Preclinical development of a molecularly-defined liposomal vaccine for cutaneous leishmaniasis

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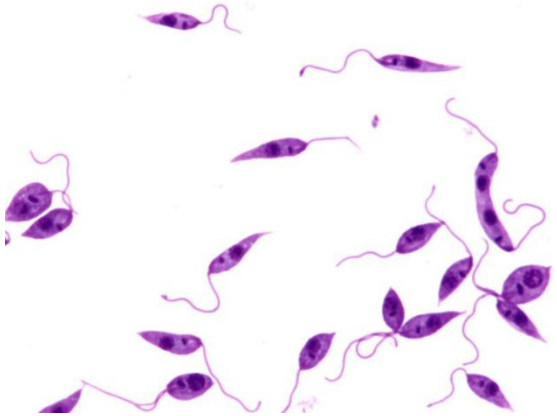
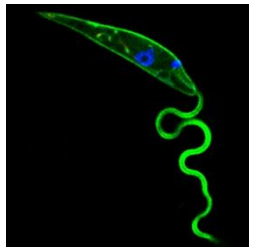
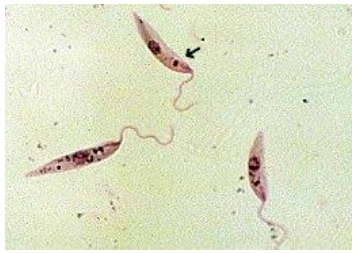
Leishmaniasis: a public health problem

Group of neglected diseases widely distributed in tropical and subtropical areas

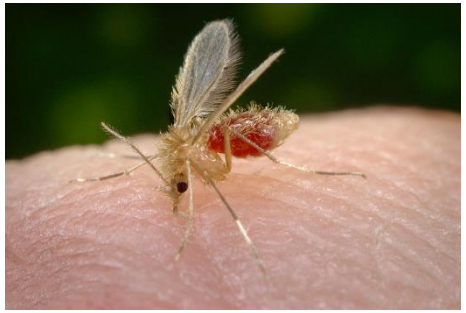
Caused by an obligate intracellular protozoan kinetoplastid from the genus *Leishmania*



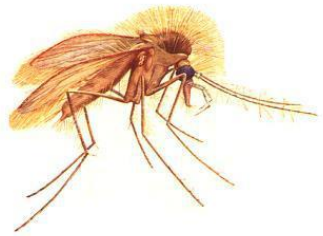




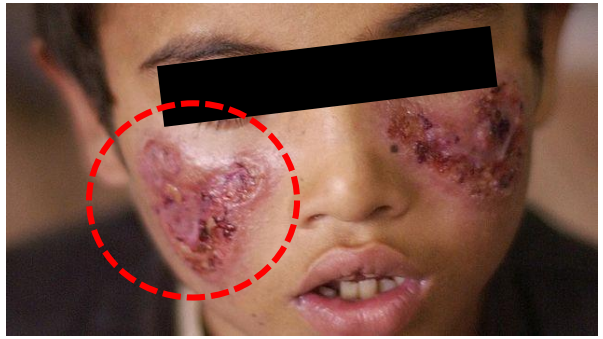
The infective form is transmitted to the human host by the bite of phlebotomineous females



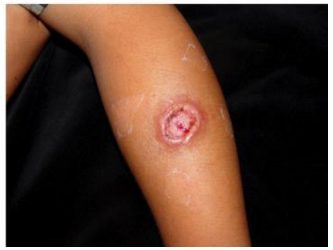
Vector: sandfly

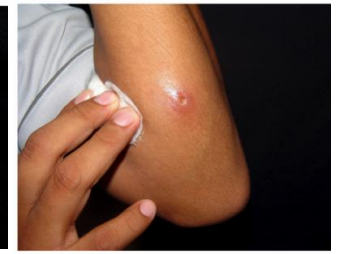


The clinical spectrum of the disease varies depending on the species involved and the immunogenic status of the host



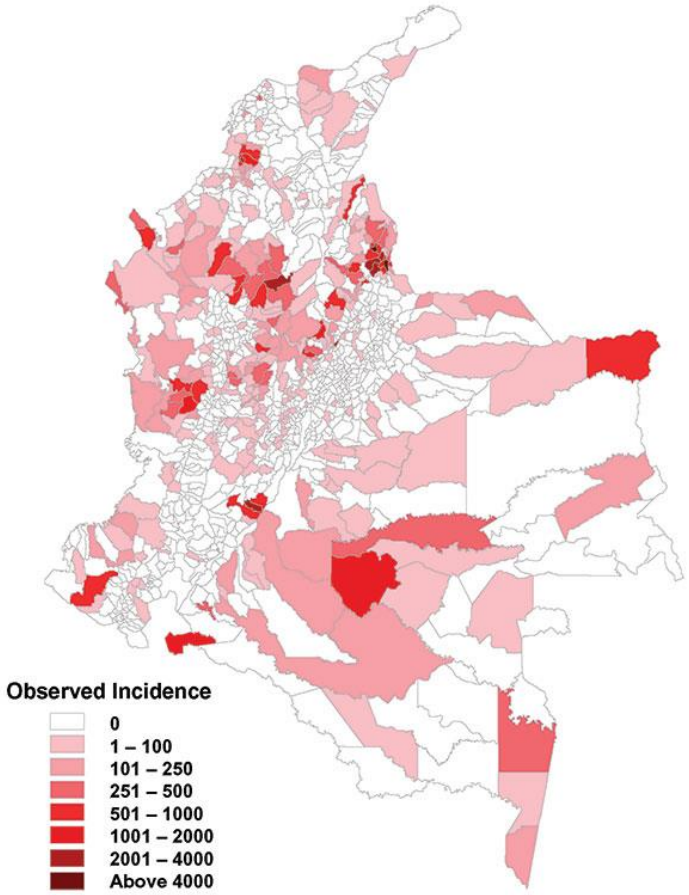
Child with cutaneous leishmaniasis



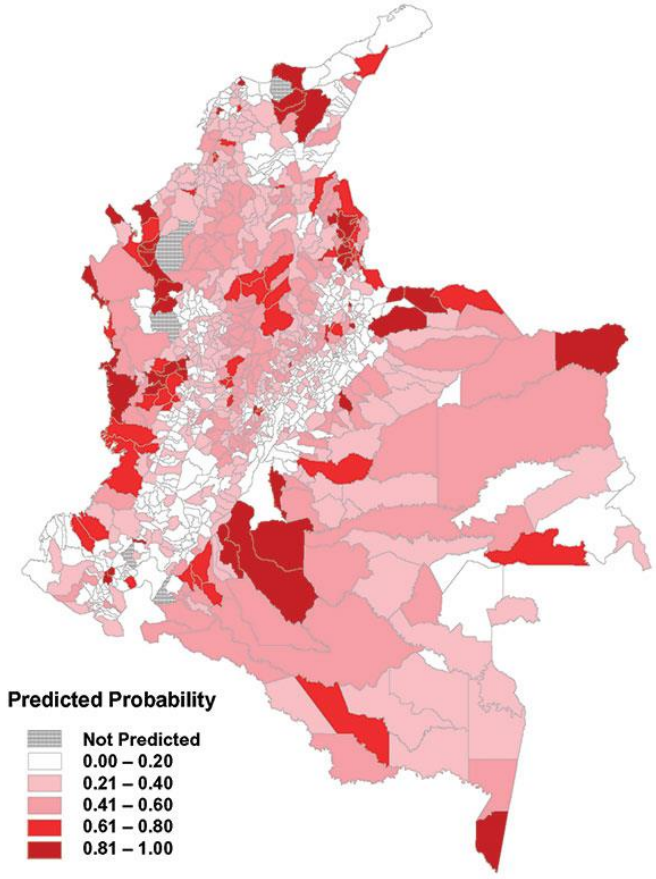


Torres-Guerrero et al. *F1000Research.* **2017**;6: 750. | CC BY Pictures

Leishmaniasis: a public health problem in Colombia



Geographic distribution of the incidence of cutaneous leishmaniasis by municipality (1994)



Map of predicted risk for the probability of transmission (2004)

Colombia is an endemic country for leishmaniasis

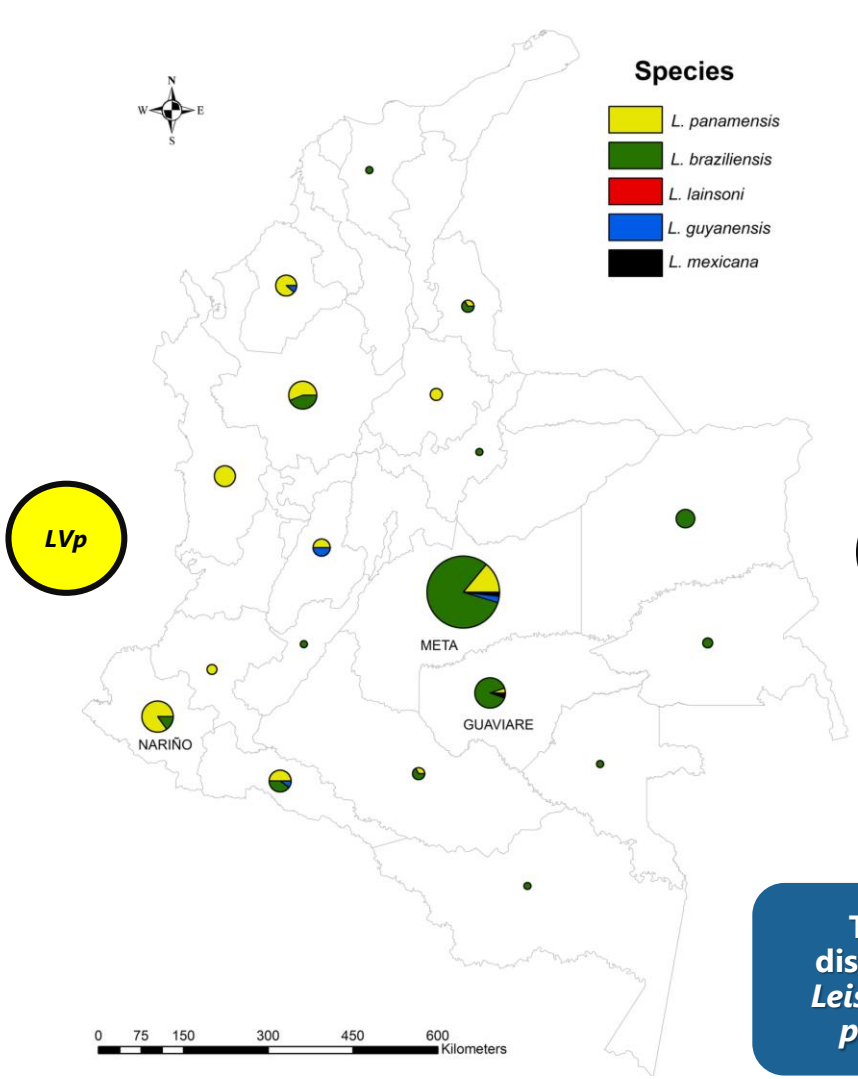
Colombia is one of the ten countries with the highest number of cases of cutaneous leishmaniasis

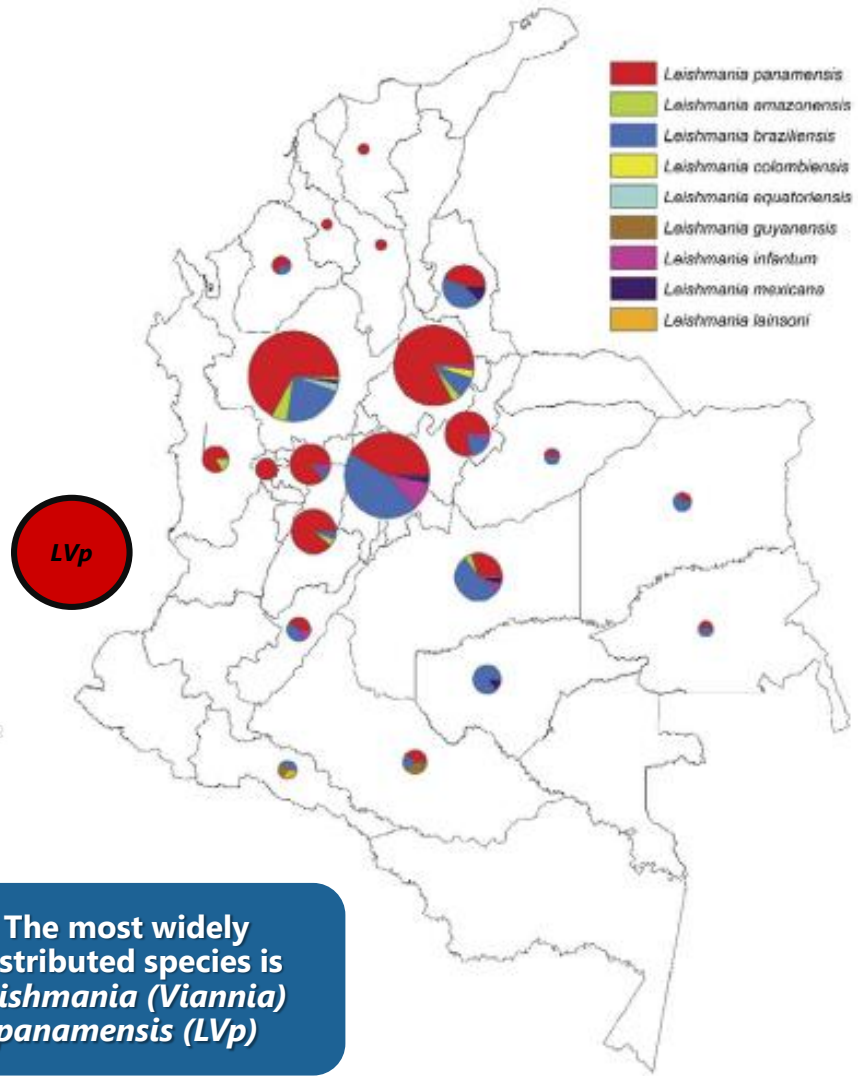
More than a half Colombians are at risk of contracting the disease

Approx. 65K new cases of cutaneous leishmaniasis are estimated annually in Colombia

Alvar et al. *PLoS One.* **2012**;7: e35671. | King et al. *Emerg Infect Dis.* **2004**;10: 598–607.

Leishmaniasis: a public health problem in Colombia





The most widely distributed species is *Leishmania (Viannia) panamensis (LVp)*

Treatment: chemotherapy with antimonial compounds, pentamidine or amphotericin B

Complicated, expensive, inefficient (it affects hard-to-reach poor population)

There is only one oral drug (miltefosine) but increasing resistance has been reported

A safe and effective vaccine is needed

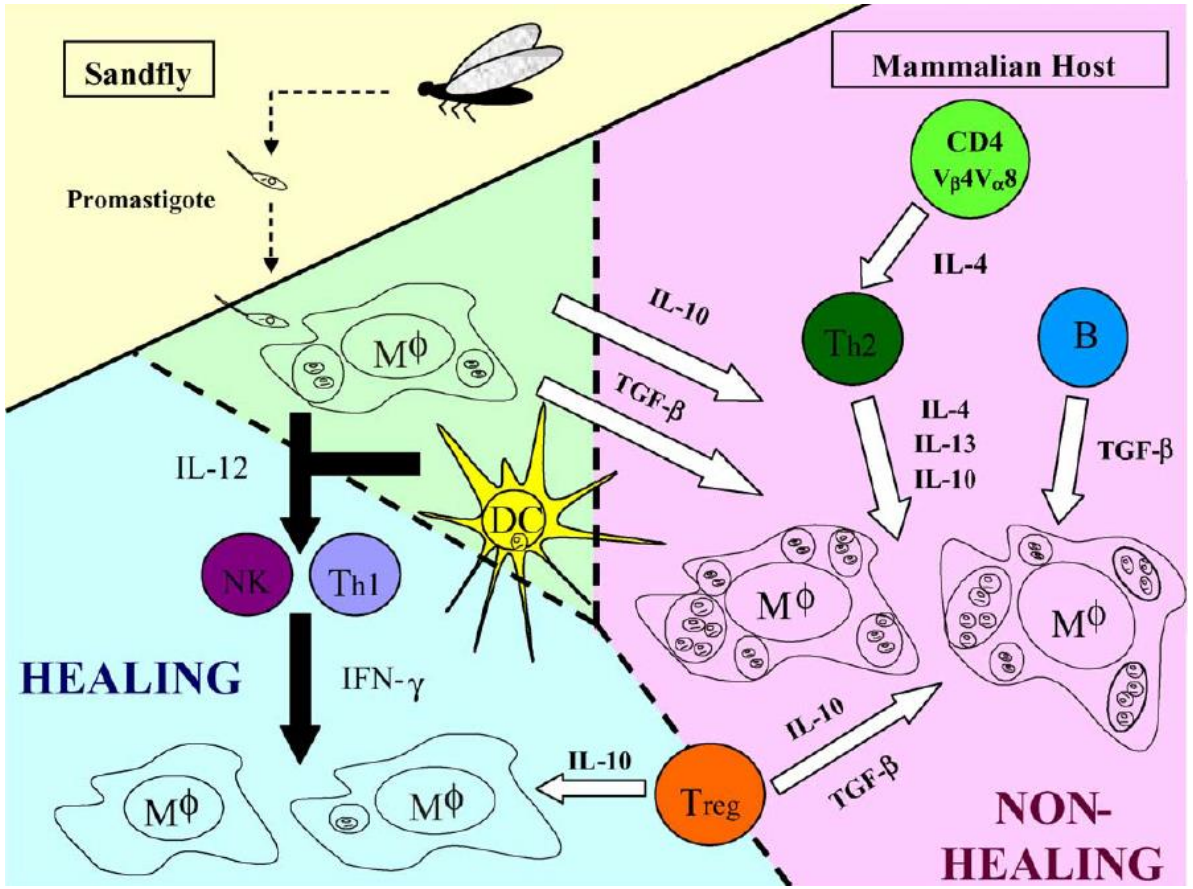
Patino et al. *PLoS Negl Trop Dis*. **2017**;11: e0005876 | Ramírez et al. *Sci Rep*. **2016**;6: 28266 | Modabber et al. *Kinetoplastid Biol Dis.* **2007**;6: 3 | Chakravarty et al. *J Glob Infect Dis.* **2010**;2: 167
Reithinger et al. *Lancet Infect Dis.* **2007**;7: 581–596 | Soto et al. *Clin Infect Dis.* **2004**;38: 1266–1272 | Rijal et al. *Clin Infect Dis.* **2013**;56: 1530–1538.

Leishmaniasis: vaccine development

What are the immune mechanisms that lead to a protective response?

Infection model with *L. major*
Th1/Th2 paradigm

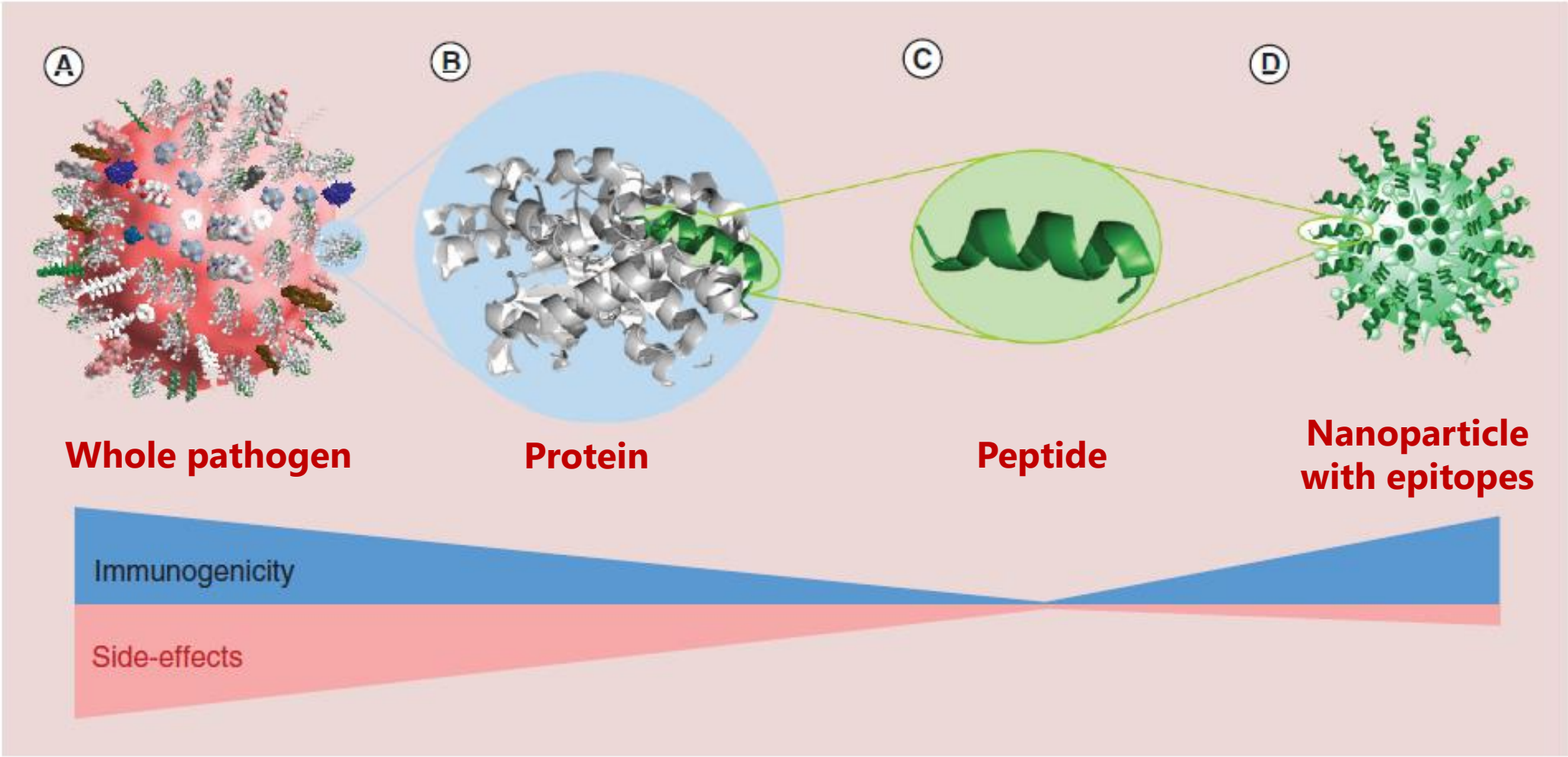
C57BL/6 Mice **Resistant**	**BALB/c Mice** **Sensitive**
CD4+ Th1 response	CD4+ Th2 response
IL-12, TNF-α, IFN-γ	IL-4
Macrophage activation	Parasite survival
Nitric oxide production	



***Leishmania (Viannia)* spp.** → **Non-polarized** → **Mixed Th1/Th2**

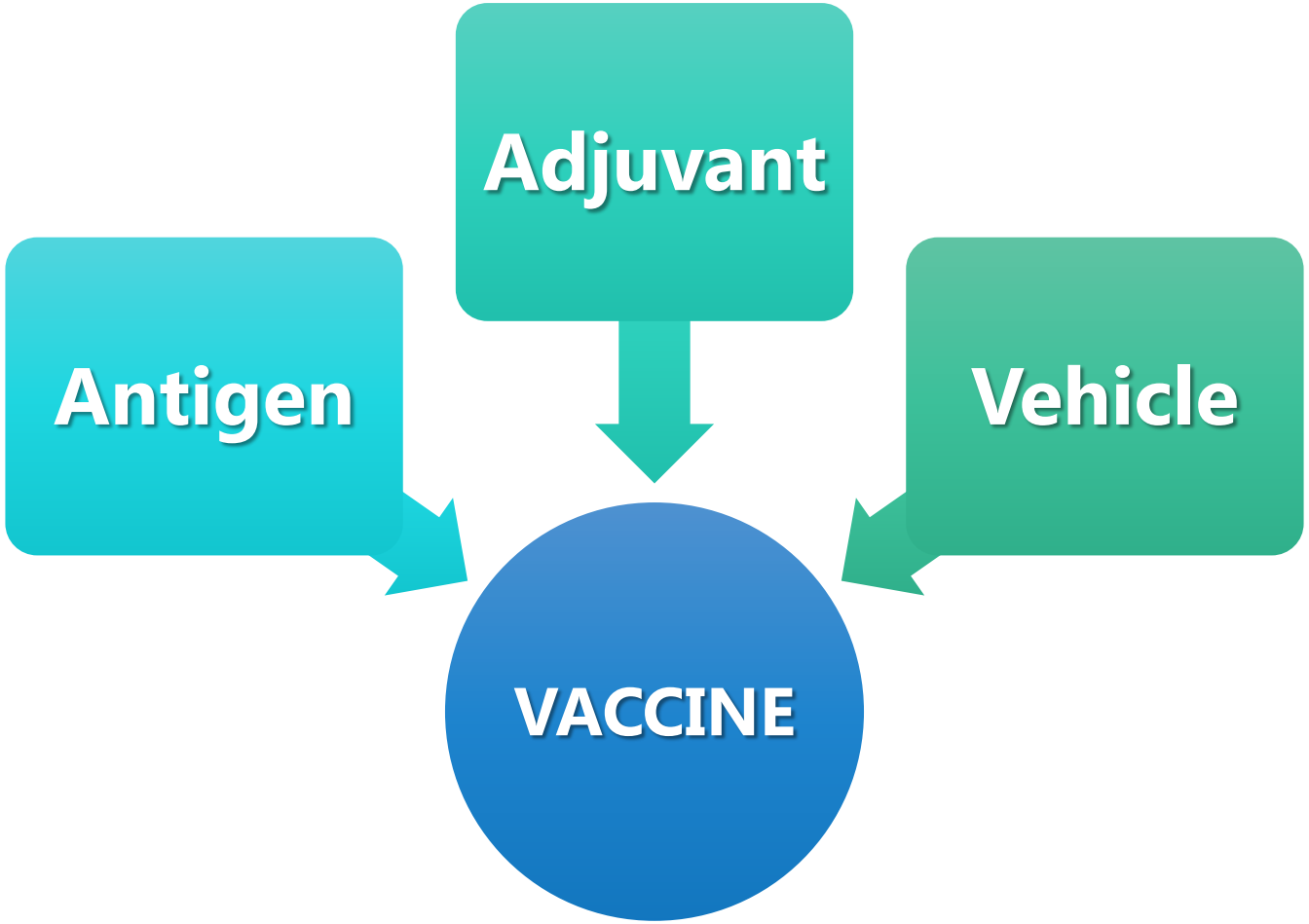
Alexander & Bryson. *Immunol Lett.* **2005**;99: 17–23. | de Moura et al. *Infect Immun*. **2005**;73: 5827–5834. | Bosque et al. *Scand J Immunol.* **2000**;51: 533–541. | Bourreau et al. *J Infect Dis.* **2001**;183: 953–959.

Leishmaniasis: vaccine development



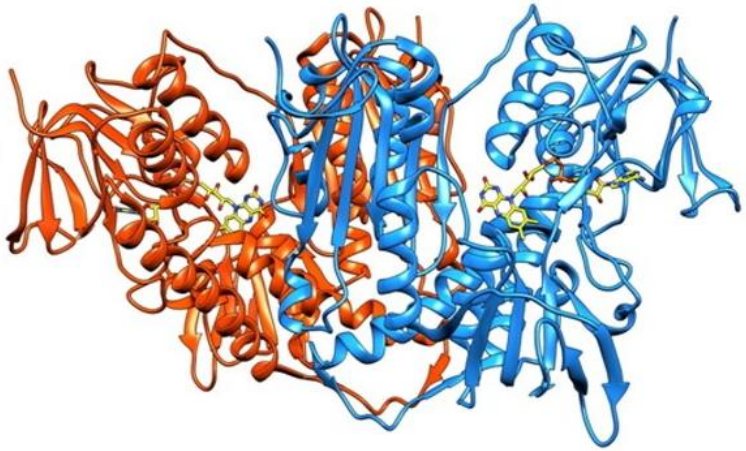
Skwarczynski & Toth. *Nanomedicine*. **2014**;9: 2657–2669.

Components of a vaccine



Trypanothione Reductase as vaccine candidate

TR from *L. infantum*



Occurs in protozoan parasites of the genus *Trypanosoma* and *Leishmania*

Oxidoreductase enzyme

Central role in the redox balance

Essential for the infectivity of the parasite

Pharmacological target

(development of inhibitory drugs)

What about its immunogenic role?

Angiulli et al. *Biochim Biophys Acta.* **2015**;1850: 1891–1897. | Fairlamb et al. *Annu Rev Microbiol.* **1992**;46: 695–729. | Krauth-Siegel & Comini. *Biochim Biophys Acta.* **2008**;1780: 1236–1248.
Tovar et al. *Mol Microbiol.* **1998**;29: 653–660. | Dumas et al. *EMBO J.* **1997**;16: 2590–2598. | Ilari et al. *Future Med Chem.* **2017**;9: 61–77.

Trypanothione Reductase as vaccine candidate

Evaluation of rTR immunogenicity

- ✓ Peripheral blood mononuclear cells (patients)
- ✓ Lymph node cells (infected and treated hamsters)

After stimulation with rTR, the proliferative response was comparable or superior to the stimulation with soluble antigen

Evaluation of the prophylactic efficacy from rTR

- ✓ Hamsters
- ✓ BCG as adjuvant
- ✓ VL model (infection by *Leishmania donovani*)

Clinical (up to 6 months)

- ⓾ 60% protection against infectious challenge.
- ⓾ Weight gain.
- ⓾ Healthy condition.

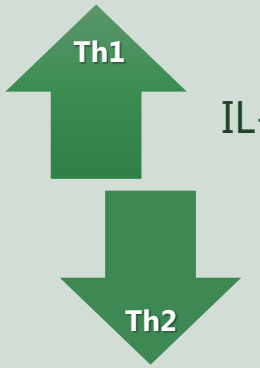
Parasitological

- ⓾ Lower parasitic load in spleen, liver and bone marrow.

Immunological

- ⓾ Higher production of IgG2a antibodies.
- ⓾ DTH and proliferative response.
- ⓾ Production of nitric oxide.

Cytokines (qPCR)



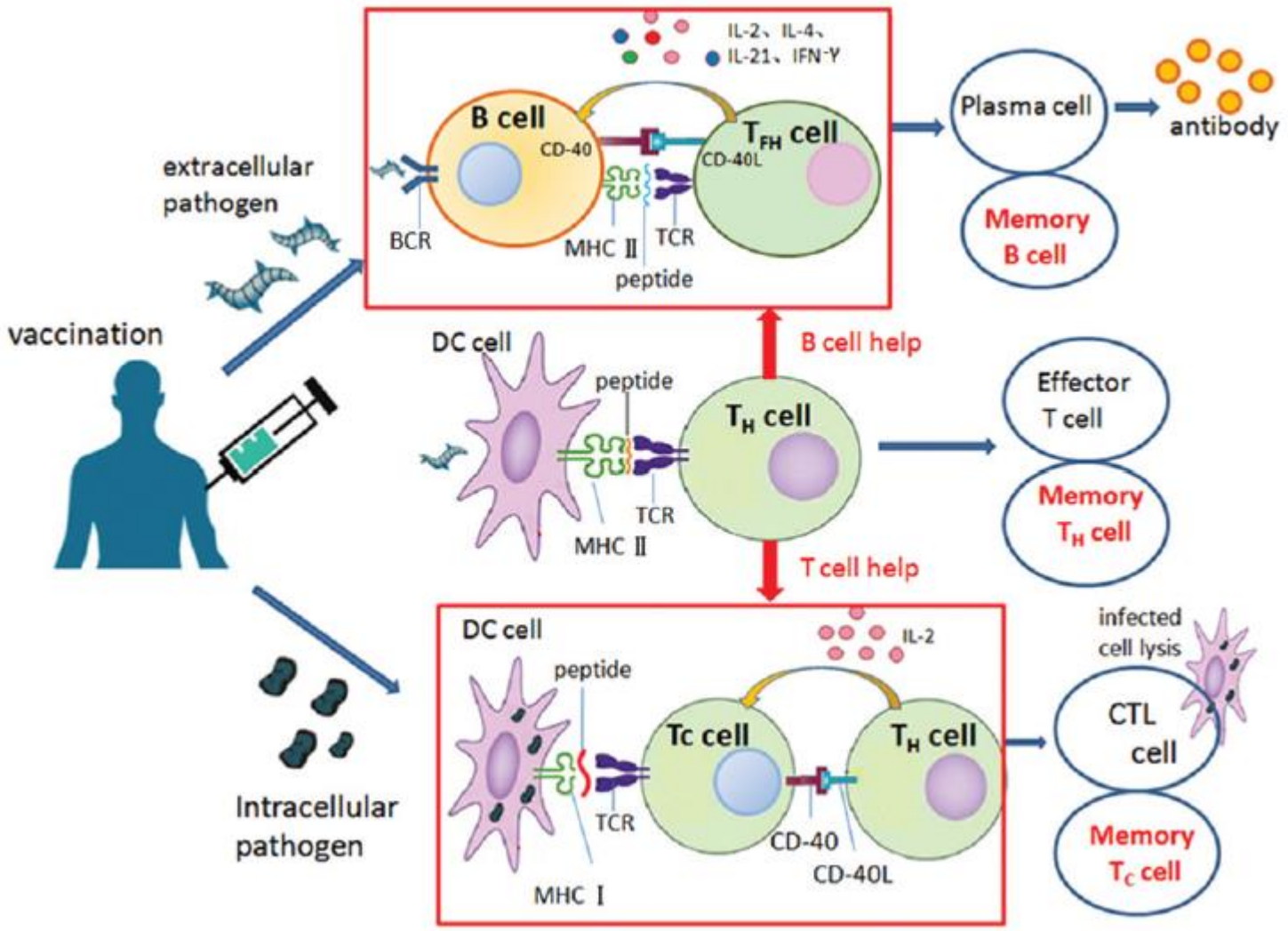
IFN-γ
IL-12, TNF-α

IL-4, TGF-β
IL-10

The design of polyvalent chimeric vaccines is suggested but an encapsulation system is not proposed

Khare et al. *Parasitol Res.* **2014**;113: 851–862. | Joshi et al. *Front Microbiol.* **2016**;7: 312.

Cellular adjuvants for vaccines



Humoral immunity

The vaccine formulations developed up to now are very efficient stimulating the production of antibodies

Cellular immunity

The protective immune response against *Leishmania* depends on the stimulation of CD4+ and CD8+ T cells

It is difficult to stimulate this type of response through conventional vaccines

For this same reason there are still no approved vaccines against HIV, malaria, tuberculosis and cancer

Yang et al. *Biomater Sci.* **2016**;4: 785–802.

Cellular adjuvants for vaccines

Receptors of innate immunity

- ✓ TLR
- ✓ NLR
- ✓ LTR

They promote crucial aspects of the antigen presentation

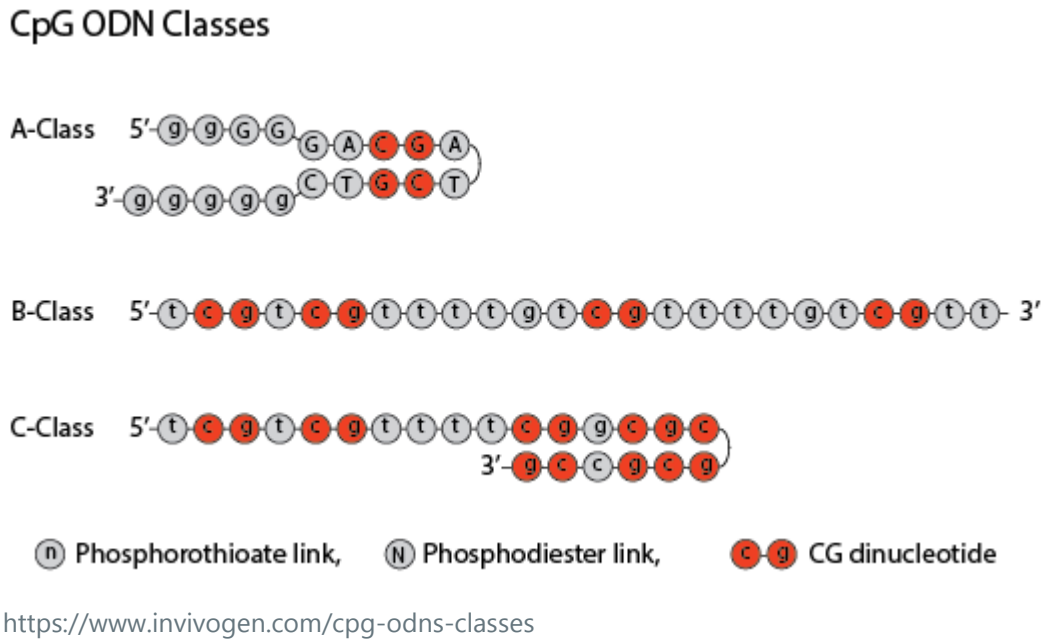
- ✓ Antigen capture and processing
- ✓ Dendritic cell maturation

Non-methylated CpG motifs

TLR9 ligand

Present in bacterial DNA

Absent in the vertebrate DNA



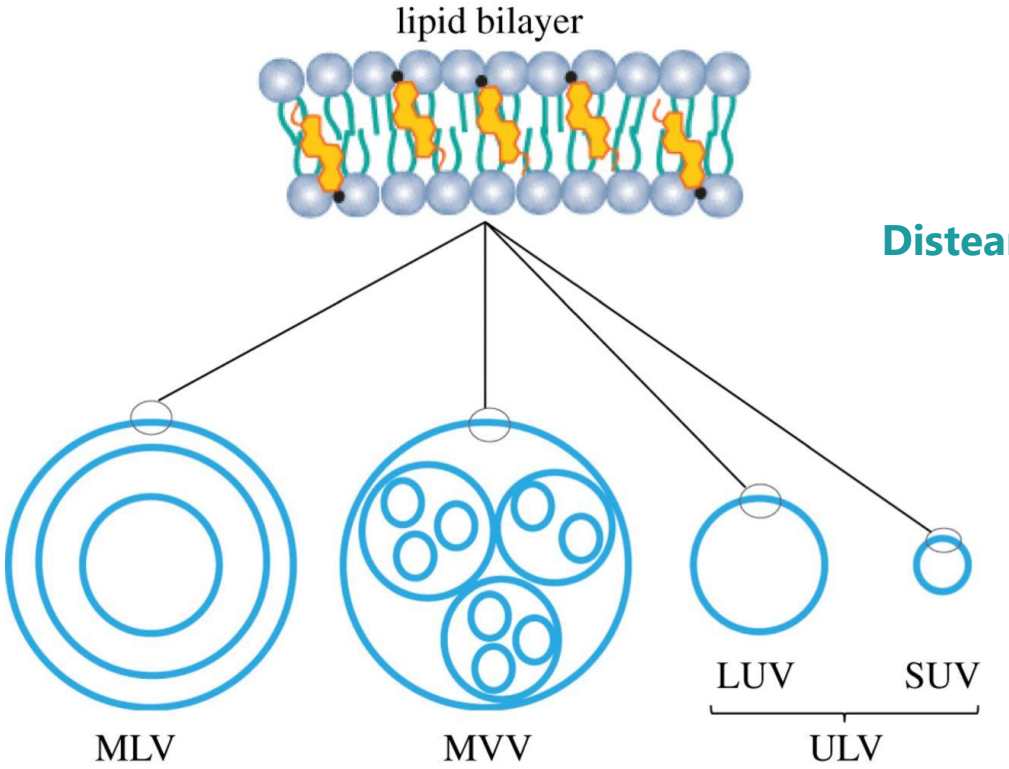
https://www.invivogen.com/cpg-odns-classes

Dowling & Mansell. *Clin Transl Immunol.* **2016**;5: e85. | Bramwell & Perrie. *Drug Discov Today.* **2005**;10: 1527–1534. | Modabber F. *Int J Antimicrob Agents.* **2010**;36: S58–S61.
Mbow et al. *Curr Opin Immunol.* **2010**;22: 411–416. | Bode et al. *Expert Rev Vaccines*. **2011**;10: 499–511.

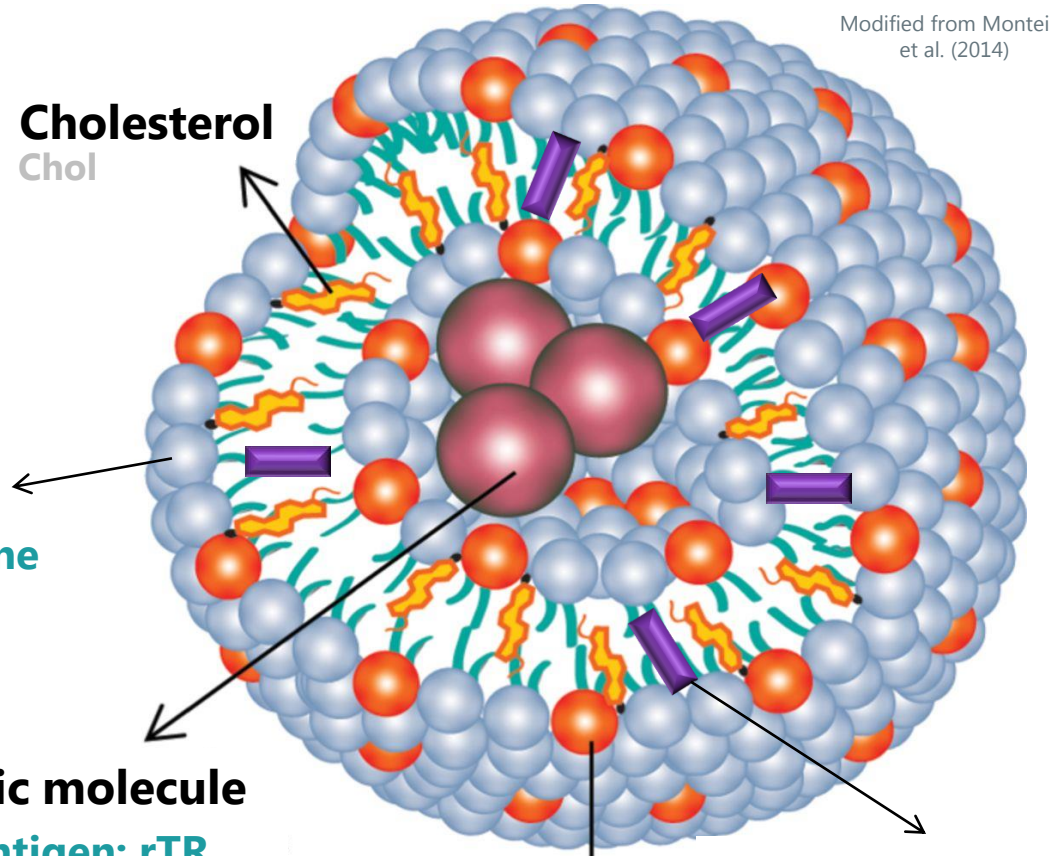
Liposomes as adjuvants/delivery system for vaccines

Micro/nanometric spherical vesicles with at least one lipid bilayer composed of phospholipids

Tunable physicochemical characteristics



Modified from Monteiro et al. (2014)



Cholesterol
Chol

Main lipid
Distearoylphosphocholine
DSPC 18:0 PC

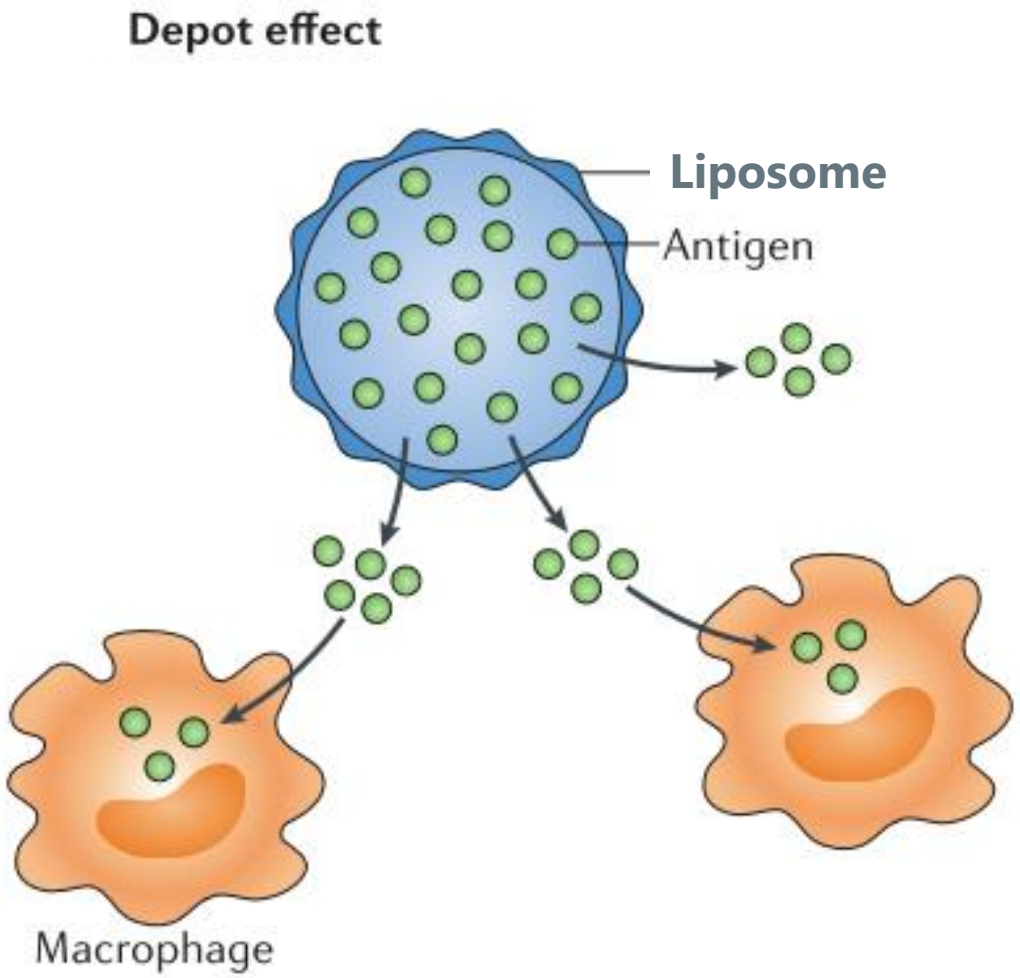
Hydrophilic molecule
Protein antigen: rTR

Cationic lipid
Stearylamine SA

Hydrophobic/ amphiphilic molecule
Adjuvant: CpG

Monteiro et al. *J R Soc Interface*. **2014**;11: 20140459. | Nekkanti & Kalepu. *Pharm Nanotechnol*. **2015**;3: 35–55. | Pattni et al. *Chem Rev*. **2015**; 10938-10966.

Liposomes as adjuvants/delivery system for vaccines



Antigen release at different times

Persistence in the injection site

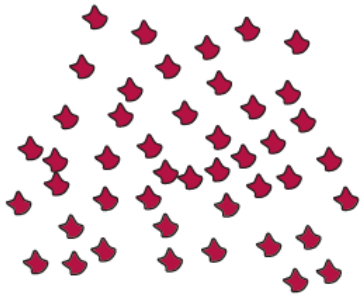
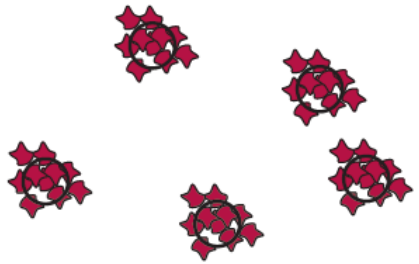
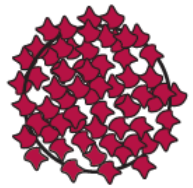
It depends on the particle size

Internalization by resident APC

Activation by intense presentation

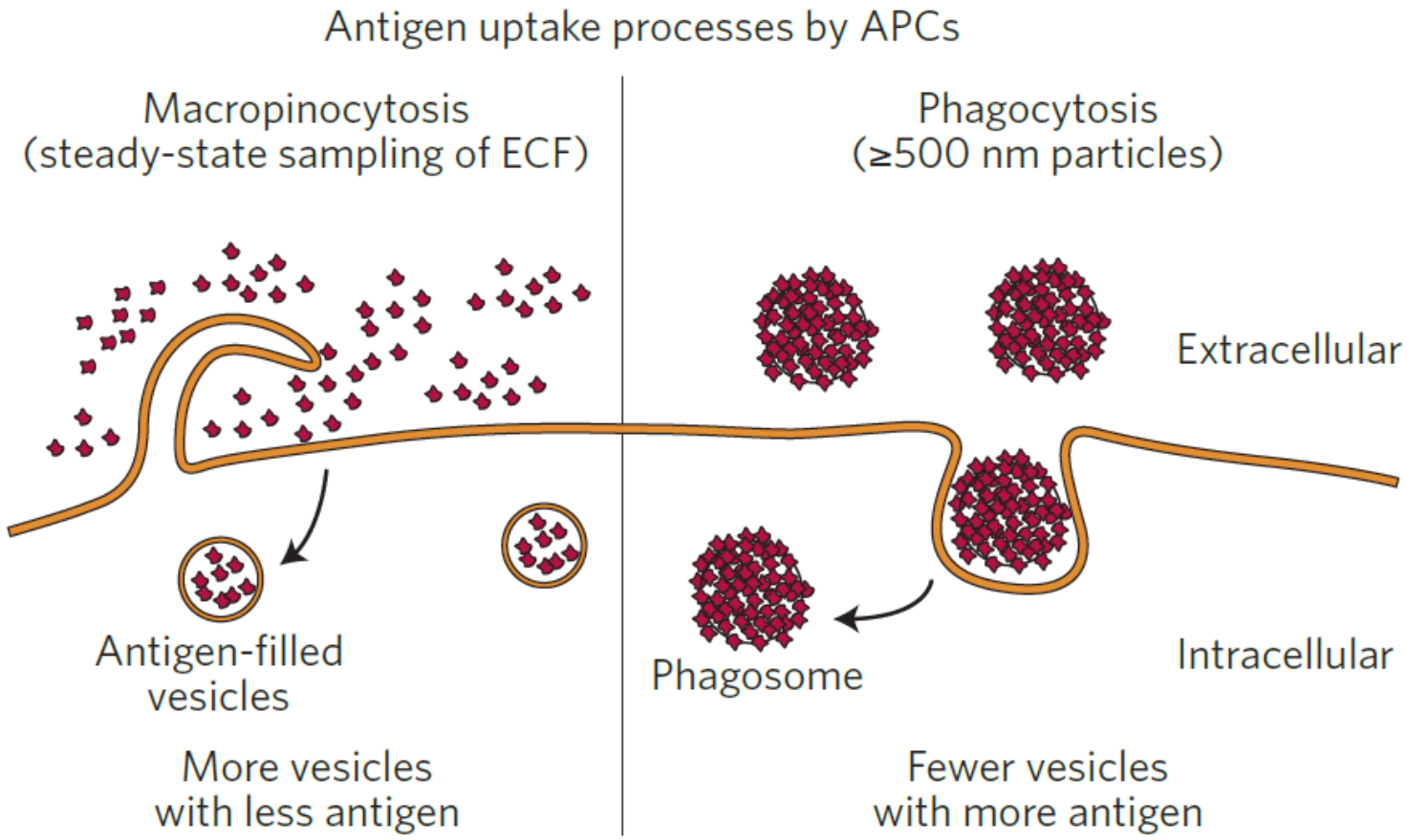
Smith et al. *Nat Rev Immunol*. **2013**;13: 592–605. | Moyer et al. *J Clin Invest*. **2016**;126: 799–808. | Park et al. *Immune Netw*. **2013**;13: 177–183.

Liposomes as adjuvants/delivery system for vaccines

	Soluble antigen	Small (for example, 30 nm) NPs	Large (for example, 300 nm) NPs
Example antigen density			
	1 unit = 1 antigen molecule	1 particle = 10 antigen molecules	1 particle = 1,000 antigen molecules
Effective (extracellular) concentration	[Particle] = [antigen]	[Particle] = 0.1 × [antigen]	[Particle] = 0.01 × [antigen]

Irvine et al. *Nat Mater*. **2013**;12: 978–990.

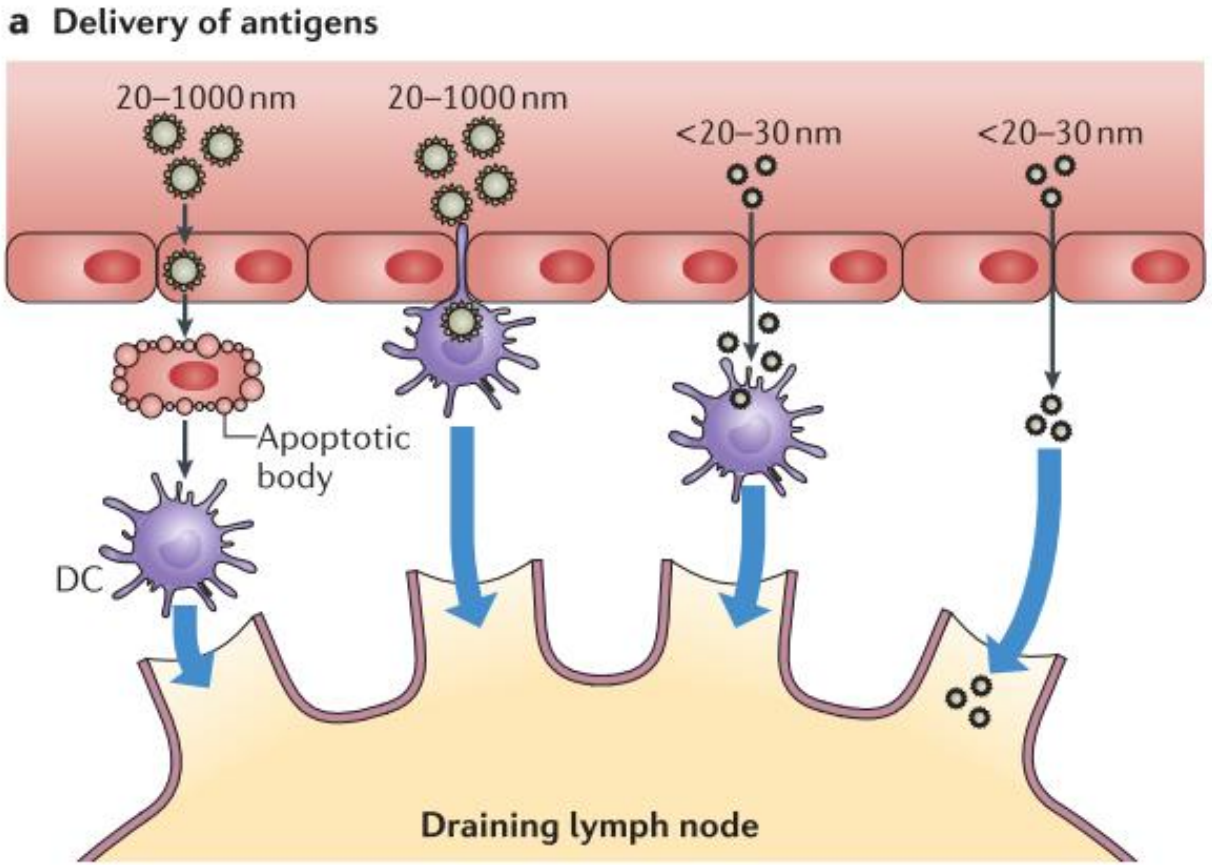
Liposomes as adjuvants/delivery system for vaccines

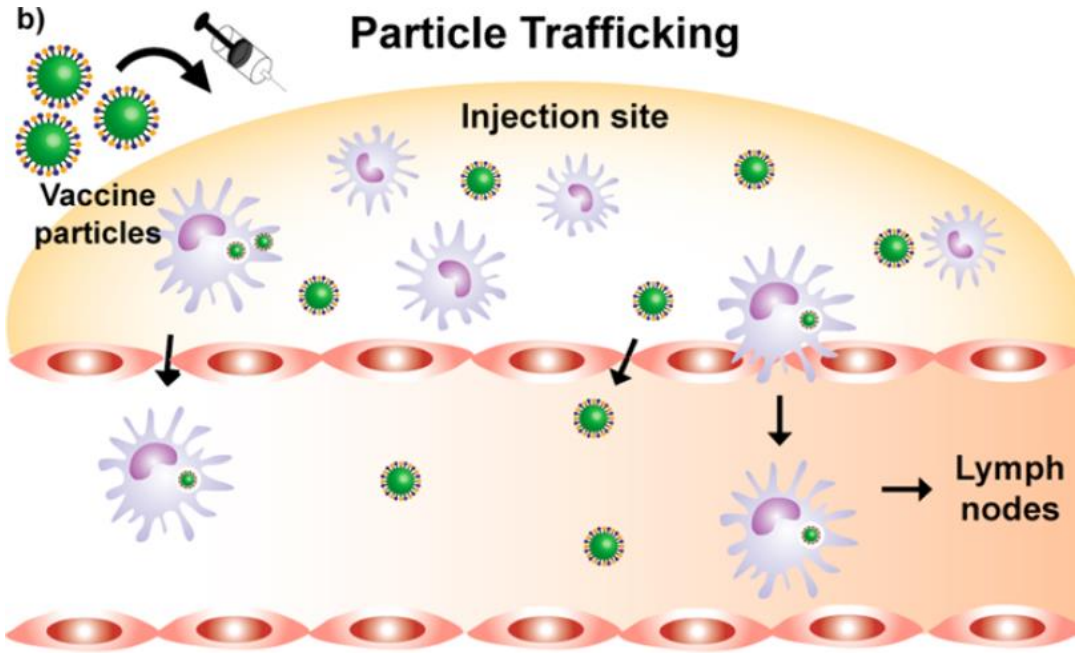


Irvine et al. *Nat Mater*. **2013**;12: 978–990.

Liposomes as adjuvants/delivery system for vaccines

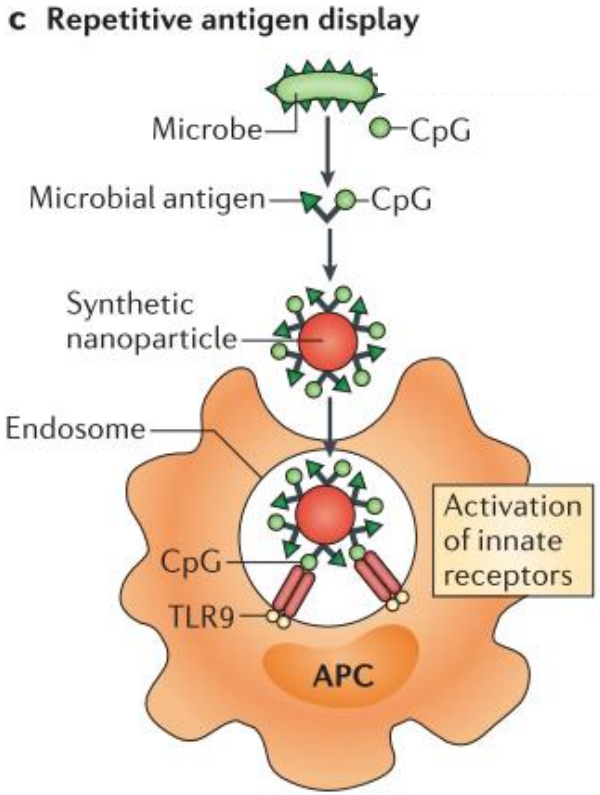
Taking and trafficking of liposomal antigens



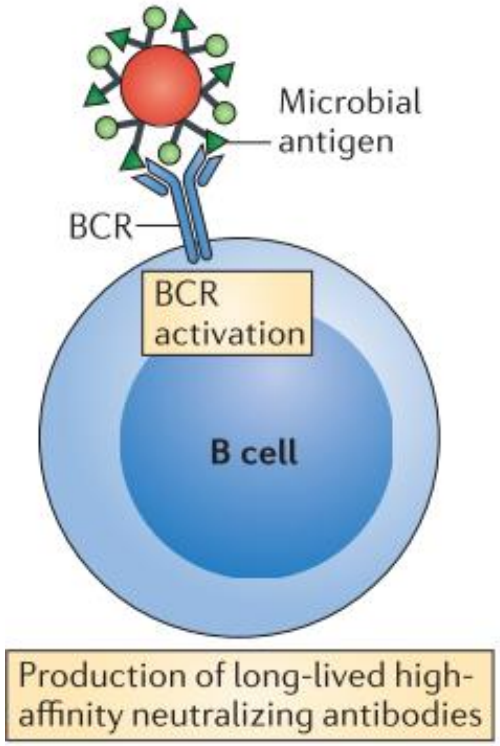


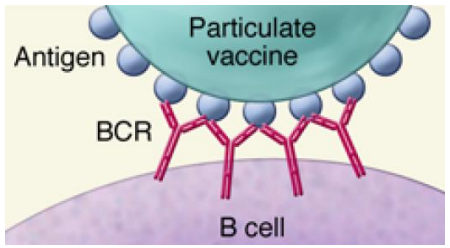
Smith et al. *Nat Rev Immunol*. **2013**;13: 592–605. | Gause et al. *ACS Nano*. **2017**;11: 54–68. | Zaman et al. *Methods*. **2013**;60: 226–231.

Liposomes as adjuvants/delivery system for vaccines

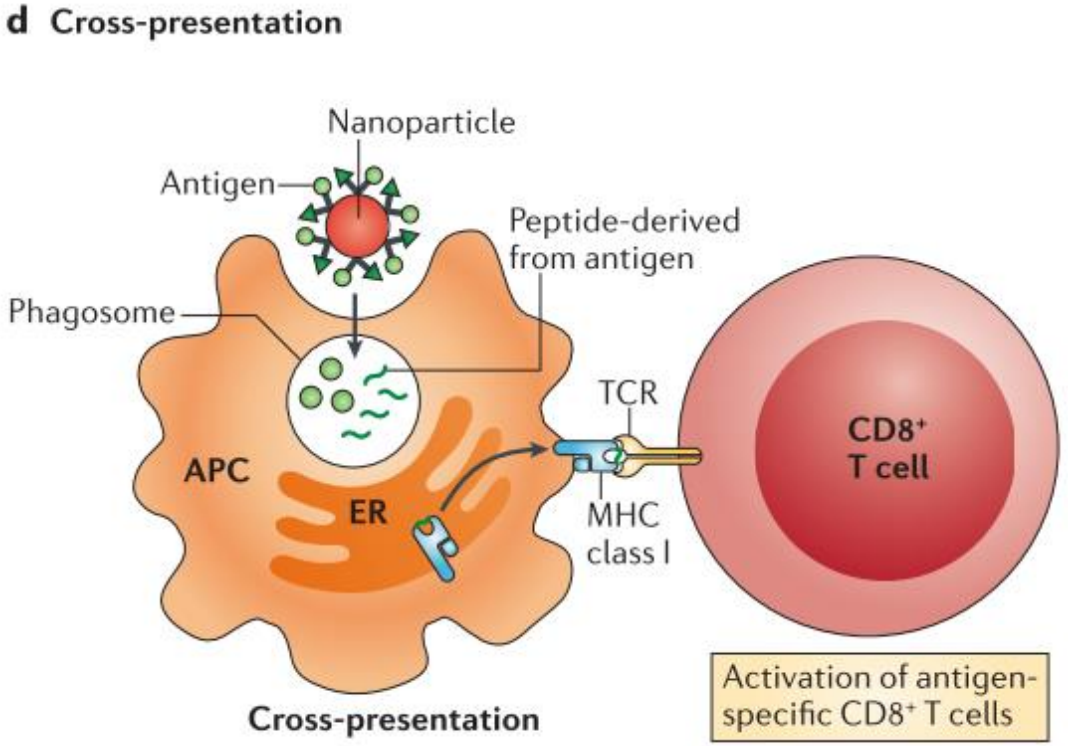


Processing of phagocytosed antigen and presentation to T cells





Presentation of the intact antigen to B cells



Cross-presentation to T lymphocytes

Smith et al. *Nat Rev Immunol.* **2013**;13: 592–605. | Moyer et al. *J Clin Invest.* **2016**;126: 799–808. | Watson et al. *Vaccine.* **2012**;30: 2256–2272. | Skwarczynski & Toth. *Nanomedicine.* **2014**;9: 2657–2669.

Subunit liposomal nanovaccines to prevent leishmaniasis

Infections caused by *L. major* and *L. donovani*

Mainly liposomes of total protein antigen

Several purified proteins have been found to be protective

Some have been encapsulated in liposomes

Nahid Ali Research Group

Subunit vaccine to prevent VL in India

Antigens encapsulated in rigid cationic liposomes are more efficient

Protection against infectious challenge

Cell-mediated immunity

Low levels of parasites

Stimulation of Th1 response

Badiee et al. *Vaccine.* **2013**;31: 735–749. | Askarizadeh et al. *Therapeutic Advances in Vaccines.* **2017**;5: 85-101.

BACKGROUND

Our ulcerative murine model of infection with *LVp* reproduces the human leishmaniasis characteristics

Synthetic ODN with CpG motifs is a protective adjuvant when combined with the total lysate of the parasite

Our liposomal formulation of soluble antigen from *Leishmania* induces protection against infectious challenge

TR is a promising antigen to formulate a molecularly-defined vaccine

HYPOTHESIS

Vaccination with a micro/nanostructured formulation (cationic liposomes) of rTR, either individually or in combination with CpG, potentiates the specific immune response needed to protect mice from an infectious challenge in the model of cutaneous leishmaniasis caused by *Leishmania (Viannia) panamensis*

GENERAL OBJECTIVE

To evaluate the prophylactic efficacy of both soluble and micro/nanostructured formulations of the rTR with/without CpG (soluble/liposomal)

METHODOLOGY Production of rTR

National Center for Genomic Sequencing

- ✓ Expression vector: pET28a (+)
- ✓ Heterologous expression system: *Escherichia coli* (DE)

Optimization of factors such as temperature, time and inductor concentration

Solubility evaluations by lysis method by sonication and separation by centrifugation

Protein labeled with polyhistidine-tag (His6-Tag)

Purification by immobilized metal affinity chromatography (IMAC)

Desalting and gel filtration chromatography

Protein integrity monitoring by SDS-PAGE

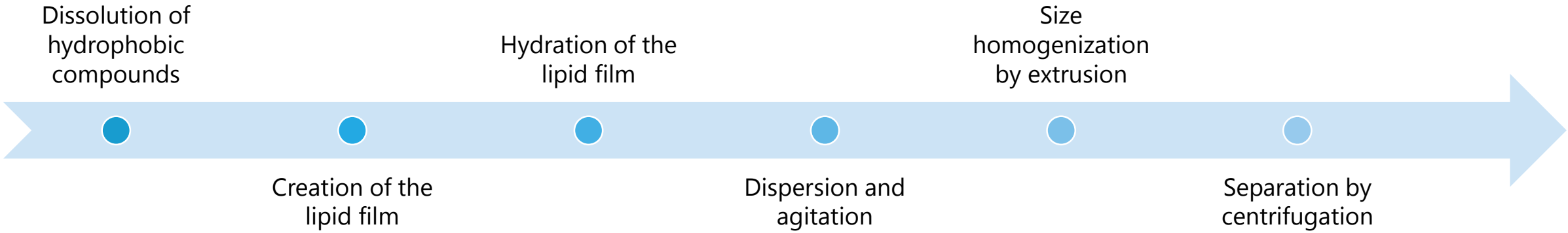
Fraction selection

METHODOLOGY

Preparation and characterization of liposomes

Conventional thin lipid film hydration technique

PC **SA** **Chol**



Physical characterization

- ✓ Particle size (hydrodynamic radius) by dynamic light scattering
- ✓ Particle charge (zeta potential) by electrophoretic mobility

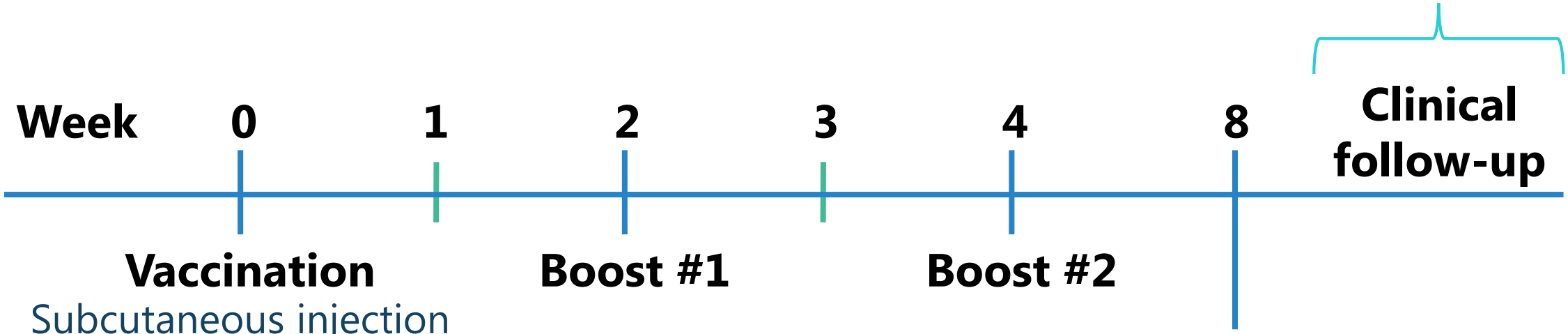
Chemical characterization

- ✓ Quantification of the protein by SDS-PAGE / densitometry
- ✓ Quantification of the adjuvant by UV-Vis spectrophotometry

METHODOLOGY Vaccination, infection and clinical follow-up

Female BALB/c mice
6-10 weeks old
***SPF* Animal Care Facility – SIU/UdeA**

Lesion area
Score (0-4), the higher the number the more severe the injury
Photographic record



METHODOLOGY

Parasitic load and antibody measurement

Parasitic load

Limiting dilution assay

Mechanical disruption of the infected ears was performed in supplemented Schneider medium → An initial dilution was performed and then 12 serial 1:3 dilutions were made in 96-well plates → Cultures were incubated for 4 weeks at 26°C and the growth of parasites was monitored weekly

Antibody measurement

Serum levels of IgG1 and IgG2a type antibodies were determined by ELISA

Overnight sensitization with rTR (antigen) was performed, then a blockade with BSA was made. Sera and, subsequently, antibody for each subclass of IgG was added → The addition of the chromogenic substrate tetramethylbenzidine (TMB) produced a colorimetric reaction whose absorbance was read at 655 nm

RESULTS Selection of quantification methods

SDS-PAGE

- ✓ Simple and reproducible
- ✓ Calibration curve with standard protein or rTR in the same gel
- ✓ Comparison of bands and estimation of quantity
- ✓ Coupled to densitometry

UV-Vis Spectro-photometry

Dissolve lipids → Avoid precipitation of CpG or rTR → Translucent homogeneous phase → Low solvent interference

Liposomal disruption with binary solvent

Mixture Chloroform:Methanol

Table 1. Dispersion of CpG or rTR liposomes with mixtures of different chloroform:methanol proportions

MIXTURE		CpG liposomes	rTR liposomes
Chloroform	Methanol		
2	1	Two turbid phases	Two turbid phases
1	1	Two translucent phases	Two phases with suspended particles
1	2	One homogeneous translucent phase	One phase with suspended particles
1	3		
1	5	One phase with suspended particles	
Ethanol		One turbid phase with suspended particles	

RESULTS Optimization of the preparation of rTR liposomes

Table 2. Determination of the optimal levels of the factors that influence the manufacturing process of rTR liposomes

PROCESS	RESULTS/FACTORS	SELECTED CONDITIONS
Lipid film formation by rotary evaporation	Synchronization of parameters such as temperature, time, rotation and pressure is required	20 min, 65°C, 180 rpm, 800 → 400 → 70 mbar
Lipid film dispersion	• Sonication produces a high percentage of small undesirable liposomes • Vortex agitation causes heterogeneous detachment of the film (aggregation of particles) • Combination of rotation while heating and agitation through vortex generates adequate hydration and homogenous detachment of the film	• Rotation/heating: 5 min, 65°C, 180 rpm • Vortex agitation: 50 s, 1500 rpm
Size homogenization by extrusion	11 extrusions through polycarbonate membranes (1000 nm)	
Separation by centrifugation	Centrifugation time depends on stability (PDI and %EE)	6 / 3 min, 4°C, 21000 rcf
Other important factors	• Lipid amount and proportion • Hydration medium • Centrifugation volume	• PC:SA:Ch 7:1,5:4 (30 mg) • PBS 0,1X (pH 5,8) • 270 µL

RESULTS

Optimization of the preparation of rTR liposomes

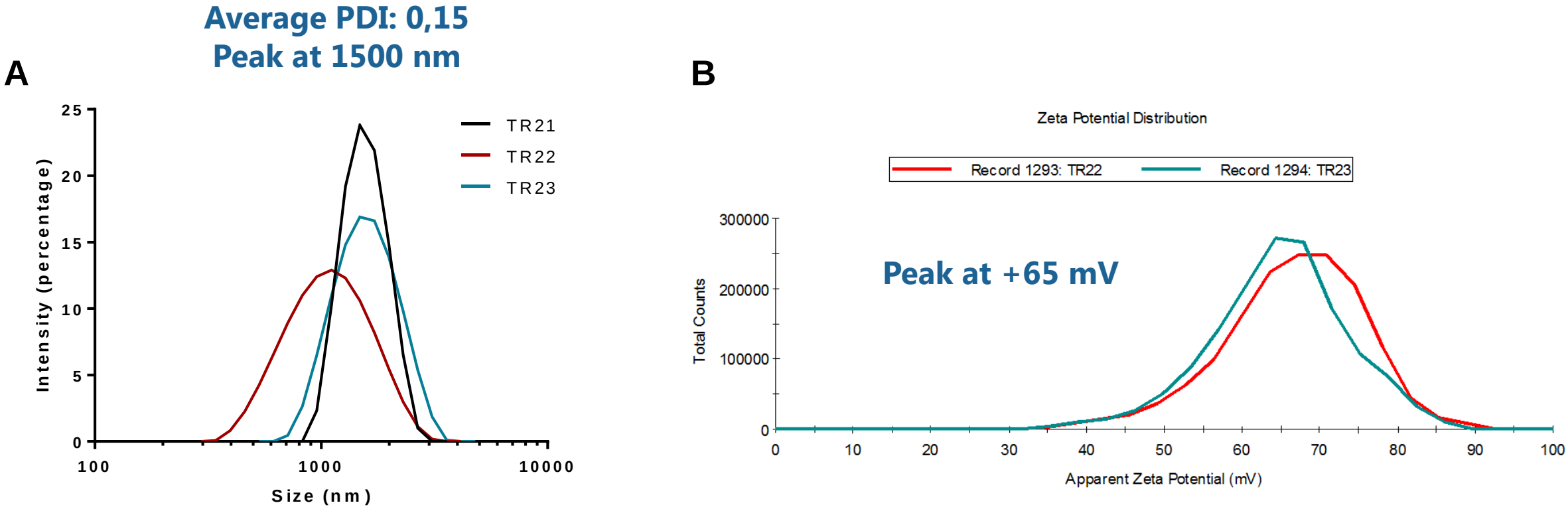
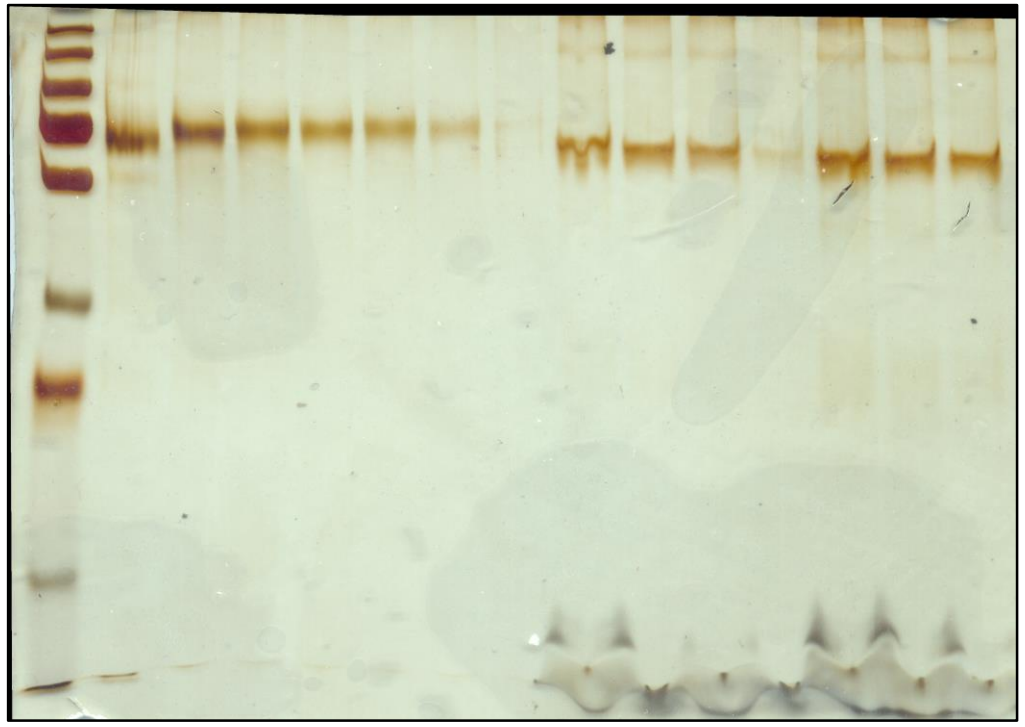


FIGURE 1. ***rTR liposomes.*** **(A)** Size distribution of three batches of rTR liposomes produced under the same conditions. Average PDI: 0.15. **(B)** Distribution of apparent zeta potential (mV) of two batches of rTR liposomes. A peak at +65 mV is appreciated in both batches.

RESULTS rTR quantification

A



B

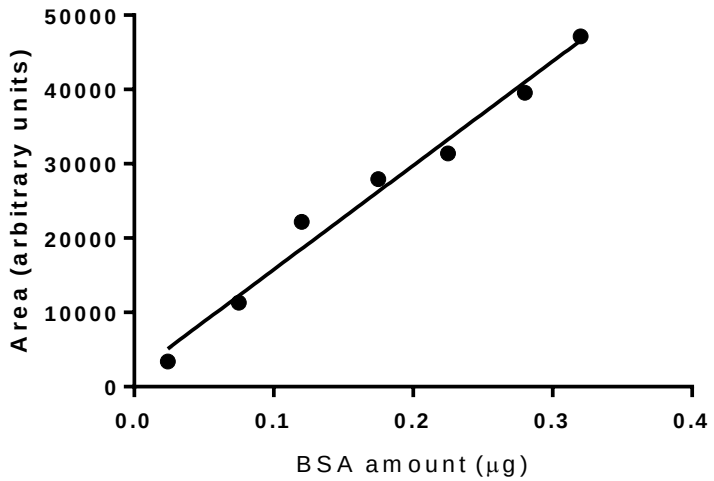


FIGURE 2. ***rTR quantification.*** **(A)** Polyacrylamide gel electrophoresis (SDS-PAGE) of liposomal samples (TR22, different stages of the process) and calibration curve with BSA. In **(B)** a calibration curve is presented for the quantification of rTR which was obtained from each SDS-PAGE for each analyzed batch.

Table 3. Summary of rTR quantification results

Parameter	rTR liposomes
Adjusted amount of analyte in 100 µL (µg)	5.0
Theoretical encapsulation efficiency (%EET)	20.00
Experimental encapsulation efficiency (%EEE)	51.20

RESULTS

Optimization of the preparation of CpG liposomes

Table 4. Determination of the optimal levels of the factors that influence the manufacturing process of CpG liposomes

Lipid proportion (PC:SA:Ch)	Lipid amount (mg)	Hydration medium	[CpG] (µg/mL)	Dispersion by rotation/heating	Size homogenization by extrusion	Separation by centrifugation
7:2:2	20,7	PBS 0.1X pH=5,8	**200**	**15 min**, 65 °C, 180 rpm	7 extrusions through polycarbonate membranes (1000 nm)	12 min, 4°C, 21000 rcf

The interaction between materials depends on the overcoming of electrostatic repulsion through modulation of temperature, mechanical agitation and progressiveness in the formation of stable vesicles that shield, as they are formed, the CpG negative charge

We standardized a liposomal formulation which reproducibly encapsulated CpG
Optimal levels were effectively adjusted and reproducible response variables were obtained: Supranometric size (peak at 900-1000 nm), cationic zeta potential (+65 mV) and physical stability for at least six weeks

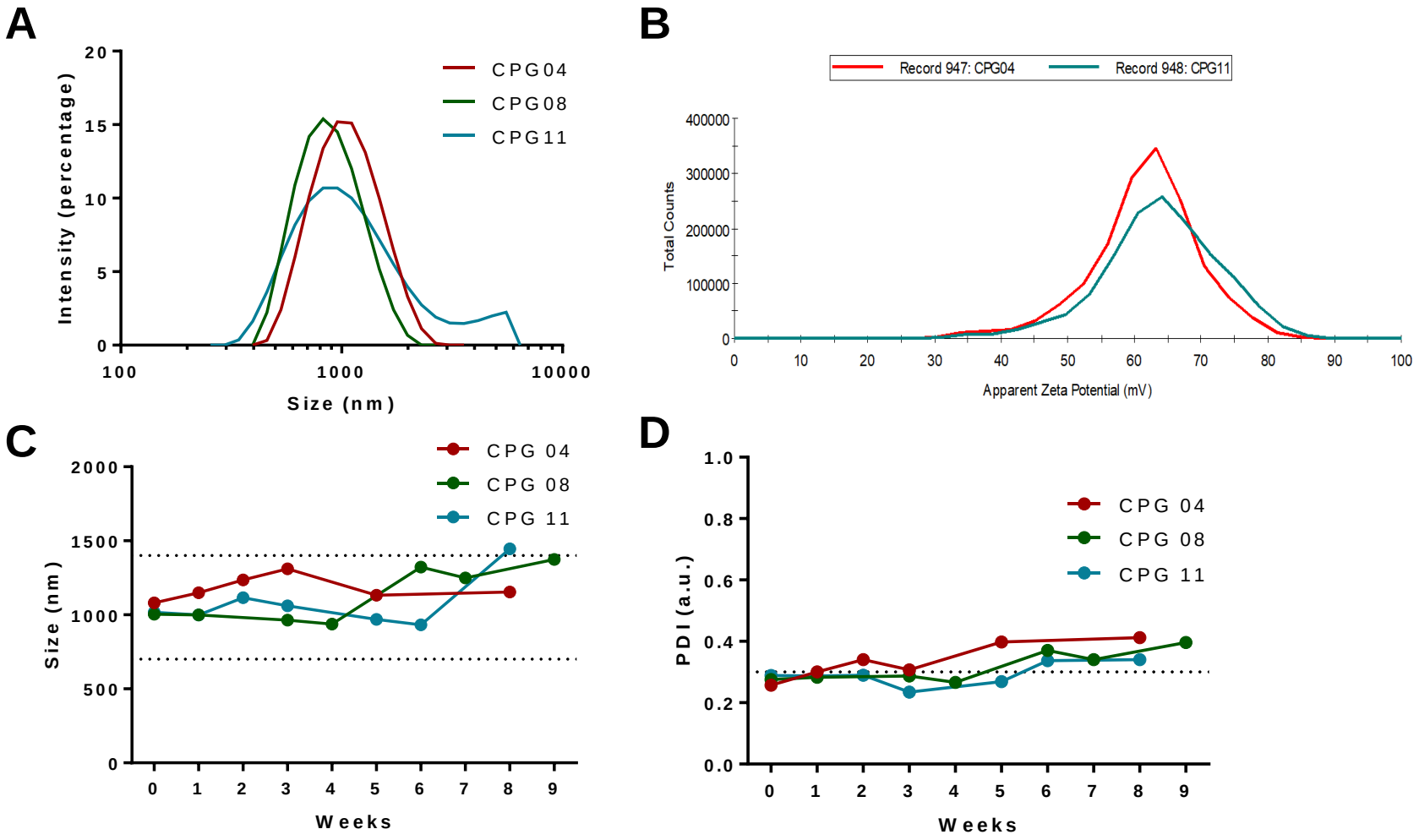


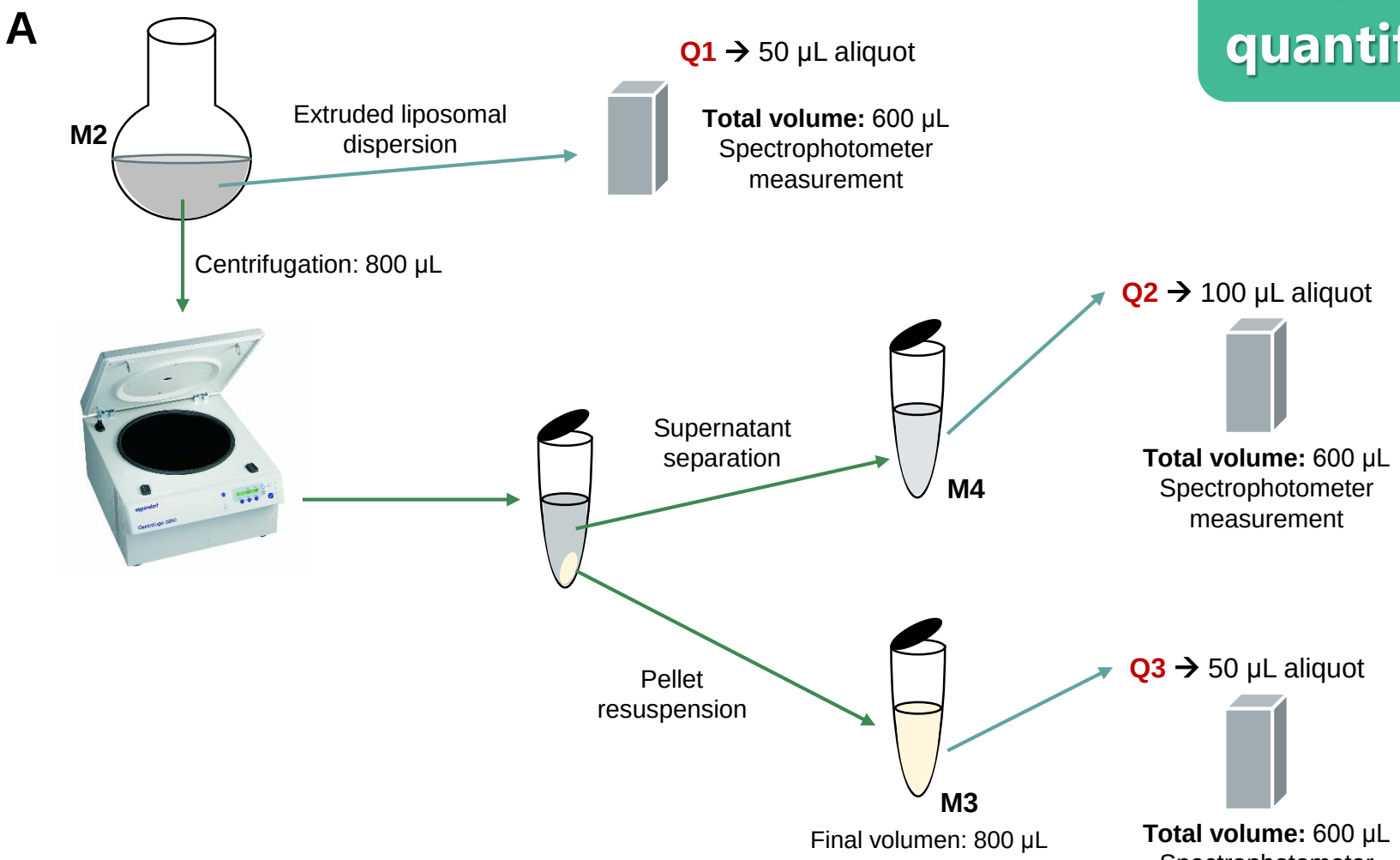
FIGURE 3. *CpG liposomes.* (A) Size distribution of three batches of CpG liposomes. **(B)** Distribution of apparent zeta potential (mV) of two batches of CpG liposomes (CPG04 and CPG11) which present a peak at +65 mV. **(C,D)** Stability of the CpG liposomes over time (weeks) according to variation of the **(C)** size (nm) and **(D)** PDI (a.u.). In **(C)**, it is indicated with dotted horizontal lines the sizes that are considered adequate: between 700 nm and 1400 nm. In **(D)**, the dotted horizontal line indicates the maximum value of PDI considered acceptable: 0.3. PDI: polydispersity index, a.u.: arbitrary unit.

RESULTS CpG quantification

Direct quantification

Evaluation of the reliability of the method

Calculations regarding theoretical and experimental values



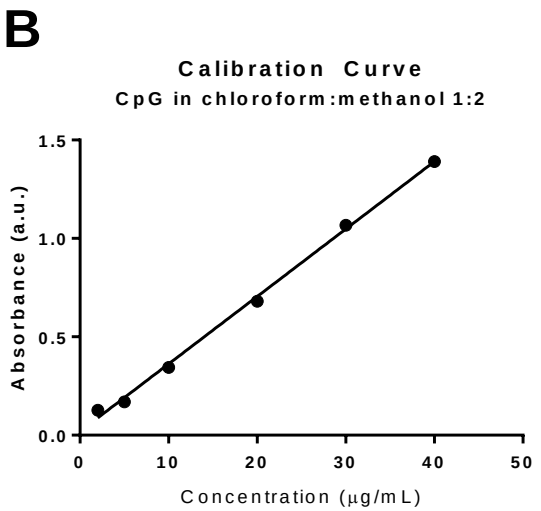


FIGURE 4. ***CpG quantification.*** **(A)** Schematic representation of the sampling system for the direct quantification of CpG in several stages of the manufacturing process of liposomes. In **(B)** a calibration curve is presented for the quantification of CpG.

RESULTS

CpG quantification

Table 5. Summary of CpG quantification results

Parameter	Mean of calculated values (n=8)
Percentage of CpG in resuspended pellet (%)	89
Percentage of CpG in dispersion (%)	94
Theoretical encapsulation efficiency (%EET)	64.53
Experimental encapsulation efficiency (%EEE)	69.16

Main effects that influence liposome preparation

- Molecule nature
- Formula and lipid amount
- Extrusion parameters
- Origin and pH of PBS
- Temperature exposure
- Centrifugation volumes and times

RESULTS Employed liposomes for *in vivo* experiment

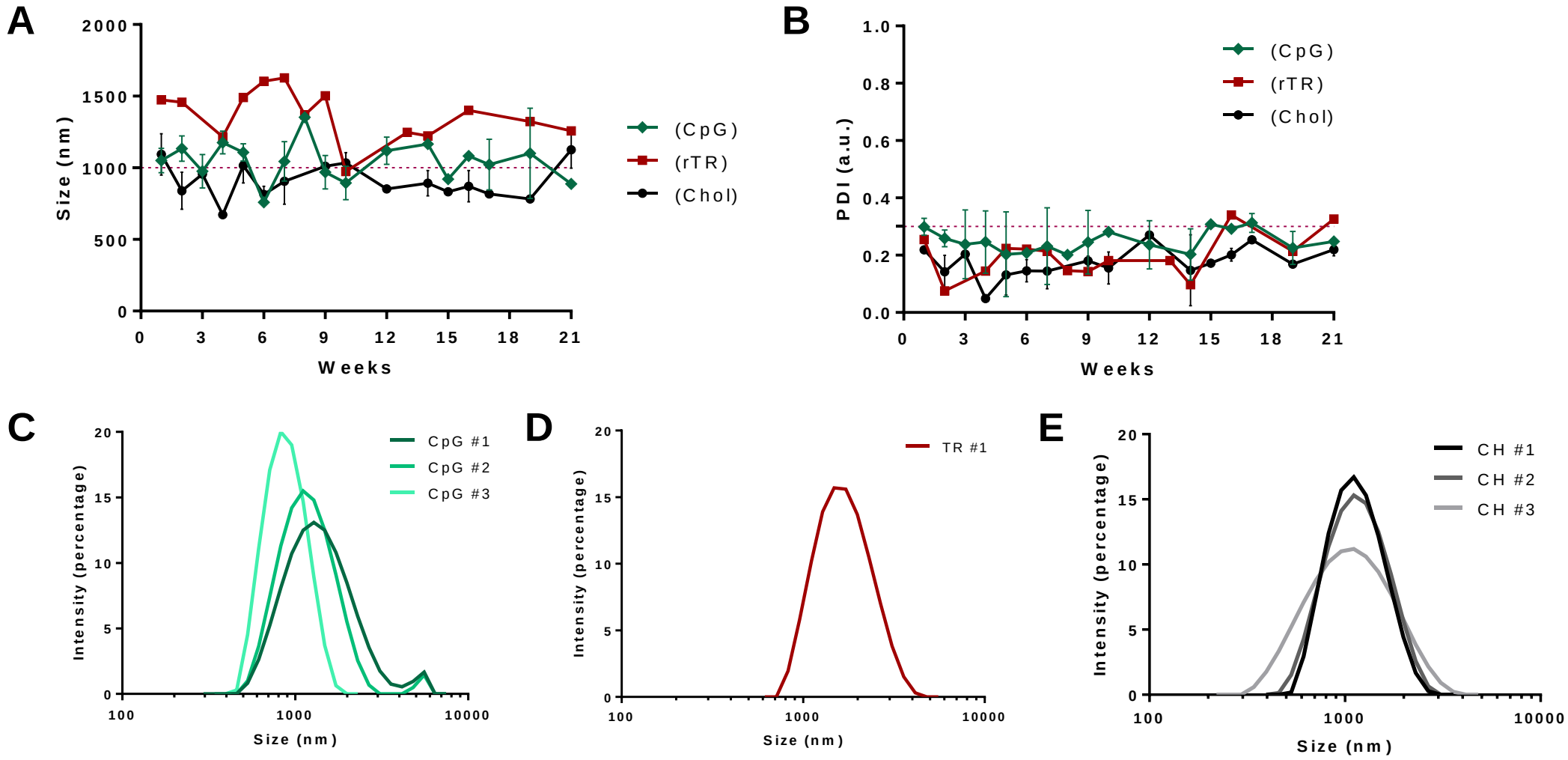


FIGURE 5. ***Employed liposomes for in vivo experiment.*** **(A-B)** Stability of the employed liposomes for *in vivo* experiment, over time (21 weeks) according to variation of the **(A)** size (nm) and **(B)** PDI (a.u.). In **(A)**, it is indicated with a dotted horizontal line the 1000 nm size as guidance. In **(B)**, the dotted horizontal line indicates the maximum value of PDI considered acceptable: 0.3. **(C-E)** Size distribution of the batches of liposomes produced under the same conditions, **(C)** CpG liposomes, **(D)** rTR liposomes, **(E)** PBS liposomes –negative control–. PDI: polydispersity index, a.u.: arbitrary unit.

RESULTS

In vivo experiment

Experimental groups of treated mice
() = liposomal; example: (CpG) = CpG encapsulated in liposomes
Dosages: rTR → 5 µg, CpG → 12,5 µg

PBS

(PBS)

(CpG)

(rTR)

(rTR) + CpG

rTR + (CpG)

(rTR) + (CpG)

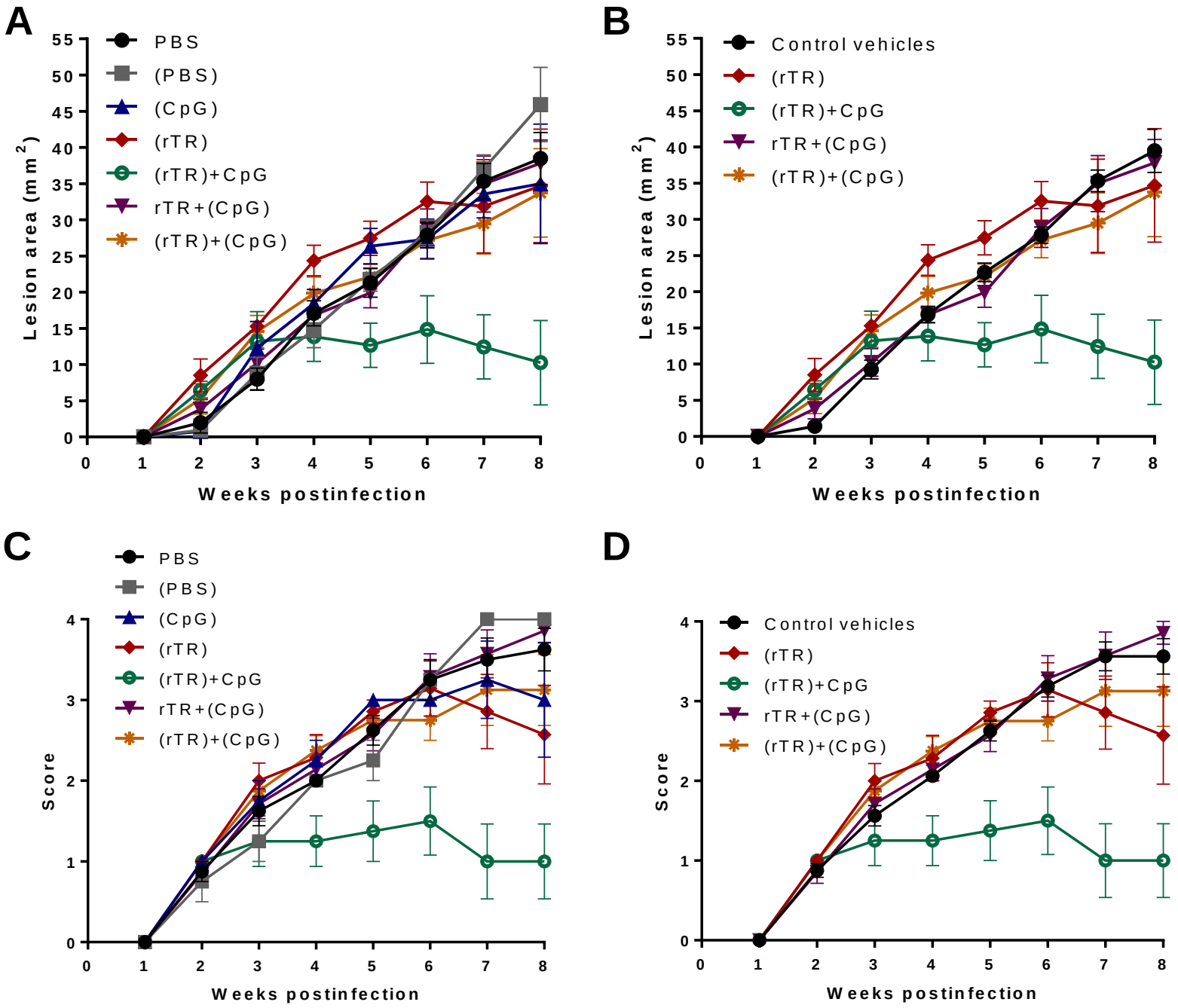


FIGURE 6. ***Lesion area kinetics and score measurements, clinical follow-up, from the in vivo experiment.*** BALB/c mice were infected in the dermis of the ear and then it was performed the clinical follow-up in terms of **(A-B)** lesion area and **(C-D)** score. In **(B,D)** negative control mice were pooled: PBS, (PBS) and (CpG).

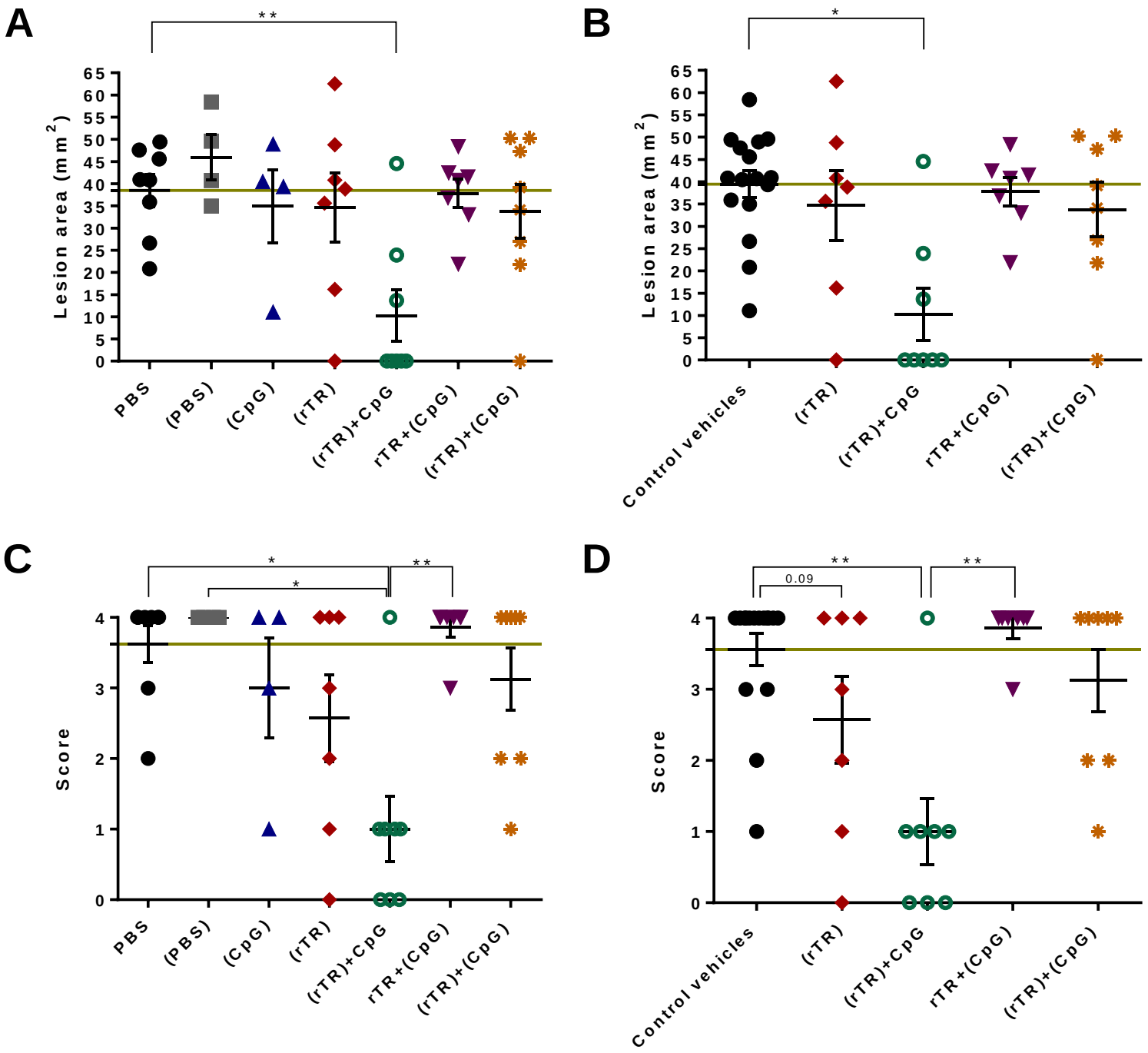


FIGURE 7. ***Lesion area and score of the mice in week 8 post-infection.*** In week 8 post-infection, clinical measurements from each mice are shown in terms of **(A-B)** lesion area and **(C-D)** score. In **(B,D)** negative control mice were pooled: PBS, (PBS) and (CpG). Statistic analysis was performed as follows: **(A)** Unpaired Mann-Whitney non-parametric *t*-test. **(B-C)** One-way ANOVA (Kruskal-Wallis non-parametric test) with Dunn's multiple comparisons test. In **(D)**, the statistical analysis between control vehicles or rTR+(CpG) and (rTR)+CpG was performed using the same approach as **(B-C)**. Also, in **(D)**, the P<0,09 between control vehicles and (rTR) was obtained as explained for **(A)**. P<0,05 in any case except for comparison between control vehicles and (rTR) in **(D)**. The olive green horizontal line for each graph is plotted to show the mean values of the PBS or control vehicles groups.

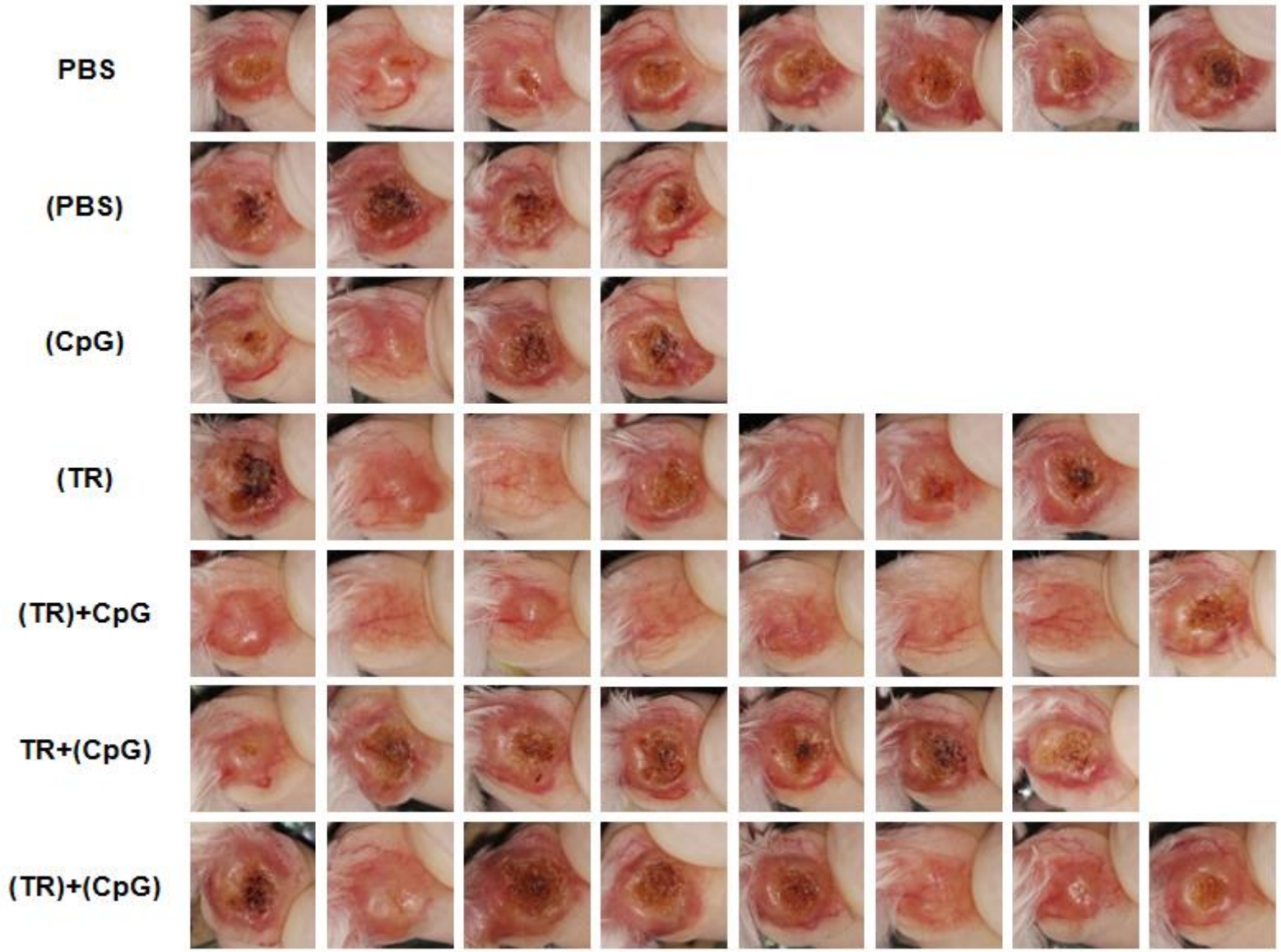


FIGURE 8. ***Clinical appearance of the lesions shown with photographs of the infected ears for each mice, in each group, in week 8 post-infection.***

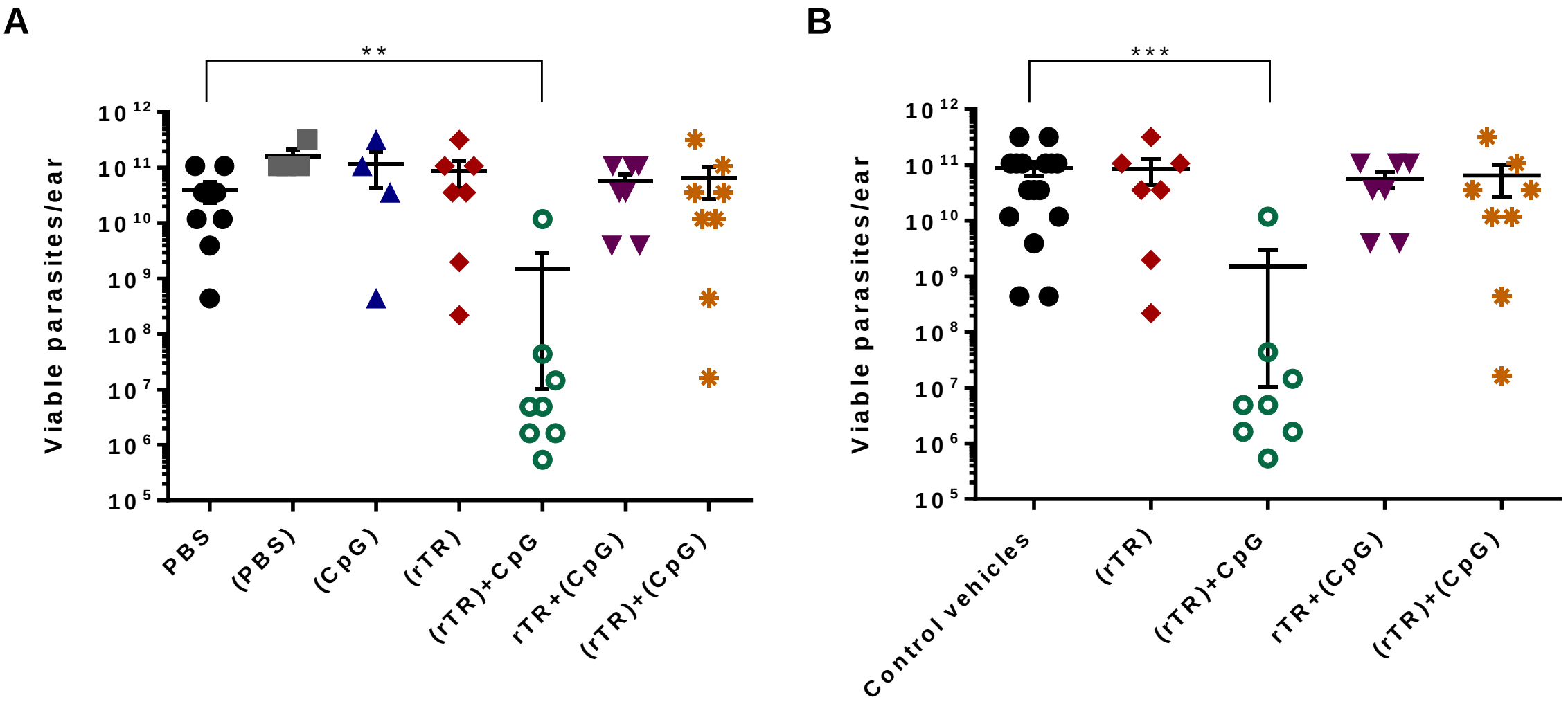


FIGURE 9. ***Parasitic loads from the in vivo experiment.*** Viable parasites per ear for each mice group with **(A)** individual negative controls or **(B)** pooled negative controls [control vehicles] – PBS, (PBS) and (CpG). Statistic analysis was performed as follows: **(A)** Unpaired Mann-Whitney non-parametric *t*-test. **(B)** One-way ANOVA (Kruskal-Wallis non-parametric test) with Dunn's multiple comparisons. P<0,05 in any case.

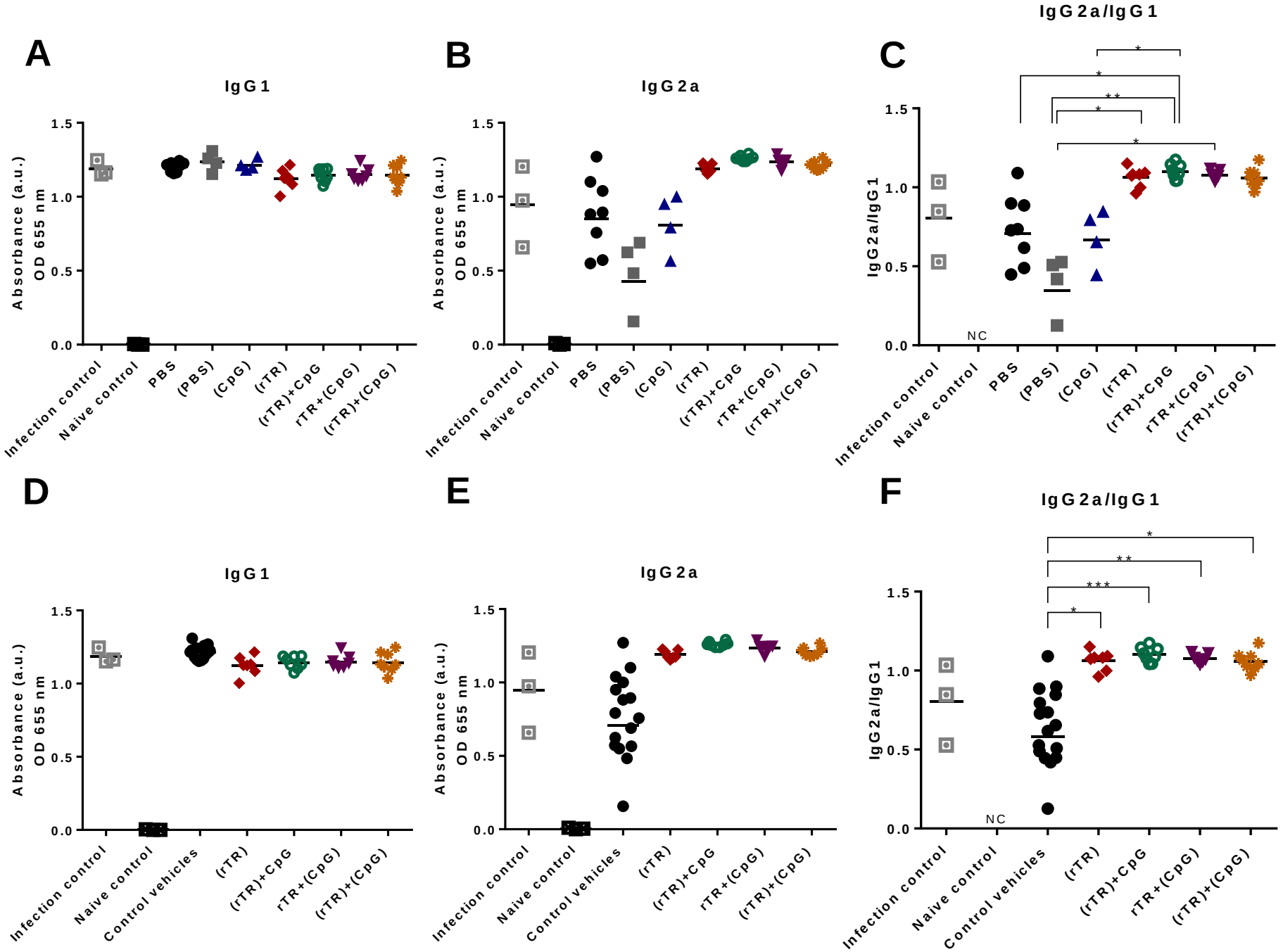


FIGURE 10. ***Serum levels of IgG1 and IgG2a type antibodies.*** **(A,B,D,E)** Absorbance (a.u.) or optical density (OD) at 655 nm of the chromogenic reaction to determine anti-rTR **(A,D)** IgG1 or **(B,E)** IgG2a antibodies. **(C,F)** calculated IgG2a/IgG1 ratio from the measurement of the anti-rTR antibody levels determined in the sera of the mice after euthanasia (8 weeks post-infection) [shown in **(A,B,D,E)**]. **(A-C)** Individual negative controls or **(D-F)** pooled negative controls [control vehicles] – PBS, (PBS) and (CpG). Statistic analysis was performed as follows: one-way ANOVA (Kruskal-Wallis non-parametric test) with Dunn's multiple comparisons. P<0,05 in any case. a.u.: arbitrary units.

CONCLUSIONS

Cationic liposomes with homogeneous supranannometric size were prepared with high CpG encapsulation efficiency

Preparation of micrometric cationic vesicles that encapsulate rTR protein was standardized without degrading nor aggregating it

Encapsulation efficiency was ~70% for CpG liposomes, and ~20% for rTR liposomes, and the procedure was reproducible enough to perform an *in vivo* experiment

87,5% [7/8] of the mice vaccinated with (rTR)+CpG were protected against the infectious challenge, with lower parasitic load and higher IgG2a/IgG1 antibody ratio vs. control vehicles

PERSPECTIVES

Co-encapsulation of CpG and rTR

Optimization the scheme and dosage of the vaccine to increase effectiveness

Advanced characterization of the most effective liposome formulation

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COLCIENCIAS

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Project CPT-1217

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THANK YOU