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Susana Cristina Roque Oliveira

Mycobacterium avium chronic infection differently affects the cytokine profile in the hippocampus from Balb/c, C57BL/6 and CD-1 mice but has no impact in behavior

junho, 2022

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TÍTULO DISSERTAÇÃO

Mycobacterium avium chronic infection differently affects the cytokine profile in the hippocampus from Balb/c, C57BL/6 and CD-1 mice but has no impact in behavior

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CONTENTS

I. DISSERTATION

Mycobacterium avium chronic infection differently affects the cytokine profile in the hippocampus from Balb/c, C57BL/6 and CD-1 mice but has no impact in behavior

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HIGHLIGHTS

M. avium chronic infection induces a cytokine imbalance in the hippocampus.

Each mouse strain presents a distinct cytokine profile upon infection.

M. avium chronic infection does not trigger hippocampal neuroplasticity alterations.

M. avium chronic infection does not induce behavioral alterations in the 3 mouse strains.

Keywords: Mycobacteria; infection; cytokine profile, mood behavior; hippocampus plasticity; corticosterone; Balb/c; C57BL/6; CD-1.

ABSTRACT

One of the most remarkable findings in the immunology and neuroscience fields was the discovery of the bidirectional interaction between the immune and the central nervous systems. This interplay is tightly regulated to maintain homeostasis in physiological conditions. Disruption in this interplay has been suggested to be associated with several neuropsychiatric disorders. Most studies addressing the impact of an immune system disruption on behavioral alterations focus on acute pro-inflammatory responses. However, chronic infections are highly prevalent and associated with an altered cytokine milieu that persists over time. Studies addressing the potential impact of mycobacterial infections on depressive-like behavior originated discordant results and the subject need to be further address. To promote our understanding on the effect of chronic infections on the central nervous system, we evaluated the impact of *Mycobacterium avium* infection. Since the profile of cytokines produce vary depending on the mouse strains, three mouse strains were analyzed (Balb/c, C57BL/6 and CD-1). We used female mice and the model of peripheral chronic infection. Our results show that *M. avium* peripheral chronic infection induces alterations not just in the peripheral immune system but also in the central nervous system, namely in the hippocampus. Interestingly these cytokine alterations vary between mouse strains. These altered cytokine profiles are not accompanied by hippocampal cell proliferation or neuronal plasticity changes. Accordingly, no differences were observed in locomotor, anxious and depressive-like behavior independently on the mouse strains used. These results show that the infection-induced alterations in the cytokine profile, both in

the periphery and the hippocampus, are insufficient to alter behavior and hippocampal plasticity.

1. INTRODUCTION

An increasing body of evidence robustly points towards a bidirectional interaction between the immune and the central nervous systems. Behavioral alterations are often associated with an imbalance in the immune system, namely in the cytokine profile (Mesquita *et al.* 2008; Monteiro *et al.* 2016; Miller *et al.* 2017; Beurel *et al.* 2020). Since infections (acute and chronic) are very frequent and induce alterations in the immune system, namely, increasing the production of pro-inflammatory cytokines, it is reasonable to infer their contributive and potential role in the pathophysiology of mood disorders. In fact, lipopolysaccharide (LPS, a cell wall component of Gram-negative bacteria) administration or *Salmonella typhi* vaccines in mice induce a pattern of behavioral changes that share similarities with those observed in mood disorders (Dunn and Swiergiel 2005; Wright *et al.* 2005; Dantzer *et al.* 2008; Harrison *et al.* 2009; DellaGioia and Hannestad 2010). These behavioral alterations, collectively termed “sickness behavior”, result from the interaction between the immune, endocrine, and central nervous systems as a normal physiological response to a danger signal, which is very important for host survival and infection clearance. Of note, those acute behavioral alterations are rapidly restored with the normalization of the immune system balance, which occurs a few days after these stimuli. Interestingly, few reports addressed chronic infections' effect on mood disorders. Chronic infections are of particular

interest since they are widely prevalent and are accompanied by a sustained altered cytokine profile.

Mycobacterial infections are among the major health threats worldwide (WHO 2021). *Mycobacterium avium* induces a chronic infection, altering the production of many pro-inflammatory cytokines, thus representing an attractive model to address the interplay between a chronically altered cytokine profile and behavior. *M. avium* is an opportunistic microorganism, frequently present in the environment, that causes serious disseminated infection in immunosuppressed patients (Brettle 1997; Field *et al.* 2004). The mouse model of *M. avium* infection is very well characterized (Appelberg 2006). The infection can last several months with no obvious clinical signs of disease (Olsson *et al.* 2010). Moreover, *M. avium* infection has been extensively used to address specific questions related to this infection and, as a mouse model, to study general features of mycobacterial infections (Appelberg 2006). Upon mycobacterial infection, an immune response is continuously present, characterized mostly by a Th1 type of response, with increased levels of pro-inflammatory cytokines such as IFN-γ and TNF (Roque *et al.* 2007).

The effect of mycobacterial infections on behavior has been previously studied by others, however, results from these studies are discordant (Lowry et al. 2007; Moreau et al. 2008). Studies performed with *Mycobacterium bovis bacillus Calmette-Guérin* (BCG) infection suggested that mycobacterial infection induces depressive-like behavior in CD1 (Moreau et al. 2008; O'Connor et al. 2009; Platt et al. 2013), in C57BL/6 (O'Connor et al. 2009; O'Connor et al. 2009; Rodriguez-Zas et al. 2015) and Balb/c mice (Kelley et al. 2013; Vijaya Kumar et al. 2014). In contrast, stimulation of the immune system with antigens from

Mycobacterium vaccae, using Balb/c mice, led the authors to conclude that mycobacterial antigens “decrease” depressive-like behavior (Lowry et al. 2007). Moreover, this immune challenge with *M. vaccae* also conferred stress resilience in mice and rats (Reber et al. 2016; Frank et al. 2018; Foxx et al. 2020; Loupy et al. 2021) and enhanced fear-extinction in rats (Fox et al. 2017). The discordant observations between studies with BCG and *M. vaccae* may originate from the different bacteria used. Thus, to further investigate the effect of the immune system imbalance occurred during mycobacterial chronic infections, we evaluated the impact of an infection by a single strain of *M. avium* using simultaneously three female mouse strains: two inbred (C57BL/6 and Balb/c) and one outbred strain (CD1). We investigated the brain cytokine profile and plasticity and mouse behavior.

2. MATERIALS AND METHODS

2.1 Animals

Specific pathogen-free Crl:CD1(ICR), C57BL/6J and BALB/cByJ 8 weeks old female mice were purchased from Charles River Laboratories (Barcelona, Spain). All mice were housed in sterile housing conditions, in groups of 5 per cage, under standard laboratory conditions (12h light/12h dark cycle, at 22°C, relative humidity of 55%; food and water *ad libitum*). Cages were environmental enriched with a soft paper as nesting material. All experimental procedures were carried out within the light period of the light/dark cycle. Even though no adverse effects were expected in this experimental procedure, mice were monitored and humanely euthanized whenever a clinical sign of a humane endpoint was observed (Burkholder *et al.* 2012).

The experiments were conducted in agreement with National guidelines (DL 113/2013 and Portaria 1005/92) and with the European Union Directive 2010/63/EU on animal care and experimentation. The study and people directly involved in animal experiments were certified by the Ethical Committee Board of the Portuguese Veterinary Directorate.

A priori power analysis for the behavioral differences between the two groups (non-infected and infected mice) were performed assuming an effect size d=0.95, an alpha of 0.05, a statistical power 0.7, a minimum of n=15 mice per group must be used (t-test; G*Power 3.1.4).

2.2 Infection and quantification of bacterial load

Mice from each strain were randomly assigned for non-infected and infected groups, using a computer based random order generator. In the infected group mice were infected intravenously (i.v.) through a lateral tail vein, with 106 colony-forming units (CFU) of *M. avium* strain 2447 (smooth transparent variant, provided by Dr F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). The non-infected group were injected (i.v.) through a lateral tail vein with saline. At 4 weeks post infection (wpi), mice were submitted to behavioral tests and three days after the end of the behavioral evaluation, animals were weighed and euthanized (mice were anesthetized with a combination of ketamine 75 mg kg−1 and medetomidine 1 mg kg−1 and lastly euthanized by decapitation, by trained certified personnel). Half of the spleen from all animals and the brain from 8-14 mice were collected, homogenized, serially diluted, and plated onto Middlebrook 7H10 agar medium. The number of CFU was counted after 1 week of incubation at 37 °C.

2.3 Behavioral tests

Behavioral tests were performed on 3 consecutive days, between 9 am and 6 pm, in the following order: open field test (OFT), forced swim test (FST) and tail suspension test (TST). All animals performed all the behavioral tests. The estrous cycle stage (proestrus, estrus, metestrus and diestrus) of each female was determined, immediately after the performance of each behavioral test (in 3 consecutive days), by vaginal smear examination for the presence of leukocytes, cornified epithelial and nucleated epithelial cells and their proportions in the smear (Byers *et al.* 2012). To evaluate the impact of the estrous cycle on the behavioral parameters analyzed, data were grouped into proestrus/estrus and metestrus/diestrus stages since it has been described that the estrogen levels of the grouped stages are very similar (Caligioni 2009). A two-way ANOVA analysis with a post hoc Fisher LSD test revealed that the estrous cycle did not impact the behavioral parameters assessed.

2.3.1 Open field test

The OFT was performed to assess locomotor and exploratory activities and anxious-like behavior. Animals were placed in the center of an arena (43.2 x 43.2 cm with transparent acrylic walls and a white floor) and their position and rearings (vertical activity) were monitored and recorded by a three 16-beam infrared system (MedAssociates, VT, USA), during 5 minutes. The total distance travelled by each animal was used to assess locomotor activity and the number and duration of rearings to determine exploratory behavior. The percentage of time spent in the central area of the OFT arena and the percentage of the distance travelled by each animal in the center of the arena (10.8 cm x 10.8 cm)

were used as an indicative measure of anxious-like behavior (Gould *et al.* 2009).

2.3.2 Forced swimming test

The FST was used to evaluate the ability of mice to cope with a stressful and inescapable situation (behavioral despair). In this test, each animal was placed in a cylinder (17 cm of diameter and 30 cm of height) filled with water (25 ºC) to a depth, so the mouse had no solid support for the rear paws or tail. The activity was recorded for 6 minutes, and the last 4 minutes were scored for mobility/immobility. Additionally, the latency to immobility, which corresponds to the time each animal takes from the beginning of the test to stop for the first time, was assessed. Mice displaying decreased latency to immobility and longer immobilization periods were considered to display higher behavioral despair, a sign of depressive-like behavior (Porsolt *et al.* 1977). The behavior parameters assessed were scored by, at least, 2 independent researchers, blind to the experimental condition. Since the results are consistent between raters the graphs present data from one rater.

2.3.3 Tail suspension test

The TST addresses, as the FST, depressive-like behavior. Mice were suspended by the tail for 6 minutes. The activity was recorded and, subsequently, the latency, mobility and immobility time were manually scored by at least two independent researchers, blind to the experimental conditions. The data presented in the graphs are from one of the raters. Displaying decreased latency to immobility and longer immobilization periods were considered traits of depressive-like behavior (Steru *et al.* 1985).

2.4. mRNA expression quantification by qPCR

Spleen and hippocampus were macroscopically dissected and stored at -80 ºC for subsequent quantification of messenger RNA (mRNA) expression levels by real-time polymerase chain reaction (qPCR).

To assess the cytokine profile in the spleen and hippocampus the expression levels of genes encoding for several pro- and anti-inflammatory cytokines (*Ifn-γ, Tnf, Il-1β, Il-6, Tgf- β and Il-10*) and for the enzyme *indoleamine 2,3-dioxygenase* (*Ido*) and *inducible nitric oxide synthase* (*iNos*) were measured by qPCR using the primer sequences described in Table 1. Total RNA (1 µg) was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The geometric mean of the mRNA expression levels of three different genes was used as reference: *hypoxanthine guanine phosphoribosyl transferase* (*Hprt*); *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) and; *18S ribosomal RNA* (*18S*) (Vandesompele *et al.* 2002). qPCR reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA) using EVA Green (Bio-Rad, CA, USA).

Table 1. Primer sequences

Gene	Sequence forward primer (5´-3´)	Sequence reverse primer (5´-3´)
Hprt	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC
Gapdh	GGGCCCACTTGAAGGGTGGA	TGGACTGTGGTCATGAGCCCTT
18S RNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Ifn-γ	CAACAGCAAGGCGAAAAAGG	GGACCACTCGGATGAGCTCA
Tnf	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
Il-1β	GTGCTGTCGGACCCATATGAG	CAGGAAGACAGGCTTGTGCTC
Il-6	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCCAGAGAAC
Tgf- β	AGCCCGAAGCGGACTACTAT	AGCCCTGTATTCCGTCTCCT
Il-10	AGGACTTTAAGGGTTACTTGGGTT	GCTCCACTGCCTTGCTCTTATT
Ido	GGCTTCTTCCTCGTCTCTCTATTG	TGACGCTCTACTGCACTGGATAC
iNos	CTCGGAGGTTCACCTCACTGT	GCTGGAAGCCACTGACACTT

2.5. Corticosterone measurement

Sera corticosterone levels were measured 3 days after the last behavioral test. Blood was collected from the tip of the tail within the first 2 min after animals were removed from their home cage. Blood collection occurred between 9, and 10 am, corresponding to the beginning of the light period (the basal time-point of the corticosterone production circadian rhythm). Corticosterone concentration was assessed using a radioimmunoassay (RIA) assay kit (Corticosterone Double Antibody RIA kit, MP Biomedicals, NY, USA), following the manufacturer's guidelines. The detection limit of the assay was 15.4 ng/mL.

2.6. Hippocampal cell proliferation: immunohistochemistry and stereological analysis

To analyze hippocampal cell proliferation, anesthetized mice with a combination of ketamine 75 mg kg−1 and medetomidine 1 mg kg−1 were transcardially perfused with saline and euthanized by decapitation and their brains removed. Brains were embedded in optimum cutting temperature compound and snap-frozen to assess cell proliferation in the dentate gyrus (DG) using stereological analysis. Serial coronal 20 µm sections were cut on a cryostat, extending over the entire length of the hippocampus. To detect Ki67, a nuclear protein expressed in all phases of the cell cycle except the resting phase G0, a mouse monoclonal anti-Ki67 (Novocastra, UK; 1:100 dilution) was used accordingly with standard procedures. The primary antibody was detected using the Ultravision Quanto Detection System (Lab Vision, CA, USA), and the reaction developed with 3,3'-diamino-benzidine substrate (Sigma Aldrich, MO, USA; DAB: 0.025% and 0.15% H_2O_2 in Tris–HCl 0.05 M, pH 7.2). Sections were then counterstained with hematoxylin.

Hippocampal cell proliferation was measured by counting the cells expressing Ki-67 in the subgranular zone (SGZ), considered as the 3-cell-body-wide zone at the border of the DG and normalized by the respective area (results are presented as number of $Ki67^+$ cells per mm^2). The use of the visiopharm integrator system software (Visiopharm, Denmark) allowed the delimitation, at low magnification (40x), of the areas of interest and the identification of the $Ki67^+$ cells within the defined areas was performed at higher magnification (400x). Counts were performed by one researcher blind to the experimental conditions.

2.7. Dendritic structure

To analyze the dendritic structure, the mouse brains were collected as described above, immersed in Golgi-Cox solution, and kept in the dark for 14 days at room temperature (Glaser and Van der Loos 1981). The brains were transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated, xylene cleared, mounted and coverslipped with entellan. All incubation steps were performed in a dark room. To minimize bias, each brain was coded to keep the experimenter blind to the experimental conditions. The arrangement of the dendritic material in the granule cells from the DG of the hippocampus was analyzed taking into consideration the following criteria: 1) full Golgi-impregnation along the dendritic tree; 2) complete dendrites without truncated branches; and 3) relative isolation from neighboring impregnated neurons, astrocytes or blood vessels to avoid interference with the analysis.

Slides containing the region of interest were randomly searched and the first 5 to 8 neurons fulfilling the criteria (maximum of 3 neurons per section) were selected.

For each selected neuron, all branches of the dendritic tree were reconstructed, at high magnification (600x), using a motorized microscope (Axioplan 2; Carl Zeiss, Germany) and the Neurolucida software (Microbrightfield, VT, USA). A 3D version of a Sholl analysis (Sholl 1956; van Pelt and Uylings 2002) of the reconstructed neurons was performed using the NeuroExplorer software (Microbrightfield, Inc.; VT, USA); the number of intersections of dendrites with concentric spheres positioned at radial intervals of 10 µm was counted. Additionally, the total length of the dendritic tree was measured. For each group of 5 mice per strain, 30 dentate granule cells were analyzed

2.8. Data analyses

Normal distribution of the variables was tested using the Shapiro-Wilk test ($p>0.05$). To compare infected with non-infected mice, the independent-sample t-test was performed for variables with normal distribution.

One-way ANOVA was used to compare the bacterial load of the 3 mouse strains. The differences between the groups were analyzed using the Tukey post-hoc test. The Sholl analysis of the reconstructed neurons was performed by ANOVA repeated measures.

The Cohen's d (*d*) effect size was calculated for all the statistical tests performed considering $d<0.50$ a small; $0.50 \leq d > 0.8$ a medium; and ≥ 0.80 a large effect size (Cohen 1988).

The results shown are expressed as mean +SEM and correspond to 1 of at least 2 independent experiments with similar results. Sample sizes are shown in the figure legends. All data were analyzed using GraphPad Prism software (v.8, GraphPad software Inc. CA, USA) or SPSS statistics software (v.27, SPSS Inc. IL, USA). Significance at $p < 0.05$ is indicated by an asterisk (*)

3. RESULTS

3.1. Chronic infection with Mycobacterium avium induces a distinct hippocampal cytokine profile in the three mouse strains

To evaluate the impact of chronic infection on the neuronal plasticity and behavior of mice we analyzed the animals at 4 wpi with *M. avium*, since it corresponds to the peak of the immune response (Pais *et al.* 2000). We infected mice from 3 mouse strains (Balb/c, C57BL/6 and CD1) also used in other studies that assessed the impact of mycobacteria infection in the brain and behavior. Moreover, these mouse strains are the most widely used in immunological and behavioral studies.

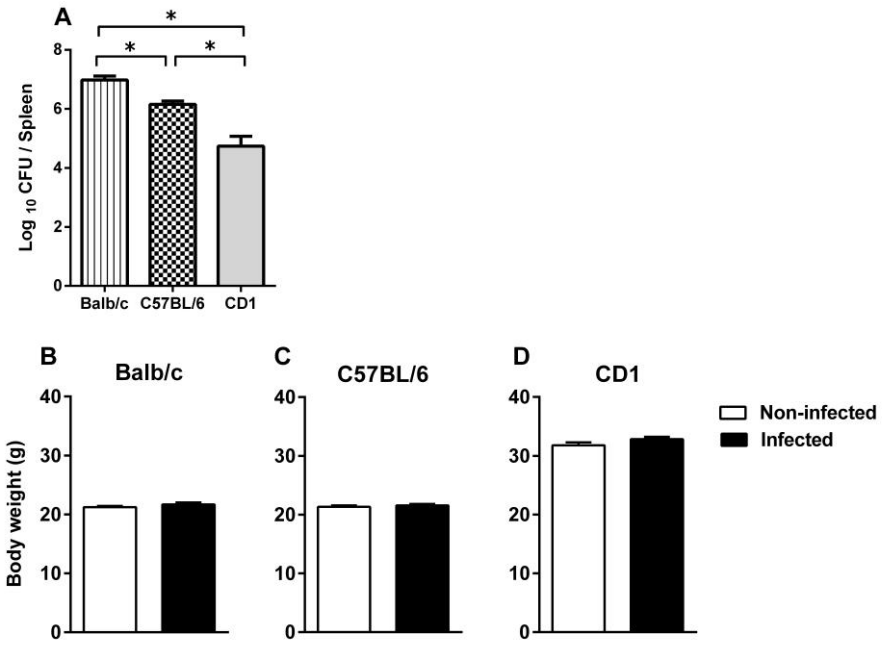

Fig. 1 - *M. avium* chronic infection does not alter mice body weight even though different susceptibilities to infection are observed. Spleen bacterial load was determined at 4wpi (A). Body weight of Balb/c (B), C57BL/6 (C) and CD1 (D) female mice were assessed in non-infected and 4 weeks infected mice. Each bar represents the mean + SEM of 15-24 mice per group, from 1 of 3 independent experiments. *p<0.05

The bacterial loads in the spleen at 4 wpi differ depending on the mouse strain (Fig. 1A; $F_{(2,57)}$=530,9; p<0.0001, *d*=0.234). Interestingly, Balb/c mice are the most susceptible whereas CD1 mice are the most resistant to bacteria growth. While the bacterial load is very high in the spleen and several other organs (Nobrega *et al.* 2007; Roque *et al.* 2007) very few bacteria are present in the brain. In CD1 mice we detected, an average of 0.3 CFU per brain (with 5/7 mice below detection limit), in C57BL/6 mice, 9 CFU per brain (with 5/14 below detection limit) and 38 CFU per brain in Balb/c mice (with 1/8 below detection limit).

None of the mouse strains revealed weight loss after infection (Fig. 1 B, C and D; Balb/c: t(32)=-1.667, p=0.105, d=0.581; C57BL/6 t(43)=-0.668, p=0.508, d=0.200; CD1: t(31)=-1.164, p=0.253, d=0.402).

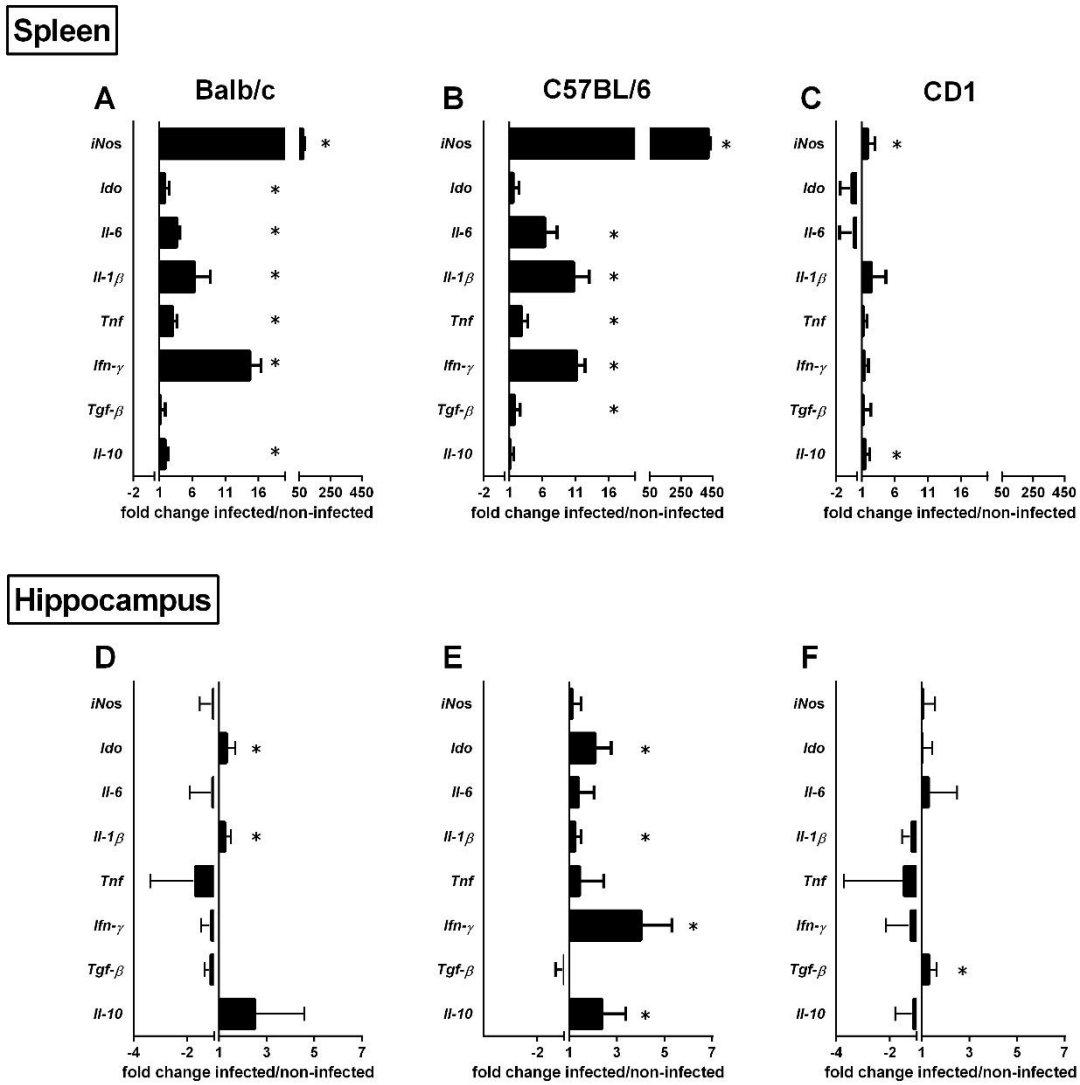

Fig. 2 - Chronic infection with *M. avium* induces a distinct cytokine/inflammatory molecules profile in the hippocampus and spleen of the different mouse strains. mRNA expression levels for the anti-inflammatory cytokines *Il-10 and Tgf-β,* pro-inflammatory cytokines *Ifn-γ*, *Tnf*, *Il-1β* and *Il-6,* and the inflammatory molecules *Ido* and *iNos* were measured in the spleen (A, B and C) and hippocampus (D, E and F) of Balb/c (A and D), C57BL/6 (B and E) and CD1 (C and F) mice at 4wpi and also non-infected. The mRNA expression levels were normalized using 3 reference genes *Hprt, Gapdh and 18S*. Each bar represents the mean of the fold change of the ratio infected/non-infected +SEM of 6-8 mice per strain from 1 of 2 independent experiments with similar results. *p<0.05

The same analysis in the hippocampus revealed that the 3 mouse strains present also a distinct cytokine profile (Fig. 2D, E and F). The hippocampus of both infected Balb/c and C57BL/6 mice present an increase in *Ido* mRNA expression (Fig. 2D and 2E; Balb/c: t(14)= -2.586, p=0.022, *d*=1.338; C57BL/6: t(13)= -3.420, p=0.005, *d*=1.828) and *Il-1β* (Balb/c: t(13)= -2.696, p=0.018, *d*=1.441; C57BL/6: t(14)= -2.640 p=0.019, *d*=1.366). Moreover *M. avium* infected C57BL/6 mice also show increased expression of *Ifn-γ* (Fig. 2E; t(14)=-4.241; p=0.001, *d*=2.195) and *Il-10* (Fig. 2E; t(8)=-2.423; p=0.042, *d*=1.625). The infected CD1 mice only present increased expression of *Tgf-β* (Fig. 2F; t(13)= -2.635, p=0.021, *d*=1.408). No differences between infected and non-infected animals are observed for the other cytokines/inflammatory molecules analyzed in the hippocampus (*Il-6, iNos* and *Tnf*).

3.2. M. avium infection does not impact on hippocampal cell proliferation

Since it has been described that inflammation can impact hippocampal neurogenesis (Zonis *et al.* 2015; Chesnokova *et al.* 2016) we next evaluated the hippocampal cell proliferation in the DG of infected and non-infected animals evaluating the expression of Ki67. *M. avium* infection does not induce alterations in hippocampal cell proliferation in none of the mouse strains analyzed Fig. 3 (Balb/c: t(14)=-0.470, p=0.690, *d*=0.243; C57BL/6 t(10)=0.113, p=0.912, *d*=0.068; CD1: t(13)=-0.258, p=0.8, *d*=0.138)

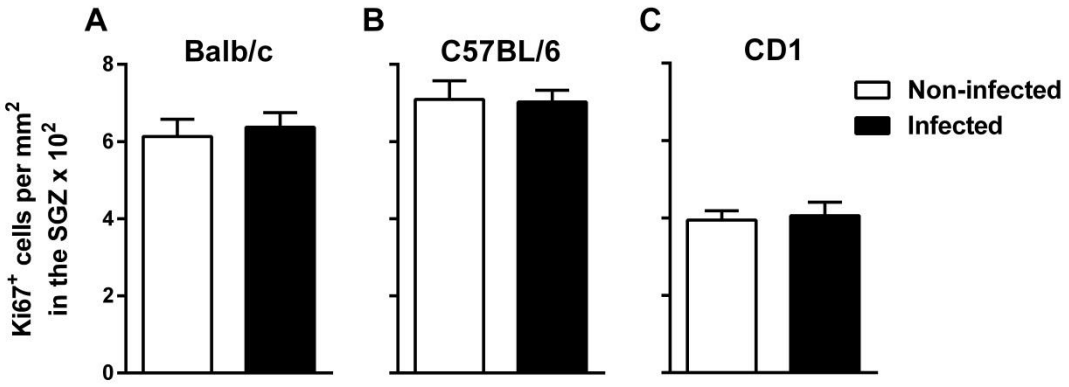

Fig. 3 - Chronic infection with *M. avium* does not alter the hippocampal cell proliferation. Hippocampal cell proliferation was assessed in *M. avium* infected and non-infected Balb/c (A) C57BL/6 (B) and CD1 (C) mice. The bars represent the mean +SEM of Ki67+ cells per mm^2 in the SGZ of each animal (6-8 mice per strain from 2 independent experiments).

3.3. M. avium infection does not impact neuronal plasticity

Even though no alterations were observed in hippocampal cell proliferation of infected animals we assessed whether infection impacts the neuronal morphology of this brain region. The morphological analysis of the DG's granule neurons revealed that infection with *M. avium* does not induce alterations in the total length of the dendrites from the 3 mouse strains (Fig. 4 A, B and C; Balb/c: t(9)=1.379, p=0.201, *d*=0.872; C57BL/6: t(8)=-1.215, p=0.259, *d*=0.815; CD1: t(8)=-0.589, p=0.572, *d*=0.395). Moreover the arrangement of the dendritic material of these same neurons, assessed by the number of intersections of dendrites as a function of their distance from the soma, also does not reveal differences between infected and non-infected animals from the 3 mouse strains (Fig. 4D, E and F; Balb/c: $F_{(1, 56)}$ = 0,2438, p= 0,6234, *d*=0.132; C57BL/6: $F_{(1, 55)}$ = 2,074, p= 0,1555, *d*=0.388; CD1: $F_{(1, 59)}$ = 0,8533, p = 0,3594, *d*=0.241).

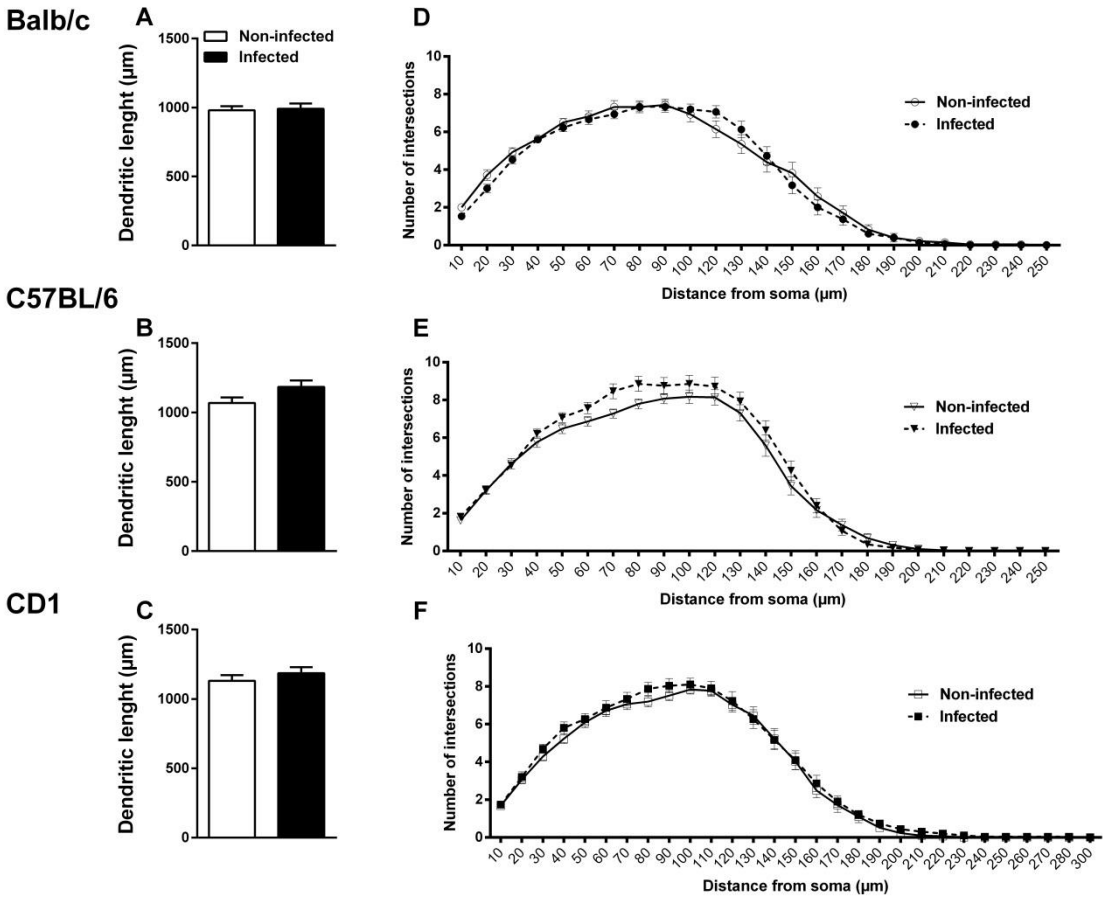

Fig. 4 - Infection with *M. avium* does not induce morphological alterations in granule neurons of the hippocampus. Dendritic morphology of granule neurons from the DG was analyzed in *M. avium* infected (4 wpi) and non-infected Balb/c, C57BL/6 and CD1 mice. The total length of the dendritic tree (A, B and C) and Sholl analysis of the number of intersections of dendrites at specific distances from the soma are displayed (D, E and F). Each bar represents the mean +SEM of the average length of 28-31 granule neurons per group, from 5-6 mice per group; the lines represent the average number of intersections of dendrite branches with consecutive 10 µm-spaced concentric spheres of the same neurons.

3.4. The basal corticosterone levels are unaltered upon M. avium infection chronic infection

Alterations in cytokine levels, namely pro-inflammatory cytokines have been associated with the activation of the HPA-axis. Thus, we also analyze the basal

levels of corticosterone. Infection does not induce alterations in the basal levels of corticosterone in the 3 mouse strains (Fig. 5; Balb/c: t(26)=1.073, p=0.293, *d*=0.413; C57BL/6: t(29)=0.544, p=0.590, *d*=0.199; CD1:t(29)=0.069, p=0.946, *d*=0.025).

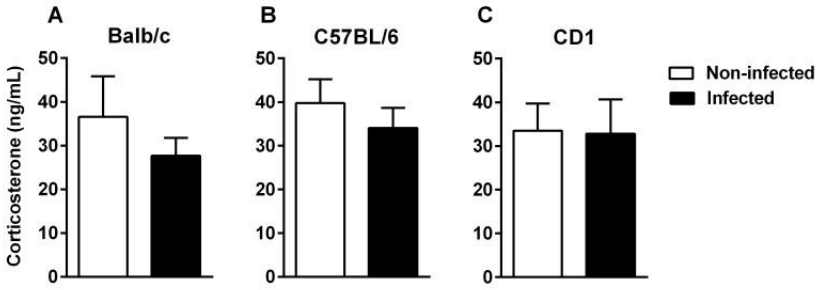

Fig. 5 - No alterations in basal corticosterone levels were observed in *M. avium* infected mice compared to non-infected mice. Basal serum corticosterone levels were measured in Balb/c (A), C57BL/6 (B) and CD1 (C) non-infected mice and infected with *M. avium* (4wpi). Each bar represents the mean +SEM from 15-16 mice per group from 1 of 2 independent experiments.

3.5 M. avium infection does not induce alterations in locomotor, exploratory, anxious-like or depressive-like behaviors

To assess the role of a chronic infection in mouse behavior we first analyzed the locomotor and exploratory behavior of 3 mouse strains in the OF arena. The locomotor and exploratory abilities are not altered by *M. avium* infection in any mouse strain, as measured by the total distance travelled (Fig. 6A, B and C; Balb/c: t(32)=0.672, p=0.506, *d*=0.234; C57BL/6: t(41)=1.216, p=0.231, *d*=0.375; CD1:t(31)=0.875, p=0.388, *d*=0.312) and the number/duration of the rearings (Fig. 6D, E and F; number of rearings: Balb/c: t(31)=1.416, p=0.167, *d*=0.501; C57BL/6: t(41)=0.660, p=0.513, *d*=0.204; CD1:t(30)=0.525, p=0.604, *d*=0.192; duration of rearings: Balb/c: t(31)=1.264, p=0.216, *d*=0.447; C57BL/6:

t(41)=-0.184, p=0.855, *d*=0.057; CD1:t(30)=-0.271, p=0.788, *d*=0.097). To evaluate anxious-like behavior we measured the time and distance spent in the center of the OF. *M. avium* infection does not alter anxious-like behavior in any of the mouse strains (Fig. 6G, H and I; center time: Balb/c: t(30)=-0.231, p=0.819, *d*=0.083; C57BL/6: t(41)=0.025, p=0.981, *d*=0.008; CD1:t(30)=1.387, p=0.176, *d*=0.498; Fig. 6J, K and L; center distance: Balb/c: t(30)=-1.478, p=0.150, *d*=0.531; C57BL/6: t(41)=-0.260, p=0.796, *d*=0.080; CD1:t(30)=0.202, p=0.841, *d*=0.073). To assess the role of chronic infection in depressive-like behavior mice were submitted to the FST (Fig. 6M, N and O) and TST (Fig. 6P, Q and R). In both tests no alteration were observed in infected compared with non-infected mice (FST: Fig. 6M, N and O; latency time: Balb/c: t(30)=0.868, p=0.392, *d*=0.312; C57BL/6: t(39)=0.214, p=0.832, *d*=0.068; CD1:t(30)=1.702, p=0.099, *d*=0.612; immobility time: Balb/c: t(30)=-1.780, p=0.085, *d*=0.640; C57BL/6: t(39)=-0.168, p=0.867, *d*=0.053; CD1:t(30)=-0.512, p=0.612, *d*=0.184); (TST: Fig. 6P, Q and R; latency time: Balb/c: t(30)=1.801, p=0.082, *d*=0.647; C57BL/6: t(39)=0.706, p=0.484, *d*=0.223; CD1:t(29)=0.897, p=0.377, *d*=0.328; immobility time: Balb/c: t(30)=-0.843, p=0.406, *d*=0.303; C57BL/6: t(39)=0.507, p=0.615, *d*=0.160; CD1:t(29)=-0.749, p=0.460, *d*=0.273).

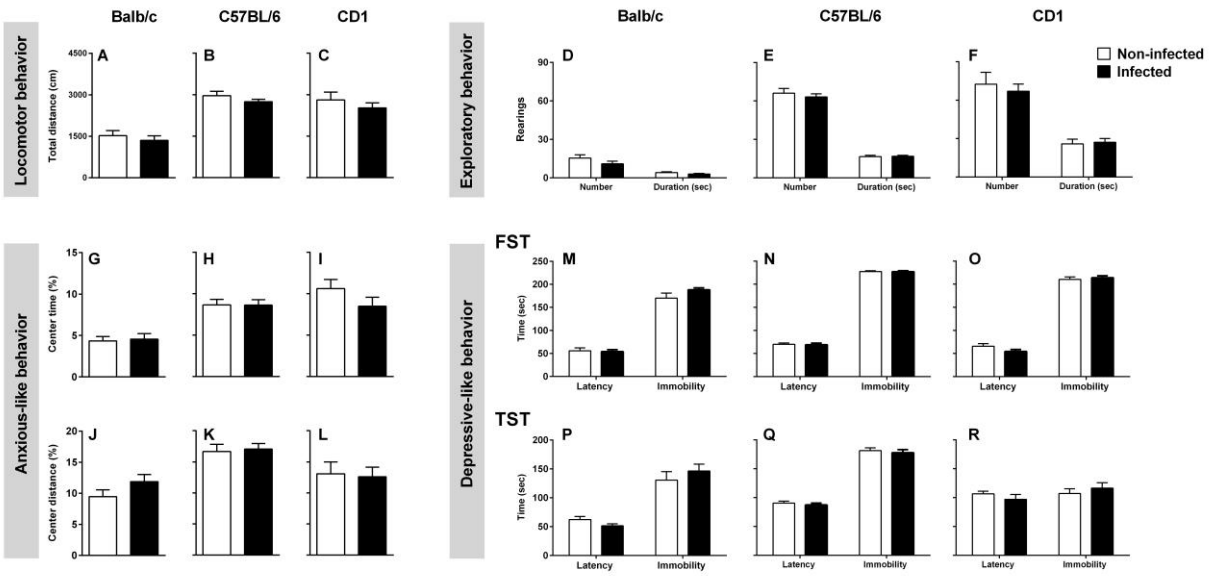

Fig. 6 - *M. avium* chronic infection does not induce alterations in locomotor, exploratory, anxious-like or depressive-like behaviors. The OF, FST and TST tests were performed with non-infected and infected (4wpi) Balb/c, C57BL/6 and CD1 female mice. In the OF arena, the total distance travelled in centimeters (A, B, and C), the number and duration of rearings (D, E and F), the percentage of time in the center of the arena (G, H, and I) and center distance (J, K and L) were scored. In the FST (M, N and O) and TST (P, Q and R) the latency until the first immobility and duration of the immobility periods were assessed. Each bar represents the mean + SEM of 15-23 mice per group, from 1 of 3 independent. * P<0.05.

4. DISCUSSION

Over the last decades, several studies demonstrated a bidirectional interaction between the immune and central nervous systems. An imbalance in the immune system is often associated with alterations in behavioral phenotypes. Taking into account the high prevalence of chronic infections, namely those caused by bacteria from the *Mycobacterium* genus (WHO 2021), we investigated whether exposure to a chronic infection with *M. avium*, during which the cytokine profile is continuously altered, triggers behavioral alterations in various mouse strains. In fact, upon infection with *M. avium*, a mouse strain specific cytokine profile is observed both in the spleen and in the hippocampus. However, this imbalanced cytokine *milieu* does not impact corticosterone production, hippocampal cell proliferation or the DG's granule neurons morphology. The chronic infection with *M. avium* does not induce alterations in locomotor, exploratory, anxious or depressive-like behaviors. Similar behavioral results were observed after 12 weeks of intravenous infection in the 3 mouse strains (with 10^6 CFU), 4 weeks of *M. avium* intraperitoneal infection (with 10^7 CFU) in C57BL/6 mice and after 4 weeks of intravenous infection (with 10^7 CFU) in Wistar rats (data no shown). With these results, we can conclude that even though infection with *M. avium* induces an imbalance in the immune system, it is not associated with alterations in the behavior of the animals nor neuronal plasticity of the hippocampus. The results also showed several interstrain differences that were already described in a previous study (de Sa-Calcada *et al.* 2015).

Infection of mice with *M. avium* 2447 induces an increase in bacterial load until 4 wpi, the peak of the immune response (Pais *et al.* 2000), after which the

bacterial load plateau for several months in organs such as the spleen and the liver (Nobrega *et al.* 2007; Roque *et al.* 2007). Thus, although the host can control bacterial growth, it cannot reduce or eliminate the bacteria within these organs (Nobrega *et al.* 2007; Roque *et al.* 2007). Since infection by mycobacteria results in a prolonged alteration of the cytokine profile, namely increased production of pro-inflammatory cytokines, we considered it to represent a good model to address how chronic infection interferes with mood behavior. The distinct susceptibility of different mouse strains to *M. avium* infection was previously described (Roque *et al.* 2007), here we also observed that it is accompanied by a different profile of cytokine expression both in spleen and hippocampus. These cytokine alterations, both in the periphery and brain, are not associated with alterations in hippocampal plasticity (cell proliferation or morphology in granule neurons of the hippocampus) as usually described (Chesnokova *et al.* 2016). Other authors have also shown that peripheral inflammation in a human TNF transgenic mouse model could be associated with hippocampal cytokine milieu and cellular plasticity (Suss *et al.* 2015). To our knowledge, these parameters of hippocampal plasticity were not previously investigated in other mycobacterial infections.

The absence of behavioral alterations in the different strains of infected mice, all with an imbalance in the cytokine *milieu* both in spleen and hippocampus, clearly indicates that increased production of pro-inflammatory molecules is insufficient to trigger changes in behavior. This evidence supports the notion that other factors contribute to triggering mood disorders. Previous studies performed in mice infected with BCG showed that infection-induced depression was associated with increased production of TNF and IFN-γ, and with IDO

activation (Moreau *et al.* 2008; O'Connor *et al.* 2009; O'Connor *et al.* 2009; Kelley *et al.* 2013). Activation of this enzyme has been proposed as one of the events responsible for the transition from a sickness state into a depressive disorder (Moreau *et al.* 2008; O'Connor *et al.* 2009; O'Connor *et al.* 2009; Kelley *et al.* 2013). In the present work, we also observed increased expression levels of *Ido* in the hippocampus at least in C57BL/6 and Balb/c mice. However, no behavioral alterations were detected which led us to hypothesize that other pathways could be involved. One consistent observation in the BCG infection model is an initial sickness behavior, accompanied by alterations in locomotion and body weight loss that were recovered after a few days (Moreau *et al.* 2008; O'Connor *et al.* 2009; O'Connor *et al.* 2009; Kelley *et al.* 2013; Platt *et al.* 2013; Vijaya Kumar *et al.* 2014; Rodriguez-Zas *et al.* 2015). However, mice can be infected with *M. avium* for several months without clinical signs of disease nor weight loss. Accordingly, it was also observed that upon *M. avium* infection, besides the absence of alterations in body weight, no variations in body temperature were present (Olsson *et al.* 2010).

Supporting the idea that other mechanisms orchestrate with the imbalance of the immune system to induce mood disorders are also the results showing a mouse-to-mouse variation within BCG-inoculated animals (Platt *et al.* 2013; Rodriguez-Zas *et al.* 2015). Actually, up to 30% of BCG-inoculated mice, despite the evidence of an activated immune system, did not exhibit increased depressive-like behavior and were categorized as ‘‘resilient’’ to BCG induced behavioral changes (Platt *et al.* 2013). Curiously, the “resilient” group does not present alterations in corticosterone levels whereas the mice “susceptible” to BCG induced behavioral changes showed elevated plasma corticosterone

levels when compared with control animals (Platt *et al.* 2013). The fact that cytokines influence the HPA axis and that increased levels of corticosterone are associated with depression, guides us to hypothesize that the imbalance in the cytokine profile should be accompanied by activation in the HPA axis to induce behavioral alterations. Our results showed that even though an increased inflammatory profile upon *M. avium* infection is present, no alterations in corticosterone levels were observed.

The relation between infection and behavioral alterations is far from being understood. It seems to be a complex interplay that could be influenced by several factors such as host, pathogen or environment, and mediated by different mechanisms (Ashley and Demas 2017; Lopes *et al.* 2021). In the opposite direction of the studies with BCG inoculation, studies with *M. vaccae* showed that this mycobacteria induces decreased depressive-like behavior and stress resilience (Lowry *et al.* 2007; Reber *et al.* 2016; Fox *et al.* 2017; Frank *et al.* 2018; Foxx *et al.* 2020; Loupy *et al.* 2021). The mechanisms underlying behavior alterations upon infection might be highly variable. Antidepressant-like behavioral alterations observed after administration of *M. vaccae* antigens are due to activation of a serotonergic subpopulation of neurons within the interfascicular part of the dorsal raphe nucleus (Lowry *et al.* 2007) and on the dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (Siebler *et al.* 2018).

In summary, with this study we conclude that peripheral chronic infection with *M. avium* leads to an altered inflammatory milieu in the hippocampus, that does not trigger neuroplasticity nor behavioral alterations in 3 mouse strains. These results highlight that other pathway(s) must synergize with the imbalance of the

immune system to trigger mood disorders. HPA axis activation and alterations in neurotransmitter homeostasis, are strong candidates that should be addressed in the future.

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Data statement

The data that support the findings of this study are available upon request.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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II. ANNEXES

1. REPORTING GUIDELINES

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	2.1 animals and 2.2 Infection and quantification of 2. Material and methods, section 2.1 -
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	2.8 Data analyses 2.1 Animals
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	No criteria were set No exclusions were done 2.8 Data analyses - Sample sizes are
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	2.2 Infection and quantification of bacterial load 2.3 Behavioral tests
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	2.3 Behavioral tests subsections
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	2. Materials and methods subsections 2.1 Animals
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	2.8 Data analyses 2.8 Data analyses
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	2.1 Animals and Fig. 1 2.1 Animals
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	2. Materials and methods sections 2. Materials and 2. Materials and 2. Materials and
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	3. Results 3. Results

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Abstract
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	1. Introduction 1. Introduction
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Last lines of the introdu
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	2.1 Animals section within 2. Materials and methods
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	2.1 Animals section with
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	2. Materials and methods 2.1 Animals section within 2. Materials and methods 2.1 Animals section within 2. Materials and methods
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	4. Discussion 4. Discussion
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	4. Discussion
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	not reported. In fact all the methods and experimental plan were predefined and
Data access	20	Provide a statement describing if and where study data are available.	data statement
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	Declaration of interest Funding

2. BBI GUIDE FOR AUTHORS

BRAIN, BEHAVIOR, AND IMMUNITY

AUTHOR INFORMATION PACK

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DESCRIPTION

Brain, Behavior, and Immunity, founded in 1987, is the official journal of the Psychoneuroimmunology Research Society (PNIRS). This innovative journal publishes peer-reviewed basic, experimental, and clinical studies dealing with **behavioral**, **neural**, **endocrine**, and **immune system** interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. The journal features online submission and review. Manuscripts are typically peer-reviewed and returned to authors within 30 days of submission, leading to timely publication of experimental results. There are no submission fees or page charges for *Brain, Behavior, and Immunity*, which is published eight times a year. Detailed instructions for authors can be found at http://www.elsevier.com/journals/brain-behavior-and-immunity/0889-1591/guide-for-authors.

Research areas include: Physiological mechanisms that convey messages between the immune and nervous systems and regulate their functionsStress and immunity, including the role of stress-related hormones and neurotransmitters on the immune system.Actions of cytokines, growth factors and PAMP activation on neuronal and glial cells that regulate behavior, learning, memory and neurogenesisRole of hormones, growth factors and cytokines in the immune and central or peripheral nervous systemsInteractions between the immune system and brain that are involved in development of neurological, psychiatric, and mental health disordersRole of immunological processes in neurodegenerative disordersThe effects of psychotropic medications on immunological mechanisms and their potential relevance to therapeutic interventionsNeuroimaging studies examining how immunological mechanisms affect brain structure and functionClinical trials and experimental studies testing the effects on both immune stimulation and immune suppression on brain and behaviorThe role of microglia in pain, psychological processes and in psychiatric disordersImmunological mechanisms involved in traumatic brain injury and its resolutionImmunologic disorders, infection and behaviorRole of the immune system in development and maintenance of inflammatory and chronic painImmune mechanisms that regulate the blood-brain-interface (BBI)Immune factors that affect health psychologySleep, exercise, immunity and healthImmune system interactions that affect behavior following use of psychotropic drugs, alcohol and other drugs of abuseHealthy aging of the immune system and brainRole of inflammation and stress during perinatal developmentCancer and its treatment, stem cells and their effects on brain behavior and immunity Reciprocal communication between the microbiome, immune and nervous systemsRegulation of nerve injury and repair by the immune systemPsychosocial, behavioral, and neuroendocrine influences on immunity and on the development and progression of immunologically-mediated diseasesNutrition, inflammation, obesity and behaviorGenomics of behavior and immunity

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Brain, Behavior, and Immunity, founded in 1987, is the official journal of the Psychoneuroimmunology Research Society (PNIRS). This innovative journal publishes peer-reviewed basic, experimental, and clinical studies dealing with behavioral, neural, endocrine, and immune system interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. The journal features online submission and review. Manuscripts are typically peer-reviewed and returned to authors within 30 days of submission, leading to timely publication of experimental results. There are no submission fees or page charges for *Brain, Behavior, and Immunity*, which is published eight times a year. Detailed instructions for authors can be found at https://www.editorialmanager.com/bbi/default.aspx.

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