Universidade do Minho
Escola de Medicina

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Cognitive performance: exploring the role of Interleukin -10 in male mice

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Trabalho efetuado sob a orientação da
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Doutora Palmira Barreira-Silva

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Performance cognitiva: estudo do papel da interleucina-10 em murganhos machos

Resumo

Nas últimas décadas a interação entre o sistema imunitário e o sistema nervoso central foi bastante investigada. Vários estudos demonstraram que o fenótipo de células T e o desequilíbrio entre citocinas pró- e anti-inflamatórias pode levar a alterações cognitivas. A presença de um perfil de citocinas pró-inflamatório em humanos foi associado ao declínio cognitivo. Em murganhos a produção de citocinas tais como o interferão (IFN) -γ, interleucina (IL)-4, IL-13 e IL-6 demostraram modular a performance cognitiva. No entanto, os mecanismos subjacentes ao papel das citocinas anti-inflamatórias no desempenho cognitivo carecem de um estudo mais aprofundado.

Estudos anteriores mostraram que a ausência da citocina anti-inflamatória IL-10 está associada a comportamentos do tipo depressivo em murganhos fêmea, mas não em machos. A administração de IL-10 recombinante reverteu este fenótipo, fortalecendo assim a associação entre IL-10 e o comportamento do tipo depressivo em fêmeas. Resultados não publicados do nosso laboratório mostram que a ausência de IL-10 está associada com uma pior performance cognitiva em murganhos fêmea, mas o fenótipo cognitivo nos machos ainda é desconhecido. O objetivo deste trabalho foi avaliar o fenótipo cognitivo e o comportamento social de murganhos machos sem expressão de IL-10. Para entender os mecanismos subjacentes a esta interação também analisamos a morfologia dos neurónios e o perfil imunológico.

Nesta dissertação mostramos que os animais machos IL-10 KO não apresentam problemas locomotores nem comportamentos do tipo ansioso ou depressivo. Os murganhos machos IL-10 KO também não apresentam alterações na memória de referência, memória de curto prazo ou memória associativa. Também não foram observados distúrbios sociais nos animais machos IL-10 KO. No entanto, os murganhos machos IL-10 KO apresentam alterações cognitivas nas estratégias utilizadas em tarefas de aprendizagem que envolvam a memória espacial. Nos neurónios granulares do giro dentado (DG) do hipocampo, não foram encontradas diferenças no número de nódulos, mas os animais IL-10 KO apresentam dendrites mais curtas e alterações no número de interseções a distâncias especificas do soma. A analise do perfil imunológico mostrou que os animais IL-10 KO apresentam uma maior percentagem de neutrófilos e células T $CD4^+$ EM, e menor percentagem de células B no sangue. Não foram encontradas diferenças nos nódulos linfáticos (LN) cervicais profundos nas populações celulares analisadas, mas os murganhos IL-10 KO apresentam maior percentagem de células T $CD4^+$ EM nos LN inguinais.

Palavras chave: Cognição; Interleucina-10; Memória espacial; Morfologia neuronal; perfil imunitário.

Cognitive performance: exploring the role of interleukin -10 in male mice

Abstract

The interaction between the immune and central nervous systems has been under investigation for many years. Several studies showed that the T cell phenotype and an imbalance between pro- and anti-inflammatory cytokines can lead to cognitive impairment. In humans, a pro-inflammatory cytokine profile has been associated with cognitive impairment. In mice interferon (IFN)-γ, interleukin (IL)-4, IL-13 and IL-6 showed to modulate cognitive performance. However, the specific mechanisms underlying the role of anti-inflammatory cytokines, in cognition must be explored.

Previous studies have demonstrated that the absence of IL-10, an anti-inflammatory cytokine, is associated with depressive-like behavior in female mice, but not in male mice. Administration of recombinant IL-10 reversed this phenotype, strengthening the association between IL-10 and depression in females. Unpublished results from our group show that IL-10 absence also induces cognitive impairment in female mice, but the cognitive phenotype in males is still unknown. The aim of this work was to assess the cognitive phenotype and social behavior of male mice lacking the expression of IL-10. To understand the mechanisms underlying this interaction we also analyzed neuronal morphology and the immune system profile.

Here we show that IL-10 KO male animals display no locomotor impairment, and no evidence of anxious or depressive-like behavior, as previously demonstrated. Similarly, we found that these animals have no alterations in reference memory, short-term memory or associative memory. No social deficits were observed in IL-10 KO male animals either. On the other hand, we show that male mice lacking IL-10 present cognitive deficits when choosing strategies to perform spatial learning and memory tasks. Studying the hippocampal dentate gyrus (DG) granular neurons, no differences were found on the number of nodes. However, IL-10 KO animals have shorter dendrites and differences in the number of intersections, at different distances from the soma, when compared with WT littermates. Finally, we observed high percentage of neutrophils and EM CD4+T cells, and lower percentage of B cells in the blood of IL-10 KO mice. No alterations were found in deep cervical lymph nodes (LN), but IL-10 KO mice present higher percentage of EM $CD4^+$ T cells in inguinal LN comparing with WT littermates.

Keywords: Cognition; Immune phenotype; Interleukin-10; Neuronal morphology; Spatial learning and memory

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Abbreviations

APC Antigen Presenting Cells

BM Barnes maze

BBB Blood-brain barrier

BCSFB Blood Cerebrospinal Fluid Barrier

BDNF Brain Derived Neurotrophic Factor

CA Cornu ammonis

CFC Contextual fear conditioning

CD Cluster of Differentiation

dcLN Deep cervical lymph nodes

CM Central Memory

CNS Central Nervous System

CSF Cerebrospinal Fluid

DC Dendritic Cells

ECS Extracellular space

EM Effector Memory

EPM Elevated plus maze

FST Forced swim test

IFN Interferon

ingLN Inguinal lymph nodes

IL Interleukin

ISF Interstitial fluid

KO Knock out

M Mean

MHC Major Histocompatibility Complex

MIP Macrophage Inflammatory Protein

MWM Morris Water Maze

NK Natural Killer Cells

NKT Natural Killer T Cells

NOR Novel object recognition

NO Nitric Oxid

OF Open field

RAWM Radial arm water maze

ROS Reactive Oxygen Species

SAS Subarachnoid space

SCID Severe Combined Immune Deficient

t T-test

TNF Tumor Necrosis Factor

TST Tail suspension test

WT Wild type

ρ Spearmans's rank correlation coefficient

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1. Introduction

1.1. Interaction between the Immune system and the Central Nervous system

For over a century the brain has been considered an immune privileged location because it was thought to be completely isolated from the immune system. Features like the existence of brain barriers that limit the entry of immune cells and immune mediators from the systemic circulation, the absence of professional antigen-presenting cells in the brain parenchyma, the low expression of major histocompatibility complex (MHC) class I and II, and the lack of a conventional drainage system all contributed to the idea that the CNS is an immune privileged location [1,2].

Nowadays the idea of CNS immune privilege is still recognized but is now clear that the nervous system and the immune system are interconnected by a wide variety of coordinated and bi-directional mechanisms. They communicate not only through physiological connections like endocrine hormones [via the Hypothalamic–pituitary–adrenal (HPA) axis][3] and neurotransmitters (via interaction with T cell receptors, the neurotransmitters dopamine and glutamate can activate or suppress different T cell functions like cytokine secretion, and proliferation)[4], but also through anatomic connections such as the autonomic nervous system (primary and secondary lymphoid organs receive direct innervation from the sympathetic division of the autonomic nervous system [5]) and the brain barriers [the blood brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (BCSFB)]. Both brain barriers are responsible for the selective passage of immune cells from the circulation and for the exchange and removal of metabolites[6].

A schematic diagram of the interaction between the immune cells and the HPA axis is represented in **figure 1**.

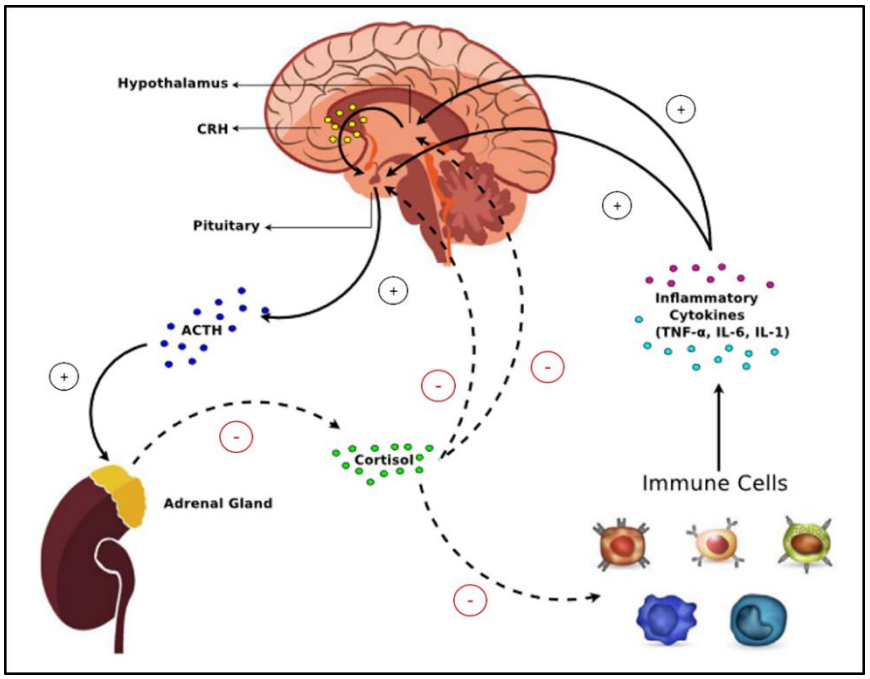

FIGURE 1. The HPA-axis interactions. Activation of the HPA axis by inflammatory cytokines and modulation of the immune response by the production of cortisol. After activation, the release of corticotropin-releasing hormone (CRH) stimulates the production of adrenocorticotropic hormone (ACTH). Secreted ACTH stimulates the adrenal cortex, releasing cortisol into the blood[3].

Cytokines represent another very important mediator of communication between the immune system and the CNS as they can signal the brain to maintain the homeostasis and also in response to diverse types of insults (injury, inflammation or infection).[7] They can exert their function internally, since they are constitutively expressed in the brain, or act via one of the following methods: passive transport using permeable regions in the BBB; active transport across the BBB and BCSFB using carrier molecules; connection with cerebral vasculature to stimulate the production of secondary messengers and activation of the peripheral afferent nerve terminals that then can transmit cytokine signals [8].

For a long time, the CNS was thought to be deprived from a drainage system, and this was one of the features that rendered the CNS as an immunological unique site. This idea was disproven when some studies, that followed the intracerebral distribution of different tracers injected in the brain parenchyma, demonstrated that these substances would exit the parenchyma via the cribriform plate and reach the lymph nodes (LN)[9,10]. Furthermore, the same exit pathway was confirmed to be used also by immune cells[11]. GFP-expressing $CD4^+$ T cells that were injected into the lateral ventricle or in the entorhinal cortex after lesion used the cribriform plate as the passage site to reach cervical LN (both deep cervical- (dcLN) and superficial cervical (scLN)). Similarly, it was shown that monocytes injected into the brain of

an entorhinal cortex lesion mouse model, migrated from the lesion site and left the CNS passing through the cribriform plate to reach dcLN[12]. As some of the injected substances would also accumulate in the perivascular spaces, it was proposed the existence of a vascularized system promoting the waste clearance of the interstitial fluid (ISF) to the CSF. This system also named glymphatic system promotes the removal of soluble proteins and metabolites from the CNS through perivascular channels formed by astroglial cells[13].

The discovery and description of a lymphatic vessel network in the dura mater of the CNS brought new insights to the mechanisms that CSF uses to access the extracranial lymphatic compartment[14,15]. The lymphatic vessels are located parallel to the dural venous sinuses and middle meningeal arteries of the mouse brain. The authors demonstrated that the functional network of vessels transports fluid into dcLN via foramina at the base of the skull. It was also shown that it absorbs CSF and ISF from the adjacent subarachnoid space[14]. Likewise, using intracerebroventricular injection of tracer dye, Louveau and colleagues also showed that the route to the dcLN is through the meningeal lymphatics. In the same work it was demonstrated, that the meningeal lymphatic vessels represent an important drainage pathway for meningeal immune cells. More specifically, they observed that T cells injected in the intra-cisterna magna drain into dcLN and scLN[16]. It has been shown that brain parenchyma and meningeal compartments communicate with each other, even though the routes of drainage seem to be different. Interestingly, CSF seems to have an undeniable importance in this immune exchange.

Using T cells specific for myelin basic protein, Schläger and colleagues observed that the decision if whether T cells enter the CNS parenchyma or not depends on activation and adhesion signals from cells in the meninges [17]. After T cells are injected intravenously in healthy rats, they exit the blood circulation and go to the nearby meningeal tissue (leptomeninges of the spinal cord). Once the CSF flows in the meningeal tissue, T cells that are on the meningeal surface can either infiltrate the nervous tissue or be washed way into the CSF. Results revealed that T cells from the leptomeninges and CNS parenchyma exhibited upregulated genes of cytokine–cytokine-receptor interaction, whereas CSF T cells lacked signs of activation[17]. Remarkably, the author also demonstrated that, the T cells that were washed way in the CSF remain fully functional, and upon inflammation they can reattach to the meninges. Taken together these results indicate that CSF can function as a storage place for T cells that did not pass the meningeal checkpoint and as a transport that delivers T cells to damaged areas of the CNS[17].

With the notion that a healthy CNS is "patrolled" by leukocytes that circulate within the CSF[18] a study used flow cytometry to compare the difference of immune cell composition between blood and CSF collected from healthy individuals (without neurological diseases)[19]. The results demonstrated that, in CSF there is prevalence of T cells – identified as activated central memory (CM) $CD4^+$ T cells - and, when compared to peripheral blood, granulocytes, B cells and Natural Killer (NK) cells are scarce in the CSF[19]. These results suggest a rigorous and very regulated control of cell migration. Which is in accordance with what Medawar and colleagues demonstrated - to reach the CNS T cells overcome two types of barriers, the BBB when the cells must pass from the blood into the CNS, and the BCSFB to exit the choroid plexus into the brain [20].

Three different routes for the entrance of leukocytes in the CNS have been previously described [21]. By one of the routes, cells enter the CSF in physiological conditions coming from the blood and cross the choroid plexus. This pathway was demonstrated using fluorescence-labelled lymphocytes injected intravenously in healthy mice, and 2 hours after injection the labeled cells were found in the choroid-plexus stroma and the meninges [22]. In the second described pathway cells flow from the blood to the subarachnoid space, and after reaching the subarachnoid and perivascular spaces, leukocytes are thought to interact with antigen presenting cell. The last described route suggests the migration of the circulating cells directly from blood to the parenchyma[21].

Roughly 150 thousand T cells enter the brain to perform immunosurveillance allowing continuous monitoring for signs of infection or tissue damage and stimulation of cognitive processes[1] However, due to the rigorous control exerted by brain barriers, this immune surveillance is circumscribed to the perivascular and subarachnoid spaces [1,23]. Additionally, T cells assist in the regulation of nervous system functions where learning, memory and neural plasticity are modulated by both immune cells and cytokines [24,25]. As described above, peripheral immune cells need to pass the BBB to enter the CNS. However, during homeostasis, CSF (produced in the choroid plexus) contains T cells and other immune cells and circulates in the parenchyma and in the subarachnoid area. While the CSF drains back to blood circulation, immune cells and proteins in CSF are drained primarily through meningeal lymphatic structures to reach dcLN **(figure 2)**[26]

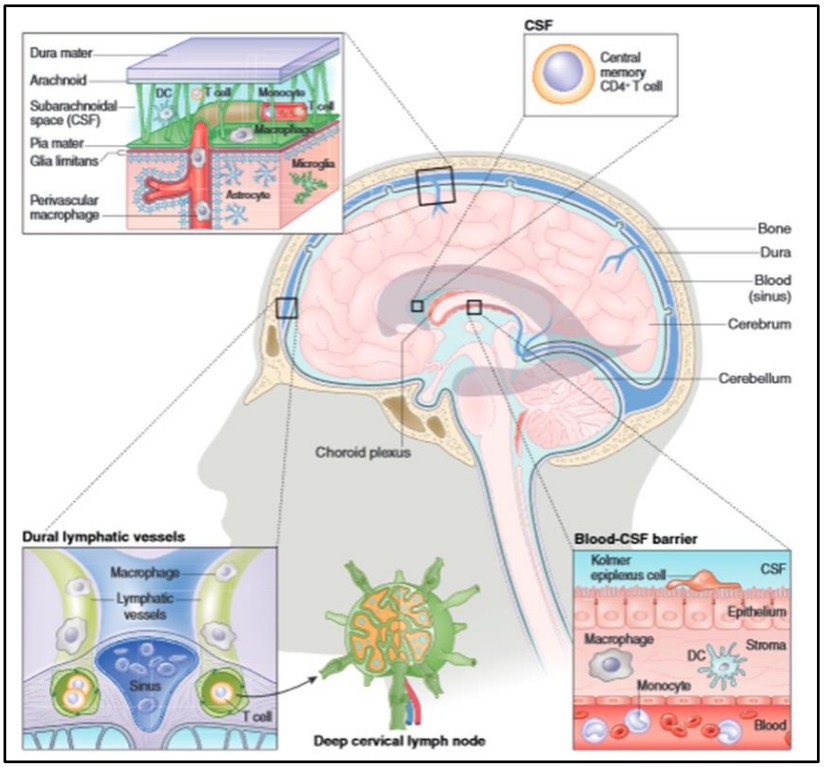

FIGURE 2. CNS immune surveillance during homeostasis. CSF contains T cells, bathes the brain and flows in the parenchyma and in the subarachnoid area (arteries and perivascular space). CSF drains back to blood circulation but immune cells and proteins in CSF are drained through meningeal lymphatic structures to the deep cervical lymph nodes[26].

1.1.1. Immune system and cognition

Aside from having a crucial role performing CNS immune surveillance, circulating T cells have been shown to be decisive in cognitive maintenance. Spatial learning and memory were tested in wild-type (WT) and severe combined immunodeficiency (SCID) mice - *i.e.* animals with deficiency on B and T cells – using six-radial arm water maze (RAWM) test [27]. When compared to the WT counterparts, SCID mice displayed longer escape latency and made more errors when attempting to reach the hidden platform. These results reveal a significant cognitive impairment when trying to resolve spatial memory and learning tasks.

Depletion of WT mice immune system with irradiation, followed by injection of bone-marrow cells from WT or SCID mice was used to study if the modulation of the immune system in adult mice has a direct effect on cognitive functions. Results showed that irradiated WT mice replenished with WT bone marrow exhibit a learning curve similar to that of non-irradiated WT mice, while irradiated WT mice replenished with SCID bone marrow exhibited impaired learning similar to that of SCID mice.

Next the authors tested if the cognitive impairment observed in animals receiving SCID bone marrow cells could be reversed by injecting T cells from WT donors. Results revealed that when compared to the control groups, the animals that were injected with T cells improved their cognitive performance demonstrating lower latency and less errors on the RAWM test[27].

The Morris water maze (MWM) behavioral test was also performed on SCID mice to assess spatial memory and learning skills (animals are expected to find an underwater hidden platform based on cues placed on the walls of the room). SCID mice showed substantial impairment in spatial learning and memory, as the latency time to find the hidden platform was higher in the these animals when compared with WT counterparts [27,28]. Additionally, SCID animals were also subjected to the Barnes Maze (BM) test, that also assesses spatial memory and learning but avoids stress related with water. Both time of latency and distance to reach the target box were significantly higher in SCID mice when compared to the WT animals confirming what was observed using the MWM[27]. Studies from Kipnis and colleagues confirmed that the cognitive impairment exhibited by SCID mice in MWM is reversed with T cell replenishment[28]. SCID mice were injected with $CD3^+$ T cells and performed RAWM after 2 weeks along with the control group (naïve SCID animals). Results showed that escape latency and the number of errors decreased significantly in the injected SCID animals, showing that replenishment with T cells improve their cognitive behavior[27]. Nude mice (*i.e.* animals that have no thymus, and so no T cells) replenished with WT T cells were compared with non-replenished nude mice in the MWM test. Accordingly with the previously referred results on SCID mice, latency time to find the hidden platform was lower and learning rates were significantly higher in animals replenished with T cells[28].These results confirm the importance of T cells in the maintenance of cognitive performance, especially in tasks related with spatial learning and memory. Similarly, to understand the importance of T cells in learning and memory, peripheral T cell lymphopenia was induced in WT mice by the administration of a drug (FTY720) that sequesters the T cells in the lymph nodes. Results demonstrated that when performing MWM, the FTY720 injected animals displayed a worst performance finding the platform than the control group[24]. These studies showed the relevance of the brain immunosurveillance by T cells on cognitive performance.

Knowing the beneficial role that adaptive immunity has in cognitive tasks, more specifically in MWM, a study from Kipnis and colleagues used brain tissue analysis, to understand what could be happening to the T cells after animals finished the test[24]. Meningeal tissue of WT mice was analyzed and, there was an increase in the $CD3^+$ $CD4^+$ T cell numbers in the meninges of the animals that performed MWM test compared to those that did not. Moreover, the results also revealed that the depletion of T cells from the

meningeal spaces induces a proinflammatory profile of meningeal myeloid cells in response to the cognitive task[24].

Schwartz and colleagues demonstrated that T cells also have an important role in neurogenesis. Their results revealed that, when compared to the WT counterparts, SCID and Nude mice present lower cell proliferation and cell differentiation in the dentate gyrus (DG), which indicates impaired neurogenesis. SCID mice also exhibited a lower number of newly formed neurons in the subventricular zone (SVZ), when compared to the control. Transferring WT mice splenocytes into SCID and Nude mice partially restored the neurogenesis in the DG[29]. Interestingly, the results also revealed that transgenic mice expressing myelin basic protein (CNS antigen) present higher neurogenesis in the DG and improved spatial learning when performing MWM, when compared with WT mice. On the other hand, transgenic mice expressing a T-cell receptor for ovalbumin (foreign antigen) present lower neurogenesis in the DG and impaired spatial learning when compared to the WT animals. Indicating that the T cells that induce neurogenesis in the adult hippocampus are CNS specific[29]. The impact of T cells in the formation of new neurons can help to explain why immunodeficient animals have impaired cognition, as neurogenesis in the DG may grant the brain enough plasticity to assure cognitive adaptability to different surrounding stimuli[30].

In humans, an association between T cells and cognition was also already described. In a cohort of elder individuals, with distinct cognitive performances but without any type of cognitive impairment, the number of effector memory (EM) $CD4^+$T cells present in the blood showed to be predictor of executive function and memory, even when controlling for variables already described to impact on cognitive function such as age, sex, education, and mood[31]. Accordingly, individuals with lower cognitive performance showed increased activated/naïve $CD4^+$T cells ratio when compared with the higher cognitive performers [32]. These results indicate an association between $CD4^+$T cell activation and cognition.

One important characteristic of activated T cells is production of several cytokines such as, IFN-γ, IL-4, IL-10 among others[33]. Many studies have provided insights on the effects that over- or underproduction of cytokines may have on behavior. Learning ability was shown to be directly associated with the production and release of IL-4. Mice lacking IL-4 expression exhibit severe cognitive impairment in the MWM test when compared to WT animals. Additionally, after task completion, IL-4 producing T cells are recruited to the meningeal spaces of WT animals. Interestingly, upon MWM the meninges of IL-4 KO mice present increased production of tumor necrosis factor (TNF) when compared with WT animals [24]. Accordingly, SCID mice, which present cognitive impairments as described before, also display increased expression levels of TNF and IL-12 in the meninges when compared with WT mice.

These findings revealed a connection between the pro-inflammatory profile of the meninges and cognitive performance.

The excessive production of pro-inflammatory cytokines is known to have damaging effects in astrocytes and to accelerate the degeneration of neurons, which may interfere with cognitive processes like learning and memory formation[34]. In fact, astrocytes cultured with IL-4 exhibit an increase of brain-derived neurotrophic factor (BDNF) mRNA levels, which suggests that IL-4 may have an impact in BDNF production[24].In fact, by being so important in synaptic plasticity BDNF is known to be involved in learning and memory consolidation[35]. BDNF effects in neurogenesis have also been reported. Infusion of adult rat hippocampus with BDNF revealed an increase in the neurogenesis of the granular cells in the dentate gyrus, when compared to infusions of phosphate-buffered saline (PBS), or bovine serum albumin (BSA).

The role of IL-13 in cognitive function was also addressed using MWW[36]. When performing MWM IL-13 deficient mice took significantly longer time than WT mice to locate the platform, especially in the reversal phase of the task, showing that spatial memory is impaired without IL-13. Additionally, meningeal cells from WT and IL-13 KO animals were analyzed to understand if IL-13 could influence T cell recruitment to the meninges. Results show that $CD3^{+}CD4^{+}$ T cells where significantly increased in WT trained mice but not in IL-13–deficient mice.[36]. Interestingly, much like what happened with IL-4[24], astrocytes cultured with IL-13 exhibit an increase in BDNF production. These results suggest that IL-13 is essential for spatial learning and memory and this is associated to the importance it has in the regulation of BDNF production and in the recruitment of T cells to the meninges[36].

Similarly, IL-6 has proven to be a relevant cytokine for the modulation of learning and memory consolidation[37]. A study revealed that when compared to the control group, IL-6 KO animals have impairments in memory related cognitive tasks like novel object recognition Test (NORT) and MWM. When performing the NORT, IL-6 KO mice were unable to discriminate between the old and the novel object as effectively as the control group. In the MWM, IL-6 KO mice revealed an impairment in both acquisition and reversal learning, when compared to the control group. These findings suggest that IL-6 might be responsible for hippocampus-dependent memory formation [38]. Furthermore IL-6 was also shown to have an impact in neurogenesis[39]. An *in vitro* study demonstrated that exposure of neuronal progenitor cells to IL-6 and to TNF - both produced by activated microglia in response to an inflammatory insult –decreased by 50% the neurogenesis. After the inhibition of only IL-6 the formation of new neurons was restored[39]. Similarly, the importance of IL-6 to adult neurogenesis was explored in an *in vivo* study. Transgenic mice that chronically express IL -6 in astroglia present neurogenesis decrease of about 63% in the hippocampal DG[40].

Interferon (IFN)-γ also showed to be crucial for cognition as IFN-γ KO mice present better cognitive performance than WT mice in the MWM test. The differences in behavior found between the two groups are associated with brain morphological alterations as IFN-γ KO animals present enhanced neurogenesis and also longer and more ramified dendrites on the dorsal DG granular neurons, when compared to WT controls [41]. Similarly, the effect that IFN-γ has in neurogenesis was also addressed in a study that compared mice with a controlled expression of the cytokine in the brain (transgenic IFN-γ animals) with WT mice. The expression of the IFN- γ in the transgenic mice brain is in amounts that do not cause damages in the tissue. Results demonstrated that, when compared to WT mice, transgenic young animals displayed an increase in DG neurogenesis and improved cognitive performance in the MWM test[42].

Social interactions are crucial for the thrive of all species. Behaviors like breeding, scavenging, providing protection are dependent of a healthy socialization, and several mental and neurological disorders (such as schizophrenia, autism and others) are characterized by causing social dysfunction. To understand the importance of the immune system in social behavior a study using SCID mice, addressed the role of T cells in sociability [43]. The results obtained show that in the three chambers test, SCID mice exhibited lower social preference when compared to the WT counterparts. A similar behavior was observed in the home cages where SCID mice spent less time exploring each other than WT mice. Finally, this social deficits could be reversed by repopulation of the immunodeficient mice with T cells [43]. A study by Filiano and colleagues, identified IFN-γ as having an important role in mediating social behavior. The socially abnormal phenotype observed in animals lacking IFN-γ was reversed with the injection of recombinant IFN-γ [43]. Curiously, similar results were obtained in a completely different species, the Zebrafish. In the social preference test, the animals were allowed to interact with other from the same species. Animals that spent less time interacting with their conspecific were shown to have lower levels of INF-γ gene expression when compared to the ones that demonstrated social preference[44].

All the above described studies support the importance adaptive immunity has in the CNS, whether by help maintaining homeostasis through immunosurveillance or by affecting memory, learning or even social interactions. Together with the relevance of T cells, that has been addressed extensively, the role cytokines play in cognition is also a subject of study. It is known that pro-inflammatory cytokines interfere with CNS and contribute to cognitive impairment via cytokine-mediated interactions between neurons and glial cells. However, the role of anti-inflammatory cytokines like IL-10 in behavior and cognition is not fully dissected. Being a cytokine also produced by T cells, and in light to all the knowledge

on the role T cells have in behavior and cognition it would be interesting to study the mechanisms that IL-10 may be playing in this context.

1.2. IL-10

IL-10 is an anti-inflammatory cytokine crucial for the homeostasis of the immune system, as it controls the immune response preventing exacerbated inflammatory responses that can cause tissue damage[45]. Because It can be produced by many different myeloid and lymphoid cells, and its receptors are basally expressed in almost all hematopoietic cells[46] the study of this cytokine can be considered of upmost interest.

Aside from limiting the immune response, IL-10 production and expression in the brain, is also a key factor. This cytokine has been associated with astrocyte protection, increased cellular survival and regulation of adult neurogenesis[47]. Studies have demonstrated that in microglial and astroglial cells, IL-10 controls its own mRNA expression, which modulates inflammation signaling sent from activated glia cells. This autoregulatory mechanism protects astrocytes from disproportionate inflammation responses[48]. In astrocytes, IL-10 potentiate the production of transforming growth factor (TGF)-β that is known to have neuroprotective effects in the CNS. This TGF-β production helps to attenuate microglial activation and prevent deleterious effects of inflammatory responses in astrocytes[49]. When exposed to excess glutamate, neurons exhibit loss of cell viability and, the addition of IL-10 to the culture medium was shown to have a neuroprotective effect, as neuronal toxicity was prevented, and the survival increased substantially[50]. In neurons, it was also observed that IL-10 regulates neurogenesis in the SVZ by maintaining neural progenitors in an undifferentiated state. This grants IL-10 the control over production of new neurons in the olfactive bulb[51].

Alongside with its immunomodulatory role and importance in the brain, IL-10 also has an impact on mood behavior phenotype since female IL-10 KO mice present depressive-like behavior. However this phenotype was observed only on IL-10 KO female but not male mice[52]. The depressive-like behavior observed in female IL-10KO mice was reversed by the administration of recombinant IL-10. Additionally, female mice overexpressing IL-10 (PMT10 animals) display an increase in the activity time on forced swim test (FST) when compared to the WT counterparts. On the other hand, males overexpressing IL-10 do not present alterations in the FST.[52] These results indicate that the modulation of IL-10 expression have an impact in mood behavior of females but not in males. In the same study, it was demonstrated the impact that IL-10 has in anxious-like behavior.

When compared to the respective WT control group, both female and male IL-10 KO mice spent less time in the center of the open field (OF) arena, suggesting an increased anxious-like behavior. Injection of recombinant IL-10 reversed the phenotype in male mice. Regarding IL-10 overexpressing animals, both genders spent more time in the center of the OF arena comparing to the basal IL-10 expressors. It is of note that no differences were observed between WT and IL-10 KO animals both in female and in male mice regarding locomotor behavior. With the OF it was also possible to evaluate the exploratory activity of the animals. IL-10 KO females revealed an increase in the number and duration of vertical activity, when compared to the WT females. To note, neither administration of IL-10 nor its overexpression were shown to have an effect in vertical activity on the OF test[52].

The impact that the modulation of IL-10 has is female mice mood behavior raised the question to whether or not the lack of this cytokine could impact cognitive behavior, more specifically the resolving of learning and memory tasks. Unpublished data from our laboratory revealed that when compared with WT animals, IL-10 KO female mice present cognitive impairment when performing Barnes Maze test. IL-10 KO females chose more random strategies to search for the target hole, when compared to WT females that displayed a preference for serial and direct strategies. IL-10 KO females also present a lower percentage of pokes in the target hole when compared to the WT group during the probe test. Alterations in the blood immune profile of IL-10 KO females were also observed. When compared to WT animals, IL-10 KO female mice present higher percentage of neutrophils, lower percentage of B cells and lower percentage $CD8^+$ T cells. Within the T cell compartments, IL-10 KO female animals present lower percentage of Central Memory (CM) $CD8^+$ and of Effector Memory (EM) $CD8^+$ T cells, and in the $CD4^+$ T cell compartments lower percentage of CM $CD4^+$ T cells and higher percentage of EM $CD4^+$ T cells. Interestingly, as it was previously mentioned, higher percentages of EM $CD4^+$ T cells and lower percentages of B cells have already been associated with a poor cognitive performance in humans[31].

It is important to mention that the impact the absence of IL-10 has in female mice cognitive performance may be influenced by other dimensions like depressive and/or anxious-like behavior. Knowing that IL-10 KO male mice do not present depressive-like it is of interest to address the role that IL-10 have in male mice cognition.

2. Aims

Considered the recent results from our lab showing that IL-10$^{-/-}$ female mice present cognitive impairment when compared with WT littermate controls, we hypothesize that the absence of IL-10 is also having an impact on male mice cognitive function.

The main objective of this project was to evaluate the cognitive performance of male mice lacking IL-10 expression and its association with neuronal morphology and alterations in immune system phenotype.

More specifically in male mice lacking IL-10 and in WT littermate controls we aim to:

1. Evaluate the cognitive performance and other behavioral dimensions associated with cognition;
2. Perform morphological analysis of hippocampal dentate gyrus granular neurons;
3. Study the immune cell populations (T cells, B cells, monocytes and granulocytes) and the activation profile of T cells, in blood and in inguinal and deep cervical lymph nodes.

3. Material and methods

3.1. Animals

BALB/c IL-10$^{-/-}$ and littermate WT mice were obtained by crossing the KO progeny (provided by Dr. A. O'Garra National Institute for Medical Research, London, UK) with BALB/c WT mice (Charles River Laboratories, Barcelona, Spain).The colony was kept by crossing heterozygous animals. Male IL-10 KO, heterozygous and WT littermates were housed together in groups of 5 or 6 per cage. Mice were kept in standardized conditions - 12h light/12h dark cycle, room temperature of 22 to 24 °C, relative humidity of 55% and with ad libitum access to sterilized water and irradiated food - in accordance with the laws concerning animal welfare (Decreto-Lei n. º 113/2013 de 7 de agosto). Mice genotype was accessed by PCR from a small sample of tissue collected after weaning. Three to four months old male mice were used in all experiments. Euthanasia was performed by the injection of anesthetic drug - "Imalgene" (Ketamine 75mg/kg) + "Dormitor" (Medetomidine 1mg/kg). Diarrheic animals or animals presenting weight loss over 20% of the initial weight were excluded from the experiment and humane endpoints were applied whenever necessary. All procedures were carried out in accordance to EU directive 2010/63/EU and Portuguese national authority for animal experimentation, Direção Geral de Veterinária guidelines on animal care an experimentation, with local ethic committee process number ORBEA EM. ICVS-I3Bs_012/2018.

3.2. Behavioral assessment

Behavioral tests were performed in four consecutive weeks, from the less stressful to the more stressful one: Elevated Plus Maze (EPM), Open Field Test (OFT), Forced Swim Test, Tail Suspension Test (TST), Novel Object Recognition Test (NORT), Y-Maze, Barnes Maze (BM), Three Chambers Test (TCT) and Contextual Fear Conditioning (CFC). Except for the set 2 of the TCT that was performed at night after 8 p.m. all other behavioral assessments took place between 8:30 a.m. and 6 p.m. All the behavioral data analysis was performed with the experimenter blinded to mice the genotype.

Elevated Plus Maze is as a protocol used to asses anxious-like behavior in rodents based on based on the animal's aversion to open spaces [41]. This test consists on placing the mouse in the central platform of a plus-shaped apparatus elevated 72.4 cm from the floor, with two opposing open arms (50.8 cm × 10.2 cm) and two opposing closed arms (50.8 cm × 10.2 cm × 40.6 cm). Each animal was allowed to explore the maze in a single trial of 5 minutes (min). After each trial the maze area was cleaned with

a 10% ethanol solution. The time spent in both open and closed arms was used to assess anxious-like behavior. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands).

Open Field test is used to access, locomotor activity, exploratory activity and anxious-like behavior in rodents [53]. The test was performed in a square arena (43.2 x 43.2 cm transparent acrylic walls and white floor). The animal was placed in the center of the arena and allowed to freely explore for 5 min while being recorded. After each trial the arena was cleaned with a 10% ethanol solution. The recorded footage was analyzed by an automated tracking system (MedAssociates) and data related to the time spent in the center of the arena (10.8 x 10.8 cm), distance traveled in the center of the arena, and number and duration of vertical exploration (rearings) was collected to assessed anxious-like behavior. To assess locomotor activity total distance traveled was used.

Tail Suspension Test: addresses depressive-like behavior by valuating the immobile posture the animal develops after subjected to inevitable stress [54]. Each animal was suspended by the tail, isolated from other animals, for 6 min. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands) and immobility time was used to assess traits of depressive -like behavior.

Forced Swim Test: assesses depressive-like behavior through the evaluation of behavioral despair [55]. The animals were placed in a cylinder (diameter: 20 cm; 55 cm of height) filled with water (25° C) and isolated from other animals. Animals were tested in a 6 min session, but only the last 4 min were considered to analyze immobility time. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands).

Novel Object Recognition: assesses recognition memory by evaluating the preference of the animal to a novel object [56]. Mice were familiarized with the empty arena for 20 min per day in three consecutive days. On the fourth day, two similar objects were placed on one side of the arena and the animals were allowed to freely explore both the objects for 10 min. Mice were then returned to their home cages and the arena and objects cleaned with 10% ethanol solution. The test session was carried out 1 hour (h) after the training phase. For the test session one of the objects was replaced by a novel one with different color and shape. Each animal was reintroduced into the arena where it freely explored the objects

for 5 min. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands). Recognition memory was assessed by the percentage of interaction with the novel object (time of interaction with novel object x 100/total interaction time).

Y-Maze was used to assess spatial reference memory and is based on the mice ability to remember previously visited places[57]. The Y shaped apparatus has three arms at 120 degrees to each other, each one having in the end a distinct visual cue (black a circle, a black square and a black triangle). The 3 arms are designated *Start arm*, *Familiar arm* and *Novel arm*. In the first trial we placed the test animal in the maze and allowed it to explore two arms for 10 min – *Start Arm* and *Familiar Arm*. The mouse was then returned to the home cage and the maze was cleaned with 10% ethanol solution. After 1h the mouse was again placed in the maze but now was allowed to explore all 3 arms of the maze for 5 min. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands) and spatial recognition information was assessed through the percentage of time spent in each arm.

Barnes Maze assesses spatial learning and memory and is based on rodents' aversion of open spaces and motivation to seek shelter [58]. For this test we used a circular platform with 80 cm of diameter, containing 12 holes equally spaced along the perimeter and elevated 105 cm from the floor. Directly above the table, we placed a bright light to create a more anxiogenic environment used to persuade the animal to escape from the open platform surface into a chamber located under one of the holes named "*target box*". The test was performed in 6 consecutive days and was divided in two phases: the *acquisition phase* with the duration of 5 days in which the animals had to perform three trials per day, 120sec each, with an inter-trial interval (ITI) of 10 min, and the *probe phase* that took place in the last day of the test in which the animals performed one single 60 sec trial.

In the beginning of each trial of the acquisition phase the animals were placed in the center of the maze under a cylindrical black chamber. After 10 sec the chamber was lifted, the light switched on and the mouse was allowed to explore the maze. The trial would end when the mouse entered the target box or after the designated time was elapsed. If in the available trial time the animal would not enter the target box, we guided them and assisted them to enter the hole. Once inside the hole, whether the mouse entered alone or with assistance, the lights were turned off. The mouse was left in the target box for 20 sec. In the probe day, we repeated the procedure, but this time we removed the target box, and the

animals were only allowed to explore for 60 sec. To assess the spatial learning and memory in the acquisition phase we analyzed the strategies used by the animals to search and reach the target hole as well as the latency to enter the target hole In the probe day we analyzed the time the animals latency to to reach the target hole, and the percentage of pokes in the target hole. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands). **Figure 3** explains, based on seven possible trajectories, the different strategies used by the mice to reach the target.

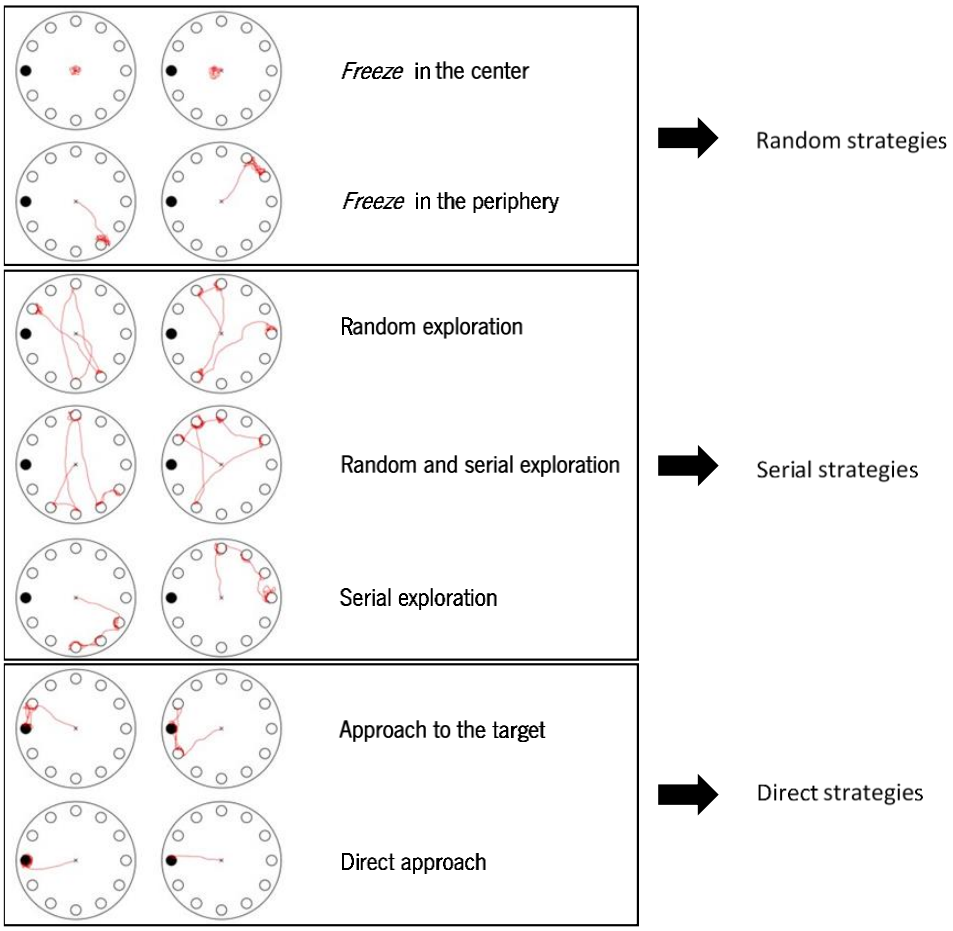

FIGURE 3. Schematic explanation of the different strategies used by the mice to reach the target hole. In the random strategy **(top)** they searched the extension of the maze in a disorganized way before reaching the target hole, in the serial strategy **(middle)** they visited serially the adjacent holes before reaching the target hole, and in the direct strategy **(bottom)** the mice traveled directly from the center of the arena to the target hole or to the adjacent hole.

Three chambers social test: this test was used to assess the mice social behavior using the natural curiosity of rodents for social novelty [59]. Knowing that Balb/c mice strain is naturally more anxious we performed a modified version of the protocol described by Van Heukelum and colleagues were, instead of a three chambered arena, they used a single chambered one[60].

We performed 2 separated pilot tests, were we optimized the test arena, light conditions, habituation periods and acquisition periods. After optimization we chose the following conditions: one chambered arena (50cm x 45cm), dim light in the room, 2 days of habituation (20 min/day), 10 min in each set of acquisition and performed at daytime.

In the first trial we assessed *Social Affiliation* - preference to spend more time with a novel animal from the same strain over spent time with an object- and in the second trial we addressed *Social Novelty* - preference for the novel subject mouse over the previously encountered one. We placed an empty perforated cup on the right and another on the left side of the arena and did a two-day habituation session (20 min per day) to the arena and the cups. On the first trial of the test day, we placed in one of the cups a same strain unfamiliar mouse while the cup on the opposite side was empty. The test animal was placed in the middle of the arena and allowed to explore for 10 min and the time spent interacting with each cup and the– *Social Affiliation*. On the second trial we maintained the familiar mouse under the cup and placed an unfamiliar one on the opposite side of the arena in the other cup. The test animal was placed again in the middle of the arena and allowed to explore for 10 min. Between each animal the arena and cups were cleaned with a 10% ethanol solution. The time spent interacting with each animal (familiar and unfamiliar) was analyzed– *Social Novelty*. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands). Schematic representation of the protocol used in the first set in **figure 4.**

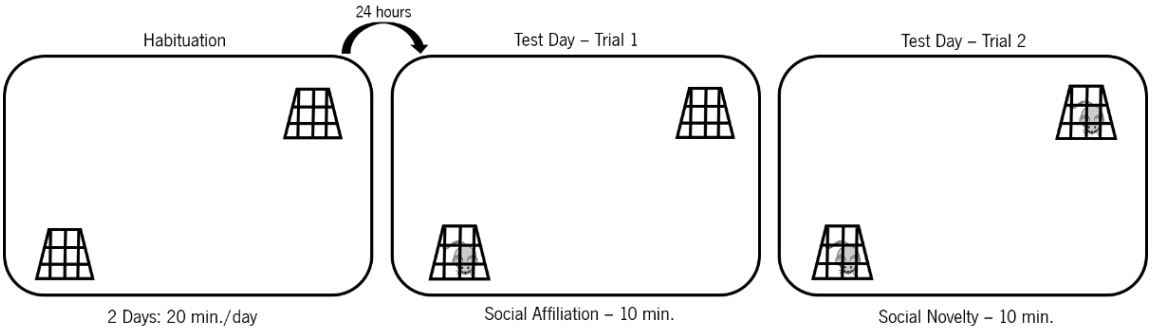

FIGURE 4. Schematic representation of Modified Three Chambers protocol. Representation of the empty arena (Habituation), arena with the stimulus mouse restrained in the cup on one side and an empty cup in the opposite side (Social Affiliation) and arena with the familiar stimulus mouse in on one side and the novel stimulus mouse in the opposite side (Social Novelty). The arena consisted of a single chamber (50cm x 40cm). The location of *Stranger 1* was alternated between the left and right sides of the social test across subjects.

After analyzing the first set, we were not able to attest the effectiveness of the protocol to evaluate social affiliation because the WT and IL-10 KO mice spent similar time exploring both *Stranger 1* and the *Object*. We decided to use a protocol adapted from Moy *et al.* and performed it during the night (starting after 8 p.m.)[59].

This protocol consisted of a single day protocol and was performed in a rectangular three-chambered box with openings between the chambers. On each of the side chambers we placed a perforated cup, and in the first trial the animal was allowed to explore for 10 min (arena and cups). After adaptation trial, we placed in one of the compartments a same strain unfamiliar mouse in the cup while in the opposite chamber we maintained the empty cup. The time spent interacting with each cup was analyzed – *Social Affiliation*. On the second trial we maintained the first mouse (familiar mouse) under the cup and placed a same strain unfamiliar mouse in the cup on the opposite side of the arena. The test animal was released again in the middle of the arena and allowed to explore for 10 min. Between each animal the arena and cups were cleaned with a 10% ethanol solution. The time spent interacting with each animal (familiar and unfamiliar) was analyzed– *Social Novelty*. The performance of the animals was recorded with a video camera and analyzed using EthoVision XT 12.0 (Noldus Information Technology, Netherlands). Schematic representation of the protocol used in the second set in **figure 5**.

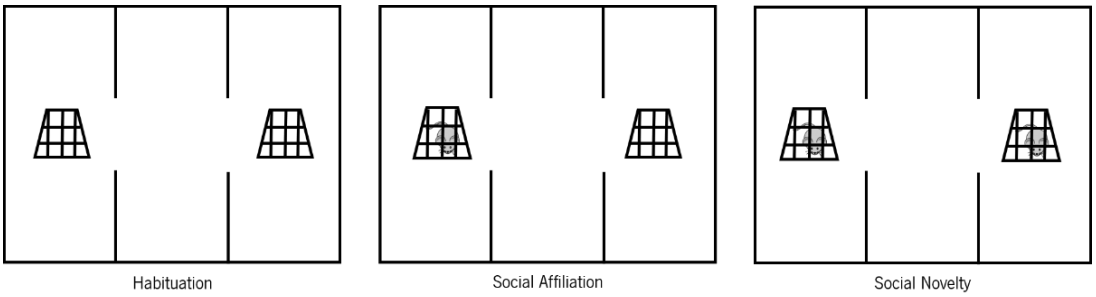

FIGURE 5. Schematic representation of Three Chambers protocol. The arena used was divided in 3 equally spaced chambers (18,7 cm/chamber). Representation of the empty arena (Habituation), arena with the stimulus mouse restrained in the cup on one side and an empty cup in the opposite side (Social Affiliation) and arena with the familiar stimulus mouse in on one side and the novel stimulus mouse in the opposite side (Social Novelty).The location of *Stranger 1* was alternated between the left and right sides of the social test across subjects.

Contextual Fear Conditioning: was used to assess the ability of mice to learn and remember an association between environmental cues and aversive experiences [61]. Mice learn to associate a particular neutral Conditioned Stimulus (CS - a tone) with an aversive Unconditioned Stimulus (US; mild electrical foot shock) and show a Conditional Response (CR - freezing). The unconditioned stimulus is the one that triggers a response automatically.

Two different contexts, Context A (white box) and B (black box), were used in CFC. In the first day, the animals were placed in Context A for 3 min and, in the last 20sec, a tone was delivered together with a mild 2 sec shock. Each animal received three CS-US pairing repetitions with an interval of 40 sec. We measured in the first day the basal freezing behavior and, after the three pairing CS+US we measured the conditional response that, in CFC consists in freezing behavior.

In the second day, mice were placed in Context A for 3 min without any tone or shocks associated. The mice were then returned to their home cages and two hours later were placed in Context B for 3 min again without any tone or shocks associated. Besides using a box with a different color, the context B was in a different room, with an odor of vanilla, and different texture covering the grid floor and walls of the box. We also wore a different color lab coat .On the second day we measure the freezing behavior in the 3 min in context A, to assess if the animal remembers the context and associates it with the aversive experience and we also measure the freezing behavior in the Context B. In the last day of the experiment, mice were placed in Context B for 2 min and 40 seconds without any CS and US. After this time, a tone (CS) was delivered (similar to the tone delivered in the first day) but no shock was associated. On the last day we measured the freezing behavior after the delivery of the tone to assess if the animal

despite being in a new context learned and remember an association between the tone and the aversive experiences. The discrimination index (DI) was calculated through the following equation[62]:

$$\frac{\%\ of\ freezing\ in\ Context\ A - \%\ of\ freezing\ in\ Context\ B}{\%\ of\ freezing\ in\ Context\ A + \%\ of\ freezing\ in\ Context\ B}$$

The performance of the animals was recorded with a video camera and analyzed using Obsevador v.0.2.7.0 (Behavioral Scoring Program). Schematic representation in **figure 6**.

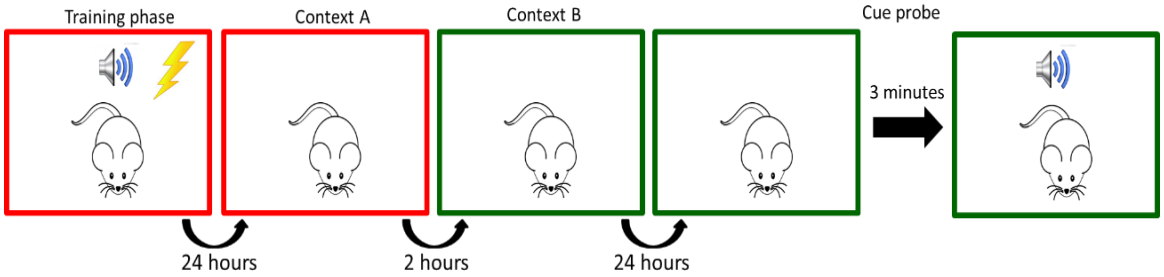

FIGURE 6. Schematic representation of the Contextual Fear Conditioning protocol. The red boxes represent context A where the mice freezing behavior was assessed basally and after conditioning. The green boxes represent context B were the cue probe was performed.

3.3. Hippocampal neurons morphological analysis

To assess the three-dimensional (3D) dendritic morphology of hippocampal neurons, we used the Golgi–Cox method. The brains were immersed in Golgi–Cox solution for 21 days, kept at RT and shook gently every day. Then they were transferred to a 30% sucrose solution and stored at 4°C protected from light. The sucrose solution was replaced every two days over a week. To a long-term storage, brains were preserved in a sucrose solution with 0,01% azide at 4°C. The brains were then cut on a vibratome. 200 µm thick coronal sections were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. Next, the sections were 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.

The morphology of neurons was evaluated in the Dentate Gyrus. From each brain 6 to 10 neurons were randomly selected using the following criteria: (1) full Golgi-impregnation along the dendritic tree; (2) complete dendrites without cut 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 searched and the neurons fulfilling the criteria were selected. For each selected neuron, all branches of the dendritic tree were reconstructed at ×1000 (immersion oil) magnification using a Zeiss

Stereology Microscope Upright AxionPlan2 and the Neurolucida software (*MicroBright Field*, Williston). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software. Total dendritic length, number of nodes and intersections were compared between experimental groups. Schematic representation of Sholl analysis in **figure 7.**

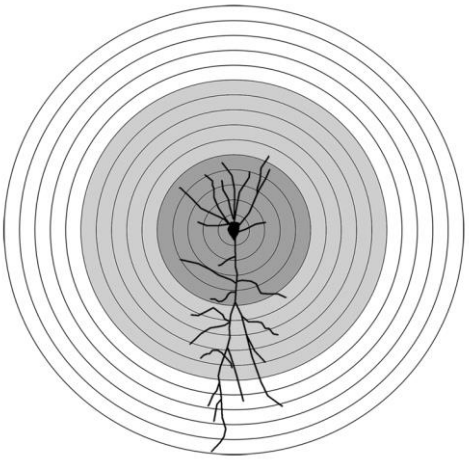

FIGURE 7. Schematic representation of a pyramidal neuron in Sholl-analysis. The NeuroExplorer software performs an overlap of concentric circles in the neuronal drawing and calculates the number of intersections between the dendrite ramifications and the concentric circles drawn[63].

3.4. Immune system characterization

3.4.1. Flow cytometry

Flow cytometry was used to analyze immune cells in blood, deep cervical and inguinal lymph nodes, and after staining, samples were acquired in a LSR II flow cytometer (BD Biosciences) equipped with the BD FACSDiva software. Analyses of the cell populations were performed using FlowJo software (BD Biosciences).

Blood: At the end of behavioral tests, blood was collected from the tail using heparin-coated capillaries. 50 µL of blood was incubated with the antibody mix present on **table 1**, in the dark, at room temperature (RT) for 15 min. Afterwards we added 2mL of ACK Lysing buffer (NH_4Cl 0,15 M, $KHCO_3$ 10 mM and $Na_2C_{10}H_{16}N_2O_8$ 0,1 mM) and incubated at RT for 10 min. Subsequently we washed twice with FACS Buffer (NaN_3 0,01%, bovine serum albumin 0.5% in PBS: NaCl 137mM, KCl 2,7mM, Na_2HPO_4 10mM and KH_2PO_4 1,8mM) (centrifuge for 5 minutes at 1200 rpm between washes). Finally, the pellet

was resuspended in 150 μL of FACS buffer. One hundred thousand leucocytes ($CD45^+$ cells) were acquired. Counting beads (AccuCheck counting beads, Molecular Probes, ThermoFisher Scientific) were used to quantify the $CD45^+$ blood cells. 10 μL of blood were incubated with 10 μL of Pe anti-CD45 antibody (BioLegend) (dilution 1/200 in FACS buffer for 15 min, RT in the dark. We then added 370 μL of ACK Lysing buffer and incubated for another 15 min before adding 10 μL of beads. Samples were acquired within the next 15 min, and to perform the quantification of cells two thousand beads were acquired. The number of cells was determined following manufacture instructions.

TABLE1. ***List of antibodies used in flow cytometry analysis of blood***

Marker	Fluorochrome	Clone	Dilution	Brand
CD49b	FITC	DX5	50	BioLegend
CD62L	PE	MEL-14	100	BioLegend
CD19	Percpcy5.5	6D5	100	BioLegend
CD3	Pecy7	145-2C11	100	BioLegend
CD44	APC	IM7	200	BioLegend
CD4	BV421	RM4-5	400	BioLegend
CD8	BV510	53-6.7	800	BioLegend
CD45	BV785	104	100	BioLegend
Ly-6G	BV650	1A8	1200	BioLegend
Ly-6C	BV711	HK1.4	1200	BioLegend

Lymph Nodes: Deep cervical and inguinal lymph nodes were harvested, placed in complete RPMI (RPMI 1640 with stable L-glutamine (Merck), sodium pyruvate 1mM (Merck), penicillin 100units/mL, streptomycin 100μg/mL (Gibco) and 10% heat inactivated FBS (Merck)) and gently mashed to have a single cell suspension. Total LN cell count was determined using the automated cell counter Countess™ (Invitrogen) by trypan blue cell death exclusion technique.

Lymph node cells were stained with the antibodies presented on **table 2**, by incubation in the dark, at 4°C for 20 min. The samples were then washed twice with FACS Buffer (centrifuge for 2 minutes at 1200 rpm) and incubated with 200 μL of Paraformaldehyde (PFA) 2% in the dark, at RT for 30 min. Finally, cells were washed twice with FACS Buffer and the pellets resuspended in 150 μL of FACS Buffer. The samples were analyzed within the next 24 h.

TABLE 2. *List of antibodies used in flow cytometry analysis of Lymph nodes*

Marker	Fluorochrome	Clone	Dilution	Brand
CD62L	PE	MEL-14	100	BioLegend
CD19	Percpcy5.5	6D5	100	BioLegend
CD3	Pecy7	145-2C11	100	BioLegend
CD44	APC	IM7	200	BioLegend
CD4	BV421	RM4-5	400	BioLegend
CD8	BV510	53-6.7	800	BioLegend
CD45	BV785	104	100	BioLegend
Ly-6C	BV711	HK1.4	1200	BioLegend

3.5. Statistical analysis

Five to nine mice were used per group in two different sets. Data from both sets were joined to present the final results (except for three chambers test were results were presented as two separate sets).

Statistical analysis was carried out using Prism GraphPad 7 (GraphPad Software, La Jolla, California, USA). Data is presented generally as group mean. Group mean ± standard error of the mean (SEM) was used to present latency times and Sholl analysis data. Statistical significance was considered whenever *p* values were lower than 0,05.

Differences between groups in Barnes Maze latency time and in Sholl-analysis were evaluated using a two-way ANOVA repeated measures, and additionally the partial eta-square (η^2p) was calculated as a measurement for effect size (small effect size if $\eta^2p < 0,13$; medium effect size if $0,13 \leq \eta^2p \leq 0,26$; large effect size if $\eta^2p \geq 0,26$). The calculations were performed using online calculator available in https://effect-size-calculator.herokuapp.com/.

T-test was used to evaluate differences between two groups whenever appropriate, and Cohen's d test was calculated as a measure of effect size (very small effect size if $d < 0,2$; small effect-size if $0,2 \leq d < 0,5$; medium effect size if $0,5 \leq d < 0,8$; big effect size if $0,8 \leq d < 1,2$; very big effect size if $1,2 \leq d < 2,0$; huge effect size if $d \geq 2,0$). The calculations were performed using online calculator available in https://www.socscistatistics.com/effectsize/.

The strategies used to perform Barnes Maze were analyzed using Pearson's χ^2 *for trend*.

4. Results

4.1. Behavioral assessment

Previous work has demonstrated the importance that the modulation of IL-10 has in dimensions like depressive-like and anxious-like behavior of female mice[52].However the impact of IL-10 on those behavioral dimensions of male mice was not as clear as in female mice. To understand if the lack of IL-10 have an impact in cognition of male mice, we performed a set of well described behavioral tests using WT littermates as control group.

4.1.1. IL-10 KO mice do not display distinct locomotor, anxious, and depressive-like behavioral traits when compared to WT animals

During the OF test both WT and IL-10 KO mice explored similar distances in the arena ($t_{(27)}$=0,5352; p=0,5969; d= 0.1981 (**Figure 8A**), and we observed no differences regarding the vertical counts ($t_{(27)}$=0,2066; p=0,8379; d= 0.8651) (**Figure 8B**) and the vertical time ($t_{(27)}$=0,3593; p=0,7222; d=0,1345) (**Figure 8C**). These results indicate that the absence of IL-10 does not impact the locomotor or the exploratory activities.

No differences were observed between IL-10 KO animals and their wild type littermates in the percentage of distance explored in the center of the OF arena ($t_{(27)}$=0,0052; p=0,9959; d=0,003) (**Figure 9A**) neither in the time spent in open arms ($t_{(27)}$=0,1927; p=0,8486; d=0,7290) (**Figure 9B**) and the frequency of entries in both closed arms ($t_{(27)}$=0,9817: p=0,3350; d=0,3704)and open arms ($t_{(27)}$=0,7026; p=0,4883; d=0,2737)(**Figure 9C**) of the EPM. Thus, we can infer that IL-10 absence does not impact on anxious-like behavior.

In both TST ($t_{(27)}$=1.331; p=0,1944; d=0,0503) and FST ($t_{(27)}$=0,4312; p=0,6698; d=0,0162), IL-10 KO and WT mice displayed similar immobility time (**Figure 9D and 9E**). Indicating that the absence of IL-10 has no implication in modulating depressive-like behavior in male mice.

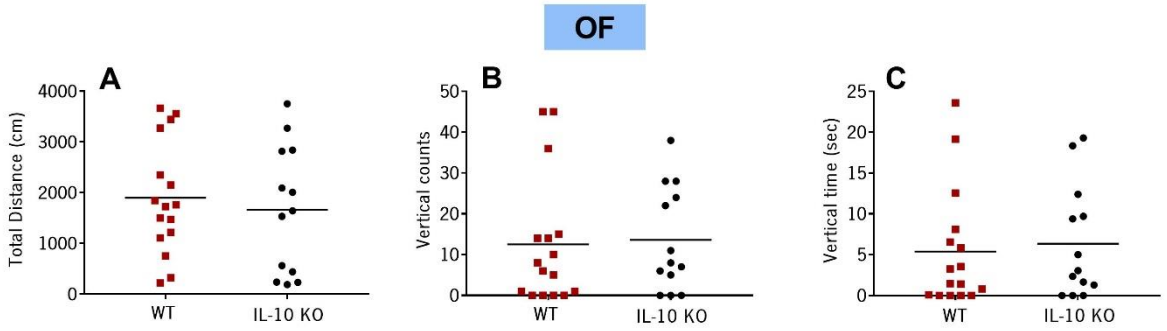

FIGURE 8. There are no differences in locomotor and exploratory behavior between WT and IL-10 KO mice. OF test was performed in WT (red squares) and IL-10 KO (black dots) male mice. The total distance traveled **(A)**, the vertical counts **(B)** and the vertical time **(C)** were used as measures of locomotion and exploratory activity. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean. Statistical analysis was determined by two-tailed Student's t test.

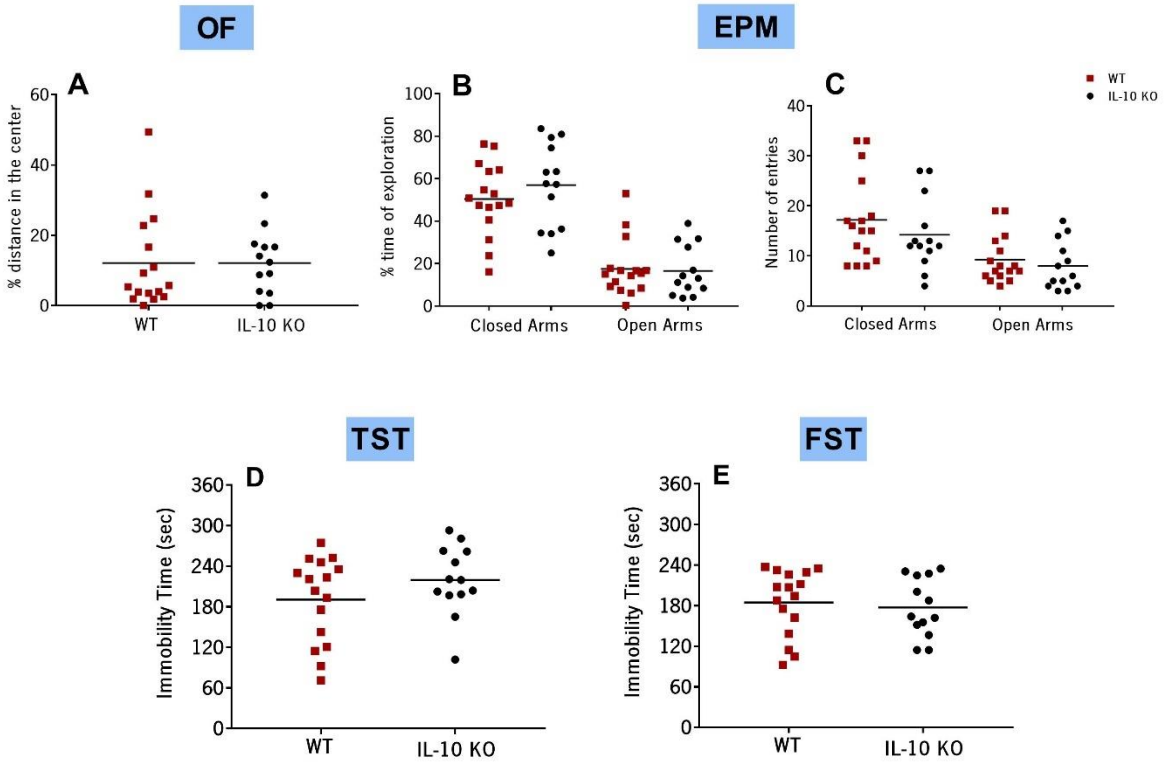

FIGURE 9. There are no differences in anxious and depressive-like behaviors between WT and IL-10 KO mice. OF, EPM, TST and FST were performed in WT (red squares) and IL-10 KO (black dots) male mice. To measure anxious-like behavior we used the percentage of distance traveled in the center of the OF arena **(A),** the percentage of time exploring the EPM arms **(B)** and the number of entries in the EPM arms **(C)**. In the TST **(D)** and FST **(E)**, the immobility time was used as measure of depressive-like behavior. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean. Statistical analysis was determined by two-tailed Student's t test.

4.1.2. IL-10 KO mice do not display alterations in short-term memory when compared to the WT littermates.

When performing NOR, WT and IL-10 KO mice spent similar percentage of time interacting with the novel object ($t_{(27)}$=0,7224; p=0,4763; d=0,2661) (**Figure 10A**). These results show that the absence of IL-10 does not affect short-term memory.

Y-maze test is based on the ability mice have to recognize visual cues in the arms of the apparatus and use them to distinguish between arms already visited and new ones. So, what is expected is that mice spend more time exploring the novel arm. As this was not the case for our control group (WT mice) we cannot attest the effectiveness of Y-maze test in our model, and the protocol requires optimization. Nevertheless, no differences were found between IL-10 KO mice and the WT littermates regarding the exploration time of the Y-Maze arms, either in the familiar arm ($t_{(27)}$=1,103; p=0,3231; d=0,3708) or the novel arm ($t_{(27)}$=1,109; p=0,2773; d=0,0411) (**Figure 10B**).

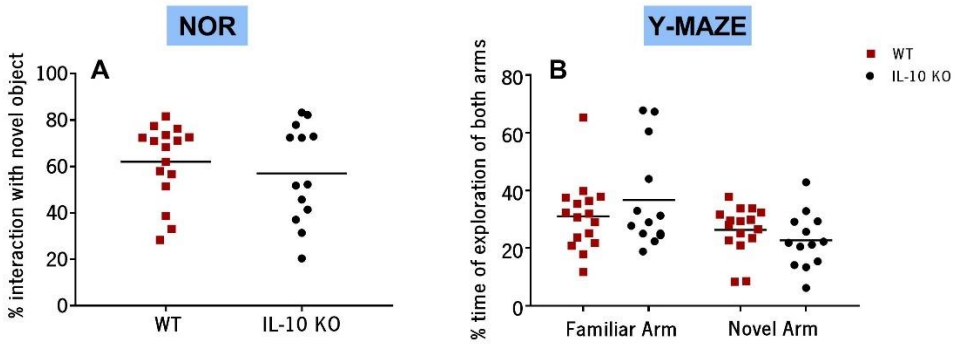

FIGURE 10. IL-10 KO and WT littermate mice do not present differences on short-term memory. NOR and Y-Maze test were performed in WT (red squares) and IL-10 KO (black dots) mice. In the NOR the percentage of time interacting with the novel object **(A)** was used as measure of short-time memory. To measure spatial reference memory, we used the percentage of time spent exploring the Y-Maze arms **(B)**. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean. Statistical analysis was determined by two-tailed Student's t test.

4.1.3. The associative memory of IL-10 KO mice is not affected when compared to the WT littermates

Both IL-10 KO and WT littermates displayed similar basal behavior ($t_{(27)}$=0,604; p=0,5509; d=0,2214) on the CFC test, and similar freezing pattern after being conditioned with the mild shocks ($t_{(27)}$=1,276; p=0,2128; d=0,7262). In *Context A* there are no differences in the performance between the two groups ($t_{(27)}$=0,2284; p=0,8210; d=0,0135), both were able to recognize the familiar context and associate it with the aversive stimuli delivered the day before - the freezing pattern resembled the one observed after conditioning. When placed in the *Context B* both IL-10 KO and WT mice were able to devaluate the context and distinguish between a noxious context (*Context A*) and a neutral one (*Context B*). The freezing behavior was similar between the two groups ($t_{(27)}$=0,4671; p=0,6441; d=0,1755) and lower than the one observed in *context A*. In the cue probe day, after the delivery of the tone, both groups presented similar percentage of freezing time ($t_{(27)}$=0,0011; p=0,9991; d=0,0005) which indicates that the animals learned and remember the association between the tone and the shock despite being in a different context. We observed no difference between IL-10 KO and WT mice in the percentage of freezing in the different contexts (**Figure 11A**) and no differences in the ability to distinguish between unrelated contexts (**Figure 11B**). These results show that the absence of IL-10 does not affect associative memory.

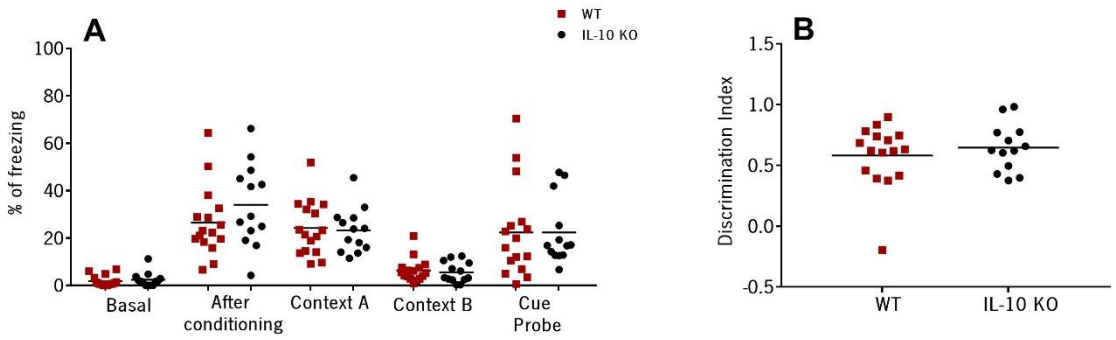

FIGURE 11. The absence of IL-10 does not affect associative learning. CFC was performed in WT (red squares) and IL-10 KO (black dots) mice. The percentage of freezing time in the different contexts **(A)** was used as measure of associative learning. Discrimination Index **(B)** was calculated to measure the ability of the animals to distinguish between context A and context B. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean. Statistical analysis was determined by two-tailed Student's *t* test.

4.1.4. IL-10 KO mice displayed an impairment in spatial learning and memory

During the five days of acquisition of the BM test, IL-10 KO mice used different search strategies to find the target hole, when compared to the control group (**Figure 12**). WT animals progressively improved and learned, using less random strategies as days pass, being these ones almost absent in the last day of the test. When compared to WT animals, IL-10 KO animals preferred more random and/or serial strategies throughout the five days (strategies explained in **figure 3**). It is of note that during the five days of acquisition phase, both WT and IL-10 KO mice were able to complete the task displaying similar latency time to enter the target hole ($F_{(1,27)}$=6,162^{-7} p=0,9994; η^2_P=0,0917) (**Figure 13A**). Results from the probe day demonstrated that there are no differences between IL-10 KO and WT mice regarding latency to reach the target hole ($t_{(18,83)}$=0,9939; p=0,3329; d=0,3801) (**Figure 13B**) and percentage of pokes in the target hole ($t_{(8,834)}$=1,171; p=0,2719; d=1,5511) (**Figure 13C**).

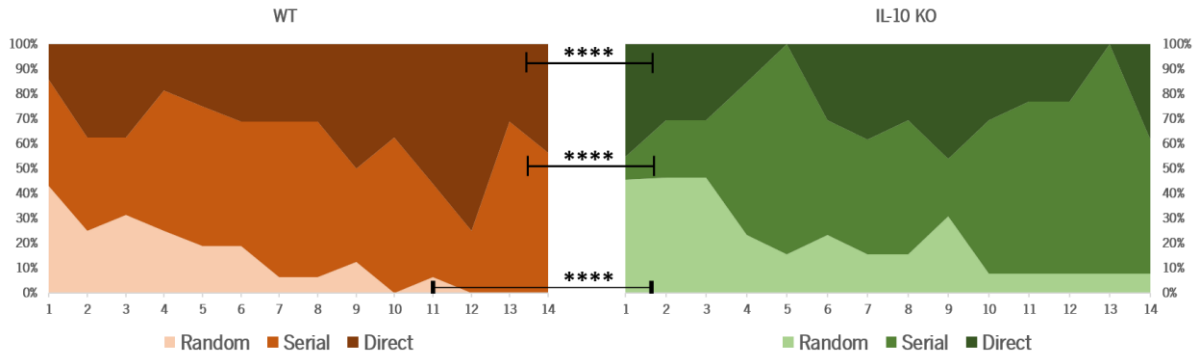

FIGURE 12. IL-10 KO mice displayed a preference for random strategies while WT mice used more direct ones. BM was performed in WT and IL-10 KO mice. The different strategies adopted by the animals to search the target hole are represented in the colored graphs (IL-10KO=green graph) and (WT=orange graph) and were used as measure of spatial learning and memory. n=16 (WT); n=13 (IL-10) KO values from two independent experiments combined. ****p<0,0001 determined by Pearson's qui-square for trend test.

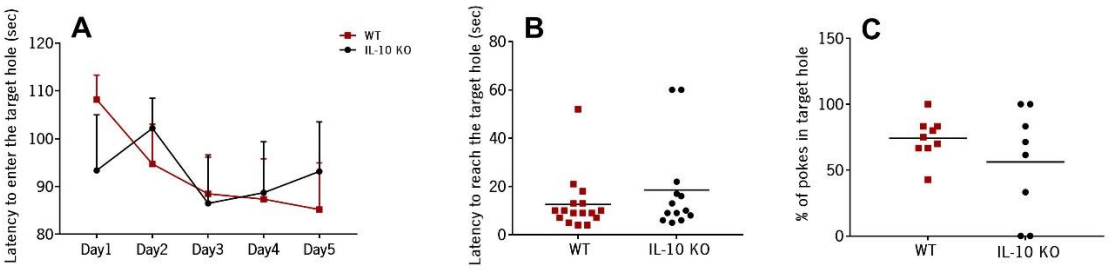

FIGURE 13. IL-10 KO and WT littermate mice do not present differences on latency time to enter the target hole. The time mice took to enter the target hole along the test days **(A)** was assessed. Data is represented as mean ± SEM. Statistical analysis was determined by repeated-measures analysis of variance (ANOVA). In Probe day latency to reach the target hole **(B)** and percentage of pokes in the target hole **(C)** were also measured. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined (except in Figure 8C were the data represents only the second experiment). Data is represented as mean. Statistical analysis was determined by two-tailed Student's t test.

4.1.5. IL-10 absence did not affect the mice social behavior

The results obtained from the three chambers test were analyzed and are presented as two separate sets because the protocols used differ between them. In the first set we used a single-chambered arena and perform the test at daytime (from 8.30 am to 6 pm); in the second set we used a 3 chambered arena and perform it at nighttime (after 8p.m). The protocol was modified between sets because during the first one we did not observe a preference to socialization on the control group, indicating that the test was not optimized for our model (**Figure 14A and B**). Each set has two trials: in *Trial 1* we assessed if mice prefer to spend more time with an unfamiliar mouse or with an object (Social Affiliation), and in *Trial 2* we assessed if mice prefer to explore a novel mouse than a familiar one (Social novelty).

The results obtained with the first set of mice were not conclusive for Social Affiliation, because both genotypes spent similar time exploring the unfamiliar mouse ($t_{(11)}$=0,5838; p=0,5711; d=0,0329) and the object ($t_{(11)}$=1,329; p=0,2109; d=0,0059) .This led us to conclude that the modified version of 3 Chambers used in the first set was not effective to evaluate sociability.

In the second set results from *Trial 1* (**Figure 14C**) showed that in both groups the animals choose to spend more time exploring the unfamiliar mouse (*Stranger 1*) ($t_{(14)}$=0,6276; p=0,5403; d=0,0314) rather than the empty cup (*Object*) ($t_{(14)}$=0,1078; p=0,9157; d=1,284) which indicated that animals present social motivation and affiliation. In *Trial 2* (**Figure 14D**) the obtained results showed that both WT and IL-10 KO mice displayed a similar preference for the cup containing the novel mouse (*Stranger 2*) (t

$_{(14)}$=1,988; p=0,0667; d=0,0994) over the cup containing the previously encountered animal (*Familiar Subject*) ($t_{(14)}$=0,9406; p=0,3629; d=0,4702) indicating not only a preference for novel experiences (Social Novelty) but also an intact social memory.

All together these results indicate that the absence of IL-10 does not affect social interactions and socially motivated behaviors in mice.

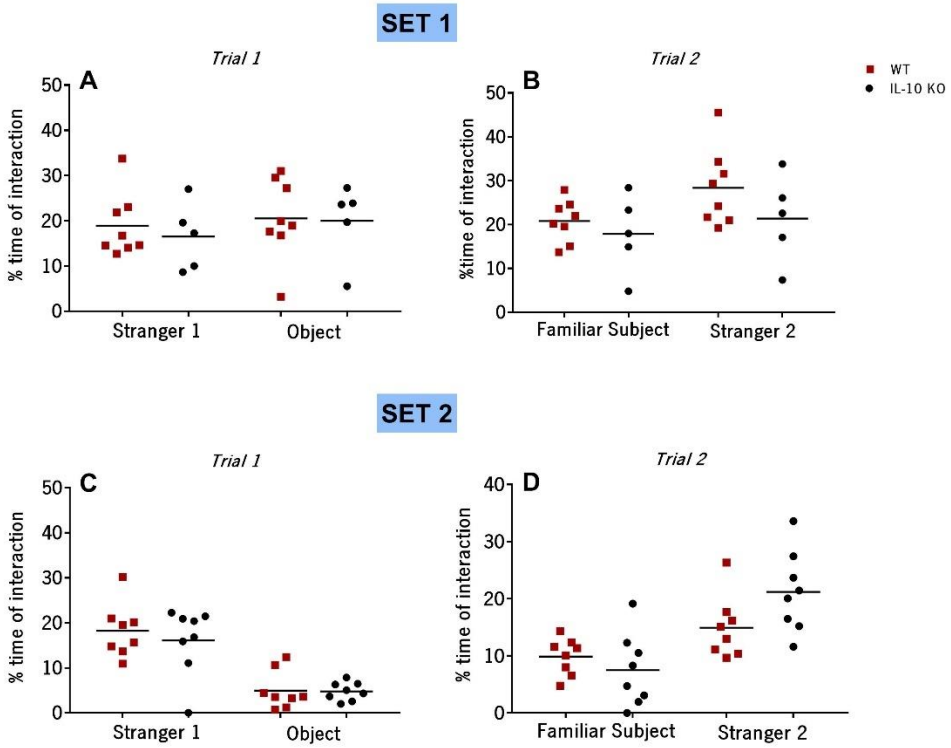

FIGURE 14.There are no differences in social behavior when comparing IL-10 KO and WT littermates. Three Chambers was performed in WT (red squares) and IL-10 KO (black dots) mice in two separated sets, using slightly different protocols. The first set **(A and B)** was performed between 8:30 a.m. and 6 p.m. n= 7 (WT); n=5 (KO). The second set **(C and D)** was performed after 8 p.m. n=8 (WT); n=8 (KO). The percentage of time interacting with the stimuli animals and the objects was used as measure of social interaction. Each point represents one animal. Data is represented as mean. Statistical analysis was determined by two-tailed Student's t test.

4.2. Neurons morphologic characterization

To assess if the absence of IL-10 induces structural alterations in the hippocampal neurons we performed Sholl analysis to quantify the complexity of the dendrites, we measured the total dendritic length and calculated the number of nodes present. From each animal we randomly analyzed 6 to 10 neurons - WT= 135 neurons/16 animals; IL-10 KO= 121/13 animals.

When compared to the WT littermates, IL-10 KO animals present less number of dendrites intersections at specific distances from the soma ($F_{(1,254)}$=3,896; p=0,0495; η^2_P=0,0151) (**Figure 15A**) and shorter dendrites ($t_{(254)}$=2,12; p=0,0350; d=2,9305) (**Figure 15B**). Even though there is a genotype associated effect in the number of interceptions along the dendrites, the interaction is also significant thus making these results difficult to interpret. No differences were found in the number of nodes of the dendrites ($t_{(254)}$=0,4319; p=0,6663; d=0,0538) (**Figure 15C**) between the two groups. These results indicate that IL-10 absence affects the length of the DG granular neurons.

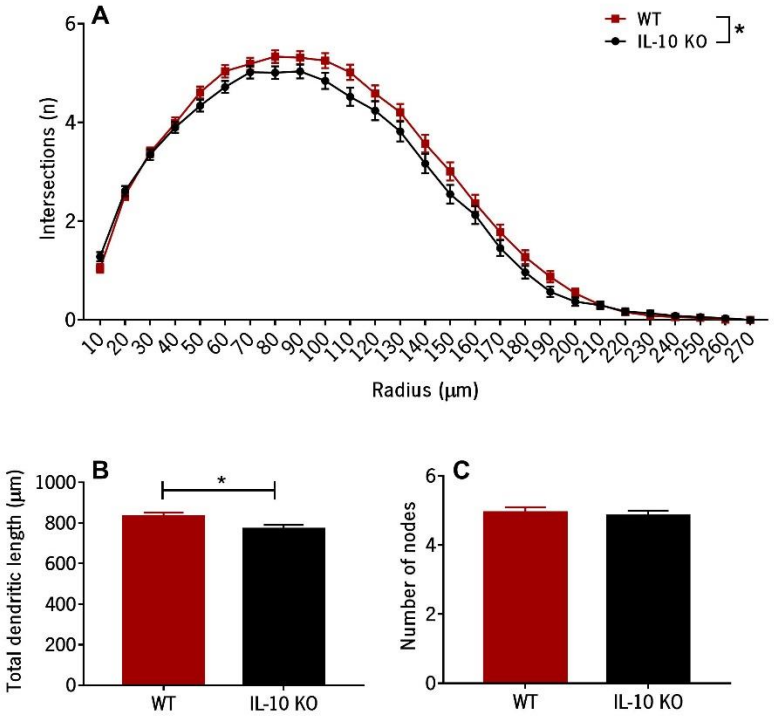

FIGURE 15. IL-10 KO animals present shorter dendrites when compared to WT littermates. Neurons morphology was assessed in WT (red) and IL-10 KO (black) male mice. Sholl analysis was performed to assess neuronal dendritic complexity by quantifying the number of intersections at specific distances from the soma **(A).** Data is represented as mean ± SEM. Statistical analysis was determined by repeated-measures analysis of variance (ANOVA). The total dendritic length **(B)** and number of nodes **(C)** were also used to assess neuronal complexity. Data is represented as mean ± SEM, *p<0.05. Statistical analysis was determined by two-tailed Student's t test. n=135 (WT); n=121 (IL-10 KO) values from two independent experiments combined.

4.3. Immune system characterization

To understand if the absence of IL-10 have an impact in mice immune profile we analyzed the immune cell populations present in blood, and in dcLN and ingLN by flow cytometry (gating strategy in ANNEX 1 and ANNEX 2).

Mice lacking IL-10, when compared with WT littermates, present lower percentages of B cells ($t_{(17,45)}$=2,227; p= 0,0394; d=0,0855), total T cells ($t_{(27)}$=2,327; p=0,0277; d=0,236), $CD4^+$ T cells ($t_{(27)}$=2,149; p=0,0408; d=0,0803), $CD8^+$ T cells ($t_{(27)}$=3,17; p=0,0037; d=2,565) and higher percentages of neutrophils ($t_{(14,36)}$=2,738; p=0,0157; d=0,3112) in the blood (**Figure 16A**). The remaining cell populations analyzed (monocytes ($t_{(27)}$=0,1401; p=0,8996; d=0,0519); NK ($t_{(27)}$=1,771; p=0,0879; d=0,6633) and NKT cells ($t_{(27)}$=0,1806; p=0,8580; d=0,0674)) do not present any differences between the two groups. No differences were found between the two groups in the total number of leukocytes ($t_{(27)}$=0,3902; p=0,6994; d=0,1467) (**Figure 16C**). Within the blood $CD4^+$ T cells, we analyzed the percentage of Naïve ($CD44^-CD62L^+$), Effector Memory (EM – $CD44^+CD62L^-$) and Central Memory (CM – $CD44^+CD62L^+$) T cells by the differential expression of the molecules CD44 and CD62L. Our results show that IL-10 KO mice, when compared with WT littermates, present higher percentage of EM $CD4^+$ T cells ($t_{(15,11)}$ =4,597; p= 0,0003; d=1,7323). Between the two groups no differences were observed in Naïve and CM $CD4^+$ T cells (**Figure 16B**).

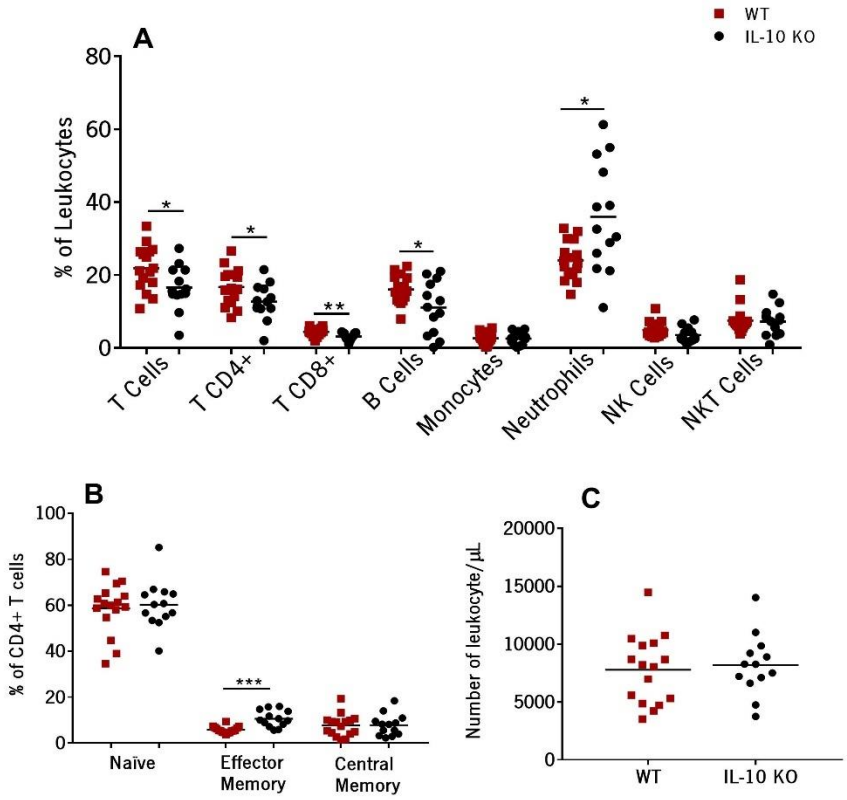

FIGURE 16. IL-10 KO animals present lower percentage of B, T and CD4+ T cells, and higher percentage of neutrophils and EM CD4+ T cells. Flow cytometry was performed in total blood of WT (red squares) and IL-10 KO (black dots) mice. The percentage of leucocyte populations **(A)** was used to characterize the mice immune profile. The percentage of distinct cell compartments of $CD4^+$ T cells (Naïve, EM and CM) was used to assess T cell activation **(B)**. The total number of leukocytes per µL was also calculated **(C)**. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean, *p<0.05; **p<0.01; ***p=0,0003. Statistical analysis was determined by two-tailed Student's t test.

We analyzed dcLN because they are responsible for the drainage of the brain. On the other hand, ingLN were used as an internal control as they play no role in this process.

We observed no differences between IL-10 KO and WT animals regarding number of total cells in both in deep cervical ($t_{(12,58)}$=1,669; p=0,1199; d=0,6526) and inguinal lymph nodes ($t_{(14,69)}$=0,8242; p=0,4230; d=0,3207) (**Figure 17A and B**). In the deep cervical lymph nodes, when comparing IL-10 KO and WT animals, we found no differences in any immune cell population analyzed within total leucocytes (B cells ($t_{(25,31)}$=1,035; p=0,3103; d=0,0713); T cells ($t_{(23,18)}$=0,597; p=0,5563; d=0,0466); $CD4^+$ T cells ($t_{(19,8)}$=0,2274; p=0,8225; d=0,0225) and $CD8^+$ T cells ($t_{(27)}$=0,9945; p=0,3288; d=0,0376)) (**Figure 17C**).

Regarding the different levels of activation of $CD4^+$ T cells, there was no differences between the two groups (Naïve - $t_{(27)}$=0,2663; p=0,7920; d=6,900); EM - ($t_{(27)}$=0,4393; p=0,6640; d=0,3065) and CM cells - ($t_{(27}$=1,288; p=0,2088; d=0,0751)(**Figure 17D**). In the inguinal lymph nodes, there are no differences in the cell populations within total leucocytes (B cells ($t_{(26,32)}$=0,942; p=0,3548; d=0,0345); T cells ($t_{(25,85)}$=0,9239; p=0,3641; d=0,0337); $CD4^+$ T cells ($t_{(21,86)}$=1,375; p=0,1829; d=0,0520) and $CD8^+$ T cells ($t_{(22,37)}$=0,1653; p=0,8702; d=0,0056), when comparing IL-10 KO and WT animals (**Figure 17F**). Between the two groups no differences were observed in Naïve ($t_{(17,81)}$=1,181; p=0,2530; d=0,0922) and CM cell ($t_{(27)}$=0,596; p=0,5561; d=0,0395) compartments of $CD4^+$ T cells (Figure 12G). IngLN from IL-10 KO animals presented higher percentage of EM $CD4^+$ T cells ($t_{(27)}$ =2,147; p=0,0410; d=1,9353) (**Figure 17G**).

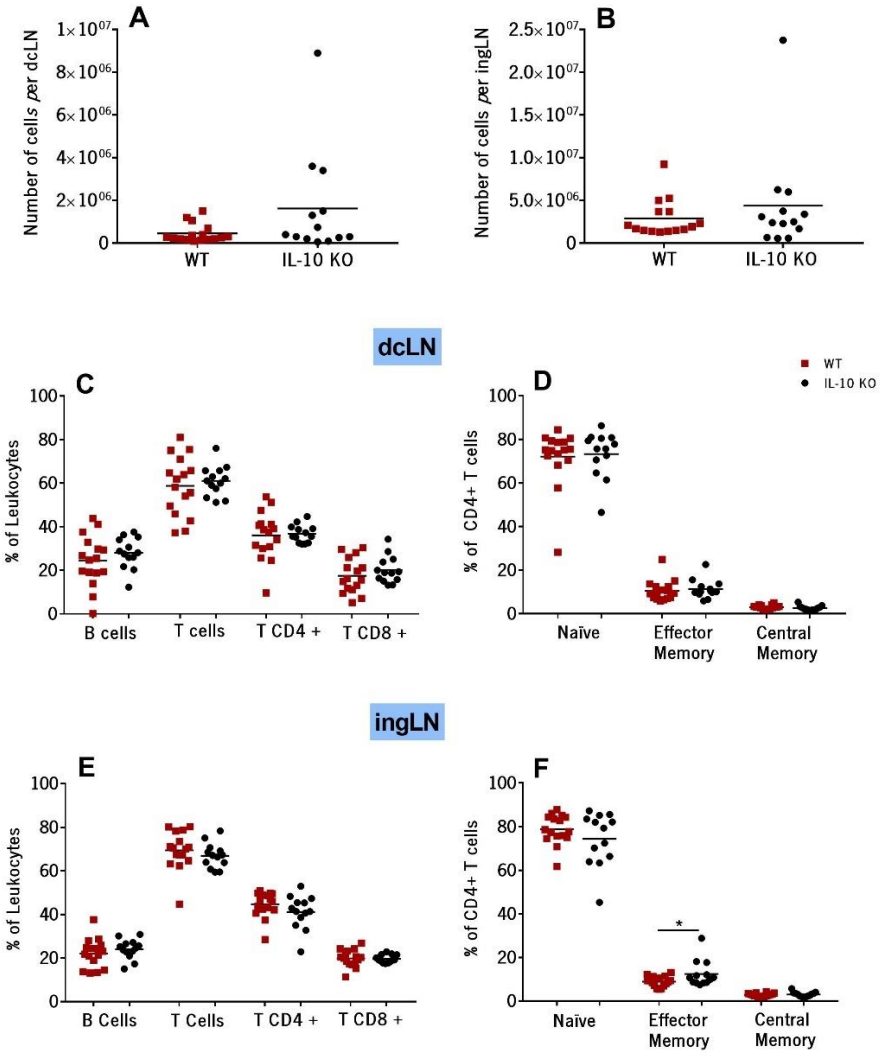

FIGURE 17. IL-10 KO animals present higher percentage of EM CD4+ T cells in inguinal lymph nodes. Flow cytometry was performed both in inguinal and deep cervical lymph nodes from WT (red squares) and IL-10 KO (black dots) mice. Total number of cells was assessed in lymph nodes **(A and B)**. The percentage of leucocyte populations **(C and E)** was used to characterize the lymph node's immune profile. The percentage of Naïve, EM and CM $CD4^+$ T cells was used to assess T cell activation profile **(D and F)**. Each point represents one animal. n=16 (WT); n=13 (IL-10 KO) values from two independent experiments combined. Data is represented as mean, *$p<0.05$, determined by two-tailed Student's t test.

In summary, our results show that the absence of IL-10 is not associated to locomotion problems and does not affect mood behavior nor causes impairment in short-term and associative memory. Socialization is also not affected by the absence of IL-10. Here we revealed that spatial learning and memory are impaired in IL-10 KO animals, thus implying the action of IL-10 in this cognitive dimension. However, is still necessary to uncover the mechanisms involved in this interaction.

On this line, we observed that the morphology of granular neurons from the DG was also different between both groups: IL-10 KO animals display shorter dendrites and less intersections along the dendrites. The number of nodes was not affected by the absence of IL-10. Finally, the immune profile of animals lacking IL-10 also presented differences when compared to the WT mice: higher percentages of neutrophils and lower percentages of B and T cells in the blood. Additionally, IL-10 KO animals also presented higher percentages of EM $CD4^+$ T cells both in blood and ingLN, although no differences were detected in the dcLN between groups.

5. Discussion

The interaction between the immune and central nervous systems has been raising interest for several years. Currently, several studies have demonstrated the role of the immune system namely, T cell phenotype and cytokine profile, in cognitive performance. In fact, unpublished results from our laboratory show that the absence of the anti-inflammatory cytokine IL-10 induces cognitive impairment in female mice. Moreover, it is known that female IL-10 KO mice also present depressive-like behavior, and that alterations in mood are sometimes associated with worst cognitive performance[64]. Since IL-10KO male mice present no alterations in depressive-like behavior, it is crucial to assess if alterations on cognitive performance induced by the lack of IL-10 are also gender dependent. Here we demonstrate that IL-10KO male mice display a preference for more random and serial strategies to find the target hole during the Barnes maze test, while WT animals use more direct strategies. This suggests that the absence of IL-10 is associated with worst cognitive performance in a spatial learning and memory task. We also show that IL-10KO male mice do not have alterations in short-term memory, reference memory or associative memory. Social behavior is also not affected in IL-10KO male mice comparing with WT littermate controls. Moreover, we observed that no locomotor impairment, anxious or depressive-like behaviors are present in IL-10KO male mice as it was previously described[52].

Taken into account the cognitive differences observed in spatial learning and memory of IL-10KO male mice, we addressed possible morphological alterations of the hippocampus structures. For this we performed morphological analysis of the neurons in the dentate gyrus (DG), a structure of the hippocampus important for memory acquisition, more specifically in tasks that require spatial orientation[65]. No differences were found in number of nodes in hippocampal neurons of animals lacking IL-10 when compared to WT animals. However, in IL-10 KO mice the total dendritic length and number of intersections are significantly lower than in WT mice.

Since the interplay between the immune profile and cognitive performance is known, and as IL-10 is a key molecule to maintain immune system homeostasis[45], we assessed if its absence is associated with alterations in the immune profile. Our results show a higher percentage of neutrophils and EM $CD4^+$T cells and lower percentages of B cells in the blood of IL-10 KO animals. Moreover, while the ingLN of IL-10 KO animals exhibited higher percentages of EM $CD4^+$T cells, no differences were found in the immune cell populations of the dcLN.

5.1. IL-10, behavior and cognition

To explore and understand the role of IL-10 in male mice behavior, we performed well-established behavioral tests that allows to evaluate distinct dimensions.

The OF test evaluates if animals have locomotor defects, that may interfere with the other tests result, and exploratory activity. The results obtained indicate that the absence of IL-10 does not influence locomotor activity nor exploratory behavior in male mice. Similarly, Mesquita and colleagues have already shown that, when compared to WT animals IL-10 KO male mice, present no differences in the total distance traveled and, in the number, and duration of "rearings"[52].

Anxious-like behavior was evaluated with EPM and by the percentage of the distance traveled on the center of the OF arena. EPM is a test used in rodents to evaluate anxiety and is based on their general aversion to open spaces. Thus, less time spent in the open arms of the apparatus, translates into higher anxious-like behavior[66]. In the OF the preference to travel close to the arena walls and away from the center can also be used as an indicative of anxious-like behavior[53]. Our results, from both EPM and OF, show that the absence of IL-10 does not influence anxious-like behavior. The impact that the absence of IL-10 has in anxious-like behavior has been previously addressed. Mesquita and colleagues demonstrated that no differences were found between IL-10 KO and WT mice when performing EPM. However, contrarily to what we observed, their results showed that, when compared to WT counterparts, IL-10 KO mice spent less time in the center of the OF arena. Being EPM the gold standard protocol used in anxiety assessment, our results strengthened the observation that the absence of IL-10 has no effect in anxious-like behavior. Discrepancies such as those mentioned above are likely to happen due to the difference in the provenience of the animals. Contrary to what occurs in the previous cited study[52], in our work we used WT littermates and this assures that the genetic background is the same between experimental and control groups[67]. This way we know that the differences between IL-10KO and WT mice observed in this work result from mechanisms triggered by the absence of IL-10 and not because of alterations in the genetic background.

The impact that the absence of IL-10 has in the ability to deal with learned helplessness was explored using TST and FST. In these tests, higher immobilization time is considered a measure of depressive-like behavior. Overall, our results from both TST and FST show that IL-10 KO male mice do not present depressive-like behavior. These results are in accordance with the literature, where no significant differences were observed between IL-10 KO and WT male animals in the immobilization time of FST[52]. In the same study it was also revealed that, unlike males, females are affected by the absence of IL-10 regarding depression-like behavior, revealing higher immobility time in the FST, which is in

agreement with the fact that women are more susceptible to develop depression[68]. These results all together indicate that IL-10 influences depressive-like behavior in a gender dependent way.

The impact that the lack of IL-10 has in short-term memory was assessed using NOR were we evaluated the animal's ability to remember an object and discriminate between the familiar and novel one. Our results indicate that the absence of IL-10 does not affect short-time memory. To our knowledge, the role of IL-10 in short-term memory was never addressed. This way, these results are the first step into a better understanding of the importance this cytokine has in cognition.

Y maze test was performed to evaluate if the lack of IL-10 affects spatial reference memory. In this test we used different reference visual cues to evaluate the ability animals have to remember places previously visited. We observed that even WT mice (our control group) spent a similar percentage of time exploring the familiar and the novel arm. This result suggests that the animals showed no memory of the familiar arm, so we could not confirm the efficacy of the Y-maze test.

The question of whether Y-Maze is effective to evaluate spatial reference memory in BALB/c strain has been addressed before, but the results obtained differ between studies. For instance, one study that compared several mouse strains in Y-maze performance stated that BALB/c is one of the strains that spent more time in the novel arm[69], while another study using the same protocol compared BALB/c and C57BL/6 strains and concluded that Y-Maze task is ineffective for BALB/c strain[70]. As an alternative to assess spatial reference memory we could use Radial Arm Maze. Is a different approach that alongside with reference memory also allows the evaluation of working memory[71].

We performed CFC to study associative memory in animals not expressing IL-10. The low levels of freezing behavior, observed prior to the conditioning shock, were expected because the context was a novel environment without anxiogenic stimulus that mice could freely explore. Both groups of mice presented similar freezing patterns at this stage. After conditioning both groups of mice learned the association between context and shock (contextual fear conditioning) and, at the cue probe day, learned the association between sound and shock (cued fear conditioning) despite being in a different context. These results show that the absence of IL-10 has no effect in either contextual fear conditioning nor in cued fear conditioning. It has been demonstrated that lesions in the amygdala reduce freezing behavior to both context and auditory stimuli[72], while lesions in the dorsal hippocampus impair the freezing response to contextual stimuli[73]. Taking this into consideration, our results also suggest that in the amygdala and dorsal hippocampus, the circuits necessary to fear memory recall, may not be affected by the lack of IL-10.

The BM test assesses spatial learning and memory by analyzing the ability mice have to learn and remember the location of a target hole using visual cues located around the testing arena. On the *probe day* both groups exhibited a similar "poking" behavior, even though there is a tendency in WT animals to poke more in the target hole, the difference did not reach significance. Of interest is that IL-10 KO animals present different strategy choices to perform the task when comparing to WT littermates. While IL-10 KO animals prefer more random and/or serial strategies, WT animals used more direct strategies to reach the target hole. This means that IL-10KO mice are not able to learn to use the spatial clues to resolve the task as well as WT littermates, indicating that the absence of IL-10 affects negatively spatial learning and memory. Similar results were also obtained previously in our lab using female IL-10 KO mice (unpublish data). As a way to assess if IL-10 has any impact in cognitive flexibility, it would be interesting to perform a reversal trial 24h after the probe trial. It would allow us to study if the animals adapt to the new situation and change the searching strategy.

Socialization plays a very important role in the progress of every specie and it has been previously demonstrated that the immune system influences social behavior. For example, it has been shown that T cells modulate social behavior[43]. Furthermore, animals lacking IFN-γ also demonstrated social deficits when compared to WT counterparts[43,44]. To further explore the role that IL-10 have in social behavior, we used the three chambers protocol and assessed social memory and the interest in social novelty.

The first protocol used in this work to assess socialization was adapted from a study by Van Heukelum and colleagues, were they used the premise that BALB/c strain is more sensitive to stress and display high levels of anxiety, to introduce an altered version of the 3 chambers protocol[60]. We performed 2 separated pilot tests, were we optimized the test arena, light conditions, habituation periods and acquisition periods. With the results obtained we were not able to attest the effectiveness of the protocol to evaluate social affiliation –WT and IL-10 KO mice spent similar time exploring both *Stranger 1* and the *Object*. Socialization however was not impaired, because in the second trial WT and IL-10 KO mice interacted with the novel stimulus mouse. One possible explanation can be that the arena is not the most adequate for our model. Contrary to what was described in the above mentioned study[60], the use of a one-chambered arena, with no dividing walls, could be causing anxiety to the animals as they were given no option to withdraw from interaction. Another valid explanation is supported by the time the animals spent on top of the perforated cups (data not shown), without interacting with animals kept underneath them.

For the second set of animals, based on the information obtained from the first one, we optimized the protocol to assess social behavior based on a well described protocol used for several mouse strains[59]. Some studies showed that social interactions are more common during the dark phase[74], whereas other

authors claimed that social behavior protocols have similar results when conducted either during light or dark phase[75]. With all this in mind, we compared the social behavior of IL-10 KO and WT mice at nighttime and using a three-chamber arena (second set). Our results showed that IL-10 KO and WT mice chose to spend more time exploring the subject mouse (*Stranger 1*) over the empty cup object. We also observed that both groups of animals have an intact social memory and a preference for novel social experiences, as they displayed similar preference for the novel subject mouse over the previously encountered one. All together these results indicate that the absence of IL-10 has no effect on social interaction and socially motivated behaviors.

Contrary to our findings, some studies described a link between socialization and IL-10. Adult rats that were kept in isolation since weaning, exhibited lower IL-10 plasma concentrations and decreased concentration of IL-10 in the hippocampus[76]. Post-weaning isolation revealed to be a stressful event that also caused aberrant behavior in adulthood (hyperlocomotion in the OF). Divergences between our findings and the previously mentioned work can be easily explained if we take in consideration that our mouse model is not a stress model and was not subjected to stress protocols that could induce either of the alterations mentioned for the post-weaning isolated rats. There are also studies with humans correlating the presence of IL-10 with lower levels of social impairment (which is translated in lower scores in the Autism Diagnostic Interview-Revised) in humans suffering from DiGeorge syndrome (autism-spectrum disorder characterized by social impairment)[77]. To note, these results also revealed that autism-related behavior is associated with a skew in the inflammatory profile, with the increase in the concentration of the cytokines IL-12, IL-6, IL-1β, and INF-γ.

5.2. IL-10 and hippocampal neuron morphology

The hippocampus is a brain region crucial for learning and memory formation in humans and rodents[78]. Here we performed the morphological analysis in the DG region of the hippocampus in both IL-10 KO and WT animals, and assessed the length of the dendritic tree, the number of nodes in each neuron and, using Sholl analysis, we were able to quantify the branching (number of intersections along each dendrite). Our results revealed no differences in the number of nodes between both genotypes. However, the absence of IL-10 have an impact in total dendritic length and suggests also a decreased neuronal complexity in the hippocampal DG. These results can help us understand the differences obtained in the BM test, where IL-10 KO and WT mice clearly choose distinct strategies to perform the task. It was previously described that, in the hippocampus, the dorsal area is responsible for cognitive functions like learning and memory activities, whereas the ventral area is more related with anxiety,

emotional regulation and affect[79]. Because IL-10 KO animals exhibit difficulties performing spatial memory and learning tasks our results suggest that in the dorsal region of the hippocampus the circuits responsible for spatial memory are affected by the lack of IL-10. To further substantiate our findings, we can discriminate between neurons from dorsal and ventral areas of the hippocampus and compare again all the parameters that were analyzed.

In the hippocampal circuit, along with the granular cells from DG there are two more cell groups responsible for the neural circuit, the pyramidal cells of the cornu ammonis (CA) 3 and the pyramidal neurons in CA1[80]. It has been described that CA3 region of dorsal hippocampus is essential for learning and memory consolidation when performing spatial tasks[81], and that CA1 region besides being the primary output of the hippocampus is also important for spatial memory[82]. To study more extensively the role of IL-10 in the hippocampal circuit we can, in the future, perform morphological analysis in the CA1 and CA3 regions.

5.3. IL-10 and immune profile alterations

When we compared leukocyte populations (lymphocytes, monocytes and neutrophils) and lymphocyte subpopulations (B cells, T cells and $CD4^+$ and $CD8^+$ T cells) of the IL-10 KO and WT animals interesting differences were observed. When compared to their WT littermates, IL-10 KO animals have a higher percentage of neutrophils in the blood. It has been described that IL-10 KO animals present a proinflammatory profile in the gut (overproduction of TNF-α, IL-1, or IFN-γ) turning them more predisposed to develop chronic enterocolitis[83] and colorectal adenocarcinoma[84]. Being one of the first defenders of the innate immune system, the natural course of action is the migration of neutrophils towards the inflammation site (gut). This means that neutrophils number increases in circulation, as they are recruited from the blood stream to work as mediators of inflammation and maintain intestinal homeostasis during the early stages of IBD[85]. This could explain the higher percentage of neutrophils we found in the blood of IL-10 KO mice.

Curiously, the IL-10 KO mice from our study did not present any major symptoms associated with chronic enterocolitis like weight loss or diarrhea and, the macroscopic observation of the gut revealed no inflammation. Because the onset of symptoms can be preceded by alterations in the gut, we consider it would be important to study inflammatory markers in the gut tissue.

The absence of IL-10 has been described to cause changes in the gut microbiome[46], and several studies demonstrated the importance of microbiome in depressive-like behavior[86], cognitive performance [87,88] and also in the immune system[89].Considering that our mice were housed together, in cages with 5-6

animals, and not separated by genotype, there is a possibility of microbial exchange between IL-10 KO, WT and heterozygous mice due to coprophagy. This exchange could be leading to the homogenization of the microbiome between the different genotypes thus assisting in the maintenance of a homeostatic immunity in IL-10 KO mice, and ultimately modeling the onset of inflammation. Conversely, during the development of our experiments a few IL-10 KO mice were euthanized due to the presence of rectal prolapses that are considered an external sign of IBD[90]. The microscopic analysis of the colon and cecum of mice used in this work could give some insights about the existence and/or stage of gut inflammation.

The higher percentage of neutrophils in IL-10 KO mice can also help us to understand the lower percentage of B cells observed in the blood of these animals in comparison to the WT counterparts. It is described that during an infection or inflammatory process, where there is high demand for neutrophils, the bone marrow hematopoietic response changes into a process named "emergency granulopoiesis". This process is characterized by an increase in the proliferation of myeloid progenitors which leads to *de novo* production of neutrophils[91]. The boost in neutrophils production has as consequence the decrease in the production of B cells. Within the bone marrow, B lymphopoiesis and granulopoiesis "compete" for space and growth factors and, upon inflammation, B cell production is disfavored and the B content reduced, leading to reactive granulopoiesis[92].

Aside from their crucial role in adaptive immunity[93], B cells have also been associated with cognition and described as one of the predictors for cognitive impairment[31]. In humans, lower counts of B cells, alongside with higher counts of EM $CD4^+$ T cells were described as prognosis for worst cognitive performances[31]. In our results the lower percentages of B cells might similarly be associated with the impairment IL-10 KO animals exhibit when performing spatial learning and memory tasks. This work also revealed that IL-10 KO animals have lower percentage of T cells and lower percentage of $CD4^+$ and $CD8^+$ T cells when compared to the WT counterparts. In IL-10 KO animals, the lower percentage of $CD4^+$ and $CD8^+$ T cells can be a direct result of the lower percentage of T cells within the total leukocyte population. Calculating the percentage of $CD4^+$ and $CD8^+$ T cells within total T cells and not in total leukocytes, would help to test this possibility.

Some studies showed that the absence of T cells has a negative impact in cognitive performance, mostly in tasks related with spatial learning and memory[27,28]. Even though mice from our study were not completely devoid of T cells, the fact that IL-10KO mice present lower percentage of T cells may be associated with its worst performance in spatial memory tasks when comparing to WT littermates.

Within the T cells compartment, IL-10 KO mice present higher percentages of EM $CD4^+$ T cells, which can be related to the cognitive impairments exhibited in spatial learning and memory tests. In fact,

higher counts of EM $CD4^+$ T cells were previously considered predictors for poor cognitive performance in humans[31].

Both T cells and EM $CD4^+$ T cells were previously described as good predictors for cognitive performance. As we have alterations in the percentage cells from both populations, it would be of interest to explore if there is a correlation between behavior dimensions addressed in this work and the alterations in immune cell populations. As it was previously mentioned, the effect that the lack of IL-10 has in the inflammatory profile of IL-10 KO mice was already addressed. The study of two of the most relevant pro-inflammatory cytokines, INF-γ and TNF-α, revealed no differences in the serum levels between WT and IL-10 KO mice[52]. Nevertheless, it would be important to determine the serum levels of other pro-inflammatory cytokines involved in the inflammatory response and that have been described as having relevance for cognition such as IL-4, IL-6, IL-8, IL-13 but also IL-1β, IL-12, , IL-17 and, IL-23.

The CNS has its own resident immune cells that are named microglia and participate in innate and adaptive immune responses. Their actions include phagocytosis, cytotoxicity, release of cytokines and chemokines to initiate neuroinflammation, and the regulation of T cell responses through antigen presentation[94]. Although contributing to the homeostasis of the brain, chronic activation of microglia may cause neurodegenerative disease through the release of cytotoxic molecules such as proinflammatory cytokines[95]. The analysis of microglia by flow cytometry could allow the identification of the state of activation (steady state or activated) and the identification of inflammatory molecules involved in the communication between microglia and peripheral immune cells.

Given the known impact of cerebral drainage to the dcLN have on cognitive performance, we analyzed the immune cells of both dc and ingLN in IL-10 KO and WT mice. Results revealed no alterations in the percentage of different cell populations (B cells, T cells and in $CD4^+$ and $CD8^+$ T cells) within total leukocytes both in ing and dcLN. However, although no differences were found between the two groups of mice in the dcLN $CD4^+$ T cell compartment (Naïve, EM and CM T cells), in ingLN, IL-10 KO animals presented higher percentage of EM $CD4^+$ T cells when comparing with WT littermates. No differences were observed in Naïve and CM cell compartments of $CD4^+$ T cells, between the two groups of animals both in ing and dcLN.

Together with the higher percentage of neutrophil, and lower percentage of T cells, $CD4^+$, $CD8^+$ and B cells in the blood, the higher percentage of EM $CD4^+$ T cells in the ingLN of the IL-10 KO mice suggests that the absence of IL-10 is altering the immune phenotype in the periphery. Even though we did not observe differences in the dcLN between groups, does not imply an absence of immune alterations in the CNS of IL-10 KO mice. There might be alterations in the CNS immune profile that are not reflected

in the LN responsible for the brain drainage. One way to test this possibility would be to analyze the immune cells and cytokines present in the meninges of IL-10 KO mice.

The work from this thesis contributed to increase the knowledge about the role the immune system plays in maintaining cognitive function. Our data revealed that IL-10, one of the most important anti-inflammatory cytokines, is involved in the mechanism of learning and memory in spatial memory tasks. We also show that, in agreement with what was previously described in humans, animals with worst cognitive performance present increased percentage of EM $CD4^+$ T cells and decreased percentage of B cells in the blood. This result suggests that IL-10 is playing a role in the peripheral immune profile. Further analysis is necessary to understand the mechanisms by which IL-10 interfere with the spatial memory and learning, which CNS cells could be affected by IL-10 action and which other immune alterations may be involved in cognitive performance.

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ANNEX 1.Gating strategies for flow cytometry analysis of peripheral blood

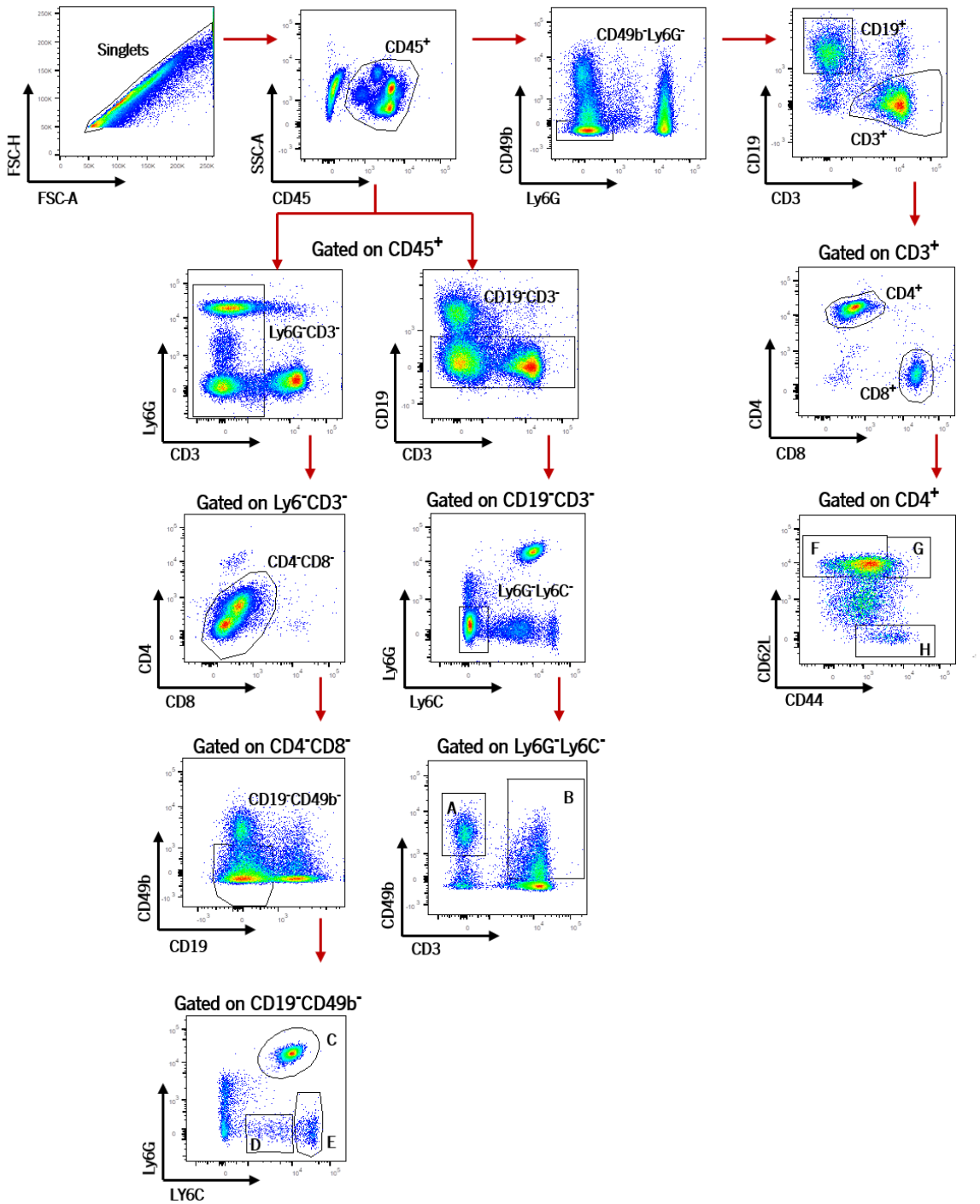

FIGURE 1: Schematic representation of the gating strategy used to analyze peripheral blood. Single cells (singlets) were selected on total events, based on the fact that the size (FSC-A) and height (FSC-H) of a single cell are proportional. Total leukocytes were gated within singlets, based on CD45 expression and debris (very low complexity (SSC-A)) were excluded.

Within $CD45^+$ gate, after excluding $CD19^+$ $CD3^+$ cells, we further selected $Ly6C^-$ $Ly6G^-$ population (within $CD19^-$ $CD3^-$ population) were we identified NK (**A**: $CD49b^+$) and NKT (**B**: $CD3^+$) cells. Additionally, within $CD45^+$ gate, we selected $Ly6G^-CD3^-$ cells, and inside this population we further selected $CD4^-CD8^-$ population. To identify granulocytes, we selected $Ly6G^+Ly6C^+$ populations within $CD19^-CD49b^-$. We identified the following populations: neutrophils (**C**: $Ly6G^+Ly6C^{low}$), macrophages (**D**: $Ly6G^-Ly6C^{low}$) and monocytes (**E**: $Ly6G^-Ly6C^{high}$). B cells were defined as $CD19^+CD3^-$ and T cells were defined as $CD19^-CD3^+$ (within $CD49b^-$ $Ly6G^-$ population). After selecting live $CD3^+$ cells, $CD4^+$ and CD8 T^+ cells were also selected for further analysis. Within $CD4^+$ T cells we identified the following populations: Naïve (**F**: $CD62L^+CD44^-$), Central memory (**G**: $CD62L^{high}CD44^{high}$) and Effector memory (**H**: $CD62L^-CD44^+$).

ANNEX 2. Gating strategies for flow cytometry analysis of lymph nodes

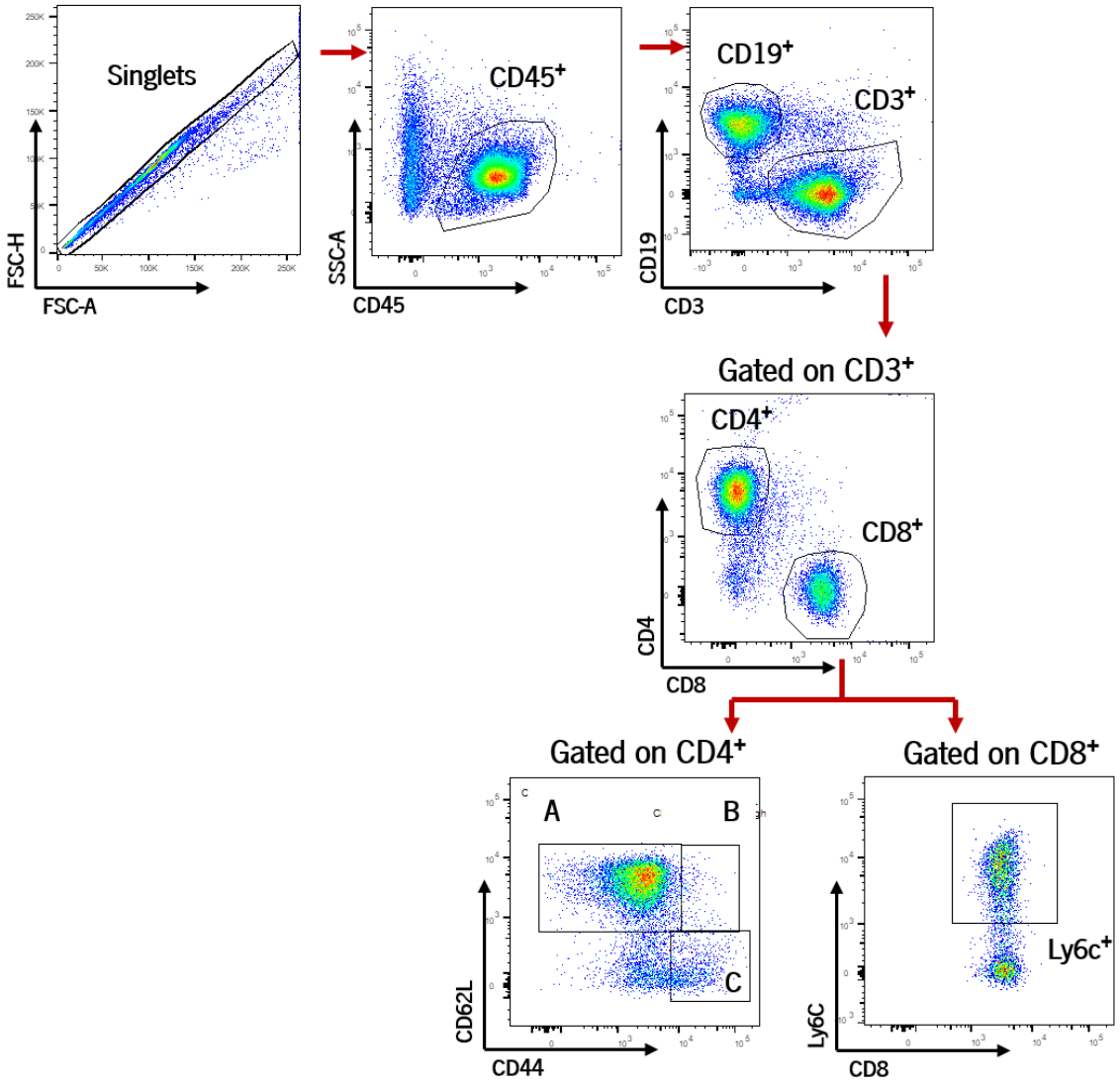

FIGURE 2: Schematic representation of the gating strategy used to analyze Lymph nodes. Single cells (singlets) were selected on total events, based on the fact that the size (FSC-A) and height (FSC-H) of a single cell are proportional. Total leukocytes were gated within singlets, based on CD45 expression and debris (very low complexity (SSC-A)) were excluded.

Within $CD45^+$ gate, after selecting $CD19^+$ $CD3^+$ cells, we identified B cells defined as $CD19^+CD3^-$ and T cells as $CD19^-CD3^+$. After selecting $CD3^+$ cells, $CD4^+$ and CD8 T^+ cells were also selected for further analysis. Within $CD4^+$ T cells we identified the following populations: Naïve (**F**: $CD62L^+CD44^-$), Central memory (**G**: $CD62L^{high}CD44^{high}$) and Effector memory (**H**: $CD62L^-CD44^+$).

ANNEX 3. Approval to perform animal experimentation from the local ethics committee

Órgão Responsável pelo Bem-Estar Animal da EM/ICVS e do I3Bs

Referência do processo: ORBEA EM/ICVS-I3Bs_012/2018

Título do Projeto: O papel do sistema imunitário em transtornos do humor e na cognição Investigador

Principal: Margarida Correia-Neves

Estabelecimento: Unidade do Biotério do Instituto de Investigação em Ciências da Vida e da Saúde (ICVS) da Escola de Medicina da Universidade do Minho

DECLARAÇÃO DE ACEITAÇÃO DO PROJETO

O Órgão Responsável pelo Bem-Estar Animal da Escola de Medicina e seu Instituto de Investigação em Ciências da Vida e Saúde, e do Instituto3Bs (Biomaterials, Biodegradables and Biomimetics) - ORBEA EM/ICVS-I3Bs- declaraterrecebidooprojetodeinvestigação, comrecursoamodelosanimais,intitulado *Opapeldosistemaimunitárioemtranstornosdohumorenacognição*, para análise.

Braga,

MAGDA JOÃO CASTELHANO CARLOS

Assinado de forma digital por MAGDA JOÃO CASTELHANO CARLOS
Dados: 2018.12.19 19:18:26 Z

(Presidente do ORBEA EM/ICVS-I3Bs)