Ageing impairs protective immunity and promotes susceptibility to murine visceral leishmaniasis

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Running Title: Senescence promotes susceptibility to visceral leishmaniasis

Abstract

It is well-accepted that the impact of diseases is generally more detrimental in elderly individuals than in younger ones. Changes in the immune system due to ageing can directly affect the ability to respond effectively to infections and may contribute to the higher morbidities and mortalities in the elderly population. Leishmaniasis is a complex of clinically unique diseases caused by obligate intracellular protozoa belonging to the *Leishmania* genus, wherein visceral leishmaniasis (VL) is the most severe form and is fatal if left untreated. In this study, aged mice (72 weeks-old) presented increased susceptibility to *L. infantum* infection compared to younger mice (4–6-week-old), with notable parasitism in both the spleen and liver, as well as exhibiting hepatosplenomegaly. A pronounced inflammatory profile was observed in the aged-infected mice, with excessive production of TNF-α and nitrite, along with diminished IFN-γ production and reduced proliferative capacity of T cells (assessed by expression of the Ki67 marker). Additionally, both CD4^{+} and CD8^{+} T cells from the aged-infected mice presented increased expression of the inhibitory receptors PD-1 and KLRG1 that strongly correlated with the parasitism found in the liver and spleen of this group. Overall, the data reported in this study suggests for the first time that ageing may negatively impact the VL outcome and provides a perspective for new therapeutic strategies involving manipulation of immunosenescence features against *Leishmania* infection.

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Introduction

As we age, physiological changes are observed in several body systems, including the immune system (Akbar *et al.*, 2016). The changes that occur within the immune system are termed immunosenescence and can be found in both the innate and adaptive immune compartments. Such alterations can be linked to different cellular processes such as loss of cellular diversity (Aiello *et al.*, 2019), impaired cell signalling (Larbi *et al.*, 2011; Solana *et al.*, 2012), telomeric erosion (Rufer *et al.*, 1999), decreased proliferative capacity (Mauch *et al.*, 1982; Rebel *et al.*, 1996), as well as a chronic inflammatory profile (Barbé-Tuana *et al.*, 2020), even in the absence of infection. Immunosenescence results in the impaired response to vaccines and increased susceptibility to pathogens and opportunistic infection in humans and animal models (Weinberger *et al.*, 2008; Leng *et al.*, 2011; Chen *et al.*, 2020).

The impact of infectious diseases on older individuals is far more significant in terms of morbidities and mortalities than on younger subjects (Norman, 2016). Elderly individuals exhibit increased susceptibility to respiratory (Bender *et al.*, 1991; Krone *et al.*, 2013; Williams *et al.*, 2015), urinary (Pavlicek *et al.*, 2017), and intra-abdominal infections caused by viruses(Murasko and Jiang, 2005), bacteria and protozoan (Krone *et al.*, 2013; Sorci *et al.*, 2021).

Leishmaniasis is a complex of clinically unique diseases caused by obligate intracellular protozoa belonging to the *Leishmania* genus (Burza *et al.*, 2018). It is considered the fifth most important parasitic disease worldwide, affecting over 12 million individuals (Alvar *et al.*, 2012). The disease manifestation ranges from self-healing cutaneous leishmaniasis (CL) to severe visceral leishmaniasis (VL), which is a

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systemic and fatal disease if left untreated (Torres-Guerrero *et al.*, 2017).

In leishmaniasis, the clinical outcome is dependent on a delicate balance between the parasite genetic background and host immunity features. Regarding the latter, a reasonable prognosis of the disease is related to the host's ability to develop a robust cellular immune response with the predominance of Th1 responses together with IFN-γ production, which is crucial for activating parasite-infected macrophages and controlling the infection (Scott *et al.*, 1989; Reiner and Locksley, 1995). Whereas, on the contrary, the exaggerated production of cytokines and inflammatory mediators such as TNF-α (Carvalho *et al.*, 2007; de Lima *et al.*, 2007) and nitric oxide (NO) (Horta *et al.*, 2012), the predominance of Th2-related or suppressive cytokines are linked to the host susceptibility and disease progression (Faria *et al.*, 2005).

The overexpression of inhibitory checkpoint receptors has been potentially deleterious in many parasitic diseases (de Freitas e Silva and von Stebut, 2021), including leishmaniasis. Of these, PD-1 and its ligands (PDL1 and PDL2) have been widely described during *Leishmania* infection and linked to the decreased proliferative ability and diminished Th1-related cytokines production by T cells. (Liang *et al.*, 2006; Covre *et al.*, 2019; da Fonseca-Martins *et al.*, 2019; de Moura *et al.*, 2021). Apart from leishmaniasis, the role of killer cell lectin-like receptor G1 (KLRG1) has been associated with T cell differentiation (Müller-Durovic *et al.*, 2016), decreased proliferative capacity (Plunkett *et al.*, 2007) and poor T cells activation through TCR stimulation (Lanna *et al.*, 2017). Although little is known about its role during leishmaniasis so far, we have recently shown that KLRG1 is increased in both CD4^{+} and CD8^{+} circulating T cells (Covre *et al.*, 2019) and its expression is found within

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lesional leukocytes of patients (de Moura *et al.*, 2021), suggesting that it may play an important role in the pathogenesis of the disease.

Although the impact of ageing on VL infection has not yet been explored, broad evidence suggests that ageing may represent a significant risk factor and affect the outcome of tegumentary CL infection (Jirmanus *et al.*, 2012). In this scenario, elderly individuals have been reported to have higher frequencies of both mucosal and disseminated CL (Cincurá *et al.*, 2017). Furthermore, both severity with higher numbers and size and longer healing times have been observed within this population (Araujo-Melo *et al.*, 2010; Oliveira *et al.*, 2011, 2013; Diniz *et al.*, 2012). Controversially, in an experimental model, aged mice were more efficient than young mice in generating a protective immune response and controlling the infection caused by *L. major*, suggesting the need for complementary studies to understand better the effect of ageing on *Leishmania* infection, particularly those caused by the different species (Ehrchen *et al.*, 2004).

Herein, we demonstrate that aged mice are more susceptible to *L. infantum* infection than younger mice, accompanied by a senescence-associated cytokine profile. Moreover, they have increased expression of inhibitory receptors that are associated with disease severity within the T cell populations. Overall, the data reported in this study suggests for the first time that ageing may negatively impact the VL outcome and provides a perspective for new therapeutic strategies involving manipulation of immunosenescence features against *Leishmania* infection.

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Materials and Methods

Animals

C57BL/6 mice were originally purchased from Jackson Laboratory (Bar Harbor, Maine, USA). They were bred and maintained at our facilities and given sterilised bedding, filtered water, and pelleted food *ad libitum*. For each experiment, 5-8 female mice at 4-6 weeks old and at 72 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.

Parasites and infection

L. infantum strain MHOM/BR/1975/PP75 promastigotes were cultured at 26°C in Grace's medium (Gibco, USA), pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 2 mM L-glutamine, 25 mM HEPES, and 20 μg/ml gentamicin (LGC, Brazil). The mice were infected by the intravenous route in the tail vein with 10^7 *L. infantum* promastigotes in the stationary growth phase.

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 (Leal *et al.*, 2015). 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

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tissue/mL) and plated in a 96-well plate (Corning, USA). Serial dilutions of the single-cell suspensions were cultured 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. The relative change values were obtained through parasite burden/mg tissue data normalization according to the following formula: Relative change (Fold change) $= \frac{\mu_A}{\mu_Y}$, where μA= Aged group experimental average of parasite burden/mg; and μY = Young group experimental average of parasite burden/mg.

Cytokines and nitrite production

Ex vivo cytokine quantification was performed in both liver and spleen of all groups. The organs were individually homogenised in 1 mL of Phosphate-Buffered Saline (PBS), pH 7.2 with the addition of protease and phosphatase inhibitors (Sigma-Aldrich, USA) using a glass tissue grinder (Thomas, USA). The cells suspension volumes were adjusted with PBS according to tissue weight (100 mg of tissue/ mL), 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 individually 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 (Green *et al.*, 1982). 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.

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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/ FITC; 1:100 dilution) or CD8$^+$ (Clone 5H10-1/PerCP-cy5-5, 1:150 dilution) cells with KLRG1 (Clone 2F1/PE; 1:50 dilution) and PD-1 (Clone J43/APC-cy7; 1:50 dilution), all from biolegend, USA. For IFN-γ (Clone XMG1.2 /PE-cy7; 1:100 dilution) intracellular staining, cells were fixed and permeabilized with the Fix & Perm Kit (Invitrogen, Life Technologies, Carlsbad, CA) after the extracellular stainings, followed 20 min incubation with the antibody. To determine the intracellular expression of Ki67 (Clone B56/PE; 1:150 dilution), cells were fixed and permeabilised for 20 min using the kit from BD Biosciences after surface staining. Following this, the cells were marked with Ki67 for 1 h. After acquisition, data were analysed using FlowJo software (Version 10). All gates were based on pooled fluorescence minus one (FMO) control samples and applied identically across all samples.

Statistical analyses

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

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significantly different when $p < 0.05$.

Results

Aged mice develop increased susceptibility to L. infantum *infection*

To compare the susceptibility of aged and young mice to *L. infantum* infection, we assessed the parasite burden in the liver and spleen at the parasitic peak, thirty days after *L. infantum* infection, as previously defined (Leal *et al.*, 2015). Our data demonstrate that aged mice exhibit a significant increase in the parasite burden in the liver and spleen (Fig 1A); that was 18- and 23-fold greater than in the young mice, respectively (Fig 1B). The parasitism was compatible with their weight loss and prostrate behavior, in comparison to both the young-infected mice and the non-infected controls, which had a healthy appearance and dynamic behaviour (data not shown). Additionally, both the liver and spleen from aged-infected mice were significantly heavier and larger dimensions than young-infected mice (Fig 1C and D), demonstrating a conspicuous hepatosplenomegaly clinical presentation.

Leishmania infection enhances the inflammatory profile in aged mice

Ageing is a key factor affecting the inflammatory profile and the host proliferative ability (Ponnappan and Ponnappan, 2011). *Ex vivo* analysis of the cytokine production in the spleen revealed increased amounts of both TNF-α (Fig 2A) and nitrite (Fig 2B), a breakdown product of nitric oxide (NO), in the aged-infected group that were not found in the respective non-infected control nor the young-infected group. Moreover, there were lower amounts of IFN-γ compared to all the other mouse groups (Fig 2C),

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suggesting that aged mice acquire a conspicuous inflammatory profile following infection.

Leishmania infection promotes enhanced expression of inhibitory receptors, diminished frequencies of IFN-γ- producing -T cells and proliferative capacity impairments of aged mice

To further investigate the acquisition of an inhibitory profile, we next assessed the expression of surface differentiation-associated receptors such as KLRG1 and PD-1, as well as the proliferative capacity of both splenic CD4$^+$ and CD8$^+$ T cells. No differences in T cell frequencies were found between young and aged mice groups, pre-and post-infection (Supplementary figure 1). However, aged-infected mice displayed increased KLRG1 (Fig 3A, B, C) and PD-1 (Fig 3D, E, F frequencies within both T cell populations compared to the young-infected mice and the non-infected groups.

During leishmania infection, the increased frequencies of PD-1 and KLRG1 receptors are linked to impairments of leishmanicidal mechanisms (Covre *et al.*, 2019; Oliveira Silva *et al.*, 2019; da Fonseca-Martins *et al.*, 2019; de Moura *et al.*, 2021). To evaluate this on aged- and young- infected mice, we next accessed the frequencies of IFN-γ - producing T cells within PD-1$^+$, and KLRG-1$^+$ subsets. Compared to young-infected control, aged-infected mice had lower frequencies of CD4$^+$ T cells expressing IFN-γ within subsets of PD-1$^+$ (Fig 3G, I) and KLRG1$^+$ cells (Fig 3H, J). These decreased frequencies were also observed within the CD8$^+$ T cell compartment, suggesting that aged mice may have more significant impairments in activating

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leishmanicidal mechanisms, and supporting our previous result demonstrating a greater susceptibility of these animals to infection.

In complement to this, analysis of their proliferative capacities conducted by staining the cell cycle-related nuclear antigen Ki67 demonstrated that aged-infected mice had decreased T cells proliferative capacity. It was a 0.5- and 0.6-fold change in the CD4+ and CD8+ T cell compartments, respectively, than we found in the young-infected controls (Supplementary figure 2). No difference in the Ki67 staining was found between the non-infected groups (data not shown), suggesting that the infection may affect the T cell compartments.

Acquisition of senescence features correlates with parasite burden of visceral leishmaniasis.

As T cells with senescence features appeared to accumulate, especially in the aged-infected mice, we next investigated if these cells were associated with the pathology during VL. To achieve this, we correlated the frequencies of KLRG1 and PD-1 expressing-CD4$^+$ and CD8$^+$ T cells with the parasite burdens observed in the liver and spleen. KLRG1 -expressing CD4$^+$ and CD8$^+$ T cells and the parasite burden in liver and spleen were strongly correlated for aged, but not young, mice (Fig 4A and B). The same correlation was also found for the expression of PD-1 for both T cell populations (Fig 4C and D), suggesting that the expression of both inhibitory receptors by T cells may contribute to the pathology of VL.

Discussion

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The immunosenescence induced by physiological processes or chronic infection has been associated with a significant increase in host susceptibility to infections (Gardner, 1980; Marston *et al.*, 1997; Barbé-Tuana *et al.*, 2020; Tsuji *et al.*, 2022). Here we extended this concept by showing for the first time that senescent C57BL/6 mice are prone to *L. infantum* infection compared to young-infected mice. In our experiments, aged mice presented increased parasitism in the liver and spleen and a propensity for developing clinical symptoms of VL such as splenomegaly. These data support the greater susceptibility of elderly mice to infections as previously reported (Toapanta and Ross, 2009; Speziali *et al.*, 2010; Fang *et al.*, 2010; Krone *et al.*, 2013) and seemingly contradict previous findings that reported an association between ageing and resistance to *L. major* infection (Ehrchen *et al.*, 2004). Differences between the experimental models that used distinct mice strains, ages analysis and the natural evolution of visceral vs cutaneous infections could account for the observed discrepancies, thus highlighting the need for further complementary studies.

In the present study, the next series of experiments tested whether the susceptibility observed in aged mice was associated with impaired anti-*Leishmania* immune mechanisms. The development of VL caused by *L. infantum* is not defined by a constant Th1/Th2 pattern of cytokines as observed in CL caused by *L. major* (Wilson *et al.*, 2005; Tripathi *et al.*, 2007). However, in both CL and VL, a good prognosis is associated with a robust IFN-γ production that mediates the clearing of intracellular *Leishmania* by activated macrophages and disease control (Squires *et al.*, 1989; Scott, 1991). In our experiments, aged-infected mice were less able to produce IFN-γ compared to young-infected mice and non-infected controls but these mice

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presented an increased ability to make TNF-α and NO. Although the last two are essential to mediate macrophage activation and *Leishmania* clearance (Liew *et al.*, 1990), their chronic production is usually associated with more extensive and unspecific inflammatory processes, defective T-cell response and severity of visceral leishmaniasis (Rodrigues *et al.*, 2014, 2016; Bogdan, 2020). Moreover, both TNF-α and NO are components of the Senescence-Associated Secretory Phenotype (SASP) (Coppé *et al.*, 2010). An array of cytokines, chemokines, and matrix metalloproteinases are associated with a pronounced pro-inflammatory secretome found in chronic stimulatory processes, which is linked with disease pathology (Childs *et al.*, 2015).

The pronounced production of inflammatory mediators, like TNF-α, NO may exacerbate the inflammatory effects on tissues (Freund *et al.*, 2010; Prata *et al.*, 2018; Cerqueira *et al.*, 2020). These can directly modulate the expression of stress ligands, including inhibitory receptors and ligands by immune and non-immune cells (Groh *et al.*, 2001; Gasser *et al.*, 2005). In our experiments, the marked pro-inflammatory profile was found in association with the increased expression of KLRG1 and PD-1 within T cell populations. Further to this, the expression of these markers positively correlated with the spleen parasitism. Both KLRG1 and PD-1 inhibit T cell function, impairing immunity and promoting disease progression in mice (da Fonseca-Martins *et al.*, 2019) and humans (Covre *et al.*, 2019; de Moura *et al.*, 2021). Moreover, further evidence has demonstrated their deleterious role in impairing microbicidal function (Wang *et al.*, 2013; Müller-Durovic *et al.*, 2016) which could contribute to the increased susceptibility to infection observed in the aged mice.

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The current study provides an overview of the global effects that ageing has on the immune system following *L. infantum* infection and demonstrates how the acquisition of a pronounced inflammatory cytokine profile and the expression of inhibitory receptors may impact the disease severity. The data presented here have important implications for understanding the differences in the immune response between young and aged individuals during infection, which could pave the way for vaccine development and immunotherapies specifically for the elderly.

Acknowledgments

Supplementary Material

The following supplementary material can be downloaded from https://www.cambridge.org/core/journals/parasitology:

Supplementary figure 1. Splenic T cell frequencies are not affected with *L. infantum* infection. Pooled data of splenic CD4$^+$ (A) and CD8$^+$ T cell (B) frequencies from young and aged mice prior and post-infection. Results are represented as arithmetic means ± S.D of pooled data (n=16 mice/group). Statistical differences and p values were determined by ANOVA and indicated in the graphs.

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Supplementary figure 2. Proliferative capacity of T cells. Fold change of cell cycle-related nuclear antigen Ki67 expression in splenic CD4$^+$ and CD8$^+$ T from aged-infected mice normalised with young controls. Results are represented as arithmetic means $\pm$ S.D of pooled data (n=16 mice/group) obtained from three independent experiments. Statistical differences and p values were determined by t-test and ANOVA and indicated in the graphs.

Ethical Standards

The Ethical Committee for Experimental Animal Use at the Universidade Federal do Espírito Santo approved all experimental protocols, registered under reference number: 014/2011.

Conflicts of Interest

The authors have no conflicts of interest.

Author Contributions

CLS, AFM, LPC and HLMG performed experiments. CLS, AFM, LPC analyzed data. DG, LPC, HLMG and AF designed the project and discussed the data. DG, CLS and

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AF wrote the manuscript.

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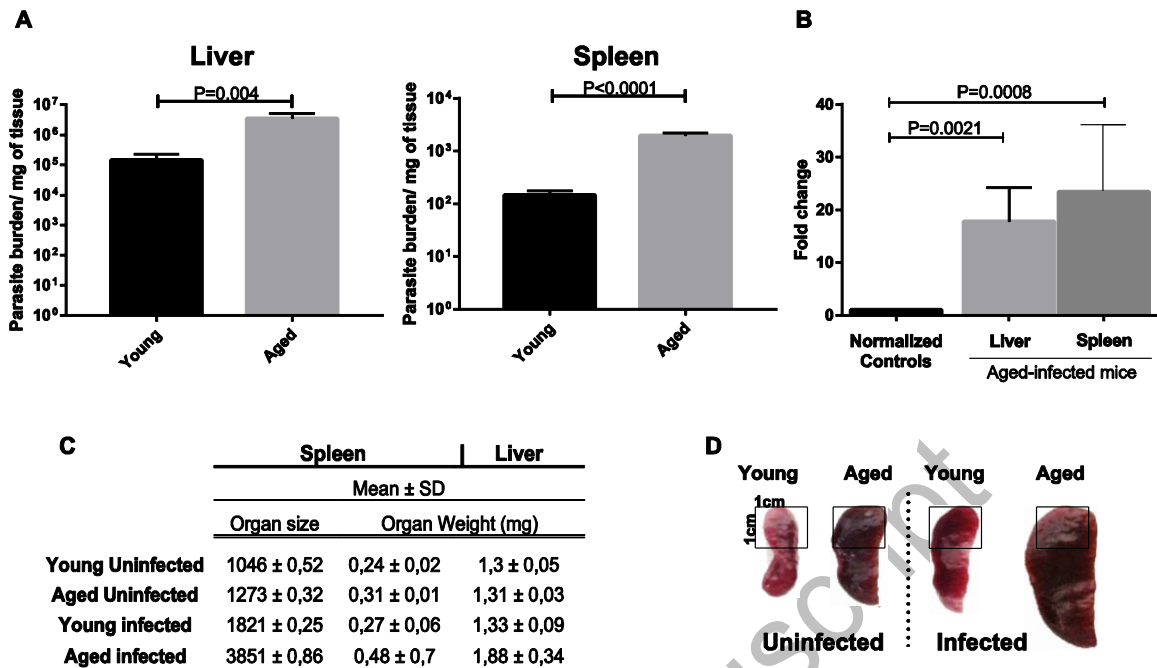
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A

Liver

P=0.004

Parasite burden/ mg of tissue

Young   Aged

Spleen

P<0.0001

Parasite burden/ mg of tissue

Young   Aged

B

Fold change

P=0.0008

P=0.0021

Normalized   Liver   Spleen
Controls

Aged-infected mice

C

	Spleen		Liver
	Mean ± SD		
	Organ size	Organ Weight (mg)	
Young Uninfected	1046 ± 0,52	0,24 ± 0,02	1,3 ± 0,05
Aged Uninfected	1273 ± 0,32	0,31 ± 0,01	1,31 ± 0,03
Young infected	1821 ± 0,25	0,27 ± 0,06	1,33 ± 0,09
Aged infected	3851 ± 0,86	0,48 ± 0,7	1,88 ± 0,34

D

Young   Aged   Young   Aged

1cm

Uninfected   Infected

Figure 1. Parasite burden and clinical features in infected aged mice. Young (4-6 weeks) and aged (72 weeks) mice were i.v.-infected with 10^7 *L. infantum* promastigotes. (A) Cumulative data of the parasite burden and (B) fold change of hepatic and splenic parasitism normalised with young controls. (C) Table with average organs size and weight and (D) representative image of spleens at the peak of parasitism. Results are represented as arithmetic means ± S.D of pooled data (n=16 mice/group). Statistical differences and p values were determined by t-test or ANOVA and indicated in the graphs.
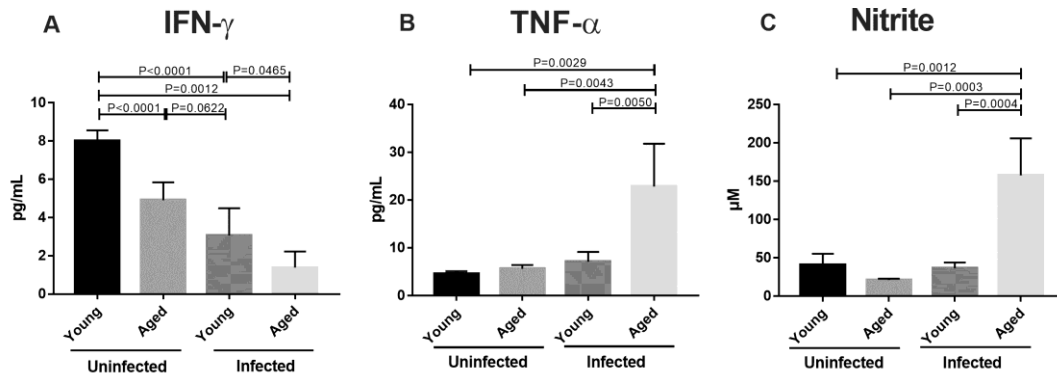
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Figure 2. Cytokines and NO production in infected aged mice. Spleens were processed individually, and the in-situ production of IFN-γ (A) and TNF-α (B) were assessed by ELISA. Nitrite production was evaluated by the Griess assay (C). Results are represented as arithmetic means ± S.D of pooled data (n=16 mice/group). Statistical differences and p values were determined by ANOVA and indicated in the graphs
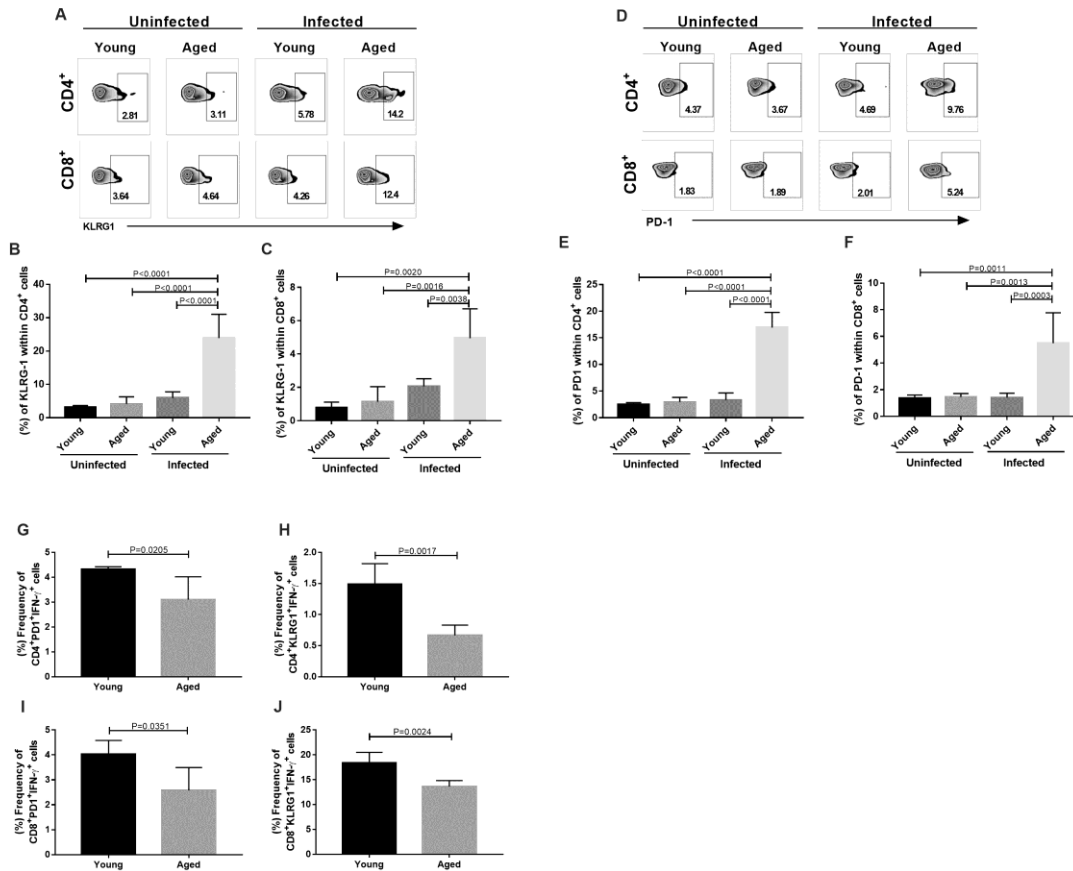
Figure 3. Increased expression of inhibitory KLRG1 and PD-1 receptors in aged mice following *Leishmania infantum* infection. Representative histograms and pooled data of ex vivo (A) KLRG1 expression on (B) CD4$^+$ and (C) CD8$^+$ T cells; (D)PD-1 expression on (E) CD4$^+$ and (F)CD8$^+$ T cells from young and aged mice. Pooled data of the ex vivo frequencies of IFN-γ- producing cells within PD-1$^+$ by (G) CD4$^+$ and (I) CD8$^+$ T cells; and KLRG-1$^+$ by (H) CD4$^+$ and (J) CD8$^+$ T cells from young and aged mice. Results are represented as arithmetic means ± S.D of pooled data (n=16 mice/group). Statistical differences and p values were determined by t-test and ANOVA and indicated in the graphs.
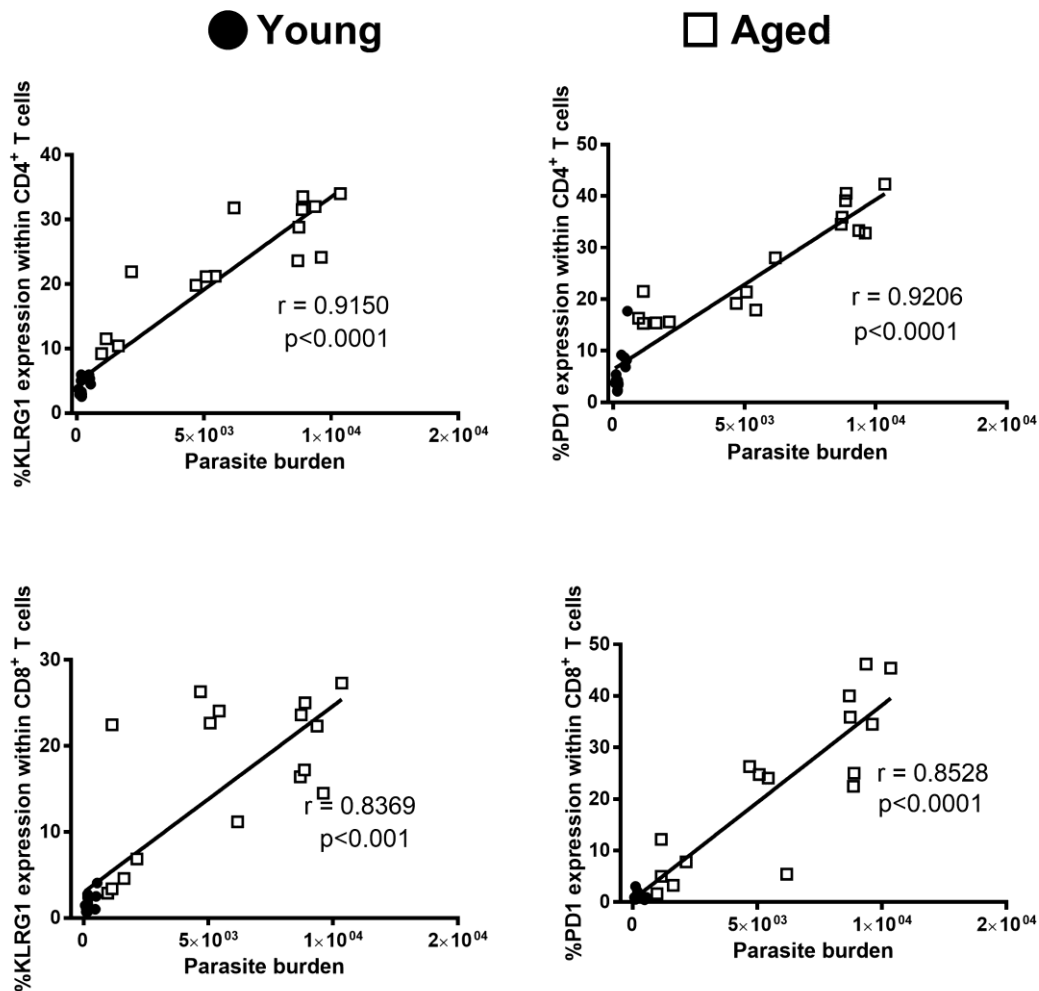
Figure 4. Inhibitory KLRG1 and PD-1 receptors correlate with the severity of visceral leishmaniasis in aged mice. Pearson's correlation test between frequencies of CD4^{+} and CD8^{+} T cells expressing KLRG1 (A and C, respectively) and PD-1 (B and E, respectively) and the splenic parasite burden of young (black dots) and aged (white dots) mice. Results are represented as arithmetic means ± S.D of pooled data (n=16 mice/group). Statistical differences and p values were determined linear regression and indicated in the graphs.

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