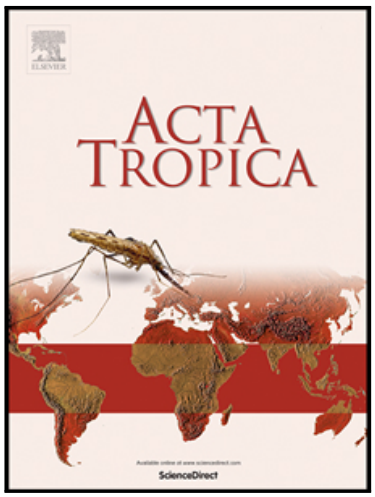

INTRANASAL DELIVERY OF LaAg VACCINE IMPROVES IMMUNITY OF AGED MICE AGAINST VISCERAL LEISHMANIASIS

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Highlights

- The age of mice directly affects Leishmania parasitemia.
- Use of the LaAg vaccine intranasally helps in the process of controlling parasite in young and elderly mice.
- The process of controlling L. infantum infection is directly related to the expression of inhibitory receptors such as PD-1 and KLRG-1

INTRANASAL DELIVERY OF LaAg VACCINE IMPROVES IMMUNITY OF AGED MICE AGAINST VISCERAL LEISHMANIASIS

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Running Title: Senescence promotes susceptibility to visceral leishmaniasis

Abstract

There are no approved vaccines yet for human visceral leishmaniasis (VL), the most severe form of the leishmaniasis clinical manifestations that is fatal in over 95% of untreated cases. It is well-accepted that immunological changes during ageing have deleterious impact on the efficacy of vaccines and response to infections. In this work, we compared the response of young and aged mice to intranasal vaccination with killed *Leishmania amazonensis*

promastigote antigens (LaAg) that were then challenged with *L. infantum* infection, a species that causes visceral leishmaniasis. Intranasal vaccination with LaAg induced a similar reduction in parasitism and hepatosplenomegaly in both young and aged mice compared to their unvaccinated counterparts. Following infection, there was also a less prominent inflammatory profile particularly in the vaccinated aged group, with lower production of TNF-α and nitrite compared to the respective unvaccinated group. Interestingly, the LaAg intranasal vaccination promoted increased production of IFN-γ that was observed in both young- and aged vaccinated groups. Additionally, $CD4^+$ and $CD8^+$ T cells from both vaccinated groups presented decreased expression of the inhibitory receptors PD-1 and KLRG1 compared to their unvaccinated controls. Interestingly, a strong positive correlation was observed between the expression of both inhibitory receptors PD-1 and KLRG1 and parasitism, which was more conspicuous in the unvaccinated-aged mice than in the others. Overall, this study helps define new strategies to improve vaccine effectiveness and provides a perspective for prophylactic alternatives against leishmaniasis.

***Key Words**:* Visceral leishmaniasis; Intranasal vaccine; *Leishmania amazonensis*; antigen; Ageing

1. Introduction

Leishmaniasis is a global vector-borne disease caused by an obligate intra-macrophage protozoan belonging to the *Leishmania* genus and for which there are still no vaccines approved for human use (Moafi et al., 2019). Visceral leishmaniasis (VL), the most severe clinical form of leishmaniasis, is a life-threatening disease that if left untreated can lead to death in over 95% of patients (World Health Organization, 2015).

In VL, the cure or disease progression is dependent on a fine interplay between the causative *Leishmania* species and distinct features of the host immunity (Torres-Guerrero et al., 2017). The host-specific cell-mediated Th1 immune response has an important role in controlling the infection, in which the pronounced production of IFN-γ is a key element for activating mechanisms associated with killing in parasite-infected macrophages (Castellano et al., 2009).

On the other hand, the acquisition of an exhausted profile, characterized by prominent expression of immune checkpoint receptors such as PD-1 and its ligands PD-L1/L2, CTLA-4, increased production of IL-10, and the accumulation of T cells expressing high levels of KLRG-1, has been associated with susceptibility to human and murine visceral leishmaniasis(da Fonseca-Martins et al., 2019; de Moura et al., 2021; Faleiro et al., 2016; Liang et al., 2006; Loureiro Salgado et al., 2022).

Compared to young subjects, elderly individuals present higher morbidity and mortality rates and increased susceptibility to acute and chronic infections, autoimmune diseases, and malignancies (Chen et al., 2020; Frasca and Blomberg, 2016; Goronzy et al., 2006; Toapanta and Ross, 2009). The changes that occur in the innate and adaptative immune system during ageing is termed immunosenescence and is associated with a cumulative decline of the immune effector functions, including altered cytokine production, poor responsiveness to antigen stimulation, and lower proliferative capacity (Caruso et al., 2009; Franceschi et al., 2000; Malaguarnera et al., 2001; Targonski et al., 2007). Moreover, the decline in the immune

system fitness with age is thought to contribute to reduced vaccine efficacy in humans and mice (Bajaj et al., 2021; Franceschi et al., 2000; Frasca and Blomberg, 2016; Goronzy and Weyand, 2013; Weinberger et al., 2008). These changes also occur in the mucosal immune system, resulting in a failure to elicit pathogen-specific humoral responses that protect the host from infectious diseases (Fujihashi et al., 2019).

Recently, we demonstrated that ageing negatively impacts the outcome of VL (Loureiro Salgado et al., 2022). Aged mice showed increased susceptibility to *L. infantum* infection compared to younger mice, presenting notable parasitism in both the spleen and liver (Loureiro Salgado et al., 2022). The parasitism was found along with a pronounced inflammatory profile, reduced proliferative capacity of T cells and increased expression of the exhaustion/inhibitory receptors on these cells (Loureiro Salgado et al., 2022). In support of this, previous studies have shown that age correlates with increased infection prevalence and lower responses to the *Leishmania* skin test (LST) during human VL (Sassi et al., 2012). Broad evidence also suggests that ageing may represent a significant risk factor for tegumentary/cutaneous leishmaniasis (CL) infection (Jirmanus et al., 2012). Elderly subjects more frequently had a previous history of CL, large lesions, longer healing times, or presented the mucosal and disseminated clinical forms than young subjects (Carvalho et al., 2015).

We also have extensively demonstrated the feasibility of the mucosal route for delivering adjuvant-free killed *L. amazonensis* promastigote antigen (LaAg) and DNA vaccines against CL and VL (Chaves et al., 2015; Leonel et al., 2014; Pinheiro et al., 2007; Pinto et al., 2004, 2003). The intranasal immunization (i.n) strategy offers advantages over the parenteral route for poorly immunogenic or disease-inducing formulations (Pinheiro et al., 2007), in addition it can induce strong long-lasting immunity that is poorly obtained in elderly individuals (de Oliveira Gomes et al., 2012). The prophylactic effect of intranasal (i.n.) LaAg has shown promising potential in inducing immunity against both CL and VL in susceptible BALB/c mice (Salgado et al., 2019). Its efficacy has been observed also in CL in C57BL/6 mice caused by

L. amazonensis (Pratti et al., 2016), as well as in golden hamsters against *L. braziliensis* (Ribeiro-Romao et al., 2014). In these instances, animals exhibited a reduced capacity to initiate early responding cutaneous hypersensitivity and displayed an enhanced ability to combat infection. However, the precise mechanism behind this effect remains incompletely understood. These findings underscore LaAg's potential as a multivalent vaccine offering cross-species protection in both young and aged mice, unexplored so far.

In this study, we analyzed the outcome of *L. infantum* infection in young and aged mice that received intranasal delivery of a LaAg vaccine. We reveal that LaAg intranasal delivery induces immunogenic and protective immunity based on the expansion of specific T cells together with the production of IFN-γ, a decrease in the expression of inhibitory receptors and control of non-specific inflammation.

2. Materials and Methods

2.1 Animals

C57BL/6 mice were originally purchased from Jackson Laboratory (Bar Harbor, Maine, USA). They were bred and maintained at our facilities, and given sterilized bedding, filtered water, and pelleted food *ad libitum*. For each experiment, 5-8 female mice at 4-6 weeks old or at 78 weeks old were used for the young and aged groups, respectively. The Ethical Committee for Experimental Animal Use at the Universidade Federal do Espírito Santo approved all experimental protocols, registered under reference number: 014/2011.

2.2 Parasites

L. infantum MHOM/BR/1975/PP75 and *L amazonensis* strain MHOM/BR/75/Josefa promastigotes were cultured at 26°C in Grace's medium (Gibco, USA), pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM HEPES, and 20 µg/ml gentamicin (LGC, Brazil).

2.3 Preparation of parasite antigen

For both *L. amazonensis* (LaAg) and *L. infantum* (LiAg) antigens, promastigotes in the late-log phase were centrifuged (4000 RPM/20 min/4°C), washed five times in phosphate-buffered saline (PBS) and disrupted by 10 rounds of freeze-thawing in liquid nitrogen. The protein content was determined by the Lowry (LOWRY et al., 1951) method and kept at 80°C until use.

2.4 Vaccination and parasite challenge

Young and aged mice received 2x 25 µg doses of adjuvant-free LaAg by nasal instillation given with a one-week interval. One week after the booster the animals were infected by the intravenous route in the tail vein with 10^7 *L. infantum* promastigotes in the stationary phase of growth in 100 µL volume, as described previously (Salgado et al., 2019).

2.5 Parasite burden

On day 30 of infection, the parasite burden in the liver and spleen of the mice were individually determined by limiting dilution assay, as described previously (Salgado et al., 2019). Briefly, each organ was weighed and homogenised in Grace's medium supplemented with 10% heat-inactivated FBS. The volume of the cell suspension was adjusted with supplemented Grace's medium according to tissue weight (100 mg of tissue/mL) and plated in a 96-well plate (Corning, USA). Serial dilutions of the single-cell suspensions were prepared and then plate was then incubated for ten days at 26°C. The original number of parasites in each organ was calculated from the reciprocal of the highest dilution containing promastigotes.

2.6 Cutaneous hypersensitivity reaction

Twenty-four hours after infection, control and vaccinated mice were injected with 10 µL of PBS containing 20 mg of LiAg in the hind footpad. Footpad swelling was measured with a dial caliper up to 48 h post-injection and the results were expressed as the difference between the thickness of the injected and pre-injected footpads.

2.7 Cytokines and nitrite production

In situ cytokine quantification was performed for both the liver and spleen of the mice as previously described in (Leal et al., 2015; Stegmiller et al., 2016a). The organs were individually homogenised in 1 mL PBS with the addition of protease and phosphatase inhibitors (Sigma-Aldrich, USA) using a glass tissue grinder (Thomas, USA). The normalised cell suspensions were centrifuged at 20,000 x g for 10 min at 4°C, and the supernatants were collected for the subsequent tests. Cytokine levels were determined by ELISA following the manufacturer's instructions, considering the sensitivity of each test (R&D Systems, USA). Nitrite production was determined using the Griess method (Bryan and Grisham, 2007). Briefly, 50 µl of the supernatants were mixed with 50 µl of Griess reagent (1% sulphanilamide and 0.1% N-1-naphthylethylenediamin dihydrochloride in 2.5% *o*-phosphoric acid) and incubated at room temperature for 10 min. The nitrite concentrations were determined at 540 nm against a standard sodium nitrite curve.

2.8 Flow cytometry

For phenotypic analysis, at least 10^6 splenocytes were stained with antibodies against cell surface markers for 30 min at 4°C. The cells were then fixed using paraformaldehyde for 30 min at room temperature. Data from 50,000 events were acquired on a BD FACSCanto II flow cytometer to determine the following: $CD4^+$ (Clone RM4-5) or $CD8^+$ (Clone 5H10-1) cells with KLRG1 (Clone 2F1) and PD-1 (Clone J43) (antibodies from BioLegend, USA). After acquisition, data were analysed using FlowJo software (Version 10). Gates for inhibitory receptors were based on pooled fluorescence minus one control samples and applied identically across all samples.

2.9 Statistics

Data were analysed using the GraphPad Prism software (Version 8.0 for Windows). Means of normally distributed variables were compared by an ANOVA simple factorial test, by one-way

ANOVA-Tukey's honestly significant difference (HSD) posthoc test, and by the Spearman's rank correlation method. Data were considered significantly different when $p < 0.05$.

3. Results

3.1 Intranasal vaccination with LaAg reduces the parasite burden and visceral leishmaniasis clinical presentation in aged mice

In both human and mouse experimental models, aging has been linked to susceptibility to infections, including influenza, Streptococcus pneumoniae, Listeria monocytogenes, and other pathogens (Alam et al., 2020; Bender et al., 1991; Ismail et al., 2017; Krone et al., 2013; Murasko and Jiang, 2005). Additionally, aging has been associated with reduced vaccine effectiveness (Boraschi and Italiani, 2014; Grubeck-Loebenstein et al., 2009).To investigate whether intranasal vaccination with LaAg could protect aged mice against *L. infantum* infection, we assessed the parasite burden in the liver and spleen on the 30th day of infection, representing the peak of parasitism (Salgado et al., 2019).. We found that both aged groups (vaccinated and non-vaccinated) had higher parasite loads than the young groups, supporting previous data from our group. However, both vaccinated groups showed a significant reduction in parasite loads in the spleens and livers compared to the corresponding unvaccinated controls (Fig. 1A). Interestingly, despite showing more significant parasitism than vaccinated young mice (Fig. 1A), vaccinated aged mice showed a comparable reduction in the parasite burden in relation to the respective control (fold difference) as that observed for the vaccinated young group (Supplementary Fig 1). In the spleen and liver, the vaccinated young mice had a 5.1- and 14.3-fold reduction and the aged group had a 3.1 - and 6.2-fold reduction in the parasite burden compared to their non-vaccinated controls, respectively (Supplementary Fig 1). In concordance with our previous findings regarding increased susceptibility to *L. infantum* infection with ageing (Loureiro Salgado et al., 2022), aged mice had larger spleens and livers than the young mice, regardless of whether they were vaccinated or not. However, both vaccinated groups displayed lower hepatosplenomegaly than their non-vaccinated controls (Fig 1B and C), suggesting a

specific effect of the LaAg vaccine.

3.2 Intranasal vaccination with LaAg enhances cellular immunity and reduces the non-specific inflammation in aged mice

The failure to stimulate specific immunity associated with the maintenance of a persistent inflammatory profile is the key factor associated with vaccine ineffectiveness during ageing in humans and mice (Cookenham et al., 2020; Franceschi et al., 2000; Franceschi and Campisi, 2014; Nanishi et al., 2022; Pereira et al., 2020; Salgado et al., 2019; Timothy et al., 2011). To investigate whether LaAg intranasal vaccination could improve the cell-mediated immune responses in aged mice, we assessed the antigen-specific delayed-type hypersensitivity and cytokine production. One day after intravenous infection with *L. infantum*, all mice were injected with LiAg in the footpad. As expected, young-vaccinated mice had the highest DTH responses starting at 18 h and extending to 48 h post-antigenic challenge (Fig 2A). Interestingly, the LaAg intranasal vaccination boosted the cellular response in aged mice LaAg at 24 and 48 h (Fig 2A), supporting the effect of LaAg vaccination in inducing a specific cellular immunity (Salgado et al., 2019). In our experiments, the analysis of the *ex vivo* splenic cytokine production revealed increased amounts of IFN-γ and IL-4 following infection in LaAg-vaccinated mice, which was more pronounced in the young than the aged-vaccinated mice (Fig 2B and C), production of which were not observed in the unvaccinated groups. Moreover, compared to their young counterparts, aged groups demonstrated increased production of TNF-α (Fig 2D) and nitrite (Fig 2E), a breakdown product of nitric oxide (NO). Interestingly, the production of both cytokines was reduced in the vaccinated group compared to the non-vaccinated group, suggesting the specific role of LaAg vaccination in protecting against VL infection. No difference in the production of either cytokine was observed between vaccinated and unvaccinated young groups (Fig. 2D and E).

3.3 LaAg vaccination contributes to reducing the expression of inhibitory receptors and

the accumulation of PD-1 and KLRG1 expressing T cells

As T cells expressing inhibitory receptors accumulate in *Leishmania* infection and are associated with the VL severity [18], we next assessed the effect of LaAg intranasal vaccination in this process. We evaluated the expression of killer cell lectin-like receptor G1 (KLRG1) and programmed cell death protein-1 (PD-1) on $CD4^+$ and $CD8^+$ T cells following *L. infantum* infection. Unvaccinated aged mice displayed the greatest frequencies of KLRG1 (Fig. 3A, B, C) and PD-1 (Fig. 3D, E, F) within both CD4 and CD8 T cell pools, which were higher than the vaccinated aged mice and both young groups. In the young groups, vaccinated mice also displayed a substantial decrease in the expression of PD-1 and KLRG1 on both T cell compartments compared to unvaccinated mice. Therefore, vaccination caused a reduction in the expression of the inhibitory receptors in both young and aged groups following infection.

We next investigated how the ability of LaAg intranasal vaccination to prevent or reduce the suppressive immunity correlates with susceptibility to *L. infantum* infection. Correlation analysis was performed between the frequencies of KLRG1 and PD-1 expressing $CD4^+$ and $CD8^+$ T cells and the parasite burdens found in the spleen. The KLRG1-expressing $CD4^+$ and $CD8^+$ T cells and the splenic parasite burden were strongly correlated for aged, but not young, mice (Fig. 4A and C). The same positive correlation was also found for the expression of PD-1 in both T cell populations (Fig. 4B and D). Overall, our results support previous data that show the correlation between the expression of inhibitory receptors and susceptibility. Thus, the greater the expression, the greater the susceptibility, with the unvaccinated aged group having both the greatest expression and greatest parasite loads (Loureiro Salgado et al., 2022). Interestingly, the aged-vaccinated mice exhibited a reduction in both of these parameters compared to their unvaccinated counterparts. The same profile was also observed in young mice. These data suggest that the vaccine may modulate the expression of inhibitory receptors on T cells, by perhaps directing the immune response towards an anti-*Leishmania* profile, resulting in reduced parasitism within the vaccinated groups.

4. Discussion

The severity of infection is generally higher in elderly individuals that also do not generate a robust enough immune response to vaccine antigens. (Ciabattini et al., 2018). Strategies to enhance vaccine-mediated protection in older individuals include increasing the dose, adding adjuvants, and utilizing new routes to deliver antigens (Leal et al., 2015; Oliveira et al., 2012; Stegmiller et al., 2016b; Vogel, 2000). In the current study, we sought to test the efficacy of the LaAg vaccine, a vaccine against *Leishmania* infection, given intranasally in aged mice compared to young mice.

Our research group has previously succeeded in inducing protective immunity against visceral leishmaniasis caused by *L. infantum* and cutaneous leishmaniasis caused by *L. amazonensis* and *L. braziliensis* infections in both mice and hamsters through the intranasal delivery of LaAg (da Silva-Couto et al., 2015; Leal et al., 2015; Salgado et al., 2019). The use of intranasal administration of antigens has been also explored in several other infection models, such as *Mycobacterium tuberculosis* and *Staphlococcus aureus*, due to its characteristics of inducing local and systemic immunity (Desheva et al., 2019; Kaushal et al., 2015; Stegmiller et al., 2016b). Nonetheless, the data presented herein demonstrate, for the first time, that the nasal LaAg vaccine can elicit protective immunity in an experimental model of aged animals.

In our experiments, aged animals exhibited heightened susceptibility to Leishmania infection, evidenced by elevated parasite burdens observed in both the spleens and livers. These findings corroborate previous research indicating their heightened vulnerability to Leishmania infection and other pathogens (Krone et al., 2013; Loureiro Salgado et al., 2022; Toapanta and Ross, 2009). While we noted higher absolute parasite loads in aged animals compared to young animals, vaccination of the aged group with LaAg led to a reduction in parasite loads comparable to the decrease observed in the vaccinated young group. This

observed consequence of the LaAg intranasal vaccination represents a significant finding with epidemiological and immunological implications, particularly when considering the heightened susceptibility to infections associated with aging (Frasca and Blomberg, 2016; Tsuji et al., 2022).

The immune response in C57BL mice infected with L. infantum differs from that observed in CL induced by L. major infection. In L. infantum infection, C57BL mice display a mixed Th1/Th2 responses profile (Duthie et al., 2017; Loureiro Salgado et al., 2022; Maioli et al., 2004), where the prevalent Th1 profile, characterized by IFN-γ production, triggers macrophage-dependent leishmanicidal activation mechanisms that are associated with protective immunity(Podinovskaia and Descoteaux, 2015). Notably, LaAg vaccination led to increased IFN-γ production and cellular immunity, indirectly accessed by the footpad swelling in both young and elderly mice post-infection. This is particularly interesting since both IFN-γ and a great DTH score in Montenegro skin test positively correlate with protection and reduced parasite burden (Keerti et al., 2018; Salgado et al., 2019), as observed in our experiments. Moreover, the same profile was observed concerning IL-4 production, suggesting its association with a protective response during infection. In fact, unlike what is observed in *L. major* infection, the presence of a Th2 along with a strong Th1 response has been associated with protection against visceral Leishmaniasis (Leal et al., 2015; McFarlane et al., 2019). Specifically, concerning IL-4, its production has been widely associated with the differentiation of dendritic cells, activation of Macrophage with M2 phenotype, which is involved in tissue repair, as well as to the formation and maintenance of granulomas, essential to contain the spread of the parasites(Poudel et al., 2020; Tomiotto-Pellissier et al., 2018). Another interesting point is that vaccinated aged mice showed significant decreases in the production of TNF-α and NO compared to the unvaccinated group following infection, which was not seen in the young groups, as these groups displayed equally low levels. Both cytokines are associated with non-specific inflammatory processes observed in elderly individuals and

termed 'inflammageing', which is associated with the pathogenic process (Santos-Moreno et al., 2021; Wang et al., 2021), including increased susceptibility to *L. infantum* infection (Loureiro Salgado et al., 2022). Therefore, vaccination with LaAg may play an important role both in preventing undesirable responses, such as non-specific inflammation, and in enhancing the production of key cytokines in parasitism control, as observed in our experiments.

The chronic production of TNF-α, NO, and other pro-inflammatory compounds can induce the expression of stress ligands, and immune checkpoint receptors and their ligands in immune and non-immune cells and promote inadequate T-cell responses (Bogdan, 2020; Rodrigues et al., 2016, 2014). A marked pro-inflammatory profile has been found in association with increased expression of KLRG1 and PD-1 within T cell populations and impaired protective immunity in other models of infection(Esch et al., 2013; Hu et al., 2018). In line with this, we demonstrated that *Leishmania* infection may positively modulate the expression of PD-1 and KLRG1 in T cells, which was more pronounced in aged mice. Moreover, a higher correlation was found between the inhibitory receptors expression and the parasitism observed in the spleen and liver of the aged group, which supports previous findings regarding the role of inhibitory receptors in promoting disease severity (Loureiro Salgado et al., 2022). A key point raised in this study is that the LaAg vaccination reduced the expression of KLRG1 and PD-1 within both $CD4^+$ and $CD8^+$ T cells of young and aged mice. The decrease is notable for the latter group, where an exacerbated inflammatory state is naturally found and presents a deleterious role in controlling the infectious process (Freund et al., 2010; Tsuji et al., 2022). The decreased production of inflammatory mediators and inhibitory receptor expression observed in the vaccinated groups may have contributed to boosting the antigen-specific immunity that was able to reduce the parasite burden in these groups.

The use of animal models, inherently more resilient to infection and that do not present

pathological/ clinical manifestations compared to human visceral leishmaniasis, holds significance, and may represent a limiting point of this study. In this context, delving into more representative models like hamsters (da Silva-Couto et al., 2015; Fiuza et al., 2016) or those aligning more closely with the disease's epidemiology (such as dogs) might yield enhanced translational insights(Gradoni et al., 2005; Morales-Yuste et al., 2022). Another pivotal aspect is the assessment of the response at a specific juncture, lacking information on the duration of the protective response post-vaccination. Establishing enduring immunity stands as a critical aspect in vaccine formulation, a facet that will be explored in forthcoming experiments within our research group. Overall, this study helps define strategies to improve vaccine effectiveness against VL. Moreover, this study also highlights the importance of considering the age of animals for infection and drug/vaccine testing models.

5. Conclusion

The study elucidates that LaAg intranasal administration elicits immunogenic and protective immunity through the amplification of T cells, concomitant with IFN-γ production, a reduction in inhibitory receptor expression, and regulation of non-specific inflammation. These findings imply that this vaccine platform holds promise for successful utilization as an alternative approach in aged populations, who exhibit heightened vulnerability to the disease.

Author statement

All authors reviewed the results and approved the final version of the manuscript.

Credit authorship contribution statement

Conceptualization: Daniel Gomes, Luciana Polaco Covre and Caio Salgado. Formal analysis: Caio Salgado, Andrés Corea, Luciana Covre and Alessandra Martins. Original draft preparation: Aloisio Falqueto, Herbert Guedes, Bartira Rossi-Bergmann and Daniel Gomes. Review: Bartira Rossi-Bergmann and Daniel Gomes. All authors have read and agreed to the

published version of the manuscript.

Data availability

Data will be made available on request.

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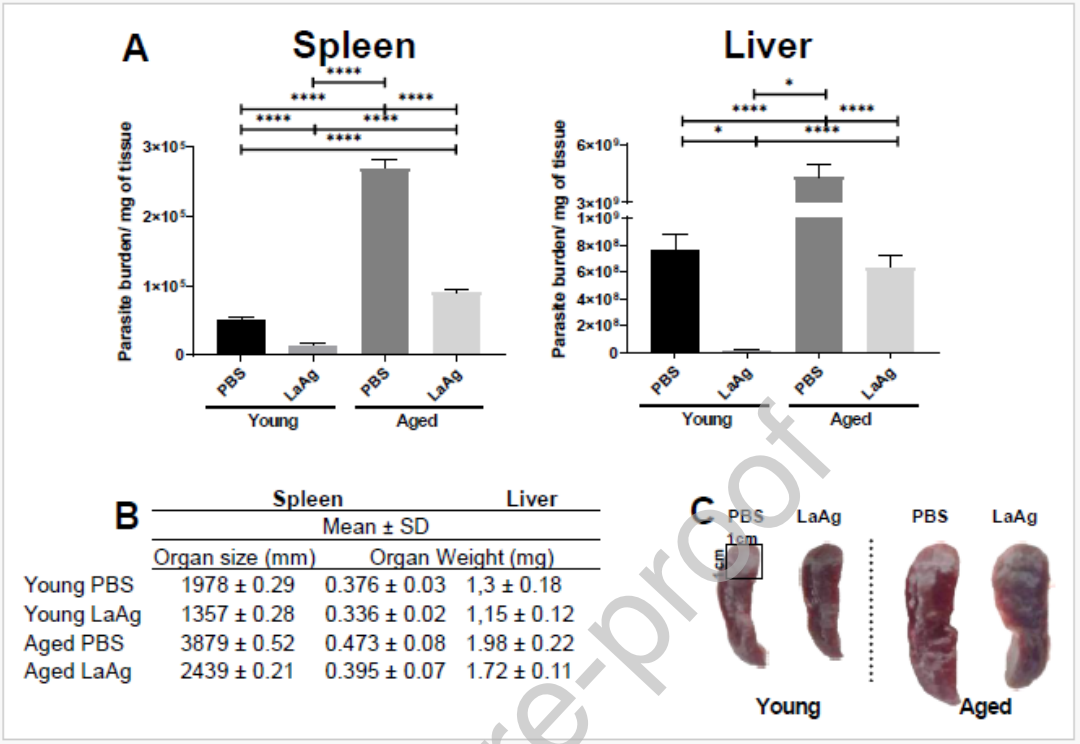


Figure 1. Parasite burden and clinical features in young and aged mice following LaAg intranasal vaccination. Young and aged mice received 2 doses of 25 µg of adjuvant-free LaAg by nasal instillation with a one-week interval, while controls received PBS alone. One week later all animals were infected i.v. with 10^7 *L. infantum* promastigotes. At 30 days post-infection the parasite burden and physical features were individually assessed in the spleen and liver. (A) Cumulative data of the parasite burden in spleen and liver. (B) Table with average organ sizes and weights, and (C) representative images of a spleen for each group at the peak of parasitism. Results are represented as arithmetic means ± S.D of pooled data (n=5 mice/group) obtained from three independent experiments. Statistical differences and p values were determined by ANOVA and are indicated in the graphs*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

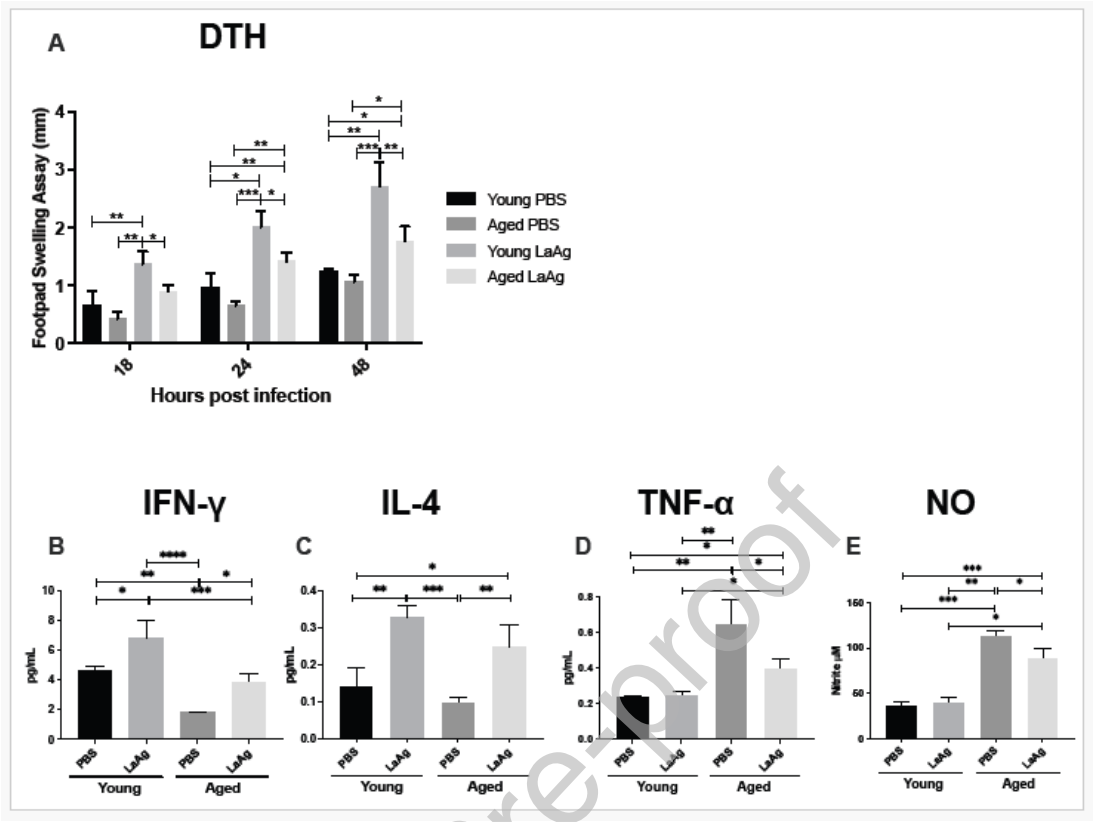


Figure 2. Cutaneous hypersensitivity, cytokine and nitrite production following infection in vaccinated mice. Mice were vaccinated with LaAg and infected with *L. infantum* as described in Fig 1. (A) The footpad swelling was determined one day after intradermal injection with LiAg. After 30 days of infection, the spleens were removed, macerated and cytokine levels in the supernatant were determined by ELISA for (B) IFN-γ, (C) IL-4, and (D) TNF-α, while (E) nitrite production was assessed by Griess assay. Results are represented as arithmetic means ± S.D of pooled data (n=5 mice/group) obtained from three independent experiments. Statistical differences and p values were determined by ANOVA and are indicated in the graphs*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

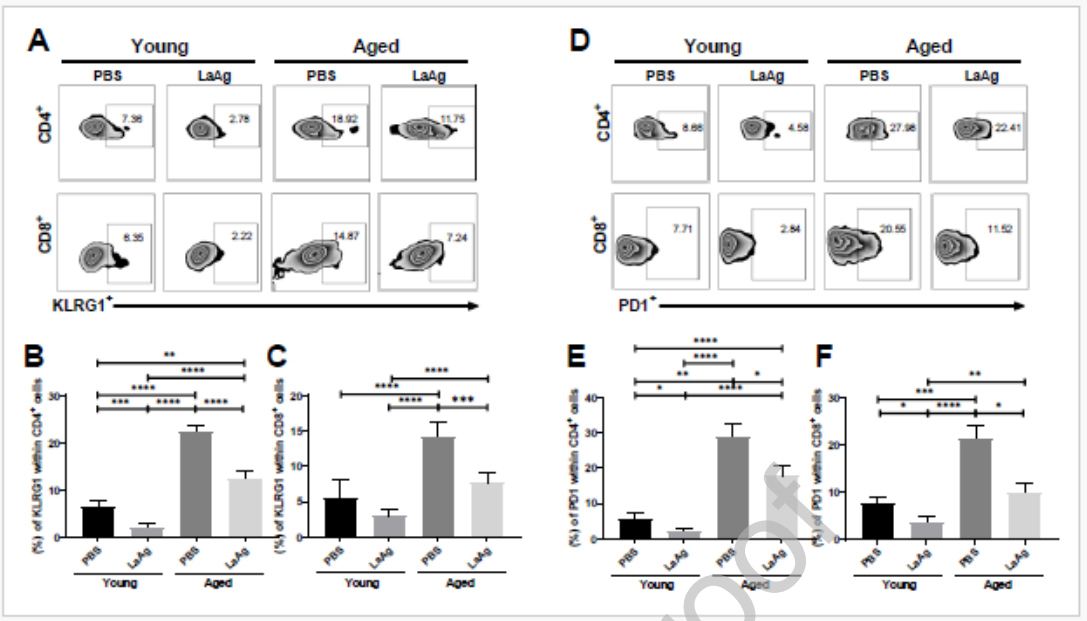


Figure 3. Expression of inhibitory KLRG1 and PD-1 receptors in vaccinated infected mice. Mice were vaccinated with LiAg and infected with *L. infantum* as described in Fig 1. On day 30 after infection, splenocytes were isolated and tested for expression of (A-C) KLRG1 and (D-F) PD-1 on $CD4^+$ and $CD8^+$ T cells by flow cytometry. (A and D) Representative dot plots with receptors expressed frequencies in the gates and (B, C, E and F) pooled data (n=5 mice/group) obtained from three independent experiments. Statistical differences and p-values were determined by ANOVA and are indicated in the graphs. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

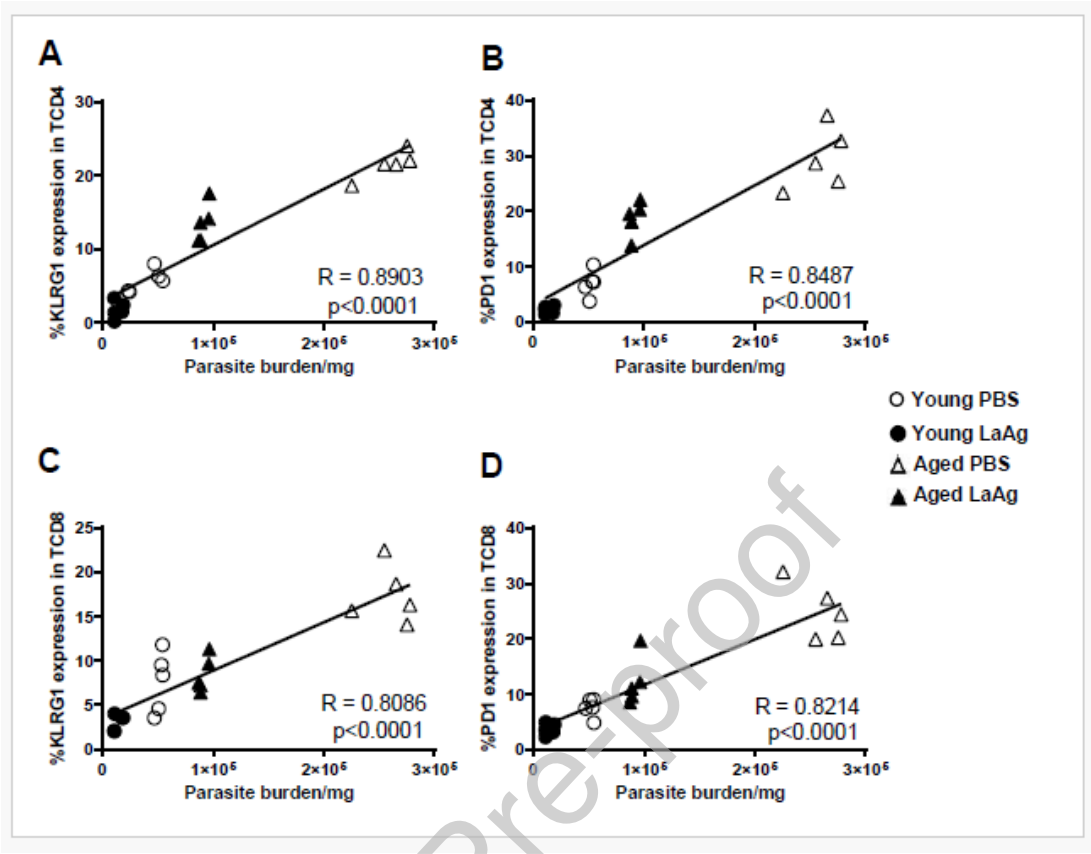
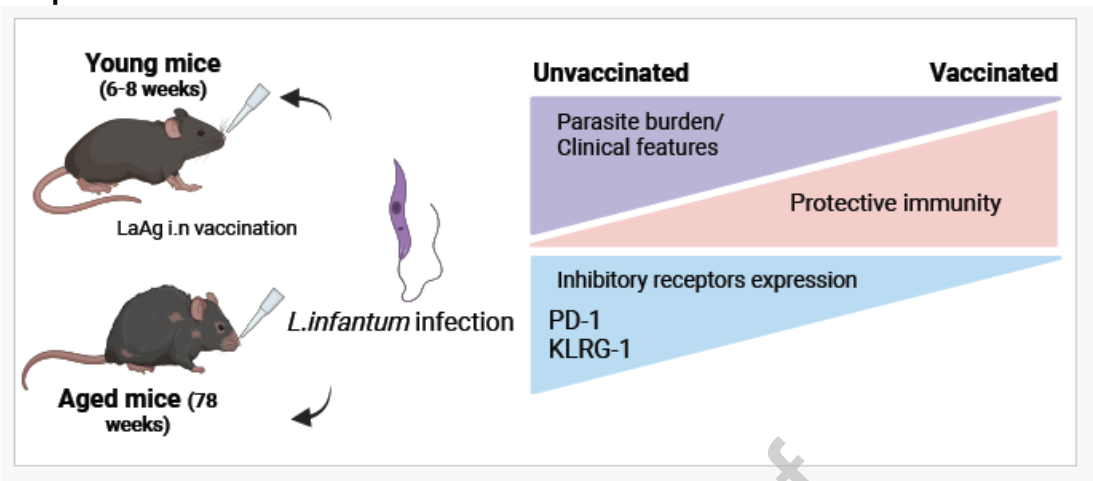


Figure 4. Correlation between the expression of inhibitory receptors and the severity of visceral leishmaniasis is reduced with intranasal vaccination with LaAg. Pearson's correlation test between frequencies of $CD4^+$ and $CD8^+$ T cells expressing KLRG1 (A and C, respectively) and PD-1 (B and D, respectively) and the splenic parasite burden of unvaccinated young (PBS; white dots), vaccinated young (LaAg; black dots), unvaccinated aged (PBS; white triangle), and vaccinated aged (LaAg; black triangle) mice. Results are presented as representative data from three independent experiments. Statistical differences and p-values were determined by linear regression and indicated in the graphs.

Graphical Abstract



Declaration of Interests

☒ 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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☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: