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Nanoencapsulated retinoic acid as a safe tolerogenic adjuvant for intranasal vaccination against cutaneous leishmaniasis

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ABSTRACT

Mucosal, but not peripheral, vaccination with whole *Leishmania amazonensis* antigen (LaAg) effectively protects mice against leishmaniasis, likely through a tolerogenic mechanism. Given the crucial role of retinoic acid (RA) in $CD4^+$ $Foxp3^+$ regulatory T cell (T_{reg}) differentiation and mucosal tolerance, here we evaluated the capacity of RA to improve intranasal (i.n.) vaccination with LaAg. To prevent degradation and possible mucosa irritation, RA was encapsulated in solid lipid nanoparticles (RA-SLN). Thus, BALB/c mice were given two i.n. doses of LaAg alone or in association with RA-SLN (LaAg/RA-SLN) prior to challenge with *L. amazonensis*. No histological sign of irritation or inflammation was produced in the nasal mucosa after RA-SLN administration. LaAg/RA-SLN vaccine was more effective in delaying lesion growth and reducing parasite burdens than LaAg alone (96% and 61% reduction, respectively). At two months after challenge, both vaccinated groups displayed similar T helper (Th) 1-skewed *in situ* cytokine responses, different from early infection where both Th1 and Th2 responses were suppressed, except for transforming growth factor (TGF)-β mRNA, that was higher in mice given RA-SLN. At the mucosa, RA-SLN promoted enhanced expression of interleukin (IL)-10 and $CD4^+$ $Foxp3^+$ T_{reg} population. In sum, these data show that RA-SLN is an effective and safe tolerogenic adjuvant for i.n. vaccination against leishmaniasis.

1. Introduction

Leishmaniases are neglected diseases caused by macrophage-infecting protozoans of the genus *Leishmania* that are transmitted by the bite of infected sandflies. Over 20 parasite species can cause a variety of clinical manifestations ranging from cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL). CL, the focus of the present study, is characterized by chronic skin ulcers frequently leaving permanent and disfiguring scars [1]. It is the most common form of the disease, affecting 1.2 million people/year worldwide [2]. Vector control is difficult to accomplish, while chemotherapy presents a series of pitfalls such as painful injections, systemic toxicity and/or prohibitive cost [3]. Therefore, prophylactic vaccination appears as the most rational means for disease control. However, as yet no human vaccine has proved sufficiently effective and/or safe for licensing.

The concept that T helper (Th) 1-type immune responses are associated with protection against CL has driven the search for Th1-biasing antileishmanial vaccines in the last decades. Most vaccines have used purified parasite antigens in the form of single or mixed recombinant proteins and/or synthetic peptides, as well as polypeptide-chimeras [4]. On their own, those defined vaccines have not been sufficiently immunogenic to overcome the dominant counter protective, Th2-biasing parasite antigens important for infection establishment [5]. For expanded protective immunity, potent pro-inflammatory adjuvants are required, but their unacceptable toxicity to humans has hampered vaccine approval [6].

Since the early demonstration that thymic tolerance induction to a single and Th2-biasing antigen, LACK (*Leishmania*-Activated C-Kinase Antigen) renders BALB/c mice resistant to *L. major* infection [7], tolerization protocols have been considered a new vaccination strategy for leishmaniasis. Mucosal sensitization is an easy way to achieve, tolerization, given that the complex Mucosa

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Associated Lymphoid Tissue (MALT) is efficiently armed to prevent immunoreactivity against the high daily exposure to exogenous antigens [8]. We have shown the feasibility of intranasal (i.n.) vaccination against *L. amazonensis* (CL) and *L. infantum* (VL) using counter-protective parasite antigens such as lipophosphoglycan (LPG) [9], serine proteases [10] and plasmid codifying the LACK protein (LACK DNA) [11–14]. However, since the majority of the antigens in crude parasite extract are Th2-biasing and disease-promoting [15], higher protection has been achieved with whole *L. amazonensis* antigens (LaAg). Different from peripheral vaccination with adjuvant-free LaAg that increased mouse [16,17] and monkey [18] susceptibility to CL and proved ineffective against human CL in Colombia [19], oral and i.n. vaccinations with LaAg have consistently increased resistance against CL and VL in different animal models [11,17,20–23].

The protective mechanism appears associated with tolerance since vaccinated animals invariably show suppressed ability to mount *Leishmania*-specific cutaneous hypersensitivity. Recently, we observed that i.n. LaAg protection is indeed related to a tolerogenic mechanism leading to increased Th1 and Treg immune response in the lesion draining lymph nodes during early infection [17]. All-trans retinoic acid (RA), a metabolite from dietary retinol, is an important co-factor in mucosal tolerance. RA produced via retinaldehyde dehydrogenase by $CD103^+$ dendritic cells (DCs) from the gut, lung and nasal [24–26] mucosa induce transforming growth factor (TGF)-β-mediated differentiation of naïve T cells into forkhead box P3 $(Foxp3)^+$ regulatory T cells (T_{regs}). Then, Tregs migrate to other mucosal and peripheral tissues to accomplish immune tolerance [27]. Previous studies have demonstrated that RA can be used as a tolerogenic adjuvant to prevent Th2 immune response in asthma [28,29].

Considering the important role of RA in mucosal tolerance, in this work we proposed to evaluate its adjuvanticity to i.n. vaccination with LaAg against CL. To prevent degradation and mucosa irritation and to increase RA uptake, this molecule was loaded into solid lipid nanoparticles (RA-SLN) [30].

2. Materials and methods

2.1. Mice

BALB/c female mice, 6–8 weeks old were bred and kept at the our animal house in Federal University of Rio de Janeiro where they received filtered water, pelleted food and sterilized bedding changed twice a week. All experiments were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH) and were approved by the Committee on the Ethics of Animal Use of the Federal University of Rio de Janeiro under the number CAUAP 180.

2.2. Parasites

L. amazonensis (strain MHOM/BR/75/Josefa) promastigotes were used at the stationary growth phase for both vaccine preparation and infection. Parasites were kept at 26 °C in Minimum Essential Medium 199 (MEM199, Cultilab, Brazil) supplemented with 10% of heat-inactivated fetal calf serum (HIFCS, Cultilab, Brazil), antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin, Stemcell Technologies, USA) and hemin (5 μg/mL, Sigma-Aldrich, USA). Parasites were periodically isolated from mouse lesions and always used up to the fourth *in vitro* passage.

2.3. Vaccine

LaAg was prepared as previously described [20]. Briefly, *L. amazonensis* promastigotes were washed 3 times in phosphate buffered saline (PBS) by centrifugation to remove HIFCS proteins. Washed cells were lysed by addition of sterile water and three cycles of freezing and thawing. The resulting lysate was lyophilized and the LaAg powder containing 40% of protein as determined by the Lowry assay was stored at −80 °C. LaAg was reconstituted in PBS immediately before use.

2.4. Retinoic acid-loaded solid lipid nanoparticles (RA-SLN)

RA-SLN consisting of Compritol 888 ATO (Gattefossé, France), α-tocopherol, cholesterol, stearylamine, glycerol, ethylenediaminetetraacetic acid (EDTA), (the last five from Sigma-Aldrich, USA), polysorbate 80 (Croda Inc, USA), distilled water, and 0.1% RA (BASF, Germany) was prepared by hot melt homogenization as previously described [30]. Such nanoparticles had 180 ± 2 nm of mean diameter, 47 ± 4 mV of zeta potential and 100% encapsulation efficiency.

2.5. Intranasal vaccination

Mice held upright were given LaAg (10 μg of protein) alone or mixed with RA-SLN (15 μg of RA/dose) in a final volume of 20 μL (10 μL in each nostril) using a micropipette adapted with a tip. After 7 days, mice were boosted with the same vaccine dosage. Controls received PBS or RA-SLN alone.

2.6. Nasal mucosa histology

Fifteen minutes, 2 h or 24 h after i.n. administration of RA-SLN (15 μL, 15 μg of RA), mice snouts were removed and fixed in 10% paraformaldehyde for 7 days. After fixation, samples were decalcified in 14% EDTA (Sigma-Aldrich, USA) solution for 10 days and after that they were dehydrated and placed in a paraffin block. Longitudinal sections of the nasal region with 4 μm-thick were made, stained with haematoxylin-eosin (Sigma-Aldrich, USA) and analyzed under the optical microscope to evaluate nasal mucosa epithelium.

2.7. Infection and follow up

Seven days after the last vaccine dose, mice were infected in the hind footpad with 2×10^5 *L. amazonensis* promastigotes in 20 μL of PBS. Footpad thicknesses were measured with a digital caliper (Mitutoyo, Brazil) once a week and lesion sizes were expressed as the difference between infected and contralateral noninfected footpads. Parasite loads were determined by Limiting Dilution Analysis as previously described [31]. Briefly, infected footpads were homogenized in 2 mL of PBS each, allowed to sediment for 10 min, and supernatants serially diluted in triplicates at 26 °C. After 15 days, the numbers of amastigotes/footpad were reversely calculated assuming that the last dilution showing promastigotes originated from a single tissue amastigote.

2.8. Cutaneous hypersensitivity

After one week of the last vaccine dose, mice were injected in the footpad wit 20 μL of live parasites (2×10^5) or PBS. Footpad swelling was measured at different times during the first 72 h of injection using a dial calliper as described above, and expressed as the difference between parasite and PBS footpad thickness.

2.9. Cytokines

Infected footpads were homogenized as above, and centrifuged at 2000*g*/10 min. The clarified supernatants were used for measurement of local cytokine production by enzyme-linked

immunosorbent assay (ELISA). The following paired monoclonal antibodies were used: rat IgG1 anti-mouse interferon (IFN)-γ (clone XMG1.2) and biotinylated rat IgG1 anti-mouse IFN-γ (clone R4-6A2); rat IgG1 anti-mouse interleukin (IL)-4 (clone 11B11) and biotinylated rat IgG1 anti-mouse IL-4 (clone BVD6-24G2); rat IgG2a anti-mouse IL-10 (clone JES5-16E3) and biotinylated rat IgG2a anti-mouse IL-10 (clone JES5-2A5); rat IgG2a anti-mouse IL-12p40 (clone C18.2) and biotinylated rat IgG2a anti-mouse IL-12p40 (clone C17.8); rat IgG2a anti-human TGF-β (clone eBioTB2F) and biotinylated rat IgG1 anti-human TGF-β1 (clone eBio16TFB). Standard curves were prepared with recombinant cytokines according to manufacturer instructions (R&D Systems, USA).

2.10. Quantitative real time PCR (qRT-PCR)

Total mRNA was extracted from cervical (cLNs) and popliteal lymph nodes (pLNs) with 4 M guanidine isothiocyanate, as previously described [17]. The cDNA was prepared using ImProm-II™ Reverse Transcription System according to manufacturer instructions (Promega, USA). qRT-PCR reactions were assayed in triplicate for each target in StepOne system (Applied Biosystems, USA) using StepOne v2.1 software. Primers were designed using Primer Express® software version 3.0 (Applied Biosystems, USA): cytokines - IFN-γF 5′-TTG GCT TTG CAG CTC TTC CT-3′, IFN-γR 5′-TGA CTG TGC CGT GGC AGT A-3′, IL-4F 5′-TTG AAC GAG GTC ACA GGA GAA -3′, IL-4R 5′-AGG ACG TTT GGC ACA TCC A-3′, IL-10F 5′-GAT GCC CCA GGC AGA GAA-3′, IL-10R 5′-CAC CCA GGG AAT TCA AAT GC-3′, IL-12F 5′-ACG CAG CAC TTC AGA ATC ACA-3′, IL-12R 5′-CAC CAG CAT GCC CTT GTC TA-3′, TGF-βF 5′-CTA TCA GGT CCT GGC ACT TTA CAA-3′, TGF-βR 5′-CCC CGT GCA TCT CTT CCA-3′; transcription factors - Foxp3F 5′-GGC CCT TCT CCA GGA CAG A-3′, Foxp3R 5′-GGC ATG GGC ATC CAC AGT-3′, GATA3F 5′-GAC CCG AAA CCG GAA GAT GT-3′, GATA3R 5′-GCG CGT CAT GCA CCT TTT-3′, TbetF 5′-GCC AGG GAA CCG CTT ATA TG-3′, TbetR 5′-AAC TTC CTG GCG CAT CCA-3′. qRT-PCR reaction conditions were: 40 cycles of 95 °C for 30 sec and 60 °C for 1 min. The expression of β-actin was used as endogenous control: β-actinF 5′-CGT GCG TGA CAT CAA AGA GAA-3′, β-actinR 5′-GGC CAT CTC CTG CTC GAA-3′.

2.11. Cell counting and phenotyping

After the indicated time after vaccination, the nasal mucosa-draining cLNs were removed and cells counted in hemocytometer. Cells (10^6) were blocked with 1% bovine serum albumin prior to incubation for 30 min at 4 °C with the following monoclonal antibodies (BD Biosciences, USA): PerCP-anti-CD4 (clone RM4-4); anti-CD8-APC (clone 53–6.7), and anti-CD3-FITC (clone 17A2), and then fixed with paraformaldehyde 4%. For Foxp3 labelling, fixed cells were washed and permeabilized with saponin 0.1% prior to incubation for 30 min at room temperature with anti-Foxp3 PE (clone 259d/C7). Samples were acquired by flow cytometry (FACSCalibur System, BD Biosciences, USA) using the Cell Quest software (BD Biosciences, USA). Data were analyzed using the software Flowing.

2.12. Statistical analysis

Results were analyzed by software *GraphPad Prism*® 6.0 using One Way ANOVA method with Tukey post-test. For lesion size kinetics, Two-way ANOVA method with Bonferroni post-test was used. Results were expressed as means ± standard deviation (SD). Groups with $p < 0.05$ were considered statistically different. All data are representative of at least 3 independent experiments.

3. Results

3.1. Intranasal administration of RA-SLN does not induce local inflammation

Since RA used on its own produce skin irritation [32], the mucosal safety of RA-SLN was evaluated in mice at different times after i.n. administration. Representative histological sections of mouse nasal mucosa at 15 min, 2 h and 24 h after RA-SLN administration revealed no alterations in the ciliated pseudostratified columnar epithelium. Importantly, no inflammatory infiltrates were seen up to 24 h in the subepithelial region (Fig. 1, arrowheads). Clinically, the animals showed no signs of nasal discomfort like sneezing or scratching after RA-SLN administration (data not shown). These results support the safe use of RA-SLN for i.n. administration.

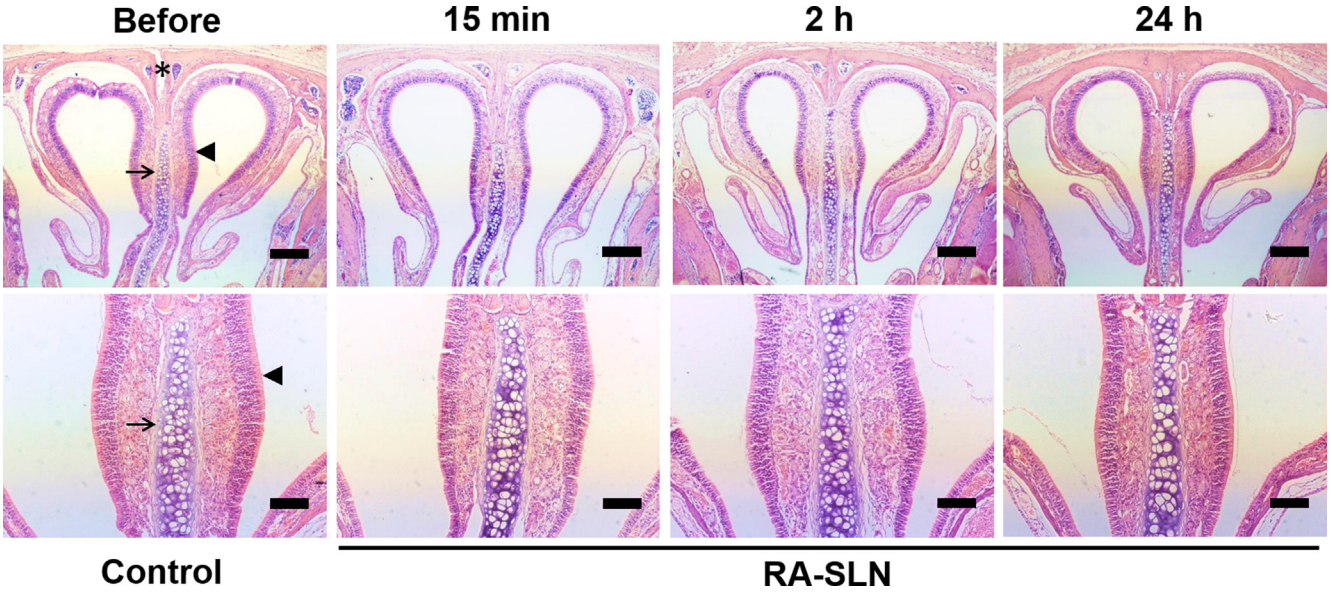

Fig. 1. RA-SLN does not induce nasal mucosal irritation. Mice received two i.n. doses of RA-SLN (15 μg of RA/10 μL/nostril) one week apart. At the indicated times after the second dose, histological sections of the nasal cavity were made and stained with H&E. Sections were imaged in lower (upper panel, bars are 400 μm), and higher magnification (lower panel, bars are 100 μm). Control is prior to instillation. Arrows and arrowheads indicate the nasal septum and the ciliated pseudostratified columnar epithelium, respectively. Asterisks indicate the snout position at the upper portion of the sections. Representative of 3 mice for each time point.

3.2. RA-SLN improves LaAg vaccine efficacy against CL

The capacity of RA-SLN to improve LaAg vaccine efficacy was evaluated in mice challenged with *L. amazonensis* infection. Expectedly, LaAg partially controlled lesion growth as compared with PBS controls (Fig. 2A). However, the onset of lesions was delayed, and their sizes were considerably smaller throughout the infection when LaAg was given together with RA/SLN. On day 63 of infection, LaAg and LaAg/RA-SLN-vaccinated mice had lesions 1.6-fold and 3.8-fold smaller than PBS controls, respectively, consistent with their appearance in Fig. 2B. For more precise evaluation, the parasite numbers were quantitated in the footpads by limiting dilution assay (Fig. 2C). Consistent with the lesion appearances, the parasite burdens in LaAg and LaAg/RA-SLN footpads were 61% and 96% smaller than PBS controls (5.1×10^4 and 5.1×10^3 *vs.* 1.3×10^7, respectively). RA-SLN alone did not affect normal lesion growth. Although the parasite burden appeared smaller, that was not statistically different from PBS ($p > 0.05$), demonstrating that the LaAg/RA-SLN is not due to RA-SLN alone. Together, these data demonstrate that RA-SLN has an adjuvant effect over LaAg, significantly improving vaccine efficacy against CL.

3.3. RA-SLN adjuvanticity is associated with suppressed early Th2 - type immune responses at the infection site

The influence of vaccination on cytokine profile was evaluated both in late (two months) as well as in the first days after parasite challenge in the footpads, when the ability to mount local cutaneous hypersensitivity was also determined. Two months (day 63) after challenge, LaAg-vaccinated mice displayed increased IL-12 concomitant with decreased IL-4 and TGF-β production in the footpads, as compared with PBS and RA-SLN controls (Fig. 3). No differences in IFN-ɣ and IL-10 levels were detected at this stage of infection. When cytokines of LaAg/RA-SLN-vaccinated mice were analyzed, they were not different from the partially Th1-skewed LaAg profile.

Thus, cellular immune responses were evaluated in earlier stages of infection, known to be more critical. We observed that the Th2-type Jones Mote-type cutaneous hypersensitivity reaction that normally follows *L. amazonensis* parasite challenge was significantly suppressed in LaAg pre-vaccinated mice (Fig. 4A), compatible with previous studies [11,17]. The LaAg suppressive effect was slightly potentiated when LaAg was associated with RA-SLN.

Analysis of transcription factors and cytokines in the lesion-draining lymph nodes of LaAg-vaccinated mice seven days after infection showed decreased Tbet (Th1) expression and enhanced GATA3 (Th2) expression in relation to PBS controls (Fig. 4B). However, the enhanced IL-12 expression together with diminished IFN-γ and IL-4 suggested a mixed Th1/Th2 phenotype. Regarding suppressor cytokines, LaAg vaccination did not significantly affect IL-10, but increased TGF-β expression. Association of LaAg with RA-SLN reduced its opposing effect on IL-12 and IFN-ɣ, and maintained IL-4 suppressed. On the other hand, the expression of Foxp3, a T_{reg} transcription factor, was significantly increased.

In summary, the above results indicate that RA-SLN adjuvanticity is associated with the peripheral suppression of an early Th2-type immune response that follows parasite challenge, and locally increased T_{reg} transcription factor and cytokine.

3.4. RA-SLN leads to increased T_{reg} cells and IL-10 production in the nasal mucosa

To better understand the immunomodulatory role of RA-SLN in the nasal mucosa, the cytokine responses and T cell phenotypes were evaluated in the mucosal cLNs 15 h and 72 h after the second vaccinating dose. Fig. 5A shows that the expression of IFN-ɣ, IL-12,

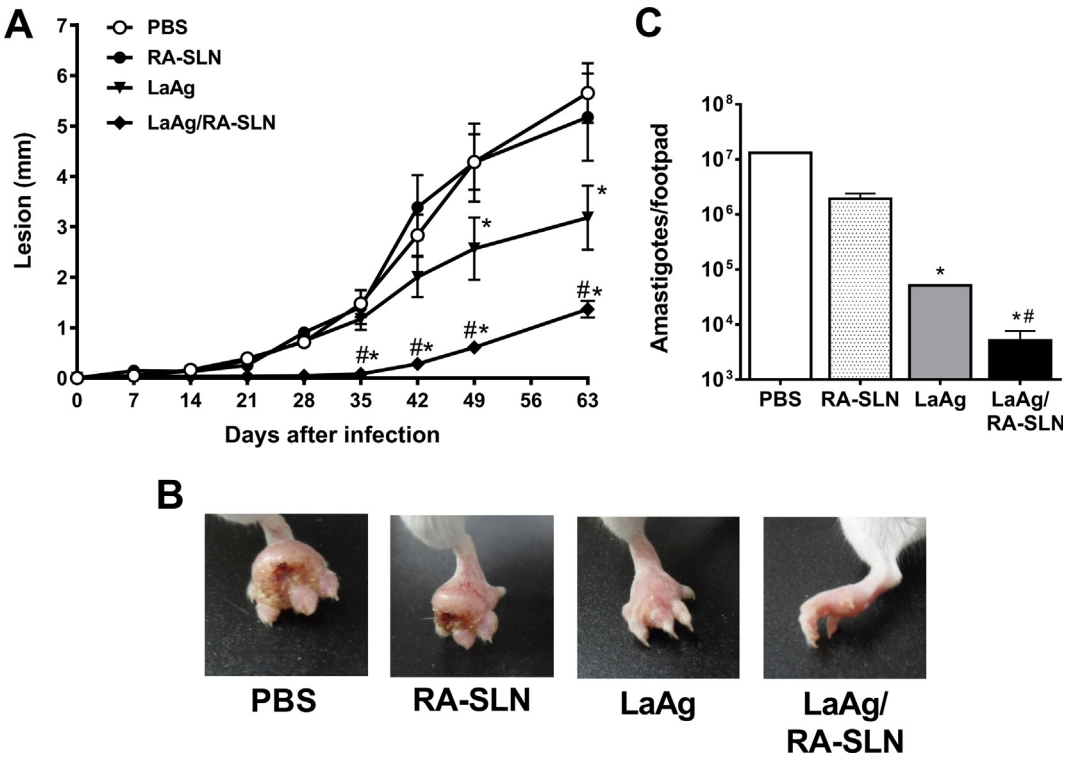

Fig. 2. Effect of RA-SLN in LaAg vaccine efficacy against CL. BALB/c mice were vaccinated with two i.n. doses of LaAg (10 μg each), LaAg/RA-SLN (10 μg LaAg + 15 μg RA), RA-SLN alone (15 μg RA) or PBS vehicle alone (10 μL) seven days apart. Seven days later, animals were infected in the footpad with 2×10^5 *L. amazonensis* promastigotes. (A) Footpad lesion sizes were measured in the indicated days. (B) Representative footpads on day 63 of infection. C) Parasite loads were determined by Limiting Dilution Assay on day 63. Results are representative of 3 different experiments. Means ± SD (n = 5). $^*p < 0.05$ (*vs.* PBS), # $p < 0.01$ (*vs.* PBS and LaAg).

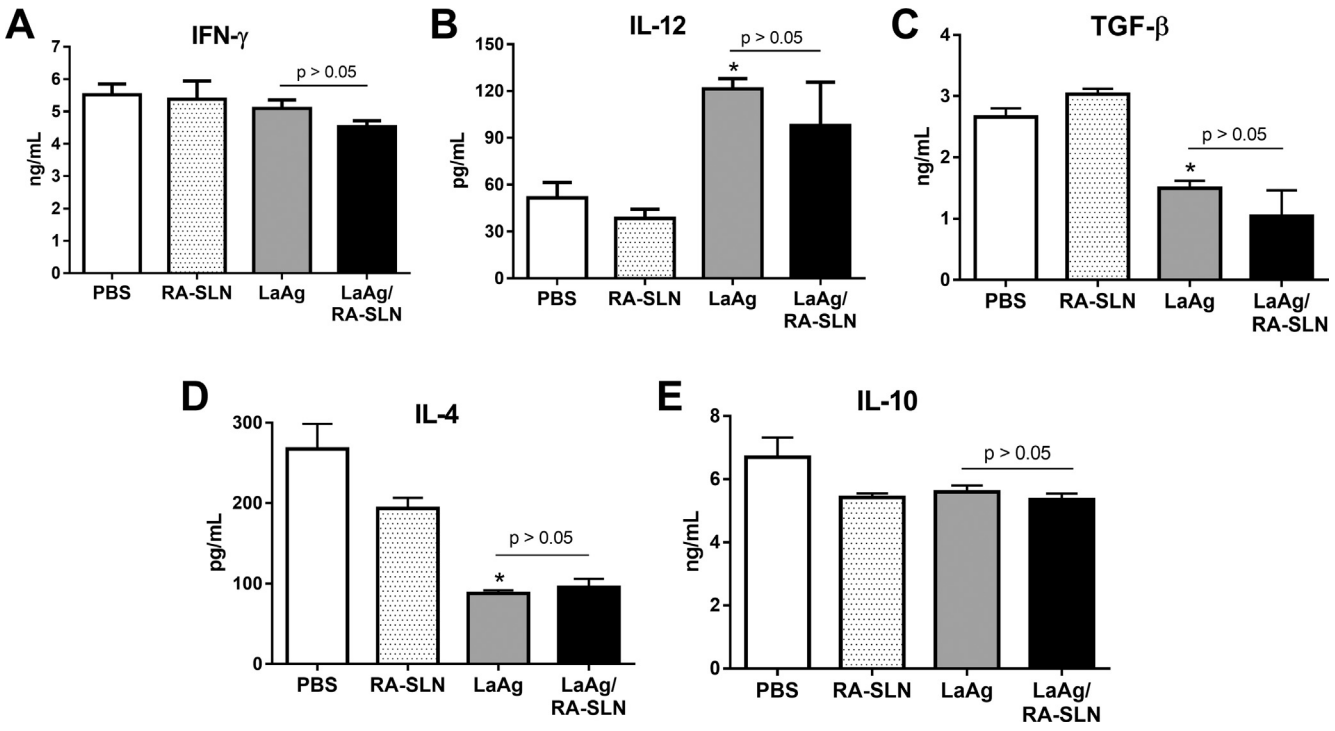

Fig. 3. Local cytokine responses in late infection. Mice were vaccinated and infected as for Fig. 2. On day 63 of infection, the indicated cytokines were measured in the infected footpad lysates. Results are representative of 3 different experiments. Means ± SD (n = 5) $^{*}p < 0.05$ *vs.* PBS.

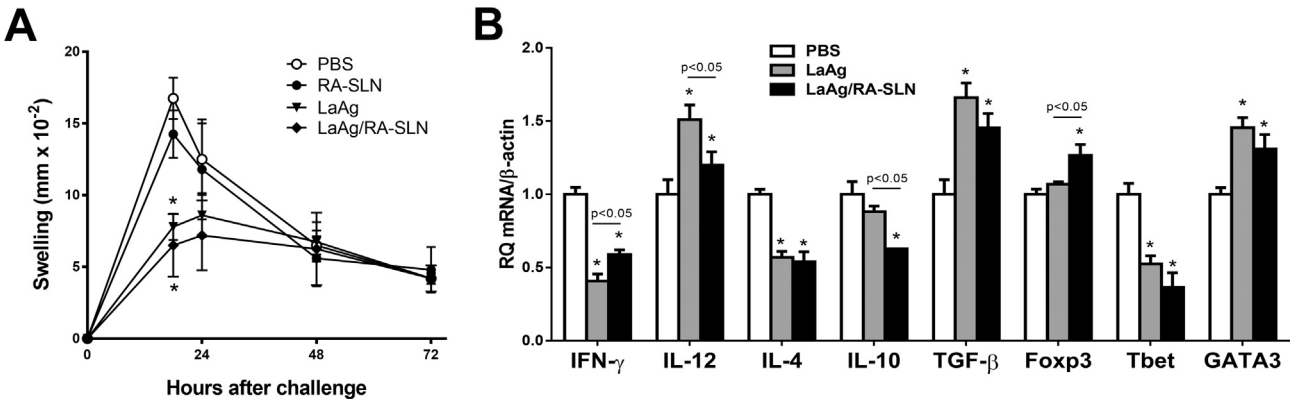

Fig. 4. Cutaneous hypersensitivity and local cytokine responses in early infection. Mice were vaccinated and infected as for Fig. 1. (A) Footpad swelling was periodically measured during the first 72 h of infection challenge. (B) On day 7 of infection, the indicated transcription factors and cytokines were assayed in lesion-draining pLNs by qRT-PCR, and expressed as relative quantities (RQ) of mRNA after normalization with β-actin. Representative of 2 experiments. Means ± SD (n = 5) $^{*}p < 0.05$ *vs.* PBS.

IL-4 and TGF-β was not affected by association of LaAg with RA-SLN. In general, all of those cytokines except IL-4 were suppressed after LaAg administration. Noteworthy, the suppressor cytokine IL-10 that was already elevated after LaAg, was further increased by RA-SLN. Vaccination with LaAg, disregard of the presence of RA-SLN, reduced total cell counts in the cLNs, compatible with cell efflux. However, RA-SLN selectively led to an increased proportion of $CD4^{+}$ and decreased $CD8^{+}$ T cells. Moreover, vaccination with LaAg/RA-SLN increased $Foxp3^{+}$ T_{reg} cell population in cLNs (Fig. 5D).

4. Discussion

A main advantage of mucosal vaccines in relation to injectable vaccines is that they do not require specialized personnel for administration, suitable for administration in least developed countries. However, few mucosal vaccines are available for human use, none of which are recombinant proteins or pathogen subunits because of lack of potent and safe mucosal adjuvants [33]. In this study, we propose the use of RA as a tolerogenic mucosal adjuvant. To prevent mucosa irritation, protect antigens from local hydrolysis and improve mucosal uptake, we used RA-SLN rather than free RA. The nasal mucosa was histologically analyzed following RA-SLN administration. No alteration in the ciliated columnar pseudostratified epithelium, or inflammatory infiltrates were seen from 15 min to 24 h after administration (Fig. 1). Although the i.n. use of free RA for treatment of olfactory loss is under clinical trial [34], SLN shall endow RA with extra safeness, as for skin use [30].

We previously showed the efficacy of i.n. vaccination with defined antigens, as LPG [9] and LACK DNA [11–14], that are likely less labile than protein antigens. However, here we opted to

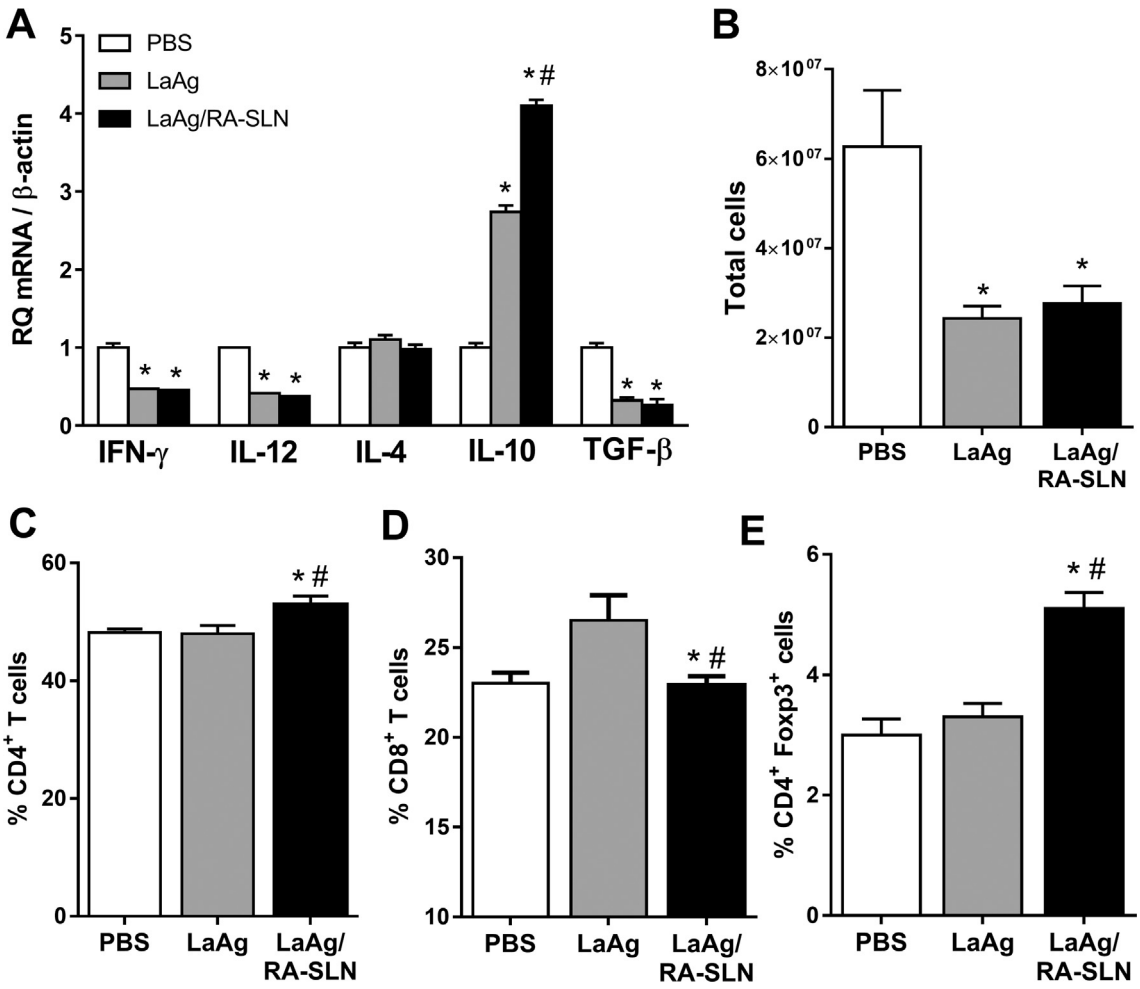

Fig. 5. Mucosal immune responses following vaccination. Mice were given two nasal doses of LaAg alone or in combination with RA/SLN, one week apart. At 15 h (A), or 72 h (B–E) after the second vaccination dose, cLNs were removed and individually assayed for the following: (A) Expression of the indicated cytokines as relative quantities (RQ) of mRNA normalization with β-actin. (B) Total cell counting; (C-E) Percentage of indicated populations in total $CD3^+$ T cells. Means ± SD (n = 5) $^*p < 0.05$ *vs.* respective PBS, #p < 0.05 *vs.* respective LaAg.

further explore the multivalent LaAg for its broad spectrum of action in different models of leishmaniasis [11,17,20–23]; ease of preparation; and reported human safety of intramuscular Leishvacin [35]. LaAg has been effective in adjuvant-free conditions probably because its intrinsic adjuvanticity and/or greater stability as compared with defined protein antigens. In a previous study, i.n. LaAg was used in association with CAF01 - a cationic liposomal system carrying a mycobacterial trehalose dimycolate analog - to improve Th1 immune response and efficacy against *L. infantum* infection in mice. However, the protection achieved was only partial [22].

Given the crucial role of RA in mucosal tolerance, in the present study, we proposed its adjuvant use to increase LaAg efficacy. The observation that RA-SLN further potentiates LaAg efficacy supports our tolerogenic mechanism hypothesis [17]. Mice that were pre-vaccinated with LaAg in the presence of RA-SLN produced significantly slower lesion growth and had lower parasite burdens, as measured two months after infection challenge (Fig. 2). Local Th1-skewed cytokines (higher IL-12, lower IL-4), concomitant with reduced TGF-β (Fig. 3) is compatible with protective immunity in *L. amazonensis* infection [16,36]. The cytokine profile and levels were similar in all vaccinated animals, irrespective of the presence of RA-SLN (Fig. 3). However, in the early and more critical stage of infection, a suppressed capacity to mount Th2-type cutaneous hypersensitivity reaction was observed (Fig. 4A). That was accompanied by suppressed IFN-γ, IL-4, IL-10 cytokine responses in the lesion draining lymph nodes (Fig. 4B), compatible with our previous study [17]. The reason why expression of Tbet and GATA-3 transcription factors did not accompany their respective Th1 and Th2 cytokine profiles is not clear, but differential expression timing should be considered. It has already been demonstrated that IFN-γ is not critical for the early control of infection in C57BL/6 mice [37]. Of relevance, increased Foxp3 expression was found when RA-SLN was used as adjuvant (Fig. 4B). Therefore, it is apparent that the RA-SLN adjuvanticity is related with suppression of important parasite-protective responses. Whether or not other responses like Th17 are also affected by RA-SLN remains to be investigated.

The mucosal effect of RA-SLN was also investigated in the local lymph nodes. Upregulated IL-10 expression and increased $CD4^+$ $Foxp3^+$ population were found in the nose-draining cLNs of mice administered with LaAg/RA-SLN (Fig. 5), compatible with a tolerogenic response. Analysis of $CD4^+$ and $CD8^+$ T lymphocyte populations showed that vaccination with LaAg alone increases $CD8^+$, whereas association with RA-SLN increases the $CD4^+$ population in the cLN (Fig. 5). Presentation of LaAg alone may favour cross-presentation of soluble exogenous antigens via MHC class I to T $CD8^+$ cells, leading to proliferation thereof. On the other hand, association with the nanoparticulated RA-SLN will favour phagocytosis and antigenic presentation via MHC class II [38], thus explaining the increased T $CD4^+$ population. Interestingly, an increased percentage of the $CD4^+$ $Foxp3^+$ T_{reg} population was verified when

RA-SLN was used as adjuvant (Fig. 5E), reinforcing the assumption that those cells are associated with the suppressive RA-SLN effect in the mucosa and early infection. Moreover, the protective role of T_{regs} in *L. amazonensis* infection is supported by the finding that $CD4^+$ $CD25^+$ T_{reg} adoptive transfer from infected mouse lesions to recipient mice leads to increased resistance against the parasite [39].

RA has been previously used as vaccine adjuvant aiming at increasing rather than suppressing mucosal immune responses, given its ability to upregulate the expression of α4β7 integrin and the chemokine receptor CCR9 in T and B lymphocytes [40,41] and to promote immunoglobulin switch to IgA isotype [40]. In this sense, RA has been explored not only as an adjuvant to oral vaccines [42] but also as parenteral vaccines aiming to redirect sensitized cells to the mucosa [43,44]. RA has also been therapeutically used to attenuate airway inflammation in a murine model of bronchial asthma by inhibiting both Th2 and Th17 cytokines, and downregulating the expression of the GATA3 in the lungs [28]. Aiming at tolerance induction also in asthma model, oral administration of ovalbumin in association with RA efficiently induced T_{reg} cells, inhibited the Th1-type inflammatory response, and decreased Th2-type eosinophilic infiltration in the lungs [29]. The concept of tolerogenic vaccine is very recent, since most vaccines are intended to enhance, not suppress host immune responses. Thus, like other Th2-driven pathologies like asthma and rhinitis, CL may also benefit from suppression of unwanted immune responses by tolerogenic vaccination in association with RA-SLN.

The protective tolerance seen here with LaAg/RA-SLN is in accord with early reports on protective tolerance induction to a single parasite antigen (LACK) in *L. major* infection achieved by transgenic expression of LACK in the thymus [7] or specific TCR deletion [45]. However, attempts to induce tolerance to LACK by i.n. vaccination with recombinant LACK protein failed to protect mice against *L. major* infection, unless cholera toxin beta subunit (CTB) was added as adjuvant [46]. In that study, i.n. LACK/CTB suppressed antigen-specific Th1, but not Th2 responses [46], different from RA-SLN used here that suppressed both Th1 and Th2 early responses, to allow subsequent expansion of effective Th1 cells.

Taken together, these data indicate that RA-SLN is an interesting adjuvant to LaAg i.n. vaccine against CL, possibly due to its capacity to increase T_{reg}-associated tolerance in the nasal mucosa. By extension, this study points to RA-SLN adjuvanticity in tolerogenic vaccination against other immunopathologies like allergy, inflammatory and autoimmune diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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