

Advancing Th1 Stimulatory Proteins Towards Development of Polypeptide Vaccine Against Kala-Azar



Anuradha Dube
Division of Parasitology
CSIR CDRI, Lucknow

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Leishmaniases

Leishmania species cause three clinical syndromes depending on the spread of the infection in the body



Cutaneous

- Self limiting but chronic skin ulcer dev at the site which may takes months to heal (scar)
- *L.major*
- *L.tropica*
- *L.mexicana* complex
- *L. aethiopica*



Mucocutaneous

- Initially causes similar skin ulcers that heal but subsequently lesions reappear primarily in mucous tissue of nose & mouth often accompanied by sec. infection & massive tissue destruction
- *L.braziliensis* complex



• Visceral

- Very severe systemic disease with the organisms homing to liver, spleen & B.M.
- Usually fatal if not treated Mortality of untreated disease 75 -90%
- *L.donovani*
- *L.infantum*
- *L.chagasi*

Post Kala Azar Dermal Leishmaniasis (PKDL)



- Occurs as a sequel of VL
- Normally develops < 2 years
- Restricted to skin
- Rare but varies geographically

Life cycle of *Leishmania*

Tropical scourge is transmitted to humans via the bite of a female phlebotomine sandfly.

Sandfly vector

Promastigote



Promastigotes:

Flagellated (2x 2-20 μm)
Procyclic (immature log phase)
Metacyclic (mature, infective phase)

Mammalian host

Differentiation

Amastigote

Macrophage

Parasite Invade reticulo-endothelial sys

Amastigotes

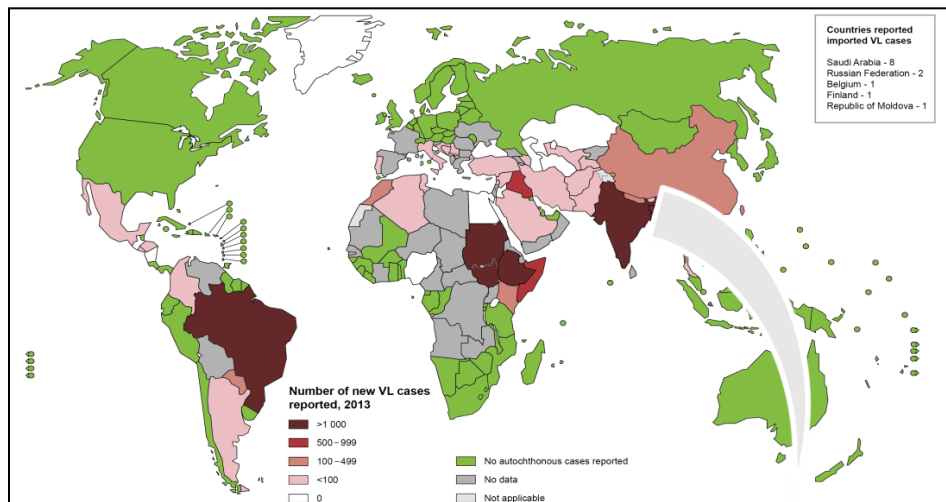
non-flagellated (2-5 μm)

Adapted from Paulo Pimenta

Dwyer, D.M.

Current Status Of Visceral Leishmaniasis (VL) – A disease of Poverty

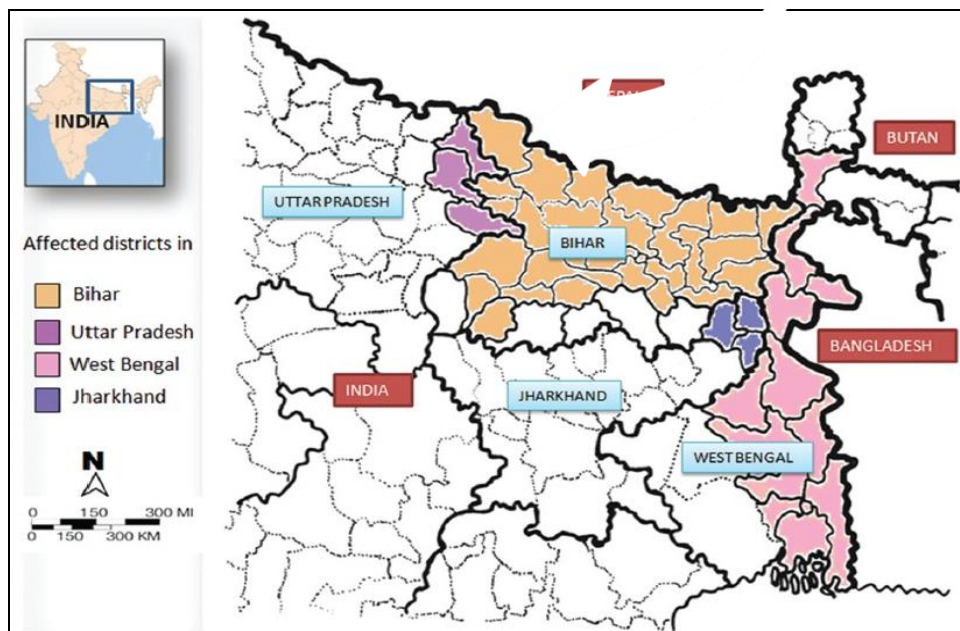
After malaria, 2nd most common parasitic cause of death



Source: WHO (<http://gamapserver.who.int/mapLibrary>)

- Annual incidence – 200,000-400,000 cases of KA (WHO report) resulting in 20,000- 30,000 deaths per year
- Endemic in 88 countries & 90% of cases in Brazil, Bangladesh, India, Nepal & Sudan.
- 35 countries around world have reported cases of VL/HIV co-infection

- KA detected in India- > 100 years.
- Big epidemic - in 1935, 1977 & 1991-92 with 94,000, 100,000 & 250,000 cases.
- Endemic in Bihar (28/37 dist), Jharkhand (4), W Bengal (10) & East UP (5)
- Bihar accounts for > 90% of the cases
- 200,000 deaths from VL so far



Source: Muniaraj M, 2014. *Trop Parasitol* 4(1):10-19.

S No	States	Cases (July, 2015)
1	Assam	1
2	Bihar	4037
3	Himachal Pradesh	0
4	Jharkhand	747
5	Madhya Pradesh	0
6	Sikkim	3
7	Uttrakhand	2
8	Uttar Pradesh	52
9	West Bengal	325

Source: NVBDCP (<http://nvbdcp.gov.in/ka-cd.html>)

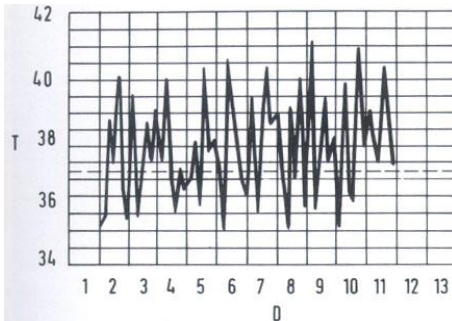
Visceral Leishmaniasis (VL) in India : Challenges.....

Parasite Invade reticulo-endothelial system
Weeks to months (3-100 weeks) incubation period

Clinical Profile

- ❖ Chronic fever - (Prolonged Low-grade fever); Often but not always fever occurs in two daily peaks- Skin dark gray hence the name kala-azar
- ❖ Wasting - Progressive weight loss (profound cachexia)
- ❖ Anemia - with Leucopenia & lymphadenopathy
- ❖ High titres of IgG (Hypergammaglobulins- mainly IgG from polyclonal B cell activation)
- ❖ Hepatosplenomegaly & Bone marrow hyperplasia
- ❖ Suppressed CMI responses in active cases
- ❖ Complicated by secondary opportunistic infections

High case fatality rate in the absence of treatment



Visceral Leishmaniasis (VL) in India : Challenges.....

Management with the current antileishmanial drugs

Drug	Dose	Duration	Cost Rs	Remarks
SAG	20 mg/kg/day	30 days	34,892	Declining activity, Drug resistance, toxicity,
Ampho B	1 mg/kg - alternate day	30 days	24,145	Costly, toxicity
ABCL	2 mg/kg/day	5 days	43,609	Costly
Lipo Ampho B/ Ambisome	5 mg/kg /10 mg/kg	1 day	36,734	Costly
Miltefosine	100 mg/daily (2.5 mg/kg/day)	28 days	13,576	Teratogenic
Inj Paromomycin	15 mg/kg/day	21 days	6,380	Costly
Paro + Milt	10 + 10 mg/Kg	10 days	4,815	Costly
AmB + Paro	5mg/Kg + 1mg/kg	1+11 days	9,180	Costly
AmB + Milt.	5mg/Kg + 7	1+8 days	8,123	Costly

Limitations: Prohibitive cost of available drugs, toxicity, requires hospitalization

Croft et al 2006 Clin Microbiol Rev 19: 111-26
Sundar et. al. 2011 Lancet, 377(9764):477-86

Visceral Leishmaniasis (VL) in India : Challenges.....

Emergence Of Resistance To Pentavalent Antimonials & Other Drugs

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2011, p. 3088–3091
0095-1137/11/\$12.00 doi:10.1128/JCM.00173-11
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Unusual Case of Resistance to Amphotericin B in Visceral Leishmaniasis in a Region in India Where Leishmaniasis Is Not Endemic^V

CLINICAL MICROBIOLOGY REVIEWS, Jan. 2006, p. 111–126
0893-8512/06/\$08.00+0 doi:10.1128/CMR.19.1.111-126.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Drug Resistance in Leishmaniasis

MAJOR ARTICLE

Resistance to Antimony and Treatment Failure
in Human *Leishmania* (*Viannia*) Infection

OPEN ACCESS Freely available online

Failure of Miltefosine Treatment for Visceral Leishmaniasis in Children and Men in South-East Asia

HIV-coinfection & increasing reports of relapse



Drug Resistance in Visceral Leishmaniasis

Tropical Medicine and International Health

VOLUME 6 NO 11 PP 849–854 NOVEMBER 2001

Drug resistance in Indian visceral leishmaniasis

PLOS ONE



Source: Pubmed (NCBI)

Visceral Leishmaniasis (VL): Challenges.....



as an alternative to chemotherapy development of vaccine is an attractive option

Vaccine - a cost-effective & safe alternative

Vaccine against VL: Requisites

1

- **Safety and ease of production at a low cost in endemic countries**

2

- **induction of robust, long-term T-cell responses**

3

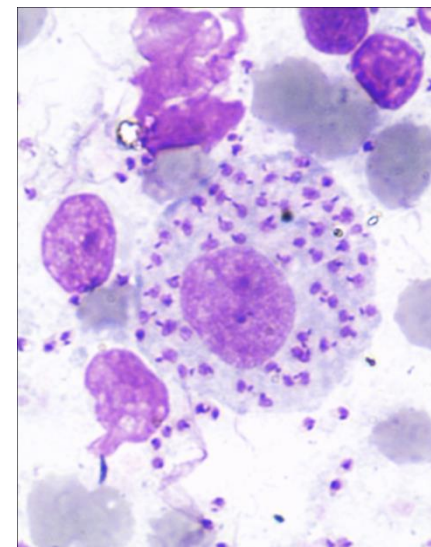
- **Exert both prophylactic and therapeutic efficacy.**

Development of a VL-specific vaccine remains an important global health priority.

Feasibility of a vaccine against VL ?

***Leishmania* parasite lends itself well to vaccine development**

- Only a small % of infected individuals develop severe disease & patients who recover from VL display resistance to re-infection
- Strong immunity after recovery
- 80 to 90% of human infections are subclinical or asymptomatic & this asymptomatic infection is associated with strong CMI.
- Parasites-Easy to grow and genetic manipulation possible



Development of clinical immunity provides a biological rationale for the development of VL vaccines

Exptl models for vaccine development against VL

Animal/strains

Parasite

Characteristics

Mouse:



BALB/b
BALB/c
C57BL/6

L. chagasi
L. donovani
L. infantum

-Self curing to non healing type
-Th1/Th2 response
- Model for acute infection

Hamster:



Golden
Chinese

L. chagasi
L. donovani
L. infantum

-Progressive fatal infection
-Severe immunosupp (Th2 response)
- Model for chronic infection

Dog:



Stray
Beagle
Mongrel

L. infantum
L. chagasi

-Natural reservoir (not in India)
-Subclinical/asympt to progr fatal infection
-Immunosupp (Th2 response)
-Good sec. model for pre clinical studies

Monkey:

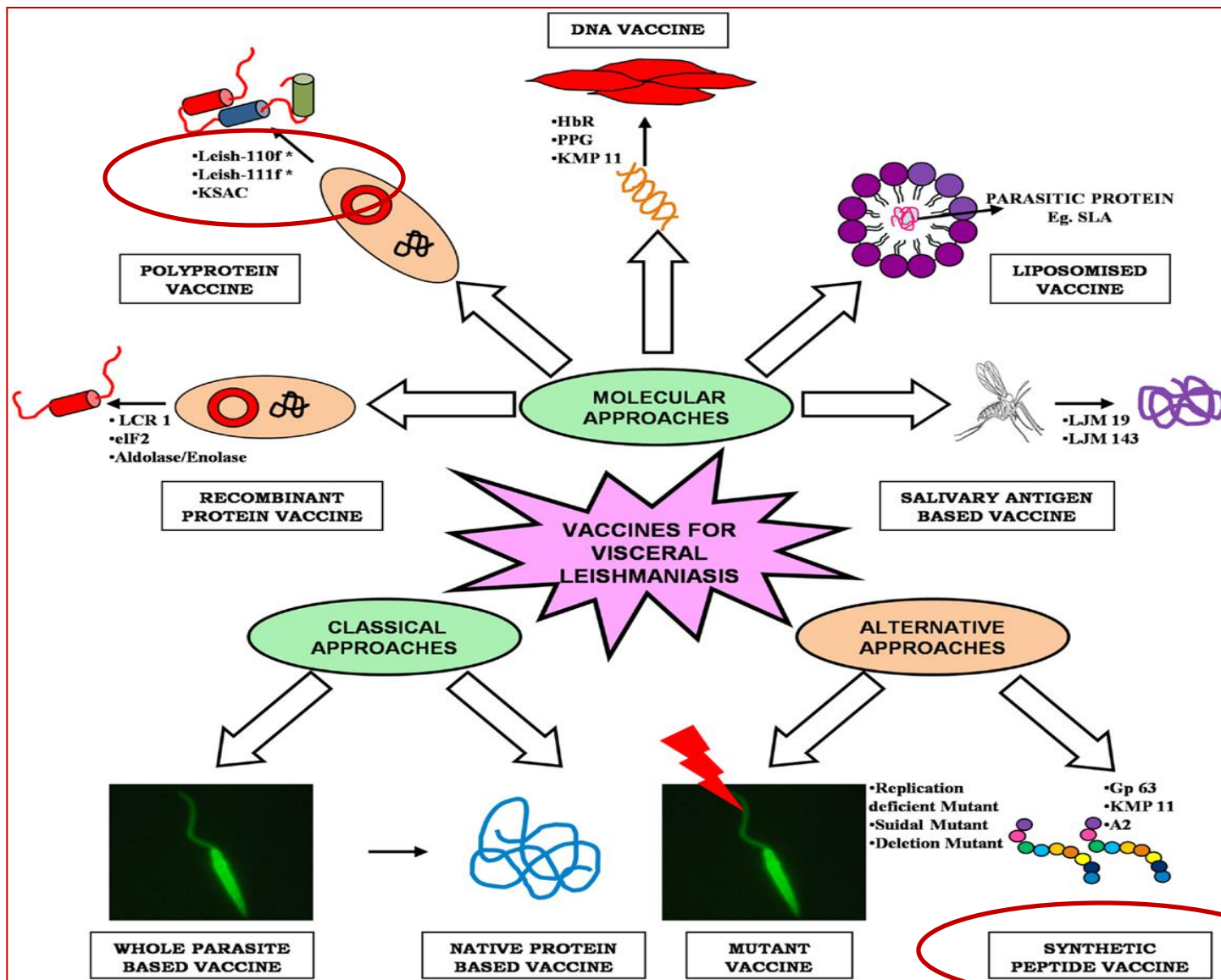


Owl
Squirrel
Vervet
Langurs

L. donovani

-Subclinical to fulminating progr fatal inf
-Severe immunosupp (Th2 response)
-Good sec. model for pre clinical studies

Approaches for vaccine development against Leishmaniasis



LEISH-F 1& LEISH-F2 - (IDRI, Seattle, WA)

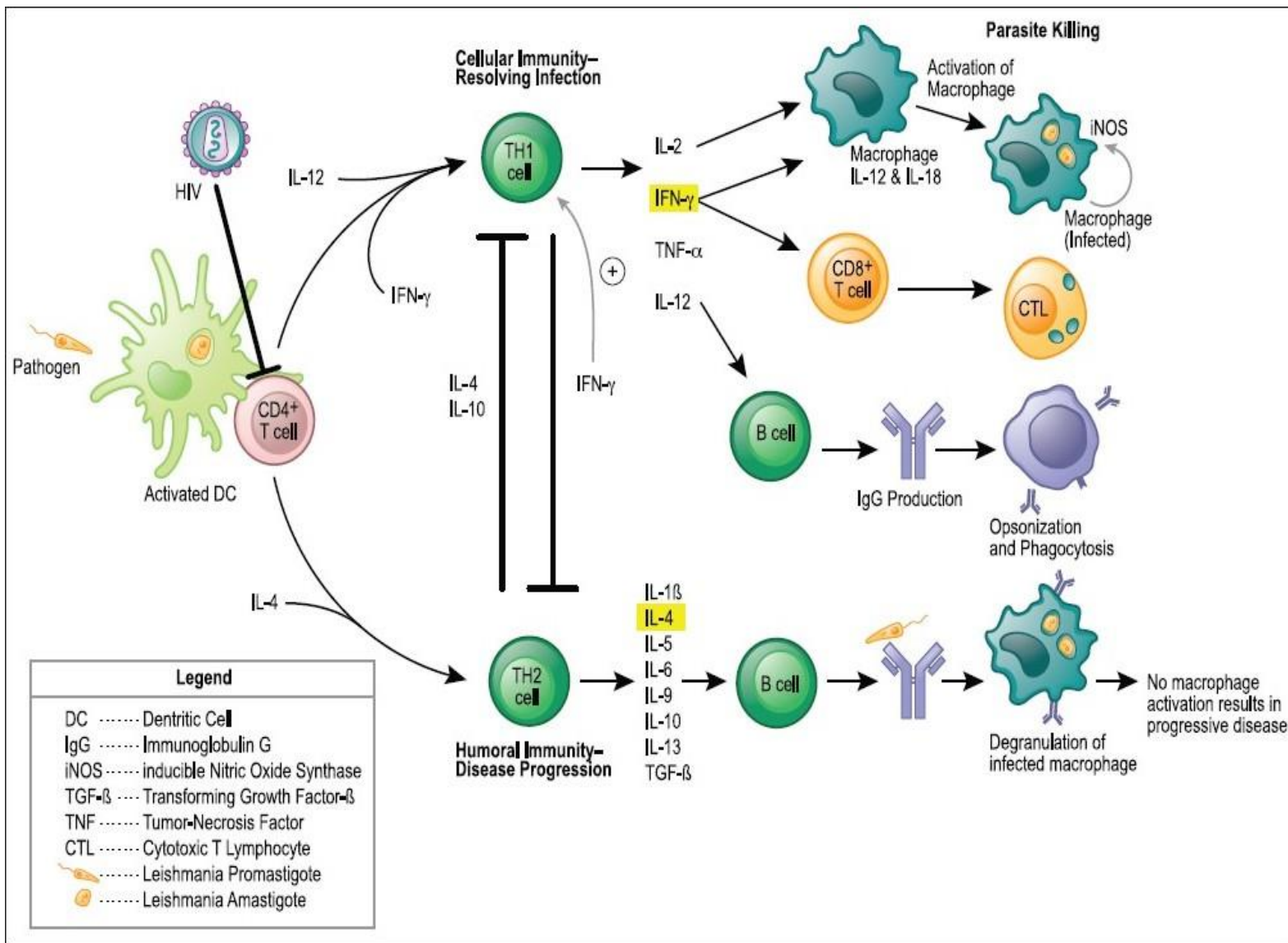
- 3 proteins, conserved, *L. donovani* & *L. chagasi*, NW VL & *L.braziliensis*, both mucosal & CL (NW)
- Phase I trials US/Colombia/Brazil/Peru & India, targeting **VL/CL**, immunogenic, safe & well tolerated in populations both with & without a seropositive subpopulation

LEISH-F3 -

- Fusion polypeptide by linking in tandem two Leishmania proteins: residues 1-314 of *Leishmania infantum/donovani* nonspecific nucleoside hydrolase (NH) protein & residues 2-353 of *Leishmania infantum* sterol 24-c-methyltransferase (SMT) protein.
- LEISH-F3 + GLA-SE (novel TLR-4-based adjuvant); 1st phase I trial in US.
2nd phase I clinical trial in Indian population (**VL**) and the outcome of this vaccination trial is yet to be seen

Zydus and IDRI - collaborate to conduct clinical development activities in India with the goal of developing, registering and marketing this vaccine candidate for the prevention of VL,

Effector mechanisms governing protection against leishmaniasis



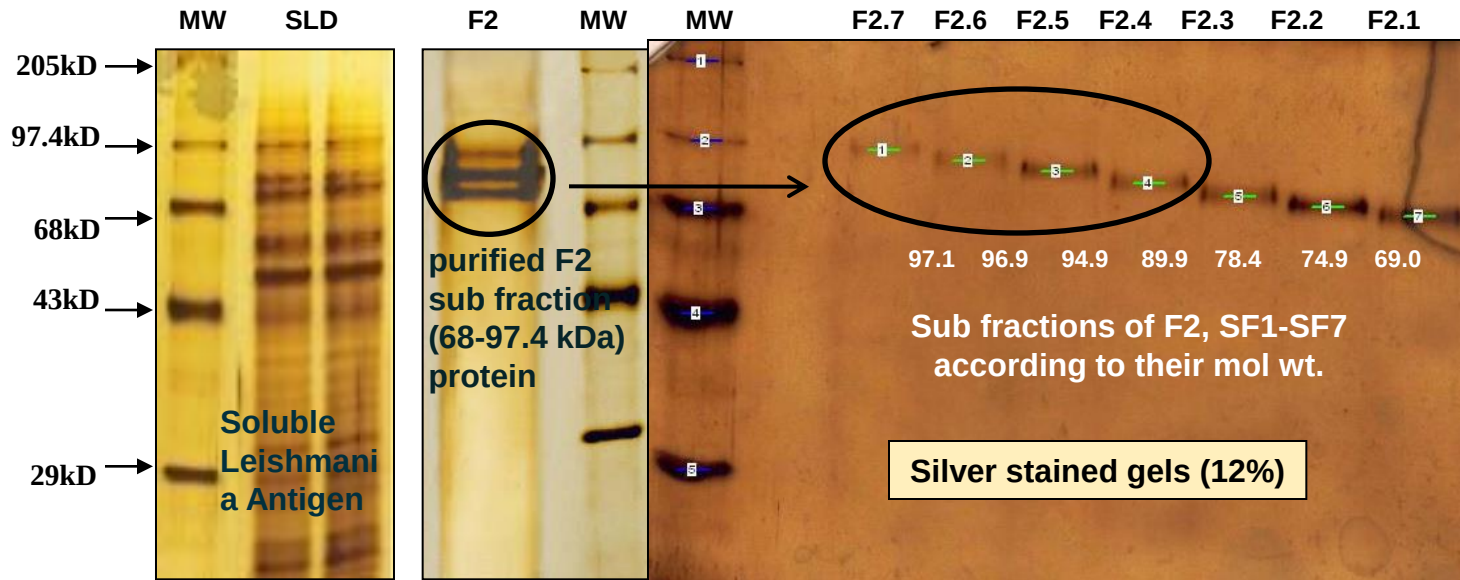
Identification of Th1 stimulatory Proteins

- It is well known that a cured *Leishmania* patient in endemic areas mounts strong Th1 stimulatory response which is responsible for lifelong immunity.
- Since, protective immunity in human and experimental leishmaniasis is predominantly of the Th1 type, immunogens with Th1 stimulatory potential would make good vaccine candidates.
- Once potent vaccine molecules are identified, the gene encoding these antigens would be cloned in an expression vector to obtain it in substantial amount for its use as polyprotein/ polytope vaccine.

Identification of potential Th1 stimulatory proteins from *Leishmania* parasites (clinical isolate 2001) of *L. donovani* for their immunoprophylactic efficacy against exptl VL

Fractionation/Sub-fractionation of soluble *L. donovani* lysate (SLD) by cell Prep

F2 fractionated into 7 sub fractions (F2.1-F2.7) acc. to MW by continuous elution SDS-PAGE (Prep Cell)



Vaccine (2008)

Study Groups

Hamsters



Assay: LTT

Lymphocytes – Lymph nodes

✓ Cured (Miltefosine at 50mg/kg x5)

Human samples



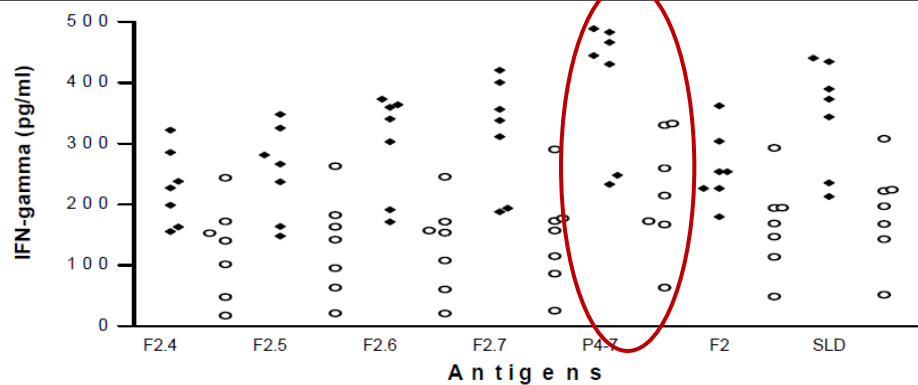
Assays: LTT & Cytokine ELISA

PBMCs- from Muzaffarpur, Bihar.

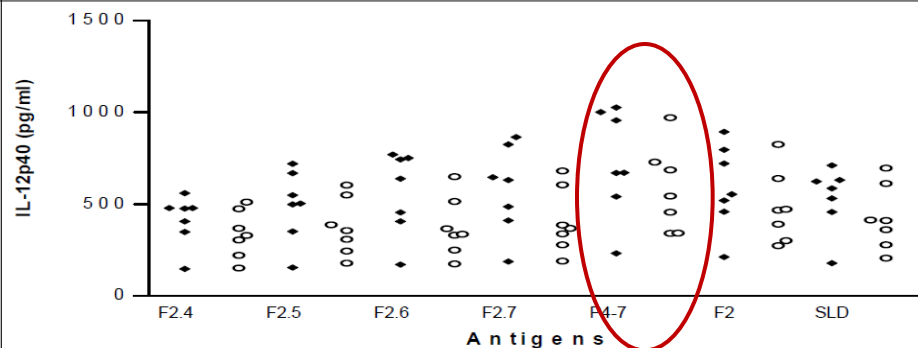
✓ Cured patients

✓ Endemic contacts

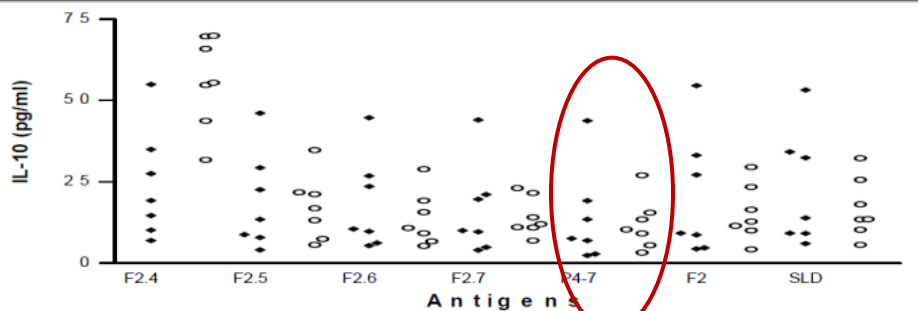
Cellular responses of Fractions/sub-fractions of *L.donovani* Promastigotes



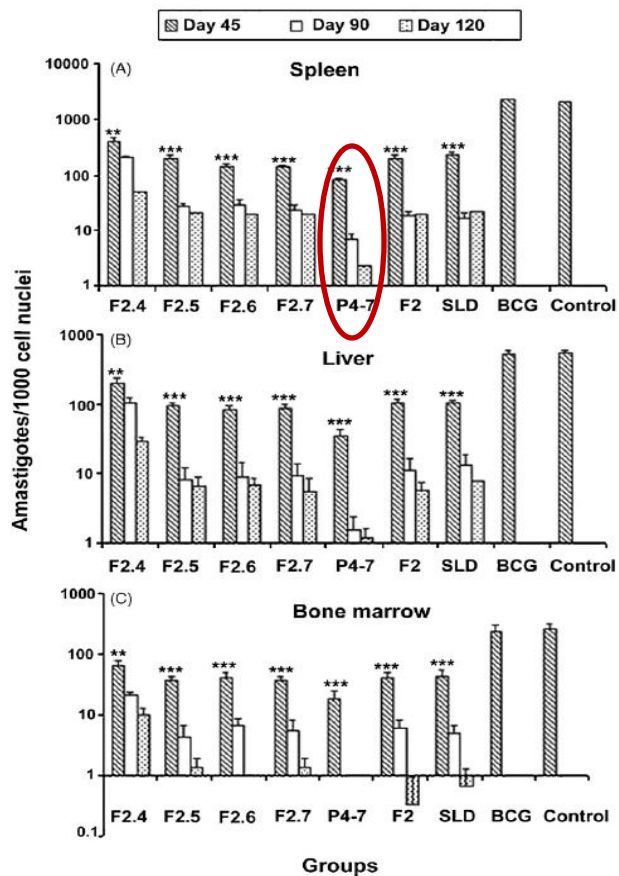
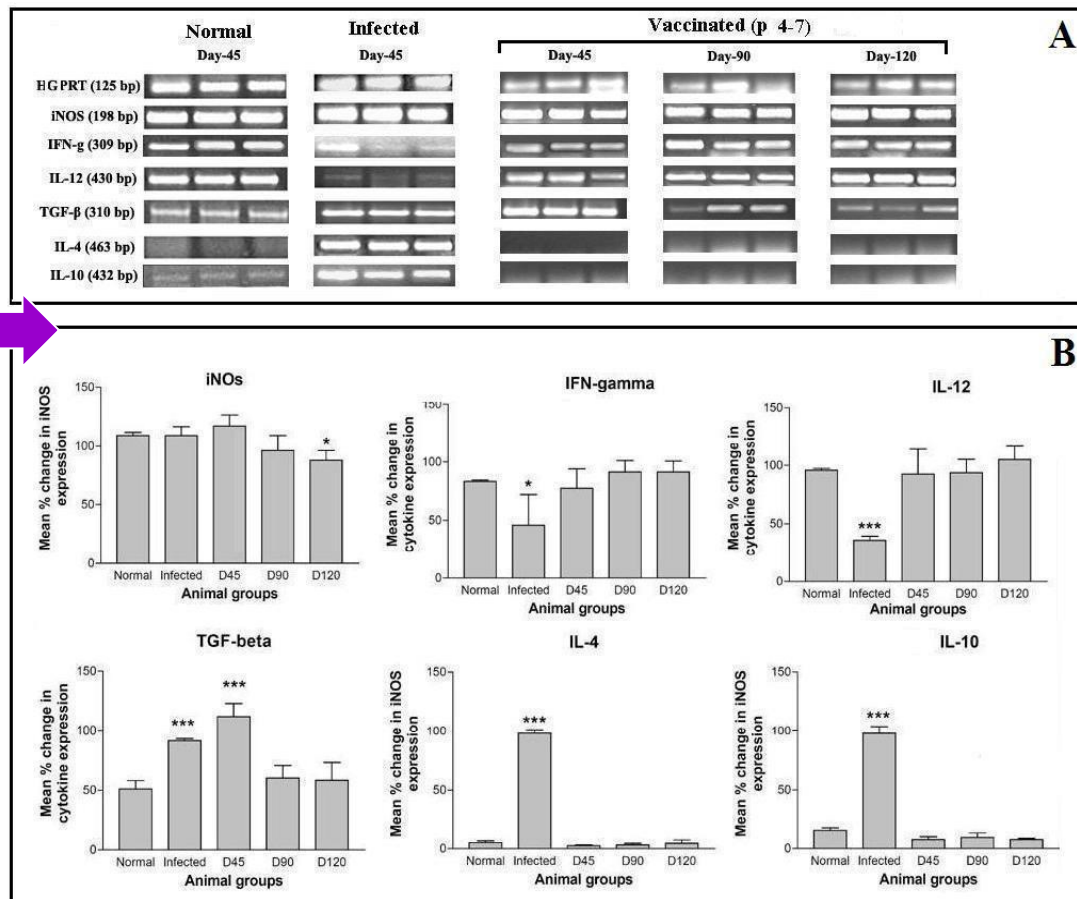
➤ F2 fraction (97.4-68 kDa) & its 4 sub-fractions (89.9 – 97.1 kDa) individually as well as pooled (P4-7) are the most potent Th1 stimulatory sub-fractions



Stimulated remarkable Th1-type (LTT, NO, IFN- γ and IL-12) response in PBMCs/lymphocytes of cured Leishmania infected patients as well as hamsters



Prophylactic potential of Fractions/sub-fractions of Promastigotes

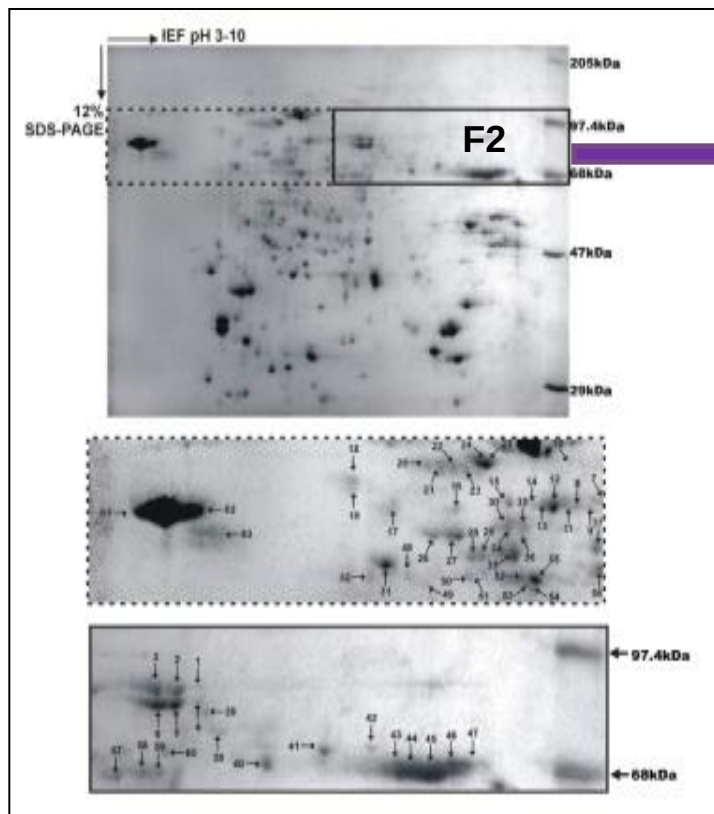


Pooled P4-7 fraction offered excellent efficacy against *L.donovani* challenge in hamster

Exhibited significant parasite inhibition in spleen & liver ; Survival : > one year.

Up-regulation of IFN- γ , IL-12, TNF- α and downregulation of TGF- β , IL-4 & IL-10

Identification of Th1 stimulatory poly-proteins through Proteomic & MALDI analysis of soluble lysate of L.donovani



33
proteins

1D MALDI : Sub-Fr-F2.4-2.7- 18 proteins

▪Elongation Factor-2	Th1
▪P45	T-cell St
▪ADHT	Th1
▪TPR	Th1
▪Aldolase	VC, DT
▪Enolase	IGP
▪TPI	VF, IGP
▪PDI	VF, DT, VC
▪ Calreticulin	VF, IGP
▪Co-factor IPG mutase	DT
▪NAD deacetylase SIR2 homolog	DT
▪Heat shock protein-70	Th1
▪Heat shock protein-83	Th1
▪ 5 Hypothetical Proteins	?

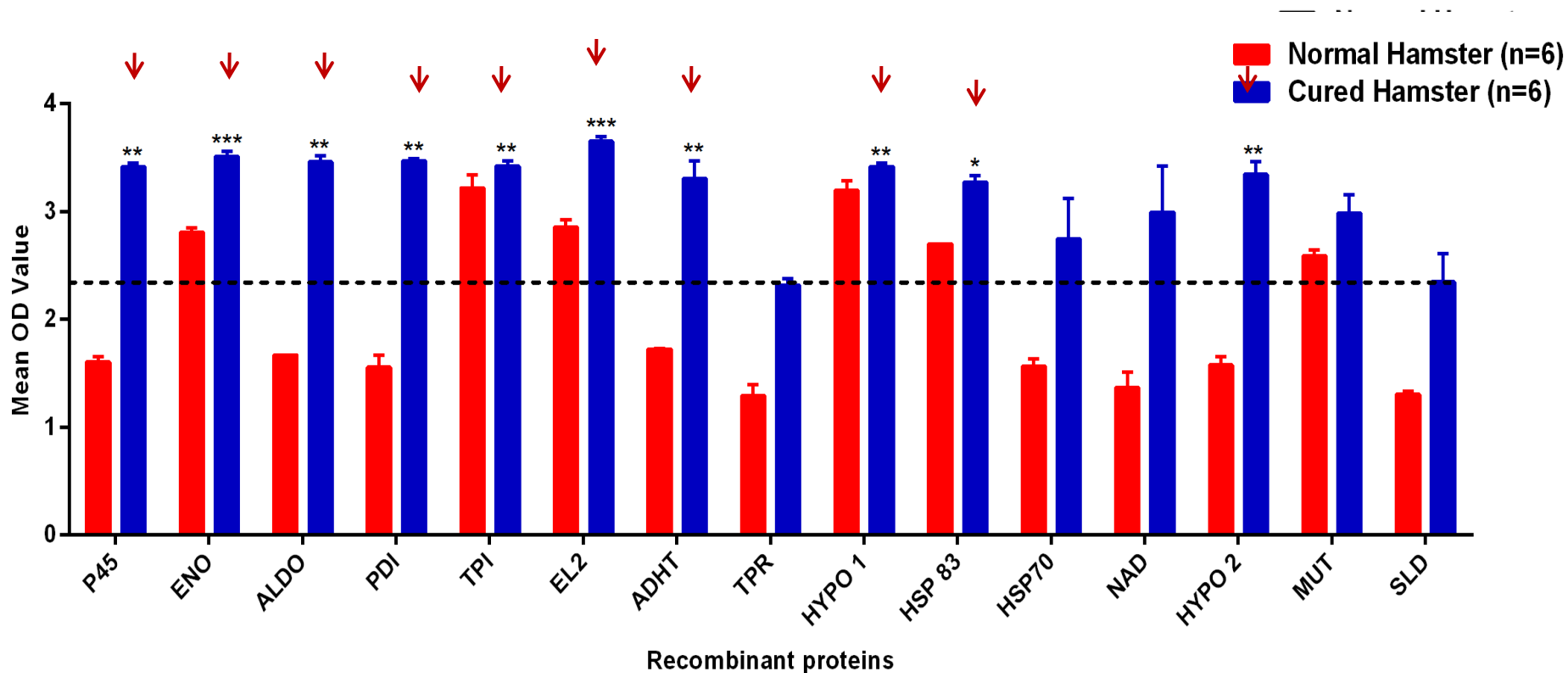
Recombinant Th1 stimulatory proteins of *L.donovani*

- 15/18 proteins developed as recombinant proteins
- Characterized for their potential as vaccine targets

S. No	Th1 stimulatory proteins	Accession No	Size bp	ClustalW	
				<i>L.major</i>	<i>L.infantum</i>
1	Calreticulin	EU723848	873	94	99
2	Protein Disulfide Isomerase	EU723849	1434	94	99
3	Enolase	EU723850	1290	97	99
4	p45	EU723851	1086	97	99
5	Triose phosphate isomerase	EU867389	759	93	99
6	Elongation factor-2	EU929069	1932	99	99
7	Aldolase	GQ 220750	1115	98	98
8	Trypanothione reductase	JN007485	1476	79	80
9	Adenosyl homocysteinase	GU353334	1314	89	90
10	Cofactor-independent phosphoglycerate mutase	HM044147	1659	93	94
11	NAD-dependent deacetylase SIR2 homolog	JN790591	1122	95	97
12	HSP70-related protein1 precursor	HQ011382	1548	97	97
13	HSP- 83	HQ230577	2109	90	90
14	Hypothetical protein # 1/5	JF957190	1254	88	89
15	Hypothetical protein # 2/5	JX855827	4074	88	89

Immunogenicity of recombinant proteins using lymphocytes of cured Leishmania infected hamsters

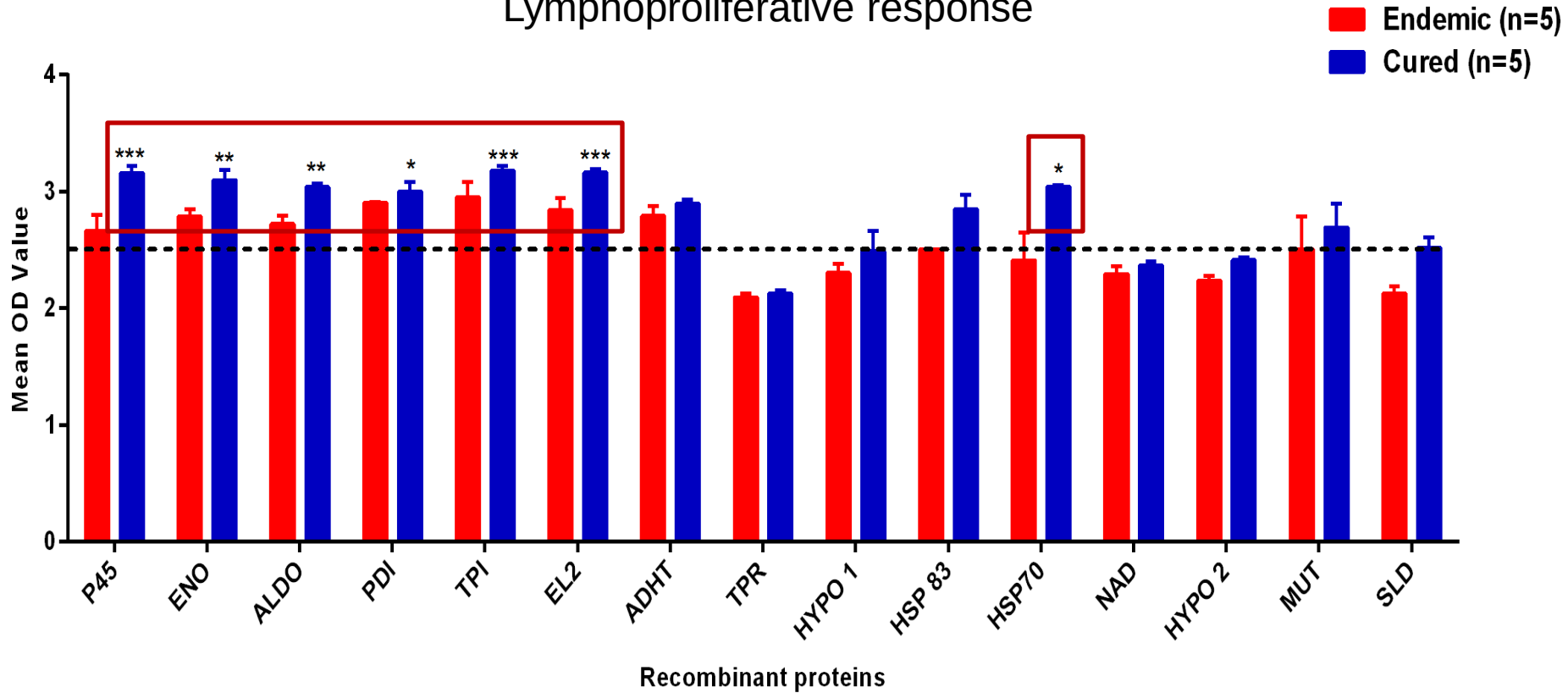
Lymphoproliferative response



10 ug/ml
mean $\pm$ SEM of triplicate wells.

Immunogenicity of recombinant proteins using PBMCs of cured Leishmania patients

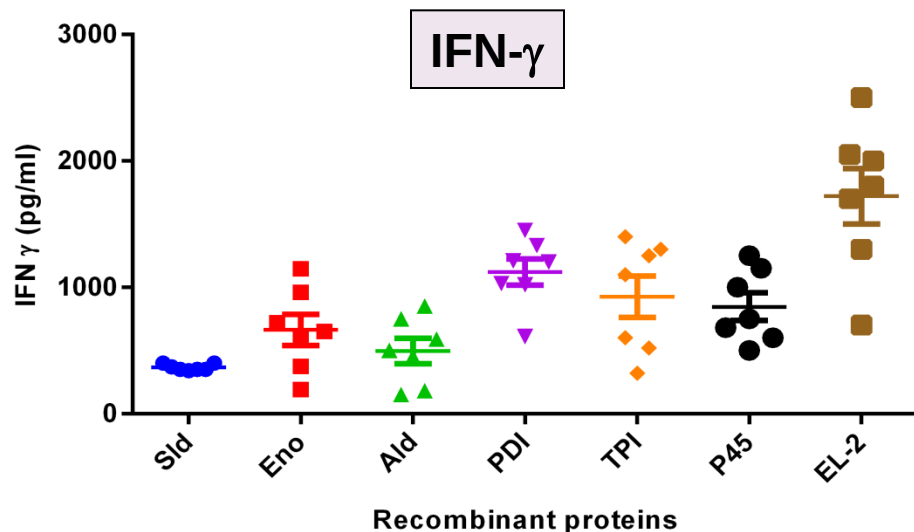
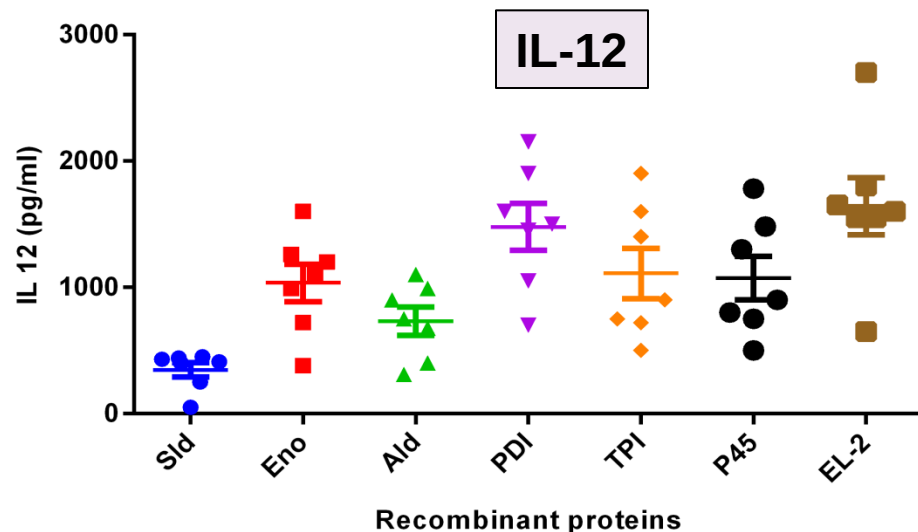
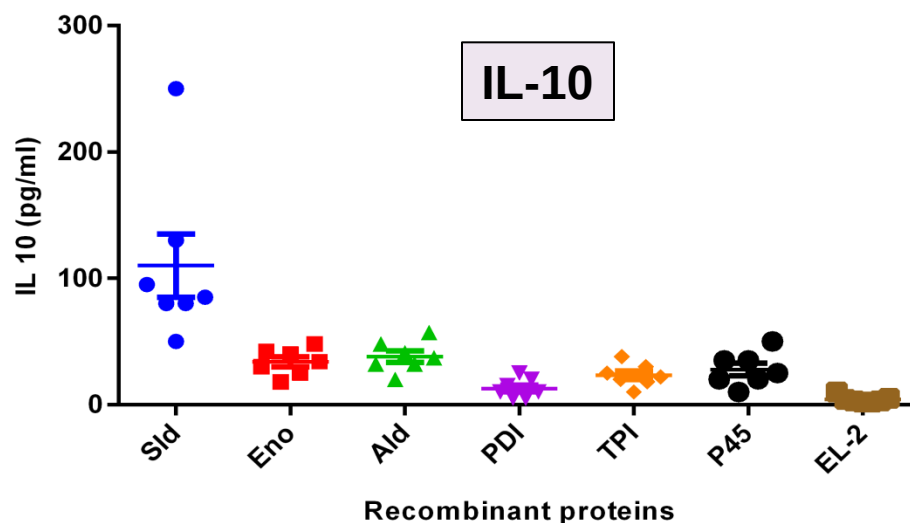
Lymphoproliferative response



10 ug/ml
 mean $\pm$ SEM of triplicate wells.

Immunogenicity of recombinant proteins using PBMCs of cured Leishmania patients

Th1/Th2 cytokines



J Immunol (2011); PLoS One (2012 a&b); IJP (2012); PLoS One (2014)

Prophylactic efficacy of recombinant proteins

Recombinant proteins	Prophylactic Efficacy (on day 120 p.c.)
Elongation factor-2	~ 75%
Protein disulfide isomerase	~85%
Triose phosphate isomerase	~90%
Enolase	~90%
Aldolase	~ 65%
p45	~85%

mRNA cytokine expression:

iNOS, IFN- γ , TNF- α , and IL-12 $\uparrow$

TGF- β and IL- 4. $\downarrow$

Development of epitope based synthetic/chimeric vaccine

As a logical progression Synthetic PolyPeptide candidate vaccines are being explored

- **Key step in vaccine designing and with emergence of immunoinformatics - identification of most appropriate vaccine candidate-protein → peptide**
- **Promiscuous T-cell epitopes - from potential Th1 stimulatory proteins of *L. donovani* which expands host protective immune responses.**
- **Selection of Peptides based on HLA polymorphism of target population in endemic regions of Bihar and adjoining areas**

In Silico Identification And Synthesis Of Antigenic Peptides

IEDB and SYFPEITHI database for T-cell epitope prediction

15-mer peptides of Eight common MHC- II HLA alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, DRB1*1501)

BlastP search to identify identity with human protein ; peptides showing >85% identity - **X**

S.No	Position	LdAld Epitopes
1.	179-193	TLARYAILSQISGLV
2.	180-194	LARYAILSQISGLVP
3.	137-151	LDGYVKRASAYYKKG
4.	1-15	MSRVTIFQSQLPACN
5.	261-275	YTVMTLARTMPAML
6.	263-277	VMTLARTMPAMLP

S.No	Position	LdEno Epitopes
7	1-15	MPIRKVYAREVLDSR
8	160-174	VLPFQEFMIAPTKAT
9	188-202	HALKVIKSKYGQDA
10	128-142	LYRYIAGLAGTKDIR
11	184-198	SEVYHALKVIKSKY
12	336-350	ACNSLLLKINQIGTI

S.No	Position	LdTPI Epitopes
13	204-218	AARLRILYGGSVSAG
14	201-215	ADVAARLRILYGGSV
15	45-59	TFVHIPLVQAKLRNP
16	138-152	NQTAKVVLSTSAIA
17	22-36	IEKLVQVLNEHNISH
18	43-57	APTFVHIPLVQAKLR

S. No.	Position	LdPDI Epitopes
19.	85-99	KYQIKGFPTLYIFRN
20.	311-325	HHVMETYPVTAESV
21.	212-236	AESVKRFLATAVL
22.	174-188	NFVFVTDAISPND
23.	166-180	ADSLRTQMNFFVTD
24.	371-385	QNVMLLFYAPWCGHC

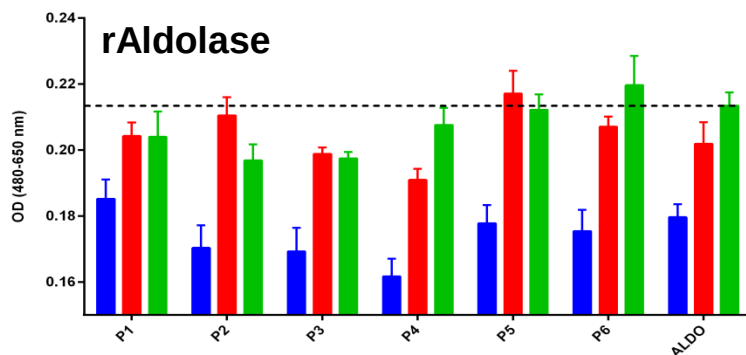
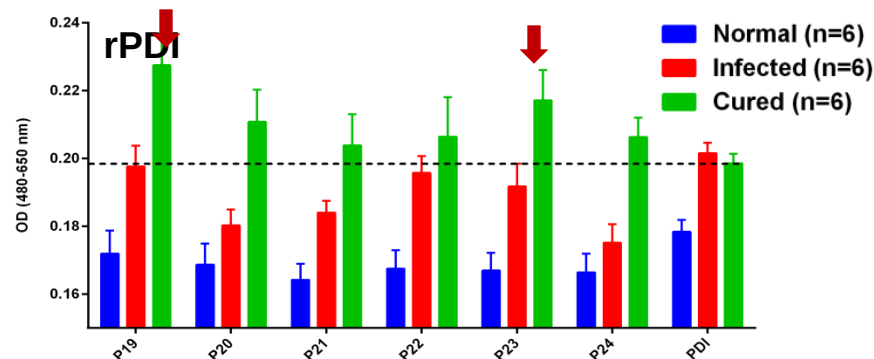
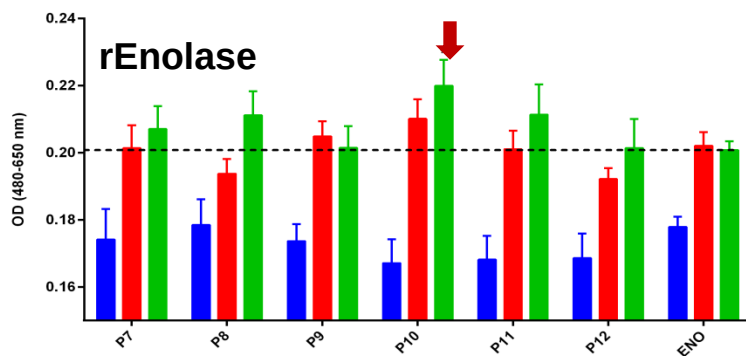
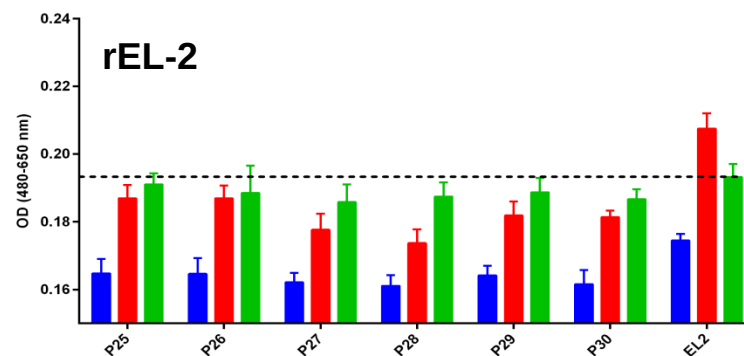
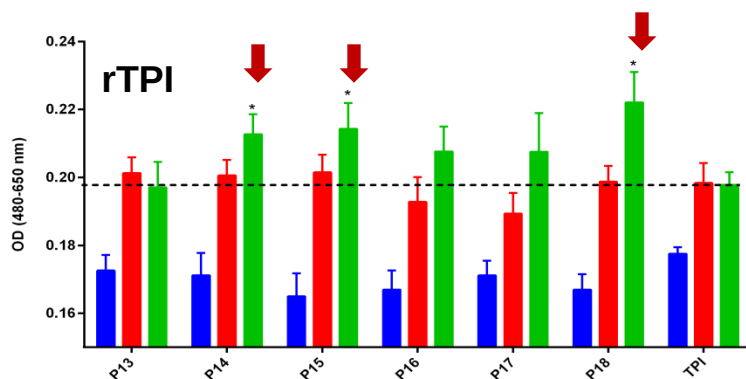
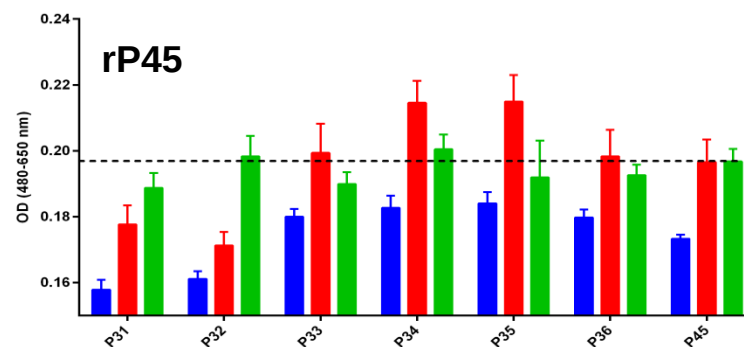
S. No.	Position	LdEIF2 Epitopes
25.	15-29	AFSLTRFANMYAAKF
26.	566-580	IYNVRAYLPVAESFG
27.	189-203	RGRFFAFGRIFSGKV
28.	2-16	KGTVAIGSGLQAWAF
29.	75-89	DPIYQIFDAVMNEKK
30.	117-131	KTVMMKFLPAAETLL

S.No	Position	Ldp45 Epitopes
31.	107-221	GIHVDGYCAVVAHTI
32.	150-164	LRQMRPGATIYQVTD
33.	172-186	HYKVTPVDGVLSHMM
34.	306-320	GEVVAHFKITVLISN
35.	320-334	NKKIEPITGLKPQKA
36.	214-228	KAQVWTLDIVMTSGK

EVALUATION OF IMMUNOGENICITY OF SYNTHETIC PEPTIDES- Lymphoproliferative response

HAMSTERS- Lymphocytes

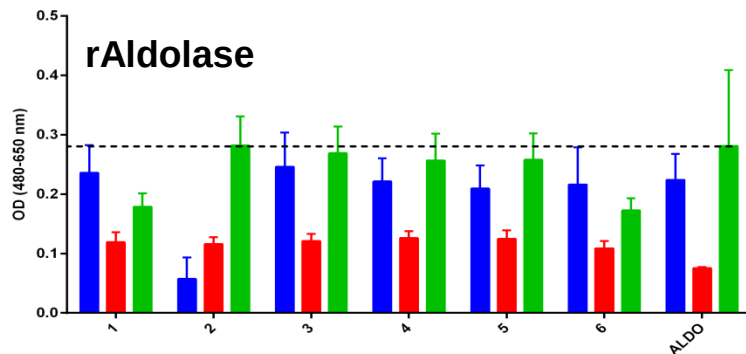
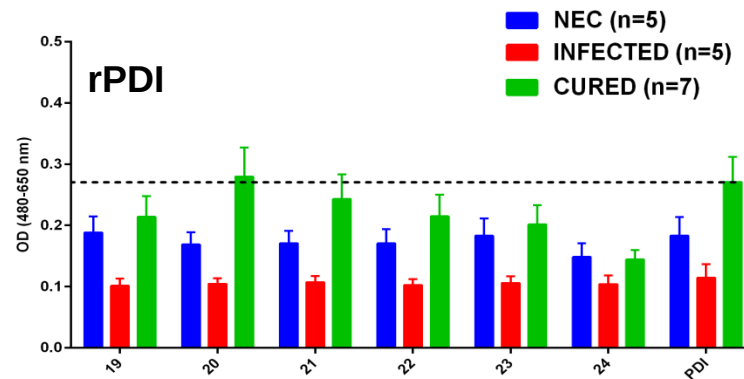
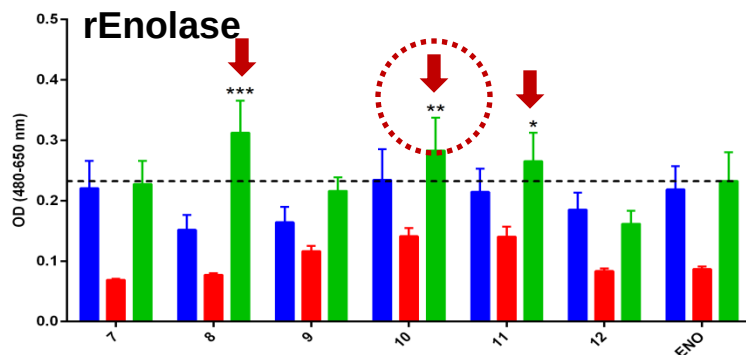
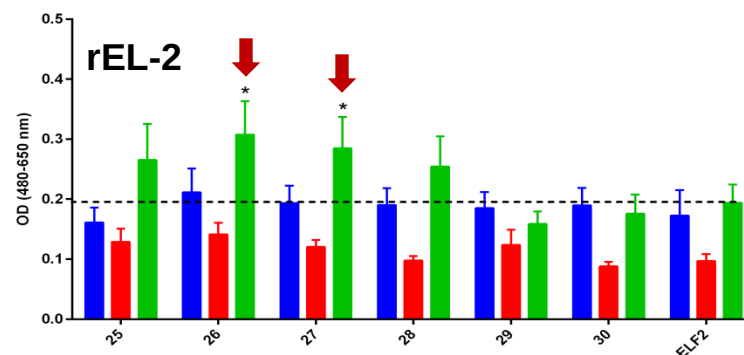
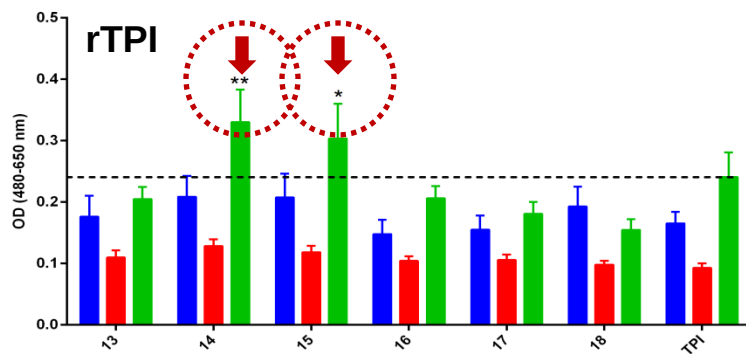
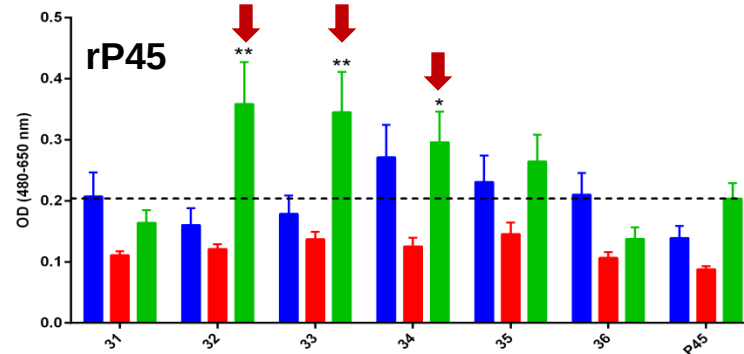
rprotein/peptide:1 ng/ml



EVALUATION OF IMMUNOGENICITY OF SYNTHETIC PEPTIDES- Lymphoproliferative response

HUMAN PATIENTS- PBMCs

rprotein/peptide:1 ng/ml



Progress towards Synthetic PolyPeptide vaccine...



Development of epitope based synthetic/chimeric vaccine: Prophylactic Evaluation of Potential peptides in hamsters against *L.donovani* challenge.. underway....

Evaluation as Therapeutic vaccine ?...



Therapeutic potential of Th1 stimulatory proteins/peptides



Expressing Th1 stimulatory proteins/peptides in non-pathogenic *Leishmania* strain, *L. tarentolae*, to exploit as a live mutant vaccine

Indian VL specific vaccine ?...

Strains of *L. donovani* in East Africa and *L. infantum* in the Mediterranean Basin are markedly heterogeneous and genetically different from Indian strains.

Abass et al 2015 PLoS one

Is a VL vaccine needed if elimination targets can be met with current control programs?

A safe and effective vaccine will be critical if success of recent VL control efforts are to be sustained.

Engwerda & Matlashewski (TRSTMH, 2015)

This is an indigenous effort for development of polyprotein/polytope vaccine from Indian clinical isolate which carries promise for a large number of people suffering from kala azar

This new vaccine approach to treat VL expands host protective immune responses, making host to produce beneficial T-cells that alleviate disease symptoms more quickly.

ACKNOWLEDGEMENTS

❖ Collaborators:

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- Prof. Sita Naik, SGPGI, LKO
- Dr Amogh Sahasrabuddhe, CDRI, LKO
- Dr W Haq, CDRI, LKO
- Dr Imran Siddiqi, CDRI, LKO

❖ Research Students:

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Reema Gupta, P Khare, R Tandon, R Baharia, A Jaiswal,
- Sumit Joshi, Narendra Yadav, Keerti Rawat, Snehapriya

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