II [30] servers were used. Selection of consensus epitopes were done on the basis of Trost theory [31,32].

2.17. Statistical analysis

One-way ANOVA and two tailed t-tests were performed for

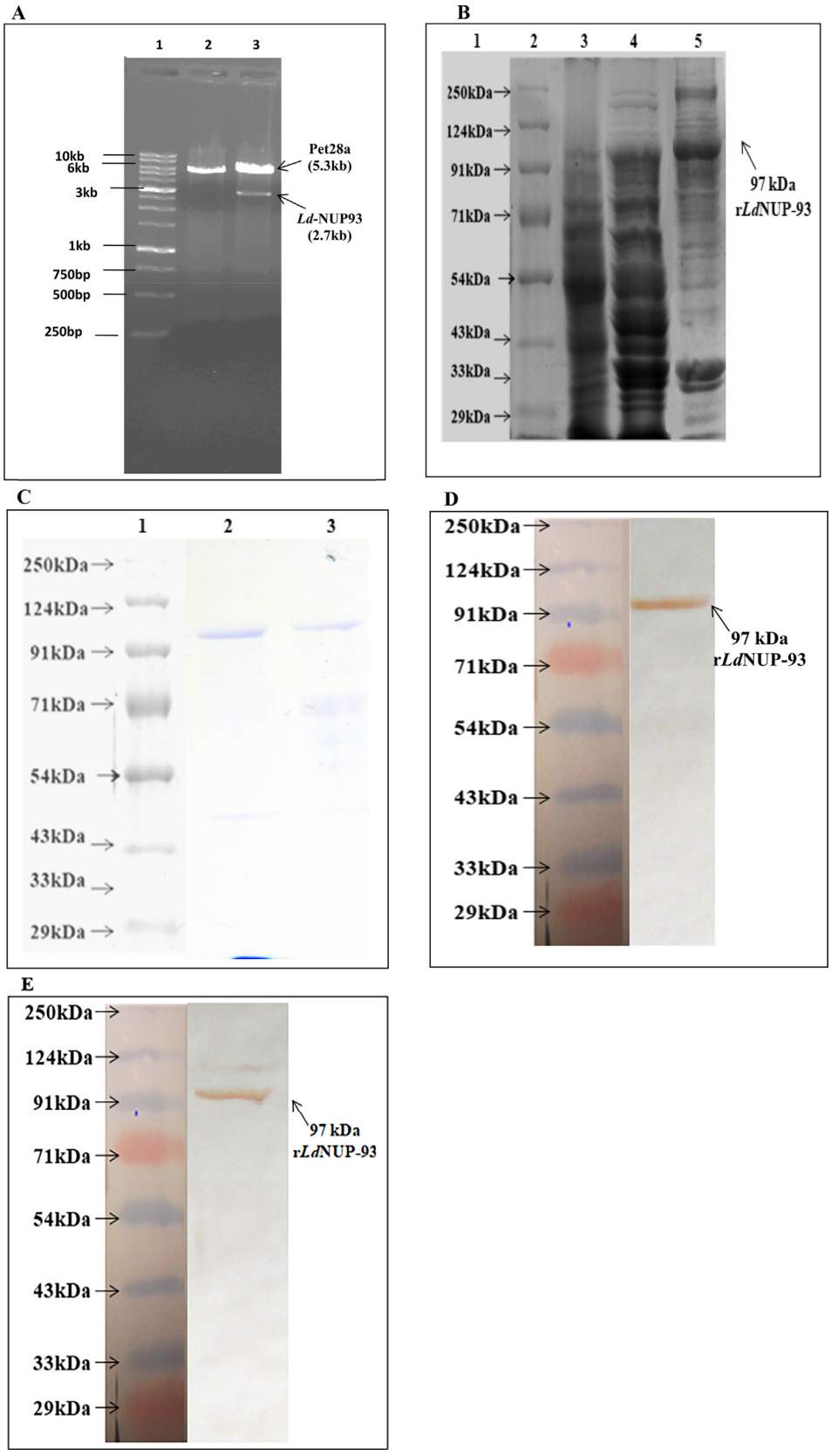

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Fig. 1. Pictures showing r*Ld*-NUP93 at the stages of cloning, expression, purification and confirmation. (A) Restriction digestion of pet28a-*Ld*-NUP93 construct showing two separate band of vector (5.3 kb) and insert (2.7 kb) (lane 1: DNA ladder, lane 2: empty vector, lane 3: restriction digestion of pet28a-*Ld*-NUP93 construct (B) SDS-12% PAGE of BL-21 lysate expressing r*Ld*-NUP93 after Coomassie Brilliant Blue R 250 staining (lane 1: protein molecular weight marker, lane 2: SLA, lane 3: uninduced transformed BL-21 and lane 4: induced transformed BL-21 expressing ~97 kDa r*Ld*-NUP93). (C) SDS-12% PAGE of Ni-NTA column purified r*Ld*-NUP93 (lane 1: protein molecular weight marker, lane 2, & 3: 1st and 2nd elutions of purified r*Ld*-NUP93). (D) r*Ld*-NUP93 protein showing reactivity with anti hexahistidine antibody after Western immune-blotting (lane 1: protein marker, lane 2: purified r*Ld*-NUP93 reactive to anti hexahistidine antibody). (E) r*Ld*-NUP93 protein showing reactivity with polyclonal antibody raised in rabbit upon r*Ld*-NUP93 immunization, through Western blot (lane 1: protein marker, lane 2: purified r*Ld*-NUP93 showed reactivity with polyclonal antibody). (F) *Ld*-NUP93 sequencing with forward primer and alignment (G) *Ld*-NUP93 sequencing with reverse primer and alignment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparison of groups and evaluation of statistical significance, respectively, using GraphPad Prism 6.

3. Results

3.1. Confirmation of cloning, expression and purification of rLd-NUP93

r*Ld*-NUP93 gene was amplified by PCR using genomic DNA of a reference *L. donovani* strain MHOM/IN/83/AG83. The amplified product was cloned, expressed and examined by SDS-PAGE. Confirmation of cloned insert in vector was shown by two separate bands of vector (5.3 kb) and *Ld*-NUP93 insert (2.7 kb) by restriction digestion using BamHI and NotI (Fig. 1A). An overexpressed ~97 kDa band of r*Ld*-NUP93 was recognized in comparison to SLA and uninduced BL-21 (Fig. 1B). The purified recombinant protein shown in Fig. 1C was confirmed by western blotting as an anti-hexahistidine monoclonal antibody-reactive ~97 kDa band (Fig. 1D). Polyclonal antibody raised in rabbit also showed reactivity to r*Ld*-NUP93 by western blot (Fig. 1E).

3.2. Sequence analysis of cloned gene insert using Sanger DNA sequencing method

The gene insert in pET-28a-*Ld*-NUP93 construct was sequenced and analysed. The pET-28a-*Ld*-NUP93 construct was double digested with their respective restriction enzymes. A chromatogram acquired by forward primer sequencing revealed 99% identity with 100% query cover (TTCCTCTG-AGCCTACC^{963}) with *Leishmania donovani* (*L. donovani*) nucleoporin interacting component (NUP93), accession number XM_003865331.1 (Fig. 1F; Supplementary data-1). Similarly, a chromatogram acquired by reverse primer sequencing exhibited 99% identity with 100% query cover (^{6}CCGCGGCG-AGTCGCTG^{600}) from *L. donovani* strain nucleoporin interacting component (NUP93) XM_003865331.1 (Fig. 1G; Supplementary data 2).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.07.005.

3.3. rLd-NUP93 induced dose-dependent expression of CD69 on lymphocytes

To evaluate the antigenicity and the optimal concentration of the antigen, r*Ld*-NUP93-induced expression of CD69 was examined in $CD4^+$ and $CD8^+$ T cells. Flow cytometry data showed maximum expression of CD69 at 5 μg/ml r*Ld*-NUP93. The expression of CD69 was dose-dependent with further dilutions of r*Ld*-NUP93 (Fig. 2A and B). There was 3.022- and 3.89-fold ($p \leq 0.005$) higher expression of CD69 on $CD4^+$ and $CD8^+$ T-cells, respectively, in response to 5 μg/ml r*Ld*-NUP93 (Fig. 2C and D). However, some diminishing effect was noticed on higher concentration of r*Ld*-NUP93. The PMA and ionomycin as a positive control induced 8.79- and 12.2-fold higher expression of CD69 in $CD4^+$ and $CD8^+$ T-cells, respectively. Therefore, the 5 μg/ml concentration of r*Ld*-NUP93 was used for further experiments (the gating strategy and dot plot data is shown in Fig. 2E and F).

3.4. rLd-NUP93 induced host protective immune activation in healthy as well as in treated VL patient

Cellular activation proceeds towards either helper cell type 1 (Th1) or type 2 (Th2). IFN-γ and IL-10 are the signalling cytokines that guide cellular immune responses either towards disease protection or disease progression. Therefore, r*Ld*-NUP93-induced IFN-γ and IL-10 secretion in cell-mediated immune responses was examined. Prompt effect of the r*Ld*-NUP93 was observed with IFN-γ, the pre-requisite for protection against leishmaniasis. r*Ld*-NUP93 stimulation induced 1.76-and 1.44-fold higher expression of IFN-γ in $CD4^+$ T cells in healthy and treated VL donors, respectively (Fig. 3A). As expected, in samples of treated VL donors, but not in healthy donors, IFN-γ expression was higher after *L. donovani* stimulation. PMA and ionomycin treatment, as a positive control, increased IFN-γ 3.35- and 1.72-fold in healthy and treated patients, respectively. At this point, evaluation of a signature regulatory cytokine IL-10 was crucial for r*Ld*-NUP93. r*Ld*-NUP93 stimulation reduced expression of IL-10 0.35- and 0.46-fold in both healthy and treated VL donors (Fig. 3C). Stimulation with *L. donovani* as well as PMA and ionomycin increased IL-10 expression in healthy samples. However, PMA and ionomycin, but not *L. donovani*, increased IL-10 expression in treated patients.

The percentage of $CD4^+$ IFN-γ^+ T-cells and $CD8^+$ IFN-γ^+ T-cells were also higher in samples from treated VL and healthy human subjects in response to r*Ld*-NUP93 (Fig. 3B). However, the percentage was reduced 0.47-fold in healthy samples and increased 1.42-fold in treated VL samples in response to *L. donovani*. On the other hand, r*Ld*-NUP93 stimulation reduced the percentage of IL-10 expressing $CD8^+$ T-cells in samples of treated VL and healthy subjects (Fig. 3D). However, *L. donovani* stimulation up-regulated IL-10 expressing $CD8^+$ T-cells in samples from healthy but not treated VL patients. PMA and ionomycin treatment showed positive responses, as expected.

3.5. rLd-NUP93 charging boosted host protective signalling of macrophages

Macrophage signalling plays a key role in providing immunity against the intracellular parasite *L. donovani*. Therefore, r*Ld*-NUP93 induced responses of macrophages were evaluated. IL-12 expressing $CD14^+$ macrophages were increased significantly after stimulation with r*Ld*-NUP93 or PMA and ionomycin in healthy and treated VL samples, respectively (Fig. 4A). Similar to r*Ld*-NUP93 or PMA and ionomycin, *L. donovani* stimulation increased the percentage of macrophages expressing IL-12 in treated VL patient samples. However, *L. donovani* stimulation reduced macrophages expressing IL-12 in healthy samples.

L. donovani up-regulates TGF-β (a negative regulator of immunity) according to its survival strategy. *L. donovani* as well as PMA and ionomycin up-regulated TGF-β expressing macrophages, also resulting in higher expression of SMAD-4. However, r*Ld*-NUP93 stimulation, showing its immunogenic strength, reduced the percentage of $CD14^+$ macrophages expressing TGF-β (Fig. 4B) and SMAD-4 (Fig. 4C).

r*Ld*-NUP93 induced immune activation was expected to release NF-κβ p50 in $CD14^+$ cells. The percentage of the macrophages showing NF-κβ p50 release was increased 2.68 fold after r*Ld*-NUP93 stimulation (Fig. 4D). *L. donovani* stimulation reduced the percentage of macrophages expressing NF-κβ p50 reactive to its monoclonal antibody. However, as expected, PMA and ionomycin stimulation up-regulated the percentage macrophages releasing NF-κβ p50.

F

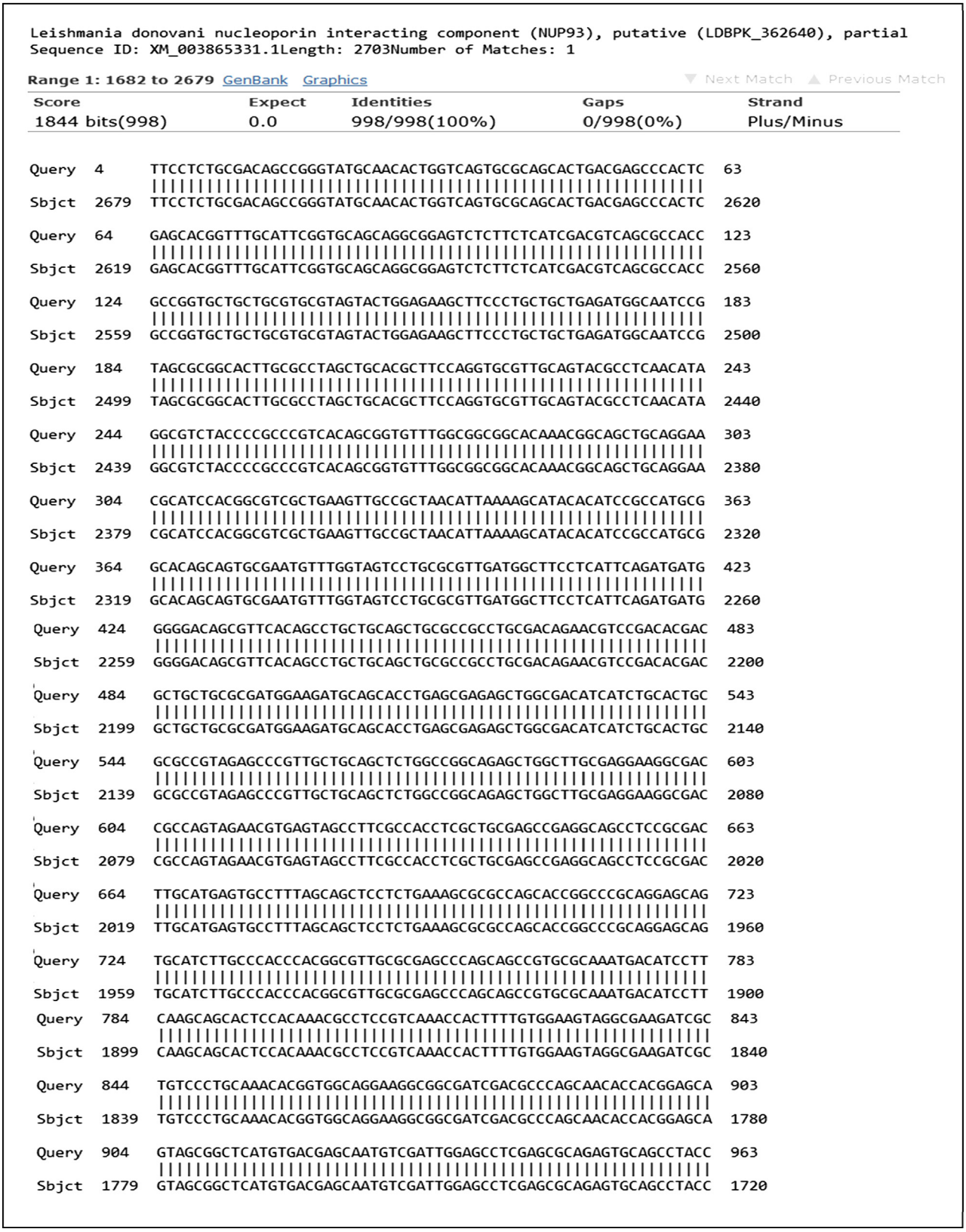

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Fig. 1. (*continued*)

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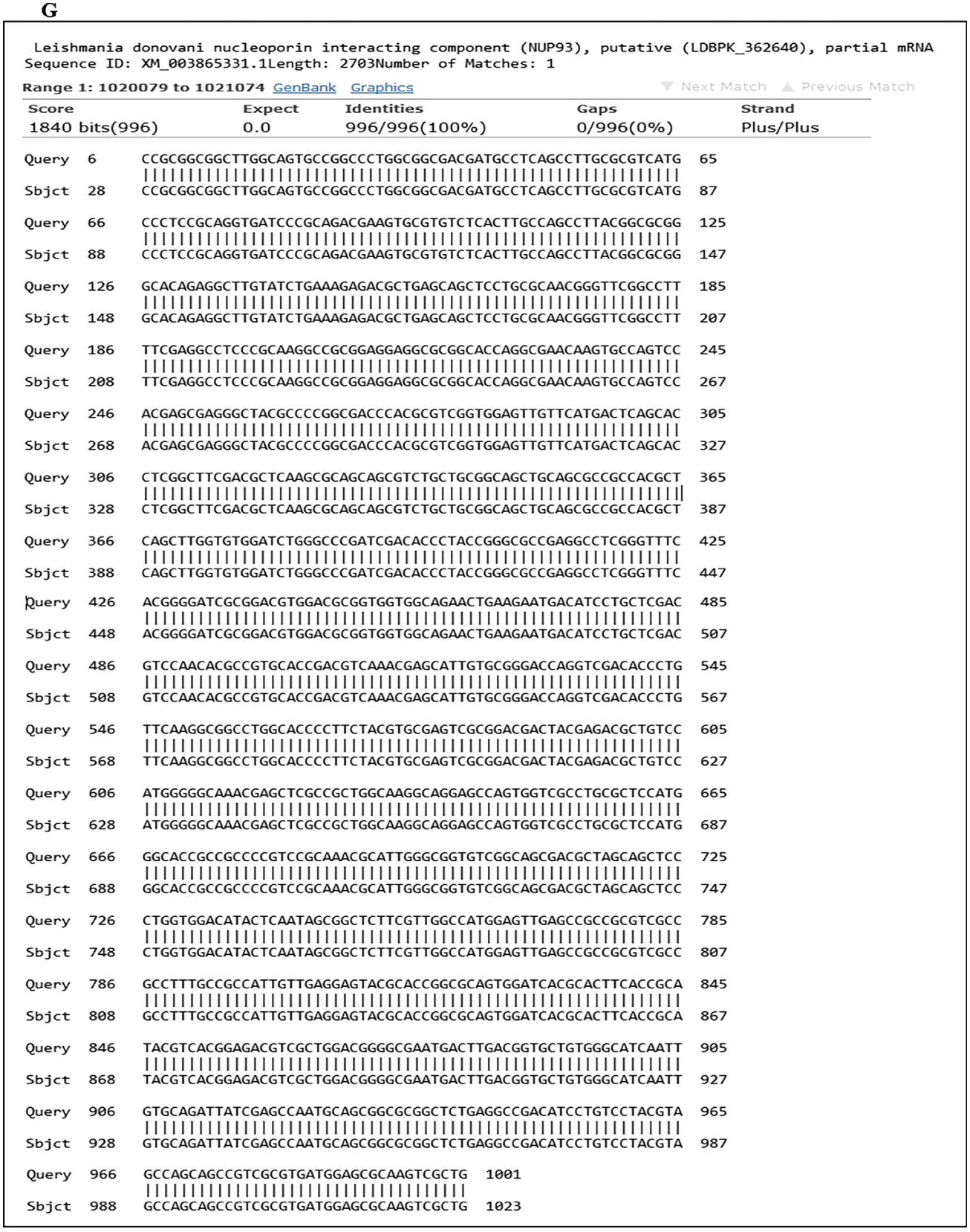

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Fig. 1. (*continued*)

3.6. Qualitative data of rLd-NUP93 induced cytokines was supported by quantitative assay by ELISA

Few of the key cytokines were also evaluated quantitatively to confirm the flow data. For this, sandwich ELISA was performed with culture supernatants of PMNCs stimulated with or without r*Ld*-NUP93. Secretion of IFN-γ, TNF-α and IL-12 was increased 2.14, 1.36 and 1.944 fold, whereas IL-10 was reduced 0.37-fold after stimulation with r*Ld*-NUP93 compared to the unstimulated control (Fig. 5A–D). In response to *L. donovani* stimulation, as predicted, IL-10 increased 1.35-fold. Unlike IL-10, the level of IFN-γ was 0.345-fold lower, TNF-α concentration was reduced to half, and the level of IL-12 decreased 0.50-fold compared with the unstimulated healthy control samples. Stimulation with PHA as a positive control increased the secretion of all these cytokines. All cytokines except IL-10 and TNF-α were increased in treated VL samples in response to r*Ld*-NUP93.

3.7. rLd-NUP93 stimulation induced strong lymphoproliferative responses (LTT assay)

PMNCs cultured in the presence or absence of SLA, r*Ld*-NUP93 and PHA were assessed by XTT. The results of the proliferative response of lymphocytes against r*Ld*-NUP93 showed significantly higher stimulation in healthy and treated VL (mean OD 1.92 ± 0.186 and 2.74 ± 0.243) than in SLA (mean OD 0.782 ± 0.105 and 1.306 ± 0.198) (Fig. 6A).

3.8. rLd-NUP93 stimulation charged macrophages to enhance ROS

To ascertain whether the immunoprotective responses to r*Ld*-NUP93 translate into parasiticidal activities of macrophages, ROS activity was evaluated. Macrophages showed higher ROS production upon r*Ld*-NUP93 stimulation than did the unstimulated control. Stimulated macrophages exhibited 2.1-fold higher ROS activity

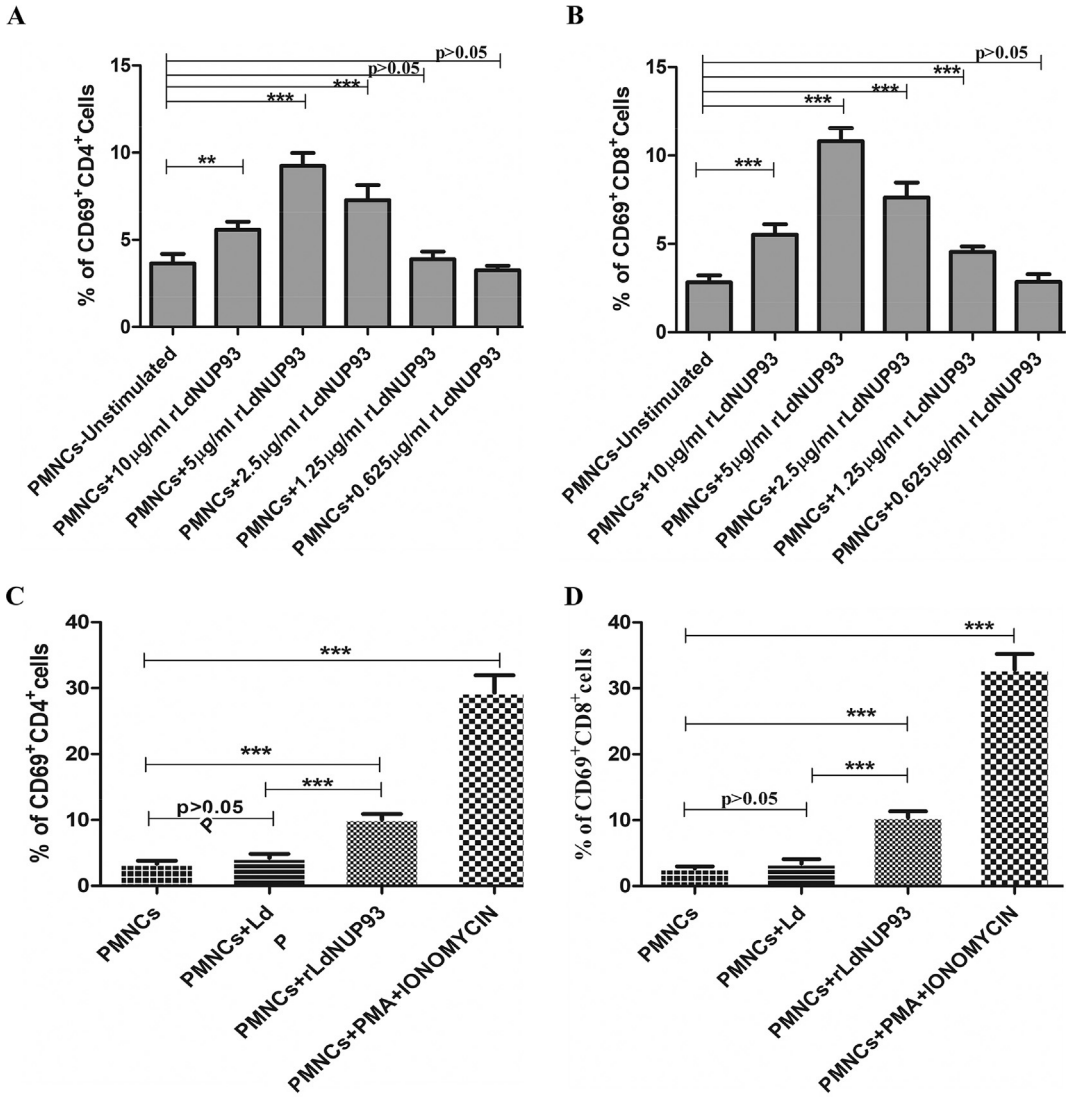
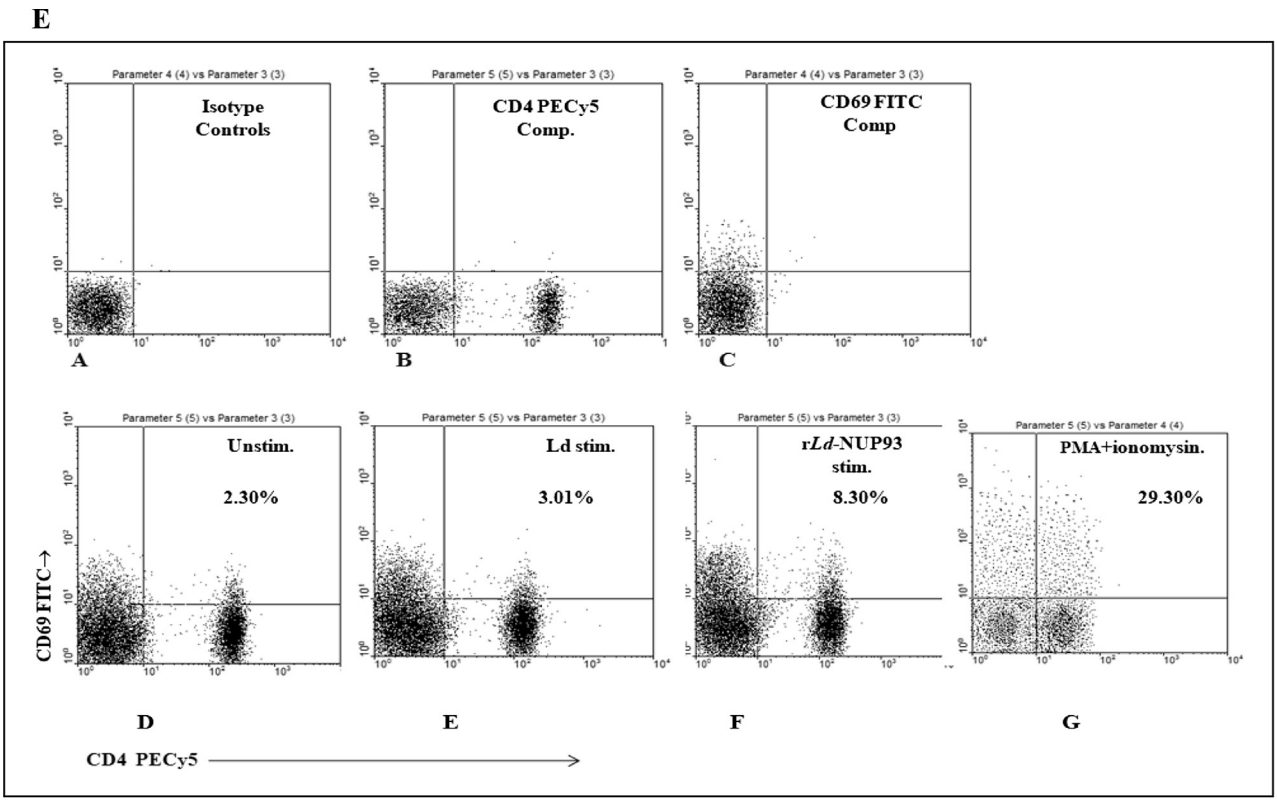


Fig. 2. Comparative bar diagram showing the activation of T-lymphocytes by expression of early activation marker CD69 on $CD4^+$ as well as $CD8^+$ T-cells. (A) Percentage of $CD4^+$ T cells expressing CD69 (gated population from PMNCs) upon stimulation with or without r*Ld*-NUP93 in a series of 2 fold serial dilution. (B) Percentage of $CD8^+$ T cells expressing CD69 (gated population from PMNCs) upon stimulation with or without r*Ld*-NUP93 in a series of 2 fold serial dilution. (C) Percentage of $CD4^+$ T cells expressing CD69 (gated population from PMNCs) after stimulation with or without *L. donovani* (*Ld*) or r*Ld*-NUP93 or PMA and ionomycin. (D) Percentage of $CD8^+$ T cells expressing CD69 (gated population from PMNCs) after stimulation with or without *L. donovani* (*Ld*) or r*Ld*-NUP93 or PMA and ionomycin. Bar diagram represent the mean ± SD of four different experiments. *$p < 0.05$, **$p < 0.005$ & ***$p < 0.0005$. (E) Gating strategy and dot plot data for the expression of CD69 on $CD4^+$ T cells. (F) Gating strategy and dot plot data for the expression of CD69 on $CD8^+$ T cells.



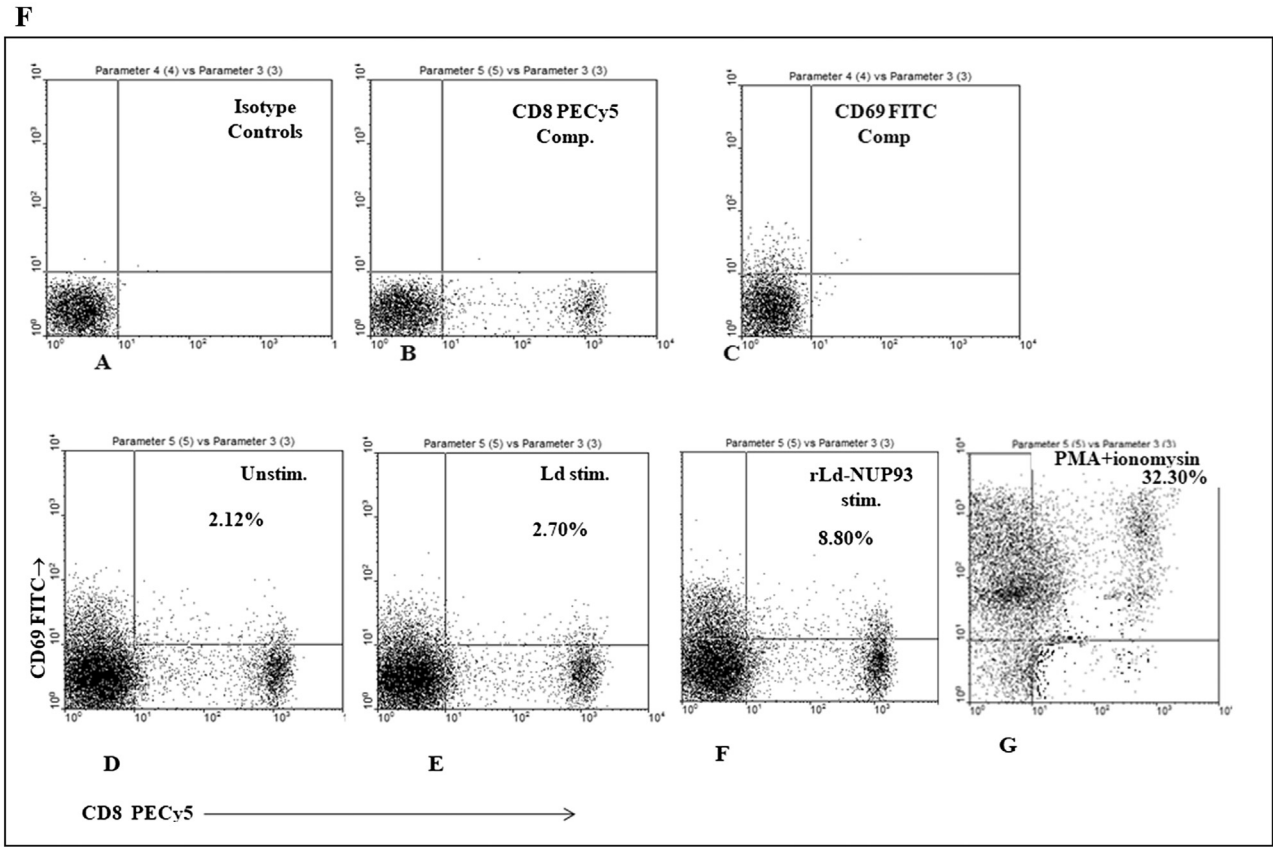


Fig. 2. (*continued*)

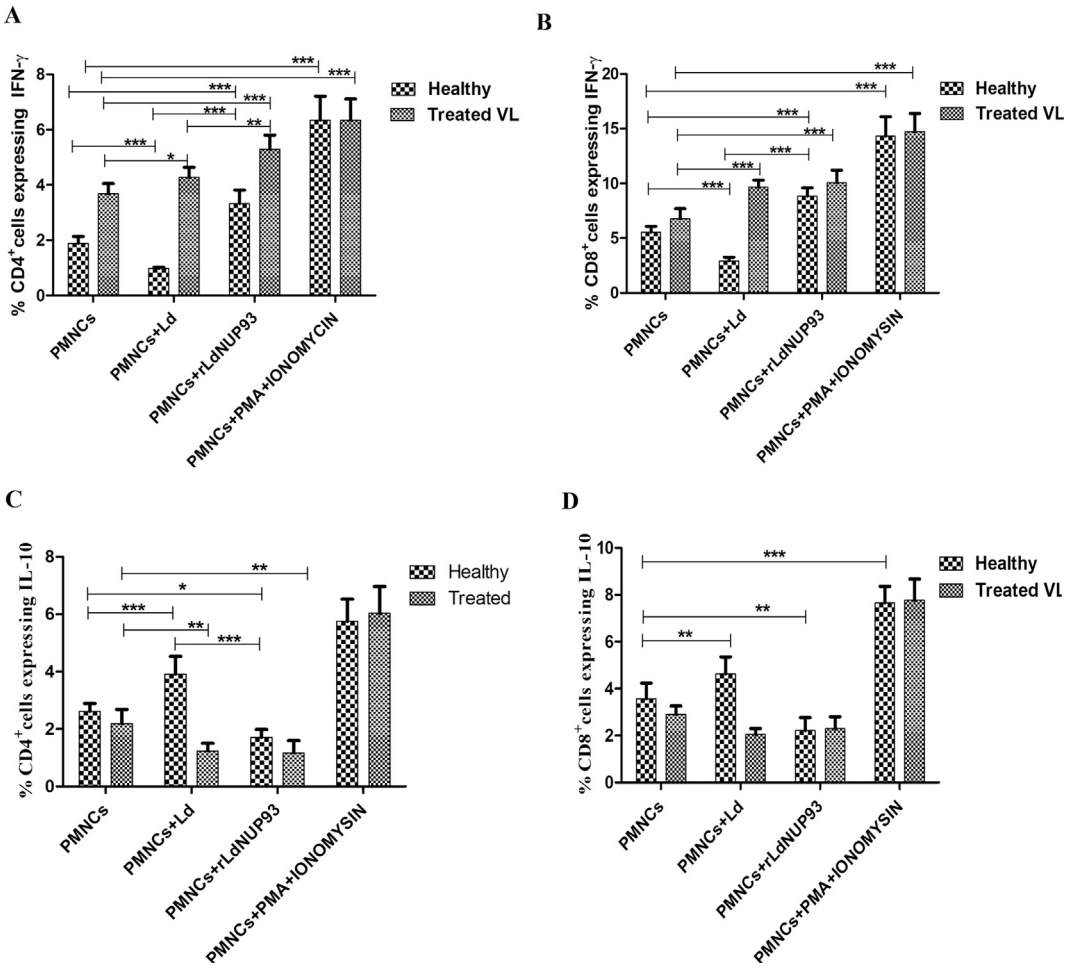


Fig. 3. Comparative bar diagram showing the secretion of intracellular cytokines IFN-γ & IL-10 by $CD4^+$ and $CD8^+$ T cells after stimulation with or without *L. donovani* (*Ld*) or r*Ld*-NUP93 or PMA and ionomycin. (A) Percentage of $CD4^+$ T cells secreting IFN-γ. (B) Percentage of $CD8^+$ T cells secreting IFN-γ. (C) Percentage of $CD4^+$ T cells secreting IL-10. (D) Percentage of $CD8^+$ T cells secreting IL-10. *p < 0.05, **p < 0.005 & ***p < 0.0005. Bar diagram represent the mean ± SD of four different experiments.

(MFI = 344.2) than did unstimulated macrophages (MFI = 158.2). However, *L. donovani* challenge decreased ROS activity (MFI = 78.2) by half and LPS stimulation increased it significantly (MFI = 566.6) (Fig. 6B).

3.9. rLd-NUP93 stimulation induced nitric oxide (NO) synthesis

NO play a crucial role in eliminating *Leishmania* amastigotes during the healing process. Leishmania is well known for reducing NO synthesis, also evidenced by the present study. r*Ld*-NUP93 stimulation induced 1.85-fold higher production of NO ($p < 0.05$; Fig. 7).

3.10. rLd-NUP93 charging directed THP-1 cells towards host protective responses

In support of the previous experiments, the immunomodulatory capacity of r*Ld*-NUP93 was also evaluated in the monocyte cell line THP-1. There was 1.93-fold higher generation of NO in THP-1 cells stimulated with r*Ld*-NUP93 than in unstimulated THP-1 cells (Fig. 8A). However, NO production was 2.88-fold higher in response to LPS. Levels of TNF-α and IL-12 were also increased 2.27- and 2.79-fold (Fig. 8B and C). Unlike TNF-α and IL-12, secretion of IL-10 was decreased by more than half compared with unstimulated THP-1 cells (Fig. 8D). However, LPS stimulation significantly increased the levels of TNF-α, IL-12 and IL -10.

3.11. Immunoactivation potential of NUP-93 was supported by an in silico study

In the present study, possible MHC class I/MHC class II restricted epitopes were predicted based on the theory of Trost et al. by combining predictions from various algorithms [32]. The epitopes that are highly conserved will be tolerated in the host. As per the prediction made by IEDB, SYFPEITHY and RANKPEP, the peptides VLWASIVQI, MMSPALAQV and ILLDVQHAV were found to have consensus high-binding affinity with MHC class I allele (Tables 2 and 3). Similarly, the 15-mer peptides STGLRLLLENNITHV, ITHFTAYVTETSLDG and PMRFEEVENMSASHL were predicted to have the best binding affinity for the MHC class II restricted epitope using IEDB, SYFPEITHY and NETMHC II database analysis (Table 4).

4. Discussion

Cell-mediated immunity is a central player in providing defence against many intracellular pathogens including *L. donovani*. *L. donovani* initiates pathogenesis by down-modulating T helper cell 1 (Th1) and managing other protective immune responses that are otherwise the major components in providing protection [33–35]. In addition, $CD8^+$ T-cells contribute to the adaptive immune response during VL [36]. Therefore, T-cell stimulating antigens are considered good vaccine targets for intracellular pathogens [37]. T-cells obtain impulses from antigen-presenting cells such as macrophages, dendritic cells and B-

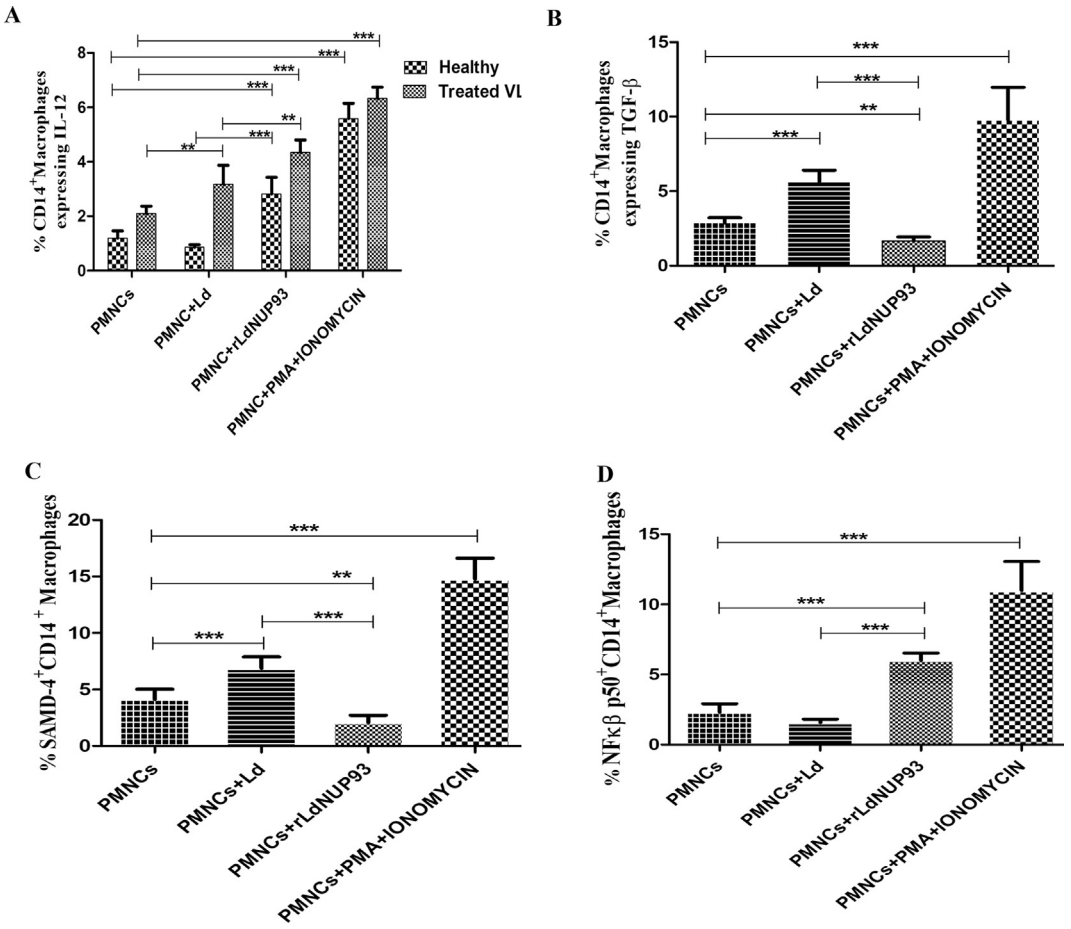


Fig. 4. Comparative bar diagram showing the secretion of intracellular cytokines and transcription factors in macrophages, stimulated with or without *L. donovani (Ld)* or r*Ld*-NUP93 or PMA and ionomycin, as positive control. (A) Percentage of $CD14^+$ macrophages secreting IL-12. (B) Percentage of $CD14^+$ macrophages secreting TGF-β. (C) Percentage of $CD14^+$ macrophages secreting SMAD-4. (D) Percentage of $CD14^+$ macrophages secreting NF-κβp50. *$p < 0.05$, **$p < 0.005$ & ***$p < 0.0005$. Bar diagram represent the mean ± SD of four different experiments.

cells. As NUP93 was isolated earlier from CICs of VL patients [38], its antigenicity was expected for B-cell activation and immunoglobulin production. Being an antigen inducing humoral immunity, NUP93 may induce B-cell dependent T-cell activation [39,40]. The immunogenic potential of r*Ld*-NUP93 was also identified by its effect on inducible cell surface glycoprotein CD69 in lymphocytes. The aim of the evaluation of CD69 expression was to find a suitable dose (5μg/ml) of r*Ld*-NUP93 for further experiments. Its up-regulated expression on $CD4^+$ and $CD8^+$ T-cells was early assurance of cellular activation, phosphorylation and proliferation [41,42].

Further, the immunoprotective efficacy of r*Ld*-NUP93 was ascertained by higher expression of IFN-γ and down-regulation of IL-10 in $CD4^+$ T cells and $CD8^+$ T cells from healthy and treated VL patients. The Th1 polarization in r*Ld*-NUP93 stimulated cells from a treated VL patient was expected due to past exposure to the *Leishmania* parasite and the presence of memory cells. However, immunoprophylactic efficacy was ascertained by its response in cells from healthy donors. These cells showed Th2 polarization in response *L. donovani* stimulation but ensured Th1 polarization when stimulated with r*Ld*-NUP93. This phenomenon suggests the potential of the antigen for vaccines, as it induces immunity in naïve cells during first exposure. Here, we have not shown the response of active VL patient cells, and this may be a limitation of this study. Again, we could not examine the r*Ld*-NUP93-induced dynamics of memory cells. Interaction of IL-12 with T cells is also a key contributor to the initiation and maintenance of Th1 responses [43,44]. IL-12 mediates essential secondary signalling between macrophages and T cells for expression of host protective cytokine IFN-γ. It was reported that *L. donovani* effectively down-modulated IL-12 expression that is otherwise needed for prompting T-cells towards Th1 type effector function. Binding of IL-12 with its receptor on antigen presenting cells induced expression of IFN-γ from T-cells as neutralizing antibodies to IL-12 abrogated IFN-γ production [44]. Macrophages from treated VL responded to both *L. donovani* and r*Ld*-NUP93, with pre-exposed hosts producing higher IL-12. However, cells from healthy subjects unexposed to either antigen responded well to r*Ld*-NUP93. As predicted, *L. donovani* stimulation enhanced the secretion of TGF-β in healthy cells due to the presence of parasite-derived factor cathepsin-B. TGF-β enhanced parasite survival in macrophages [45] by down-modulating nitric oxide (NO) production [46]. The down-regulated expression of TGF-β upon r*Ld*-NUP93 stimulation may be due to either lack of cathepsin-B or host immune pressure. This r*Ld*-NUP93-induced down-regulation of TGF-β resulted in the decreased accumulation of phosphorylated SMAD-4 in the nucleus [47]. Under-expressed SMAD-4 fails to promote IL-1 receptor-associated kinase-M (ITRAK-M), ultimately leading to a concomitant up-regulation of downstream NF-κβ p50 release and its translocation across the nuclear membrane [48].

Flow cytometry based qualitative evaluation of cytokines was strengthened by the data obtained after quantitative evaluation of cytokines such as IFN-γ, IL-10, TNF-α and IL-12 through ELISA. This extracellular cytokine data generated through ELISA supported the host protective nature of this protein. IFN-γ acted synergistically with another macrophage-derived cytokine, TNF-α, to induce nitric oxide [49]. Binding of IFN-γ and TNF-α with their receptors on macrophages activates NOS via the tetrahydrobiopterin cascade. NOS using oxygen and

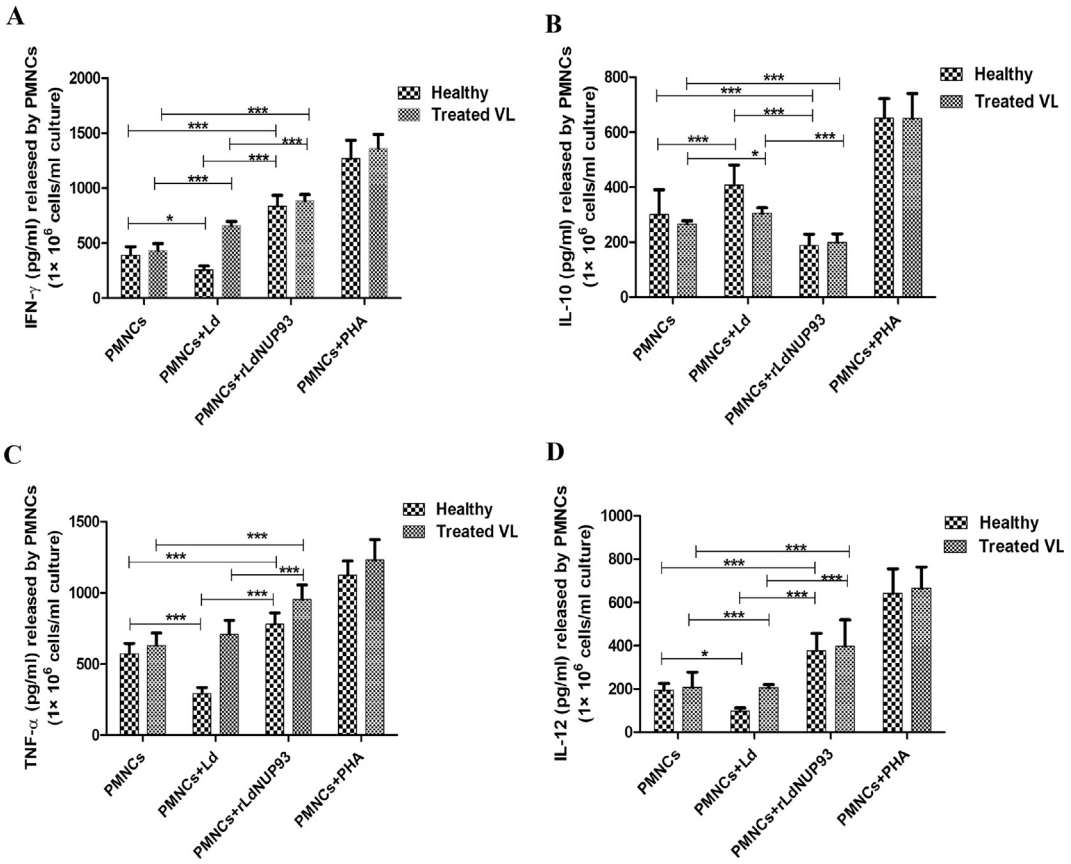


Fig. 5. Comparative bar diagram showing the quantitative data of IFN- γ, IL-10, TNF-α and IL-12 evaluated by ELISA using culture supernatant of 1 × 10^6 PMNCs/ml incubated with or without *L. donovani (Ld)* or r*Ld*-NUP93 and PHA, as positive control. (A) IFN-γ in pg/ml. (B) IL-10 in pg/ml. (C) TNF-α in pg/ml. (D) IL-12 in pg/ml. Bar diagram represent the mean ± SD of four different experiments. *p < 0.05, **p < 0.005 &***p < 0.0005.

guanidine nitrogen of L-arginine produce NO that is catalysed by tetrahydrobiopterin [50]. Hence, up-regulated IFN-γ and TNF-α upon r*Ld*-NUP93 stimulation increase production of NO and promote *L. donovani* clearance as well as elevated expression of IFN-γ, TNF-α and IL-12; r*Ld*-NUP93 down-regulated IL-10, which may otherwise bind with IL-12 receptor to induce T-cell anergy and apoptosis.

Oxidative bursts account for leishmanicidal activity [51–53]. it has been reported that *L. donovani* inhibits the production of O_2^- inside host cells. The levels of O_2^- and H_2O_2 were significantly lower in monocytes from VL patients than in healthy subjects [54,55]. *L. donovani* evades the host immune response and assures its survival inside the host by either using the antioxidant system or by suppressing macrophage ROS production [52]. In the absence of *L. donovani* bearing pathogenic components, antigenic potential of r*Ld*-NUP93 probably assisted ROS generation. r*Ld*-NUP93 stimulation mediated higher secretion of NO in comparison to control. The Th1 stimulatory nature of r*Ld*-NUP93 was also validated in experiments on THP-1, showing higher expression of NO, TNF-α IL-12 and simultaneously decreasing IL-10.

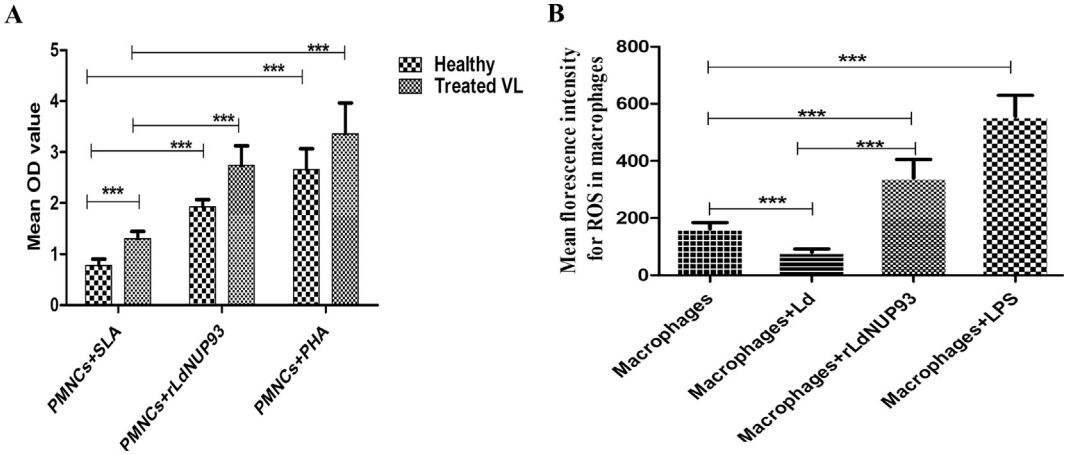


Fig. 6. Comparative bar diagram showing the lymphocyte proliferation response of PMNCs and the ROS released by active macrophage. (A) LTT response of PMNCs from healthy donors against SLA, r*Ld*-NUP93 and PHA. Proliferation was represented as the mean O.D. of stimulated culture − (minus) mean O.D. of unstimulated culture, as negative control. (B) The ROS produced by macrophages stimulated with or without *Ld* or r*Ld*-NUP93 or LPS, as positive control. The data of ROS were measured via mean fluorescence intensity (MFI). Bar represent the mean ± SD of two different experiments. ***p < 0.0005. (O.D. – optical density).

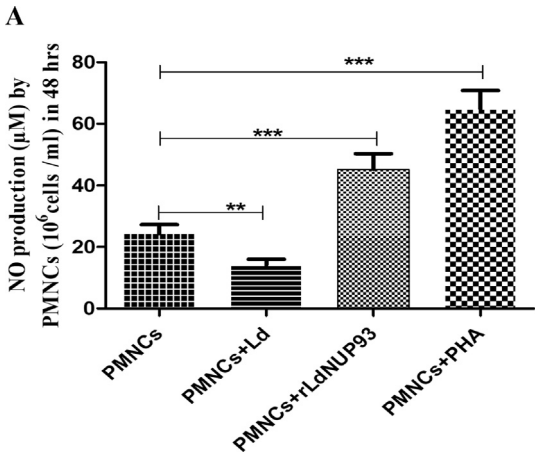


Fig. 7. Comparative bar diagram showing the secretion of nitric oxide (NO) in PBMCs from healthy donor treated with or without *L. donovani* (*Ld*) or r*Ld*-NUP93 or PHA. Bar represent the mean ± SD of single independent experiments. **p < 0.005 & ***p < 0.0005.

The data generated by the *in vitro* experiments with r*Ld*-NUP93 were supported by the *in silico* study, suggesting the presence of MHC class I and class II restricted epitopes that could trigger the desired immune response in the host. In addition to the presence of HLA-A*0201 (very common in European populations), 0207, 0205 and A1101 (frequent in Asian and Indian populations), specific epitopes in *Ld*-NUP93 ascertain its expression by antigen presenting cells, essential to boost host protective immunogenic responses [56–58]. Based on previous reports, the 10% top-scoring peptides predicted by SYFPEITHI and BIMAS, 85% peptides had the ability to trigger the desired immune response [59,60].

Table 2
HLA A0201 restricted 9 mer epitopes. Antigen specific cytotoxic T cell epitopes was predicted by SYFPEITHY using matrix-based algorithm. Peptide binders to MHC-I molecules from protein sequences or sequences alignment was predicted using RANKPEP which is a Position Specific Scoring Matrices (PSSMs) based bioinformatics tool. Immune Epitope Database (IEDB, a database which includes the tool that predicts the MHC class I and class II binding epitopes) was used to experimentally measure immune epitopes.

Peptide	Start position	SYFPEITHY	RANKPEP	IEDB
VLWASIVQI	304	26	101	0.5
MMSPALAQV	717	27	96	0.3
ILLDVQHAV	166	27	86	0.1
SLSRLLERV	340	27	93	1.5
ELLKALMQV	666	23	102	2.6
LLENNITHV	549	25	91	2.7

Bearing epitopes for major types of MHC I and MHC II and their associations with humoral immune response, r*Ld*-NUP93 prompted antigen presentation followed by secondary signalling (IL-12) and activation of both $CD4^+$ and $CD8^+$ T-cells. r*Ld*-NUP93 also did not promote the regulatory functions of T-cells or regulate ITRAK-M by down-regulating SMAD-4. It probably assisted in the concomitant up-regulation of NF-κβ p50 translocation in the nucleus. The binding of IFN-γ and TNF-α with its receptor on macrophages promoted NO production. In

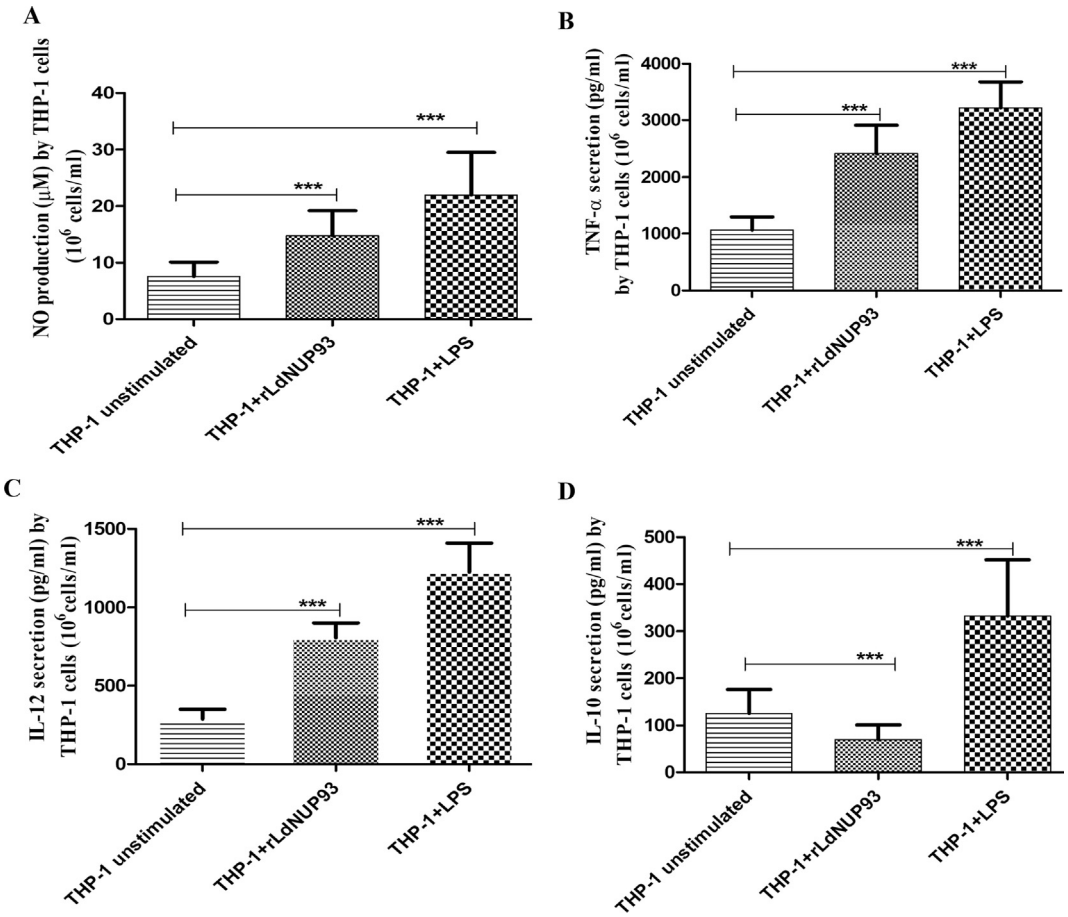


Fig. 8. Comparative bar diagram showing the various responses shown by THP-1 cell line stimulated with or without r*Ld*-NUP93 or LPS. (A) NO (in μM) production by THP-1 cell line. (B) TNF-α (in pg/ml) secreted by THP-1 cell line. (C) IL-12 (in pg/ml) secreted by THP-1 cell line. (D) IL-10 (in pg/ml) secreted by THP-1 cell line. Bar represent the mean ± SD of four different experiments. ***p < 0.0005.

Table 3
Showing the 9 mer conserved epitopes of r*Ld*-NUP93 based on other allele HLA 0207, 0205 & A1101, frequent in Indian and Asian population. These 9 mer conserved epitopes was predicted using RANKPEP and IEDB.

Alleles	Start sequence	Peptides	IEDB	RANKPEP
HLA 0207	i. 304	i. VLWASIVQI	i. 0.5	i. 41.36%
	ii. 666	ii. ELLKALMQV	ii. —	ii. 21.53%
	iii. 549	iii. LLENNITHV	iii. —	iii.9.71%
	iv. 166	iv. ILLDVQHAVH	iv. 0.6	iv. —
	v. 717	v. MMSPALAVQ	v. 0.3	v. —
	vi. 340	vi. SLSRLLERV	vi. 2.7	vi. —
HLA 0205	i. 717	i. MMSPALAQV	i. 0.2	i. —
	ii. 166	ii. ILLDVQHAV	ii. 0.4	ii. 32.53%
	iii. 340	iii. SLSRLLERV	iii. 1.2	iii. —
	iv. 549	iv. LLENNITHV	iv. 3	iv. —
	v. 304	v. VLWASIVQI	v.	v. 39.85
HLA A1101	i. 666	i. ELLKALMQVA	i. —	i. 13.76%
	ii. 166	ii. ILLDVQHAVH	ii. 17.85	ii. 11.16%

Table 4
HLA DRB1 0401 restricted 15 mer epitopes. Immune Epitope Database (IEDB, a database which includes the tool that predicts the MHC class I and class II binding epitopes) was used to experimentally measure immune epitopes. Antigen specific cytotoxic T cell epitopes was predicted by SYFPEITHY using matrix-based algorithm. NETMHC II is a server, which predicts binding of peptides to HLA-DR using artificial neuron networks.

Peptide	Start position	IEDB	SYFPEITHI	NETMHC II
STGLRLLLENNITHV	543	0.52	26	SB (0.12%)
ITHFTAYVTETSLDG	284	0.31	22	SB (0.60%)
PMRFEEVENMSASHL	353	4.14	28	SB (0.90)
SGRLRSMGTAAPSAN	223	0.89	26	WB (3%)
HMALCFSAQNLLQGR	561	10.0	20	WB (2%)
SSLVDILNSGSSLAM	248	1.49	26	WB (4.5%)

addition, it promoted innate macrophage responses [61]. Based on the data obtained, this study recommends r*Ld*-NUP93 as a potential immunomodulating agent. It may be explored further for vaccine utility.

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