Development of genetically modified live attenuated parasites as potential vaccines against visceral leishmaniasis

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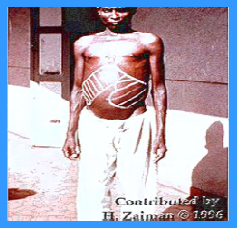
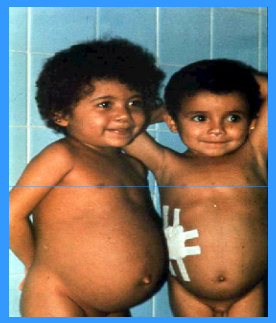
Impact of Visceral Leishmaniasis

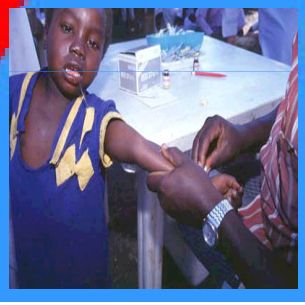


L. infantum

L. chagasi

L. donovani





VL Fact sheet

- Post Kala-azar Dermal Leishmaniasis.

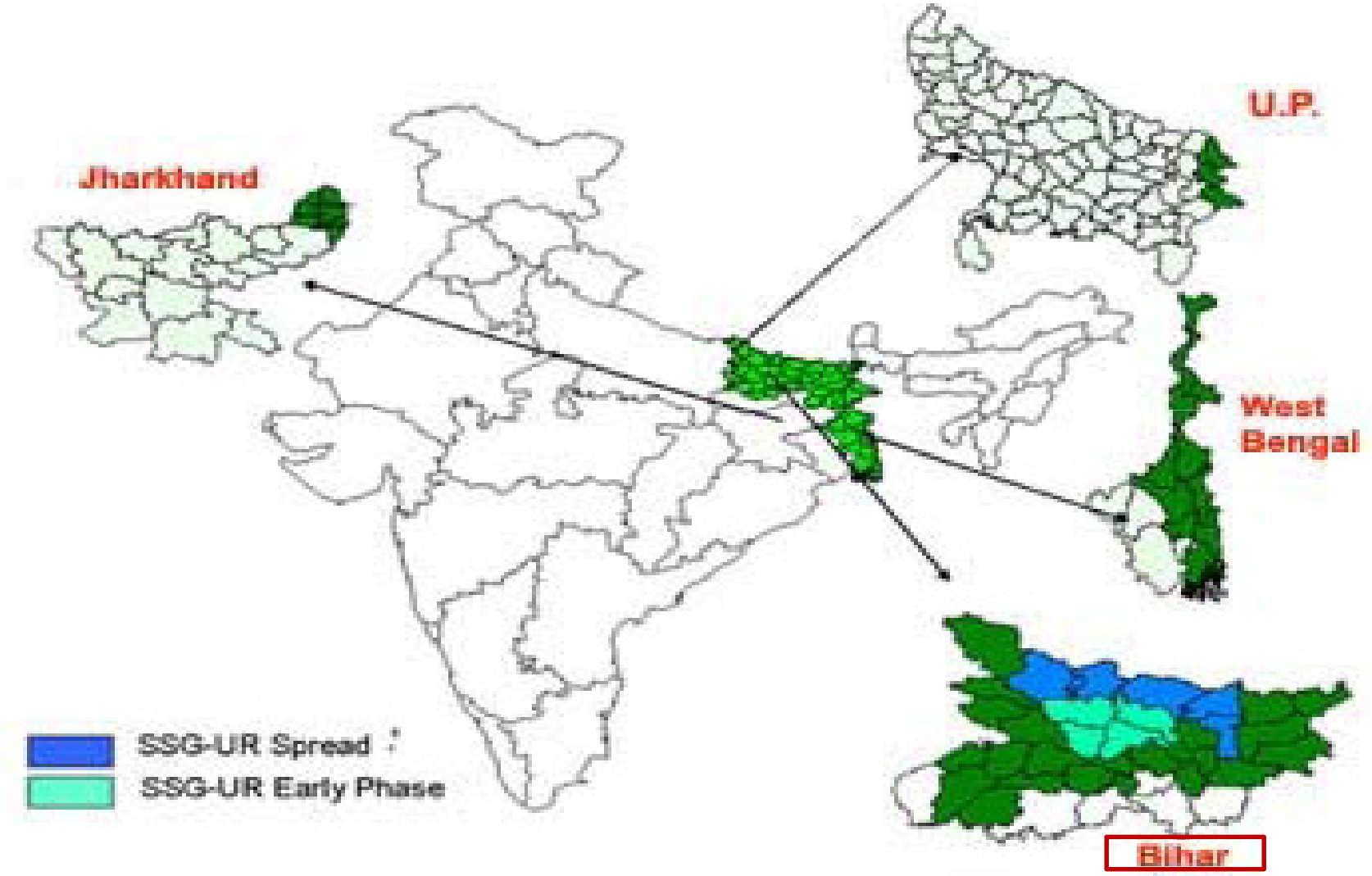
200 Million People currently at risk for contracting VL

62 Countries endemic for VL
400,000 New cases per year

1.5-9% cases of AIDS with VL

90% of VL cases are from India, Sudan, Nepal, Bangladesh and Brazil

VL endemic areas in India



165 million people at risk reports from other states

Need for vaccine against VL

- No effective vaccine is available for VL

- Drugs are costly, limited, toxic and associated with high relapse rate. Development of drug resistant parasite due to prolonged use.

Strategies for vaccine development

- Whole cell lysates/enriched fractions
- Attenuation through long term culture
- Irradiated parasites
- Killed parasites + Adjuvants
- Recombinant and Synthetic antigens + Adjuvant
- DNA vaccines and CpG ODNs
- Leishmanization

Outcome

- Effective in animal models, little protection in humans
- Degree of effectiveness correlated with combined effect of several different antigens
- Reversion and or loss of attenuation
- Parasite persistence may be required to maintain the immunological memory to prevent reinfection

Possible to achieve by live-attenuated parasite immunization that could persist w/o inducing disease

Leishmanization

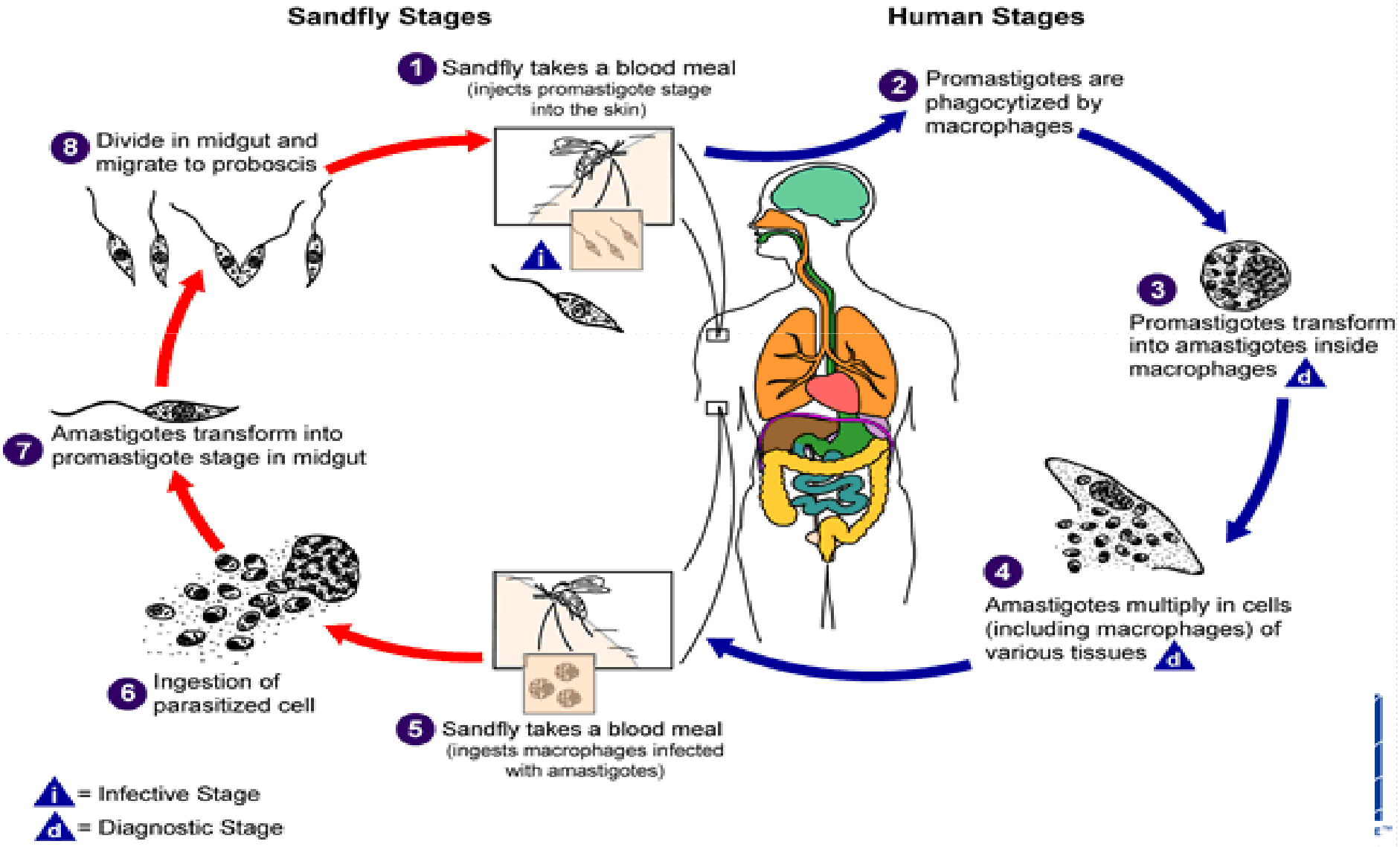
- Leishmanization experience in CL showed that some degree of parasite persistence may be required to maintain the immunological memory to prevent reinfection

- Possible to achieve by live-attenuated parasite immunization that could persist w/o inducing disease

- Persons once infected with *Leishmania*, after recovery are protected for life long.

Life cycle of *Leishmania*

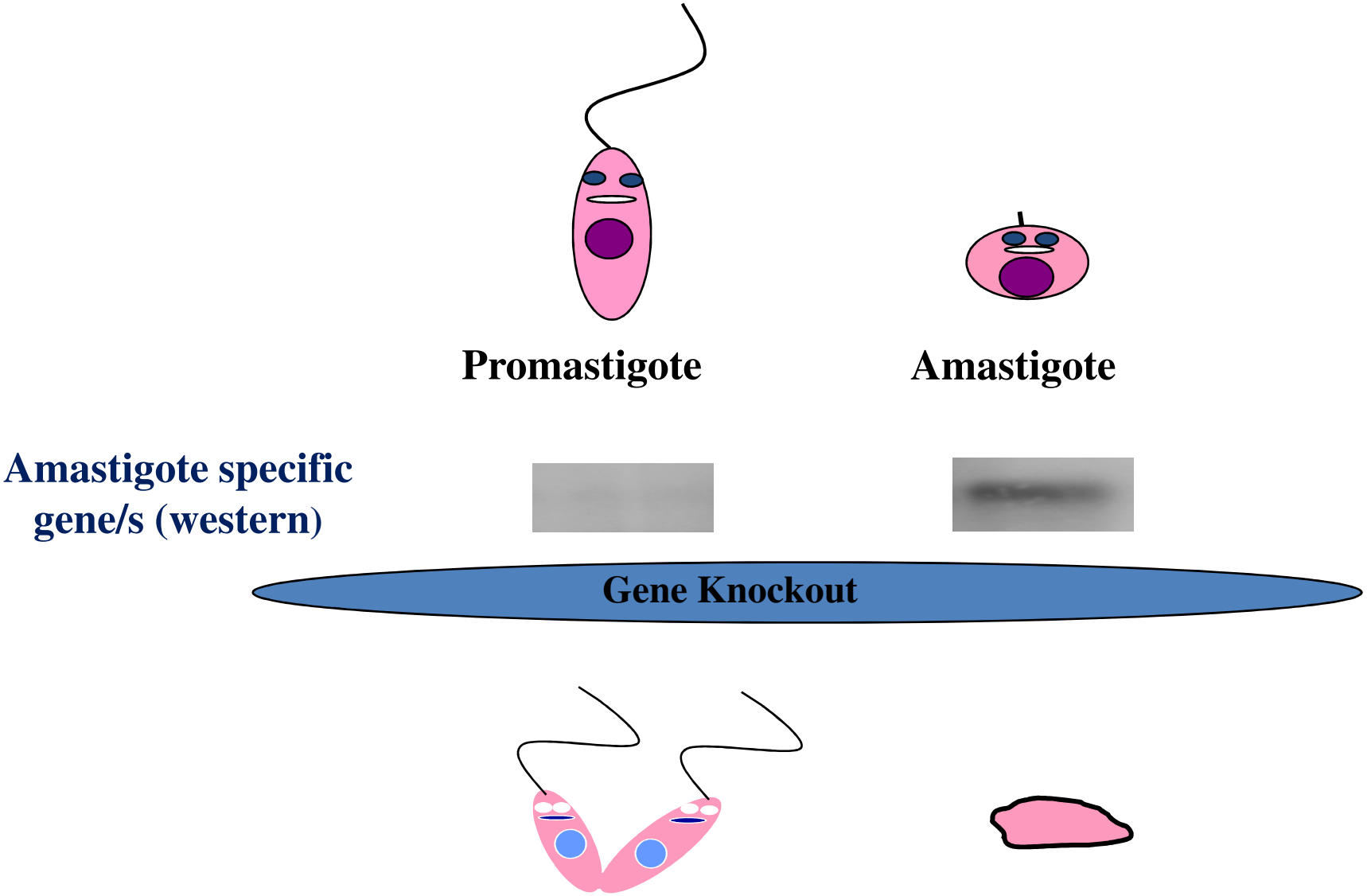
VL is caused by protozoan parasites *Leishmania donovani*.



A survey of gene knockout studies

Organism	Gene/s knocked out	Attenuation/ Protection
L. major	*dhfr-ts*	Mutant eliminated in mice and monkeys, No protection in monkeys.
L. major	*Galactofuranosyl* transferase (*lpg1)*	Did not infect sand fly, mouse or macrophages
L. mexicana	*Cysteine proteases*	Reduced infectivity in macrophages, Attenuated virulence in mice Protection in mice
L. donovani	Partial knockout of *A2-A2rel gene clusters*	Attenuated virulence in mice
L. donovani	*Biopterin transporter* (*BT1)*	Reduced infectivity, Cellular immunity -TH1 type response Provided protection upon challenge
L. donovani	p27	Reduced parasite survival in macrophage and mice

Amastigote specific genes are considered as potential virulence genes



Advantages of genetically defined live-attenuated vaccines

I. Immunogenicity:

- Mimic natural course of infection
- Provides complete array of antigens for presentation to immune cells
- Controlled persistence without causing disease allows generation of memory response

II. Safety:

- Genetically defined mutations
- Amenable to further manipulations
- Biomarkers of safety

Generation of genetically defined live-attenuated Parasites as vaccine candidates

Genes selected for deletion :

A. Growth regulating gene:- *Ld*Centrin1
B. Upregulated in amastigote stage:-

(1) *Ld*HP30b (2) *Ld*AKLP (3) *Ld*A1

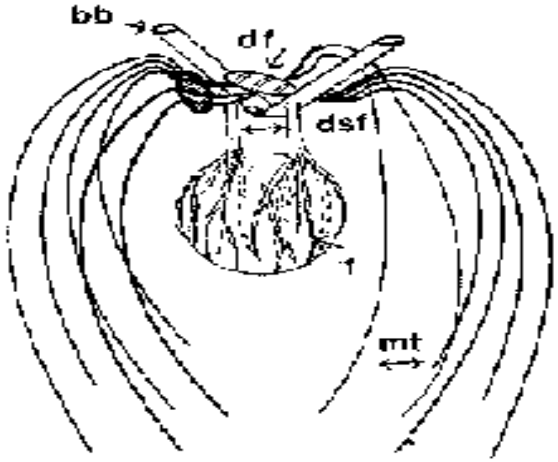
- Can be propagated in large quantity as promastigote. When differentiated into the amastigote in the mammalian host will have a limited capability to replicate and cause no pathology

- Limited replication should be sufficient to draw out a long lasting protective immune response in the host.

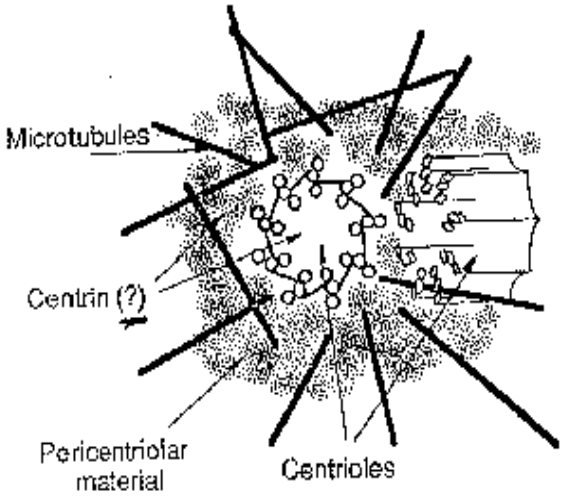
Leishmania donovani Centrin1 gene

- Annotated by TriTrypDB as: Centrin, putative,Ca2 -binding EF- hand protein
- Location in Chromosome: 36
- Predicted protein size: 17 kDa
- Motif(s) analysis through NCBI: $Ca2^{+}$ binding sites

Centrin and its functions



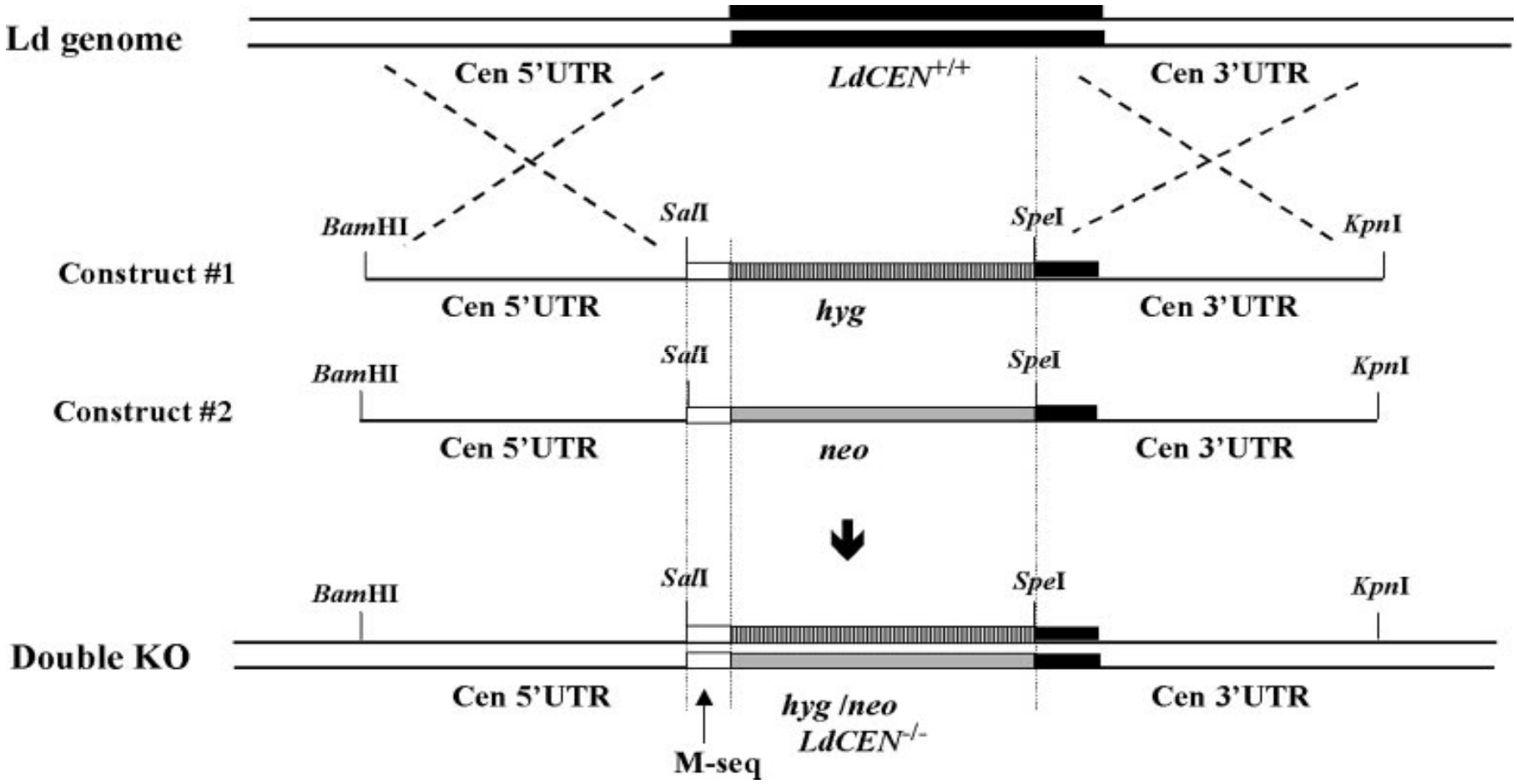
Unicellular Organisms



Higher Eukaryotes

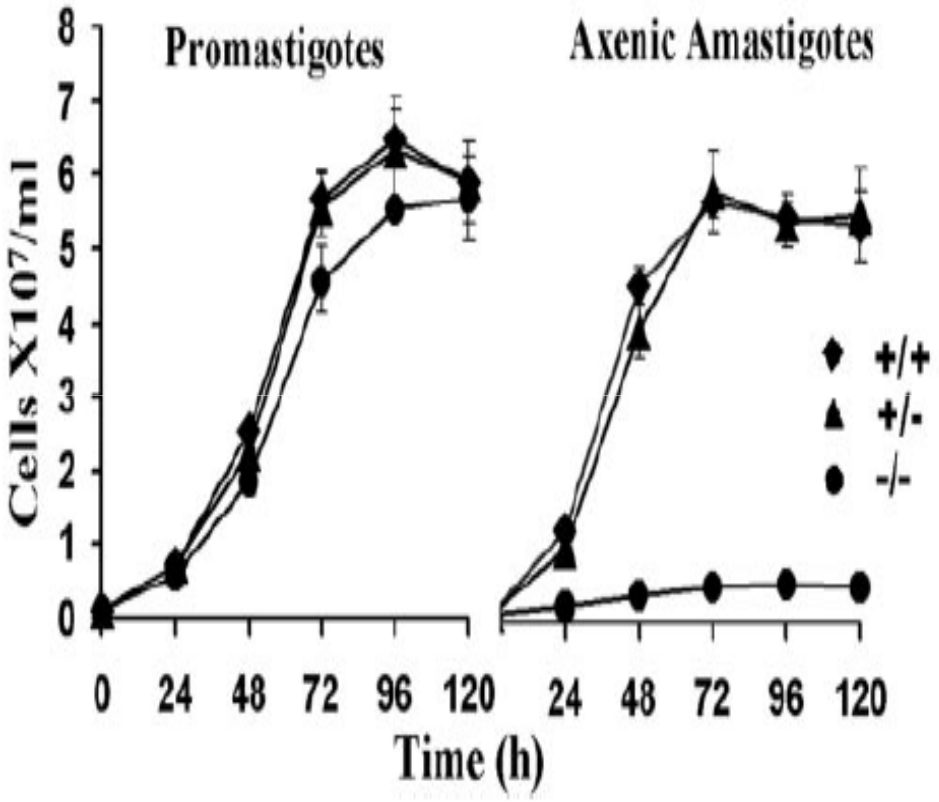
- Centrin is a calcium-binding cytoskeletal protein found in all eukaryotes.
- Localized mainly to centrosome or basal bodies,
- Participate in contraction of fibers, duplication and segregation of centrosome

*Ld*CEN gene disruption in *L. donovani* genome by homologous recombination



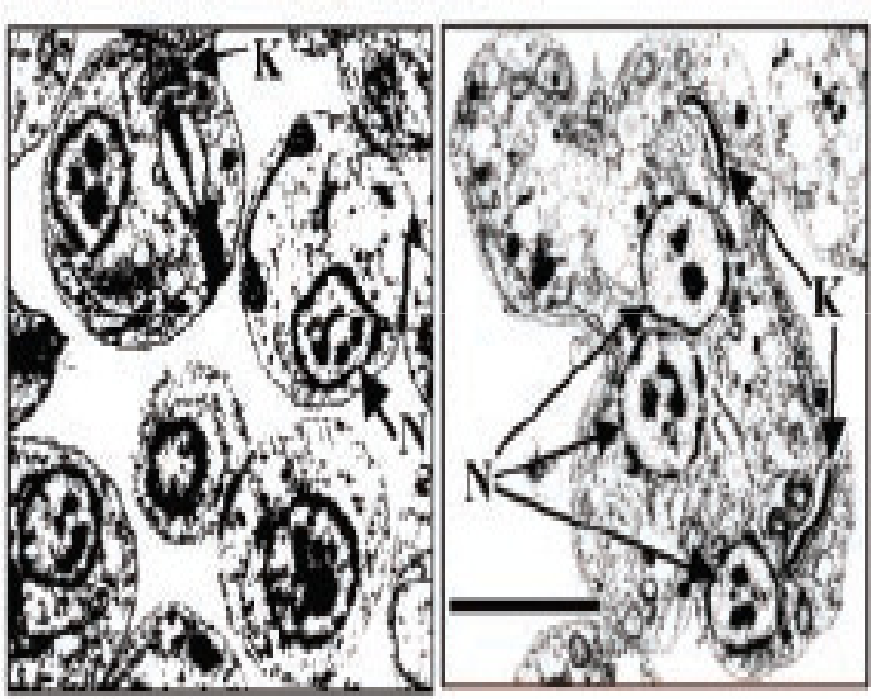
Design and use of constructs 1 and 2 with hyg and neo resistance genes, respectively, flanked on the 5' and 3' UTR

Centrin 1 gene deleted parasites (*LdCen1*$^{-/-}$) are specifically attenuated at amastigote stage



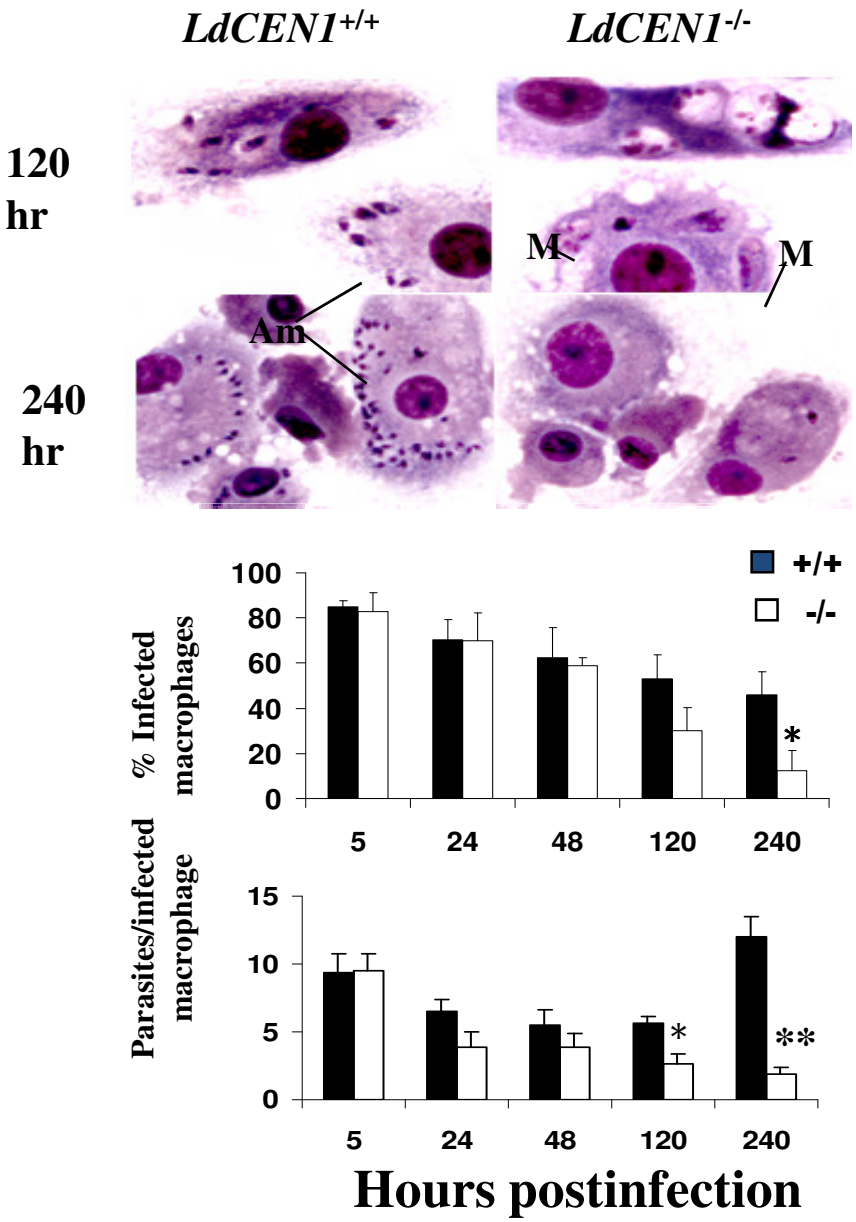
Centrin 1 gene deletion inhibit growth at amastigote stage

*Ld*Cen1$^{+/+}$ *Ld*Cen1$^{-/-}$



Cytokinesis arrest in *LdCen1*$^{-/-}$ results in large cell with multiple nuclei and kinetoplasts

Features of Centrin KO parasites



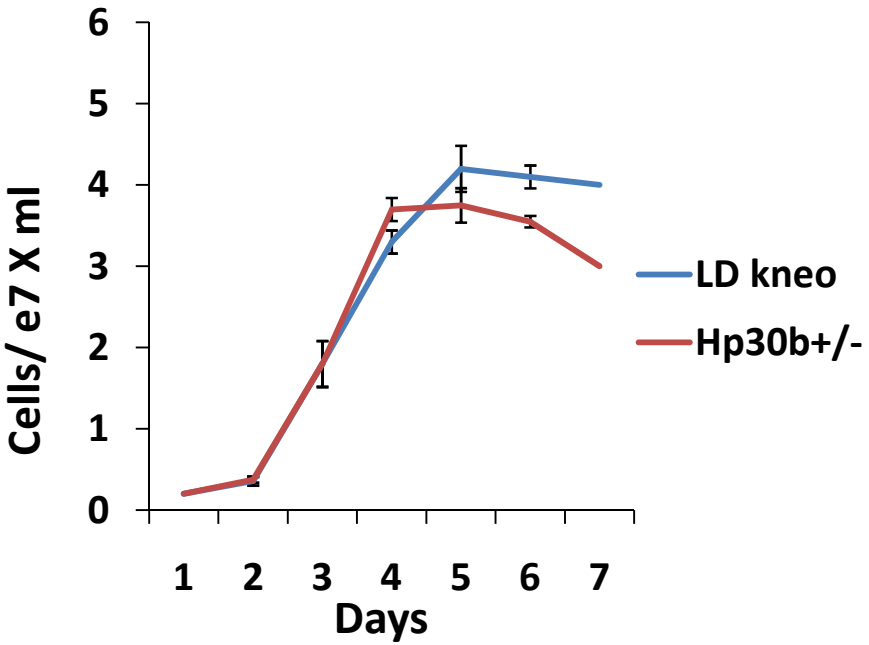
- Centrin-1 null mutants show growth arrest as amastigotes and not as promastigotes
- Centrin KO are growth arrested in the G2/M phase, have multiple nuclei and are prone to cell death.
- Basal body duplication does not occur in the amastigotes
- Centrin deleted amastigotes do not survive in the macrophages
- *Leishmania* centrin null mutants have a potential as live attenuated vaccine candidates.

*Ld*HP30b gene upregulated at amastigote stage

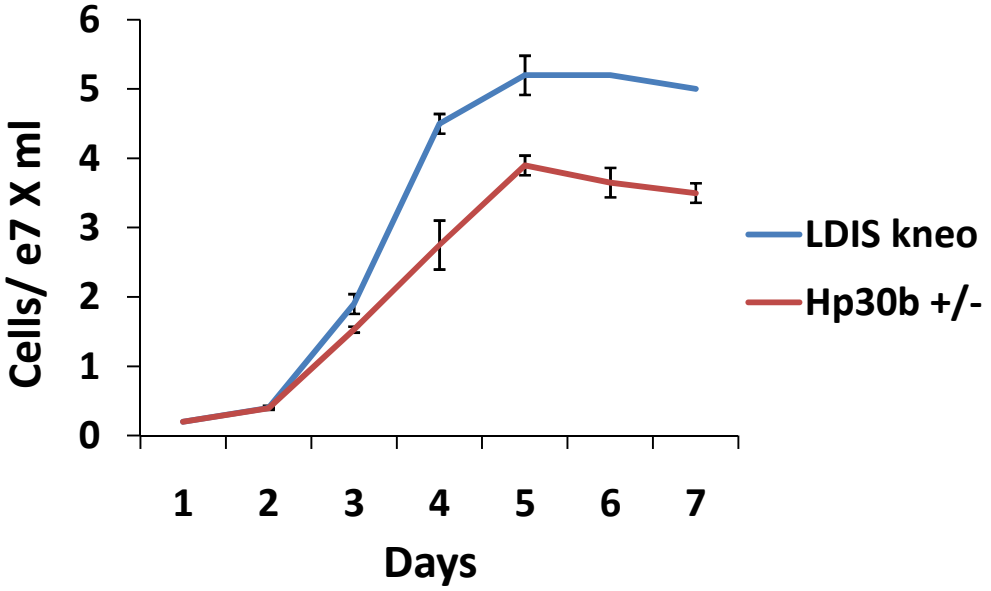
- Annotated by TriTrypDB as: hypothetical protein
- Location in Chromosome: 30
- Number of amino acids: 124
- Predicted protein size: 14 kDa
- Motif(s) analysis through NCBI: - no motif recognized
- Uniqueness: Homologs present in all Trypanosomatids

Growth of Ld-HP30b single allele deleted *L.donovani* parasites

Promastigotes



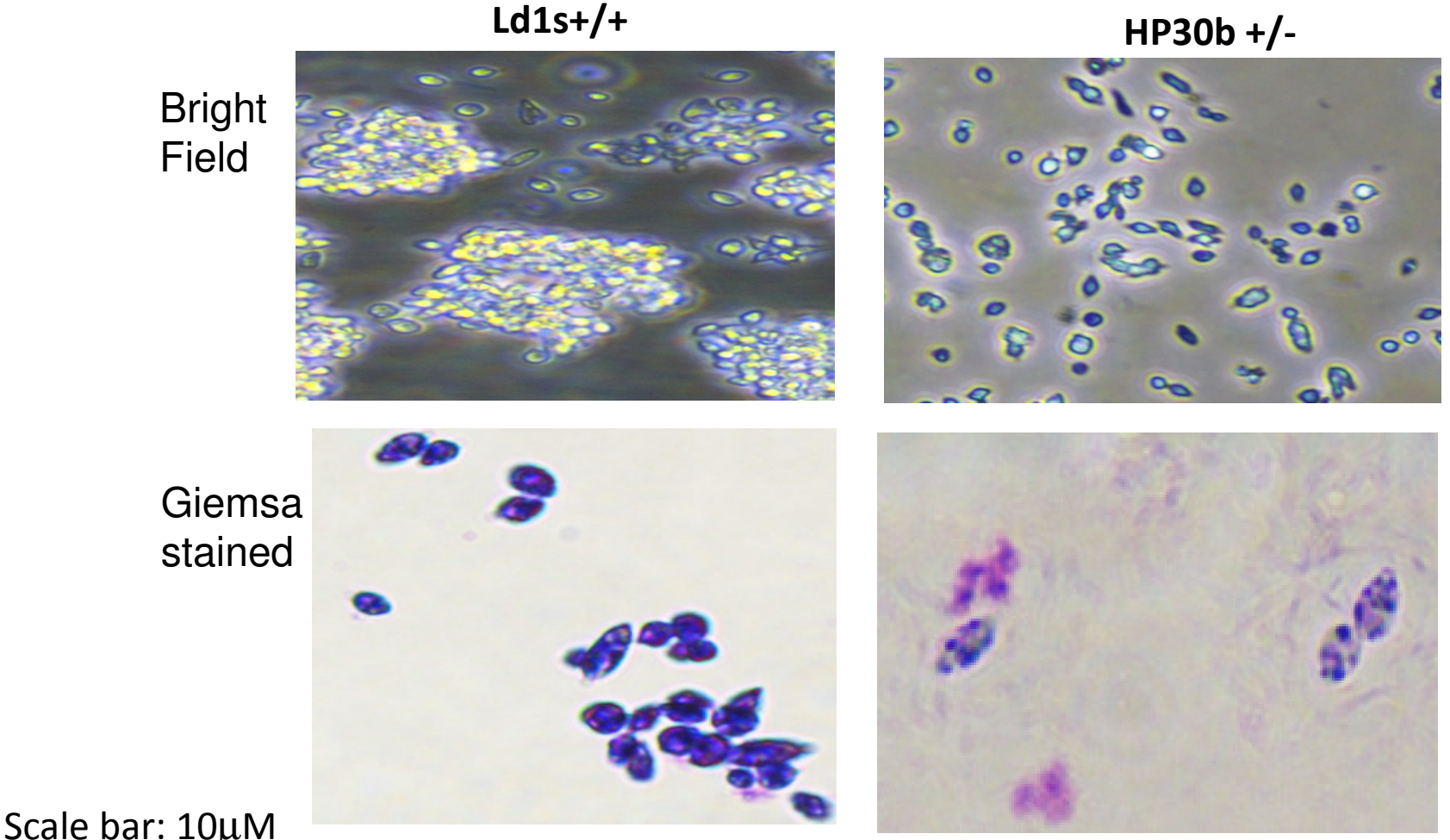
Axenic amastigotes



Growth curve of *Ld-H*P30b single allele deleted parasites

Ld-HP30b single allele deleted amastigotes showed attenuated growth, promastigotes growth not affected

Ld-HP30b single allele deletion gives multinucleated amastigotes



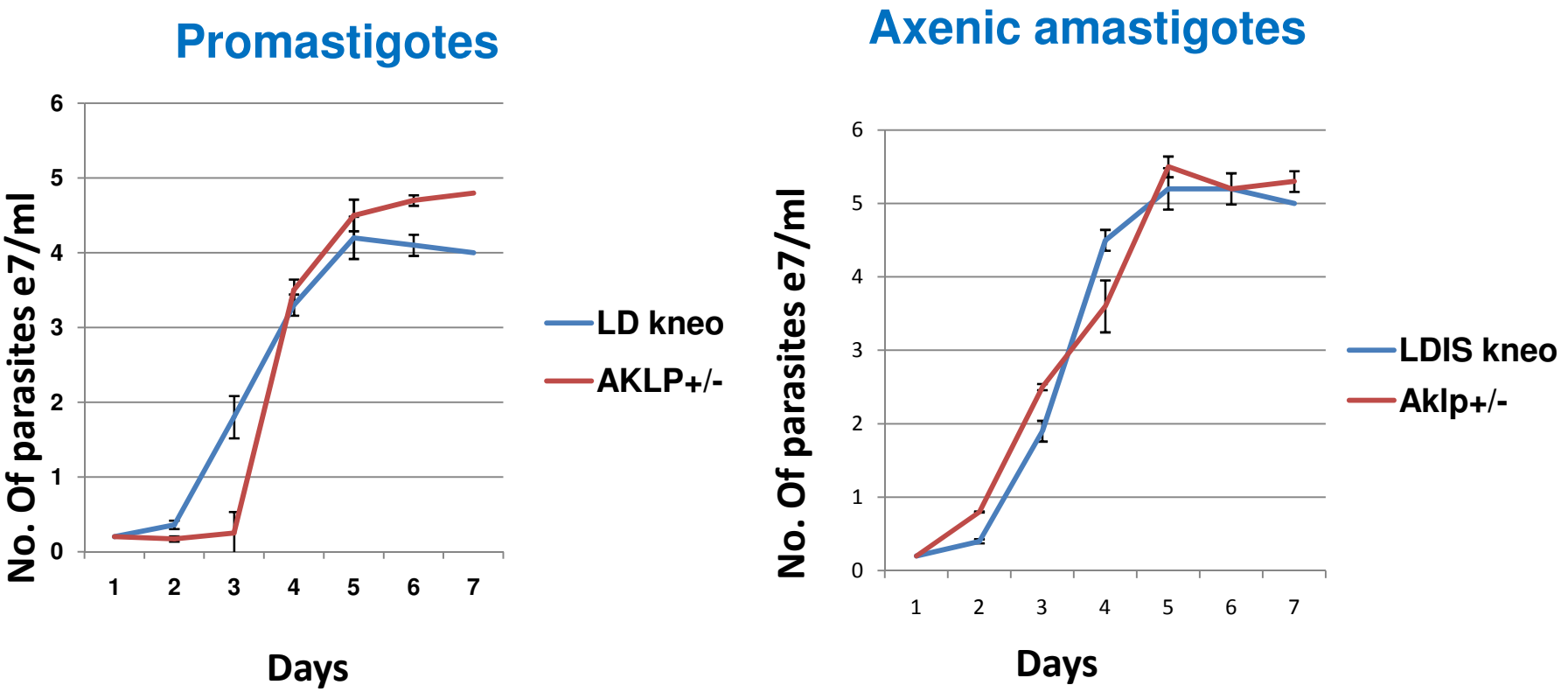
Microscopy of Ld-HP30b single allele deleted parasites

Multinucleated amastigotes confirm defect in cytokinesis

*Ld*AKLP gene over-expressed at amastigote stage

- Annotated by TriTrypDB as: Adenosine kinase-like protein
- Location in Chromosome: 30
- Number of amino acids: 214
- Predicted protein size: 24.4 kDa
- Motif(s) analysis through NCBI: Adenosine kinase. Catalyzes phosphorylation of ribofuranosyl
- Uniqueness: Homologs present only in *Leishmania* sp.

Growth of *Ld*-AKLP single allele deleted parasites



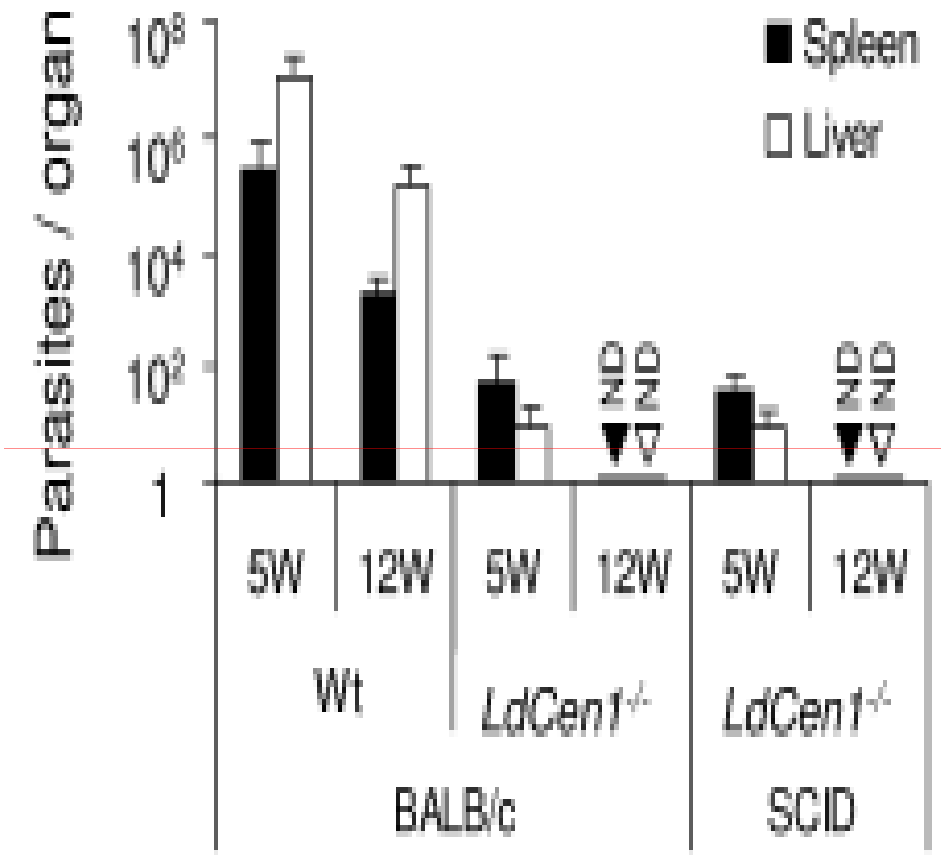
Growth curve of *Ld*-AKLP single allele deleted parasites

Ld-AKLP single allele deleted parasite show unchanged growth as promastigotes or as amastigotes

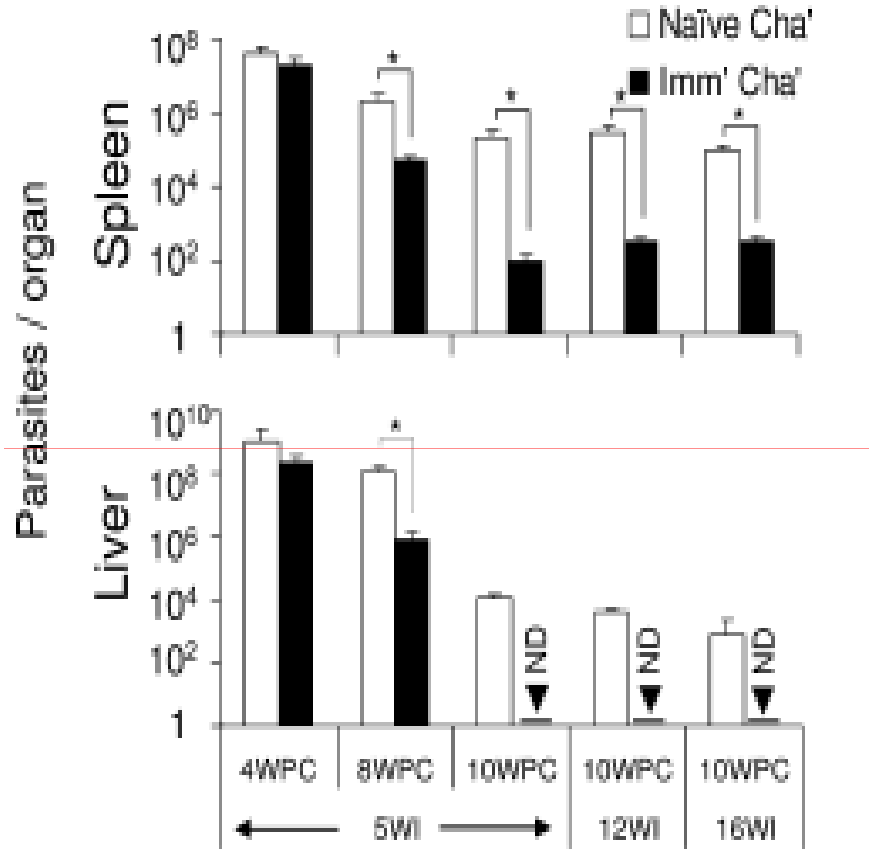
*Ld*A1gene upregulated at amastigote stage

- Annotated by TriTrypDB as: hypothetical protein
- Location in Chromosome: 29
- Number of amino acids: 175
- Predicted protein size: 20 kDa
- Motif(s) analysis through NCBI: no motif recognized
- Uniqueness: Homologs present only in *Leishmania* sp.

*Ld*Cen1$^{-/-}$ is safe and protective in mice model

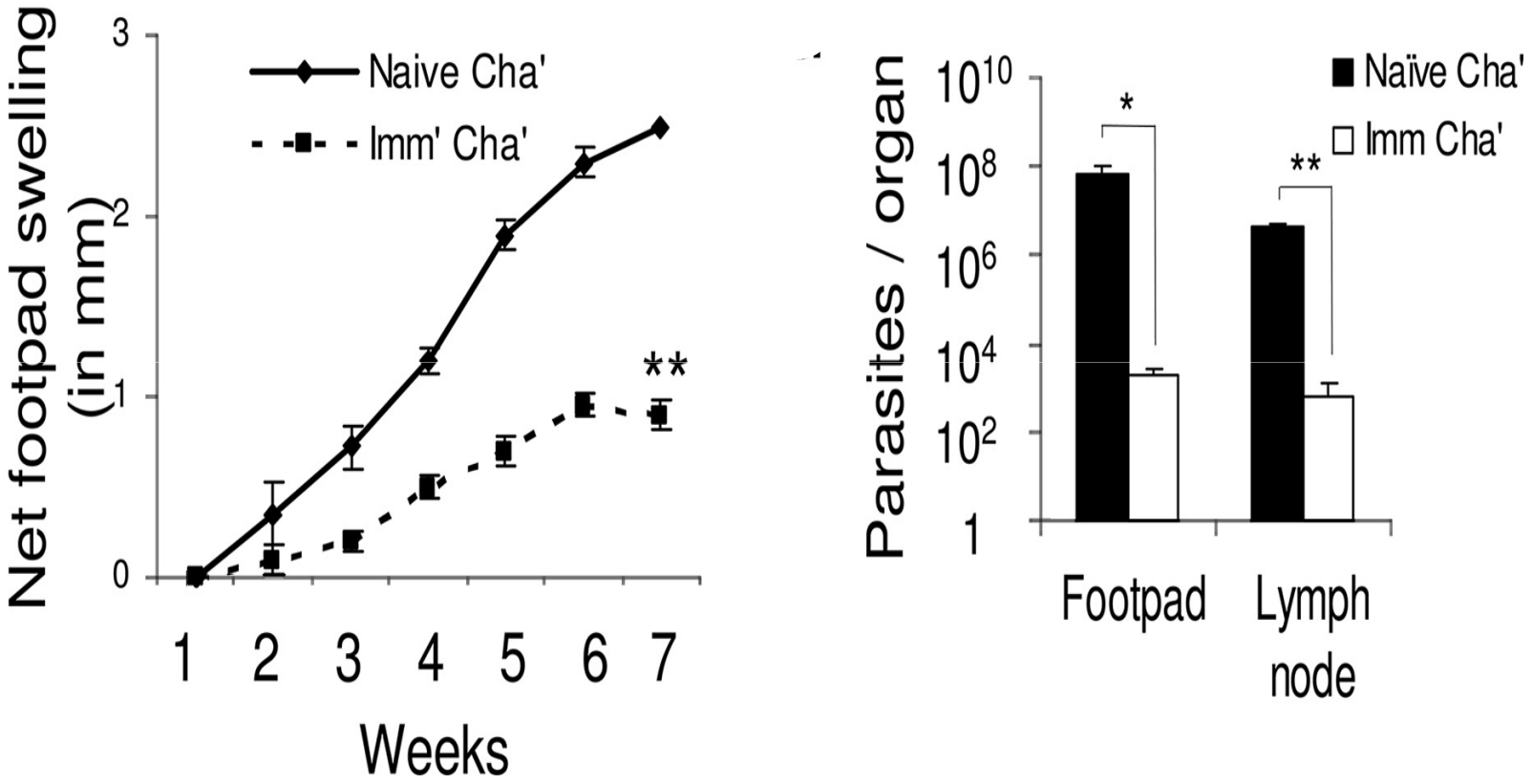


Avirulence and limited persistence of *Ld*Cen1$^{-/-}$ in BALB/c and SCID mice



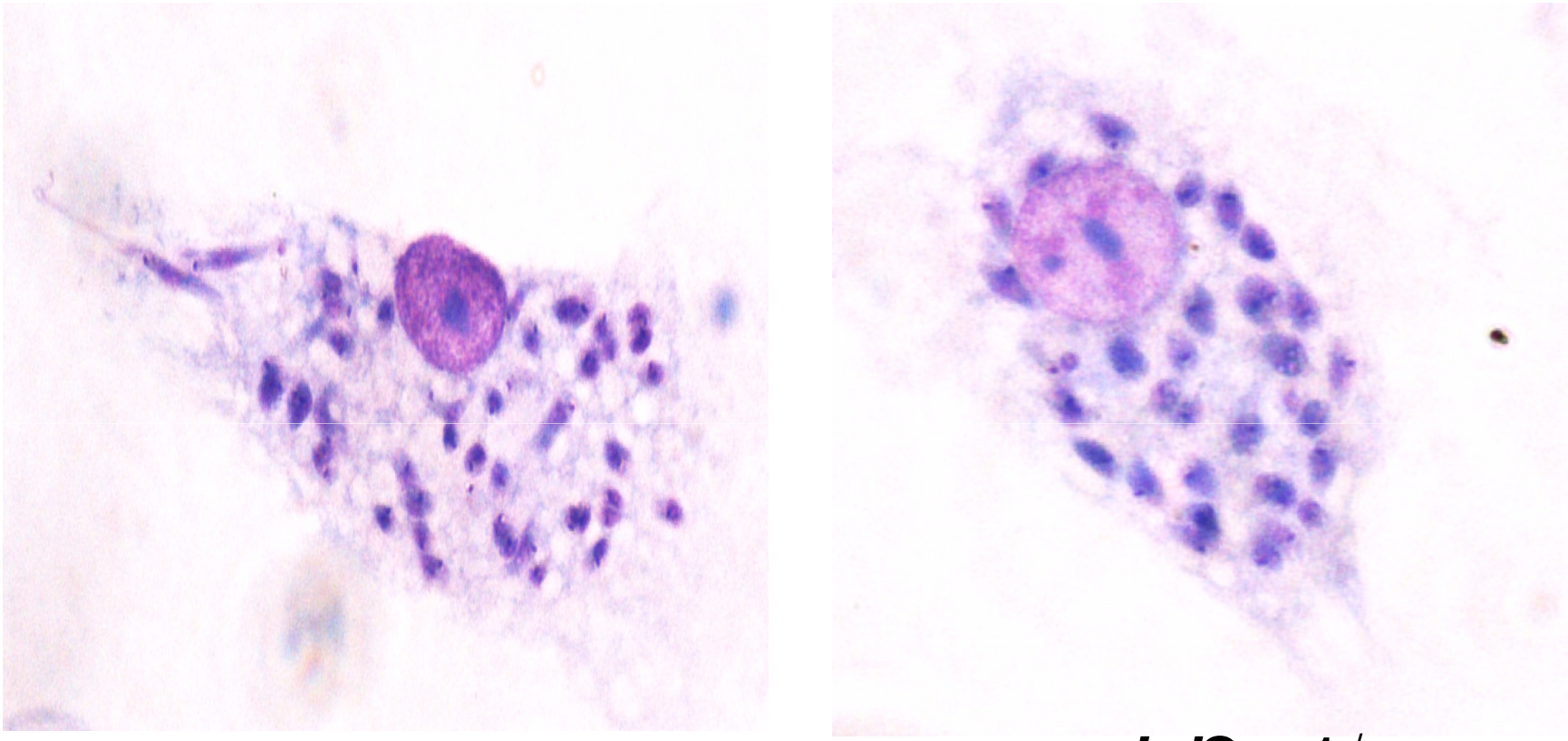
***Ld*Cen1$^{-/-}$ parasite protects mice against challenge**

*Ld*Cen1$^{-/-}$ cross-protects mice against other *Leishmania* species



Mice immunized with *Ld*Cen1$^{-/-}$ showed significant lower parasite burden against *L. brazilensis* challenge

*Ld*Cen1$^{-/-}$ parasites infect human macrophages similar to wild type

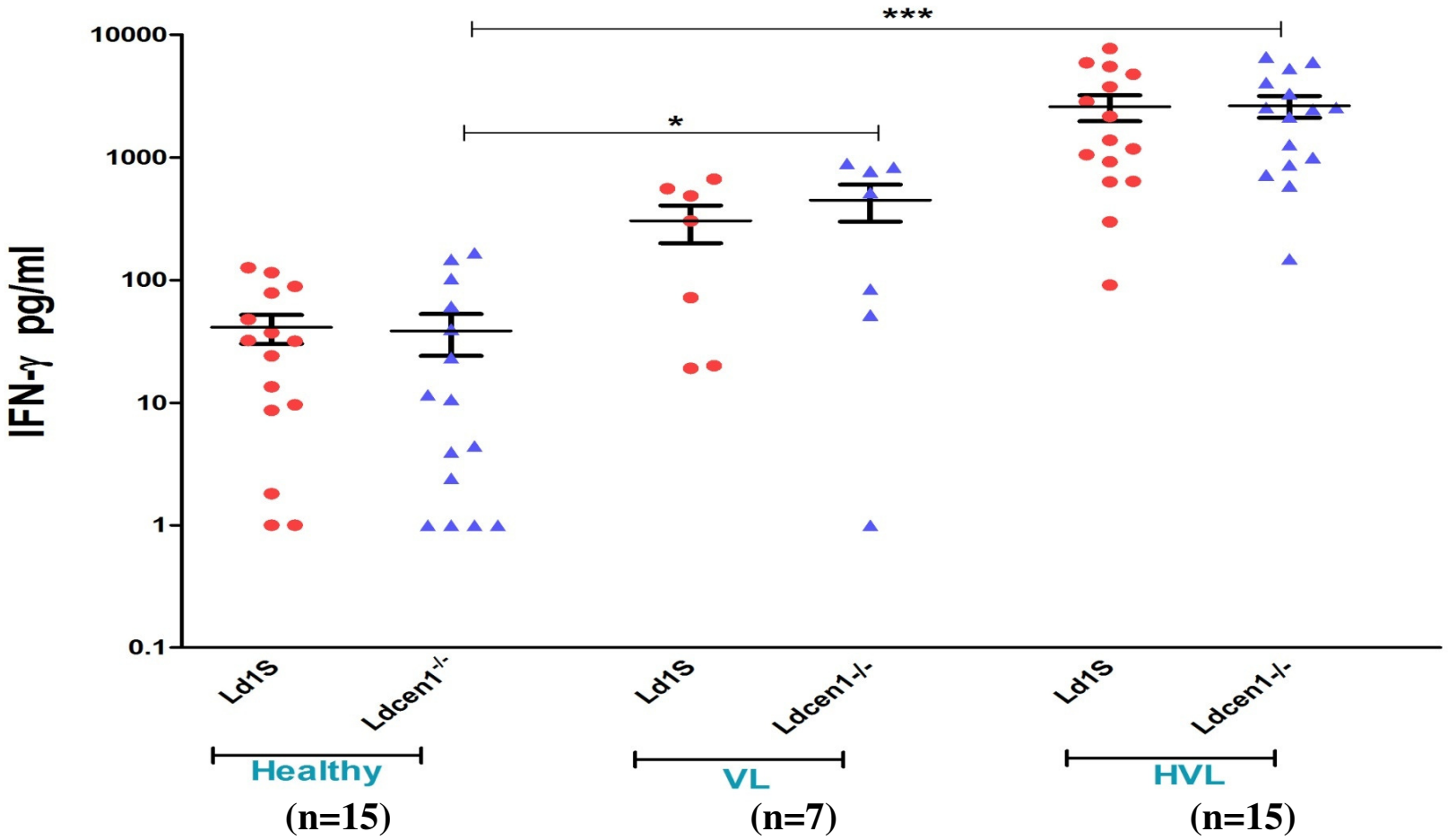


Wild type **LdCen1$^{-/-}$**

Infection of human PBMCs derived macrophages at 16 hr

Infectivity of macrophages was 70-80% with both

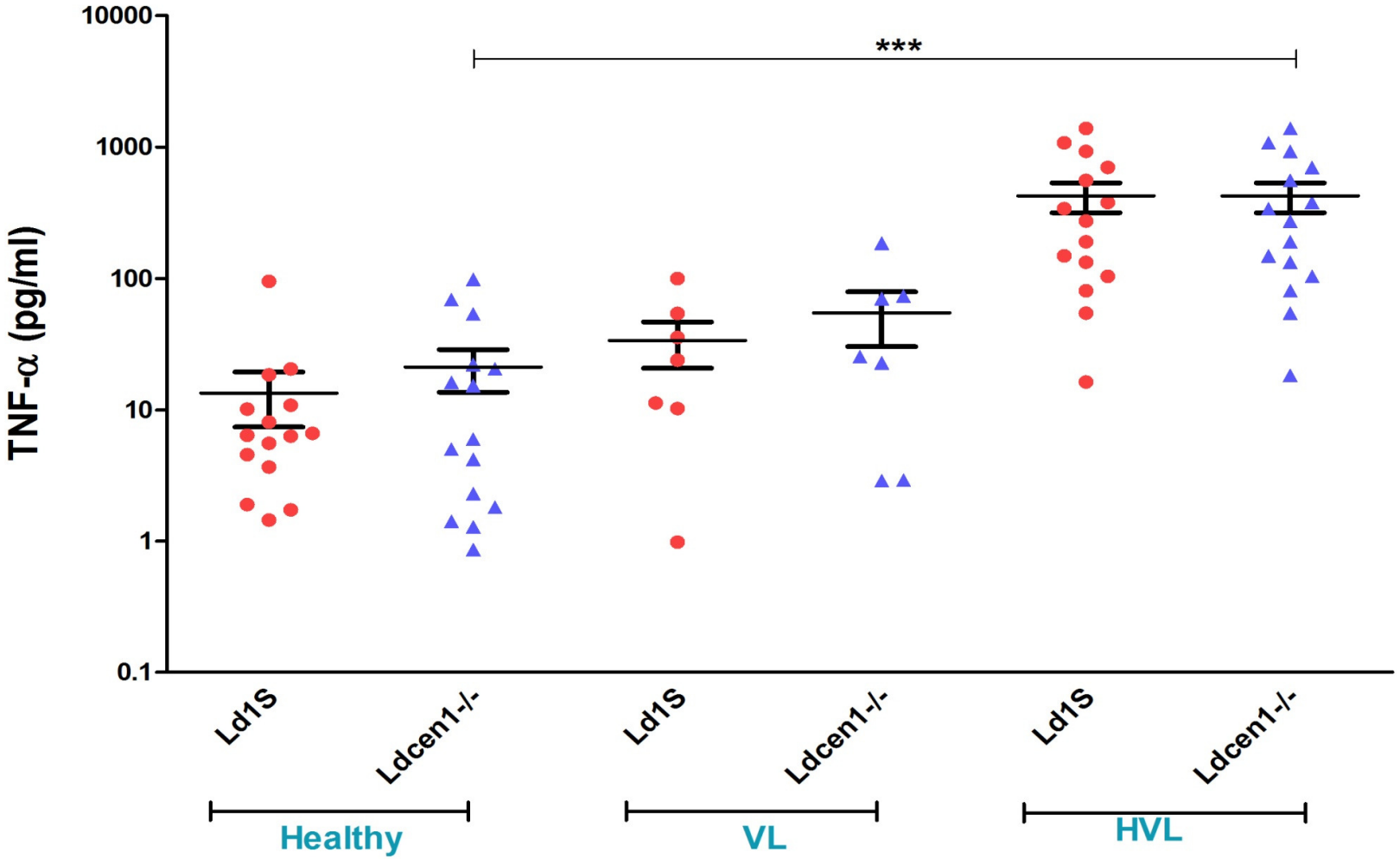
*Ld*Cen1$^{-/-}$ induces strong Th1 response in PBMCs of HVL



IFN -γ in supernatant of PBMCs in response to Ld1S and *Ld*Cen1$^{-/-}$ parasite. * = P<0.05, ***=P<0.001

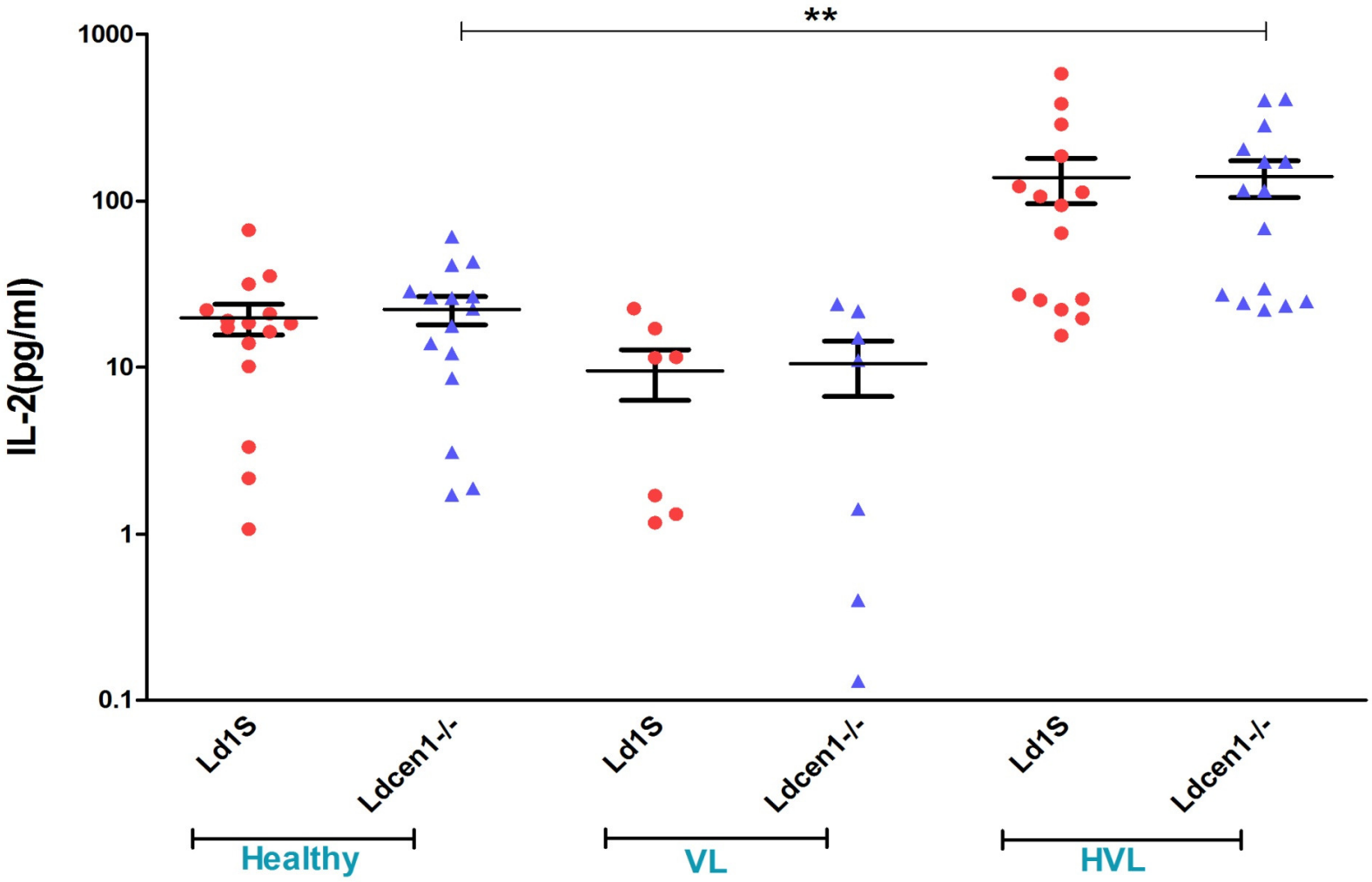
Stimulation of IFN -γ in PBMCs of HVL with *Ld*Cen1$^{-/-}$

Stimulation of TNF- α in PBMCs of HVL infected with *Ld*Cen1$^{-/-}$



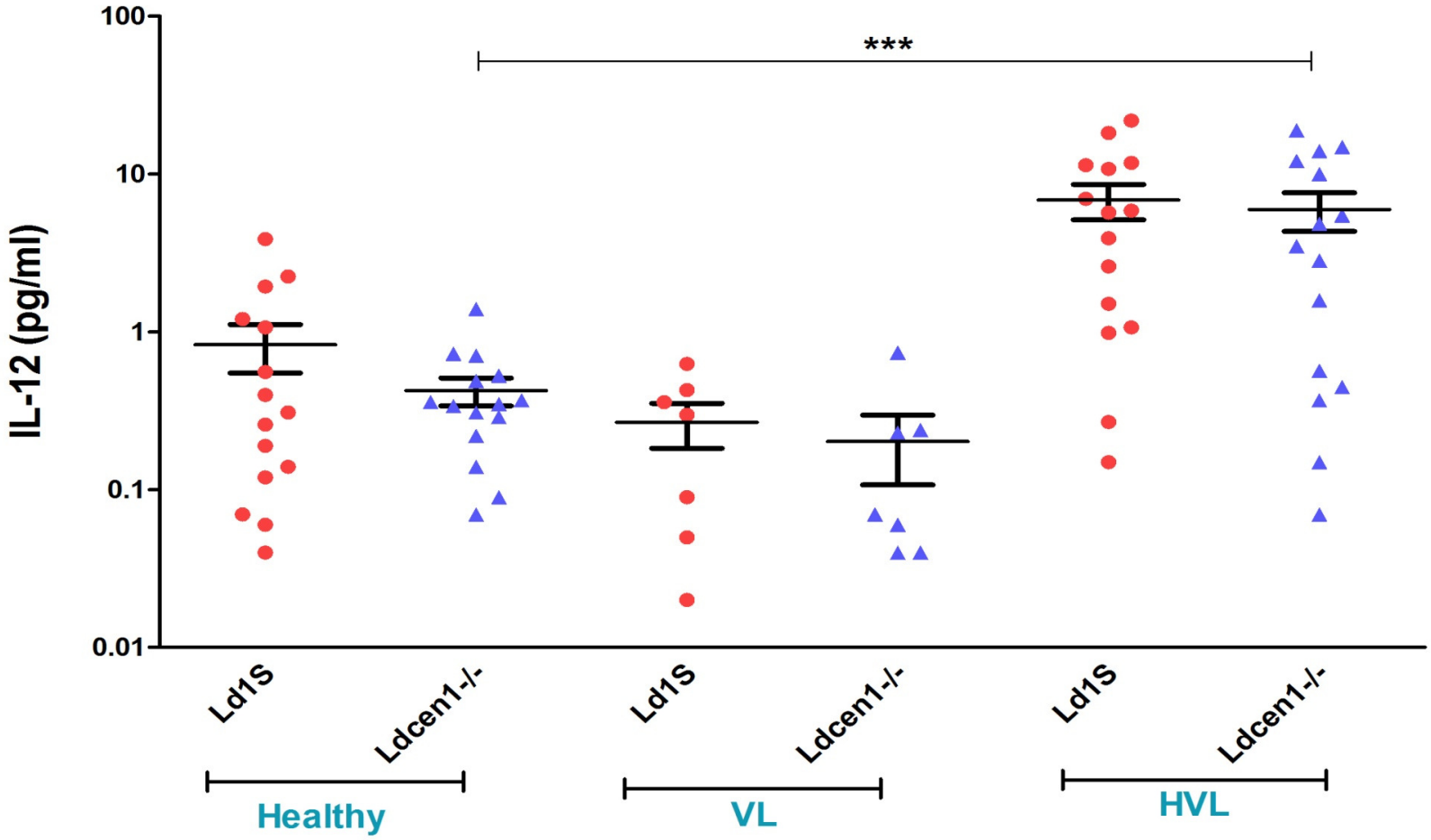
Level of TNF- α in supernatant of PBMCs in response to Ld1S and *Ld*Cen1$^{-/-}$ parasite. ***=P<0.001

Stimulation of IL-2 in PBMCs of HVL infected with *Ld*Cen1$^{-/-}$



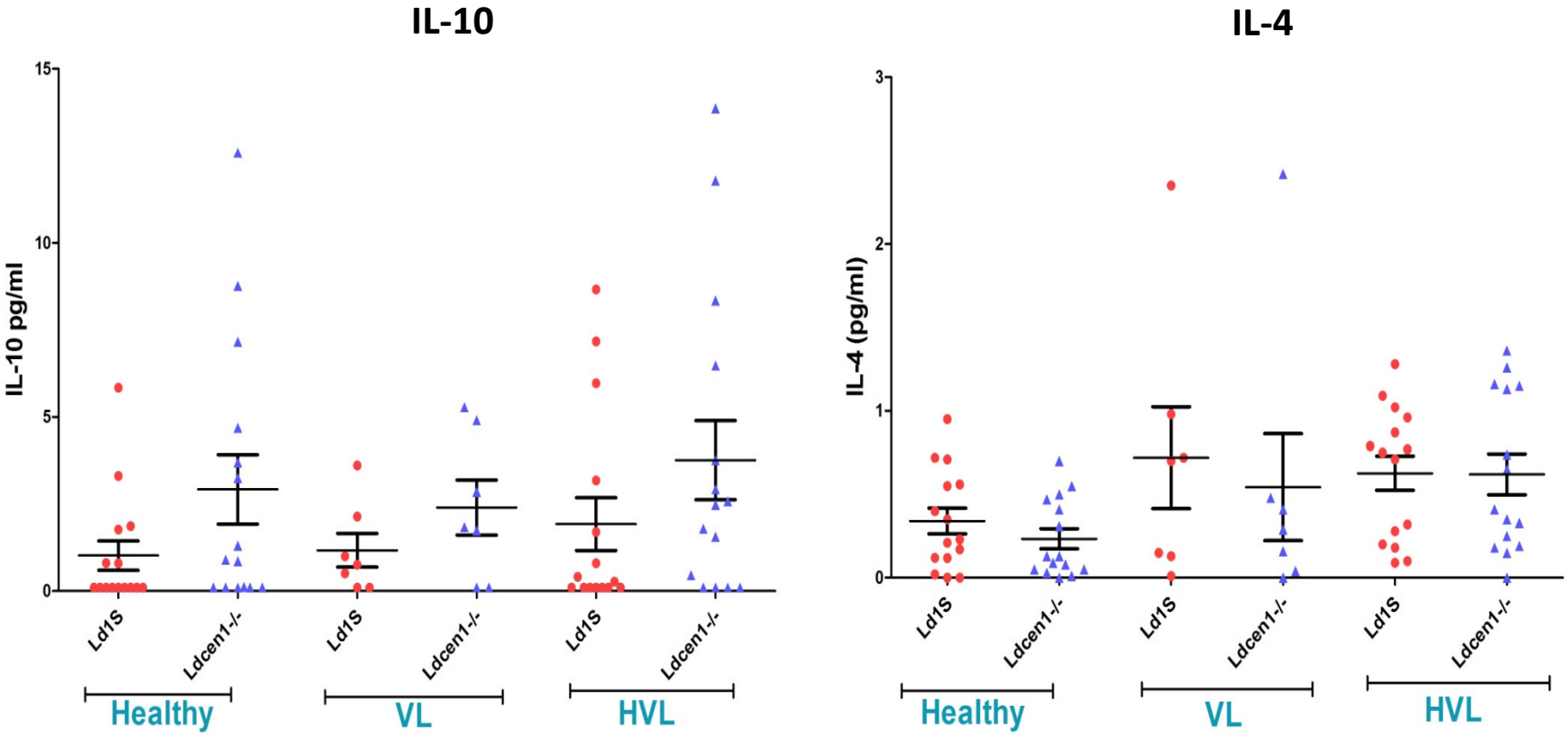
Level of IL-2 in supernatant of PBMCs from Healthy VL and HVL groups in response to Ld1S and *Ld*Cen1$^{-/-}$ parasite. **=P<0.01

Stimulation of IL-12 in PBMCs of HVL infected with *Ld*Cen1$^{-/-}$



Level of IL-12 in supernatant of PBMCs from Healthy VL and HVL groups in response to Ld1S and *Ld*Cen1$^{-/-}$ parasite. ***=P<0.001

*Ld*Cen1$^{-/-}$ parasites do not induce Th2 response



Level of IL-10 and IL-4 in supernatant of PBMCs from Healthy VL and HVL groups in response to Ld1S and *Ld*Cen1$^{-/-}$ parasite

No significant stimulation of IL-10 or IL-4 upon infection with *Ld*Cen1$^{-/-}$ in any group

Pre-clinical evaluation of *LdCen1*$^{-/-}$ live attenuated vaccine candidate

Immunogenicity

- Protective immune response in mice, hamsters, dogs and ex vivo in human PBMCs
- Cross protection across VL, CL and MCL

Safety

- Lack of pathogenicity
- Lack of long term persistence
- Lack of reversion to virulent form
- Lack of survivability in sand fly
- Can grow in non-animal derived materials

Summary

- Development of live attenuated parasite through gene deletion is a sound approach to develop vaccine against VL

- Pre-clinical evaluation of *Ld*Cen1$^{-/-}$ establishes its potential as a live attenuated vaccine against VL

- Generation of new vaccine candidates attenuated at amastigote stage

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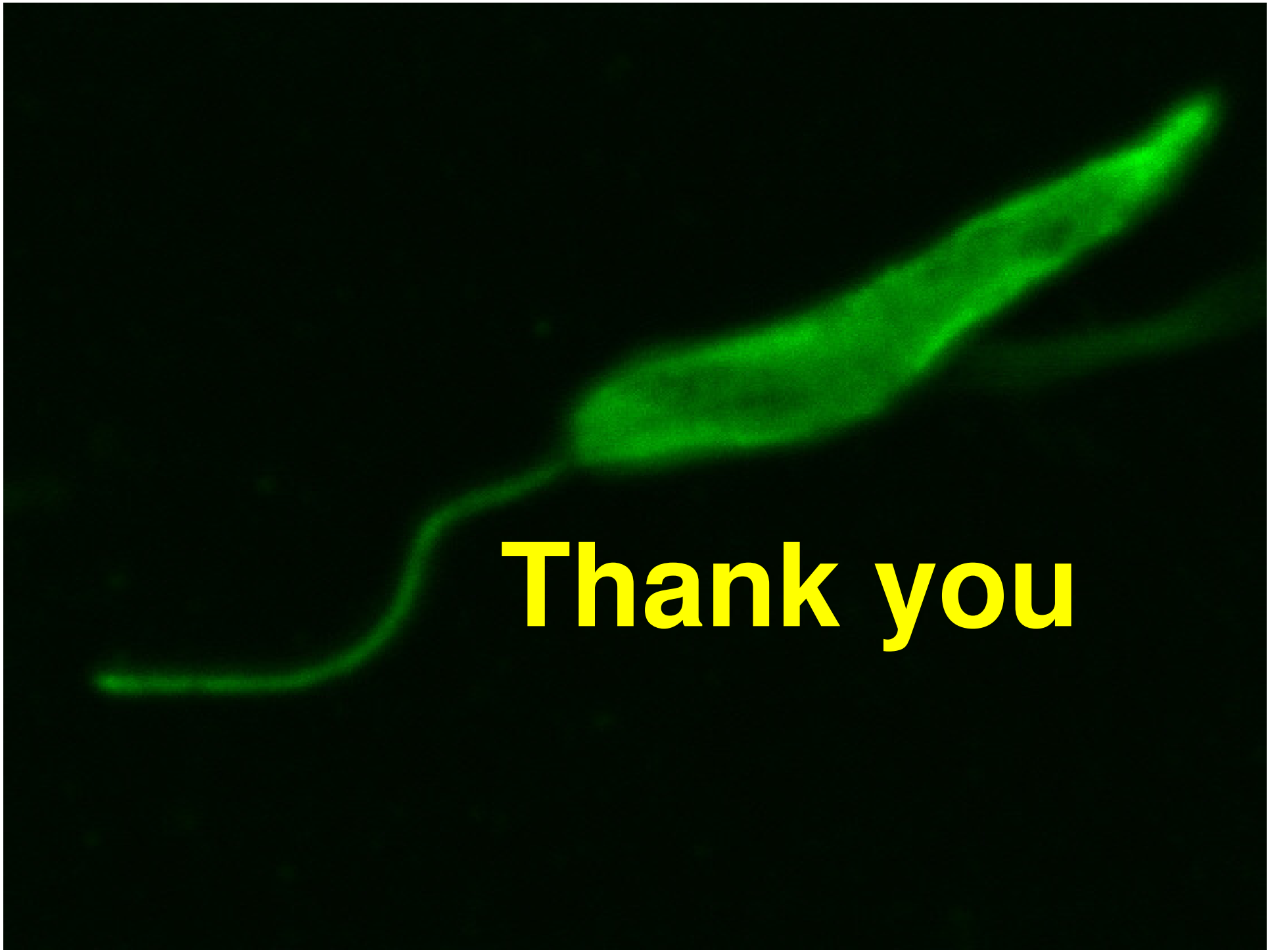
Thank you