**Universidade do Minho**
Escola de Medicina

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# The crosstalk between immune mediators and cognition

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# The crosstalk between immune mediators and cognition

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Trabalho efetuado sob a orientação da
**Professora Doutora Joana Almeida Palha**
e da
**Professora Doutora Margarida Correia Neves**

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## DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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

**TÍTULO:** A COMUNICAÇÃO ENTRE MEDIADORES IMUNITÁRIOS E COGNIÇÃO

## RESUMO

O envelhecimento é um processo complexo que envolve acumulação progressiva de danos celulares em diversos sistemas de órgãos, influenciando a sua função. Este processo decorre de forma variável, como se pode constatar pelo efeito da idade na detioração da função cognitiva de diferentes indivíduos. Compreender as diversas trajetórias de envelhecimento e as variáveis associadas torna-se crucial para se poder projetar estratégias que permitam melhorar a qualidade de vida ao longo do envelhecimento. Sabendo que o cérebro requer constante vigilância imunitária, e que o envelhecimento também está associado a alterações no sistema imunitário, propusemos dissecar a relação entre as alterações no sistema imunitário associadas à idade e a cognição. Para tal avaliámos, transversalmente, uma coorte de indivíduos seniores saudáveis e fizemos uma análise longitudinal em murganhos.

Analisando os mesmos animas desde os 3 aos 18 meses de idade, caracterizámos a progressão da frequência relativa das principais células do sangue, tanto do sistema imunitário adaptativo como do inato. Verificámos que o envelhecimento é acompanhado por um aumento da frequência de células do sistema imunitário inato e diminuição das do adquirido, num efeito que é moderado pelo sexo. Também explorámos o papel das alterações do sistema imunitário associadas à idade na heterogeneidade cognitiva. Através de uma caracterização comportamental multi-paramétrica, dividimos os animais velhos em 2 grupos, referentes a bom e mau desempenho cognitivo. Observámos que machos com desempenhos cognitivos distintos apresentam diferenças moderadas na progressão de algumas células imunitárias ao longo do envelhecimento. Adicionalmente, observámos que a percentagem de células T $CD4^+$ e $CD8^+$ e as células NK $Ly6C^-$ aos 18 meses também está associada ao desempenho cognitivo de murganhos, mesmo controlando para o sexo.

Dada a ligação já conhecida entre os componentes solúveis do sangue e o envelhecimento cognitivo, quantificámos 30 moléculas relacionadas com o sistema imunitário em indivíduos seniores saudáveis. Não só confirmámos associações entre moléculas imunitárias periféricas e heterogeneidade cognitiva já previamente descritas, como a IL-6; como fortalecemos outras associações, como a IL-1β, IL-8, IP-10 (CXCL10) e o TNF; e revelámos novos mediadores, como a IL-13.

Em suma, os presentes estudos enriquecem o conhecimento sobre as alterações do sistema imunitário periférico durante o envelhecimento e a sua associação com a cognição. Os resultados apresentados corroboram uma interação próxima entre o sistema imunitário e a cognição que merece uma investigação mais detalhada.

**Palavras-chave:** cognição, envelhecimento, mediadores imunitários, sistema imunitário.

**TITLE:** THE CROSSTALK BETWEEN IMMUNE MEDIATORS AND COGNITION


## ABSTRACT

Aging is a complex phenomenon that encompasses a progressive accumulation of cellular damage impacting in multiple systems and affecting individuals differently. For instance, the cognitive function, which commonly deteriorates with age, presents inter-individual variability. Understanding such diverse trajectories in aging, and the associated variables, is crucial to design strategies to overcome the natural effect of aging and improve quality of life. Knowing that throughout aging the brain requires constant immune surveillance to maintain its normal functioning and that aging is also associated with alterations in the immune system, we proposed to further dissect the relation between age-associated alterations in the immune system and cognition. For that purpose, we took advantage of a cohort of healthy senior individuals using a cross-sectional approach, and launch a longitudinal evaluation of mice.

By following the same mice from 3 to 18 months of age we characterized the progression of the relative frequency of the main blood immune cells, both from adaptive and innate immune system. We observed that aging promotes an imbalance towards the innate immune system and that sex moderates the relation between the frequency of cells and age. We also explored the role of age-associated alterations of the immune system as a potential contributor for cognitive heterogeneity. By performing a multiparametric behavioral characterization we segregated old mice as good and poor cognitive performers. We found that males with distinct cognitive performances at old age present moderate differences on specific immune cells progression over time. The percentage of $CD4^+$ and $CD8^+$ T cells among T cells and of NK $Ly6C^-$ cells, is also associated with mice cognitive performance, even controlling for sex at 18 months.

Given the known link between the soluble components of the blood and cognitive aging, 30 immune-related molecules were quantified in healthy senior individuals. We not only confirmed already well described associations between peripheral immune molecules and cognitive heterogeneity, such as IL-6, but also strengthen others, like IL-1β, IL-8, IP-10 (CXCL10) and TNF, and revealed new players, such as IL-13.

Altogether, the present studies enrich the knowledge on the peripheral immune system changes with aging and their association with cognition. These findings are supportive of a close interplay that deserve to be further investigated.



**Keywords:** aging, cognition, immune mediators, immune system.

## TABLE OF CONTENTS

# ABBREVIATIONS LIST

**Units/Greek letters**

| | |
|---|---|
| α | Alpha |
| B | Beta |
| γ | Gamma |
| δ | Delta |
| s | Second |
| min | Minute |
| h | Hour |
| cm | Centimeter |
| $m^2$ | Square Meter |
| g | Gravitational Force |
| °C | Degree Celsius |
| % | Percentage |
| µg | Microgram |
| mg | Milligram |
| kg | Kilogram |
| mM | Millimolar |
| M | Molar |
| mA | Milliampere |
| dB | Decibel |

**A**

| | |
|---|---|
| ACK | Ammonium–Chloride–Potassium Buffer |
| AD | Alzheimer's Disease |
| ANOVA | Analysis Of Variance |
| APC | Antigen Presenting Cell |

**B**

| | |
|---|---|
| B2M | Beta2-Microglobulin |
| BBB | Blood-Brain Barrier |
| BEC | Brain Endothelial Cell |
| BSA | Bovine Serum Albumin |

**C**

| | |
|---|---|
| CCL | Chemokine C-C Motif Ligand |
| CCL11 | Chemokine C-C Motif Ligand 11/Eotaxin 1 |
| CD | Cluster of Differentiation |
| cDNA | Complementary Deoxyribonucleic Acid |
| CFC | Contextual-Fear Conditioning |
| CI | Confidence Interval |
| CM | Central Memory |
| CMV | Cytomegalovirus |
| CNS | Central Nervous Systems |
| COWAT | Controlled Oral Word Association Test |
| CP | Choroid Plexus |
| CRP | C-Reactive Protein |
| CSF | Cerebrospinal Fluid |
| CSF2 | Colony-Stimulating Factor 2 |

| | |
|---|---|
| CTLS | Consistent Long-Term Retrieval |
| CV | Coefficient Of Variation |
| CXCL | C-X-C Motif Chemokine |

**D**

| | |
|---|---|
| DALYs | Disability-Adjusted Life Years |
| DAMP | Damage-Associated Molecular Patter |
| DC | Dendritic Cells |
| dCLN | Deep Cervical Lymph Node |
| df | Degrees Of Freedom |
| DGAV | Direção Geral De Alimentação E Veterinária |
| DNA | Deoxyribonucleic Acid |
| DSST | Digit Symbol Substitution Test |

**E**

| | |
|---|---|
| EM | Effector Memory |
| EPM | Elevated Plus Maze |

**G**

| | |
|---|---|
| G-CSF | Granulocyte Colony-Stimulating Factor |
| GDF11 | Growth Differentiation Factor 11 |
| GDS | Geriatric Depression Scale |
| GENEXEC | General And Executive Function Dimension |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |

**H**

| | |
|---|---|
| hsCRP | High-Sensitivity C-Reactive Protein |

**I**

| | |
|---|---|
| i.p. | Intraperitoneally |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILC2 | Thype 2 Innate Lymphoid Cell |
| IP-10 | Interferon-$\gamma$-Induced Protein-10 |
| ISF | Interstitial Fluid |

**L**

| | |
|---|---|
| LLOQ | Lower Limit Of Quantification |
| LTS | Long-Term Storage |

**M**

| | |
|---|---|
| MCP | Monocyte Chemoattractant Protein |
| MEM | Memory Dimension |
| MHC | Major Histocompatibility Complex |
| MIP | Macrophage Inflammatory Protein |
| MMSE | Mini-Mental State Examination |
| Moca | Montreal Cognitive Assessment |
| MWM | Morris Water Maze |

**N**

| | |
|---|---|
| N | Sample Size |
| n.s. | Non-Significant |
| NK | Natural Killer |
| NKT | Natural Killer T |
| NLR | Novel Location Recognition |
| NOR | Novel Object Recognition |

**O**

| | |
|---|---|
| OF | Open Field |

**P**

| | |
|---|---|
| PAMP | Pathogen-Associated Molecular Patter |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate Buffered Saline |
| PCA | Principal Component Analysis |
| PRR | Pattern Recognition Receptor |

**Q**

| | |
|---|---|
| qRT-PCR | Quantitative Real-Time Polymerase Chain Reaction |

**R**

| | |
|---|---|
| RA | Receptor Antagonist |
| RNA | Ribonucleic Acid |
| RT | Room Temperature |

**S**

| | |
|---|---|
| SAA | Serum Amyloid A |
| sCD40L | Soluble CD40 Ligand |
| SCID | Severe Combined Immune Deficiency |
| SD | Standard Deviation |
| SE | Standard Error |
| SRT | Selective Reminding Test |
| sTNF-R | Soluble Tumor Necrosis Factor Receptor |

**T**

| | |
|---|---|
| Tc | T Cytotoxic |
| TCR | T-Cell Receptor |
| TD | Terminally (Or Late) Differentiated |
| TGF | Transforming Growth Factor |
| Th | T Helper |
| TIMP2 | Tissue Inhibitor Of Metalloproteinase 2 |
| TLR | Toll-Like Receptors |
| TNF | Tumor Necrosis Factor |
| TREC | T-Cell Receptor Excision Circle |
| TST | Tail Suspension Test |

**V**

| | |
|---|---|
| VCAM1 | Vascular Cell Adhesion Molecule 1 |

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## CHAPTER I. INTRODUCTION



## CHAPTER II. LONGITUDINAL EVALUATION OF PERIPHERAL IMMUNE SYSTEM IN MICE: THE MODERATION EFFECT OF SEX

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## THESIS LAYOUT

This thesis is organized in 5 chapters, the first one dedicated to a general introduction, 3 chapters where the results obtained are presented and discussed and a fifth chapter addressing a general discussion, specialty:

**Chapter I** presents a general introduction on the impact of aging both in the cognitive function and in the immune system. It provides an overview of the crosstalk between immune mediators and functions of the central nervous system, with a particular emphasis on cognition. Thesis aims will be also presented in this chapter.

**Chapter II** displays the results on the characterization of the cellular composition of peripheral blood in male and female mice during aging.

**Chapter III** addresses the association between age-associated alterations of the immune system, assessed through variations in cellular compositions during aging, and heterogeneous cognitive performance in old mice.

**Chapter IV** presents a comprehensive characterization of peripheral immune molecules on healthy senior individuals in relation to their cognitive performance. The results presented in this chapter are contained in a original research paper published in Frontiers in Immunology in 2020 (Serre-Miranda *et al.* 2020).

**Chapter V** contains a general discussion, giving an overview of the major findings in the context of the known literature. The main limitations of this work along with future perspectives are also herein briefly discussed.

# CHAPTER I
## INTRODUCTION

# 1. AGING AT A GLANCE

## 1.1. POPULATION AGING

The improvement in health care services, medicine, economic and social development and quality of life are allowing people to live longer. Globally, life expectancy at birth has reached 72.3 years, with women on average living five years longer than men, and it is expected to increase 2 years by 2045-2050 (United Nations, DESA, 2020). However, this increase in life expectancy combined with a reduced natality/fertility is leading to a shift in the populations' age structure: successive cohorts are living longer and having fewer children (United Nations, DESA, 2020). This global phenomenon has been characterized as one of the last decade's hallmarks and it is expected to increase in the forthcoming years.

According to the United Nations Program on Aging, in 2019 there were 703 million people 65+ worldwide, number that is expected to double (1.5 billion) by the year of 2050, when it is expected that one in six people worldwide will be older than 65 years of age (Figure 1) (United Nations, DESA, 2020). The octogenarian population (or older) nearly tripled in the last 30 years, growing from 54 to 143 million, and it expected to triple again in the next 30 years, reaching 426 million (United Nations, DESA, 2020). The Portuguese population is not an exception to this trend. The 65+ individuals represent nowadays approximately 20% of the overall population, however, it is estimated that in 60 years from now they will represent 37% of the population and for every 100 young people we will have 317 seniors (65+) (Figure 1) (Instituto Nacional de Estatística, 2017). Though, these traditional metrics do not discriminate between extending life expectancy and adding healthy years. More than just living longer, it is important to understand if we are doing this progress in "good shape". A report based on the Global Burden of Disease Study 2017 developed a metric that reflects age-related morbidity and mortality at the population level: an age-related burden defined by the sum of the disability-adjusted life years (DALYs) of several age-related diseases. It was estimated that age-related diseases account for 51.3% of all global burden among adults in 2017, and has been declining since 1990 (Chang et al., 2019).

Even though the age-related burden has decreased in the last 30 years, the current trends on population aging are a concern given various age-associated pathologies and decline of several abilities. For instance, people are living longer but are also being more prone to develop cognitive decline, ranging from normative cognitive aging to mild and severe forms of dementia, such as Alzheimer's disease (AD) (Paulo et al., 2011; Prince et al., 2016; Salthouse, 2010). Moreover, the economic impact on health care of those who have lost independence are expected to rapidly

increase in the years to come as a result of the projected increase in life expectancy. As such, the scientific community faces the challenge to identify predictive factors and strategies to promote a healthy aging that would ultimately allow an independent and functional aging, positively reflected in a continued overall quality of life.

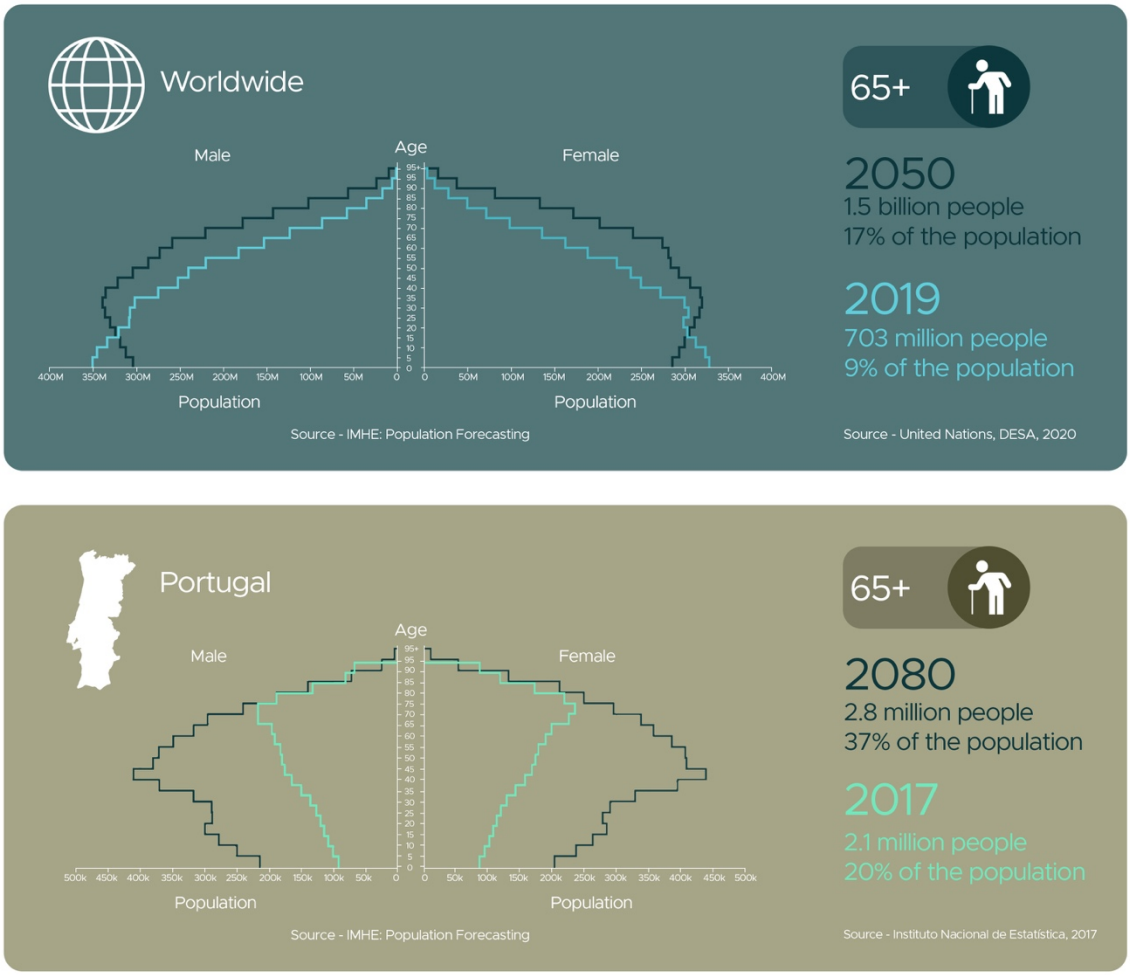


**FIGURE 1. Population aging forecasting.** Population age structure pyramid for the entire world population (upper panel) and for Portugal (bottom panel) at the present time and the forecasting in 30 and 60 years, respectively. It is estimated that worldwide the 65+ population will increase from 9% to 17% in 30 years. Portugal, having already 20% of the population with 65+, in 60 years will have around 37%.

Curiously, the constant desire to keep youth over the years gave rise to myths and tales that have perpetuated themselves for centuries. The Fountain of Youth is a mythical spring that restores the youth of everyone who bathes or drinks in its waters. Interestingly, in the last decade Tony Wyss-Coray and collaborators have been clamming that young blood have anti-aging and other beneficial properties, which have been constantly compared to a Fountain of Youth. They have demonstrated that young blood has the capacity to rejuvenate cognitive function in older mice or even to ameliorate disease in a model of AD - maybe Count Dracula was right (Middeldorp et al., 2016;

Pluvinage and Wyss-Coray, 2020; Villeda et al., 2014; Wyss-Coray, 2016). The potential link between circulating factors and the modulation of brain function, mainly cognitive function, will be further explored in this chapter.

### 1.2. HALLMARKS OF AGING

Aging, as a physiological mechanism, has been characterized by a progressive loss of integrity, ultimately leading to impaired function and increased susceptibility to death. It results from a cellular loss of fitness due to a time-dependent accumulation of cellular damage (Gems and Partridge, 2013; Kirkwood, 2005; López-Otín et al., 2013; Vijg and Campisi, 2008). Recently, nine cellular and molecular candidate hallmarks were proposed as common denominators of aging in different organisms (best characterized in mammals). These can be grouped in three categories: 1) primary cause of damage - genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis; 2) compensatory or antagonistic responses to damage - deregulated nutrition sensing, mitochondrial dysfunction and cellular senescence; and 3) integrative hallmarks, which are the end result of the previous two and are responsible for the age-associated functional decline - stem cell exhaustion and altered intracellular communication (López-Otín et al., 2013).

To holistically understand human body aging, a multidisciplinary approach is required. The above described age-related cellular and molecular alterations affect all systems, including the immune and the central nervous systems (CNS). In the next two sections the major alterations occurring with age in cognitive function (one of the major higher brain functions) and in the immune system will be addressed.

## 2. COGNITIVE AGING

### 2.1. COGNITION: AN OVERVIEW

Cognitive functioning is a multiparametric brain function that encompasses many mental (or intellectual) abilities such as attention, learning, thinking, reasoning, memory, problem solving, decision making (Fisher et al., 2019). Among them, two main different types of cognitive abilities can be distinguished: the “crystallized” abilities, which reflect the acquired knowledge throughout life, usually measured through vocabulary and general information tests; and the “fluid” abilities that comprise reasoning, memory, processing speed and novel problem solving, which are features related with executive function (Salthouse, 2012).

Cognitive function assessment is one of the core aims of behavioural neurosciences. Whereas in humans there are several proxies to assess the overall cognitive function, most of the behavioural paradigms used in rodents rely on their ability to learn a task (the ability to acquire knowledge) and to encode, store and retrieve that information, which is commonly referred as memory. Some of the behavioural paradigms also allow the assessment of working memory and behavioural flexibility (Bizon et al., 2012; Shepard et al., 2017).

It is important to keep in mind that when comparing studies conducted in humans with animal models (commonly rodents), the concept of cognition is, in most of the cases, oversimplified: memory function in rodents is often addressed as a synonym of cognition function.

## 2.2. TOOLS TO EVALUATE COGNITIVE FUNCTION IN RODENTS

A diversity of behavioural paradigms is used to infer the cognitive function status of rodents. Among these, and in the context of the present thesis, are those that explore learning and memory. It is important to keep in mind that behavioural test are, in most cases, not specific for a single cognitive domain or brain area. They rely on complex brain circuits, and are influenced by non-cognitive parameters.

In a simplistic perspective, learning and memory tasks can be divided in 1) tasks that require a stimulus with an emotional component (an aversive stimuli), such a foot shock used in the contextual-fear conditioning (CFC) test (Curzon et al., 2009); or get advantage of a negative reinforcement, such as the cold water in Morris Water Maze (MWM) test (Morris, 1984) or a bright-lit platform in its dry version Barnes Maze (Barnes, 1979); and 2) tasks that take advantage of rodents' natural preference to investigate novel stimuli, either with a spatial component, such as the novel location recognition (NLR) test (Denninger et al., 2018) and Y-maze (Kraeuter et al., 2019), or with a non-spatial component, such as the novel object recognition (NOR) test (Bevins and Besheer, 2006).

This chapter will cover some of the most widely used behavioural tests to study cognitive status in rodents.

**Novel location and novel object recognition tests** are both based on rodents' natural curiosity to explore the environment. It relies on animals natural preference for novelty (Berlyne, 1950; Ennaceur and Delacour, 1988). Both tests are composed by two phases: training and testing, which are preceded by a habituation phase to the apparatus prior to the test day. In the

training session, rodents are exposed to two identical objects, which are called familiar or sample objects. After the training session, they return to their home cage. After a retention period, that can range from 1h to 24h, animals return to the apparatus (testing phase) and: 1) in the NOR are exposed to one familiar object and a new one (Bevins and Besheer, 2006) ; and 2) in the NLR are exposed to one of the familiar objects placed in a different location in the apparatus (Denninger et al., 2018). Recognition memory is denoted when animals spend more time exploring the novel object or the object in the new location. A commonly used measure is the discrimination ratio (or the % of novelty preference) that is obtained dividing the time spent exploring the new object (or the object in the new location) by the total interaction time (Bevins and Besheer, 2006).
Object recognition tasks rely on a neural circuitry involving the perirhinal cortex, medial prefrontal cortex and hippocampus (Warburton and Brown, 2015). While the involvement of the hippocampus in the NLR test (spatial task) is widely accepted, its involvement in the NOR test (non-spatial tasks) is still being debated (Cohen et al., 2013; Warburton and Brown, 2015).

**Y-maze test** also takes advantage of the rodents' innate curiosity to explore the environment. They usually spend more time exploring previously unvisited areas than a place they already know. This test is used to assess short-term memory and, depending on the protocol used, it can measure either spatial working memory (spontaneous alternation protocol) or spatial reference memory (2-trial protocol) (Kraeuter et al., 2019). In the spontaneous alternation protocol rodents are allowed to freely explore the Y-shaped maze and the number of alternations between arms are counted. A good working memory is characterized by consecutive entries into different arms, which suggest the animal remembers the arm previously visited. This type of behaviour involves different brain areas such as the prefrontal cortex and the hippocampus (Lalonde, 2002). In the 2-trial Y-maze protocol, rodents explore the maze in a training session where one of the arms is closed. After a retention period that can range from 1h to 24h, the previously closed arm is opened and the animal is allowed to freely explore the maze. A good reference memory is reflected by the preference for the novel arm (Kraeuter et al., 2019).

**Spatial learning tasks** (or spatial reference memory tests) have long been used in rodents as reliable measures of hippocampal-dependent spatial navigation and reference memory (Bannerman et al., 1999; Garthe and Kempermann, 2013). Two of the most widely used tests to assess long-term memory are the MWM and the Barnes maze test. Both use a negative reinforcement to promote spatial learning (cold water and brightly-lit platform, respectively).

The **MWM**, which was developed in the early 80's by Richard Morris and became a test of reference, measures spatial navigation and memory in rodents (Morris, 1984; Vorhees and Williams, 2006). In this task rodents use spatial cues to navigate in an open swimming arena to find a hidden platform. Rodents are allowed to freely explore the swimming arena for a certain period of time in a series of repeated trials in the same day, replicating this procedure for several days. Rodents with an intact spatial and reference memory remember the locations of the platform and take less time over the days to find the hidden platform (Morris, 1984; Vorhees and Williams, 2006).

The **Barnes maze test** was first described by Carol Barnes in 1979 (Barnes, 1979). It is a dry version of the MWM and consists in an elevated circular platform with holes along the perimeter. A dark box is installed in one of the holes to provide rodents an escape from an aversive situation (such as a bright light). As in MWM, animals use spatial cues to learn and remember where the target box is hidden and over the days they take less time to find the hidden box. As a readout, the number of errors are evaluated through the number of nose pokes and head deflections in the holes that do not have the target box (Gawel et al., 2019; Sunyer et al., 2007).

**Fear conditioning** (to a context and/or a cue) represents a form of associative memory, which is an adaptive process that allows organisms to anticipate specific events. In this task, rodents learn the association between an aversive stimulus (such as a mild foot shock) and a specific context and/or a cue (such as a tone or a light). Freezing behaviour, a natural response to fear, is a direct readout of memory. Animals with intact memory freeze upon being exposed to the same context and/cue (Curzon et al., 2009).

In the CFC paradigm, animals must first form the representation of the context and then associate that representation with an aversive stimuli (reviewed in Maren et al., 2013). To encode and integrate all that information, several brain regions are involved. For instance, the hippocampus has a crucial role in learning/encoding and remembering the contexts (Holland and Bouton, 1999; Saxe et al., 2006), while the amygdala has a fundamental role in encoding, storing and retrieving the associations between contexts (or cues) and aversive stimuli (Fanselow and Poulos, 2005; Maren and Quirk, 2004).

## 2.3. TOOLS TO EVALUATE COGNITIVE FUNCTION IN HUMANS

Cognitive profiling requires a multiparametric evaluation to cover the different domains that influence cognitive ability. There is a great diversity of cognitive tests to evaluate different functions, such as memory, attention, language, processing speed and learning. The following paragraphs highlight those more usually used in research.

**Mini-Mental State Examination (MMSE)** is the most widely used test to evaluate global cognitive status (Folstein et al., 1975; Freitas et al., 2015). The test is composed by 30 items and it assesses word recall, orientation, language, visuo-construction abilities, attention, and calculation. The thresholds used to define cognitive impairment depends on factors such as education (Folstein et al., 1975; Freitas et al., 2015; Grigoletto et al., 1999). For the Portuguese population, a threshold of MMSE score <17 is used for individuals with 4 or less years of formal education or/and 72 years old and older; a threshold of MMSE score <23 is used for individuals with more than 5 years of formal education or younger than 72 years of age (Paulo et al., 2011).

The **Montreal Cognitive Assessment (MoCA)** test is also used to evaluate global cognitive status and has higher sensitivity and specificity than MMSE to detect mild cognitive impairment (Freitas et al., 2011; Nasreddine et al., 2005). This instrument comprises 30 items, with higher scores corresponding to better performance, with a cut-off for the general population of 26. It evaluates several cognitive domains such as short-term memory recall, visuospatial abilities, multiple aspects of executive function, attention, concentration, working memory and language.

The Buschke **Selective Reminding Test (SRT)** is a multiple trial verbal learning and memory task that involves episodic verbal memory (Buschke, 1973; Buschke et al., 1995). It is composed by a list of 12 words that is read to the participants. In the first trial participants are asked to recall as many items as possible. In the subsequent 5 trials, the items not recalled in the previous trial are read back to the participants. Several parameters can be extracted from this test: long-term storage (LTS), when a word is recalled in two consecutive trials; consistent long-term retrieval (CLTS), when words are consistently recalled in all subsequent trials; delay recall, when words are recalled after 20 mins; and intrusions, when a word is incorrectly named or is not part of the list.

The **Stroop Test** evaluates response inhibition and cognitive flexibility (Stroop, 1935). It can be divided in 3 variations: Words – where participants are asked to read words denoting colors but printed in black; Colors – where participants are asked to name the color of a series of words printed in the denoted color; and Word/Colors - where participants are asked to name the ink-color of a series of words denoting a different color. For each test, individuals are asked to process in 45 s as many items as possible, and the performance is measured by the number of correct answers. The Word/Color condition is based on the inhibition of an over-learned response by a competing response and it is used as an indicator of selective attention, cognitive flexibility, and response inhibition. It is used as a tool to measure executive function (Scarpina and Tagini, 2017).

The **Digit Span Test** is a subset of the Wechsler Adult Intelligence Scale and it measures short-term verbal working memory and attention. It is composed by two subtests: digits forward – where individuals are asked to repeat a list of numbers in the correct order immediately after presentation; and digits backwards – where individuals are asked to repeat a list of numbers in the reverse order (Hester et al., 2004; Ryan and Lopez, 2001; Wechsler, 1997).

The **Controlled Oral Word Association Test F-A-S** (COWAT, FAS) is the most commonly used verbal fluency test and it measures the number of words beginning with a given letter, i.e. F, A and S, that a person can name. Individuals are given 60 s to produce as many words as possible (Lezak et al., 2004). Two measures can be extracted: admissible - the number of the words named correctly by participants; and non-admissible – the number of incorrectly named or repeated words named by participants (Strauss et al., 2006).

The **Digit Symbol Substitution Test** (DSST) is used to measure high-level information processing speed, associative learning, attention and visuo-perceptual functions (Jaeger, 2018; Wechsler, 1997). In this test, subjects are given a key grid with numbers and the matching symbols, and a test box with only numbers and an empty space that they need to fill with the matching symbol. Subjects are asked to fill as much boxes as possible, and the number of correct answers is the readout of the test.

## 2.4. COGNITIVE AGING IN HUMANS

The normal age-associated cognitive decline, in the absence of dementia or other types of mild cognitive impairment, is not transversal to all cognitive domains. Some mental functions are stably

maintained throughout life, such as verbal and numerical abilities and general knowledge ("crystallized" abilities), while other mental capabilities, such as particular aspects of memory, executive function, reasoning and processing speed, decline from middle age onwards (Hedden and Gabrieli, 2004; Park and Reuter-Lorenz, 2009; Salthouse, 2019, 2012). Thus, the ability of solving novel problems is more sensitive to age than knowledge acquired throughout life. Those "fluid" abilities are important for carrying out everyday activities and to maintain an independent and fulfilling life. The level of cognitive ability is also one of the strongest predictors of work success, which affects other domains of the daily life (reviewed in (Salthouse, 2012).

The increasingly aged population raises several health and socio-economic concerns, being cognitive deterioration one of the most debilitating conditions leading to institutionalization. Facing this reality, the scientific community has been joining efforts to understand the individual differences in normal cognitive aging. Besides the intra-individual variability (dispersion), which is characterized by the variability observed in a single's person performance across different task covering different cognitive domains, there are also inter-individual differences (Deary et al., 2009a; Hilborn et al., 2009).

Which are the factors acting as protectors (or helping people to maintain or enhance their cognitive ability) and which are the risk factors, leading to a progressive cognitive decline? The identification of those risk factors and possible mechanisms for the individual differences in cognitive decline are among the greatest challenges to improve the wellbeing of senior population (Figure 2) (Deary et al., 2009a). Sociodemographic characteristics, such as educational level, have been associated to cognitive performance later in life, being a good predictor of cognitive functioning (Ardila et al., 2000; Josefsson et al., 2012; Paulo et al., 2011).

The brain is a highly irrigated organ and a disruption or impaired blood supply may affect normal brain function. Thus, some cardiovascular parameters have been identified as risk factors for decreased cognitive performance in senior individuals (Gardener et al., 2016; Schievink et al., 2017; Takeda et al., 2017). Furthermore, the brain undergoes a profound structural alteration during aging; the most evident being a decrease in brain size accompanied by an increase in the ventricular spaces and cerebrospinal fluid to balance for the total cranial volume. The white matter integrity is also an important factor for normal cognitive ageing, since its loss leads to inefficient information transfer between brain areas (Deary et al., 2009a; Marques et al., 2016).

Genetic traits have been explored as factor influencing life-long intelligence and age-associate cognitive decline (Deary et al., 2009b). One of the most consistent and replicable results is the

association between apolipoprotein E (APOE), a risk factor for Alzheimer's disease, and cognitive aging. Two meta-analysis revealed that carriers of APOE allele (ε)4 have worse episodic memory, executive function and general cognitive abilities, than non-ε4 carriers (Deary et al., 2009a; Small et al., 2004; Wisdom et al., 2011).

Emotions, mood and motivational abilities have also demonstrated a significant impact on the rate of the cognitive decline in older adults (Forstmeier and Maercker, 2008; Kinugawa et al., 2013; Santos et al., 2013). In a cross-sectional study, mood was found to be the principal factor contributing to the discrimination between very good and good, as well as between poor and very poor cognitive performers (Santos et al., 2013).

Social engagement has a clear impact on cognitive function across older adult (Josefsson et al., 2012; Paulo et al., 2011; Stine-Morrow et al., 2008). Other lifestyle factors such as diet, smoking, drinking, physical activity and sleep influence cognitive ageing (Deary et al., 2009a; Josefsson et al., 2012; Santos et al., 2014). Other factors that have been calling attention in the last decades to account for cognitive variability are the low-grade inflammation, usually observed in senior individuals and also known as "inflammaging" (Franceschi et al., 2017), and the age-associated alterations of the immune system (this topic will be extensively explored in another section of this thesis). The increased peripheral inflammatory profile has been associated with worse cognitive performances, being IL-6 and C-reactive protein the most commonly studied inflammatory markers (Baune et al., 2008; Heringa et al., 2014; Trollor et al., 2012). We have also previously demonstrated that effector memory $CD4^+$ T cells, which are one of the major cytokine producers among T cells, are associated with memory and executive functions in cognitively healthy senior individuals, even accounting for age, sex, years of formal education and mood (Serre-Miranda et al., 2015).

Altogether, the evidence is clear on the complex interplay of mediators/pathways/behaviours that regulate aging and influence cognition. However, the causality of the aforementioned factors in the explanation of cognitive variability is still on debate. Deary and colleagues also raise the awareness for the over simplified causal models, in some of the cases extrapolated from cross-sectional experimental designs. They state that in some cases the probable cause of cognitive deteriorations might be a consequence of that deterioration, or even the existence of a third inter-player (confounder) affecting both the apparent cause and effect (consequence) (Deary et al., 2009a). Although exigent and difficult, more robust longitudinal studies should be performed to investigate the possible mechanisms for this causal relationship.

## 2.5. COGNITIVE AGING IN RODENTS

Rodents have been used for a long time as models to study cognitive aging. Their short lifespan and the possibility for genetic manipulation, pharmacological, interventional and mechanistical studies allow researchers to explore and unravel the process of cognitive aging and how can it be modified. For instance, on average mice can survive until 24 months of age (depends on the strain). Between 3 to 6 months of age they are considered mature adults, from 10 to 14 months of age are considered middle-aged and with more than 18 months of age are considered old (Hagan, 2017).

Like humans, rodents experience a progressive and heterogeneous decline in cognitive function, mostly reflected in learning and memory deterioration. As explored above, most of the cognitive paradigms used in rodents rely on three types of memory: spatial, recognition and associative memory. Old rats and mice present poorer spatial memory and behavioral flexibility than younger animals in the MWM test (De Fiebre et al., 2006; Da Mesquita et al., 2015; Mota et al., 2019) and in the Y-Maze (Aggleton et al., 1989; Sooy et al., 2010) and Barnes Maze (Bach et al., 1999; Barrett et al., 2009). The ability of mice to recognize a familiar object in a different location is also affected by aging, as demonstrated by the worse performance of old mice (15 to 18 months old) in the NLR test than young animals. However, their ability to recognize a non-familiar object in a NOR test (non-spatial) seems only to be affected near 24 months of age (Baruch et al., 2014; Ron-Harel et al., 2008; Stilling et al., 2014). Regarding associative memory, it was demonstrated that 30% of middle age mice (8 months old) already present decreased context discrimination (Kaczorowski and Disterhoft, 2009), being the phenotype more evident when they get older (20 months old) (Peters et al., 2014). Similarly to what happens in mice, rats also present less freezing in the contextual-fear conditioning protocol, starting to present memory deterioration already at 16 to 20 months old (Moyer and Brown, 2006).

While some of the animals present reduced spatial reference and working memory abilities throughout aging, others maintain their capacity to acquire spatial memory as young animals (Foster et al., 2012; Mota et al., 2019). As demonstrated by Mota *et al.*, it is possible to discriminate rats that present a good performance from those who present a bad performance by a clustering method. The authors also showed that performance differences are accompanied by structural and functional differences. While in younger rats better cognitive performance is associated with longer dendritic trees and increased levels of synaptic markers in the hippocampus

and the pre-frontal cortex, in older rats the opposite was observed: better performance is associated with shorter dendrites and lower synaptic markers. They conclude that "while in younger individuals bigger is better, smaller is better is a more appropriate aphorism for older subjects" (Mota et al., 2019). Another group also demonstrated that 30% of aged mice (18 months old) tested on NLR test (another spatial task) retained memory function (Baruch et al., 2014). Curiously, the onset of cognitive deficits start already at 8 months old, where 30% of mice already present decreased context memory, than 2 months old mice in the CFC test (Kaczorowski and Disterhoft, 2009).

Similarly to humans, several factors based on habits or social environment that can preserve or ameliorate memory deficits in rodents have also been reported (Figure 2). For instance, diet has an impact on cognitive performance of aged mice, shown by the impaired memory in a Y-maze spontaneous alternation test caused by high-fat diet already at 11-12 months old (Knight et al., 2014). On the other hand, ketogenic diet, which recapitulates certain metabolic aspects of dietary restriction, has been shown to improve memory performance in the NOR test in aged mice with 28 to 30 months old (Newman et al., 2017). Social interaction also plays a role in the aged associated cognitive deterioration. Old mice maintained in isolation (either single or pair-housed) showed worse performance in spatial memory tasks when compared with group-housed aged-match controls (Smith et al., 2018; Wang et al., 2018). The memory deficits induced by social isolation can be mitigated by environmental enrichment placing aged mice in large cages with toys and running wheels, that can also count as physical exercise benefiting the maintenance of a healthy brain function (Wang et al., 2018).

The possibility to use rodents, where experiments to infer causal relationships are easier to design than in humans, to dissect the association between the different factors that contribute to the heterogeneous cognitive aging will allow to faster unravel the mechanisms behind those associations. For instance, while in humans several studies have been associating peripheral age-related alterations in the immune mediators with cognitive deterioration, in rodents this association is still poorly studied. Comprehensively understand the aging of the immune system in rodents, how similar is to humans and how it affect cognitive aging will allow the establishment of causal relationships.

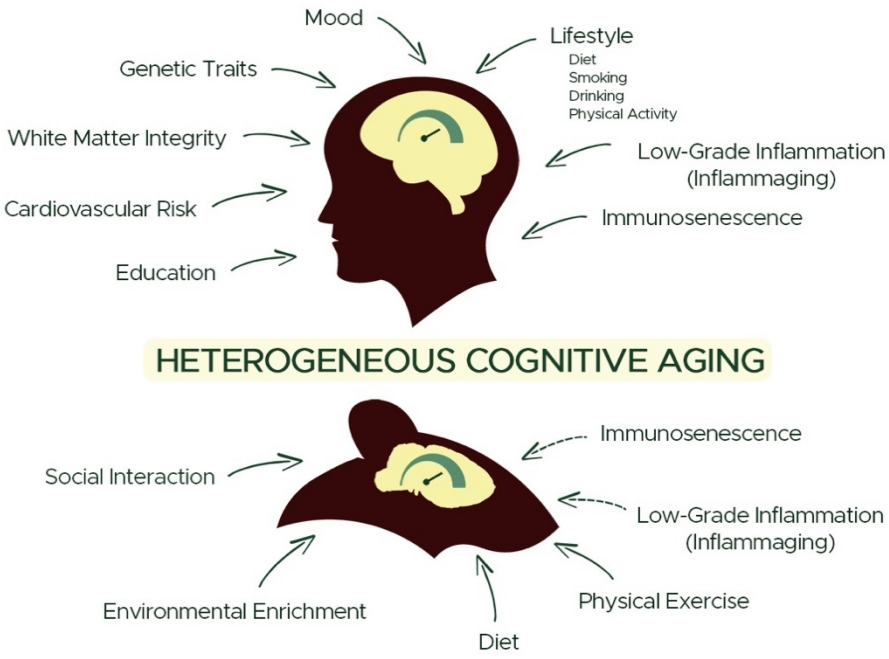


**Figure 2. Factors that are associated with an heterogenous cognitive aging both in humans and in rodents.** In humans factors such as education, cardiovascular risk, white matter integrity, genetic traits, emotions and mood-related states, lifestyle indicators, the low-grade inflammation and the immunosenescence have been associated with inter-individual differences in cognitive performance of senior individuals. Similarly, in rodents several factors based on habits or social environment, such as social interaction, environmental enrichment, diet and physical exercise have an impact on cognitive performance in aged animals. However the role of immunosenescence and of low-grade inflammation in still poorly studied in rodents.

# 3. IMMUNE SYSTEM AGING

## 3.1. IMMUNE SYSTEM AT A GLANCE

The immune system is a host defense system that interacts with all the other systems in the human body. The surveillance and defense capacity of the immune system is pivotal to maintain body homeostasis, for instance to fight against infections and to mount an immune response against tumor cells. In several species, the immune system can be classically divided in two main branches: the innate and the adaptive immune system. In a simplistic perspective, the innate immune system is the first "line of defense" responding to patterns common to several pathogens, while the adaptive immune system recognizes specific antigens and develops immune memory (Owen et al., 2013). Of notice, in the last few years, it has been described that the innate immune system has the ability to create a non-specific immune "memory" that has been named "trained immunity", and is mediated mostly by epigenetic alterations (Netea et al., 2016). The communication between the two components of the immune system is essential to establish an

effective immune response. However due to cumulative alterations of the immune system during aging, in a process referred as immunosenescence, older individuals experience increased susceptibility to certain infections, and reduced response to vaccination among other morbidities (Bupp et al., 2018; Klein and Flanagan, 2016; Nikolich-Žugich, 2018). Both innate and adaptive immunity display age related alterations in cell frequency and function that will be explored in the next topics.

### 3.2. ADAPTIVE IMMUNITY

One of the main, and most classical, functions attributed to the adaptive immune system is its ability to distinguish specific antigens when presented by antigen-presenting cells (APC). The adaptive immune response develops immunological memory after the first encounter with an antigen, allowing a faster and stronger response in a second "insult" (Owen et al., 2013). This response is mediated mainly by B and T lymphocytes, also known simply as B and T cells. One of the most common alterations during aging in the adaptive immunity is the increased proportion of T and B cells with a memory phenotype in respect to the naïve cells (Figure 3) (Britanova et al., 2014; Chong et al., 2005; Cossarizza et al., 1996; Frasca et al., 2008; Saule et al., 2006; Thome et al., 2014).

**T cells** are responsible for cell-mediated immunity through the recognition by the T-cell receptor (TCR) of specific antigens in the context of major histocompatibility complex (MHC). T cell precursors are produced in the bone marrow (hematopoiesis) and differentiate in the thymus (Owen et al., 2013). During differentiation T cells commit to a Th (T helper, also known as $CD4^+$ T cells) phenotype or a Tc (T cytotoxic, also known as $CD8^+$ T cells) phenotype (Taniuchi, 2018). $CD4^+$ T cells are important immune mediators, helping in the activation of B cells, $CD8^+$ T cells, and also innate cells (Taniuchi, 2018). On other hand, $CD8^+$ T cells, known by their cytotoxicity, induce cell death in cells infected with virus (or other pathogens), or in cells that are damaged and dysfunctional (such as tumor cells) (Gupta et al., 2004; Owen et al., 2013).

The total T cell poll of $CD4^+$ and $CD8^+$ T cells can be divided in several subsets/compartments based on their phenotype and activation state: 1) naive T cells (in humans characterized as $CD45RA^+$ $CD45RO^-$ $CCR7^+$ and in mice (described mainly in C57BL/6 mice) as $CD44^{low/-}$ $CD62L^{high}$), are cells that have not yet encountered their cognate antigen; 2) central memory (CM) T cells (in humans characterized as $CD45RA^-$ $CD45RO^+$ $CCR7^+$ and in mice as $CD44^{high}$

CD62L$^{high}$), are cells that were previously activated and present the ability to expand rapidly upon secondary challenge; 3) effector memory (EM) T cells (in humans characterized as CD45RA$^-$ CD45RO$^+$ CCR7$^-$ and in mice as CD44$^{high}$ CD62L$^{low/-}$) are cells actively responding to a stimulus; and 4) terminally (or late) differentiated (TD) T cells (also known as $T_{EMRA}$ cells and characterized in humans by CD45RA$^+$ CCR7$^-$) are considered end-stage differentiated effectors with a major representation in the CD8$^+$ T cell subset (Appay et al., 2008; Okada et al., 2008; Rosenblum et al., 2016; Saule et al., 2006); in mice these cells have been less explored in physiological conditions, but have been characterized as CD44$^{low/-}$ CD62L$^{low/-}$ (Abu Eid et al., 2017).

During aging, as a result of the progressive thymic involution and a constant challenge of the immune system, the naive compartment progressively decreases over time, while the memory and effector compartments increase (Cossarizza et al., 1996; Goronzy and Weyand, 2005; Jiao et al., 2009; Nikolich-Žugich, 2014; Saule et al., 2006; Wikby et al., 2008). In humans, the increase of memory T cells in the CD4$^+$ subset is mainly due to an increase in CM and EM cells, whereas the increase in the CD8$^+$ subset is due to accumulation of EM and TD (Saule et al., 2006; Thome et al., 2014). The accumulation of terminally differentiated cells within the CD8$^+$ T cells compartment is considered a hallmark of adaptive immune aging, and has been attributed to chronic and latent viral infections, such as CMV (cytomegalovirus) (Tu and Rao, 2016; Wertheimer et al., 2014).

Besides the alterations in the T cells' compartments, the relative and absolute numbers of T cells, as well as of CD4$^+$ and CD8$^+$ T cells, have been reported to decrease over time (Jiao et al., 2009; Márquez et al., 2020; Patin et al., 2018; Saule et al., 2006; Wikby et al., 2008). This phenomena has been extensively characterized in humans, while the information in rodents is still scarce (El-naseery et al., 2020). It is important to notice that thymic involution clearly contributes to the loss of naive T cells in mice. However, in humans, T cell homeostasis is different and depend on peripheral proliferation of naive T cells (reviewed in Goronzy and Weyand, 2017). Thus, we need to be cautious when assuming a direct parallelism between mice and humans when describing alterations in T cells' compartments.

TCR repertoire, which reflects the diversity of antigens that T cells can recognize, also decrease during aging due to the reduced export of newly differentiated T cells from the thymus and expansion of memory cells (Blackman and Woodland, 2011; Britanova et al., 2014; Shifrut et al., 2013). It has been reported that the TCR diversity in senior individuals undergoes a two- to fivefold reduction in peripheral blood (Qi et al., 2014). Moreover, TCR signaling intensity is also affected in CD4$^+$ T cells, there is a reduction it T cells expansion upon stimulation, they present a different

cytokine secretion profile, and a reduction of Th1 and Th2 effector differentiation in favor of Th17 and regulatory T cells (Haynes and Maue, 2009; Lefebvre and Haynes, 2012; Pieren et al., 2019; van der Geest et al., 2014). $CD8^+$ T cells also experience reduced proliferative capacity and anti-tumor response in older individuals (Haynes and Maue, 2009; Pieren et al., 2019).

It is important to call the attention to the fact that most studies and reviews investigating T cell compartments dynamics during aging explore mostly what happens in blood. However, the majority of T cells reside within lymphoid tissues, such as the bone marrow, spleen, tonsils and lymph nodes, and it is estimated that only 2 to 3% of total T cells in the human body are found in blood (Ganusov and De Boer, 2007). Interestingly, in a study exploring T cell homeostasis at different ages in different organs (blood, lymphoid organs and mucosa), it was demonstrated that the compartmentalization and activation state of T cells varies greatly over time and is dependent on tissue location. Thus, it cannot be simply inferred from blood (Thome et al., 2014).

**B cells** are generated in the bone marrow and their main function is the production of antibodies (also known as immunoglobulins [Igs]) upon stimulation in a T cell-dependent or independent manner, making them vital for the humoral immunity. They act as APCs and have the capacity to develop into memory cells upon activation (Lebien and Tedder, 2008). During aging, B cells experience both functional and relative and absolute abundance alterations. It has been demonstrated for a long time that human B cells frequency and absolute numbers decrease with aging (Chong et al., 2005; El-naseery et al., 2020; Frasca et al., 2008; Jiao et al., 2009; Muggen et al., 2019; Ongrádi and Kövesdi, 2011; Paganelli et al., 1992). An hypothesis is that such decrease might be a reflex of impaired generation of B cells in the bone marrow with aging (Frasca and Blomberg, 2011; Scholz et al., 2013). Regarding the naive to memory dynamics alteration during aging, conflicting reports have been published. While some researchers describe a decrease in the proportion of naive B cells ($CD27^-$) and an increase in memory B cells ($CD27^+$) (Bancos and Phipps, 2010; Frasca et al., 2008), others report the opposite, even though the absolute counts decrease in both populations (Chong et al., 2005). This controversy may be caused by a disparity in the classification used to identify the memory subsets in different studies. Interestingly, it has been reported that age-associated B cells, which is a specific subset of exhausted mature B cells comprising 30% of the pool, increase with age (Hao et al., 2011; Rubtsova et al., 2015). B cells' functional alterations are also observed in aged individuals, such as the decreased antibody diversity and reduced B cell repertoire (Frasca et al., 2005; Gibson et al., 2009; Tabibian-Keissar

et al., 2016). After an immune challenge with specific antigens, old mice also present a reduction in IgG and IgA production (Speziali et al., 2009).

### 3.3. INNATE IMMUNITY

The innate immune system is composed by several cell types including neutrophils, monocytes, macrophages, dendritic cells (DC), natural killer (NK) and natural killer T (NKT) cells, which present different roles in the immune response. Cells of the innate immune system are activated when pathogen-associated molecular patters (PAMPs) or damage-associated molecular patters (DAMPs), are recognized by pattern recognition receptors (PRR), such as toll-like receptors (TLR). PAMPs are molecules conserved among an extensive group of pathogens while DAMPs are released during tissue injury, (Kubelkova and Macela, 2019; Owen et al., 2013). As we age, the innate immunity undergoes both quantitative and qualitative alterations that will be further discussed ahead (Figure 3).

**Neutrophils** constitute the first line of defense against bacteria, yeast and fungi, being the first cells to arrive at the sites of infection. These cells compose more than 50% of the human immune cells in circulation and are short lived (Mayadas et al., 2014). In the sites of infection neutrophils recognize and phagocyte microorganisms, and kill pathogens through several cytotoxic mechanisms, such as the generation of reactive oxygen and nitrogen species, release of proteolytic enzymes, and microbicidal peptides from cytoplasmic granules (Mayadas et al., 2014; Shaw et al., 2010).

While the impact of aging in the number of circulating neutrophils is still controversial, being described to remain unaltered or to increase with aging (Cakman et al., 1997; Chatta et al., 1993; Valiathan et al., 2016), some of their functions are consensually described as being reduced. Among others, it has been described a reduction on chemotactic activity, phagocytic capacity, cytokine and PRR signaling and free radicals production (Brubaker et al., 2013; Fulop et al., 2004; Hakim et al., 2004; Larbi et al., 2008; Schröder and Rink, 2003; Shaw et al., 2010; Wenisch et al., 2000). These changes result in a reduced ability to control infections both directly and through activation of the adaptive immune system (Wessels et al., 2010).

**Monocytes** are important in recognition and clearance of invaders through their PRR. They represent a highly mobile component of the innate immune system located mainly in the spleen

and blood and play a crucial role in tissue homeostasis (Ginhoux and Jung, 2014; Jakubzick et al., 2017). Monocytes respond to inflammation differentiating into APC, such as macrophages and DCs, that process antigens and present them to T cells in the context of MHC (Ginhoux and Jung, 2014; Jakubzick et al., 2017).

With age, whether the absolute and relative numbers of total monocytes increase or remain constant over time is still debatable (Della Bella et al., 2007; Puchta et al., 2016; Seidler et al., 2010; Shaw et al., 2013). However, the effect of aging on monocytes seems to be phenotype-dependent since, in humans, the abundance of circulating $CD16^{high}$ $CD14^{low}$ monocytes (non-classical monocytes) and $CD16^{+}$ $CD14^{+}$ monocytes (intermediate monocytes) has been reported to increase with age, whereas $CD16^{low}$ $CD14^{high}$ monocytes (classical monocytes) decrease or maintain (Hearps et al., 2012; Márquez et al., 2020; Patin et al., 2018; Seidler et al., 2010). On the other hand, the monocytes' function has been consistently reported as becoming impaired with aging. They present a decrease in phagocytosis, reduced reactive oxygen species production and dysregulated cytokine production upon TLR stimulation (Della Bella et al., 2007; Hazeldine and Lord, 2015; Hearps et al., 2012). The alterations in monocyte numbers also aligns with alterations in macrophages function such as reduced chemotaxis and phagocytic activity, activation through TLR, antigen presentation (possibly due the reduction of MHC class II expression with age) and also increased pro-inflammatory cytokine production (Larbi et al., 2008; Panda et al., 2009; Shaw et al., 2010; Villanueva et al., 1990).

**Dendritic cells** are professional APCs that play critical roles in the initiation and definition of the immune response profile linking the innate and adaptive branches of the immune system. DCs capture and process antigens in the periphery and migrate to the draining lymph nodes where they present the antigens to T cells, inducing an antigen-specific response by the adaptive immune system (Banchereau et al., 2000). In general, aging induces a decrease in DC function, such as impaired phagocytic and migration functions, and reduced antigen presentation capacity (Della Bella et al., 2007; Panda et al., 2009; Shaw et al., 2010; Wong and Goldstein, 2013). DCs present a decrease in basal cytokine production, however the TLR–induced cytokine production increases with aging, both in myeloid and plasmacytoid DCs (Agrawal et al., 2007; Panda et al., 2010; Shaw et al., 2013). Regarding the age-related numerical alterations, the literature displays controversial results: while some studies report no alterations in total number, others suggest that aging is

associated with a decrease in the total number of blood DCs, as well as in their myeloid and plasmacytoid subsets (Agrawal et al., 2007; Jing et al., 2009; Orsini et al., 2012).

**NK cells** are cytotoxic cells involved in the innate defense against viral infections and tumor cells. Besides their cytotoxic activity, NK cells express cytokines and chemokines that are required to drive the adaptive immune response (Mocchegiani et al., 2009; Solana and Mariani, 2000). In humans, it has been described an increased frequency of total NK cells ($CD56^+$) in circulation with aging (Jiao et al., 2009; Schindowski et al., 2002; Yan et al., 2010). Human NK cells can be subdivided taking into account the expression of CD56: $CD56^{bright}$ cells (responsible for cytokine production, presenting immunoregulatory functions) and $CD56^{dim}$ NK cells (that have cytotoxic functions) (Chidrawar et al., 2006). Curiously, older individuals present increased levels of $CD56^{dim}$ NK cells and, despite the total stability of NK cytotoxicity, the "per-cell" basis cytotoxicity seems hampered (Almeida-Oliveira et al., 2011; Borrego et al., 1999; Hazeldine et al., 2012; Mocchegiani et al., 2009; Patin et al., 2018; Solana and Mariani, 2000). On the other hand, the number of $CD56^{bright}$ NK cells within the peripheral blood declines with aging, disturbing the communication with the adaptive immune system (Almeida-Oliveira et al., 2011; Chidrawar et al., 2006; Hazeldine et al., 2012). Similarly, in mice, different populations of NK cells ($CD49b^+$) have been characterized based on the expression of CD11b and CD27: R1 NK cells ($CD27^+$ $CD11b^-$) are immature cells; R2 NK cells ($CD27^+$ $CD11b^+$) are intermediate-mature cells; and R3 NK cells ($CD27^-$ $CD11b^+$) are the most mature and cytolytic NK cells (Hayakawa and Smyth, 2006). When comparing to young, aged mice present lower percentages of total NK cells in blood and in spleen, lower percentages of R3 NK cells and higher percentages of R1 NK cells with poor proliferative potential (Nair et al., 2015). NK cells during aging are also characterized by a reduction on cytokines and chemokines production upon cell activation and significantly less perforin release into the immunological synapse (Hazeldine et al., 2012; Mocchegiani et al., 2009).

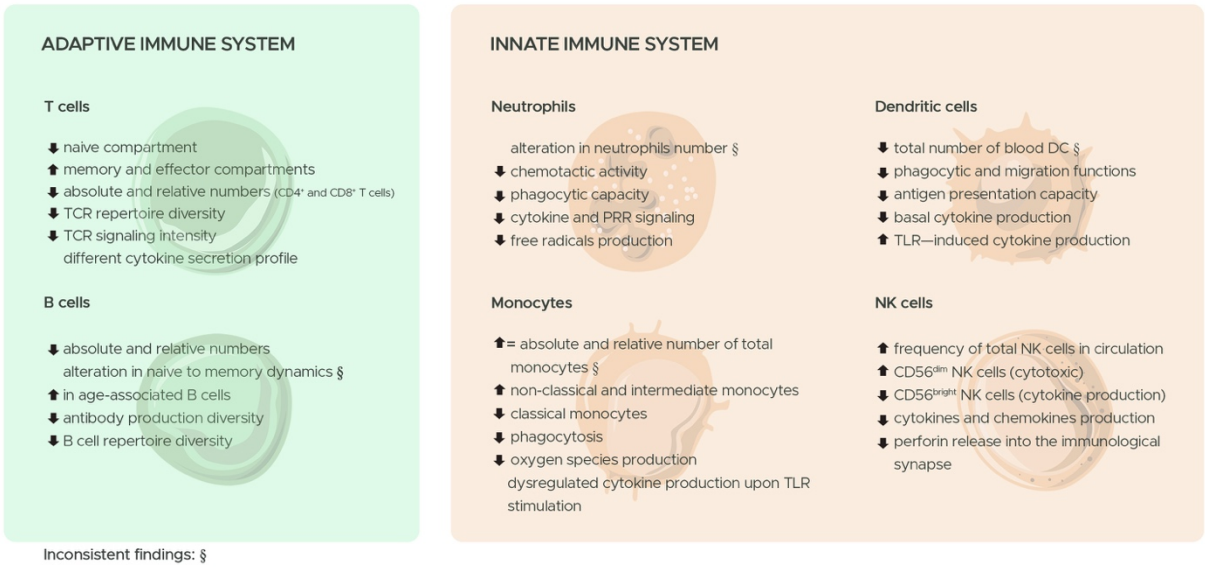


**Figure 3. Age-associated alterations of the immune system.** Functional and frequency of cells (and also absolute numbers) alterations common with aging both in adaptive and innate immune system.

## 3.4. INFLAMMAGING

Besides the age-related cellular alterations, old age is also characterized by a low-grade inflammation, a subclinical inflammatory state much weaker than an acute inflammatory response. This phenomenon has been named "Inflammaging" – a term first described in the early 2000' by Francheschi and colleagues (Franceschi et al., 2000). Although inflammation is essential to mount a proper immune response, inflammaging has been associated with increased mortality and morbidity in the elderly (Bruunsgaard et al., 2003b, 2003a; Reuben et al., 2002). Interleukin (IL)-6, tumor necrosis factor (TNF) and C-reactive protein (CRP) have been the most reported inflammatory mediators shown to present increased levels in blood with age (Bruunsgaard, 2002; Bruunsgaard et al., 2003a; Ferrucci et al., 2005; Franceschi et al., 2017; Hager et al., 1994; Márquez et al., 2020; Puzianowska-Kuźnicka et al., 2016; Wang et al., 2017; Wei et al., 1992). Other inflammatory mediators have also been associated with aging, presenting increased levels in older individuals: IL-1 receptor antagonist (RA), IL-2, IL-8, IL-12, IL-13, IL-15, IL-18, interferon-γ-induced protein-10 (IP-10), interferon (IFN)γ, chemokine C-C motif ligand (CCL)11, soluble TNF receptor (sTNF-R)I and sTNF-RII (Ferrucci et al., 2005; Gerli et al., 2001; Villeda et al., 2011; Wang et al., 2017).

Using diverse approaches, several studies have been addressing the complex process of inflammaging (Cohen et al., 2018). One good example is the work of Morrisette-Thomas and collaborators using samples from the inCHIANTY study where 19 inflammatory markers were

measured in serum (Morrisette-Thomas et al., 2014). This cohort is composed of about 1500 participants with ages ranging from 20 to 102 years , 75% of which being 65+. The authors found several positive correlations with age (hsCRP, IL1-RA, IL-6, IL-15, IL-18, macrophage inflammatory protein (MIP), sTNF-RI and sTNF-RII) and a negative correlation with IL-10 . They also used a principle component analysis to group the inflammatory markers, and one of the components encompassed inflammatory and anti-inflammatory markers and presented a strong correlation with age (Morrisette-Thomas et al., 2014). Hyun Ok Kim and collaborators, measured 22 cytokines (including chemokine and growth factors) in 55 participants <45yo and 55 participants >65yo (Kim et al., 2011). Soluble CD40 ligand (sCD40L) and transforming growth factor alpha (TGF-α) levels were significantly higher in the elderly participants, whereas granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein-1 (MCP-1) levels were significantly lower. No differences were observed in the other cytokines that are usually reported augmented in aged individuals, such as IL-6, TNF and IL-1β. The discrepancies observed between studies may be due to variations in the sensitivity of the assays used, different age ranges in the cohorts and the health status of the participants. Not all studies control for medical conditions that might have an impact on the immune profile of the individuals, such as hypertension and diabetes.

Even though most studies explore the inflammaging phenomenon in humans, some reports in rodents also support the same evidences. Aged mice have increased concentrations of IL-2, IL-1β, TNF and IL-6 (Dugan et al., 2009; Scott et al., 2017).

There are several proposed mechanisms contributing to the low-grade inflammation (reviewed in Deleidi et al., 2015): 1) the increased secretion of inflammatory cytokines and chemokines by senescent cells; 2) hormonal changes; 3) and alterations in mitochondrial and metabolic function in the adipose tissue (knowing that adiposity in excess drives low-grade inflammation and that cytokines can be produced by hypertrophic adipocytes).

### 3.5. SEX-RELATED DIFFERENCES

Sex-related differences in the immune system have been extensively described, both in humans and in some animal models (Giefing-Kröll et al., 2015; Klein and Flanagan, 2016; Piasecka et al., 2018). The sex-differences have been attributed to the possible involvement of sex hormones on the immune system, such as estrogen and androgen; to genetic factors related to the sex chromosomes; and to gender-specific behaviors and exposures (extensively reviewed in Gubbels

Bupp, 2015; Klein and Flanagan, 2016).For instance, females usually present stronger immune responses resulting in a more efficient bacterial clearance and increased susceptibility to autoimmunity (Giefing-Kröll et al., 2015; Klein and Flanagan, 2016; Piasecka et al., 2018). Moreover, the aging of the immune system seems to occur at a higher accelerated rate in males than in females (Márquez et al., 2020; Yan et al., 2010). In an comprehensive study that evaluated genomic (gene expression and chromatin accessibility) and cellular alterations in peripheral blood mononuclear cells (PBMCs) in an healthy adult cohort with ages ranging from 22 to 93 years old, it was demonstrated that even though age-related epigenomic and transcriptomic changes accumulate over time, there were 2 periods during which the immune system abruptly changes (Márquez et al., 2020). Márquez and colleagues demonstrated that the first peak of age-related alterations in the immune system (around late-thirties) is similar in both sexes in terms of timing and magnitude, however the second peak occurs earlier and stronger in men (around early-sixties in men and late-sixties in women) (Márquez et al., 2020). They also reported that the genomic differences between sexes increase after 65 years of age, with men having higher innate and pro-inflammatory activity while women present higher adaptive activity (Márquez et al., 2020).

Furthermore, sexual-dimorphism in age-related changes in blood cell composition have been reported (Abdullah et al., 2012; Márquez et al., 2020; Patin et al., 2018; Strindhall et al., 2013). Men present greater decline in the percentage of $CD4^+$ and $CD8^+$ T cells than women (Márquez et al., 2020; Patin et al., 2018; Strindhall et al., 2013; Wikby et al., 2008; Yan et al., 2010). The relative decrease in naive T cell pool is more pronounced in $CD8^+$ T cells, with women displaying higher relative amounts of naive T cells than men (Márquez et al., 2020; Yan et al., 2010) and men present higher proportion of effector T cells than women (Yan et al., 2010), mostly in younger ages (Márquez et al., 2020). Interestingly, it was also reported that women have higher thymic function than men, and that the decrease of newly exported T cells from the thymus with ageing is more pronounced in men (Clave et al., 2018). This evidence can potentially explain some of the sex differences reported for T cells aging. The CD4/CD8 ratio, which in homeostatic conditions is >1, is higher in women than in men and the proportion of individuals with inverted ratio, which is associated with a chronic viral infection (such as CMV), has also been reported to be greater in men (Strindhall et al., 2013; Wikby et al., 2008). However, the sex-related differences in the different T cell subsets appear not to be consistent in all human cohorts, as other study reported no significant differences in relative and absolute measures in T cells subsets (Saule et al., 2006).

Similarly, Abdullah and collaborators reported that young adult women (20 – 35 years of age) have higher B cells (absolute and relative counts) than men (Abdullah et al., 2012), which was also reported in older individuals (70+ years of age) (Al-Attar et al., 2016). However, other studies report only a male specific decline on B cells after the age of 65 years, while females maintain the proportion of these cells stable over time (Márquez et al., 2020; Strindhall et al., 2013).

Regarding innate immunity, men present more "non-classical" monocytes than women (Hearps et al., 2012), while older women present higher proportions of "intermediate" monocytes than men (Al-Attar et al., 2016). Concordantly, it was also reported that older men have higher epigenetic changes for monocytes and inflammation (Márquez et al., 2020). Young adult men present also higher NK cells counts than women (Abdullah et al., 2012).

## 4. CROSS-TALK BETWEEN SYSTEMIC IMMUNE MEDIATORS AND THE CENTRAL NERVOUS SYSTEM

For decades, the brain was classically viewed as an immune-privilege organ with tolerance to normally rejected tissue grafts in the periphery. Over the last decades the paradigm has shifted and nowadays it is well established that throughout life the brain more than being "immune-privilege", rather "enjoys the privilege" of an immune-dependent maintenance. Research advances in the last decades brought new insights by demonstrating that a variety and heterogeneous population of immune cells, both adaptive and innate, populate the meninges and the choroid plexus (CP), from where they can infiltrate into the brain parenchyma (Brynskikh et al., 2008; Derecki et al., 2011, 2010; Filiano et al., 2016; Korin et al., 2017; Mrdjen et al., 2018; Schwartz and Shechter, 2010).

Interestingly, this holistic view of the body-mind interaction is not a novel concept of modern societies. It remotes to ancient eastern medicine philosophies where traditional Chinese medicine postulated that a vital energy ("qi") flows in specialized routes connecting the body and mind, and Ayurvedic texts from ancient India postulated a similar concepted called "prana" (Pluvinage and Wyss-Coray, 2020).

Understanding how systemic factors (both immune cells and circulating factors – cytokines, chemokines and other plasma proteins) might affect the brain is essential to understand how systemic aging can affect brain function, and therefore affect cognition.

It has been proposed that immune cells and soluble factors in the periphery can interact directly with brain through the brain borders [at the meninges, the blood-brain barrier (BBB) and the Blood-

cerebrospinal fluid (CSF) barrier at the CP], modulating brain function by targeting several cells (neurons, astrocytes, endothelial cells, pericytes, microglia and other glia cells). Proteins from the brain can be then cleared from the parenchyma/CSF back into the periphery through the brain clearance system (the meningeal lymphatics and perivascular glymphatic system). Some of these topics will be further explored.

## 4.1. IMMUNE CELLS AND THEIR ROLE IN THE MAINTENANCE OF BRAIN HOMEOSTASIS

### COGNITIVE FUNCTION

Experimental evidence have been supporting the essential role of immune cells in the maintenance of brain function, focusing mostly on cognitive performance. Studies in animal models showed that the lack of a fully functional immune system affects cognitive performance. For instance nude mice, which are devoid of T cells due a genetic ablation of FOXN1, that is essential for thymic development, and severe combined immune deficiency (SCID) mice, which are devoid of T and B cells, present a significant impairment in the hippocampal-dependent spatial learning and memory in several cognitive tasks (Brynskikh et al., 2008; Kipnis et al., 2004; Ron-Harel et al., 2008). Conversely, by replenishing those mice with T cells (Brynskikh et al., 2008; Kipnis et al., 2004; Ron-Harel et al., 2008), but not with other immune cells (Brynskikh et al., 2008), the cognitive deficits can be reverted. Moreover, other studies have demonstrated that not only a lifelong immune deficiency, which can affect neurodevelopment in young mice, but also an immune deficiency imposition in adulthood is sufficient to cause cognitive deficits. The irradiation of wild-type mice followed by transplantation with SCID-bone marrow induces spatial learning and memory loss, compared to WT-bone marrow transplanted mice (Brynskikh et al., 2008; Ron-Harel et al., 2008). Likewise, acute suppression of T cells using immunosuppressive drugs, currently used in clinical trials or in disease treatment, yielded similar phenotype of cognitive impairment (Derecki et al., 2010). Furthermore, depletion of $CD4^+$ T cells in mice, but not of $CD8^+$ T cells, significantly impairs neurogenesis in the hippocampus and the performance during the reversal learning of MWM (Wolf et al., 2009), which leads to consider that $CD4^+$ T cells are key players in the orchestration between adaptive immunity and cognitive function. Besides the most prevalent T cell population in circulation ($CD4^+$ and $CD8^+$ $\alpha\beta$ T cells), $\gamma\delta$ T cells, another subtype of T cells mainly resident in mucosa, do also infiltrate the meninges from birth, decreasing percentualy over time together with other myeloid cells, oppositely to an increase in the percentage of $\alpha\beta$ T cells (Figure

4) (Mrdjen et al., 2018; Ribeiro et al., 2019). Curiously, the absence of γδ T cells seems also to be associated with short-term spatial memory deficits, in a process suggested to be mediated by IL-17.

It has been proposed that the pro-cognitive properties of T cells are due to non-pathological T cell recognition of CNS-specific molecules. More precisely, transgenic mice expressing mostly TCRs specific to myelin-based protein perform better in MWM than wild type controls. Contrastingly, transgenic mice for ovalbumin-specific TCRs present a worse performance and decreased neurogenesis (Jeon et al., 2016; Ziv et al., 2006). Moreover, cognitive impairment induced by neurotransmitter dysfunctions in animal models can also be reverted boosting T cell activation by vaccination, using copolymer-1, which is a random polymer of four amino acids enriched in myelin based protein (Kipnis et al., 2004). These findings reinforce the theory of a "protective autoimmunity", where it is proposed that T cells that recognize self and CNS-specific molecules are needed to maintain a proper brain function, including cognition (Schwartz and Shechter, 2010). Alterations in homeostasis, for instance caused by a learning associated stress and brain activity, may release brain-derived molecular cues (mediators) into CSF and/or blood that function as a trigger for immune cells to assume a pro-cognitive phenotype (Kipnis et al., 2012). Evidence from human studies also corroborate the importance of T cells in the maintenance of proper cognitive function: immunocompromised individuals (immunodeficiency caused by HIV and chemotherapy) present cognitive dysfunction, which is reverted when the levels of immune cells return to normality (Cysique et al., 2009; Hess and Insel, 2007; Kipnis et al., 2008). However, care should be taken in the interpretation of these results due to the potential confounding factors inherent to the health state of the individuals.

It has been proposed that the peripheral immune modulation or the performance of a cognitive paradigm induces substantial immunological changes in the brain borders, mainly in the meninges. A constant intracranial immune surveillance and a supportive immune response within the brain's barriers are essential to maintain brain homeostasis (Ron-Harel et al., 2011; Schwartz and Shechter, 2010). The pharmacological inhibition of T cells migration into the CNS in mice, which results in the elimination of T cells from the meningeal spaces, induces cognitive deficits similar to that observed upon peripheral depletion of T cells (Derecki et al., 2010). Additionally, the depletion of T cells from the meningeal spaces drastically changed the profile of meningeal myeloid cells toward a pro-inflammatory profile (IL-12 and/or TNF increased production), similarly to what happens in SCID mice (Derecki et al., 2010). After challenging mice with a cognitive task,

recruitment of activated $CD4^+$ T cells, mainly with an IL-4-production phenotype (but not $CD8^+$ T cells) to the meninges occurs. No differences are observed with respect to IFNγ-producing T cells (Derecki et al., 2010). Likewise, the absence of IL-4, both constitutively and specifically on T cells, induces cognitive deficits (Derecki et al., 2010), while the absence of IFNγ enhances cognitive performance and promotes hippocampal plasticity (Monteiro et al., 2016). The intravenous injection of "alternatively" activated M2 myeloid cells (previously stimulated in *in vitro* with IL-4 and characterized by the production of IL-10 and by the expression of CD206) into mice devoid of T cells, resulted in the improvement of learning and memory and the reverse of pro-inflammatory meningeal phenotype (Derecki et al., 2011). Apparently, the modulatory effect of immune cells is not exclusive to T-cell dependent mechanisms, widening the possibility for potential therapeutic targets.

Although not explored in this chapter, the immune system "dysbiosis" affects not only cognitive performance but also a broad spectrum of behaviors. For instance, mice with a deficiency in adaptive immunity (SCID mice) exhibit a social deficit and a hyper-connectivity of fronto-cortical brain regions in a process that appears to be modulated by IFNγ, demonstrating that meningeal immunity is also critical for social behavior (Filiano et al., 2016). Interestingly, it was recently found that meningeal γδ T cells are important regulators of anxiety-like behavior, in a process partially mediated by IL-17. The absence of meningeal γδ T cells was associated with the absence of the natural and basal anxious behavior in mice (Alves de Lima et al., 2020).

## AGE-RELATED COGNITIVE DEFICITS

Brain aging is caused by a deviation from homeostasis, when cells become less capable to cope and respond to stress and cell damage. It was hypothesized that the age-related brain impairment can be partially attributed to immunosenescence (Ron-Harel and Schwartz, 2009). The rational is that immune alterations with aging results in a defective CNS immunosurveillance leading to a reduced ability to cope and restore brain homeostasis (Schwartz and Shechter, 2010).

Modulating and changing the interactions of the immune system with the aged brain seems to have the potential to alleviate the age-associated cognitive decline. For instance, the intracerebroventricular transfer of activated group 2 innate lymphoid cells (ILC2), which are innate effector cells resembling T cells that do populate both CP and meninges (Fung et al., 2020; Gadani et al., 2017), alleviates age-associated cognitive decline in mice (Fung et al., 2020). A phenotype that is replicated by the administration of IL-5, a major ILC2 product (Fung et al., 2020). Ron-Harel

and colleagues also suggest that mice age-related spatial memory loss could be partially restored by boosting peripheral T-cells activation through homeostatic-driven proliferation (Ron-Harel et al., 2008).

The profile of peripheral immune cells has been also correlated with cognitive aging. Interestingly, results from our group found that circulating EM $CD4^+$ T cells are augmented in blood of elders with worse cognitive performance. EM $CD4^+$ T cells, which gradually increase with aging (Saule et al., 2006), were found to be significantly associated with memory and executive function, even controlling for education level, age, sex and mood (Serre-Miranda et al., 2015). However, a causal effect and the precise mechanism through which those cells impact on cognitive deterioration remains unknown. Similarly, demented individuals diagnosed with probable AD present significantly lower levels of $CD4^+$ naive T cells ($CD45RA^+$) and an increase in the activated/naive $CD4^+$ T cell ratio ($CD45RO^+/CD45RA^+$) compared with age-matched cognitively healthy individuals (Tan et al., 2002). Another multicohort study revealed that PBMCs from AD and mild cognitive impairment patients present increased numbers of $CD8^+$ T effector memory $CD45RA^+$ ($T_{EMRA}$) cells, which were negatively associated with cognition, when compared to healthy controls (Gate et al., 2020). This association between the peripheral activation profile of T cells and cognitive aging lead us to speculate about the use of peripheral immune system as an early detection tool for malfunctions of the brain and a potential treatment vehicle.

Hippocampal neurogenesis is a neuroplasticity mechanisms strictly linked with memory and learning that gradually decreases with aging (Galvan and Jin, 2007). As stated previously, neurogenesis is highly dependent on peripheral immune cells (Wolf et al., 2009; Ziv et al., 2006), being speculated that age-related neurogenesis reduction might be an outcome of, among other factors, reduced immune surveillance or an over powering local inflammation in the elderly (Ron-Harel and Schwartz, 2009). In fact, clonally expanded IFNγ-producing $CD8^+$ T cells can be found in aged subventricular zone, a neurogenic niche, possible contributing to the impaired neurogenesis observed in aged individuals (Figure 4) (Dulken et al., 2019). Similarly, in a neurodegenerative mouse model (tau transgenic mice), $CD8^+$ T cell infiltration was also observed in the hippocampus, where another neurogenic niche is located, and the depletion of T cells reverted the age-associated spatial memory deficits (Laurent et al., 2017).

It is widely accepted by the scientific community that the brain, to maintain its normal function, needs a constant immunosurveillance. However, the precise signals that trigger immune cells to infiltrate the CNS and affect its normal function remains to be elucidated. Curiously, this year, Gate

and collaborators demonstrated that clonally expanded CD8⁺ $T_{EMRA}$ cells specific to two separate Epstein-Barr virus antigens patrol the CSF in AD patients (Gate et al., 2020). This discovery provided evidence that antigen-experienced T cells patrol the intrathecal space of brains affected by age-related neurodegeneration and that viral infections might be a potential trigger to their infiltration into the CSF and undertake detrimental functions.

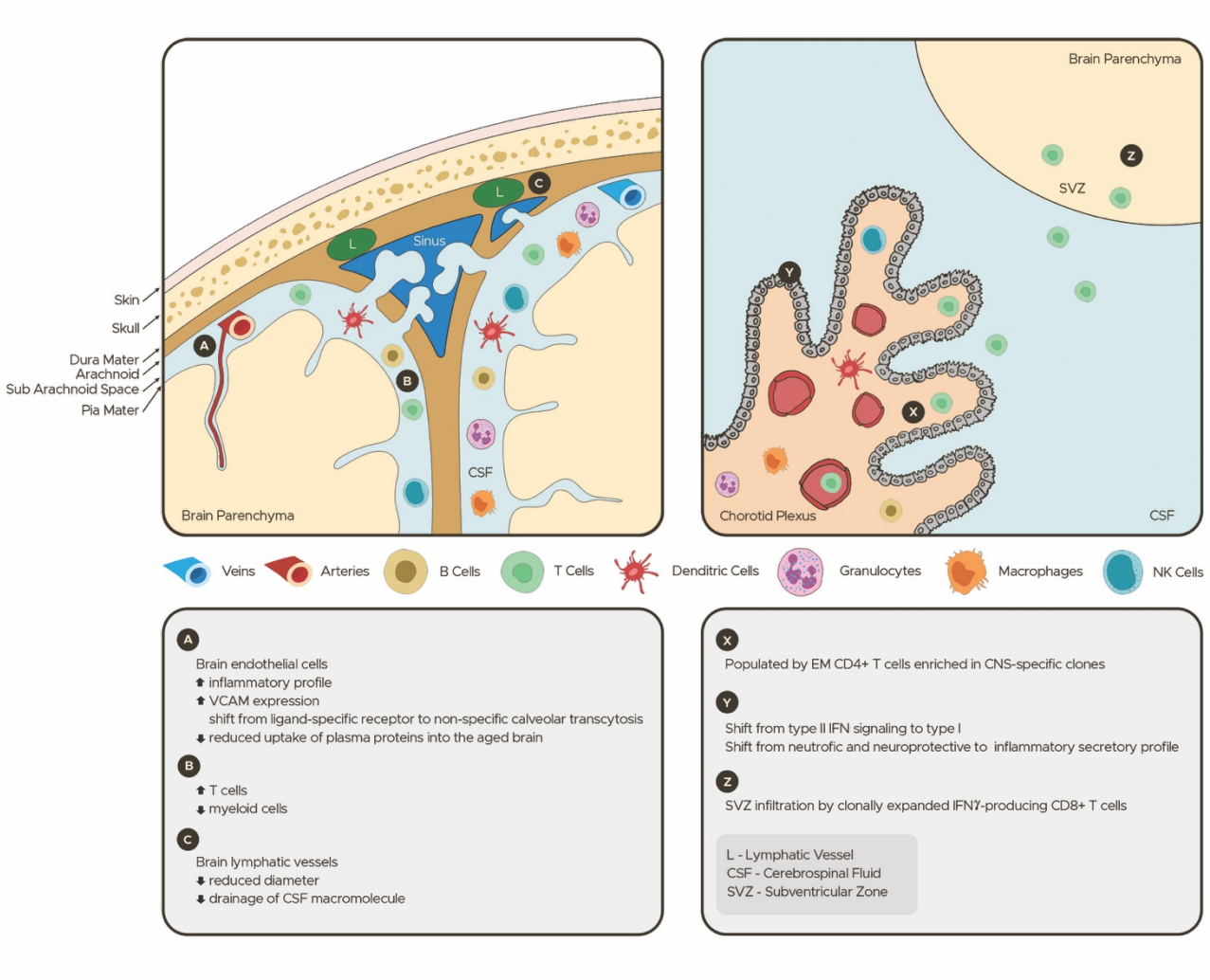


**Figure 4. Neuro-immune communication in the brain borders at old age.** Schematic representation of some described neuro-immune interactions in the brain borders (meninges, upper left panel, and choroid plexus, upper right panel) at old age. Aging affects the capacity of immune mediators to reach the brain (A), the proportion of cells circulation in the CSF get altered (B), the drainage capacity of meningeal lymphatics get impaired (C), CNS-specific T cells populate the choroid plexus (X), whose inflammatory and secretory profile shifts from a neurotrophic and neuroprotective to inflammatory (Y), and clonally expanded T cells infiltrate the brain parenchyma (Z).

## 4.2. BLOOD SOLUBLE FACTORS THAT AFFECT COGNITIVE FUNCTION

### INFLAMMAGING

The low-grade inflammation usually observed in elderly, also referred as inflammaging, has been associated with inter-individual cognitive diversity and cognitive decline, being IL-6 and CRP the two most explored markers (Baune et al., 2008; Heringa et al., 2014; Serre-Miranda et al., 2020; Trollor et al., 2012). Elevated concentrations of peripheral IL-6 have been commonly associated with a worse cognitive outcome (Mooijaart et al., 2013; Tegeler et al., 2016; Trollor et al., 2012; Yaffe et al., 2003), mainly with measures of executive function, and not of memory (Mooijaart et al., 2013; Tegeler et al., 2016; Trollor et al., 2012). Yet, some authors reported no association between serum/plasma concentrations of IL-6 and cognitive performance in non-demented senior individuals (Baune et al., 2008; Dik et al., 2005). Similarly, CRP, an acute phase protein vastly associated with an increased risk for cerebrovascular disease, AD and vascular dementia (Schmidt et al., 2002), has been related with cognitive deterioration in elderly. While some studies describe no association between levels of CRP and cognitive performance in healthy senior individuals (Dik et al., 2005; Lin et al., 2018; Trollor et al., 2012), others demonstrate an association between higher levels of CRP and worst cognitive performance (Ravaglia et al., 2005; Tegeler et al., 2016; Wersching et al., 2010; Yaffe et al., 2003). As evidenced here, epidemiological studies exploring the relationship between immune mediators and cognition have often produced mixed and inconsistent results. Some of the inconsistencies observed among studies might be caused by differences in selection criteria and cohorts' characterization. The age ranges, sample sizes, cognitive evaluation tools used as well the concentration range of the immune molecules vary greatly among studies, and not all studies check or control for possible active inflammatory processes or for the intake of anti-inflammatory drugs (Baune et al., 2008; Lekander et al., 2011). Other immune molecules have also been negatively associated with normal age-related cognitive decline (non-dementia). These include IL-12 (Trollor et al., 2012), IL-10 (Tegeler et al., 2016), IL-1β (Simpson et al., 2013), IL-8 (Baune et al., 2008; Goldstein et al., 2015) and haptoglobin (Teunissen et al., 2003), evidencing that an overall inflammatory profile and a deviation from homeostasis are implicated in the relation between these two phenomena. Unfortunately, the majority of studies explore a limited number of inflammatory molecules.

A large study reported that the low-grade inflammation, based on a composite score composed by CRP, IL-6, TNF, IL-8, serum amyloid A (SAA), MPO (myeloperoxidase) and sICAM-1, is associated

with worse performance on information processing speed and attention and executive function, but not with memory, in senior individuals (Heringa et al., 2014). One of the aims of this thesis pertains with the comprehensive characterization of peripheral immune molecules in relation to cognitive performance in senior individuals (Chapter IV)

## OTHER PLASMA PROTEINS

Besides cytokines and chemokines, other circulatory factors, like other plasma proteins and metabolites (microbial and metabolic by-products), have been documented to increase or decrease during aging. Some of them can even contribute to the low-grade inflammation frequently observed in older individuals. Those factors, also referred as chronokines (plasma proteins that increase or decrease as we age), represent another potential link between peripheral aging and the CNS aging (Wyss-Coray, 2016).

Drastic changes in plasma proteome with aging have been documented by several researchers (Johnson et al., 2020). A systematic review analysis revealed that a subset of human proteins have been consistently reported to increase during aging, being heavily related in processes relevant to inflammation, extracellular matrix and gene regulation, and a proportion of them have relevant connections to aging and age-related disease (Johnson et al., 2020). A group of those proteins can, indeed, be used as an aging clock, predicting accurately biological age (Johnson et al., 2020; Lehallier et al., 2019). Some of the aging-related proteins were also found to be conserved between humans and mice, reinforcing that some biological pathways are conserved among species and increasing the degree of confidence when using mouse models to study aging phenomena (Lehallier et al., 2019).

Recent studies using heterochronic parabiosis models, where old and young mice share their circulatory system, have shed some light on the potential rejuvenation capacity of young blood. Blood and/or plasma from old mice impair spatial learning and memory, as well as neurogenesis and synaptic plasticity, in young animals (Rebo et al., 2016; Villeda et al., 2011). Conversely, blood from young animals reverse age-related impairments (Castellano et al., 2017; Katsimpardi et al., 2014; Villeda et al., 2014, 2011). The human young plasma has also been tested in clinical trials as a potential treatment for AD. Its safety was already reported (Sha et al., 2019), and a phase 2 clinical trials was conducted using a plasma fraction without clotting factors or immunoglobulins (GRF6019) to improve its safety and efficacy treating Alzheimer's Disease patients (ClinicalTrials.gov Identifiers: NCT03520998 and NCT03765762). However, the rejuvenation

effect of young blood is not limited to the brain. Youthful circulating factors have the capacity to reverse aspects of aging in multiple tissues/organs, such as bone, skeletal muscle, liver, kidney, heart and pancreas (Baht et al., 2015; Conboy et al., 2005; Huang et al., 2018; Loffredo et al., 2013; Salpeter et al., 2013; Sinha et al., 2014).

The regenerative properties of young plasma might be mediated by several soluble proteins, which has been the focus of interest of several researchers. The discovery of the so desired "fountain of youth", the mythical spring that populated several story tales and rejuvenates anyone that drinks or baths on its waters, is maybe closer than we thought and resides within our own blood, as proposed by several researchers. The identification of the factors that mediate this beneficial or detrimental effect of human blood on brain aging, and consequently on cognitive function, constitute a possible therapeutic target to revert some of the detrimental effects of aging. In fact, some researchers have already demonstrated that specific mouse and human proteins recapitulate the effects of (young and aged) plasma on brain function. They can present either a pro-aging effect, such as B2M (beta2-microglobulin, a non-polymorphic subunit of the type 1 major histocompatibility complex) and CCL11 (eotaxin 1, an eosinophil-recruiting chemokine), which are increased in aged mice and humans (Smith et al., 2015; Villeda et al., 2011; Villeda and Wyss-Coray, 2013), or an anti-aging (or pro-youth) action, such as TIMP2 (tissue inhibitor of metalloproteinase 2) and CSF2 (colony-stimulating factor 2), enriched in human cord plasma and young mouse plasma (Castellano et al., 2017), GDF11 (growth differentiation factor 11, member of the transforming growth factor beta family) (Katsimpardi et al., 2014), osteocalcin (Khrimian et al., 2017; Pluvinage and Wyss-Coray, 2020).

### 4.3. HOW DO SYSTEMIC FACTORS AFFECT (AGED) BRAIN FUNCTION?

Five potential communication pathways between the central nervous and immune systems have been proposed: 1) passage through leak regions in the BBB, such as the circumventricular organs; 2) active transport through saturable molecules; 3) activation of cells lining the cerebral vasculature, such as endothelial cells and perivascular macrophages; 4) binding to cytokine receptors associates with peripheral afferent nerves, such as the vagus nerve; and 5) recruitment of activated cells to the brain where they can produce cytokines (to explore more this issue read Bodles and Barger, 2004; Haroon et al., 2012; Wilson et al., 2002). Some of these potential pathways will be further explored in this section.

Once cytokine signals reach the brain, they can act through a rich cytokine network comprised by several cellular populations, including astrocytes, microglia and neurons, which themselves produce cytokines and express cytokine receptors (Miller et al., 2009). In the brain, the immune mediators can modulate brain function, which will ultimately influence behaviour, by altering neurotransmitters metabolism (Miller et al., 2013), impacting on neuronal plasticity, such as neurogenesis (Borsini et al., 2015), affecting neuroendrocrine function (Correa et al., 2007) and the neurocircuitry (Miller et al., 2013).

## BLOOD-BRAIN BARRIER

The brain vasculature has two paradoxical functions: it is essential to supply nutrients and oxygen to one of the most metabolic active organs of the body, but also to function as a barrier by blocking peripheral inflammatory insults and deleterious molecules to reach the brain. The neurovascular unit it is composed by endothelial cells, pericytes, astrocyte end-feet and neurons, and is the fundamental building block of the BBB (McConnell et al., 2017). BBB, that was initially viewed as a simple passive diffusion barrier, is now known as a fundamental mediator of the peripheral-central interaction that has a spatiotemporal heterogeneity (Pluvinage and Wyss-Coray, 2020). Therefore, alterations on its function affect how peripheral mediators modulate brain function.

As stated previously, the aged circulatory environment can impair cognition, reduce neural precursor activity and activate microglia in mice (Pluvinage and Wyss-Coray, 2020; Villeda and Wyss-Coray, 2013; Wyss-Coray, 2016). However, it is not yet fully elucidated how circulating factors modulate brain function considering the tight regulation of transport and permeability of macromolecules across the BBB (Engelhardt and Liebner, 2014). Theoretically, some of those effects can be partially mediated by brain endothelial cells (BECs). It was demonstrated that BECs in aged mouse hippocampus present an inflammatory transcriptional profile and upregulation of vascular cell adhesion molecule 1(VCAM1), a protein essential for the vascular-immune interaction (Figure 4). The authors have also shown that aged human and mouse plasma, which has great amounts of soluble VCAM1, is sufficient to increase VCAM1 expression in cultured BECs in hippocampi of young mice. Interestingly, the detrimental effects of aged plasma (activation of microglia and inhibition of hippocampal neurogenesis) can be reverted by blocking the action of soluble VCAM1. So, the VCAM1 expression on BECs, at some extent, mediate the detrimental effects of aged plasma on the brain and can act as a potential target to treat age-related neurodegeneration (Yousef et al., 2019). Interestingly aged and young plasma have the ability to

mimic and reverse BECs signatures of normal aging, respectively, demonstrating that they are sensitive sensors of age-related circulatory cues (Chen et al., 2020).

Another possible mechanism is the transcytosis, a type of transcellular transport, of plasma proteins. In a healthy brain, plasma proteins readily permeate the parenchyma, however the uptake of plasma proteins in the aged brain is diminished, driven by an age-related shift in transport from ligand-specific receptor-mediated to non-specific caveolar transcytosis, altering the type of proteins that can cross the BBB (Figure 4) (Yang et al., 2020).

### BLOOD-CSF BARRIER

The CP, a highly vascularized epithelial tissue present in the four brain ventricles, is the place where the most intimate contact between the CNS and blood occurs. It is responsible for the CSF production and consequently an important information transducer from the periphery to CNS (Baruch and Schwartz, 2013; Marques et al., 2009).

Throughout aging the CP is populated by effector memory $CD4^+$ T cells with a TCR repertoire enriched for CNS antigens (Figure 4). However, there is a shift in their profile in favour of Th2 response mediated by IL-4 (Baruch et al., 2013). CP also faces unavoidable alterations during aging. It experiences a shift from type II interferon (IFN-II) to type I (IFN-I) signalling, from a neurotrophic and neuroprotective profile to a proinflammatory secretory profile (Figure 4) (Baruch et al., 2014). Aged mice present increased expression of IFN-I at the CP and higher protein amounts at the CSF, while the expression of IFN-II at the CP decreases with aging (Baruch et al., 2013; Da Mesquita et al., 2015). Blocking IFN-I signalling within the aged brain partially restores age-related cognitive dysfunction and hippocampal neurogenesis and the IFN-II-dependent CP activity (Baruch et al., 2014), which seem to be essential for the CNS immunosurveillance and repair (Kunis et al., 2013). Curiously, in a mouse model of AD this shift from IFN-II to IFN-I signalling profile of the CP appears earlier (Da Mesquita et al., 2015).

## 4.4. FROM BRAIN SOLUBLE FACTORS (AND CELLS) CLEARANCE TO IMMUNE SURVEILLANCE

Even going back 200 years to first reports of putative existence of brain lymphatic system, only recently, and due the technological advance, the function and structure of a meningeal lymphatic system was demonstrated (Aspelund et al., 2015; Da Mesquita et al., 2018a; Louveau et al., 2015). This breakthrough discovery is now challenging the understanding of fluid drainage from

the brain, which seems to be conserved among species. Similar structures were also found in post mortem human meninges and visualized *in vivo* through neuroimaging techniques in human and non-human primates, in fish and rats (Absinta et al., 2017; Bower et al., 2017; Jung et al., 2017; Louveau et al., 2015).

This extensive lymphatic network has been discovered in the dura mater (the outer meningeal layer) along with the transverse and sagittal sinuses, exiting together with the jugular vein (Aspelund et al., 2015; Louveau et al., 2015). The meningeal lymphatic system, together with the perivascular glymphatic system (Iliff et al., 2012; Raper et al., 2016), are two of the major clearance pathways of the CNS. They are essential to balance the influx and efflux of CSF and interstitial fluid (ISF), which are extracellular fluid compartments within the CNS essential to wash out most of systemic factors, waste products, cytokines and other molecules from neurons and glia (Da Mesquita et al., 2018a; Raper et al., 2016). CSF baths the brain occupying the ventricles and the subarachnoid space, a space between the two most inner meningeal layers - the arachnoid mater and the pia mater, while ISF perfuses the brain parenchyma. The interaction between these two systems, in health and disease, was already extensively reviewed elsewhere (Da Mesquita et al., 2018a; Louveau et al., 2016).

Considering an immunological function of the meningeal lymphatic system, it has been proposed that the immune surveillance of the CNS and the lymphatic collection of antigens from the CNS is an active part to maintain brain homeostasis (Papadopoulos et al., 2020). The priming of immune cells toward the brain-related molecules relies on the capacity of antigens to move from brain parenchyma to the CSF/ISF to lymph nodes [in this case to deep cervical lymph nodes – dCLN (Aspelund et al., 2015; Louveau et al., 2015)] where peripheral cells can be primed (Papadopoulos et al., 2020). Interestingly, meningeal lymphatic vessels can efficiently drain both immune cells and molecules from the subarachnoid space into the dCLN (Aspelund et al., 2015; Louveau et al., 2015). The surgical removal of dCLN interrupt the normal flow of T cells from the meninges to the lymph node, resulting in an increased number of meningeal $CD4^+$ T cells and an impairment in the cognitive performance compared to sham-operated mice (Radjavi et al., 2014). More recently it was also shown that pharmacological ablation of meningeal lymphatics in young mice is sufficient to induce cognitive deficits. By contrast, by enhancing meningeal lymphatic drainage in aged mice, which was demonstrated to be impaired (Figure 4), improved learning and memory performance (Da Mesquita et al., 2018b).

The meningeal lymphatic system is a structural link between the “protective autoimmunity” theory and cognitive function. However, the precise mechanisms and molecules that prime immune cells to be recruited to the brain borders (or even brain parenchyma) with the capacity to modulate cognitive function, needs to be further elucidated.

## THESIS AIMS

Given the state of the art at the launching of this project, we hypothesized that, in physiological aging, alterations in the profile of immune mediators, either cellular or plasma-molecules, are associated with age-related cognitive heterogeneity. To contribute for a better understanding of the crosstalk between immune mediators and healthy cognitive aging the following aims were established:

**AIM1. Dissect the cellular composition of peripheral blood in male and female mice during aging.** Despite the widespread use of mouse models to study immune system aging and its interplay with several other systems, there was scarce information regarding the detailed evolution of the blood immune cellular composition during aging in this animal model.

**AIM2. Determine how the inter-individual differences in cognitive function at old age correlate with age-associated alterations of the immune system, using a longitudinal study design in mice.** Cognitive heterogeneity has been attributed to several factors. However, the precise association between the aging of the immune cell populations and cognitive performance was poorly characterized/studied.

**AIM3. Determine how plasma immune molecules correlate with age-related cognitive heterogeneity in healthy senior individuals.** The association between peripheral low-grade inflammation and cognitive performance was already described by several authors. However, most studies focused on a reduced set of immune molecules. A comprehensive and broader characterization of the peripheral inflammatory profile, was missing.

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# CHAPTER II

## LONGITUDINAL EVALUATION OF PERIPHERAL IMMUNE SYSTEM IN MICE: THE MODERATION EFFECT OF SEX

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# LONGITUDINAL EVALUATION OF PERIPHERAL IMMUNE SYSTEM IN MICE: THE MODERATION EFFECT OF SEX


## ABSTRACT

Mouse models have been widely used as surrogates to understand the physiology of aging, including that of the immune system. Interestingly, while the sexual dimorphism in humans has been broadly characterized, the impact of sex in the immune system of animal models, particularly during aging, is poorly studied. Here we describe the longitudinal evaluation of male and female Balb/c mice with respect to the main populations of both innate and adaptive immune system, assessed by flow cytometry. To overcome listwise deletions due to the loss of animals during the course of the 18 months evaluation, linear mixed models analysis was used to account for missing values. The percentage of neutrophils, monocytes, eosinophils, NK cells $Ly6C^-$ increase with aging; while that of NK cells $Ly6C^+$, B and T cells (including $CD4^+$ and $CD8^+$ subsets) decrease. Interestingly, males present overall higher percentages of neutrophils and activated monocytes $Ly6C^{high}$ compared to females. By contrast, females present higher percentages of total T cells, both $CD4^+$ and $CD8^+$ T cells, as well as of eosinophils and NK cells. Both sexes display similar percentages of B cells, even though their progression over time differs.

Altogether this study dissects how aging promotes an imbalance towards the innate immunity. It also reveals that sex moderates the relation between the proportion of cells and age. These observations further strengthen the need to use both males and females when addressing the immune system in animal models.


**Keywords:** immune system; aging; immunosenescence; sex; innate immune system; adaptive immune system; linear mixed models.

# 1. INTRODUCTION

The increasingly aged population challenges the scientific community to better understand the physiology of aging. One of the central body systems, given the myriad of interactions, is the immune system. Understanding how the immune system ages, and how the process influences and is influenced by age is therefore of great relevance.

Most publications addressing the senescence of the immune system, also known as immunosenescence, study changes in cell function, such as the reduction in antigen presentation and of phagocytic capacity in the innate immune system (Della Bella et al., 2007; Mocchegiani et al., 2009; Panda et al., 2009; Shaw et al., 2013, 2010). Additionally, relevant features of aging of the adaptive immune system are the reduction on T and B repertoire diversity and the increase of the memory and activated cells in comparison to naïve cells (Britanova et al., 2014; Frasca et al., 2008; Nikolich-Žugich, 2014; Saule et al., 2006; Thome et al., 2014). In addition, the relative and absolute numbers of T cells, of both the $CD4^+$ and $CD8^+$ subsets, have been shown to decrease over time in humans (Jiao et al., 2009; Patin et al., 2018; Saule et al., 2006; Wikby et al., 2008). Similarly, B cells experience functional alterations and a decrease in both cell numbers and frequency among leukocytes with aging (Frasca et al., 2008; Jiao et al., 2009; Muggen et al., 2019).

The cumulative alterations in the immune system over time ultimately lead to increased susceptibility to infection, increased predisposition to autoimmune disorders, reduced response to vaccination, among other morbidities (Bupp et al., 2018; Klein and Flanagan, 2016; Nikolich-Žugich, 2018). Of interest, men and women experience those alterations differently, being the immune response typically stronger in women than in men (Giefing-Kröll et al., 2015; Klein and Flanagan, 2016; Piasecka et al., 2018). Similarly, in other species (insects, reptiles, mammals) the immune response is also varies among males and females (Klein and Flanagan, 2016). Studies in humans have further denoted sexual-dimorphism in age-related changes in blood cellular composition, however information on other animal models is still scarce (Abdullah et al., 2012; Márquez et al., 2020; Patin et al., 2018; Strindhall et al., 2013).

Mouse models are widely used to study aging due to their short lifespan, and the immune system given its similarities with humans. Most published reports in immunosenescence compare young vs aged mice, with a cross-sectional experimental approach, not following the same mice longitudinally throughout aging. Here we performed the first longitudinal study in a mouse model

to dissect the individual progressions on the frequency of the main adaptive and innate immune cell populations.

## 2. METHODS

### 2.1. ANIMALS

Male and female BALB/cByJ mice, 6 weeks old, were purchased from Charles River and maintained until 18 months of age at the Life and Health Sciences Research Institute (ICVS) animal facility. Mice were initially housed in groups of 5 per cage and males were re-grouped whenever aggressive behaviors were observed; under standard laboratory conditions (light/dark cycle of 12/12 h; 22 °C; 55% humidity); food and water *ad libitum*. Health monitoring was performed according to FELASA guidelines, confirming the Specified Pathogen Free health status of sentinel animals maintained in the same animal room. To avoid excessive and unnecessary suffering of animals, humane endpoints were applied; mice were sacrificed when reaching 20% weight loss and whenever the overall well-being was compromised (wounds from aggressions, appearance of tumors). At the end of the study, mice were anesthetized with a mixture of ketamine (75 mg/kg, intraperitonealy [i.p.]) and medetomidine (1 mg/kg, i.p.) and were transcardially perfused with 0.9% saline. All procedures were carried out in accordance with European Regulations (European Union Directive 2010/63/EU). The animal facility and people directly involved in animal experiments were certified by the Portuguese regulatory entity—Direção Geral de Alimentação e Veterinária (DGAV). All experiments were approved by the Ethics Committee of the University of Minho. The experiments were also authorized by the national competent entity DGAV (#009458).

### 2.2. FLOW CYTOMETRY

Peripheral blood was collected from a small incision on the tail to heparin coated capillaries. Samples were longitudinally collected from the same animals approximately at 3, 6, 10, 12, 15 and 18 months of age. Three independent experiments (set1, set2 and set3) were performed and the number of mice used is listed in Table 1. A total sample size of 211 mice (approximately 35 mice per sex/per experiment) was previously determined using G*Power 3.1.2 and assuming a medium effect size (f(V)=0.25), an alpha of 0.05, a statistical power of 0.8, two groups with six measurements. Fifty microliters of blood were incubated for 20 min at room temperature (RT) in the dark with the following combination of antibodies: anti-mouse CD49b FITC (clone: DX5); anti-mouse CD62L PE (clone: MEL-14); anti-mouse CD19 PercpCy5.5 (clone: 6D5); anti-mouse CD3

PECy7 or APC (clone: 145-2C11); anti-mouse CD44 APC or BV605 (clone: IM7); anti-mouse CD4 BV421 (clone: RM4-5); anti-mouse CD8 BV510 (clone: 53-6.7); anti-mouse Ly6G BV650 (clone: 1A8); anti-mouse Ly6C BV711 (clone: HK1.4) and anti-mouse CD45.2 BV785 or PECy7 (clone: 104) [all from Biolegend, San Diego, CA, USA]. Erythrocytes were then lysed with ammonium–chloride–potassium (ACK) buffer (0.15M NH4CL, 10mM KHCO3, 0.1 mM Na2EDTA) for 10 min at RT in the dark. Samples were washed twice with FACS buffer (0.5% BSA and 0.01% sodium azide in PBS) and centrifuged at 300g for 5 min at RT. Samples were acquired (minimum of 100000 events among leukocytes/sample) on a BD LSRII flow cytometer using the FACS DIVA software (BD Biosciences). Data was analyzed using FlowJo software (BD Biosciences) version 10.6.1. The used gating strategy is represented in Supplementary Figure 1 and 2. Due to an atypical leukocyte profile 4 females were excluded from the analysis (1 from set1, 1 from set2 and 2 from set3).

**Table 1.** Number of mice analyzed at each timepoint.

| Timepoint (months) | SET1 | | SET2 | | SET3 | | TOTAL | | |
|---|---|---|---|---|---|---|---|---|---|
| | Males | Females | Males | Females | Males | Females | Males | Females | Both |
| 3 | 35 | 34 | 35 | 35 | 39 | 40 | 109 | 109 | 218 |
| 6 | 35 | 35 | 35 | 35 | 35 | 39 | 105 | 109 | 214 |
| 10 | 31 | 35 | 35 | 34 | 32 | 39 | 98 | 108 | 206 |
| 12 | 24 | 32 | 31 | 33 | 26 | 38 | 81 | 103 | 184 |
| 15 | 22 | 32 | 25 | 31 | 20 | 38 | 67 | 101 | 168 |
| 18 | 15 | 31 | 18 | 29 | 15 | 37 | 48 | 97 | 145 |

## 2.3. STATISTICAL ANALYSIS

Linear mixed models for repeated measures were used to explore the progression/trajectory of the main cell populations of the peripheral immune system over time/during aging, and to dissect the impact of sex in that progression. This approach prevented listwise deletion due to missing data (considering that some mice died during the course of the 18 months evaluation, as shown in Table 1). The survival rate in all sets combined was 89% for females and 44% for males, comprising an overall survival rate of 67%. All the analyses were performed with results from the 3 sets combined.

The relation between time and percentage of cells can be either linear or quadratic. To account for that, time was introduced in the models either as a linear variable (Time) or as a quadratic variable by calculating the time square ($Time^2$). To avoid collinearity, time was centered to the mean (cTime)

and only then computed $cTime^2$. cTime, sex, cTime x sex (interaction), $cTime^2$ and cTime2 x sex (interaction) were used as fixed factors. To analyze the changes in the percentages of immune cells over time taking into account the within-subject correlations between the percentages of immune cells, a random intercept per subject was introduced in the model. Models were tested for various covariance structures (level 1: repeated covariance type and level 2: random covariance type) and the pair with the lowest Akaike's Information Criteria was used. The estimation method used was the restricted maximum likelihood.

To account for inter-experiments variability, the variable "sets" was also included as covariate in the models (results in supplementary information). Additionally, the same models were tested with standardized measures: z-scores were computed considering the mean of each population for all the timepoints at each set independently (data not shown). Death rate was 50% on males and 11% on females in all sets; to assess the possible bias caused by missing data (mice that died before the last timepoint), the same analysis were performed only with survivors.

The statistical procedures were performed in IBM SPSS Version 25 (IBM Corp, USA) and the graphs were designed using Prism7 (GraphPad Software, USA).

## 3. RESULTS

To explore the age-related alterations on the proportion of immune cells, the main circulating cells of the innate immune system were evaluated: eosinophils, NK cells ($Ly6C^-$, less mature cells, and $Ly6C^+$, more mature phenotype/memory cells (Cerwenka and Lanier, 2016; Omi et al., 2014)), neutrophils and activated monocytes (expressing $Ly6C^{high}$). The proportion of circulating innate immune cells varies with age (eosinophils, NK cells $Ly6C^-$, neutrophils and activated monocytes increase, while NK cells $Ly6C^+$, decrease), being in most of cases moderated by sex. Specifically:

1) The percentage of eosinophils (Figure 1A and Table 2) increases over time (B[cTime]=0.225, indicating that eosinophils increase 0.225% per month) almost exclusively on females (B[cTime * Sex]= -0.183; if males 0.225 - 0.183= 0.042); with males presenting lower percentages than females (B[sex]= -0.889, reference category is females).

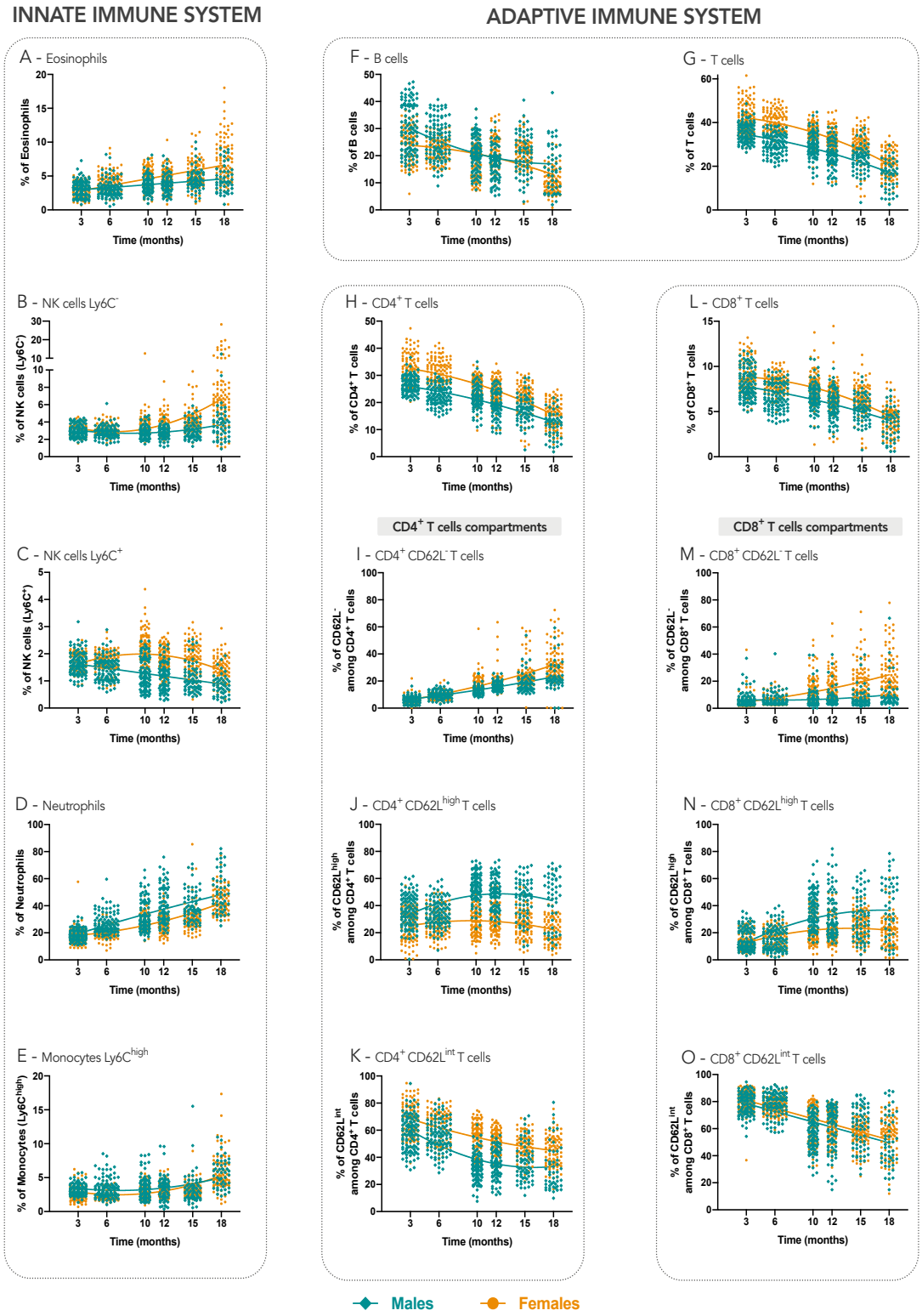


**FIGURE 1.** Longitudinal evaluation of the circulating immune cells. Percentages of the main innate [Eosinophils (A), NK cells Ly6C$^-$ (B) and Ly6C$^+$ (C), neutrophils (D), and monocytes Ly6C$^{high}$ (E)] and adaptive immune system populations over time [total B and T cells (F and G, respectively), and its subpopulations CD4$^+$ and CD8$^+$ T cells (H and L, respectively) and the corresponding activation compartments: for CD4$^+$ T cells – CD62L$^-$ (I), CD62L$^{high}$ (J) and CD62L$^{int}$ (K); for CD8$^+$ T cells – CD62L$^-$ (M), CD62L$^{high}$ (N) and CD62L$^{int}$ (O)]. The representation combines the results of the 3 experimental sets. Each dot represents an animal, where males are depicted as mint blue diamonds and females are depicted as orange circles. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) functions].

**TABLE 2.** Linear mixed models testing the effect of time (aging) and sex on the percentages of the main innate immune system cell populations.

| Dependent variable | Parameters | Estimate (B) | SE | df | t | Sig. | 95% CI | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower Bound | Upper Bound |
| **Eosinophils** | Intercept | 4,582 | 0,101 | 300,645 | 45,431 | **<0,001** | 4,383 | 4,780 |
| | cTime | 0,225 | 0,017 | 213,546 | 13,089 | **<0,001** | 0,191 | 0,259 |
| | Sex[a] | -0,889 | 0,146 | 309,630 | -6,071 | **<0,001** | -1,177 | -0,601 |
| | cTime * Sex[a] | -0,096 | 0,028 | 251,108 | -3,464 | **0,001** | -0,150 | -0,041 |
| | $cTime^2$ | 0,002 | 0,003 | 496,369 | 0,799 | 0,425 | -0,003 | 0,008 |
| | $cTime^2$ * Sex[a] | 0,004 | 0,005 | 561,221 | 0,974 | 0,331 | -0,005 | 0,013 |
| **Neutrophils** | Intercept | 27,114 | 0,660 | 279,564 | 41,096 | **<0,001** | 25,815 | 28,412 |
| | cTime | 1,639 | 0,075 | 165,311 | 21,821 | **<0,001** | 1,490 | 1,787 |
| | Sex[a] | 8,308 | 0,951 | 289,201 | 8,734 | **<0,001** | 6,436 | 10,181 |
| | cTime * Sex[a] | 0,311 | 0,123 | 193,164 | 2,527 | **0,012** | 0,068 | 0,554 |
| | $cTime^2$ | 0,055 | 0,013 | 374,968 | 4,259 | **<0,001** | 0,030 | 0,081 |
| | $cTime^2$ * Sex[a] | -0,080 | 0,020 | 408,355 | -3,989 | **<0,001** | -0,120 | -0,041 |
| **NK cells Ly6C$^+$** | Intercept | 1,913 | 0,037 | 273,096 | 51,231 | **<0,001** | 1,839 | 1,986 |
| | cTime | -0,017 | 0,004 | 139,429 | -4,610 | **<0,001** | -0,024 | -0,010 |
| | Sex[a] | -0,681 | 0,054 | 291,986 | -12,595 | **<0,001** | -0,787 | -0,575 |
| | cTime * Sex[a] | -0,032 | 0,006 | 160,123 | -5,403 | **<0,001** | -0,043 | -0,020 |
| | $cTime^2$ | -0,007 | 0,001 | 340,715 | -10,764 | **<0,001** | -0,008 | -0,006 |
| | $cTime^2$ * Sex[a] | 0,007 | 0,001 | 372,631 | 7,281 | **<0,001** | 0,005 | 0,009 |
| **NK cells Ly6C$^-$** | Intercept | 3,544 | 0,062 | 284,796 | 57,327 | **<0,001** | 3,422 | 3,666 |
| | cTime | 0,130 | 0,014 | 309,017 | 9,428 | **<0,001** | 0,103 | 0,157 |
| | Sex[a] | -0,761 | 0,090 | 290,551 | -8,480 | **<0,001** | -0,937 | -0,584 |
| | cTime * Sex[a] | -0,099 | 0,021 | 324,288 | -4,645 | **<0,001** | -0,142 | -0,057 |
| | $cTime^2$ | 0,009 | 0,002 | 550,753 | 3,981 | **<0,001** | 0,005 | 0,014 |
| | $cTime^2$ * Sex[a] | -0,001 | 0,003 | 542,784 | -0,376 | 0,707 | -0,008 | 0,005 |
| **Monocytes Ly6C$^{high}$** | Intercept | 2,751 | 0,085 | 231,722 | 32,545 | **<0,001** | 2,584 | 2,917 |
| | cTime | 0,176 | 0,066 | 79535,108 | 2,663 | **0,008** | 0,047 | 0,306 |
| | Sex[a] | 0,617 | 0,123 | 246,180 | 5,027 | **<0,001** | 0,375 | 0,858 |
| | cTime * Sex[a] | -0,060 | 0,093 | 69043,094 | -0,646 | 0,518 | -0,243 | 0,123 |
| | $cTime^2$ | 0,024 | 0,001 | 1139,367 | 15,880 | **<0,001** | 0,021 | 0,027 |
| | $cTime^2$ * Sex[a] | -0,009 | 0,002 | 1329,631 | -3,884 | **<0,001** | -0,014 | -0,005 |

[a]Reference category is female; SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

2) The percentage of NK cells Ly6C$^-$ (Figure 1B and Table 2) increases with a mild acceleration over time almost exclusively on females (B[cTime]=0.130 and B[cTime$^2$]= 0.009, indicate that this NK cells subpopulation increases 0.130% per month and for each month there is an increment (acceleration) of 0.009 for the first month, 0.036 for the second, 0.081 for the third, and so forth – same rational for the other models; and B[cTime * Sex]= -0.099); with males presenting lower cell percentages than females (B[sex]= -0.761).

3) The percentage of NK cells Ly6C$^+$ (Figure 1C and Table 2) decreases over time (B[cTime]= -0.017, indicating that NK cells Ly6C$^+$ decrease 0.017% per month) and is more pronounced on males (B[cTime * Sex]= -0.032). Males present lower percentages than females (B[sex]= -0.68). Females display an accelerated decrease over time, while males present only a linear decrease (B[cTime$^2$]= -0.007 and B[cTime$^2$ * Sex]= 0.007).

4) The percentage of neutrophils (Figure 1D and Table 2) increases with a slight acceleration over time (B[Time]= 1.639, indicating that neutrophils increase 1.639% per month; B[cTime$^2$]= 0.055); with males presenting higher percentages than females (B[sex]= 8.308) and having a faster increase than females B[cTime * Sex]= 0.311; the slight accelerated increase in the percentage of neutrophils during aging is almost exclusive of females [B[cTime$^2$ * Sex]= -0.080; if males 0.055 - 0.080= -0.025]).

5) The percentage of activated monocytes (Ly6C$^{high}$) (Figure 1F and Table 2) increases over time, 0.176% per month (B[Time]= 0.176); with males presenting higher percentages than females (B[sex]= 0.617). The trajectory's slope of the percentages over time is similar between males and females (no significant interaction between time and sex), however the percentual increase of monocytes gradually accelerates over time, more pronouncedly in females than in males (B[cTime$^2$]= 0.024 and B[cTime$^2$ * Sex]= -0.009).

In respect to adaptive immune system, total B cells and T cells (total, CD4$^+$ and CD8$^+$ subpopulations) were analyzed. Alike the innate immune cells, there is a percentage's variation in most populations during the aging process (the percentage of total B cells and T cells [total, CD4$^+$ and CD8$^+$ subpopulations decrease with aging] that is commonly moderated by sex. Specifically:

1) The percentage of B cells (Figure 1F and Table 3) decreases over time approximately 0,7% per month (B[Time]= -0.697); with females presenting an accelerated decrease and males presenting a decelerated decrease over time (B[cTime$^2$]= -0.035 and B[cTime$^2$ * Sex]= 0.096, suggesting a quadratic effect of -0.035 for females and of 0.061 for males).

**TABLE 3.** Linear mixed models testing the effect of time (aging) and sex on the percentages of the main adaptive immune system cell populations.

| Dependent variable | Parameters | Estimate (B) | SE | df | t | Sig. | 95% CI | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower Bound | Upper Bound |
| **B cells** | Intercept | 20,193 | 0,437 | 372,646 | 46,174 | **<0,001** | 19,333 | 21,053 |
| | cTime | -0,697 | 0,056 | 427,239 | -12,515 | **<0,001** | -0,806 | -0,587 |
| | Sex[a] | -0,230 | 0,635 | 393,518 | -0,362 | 0,718 | -1,477 | 1,018 |
| | cTime * Sex[a] | -0,211 | 0,086 | 380,324 | -2,440 | **0,015** | -0,381 | -0,041 |
| | $cTime^2$ | -0,035 | 0,011 | 338,902 | -3,085 | **0,002** | -0,058 | -0,013 |
| | $cTime^2$ * Sex[a] | 0,096 | 0,017 | 343,783 | 5,512 | **<0,001** | 0,062 | 0,130 |
| **T cells** | Intercept | 34,433 | 0,417 | 289,023 | 82,560 | **<0,001** | 33,612 | 35,254 |
| | cTime | -1,474 | 0,052 | 162,269 | -28,433 | **<0,001** | -1,577 | -1,372 |
| | Sex[a] | -6,995 | 0,604 | 299,996 | -11,575 | **<0,001** | -8,184 | -5,806 |
| | cTime * Sex[a] | 0,316 | 0,082 | 193,271 | 3,871 | **<0,001** | 0,155 | 0,477 |
| | $cTime^2$ | -0,058 | 0,009 | 328,827 | -6,377 | **<0,001** | -0,075 | -0,040 |
| | $cTime^2$ * Sex[a] | 0,017 | 0,014 | 351,538 | 1,226 | 0,221 | -0,010 | 0,045 |
| **$CD4^+$ T cells** | Intercept | 25,802 | 0,323 | 294,147 | 79,777 | **<0,001** | 25,165 | 26,438 |
| | cTime | -1,174 | 0,038 | 163,104 | -30,510 | **<0,001** | -1,250 | -1,098 |
| | Sex[a] | -5,403 | 0,469 | 305,722 | -11,520 | **<0,001** | -6,326 | -4,480 |
| | cTime * Sex[a] | 0,279 | 0,061 | 194,527 | 4,577 | **<0,001** | 0,159 | 0,399 |
| | $cTime^2$ | -0,037 | 0,007 | 314,940 | -5,317 | **<0,001** | -0,050 | -0,023 |
| | $cTime^2$ * Sex[a] | 0,010 | 0,011 | 338,917 | 0,918 | 0,359 | -0,011 | 0,031 |
| **$CD62L^-$ among $CD4^+$ T cells** | Intercept | 17,174 | 0,402 | 220,193 | 42,695 | **<0,001** | 16,381 | 17,966 |
| | cTime | 1,789 | 0,072 | 324,665 | 24,759 | **<0,001** | 1,647 | 1,932 |
| | Sex[a] | -2,682 | 0,581 | 226,822 | -4,620 | **<0,001** | -3,826 | -1,538 |
| | cTime * Sex[a] | -0,749 | 0,113 | 387,335 | -6,634 | **<0,001** | -0,971 | -0,527 |
| | $cTime^2$ | 0,051 | 0,009 | 455,221 | 5,691 | **<0,001** | 0,034 | 0,069 |
| | $cTime^2$ * Sex[a] | -0,051 | 0,014 | 490,849 | -3,628 | **<0,001** | -0,078 | -0,023 |
| **$CD62L^{int}$ among $CD4^+$ T cells** | Intercept | 53,645 | 0,756 | 404,363 | 70,915 | **<0,001** | 52,158 | 55,133 |
| | cTime | -1,547 | 0,089 | 205,230 | -17,340 | **<0,001** | -1,723 | -1,371 |
| | Sex[a] | -16,141 | 1,115 | 424,314 | -14,472 | **<0,001** | -18,333 | -13,949 |
| | cTime * Sex[a] | -0,073 | 0,139 | 240,834 | -0,523 | 0,601 | -0,347 | 0,201 |
| | $cTime^2$ | 0,048 | 0,018 | 636,172 | 2,755 | **0,006** | 0,014 | 0,083 |
| | $cTime^2$ * Sex[a] | 0,115 | 0,027 | 693,879 | 4,236 | **<0,001** | 0,062 | 0,168 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **CD62L$^{high}$ among CD4$^{+}$ T cells** | Intercept | 28,844 | 0,800 | 318,138 | 36,051 | **<0,001** | 27,270 | 30,418 |
| | cTime | -0,198 | 0,101 | 169,635 | -1,965 | 0,051 | -0,396 | 0,001 |
| | Sex[a] | 19,334 | 1,171 | 329,443 | 16,506 | **<0,001** | 17,030 | 21,639 |
| | cTime * Sex[a] | 0,748 | 0,158 | 199,149 | 4,735 | **<0,001** | 0,436 | 1,059 |
| | cTime$^2$ | -0,091 | 0,019 | 377,745 | -4,894 | **<0,001** | -0,128 | -0,055 |
| | cTime$^2$ * Sex[a] | -0,090 | 0,029 | 415,860 | -3,104 | **0,002** | -0,146 | -0,033 |
| **CD8$^{+}$ T cells** | Intercept | 7,480 | 0,149 | 127,940 | 50,290 | **<0,001** | 7,186 | 7,774 |
| | cTime | -0,296 | 0,067 | 6507,475 | -4,397 | **<0,001** | -0,428 | -0,164 |
| | Sex[a] | -1,390 | 0,213 | 132,918 | -6,538 | **<0,001** | -1,811 | -0,970 |
| | cTime * Sex[a] | 0,022 | 0,095 | 6626,849 | 0,232 | 0,816 | -0,164 | 0,209 |
| | cTime$^2$ | -0,018 | 0,002 | 681,585 | -10,809 | **<0,001** | -0,021 | -0,015 |
| | cTime$^2$ * Sex[a] | 0,009 | 0,003 | 787,119 | 3,569 | **<0,001** | 0,004 | 0,015 |
| **CD62L$^{-}$ among CD8$^{+}$ T cells** | Intercept | 11,359 | 0,646 | 187,872 | 17,576 | **<0,001** | 10,084 | 12,633 |
| | cTime | 1,372 | 0,092 | 162,046 | 14,833 | **<0,001** | 1,189 | 1,554 |
| | Sex[a] | -4,240 | 0,928 | 197,321 | -4,567 | **<0,001** | -6,071 | -2,409 |
| | cTime * Sex[a] | -1,061 | 0,146 | 184,883 | -7,278 | **<0,001** | -1,349 | -0,774 |
| | cTime$^2$ | 0,062 | 0,014 | 144,032 | 4,437 | **<0,001** | 0,034 | 0,089 |
| | cTime$^2$ * Sex[a] | -0,033 | 0,021 | 174,946 | -1,567 | 0,119 | -0,075 | 0,009 |
| **CD62L$^{int}$ among CD8$^{+}$ T cells** | Intercept | 65,384 | 0,898 | 378,958 | 72,783 | **<0,001** | 63,618 | 67,150 |
| | cTime | -1,950 | 0,103 | 167,263 | -18,919 | **<0,001** | -2,153 | -1,746 |
| | Sex[a] | -1,263 | 1,309 | 389,304 | -0,965 | 0,335 | -3,837 | 1,311 |
| | cTime * Sex[a] | 0,121 | 0,165 | 197,215 | 0,733 | 0,464 | -0,204 | 0,446 |
| | cTime$^2$ | 0,032 | 0,019 | 381,651 | 1,690 | 0,092 | -0,005 | 0,070 |
| | cTime$^2$ * Sex[a] | 0,002 | 0,029 | 417,327 | 0,081 | 0,936 | -0,055 | 0,060 |
| **CD62L$^{high}$ among CD8$^{+}$ T cells** | Intercept | 22,334 | 0,932 | 334,796 | 23,972 | **<0,001** | 20,501 | 24,167 |
| | cTime | 0,588 | 0,095 | 127,900 | 6,211 | **<0,001** | 0,401 | 0,775 |
| | Sex[a] | 6,978 | 1,352 | 346,854 | 5,160 | **<0,001** | 4,318 | 9,638 |
| | cTime * Sex[a] | 0,975 | 0,152 | 150,909 | 6,404 | **<0,001** | 0,674 | 1,276 |
| | cTime$^2$ | -0,087 | 0,018 | 348,568 | -4,907 | **<0,001** | -0,121 | -0,052 |
| | cTime$^2$ * Sex[a] | 0,024 | 0,027 | 381,250 | 0,873 | **<0,001** | -0,030 | 0,077 |

[a]Reference category is female; SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

2) The percentage of total T cells (Figure 1G and Table 3) decreases with a mild acceleration over time of approximately 1.5% per month (B[Time]= -1.474; B[cTime$^2$]= -0.058). Males present, on average, 7% less cells than females (B[sex]= -6.995) and have a slower decrease than females (B[cTime * Sex]= 0.316).

3) The percentages of both CD4$^+$ or CD8$^+$ T cells subsets (Figure 1H and 1L and Table 3) decrease during aging ($B_{CD4+}$[Time]= -1.174 and $B_{CD8+}$[Time]= -0.296) with an acceleration profile in both sexes in CD4$^+$ T cells percentages. In CD8$^+$ T cells the acceleration is more pronounced in females than in males; globally males present less cells than females, 5.4% and 1.4%, respectively ($B_{CD4+}$[Sex]= -5.403 and $B_{CD8+}$[Sex]= -1.390) and have a slower decrease in CD4$^+$ T cells percentages (B[cTime * Sex]= 0.279), while the decrease on the percentage of CD8$^+$ T cells is similar between males and females (B[cTime * Sex] not significant).

CD4$^+$ and CD8$^+$ T cells can be divided in subpopulations/compartments based on their activation state. Mouse studies, based mostly on C57BL/6 mice, define naïve T cells as CD44$^{low/-}$CD62L$^{high}$, memory T cells as CD44$^{high}$ CD62L$^{high}$ and effector memory T cells as CD44$^{high}$ CD62L$^{low/-}$ (Rosenblum et al., 2016). However, the expression of CD44 and CD62L is distinct in Balb/c mice (data not shown). In this study it was used exclusively the expression of CD62L: CD62L$^-$ T cells are defined as effector memory/activated cells, while the precise definition of cells expressing intermediate and high levels of CD62L is not fully elucidated.

Again, aging is associated with changes in the several T cell compartments, which is also sex-dependent: CD62L$^-$ T cells (effector memory cells) increase over time (both in males and females among CD4$^+$ T cells and exclusively for females on CD8$^+$ T cells); CD62L$^{int}$ T cells decrease with aging both in CD4$^+$ and CD8$^+$ T cells, where females have clearly more cells than males among CD4$^+$ T cells; and CD62L$^{high}$ T cells increase almost exclusively on males. Specifically:

1) As expected the percentage of CD62L$^-$ T cells increases during aging, for both for CD4$^+$ and CD8$^+$ subsets (Figure 1I and 1M and Table 3) ($B_{CD4+CD62L-}$[Time]= 1.789 and $B_{CD8+CD62L-}$[Time]= 1.372); with males presenting lower percentages than females ($B_{CD4+CD62L-}$[Sex]= -2.682 and $B_{CD8+CD62L-}$[Sex]= -4.240. The increase in CD4$^+$CD62L$^-$ T cells percentages is slower in males than in females ($B_{CD4+CD62L-}$ [cTime * Sex] = -0.749), while the increase in the CD8$^+$CD62L$^-$ T cells percentages is almost exclusive for females ($B_{CD8+CD62L-}$ [cTime * Sex] = -1.061; if males 1.372 - 1.061= 0.311).

2) The percentage $CD4^+CD62L^{high}$ T cells (Figure 1J and Table 3), decreases (with a mild acceleration) by 0.2% per month in females (B[cTime]= -0.198; p=0.051), while males present a decelerated increase by 0.55% monthly (B[cTime * Sex]= 0.748; -0.198+0.748 = 0.550). Males also present higher percentages than females (B[sex]= 19.334). On the contrary, the percentage of $CD8^+CD62L^{high}$ T cells (Figure 1N and Table 3) presents an decelerated increase over time that is more prominent in males (B[cTime]= 0.588; B[cTime * Sex]= 0.975; $B[cTime^2]$= -0.087); with males presenting 7% more cells than females (B[Sex]= 6.978).

3) The percentages of $CD4^+CD62L^{int}$ (Figure 1K and Table 3) and of $CD8^+CD62L^{int}$ T cells (Figure 1O and Table 3] decreases during aging approximately 1.5% and 2%, respectively ($B_{CD4+CD62Lint}$[Time]= -1.547 and $B_{CD8+CD62Lint}$[Time]= -1.959); with males presenting lower percentages of $CD4^+CD62L^{int}$ T cells than females (B[Sex]= -16,141); males and females present similar profiles regarding the percentage of $CD62L^{int}CD8^+$ T cells.

The natural biological diversity, even in inbred strains, introduces some heterogeneity between sets. To control and account for this heterogeneity in the analysis, the percentages of cells from each of the 3 sets were standardized. As shown in Supplementary Figure 3, the progression for standardized measures is similar to that presented in Figure 1 (unstandardized measures). The linear mixed models are extremely similar between standardized and unstandardized measures (except for B cells: sex is a significant parameter and the interaction between time and sex is no longer significant when considered standardized measures; and for $CD4^+CD62L^{int}$ T cells: the quadratic effect of time is no longer significant when considered standardized measures – data not shown).

To further control for a potential effect of individual sets in the models with unstandardized values, the variable “set” was also introduced. As seen in Supplementary Tables 1 and 2, even when controlling for the “set” effect, the significant parameters remain the same with estimates within the 95% confidence interval of the first model (Tables 2 and 3). The survival rate, especially in males, is relatively low – 44%. Thus, it is conceivable that mice that died before the last evaluation are different and not representative of the overall population. To explore the possible impact of a listwise exclusion of all mice that died over the course of experiment, linear mixed models were also built using only the survivors, which did not alter the main conclusions (data not shown).

# 4. DISCUSSION

This longitudinal study shows that the percentage of immune cells varies with aging and differs among sexes. More specifically, the frequency of neutrophils, monocytes, eosinophils and NK cells Ly6C$^-$ increases with age, while that of NK cells Ly6C$^+$, B and T cells, as well as CD4$^+$ and CD8$^+$ T cells, decreases. These observations show an overall decrease of adaptive immune populations and an increase in innate immunity. Notably, this signature is in line with the known age-related changes in the immune system described in humans, which are characterized by a decline in the adaptive immune response and increase in systemic inflammation often attributed to the innate immunity (Franceschi et al., 2017; Fulop et al., 2018; Goronzy and Weyand, 2013; Puchta et al., 2016). Interestingly, a myeloid skew in hematopoiesis has been shown both in humans and in rodents, which can contribute to the observed increased proportion of circulating innate cells (Cho et al., 2008; Rundberg Nilsson et al., 2016). In the same line of evidence, both old humans and mice present increased levels of activated monocytes (Della Bella et al., 2007; Puchta et al., 2016; Seidler et al., 2010). However, the effect of aging on human monocytes seems to be phenotype-dependent, since the abundance of circulating CD16$^{high}$ monocytes was reported to increase with age, whereas CD14$^{high}$ monocytes decrease or remain constant (Márquez et al., 2020; Patin et al., 2018). Besides the absolute and relative abundance alterations with aging, monocytes' function is also affected by aging. A reduction on monocyte's chemotaxis capacity has been described (Panda et al., 2009), which might result in the accumulation of these cells in circulation. Similarly, the percentage of human neutrophils increases with age, as we also observed here in mice (Valiathan et al., 2016). Neutrophils' capacity to migrate to inflamed tissues is also impaired in aged animals, providing a potential explanation for their accumulation in blood (Brubaker et al., 2013; Wenisch et al., 2000).

Concerning NK cells, we observed an overall decrease of NK cells Ly6C$^+$ and an increase (almost exclusive for females) in the NK cells Ly6C$^-$, which is in accordance with reports showing that NK cells CD27$^-$ CD11b$^+$, also defined as the most mature ones, are diminished in aged animals (Nair et al., 2015). Similarly, in humans, there is an increase in CD56$^{dim}$ NK cells (less mature) and a decrease in CD56$^{bright}$ NK cells (more mature) with aging, even though the overall NK cell population increases (Almeida-Oliveira et al., 2011; Borrego et al., 1999; Hazeldine et al., 2012; Jiao et al., 2009; Mocchegiani et al., 2009; Nikolich-Žugich, 2018; Patin et al., 2018; Schindowski

et al., 2002; Solana and Mariani, 2000; Yan et al., 2010). As for the adaptive immune system, it has been known for long that human B and T (also $CD4^+$ and $CD8^+$ T) cells frequency and absolute number decrease with aging (Chong et al., 2005; Frasca et al., 2008; Jiao et al., 2009; Márquez et al., 2020; Muggen et al., 2019; Paganelli et al., 1992; Patin et al., 2018; Wikby et al., 2008). A recent study in rodents also evidenced a decrease in splenic T cells in old animals (El-naseery et al., 2020).

Interestingly, sex influences the percentage of immune cells. Males present overall higher percentages of neutrophils and activated monocytes ($Ly6C^{high}$) than females. By contrast, females present higher percentages of T cells, as well as $CD4^+$ and $CD8^+$ T cells, eosinophils and NK cells. Additionally, even though males and females present similar percentages of B cells, their progression over time is different. Altogether, this clearly demonstrates an age-related sexual dimorphism in the proportion of immune cells in circulation. Sex differences in immune response have been extensively described and are well-characterized both in humans and in animal models. Females usually present a stronger immune response resulting in a more efficient bacterial clearance, as well as increased susceptibility to autoimmunity (Giefing-Kröll et al., 2015; Klein and Flanagan, 2016; Piasecka et al., 2018). Concordantly, older women have higher genomic accessibility for genes (ATAC-seq) related with adaptive immunity and lower for monocytes and inflammation activity than men (Márquez et al., 2020). Abdullah and collaborators reported that adult females have higher B cell counts than males (Abdullah et al., 2012). However, if considering the progression of cells throughout time, males present a specific decline after the age 65, while females maintain the proportion of B cells stable over time (Márquez et al., 2020; Strindhall et al., 2013). Yet, all the reports describe alterations in overall B cells. The dynamics of aging on naïve to memory B cells deserve to be explored to further characterize and understand the effect of time and sex on these populations.

As described here, the relative frequency of T cells and theirs $CD4^+$ and $CD8^+$ subpopulations are lower in males and decreases during aging in both sexes. Concordantly, human cross-sectional studies demonstrated an identical profile, even though the impact of sex was only demonstrated for T cells and $CD4^+$ T cells (Márquez et al., 2020; Patin et al., 2018; Wikby et al., 2008). The detection of T-cell receptor excision circles (TRECs) in peripheral blood T cells has been used as a surrogate marker for thymopoiesis. Interestingly, not only the amount of TRECs decrease with age, as a result of a well described age-related decrease in thymic function, but also women present

higher amount of TRECs than men. This is suggestive of higher thymic function in women that might explain the increased percentages of T cells in females (Clave et al., 2018).

The most well described age-related alteration in the adaptive immune system is the shift in the T cell compartments from a naïve into a memory and effector phenotype (Goronzy and Weyand, 2005; Jiao et al., 2009; Nikolich-Žugich, 2014; Saule et al., 2006; Thome et al., 2014; Wikby et al., 2008). We observed that $CD62L^-$ T cells (effector memory cells) increase over time (both in males and females among $CD4^+$ T cells and exclusively for females on $CD8^+$ T cells); $CD62L^{int}$ T cells decrease with aging both in $CD4^+$ and $CD8^+$ T cells, where females have clearly more cells than males among $CD4^+$ T cells, suggesting that this population represents naïve cells; and $CD62L^{high}$ T cells increase almost exclusively on males, suggesting that these cells represent memory cells. In humans, it has been demonstrated that the relative decrease in naive T cells is more pronounced in $CD8^+$ T cells, with women displaying higher relative amounts of naive T cells than men, as well as more central memory $CD4^+$ T cells (Márquez et al., 2020; Patin et al., 2018; Yan et al., 2010). On the other hand, men present higher proportion of effector T cells than women (Yan et al., 2010), mostly when they are young (Márquez et al., 2020). To note, here we present the proportion of cells among either $CD4^+$ or $CD8^+$ T cells, while human studies present both percentages among total peripheral blood mononuclear cells or absolute counts, which can explain some of the inconsistencies found (Márquez et al., 2020; Yan et al., 2010). Overall, irrespectively from the T cell compartments analyzed, the alterations in the proportion of cells are highly influenced by sex, meaning that a sexual dimorphism needs to be taken into account when addressing the impact of aging on T cells.

The concordance across human and mouse studies suggests that some of the sex-specific aging signatures described here might be conserved across species, which gives confidence when using animal model in the seeking for a further understanding of the sex-related discrepancies in immune system aging.

A final note on the advantage of using the linear mixed model for the longitudinal analysis here. Mixed design ANOVA, the most used statistical approach in biological sciences to explore between-subject differences in repeated measures datasets, does not allow missing values. So, in a longitudinal experimental design losing subjects over time (in this case due to mice death) results in extra loss of information. Hereupon, to overcome this gap we took advantage of the linear mixed model, as described in methods section, which is novel and should be further considered in alike studies.

Taken together, this longitudinal study reveals that sex plays an essential role in mouse's immune system aging that might reflect the already described differences in the immune response and must be taken into account when designing and planning experiments in rodents.

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### AUTHORS CONTRIBUTION

CS-M, JAP and MCN conceptualized, designed and supervised the study; CS-M performed the experiment and processed the samples, analyzed the data, prepared the figures, performed the statistical analysis and drafted the first version of the manuscript; SR, PB-S, CN and NV collected and processed samples; PC, supervised the statistical analysis; and all authors discussed the results and contributed to the final version of the manuscript.

### DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### CONFLICT OF INTEREST

The authors declare no competing interests.

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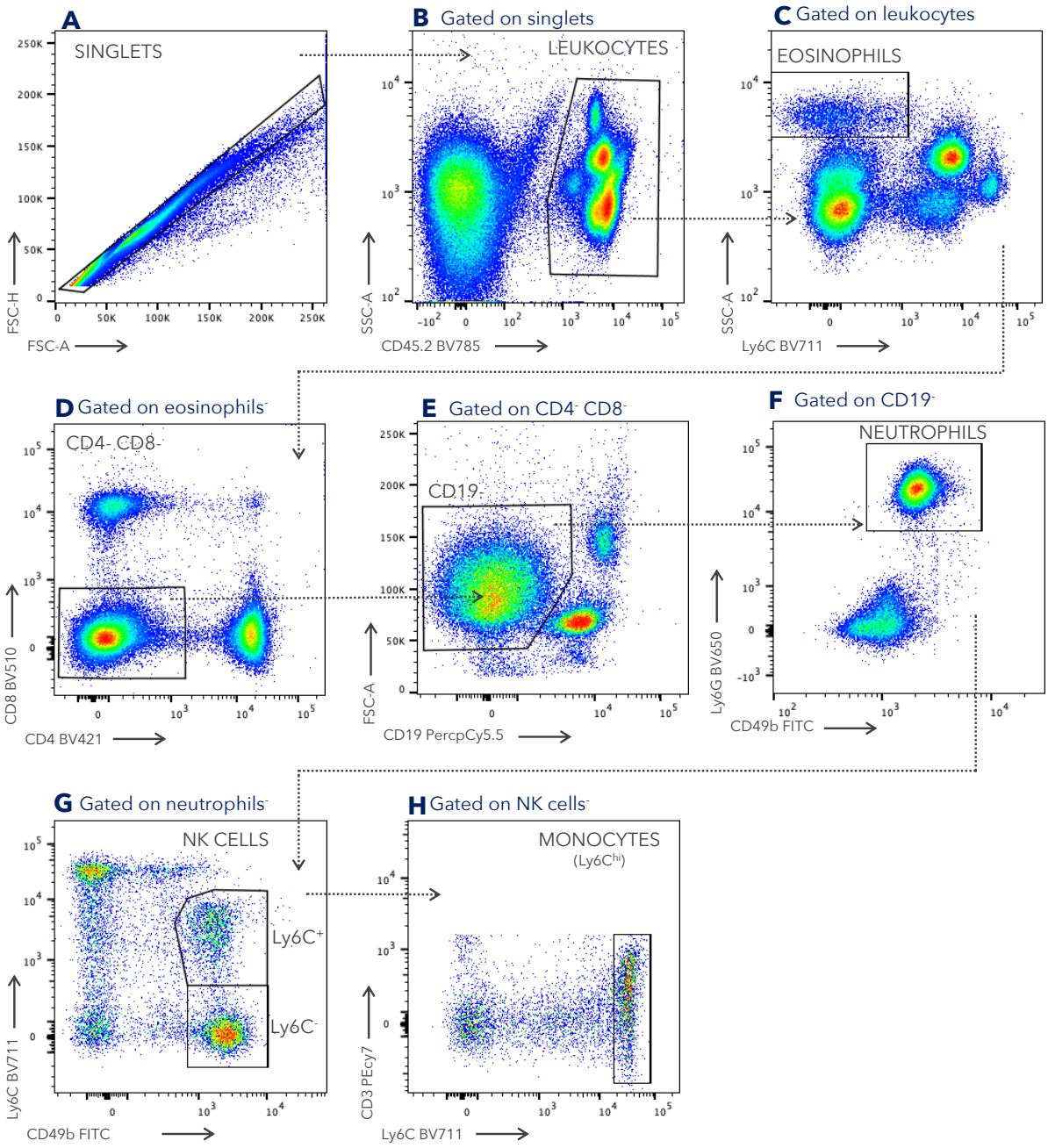
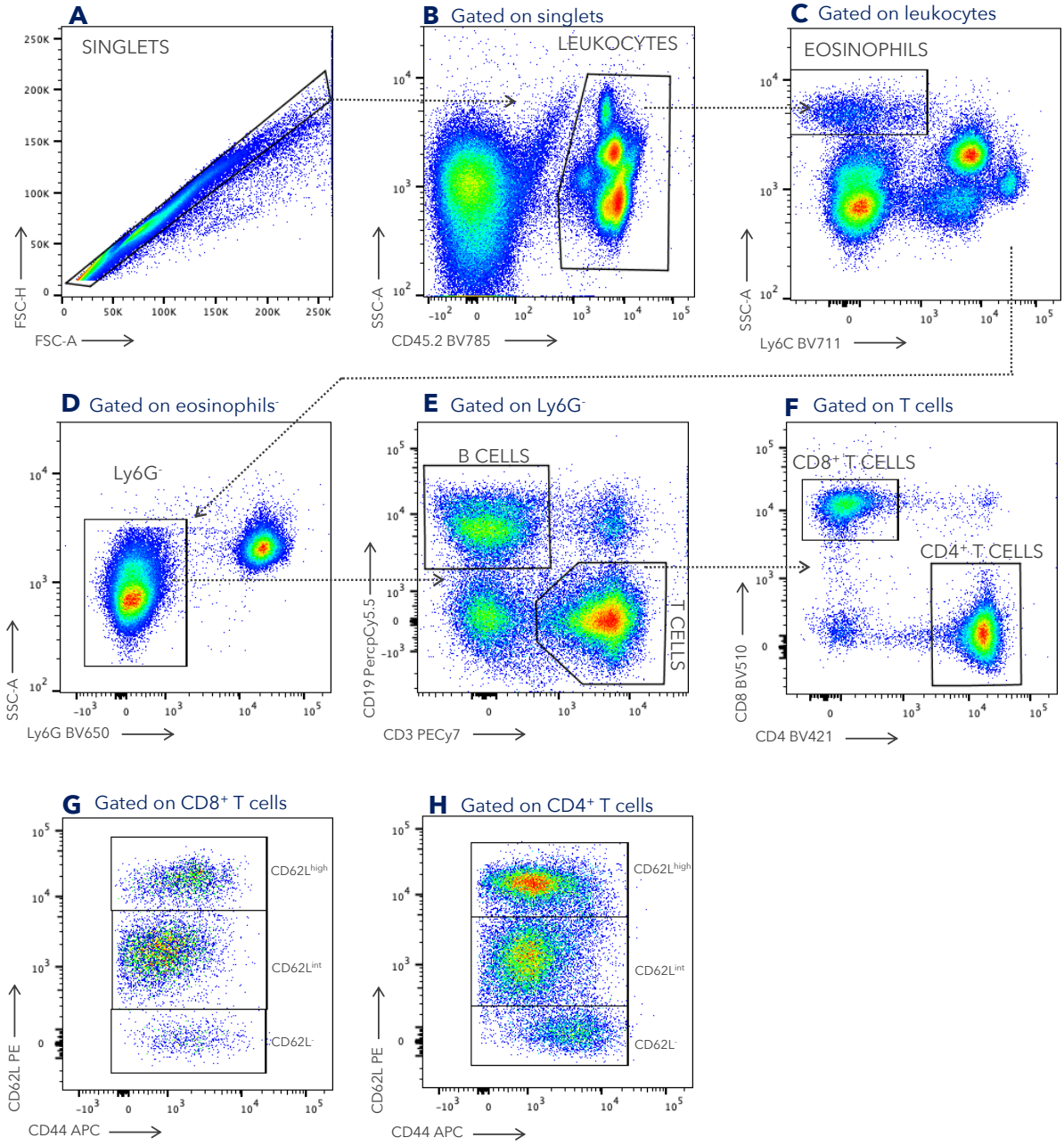
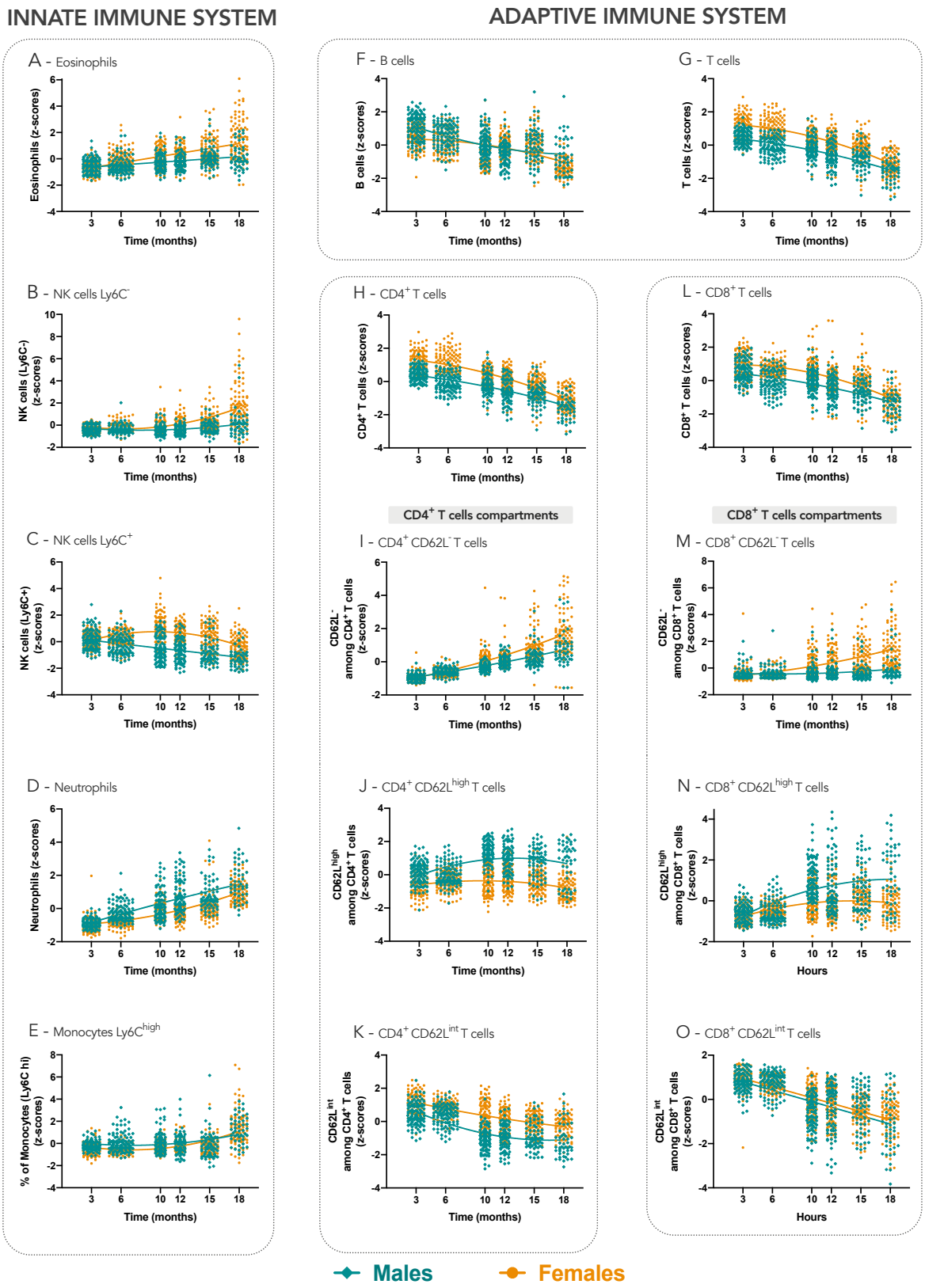
## SUPPLEMENTARY INFORMATION




**SUPPLEMENTARY FIGURE 1.** Flow cytometry's gating strategy of the whole blood main innate immune cells populations. The analysis initiated by selecting singlets (A); leukocytes were defined as CD45.2$^{+}$ cells (B); eosinophils were defined as Ly6C$^{-}$ and SSC$^{high}$ (C); to exclude possible interferences from other populations/markers the following populations were defined after excluding eosinophils, and selecting CD4$^{-}$CD8$^{-}$ (D) and CD19$^{-}$ (E): neutrophils are defined as Ly6G$^{+}$ cells (F), among the neutrophils$^{-}$ cells, NK cells were subdivided as CD49b$^{+}$Ly6C$^{+}$ and CD49b$^{+}$Ly6C$^{-}$ (G), after excluding all the aforementioned cells, activated monocytes were defined as Ly6C$^{high}$ cells (H).




**SUPPLEMENTARY FIGURE 2.** Flow cytometry's gating strategy of the main whole blood adaptive immune cell populations. The analysis started by selecting singlets (A); leukocytes were defined as CD45.2$^+$ cells (B); to exclude possible interferences from other populations/markers the following populations were defined after excluding eosinophils (Ly6C$^-$SSC$^{high}$) (C) and selecting Ly6G$^-$ cells (D): B cells were defined as CD19$^+$CD3$^-$ and T cells as CD19$^-$CD3$^+$ (E); among T cells, CD4$^+$ T cells were defined as CD4$^+$CD8$^-$ and CD8$^+$ T cells were defined as CD4$^-$CD8$^+$ (F). The different activation state/cellular compartments among either CD8$^+$ T cells or CD4$^+$ T cells were defined based on the expression of CD62L (G and H, respectively).




**SUPPLEMENTARY FIGURE 3.** Longitudinal evaluation of the immune system. Representation of standardized values (z-scores) from the percentages of the main innate [Eosinophils (A), NK cells Ly6C$^-$ (B) and Ly6C$^+$ (C), neutrophils (D), and monocytes Ly6C$^{high}$ (E)] and adaptive immune system populations over time [overall B and T cells (F and G, respectively), and its subpopulations CD4$^+$ and CD8$^+$ T cells (H and L, respectively) and the corresponding activation compartments: for CD4$^+$ T cells – CD62L$^-$ (I), CD62L$^{high}$ (J) and CD62L$^{int}$ (K); for CD8$^+$ T cells – CD62L$^-$ (M), CD62L$^{high}$ (N) and CD62L$^{int}$ (O)]. The representation combines the results from the 3 experimental sets and z-scores were calculated based on the mean and standard deviation of all time points combined for each set independently. Each dot represents an animal, where males are depicted as mint blue diamonds and females are depicted as orange circles. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) functions].

**SUPPLEMENTARY TABLE 1.** Linear mixed models testing the effect of time (aging) and sex on the percentages of the innate immune system main cell populations, controlling for experimental set differences.

| | | | | | | | 95% CI | |
|---|---|---|---|---|---|---|---|---|
| **Dependent variable** | **Parameters** | **Estimate (B)** | **SE** | **df** | **t** | **Sig.** | **Lower Bound** | **Upper Bound** |
| **Eosinophils** | Intercept | 4,178 | 0,118 | 351,208 | 35,320 | **<0,001** | 3,945 | 4,410 |
| | cTime | 0,227 | 0,018 | 220,996 | 12,900 | **<0,001** | 0,192 | 0,261 |
| | Sex[a] | -0,902 | 0,142 | 310,178 | -6,359 | **<0,001** | -1,180 | -0,623 |
| | cTime * Sex[a] | -0,100 | 0,028 | 260,613 | -3,546 | **<0,001** | -0,156 | -0,045 |
| | $cTime^2$ | 0,002 | 0,003 | 486,314 | 0,687 | 0,493 | -0,004 | 0,008 |
| | $cTime^2$ * Sex[a] | 0,005 | 0,005 | 552,211 | 1,080 | 0,281 | -0,004 | 0,014 |
| | Set2 vs others | 0,828 | 0,114 | 214,356 | 7,255 | **<0,001** | 0,603 | 1,053 |
| | Set3 vs others | 0,376 | 0,112 | 218,615 | 3,351 | **0,001** | 0,155 | 0,597 |
| **Neutrophils** | Intercept | 24,734 | 0,742 | 351,755 | 33,342 | **<0,001** | 23,275 | 26,193 |
| | cTime | 1,638 | 0,075 | 166,135 | 21,816 | **<0,001** | 1,490 | 1,787 |
| | Sex[a] | 8,481 | 0,909 | 302,329 | 9,326 | **<0,001** | 6,692 | 10,271 |
| | cTime * Sex[a] | 0,312 | 0,123 | 194,102 | 2,535 | **0,012** | 0,069 | 0,555 |
| | $cTime^2$ | 0,056 | 0,013 | 377,593 | 4,305 | **<0,001** | 0,030 | 0,081 |
| | $cTime^2$ * Sex[a] | -0,083 | 0,020 | 409,877 | -4,126 | **<0,001** | -0,122 | -0,043 |
| | Set2 vs others | 1,907 | 0,673 | 210,059 | 2,833 | **0,005** | 0,580 | 3,233 |
| | Set3 vs others | 4,788 | 0,658 | 212,695 | 7,275 | **<0,001** | 3,491 | 6,085 |
| **NK cells $Ly6C^+$** | Intercept | 1,901 | 0,049 | 269,388 | 39,013 | **<0,001** | 1,805 | 1,997 |
| | cTime | -0,017 | 0,004 | 140,783 | -4,528 | **<0,001** | -0,024 | -0,009 |
| | Sex[a] | -0,683 | 0,054 | 284,206 | -12,639 | **<0,001** | -0,789 | -0,577 |
| | cTime * Sex[a] | -0,032 | 0,006 | 161,685 | -5,479 | **<0,001** | -0,044 | -0,020 |
| | $cTime^2$ | -0,007 | 0,001 | 340,997 | -10,740 | **<0,001** | -0,008 | -0,006 |
| | $cTime^2$ * Sex[a] | 0,007 | 0,001 | 373,166 | 7,292 | **<0,001** | 0,005 | 0,009 |
| | Set2 vs others | 0,065 | 0,054 | 197,770 | 1,208 | 0,229 | -0,041 | 0,170 |
| | Set3 vs others | -0,026 | 0,052 | 200,297 | -0,494 | 0,622 | -0,129 | 0,077 |
| **NK cells $Ly6C^-$** | Intercept | 3,343 | 0,075 | 322,412 | 44,722 | **<0,001** | 3,196 | 3,490 |
| | cTime | 0,131 | 0,014 | 300,060 | 9,277 | **<0,001** | 0,103 | 0,158 |
| | Sex[a] | -0,761 | 0,091 | 265,041 | -8,371 | **<0,001** | -0,941 | -0,582 |
| | cTime * Sex[a] | -0,101 | 0,022 | 315,266 | -4,595 | **<0,001** | -0,144 | -0,057 |
| | $cTime^2$ | 0,009 | 0,002 | 553,950 | 4,021 | **<0,001** | 0,005 | 0,014 |
| | $cTime^2$ * Sex[a] | -0,001 | 0,003 | 547,392 | -0,392 | 0,695 | -0,008 | 0,005 |
| | Set2 vs others | 0,296 | 0,069 | 206,511 | 4,264 | **<0,001** | 0,159 | 0,433 |
| | Set3 vs others | 0,287 | 0,068 | 210,765 | 4,205 | **<0,001** | 0,153 | 0,422 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Monocytes (Ly6C$^{high}$)** | Intercept | 3,157 | 0,117 | 172,169 | 27,093 | **<0,001** | 2,927 | 3,387 |
| | cTime | 0,173 | 0,067 | 4780,026 | 2,567 | **0,010** | 0,041 | 0,305 |
| | Sex[a] | 0,592 | 0,121 | 188,285 | 4,907 | **<0,001** | 0,354 | 0,830 |
| | cTime * Sex[a] | -0,060 | 0,095 | 4460,274 | -0,634 | 0,526 | -0,247 | 0,126 |
| | cTime$^2$ | 0,024 | 0,001 | 1156,684 | 16,044 | **<0,001** | 0,021 | 0,027 |
| | cTime$^2$ * Sex[a] | -0,009 | 0,002 | 1363,905 | -3,898 | **<0,001** | -0,014 | -0,005 |
| | Set2 vs others | -0,379 | 0,138 | 155,054 | -2,738 | **0,007** | -0,652 | -0,105 |
| | Set3 vs others | -0,726 | 0,137 | 156,398 | -5,292 | **<0,001** | -0,997 | -0,455 |

[a]Reference category is female; SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

**SUPPLEMENTARY TABLE 2.** Linear mixed models testing the effect of time (aging) and sex on the percentages of the main adaptive immune system cell populations, controlling for experimental set differences.

| | | | | | | | 95% CI | |
|---|---|---|---|---|---|---|---|---|
| **Dependent variable** | **Parameters** | **Estimate (B)** | **SE** | **df** | **t** | **Sig.** | **Lower Bound** | **Upper Bound** |
| **B cells** | Intercept | 24,319 | 0,530 | 323,832 | 45,894 | **<0,001** | 23,277 | 25,362 |
| | cTime | -0,793 | 0,046 | 302,055 | -17,233 | **<0,001** | -0,884 | -0,703 |
| | Sex[a] | -1,137 | 0,603 | 373,130 | -1,884 | 0,060 | -2,323 | 0,050 |
| | cTime * Sex[a] | -0,156 | 0,074 | 294,789 | -2,117 | **0,035** | -0,301 | -0,011 |
| | cTime$^2$ | -0,051 | 0,010 | 417,571 | -5,197 | **<0,001** | -0,070 | -0,032 |
| | cTime$^2$ * Sex[a] | 0,110 | 0,015 | 422,691 | 7,254 | **<0,001** | 0,080 | 0,139 |
| | Set2 vs others | -3,603 | 0,565 | 206,407 | -6,372 | **<0,001** | -4,718 | -2,488 |
| | Set3 vs others | -6,773 | 0,557 | 209,523 | -12,160 | **<0,001** | -7,870 | -5,675 |
| **T cells** | Intercept | 33,269 | 0,548 | 283,583 | 60,710 | **<0,001** | 32,190 | 34,347 |
| | cTime | -1,472 | 0,052 | 160,103 | -28,141 | **<0,001** | -1,575 | -1,369 |
| | Sex[a] | -6,969 | 0,619 | 262,682 | -11,252 | **<0,001** | -8,189 | -5,750 |
| | cTime * Sex[a] | 0,304 | 0,083 | 189,172 | 3,678 | **<0,001** | 0,141 | 0,467 |
| | cTime$^2$ | -0,058 | 0,009 | 314,024 | -6,461 | **<0,001** | -0,076 | -0,040 |
| | cTime$^2$ * Sex[a] | 0,018 | 0,014 | 339,029 | 1,320 | 0,188 | -0,009 | 0,046 |
| | Set2 vs others | 1,521 | 0,586 | 202,712 | 2,596 | **0,010** | 0,366 | 2,675 |
| | Set3 vs others | 1,846 | 0,576 | 205,743 | 3,203 | **0,002** | 0,710 | 2,982 |
| **CD4$^+$ T cells** | Intercept | 25,317 | 0,428 | 282,882 | 59,210 | **<0,001** | 24,475 | 26,159 |
| | cTime | -1,171 | 0,039 | 160,739 | -30,205 | **<0,001** | -1,248 | -1,094 |
| | Sex[a] | -5,369 | 0,476 | 282,531 | -11,282 | **<0,001** | -6,305 | -4,432 |
| | cTime * Sex[a] | 0,270 | 0,061 | 190,259 | 4,398 | **<0,001** | 0,149 | 0,391 |
| | cTime$^2$ | -0,037 | 0,007 | 298,302 | -5,408 | **<0,001** | -0,050 | -0,023 |
| | cTime$^2$ * Sex[a] | 0,010 | 0,011 | 324,429 | 0,966 | 0,335 | -0,011 | 0,031 |
| | Set2 vs others | 0,478 | 0,468 | 207,012 | 1,022 | 0,308 | -0,444 | 1,401 |
| | Set3 vs others | 0,882 | 0,460 | 210,468 | 1,916 | 0,057 | -0,025 | 1,790 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **CD62L$^{-}$ among CD4$^{+}$ T cells** | Intercept | 15,787 | 0,431 | 262,834 | 36,590 | **<0,001** | 14,937 | 16,636 |
| | cTime | 1,800 | 0,072 | 321,929 | 25,021 | **<0,001** | 1,659 | 1,942 |
| | Sex[a] | -2,461 | 0,572 | 213,031 | -4,302 | **<0,001** | -3,589 | -1,334 |
| | cTime * Sex[a] | -0,759 | 0,112 | 383,567 | -6,754 | **<0,001** | -0,980 | -0,538 |
| | cTime$^2$ | 0,054 | 0,009 | 457,266 | 5,949 | **<0,001** | 0,036 | 0,071 |
| | cTime$^2$ * Sex[a] | -0,055 | 0,014 | 493,309 | -3,913 | **<0,001** | -0,082 | -0,027 |
| | Set2 vs others | 0,732 | 0,292 | 202,006 | 2,505 | **0,013** | 0,156 | 1,308 |
| | Set3 vs others | 2,928 | 0,287 | 205,647 | 10,212 | **<0,001** | 2,363 | 3,494 |
| **CD62L$^{int}$ among CD4$^{+}$ T cells** | Intercept | 57,077 | 0,920 | 442,224 | 62,056 | **<0,001** | 55,270 | 58,885 |
| | cTime | -1,536 | 0,101 | 135,951 | -15,207 | **<0,001** | -1,735 | -1,336 |
| | Sex[a] | -16,109 | 1,112 | 521,635 | -14,490 | **<0,001** | -18,293 | -13,925 |
| | cTime * Sex[a] | -0,005 | 0,158 | 165,394 | -0,032 | 0,975 | -0,318 | 0,307 |
| | cTime$^2$ | 0,044 | 0,020 | 241,716 | 2,192 | **0,029** | 0,004 | 0,084 |
| | cTime$^2$ * Sex[a] | 0,117 | 0,031 | 289,134 | 3,764 | **<0,001** | 0,056 | 0,178 |
| | Set2 vs others | 0,400 | 0,902 | 184,987 | 0,444 | 0,658 | -1,379 | 2,180 |
| | Set3 vs others | -9,440 | 0,889 | 186,771 | -10,617 | **<0,001** | -11,193 | -7,686 |
| **CD62L$^{hi}$ among CD4$^{+}$ T cells** | Intercept | 26,643 | 0,992 | 351,926 | 26,852 | **<0,001** | 24,692 | 28,595 |
| | cTime | -0,217 | 0,115 | 96,671 | -1,893 | 0,061 | -0,444 | 0,011 |
| | Sex[a] | 18,920 | 1,200 | 331,581 | 15,771 | **<0,001** | 16,560 | 21,280 |
| | cTime * Sex[a] | 0,802 | 0,179 | 117,564 | 4,487 | **<0,001** | 0,448 | 1,155 |
| | cTime$^2$ | -0,093 | 0,018 | 317,141 | -5,192 | **<0,001** | -0,129 | -0,058 |
| | cTime$^2$ * Sex[a] | -0,065 | 0,028 | 361,297 | -2,322 | **0,021** | -0,120 | -0,010 |
| | Set2 vs others | -1,839 | 0,958 | 175,006 | -1,918 | 0,057 | -3,730 | 0,053 |
| | Set3 vs others | 7,931 | 0,944 | 177,639 | 8,400 | **<0,001** | 6,068 | 9,794 |
| **CD8$^{+}$ T cells** | Intercept | 7,113 | 0,134 | 436,646 | 53,070 | **<0,001** | 6,850 | 7,377 |
| | cTime | -0,297 | 0,036 | 3194,849 | -8,130 | **<0,001** | -0,368 | -0,225 |
| | Sex[a] | -1,393 | 0,160 | 317,103 | -8,704 | **<0,001** | -1,708 | -1,078 |
| | cTime * Sex[a] | 0,032 | 0,052 | 4209,337 | 0,612 | 0,541 | -0,070 | 0,134 |
| | cTime$^2$ | -0,021 | 0,002 | 363,630 | -9,541 | **<0,001** | -0,025 | -0,016 |
| | cTime$^2$ * Sex[a] | 0,011 | 0,003 | 448,171 | 3,364 | **0,001** | 0,005 | 0,018 |
| | Set2 vs others | 0,470 | 0,130 | 374,054 | 3,627 | **<0,001** | 0,215 | 0,725 |
| | Set3 vs others | 0,486 | 0,128 | 373,850 | 3,804 | **<0,001** | 0,235 | 0,737 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **CD62L$^{-}$ among CD8$^{+}$ T cells** | Intercept | 13,644 | 0,673 | 375,892 | 20,278 | **<0,001** | 12,321 | 14,967 |
| | cTime | 1,370 | 0,390 | 150516,708 | 3,515 | **<0,001** | 0,606 | 2,134 |
| | Sex[a] | -6,212 | 0,694 | 413,589 | -8,944 | **<0,001** | -7,577 | -4,847 |
| | cTime * Sex[a] | -1,133 | 0,549 | 173607,711 | -2,061 | **0,039** | -2,210 | -0,056 |
| | cTime$^2$ | 0,042 | 0,008 | 796,160 | 5,361 | **<0,001** | 0,027 | 0,057 |
| | cTime$^2$ * Sex[a] | -0,016 | 0,013 | 905,664 | -1,284 | 0,199 | -0,041 | 0,009 |
| | Set2 vs others | 0,904 | 0,800 | 325,726 | 1,129 | 0,260 | -0,671 | 2,478 |
| | Set3 vs others | -3,087 | 0,794 | 327,642 | -3,887 | **<0,001** | -4,649 | -1,525 |
| **CD62L$^{int}$ among CD8$^{+}$ T cells** | Intercept | 69,898 | 0,975 | 448,671 | 71,686 | **<0,001** | 67,982 | 71,815 |
| | cTime | -1,965 | 0,109 | 113,907 | -18,062 | **<0,001** | -2,181 | -1,749 |
| | Sex[a] | -0,945 | 1,239 | 403,207 | -0,763 | 0,446 | -3,380 | 1,490 |
| | cTime * Sex[a] | 0,086 | 0,172 | 142,395 | 0,503 | 0,616 | -0,254 | 0,427 |
| | cTime$^2$ | 0,029 | 0,019 | 347,350 | 1,563 | 0,119 | -0,008 | 0,066 |
| | cTime$^2$ * Sex[a] | -0,008 | 0,029 | 379,691 | -0,278 | 0,781 | -0,064 | 0,048 |
| | Set2 vs others | -4,366 | 0,817 | 144,390 | -5,343 | **<0,001** | -5,981 | -2,751 |
| | Set3 vs others | -7,901 | 0,804 | 145,217 | -9,832 | **<0,001** | -9,489 | -6,313 |
| **CD62L$^{hi}$ among CD8$^{+}$ T cells** | Intercept | 16,737 | 0,947 | 337,964 | 17,678 | **<0,001** | 14,875 | 18,600 |
| | cTime | 0,587 | 0,103 | 1162,293 | 5,703 | **<0,001** | 0,385 | 0,790 |
| | Sex[a] | 6,507 | 1,207 | 707,010 | 5,390 | **<0,001** | 4,136 | 8,877 |
| | cTime * Sex[a] | 1,035 | 0,163 | 1018,315 | 6,343 | **<0,001** | 0,715 | 1,355 |
| | cTime$^2$ | -0,087 | 0,017 | 527,331 | -5,000 | **<0,001** | -0,121 | -0,053 |
| | cTime$^2$ * Sex[a] | 0,038 | 0,027 | 539,166 | 1,436 | 0,152 | -0,014 | 0,091 |
| | Set2 vs others | 4,735 | 0,782 | 71,149 | 6,052 | **<0,001** | 3,175 | 6,295 |
| | Set3 vs others | 11,035 | 0,769 | 72,375 | 14,351 | **<0,001** | 9,502 | 12,568 |

[a]Reference category is female; SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

# CHAPTER III

## COGNITIVE HETEROGENEITY IN ELDERLY IS ASSOCIATED WITH THE PERIPHERAL IMMUNE SYSTEM PROFILE: LONGITUDINAL STUDY IN MICE

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# COGNITIVE HETEROGENEITY IN ELDERLY IS ASSOCIATED WITH THE PERIPHERAL IMMUNE SYSTEM PROFILE: LONGITUDINAL STUDY IN MICE


## ABSTRACT

**Background.** Aging is commonly associated with cognitive decline. However, individual's cognitive performance is quite diverse. Aging is also accompanied by alterations in the immune system. Given the recognized interactions between the immune and the central nervous systems, here we explore how the profile of age-associated alterations of the immune system correlate with cognition and are influenced by sex.

**Methods**. Longitudinal characterization of the immune system of male and female BALB/cByJ mice (n=136), from 3 to 18 months of age. The major innate and adaptive immune cells were assessed by flow cytometry. Behavior of old mice (17-18 months old) was evaluated using tests that assess associative, spatial and recognition memories.

**Results.** Using a clustering analysis, old mice were classified as good and poor cognitive performers. The relative abundance of the main blood populations in old mice was associated with their cognitive performance: the % of $CD4^+$ T cells within the T cell pool was negatively associated with spatial memory and decreased the odds for a mouse to belong to the good performance cluster; conversely, the % of $CD8^+$ T cells within the T cell pool and NK cells were positively associated with spatial memory. Moreover, males with better cognitive performance had higher percentages of monocytes $Ly6C^{high}$, a moderate decrease in the percentage of $CD4^+$ T cells among T cells and higher percentages of $CD8^+$ T cells among T cells. Females with distinct cognitive performances present similar progressions over time.

**Conclusions.** This study shows, for the first time, that the progression over time of specific immune cells proportion is associated with distinct cognitive performances in old male mice. This association deserves further investigation in order to understand the mechanisms behind the crosstalk of the immune and central nervous systems throughout aging and to develop therapies to delay or even ameliorate age-related cognitive malfunctioning.


**Keywords:** immune system; immunosenescence; cognition; memory; aging; sex.

# 1. INTRODUCTION

For a long time, the brain was merely viewed as an immune-privileged organ with tolerance to tissue grafts normally rejected in the periphery. However, over the last two decades the paradigm has shifted. Nowadays it is accepted that the brain requires a constant immune surveillance to maintain its normal functioning. A variety of immune cells, generally playing supportive role, populate the brain-borders (meninges and choroid plexus) and modulate brain function in health and in disease (Brynskikh et al., 2008; Derecki et al., 2011, 2010; Filiano et al., 2016; Korin et al., 2017; Mrdjen et al., 2018; Schwartz and Shechter, 2010).

Several studies indicated that the immune system is essential for the maintenance of cognitive function. Mice devoid of T and B cells, the immune deficiency imposed by irradiation or the acute suppression of T cells using immunosuppressive drugs, resulted in cognitive deficits reverted by the replenishment of the T cell poll (Brynskikh et al., 2008; Derecki et al., 2010; Kipnis et al., 2004; Ron-Harel et al., 2008). Interestingly the performance of a cognitive task was shown to be accompanied by an increased recruitment of T cells, mainly $CD4^+$ T cells, to the meninges (Derecki et al., 2010). Moreover, the depletion of T cells from the meningeal spaces, either pharmacologically induced or using immunodeficient mice, was shown to drastically change the profile of meningeal myeloid cells toward a pro-inflammatory profile, affecting mice cognitive performance (Derecki et al., 2010).

Aging is characterized by a progressive loss of integrity and cellular fitness due to a time-dependent accumulation of cellular damage, leading ultimately to impaired functions that will affect multiple systems (Gems and Partridge, 2013; Kirkwood, 2005; López-Otín et al., 2013; Vijg and Campisi, 2008). Both the central nervous and the immune systems undergo countless alterations during aging. For instances, the cognitive function gets impaired in multiple domains (Da Mesquita et al., 2015; Mota et al., 2019; Salthouse, 2012), which varies among individuals (Franceschi et al., 2017; Marques et al., 2016; Paulo et al., 2011; Santos et al., 2014, 2013); and the immune system, in a process known as immunosenescence, suffers both functional and cell frequency alterations both in the adaptive and innate immune systems (Hazeldine and Lord, 2015; Nikolich-Žugich, 2018, 2014; Shaw et al., 2013).

Moreover, it has been proposed that the age-related cognitive dysfunction can be attributed, at least in part, to immunosenescence (Ron-Harel and Schwartz, 2009). Ron-Harel and colleagues suggested that age-related spatial memory loss could be partially restored by boosting peripheral T-cells activation through homeostatic-driven proliferation (Ron-Harel et al., 2008). Serre-Miranda

*et al* showed that circulating EM $CD4^+$ T cells, which gradually increase with aging (Saule et al., 2006), are augmented in blood of healthy elders with worse cognitive performance (Serre-Miranda et al., 2015). A similar unbalanced activated/naive $CD4^+$ T cell ratio and increased numbers of $CD8^+$ T effector memory $CD45RA^+$ ($T_{EMRA}$) cells in circulation were described in demented individuals (Gate et al., 2020; Tan et al., 2002).

Given the known crosstalk between the immune system and cognitive function here we performed a longitudinal study in mice to evaluate whether peripheral alterations of the immune system that occur over aging are associated with the heterogeneous cognitive performance in old mice, which is novel.

## 2. METHODS

### 2.1. ANIMALS

Male and female BALB/cByJ mice, 6 weeks old, were purchased from Charles River and maintained until 18 months of age at the Life and Health Sciences Research Institute (ICVS) animal's facility. Mice were initially housed in groups of 5 animals per cage and males were re-grouped whenever aggressive behaviors were observed; under standard laboratory conditions (light/dark cycle of 12/12 h; 22 °C; 55% humidity); food and water *ad libitum*. Health monitoring was performed according to FELASA guidelines, confirming the Specified Pathogen Free health status of sentinel animals maintained in the same animal room. To avoid excessive and unnecessary suffering of animals, humane endpoints were applied; mice were sacrificed when reaching 20% weight loss and whenever the overall well-being was compromised (wounds resulted from aggressions, appearance of tumors). At the end of the study mice were anesthetized with a mixture of ketamine (75 mg/kg, intraperitonealy [i.p.]) and medetomidine (1 mg/kg, i.p.) and were transcardially perfused with 0.9% saline. All procedures were carried out in accordance with European Regulations (European Union Directive 2010/63/EU). The animal facility and people directly involved in animal experiments were certified by the Portuguese regulatory entity—Direção Geral de Alimentação e Veterinária (DGAV). All experiments were approved by the Ethics Committee of the University of Minho. The experiments were also authorized by the national competent entity DGAV (#009458).

### 2.2. FLOW CYTOMETRY

Peripheral blood was collected from a small incision on the tail to heparin coated capillaries. Samples were longitudinally collected from the same animals approximately at 3, 6, 10, 12, 15

and 18 months of age. Three independent experiments (set1, set2 and set3) were performed and the number of mice used is listed in Table 2. Fifty microliters of blood were incubated for 20 min at room temperature (RT) in the dark with the following combination of antibodies: anti-mouse CD49b FITC (clone: DX5); anti-mouse CD62L PE (clone: MEL-14); anti-mouse CD19 PercpCy5.5 (clone: 6D5); anti-mouse CD3 PECy7 or APC (clone: 145-2C11); anti-mouse CD44 APC or BV605 (clone: IM7); anti-mouse CD4 BV421 (clone: RM4-5); anti-mouse CD8 BV510 (clone: 53-6.7); anti-mouse Ly6G BV650 (clone: 1A8); anti-mouse Ly6C BV711 (clone: HK1.4) and anti-mouse CD45.2 BV785 or PECy7 (clone: 104) [all from Biolegend, San Diego, CA, USA]. Erythrocytes were then lysed with ammonium–chloride–potassium (ACK) buffer (0.15M NH4CL, 10mM KHCO3, 0.1 mM Na2EDTA) for 10 min at RT in the dark. Samples were washed twice with FACS buffer (0.5% BSA and 0.01% sodium azide in PBS) and centrifuged at 300 g for 5 min at RT. Samples were acquired (minimum of 100000 events among leukocytes/sample) on a BD LSRII flow cytometer using the FACS DIVA software (BD Biosciences). Data was analyzed using FlowJo software (BD Biosciences) version 10.6.1. The used gating strategy is the same of the Chapter II (represented in Supplementary Figure 1 and 2). Due to an atypical leukocyte profile 4 females were excluded from the analyses (1 from set1, 1 from set2 and 2 from set3).

### 2.3. BEHAVIORAL CHARACTERIZATION

A multiparametric behavioral assessment was performed between ages of 17 and 18 months, covering different dimensions ranging from locomotor activity, anxious and depressive-like behavior, to memory and learning (cognitive function). The timeline for the behavioral characterization is represented in Figure 1A. The behavioral assessment was performed during the light-phase (between 8am and 8pm). To familiarize mice with the experimenter, 2 weeks prior the behavioral testing mice were handled 3 to 4 times a week for 5 min in their living room. On the testing day, animals were left in the testing room 10 min prior the test for habituation. All apparatuses were cleaned with 10% ethanol between each mouse trial.

**Elevated Plus Maze (EPM).** Animals were placed in the center of a plus-shape apparatus containing two open (50.8 cm x 10.2 cm) and two closed arms (50.8 cm x 10.2 cm x 40.6 cm) elevated 72.4 cm above the floor (ENV560; Med Associates Inc, Vermont, USA). Mice were allowed to freely explore the maze for 5 min. Each trial was recorded and analyzed using Ethovision XT 13 software (Noldus, Netherlands). Time spent on each arm and the number of entries were counted. As a readout of the test, the percentage of entries and the time spent in closed arms were used as

a measure of anxious-like behavior (Walf and Frye, 2007). One animal was excluded for spending more than 75% of the time immobile in the center of the arena.

**Open Field (OF).** Mice were placed in the center of a square arena (43.2 cm x 43.2 cm x 30.5 cm) and allowed to freely explore the apparatus for 5 min. The equipment has two 16-beam infrared arrays and the activity was monitored using the Activity Monitor Software (Med Associates Inc, Vermont, USA). The total distance traveled was used as an indicator of locomotor activity (Seibenhener and Wooten, 2015). Due to tracking problems 3 mice were excluded from the analyses.

**Tail Suspension Test (TST).** Mice were suspended by their tail (using adhesive tape), for 6 min, on a beam elevated 150 cm from the floor. Each trial was recorded and analyzed using Ethovision XT 13 software (Noldus, Netherlands). Total immobility time was used as a measure of depressive-like behavior (Steru et al., 1985).

**Novel Object Recognition (NOR) Test.** The test was performed in black acrylic boxes (33 cm x 33 cm x 33 cm) under dim illumination. During the first three days mice were allowed to explore the boxes during 20 min for habituation. On the fourth day, a two-trial session was performed: a training session and a test session. During the training session mice were exposed to two identical objects aligned in the same wall during 10 min. One hour afterwards, in the test session, one of the familiar objects was replaced for a new one and the animals were allowed to freely explore the objects for 5 min. The test session was recorded and the objects interaction time was evaluated using Ethovision XT 13 software (Noldus, Netherlands). Three mice that presented less than 3 s of total object exploration time were excluded from the analyses by not performing the test. The percentage of exploration of the new object was calculated dividing the time each animal spent exploring the novel object by the total object exploration time. Object recognition memory is reflected by more time interacting with the new object (>50% interaction with new object) (Bevins and Besheer, 2006).

**Y-Maze.** The Y-shaped maze is composed by 3 equal size arms (29.5 cm x 7.5 cm x 15.5 cm) with spatial cues printed on the end of each arm (three horizontal lines, a circle and a triangle). The two-trial session protocol was performed under dim illumination. In the first trial mice were allowed to freely explore the maze for 10 min with one of the arms blocked. One hour later mice were returned to the maze, now with all the arms unblocked, and allowed to explore the maze for 5 min. This session was recorded and the time and number of entries on each arm was evaluated

using Ethovision XT 13 software (Noldus, Netherlands). Novelty preference was assessed by the percentage of time spent exploring the novel arm, and the percentage of entries in the novel arm were also evaluated. A spatial memory is reflected by explorations higher than 33%, which represents a random exploration (Kraeuter et al., 2019). One mouse was excluded from the analyses for being considered an outlier.

**Contextual-fear Conditioning (CFC) Test** was performed in three consecutive days as follows:
Day1. Training session. Mice were placed in a sound-attenuated chamber containing a transparent box (Context A – 22 cm x 19 cm x 12.5 cm, Med Associates Inc, Vermont, USA) with a stainless steel shock grid at the bottom. The chamber has a window through which mice could be recorded without disturbance. After 2 min and 40 s of free exploration, a conditioned stimulus (a white noise 80 dB sound) was presented for 20 s co-terminating with a mild foot shock (unconditioned stimulus, 2 s, 0.5 mA). Two more consecutive shocks were presented with 1 min interval, always pairing the 20 s conditioned stimulus co-terminating with the unconditioned stimulus. After the last shock, mice remained in the apparatus for additional 30s. The full session lasted 5 min and 30 s. The baseline freezing behavior was assessed in the first 60 s and the foot shock conditioned freezing in the last 30 s.
Day2. Context probe. Twenty-four hours after the training session, mice returned to the Context A chamber (without any conditioned and unconditioned stimulus) for 3 min. Freezing behavior was assessed during the entire session. Two to 3 h later, mice were placed in a novel context (Context B – 24 cm x 32 cm x 18.5 cm). The novel chamber was located in a different room, the transparent box was completely covered with blue acrylic, the chamber was scented with a vanilla extract and the experimenter wore a different color lab coat and gloves. The session lasted 3 min and freezing behavior was assessed during the entire session.
Day3. Cue probe. On the following day, mice were re-exposed to context B. Two min and 40 s from the beginning of the session the conditioned stimulus was presented (20 s). After turning off the tone, the freezing behavior was scored during 1 min.
The chambers were illuminated during the entire protocol. After the end of each session, mice returned to their home cage. The freezing behavior was manually scored using Observador v0.2.7. Context discrimination index (Day2 Context A/ [Day2 Context A + Day2 Context B]) and a Cue discrimination index (Day3 Context B+Cue/ [Day3 Context B+Cue + Day2 Context B]) were calculated (Wiltgen and Silva, 2007). An index of 1 indicates that mice were able to discriminate the contexts/cue perfectly (associative memory retention), and a ratio of 0.5 means that mice were

unable to discriminate (Curzon et al., 2009). Two animals were excluded from the analyses for being considered outliers.

## 2.4. STATISTICAL ANALYSIS

### Principal component analysis

The sample with no missing values is composed of 136 mice from 3 independent experiments (Set1 [n=45]; Set2 [n=43]; Set3 [n=48]), with 89 females (65,4%) (Table 2) (Sampling adequacy, KMO= 0.527). Outliers were previously excluded based on mean ± 3 standard deviations. Only mice with information in all the cognitive tests were inserted in the analyses (listwise deletion). As described in Chapter II, some animals died during the course of the experiment.

To perform a dimensionality reduction, a principal component analysis (PCA) was performed (Table 1). The standardized variables (z-scores calculated for each set independently) initially introduced in the model were: % entries in the novel arm and novelty preference from the Y-maze test, % interaction with the novel object from the NOR test, and context and cue discrimination indexes from the CFC test. Due to low communality value (<0.300), % interaction with the novel object in the NOR test was excluded from the PCA analysis. This variable was considered as a unique single dimension in further analysis. Cronbach's alpha was used as a reliability measure. A direct oblimin rotation methods was applied (assuming a correlation between the components extracted) and each component score was computed based on regression method. An independent sample t-test was used to compare the performance in each behavior test and, within each group, between males and females. Levene's test was used to test for equality of variance. All variables presented a normal distribution (skewness below 3 and of kurtosis below 8).

### Cluster analysis

To classify mice based on their cognitive performance, a K-means cluster analysis was performed considering, *a priori*, a two clusters solution. Associative memory and short-term spatial memory obtained in the PCA analysis and % interaction with new object from NOR test were used as independent variables in the model. To test whether there was any difference in sample size distribution by cluster per set and sex, a Chi-square test was performed.

Linear **regression models** were performed to explore whether the main circulating immune cells evaluated at 18 months of age (independently) explain the variance of various memory dimensions (dependent variable: recognition, spatial and associative memories) and binary logistic regression models to explore whether immune cells are able predict to which cognitive performance group

each mouse belongs to (good vs poor), controlling for sex. Multicollinearity between variables was assessed by the tolerance values (all the variables had a tolerance >0.5).

**Linear mixed models** for repeated measures (as previously described in Chapter II) were used to explore the impact of cognitive performance in the progression of the main circulating immune cells during aging. Considering the previous described sex-related differences in the progression of immune cells over time (Chapter II), males and females were analyzed independently. All analyses were performed with results from the 3 sets combined - standardized variables (z-scores) were calculated for each set, independently.

cTime (time centered), Cognitive Cluster, cTime x Cognitive Cluster (interaction), $cTime^2$ (time square centered) and $cTime^2$ x Cognitive Cluster (interaction) were used as fixed factors. To analyze the changes in the percentages of immune cells over time, considering the within-subject correlations between the percentages of immune cells, a random intercept per subject was introduced in the model. Models were tested for various covariance structures (level 1: repeated covariance type and level 2: random covariance type), and the pair with the lowest Akaike's Information Criteria. The restricted maximum likelihood was used as the estimation method.

All statistical procedures were performed in IBM SPSS Version 25 (IBM Corp, USA) and the graphs were designed using Prism7 (GraphPad Software, USA).

## 3. RESULTS

### Aged mice can be grouped as good and poor cognitive performers

To simplify the model and reduce the number of variables in the cluster analysis, a dimensionality reduction achieved by PCA identified two significant components (with eigenvalues >1): an associative memory dimension (composed by context and cue discrimination indexes from the CFC), and a short-term spatial memory dimension (composed by the % of entries in the novel arm and novelty preference from the Y-maze tests) (Table 1). Bartlett's sphericity test revealed a significant correlation between the variables used in the model ($\chi^2_{(6)}$=158.892; p<0.001). The % interaction with the novel object in the NOR test (low communality value [<0.300]) was considered an independent dimension, which we called recognition memory.

The cluster analysis allowed to classify mice based on their cognitive performance. Good cognitive performers present higher scores in all the memory paradigms/domains tested [Figure 1B -

recognition memory: t(78.067)= -4.992, p<0.001; associative memory: t(134)= -7.046, p<0.001; spatial memory: t(77.742)= -8.397, p<0.001; and Supplementary Figure 1 - % interaction with new object: t(78.067)= -4.992, p<0.001; context discrimination index: t(134)= -6.984, p<0.001; cue discrimination index: t(134)= -5.960, p<0.0001; % entries in novel arms: t(72.710)= -6.289, p<0.001; novelty preference: t(134)= -8.119, p<0.001]. Interestingly, none of the dimensions correlates significantly with each other, even though the majority of good performers present scores above the average for the three dimensions combined and bad performers below the average (Supplementary Table 1 and Supplementary Figure 2A). As expected, variables composing the same component present strong correlations (r>0.5) with each other, and with the corresponding dimension (r>0.87) (Supplementary Table 1 and Supplementary Figure 2B). Novelty preference in the Y-Maze correlates positively with the % of interaction with the new object in the NOR, context discrimination index from the CFC and associative memory dimension. Similarly, context discrimination index from the CFC correlate positively with the % of entries in the novel arm of the Y-Maze and with the spatial memory dimension. However, those correlations are relatively small (r>0.3), explaining less than 5% ($r^2$) of the variance.

**TABLE 1.** Dimensionality reduction using principal component analysis (PCA): identification of composite dimensions.

| | Communalities | Component | |
|---|---|---|---|
| | | 1 | 2 |
| zCue Discrimination Index (CFC) | 0.88 | **0.932** | 0.07 |
| zContext Discrimination Index (CFC) | 0.865 | **0.922** | 0.287 |
| zNovel arm entries (Y-Maze) | 0.771 | 0.139 | **0.878** |
| zNovelty preference (Y-Maze) | 0.761 | 0.184 | **0.872** |
| Eigenvalue | | 1.962 | 1.315 |
| % of Variance (cumulative %) | | 49.0 | 32.9 (81.9) |
| Cronbach's alpha | | 0.840 | 0.694 |

Notes: The table shows results from the PCA where two significant components were identified (with eigenvalues >1): an associative memory dimension (component 1 and variables highlighted in bold) and a short-term spatial memory dimension (component 2 and variables highlighted in bold). Cronbach's alpha >0.7 revealed good reliability.

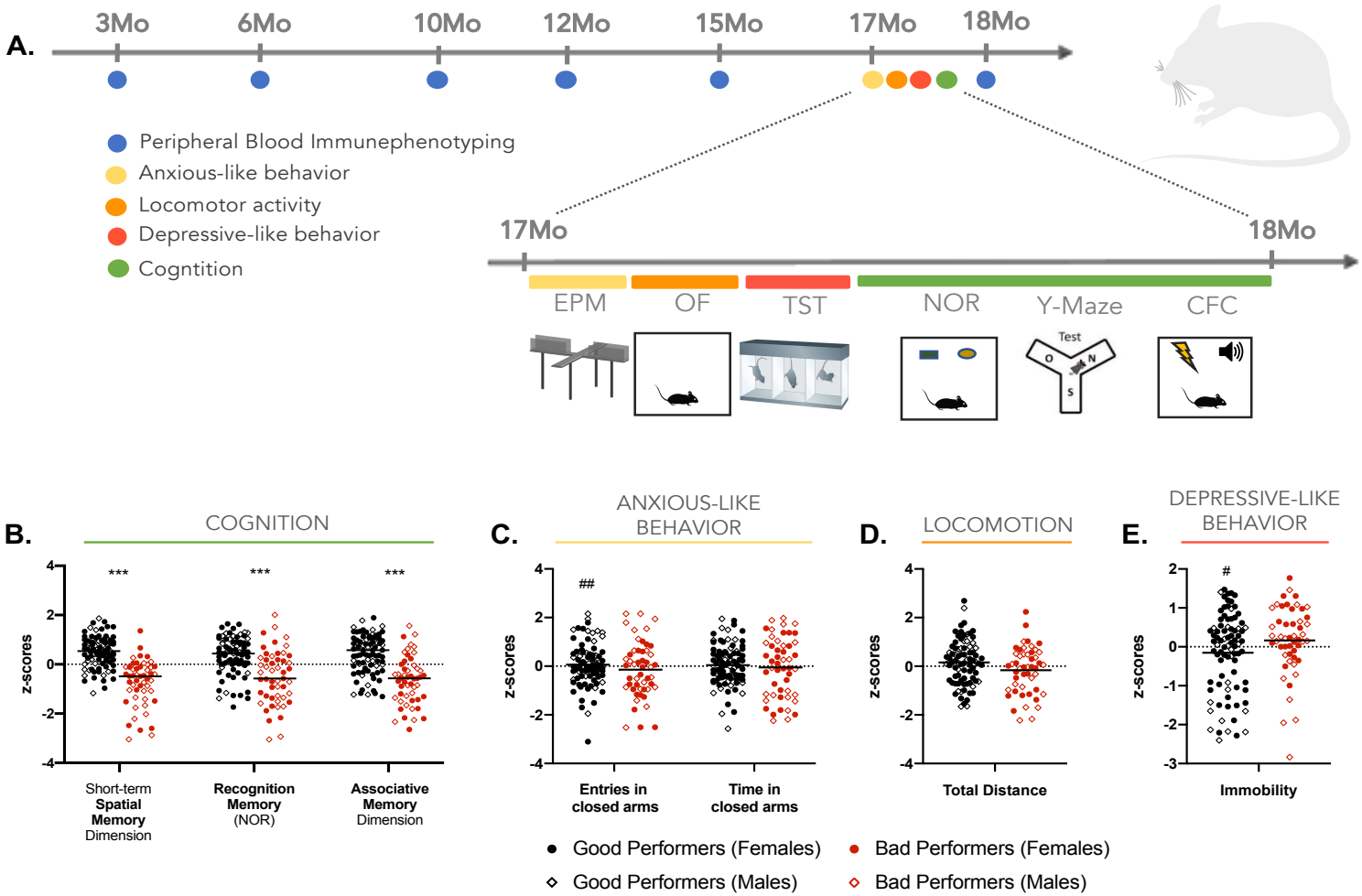


**FIGURE 1.** Timeline of the longitudinal experimental design and behavioral characterization. Blood was collected at 3, 6, 10, 12, 15 and 18 months of age to characterize the main populations of the immune system. Behavioral characterization was done between the age of 17 and 18 months (A). Representation of good and poor cognitive performers divided by sex in the various behavioral dimensions: cognition, assessed by short-term spatial memory and associative memory dimensions obtained in the PCA analysis and recognition memory assessed by NOR test (B); anxious-like behavior assessed by the EPM (C), locomotion by the OF (D) and depressive-like behavior assessed by the TST (E). Representation of standardized values (z-scores) calculated by set. T-test was used to compare good and poor cognitive performers: *** $p<0.001$; and to compare males and females within each group: # $p<0.05$; ## $p>0.01$. Female good performers are represented in black circles, male good performers in black diamonds, female bad performers in red circles and male bad performers in red diamonds.

The mice's clustering in two cognitive groups explains approximately 28% of the variables' variance ($\eta 2 = 0.280$ - effect size measure). The cluster analysis equally distributed mice from different sets in the good and poor performance groups ($\chi^2(2)=2.333$; p=0.311). However, while males are equally distributed among good and poor cognitive performance groups, 75% of the females are in the good performance group ($\chi^2(1)=7.545$; p=0.006) (Table 2). Belonging to a specific cognitive performance group is not associated with the performance in other behavior domains such as locomotor, anxious and depressive-like behavior. The percentage of entries and time spent in the closed arms of the EPM (Figure 1C; anxious-like behavior), the total distance travelled in the OF (Figure 1D; locomotor behavior) and total immobility time in the TST (Figure 1E; depressive-like behavior) is similar between good and poor cognitive performers. However, among the good performers, male present higher scores than female in the number of entries in the closed arms, indicating a more anxious-like behavior [Figure 1C: t(83)=-2.790, p=0.007], and female present higher scores than male in the immobility of the TST [Figure 1E: t(30.673)=2.176, p=0.037], indicating a more depressive-like behavior.

**TABLE 2.** Sample size distribution of the different cognitive clusters per set and sex.

| | | Set1 | Set2 | Set3 | Total |
|---|---|---|---|---|---|
| **Male** | Good Performers | 11 | 8 | 3 | 22 |
| | Bad Performers | 5 | 10 | 10 | 25 |
| | Total | 16 | 18 | 13 | 47 |
| **Female** | Good Performers | 21 | 16 | 26 | 63 |
| | Bad Performers | 8 | 9 | 9 | 26 |
| | Total | 29 | 25 | 35 | 89 |
| **Total** | Good Performers | 32 | 24 | 29 | 85 |
| | Bad Performers | 13 | 19 | 19 | 51 |
| | Total | 45 | 43 | 48 | 136 |

## Peripheral immune system profile at 18 months is associated with cognitive performance

Given the previously described association between peripheral immune profile and cognitive heterogeneity in healthy senior individuals (Serre-Miranda et al., 2015), we hypothesized that the same would occur in rodents. For that purpose, we performed several regression models using each immune cell population independently (covering both innate and adaptive immune system)

evaluated at 18 months old as covariates to explain the variance of the different memory dimensions. For the analysis, sex was included in the models to take into account the already described sex-dimorphism in the immune system aging (Chapter II) and the unbalanced distribution of males and females by the good and poor performance groups (Table 2). At 18 months the increased % of $CD4^+$ T cells (among T cells) decreases the odds of a mouse to belong to the good performance group [indicating that 13.3% ($R^2_{Nagelkerke}$ = 0.133) of the chance to belong to a particular cognitive group is predicted by the model (% of cells and sex; Figure 2A and Supplementary Table 2)], and is negatively associated with spatial memory [explaining 8.4% of the spatial memory variance ($R^2$=0.084; Figure 2B and Supplementary Table 3)]. On the other hand, the % of $CD8^+$ T cells (among T cells) and NK $Ly6C^-$ cells is positively associated with spatial memory function [explaining 8.7 to 8.8% of the spatial memory variance (Figure 2B and Supplementary Table 3)]. None of immune cell populations evaluated at 18 months were associated with associative (Figure 2C) or recognition memories (Figure 2D).

**Males with distinct cognitive performances present moderate differences in the immune cells' progression over time**

We tested whether age-associated alterations of the immune system, reflected by different cellular proportions over time, could be associated and explain some of the age-associated cognitive heterogeneity in mice using linear mixed models. As specified below good and poor cognitive performers present different trajectories over time in the percentage of specific immune cells of both innate (A) and adaptive immune system (B), as well as in the T cells' compartments (C)

A) The male's increased percentage of eosinophils over time has a mild acceleration in poor cognitive performers (B[cTime]= 0.076; B[$cTime^2$]=0.007; B[$cTime^2$ * Cognitive Cluster]= -0.010; in the case of good performers: 0.007-0.010 = -0.003, indicating they only present a linear increase [reference category is bad performance]; Figure 3A and Supplementary Table 4). Males with better cognitive performance have higher percentages of monocytes $Ly6C^{high}$ (B[Cognitive Cluster]= 0.613); while good performers present a linear increase over time, poor performers present a moderate accelerated increase (B[cTime]= 0.077; B[$cTime^2$]=0.013; B[$cTime^2$ * Cognitive Cluster]= -0.012; Figure 3B and Supplementary Table 4). Males' decreased percentage of NK cells $Ly6C^+$ accelerate over time in good performers (B[cTime]= -0.083; B[$cTime^2$ * Cognitive Cluster]= -0.010; Figure 3E and Supplementary Table 4). However, in females, the trajectories of

innate immune cells over time present similar profiles between good and poor performers (Figure 3A-E and Supplementary Table 4).

B) Males with good performance have a moderate decrease in the percentage of $CD4^+$ T cells among T cells (B[cTime * Cognitive Cluster]= -0.050; Figure 4E and Supplementary Table 5) and have higher percentages of $CD8^+$ T cells among T cells (B[Cognitive Cluster]= 0.342; Figure 4F and Supplementary Table 5). Considering the percentage of $CD8^+$ T cells among total leukocytes, males with good performance have a slower decrease than poor performers (B[cTime]= -0.133; B[cTime * Cognitive Cluster]= 0.042; Figure 4D and Supplementary Table 5). In females, the trajectories of adaptive immune cells over time present similar profiles between good and poor performers (Figure 4A-F and Supplementary Table 5).

C) In males the decreased percentage of $CD4^+$ T cells $CD62L^{int}$ has a stronger deceleration in good cognitive performers (B[cTime]= -0.106; B[$cTime^2$]= 0.009 ; B[$cTime^2$ * Cognitive Cluster]= 0.007, Figure 5C and Supplementary Table 6). The progression of the other T cell compartments among good and poor performers is similar over time, both in males and in females (Figure 5A-F and Supplementary Table 6).

Even considering the inter-set variability, doing a similar analysis with unstandardized measures the profiles are similar to standardized measures, as shown here (Supplementary Figures 3-5).

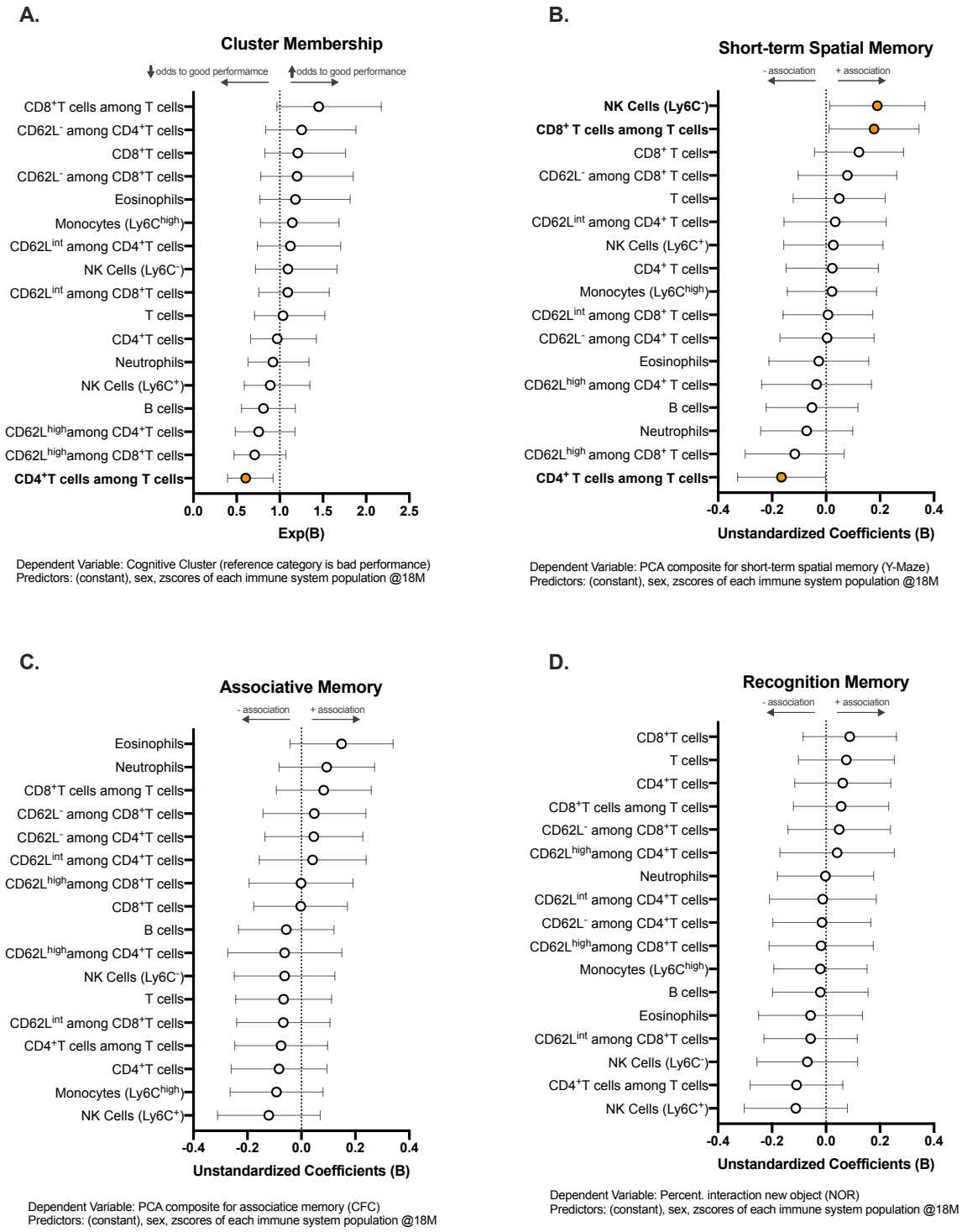


**FIGURE 2.** Parameter estimates from regression analysis to explain the variance of various memory dimensions using blood immune cells evaluated at 18 months of age and sex as independent variables. To predict cluster membership a logistic regression was performed and the Exp[B] for each individual population is represented in (A). To explain the variance of short-term spatial memory (B), associative memory (C) and recognition memory (D) a linear regression analysis was performed and the unstandardized coefficients [B] represented for each individual population. Circles represent the mean and bars the 95% CI and orange circles represent the significant predictors.

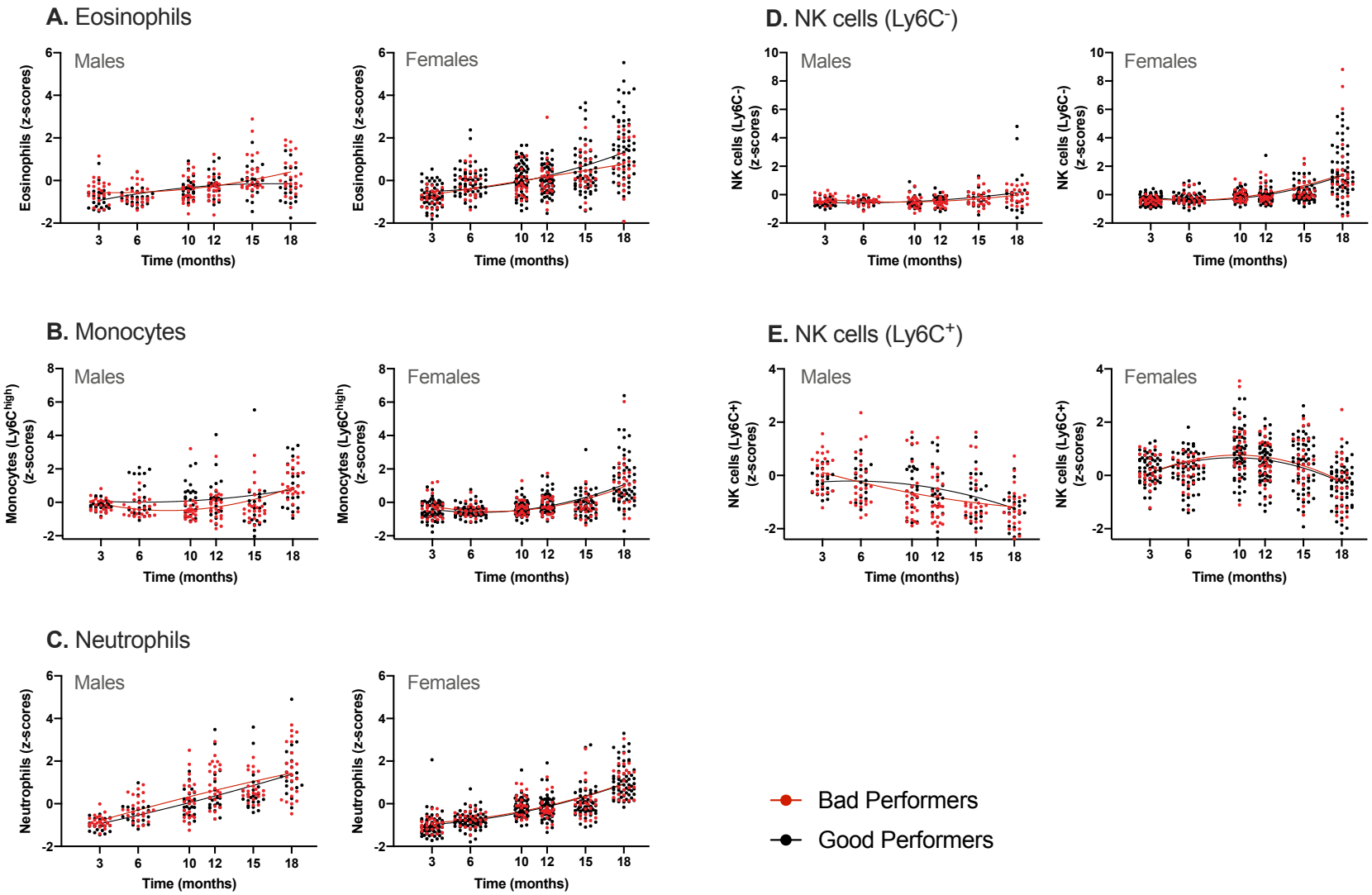


**FIGURE 3.** Longitudinal evaluation of the main circulating innate immune cells in accordance to cognitive performance: Eosinophils (A), monocytes Ly6C$^{high}$ (B), neutrophils (C) and NK cells Ly6C$^{-}$ (D) and Ly6C$^{+}$ (E). The representation combines standardized values (z-scores) of the results of the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].

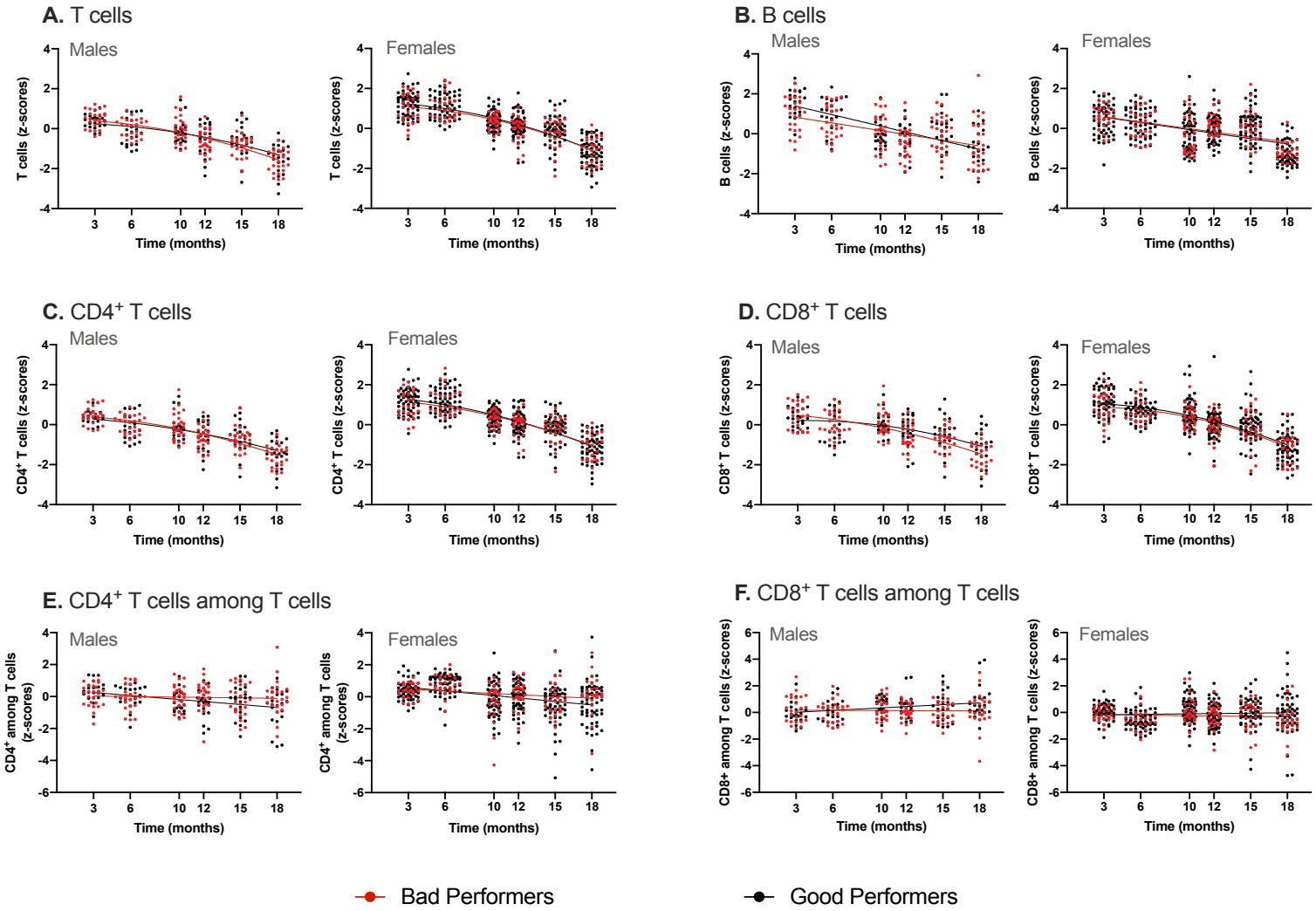


**FIGURE 4.** Longitudinal evaluation of the main circulating adaptive immune cells in accordance with cognitive performance: T cells (A), B cells (B), $CD4^+$ T cells (C), $CD8^+$ T cells (D), and their percentages among T cells, respectively (E and F). The representation combines standardized values (z-scores) of the results of the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].

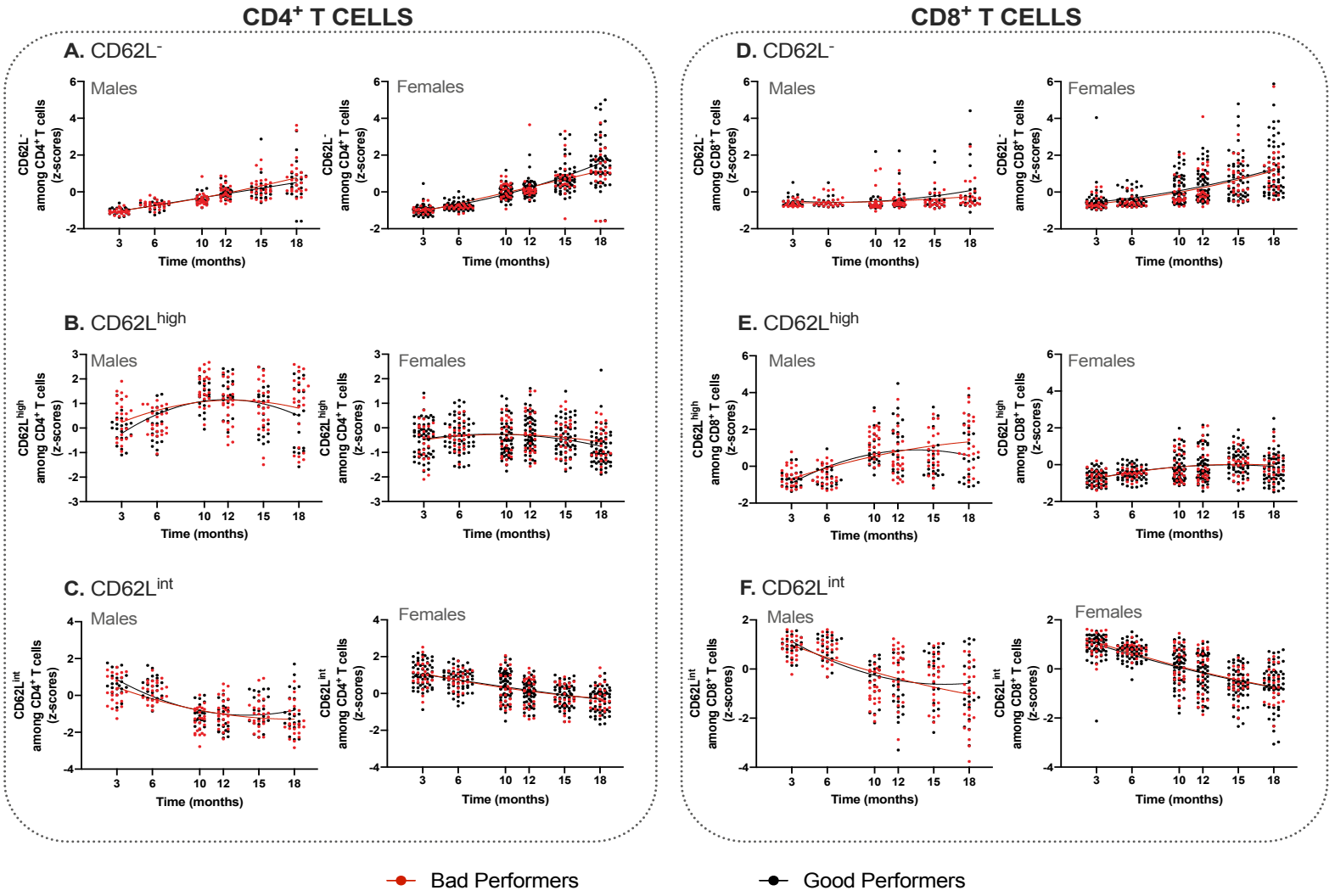


**FIGURE 5.** Longitudinal evaluation of the T cell compartments in accordance to cognitive performance. Percentages over time: for $CD4^+$ T cells – $CD62L^-$ (A), $CD62L^{high}$ (B) and $CD62L^{int}$ (C); for $CD8^+$ T cells – $CD62L^-$ (D), $CD62L^{high}$ (E) and $CD62L^{int}$ (F). The representation combines standardized values (z-scores) of the results of the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].

## 4. DISCUSSION

The cognitive heterogeneity at old age has been attributed to several factors (e.g. education, cardiovascular risk, genetics, lifestyle) (Ardila, 2000; Deary et al., 2009; Gardener et al., 2016; Josefsson et al., 2012; Paulo et al., 2011; Santos et al., 2014; Schievink et al., 2017; Small et al., 2004; Takeda et al., 2017; Wisdom et al., 2011) and the association with immunosenescence, even though already speculated by others (Ron-Harel and Schwartz, 2009), is poorly studied and understood. The present study contributes to fill this gap. We have demonstrated, in a longitudinal study that some of the whole blood immune cell populations at 18 months of age are associated with associative memory and with the likelihood of a mouse to be considered a good or poor cognitive performer. Moreover, mice with distinct cognitive performances present moderate differences on the immune cells' progression over time, which is seen only in males.

Age-related cognitive heterogeneity in rodents has already been reported (Baruch et al., 2014; Foster et al., 2012; Mota et al., 2019). Yet, the classification of mice and rats as having impaired cognitive function is usually based on a single behavioral paradigm (Baruch et al., 2014; Foster et al., 2012; Mota et al., 2019). For instance, 30% of aged mice (18 months old) present retained memory function in a spatial memory task (novel location recognition test) (Baruch et al., 2014). Here we took this approach further and used a broad characterization of the cognitive performance in mice similarly to what has been done in humans (Paulo et al., 2011; Santos et al., 2013). Of notice, mice with good and poor cognitive performances have similar locomotor activity, anxious and depressive-like behaviors.

Interestingly, males are equally distributed between good and poor cognitive performers, while 70% of females cluster as good performers (Table 2). Despite this disproportionate segregation in good and poor cognitive performers, males and females have similar behavioral performances within each cognitive group. Curiously, others have demonstrated that males usually outperform females on spatial and working memory tasks (Yagi and Galea, 2019). This discrepancies only emphasizes the need to explore and take into account the sex-related differences that have already been described in multiple systems: from immune response to cognitive function, both in humans or other animals (Klein and Flanagan, 2016; Yagi and Galea, 2019).

The relative abundance of the main blood populations in old mice is associated with their cognitive performance. Namely, the percentage of $CD8^+$ T cells within the T cell pool and NK cells are positively associated with spatial memory. Conversely, the percentage of $CD4^+$ T cells within the T

cell pool is negatively associated with spatial memory and decreases the odds for a mouse to belong to the good performance cluster. This is in accordance with data obtained in healthy senior individuals (Serre-Miranda et al., 2015): seniors with worse cognitive performance presented higher levels of effector memory $CD4^+$ T cells, even accounting for education, sex, age and mood (Serre-Miranda et al., 2015). However, a causal relationship and the precise mechanism through which those cells impact cognitive aging cannot be inferred from these studies.

In addition, males with better cognitive performance have higher percentages of monocytes $Ly6C^{high}$, have a moderate decrease in the percentage of $CD4^+$ T cells among T cells and have higher percentages of $CD8^+$ T cells among T cells. The decrease over time of the proportion of $CD4^+$ T cells and subsequent increase in the proportion of $CD8^+$ T cells in male mice classified as good performers lead us to hypothesize that an alteration in the cell recruitment maybe present. Curiously, it was already demonstrated that $CD4^+$ T cells can be recruited to the brain borders, such as the meninges and the choroid plexus, were they exert crucial homeostatic functions to maintain brain function and plasticity (Derecki et al., 2010; Wolf et al., 2009; Ziv et al., 2006). We found that male good cognitive performers present higher percentages of monocytes $Ly6C^{high}$ in circulation than poor performers. Curiously, Möhle and collaborators proposed that monocytes $Ly6C^{high}$ are critical for brain homeostasis and plasticity. They demonstrated that peripheral adoptive transfer of monocytes $Ly6C^{high}$ rescues the decreased neurogenesis caused by antibiotic treatment (Möhle et al., 2016).

The major strength of this study is its longitudinal design. By following the same mice over the course of 18 months we were able to explore the effect of intra-individual progressions that would be impossible by simply comparing a young vs an old group. By performing 3 independent experiments we obtained a more robust sample size and diluted the experiment bias that can randomly appear in one experiment and not in the others. Moreover, by analyzing both males and female we were able to detect a sex-specific, even though moderate, association between age-associated alterations of the immune system and cognitive heterogeneity in old mice. On the other hand, one of the limitations of this study is using mice living in specific pathogen free conditions, which does not mimic real life conditions (Rosshart et al., 2019). The recently developed mouse model, which acquired the microbes and pathogens of wild mice while maintaining the genetic background of C57BL/6 called wildlings, was proven to mirror better the human immune response than a conventional lab strain (in this case C57BL/6) does (Rosshart et al., 2019). Additionally, a complete and robust assessment of the cognitive function in rodents would also require the

evaluation of executive-related functions, such as working memory and behavioral flexibility (Shepard et al., 2017), that was not assessed in this study.

Immunosenescence is not limited to alterations in cell frequency and absolute numbers, as investigated here. The present finds suggest that a broader characterization of the immune system may further unravel other age-related alterations with cognition.

The association between the immune system aging and the cognitive heterogeneity in old mice, even being moderate, reinforces the notion that systemic aging might affect the brain function and therefore affect cognition. It is imperative to deeper explore this association and understand the mechanisms behind this crosstalk to develop therapies to delay or even ameliorate age-related cognitive malfunctioning.

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## AUTHORS CONTRIBUTION

CS-M, JAP and MCN conceptualized, designed and supervised the study; CS-M performed the experiments and processed the samples, analyzed the data, prepared the figures, performed the statistical analysis and drafted the first version of the manuscript; SR, NV and GA assisted the behavioral characterization; SR, PB-S, CN, NV, JA, JC-G and CS collected and processed samples; PC, supervised the statistical analysis; and all authors discussed the results and contributed to the final version of the manuscript.

## DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## CONFLICT OF INTEREST

The authors declare no competing interests.

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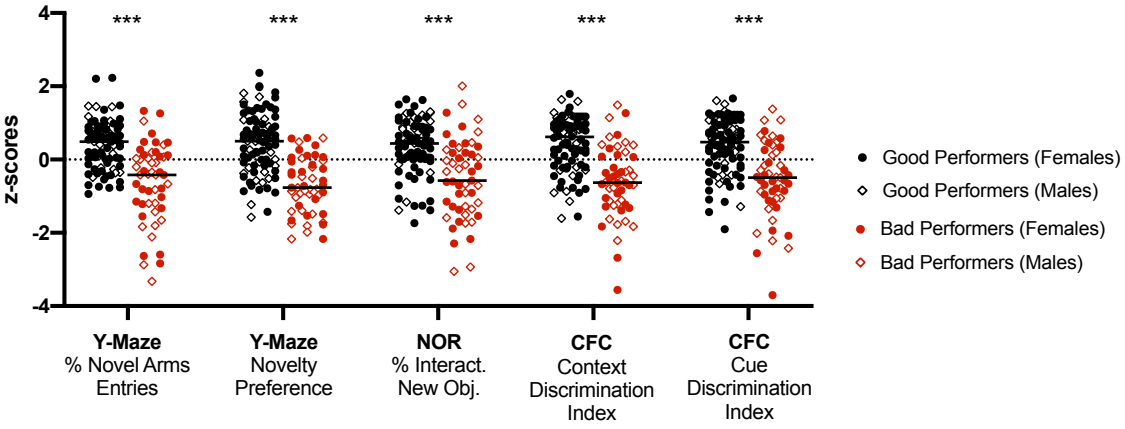
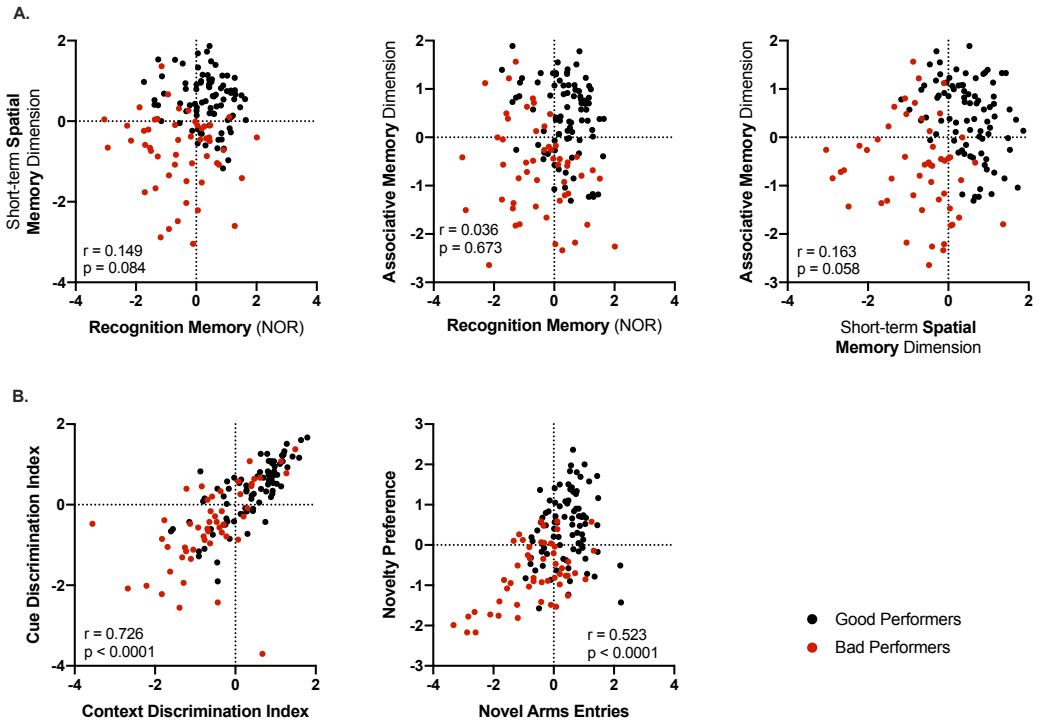
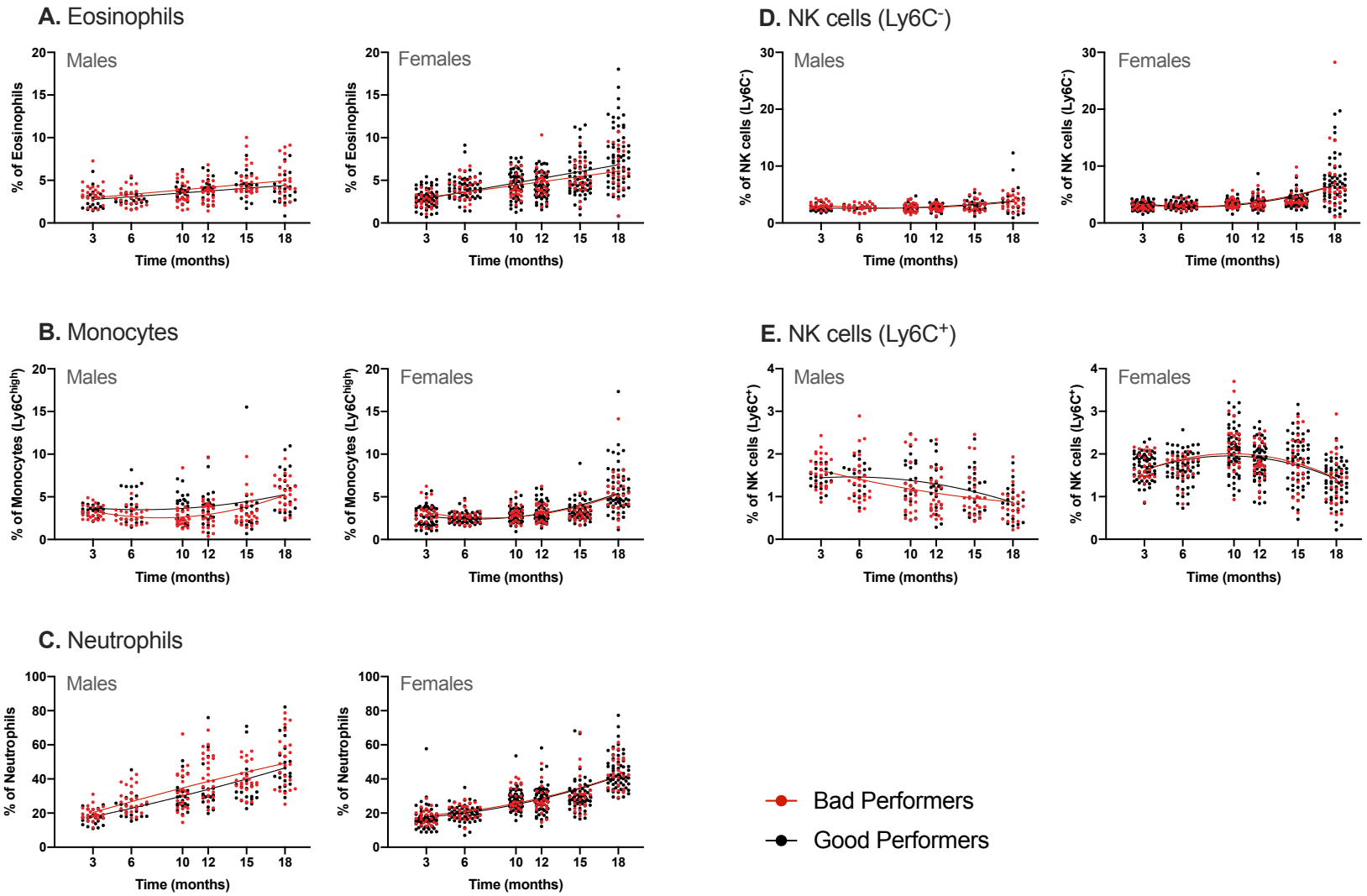
## SUPPLEMENTARY INFORMATION



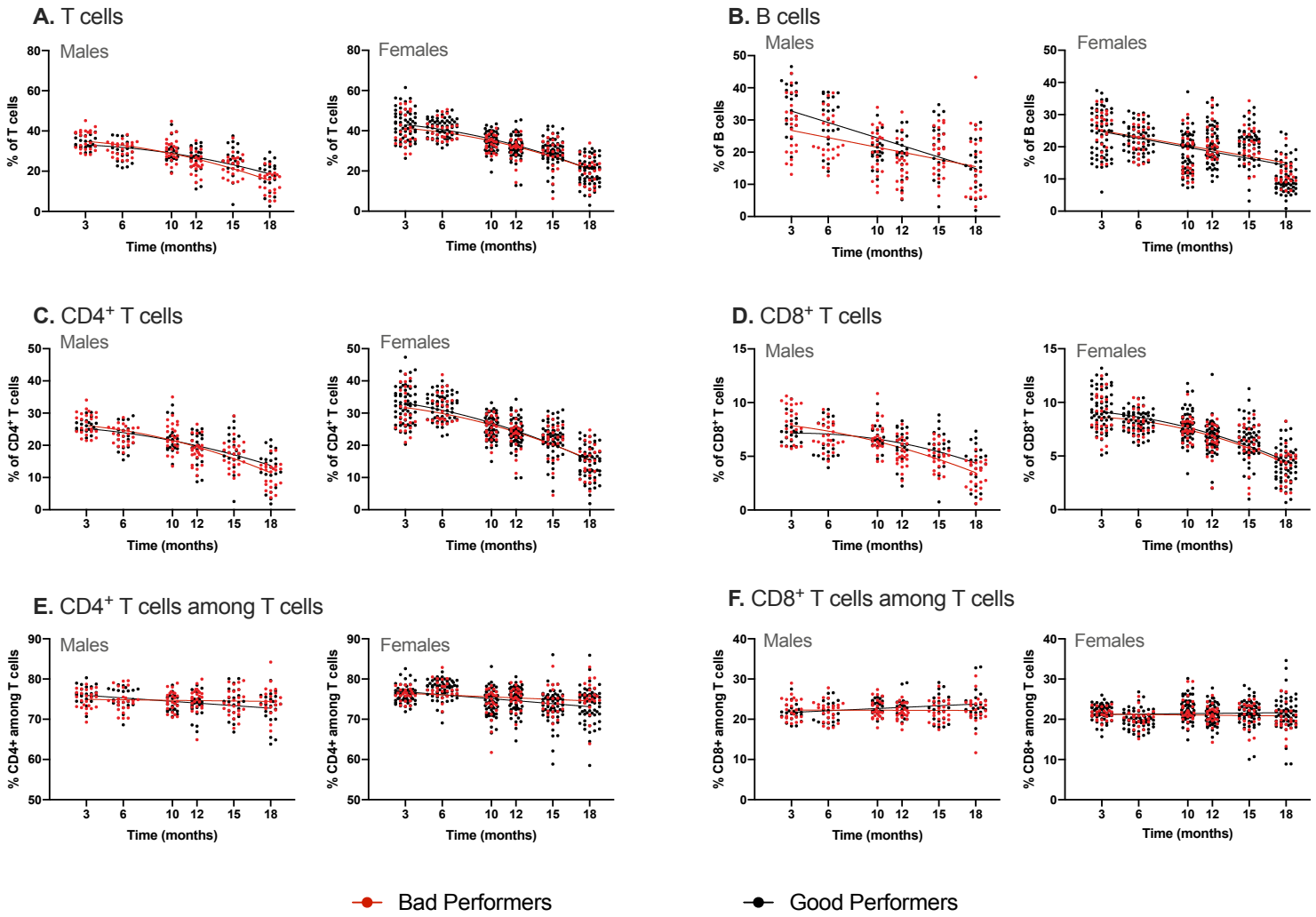

**SUPPLEMENTARY FIGURE 1.** Representation of good and poor cognitive performers in accordance to sex in the various behavioral paradigms: novel arms entries and novelty preference by the Y-maze test; interaction with the new object by the NOR test and context and cue discrimination indexes by the CFC test. Representation of standardized values (z-scores) calculated by set. T-test was used to compare good and poor cognitive performers: *** p<0.001. Female good performers are represented as black circles, male good performers as black diamonds, female bad performers as red circles and male bad performers as red diamonds.



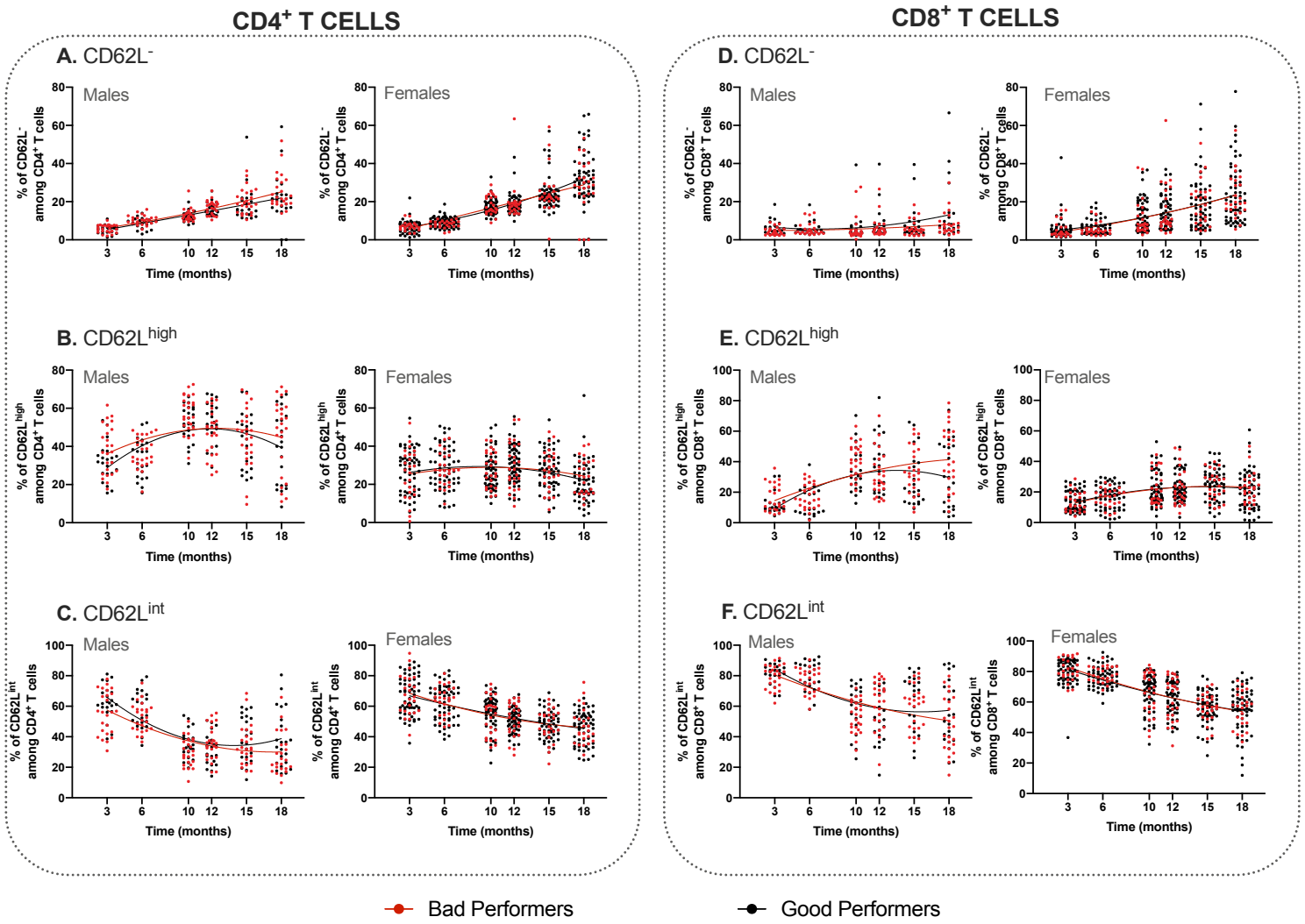

**SUPPLEMENTARY FIGURE 2.** Scatter plots and correlations between short-term spatial, associative and recognition memory dimensions (A) and between variables composing the associative and short-term memory dimensions (B). Representation of standardized values (z-scores) calculated by set. Person's correlation coefficient and p-value represented in the graphs. Good performers are represented as black circles and bad performers as red circles.




**SUPPLEMENTARY FIGURE 3.** Longitudinal evaluation of the main circulating innate immune cells in accordance to cognitive performance: Eosinophils (A), monocytes Ly6C$^{high}$ (B), neutrophils (C) and NK cells Ly6C$^{-}$ (D) and Ly6C$^{+}$ (E). The representation combines the results of the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].




**SUPPLEMENTARY FIGURE 4.** Longitudinal evaluation of the main circulating adaptive immune cells in accordance to cognitive performance: T cells (A), B cells (B), $CD4^+$ T cells (C), $CD8^+$ T cells (D), and their percentages among T cells, respectively (E and F). The representation combines the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].




**SUPPLEMENTARY FIGURE 5.** Longitudinal evaluation of the T cell compartments in accordance to cognitive performance. Percentages over time: for $CD4^+$ T cells – $CD62L^-$ (A), $CD62L^{high}$ (B) and $CD62L^{int}$ (C); for $CD8^+$ T cells – $CD62L^-$ (D), $CD62L^{high}$ (E) and $CD62L^{int}$ (F). The representation combines the results of the 3 experimental sets. Males and females are independently represented to highlight the effect of sex. Each dot represents an animal: good performers are depicted as black dots and bad performers as red dots. Lines represent the best fit equation representative of the data [either linear regression or second order polynomial (quadratic) function].

**SUPPLEMENTARY TABLE 1.** Person's correlation coefficients between the various cognitive behavioral paradigms/dimensions.

| | | Novelty Preference(Y-MAZE) | Novel Arm Entries (Y-MAZE) | Interaction New Object (NOR) | Context Discrimination Index (CFC) | Cue Discrimination Index (CFC) | Associative Memory Dimension | Short-term Spatial Memory Dimension |
|---|---|---|---|---|---|---|---|---|
| **Novelty Preference (Y-MAZE)** | Pearson Correlation | 1 | **0,523** | **0,211** | **0,229** | 0,093 | **0,171** | **0,872** |
| | $r^2$ | | **0,274** | **0,045** | **0,052** | 0,009 | **0,029** | **0,760** |
| | Sig. (2-tailed) | | >0,001 | 0,013 | 0,007 | 0,283 | 0,047 | >0,001 |
| **Novel Arm Entries (Y-MAZE)** | Pearson Correlation | | 1 | 0,062 | **0,193** | 0,055 | 0,119 | **0,871** |
| | $r^2$ | | | 0,004 | **0,037** | 0,003 | 0,014 | **0,759** |
| | Sig. (2-tailed) | | | 0,476 | 0,024 | 0,524 | 0,169 | >0,001 |
| **Interaction New Object (NOR)** | Pearson Correlation | | | 1 | -0,008 | 0,071 | 0,036 | 0,149 |
| | $r^2$ | | | | 0,000 | 0,005 | 0,001 | 0,022 |
| | Sig. (2-tailed) | | | | 0,926 | 0,412 | 0,673 | 0,084 |
| **Context Discrimination Index (CFC)** | Pearson Correlation | | | | 1 | **0,726** | **0,922** | **0,259** |
| | $r^2$ | | | | | **0,527** | **0,850** | **0,067** |
| | Sig. (2-tailed) | | | | | >0,001 | >0,001 | 0,002 |
| **Cue Discrimination Index (CFC)** | Pearson Correlation | | | | | 1 | **0,935** | 0,063 |
| | $r^2$ | | | | | | **0,874** | 0,004 |
| | Sig. (2-tailed) | | | | | | >0,001 | 0,464 |
| **Associative Memory Dimension** | Pearson Correlation | | | | | | 1 | 0,163 |
| | $r^2$ | | | | | | | 0,027 |
| | Sig. (2-tailed) | | | | | | | 0,058 |
| **Short-term Spatial Memory Dimension** | Pearson Correlation | | | | | | | 1 |
| | $r^2$ | | | | | | | |
| | Sig. (2-tailed) | | | | | | | |

**SUPPLEMENTARY TABLE 2.** Binary logistic regression model to investigate the percentage of immune cells at 18 months of age that discriminate between good and poor cognitive performers.

| | B | SE | Wald | Exp(B) |
|---|---|---|---|---|
| **Sex**[a] | -1.114 | 0.395 | 7.951** | 0.328 |
| **zCD4+ T cells among T cells** | -0.505 | 0.216 | 5.458* | 0.604 |
| $\chi^2$ **(df)** | 13.408 (2)*** | | | |
| $R^2$ **Nagelkerke** | 0.133 | | | |
| **Total Hit Rates (%)** | 65.600 | | | |

Abbreviations: SE (standard error)

a - reference category is female

*p=<0.05. **p<0.01. ***p<0.001

**SUPPLEMENTARY TABLE 3.** Linear regression models to explain the variance of short-term spatial memory dimension based on circulating immune cells at 18 months of age.

| | zCD4+ T cells among T cells | | | | zCD8+ T cells among T cells | | | | zNK cells (Ly6C-) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | SE | β | t | B | SE | β | t | B | SE | β | t |
| **Sex**[a] | -0.548 | 0.171 | -0.270 | -3.209** | -0.615 | 0.174 | -0.303 | -3.528*** | -0.386 | 0.185 | -0.190 | -2.09* |
| **zCD4$^+$ T cells among T cells** | -0.165 | 0.082 | -0.168 | -1.999* | | | | | | | | |
| **zCD8$^+$ T cells among T cells** | | | | | 0.178 | 0.084 | 0.181 | 2.110* | | | | |
| **zNK cells (Ly6C$^-$)** | | | | | | | | | 0.190 | 0.089 | 0.193 | 2.129* |
| **F(df1. df2)** | 6.947 (2; 128)*** | | | | 7.191 (2; 128)*** | | | | 7.235 (2; 128)*** | | | |
| **R$^2$** | 0.098 | | | | 0.101 | | | | 0.102 | | | |
| **adjusted R$^2$** | 0.084 | | | | 0.087 | | | | 0.088 | | | |

Abbreviations: SE (standard error)

a - reference category is female

*p=<0.05. **p<0.01. ***p<0.001

**SUPPLEMENTARY TABLE 4.** Linear mixed models testing the effect of time (aging) and cognitive performance (clusters) on the standardized percentages of the main populations of the innate immune system (z-scores).

| Dependent variable | Sex | Parameters | Estimate (B) | SE | df | t | Sig. | 95% CI | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower Bound | Upper Bound |
| **Eosinophils** (z-scores) | Females | Intercept | 0.066 | 0.102 | 143.465 | 0.643 | 0.521 | -0.136 | 0.268 |
| | | cTime | 0.091 | 0.018 | 121.839 | 5.147 | **<0.001** | 0.056 | 0.127 |
| | | Cogntive Cluster | 0.026 | 0.121 | 143.461 | 0.218 | 0.828 | -0.214 | 0.267 |
| | | cTime * Cognitive Cluster | 0.019 | 0.021 | 121.002 | 0.909 | 0.365 | -0.023 | 0.061 |
| | | $cTime^2$ | -0.002 | 0.003 | 249.572 | -0.733 | 0.464 | -0.008 | 0.004 |
| | | $cTime^2$ * Cognitive Cluster | 0.004 | 0.003 | 248.466 | 1.182 | 0.238 | -0.003 | 0.011 |
| | Males | Intercept | -0.399 | 0.078 | 60.186 | -5.132 | **<0.001** | -0.555 | -0.244 |
| | | cTime | 0.076 | 0.013 | 23.662 | 5.985 | **<0.001** | 0.050 | 0.103 |
| | | Cogntive Cluster | 0.066 | 0.114 | 60.187 | 0.579 | 0.565 | -0.162 | 0.293 |
| | | cTime * Cognitive Cluster | -0.017 | 0.019 | 23.313 | -0.907 | 0.373 | -0.055 | 0.021 |
| | | $cTime^2$ | 0.007 | 0.002 | 97.026 | 3.171 | **0.002** | 0.003 | 0.012 |
| | | $cTime^2$ * Cognitive Cluster | -0.010 | 0.003 | 96.471 | -3.028 | **0.003** | -0.017 | -0.003 |
| **Monocytes (Ly6C$^{high}$)** (z-scores) | Females | Intercept | -0.332 | 0.065 | 122.853 | -5.093 | **<0.001** | -0.461 | -0.203 |
| | | cTime | 0.058 | 0.014 | 126.105 | 4.188 | **<0.001** | 0.031 | 0.085 |
| | | Cogntive Cluster | 0.012 | 0.077 | 122.896 | 0.159 | 0.874 | -0.141 | 0.166 |
| | | cTime * Cognitive Cluster | 0.023 | 0.016 | 125.621 | 1.403 | 0.163 | -0.009 | 0.055 |
| | | $cTime^2$ | 0.008 | 0.003 | 262.195 | 2.964 | **0.003** | 0.003 | 0.013 |
| | | $cTime^2$ * Cognitive Cluster | 1.45E-04 | 0.003 | 261.754 | 0.047 | 0.963 | -0.006 | 0.006 |
| | Males | Intercept | -0.350 | 0.117 | 104.570 | -2.978 | **0.004** | -0.582 | -0.117 |
| | | cTime | 0.077 | 0.016 | 57.983 | 4.741 | **<0.001** | 0.045 | 0.110 |
| | | Cogntive Cluster | 0.613 | 0.172 | 104.581 | 3.573 | **0.001** | 0.273 | 0.953 |
| | | cTime * Cognitive Cluster | -0.015 | 0.024 | 57.434 | -0.655 | 0.515 | -0.063 | 0.032 |
| | | $cTime^2$ | 0.013 | 0.003 | 104.456 | 4.565 | **<0.001** | 0.008 | 0.019 |
| | | $cTime^2$ * Cognitive Cluster | -0.012 | 0.004 | 104.063 | -2.854 | **0.005** | -0.021 | -0.004 |
| **Neutrophils** (z-scores) | Females | Intercept | -0.229 | 0.072 | 142.195 | -3.191 | **0.002** | -0.371 | -0.087 |
| | | cTime | 0.122 | 0.011 | 90.590 | 11.586 | **<0.001** | 0.101 | 0.143 |
| | | Cogntive Cluster | -0.048 | 0.085 | 142.226 | -0.568 | 0.571 | -0.217 | 0.120 |
| | | cTime * Cognitive Cluster | 0.005 | 0.012 | 90.005 | 0.404 | 0.687 | -0.020 | 0.030 |
| | | $cTime^2$ | 0.004 | 0.002 | 181.423 | 2.068 | **0.040** | 0.000 | 0.008 |
| | | $cTime^2$ * Cognitive Cluster | -8.62E-06 | 0.002 | 181.150 | -0.004 | 0.997 | -0.005 | 0.005 |
| | Males | Intercept | 0.436 | 0.108 | 73.889 | 4.042 | **<0.001** | 0.221 | 0.651 |
| | | cTime | 0.144 | 0.013 | 69.095 | 10.815 | **<0.001** | 0.117 | 0.171 |
| | | Cogntive Cluster | -0.236 | 0.158 | 73.889 | -1.494 | 0.139 | -0.550 | 0.079 |
| | | cTime * Cognitive Cluster | 0.007 | 0.019 | 68.281 | 0.386 | 0.701 | -0.031 | 0.046 |
| | | $cTime^2$ | -0.003 | 0.002 | 113.542 | -1.239 | 0.218 | -0.007 | 0.002 |
| | | $cTime^2$ * Cognitive Cluster | 0.002 | 0.003 | 112.836 | 0.624 | 0.534 | -0.004 | 0.008 |
| **NK cells (Ly6C$^-$)** (z-scores) | Females | Intercept | -0.015 | 0.064 | 101.284 | -0.242 | 0.809 | -0.141 | 0.111 |
| | | cTime | 0.080 | 0.015 | 144.196 | 5.420 | **<0.001** | 0.051 | 0.109 |
| | | Cogntive Cluster | -0.058 | 0.075 | 101.294 | -0.766 | 0.446 | -0.208 | 0.092 |
| | | cTime * Cognitive Cluster | -0.020 | 0.017 | 144.383 | -1.131 | 0.260 | -0.054 | 0.015 |
| | | $cTime^2$ | 0.004 | 0.002 | 240.135 | 1.811 | 0.071 | 0.000 | 0.009 |
| | | $cTime^2$ * Cognitive Cluster | 7.41E-05 | 0.003 | 240.791 | 0.027 | 0.978 | -0.005 | 0.005 |
| | Males | Intercept | -0.508 | 0.059 | 55.181 | -8.605 | **<0.001** | -0.626 | -0.390 |
| | | cTime | 0.025 | 0.012 | 68.855 | 2.184 | **0.032** | 0.002 | 0.048 |
| | | Cogntive Cluster | 0.077 | 0.086 | 55.171 | 0.897 | 0.374 | -0.096 | 0.250 |
| | | cTime * Cognitive Cluster | 0.012 | 0.017 | 68.599 | 0.690 | 0.492 | -0.022 | 0.045 |
| | | $cTime^2$ | 0.006 | 0.002 | 129.206 | 4.000 | **<0.001** | 0.003 | 0.010 |
| | | $cTime^2$ * Cognitive Cluster | -0.004 | 0.002 | 129.784 | -1.726 | 0.087 | -0.009 | 0.001 |
| **NK cells (Ly6C$^+$)** (z-scores) | Females | Intercept | 0.705 | 0.098 | 210.651 | 7.219 | **<0.001** | 0.513 | 0.898 |
| | | cTime | -0.017 | 0.067 | 28479.607 | -0.258 | 0.796 | -0.149 | 0.115 |
| | | Cogntive Cluster | -0.091 | 0.116 | 210.681 | -0.788 | 0.432 | -0.320 | 0.137 |
| | | cTime * Cognitive Cluster | -0.003 | 0.080 | 28479.761 | -0.033 | 0.973 | -0.159 | 0.154 |
| | | $cTime^2$ | -0.014 | 0.002 | 375.974 | -8.857 | **<0.001** | -0.017 | -0.011 |
| | | $cTime^2$ * Cognitive Cluster | 0.001 | 0.002 | 375.045 | 0.729 | 0.467 | -0.002 | 0.005 |
| | Males | Intercept | -0.804 | 0.177 | 45.000 | -4.538 | **<0.001** | -1.161 | -0.447 |
| | | cTime | -0.083 | 0.009 | 45.421 | -9.142 | **<0.001** | -0.102 | -0.065 |
| | | Cogntive Cluster | 0.355 | 0.259 | 45.000 | 1.369 | 0.178 | -0.167 | 0.876 |
| | | cTime * Cognitive Cluster | 0.014 | 0.013 | 45.031 | 1.050 | 0.299 | -0.013 | 0.041 |
| | | $cTime^2$ | 0.003 | 0.003 | 43.829 | 1.272 | 0.210 | -0.002 | 0.009 |
| | | $cTime^2$ * Cognitive Cluster | -0.010 | 0.004 | 43.603 | -2.388 | **0.021** | -0.018 | -0.001 |

SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

**SUPPLEMENTARY TABLE 5.** Linear mixed models testing the effect of time (aging) and cognitive performance (clusters) on the standardized percentages of the main populations of the adaptive immune system (z-scores).

| Dependent variable | Sex | Parameters | Estimate (B) | SE | df | t | Sig. | 95% CI | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower Bound | Upper Bound |
| **B cells** (z-scores) | Females | Intercept | 0.082 | 0.090 | 52.204 | 0.910 | 0.367 | -0.099 | 0.264 |
| | | cTime | -0.083 | 0.074 | 2579.504 | -1.126 | 0.260 | -0.228 | 0.062 |
| | | Cogntive Cluster | -0.010 | 0.107 | 52.198 | -0.093 | 0.926 | -0.226 | 0.205 |
| | | cTime * Cognitive Cluster | -0.004 | 0.088 | 2609.656 | -0.048 | 0.961 | -0.176 | 0.168 |
| | | $cTime^2$ | -0.006 | 0.002 | 474.312 | -3.635 | **<0.001** | -0.009 | -0.003 |
| | | $cTime^2$ * Cognitive Cluster | -0.002 | 0.002 | 473.266 | -1.065 | 0.287 | -0.005 | 0.002 |
| | Males | Intercept | -0.018 | 0.101 | 76.704 | -0.174 | 0.862 | -0.219 | 0.184 |
| | | cTime | -0.101 | 0.075 | 5135.512 | -1.333 | 0.183 | -0.249 | 0.047 |
| | | Cogntive Cluster | 0.111 | 0.148 | 76.650 | 0.747 | 0.457 | -0.184 | 0.406 |
| | | cTime * Cognitive Cluster | -0.037 | 0.110 | 5213.865 | -0.339 | 0.734 | -0.254 | 0.179 |
| | | $cTime^2$ | 0.005 | 0.002 | 248.386 | 3.179 | **0.002** | 0.002 | 0.009 |
| | | $cTime^2$ * Cognitive Cluster | 0.003 | 0.003 | 246.841 | 1.200 | 0.231 | -0.002 | 0.008 |
| **T cells** (z-scores) | Females | Intercept | 0.305 | 0.080 | 131.592 | 3.822 | **<0.001** | 0.147 | 0.463 |
| | | cTime | -0.150 | 0.010 | 81.962 | -14.447 | **<0.001** | -0.170 | -0.129 |
| | | Cogntive Cluster | 0.062 | 0.095 | 131.593 | 0.651 | 0.516 | -0.126 | 0.249 |
| | | cTime * Cognitive Cluster | -0.008 | 0.012 | 81.561 | -0.674 | 0.502 | -0.033 | 0.016 |
| | | $cTime^2$ | -0.006 | 0.002 | 220.226 | -3.338 | **0.001** | -0.010 | -0.002 |
| | | $cTime^2$ * Cognitive Cluster | 7.20E-05 | 0.002 | 219.994 | 0.034 | 0.973 | -0.004 | 0.004 |
| | Males | Intercept | -0.293 | 0.090 | 90.998 | -3.271 | **0.002** | -0.472 | -0.115 |
| | | cTime | -0.135 | 0.010 | 49.782 | -13.963 | **<0.001** | -0.154 | -0.116 |
| | | Cogntive Cluster | -0.019 | 0.131 | 90.989 | -0.145 | 0.885 | -0.279 | 0.241 |
| | | cTime * Cognitive Cluster | 0.025 | 0.014 | 49.205 | 1.746 | 0.087 | -0.004 | 0.053 |
| | | $cTime^2$ | -0.005 | 0.002 | 82.865 | -2.917 | **0.005** | -0.008 | -0.002 |
| | | $cTime^2$ * Cognitive Cluster | 0.002 | 0.002 | 82.374 | 0.622 | 0.536 | -0.003 | 0.006 |
| **CD4⁺ T cells** (z-scores) | Females | Intercept | 0.273 | 0.079 | 130.442 | 3.439 | **0.001** | 0.116 | 0.430 |
| | | cTime | -0.147 | 0.010 | 80.598 | -14.331 | **<0.001** | -0.168 | -0.127 |
| | | Cogntive Cluster | 0.052 | 0.094 | 130.438 | 0.551 | 0.583 | -0.134 | 0.238 |
| | | cTime * Cognitive Cluster | -0.012 | 0.012 | 80.240 | -0.998 | 0.322 | -0.036 | 0.012 |
| | | $cTime^2$ | -0.005 | 0.002 | 195.662 | -2.731 | **0.007** | -0.008 | -0.001 |
| | | $cTime^2$ * Cognitive Cluster | -4.87E-05 | 0.002 | 195.432 | -0.023 | 0.982 | -0.004 | 0.004 |
| | Males | Intercept | -0.306 | 0.089 | 90.496 | -3.428 | **0.001** | -0.483 | -0.128 |
| | | cTime | -0.131 | 0.009 | 49.469 | -14.044 | **<0.001** | -0.149 | -0.112 |
| | | Cogntive Cluster | -0.053 | 0.130 | 90.490 | -0.405 | 0.687 | -0.312 | 0.206 |
| | | cTime * Cognitive Cluster | 0.018 | 0.014 | 48.900 | 1.303 | 0.199 | -0.010 | 0.045 |
| | | $cTime^2$ | -0.004 | 0.002 | 82.090 | -2.677 | **0.009** | -0.008 | -0.001 |
| | | $cTime^2$ * Cognitive Cluster | 0.002 | 0.002 | 81.609 | 0.912 | 0.364 | -0.003 | 0.007 |
| **CD4⁺ T cells among T cells** (z-scores) | Females | Intercept | 0.215 | 0.139 | 135.148 | 1.549 | 0.124 | -0.060 | 0.490 |
| | | cTime | -0.038 | 0.019 | 92.226 | -1.929 | 0.057 | -0.076 | 0.001 |
| | | Cogntive Cluster | -0.073 | 0.165 | 135.135 | -0.443 | 0.659 | -0.400 | 0.253 |
| | | cTime * Cognitive Cluster | -0.038 | 0.023 | 91.640 | -1.638 | 0.105 | -0.084 | 0.008 |
| | | $cTime^2$ | -0.001 | 0.003 | 190.757 | -0.418 | 0.677 | -0.006 | 0.004 |
| | | $cTime^2$ * Cognitive Cluster | -0.004 | 0.003 | 189.859 | -1.109 | 0.269 | -0.010 | 0.003 |
| | Males | Intercept | -0.064 | 0.124 | 64.059 | -0.517 | 0.607 | -0.312 | 0.184 |
| | | cTime | -0.009 | 0.016 | 48.913 | -0.543 | 0.589 | -0.042 | 0.024 |
| | | Cogntive Cluster | -0.246 | 0.182 | 64.058 | -1.356 | 0.180 | -0.609 | 0.117 |
| | | cTime * Cognitive Cluster | -0.050 | 0.024 | 48.420 | -2.103 | **0.041** | -0.098 | -0.002 |
| | | $cTime^2$ | 0.001 | 0.002 | 104.022 | 0.360 | 0.720 | -0.004 | 0.006 |
| | | $cTime^2$ * Cognitive Cluster | 0.003 | 0.003 | 103.217 | 0.786 | 0.434 | -0.004 | 0.009 |
| **CD8⁺ T cells** (z-scores) | Females | Intercept | 0.293 | 0.084 | 150.300 | 3.474 | **0.001** | 0.126 | 0.459 |
| | | cTime | -0.136 | 0.066 | 25118.011 | -2.052 | **0.040** | -0.267 | -0.006 |
| | | Cogntive Cluster | 0.069 | 0.100 | 150.410 | 0.692 | 0.490 | -0.128 | 0.267 |
| | | cTime * Cognitive Cluster | -0.003 | 0.079 | 25299.837 | -0.034 | 0.973 | -0.157 | 0.152 |
| | | $cTime^2$ | -0.009 | 0.001 | 480.248 | -6.204 | **<0.001** | -0.011 | -0.006 |
| | | $cTime^2$ * Cognitive Cluster | 0.001 | 0.002 | 478.618 | 0.893 | 0.372 | -0.002 | 0.005 |
| | Males | Intercept | -0.149 | 0.105 | 42.193 | -1.419 | 0.163 | -0.362 | 0.063 |
| | | cTime | -0.133 | 0.012 | 46.450 | -11.206 | **<0.001** | -0.156 | -0.109 |
| | | Cogntive Cluster | 0.187 | 0.154 | 42.113 | 1.217 | 0.230 | -0.123 | 0.497 |
| | | cTime * Cognitive Cluster | 0.042 | 0.017 | 46.023 | 2.412 | **0.020** | 0.007 | 0.076 |
| | | $cTime^2$ | -0.007 | 0.002 | 40.616 | -3.106 | **0.003** | -0.012 | -0.002 |
| | | $cTime^2$ * Cognitive Cluster | -0.002 | 0.003 | 40.175 | -0.556 | 0.581 | -0.009 | 0.005 |
| **CD8⁺ T cells among T cells** (z-scores) | Females | Intercept | -0.311 | 0.141 | 79.201 | -2.210 | **0.030** | -0.591 | -0.031 |
| | | cTime | -0.029 | 0.017 | 115.162 | -1.668 | 0.098 | -0.063 | 0.005 |
| | | Cogntive Cluster | 0.036 | 0.167 | 79.208 | 0.218 | 0.828 | -0.296 | 0.369 |
| | | cTime * Cognitive Cluster | 0.021 | 0.021 | 114.561 | 1.011 | 0.314 | -0.020 | 0.061 |
| | | $cTime^2$ | -0.001 | 0.003 | 62.146 | -0.388 | 0.700 | -0.008 | 0.005 |
| | | $cTime^2$ * Cognitive Cluster | 0.005 | 0.004 | 62.131 | 1.193 | 0.238 | -0.003 | 0.012 |
| | Males | Intercept | 0.108 | 0.106 | 37.192 | 1.016 | 0.316 | -0.107 | 0.323 |
| | | cTime | -0.001 | 0.080 | 939.411 | -0.008 | 0.994 | -0.158 | 0.156 |
| | | Cogntive Cluster | 0.342 | 0.155 | 37.131 | 2.209 | **0.033** | 0.028 | 0.656 |
| | | cTime * Cognitive Cluster | 0.047 | 0.117 | 949.731 | 0.401 | 0.688 | -0.183 | 0.277 |
| | | $cTime^2$ | 0.002 | 0.002 | 230.745 | 0.933 | 0.352 | -0.002 | 0.005 |
| | | $cTime^2$ * Cognitive Cluster | -0.004 | 0.003 | 227.926 | -1.438 | 0.152 | -0.009 | 0.001 |

SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

**SUPPLEMENTARY TABLE 6.** Linear mixed models testing the effect of time (aging) and cognitive performance (clusters) on the standardized percentages of the T cell compartments (z-scores).

| Dependent variable | Sex | Parameters | Estimate (B) | SE | df | t | Sig. | 95% CI | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Lower Bound | Upper Bound |
| **CD62L$^{-}$ among CD4$^{+}$ T cells** (z-scores) | Females | Intercept | -0.043 | 0.067 | 84.586 | -0.641 | 0.523 | -0.177 | 0.091 |
| | | cTime | 0.170 | 0.013 | 162.597 | 12.983 | **<0.001** | 0.144 | 0.196 |
| | | Cogntive Cluster | 0.028 | 0.080 | 84.613 | 0.346 | 0.730 | -0.131 | 0.186 |
| | | cTime * Cognitive Cluster | 0.001 | 0.016 | 161.757 | 0.047 | 0.963 | -0.030 | 0.031 |
| | | cTime$^{2}$ | 0.005 | 0.002 | 219.635 | 3.064 | **0.002** | 0.002 | 0.009 |
| | | cTime$^{2}$ * Cognitive Cluster | 0.000 | 0.002 | 220.005 | -0.012 | 0.990 | -0.004 | 0.004 |
| | Males | Intercept | -0.234 | 0.052 | 47.670 | -4.529 | **<0.001** | -0.338 | -0.130 |
| | | cTime | 0.117 | 0.010 | 87.410 | 11.198 | **<0.001** | 0.096 | 0.138 |
| | | Cogntive Cluster | -0.020 | 0.076 | 47.670 | -0.267 | 0.791 | -0.172 | 0.132 |
| | | cTime * Cognitive Cluster | -0.008 | 0.015 | 86.499 | -0.518 | 0.606 | -0.038 | 0.022 |
| | | cTime$^{2}$ | 0.001 | 0.001 | 118.785 | 0.988 | 0.325 | -0.001 | 0.004 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.001 | 0.002 | 117.939 | -0.283 | 0.777 | -0.005 | 0.003 |
| **CD62L$^{hi}$ among CD4$^{+}$ T cells** (z-scores) | Females | Intercept | -0.266 | 0.077 | 103.551 | -3.480 | **0.001** | -0.418 | -0.115 |
| | | cTime | -0.007 | 0.058 | 13327.959 | -0.114 | 0.910 | -0.120 | 0.107 |
| | | Cogntive Cluster | -0.007 | 0.091 | 103.641 | -0.082 | 0.935 | -0.188 | 0.173 |
| | | cTime * Cognitive Cluster | -0.009 | 0.069 | 13364.847 | -0.127 | 0.899 | -0.143 | 0.126 |
| | | cTime$^{2}$ | -0.005 | 0.001 | 223.748 | -3.668 | **<0.001** | -0.008 | -0.003 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.001 | 0.002 | 223.418 | -0.842 | 0.401 | -0.005 | 0.002 |
| | Males | Intercept | 1.072 | 0.101 | 45.689 | 10.590 | **<0.001** | 0.868 | 1.275 |
| | | cTime | 0.040 | 0.080 | 4335.859 | 0.509 | 0.611 | -0.115 | 0.196 |
| | | Cogntive Cluster | 0.031 | 0.148 | 45.681 | 0.211 | 0.834 | -0.267 | 0.329 |
| | | cTime * Cognitive Cluster | 0.008 | 0.116 | 4581.649 | 0.069 | 0.945 | -0.220 | 0.236 |
| | | cTime$^{2}$ | -0.010 | 0.002 | 101.196 | -6.086 | **<0.001** | -0.014 | -0.007 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.006 | 0.002 | 99.932 | -2.395 | **0.018** | -0.011 | -0.001 |
| **CD62L$^{int}$ among CD4$^{+}$ T cells** (z-scores) | Females | Intercept | 0.224 | 0.077 | 332.821 | 2.911 | **0.004** | 0.073 | 0.376 |
| | | cTime | -0.094 | 0.009 | 130.817 | -10.953 | **<0.001** | -0.111 | -0.077 |
| | | Cogntive Cluster | 0.050 | 0.091 | 332.712 | 0.552 | 0.582 | -0.129 | 0.230 |
| | | cTime * Cognitive Cluster | 0.004 | 0.010 | 130.614 | 0.403 | 0.688 | -0.016 | 0.024 |
| | | cTime$^{2}$ | 0.002 | 0.002 | 269.564 | 1.117 | 0.265 | -0.002 | 0.007 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.001 | 0.003 | 268.696 | -0.415 | 0.678 | -0.006 | 0.004 |
| | Males | Intercept | -0.969 | 0.127 | 50.593 | -7.618 | **<0.001** | -1.224 | -0.714 |
| | | cTime | -0.106 | 0.013 | 197.895 | -8.381 | **<0.001** | -0.130 | -0.081 |
| | | Cogntive Cluster | 0.068 | 0.186 | 50.515 | 0.367 | 0.715 | -0.305 | 0.442 |
| | | cTime * Cognitive Cluster | 0.007 | 0.018 | 198.666 | 0.388 | 0.698 | -0.029 | 0.043 |
| | | cTime$^{2}$ | 0.009 | 0.002 | 87.058 | 4.315 | **<0.001** | 0.005 | 0.014 |
| | | cTime$^{2}$ * Cognitive Cluster | 0.007 | 0.003 | 87.157 | 2.093 | **0.039** | 0.000 | 0.013 |
| **CD62L$^{-}$ among CD8$^{+}$ T cells** (z-scores) | Females | Intercept | 0.007 | 0.126 | 103.870 | 0.053 | 0.958 | -0.242 | 0.256 |
| | | cTime | 0.132 | 0.018 | 85.802 | 7.206 | **<0.001** | 0.096 | 0.169 |
| | | Cogntive Cluster | 0.114 | 0.149 | 103.914 | 0.764 | 0.447 | -0.182 | 0.410 |
| | | cTime * Cognitive Cluster | -0.005 | 0.022 | 85.417 | -0.213 | 0.832 | -0.048 | 0.039 |
| | | cTime$^{2}$ | 0.005 | 0.002 | 200.780 | 2.335 | **0.021** | 0.001 | 0.010 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.001 | 0.003 | 200.305 | -0.389 | 0.697 | -0.006 | 0.004 |
| | Males | Intercept | -0.471 | 0.088 | 48.943 | -5.339 | **<0.001** | -0.649 | -0.294 |
| | | cTime | 0.009 | 0.011 | 49.151 | 0.822 | 0.415 | -0.013 | 0.030 |
| | | Cogntive Cluster | -0.005 | 0.129 | 48.937 | -0.039 | 0.969 | -0.264 | 0.254 |
| | | cTime * Cognitive Cluster | 0.024 | 0.016 | 49.170 | 1.514 | 0.136 | -0.008 | 0.055 |
| | | cTime$^{2}$ | -0.001 | 0.001 | 129.336 | -1.045 | 0.298 | -0.004 | 0.001 |
| | | cTime$^{2}$ * Cognitive Cluster | 0.005 | 0.002 | 129.985 | 2.710 | **0.008** | 0.001 | 0.009 |
| **CD62L$^{hi}$ among CD8$^{+}$ T cells** (z-scores) | Females | Intercept | -0.117 | 0.088 | 194.724 | -1.322 | 0.188 | -0.290 | 0.057 |
| | | cTime | 0.047 | 0.011 | 87.158 | 4.240 | **<0.001** | 0.025 | 0.069 |
| | | Cogntive Cluster | 0.004 | 0.105 | 194.898 | 0.037 | 0.970 | -0.203 | 0.210 |
| | | cTime * Cognitive Cluster | -0.006 | 0.013 | 86.702 | -0.459 | 0.647 | -0.032 | 0.020 |
| | | cTime$^{2}$ | -0.005 | 0.002 | 137.541 | -2.331 | **0.021** | -0.008 | -0.001 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.001 | 0.002 | 137.691 | -0.220 | 0.826 | -0.005 | 0.004 |
| | Males | Intercept | 0.620 | 0.163 | 598.221 | 3.810 | **<0.001** | 0.300 | 0.940 |
| | | cTime | 0.130 | 0.020 | 94.043 | 6.370 | **<0.001** | 0.089 | 0.170 |
| | | Cogntive Cluster | -0.105 | 0.238 | 598.973 | -0.442 | 0.659 | -0.572 | 0.362 |
| | | cTime * Cognitive Cluster | -0.039 | 0.030 | 91.512 | -1.310 | 0.194 | -0.098 | 0.020 |
| | | cTime$^{2}$ | -0.005 | 0.003 | 9.957 | -1.771 | 0.107 | -0.012 | 0.001 |
| | | cTime$^{2}$ * Cognitive Cluster | -0.006 | 0.004 | 9.916 | -1.338 | 0.211 | -0.016 | 0.004 |
| **CD62L$^{int}$ among CD8$^{+}$ T cells** (z-scores) | Females | Intercept | 0.082 | 0.088 | 200.296 | 0.924 | 0.356 | -0.093 | 0.256 |
| | | cTime | -0.129 | 0.012 | 85.968 | -10.562 | **<0.001** | -0.153 | -0.104 |
| | | Cogntive Cluster | -0.069 | 0.105 | 200.443 | -0.654 | 0.514 | -0.276 | 0.138 |
| | | cTime * Cognitive Cluster | 0.005 | 0.014 | 85.624 | 0.378 | 0.706 | -0.023 | 0.034 |
| | | cTime$^{2}$ | 0.001 | 0.002 | 175.842 | 0.410 | 0.682 | -0.003 | 0.005 |
| | | cTime$^{2}$ * Cognitive Cluster | 0.001 | 0.003 | 175.825 | 0.215 | 0.830 | -0.004 | 0.006 |
| | Males | Intercept | -0.222 | 0.143 | 129.804 | -1.556 | 0.122 | -0.504 | 0.060 |
| | | cTime | -0.128 | 0.017 | 269.110 | -7.671 | **<0.001** | -0.161 | -0.095 |
| | | Cogntive Cluster | 0.094 | 0.208 | 129.748 | 0.451 | 0.652 | -0.318 | 0.506 |
| | | cTime * Cognitive Cluster | 0.023 | 0.024 | 271.823 | 0.965 | 0.336 | -0.024 | 0.071 |
| | | cTime$^{2}$ | 0.004 | 0.003 | 137.693 | 1.397 | 0.165 | -0.002 | 0.009 |
| | | cTime$^{2}$ * Cognitive Cluster | 0.003 | 0.004 | 137.118 | 0.636 | 0.526 | -0.005 | 0.010 |

SE (standard error); df (degrees of freedom); CI (confidence interval); Statistically significant results are highlighted in bold.

# CHAPTER IV

## COGNITION IS ASSOCIATED WITH PERIPHERAL IMMUNE MOLECULES IN HEALTHY OLDER ADULTS: A CROSS-SECTIONAL STUDY

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# COGNITION IS ASSOCIATED WITH PERIPHERAL IMMUNE MOLECULES IN HEALTHY OLDER ADULTS: A CROSS-SECTIONAL STUDY

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## ABSTRACT

**Background.** Cognition in the elderly is heterogeneous. Senescence of the immune system is increasingly considered as a potential player in cognitive performance. We explored here the interplay between cognitive performance and peripheral immune molecules in healthy older individuals.

**Methods.** Cross-sectional study of clinically well characterized senior healthy individuals (120; 51 to 87 years old) previously clustered as "Good" and "Poor" performers based on established tests that evaluate memory and executive function. Plasma concentration of 30 immune molecules was assessed by multiplex analysis and correlated with parameters of cognitive performance.

**Results.** Participants with worse cognitive performance ("Poor") exhibited increased concentrations of IL-1β, IL-8, IL-13 and TNF when compared to individuals with a better cognitive performance ("Good"). The cognitive dimensions memory and executive function, when considered separately, displayed a negative association with several immune molecules (IL-1β, IL-1RA, IL-6, IL-13, IP-10 and TNF with memory and only IL-1β with executive function), even controlling for age, sex, years of formal education, mood and use of anti-inflammatory drugs. Regression analysis showed that several of these molecules (IL-1β, IL-6, IL-8 and IL-13) contribute to predict whether an individual belongs to the "Good" or "Poor" cognitive performance group.

**Conclusions.** These results strengthen the hypothesis that increased concentration of peripheral immune molecules, like IL-1β, are associated with worse cognitive performance in senior healthy individuals. It further highlights that some poorly studied immune molecules should be considered in the context of cognitive aging, such as IL-13, here revealed as a new player in such interaction.



**KEYWORDS**

Healthy aging; cognition; immune molecules; cytokines; chemokines.

# 1. INTRODUCTION

Understanding how peripheral immune mediators interact with the central nervous system (CNS) and influence cognition is of great relevance to provide clues on strategies to reduce or delay aging-associated cognitive decline in an increasingly aged population.

Several studies associate peripheral T lymphocytes and the cytokines they produce with cognitive performance, both in humans and in animal models (Kipnis et al., 2012; Serre-Miranda et al., 2015). Moreover, it was previously shown, in the same cohort of senior individuals studied here, that those displaying worse cognitive performance have a higher number of circulating effector memory (EM) $CD4^+$ T cells (Serre-Miranda et al., 2015). In fact, using regression models, EM $CD4^+$ T cells were shown to contribute in predicting both memory and executive functions, controlling for age, sex, years of formal education and mood (Serre-Miranda et al., 2015). Interestingly, among T cells, EM $CD4^+$ T cells are major cytokine producers in the periphery and possibly contribute to the increased inflammatory profile (also known as "inflammaging") usually observed with aging (Franceschi et al., 2017). In accordance, it has been evidenced an association between increased peripheral inflammatory profile with worse cognitive performances (Baune et al., 2008a; Heringa et al., 2014; Trollor et al., 2012), with few studies addressing more than 3 immune molecules in cognitively healthy senior cohorts (IL-6 and C-reactive protein (CRP) are most commonly studied (Baune et al., 2008a; Heringa et al., 2014; Trollor et al., 2012)). Interestingly, studies in rodents revealed that bloodborne factors from old mice are able to impair spatial learning and memory as well as neurogenesis and synaptic plasticity in young animals (Villeda et al., 2011). By contrast, blood from young animals is able to reverse age-related impairments (Villeda et al., 2014). Even though the precise bloodborne factors that have the capacity to modulate and influence cognitive function are still under investigation, they likely relate to immune cells and/or immune mediators. Hereupon, the aim of the present study was to perform an extensive analysis of peripheral immune molecules and explore their association with cognitive performance in a senior cognitively healthy population.

# 2. METHODS

## 2.1. PARTICIPANTS CHARACTERIZATION

The sample of this study originates from a larger study in which a cohort representative of the older Portuguese population, in terms of sex and education, was selected from two districts in the North of Portugal (Guimarães and Vizela) (n=1051). These individuals were extensively clinically and

cognitively characterized (Santos et al., 2014). Cluster analysis identified neurocognitive/psychological performance patterns, and the two extreme groups were termed "Good" and "Poor" cognitive performers. For further characterization a total sample of 120 subjects was selected, from both the "Good" and "Poor" cognitive performance groups, balanced for sex and age (sample size estimated assuming a two-tailed sample size, a medium effect size (d=0.5), an alpha of 0.05, a statistical power of 0.8 and an equal sample size for each group). The socio-demographic and clinical characterization of the participants is presented in Table 1. The recruitment and data acquisition took place between March 2012 and March 2013. Participants with incapacity and/or inability to attend the clinical and neuropsychological sessions, diagnosed with cognitive impairment or dementia and/or unable to understand informed consent, with disorders of the central nervous system or with overt thyroid pathology were not recruited. One participant was *a posteriori* excluded from the analysis due to impossibility of conducting blood collection.

Considering the high prevalence of cytomegalovirus (CMV) infection in the Portuguese population and the impact of this chronic viral infection in the immune system, the presence of anti-CMV IgG was determined. Only 7 out of the 119 participants were non-immune to CMV; no correlations were observed between CMV antibody titers and the cognitive performance of the participants (data not shown).

## 2.2. COGNITIVE CHARACTERIZATION

Trained psychologists evaluated the cognitive and mood profile of the participants as previously described (Santos et al., 2014). Briefly, the cognitive profile was established using a battery of neurocognitive and psychological tests selected to evaluate short-term verbal memory, verbal working memory, response inhibition/cognitive flexibility, verbal fluency, multiple trial verbal learning and memory, high level information processing speed, global cognitive status and mood. Using a principal component analysis, the neurocognitive/psychological test variables were grouped in 3 dimensions: memory (MEM), general and executive function (GENEXEC) and mood (Geriatric Depression Scale - GDS). The cognitive groups, identified using cluster analysis, were classified as "Good" and "Poor" cognitive performers (Santos et al., 2014). Descriptive information with respect to scores for GDS, MEM and GENEXEC of the total participants and of the "Good" and "Poor" cognitive performance groups is described in Table 1.

### 2.3. PERIPHERAL IMMUNE MOLECULES QUANTIFICATION

Blood was collected in EDTA tubes and processed for plasma collection and for standard hospital biochemical analysis. Plasma was obtained by gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, USA) for 30 min, at room temperature, according to the manufacturer's instructions, and stored at -80 °C. Prior to use, plasma samples were centrifuged at 10000 g for 10 min at 4 °C to remove platelets and precipitates. Each participant was assigned a code and all analyses were assessed blindly.

**TABLE 1. Socio-demographic, clinical and neuropsychological characterization**

| | All Participants | "Good" Cognitive Performers | "Poor" Cognitive Performers | "Good" vs "Poor" |
|---|---|---|---|---|
| **Total sample** | 119 | 64 (53.8%) | 55 (46.2%) | n.s |
| **Sex** | | | | |
| Male | 63 (52.9%) | 37 (57.8%) | 26 (47.3%) | n.s |
| Female | 56 (47.1%) | 27 (42.2%) | 29 (52.7%) | |
| **Age** | | | | |
| Mean [Range] | 65.9 [51-87] | 64.3 [51-82] | 67.7 [52-87] | * |
| SD | 8.4 | 8.3 | 8.2 | |
| **School Years** | | | | |
| Median | 4 | 4 | 4 | *** |
| 0 | 3.4% | – | 7.3% | |
| 1-2 | 10.9% | 6.3% | 16.4% | |
| 3-4 | 63% | 56.3% | 70.9% | |
| 5-8 | 4.2% | 6.3% | 1.8% | |
| 9-12 | 14.3% | 23.4% | 3.6% | |
| 13+ | 4.2% | 7.9% | – | |
| **Anti-Inflammatory Drugs** | | | | |
| Yes | 18 (15.1%) | 8 (12.5%) | 10 (18.2%) | n.s |
| **Immune to CMV** | | | | |
| Yes | 112 (94.1%) | 60 (93.8%) | 52 (94.5%) | n.s |
| **Neuropsychological evaluation** | | | | |
| MEM [Mean; SD] | 0.297; 1.209 | 1.245; 0.732 | -0.807; 0.516 | |
| GENEXC [Mean; SD] | 0.031; 1.318 | 1.007; 0.923 | -1.126; 0.557 | |
| GDS [Mean; SD] | -0.023; 1.029 | -0.343; 0.931 | 0.349; 1.021 | |

*p =< 0.05, ***p < 0.001, n.s.: non-significant, SD: standard deviation.

Cytokines, chemokines and other immune molecules were quantified using multiplex magnetic bead-based immunoassays: Macrophage Inflammatory Protein-3β (MIP-3β)/Chemokine (C-C motif) ligand 19 (CCL19) using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel III - Immunology Multiplex Assay (Merck Millipore, USA); Interleukin (IL)-1β, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, Interferon (IFN)-γ, Tumor Necrosis Factor (TNF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, Interferon-γ-induced protein (IP)-10/C-X-C motif chemokine (CXCL)10, monocyte chemoattractant protein (MCP)-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Eotaxin/CCL11 using the Bio-Plex Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Lda, USA); IL-33, IL-37, IFN-α, IFN-β and IFN-ω using a Human ProcartaPlex Mix & Match (Invitrogen, USA). The plates were read at Bio-Plex MAGPIX Multiplex Reader and the data analyzed using the Bio-Plex Manager™ MP Software (both from Bio-Rad Laboratories, Lda, USA). Lower limit of quantification (LLOQ) and the percentage of detection for each analyte are listed in supplemental Table 1. Inter-assays coefficient of variation was lower than 4.5%. Samples bellow LLOQ were extrapolated from the standard curve. The quantification of high-sensitivity C-reactive protein (hsCRP) was conducted at the certified Pathology Laboratory of Braga's Hospital. Due to technical problems 3 samples were not quantified for some of the immune molecules measured (sample size is presented in supplemental Table 1).

## 2.4. STATISTICAL ANALYSIS

Only analytes with a percentage of detection above 50% were considered for analysis. Outliers were defined by mean +/- 3 standard deviations (SD) and excluded from the sample (supplemental Table 1). Data for MEM, GENEXEC and GDS were used in the analysis as z-scores, as previously determined (Santos et al., 2014). To evaluate normal distribution of the variables, skewness and kurtosis values were calculated and the approximate normal distribution was defined for variables with absolute values of skewness below 3 and of kurtosis below 8 (Kline, 2011). Considering the overall population, all analytes, except IL-6, followed a normal distribution. IL-6 concentration values were log10 transformed to comply with normality. Considering "Good" and "Poor" cognitive performance groups independently, IL-6 and G-CSF did not follow a normal distribution. Levene's test was used to evaluate equality of variances.

To compare the peripheral concentration of immune molecules between "Good" and "Poor", an independent-sample t-test was performed for variables with normal distribution and a Mann-

Whitney U test for variables with non-normal distribution. Significance was considered for p-values equal or below 0.05. To quantify the strength of the differences, Cohen´s *d* was calculated as a measure of effect size (0.2 considered a small effect size, 0.5 a medium effect size and 0.8 a large effect size) (Kotrlik and Williams, 2003).

Linear regression analyses were performed to explore whether the various immune molecules (independently) were able to predict MEM or GENEXEC dimensions (dependent variables) and a binary logistic regression to explore whether the immune molecules were able predict to which cognitive group each individual belongs to ("Good" vs "Poor"), controlling for socio-demographic (age, sex and school years) and clinical (GDS and anti-inflammatory drugs) variables. Multicollinearity between variables was assessed by the tolerance values (all the variables had a tolerance >0.5). The statistical procedures were performed in IBM SPSS Version 25 (IBM Corp, USA) and the graphs were designed using Prism7 (GraphPad Software, USA).

### 2.5. DATA AVAILABILITY STATEMENT

Anonymized data will be shared by request from any qualified investigator.

### 2.6. STANDARD PROTOCOL APPROVALS, REGISTRATIONS AND PATIENT CONSENTS

The study was performed in accordance with the Declaration of Helsinki and approved by ethics review boards (Hospital de Braga, Centro Hospitalar do Alto Ave and Unidade Local de Saúde do Alto Minho) and by the national data protection entity (Comissão Nacional de Proteção de Dados). All study goals and nature of the tests were explained, and informed signed consent obtained from all participants.

## 3. RESULTS

### "POOR" AND "GOOD" COGNITIVE PERFORMERS PRESENT A DISTINCT PERIPHERAL IMMUNE MOLECULES PROFILE

From the 31 analytes measured, 19 were detected in more than 50% of the samples (descriptive statistics and percentages of detection of each analyte for both groups are described in supplement Table 1). "Poor" cognitive performers had higher plasma concentration of IL-1β, IL-8, IL-13 and TNF compared to "Good" cognitive performers (Figure 1 and detailed statistics in supplemental Table 2). No differences were observed in the other analytes measured (supplemental Table 2).

As an internal control, it was validated that all participants presented CRP levels below the limit associated with active inflammation/infection (10 mg/L) (Clyne and Olshaker, 1999).

## IL-1β IS A SIGNIFICANT PREDICTOR OF EXECUTIVE FUNCTION, MEMORY AND THE COGNITIVE CLUSTERS

Cognitive performance has been described to be directly, or indirectly, influenced by socio-demographic variables such as age, sex and years of formal education (Paulo et al., 2011; Santos et al., 2014; Serre-Miranda et al., 2015) and by emotional health (Harvey et al., 2006; Santos et al., 2013). Considering also that the inflammatory profile can be influenced by age (a process known as "inflammaging"), sex, anti-inflammatory drugs and mood (Franceschi et al., 2017; Klein and Flanagan, 2016; Krogh et al., 2014), we next explored the association between cognitive function and the concentration of immune molecules controlling for these variables. Linear regression models were used to explain the variation of executive function (GENEXEC) and memory (MEM) dimension performances and binary logistic regression to discriminate between "Good" and "Poor" cognitive performers using each of the measured plasma molecules independently.

Regarding GENEXEC, only IL-1β, in addition to age, years of formal education and mood, as previously reported (Serre-Miranda et al., 2015), was found as significant predictor (Table 2). The model explained approximately 46% (adjusted $R^2$) of the variance observed in the GENEXEC dimension. Altogether, these observations indicate that, in the GENEXEC, "Good" performers have lower peripheral concentration of IL-1β and, as shown previously, they are also younger, have more years of formal education and better mood than "Poor" performers. To infer about the increment in the predictive power of the model due to the immune molecules, a hierarchical regression was performed. The first block was composed by variables needed to control for - sex, age, school years, mood and anti-inflammatory drugs. The second block was composed by the immune molecules, independently. Data on the concentration of IL-1β increased the predictive power of the model ($R^2$ change) by 1.8%.

Regarding MEM, IL-1β, IL-1RA, IL-6, IL-13, IP-10 and TNF, in addition to age, mood and number of years of formal education, were found as significant predictors (Table 3). Models (Table 3) (a model per molecule) explained between 29% to 32% (adjusted $R^2$) of the variance observed in the MEM dimension. Altogether these observations indicate that "Good" performers in the MEM dimension have diminished peripheral concentration of some immune molecules. To infer about the increment due to the immune molecules in the predictive power of the models, a hierarchical regression was performed as described for GENEXEC. The levels of peripheral immune molecules

increased the predictive power of the model ($R^2$ change): IL-13 by 5.6%, followed by IL-1RA (3.2%), IL-1β and TNF (2.9%), IP-10 (2.8%) and IL-6 (2.7%). Body fat content can influence inflammatory profile, however in this cohort body mass index, whose values range from 19.5 to 38.2 kg per $m^2$, did not influenced the association between immune molecules and MEM and GENEXEC (data not shown).

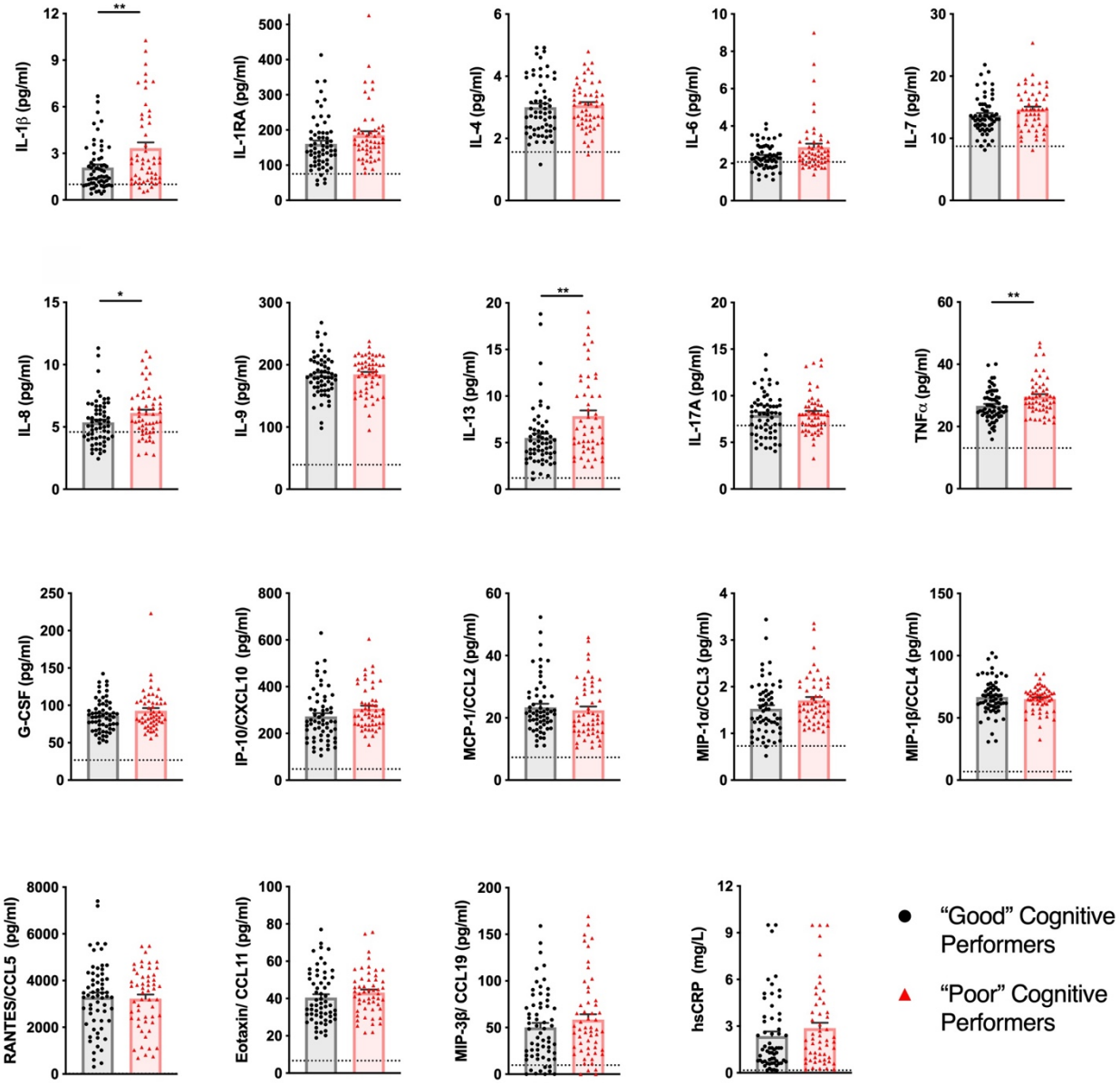


**FIGURE 1. Healthy senior individuals with distinct cognitive performances present differences in the concentration of peripheral immune molecules.** The profile of cytokines, chemokines and other immune molecules in the plasma of "Good" (black circles) and "Poor" (red triangles) cognitive performers. Dashed lines represent LLOQ (lower limit of quantification) and values below LLOQ were extrapolated from the standard curve. Dots represent each participant, columns represent the mean of the group and bars the standard error of the mean (*p<0.05, **p<0.01).

The classification as "Good" or "Poor" performers was defined based on the individual scores in GENEXEC and MEM. To examine whether the immune molecules measured in plasma, together with sex, age, years of formal education, mood and anti-inflammatory drugs, could account for the group delineation ("Good" vs "Poor"), a binary logistic regression was performed (Table 4). In addition to years of formal education and mood, IL-1β, IL-6, IL-8 and IL-13 (independently) were found as significant predictors. $R^2_{Nagelkerke}$ values ranged from 0.348 to 0.407, indicating that about 35 to 41% of the chance to belong to a particular cognitive group could be predicted by the model. The correct classifications of the subjects (% of total hit rates), based on the independent variables added in the model, ranged from 69 to 71%. To infer about the increment of the immune molecules in the predictive power of the models, the same rational of the linear regression models was followed and a hierarchical regression performed. The levels of peripheral immune molecules increased the predictive power of the model ($R^2_{Nagelkerke}$ change): IL-13 (10.7%), followed by IL-1β (7.4%), IL-6 (4.4%) and IL-8 (3.5%).

Of notice, inflammasome activation has been associated with cognitive symptoms in several human diseases (Cheon et al., 2020) and with cognitive deficits in autoimmune experimental models (Hou et al., 2020). As such, we further investigated inflammasome activation as a possible mechanism behind the low-grade inflammation observed and the cognitive deterioration. To do so, the expression of 5 inflammasome activation markers (*nlrp3, aim2, pycard, casp1*and *nlrc4*) (He et al., 2016) was analyzed in peripheral blood mononuclear cells from the same participants (Supplemental Methods and Supplemental Table 3). No differences were detected in the expression levels of these genes between "Good" and "Poor" cognitive performance groups (Supplemental Figure 1).

**TABLE 2. Linear regression models to explain the variance of general and executive function (GENEXEC)**

| | IL-1β | | | |
|---|---|---|---|---|
| | **B** | **SE** | **β** | **t** |
| **Sex**[a] | -0.141 | 0.196 | -0.054 | -0.722 |
| **Age** | -0.047 | 0.011 | -0.302 | -4.161*** |
| **School years** | 0.133 | 0.029 | 0.367 | 4.528*** |
| **GDS** | -0.353 | 0.100 | -0.279 | -3.518** |
| **Anti-inflammatory drugs** | 0.166 | 0.265 | 0.044 | 0.628 |
| **IL-1β** | -0.084 | 0.043 | -0.139 | -1.945* |
| **F(df1. df2)** | 15.635 (6; 106)*** | | | |
| **$R^2$ (change)** | 0.489 (0.018) | | | |
| **adjusted $R^2$** | 0.460 | | | |

Abbreviations: SE (standard error); GDS (geriatric depression scale); a - reference category is male; *p=<0.05, **p<0.01, ***p<0.001

**TABLE 3. Linear regression models to explain the variance of memory (MEM)**

| | IL-1β | | | | IL-1RA | | | | IL-6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **B** | **SE** | **β** | **t** | **B** | **SE** | **β** | **t** | **B** | **SE** | **β** | **t** |
| **Sex**[a] | 0.186 | 0.208 | 0.077 | 0.896 | 0.298 | 0.212 | 0.123 | 1.403 | 0.104 | 0.209 | 0.043 | 0.499 |
| **Age** | -0.036 | 0.012 | -0.253 | -3.043** | -0.033 | 0.012 | -0.226 | -2.743** | -0.026 | 0.012 | -0.178 | -2.084* |
| **School years** | 0.061 | 0.031 | 0.182 | 1.955* | 0.070 | 0.031 | 0.209 | 2.262* | 0.070 | 0.031 | 0.209 | 2.274* |
| **GDS** | -0.425 | 0.106 | -0.364 | -3.995*** | -0.463 | 0.107 | -0.390 | -4.340*** | -0.446 | 0.105 | -0.380 | -4.262*** |
| **Anti-inflammatory drugs** | 0.017 | 0.281 | 0.005 | 0.061 | 0.102 | 0.280 | 0.030 | 0.363 | -0.009 | 0.283 | -0.003 | -0.031 |
| **IL-1β** | -0.098 | 0.046 | -0.175 | -2.131* | | | | | | | | |
| **IL-1RA** | | | | | -0.003 | 0.001 | -0.186 | -2.253* | | | | |
| **IL-6 (log10)** | | | | | | | | | -1.470 | 0.702 | -0.175 | -2.096* |
| **F(df1. df2)** | 8.595 (6; 106)*** | | | | 8.821 (6; 107)*** | | | | 8.914 (6; 108)*** | | | |
| **$R^2$ (change)** | 0.327 (0.029) | | | | 0.331 (0.032) | | | | 0.331 (0.027) | | | |
| **adjusted $R^2$** | 0.289 | | | | 0.293 | | | | 0.294 | | | |

| | IL-13 | | | | IP-10 | | | | TNF | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **B** | **SE** | **β** | **t** | **B** | **SE** | **β** | **t** | **B** | **SE** | **β** | **t** |
| **Sex**[a] | 0.184 | 0.202 | 0.076 | 0.911 | 0.173 | 0.208 | 0.071 | 0.831 | 0.167 | 0.207 | 0.068 | 0.807 |
| **Age** | -0.033 | 0.012 | -0.228 | -2.793** | -0.023 | 0.013 | -0.157 | -1.792 | -0.030 | 0.012 | -0.206 | -2.493* |
| **School years** | 0.072 | 0.031 | 0.214 | 2.332* | 0.067 | 0.031 | 0.200 | 2.158* | 0.062 | 0.031 | 0.186 | 2.008* |
| **GDS** | -0.402 | 0.104 | -0.343 | -3.865*** | -0.453 | 0.105 | -0.387 | -4.320*** | -0.450 | 0.105 | -0.381 | -4.291*** |
| **Anti-inflammatory drugs** | -0.040 | 0.277 | -0.012 | -0.144 | -0.047 | 0.279 | -0.014 | -0.170 | 0.033 | 0.276 | 0.010 | 0.118 |
| **IL-13** | -0.074 | 0.024 | -0.244 | -3.026** | | | | | | | | |
| **IP-10** | | | | | -0.002 | 0.001 | -0.180 | -2.112* | | | | |
| **TNF-α** | | | | | | | | | -0.038 | 0.017 | -0.175 | -2.174* |
| **F(df1. df2)** | 9.745 (6; 105)*** | | | | 8.575 (6; 107)*** | | | | 9.079 (6; 107)*** | | | |
| **$R^2$ (change)** | 0.358 (0.056) | | | | 0.325 (0,028) | | | | 0.337 (0.029) | | | |
| **adjusted $R^2$** | 0.321 | | | | 0.287 | | | | 0.300 | | | |

Abbreviations: SE (standard error); GDS (geriatric depression scale)

a - reference category is male

*p=<0.05, **p<0.01, ***p<0.001

**TABLE 4. Binary logistic regression models to investigate the variables that discriminate between "Good" and "Poor" cognitive performers**

| | IL-1β | | | | IL-6 | | | |
|---|---|---|---|---|---|---|---|---|
| | **B** | **SE** | **Wald** | **Exp(B)** | **B** | **SE** | **Wald** | **Exp(B)** |
| **Sex**[a] | -0.551 | 0.484 | 1.296 | 0.577 | -0.452 | 0.481 | 0.884 | 0.636 |
| **Age** | 0.043 | 0.029 | 2.141 | 1.043 | 0.012 | 0.029 | 0.159 | 1.012 |
| **School years** | -0.313 | 0.120 | 6.778** | 0.732 | -0.342 | 0.123 | 7.700** | 0.710 |
| **GDS** | 0.506 | 0.240 | 4.452* | 1.659 | 0.594 | 0.236 | 6.344* | 1.811 |
| **Anti-inflammatory drugs** | -0.345 | 0.625 | 0.305 | 0.708 | -0.507 | 0.626 | 0.657 | 0.602 |
| **IL-1β** | 0.326 | 0.125 | 6.777** | 1.385 | | | | |
| **IL-6 (log10)** | | | | | 3.906 | 1.851 | 4.453* | 49.708 |
| $\chi^2_{(df)}$ | 36.653 (6)*** | | | | 36.505 (6)*** | | | |
| **$R^2_{Nagelkerke}$ (change)** | 0.370 (0.074) | | | | 0.363 (0.044) | | | |
| **Total Hit Rates (%)** | 69.000 | | | | 68.700 | | | |
| | **IL-8** | | | | **IL-13** | | | |
| | **B** | **SE** | **Wald** | **Exp(B)** | **B** | **SE** | **Wald** | **Exp(B)** |
| **Sex**[a] | -0.558 | 0.475 | 1.384 | 0.572 | -0.694 | 0.499 | 1.937 | 0.499 |
| **Age** | 0.030 | 0.028 | 1.139 | 1.031 | 0.039 | 0.029 | 1.847 | 1.040 |
| **School years** | -0.333 | 0.125 | 7.095** | 0.717 | -0.371 | 0.122 | 9.178** | 0.690 |
| **GDS** | 0.558 | 0.233 | 5.706* | 1.747 | 0.498 | 0.243 | 4.213* | 1.646 |
| **Anti-inflammatory drugs** | -0.254 | 0.614 | 0.172 | 0.775 | -0.173 | 0.634 | 0.074 | 0.841 |
| **IL-8** | 0.231 | 0.119 | 3.767* | 1.260 | | | | |
| **IL-13** | | | | | 0.225 | 0.071 | 9.926** | 1.252 |
| $\chi^2_{(df)}$ | 34.754 (6)*** | | | | 40.648 (6)*** | | | |
| **$R^2_{Nagelkerke}$ (change)** | 0.348 (0.035) | | | | 0.407 (0.107) | | | |
| **Total Hit Rates (%)** | 68.7 | | | | 70.500 | | | |

Abbreviations: SE (standard error); GDS (geriatric depression scale)

a - reference category is male

*p=<0.05, **p<0.01, ***p<0.001

## 4. DISCUSSION

This study shows that, in a healthy senior cohort, the individuals with worse cognitive performance have higher plasma concentrations of IL-1β, IL-8, IL-13 and TNF. In addition, several immune molecules present a negative association with both memory (IL-1β, IL-1RA, IL-6, IL-13, IP-10 and TNF) and executive function (IL-1β), even controlling for age, sex, number of years of formal education, mood and use of anti-inflammatory drugs. Interestingly, some of the immune molecules (IL-1β, IL-6, IL-8 and IL-13) are able to discriminate between "Good" or "Poor" cognitive performers.

Higher plasma concentrations of IL-1β negatively predicts both memory and executive function, and increases the odds of belonging to the "Poor" cognitive performance group in the regression models. These observations are in line with several previous studies, namely: i) IL-1β expression by activated monocytes predicts, negatively, cognitive performance in working memory in healthy senior individuals (Simpson et al., 2013); ii) IL-1β administration in rodents (both in the central nervous system and in the periphery) induces hippocampal-dependent memory deficits (reviewed in (Huang and Sheng, 2010)). However, some studies found no association or beneficial effect of IL-1β in cognitive function: i) serum concentration of IL-1β failed to demonstrate association with several cognitive domains in healthy senior individuals (Baune et al., 2008a); ii) higher serum levels of IL-1β were associated with better semantic memory in older women even controlling for age, education, body mass index and the presence of disease (Lekander et al., 2011); iii) blocking IL-1β signaling in rodents, both by administering IL-1RA (an IL-1β receptor antagonist) or by knocking out IL-1β receptor, impaired memory and learning (Avital et al., 2003; Goshen et al., 2007); iv) IL-1β administration in rodents had no effect or was beneficial on cognitive function (reviewed in (Huang and Sheng, 2010)).

In addition to IL-1β, other immune molecules were found to associate with a specific cognitive domain: IL-1RA, IP-10 and TNF negatively associate with memory function; high concentrations of IL-8 increase the odds of belonging to the "Poor" performance group; and IL-6 and IL-13 both negatively associate with memory and increase the odds of belonging to the "Poor" performance group.

The role of IL-1RA on cognition has not been extensively addressed and most of the available evidence originates from animal studies. To our knowledge, the present report is the first to describe a negative association between peripheral IL-1RA and memory in healthy older adults. Interestingly, serum concentration of IL-1RA was also inversely correlated with cognitive function in a senior population with bipolar disorder (Lotrich et al., 2014); however, it may not necessarily affect cognition through the same mechanisms. Plus, intracerebral injection of IL-1RA, or its over expression exclusively in the CNS, was shown to cause learning and memory impairments in various rodent models (Goshen et al., 2007; Yirmiya et al., 2002). The overall evidence favors the hypothesis of a negative role of IL-1RA in the modulation of cognition. Most of the literature support that the pro-inflammatory profile, usually observed in senior individuals, is associated with worse cognitive profile (Baune et al., 2008a; Heringa et al., 2014; Trollor et al., 2012). Interestingly, IL-

1RA has mainly an anti-inflammatory action (by blocking one of the IL-1β receptors (Dinarello, 2018)) and seems to be negatively associated with cognition.

IP-10, also known as IFNγ-induced protein 10 or CXCL10, is secreted by several cell populations in response to IFNγ. Here we observed that IP-10 concentration negatively associates with working memory performance after controlling for age and other confounders (as also shown by others (Bradburn et al., 2018)). More so, IFNγ was below detection limit for most individuals in our cohort; but, interestingly, IFNγ was identified as a negative regulator of cognitive functioning in rodents (Monteiro et al., 2016).

Regarding TNF, a negative association with memory is here shown. Another study in which TNF was part of a composite score of various immune biomarkers, showed a negative association with processing speed, attention and executive functioning, but not with memory (Heringa et al., 2014). In addition, most reports in healthy human elders (mostly older than 70 years of age and based on cognitive assessment diverse from ours) found no association between peripheral TNF concentration and cognitive performance (Baune et al., 2008a; Trollor et al., 2012; Yaffe et al., 2003) Discordant findings on the role of TNF in cognition emerge as well from rodent studies (Baune et al., 2008b; Golan et al., 2004; McAfoose et al., 2009). Nevertheless, it is possible that the role of TNF on cognition may be age-dependent. In accordance, McAfoose and colleagues (McAfoose et al., 2009) showed that TNF-/- mice perform worse than WT when they are young and better when they are old (McAfoose et al., 2009).

IL-8, although poorly studied in the context of cognition, was one of the immune molecules that increased the odds to belong to the "Poor" cognitive performance group in the regression model, which is in accordance with other studies (Baune et al., 2008a; Goldstein et al., 2015). Still, in a large sample, another report showed no association between IL-8 and cognitive function in a non-demented community-dwelling elderly individuals (Trollor et al., 2012).

IL-6 is one of the best studied cytokines in aging. The vast majority of studies report a negative association between peripheral concentration of IL-6 and cognitive performance (Mooijaart et al., 2013; Tegeler et al., 2016; Trollor et al., 2012; Yaffe et al., 2003), mainly with measures of executive function, and not of memory (Mooijaart et al., 2013; Tegeler et al., 2016; Trollor et al., 2012). In this study we show no association between the concentration of IL-6 and executive function; but higher levels of IL-6 associate with worst memory and increase odds of belonging to the "Poor" cognitive performance group. Of note, in several other studies it has also been shown to predict cognitive decline (Singh-Manoux et al., 2014; Weaver et al., 2002; Yaffe et al., 2003).

The majority of the literature, including ours, associates an increase in peripheral IL-6 with worst memory function in seniors (although two studies reported no association (Baune et al., 2008a; Dik et al., 2005)).

To our knowledge, only one publication indicated that IL-13 deficiency in rodents significantly impairs working and reference memory (Brombacher et al., 2017). Here we observed the opposite - IL-13 not only negatively associates with memory, but also increases the odds of belonging to the "Poor" cognitive performance group. These results highlight the need to perform more studies using cohorts composed by healthy aging individuals and addressing less explored immune molecules like IL-13 and others.

The major study strengths are its extensive evaluation of peripheral immune molecules and the cohort being composed by extremely well characterized healthy seniors; the comprehensive neuropsychological characterization assessing two distinct cognitive domains (memory and executive function); and the control for active inflammatory responses (through quantification of CRP) and for the use of anti-inflammatory drugs. Nonetheless, the cross-sectional design does not allow causal conclusions about the relationship between peripheral profile of immune molecules and age-associated cognitive decline.

A note on the inconsistencies observed between human studies is warranted. These may possibly occur due to differences in selection, characterization and inclusion/exclusion criteria. For instance, the age ranges and the sample sizes vary greatly, as well as in the diversity in the cognitive evaluation tools and in the concentration range of the immune molecules measured. More so, not all studies check or control for possible active inflammatory processes or the intake of anti-inflammatory drugs (Baune et al., 2008a; Lekander et al., 2011). CRP, an acute phase protein, is vastly studied in geriatric populations and has been associated with an increased risk for cerebrovascular disease, Alzheimer's Disease and vascular dementia (Schmidt et al., 2002). Some studies described no association between levels of CRP and cognitive performance in healthy senior individuals (Dik et al., 2005; Lin et al., 2018; Trollor et al., 2012) as we do here, while others demonstrate an association between higher levels of CRP and worst cognitive performance (Ravaglia et al., 2005; Tegeler et al., 2016; Wersching et al., 2010; Yaffe et al., 2003). Interestingly, in those studies the CRP range is lower than in this study, indicating that other factors that vary between cohorts may mediate or dilute its impact on cognition.

In summary, this study presents an extensive evaluation of peripheral immune molecules in a healthy senior population comprehensively characterized for cognitive performance, allowing a

broader perspective on the association between the peripheral profile of immune molecules and cognitive performance. The data support the hypothesis that the peripheral profile of immune molecules at older ages is related with cognitive performance. Furthermore, it reinforces the pertinence to explore the possible interactions between cytokines in the modulation of cognitive function. It seems that an overall increased concentration of immune molecules, both pro- and anti-inflammatory, associates with stronger cognitive deterioration during aging. Additionally, a reasonable set of these studies, preferentially performed in distinct geographic regions are necessary to feed secondary research, like meta-analysis, to be able to integrate the data from independent studies and clearly define the immune molecules that coherently associate with cognitive functions. Furthermore, the precise mechanisms behind the impact of peripheral immune mediators in the cognitive function are still unknown. Animal models are likely to shed some light on those mechanisms.

Understanding how the immune mediators interact with the CNS and influence cognition is of great relevance to provide clues on potential strategies to reduce or delay aging-associated cognitive decline in an increasingly aged population.

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## CONFLICT OF INTEREST

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

## AUTHORS CONTRIBUTION

CSM had a major role in acquisition of data, performed the statistical analysis of the data, interpreted the data, wrote the first draft of the manuscript; SR designed and conceptualized the study, and revised the manuscript for intellectual content; NCS established the cohort and revised the manuscript for intellectual content; PC participated in establishment of the cohort, assessment and statistic advisory; NS designed and conceptualized the study, revised the manuscript for intellectual content; JAP and MCN designed and conceptualized the study, interpreted the data, and revised the manuscript for intellectual content.

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# SUPPLEMENTARY INFORMATION

## SUPPLEMENTAL METHODS

### QUANTITATIVE REAL-TIME PCR ANALYSIS

Total RNA was isolated from PBMCs (peripheral blood mononuclear cells), previously collected and stored in liquid nitrogen (plasma and cells were isolated from the same blood samples), using a PureLink® RNA Mini Kit (ThermoFisher Scientific) according to the manufacturer's instructions. For cDNA synthesis 1µg of RNA was converted into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed in a CFX96 System (Bio-Rad) using the SsoFast™ EvaGreen® Supermix (Bio-Rad). A melting curve analysis was also carried out to verify the specificity of amplicons. The analysis was performed in the CFX Maestro™ Software and the ΔΔCt method was used to quantify the amount of mRNA level relative to housekeeping genes: *actin*, *b2m* and *gapdh*. All the housekeeping genes presented an average M value inferior to 0.5. The oligonucleotides used for qRT-PCR analysis are listed on Supplemental Table 3. To normalize for the 2 independent runs performed for each gene due the sample size, a z-score method was applied. Samples from the "Good" and "Poor" Cognitive Performance groups were distributed equally by the 2 runs. The inter-run CV (%) was below 10% for all genes, except for *casp1* (13%) and *nlrp3* (19%). To compare the relative expression of genes associated with inflammasome activation between "Good" and "Poor" cognitive performers, an independent-sample t-test was performed.

**SUPPLEMENTAL TABLE 1. Descriptive statistics of the immune molecules measured in plasma of "Good" and "Poor" cognitive performers.**

| | | | All Participants | | "Good" Cognitive Performers | | | | | | "Poor" Cognitive Performers | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Molecules | LLOQ (pg/mL) | Outliers (n) | % Detection | N | % Detection | Mean | SD | Min. | Max. | N | % Detection | Mean | SD | Min. | Max. |
| Molecules with >50% samples above LLOQ | IL-1β | 1.00 | 3 | 84% | 61 | 77% | 2.09 | 1.47 | 0.40 | 6.68 | 52 | 93% | 3.34 | 2.61 | 0.52 | 10.28 |
| | IL-1RA | 74.84 | 2 | 96% | 65 | 92% | 153.00 | 80.25 | 0.00 | 413.20 | 55 | 100% | 174.98 | 90.04 | 0.00 | 525.88 |
| | IL-4 | 1.56 | 2 | 98% | 61 | 98% | 3.01 | 0.91 | 1.16 | 4.92 | 53 | 98% | 3.06 | 0.73 | 1.48 | 4.80 |
| | IL-6 | 2.07 | 1 | 75% | 62 | 72% | 2.37 | 0.66 | 1.12 | 4.12 | 53 | 78% | 2.86 | 1.40 | 1.40 | 9.00 |
| | IL-7 | 8.71 | 4 | 98% | 61 | 98% | 13.67 | 2.95 | 8.12 | 21.84 | 51 | 98% | 14.64 | 3.38 | 8.12 | 25.36 |
| | IL-8 | 4.59 | 1 | 72% | 61 | 66% | 5.37 | 1.79 | 2.44 | 11.32 | 54 | 78% | 6.12 | 1.98 | 2.76 | 11.08 |
| | IL-9 | 39.33 | 1 | 100% | 61 | 100% | 183.43 | 33.13 | 98.08 | 267.88 | 54 | 100% | 184.57 | 29.99 | 95.16 | 238.16 |
| | IL-13 | 1.20 | 4 | 99% | 60 | 98% | 5.51 | 3.34 | 1.08 | 18.80 | 52 | 100% | 7.85 | 4.37 | 2.44 | 19.04 |
| | IL-17A | 6.80 | 2 | 72% | 61 | 69% | 7.94 | 2.29 | 4.04 | 14.40 | 53 | 76% | 8.08 | 2.17 | 3.28 | 13.88 |
| | TNF | 13.07 | 2 | 100% | 61 | 100% | 26.64 | 5.01 | 15.88 | 40.04 | 53 | 100% | 29.58 | 6.08 | 21.24 | 46.96 |
| | G-CSF | 26.75 | 1 | 100% | 61 | 100% | 86.26 | 21.72 | 49.84 | 142.44 | 54 | 100% | 92.73 | 26.56 | 55.84 | 223.28 |
| | IP10/CXCL10 | 47.71 | 2 | 100% | 61 | 100% | 272.56 | 110.21 | 105.52 | 629.92 | 53 | 100% | 305.97 | 91.93 | 151.52 | 604.28 |
| | MCP-1/CCL2 | 7.29 | 2 | 100% | 60 | 100% | 23.39 | 8.74 | 11.00 | 52.36 | 54 | 100% | 22.43 | 8.86 | 10.48 | 45.92 |
| | MIP-1α/CCL3 | 0.73 | 1 | 99% | 61 | 98% | 1.53 | 0.57 | 0.52 | 3.44 | 54 | 100% | 1.71 | 0.51 | 1.04 | 3.36 |
| | MIP-1β/CCL4 | 6.76 | 0 | 100% | 62 | 100% | 66.80 | 14.59 | 30.80 | 102.16 | 54 | 100% | 65.13 | 10.19 | 32.52 | 85.60 |
| | RANTES/CCL5 | 12.63 | 0 | 100% | 62 | 100% | 3293.75 | 1495.24 | 309.12 | 7401.32 | 54 | 100% | 3226.14 | 1300.56 | 686.28 | 5484.64 |
| | CCL11/Eotaxin | 6.68 | 2 | 100% | 61 | 100% | 40.51 | 13.88 | 18.92 | 77.04 | 53 | 100% | 43.19 | 11.96 | 21.72 | 75.64 |
| | CCL19 | 9.67 | 1 | 89% | 63 | 86% | 50.25 | 37.64 | 0.00 | 158.93 | 55 | 93% | 58.45 | 43.20 | 0.00 | 169.20 |
| | hsCRP | 1.60E+05 | 1 | 99% | 61 | 98% | 2.36E+06 | 2.31E+06 | 1.60E+05 | 9.50E+06 | 54 | 100% | 2.87E+06 | 2.54E+06 | 2.30E+05 | 9.50E+06 |
| Molecules with <50% samples above LLOQ | IL-2 | 4.64 | 1 | 8% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-5 | 14.79 | 2 | 13% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-10 | 6.75 | 2 | 16% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-12p70 | 4.88 | 1 | 8% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-15 | 141.47 | 1 | 5% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-33 | 22.79 | 1 | 0% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IL-37 | 4.20 | 1 | 29% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IFN-γ | 7.08 | 1 | 7% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IFN-α | 2.43 | 3 | 0% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IFN-β | 7.11 | 2 | 0% | – | – | – | – | – | – | – | – | – | – | – | – |
| | IFN-ω | 5.11 | 1 | 31% | – | – | – | – | – | – | – | – | – | – | – | – |
| | GM-CSF | 3.33 | 2 | 7% | – | – | – | – | – | – | – | – | – | – | – | – |

Abbreviations: LLOQ (Lower limit of quantification); N (sample size); SD (standard deviation).

**SUPPLEMENTAL TABLE 2. Detailed statistics on the comparison between the immune molecules measured in plasma of "Good" and "Poor" cognitive performers.**

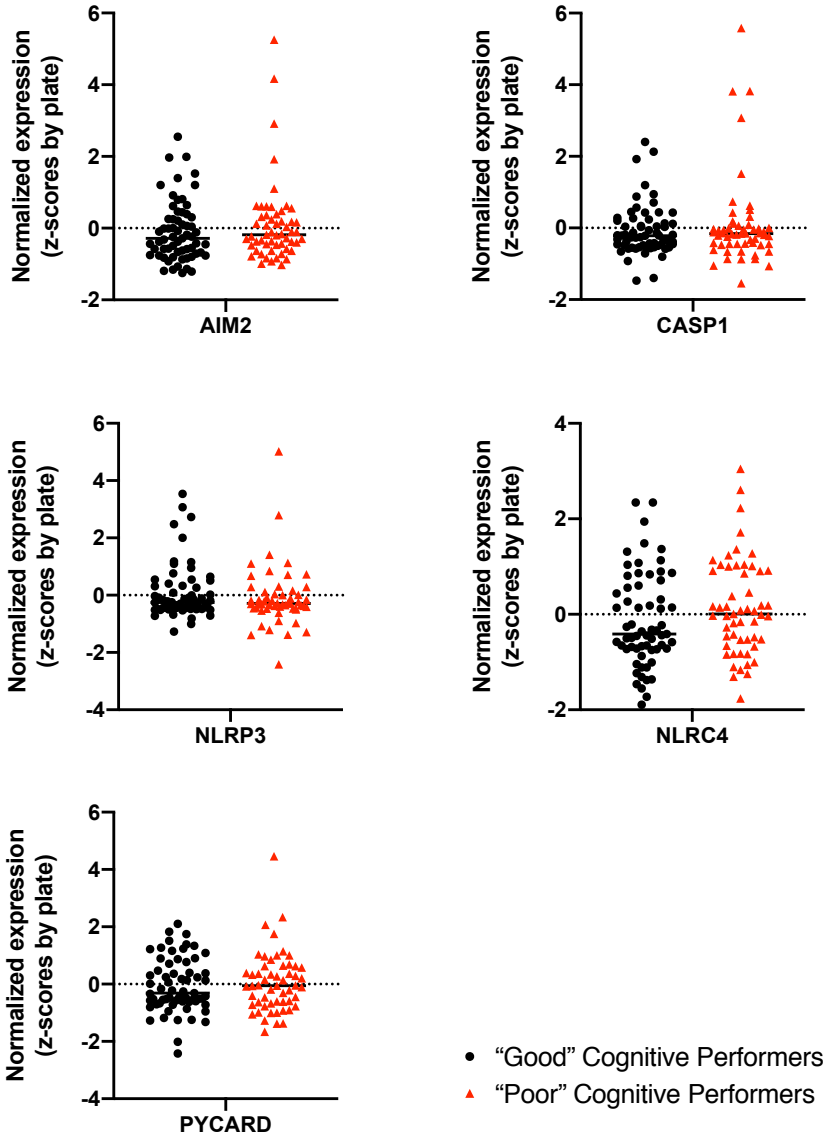
| Independent-sample t-test | | | | |
|---|---|---|---|---|
| **Molecules** | **t** | **df** | **p value** | **d** |
| **IL-1β** | -3.049 | 77.409 | **>0.01** | 0.590 |
| IL-1RA | -1.685 | 112 | 0.10 | – |
| IL-4 | -0.374 | 112 | 0.71 | – |
| IL-7 | -1.608 | 110 | 0.11 | – |
| **IL-8** | -2.128 | 113 | **0.04** | 0.397 |
| IL-9 | -0.193 | 113 | 0.85 | – |
| **IL-13** | -3.137 | 94.591 | **>0.01** | 0.602 |
| IL-17A | -0.342 | 112 | 0.73 | – |
| **TNF** | -2.833 | 112 | **>0.01** | 0.348 |
| IP10/CXCL10 | -1.742 | 112 | 0.08 | – |
| MCP-1/CCL2 | 0.584 | 112 | 0.56 | – |
| MIP-1α/CCL3 | -1.762 | 113 | 0.08 | – |
| MIP-1β/CCL4 | 0.721 | 109.089 | 0.47 | – |
| RANTES/CCL5 | 0.258 | 114 | 0.80 | – |
| CCL11/Eotaxin | -1.096 | 112 | 0.28 | – |
| CCL19 | -1.103 | 116 | 0.27 | – |
| hsCRP | -1.113 | 113 | 0.27 | – |

| Mann-Whitney U test | | | |
|---|---|---|---|
| **Molecules** | **U** | **Z** | **p value** |
| IL-6 | 1339.5 | -1.704 | 0.088 |
| G-CSF | 1430.5 | -1.213 | 0.225 |

Abbreviations: df (degrees of freedom); d (Cohen's d).

**SUPPLEMENTAL TABLE 3. List of primers used for cDNA amplification.**

| Genes | Forward primer | Reverse primer |
|---|---|---|
| *actin* | GCC GTC TTC CCC TCC ATC GTG | GGA GCC ACA CGA GCT CAT TGT AGA |
| *b2m* | AGC AGC ATC ATG GAG GTT TGA | TCA AAC ATG GAG ACA GCA CTC A |
| *gapdh* | CCT CCT GTT CGA CAG TCA G | CGA CCA AAT CCG TTG ACT C |
| *nlrp3* | AAG GGC CAT GGA CTA TTT CC | ACT CCA CCC GAT GAC AGT TC |
| *pycard (asc)* | AAG CCA GGC CTG CAC TTT AT | GGT ACT GCT CAT CCG TCA GG |
| *aim2* | CGT GCT GCA CCA AAA GTC TC | GGC AAA CAG CTT CTG AAA |
| *casp1* | CCA CAA TGG GCT CTG TTT TT | CAT CTG GCT GCT CAA ATG AA |
| *nlrc4* | AAC TCG AGC TTG GGT TG | TTC CCG CCA AAT TCA ACT GC |




**SUPPLEMENTAL FIGURE 1. No differences were found in the expression levels of inflammasome-related genes between "Good" and "Poor" cognitive performers.** The profile of normalized expression (relative to *actin*, *b2m* and *gapdh*) of 5 genes associated with the inflammasome activation in PBMCs collected from "Good" (black circles) and "Poor" (red triangles) cognitive performers. Standardized values are presented to account for inter-run variations. Dots represent each participant and lines the mean for each gene.

# CHAPTER V

## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The data presented in this thesis dissected the relation between the age-associated alterations of immune parameters and the age-related cognitive heterogeneity both in humans and in mice. Our studies addressed a longitudinal evaluation of mice and a cross-sectional study with a cohort of healthy senior individuals.

Given the lack of information concerning the progression of the main immune cells in circulation in healthy aging mice, in CHAPTER II we characterized for the first time the relative frequency progression over time of the main blood immune cells, both from adaptive and innate immunity, by following the same animals from 3 to 18 months of age. The described sex-related differences in immune response in different species, including humans and mice (Giefing-Kröll et al., 2015; Klein and Flanagan, 2016; Piasecka et al., 2018), and the scarce reported sex-related changes in blood cellular composition in humans (Abdullah et al., 2012; Márquez et al., 2020; Patin et al., 2018; Strindhall et al., 2013), prompted us to explore the effect of sex on this interaction. We observed that aging promotes an imbalance towards the frequency of innate immune cells and that sex moderates the relation between the frequency of cells and age, reinforcing that sex must be considered when designing and planning experiments in rodents. Namely, aging is accompanied by an increase in the percentages of neutrophils, monocytes, eosinophils, NK cells $Ly6C^-$, concomitantly with a decrease in NK cells $Ly6C^+$, B and total T cells (including $CD4^+$ and $CD8^+$ subsets), which resembles some of the alterations described in humans (Chong et al., 2005; Frasca et al., 2008; Jiao et al., 2009; Márquez et al., 2020; Muggen et al., 2019; Ongrádi and Kövesdi, 2011; Paganelli et al., 1992; Patin et al., 2018; Saule et al., 2006; Wikby et al., 2008). Interestingly, while males have higher percentages of neutrophils and activated monocytes $Ly6C^{high}$, females present higher percentages of total T cells, both $CD4^+$ and $CD8^+$ T cells, as well as eosinophils and NK cells. The similarities across human and mouse studies, suggest that some sex-specific aging signatures are conserved across species, however those similarities and discrepancies between humans and mouse models need to be further investigated. Extending this approach to studies on cell function and cytokine production profile of each population over time will wider our comprehension of the immunosenescence process.

In Chapter III we explored the role of age-associated alterations of the immune system as a potential contributor for cognitive heterogeneity at old age. By performing a multiparametric behavioral characterization in old mice (same cohort of mice explored in Chapter II), we dissected their heterogenous cognitive performance, with a focus on different memory dimensions, and segregate them as good and poor performers. Taking into consideration the previously described sex-

dimorphisms on the cellular composition of the circulating immune system over time (Chapter II), males and females were analyzed independently. We observed that male mice with distinct cognitive performances at old age present moderate differences on specific blood immune cells progression over time, while in females we could not detect differences between good and poor cognitive performances. This moderate association reinforce that systemic immune aging might affect the brain function and therefore affect cognitive performance in a sex-dependent manner, where males and females might experience the impact of immune system aging on cognitive performance differently. We also found that the peripheral immune system profile at 18 months old, such as the percentage of $CD4^+$ and $CD8^+$ T cells among T cells and NK $Ly6C^-$ cells, is associated with mouse cognitive performance. Curiously, previous results from our group also showed an association between an immune profile in blood and cognitive performance in healthy senior individuals (Serre-Miranda et al., 2015). Specifically, higher levels of effector memory $CD4^+$ T cells in blood were associated with worse cognitive performance, even when accounting for education, sex, age and mood (Serre-Miranda et al., 2015). While the association between immune cell profiles and cognitive performance reported here in mice, as the previously reported in humans, do not allow to infer about causation, they support further mechanistic research on if and how these alterations in the proportion of immune cells impacts on cognitive aging. In fact, it has been demonstrated that $CD4^+$ T cells exert fundamental homeostatic functions to maintain brain function and plasticity throughout life (Derecki et al., 2010; Pasciuto et al., 2020; Wolf et al., 2009; Ziv et al., 2006). Curiously, we observed a decreased proportion of circulating $CD4^+$ T cells among T cells over time in old mice with a better cognitive performance (only on male); it will next be of interest to investigate whether some of those cells are being recruited to the brain and somehow affect brain function throughout life. Moreover, while we reported a decreased frequency of T cells in circulation with aging in mice (Chapter II), others have seen an increased frequency of those cells in the brain and meninges (Mrdjen et al., 2018). This suggests that aging might affect immune cells in blood circulation and in the brain borders differently. It will be interesting to further investigate what is the phenotype of T cells and which are the signals responsible for the recruitment of immune cells to the brain barriers, and how they vary over time. Of interest, the CP is populated by effector memory $CD4^+$ T cells with a TCR repertoire enriched for CNS antigens (Baruch et al., 2013). This enrichement could possibly be driven by peripheral immune activation by brain-derived antigens. Interestingly, the frequency of T cells in circulation reactive to CNS antigens increases with aging both in human blood and mouse spleen (Baruch et al., 2013;

Monsonego et al., 2003). However, the association between the presence of those cells in circulation and cognitive heterogeneity in healthy individuals is still poorly understood. Nonetheless the potential to use those cells as predictors of age-related cognitive impairment is promising.

The recently "re-discovery" of a meningeal lymphatic system shed some light on how the brain-derived cues can reach the periphery and activate immune cells (Aspelund et al., 2015; Louveau et al., 2015). This set of lymphatic vessels, which are found in the dura mater along the transverse and sagittal sinuses, present the capacity to efficiently drain both immune cells and molecules from the subarachnoid space into the deep cervical lymph nodes (dCLN) (Aspelund et al., 2015; Louveau et al., 2015). With aging the diameter of those vessels decreases, as well as their capacity to drain macromolecules (Da Mesquita et al., 2018). Furthermore, cognitive deficits can be induced by disrupting the communication between brain lymphatics and the dCLN or by ablating meningeal lymphatics (Da Mesquita et al., 2018; Radjavi et al., 2014). Thus, exploring the profile and repertoire of immune cells at the dCLN could give an indirect measure of the profile of immune cells being drained from the brain back to the periphery.

In the results presented in Chapters II and III, we addressed the cellular composition of the adaptive and innate immune systems, and how their percentages change with age. In addition to alterations in cell frequencies, aging of the immune system also encompasses alterations in cell functions (Brubaker et al., 2013; Della Bella et al., 2007; Frasca et al., 2005; Fulop et al., 2004; Hakim et al., 2004; Haynes and Maue, 2009; Hazeldine et al., 2012; Hazeldine and Lord, 2015; Hearps et al., 2012; Larbi et al., 2008; Lefebvre and Haynes, 2012; Mocchegiani et al., 2009; Ongrádi and Kövesdi, 2011; Schröder and Rink, 2003; Shaw et al., 2010; van der Geest et al., 2014; Wenisch et al., 2000), which require further studies to explore its relation with cognitive aging. Techniques such as mass cytometry (CyTOF) or even single cell RNA sequencing, complemented with in vitro studies, would allow a more detailed characterization, covering from cellular compartmentalization to function. For instance it would be interesting to stimulate T cells, both from humans and mice, with non-specific stimuli (i.e. phorbol myristate acetate + ionomycin) vs. CNS-specific molecules and evaluate their cytokine profile throughout aging. Thus, we could compare whether cells from humans and rodents respond similarly, and explore to what extent the T cell cytokine profile correlates with cognitive heterogeneity in old individuals/mice.

In the present study, mice were maintained in specific pathogen free conditions. Therefore, the changes observed may be different in real life scenarios where different microorganisms/pathogens are present. It is know that the interaction between microbiota and the

immune system is critical for a proper maturation of the host's immune system (Zheng et al., 2020). While maintain mice in similar conditions is beneficial for standardizing experiments between laboratories across the world, by artificially selecting the microorganisms that a mouse is in contact with, we are potentially biasing the profile of the immune system. For instance, the frequency of blood memory $CD8^+$ T cells of adult laboratory mice is closer to a neonatal human than to an adult (Beura et al., 2016), possibly explaining some of the inconsistencies observed between mice and humans' immune system aging. In fact, studies using C57BL/6 mice that acquired microorganisms from wild mice, both commensal flora and pathogens, demonstrated that their immune system mimics better the human immune response than conventional C57BL/6 mice (Rosshart et al., 2019). Those evidences reinforce that we need always to be cautious when translating findings related with the immune system function from laboratory mice to humans.

In Chapter IV we explored the association between the composition of the soluble component of the blood and cognitive heterogeneity in healthy senior individuals. Even though the association between peripheral low-grade inflammation was already described by several authors (Baune et al., 2008; Heringa et al., 2014; Trollor et al., 2012), most of the studies only addressed a reduced number of immune molecules in cognitively healthy senior cohorts. In an attempt to explore this association in a less bias approach to specific molecules, we analyzed up to 30 immune-related molecules. With this characterization we not only confirmed already well described associations between peripheral immune molecules and cognitive heterogeneity, such as IL-6 (Mooijaart et al., 2013; Tegeler et al., 2016; Trollor et al., 2012; Yaffe et al., 2003), but also strengthen others, like IL-1β, IL-8, IP-10 (CXCL10) and TNF (Baune et al., 2008; Bradburn et al., 2018; Goldstein et al., 2015; Heringa et al., 2014; Simpson et al., 2013), and revealed new players in such association, such as IL-13. Saying so, we further highlight that some poorly studied immune molecules should be considered in the context of cognitive aging. As a complex system, the effect of peripheral immune molecules will not be singular but rather the interplay between several players. Even though not explored in this thesis, it would be interesting to investigate how the immune mediators here measured associate in groups, for instance by performing a principal component analysis, and how the different group of molecules associate with cognitive heterogeneity. This way, we would not only explore the potential association with each individual molecule with cognition, but the association with profiles composed by different molecules.

A technical aspect on the quantification of immune mediators deserves consideration here. From the 31 immune mediators measured, for only 19 the concentration values obtained were above

the detection level in more than 50% of the samples, which is a common phenomenon in healthy individuals. Not being able to detect due low concentration does not necessarily mean lack of relevance, we just don't have the capacity to quantify. Exploring the role of immune mediators in cognitive aging using higher sensitivity techniques will clarify whether there are any differences in the molecules for which the detection level was insufficient to quantify in a great proportion of individuals.

Knowing that sex has a mediator effect on the age-associated alterations of the immune system (CHAPTER II) and on the association between immune mediators and cognitive heterogeneity in old mice (CHAPTER III), it would be of interest to also explore the effect of sex on the association between blood immune molecules profile in senior individuals and cognitive variability. A replication cohort with a larger sample size would allow not only to evaluate if our finding can be replicated and validated, but also explore the effect of sex on the association between immune mediators and cognitive variability in elderly. In this particular scenario assuming 4 groups (men and women with good and poor cognitive performance analyzed independently) it would be necessary at least 270 participants equally distributed by the groups (calculated using G*Power 3.1, assuming a medium effect size (f=0.25) and a power of 0.8). A comprehensive characterization of a cohort of this magnitude is extreme valuable but also extremely time and resources consuming. Performing this type of evaluation in a collaborative approach and establishing multi research centers cohorts, will not only dilute the cohort effect bias, but also reduce the time necessary to achieve specific goals. The prospective to use an inflammatory state profile to predict how individual cognitive performances will evolve with aging could revolutionize our perception of aging. In fact, some authors have already described in human longitudinal studies that the plasma concentration of IL-6 predicts cognitive decline (Mooijaart et al., 2013; Singh-Manoux et al., 2014; Weaver et al., 2002; Yaffe et al., 2003). However, most of these studies are restricted to specific molecules. Doing a broader and longitudinal characterization of the peripheral inflammatory (immune related) profile will give a wider picture and will certainly improve the understanding of the interplay between peripheral immune-realted factors and the aged brain. Whether additional understanding of immune cell functional alterations during aging will provide novel targets for intervention, is still speculative. In fact, it must be viewed with caution given the pleiotropic effects well described for several immune mediators. Still, the knowledge of a potential pro-inflammatory profile associated with cognitive aging is suggestive of the potential benefits of attempts to reduce the low-grade inflammation. The use of Mediterranean diet, caloric restriction and metformin (that can be

considered a caloric restriction mimetic and has been proposed as an anti-aging drug), have demonstrated to down-regulate the levels of several peripheral inflammatory markers (reviewed in Aiello et al., 2019; Xia et al., 2016). Moreover, rejuvenating human body with young plasma has already demonstrated promising results in animal models, reversing aspects of aging in multiple tissues (Baht et al., 2015; Conboy et al., 2005; Huang et al., 2018; Loffredo et al., 2013; Salpeter et al., 2013; Sinha et al., 2014), including the brain, which is reflected in an improvement of cognition (Castellano et al., 2017; Katsimpardi et al., 2014; Villeda et al., 2014, 2011).

Still to be understood is how much of this inflammatory state "reaches" the brain and how it will influence and will be integrated with all the other stimuli that modulate cognitive performance. Several pathways for the communication of plasma immune mediators and the central nervous system have been proposed: the entrance in the brain parenchyma through the brain barriers acting directly on neurons and glia cells, the activation of cells lining the cerebral vasculature, such as endothelial cells and choroid plexus epithelial cells, and also the peripheral activation of afferent nerves, such as the vagus nerve (Haroon et al., 2012).

Another interesting question that remains to be clarified pertains with the similarity/differences between the peripheral immune mediators profile and that of the central nervous system, such as in the CSF. Bettcher and collaborators explored the relationship between CSF and plasma levels of inflammatory markers in middle-aged and older adults (asymptomatic but with parental history of dementia) and observed that, from a set of 5 markers measured, only plasma levels of MIP-1b correlated with CSF level (no association was seen for IL-6, IL-8, IP-10 and MCP-1) (Bettcher et al., 2018). Due to ethical issues we cannot collect CSF in healthy humans, so it will be difficult to explore the profile's similarities between CSF and plasma in healthy senior cohorts.

To use animal models to explore specific questions regarding human physiology is crucial also to explore the similarities and differences between humans and animal models. Regarding the association between peripheral immune molecules profile and cognitive heterogeneity in humans, it would be crucial to explore if similar findings can be achieved in mice. To further explore in this direction we have collected plasma throughout mice aging (and also CSF at 18 months of age) and we plan to analyse: 1) the profile of immune molecules over time, whether the age-associated progression towards low-grade inflammation profile is also present in this mouse model; 2) if the inflammatory profile at 18 months of age is associated with mice cognitive performance, as demonstrated in humans (CHAPTER IV); and 3) the similarities between peripheral and central immune molecules profile.

Of notice, even though cytokines and other plasma proteins have the ability to cross the blood-brain barrier (Yang et al., 2020), they may also originate from cells in the brain parenchyma and from cells of the brain barriers themselves. In addition, a peripheral inflammatory state might modulate cells at the brain barriers, such as brain endothelial cells (BECs) and the choroid plexus to produce and secrete molecules into the brain parenchyma (Marques et al., 2009) that ultimately influence brain function. BECs in aged mouse hippocampus present an inflammatory transcriptional profile and upregulation of vascular cell adhesion molecule 1(VCAM1), a protein essential for the vascular-immune interaction (Yousef et al., 2019). Interestingly aged plasma has the ability to mimic BECs signatures of normal aging, while young plasma can reverse those effects, demonstrating that BECs are sensitive sensors of age-related circulatory cues (Chen et al., 2020). Similarly, choroid plexus also changes its inflammatory signature with aging (Baruch et al., 2014, 2013; Da Mesquita et al., 2015). However it has not been explored yet whether the inflammatory profile at the brain barriers correlates with cognitive variability in aged mice. The precise peripheral molecules that might potentially mediate these relations needs also to be further investigated.

Altogether, the studies presented in this thesis enrich the field with knowledge of the peripheral immune system changes with aging and their association with cognition. The findings are supportive of a close interplay that deserve to be further investigated.

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