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Article

A T-Cell Epitope-Based Multi-Epitope Vaccine Designed Using Human HLA Specific T Cell Epitopes Induces Sterile Immunity against Experimental Visceral Leishmaniasis in Hamsters

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Abstract: Visceral Leishmaniasis is a neglected tropical disease affecting 12 million people annually. Even in the second decade of the 21st century, it has remained without an effective vaccine for human use. In the current study, we have designed three multiepitope vaccine candidates by the selection of multiple IFN-γ inducing MHC-I and MHC-II binder T-cell specific epitopes from 3 previously identified antigen genes of *Leishmania donovani* from our lab, by immune-informatic approach using IFNepiotpe, NET-MHC-1 and NET MHC-2 webservers. We have tested the protective potential of these three multiepitope proteins as vaccine in a hamster model of visceral leishmaniasis. The immunization data revealed that the vaccine candidates induced a very high level of Th-1 biased protective immune response *in-vivo* in a hamster model of experimental visceral Leishmaniasis, with one of the candidates inducing a sterile immunity. The vaccinated animals displayed highly activated monocyte macrophages with the capability of clearing intracellular parasites due to increased respiratory burst. Additionally, these proteins induced activation of polyfunctional T cells secreting INF-γ, TNF-α, and IL-2 in *ex-vivo* stimulation of human peripheral blood mononuclear cells, further supporting the protective nature of designed candidates.

Keywords: *Leishmania*; Vaccine; T cell; Epitopes; Hamster; IFN-γ epitope; Sterile Immunity

Introduction

After 100 years of discovery, Visceral Leishmaniasis (VL) remains elusive to a vaccine candidate for humans. The development of a vaccine for kala-azar has manifested itself into an immunological challenge of its own [1]. According to WHO estimates, annually 12 million new cases are being registered worldwide [2,3]. VL is a systemic infection of the reticuloendothelial system with macrophages being the primary host, if left untreated the condition easily becomes perilous. The immunology of the disease is centered around the Th1 cytokine axis[4]. In the natural course of infection, there is polyclonal hypergammaglobulinemia with non-specific antibody response along with elevated levels of immunosuppressive cytokines like IL-10 and TGF-β, while recovering patients develop elevated levels of IFN-γ and TNF-α [5,6]. Acute infection in recovered patients results in lifelong immunity, which indicates that an effective vaccine is possible[7].

The efficacy of a T cell-based prophylactic vaccine for any infection depends upon multiple factors including primary signal generated through TCR (CD3 complex) and peptide conjugated major histocompatibility complex (MHC) molecules [8,9], while the secondary stimuli are based on coreceptor activation like CD80/86-CD28 [10]. Additionally, the generation of specific cytokine milieu affecting the outcome depends upon PAMP (Pathogen-associated molecular pattern), & Toll-like receptors (TLR) associated with Antigen-presenting cells(APC) like dendritic cells [8]. In the events leading to T cell activation, besides the role of T cell Receptor (TCR) and coreceptors, the role of a core epitope molecule is not completely understood [11]. Many theories have been proposed to account

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for antigen discrimination for T cell selectivity and sensitivity, but the extent of the role of epitope has remained unexplored [12–17]. In this regard, many researchers have categorized T cell epitopes as dominant and subdominant or optimal and suboptimal epitopes[18–22]. Dominant epitopes are strong binders of MHC molecules and create a strong signal when linked in an MHC-p-TCR complex [23,24]. Optimal epitopes and strong TCR signal strength have also been linked with Th1 immune profile generation specifically MHC-I epitopes [25–29].

In the past, many vaccine candidates have been proposed for VL, which either have utilized the whole parasite in attenuated form or complete antigenic protein [30]. Regarding novel vaccine candidates with a tailored response, immunoinformatic-predicted T cell-specific epitopes can be used to generate newer candidates [31,32]. Advancement in prediction models of linear and conformational epitopes has significantly boosted vaccine research in the last decades with the emergence of many epitope-based vaccine candidates for *L. donovani*, *L. major* and, *L. infantum* [33–39], and for other diseases [40–44]. Keeping in mind the immune-correlates of protection in *Leishmania donovani* infection, we have used the strategy of designing multiple vaccine candidates either by addition of various T cell epitopes in a single multi-epitope candidate or with the use of multiple IFN-γ inducing epitopes to generate different multi-epitope constructs. Three previously identified and tested indigenous vaccine candidates were selected as parent antigens for epitope mapping based on their IFN-γ inducive protective nature [45–47]. We have used various online servers like IEDB T cell epitope identification tools for MHC-I and MHC-II, NET MHC-1 & NET MHC-2 including 'IFNepiotpe' to map T cell epitopes [48]. T cell-stimulating nature of vaccine candidates was tested on healthy Human PBMC for Polyfunctional T cell activation along with IFN-γ, TNF-α, IL-2, and IL-10 cytokine generation, the results of which pointed towards the generation of polyfunctional T cells and strong IFN-γ and TNF-α responses. We selected the Syrian golden hamster as a disease model for protective efficacy evaluation *in-vivo*. Syrian golden hamster makes a good disease model for visceral leishmaniasis because the clinical features are similar to humans and the animals succumb to the disease in 10-12 weeks if not treated [49]. The protective potential and immunogenicity data in immunized hamsters revealed a very high level of protective efficacy against a virulent challenge along with induction of a strong Th-1 biased immune response.

Material and Methods

The study protocol was approved by Institutional Ethics Committee (IEC) of Post Graduate Institute of Medical Education & Research (PGIMER) Chandigarh India on August 8, 2016 vide ref. No. MK/2867/PhD/7754. And also by the Institute Animal Ethics Committee of PGIMER, Chandigarh, India on 13th Dec, 2016 vide Ref No. 85/IAEC/559.

Epitope Identification and Multiepitope candidate designing

Three novel antigen genes of *Leishmania donovani,* A2/1(AY-377788), B4/1 (AY-161269), and F2/1(AY-180912), were selected for epitope mapping [45]. We identified T cell epitopes using 3 different web servers, IEDB T cell epitope identification tools for MHC-I and MHC-II, NET MHC-1[50,51], NET MHC-2 [52], & IFNepiotpe [48].

For IEDB web server-based MHC-I T cell epitope identification tool, the query was submitted in FASTA format for all three genes. Other parameters were set at default. For each gene following MHC-I alleles were selected: HLA:A01, HLA:A02, HLA:A03, HLA:A24, HLB:B07, HLB:B08, HLB:B27, HLB:B39, HLB:B44, HLB:B58, HLAC:C04 & HLAC:C06 to maximize the population coverage. Similarly, for MHC-II specific T cell epitopes following MHC-II alleles were selected for each gene, DP1 DP2, DQ7, DR4, DRB1, DRB3, DRB5, and main DQ. The parameters were set at default values. All the results were downloaded in .XLS format. Epitopes were selected based on IC50 values of <50nM.

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For NETMHC-1 4.0 input sequence was given in FASTA format, with other parameters set at the default value. HLA allele selection was as following: HLA: A01, HLA: A02, HLA: A03, HLA: A24, HLA: A25, HLA: B07, HLA: B15, HLA: B27, HLA: B39, HLA: B40, and HLA: B58, similarly for NETMHC-2 4.0 MHC-II alleles selected were as following DP, DQ, DR4, DRB3, & Main-DR. The results were obtained in .XLS format. The selection was based on strong binders defined as having a %rank<0.5.

For IFNepitope the input sequences were submitted in FASTA format and parameter values were set at default. Since IFNepitope does not require HLA allele submission for epitope prediction, the predicted epitopes were checked for HLA alleles by Propred and Propred1 (MHC class 1 and class 2 binding peptide prediction servers respectively) later on [53,54].

Based on epitope mapping results, 3 different multiepitope candidate proteins were designed: **(Table 1) (Supplementary Figure 1-4 and Supplementary Table 1)**

Construct 1: The design was based on epitopes identified through NETMHC-1 and NETMHC-2 and IEDB T-cell epitope prediction servers for both MHC1 and MHC-II. For each HLA allele high-affinity epitopes (strong binder) with <50nM IC50 were selected from the IEDB epitope server, similarly epitopes with percent rank <0.5% were selected using NETMHC-I and NETMHC-II. Integration of results from both NETMHC-1&2 and IEDB for MHC-I and MHC-II gave large sequences or island regions containing multiple overlaps of partially overlapping epitopes. These regions of multiple epitopes were linked by diglycine (-G-G-) residues to provide optimum steric flexibility and avoid any scrambling of epitope sequences by additional amino acids from the linker sequence. The sequence order of epitopes was kept similar to their native sequential order as in the parent antigen molecule **(Supplementary Figure 5).**

Construct 2: The second construct was designed based on IFN-γ inducing MHC-I binders. All IFNepiotpe predicted IFN-γ epitopes were additionally screened for MHC-I affinity by Propred1. The final epitopes were IFN-γ inducing epitopes, able to bind CTL of specific HLA allele. Similar to Construct 1, the epitope-rich regions were kept in native order and linked by diglycine linker sequence **(Supplementary Figure 6).**

Construct 3: The third construct was designed based on the results of IFNepiotpe Propred, and NetChop3.0. Epitopes picked were IFN-γ inducers and contained multiple Propred2 binders were selected. The flanking N-terminal and C-terminal regions of the epitope sequences were selected based on NETChop3.0 predicted cleavage sites of proteasome complex [55,56], these native N-terminal and C-terminal sequences were used to link epitopes **(Supplementary Figure 7)**.

Molecular Cloning and Recombinant Protein Production

For the production of multi-epitope vaccine candidate protein, the multiepitope sequences were reverse translated into nucleotide sequence and obtained in pMAT cloning vectors through Gene Art (ThermoFisher USA). The construct sequences were sub-cloned into pQE30 expression vector (Qiagen GmbH, Hilden, Germany), and transformation was carried out in M15 *E. coli* for protein expression. Protein expression was carried at 37°C by induction with 0.1mM IPTG and the over-expressed protein was isolated from inclusion bodies by solubilizing in tris buffer(pH 8.0). Membrane-bound protein was repeatedly washed in a buffer containing Deoxycholic acid and Tris-HCl with EDTA and finally dissolved in 6M Guanidium HCL (GuHCl). The excess GuHCl was removed by overnight dialysis against double distilled water(ddH20). The purified product was tested for endotoxin levels using Endotoxin detection kit (THG10-0250, Hi-Media, India), the levels were within acceptable range i.e., less than 0.25 EU. The final product was lyophilized, weighed, and stored at -20°C. The purified proteins were confirmed for their molecular weight by SDS-PAGE separation and western blot. Individual bands were visualized by staining with Horse-radish peroxidase (HRP) conjugated anti-histidine (poly-HIS) antibody. **(Details available in supplementary data)**

Protective efficacy study In Hamster model of Experimental Visceral Leishmaniasis

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The protective efficacy of the multiepitope vaccine proteins was evaluated in a hamster (*Mesocricetus auratus*) model of VL. A total of 48 hamsters were divided into 6 groups. Different groups of animals were immunized intraperitoneally (i.p.) with 50 µg each of proteins expressed from construct 1 (64 kDa), construct 2 (36 kDa), and construct 3 (29 kDa), admixed 1:1 (v/v) with Complete Freund's adjuvant (CFA). The control groups of animals included an adjuvant control group, an unimmunized but infected group, and one healthy (unimmunized uninfected) group. Freund's adjuvant was selected to generate a sustained and slow release of antigen [57,58]. The animals in vaccinated groups were given 2 booster doses of proteins i.p. at day 14 and 28 in emulsion form with Incomplete Freund's adjuvant (IFA) [59]. **(Details in supplementary table 4).** Additionally, promastigotes for any *in-vitro* challenge were obtained from an infected hamster by culturing the crushed spleen in RPMI-1640 culture media supplemented with 10% FBS. and keeping at 25°C.

The hamsters were challenged with 1×10^7 amastigotes (*Leishmania donovani* Dd8) intra-cardinally (i.c.) on day 42 post-immunization and were maintained in Animal Biosafety Level 2 (ABSL-2) cages for 60 days post-challenge before termination of the experiment. The immunization experiments with all three multi-epitope candidate proteins were repeated twice with a fresh batch of hamsters using the same immunization protocol.

Parasitological parameters

On the day of the termination of the experiment, the hamsters were euthanized in a sterile environment and the peritoneal cavity was dissected to reveal the spleen aseptically. The spleen was weighed and bisected into two halves such that a small portion from the middle was taken for touch impressions on a glass slide, while the other half of the spleen was used for splenic culture for viable parasite detection. The touch imprints were fixed with methanol and stained with Giemsa stain for 30-40 minutes. The stained touch impressions were analyzed for intracellular amastigotes under an oil immersion lens in an inverted bright field microscope (Olympus BX-51, Japan). The data were represented as Leishman Donovan units (LDU) calculated as Number of amastigotes/Number of Nucleated Cells × Weight of the organ(mg) (Bradley & Kiskley 1977).

Physical Parameters

Basic physical parameters of hamsters were analyzed in terms of body weight, spleen size, and spleen weight. Taken together these parameters were used to calculate splenic index which is spleen weight to whole body weight ratio multiplied by 100.

Spleen culture assay

The sterile portion of resected spleen from the euthanized hamsters was crushed to a single cell suspension in 2 ml RPMI-1640 medium (Sigma Aldrich, USA). Fifty µl of the suspension was used to inoculate 1 ml RPMI-1640 complete medium containing 10% Fetal bovine serum (FBS) (US origin, Gibco USA) and antibiotics (ampicillin & streptomycin) in a sterile culture tube. The cultures were incubated at 25°C in a BOD incubator and an aliquot was observed every alternate day for the development of promastigotes. The promastigote population was counted using a hemocytometer.

Immunological parameters

Macrophage Function Assays

The functional status of macrophages obtained from the peritoneum of experimental hamsters was assessed for intracellular parasite clearance and generation of the oxidative burst in terms of ROS production. For obtaining the macrophages, the peritoneal cavity of experimental hamsters was washed with a cold RPMI1640 culture medium before the removal of the spleen. The cells obtained from wash were seeded in a 24 well plate and

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left for 6 hours at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 10% FBS. After 6 hours the cultures were agitated to remove the non-adherent cells and the culture media was replaced with fresh medium, leaving only adherent cells, mainly monocytes which differentiated into macrophages overnight, behind. For parasite clearance assay metacyclic promastigotes were obtained by isolating amastigotes from infected hamster spleen and culturing them in blood agar slants overlaid with RPMI-1640 supplemented with 10% FBS at 25°C temperature. After promastigote formation metacyclic form was obtained by culturing the promastigotes for 7-9 days without changing the culture medium (M199, 10% FBS).

- **Parasite clearance:** To check the ability of macrophages to clear the intracellular parasites, the adherent macrophages (50,000 cells/well) were layered with live metacyclic CFSE-stained promastigotes in a 1:10 ratio and incubated at 34°C for 10 hours. The wells were washed to remove any uninternalized promastigotes. The cells were de-adhered using Trypsin EDTA solution, at specific time intervals of 12 hours, 24 hours & 48 hours, washed twice in cold sheath fluid, and acquired on a flow cytometer (FACS Calibur, BD Biosciences, USA) for CFSE signal. Quantitative assessment of internalized promastigotes was made in terms of Mean Fluorescence Intensity (MFI) at 12 hours, 24 hours & 48 hours. A decrease in MFI would indicate parasite clearance. The data analysis was done by BD CellQuest™ Pro software, by gating granulocytes and monocyte based on forward scatter (FSC) and side scatter (SSC) pattern. The baseline fluorescence was set based on uninfected unstained healthy cells.
- **Oxidative burst:** The capability of macrophages from the immunized hamsters for the production of reactive oxygen species (ROS) would indicate the restoration of normal function in terms of generation of oxidative burst. To assess that, 50,000 peritoneal adherent cells were cultured in a 24 well culture plate in presence of RPMI-1640 medium supplemented with 10% FBS. At the beginning of the culture, the cells were stimulated with bacterial lipopolysaccharide (LPS, 5 μg/ml) or 10 μg/ml of *Leishmania* Promastigote Soluble Antigen (LPSA). At 12 & 24 hours the cells were stained with 2',7' –dichlorofluorescein diacetate (DCFDA) for 35 minutes and acquired on a flow cytometer (BD FACS Calibur) for change in MFI due to ROS-mediated conversion of DCFDA to 2',7'-dichlorofluorescein (DCF). Post-acquisition analysis was done by BD CellQuest™ Pro and the baseline was set by unstimulated-stained cells.

Post-immunization Immune-profiling

To assess the type and quantum of immune-response generated post-immunization, the immunological parameters were analyzed from the spleen tissue by evaluating the gene expression of a set of immune-response related genes **(List of genes selected for study- supplementary table 7)** by real-time quantitative PCR. The RNA was isolated from splenic tissue using Trizol (Sigma) and cDNA was prepared using a first-strand synthesis kit (iScript cDNA Synthesis Kits, Bio-Rad USA). Primers for selected genes of interest were designed using Primer-BLAST (NCBI).

The RT qPCR was carried out on the LC480 platform (Roche diagnostics Nederland BV). The reaction parameters were set for an annealing temperature of 59°C for 60 seconds with a consecutive 45-second amplification step at 72°C. The amplification reaction was programmed for 37 cycles. The data was obtained in the form of cycle threshold (Ct) values. The expression levels were normalized to the reference gene (γ-Actin). The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative expression of each gene. The ΔCT values were calculated using the formula: ΔCt = Ct (Gene of interest) – Ct (Reference gene), and then, ΔΔCt was calculated using the equation: ΔΔCt = ΔCt (Treated sample i.e., Immunized or Unimmunized animals) – ΔCt (Untreated sample i.e., Healthy uninfected animals), and final values were given in terms of fold change ($2^{-\Delta\Delta CT}$) to healthy control.

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Polyfunctional T cell activation

Polyfunctional T cells are defined by their secretion of multiple cytokines at the same time. IFN-γ, TNF-α, and simultaneously IL-2 secreting T cells are directly correlated to protective response (41). To assess the capability of multiepitope protein constructs in inducing a polyfunctional T-cell response, the peripheral blood mononuclear cells (PBMC) from healthy human volunteers were stimulated *in-vitro* with 64 kDa, 36 kDa, 29 kDa proteins. Phorbol 12-myristate 13-acetate (PMA at 100 ng/ml) (Sigma Aldrich USA) along with 50 ng/ml ionomycin (Sigma Aldrich USA) and Phytohemagglutinin (PHA at 10µg/ml) (Sigma Aldrich USA) were used as control mitogen activators in separate wells. Total *Leishmania* promastigote soluble antigen (LPSA) at 20 µg/ml concentration was used as antigen control. At 14 hours post-stimulation, 5µg/ml Brefeldin A (BFA) (Sigma Aldrich USA) was added, and the cells were harvested after a further 2 hours incubation, washed with sterile PBS, and fixed with Cytoperm Cytofix buffer (BD Biosciences-US). The cells were then stained with PE Mouse Anti-Human CD3 (552127, BD Biosciences-US) for surface staining and with PE-Cy™7 Mouse Anti-Human IFN-γ (557643, BD Biosciences-US), BV421 Mouse Anti-Human TNF (562783, BD Biosciences-US), FITC Mouse Anti-Human IL-2 (340448, BD Biosciences-US) and APC Rat Anti-Human IL-10 (554707, BD Biosciences-US) for intracellular cytokine staining. Finally, the cells were acquired on a flow cytometer (FACS CANTO, BD Biosciences-USA). The lymphocytes were gated based on the forward scatter (FSC) and side scatter (SSC) pattern. The gating and baseline settings were based on unstimulated-stained controls. Frequency of Triple positive (INF-γ^+, TNF-α^+ & IL-2^+) and dual positive (INF-γ^+, & TNF-α^+) cells was calculated based on Boolean gating for INF-γ^+, TNFα^+ & IL-2^+ triple-positive $CD3^+$ T-cells, using FlowJo version 10.1 **(Supplementary Figure 19, -22)**.

Statistical analysis

Statistical analysis was done on GraphPad Prism Version 6.0. Student's T-test was employed to check the statistical difference between the two groups of treatments. $P<0.05$ was considered significant.

Results

Multiepitope vaccine designing

Three multiepitope constructs were designed utilizing the results obtained from the prediction tools. The first construct of molecular mass 64 kDa was based on the dominant epitopes of the high affinity for both MHC-I and MHC-II alleles. It included multiple overlaying epitopes from the regions aa 1-83 from B4/1(AY161269), aa 8-65, aa 77-206 from F2/1(AY180912) & aa 12-103, aa 125-223, aa 242-310, & aa 264-403 from A2/1(AY 377788). The second construct of 36 kDa mass was based on MHC-I (CTL) specific to the IFN-γ inducing epitope as predicted by IFNepitope and Propred1 results. A total number of 63 epitopes were used out of which 17 were dual binders for MHC-I and MHC-II alleles. The third construct of 29 kDa was designed using 24 epitopes positive for MHC-II alleles out of which 7 were also MHC-I(CTL) binders **(Table 1)**.

Table 1. Multiple overlaying regions of epitopes used in designing of multiepitope constructs.

64 kDa	**B4/1 (AY 161269)**	**F2/1 (AY 180912)**	**A2/1(AY 377788)**
1317 eptiopes	aa 1-83	aa 8-65	aa 12-103
		aa 77-206	aa 125-223
			aa 242-310
			aa 364-403
36 kDa	**B4/1 (AY 161269)**	**F2/1 (AY 180912)**	**A2/1(AY 377788)**
63 epitopes	aa 23-52	aa 41-58	aa 14-28
	aa 69-89	aa 186-196	aa 46-70
	aa 99-109		aa 81-94
			aa 98-119

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			aa 139-178
			aa 185-225
			aa 242-283
			aa 372-384

29 kDa: Core multiepitope sequence & flanking regions included in 29 kDa construct as indicated in brackets			
	B4/1 (AY 161269)	**F2/1 (AY 180912)**	**A2/1(AY 377788)**
24 epitopes	aa 36-66 (30-69)	aa 1-9 (1-12)	aa 15-27 (1-31)
		aa 41-53 (38-56)	aa 63-106 (60-109)
			aa 166-176 (166-179)
			aa 239-271 (236-277)
			aa 288-295 (281-305)
			aa 392-399 (389-403)

Multi-epitope construct proteins induced significant protection in the Hamster Model of Experimental Visceral Leishmaniasis

Survival efficacy: Post-challenge all the animals in all 3 immunized groups survived, while 2 animals in the un-immunized infection-alone group and 1 animal from the CFA-only group died due to disease-induced morbidity. Postmortem analysis revealed enlarged spleen with a high parasite burden.

Parasitological parameters and parasite load.

The animals from all the immunized groups resisted an increase in parasite burden as observed by a significantly lower parasite number (LDU) in spleen tissue touch imprints : 178 (± 47.03 SEM, p=0.0002) in 64 kDa immunized group, 332(± 105.32 SEM, p=.0002) in 36 kDa immunized group and 478(± 50.10 SEM, p=0.0002) in 29 kDa immunized group, compared to 4277(±661.1 SEM) and 4602(±931.02 SEM) in unimmunized infected group and adjuvant control group respectively (**Figures 1A, 1B & 2**). The splenic index of immunized animals also corresponded with the parasite burden **(Table 2)**.

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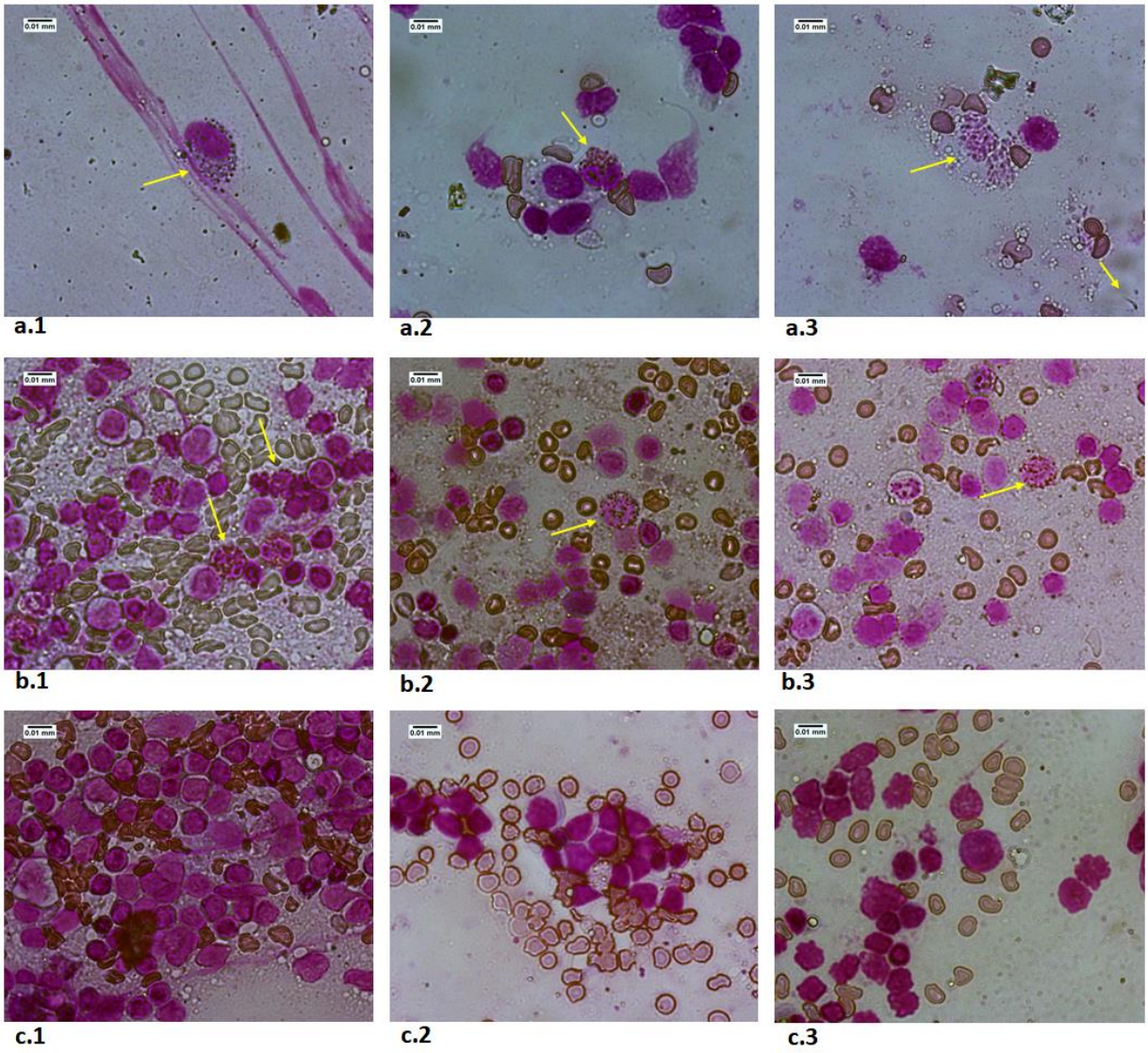

Figure 1. A: Giemsa-stained touch impressions of spleen from different animal groups. **a.1-3)** Unimmunized Infected animal, **b.1-3)** Complete Freund's adjuvant (CFA) only, & **c.1-3)** healthy animal. Arrows indicating the infected cells. *Magnification 10×100X* (oil immersion) (Olympus Japan).

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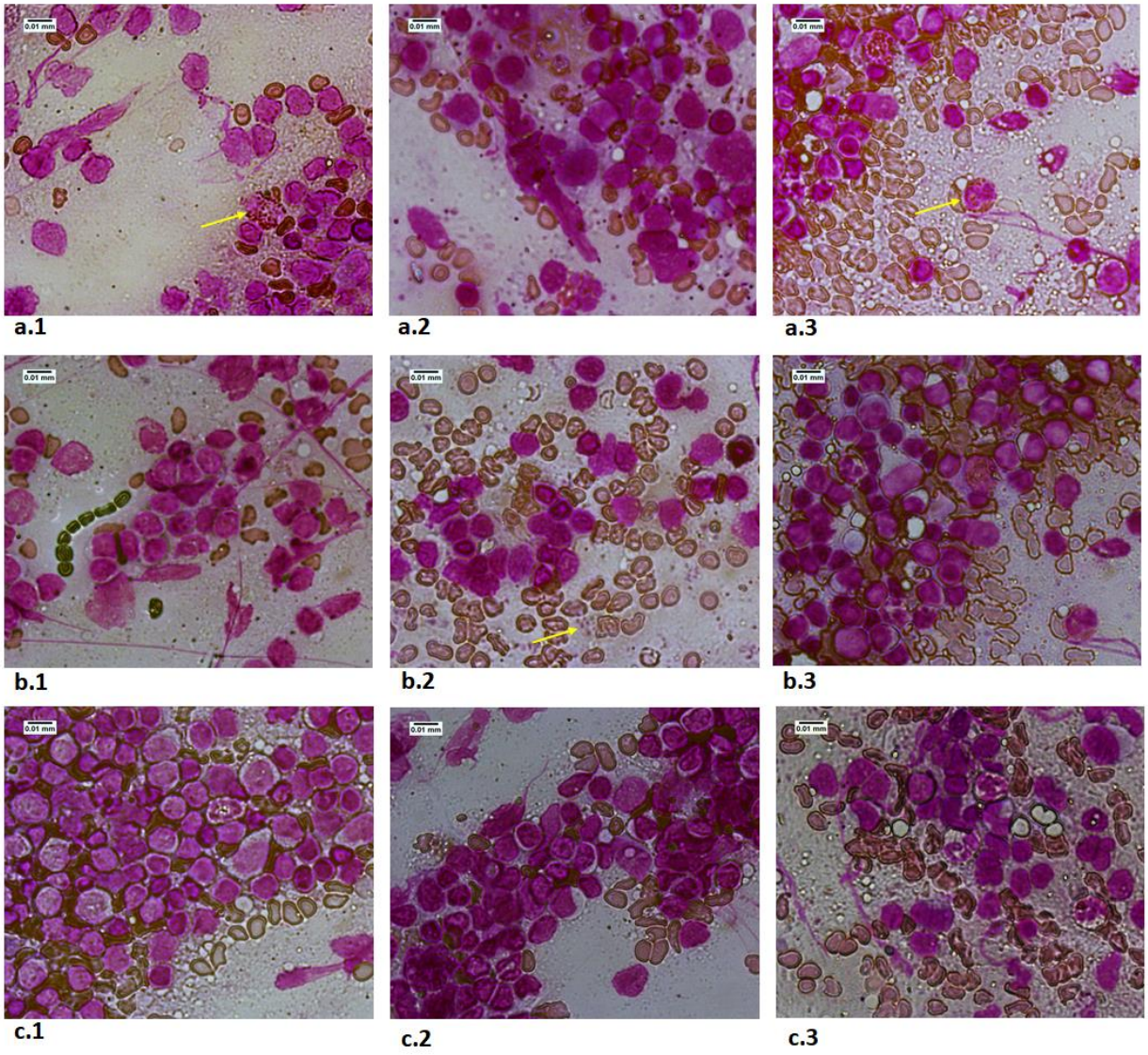

Figure 1. B: Giemsa-stained touch impressions of spleen from different animal groups. **a.1-3)** 29 kDa immunized animal, **b.1-3)** 36 kDa immunized animal, & **c.1-3**) 64 kDa immunized animal. Arrows indicating the infected cells. *Magnification 10×100X* (oil immersion) (Olympus Japan).

Table 2. Parasite loads in the spleen of infected and immunized animals.

Group (n=8)	Mean LDU (Amount of amastigotes/Number of Nucleated cells) × Weight of organ(mg))	Mean Splenic Index Splenic Index is measured as the ratio of spleen weight to whole body weight multiplied by a hundred
Unimmunized-Infected	4277.3 ±661.1 SEM	0.322 ±0.0567 SEM
CFA only	4602.6 ±931.02 SEM	0.368 ± 0.0534 SEM
Immunized with 64 kDa	178.71 ± 47.03 SEM (p=0.0002)	0.145 ± 0.048 SEM (p=0.0050)
Immunized with 36 kDa	332.2 ± 105.32 SEM (p=0.0002)	0.216 ± 0.067 SEM (p=0.0303)
Immunized with 29 kDa	478.5 ± 50.10 SEM (p=0.0002)	0.151 ± 0.013 SEM (p=0.0012)
Healthy	NA	0.168 ± 0.0548 SEM

Statical comparison between Infected and test group. Statistics Mann-Whitney t-test. Level significance, ns $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

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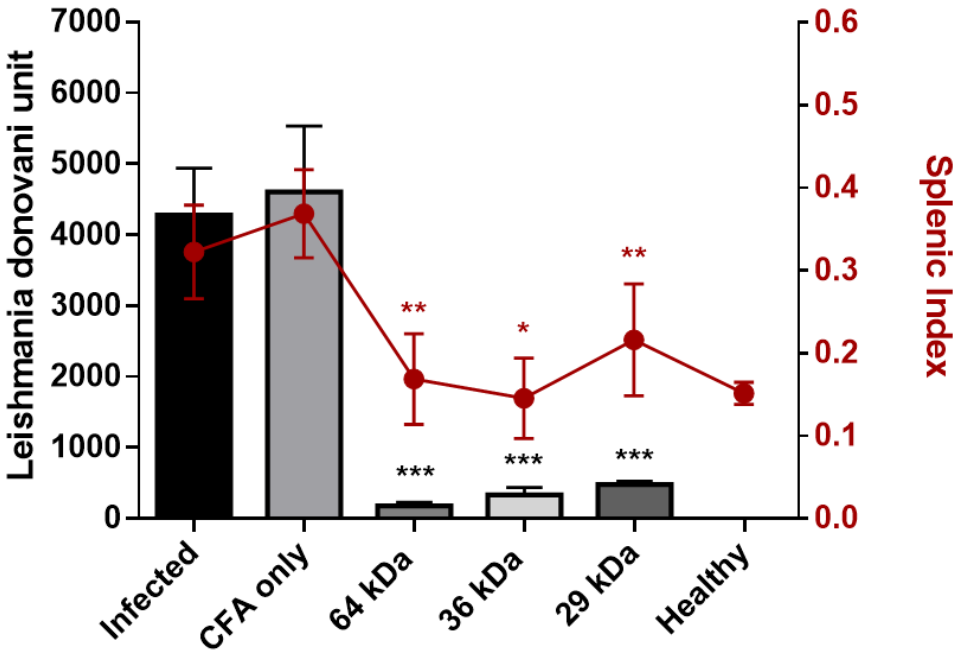

Figure 2. Mean parasite load and splenic index from the spleen of different animal groups. LDU= *Leishmania donovani* unit. (Bradley & Kiskley 1977). Splenic index, the ratio of (spleen weight/whole body weight) × 100. N=8. Mean ±SEM. Statistical comparison between Infected and test group. Mann-Whitney t-test. Level of significance, ns P > 0.05, * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Splenic culture, parasite growth

To account for any residual viable parasites in the spleen of immunized animals, we conducted the spleen tissue culture. The spleen cultures from unimmunized animals showed motile promastigotes as early as day 5 post-inoculation in concordance with a high parasitic load. **(Figure 3)**. The promastigotes became visible in animals immunized with 36 kDa and 29 kDa proteins by day 14, indicating a significantly lower number of viable parasites in these animals as compared to unimmunized groups. However, no promastigotes could be detected in 64 kDa immunized group animal spleen cultures throughout 14-day culture, indicating no viable parasites in spleen. **(Supplementary Table 5)**.

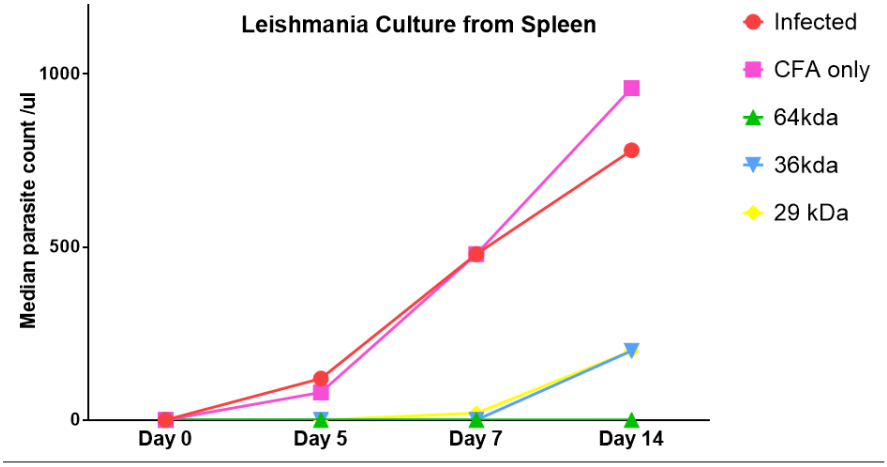

Figure 3. Median promastigote growth in different groups at day zero, day 5, day 7, and day 14th.

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The immunization with multiepitope antigens induced normalization of macrophage functions for effective clearance of intracellular parasites

To further evaluate the mechanism of protection in immunized animals, we checked the functional status of peritoneal macrophages from these animals in terms of their capability to clear the intracellular parasites and produce Reactive oxygen species (ROS) as a signature of normalized oxidative burst.

Parasite clearance assay

CFSE-stained parasite clearance assay showed a significant difference in the level of infection at 12 hours, 24 hours & 48 hours post-infection *in vitro* compared to cells from unimmunized-infected hamsters (**Figure 4A) (Supplementary Image 16A-16C & Supplementary Table 6)**. At 12 hours, the mean fluorescence intensity (MFI) from cells of all three immunized groups were significantly lower compared to MFI values from uninfected or unimmunized-infected animals, indicating facilitated parasite clearance by cells from immunized animals. The parasite clearance was significant at 24 hours and 48 hours also, indicating the normalized functional status of macrophages to clear the intracellular parasites after immunization.

The ability of macrophages to clear the intracellular parasites depends upon their ability to produce reactive oxygen species (ROS). The *Leishmania*-infected macrophages during active disease are shown to have a stunted ability to produce ROS due to *Leishmania*-mediated down-modulation of the oxidative burst in infected macrophages. Therefore, the restoration of macrophage functions can be determined by their capability to induce ROS upon stimulation. We assessed the functional state of macrophages from immunized animals for the production of ROS as determined by the DCFDA assay. For estimation of ROS, we estimated the level of fluorescence emitted due to 2'7-dichlorofluorescein (DCF) which is directly proportional to the production of ROS **(Supplementary Figure 17A & 17B),** interpreted in the terms of the relative level of ROS production in cells from immunized compared to unimmunized animals, termed as 'ROS production Index (RPI)'. The difference in ROS production in cells from immunized animals was significantly higher compared to unimmunized-infected samples from 12 hours onwards for 64 kDa (2.24±1.12 SEM, p=0.0011), while this difference became statistically significant for all the three proteins at 24 hours with an RPI of 1.23(±0.68 SEM p= 0.0019) for 64 kDa group; 1.11(±0.32 SEM p=0.0002) for 36 kDa and 0.93 (±0.31 SEM, p= 0.0002) for 29 kDa **(Figure 4B) (Supplementary Table 6)**. A close to 1 value of RPI was equivalent to ROS produced by naïve cells, and an indicator of the normalized functional status of macrophages from immunized groups. These findings were in perfect correlation with parasite clearance assay, where higher parasite clearance levels from macrophages of immunized animals were achieved, indicating the enhanced leishmanicidal activity due to enhanced oxidative burst against *Leishmania donovani* in immunized animals.

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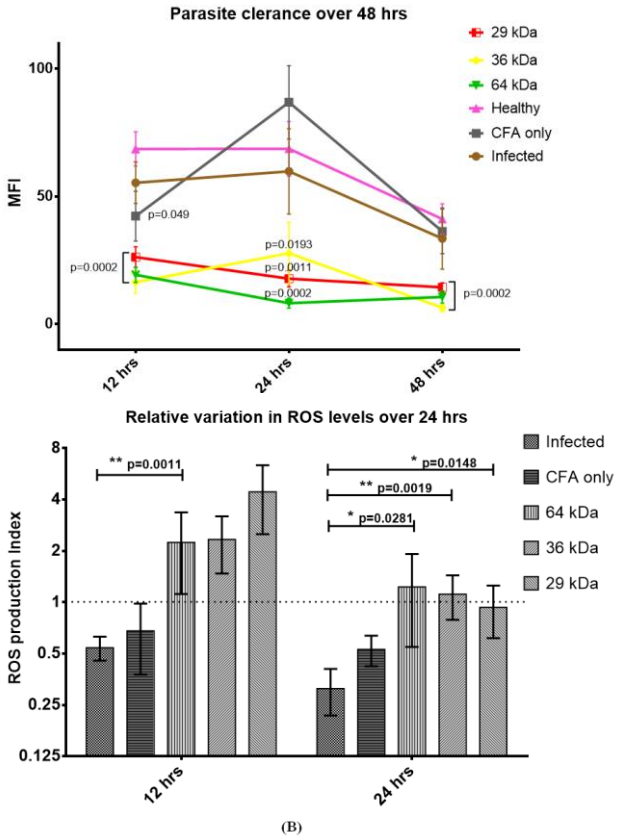

Figure 4. A) Parasite clearance observed at different time intervals. Statistical comparison between healthy and test groups. Average MFI (Mean Fluorescent Intensity) at different time points from adherent peritoneal cells of different animal groups. **B)** Variation in ROS production levels over 24 hours. ROS is expressed relative to healthy control. The dashed line indicates the expression levels of healthy cells. CFA: Freund's adjuvant. N=8, Mean values ± SEM. Statistics Mann-Whitney t-test level significance, ns $P > 0.05$, * $P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Immunization with multiepitope protein (64 kDa) induces a strong Th-1 polarized response in the spleen of immunized animals

To further understand the nature of protective responses generated in the spleen of immunized animals, we conducted a quantitative real-time PCR analysis for Th1 cytokines and genes related to T-cell activation. The results for real-time PCR were interpreted in terms of fold change with respect to unimmunized animals **(Figure 5)**. Real-time PCR-based immuno-profiling revealed a strong Th1-biased immune profile of the spleen for all the three immunized groups, indicating a significant upregulation of IFN-γ (33-fold in case of 64 kDa immunized animals) compared to unimmunized controls **(Figure 5A)**. A similar trend was observed for the 36 kDa immunized group (11.6-fold) and for 29 kDa the increase was 6.9-fold. The expression of signature Th-1 cytokines like IFN-γ, IL-12, and TNF-α, was found to be significantly upregulated in immunized animals with strikingly better in the 64 kDa group **(Figure 5B-5C)**, which is in coherence with macrophage activation and ROS production [4,60,61]. The IL-21, which is an activator of cytotoxic T-lymphocytes (CTL) and has a significant role in the clearance of intracellular infection, was also found to be significantly upregulated in 64 kDa and 36 kDa at 16 and 9.8-fold respectively **(Figure 5D)**. The IL-21 is produced by T helper cells and affects NK cells along with $CD8^+$ T cells. Additionally, the IL-21 has also been reported to revert CTL exhaustion and promote antigen-specific T cell activation [62–65]. Further, immune-profiling suggested a high level of inflammation and recruitment of activated T cells in the case of the 64 kDa group based on elevated levels of Chemokine (C-X-C motif) ligand 9 (CXCL9), the Chemokine receptor CXCR3 & C-C chemokine receptor type 7 (CCR7). The levels of CXCL-9 were 41 times higher in the 64 kDa immunized group and 6-fold higher in the

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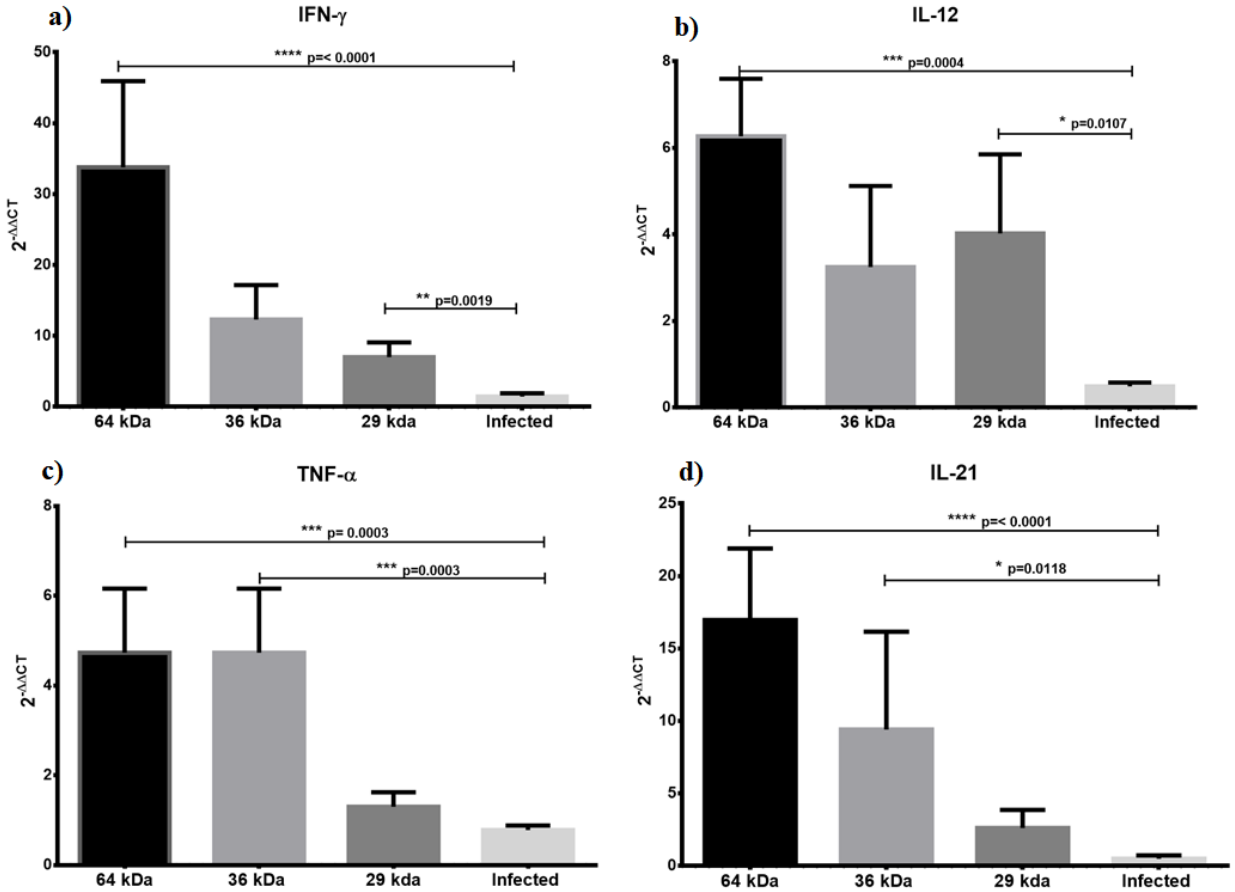
case of 36 kDa, although the levels for 29 kDa remained at only 3.5-fold higher yet were better compared to the infection alone group (0.8-fold change). Statistically, the data was significant only for 64 kDa protein. The IL23R, which is an indicator of the inflammatory profile, was 11-fold higher in 64 kDa immunized animals, but only 1.6-fold and 2.6-fold higher in for 36 kDa group and 29 kDa group respectively, not being statistically significant. The level of CCR7, a lymphoid homing receptor, was a 12-fold increase in the 64 kDa immunized group. 2.7-fold higher for the 29 kDa immunized group and 1.3-fold higher for the 36 kDa immunized animal group **(Supplementary Figure 18)**. The expression levels of (T-box transcription factor) TBX21 for 64 kDa were 26-fold higher along with 2.8-fold for 36 kDa and 1.7-fold for 29 kDa, all of which were significantly higher than the unimmunized group, further indicating the development of Th1 biased immune-response with immunization **(Figure: 5E)**. The expression level of L-selectin which is a secondary lymphoid tissue homing receptor, an indicator of memory T cell response, was significantly upregulated at 6.6, 1.6 and 2.6-fold change in 64 kDa, 36 kDa, and 29 kDa immunized animals compared to infected unimmunized animal groups, indicating T cell activation and formation of memory component **(Figure: 5F)**. In contrast to the Th1 profile, the markers for the Th2 cytokine profile showed a low level of expression in the case of immunized animals with all three antigen constructs. Most crucially, the levels of IL-10 and TGF-β remained significantly low for all immunized groups except 29 kDa (at a 3.7-fold increase) **(Figure 5G** & **5H)**, further indicating the predominance of Th1 polarized response in immunized animals compared to the unimmunized-infected group.

Further, the levels of programmed death-ligand 1(PD-L1: CD-274), a major immunosuppressor was significantly lower in all the immunized groups compared to unimmunized infected animals **(Supplementary Figure 18)**.



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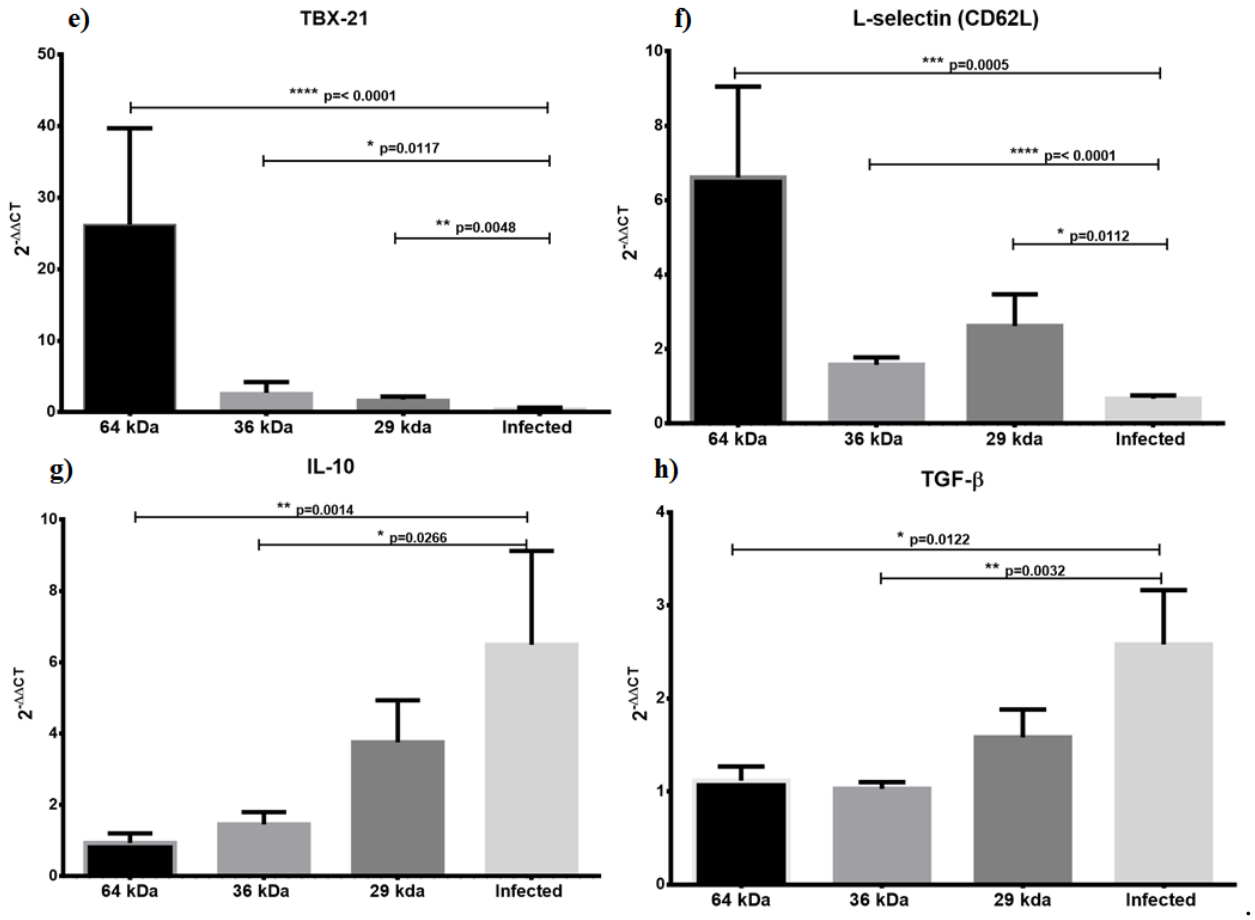

Figure 5. Relative fold change in gene expression levels of **a)** IFN-γ, **b)** IL-12, **c)** TNF-α, **d)** IL-21, **e)** TBX21, **f)** L selectin, **g)** IL-10 & **h)** TGF-β from immunized and unimmunized animal's spleen. n=8 for 64 kDa & 36 kDa, n=7 for 29 kDa & n= 5 for infected. Mean values ± SEM. Statistics Mann-Whitney t-test Level significance, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The multiepitope proteins induced activation of human polyfunctional T-cells ex-vivo:

To evaluate the T-cell activation potential of the vaccine proteins, we checked the *ex vivo* stimulation of polyfunctional T-cells in healthy human PBMCs with these proteins. Based on cytokine profile of individual T cells as calculated using Boolean Gating for INF-γ, TNF-α & IL-2, and IL-10 secretion, we found 36 kDa construct to be the highest inducer of triple-positive T cells (cells making IFN-γ, TNF-α, and IL-2 simultaneously) at 0.96% (mean ± 0.47 SD), which was higher than LPSA induced T cells at 0.55% (mean ± 0.073 SD) respectively. The 29 kDa and 64 kDa antigens were also able to induce the cells at a comparable frequency of 0.78 % (mean ± 0.35 SD) & 0.49 % (mean ±0.10 SD) respectively **(Figure 6A)**. These values were higher than PMA-ionomycin induced T cells and comparable to PHA-induced T cells at 0.46 (mean ±0.11 SD) and 0.84 % (mean ±0.64 SD) respectively. Besides triple-positive cells, the frequency of dual positive (INF-γ & TNF-α) T cells were also significantly higher for all the 3 proteins when compared to LPSA. The highest frequency of dual positive cells, for IFN-γ and TNF-α, was present for 64 kDa protein at an average 3.97% (mean ± 0.71 SD, p=0.0286) compared to LPSA induced population of 0.53% (mean ± 0.11 SD) T cells. Similarly, a significantly higher number was observed for 36 kDa protein at average frequency of 3.39% (mean ±1.4 SD, p=0.0286) cells and 1.67% (mean ± 0.20 SD, p=0.0286) cells for 29 kDa **(Figure: 6B).**

The frequency of T cells producing IFN-γ alone was found to be very high post-stimulation with 36 kDa and 64 kDa at an average of 10% (mean ± 1.54 SD, p=0.0286) and 8.27% (mean ± 0.8 SD, p=0.0286) of total T cells respectively, while the 29 kDa induced 5.5% cells (mean ± 0.52 SD, p=0.0286) **(Figure 6C).** This was further supported by high level of IFN-

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γ in culture supernatant as checked by ELISA **(Supplementary Image 23)**. This data supports our premise of selecting T cell-specific epitopes using IFNepiotpe tool in the case of 36 kDa and 29 kDa being strong IFN-γ inducers. Besides IFN-γ, we detected a high frequency of T cells producing TNF-α also, which is a protective cytokine and supports IFN-γ based Th1 cytokine axis through IL-12 and enhances IFN-γ production [66,67]. The 64 kDa antigen was able to induce an average 21% (mean ±2.4 SD, p=0.0079) of T cells for TNF-α production, while 36 kDa induced 18% (mean ±5.0 SD, p=0.0079) and 29 kDa induced only 7.35% (mean ±3.06 SD) of cells **(Figure 6D)**. Further, IL-2 production was also found to be significant for 36 kDa at mean frequency of 9.2% (mean ±1.4 SD, p=0.0286) T cells, which was 4.3% for 64 kDa (mean ± 0.37 SD, p=0.0286) and 3.9% for 29 kDa (mean ± 0.93 SD) **(Figure 6E)**. It was interesting to note that the frequency of IL-10 producing T-cells in the same experiment was very low at only 0.9% (±0.33 SD) for 64 kDa and 1.39% (±0.57 SD) for 36 kDa, which was comparable to the number of cells induced by LPSA [1.2%(±0.29SD)] **(Figure 6F)**. The low levels of IL-10 correlated with higher levels of protection as in case of both 64 kDa and 36 kDa minimal IL-10 presence was detected in both human PBMC stimulation experiment as well as in spleens of both 64 kDa and 36 kDa immunized animals.

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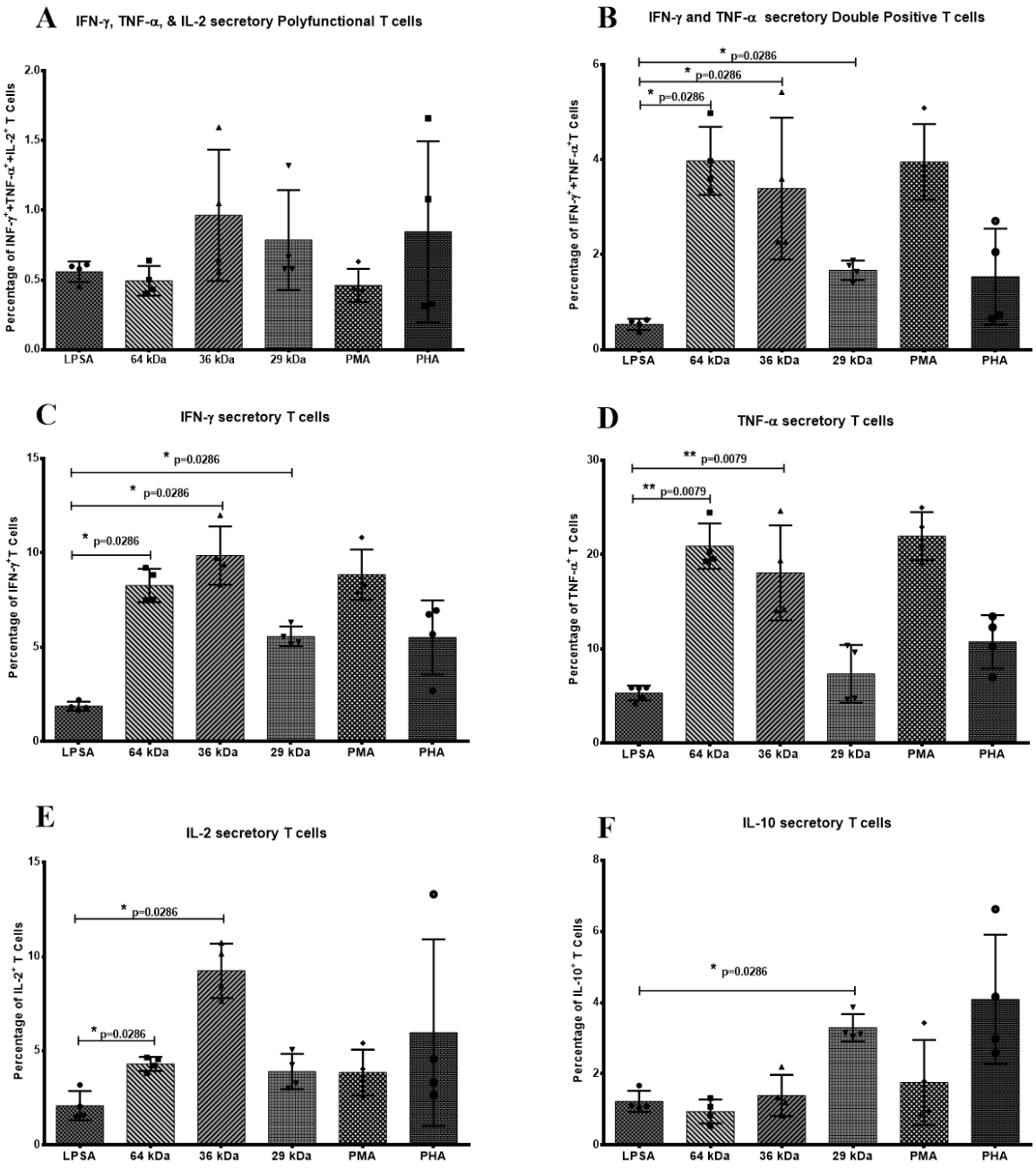

Figure 6. Bar graph comparing the frequency of CD3+ T cells secreting IFN-γ, TNF-α, IL-2 & IL-10. **A)** Frequency of poly-functional CD3+ T cells expressing IFN-γ, TNF-α, and IL-2 simultaneously. **B)** Frequency of dual positive CD3+ T cells expressing IFN-γ and TNF-α simultaneously. **C**) Frequency of CD3+ T cells expressing IFN-γ. **D)** Frequency of CD3+ T cells expressing TNF-α. **E**) Frequency of CD3+ T cells expressing IL-2. **F)** Frequency of cells expressing IL-10. N= 4. Mean values and SD. Statistics Mann-Whitney t-test Level significance, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Discussion

The previous generation of vaccines for visceral leishmaniasis has mainly relied on the generation of enhanced IFN-γ response, with few attempts at a possible suppression of IL-10 [30]. So far, the vaccine designs have been based on either whole attenuated parasite form or multiple antigens and due to partial efficacy, none is yet licensed for human use [68]. These whole antigen-based vaccines could carry a mix of high affinity and low-affinity random epitopes. In this regard, the selection of strong binders for vaccine designing has been shown to induce inflammatory responses and the generation of memory T cells [69–74]. An epitope has a quantitative and qualitative role in immune-response

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generation, specifically T-cell receptor (TCR) dependent memory cell formation [14]. The role of a single epitope in TCR activation and its downstream effect is a matter of debate and research [9], because the substitution of a single amino acid in epitope sequence can alter the immune response to a great extent [75,76]. The prevailing qualitative model of memory formation depends on the cytokine environment generated by APC and other supportive cells [14,77]. The quantitative model however relies on TCR and immune-signalosome complex involving peptide-MHC-TCR interaction strength, amount of antigen, and costimulatory molecule interaction [28,78–80]. In vaccine designing the level of control on the quantitative model is debatable and cannot be controlled directly. In the context of the qualitative model, the modern immunoinformatic approach can be utilized to dictate the choice of the epitope, which can affect the TCR-peptide-MHC signalosome response by utilization of either high or low-affinity epitopes [81,82]. The approach of MHC-I and MHC-II specific epitope identification has been utilized by researchers in the last 5 years for Leishmaniasis vaccine research [83]. However, most of these vaccine ideas are limited to *in-silico* docking models. Additionally, the number of epitopes included in a majority of research has remained limited to selective few epitopes, limiting the overall size of vaccine candidates [83–86]. Since larger size antigen is known to generate a higher degree of immunogenicity, this is a limitation in the case of designed vaccines because the smaller size antigens are generally poor immunogens [87]. On the other hand, the variants with a large number of epitopes as in chimeric molecules have shown promising results [88].

Previous studies from our lab have identified three antigenic genes of *Leishmania donovani* from an indigenously made cDNA library [45]. When repeatedly tested as potential vaccines in hamster and mice model, the responses indicated strong IFN-γ induction and an optimal level (70-80%) of protection. Although a significant reduction in splenic parasite burden was observed, yet sterile immunity could not be achieved, probably due co-induction of IL-10 in the immunized animals [46,47,89]. Our current work is a refinement of the previous work and takes one step further from whole antigen-based subunit vaccine to T cell epitope-based vaccine. In the present proof-of-concept study, we have tried the possibility of developing T cell epitope-based vaccine candidates based on multiple MHC-I and MHC-II epitopes, along with candidates exclusively based on multiple INF-γ specific T cell epitopes, from the three previously identified cDNA clones of *Leishmania donovani* [45]. Based on the immunoinformatic mapping, the data gave a large number of MHC-I and MHC-II binding epitopes with the majority of epitopes being overlapping and present in a cluster of epitope-rich islands. Similar results were obtained with usage of IFNepitope tool. The presence of T-cell epitopes in clusters could be of some evolutionary significance or just a chance occurrence with our 3 antigen genes, which needs to be studied and resolved further. We couldn't come up with any previous study in literature to account for the clustering of T cell epitopes. In our opinion, the clustering of immunodominant epitopes could occur due to evolutionary selective pressure in which certain regions of antigens escape the MHC affinity due to reasons not known yet. One study does point towards common amino acid polymorphisms and epitope binding repertoires in HLA-DRB1 associated with lower Leishmaniasis susceptibility [90]. Our study further raises the question regarding epitope representation on MHC molecule, since the epitope identification done on human MHC-binding based selection, showed their ability to raise a T cell-based immunity in hamster model also, points towards the conserved nature of high-affinity epitopes across species. The hypothesis of specific epitopes, both conformational and linear, being conserved through evolution and shared in antigens from different species, have been validated by different publications [91–94].

The three designed multi-epitope constructs in this study, when used as vaccine in a hamster model of experimental visceral Leishmaniasis along with FCA, induced a significant protective response to a virulent challenge along with a strong Th-1 biased immune-response. Our observations for the designed vaccine candidates, especially 64 kDa and 36 kDa, indicated a significant improvement over the parent antigens identified earlier and used as base genes for selection of epitopes in this study, as evident from a significant

increase in the protection level with even achieving a sterile immunity with 64 kDa protein and a notable reduction of IL-10 cytokine production also correlating with increased vaccine efficacy [46,47]. Scanty parasites seen in splenic touch imprints of 64 kDa immunized animals could be most likely be remnants of dead parasites, as no motile promastigotes could be observed in the splenic culture assay with these spleen tissues. Splenic culture assay is considered a gold-standard method of detecting the viable parasites in the spleen as only living and viable parasites would be able to differentiate to promastigote form [95,96]. Active *Leishmania* infection would normally lead to hepato-splenomegaly with an increase in spleen size and weight, which could be observed in the infected but unimmunized animals. However, the parasite load as well as the splenic index, which is a proxy indicator of spleen weight, was consistently low in animals immunized with all the three multiepitope proteins designed in the current study, which primarily indicated the protective efficacy of these vaccine candidates in terms of restricting the parasite growth.

The *Leishmania* parasite completely hijacks the killing machinery of host cells, i.e., macrophages. A predominance of immuno-suppressive macrophages with a subdued capacity to generate optimum oxidative burst, which is necessary for clearance of parasites, is seen in active *Leishmania* disease [97]. Thus, the protective response of any vaccine would envisage the reversal of this process and conversion of immuno-suppressive to activated macrophages having enhanced capacity to generate enough reactive oxygen species to clear the intracellular parasites. The peritoneal macrophages from the immunized animals displayed the characteristics of normalized functions with enhanced capability to clear the intracellular parasites very effectively along with the generation of significantly higher amounts of ROS, indicating the restoration of the killing mechanisms and generation of oxidative burst (87-89). Hyper responsive adaptation in macrophages is an increased expression of inflammatory genes after re-stimulation of macrophages. This depends upon increased expression of signaling molecules, metabolic reprogramming, and the epigenetic change at *cis*-regulatory elements. Perseverance of these features even after elimination of stimulus can facilitate or potentiate secondary activation [98,99]. Our results can be inferred as hyperresponsive adaptation arising due to immunization by the multiepitope proteins, when used as a vaccine, in the experimental model [99–101]. Although, the details of the exact mechanism for this state in our study remains to be explored, yet the functioning of the iNOS pathway in immunized animals seems evident for their ability to clear *in-vitro* reinfection, as the ROS pathway, in turn, is connected to inflammasome activity in macrophage [102,103]. This is in coherence with previous studies where ROS generation has been shown in support of *Leishmania donovani* clearance [104,105]. The protective response in our study is supported by a high level of inflammation and T cell activation in hamsters immunized with the designed multiepitope proteins. The data indicates an upregulated expression of IFNγ, IL-12, and TNF-α, which forms the signature for Th-1 type of immune-response being induced in the immunized animals, leading to a protective response as seen in these animals. This type of response was most evident in animals immunized with 64 kDa protein, which induced a sterile immunity. . Development of memory T cell responses was supported by elevated expression of genes like TBX21, IL-21, IL-23r, IL-12, and L-Selectin in the spleen tissue of all the immunized animals, especially with 64 kDa protein **(Supplementary Figure 18)**. Among these, the IL-21 has been shown to mediate inflammatory axis in parasite-infected organs, mice deficient in IL-21 are shown to be unable to clear parasite in a TBX21, STAT-4 and IL-12 dependent manner [106,107]. IL23r is present on T cells and NK cells & has been shown to have a protective role in VL infection via IL-17 mediated pathways [108]. Further T cell functioning and development of proliferative T cells was evident from high levels of CXCL9, CXCR3 in the case of 64 kDa immunized group, further indicative of Th1 polarization **(Supplementary Figure 18)**. The CXCL9 increases transcription of T-bet and RORγ, leading to the polarization of Foxp3 type 1 regulatory (Tr1) cells or T helper 17 (Th17) cells from naive T cells via STAT1, STAT4, and STAT5 phosphorylation [109]. The Treg component was low in immunized animals in our study as indicated by significantly downregulated expression of IL-10 and TGF-β. This type of response is important for any

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vaccine to be efficacious against this parasite, since the pathogenesis of *Leishmania* infection is marked by presence of Treg cells, which cause a broad level of immune suppression (92,93).

A further elaboration of protective responses in the human context was shown by the activation of polyfunctional T cells. Polyfunctional T cells have been correlated with protection in multiple studies [110,111]. It was interesting to find that the designed protein constructs were able to induce the polyfunctional T-cells in the PBMC of a healthy human donor to produce IFN-γ, TNF-α, and IL-2 simultaneously. Similarly, cytokine levels and frequency of IFN-γ producing T cells were significantly high for all the proteins asserting the role of dominant and IFN-γ specific epitopes as the inducer of Th1 responses, justifying our epitope-selection process. . The induction values were equivalent and higher in some cases when compared to other candidates tested in other studies [112–114]. This data further emphasizes the protective potential of our antigen constructs via the induction of polyfunctional T cells.

In summary, among the three designed multi-epitope constructs, the 64 kDa candidate contained the largest number of dominant epitopes, which were both strong binders of MHC-I and MHC-II along with regions of IFN-γ specific epitopes and this protein induced a sterile immunity in hamsters when used as a vaccine. The protective nature of 64 kDa was also evident by the generation of polyfunctional T cells and very high frequency of IFN-γ and TNF-α producing dual-positive T-cells on activation of human PBMC with this protein. High-affinity dominant epitopes are generally considered immune-responsive with the potential to activate cytotoxic T lymphocytes (CTLs), and a skewness towards the Th1 cytokine profile [25,26]. The 36 kDa protein, designed with IFN-γ specific epitopes with a preference for epitopes that had an affinity towards MHC-I, but also contained some dual-specificity epitopes for both MHC-I and MHC-II, was also able to reduce parasite burden significantly with inflammatory response and absence of any immune-suppressive responses, while the sterile immunity could not be achieved with this protein. The results from human PBMC stimulation indicated polyfunctional T cell activation along with IFN-γ and TNF-α production capability. The levels of IFN-γ in suboptimal concentrations have been shown to have a vital role in the optimization of innate immune cell functions including phagocytosis and destruction of reminiscent pathogens [115]. Previously, other researchers have also shown a limited role of CTL responses in the clearance of parasites [116]. The 29 kDa protein was designed from IFN-γ inducing epitopes with affinity to MHC-II, it also contained some dual-specificity epitopes for both MHC-I and MHC-II. Immunization with 29 kDa induced a lower level of protection, although showed increased IFN-γ production, yet had very insignificant effect on IL-10 producing T cells. However, 29 kDa contained only 24 core epitopes, compared to 63 epitopes in 36 kDa and 1317 epitopes in 64 kDa in various overlapping configurations, which seem to have limited its protective efficacy.

Conclusion

Visceral Leishmaniasis has remained without a protective vaccine probably because of the lack of a candidate that could generate a predominant Th1 polarized response while keeping the immuno-suppressive responses to a minimum. We have shown the possibility to develop a vaccine candidate, the 64 kDa protein, with tailored Th1 responses by the selection of T cell-specific high-affinity and IFN-γ inducing epitopes, which could induce a sterile immunity against a lethal challenge. Our other two proposed candidates were also able to induce a significant level of protection in the hamster model but with limited efficacy. The exact mechanism of these responses along with the kinetics of T cell epitopes remains to be further evaluated. This approach opens the possibility of developing tailored immune response-specific vaccines against other infectious agents also such as HIV, malaria, coronavirus or even cancer.

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Conflicts of Interest: Authors declare no conflict of interest.

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