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# The adjuvanticity of *Chiococca alba* saponins increases with the length and hydrophilicity of their sugar chains

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ABSTRACT

The saponins of *Chiococca alba* are triterpene bidesmosides that contain glycidic moieties attached to the C-3 and C-28 carbon of their aglycone. We describe that their adjuvant potential increases in direct relationship to the length and hydrophilicity of the C-28 attached sugar chain which contains: arabinose–rhamnose in the CA2, arabinose–rhamnose–xylose in the CA3X; arabinose–rhamnose–apiose in the CA3 and arabinose–rhamnose–apiose–apiose in the CA4 saponin. The hydrophile/lipophile balance calculated for CA2 was 12.7, for CA3 and CA3X was 15.8 and for CA4 19.9. All saponins were formulated with the FML antigen for mice prophylaxis against visceral leishmaniasis. The immune response was studied using an ELISA-antibody assay and monitoring of the intradermal response (IDR) to *Leishmania* antigens, the cytokine expression in supernatants and the intracellular staining of *in vitro* cultured splenocytes. After challenge, significant increases of IgG and IgG2a antibodies were noted only in the CA4 vaccinated mice that showed extended IDR, higher IFN-γ production by CD8+ and TNF-α production by CD4+ T cells, higher TNF-α secretion and the highest reduction of the parasite load (78%). The increases in IDR, CD4–TNF-α, CD8–IFN-γ and CD8–TNF-α by the CA4 vaccine were strong correlates of protection and were significantly correlated to the decrease of parasite load ($p = -0.007$). Protection generated by the CA4 vaccine was mainly mediated by a CD4+ T cell and a TNF-α driven response with a lower contribution of CD8+ T cells, as confirmed by an *in vivo* depletion with monoclonal antibodies and by vaccination assays in TNF-α-receptor knock-out mice. Our results confirm that the superiority of the CA4 saponin is related to the higher hydrophilicity of its longer carbohydrate chain. *C. alba* saponins were non-toxic and only the xylose-containing saponin CA3X was hemolytic ($HD_{50} = 87$ μg/ml). The increase in sugar units of the saponins is positively correlated to the increase of IDR and to the decrease of parasite load.



## 1. Introduction

Leishmune®, the first licensed vaccine against visceral leishmaniasis has a 76–80% of vaccine efficacy. This veterinary vaccine protects 98% of vaccinated dogs and blocks the transmission of the disease in endemic areas [1–3]. In the Americas and the Mediterranean, visceral leishmaniasis is an immunosuppressive zoonotic disease transmitted from dogs to humans through the byte of a sand fly vector [4]. The disease is fatal in humans and dogs if untreated and treatment is highly toxic and not always efficient. The epidemiological control of the disease includes the treatment of human cases, insect vector control with insecticides and the culling of seropositive/infected dogs. Human or canine vaccines are expected to be effective tools for the prophylactic control of epidemics [5]. The recent canine vaccinations with the Leishmune® vaccine in Brazil reduced the incidence of human cases, human deaths and dog prevalence of visceral leishmaniasis in endemic areas [6]. In districts where the vaccinations occurred the canine and human incidence decreased or achieved a stabilized plateau while in non-vaccinated districts the incidences rose [6].

Leishmune® is the FML-saponin vaccine [1,3,7,8] composed of the FML (Fucose Mannose ligand) antigen [9], a complex glycoproteic fraction of *Leishmania donovani*, and a *Quillaja saponaria* saponin adjuvant (Riedel de Haën-Sigma) [revised in 3]. The main

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active components of the Leishmune® adjuvant are the well known QS21 saponin and the two deacylated saponins that only differ from the QS21 due to the absence of the hydrophobic moiety [10].

Saponins are a structurally diverse class of natural compounds occurring in several plant species. According to previous reports the most common components of the saponin core are the triterpenoid and steroid aglycones to which carbohydrate chains are attached [11]. They exhibit from one to three straight or branched sugar chains which most often include D-glucose, L-rhamnose, D-galactose, D-glucuronic acid, L-arabinose, D-xylose or D-fucose. The sugar chain can contain from one or more monosaccharide residues, and is usually attached at the C-3 of the triterpene [11].

The correlation between structure and function of saponins has been the focus of intensive research in order to define the essential moieties for the development of the adjuvant activity [10–15]. Saponins with steroid but not with triterpene aglycones are considered to be the most hemolytic [12,16]. Alternatively, the hemolytic or membranolytic activity has been attributed to the oligosaccharide moiety of saponins [13,17–22]. And the saponins with two glycidic chains attached to the aglycone, called bidesmosidic [10,14], have been shown to be more immunogenic than the monodesmosidic ones [14].

In the QS21 saponin of *Q. saponaria* Molina, the induction of a lymphoproliferative CD8+ T cell response is attributed to the normonoterpene hydrophobic moiety while the induction of a TH1 response is related to the C-23-aldehyde of the triterpene nucleus [15,23]. The different spatial conformation of the C-23 aldehyde group defines the type of induced immune response [17]. An enhanced humoral immune response was obtained using an enriched axial aldehyde-containing sapogenin while an enhanced cellular immune response (increased DTH and IFN-γ sera levels) that determined a 77% reduction of liver parasitic load was obtained using an enriched equatorial aldehyde-containing QuilA-sapogenin [17].

The *Q. saponaria* saponins, which lack the hydrophobic moiety of QS21, are capable of inducing increases in DTH, CD4+ T lymphocytes in spleen, IFN-γ *in vitro*, body weight gain and a pronounced reduction of parasite burden in the liver, suggesting that the immunoprotective potential of the saponin relies more on its carbohydrate chains than on its hydrophobic attached moiety [10]. Similar to QS21, the CP05 saponin of *Calliandra pulcherrima* is composed of a triterpene nucleus with two carbohydrate fractions attached to C-3 and C-28, respectively, and one hydrophobic moiety acylated to a sugar attached to C-28 [24]. The chemical removal of the hydrophobic monoterpene moiety of CP05 did not interfere with the protection but the removal of one or two of the carbohydrate chains, however, abolished protection and determined an increase of the parasite load indicating that, as postulated for other saponins [25–27], and in the case of the CP05 saponin also, the induction of protection is directly related to the presence of the carbohydrate moieties [14].

Considering the relevance of the carbohydrate moieties to the adjuvant potential of saponins, and the evidence that the immunoprotective potential increases in direct relation to the number of sugar units on the carbohydrate chains [19,22] this work investigated, two saponins of *Chiococca alba* (CA3 and CA4) [28] which differ only in one sugar unit. These two saponins were compared in the murine vaccination against visceral leishmaniasis with the FML antigen. The QS21-containing saponin adjuvant of the Leishmune® vaccine (saponin R) was used as a positive control.

The CA3 and CA4 saponins of *C. alba* are two typical Glucuronide Oleanane-type Triterpene Carboxylic Acid 3,28-*O*-Bisdesmosides (GOTCAB). Their structures were recently elucidated [28]. Both share a triterpene nucleus to which a glucuronic acid is attached at C-3 and an apiose–rhamnose and arabinose chain is attached at C-28 (Fig. 1). The CA4 shows the same triterpene and sugar chains with one additional apiose unit 1 → 3 linked to the rhamnose unit of the C-28 carbohydrate chain (Fig. 1). The QS21 saponin on the other hand is more complex but also, similar to the *C. alba* saponins, shows a triterpene nucleus to which is attached one glucuronic acid at C-3, substituted with galactose and xylose and a 5-sugar-unit chain (rhamnose–xylose–apiose and fucose and arabinose) at C-28 that also includes a hydrophobic moiety composed of 2 normonoterpenes [29].

In this investigation we pursued the analysis of the adjuvant potentials of CA3 and CA4 saponins of *C. alba* aiming to identify if the addition of one sugar unit has any impact on the immunoprotective potential of the saponin.

## 2. Materials and methods

### 2.1. Ethical statements

All mouse studies followed the guidelines set by the National Institutes of Health, USA and the Institutional Animal Care and Use Committee approved the animal protocols (Biophysics Institute-UFRJ, Brazil, protocol IMPPG-007).

### 2.2. Isolation of saponins of C. alba (L.) Hitch

Samples of *C. alba* were collected in Nova Friburgo, Rio de Janeiro, Brazil. The botanical identification was made by Dr. Sebastião Neto, and a voucher specimen (RB395399) has been deposited in the Herbarium of the Rio de Janeiro Botanical Garden. Air-dried and powdered roots of *C. alba* (400 g) were extracted with ethanol. The extract was evaporated and the residue obtained (12 g) was suspended in water and successively partitioned with methylene chloride and butanol. The butanol fractions were combined, evaporated and the residue (4 g) was suspended in methanol and subjected to controlled precipitation with diethyl ether. The precipitate (2 g) was fractionated by column chromatography (octadecylsilane, 60 cm × 20 cm) using $H_2O$ with increasing proportions of methanol (0–100%) to obtain 10 fractions. TLC tests carried out with Liebermann–Bouchard and sulfuric orcinol reagents together with the observation of an abundant foam formation, allowed the identification of the saponin enriched fractions. Further purification was carried out with reversed-phase (octadecylsilane) preparative HPLC using methanol: 0.02% aqueous trifluoroacetic acid (60:40; v/v) to obtain 48 mg of CA3 (Chiococca saponin II) and 78 mg of CA4 (Chiococca saponin I) [28]. We also collected and identified two other saponins of *C. alba* to be used as controls: the CA2 (18 mg) and the CA3X (10 mg) (Fig. 1). All saponins (CA4, CA3, CA3X and CA2) share a triterpene nucleus to which a glucuronic acid is attached at C-3 and a rhamnose and arabinose containing chain is attached at C-28 (Fig. 1). The CA3X and CA3 have a third sugar attached 1 → 4 to the rhamnose unit. This third sugar is xylose in CA3X and apiose in CA3. The CA4 saponin has, in addition to the 1 → 4 linked apiose present in CA3, a fourth apiose unit, 1 → 3 linked to the rhamnose unit of the C-28 carbohydrate chain (Fig. 1). The hydrophile–lipophile balance (HLB) value of the saponins was calculated theoretically by the Davies and Riedel method [30] considering their chemical structure as previously described by Borges et al. [28] and represented in Fig. 1. The value was calculated by integrating the number of each functional group composing the saponin molecule with the group unit defined by the Davies method ($HLB = 7 + \sum$ hydrophilic groups $- \sum$ lipophilic groups) [30].

### 2.3. Hemolytic assay

Normal human red blood cell suspension (0.1 ml of 0.5%) was mixed with 0.1 ml diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 μg/ml concentrations of the Riedel de Haën and the CA4,

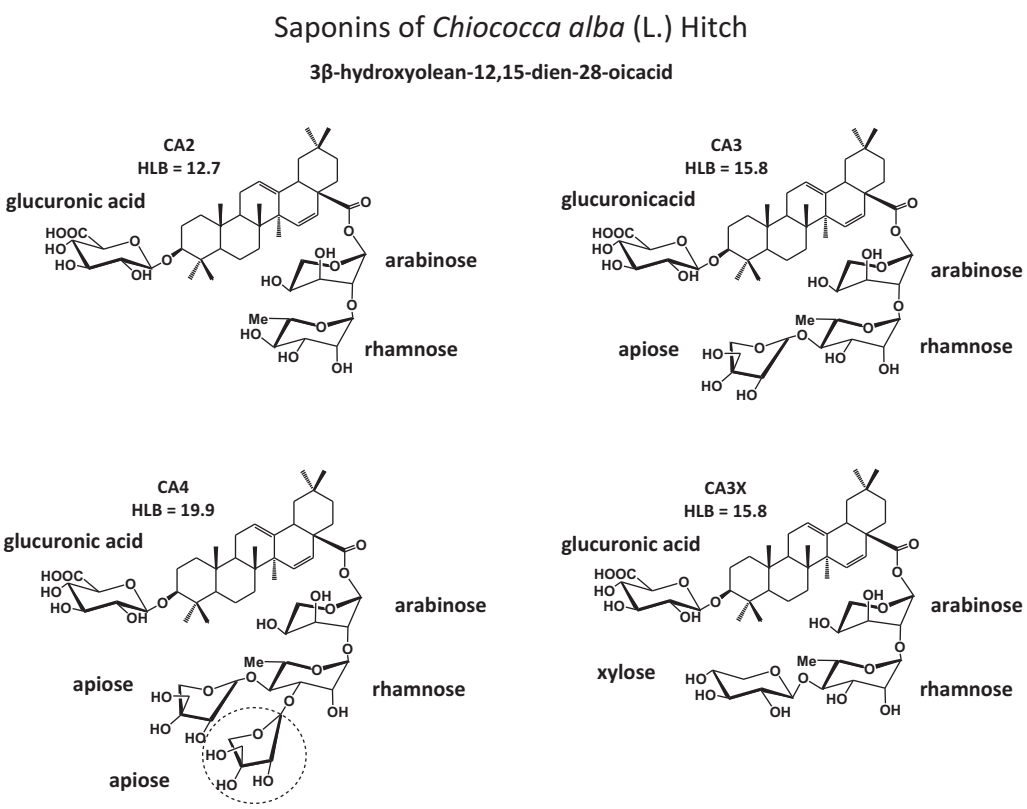


**Fig. 1.** Chemical structure of the CA4, CA3, CA3X and CA2 saponins isolated from the roots of *Chiococca alba* (L.) Hitch.

CA3, CA3X and CA2 saponins in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70 × *g* for 10 min. Free hemoglobin in the supernatants was measured by absorbance at 415 nm [21]. Saline and distilled water were included as minimal and maximal hemolytic controls. The hemolytic percent developed by the saline control was subtracted from all groups. The adjuvant concentration inducing 50% of the maximum hemolysis was considered as the $HD_{50}$ (graphical interpolation). Each experiment included triplicates at each concentration. A series of 3 independent experiments was performed for the analysis of each $HD_{50}$. Human red blood cells for the hemolytic assay were obtained from healthy adult blood bank donors (Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, RJ, Brazil). The red blood cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution.

### 2.4. In vivo saponin toxicity

Toxicity (assessed by lethality, local pain, local swelling, and loss of hair) was tested in the vaccinated mice that received 100 μg of either Riedel de Haën or each one of the *C. alba* saponins formulated with the FML antigen, as three weekly doses. The mice were monitored for seven days after each vaccine dose.

### 2.5. Immunization and parasite challenge by Leishmania chagasi

Eight-week-old female Balb/c mice, received 3 doses of 150 μg of the FML antigen [9] and 100 μg of either the CA3, CA4 saponins of *C. alba* or of the Sigma-Riedel de Haën 16109 saponin [reviewed in 3] on the back, through the sc route, at weekly intervals. At the beginning of week 4, mice were challenged with $3 \times 10^7$ *L. chagasi* amastigotes obtained from infected hamster spleens. The strain used for challenge in this study (IOC-L 3324) was originally isolated from the spleen of an infected dog of Andradina, São Paulo, Brazil and taxonomically characterized as *Leishmania L. chagasi* by the CLIOC-WDCM 731 (Instituto Oswaldo Cruz *Leishmania* collection, Rio de Janeiro, Brazil). Fifteen days after infection, mice were euthanized with ether and the parasite load was evaluated in Giemsa-stained liver smears and expressed in LDU values (Leishman Donovan units of Stauber = number of amastigotes per 600 liver cell nuclei/mg of liver weight) as described [reviewed in 3]. The increase in total body weight and liver/corporal relative weight were also recorded as clinical signs of VL. Control experiments in Balb/c female mice also included groups treated with saponins CA2 and CA3X.

### 2.6. Detection of antibodies

Seven days after immunization and 15 days after infection with *L. chagasi*, antibodies of sera were measured by an ELISA assay against FML antigen as previously described [31], using 2 μg antigen per well and Protein-A peroxidase (KPL, Kirkegaard & Perry Laboratories, Inc.) or goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA horseradish peroxidase conjugated antibodies (Southern, Biotechnology Associates, Birmingham, AL, USA) in a 1:1000 dilution in blocking buffer. The reaction was developed with *O*-phenyldiamine (Sigma), interrupted with 1 N sulfuric acid, and monitored at 492 nm. Each individual serum was analyzed in triplicate in double-blind tests. Positive and negative control sera were included in each test. Results were expressed as the mean of the absorbance values (492 nm) of the 1/100 diluted sera of each animal.

### 2.7. Assessment of the specific T cell immunity

Seven days after immunization and 15 days after infection with *L. chagasi*, the intradermal response against *L. donovani* lysate (IDR) was measured in the footpads as described earlier [32]. Briefly, mice

were injected intradermally, in the right hind footpad, with $10^7$ freeze–thawed stationary phase *Leishmania donovani* promastigotes (LD-1S Sudan strain) (200 μg of protein) in 0.1 ml sterile saline solution. The footpad thicknesses were measured with a Mitutoyo apparatus, both before and 0, 24 and 48 h after injection. Injecting each animal with 0.1 ml saline in the left hind footpad served as control. At each measurement, the values of the saline control were subtracted from the reaction due to the *Leishmania* antigen. Previous experiments carried out in Balb/c mice and CB hamsters demonstrated that 24 h after inoculation saline treated footpads returned to base levels [32].

We also compared the IDR induced in immunized and in challenged mice by the injection of either the promastigote lysate (200 μg of protein), or the FML antigen (100 μg), or the NH36 recombinant protein (100 μg), in 0.1 ml of saline solution.

Further analyses of cellular immune responses was carried out using $10^6$ splenocytes after 5 days of *in vitro* culturing at 37 °C and 5% $CO_2$ in RPMI medium and/or 5 μg of recombinant NH36, the main antigenic component of the FML antigen [31]. Secretion of IFN-γ and TNF-α was evaluated in the supernatants of *in vitro* cultured splenocytes by an ELISA assay, using the Biotin Rat anti-mouse IFN-γ (clone XMG1.2), the purified Rat anti-mouse IFN-γ (clone R4-6A2) and the Mouse TNF ELISA Set II kit (BD Bioscience Pharmingen) according to the manufacturer's instructions. Flow cytometry analysis (FACS analysis) in a FACScalibur apparatus was performed after splenocyte immunostaining with anti-CD4 (clone GK1.5) or anti-CD8-FITC (clone 53-6.7) monoclonal antibodies (R&D systems, Inc.). The intracellular production of IFN-γ, TNF-α and IL-10 cytokines by CD4+ and CD8+ T cells was determined using 10 mg/ml brefeldin (Sigma) for 4 h at 37 °C and 5% $CO_2$ followed by washing with FACS buffer (2% fetal calf serum, 0.1% sodium azide in PBS). Cells were labeled for 20 min at 4 °C in the dark with rat anti-mouse CD4FITC and CD8FITC (R&D systems) in FACS buffer (1/100). After that they were fixed with 4% paraformaldehyde, washed and treated with FACS buffer with 0.5% saponin (Sigma) for 20 min at room temperature and then further stained with IFN-γ-APC, TNFPE and IL-10PE monoclonal antibodies (BD-Pharmingen), 1/100 diluted in FACS buffer with 0.5% saponin for 20 min, and finally washed and resuspended in FACS buffer. A total of 30,000 cells were analyzed by flow cytometry in a Becton Dickinson FACScalibur apparatus, and further analyzed using WinMDI (Windows Multiple Document Interface Flow Cytometry Application) Version 2.8 software [31].

*In vivo* depletion of CD4+ or CD8+ T cells was performed by treating CA4 saponin and FML vaccinated mice with GK1.5 or 53.6.7 rat IgG MAb on days 2, 4 and 6 before challenge and on day 7 after challenge. Control mice received the CA4-FML vaccine and 0.05 mL of rat serum through the intraperitoneal route, equivalent to 0.25 mg of IgG, or nude mice ascitic fluids containing 0.25 mg of anti-CD4+ and/or anti-CD8+ antibodies. As determined by FACS analyses, the efficacy of depletion of CD4+ or CD8+ spleen cells before challenge was of 99.94% or 96% in anti-CD4+ or anti-CD8+ treated mice, respectively. The efficacy of depletion treatment was monitored by the increase in liver parasite load and liver relative weight, 15 days after infection.

Randomly selected female TNF KO mice ($n = 15$) and their wild-type (WT) littermates ($n = 15$), generated on a C57BL/6 background, were used in these experiments. Groups of five mice were vaccinated with CA3 or CA4 saponin in combination with FML-antigen or with saline and were injected *via* the tail vein with $3 \times 10^7$ hamster spleen-derived *L. chagasi* amastigotes (IOC-L 3324). The IDR was determined after immunization and 15 days after infection, visceral infection was monitored microscopically using Giemsa-stained liver imprints, and liver parasite burdens were measured in livers by counting in a blinded fashion the amastigotes per 600 cell nuclei and multiplying this number by the liver weight in milligrams (LDU units).

### 2.8. Statistical analysis

Differences between means were compared by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests (Analyze-it). For the analysis of dependent data of the same individuals before and after infection the Wilcoxon Signed-Rank two-tailed test was used, which is the non-parametric alternative of the *t*-test for correlated samples of the VassarStats program (http://faculty.vassar.edu/lowry/wilcoxon.html) [33]. Correlation coefficient analysis was determined using a Pearson bivariate, two tailed test of significance (SPSS for windows).

## 3. Results

### 3.1. Assessment of the anti-FML humoral response

After complete immunization significant differences in anti-FML antibodies were found among treatments for IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 ($p < 0.01$ for all antibody types) but not for IgA antibodies ($p = 0.7331$). The CA3, CA4 and R saponins raised the IgM, IgG1 and IgG3 antibody levels above the respective saline controls (Fig. 2). The CA3 vaccine induced 54% and 76% of the IgM and the IgG1 absorbency values induced by the saponin R positive control, respectively. The CA4 vaccine, on the other hand, induced 62% and 82% of the total IgM and IgG1 response generated by saponin R, respectively. We conclude that after immunization both *C. alba* saponins induced a predominant IgM, IgG3 and IgG1 anti-FML antibody response.

After challenge, on the other hand, significant differences between groups were found in anti-FML IgG ($p < 0.0001$), IgG1 ($p < 0.0001$), IgG2a ($p < 0.0001$), IgG2b ($p = 0.0094$) and IgG3 ($p = 0.0003$) but not for IgA ($p = 0.5164$) or IgM ($p = 0.0783$) antibodies. As disclosed before challenge, the IgG1 and the IgM antibodies were strongly enhanced by all the saponins (Fig. 2). In the case of IgM, a significant enhancement was also noted after infection in the saline controls. Following the R saponin positive control, the CA4 saponin raised more IgG and IgG2a antibodies to the FML antigen than the CA3 saponin (Fig. 2). Indeed, the average absorbance of

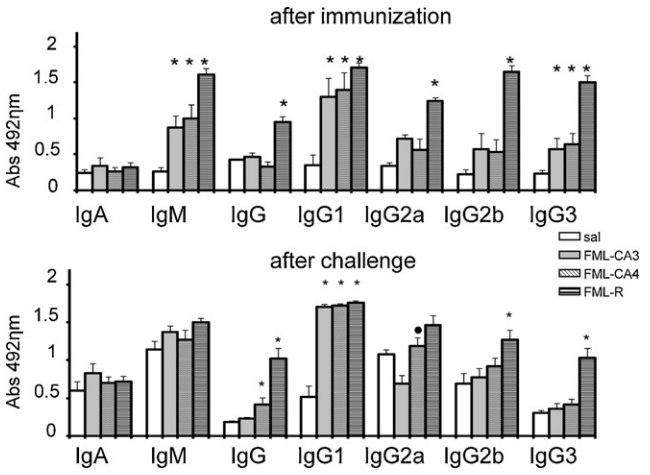


**Fig. 2.** Antibody analysis. Bars represent the mean ± SE of the absorbance values of anti-FML antibodies from 1/100 diluted serum of two independent experiments using $n = 6$–8 mice per treatment in each experiment, after immunization and after challenge with $3 \times 10^7$ amastigotes. Significant differences to the saline controls (*); to the saponin R vaccine positive control (○); and to the FML-CA3 vaccine treatment (●) as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

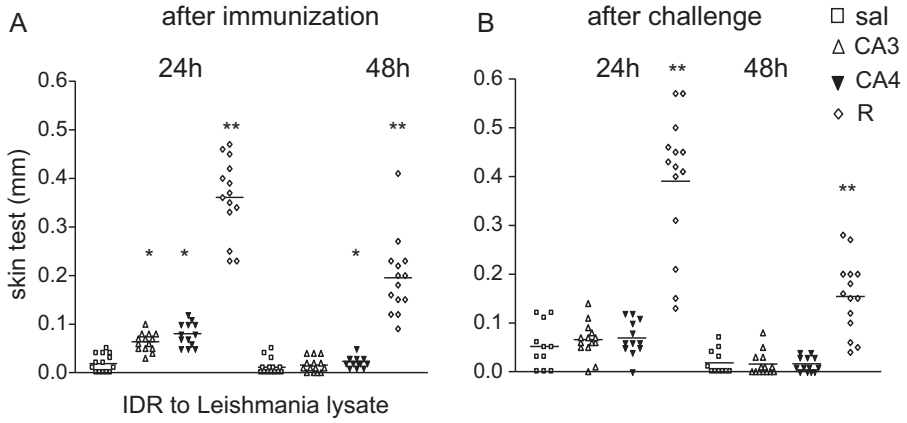


**Fig. 3.** Intradermal response to the leishmanial antigen, 24 h (left) and 48 h (right) after complete immunization (A) and after challenge (B) with $3 \times 10^7$ amastigotes of *L. chagasi*. Results of 2 independent experiments using $n = 6–8$ mice per treatment in each experiment, are shown as individual IDR measures. Horizontal full lines represent the mean values. *$p < 0.05$ indicates significant differences from the saline treated controls and ** from all treatments as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

CA4 increased from 0.564 before to 1.189 after infection ($p = 0.0079$) while the average for CA3 vaccinated mice did not significantly changed (from 0.718 to 0.689; $p = 0.114$). Furthermore, the CA4sap vaccine IgG2a response after infection was not statistically different from the saponin R vaccine. All saponins raised equivalent levels of IgG1 above the saline control and only the R saponin significantly enhanced the IgGb and IgG3 antibodies above saline controls (Fig. 2). The IgA antibodies, on the other hand, were enhanced in all groups after challenge (Fig. 2).

The predominance of the CA4 saponin, although only modest after immunization, was more evident after infection. Indeed, compared to the respective antibody titers before infection, significant increases were detected in the CA4 saponin vaccinated mice after challenge for IgA ($p = 0.0032$), IgM ($p = 0.0124$), IgG ($p = 0.0414$), IgG2a ($p = 0.0061$) and IgG2b ($p = 0.0349$) antibodies while the CA3 saponin vaccine only showed an increase of the IgA ($p = 0.0016$) and IgM antibodies ($p = 0.0045$). These results confirm the higher potency of the 4 sugar chain CA4 saponin (Fig. 1) in the induction of anti-FML specific antibodies that was further enhanced after the infective challenge.

### 3.2. Assessment of the intradermal response to leishmanial antigen (IDR)

The cellular immune response was initially evaluated by the intradermal reaction against *Leishmania* lysate (IDR) (Fig. 3). IDR was measured in the right hind footpads and subtracted from the values of the left hind footpad injected only with saline. At 24 h after immunization, the IDR response was significantly higher for the R saponin compared to all the other groups and also higher for the CA3 (mean = 0.06 mm) and CA4 (mean = 0.08 mm) than for the saline control (mean = 0.02 mm) (Fig. 3A). At 48 h only the R and CA4 sustained this response indicating the superiority of CA4 over the CA3 saponin of *C. alba*. After challenge, only the R saponin vaccine sustained the enhanced IDR (Fig. 3B). There was no significant variation, before and after infection, in the magnitude of the IDR response induced by the CA3 ($p = 0.8103$ at 24 h and $p = 0.6818$ at 48 h) or by the CA4 vaccines ($p = 0.3898$ at 24 h and $p = 0.2801$ at 48 h) (Fig. 3A and B).

In an additional experiment we compared the abilities of the *Leishmania* promastigote lysate, the FML antigen that was used for vaccination and the NH36 recombinant protein, which is the main antigenic component of FML for the induction of IDR (Fig. 4). Although the same trend described in Fig. 3A was observed, the predominance of the CA4 IDR against the *Leishmania* lysate was in this experiment even more pronounced (mean = 0.416 mm and 0.430 at 24 h, before and after challenge, respectively) (Fig. 4A and C). The CA3 vaccine, on the other hand, showed means = 0.202 and 0.217 at 24 h, before and after challenge, respectively (Fig. 4A and C). In this experiment, the predominance of the CA4 saponin vaccine was sustained even after challenge. IDR reactions after injection with either FML or NH36 antigens were higher in mice vaccinated with CA4 than with CA3 saponin. While all reactions to promastigote lysate were sustained after challenge, the IDR to FML or NH36 antigens showed to be reduced (Fig. 4C and D).

### 3.3. Assessment of the specific T cell immunity by flow cytometry analysis and intracellular production of cytokines

Following the analysis of the cellular immune response, the increase of the percents of spleen *Leishmania*-specific T cells after challenge was evaluated by fluorescent cytometry analysis (Fig. 5). We observed that only the CA4 vaccine increased both the CD4+ and the CD8+ *Leishmania*-specific T cell proportions over the saline controls while the CA3 vaccine increased only the CD8+ specific T cell proportions (Fig. 5). There was no difference between the CA3 and CA4 vaccines to the gold standard R. Finally, the splenocytes were also labeled through the ICS method and the results are shown as double positive cells (Fig. 6). We observed that the CA4 vaccine induced enhancements of the TNF-α-producing CD4+ T cells and of the IFN-γ-producing CD8+-T cells while the CA3 vaccine induced the increase of the IFN-γ-producing CD4+-T cell proportions. No significant variations among treatments were observed in the proportions regarding the TNF-α or the IL-10 production by the CD8+ T cells.

### 3.4. Protective efficacy to infection by L. chagasi

The analysis of the parasite load in livers showed that all vaccines induced protection when compared to saline controls ($p < 0.0001$) (Fig. 7). Besides the QS21 containing saponin positive control which induced a 89% significant reduction, in agreement with the above described results of the analysis of the immune response, the *C. alba* CA4 induced the highest protection (78%, $p < 0.0001$) that was followed by the CA3 saponin with 57% ($p < 0.0001$) of parasite load reduction. The difference between CA4 and CA3 was significant

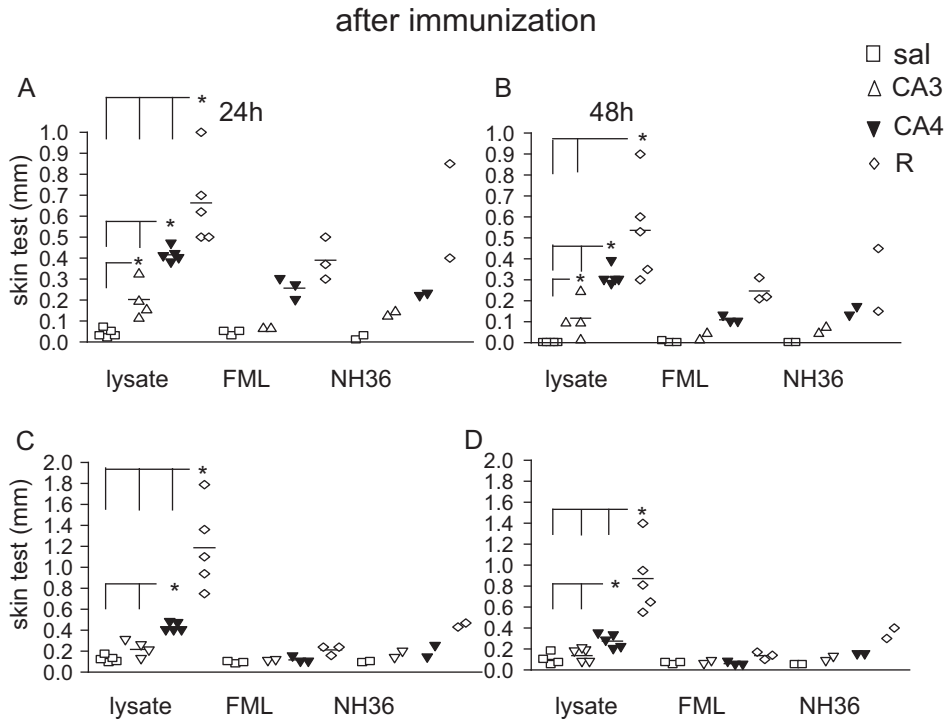


**Fig. 4.** Comparison of antigens for the intradermal response, at 24 h (A) and 48 h (B) after complete immunization and 24 h (C) and 48 h (D) after challenge with $3 \times 10^7$ amastigotes of *L. chagasi*. Results of one experiment using $n = 4$–5 mice per treatment for the *Leishmania* promastigote lysate, $n = 2$–3 for the FML antigen and $n = 2$ for the NH36 recombinant protein. Results are shown as individual IDR measures. Horizontal full black lines represent the mean values. $^*p < 0.05$ indicates significant differences among treatments, as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

($p < 0.0125$) hence confirming the superiority of the CA4 saponin in protection against visceral leishmaniasis (Fig. 7). The gain in body weight along the experiment induced by R saponin was superior to that of the saline controls ($p = 0.0407$) but not significantly different from the increases in the CA3 and CA4 saponin vaccinated mice (not shown).

The increases in IDR after vaccination and infection were strong correlates of protection and were significantly correlated to the decrease of parasite load ($p = -0.007$) and to the gain in corporal weight ($p = 0.0001$). The increases in CD4–TNF-α ($p < -0.001$), CD8–IFN-γ ($p < -0.002$) and CD8–TNF-α ($p < -0.015$) expressing T cell proportion were also correlated to the decrease of parasite load.

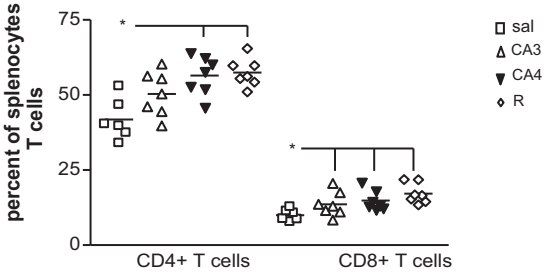


**Fig. 5.** Analysis of the NH36-specific cellular immune response after challenge. Results are shown as the individual percent of splenocytes, stained with anti-CD4 (clone GK1.5) or anti-CD8-FITC monoclonal antibodies, after 5 days *in vitro* culture with 5 μg of recombinant NH36 protein. Horizontal bars represent the means of one experiment (n = 6–7 mice per treatment). $^*p < 0.05$ indicates differences from the saline treated controls, as disclosed by the Kruskall Wallis (KW) and Mann Whitney (MW) non-parametrical tests.

### 3.5. *In vivo depletion assay of CD4+ and CD8+ T cells*

The relative contribution of the NH36-specific-CD4+ and CD8+ T cell producing cells was evaluated in an *in vivo* depletion assay with monoclonal antibodies (Fig. 8). In correlation to what was detected for the specific increase of the CD4+ T cells (Fig. 5), the TNF-α–CD4+ producing T cells (Fig. 6), only the treatment with anti-CD4+ monoclonal antibody induced a 66% increase in the total LDU counts of mice vaccinated with CA4 saponin, indicating a main contribution of CD4+ T cells (Fig. 8; $p > 0.05$) to the vaccine induced protection. On the other hand, the protective effect of the CA3-vaccine is mediated by both CD4+ and the CD8+ T cell contributions since the anti-CD4+ antibody treatment induced a 43% and the anti-CD8+ antibody induced a 16% increase of the total LDU counts of CA3 vaccinated mice, respectively (Fig. 8). This is in agreement with the increase of the percent of CD8+ NH36-specific T cells by the CA3 vaccine (Fig. 5) and of the IFN-γ-CD4+ producing T cells (Fig. 6). The increases in IDR, CD4–TNF-α, CD8–IFN-γ and CD8–TNF-α by the CA4 vaccine were strong correlates of protection and were significantly correlated to the decrease of parasite load ($p = -0.007$).

### 3.6. *TNF-α contribution to protection*

To confirm the relevance of TNF-α in the protection induced by *C. alba* we vaccinated C57BL6 wild-type and TNF-α-receptor knock-out mice and challenged them with *L. chagasi* amastigotes. The IDR response against *Leishmania* lysate was significantly increased (81%) only by the CA4 saponin vaccine in wild type mice above their respective saline control (Fig. 9). No increases in IDR were observed however in vaccinated TNF-α-knock-out mice (Fig. 9). Different from what was detected in Balb/c mice treated with saline (mean = 415 LDU units) (Fig. 7) the C57Bl6 strain was more sensitive (mean = 1200 LDU units). Confirming the role of IDR as a correlate of

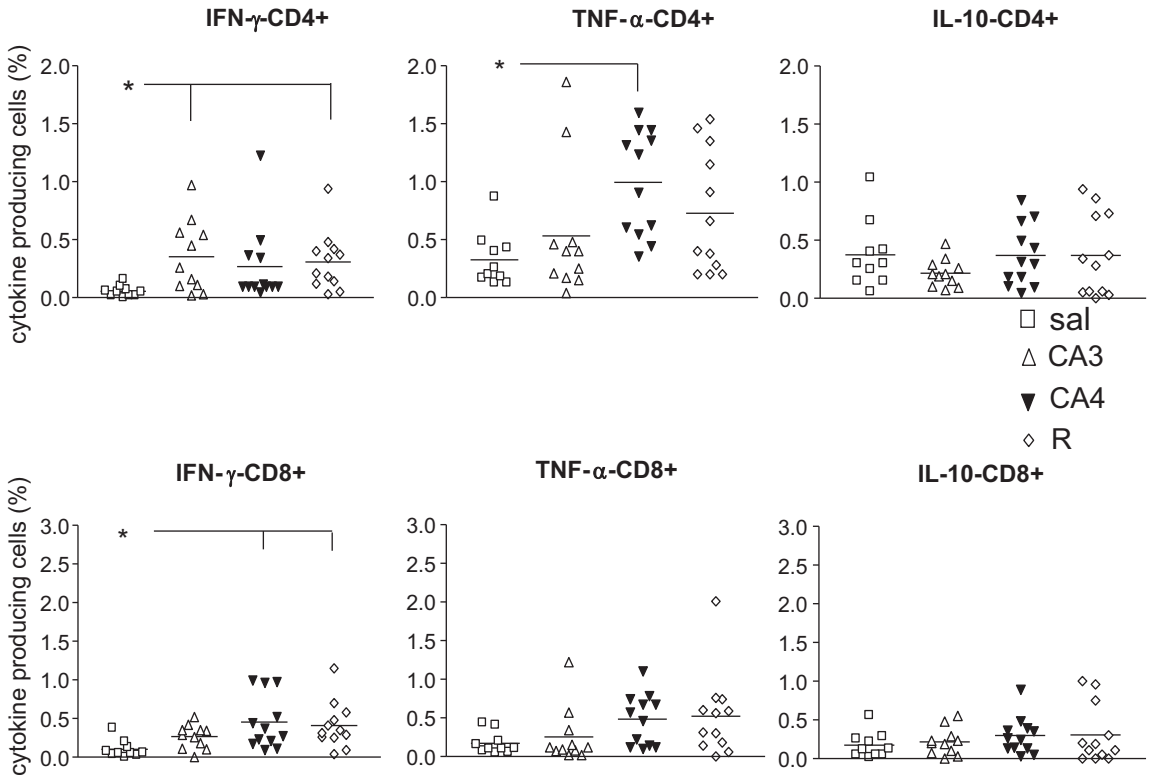


**Fig. 6.** Development of NH36-specific cellular immune response as disclosed by intracellular staining analysis of splenocytes *in vitro* culture with NH36 after *L. chagasi* infection. Anti-CD4-FITC and anti-CD8-FITC antibodies were used for labeling the cell surfaces and anti-IFN-γ-APC, anti-TNF-α-PE and anti-IL-10-PE for intracellular staining. Dots represent the individual results of: IFN-γ–CD4+, TNF-α–CD4+, IL-10–CD4+, IFN-γ–CD8+, TNF-α–CD8+ and IL-10–CD8+ producing T cells. Horizontal full line bars represent means of two independent experiments ($n = 6$–7 mice per treatment for each experiment). A single measure was obtained for each antibody and each mouse. $^*p < 0.05$ indicates differences from the saline treated controls as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

protection in visceral leishmaniasis, only the CA4-saponin vaccine (mean = 596 LDU units) induced a significant reduction of 50% of the parasite load (Fig. 9). The TNF-α-receptor deficient mice lost the ability to clear amastigotes from the liver and showed a mean control value (2185 LDU) 56% greater than the control wild type group (1200 LDU). Protection due to the CA4 saponin was not observed in the TNF-α-receptor deficient mice.

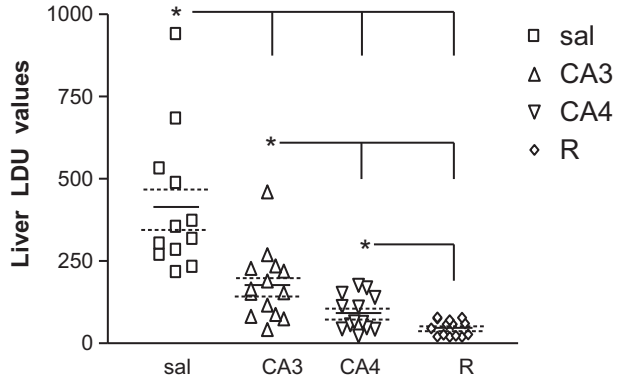


**Fig. 7.** Protective efficacy of saponin-vaccinated mice against *L. chagasi* infection. The individual *L. chagasi* liver parasite load of vaccinated and control groups is expressed in LDU values (number of amastigotes per 600 liver cell nuclei/mg of liver weight). Results represent the individual liver LDU values of mice from two independent experiments ($n = 6$–7 mice per treatment for each experiment). Horizontal full lines represent the mean values and dashed horizontal lines represent the SE values. $^*p < 0.05$ indicates significant differences as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

### 3.7. Correlation between the increase of the sugar chain and cytokine secretion

To confirm that the presence of an extra-apiose in CA4 is responsible for its increased adjuvant potential, we compared the protective efficacy of the CA3 and CA4-vaccines to the one of vaccines formulated with the CA3X and CA2 saponins of *C. alba* (Fig. 1). All these saponins are naturally produced through a glycosylation series by the *C. alba* plant. The shorter chain is present in CA2 which has only an arabinose and a rhamnose unit attached to C-28 (Fig. 1) and is followed by the CA3X and CA3 saponins, both with three sugars attached to the C-28 chain. The third sugar is xylose for CA3X and apiose for CA3. Finally, the CA4 shows the same arabinose, rhamnose and apiose chain present in CA3 and an additional extra apiose (Fig. 1). The cellular immune response was also analyzed by monitoring the secretion of cytokine by splenocytes of vaccinated and challenged mice after *in vitro* incubation with the NH36 antigen. The results are summarized in Fig. 10. The ELISA-analysis of the cytokines secreted by splenocytes after *in vitro* incubation with NH36 antigen was performed after challenge (Fig. 10). The secretion of TNF-α was increased by the CA3X, CA4 and the control R vaccines while the secretion of IFN-γ was enhanced above the saline control only by the control R vaccine. The IL-10 secretion was enhanced only by the CA4 vaccine. It is worth noting that the increase in the number of sugar units of the C-28 attached to the carbohydrate chain of saponins is positively correlated to the increase in secretion of TNF-α ($p < 0.001$) and of IFN-γ ($p = 0.026$) and to the decrease in secretion of IL-10 ($p = -0.008$). Secretion of TNF-α was more intense than that of IFN-γ. Our results disclose the protective adjuvant potential of CA3 and CA4 saponins and suggest that the addition of one sugar unit on the C-28 attached chain of CA4 determines a significant increase in its adjuvant potential.

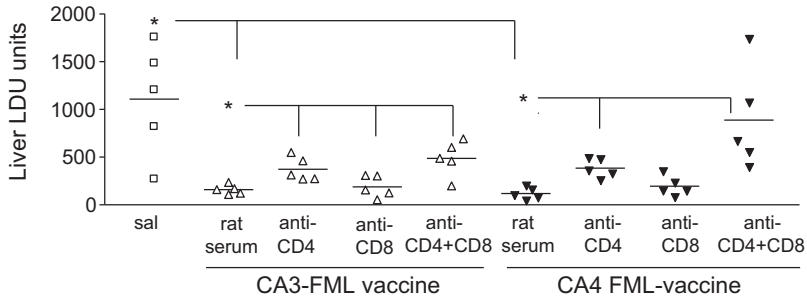


**Fig. 8.** Development of cell-mediated immune response as disclosed by *in vivo* depletion with anti-CD4+ and anti-CD8+ monoclonal antibodies. *Leishmania chagasi* parasite-load in mice vaccinated with CA3-FMl and CA4-FML vaccines and treated with rat serum, anti-CD4+ or anti-CD8+ or the combination of anti-CD4+ and anti-CD8+ MAbs. Mice that receive the vaccines were also treated with rat serum (rat IgG) as controls for antibody treatment. Results represent the individual liver LDU values of mice from one experiment ($n = 5$ mice per treatment). Horizontal full lines represent the mean values. $^*p < 0.05$ indicates significant differences between treatments as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

### 3.8. Correlation between the increase of the sugar chain, IDR and the decrease of parasite load

Furthermore, the impact of the increase of the C-28 attached sugar chain of *C. alba* was compared in the Balb/c mice model, using the CA2 and the CA3X saponins (Fig. 1) as controls. The IDR response was enhanced only by the CA4 and the R saponin above the saline controls (Fig. 11). In correlation to that, only the CA4 and the R saponin reduced the parasite load when compared to saline control (Fig. 11), confirming the superiority of CA4. The reduction determined by CA4 was stronger than that of CA2 and CA3X, and, as described in Fig. 7, not different from the protection induced by CA3. Maximal parasite load reduction was achieved by the R saponin control group (Fig. 11). There was a positive correlation between the increase in IDR measures and in the number of sugar units attached to the triterpene-C-28 ($p < 0.0001$). Supporting our hypothesis of the superiority of the CA4 saponin, on the other hand, the LDU values decreased with the increase of the sugar chain ($p = -0.014$).

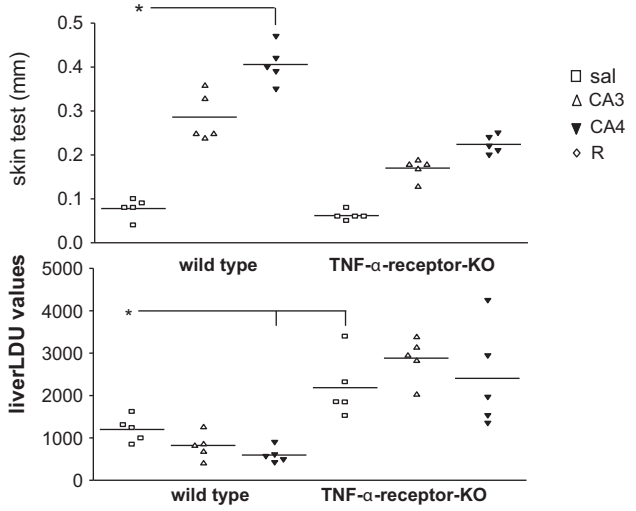


**Fig. 9.** TNF-α is required for efficient parasite clearance in the liver. C57BL/6 mice wild type and TNF-α-receptor knock-out (TNF-α-KO) were vaccinated with CA3 or CA4 saponin and FML or treated with saline only and further challenged with *L. chagasi* amastigotes. The intradermal reaction to promastigote lysate, at 24 h after antigen injection, is expressed in millimeters. The individual *L. chagasi* liver parasite load values are expressed in LDU values (number of amastigotes per 600 liver cell nuclei/mg of liver weight) ($n = 5$ mice per treatment). Horizontal full lines represent the mean values. $^*p < 0.05$ indicates significant differences as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

### 3.9. Assessment of the hydrophile–lipophile balance (HLB) value, hemolytic index and in vivo toxicity of saponins

The hydrophile/lipophile balance calculation performed according to the Davies and Riedel method disclosed an HLB = 12.7 for CA2, HLB = 15.8 for both CA3 and CA3X and an HLB = 19.9 for CA4 saponin confirming its higher hydrophilicity.

The analysis of the hemolytic capacity of *C. alba* saponins (Table 1) disclosed that saponins CA2, CA3 and CA4 share a high $HD_{50}$ (175 μg/ml) which means that they are poorly hemolytic and that the hemolytic capacity, differently from what happens with the HLB, does not increase in positive correlation with the number of sugar units linked to the sapogenin. The saponin CA3X, with 3 sugar units attached to C-3 of triterpene, but not apiose, induced, on the other hand, a strong hemolysis ($HD_{50} = 87$ μg/ml) (Table 1). The control saponin R, was as expected the most hemolytic ($HD_{50} = 35$ μg/ml). Furthermore, the safety analysis detected neither lethality nor local pain or swelling (Table 1) for any of the *C. alba* vaccines. Only loss of hair at the local of injection was detected in the 5 mice treated with the QS21 containing saponin R.

## 4. Discussion

The increase in hemolytic activities of *C. alba* saponins was not correlated to the increase in the size of the C-28 attached carbohydrate chain. In contrast, the CA3 and CA3X saponins that both have three sugar units in that chain strongly differed in their hemolytic capabilities. Saponin CA3X which has a xylose terminal unit induced strong hemolysis while saponin CA3 that shows an apiose unit instead was much less hemolytic. In correlation with our findings, the QS21 adjuvant is composed of two isomers that include either apiose (QS21-Api) or xylose (QS21-Xyl) as the terminal sugar residue within the linear tetrasaccharide segment, in a ratio of 65:35, respectively [34]. The saponin QS21-Xyl was marginally more toxic than QS21-Api or the QS21 mixture. Overall mice weight loss was greatest in the SQS21-Xyl groups and although one mouse of both groups died over the course of immunizations, the mice in the QS21-Xyl group showed the worst clinical status. On the other hand, the QS21-Xyl treated mice induced a higher IgM and IgG response [34].

In our investigation we demonstrated that the adjuvant potential of *C. alba* saponins is correlated to the increase of their C-28 attached sugar chain. We also demonstrated that the addition of an extra apiose unit in CA4 saponin is determinant of its enhanced adjuvant potential. Both the CA3 and CA3X saponins have three sugar chains and three exposed hydroxyl groups on the terminal sugar unit, therefore sharing the same HLB. However the spatial configuration and exposition of the HO groups on the apiose

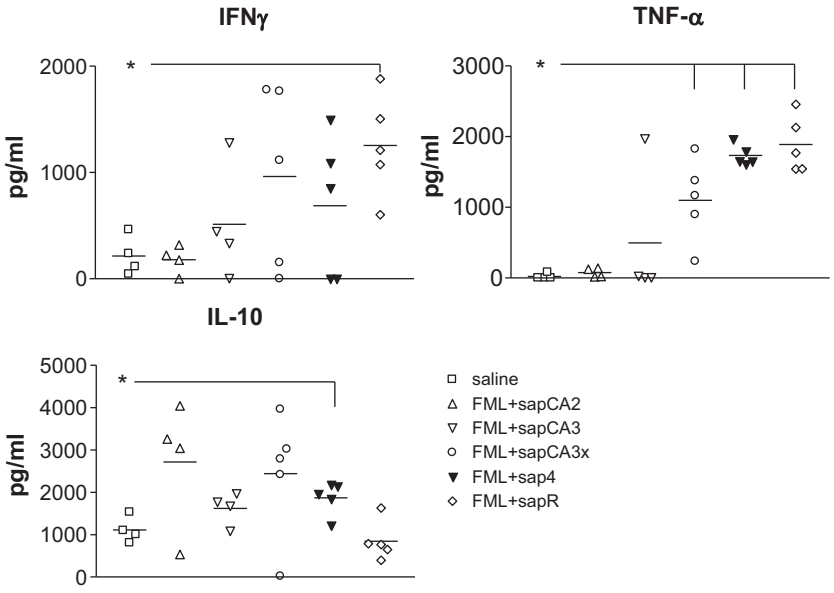


**Fig. 10.** Development of NH36-specific cellular immune response. ELISA of cytokines in supernatants of mice splenocytes. IFN-γ, TNF-α and IL-10 were assayed in the supernatants of splenocytes of control and vaccinated mice after 5 days of *in vitro* culture with the NH36 antigen. Data correspond to individual results of one experiment with 4–5 mice of each vaccine group and saline control obtained after sacrifice. Horizontal full lines represent the mean values. $^*p < 0.05$ indicates significant differences to the saline control as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

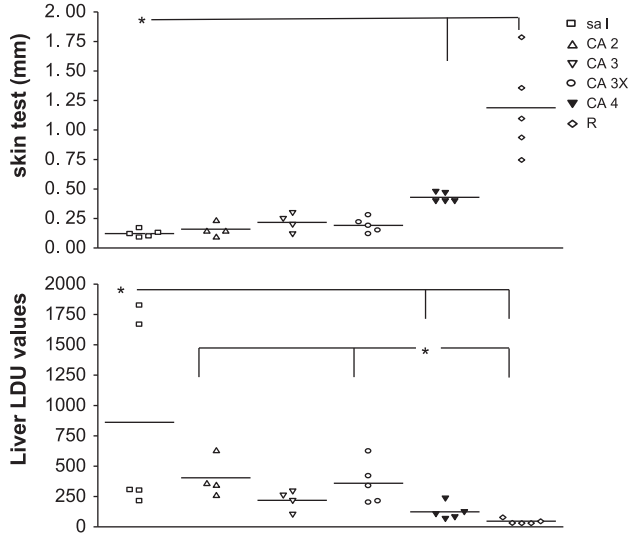


**Fig. 11.** Parasite load reduction increases with sugar chain of saponins. Balb/c mice were vaccinated with the CA2, CA3, CA3X, CA4 or R saponin and FML or treated with saline only and further challenged with *L. chagasi* amastigotes. The intradermal reaction to promastigote lysate, at 24 h after antigen injection, is expressed in millimeters. The individual *L. chagasi* liver parasite load values are expressed in LDU values (number of amastigotes per 600 liver cell nuclei/mg of liver weight) ($n = 5$ mice per treatment). Horizontal full lines represent the mean values. $^*p < 0.05$ indicates significant differences as disclosed by the Kruskall–Wallis (KW) and Mann–Whitney (MW) non-parametrical tests.

**Table 1**
Toxicity *in vivo* and hemolytic effect of saponins.

| Saponin | Local pain | Swelling | Loss of hair | Lethality | $HD_{50}$ |
|---|---|---|---|---|---|
| R (QS21) | 0/5 | 0/5 | 5/5 | 0/5 | 35 μg/ml |
| CA4 | 0/5 | 0/5 | 0/5 | 0/5 | 175 μg/ml |
| CA3 | 0/5 | 0/5 | 0/5 | 0/5 | 175 μg/ml |
| CA3X | 0/5 | 0/5 | 0/5 | 0/5 | 87 μg/ml |
| CA2 | 0/5 | 0/5 | 0/5 | 0/5 | 175 μg/ml |

*Note*: Results are expressed as number of mice per group that showed any reactivity within 7 days after each one of the three subcutaneous injections of each vaccine containing 100 μg of saponin in the back of each animal. $HD_{50}$ is the adjuvant concentration inducing 50% of the maximum hemolysis.

terminal sugar unit is optimized when compared to the configuration of the same groups in xylose. This would explain also the reason for the increased adjuvant potential of CA4 which has an additional apiose unit.

The CA4 saponin of *C. alba* in formulation with FML induced a higher response after challenge, significant increases in IgG and IgG2a anti-FML antibodies which were absent in the CA3-saponin. These results confirm the relevance of the addition of a fourth unit of apiose $1 \rightarrow 3$ linked to the rhamnose residue of the C-28 attached sugar chain in the induction of the anti-FML humoral response. As expected for a positive adjuvant control, the global humoral response induced by the saponin QS21 containing saponin R vaccine was the highest. The intensity of the humoral response generated by saponins has been shown to be related to the presence of carbohydrate moieties attached to the triterpene nucleus [14,17,25] and this response increases in direct proportion to their length [22]. This explains why, even in a saponin with only 4 sugar units chain (CA4), the difference of just one sugar residue [28] may account for a significant increase in IgG and IgG2a specific antibodies which are the subclasses of antibodies previously shown to be correlated to protection.

In our investigation, all saponins increased the IgG1 antibodies. This humoral response is induced by whole saponins [23] but seems to be correlated to the carbohydrate deprived sapogenin nuclei [14,17]. A global increase of IgM and IgG3 antibodies by all adjuvants was described which is expected to occur in response to carbohydrate enriched antigens [35] and saponins [14,17].

The sugar side chain in saponins may be essential to their adjuvanticity [reviewed in 22]. Soyasaponins that comprise sugar chain(s) have shown adjuvanticity stimulating anti-OVA total-IgG and IgG1 antibody responses while their corresponding aglycones soyasapogenols A and B, did not. The CP05 saponin of *C. pulcherrima* induced a strong antibody response that was maintained after removal of its monoterpene hydrophobic moiety but not after removal of the C-28 and or the C-3 attached glycosidic chains [14]. With the removal of these glycosidic chains the CP05 aglycone only sustained the IgG1 and the IgM response [14]. Oda et al. [25] described that the adjuvanticity of saponins increases with their hydrophile–lipophile balance (HLB). Indeed,

the capability of saponins to induce antibody responses increases with their hydrophilicity. Among bidesmosidic (two sugar chains) soyasaponins, soyasaponin A1 with three sugars attached to C-3 induced stronger total-IgG and IgG1 antibody responses than soyasaponin A2 with only two sugar attached to C-3 [25]. An identical conclusion was obtained by Bernardo et al. [19] working with the PSAGLE saponin of *Albizia saman*. For monodesmosidic (one sugar chain) soyasaponins, the ranking in terms of antibody response was soyasaponin I (-glcA-gal-rha) > soyasaponin II (-glcA-ara-rha) > soyasaponin III (-glcA-gal) [25]. This means that a trisaccharide (soyasaponin I and II) chain is more potent than a disaccharide one (soyasaponin I), and that a residue of galactose in the trisaccharide chain of soyasaponin I that exposes one OH group turns the saponin more potent than a residue of arabinose which lacks this OH group (soyasaponin II) [25]. Therefore, among saponins of the same sugar chain length, the more hydrophilic the sugar components are, the more potent the humoral response is. The C-28 attached chain of the *C. alba* CA3 saponin is composed of arabinose–rhamnose–apiose. The addition of one additional apiose sugar unit in the CA4 saponin is then expected to add hydrophilicity to the saponin [25] increasing its adjuvant potential. Our results with saponins of *C. alba* therefore, strongly support the previous conclusions of Oda et al. [25] stating that the adjuvant activity tended to increase with the sugar side chain length and the HLB value. Indeed, this investigation reported HLB values of 15.8 and 19.9 for CA3 and CA4 saponins, respectively. The HLB of QS21 saponin, which is a highly potent standard adjuvant, is 36.3 [25]. The saponins of *C. alba* display lower HLB values than QS21 and bidesmosidic soyasaponins but higher HLB values than monodesmosidic soyasaponins [25].

The extra-apiose of CA4 saponin determines the increase of the C-28 sugar chain length and, in this way, of the saponin hydrophilicity. In order to confirm that HLB is a crucial factor influencing the adjuvanticity of the CA4 saponin we used as controls, the CA2 and CA3X saponins of *C. alba*, that have shorter sugar chains since they are synthesized in earlier steps of the biosynthetic pathway. Indeed, the triterpene nucleus is synthesized first and the sugar units added in sequence to its C-28 by specific glycosyltransferases [36]. Our results confirmed the correlation between increased HLB and increased adjuvant capabilities.

As expected for protection generated against visceral leishmaniasis [31,37–44] protection induced by CA4 saponin determined the decrease of liver LDU and the increases of IDR and of TNF-α–CD4+ producing cells and TNF-α secretion. The protection induced by the CA4-vaccine was mediated a CD4+ T cell and TNF-α-driven response. This could indicate the existence of an early effector cell-response generated by the vaccine [45]. TNF-α is considered to be the most ubiquitous cytokine and it is produced by most activated CD4+ T cells [reviewed in 45] generated under conditions that favor TH1-cell differentiation. It has proved to be important in protection against visceral leishmaniasis [37–44,46,47]. A sustained or an overall increased global or *Leishmania*-specific CD4+ T cell expansion is expected in protection against visceral leishmaniasis [31,48]. In our investigation, the CA4 saponin, with the longest sugar chain was the only one capable of enhancing the CD4+ *Leishmania*-specific T cell population.

Also, supporting our results, a study of the relationship between hemolytic and adjuvant activity and structure of protopanaxadiol and protopanaxatriol-type saponins from the roots of *P. notoginseng* showed that the number, length and position of sugar side chains, and the type and the linkage of the glycosyl group in the structure of these saponins did not only affect the adjuvant potentials, but also had significant effects on the nature of the immune responses [20,21].

We conclude that the addition of one sugar unit in the C-28 attached glycosidic moiety provides a significant increase of adjuvanticity and protective capability of the *C alba* saponins. Our results encourage the new synthesis or remodeling of natural saponins by additional glycosylation, aiming the rationale development of effective adjuvants. This has proven to be feasible since the most frequent isomer of QS21 saponin which has also apiose as the terminal sugar unit of its C-28 chain, was obtained by chemical synthesis while maintaining the adjuvant capability of the natural product [34].

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