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Regulation of meningeal IL-17^{+} immune cell homeostasis: (patho)physiological implications

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Abstract

The crosstalk between the Central Nervous System (CNS) and the immune system prompted the Neuroimmunology field towards the relevance of immune cells in supporting steady-state brain physiology. The recent (re)discovery of meningeal lymphatic vessels containing a wide repertoire of immune cells in the steady state that drain into the cervical lymph nodes established meninges as a strategic anatomical site for the neuroimmune interface.

A pioneer publication from the host laboratory revealed an unexpected role for meningeal IL-17 in the regulation of short-term working memory and revealed a novel foetal thymic-derived population of meningeal γδ T cell as the major source of IL-17 in the steady state CNS. Further studies highlighted the role of this cytokine in pathogen-avoidance behaviours and sociability.

Notwithstanding, an IL-17 contribution to neuroinflammation is emphasized by its role during the development and progression of experimental autoimmune encephalomyelitis (EAE) and brain ischemia upon injury. Furthermore, unpublished results from the host laboratory revel that exacerbated levels of meningeal IL-17 seemingly promote the onset of Alzheimer's Disease (AD). IL-17-dependent accelerated neurodegeneration was also reported in a Parkinson's disease (PD) mice model.

This suggests a dual role for IL-17 – pro-cognitive at steady state and anti-cognitive in the context of neurodegeneration – and implies a hypothetic meningeal IL-17 threshold, below which cognitive deficits would appear and above which pathogenic neuroinflammation would be triggered. Therefore, a fine-tuned regulation of meningeal IL-17 homeostasis is seemingly required to maintain brain integrity and cognitive functions.

Built on this background, this project aims to decipher the cellular and molecular mechanisms that control such an important - yet unexplored - process. Based on the literature and RNA-sequencing data from the host laboratory, we postulated a potential contribution of stress, circadian clock and sleep to meningeal IL-17 immune cell homeostasis in pathophysiological conditions.

To test this hypothesis, we resorted to flow cytometry techniques to analyse the survival, proliferation and cytokine response of purified γδ T cells stimulated *in vitro* with different concentrations of glucocorticoids and *in vivo* glucocorticoid modulatory functions. Meningeal lymphocytes were also isolated from wild-type (WT) mice and from the 3xTg-AD mice model of AD for the characterization of IL-17 diurnal oscillatory pattern. Finally, the effect of sleep on meningeal IL-17 was tested by anesthetizing mice through intraperitoneal injections of ketamine/xylazine (K/X). We observed an increased percentage, survival and proliferation of γδ T cells IL-17 producers (γδ17), the major contributor to IL-17 production in the meninges, to glucocorticoid stimulation *in vitro* in a dose-dependent manner. However, no effect was observed in meningeal IL-17-producing cells after *in vivo* injection of glucocorticoids. Additionally, a diurnal oscillation of the percentage of meningeal IL-17-producing cells, with a minimum during the light phase (bathyphase) and a peak during the dark phase (acrophase) was observed in WT mice. Conversely, the AD mice model and their littermate controls displayed no such oscillatory pattern. Our data also point at a reduction in meningeal IL-17-producing cells upon sleep.

Although a stress contribution was not observed *in vivo*, our results suggest that circadian rhythms and sleep can regulate the homeostasis of IL-17-producing immune cells in the meninges. Importantly, both circadian clock and sleep are integrated systems that contribute to higher mental processes and neurodegenerative disorders.

Taken together, this exploratory study is a further step towards the characterization of meningeal immune subset biology. The modulation of these cues might give further insight into the cellular and molecular mechanisms that underlie learning and memory with a biomedical potential for neurodegenerative diseases.

Keywords: Meninges | IL-17 | Stress | Circadian Rhythms | Sleep

Resumo

Recentemente o campo da Neuroimunologia alargou o seu clássico foco patológico e entrou numa era focada nas implicações desta interação num contexto não inflamatório. A interação entre o sistema nervoso e o sistema imune durante o desenvolvimento, a vida adulta e o envelhecimento são importantes marcos a explorar que nos darão um vislumbre de questões desafiantes.

A (re)descoberta dos vasos linfáticos meníngeos levou a uma reavaliação do conceito de cérebro como órgão imunologicamente privilegiado. Estudos recentes revelam que estes vasos linfáticos possuem um amplo repertório de células imunológicas qualificadas para monitorizar e proteger o sistema nervoso central (SNC) e influenciar as funções cerebrais através de moléculas solúveis, sem que ocorra infiltração no parênquima cerebral.

Diferentes citocinas produzidas por células imunes a partir dos espaços meníngeos demonstraram um efeito surpreendente na modulação das funções cerebrais. Particularmente, a produção de IL-4 por células T $CD4^+$ após a realização de tarefas cognitivas foi determinante na regulação da aprendizagem e memória a longo prazo. Uma recente publicação do laboratório de acolhimento demonstrou que a produção de IL-17 nas meninges, uma citocina frequentemente implicada em contextos inflamatórios, tem um papel surpreendente na regulação da memória de trabalho espacial de curto prazo através da modulação da plasticidade sináptica no hipocampo. Esta publicação revelou ainda que uma nova população derivada do timo fetal – células T γδ – são a principal fonte de IL-17 no SNC saudável.

Estes dados sugerem uma possível regulação diferencial de circuitos neuronais pelas diferentes citocinas presentes nos espaços meníngeos. A estas funções mediadas por IL-17 acrescem ainda as suas propriedades neuromoduladoras envolvidas em comportamentos de evasão a patógenos e na promoção da sociabilidade. Esta citocina emerge assim como novo ator em neurofisiologia.

Apesar deste importante papel fisiológico, níveis exacerbados de IL-17 no SCN estão associados a morte neuronal e anomalias comportamentais. Para além disto, num contexto neuroinflamatório, a produção desta citocina por células T γδ tem um papel crucial no desenvolvimento de encefalomielite autoimune experimental (EAE) e na progressão de isquemia cerebral e défices neurológicos num modelo de lesão de isquemia-reperfusão (I/R) cerebral. Em ambos os modelos, as células T γδ produtoras de IL-17 (γδ17) presentes nas meninges desencadeiam um ciclo de amplificação imunológica local que irá alterar o microambiente estromal do cérebro inflamado e causar ruturas na barreira hematoencefálica que contribuirão para a progressão da doença.

Resultados do laboratório de acolhimento demonstram ainda que num contexto neurodegenerativo, a produção exacerbada de IL-17 por células T γδ nas meninges está associada ao desencadeamento dos défices cognitivos observados num modelo de ratinho da doença de Alzheimer (3xTg-AD). A neutralização desta citocina foi suficiente para prevenir os défices da memória de trabalho espacial de curto prazo e disfunção da plasticidade sináptica observados nas fases iniciais da doença. Concentrações elevadas de IL-17 no SCN estão ainda associadas à promoção da neurodegeneração num modelo de ratinho da doença de Parkinson.

Estes dados sugerem um duplo papel da IL-17 no SCN. Enquanto a produção desta citocina em condições fisiológicas é indispensável para as funções cognitivas, a sua produção exacerbada contribui para a propagação da neuroinflamação e neurodegeneração. Isto implica um limiar hipotético ideal da concentração de IL-17 nas meninges, abaixo do qual surgiriam défices cognitivos e acima do qual se verificaria um desencadeamento de processos neuroinflamatórios. Assim, o controlo rigoroso da concentração desta citocina nas meninges parece indispensável para manter a integridade cerebral e as funções cognitivas.

Com essa ideia em mente, este estudo exploratório tem como objetivo desvendar possíveis mecanismos homeostáticos que, em condições fisiológicas, regulariam diferentes produtores imunes de IL-17 presentes nos espaços meníngeos de forma a otimizar os níveis de IL-17. A desregulação de tais

mecanismos poderá potencialmente contribuir para a acumulação desta citocina observada nas doenças neuroinflamatórias.

Stress, ritmos circadianos e sono são processos com importantes implicações na modulação das respostas imunes e cerebrais em contextos patofisiológicos. Considerando os dados da literatura, recorremos à análise de dados obtidos em ensaios de sequenciação de RNA realizados em diferentes populações de linfócitos T γδ isolados a partir de nódulos linfáticos periféricos. Estes dados permitiram observar que células T γδ produtoras de IL-17 (γδ17) sobreexpressam recetores de catecolaminas e glucocorticoides, assim como genes que codificam os principais reguladores dos ritmos circadianos, comparativamente com as populações de células T γδ produtoras de IFN-γ ou duplas negativas.

Para testar uma potencial contribuição do stress na modulação das propriedades efetoras das células T γδ17, células T γδ totais ou as suas diferentes populações foram isoladas e colocadas em cultura com diferentes concentrações de glucocorticoides. Esta estimulação resultou num aumento da percentagem de células T γδ17 de forma proporcional à concentração, mediada pela estimulação da sua sobrevivência e proliferação. Estes dados corroboraram os resultados obtidos por sequenciação de RNA, ao sugerir que células T γδ17 são preferencialmente afetadas por glucocorticoides comparativamente com as restantes populações.

Para analisar uma possível contribuição do stress na modulação de células meníngeas produtoras de IL-17 recorremos à injeção intraperitoneal de glucocorticoides *in vivo*. Surpreendentemente, não foi observado nenhum efeito significativo nos espaços meníngeos quatro horas após a injeção, embora uma modulação de células produtoras de IL-17 tenha sido observada em órgãos linfoides periféricos, corroborando os resultados obtidos *in vitro*.

Para avaliar um possível envolvimento dos ritmos circadianos, células meníngeas foram isoladas de ratinhos em diferentes fases do dia para avaliar a produção de IL-17. Observou-se uma oscilação diurna da percentagem de células meníngeas produtoras de IL-17 que atingiu o seu valor mais baixo a meio do dia (batifase) e o seu valor mais alto durante a noite (acrofase). Estes dados indicam um potencial efeito dos ritmos circadianos na produção de IL-17 nas meninges.

Considerando que as doenças neurodegenerativas estão geralmente associadas a distúrbios circadianos e a acumulação de IL-17 nas meninges está associada ao início dos défices cognitivos observados num modelo de ratinho da doença de Alzheimer, em seguida postulámos que estes distúrbios poderiam contribuir para a acumulação de IL-17 verificada neste modelo que se traduziriam numa desregulação da oscilação diurna das células produtoras de IL-17 previamente observada. Inesperadamente nem os ratinhos controlo nem os ratinhos 3xTg-AD utilizados demonstraram uma oscilação diurna nestas células. Estes dados indicam que outras variantes como o contexto genético, idade ou sexo possam ter influenciado os resultados obtidos.

Considerando a interação entre os ritmos circadianos e o sono, em seguida postulámos que este último poderia regular a homeostase das células imunes produtoras de IL-17 das meninges. Para testar esta hipótese foi injetada uma solução anestésica de cetamina/xilazina que reproduz as principais características do sono e as células meníngeas foram isoladas uma hora e meia depois. Esta técnica permitiu-nos observar uma redução nas células produtoras de IL-17 nas meninges, sugerindo que o sono poderá ser outro mecanismo de regulação destas células em contextos fisiológicos.

No seu conjunto, embora uma contribuição do stress não tenha sido observada *in vivo*, os nossos resultados sugerem que os ritmos circadianos e o sono podem regular a homeostase das células imunes produtoras de IL-17 nas meninges. É importante destacar que estes dois processos estão fortemente interligados e ambos contribuem para processos mentais superiores e distúrbios neurodegenerativos.

Este estudo exploratório é assim mais um passo para a caracterização da biológica das células imunes meníngeas e, se validados, estes resultados poderão ter grandes implicações na manipulação da produção de IL-17 nas meninges. A modulação destes processos homeostáticos poderá contribuir para aprofundar

os conhecimentos sobre os mecanismos celulares e moleculares que fundamentam a aprendizagem e a memória com um potencial biomédico para doenças neurodegenerativas.

Palavras Chave: Meninges | IL-17 | Stress | Ritmos Circadianos | Sono

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List of Abbreviations

6-OHDA: 6-hydroxydopamine

Abs: antibodies

ACTH: adrenocorticotropic hormone

AD: Alzheimer's disease

Adrb2: Adrenergic Receptor Beta 2

Ag: antigen

ANS: autonomic nervous system

APPswe: amyloid precursor protein

AQP4: aquaporin-4

AVP: arginine vasopressin

Aβ: amyloid-β

BBB: blood-brain barrier

BDNF: brain-derived neurotrophic factor

BFA: Brefeldin A

BM: bone marrow

BMAL1: brain and muscle ARNT-like 1

CCGs: clock-controlled genes

CCR7: C-C chemokine receptor type 7

CD: cluster of differentiation

CLOCK: circadian locomotor output cycles kaput

CNS: central nervous system

CRH: corticotrophin-releasing hormone

CRY: cryptochrome

CSF: cerebrospinal fluid

CTV: Cell Trace Violet

CXCR4: C-X-C chemokine receptor type 4

dcLNs: deep cervical lymph nodes

DCs: dendritic cells

DETCs: dendritic epidermal γδ T cells

DG: dentate gyrus

DN: double negative

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

EDTA: Ultra-Pure Ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FCS: fetal calf serum

GH: growth hormone

gMFI: geometric mean fluorescence intensity

GRs: glucocorticoid receptors

HPA: hypothalamic–pituitary–adrenal

HSC: hematopoietic stem cell

I/R: ischemia-reperfusion

IFN: interferon

IL: interleukin

IL-17R: Interleukin-17 receptor

IL-7R: interleukin-7 receptor

ILC3s: group 3 innate lymphoid cells

ILCs: innate lymphoid cells

Ip: intraperitoneal

ISF: Interstitial fluid

K/X: ketamine/xylazine solution

LNs: lymph nodes

LPS: lipopolysaccharide

mAbs: monoclonal antibodies

MACS: magnetic cell sorting

MAIT: mucosal-associated invariant T

MAPK: mitogen-activated protein kinase

MAPT: microtubule-associated protein tau

MCMV: mouse cytomegalovirus

MHC: major histocompatibility complex

MIA: maternal immune activation

MMP: matrix metalloproteinase

Mo: month-old

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS: multiple sclerosis

NFIL3: nuclear factor IL-3-regulated protein

NF-κB: nuclear factor-κB

NK: natural killer

***Nr3c1*:** Nuclear Receptor Subfamily 3 Group C Member 1

PBS: phosphate-buffered saline

PD: Parkinson's disease

PER: period circadian protein

PFC: prefrontal cortex

PNS: peripheral nervous system

PSEN1: presenilin-1

RBC: red blood cell

REM: rapid-eye-movement

RHT: retinohypothalamic tract

RNA-seq: RNA sequencing

ROR: retinoic acid receptor-related orphan receptor

RT-PCR: reverse transcription polymerase chain reaction

SCID: severe combined immunodeficiency

scLN: superficial cervical lymph nodes

SCN: suprachiasmatic nucleus

SN: substantia nigra

SNPs: single nucleotide polymorphisms

SNS: sympathetic nervous system

SWS: slow wave sleep

TCR: T cell receptor

TGF: tumour growth factor

T_H: T helper

TLRs: Toll-like receptors

TNF: tumour necrosis factor

TRAF6: TNF receptor-associated factor 6

Tregs: regulatory T cells

VEGF-C: vascular endothelial growth factor C

Wo: week-old

WT: with type

ZTs: zeitgebers

αSyn: alpha-synuclein

β_2ARs: β_2-adrenergic receptors

γδ17: IL-17A-producing γδ

1. Introduction

1.1 The Nervous System and the Immune System

1.1.1 The Nervous System

The nervous system is a complex, highly specialized network that is perceived as the command centre of the body[1]. In mammals, this system is anatomically divided in two main components: the central nervous system (CNS) and the peripheral nervous system (PNS)[2].

The structure and function of the CNS has been the focus of extensive research. In a simplistic way, the CNS can be divided into brain and spinal cord that will combine information and coordinate the response to environmental stimuli. Two main classes of cells are present: neurons – transporting information in the form of electrical impulses – and glial cells – mostly responsible for the support of neuronal cells. The signalling between these cell-types enables the different cognitive processes such as thinking, communicating, learning and memory storage[2].

The PNS conveys neuronal messages to and from the brain through the cranial and spinal nerves, connecting the CNS to the rest of the organism. Functionally, the PNS can be further divided into the somatic nervous system – associated with the voluntary control of movements – and the autonomic nervous system – responsible for the involuntary body functions and homeostasis maintenance[3,4]. The constant interaction between these two systems allows a continuous monitoring of the internal and external environmental cues, providing an adaptive advantage to the organism.

1.1.2 The Immune System

The immune system consists in a complex network of different organs, cells and proteins that work together to protect the human body from pathogens, including bacteria, viruses, parasites and fungi, as well as environmental and physiological perturbations that could disrupt tissue homeostasis and induce malignant changes[5]. The crucial role of the immune system for health and survival is emphasized when its function is impaired, resulting in inflammatory diseases, such as autoimmunity and cancer[6]. The immune response is driven by two main branches: the innate and the adaptive systems[7].

The innate system is considered the first defence barrier present in primitive animals and invertebrates. It is composed by granulocytes, monocytes, macrophages, dendritic and natural killer (NK) cells, short-lived cells that respond immediately to a wide range of pathogens with relatively poor diversity of antigen (Ag)-specific recognition elements. In contrast, the adaptive system shows a delayed response but performs specialized responses by generating long-lived, Ag-specific cells after a first exposure to pathogens, while maintaining tolerance to self-constituents. The latter is present in more complex animals, including humans[7] and its ability to create immunological memory results in a faster and more efficient response to subsequent encounters with specific pathogens. The adaptive immune system comprises both humoral immunity - mediated by antibodies (Abs) and antibody-secreting B cells (also known as plasma cells), that protect the human body against extracellular pathogenic agents and toxins – and cell-mediated immunity – composed mainly by T cells that work primarily against intracellular pathogens and cancerous cells. A precise orchestration of these two systems is fundamental to achieve maximum protection for the organism[8].

To ensure tissue homeostasis, a strict balance must be maintained between the detection of potentially harmful factors and the resolution of immune responses that, if unrestrained, lead to massive damages[9].

1.1.3 Neuro-immune crosstalks

The blooming field of neuroimmunology studies the bidirectional interaction between the nervous and the immune system, both under homeostatic and pathological conditions[10,11]. These two systems, assumed to work in an independent manner for decades, form elegant bidirectional brain–immune communications, critical for homeostasis maintenance. Their close interaction is easily perceived

during non-physiological events: an acute mental or physical stressor that activates the CNS can also induce an inflammatory response, while a microbial challenge that activates the immune system will prompt neurobehavioral, neuroendocrine and autonomic nervous system (ANS) responses[12].

Notwithstanding, the classical dogma of cytokines as immune communicator molecules and neurotransmitters as neuronal communicators is fading. Neurons and immune cells share intercellular signals and their respective receptors[13]. Cytokines participation in normal CNS function ranges from the regulation of sleep[14], a variety of neuroendocrine functions[15] and neuronal development[16] to a possible implication in ageing[17]. On the other hand, immune cells contain the molecular machinery required to respond to neural factors including receptors for neurotransmitters[18], neuropeptides[19], hormones[20] and the components of their signalling pathways[21], but also the ability to synthesize and store neurotransmitters[22]. The nervous system-mediated regulation of immunity can occur through systemic, regional, or local routes[21] with important implication for the development, homeostasis and functions of immune cells[4]. Within the several neuronal factors involved in the modulation of immune cell responses, a fundamental role for the PNS and the neuroendocrine modulation of immunity at a systemic level by the hypothalamic–pituitary–adrenal (HPA) axis must be acknowledged[21]. The effects of these neuronal factors will differ according to the time course of an immune response or the stage of immune-cell maturation[23,24].

The neuroimmune crosstalk provides a mean to optimize the effectiveness of the immune response and an enhanced preparation for an upcoming danger by synchronizing behaviour, metabolism and immune activity, increasing the potential for survival. Additionally, a faster homeostatic restoration will be achieved following pathogenic attacks, development of malignant cells or tissue damage[25].

1.2 Immune surveillance in the CNS

The body's most complex system requires exceptional protection[1]. As such, the skull and the vertebral column constitute the CNS primary protective layer against external damages and the blood-brain barrier (BBB) controls the communication with the periphery at an internal level[26]. Another level of protection is given by the meninges, a triple-layered membrane that comprises the dura mater (adjacent to the skull), the arachnoid mater and the pia mater (adjacent to the brain and spinal parenchyma) (**Figure 1.1A**). The dura mater consists in a dense, collagenous, highly innervated and vascularized membrane. The arachnoid mater is a non-vascularized layer that regulates the transport of molecules. It encloses the sub-arachnoid space filled with cerebrospinal fluid (CSF) produced by the choroid plexus epithelium, which provides a mechanical barrier against shock and maintains a constant pressure within the cranium[27]. Within this space, thin projections called trabeculae extend into the pia mater, a highly vascularized layer composed of permissive flat cells adherent to the CNS parenchyma surface. Together, the arachnoid and pia mater form the leptomeninges. Juxtaposed to the pia mater, the glia limitans composed of astrocytic endfeet form a dense membrane of selective permeability[28,29].

The CNS critical role for survival and its limited regenerative capacity led to the long-lasting concept of the brain as an immune-privileged organ[30,31]. This view was mostly supported by the prolonged survival of homologous tissues grafted into the brain[30] and the presence of immune cells in the CNS parenchyma was perceived as a hallmark of neuropathology[10]. However, recent studies have revealed that the CNS is neither isolated nor passive in its interactions with the immune system[32,33]. Rather, constitutive immune surveillance is provided by cells patrolling around and residing within the CNS[9]. Despite the limited access, the brain parenchymal tissue comprises resident microglia with fundamental roles in brain homeostasis throughout life and in disease contexts[34].

This new concept was also supported by the recent (re)discovery of meningeal lymphatic vessels. Although first described in the eighteenth century[35], only recently a definite structural and functional characterization of meningeal lymphatic vessels was provided in mice[26,36–38] and humans[39]. The lymphatic system is fundamental for tissue homeostasis and immune control, and such a metabolic

active organ as the brain requires a constant drainage to prevent the accumulation of potentially toxic compounds and ensure a correct performance[40]. This conventional and functional lymphatic system located parallel to the venous sinus in the dura mater provides, for the first time, a direct link between the brain parenchyma and the periphery[41] (**Figure 1.1A**). Its privileged location allows the continuous drainage and clearance of fluid, macromolecules and immune cells from the brain into the deep cervical lymph nodes (dcLNs) and later to the superficial cervical lymph nodes (scLN)[38,41,42].

Through the glymphatic route, the CSF is prompt into the brain by arterial pulsation through aquaporin-4 (AQP4) water channels of the astrocytic end feet of glia limitans, creating a convective bulk flow of brain interstitial fluid (ISF) that allows an exchange of neurotransmitters, hormones, proteins, cytokines, antigens and waste products[43,44]. Impairments in this cleaning route have harmful consequences for brain physiology[40]. The identification of meningeal lymphatic vessels as a cleaning route of CSF content highlighted its importance for brain CNS physiology[37,45].

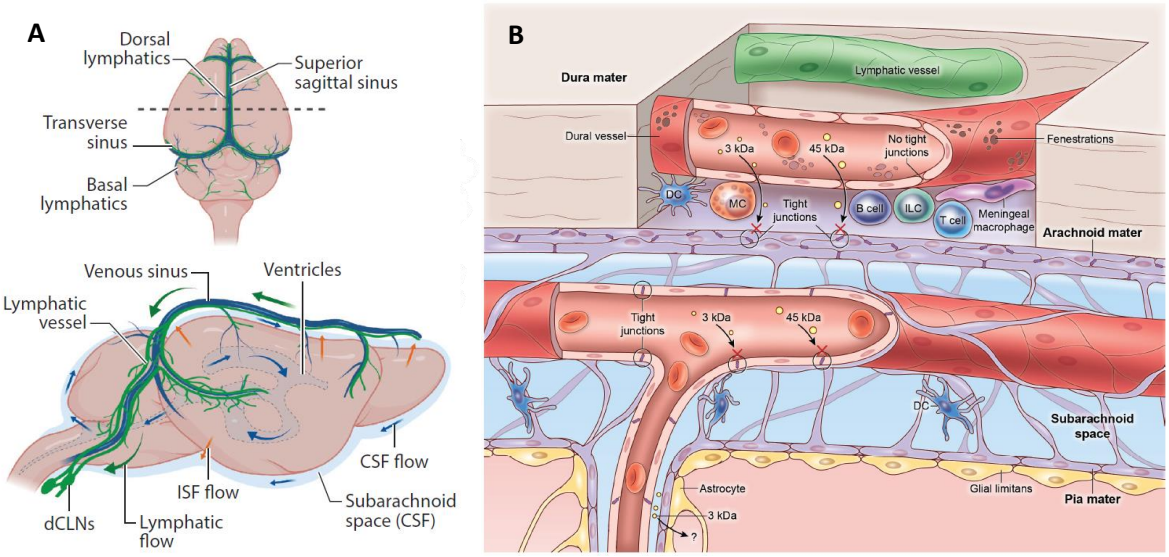

Figure 1.1 - Steady state meningeal anatomy and immune composition. (A) Meningeal lymphatics are located parallel to the venous sinus in the dura mater, follow the transverse sinus until the base of the skull to form the basal lymphatics and surround the cerebellum. The CSF is produced in brain ventricles, fills the subarachnoid space and can be drained via meningeal lymphatics toward the dcLNs. [Extracted from Alves de Lima, K. et al., *Annual Review of Immunology* 2020[28]] (B) Schematic representation of the three meningeal membranes, the dura, arachnoid and pia maters. The latest forms the final barrier before the parenchyma, restricting the diffusion of molecules to 3 kDa. [Extracted from Rua, R., & McGavern, D. B. *Trends in Molecular Medicine* 2018[29]]

Importantly, a progressive dysfunction of meningeal lymphatic vessels is observed with age. The disruption of meningeal lymphatic function in young-adult mice impairs the macromolecule removing from the interstitial fluid to the dcLNs and recapitulates the learning and memory deficits observed during aging[46]. Treatment of old mice with recombinant vascular endothelial growth factor C (VEGF-C) improved the drainage of CSF macromolecules and cognitive function[37,46], through a VEGF-C-dependent increase of the diameter of meningeal lymphatic vessels[42]. Additionally, the induction of meningeal lymphatic disfunction in mouse models of Alzheimer's disease (AD) worsened amyloid-β (Aβ) pathology in the meninges and in the brain[46].

1.2.1 Impact of Meningeal Immunity on Neurophysiology

Under steady state conditions and in opposition to the brain parenchyma (apart from the choroid plexus), meninges are populated by many different immune cell populations (e.g., macrophages, dendritic cells (DCs), innate lymphoid cells (ILCs), mast cells, neutrophils, B cells and T cells) (**Figure 1.1B**). Due to their strategic location at the interface between the periphery and the brain, the meninges function as the first line of protection of the CNS and represent a major site of immune cell residency and recruitment[29]. Importantly, T cells can travel from the blood into the meninges to scan the tissue for possible antigens

before entering the dcLN where they can be primed[47,48]. All these features establish the meninges as a unique and complex microenvironment, with the potential to regulate brain homeostasis.

The role of immune cells to guarantee CNS functions and homeostasis has become indisputable[26]. T cells emerged as protagonists that contribute to physiological brain functions[49], including sensory responses[50], stress responses[51] and adult neurogenesis[52]. Since these cells do not penetrate the brain parenchyma at steady state[53], their effects are seemingly mediated through the secretion of soluble factors from the meninges[49,54]. Remarkably, reports of severe combined immune-deficient (SCID) mice impaired ability to perform spatial learning and long-term memory tasks, along with defective adult neurogenesis, turn a new leaf in the comprehension of cognition and behaviour[55,56]. It was latter demonstrated that the underlying mechanism was cluster of differentiation (CD)4^+ T cell dependent[47,57]. More specifically, T cell derived interleukin (IL)-4 production after engaging in learning/long-term memory tests enhances the production of glial neurotrophic factor[58]. This study showed, for the first time, the importance of the cytokine content of the meningeal milieu for brain physiology. Another important finding concerning meningeal immunity participation in neuronal functions and complex behaviours was the observation of a deficient social behaviour in SCID mice. Similarly, interferon (IFN)-γ-deficient mice also exhibited social deficits and the repopulation of SCID mice with T cells or injections of IFN-γ could restore social interactions. This reveals IFN-γ as a molecular link between meningeal immunity and neural circuits, necessary for social behaviour[59].

1.3 IL-17: A Central Player in Pathophysiology

IL-17A, generally called IL-17, is a pro-inflammatory cytokine highly conserved across the evolution of the vertebrate immune system and part of a family composed of six members — from IL-17A to IL-17F. IL-17A and IL-17F share the highest structural identity and are the main IL-17 family members that contribute to host defence against extracellular bacterial and fungal pathogens. The IL-17 receptors (IL-17R) family is composed of 5 receptors - IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. IL-17A and IL-17F bind to the heterodimeric receptor complex composed of IL-17RA and IL-17RC subunits. In the presence of the adaptor protein Act1 and tumour necrosis factor receptor-associated factor 6 (TRAF6), IL-17A and/or IL-17F canonical signalling will activate the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) and CCAAT-enhancer-binding protein pathways[60,61], leading to downstream target genes activation. IL-17 is a potent inducer of inflammation and its effects are mediated via the recruitment of immune cells such as neutrophil and monocytes, antimicrobial peptide secretion and complement responses to stimulate epithelial barrier function[60,62]. An active involvement in immune cell recruitment is accomplished by an induction of proinflammatory cytokines and chemokines, and matrix metalloproteinase (MMP) production that, in turn, will create a positive feedback loop that enhances the production and strengthens the effects of IL-17[63,64]. Among its epithelial protective roles, IL-17 is involved in the maintenance of intestinal barrier integrity through the regulation of claudins, main components of tight junctions, and antimicrobial peptides[65].

Different cellular sources of IL-17 have been discovered over the years. The observation that $CD3^+$ T cell receptor (TCR)$\alpha\beta^+$ $CD4^+$ cells require different cytokines and transcription factors to produce IL-17 than those involved in the development of the classic type 1 and type 2 T cells (T_H1 and T_H2 cells, respectively), led to the discovery of the new T_H17 subset[66,67]. This subset requires IL-6 and tumour growth factor (TGF)-β[67] to differentiate from naive T cells, with potentiating effects from the combination of IL-1β and tumour necrosis factor (TNF); IL-21 and TGF-β; and IL-6, IL-1β and IL-23[63,64,68]. Besides T_H17, several other immune cell types can produce IL-17 which share IL-23R and CCR6 surface expression, and have retinoic orphan receptor (ROR)γt as their master transcription factor that is both necessary and sufficient for IL-17-producing cell development[69]. These populations include

$CD3^+TCR\gamma\delta^+$ γδ T cells, $CD3^+NK1.1^+$ NKT cells, $CD3^-NK1.1^+$ NK cells and $CD3^-$ ILC3s[63]. Additionally, $CD3^+TCR\alpha\beta^+CD8^+$ cells, $CD3^+CD4^{-/+}CD8^-$ mucosal-associated invariant T (MAIT) cells, KIT^+LIN^- lymphoid-tissue inducer (LTi)-like and myeloid cells have also shown to produce IL-17[70–72]. A major source of IL-17 in steady-state tissue physiology is provided by γδ T cells, a particular subset that blend innate and adaptive immune cell functions – termed as an "adaptate" biology[73]. These cells acquire their effector functions during thymic development and their activation is typically independent of antigen presentation by major histocompatibility complex (MHC) molecules[74], unlike αβ $CD4^+$ T cells that mostly leave the thymus as 'naive' cells[75]. γδ T cells are able to sense and respond immediately to infections or non-microbial stress without the need of clonal expansion or *de novo* differentiation[76]. Critically, this places them as the first line of defence that precedes antigen-specific αβ T cell responses[76]. For instance, γδ T cell-derived IL-17 is fundamental for the early response to *Mycobacterium tuberculosis* pulmonary infection[77]. Additionally, dendritic epidermal γδ T cells (DETCs) IL-17 production in critically involved in wound healing by the promotion of multiple host-defence molecules in epidermal keratinocytes[78] and in the protective response to UV radiation through the stimulation of deoxyribonucleic acid (DNA) repair[79]. Beside host defence, γδ T cell-derived IL-17 has crucial functions in (non-barrier) tissue physiology. By instance, an important homeostatic control of body temperature is exerted in the adipose tissue via IL-17A and TNF. The production of IL-33 in response to these cytokines by adipose stromal cells is fundamental to the maintenance of regulatory T cells (Tregs) and for the metabolic adaptation to cold temperatures[80]. Interestingly, it was later described that IL-17F, not IL-17A, was crucially involved in thermogenesis by the promotion of sympathetic innervation through a mechanism partly mediated by TGFβ expression in adipocytes[81]. Additionally, IL-17A-producing γδ (γδ17) responses are involved in skeletal tissue regeneration. The stimulation of proliferation and osteoblastic differentiation of mesenchymal progenitor cells by IL-17A accelerates osteoblastic bone formation and aids fracture healing[82].

1.3.1 Pathophysiological implications of IL-17 in the CNS

Inspired by important reports of an immune cell support of brain physiology, a pioneer publication from the host laboratory revealed an unexpected role for meningeal IL-17 in the regulation of short-term working memory (**Figure 1.2**).

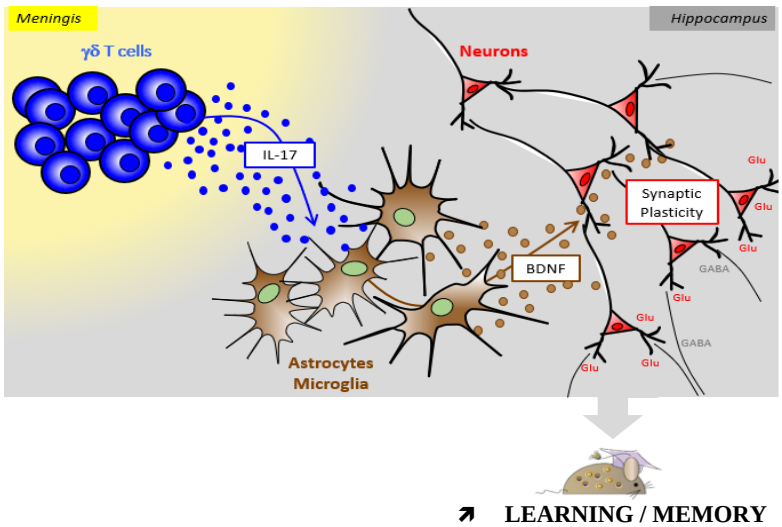

Figure 1.2 – Steady state meningeal γδ17 promote short-term working memory. The production of IL-17 in the meningeal spaces by γδ T cells induces the production of brain-derived neurotrophic factor (BDNF) by glial cells that promotes hippocampal synaptic plasticity, a fundamental process to spatial learning and memory formation. [Adapted from Ribeiro, M. et al., *Science Immunology* 2019[53]]

When tested in the Y-maze (a classical paradigm to analyse short-term working memory), animals deficient in IL-17 displayed clear cognitive impairments in contrast to their littermate controls. A thorough

analysis of meningeal immune cell populations of WT mice revealed a novel foetal thymic-derived population of meningeal γδ T cell as the major source of IL-17 (γδ17) in the steady state CNS. At the functional level, IL-17 promotes learning and memory by favouring the glutamatergic synaptic plasticity of hippocampal neurons. This effect was mediated brain-derived neurotrophic factor (BDNF) production by glial cells[53] (**Figure 1.2**). Importantly, this study added an innate like population - γδ T cells - to the repertoire of immune populations infiltrating the CNS that regulate neurophysiology.

Further studies have reported a role for IL-17 in brain physiology. IL-17 direct action on *Caenorhabditis elegans* neurons regulates pathogen-avoidance behaviour through a potentiation of circuit function[83]. Furthermore, in a mouse model of maternal immune activation (MIA), the activation of IL-17RA^{+} neurons in the CNS rescued the sociability behaviour deficits exhibited by these mice[84].

Conversely, IL-17 has been shown to promote neuronal death and behavioural abnormalities. IL-17 was found to negatively regulate adult neurogenesis in the dentate gyrus (DG) of the hippocampus under physiology conditions[85]. Moreover, maternally derived IL-17 action on the developing fetal brain mediates an autism-like phenotype in the offspring[86]. The risk of neurodevelopmental disorders in the offspring increases with the presence of gut microbiota-dependent inflammatory IL-17 in pregnant mothers[87]. Additionally, an important contribution of IL-17 to neuroinflammation and neurodegenerative diseases is under the spotlight. Using non-physiological stimuli, IL-17 demonstrated to be involved in lipopolysaccharide (LPS)-induced systemic inflammation, consequently promoting neuroinflammation and memory impairments via microglial activation[88]. In a mouse model of cerebral ischemia-reperfusion (I/R) injury, IL-17 has a pivotal role in the progression of brain infarction and neurological deficits, with γδ T lymphocytes as the major source of this cytokine[89]. This subset was shown to be regulated by intestinal dysbiosis and a microbiota–γδ17 T cell–brain axis was identified, having meninges as the preferential destination[90]. Furthermore, a crucial role for IL-17 in the development of experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS), is evidenced by the delayed onset, reduced severity and early recovery of EAE induced in IL-17$^{-/-}$ mice[91]. Also in this context, γδ T cells are an important source of IL-17 that will then mediate the recruitment of IL-1β-secreting myeloid cells that prime pathogenic γδ17 and T helper (T_H)17 cells[92,93]. In both I/R injury and EAE pathology, γδ17 T cells in the meningeal spaces unleash a local immune amplification loop that will alter the stromal microenvironment of the inflamed brain, cause BBB disruptions and ultimately contribute to disease progression[94,95]. Interestingly, clinical studies in patients support a pathogenic role of IL-17 by reporting an increased amount of this cytokine in the brain, blood and CSF of stroke[96,97] and MS patients[98,99].

Last but not least, a pathogenic role for IL-17 in a neurodegenerative context has also been suggested[100,101]. Unpublished work from the host laboratory reveals an exacerbation of IL-17 levels in the meningeal compartment of a progressive mouse model of AD (3xTg-AD) at the onset of cognitive decline (5-6 months of age), mainly provided by γδ T cells (**Figure S1**). Critically, IL-17 neutralization with anti-IL17 monoclonal Abs (mAbs) was sufficient to prevent short-term reference memory deficits and synaptic plasticity dysfunction observed at early stages of disease (Brigas, H. et al., *under review*). Additionally, using a genetically modified progressive mouse model of Parkinson's disease (PD) characterized by an overexpression of human alpha-synuclein (αSyn) (Thy1-αsyn mice), we observed a putative accumulation in meningeal IL-17 levels in transgenic mice compared with littermate controls, at 6-months of age (**Figure S2**). At this stage of disease, Thy1-αsyn mice display impaired coordinated motor tasks[102] that are dependent on gut microbial colonization and exacerbated with gut dysbiosis[103], and exhibit memory and synaptic impairments[104]. As observed in the 3xTg-AD, this cytokine was mainly provided by γδ T cells, implying a homeostasis dysregulation of γδ17 T cells in the meninges in pathological settings. Concomitantly, a pathogenic role for IL-17 has also been suggested in a model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD, contributing to BBB disruption, death of dopaminergic neurons and motor impairments, in a microglia-dependent manner [105]. Altogether,

these findings support the idea of a dual role for IL-17 in physiological versus pathological conditions. Whereas at steady state the production of IL-17 by meningeal γδ T cells is fundamental to ensure learning and memory, its exacerbated production upon inflammation contributes to the pathogenesis of neuroinflammation[53]. Therefore, a tight control of meningeal IL-17 homeostasis is required to maintain brain integrity and cognitive functions. Based on data from the literature, we hypothesize that neuromodulators, circadian rhythms and sleep might contribute to this control.

1.4 Potential Mechanisms Regulating Meningeal Immunity

1.4.1 Stress

Stress is a natural and fundamental physiological response that prepares the organism for an upcoming challenge[106]. During a stressful event, the amygdala will send distress signals to the hypothalamus, perceived as the command centre of the body. This leads to the activation of the sympathetic nervous system (SNS) that releases catecholamines, such as norepinephrine and adrenaline, triggering a "fight-or-flight" response[107,108]. Additionally, the activation of the HPA axis (**Figure 1.3**) will orchestrate and synchronize complex physiological processes through changes in the internal and external environments mediated by hormones. Upon exposure to a stressor, the secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) by the hypothalamus will induce the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. Circulatory ACTH will stimulate the adrenal cortex to produce glucocorticoids - cortisol in humans, corticosterone in rodents -, the final product of the HPA axis. In addition to stress, a constant regulation of the HPA axis is provided by the circadian clock in steady state conditions[109,110]. Consequently, a peak of glucocorticoids anticipates wakening and the activity cycle: early morning in diurnal and early night in nocturnal animals[111].

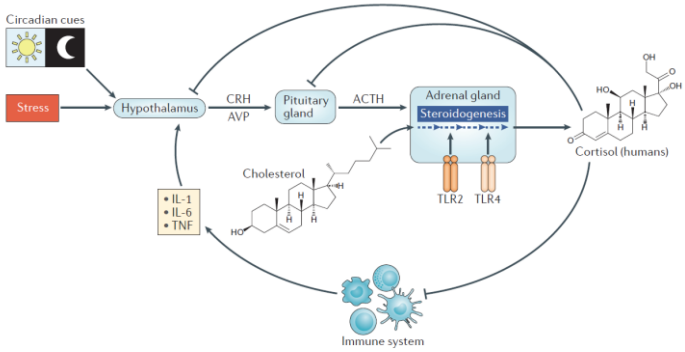

Figure 1.3 - Regulation of glucocorticoid production by the hypothalamic–pituitary–adrenal axis. Glucocorticoid production is an autoregulated process that depends on hypothalamic activation by several endogenous and exogenous cues (such as stress, circadian cues and cytokines) that results in the production of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). These hormones stimulate secretion of adrenocorticotropin hormone (ACTH) that induce the steroidogenic process using cholesterol. Glucocorticoids can control their own synthesis by acting on the hypothalamus, pituitary gland or on the immune system. [Extracted from Cain, D. W. & Cidlowsji, J. A. *Nat. Rev. Immunol* 2017[109]]

Glucocorticoids will exert their functions by binding to the glucocorticoid receptors (GRs) that are encoded by the Nuclear Receptor Subfamily 3 Group C Member 1 (*Nr3c1*) and spread throughout the body. Glucocorticoid secretion will induce a negative feedback loop due to the activation of GRs expressed by cells in the hypothalamus and pituitary gland[109]. Furthermore, an immune regulation of glucocorticoid production is exerted by the direct activation of Toll-like receptors (TLRs) expressed in human and mouse adrenals[112,113]. During bacterial infections TLRs activation directly induces adrenocortical steroid release, enabling the body to immediately respond to external and internal stimuli with an efficient endocrine response. Pro-inflammatory cytokines, such as IL-1, TNF[114] and IL-6[115] also have modulatory effects on the HPA axis. The release of these cytokines during inflammatory events results in the direct activation of the hypothalamus and pituitary inducing CRH and ACTH release and, consequently, corticosteroid production[116]. This HPA-axis immune control provides a second feedback loop that drives the suppression of pro-inflammatory responses[109] and emphasizes the role of cytokines

as potent neuromodulators. Thus, a crosstalk between the endocrine and the immune systems is fundamental for the control of inflammatory responses.

1.4.1.1 Stress and Immune Function

Although often seen as noxious, stress effects will differ according to the duration, intensity and chronicity[117]. An acute stress response triggers immune responses[118] by enhancing leukocyte circulation to the blood and infiltration to inflamed tissues, as well as by promoting the proliferation and functions of several immune populations[119–121]. On the other hand, chronic stress is often associated with immunosuppressive effects and higher risk of infection illnesses[122]. Increased levels of pro-inflammatory genes, decrease in genes involved in innate antiviral responses and antibody synthesis[123], accumulation of pro-inflammatory leukocytes[124], a shift towards a type 2 immune response which is associated with an increased susceptibility to autoimmune disorders[125] and restructure of lymphatic networks within and around tumours to provide pathways for tumour cell escape[126] are some of the described effects of chronic stress[25].

Owing to the innervation of primary and secondary lymphoid organs by sympathetic nerves[127], the PNS exert important roles through the release of catecholamines and neuropeptides[4]. For instance, these signals are involved in haematopoiesis regulation through hematopoietic stem cell (HSC) retention in the bone marrow (BM)[128,129] and in adaptive cell priming in the lymph nodes (LNs) through dendritic cell (DC) regulation[130,131]. Additionally, a modulation of immune cell trafficking is achieved through a physical interaction of β_2-adrenergic receptors (β_2ARs) with the chemokine receptors C-C chemokine receptor type 7 (CCR7) and chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) expressed on lymphocyte surface that inhibits their egress from the LNs[132]. Furthermore, a diurnal lymphocyte recirculation through LNs controlled by the adrenergic nerves has important implications for the adaptive immune response efficiency[133]. On the other hand, glucocorticoids released from the adrenal cortex are mainly perceived as immunosuppressive and anti-inflammatory[109,122]. Accordingly, synthetic glucocorticoid agonists, such as dexamethasone, are used in several inflammatory conditions, including multiple sclerosis and asthma[134,135]. Glucocorticoid signalling can exert potent regulatory effects on cellular immunity[136] by negatively affecting DC activity[137], as well as T cell development, activation, cytokine production, polarization and survival[138]. Importantly, dysregulation the HPA axis induced by chronic life stress during prenatal life can determine the permanent reprogramming of the neuroendocrine stress pathway[139] that results in reduced glucocorticoid levels in adults, leading to attenuated anti-tumour and antibacterial $CD8^+$ T cell responses[140]. It was recently shown that Tregs are direct targets and key players of glucocorticoid anti-inflammatory effects[141]. Last but not least, a recent study reports that glucocorticoids influences diurnal oscillations of T cell survival and distribution among lymph nodes, spleen, and blood by modulating the expression of interleukin-7 receptor (IL-7R) and CXCR4 on T cells, thus contributing to differential (diurnal) responses against pathogenic insults[142].

1.4.1.2 Stress in Brain Pathophysiology

Glucocorticoids are involved in the consolidation of memories during emotional stressful events[143], by diffusing into the brain and binding to the GRs expressed in most brain regions, namely in hippocampal CA1 neurons[144,145]. Since the hippocampus is fundamental for the formation of spatial and episodic memory, glucocorticoid can easily modulate this process[143] and circadian glucocorticoid peaks have shown a fundamental role in the stabilization of newly formed spines that are important for long-term memory retention[146]. Notwithstanding, excessive levels or long-term exposure to glucocorticoids can have hazard effects[147]. While acute stress enhances synaptic plasticity and facilitates spatial learning by an increase in glutamate release, chronic stress is a major risk factor for accelerating the onset of mental health disorders[148,149]. By instance, chronic exposure to glucocorticoids eliminates this learning-associated new spine and disrupts previously acquired memories[146]. Additionally, maternal

glucocorticoid dysregulation increases the offspring's risk of developing psychiatric disorders in humans later in life[150], such as autism spectrum disorder (ASD)[151], depression[152], anxiety[153], amongst others, with more profound effects if maternal exposure to prenatal GCs occurs out of circadian phase/time of the day[154]. Of note, blockage of GR signalling can attenuate post-traumatic behavioural deficits induced after stress exposure, by enabling the recruitment of *Gata3*- and *Foxp3*-expressing T cells to the brain[155]. In human, the importance of stress for brain physiology is evidenced by Cushing syndrome and Addison's disease, that are associated with high levels or low levels of corticosterone, respectively. Both conditions present severe psychological in addition to physiological symptoms. Altogether, this glucocorticoid-regulated neuroendocrine stress axis shows fundamental implications for brain physiology.

1.4.2 Circadian Rhythms

The circadian system comprises a set of endogenous autonomous oscillators that modulate the physiological activities of different tissues according to the 24-hour periodicity in the Earth's rotation, providing an adaptive advantage to organisms[156]. In this system, the suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the master pacemaker that oscillates autonomously in response to several environmental cues or "zeitgebers" (ZTs) (**Figure 1.4A**). The main entrainment pathway of this system are photic cues (the light/dark cycles) processed through the eye and transmitted via the retinohypothalamic tract (RHT) to neurons of the SCN[157,158]. As an output, synchronizing signals will regulate the oscillation of the peripheral clocks present in most (if not all) cells throughout the body to coordinate tissue-specific rhythms[159]. This synchronization is maintained by a combination of neuronal signalling, secretion of hormones and metabolic cues provided by rhythmic feeding behaviour[160].

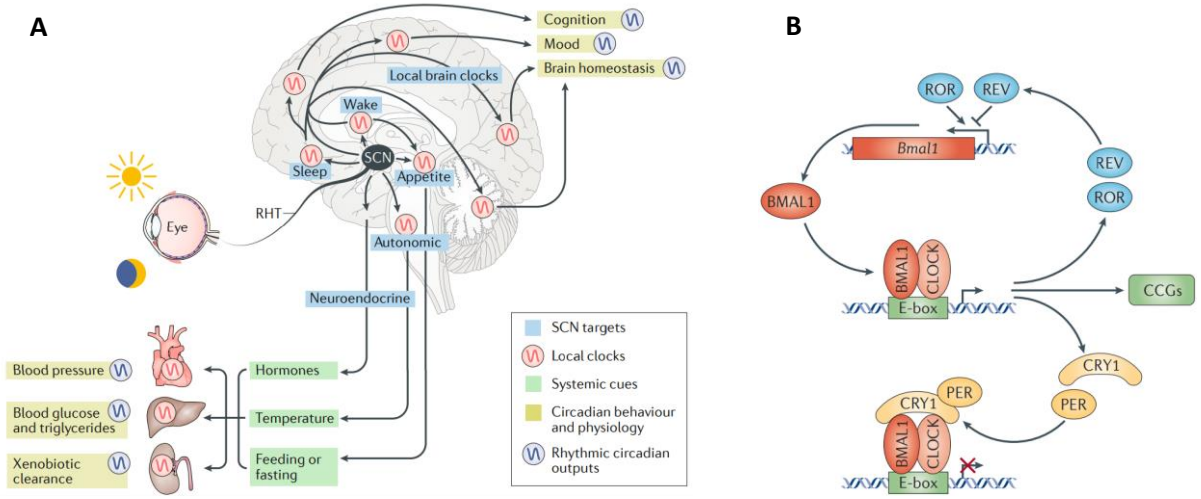

Figure 1.4 – The circadian clock in mammals. (A) The light/dark cycles received via the retinohypothalamic tract (RHT) synchronize the SCN that coordinates the circadian oscillations of several clocks within the brain (that regulate sleep, appetite, feeding behaviour, cognition, mood, etc.) and the periphery (that regulate blood pressure, triglyceride metabolism, renal function, etc.) through neuroendocrine and autonomic signalling. [Extracted from Hastings, M. H., Maywood, E. S. & Brancaccio, M. *Nat. Rev. Neurosci.* 2018[158]] **(B)** The molecular clock comprises several transcriptional–translational feedback loops that include positive (red), negative (yellow) and stabilizing (blue) elements. The final product of this regulation is a rhythmic expression of circadian-clock-controlled genes (CCGs) that will control complex physiological processes throughout the body according to several environmental cues. [Extracted from Kondratova, A. A. & Kondratov, R. V. *Nat. Rev. Neurosci.* 2012[161]]

At the molecular level, these rhythms result from complex circadian transcription–translation feedback loops (**Figure 1.4B**). The main loop takes 24h to be completed and is comprised by the core clock proteins: Brain and Muscle ARNT-Like 1 (BMAL1) encoded by *Arntl* gene and circadian locomotor output cycles kaput (CLOCK) encoded by the *Clock* gene. The transcription and heterodimerization of BMAL1 and CLOCK drive circadian processes by inducing their own expression, the expression of negative regulators of this cycle, such as period circadian protein (PER) and cryptochrome (CRY), the expression of positive regulators, such as retinoic acid receptor-related orphan receptor (ROR) family

members and their bind to canonical E-box sequences (CACGTG) in clock-controlled genes (CCGs). In this main loop, the PER–CRY complex will repress the binding of BMAL1–CLOCK to target genes until their own degradation occurs and the cycle starts anew. A second autoregulatory feedback loop is prompted by the REV-ERBα–REV-ERBβ complex that represses *Arntl* transcription[158,159,161].
This internal circadian clock controls all aspects of physiology, including sleep–wake cycles, behaviour and locomotor activity, body temperature cycles, cardiovascular and digestive processes, the endocrine system, metabolism and immune functions[156].

1.4.2.1 Circadian clock and Immune Function

A circadian control of innate and adaptive immune cells is interpreted as a mechanism to enhance sensitivity and immunosurveillance ahead of activity and feeding during the active phase, when the risk of infection would be highest[162]. For instance, administration of LPS during the active phase of mice significantly reduces the mortality rate compared with administration during the resting period[163]. Accordingly, in humans, the antibody response induced by vaccination is higher in the morning than in the afternoon[164]. These intrinsic timers can affect several features of the immune system. A fluctuation of lymphocytes in the blood and LNs was described as the result of a cell-intrinsic oscillation in trafficking molecules, such as adhesion molecules and chemokine receptors, and lineage- and tissue-specific oscillations of promigratory factors, such as endothelial cell-adhesion molecules[165]. This results in a lymphocyte homing to LNs that peaks at the active phase onset and is affected by the modification of photic cues[166]. In addition to immune cell trafficking, circadian oscillations can also modulate the effector functions of group 3 innate lymphoid cells (ILC3s) reflected by *Rorc*, *Il17a* and *Il22* diurnal oscillatory expression[167]. Circadian disruptions disturb ILC3s homeostasis in the gut which results in number and cytokine secretion reduction. This regulation is fundamental to ensure intestinal homeostasis, lipid metabolism, microbiota composition and enteric defence against bowel infections induced by pathogenic bacteria[168,169]. Additionally, a circadian regulation of intestinal IL-17–producing $CD4^+$ T helper (T_H17) cells by the basic leucine zipper transcription factor nuclear factor IL-3-regulated protein (NFIL3) was described. Rhythmic expression of NFIL3 induced by the circadian clock protein REV-ERBα regulates T_H17 cell frequency via the suppression of *Rorc*, which encodes RORγt, a key transcription factor necessary for IL-17-producing cell differentiation[170].
Taking together, these reports emphasize the importance of a healthy intact clock for immune homeostasis and the prevention of several pathologies. In fact, a negative impact of shift-work on health has been largely described[171] and is associated with increased risk of cancer, obesity, heart disease, gastrointestinal dysfunction, sleep disorders, diabetes and depression[172].

1.4.2.2 Circadian Clock in Brain Pathophysiology

Notably, the phase coherence between brain circadian clocks is critical for brain homeostasis, influencing several mental processes such as cognitive function, adaptation to novelty and mood[161]. By instance, cognitively complex tasks requiring short-term memory show a circadian rhythm dependence that peaks around midday in humans[173]. The potential mechanisms underlying the role of circadian rhythms on cognition and memory formation include synaptic plasticity[174], neurogenesis[175] and sleep[176].
Importantly, a link between circadian rhythm disruptions and the pathophysiology of neurodegenerative diseases can be anticipated. Since sleep and circadian rhythms disruption is one of the earliest and most common signs of neurodegenerative diseases - such as Alzheimer, Parkinson and Huntington's diseases - that worsen with disease progression, a possible circadian clock control of neurodegeneration has been discussed[161]. In accordance, single nucleotide polymorphisms (SNPs) in *Clock* and *Arntl* are associated with increased susceptibility to AD, while SNPs in *Arntl* and *Per1* are associated with PD[177–179]. Interestingly, the deletion of REV-ERBα, a circadian clock component directly regulated by BMAL1, induced spontaneous hippocampal microglial activation, neuronal injury and exacerbated LPS-induced

neuroinflammation[180]. Within the potential mechanisms of circadian clock-dependent regulation of neurodegeneration is the circadian control of the antioxidant defence[181], of nucleotide excision repair systems[182] and the regulation of autophagy activity[183]. Disruptions in the normal clock function may result in the failure of these brain cleaning systems followed by increased oxidative damage, reduced removal of DNA lesions, increased neuronal death and decreased autophagy, thus contributing to neurodegenerative diseases development[161].

1.4.3 Sleep

Sleep is a physiological process regulated by a homeostatic process – that prolongs the sleep period according to the wakefulness duration – and the circadian system – responsible for synchronizing the propensity to sleep or be awake along the 24-h sleep-wake cycle, according to the two-process model of sleep regulation[184]. In mammals, the two core stages of sleep are the slow wave sleep (SWS) and the rapid-eye-movement (REM) sleep, which alternate in a cyclic manner. On an evolutionary perspective, the vital function of sleep is strengthened by its conservation across all species[185]. Indeed, this biological process is critical to ensure optimal brain and body health, given its implications in metabolism, appetite regulation and the functioning of immune, hormonal and cardiovascular systems[186]. Consequently, sleep deprivation is associated with cardiovascular diseases, obesity, diabetes and cancer[187,188].

1.4.3.1 Sleep and Immune Function

During an inflammatory event, the induction of fatigue and sleepiness (among other symptoms) is triggered by an immune system activation of the CNS, likely facilitating recovery[12]. It is not surprising that if the immune system can affect sleep, sleep can also cause major changes in the immune system response[189,190]. Although the effects of sleep and the circadian rhythms are difficult to dissociate, it is known that the nocturnal period is characterized by a downregulation of the HPA axis and SNS, as well as an upregulation of pro-inflammatory signals like the growth hormone (GH), prolactin and melatonin[191]. Consequently, immune cell redistribution, activation, proliferation, differentiation and the production of pro-inflammatory cytokines like IL-1, IL-12, TNF-α and of T_H1 cytokines like IFN-γ is stimulated, with beneficial effects for adaptive immunity[13]. In contrast, upon wakening, a rise of stress hormones with potent anti-inflammatory potential is observed. This system optimizes immune cell function by boosting adaptive immune memory response during the resting time when destabilizing influences (stress or immunosuppressants) reach their minimum levels, while during the active time, the activity and stress support immediate cytotoxic effector functions against invading pathogens and tumour cells[192]. Such orchestration will avoid a competition for resources that will optimize these important physiological processes and allow coordination between the organism and the environment[25].

1.4.3.2 Sleep in Brain Pathophysiology

Sleep contribution for memory formation and consolidation has long been recognized[193]. According to the synaptic homeostasis hypothesis, sleep enhances the strength of synapses formed during information acquisition in the prior awaken time[194]. Recently, another important role of sleep in the removal of toxic waste metabolites from the brain was described. During sleep, a 60% increase in the cortical interstitial space results in an increase on the convective exchange of CSF and ISF and the removal of interstitial toxic proteins resultant from neural activity that accumulate during wakefulness, including Aβ and Tau in mouse and humans[185,195]. Interestingly, this clearance route in under a circadian control, with the CSF drainage to the LNs exhibiting diurnal oscillations that are supported by the differential polarization of AQP4[196]. Consequently, sleep deprivations or disturbances have hazard consequences for cognition and emotions, reducing learning, impairing cognitive performance[193], prolonging reaction time[197], provoking seizures[198] and promoting neurological diseases[185,199]. Extreme cases of sleep deprivation can also lead to dementia and death of rodents[200] and humans[201].

2. Aims of the Thesis

An exciting new era for neuroimmunology is emerging and recent advances reshaped our understanding of meninges as gatekeepers that direct and coordinate immune traffic throughout the CNS[29]. In line with this, data from the host laboratory reported new clues on the evolutionary link between the immune and nervous systems, by revealing a previously unknown meningeal γδ T cell subset that produces IL-17 in the steady state CNS. This subset was shown to regulate short-term working memory, by enhancing neuronal synaptic plasticity in the hippocampus, namely favouring excitatory glutamatergic over inhibitory GABAergic pathway[53].

On the other hand, unpublished data from the host laboratory also reported a significant increase of meningeal IL-17-producing cells – mainly γδ T cells - in a transgenic mouse model of AD, associated with the onset of the disease. Importantly, local neutralization of IL-17 in 3xTg-AD mice prevented the development of short-term cognitive deficits and synaptic plasticity dysfunction (Brigas, H. et al., *under review*). These results underpin IL-17 as an early trigger of neuroinflammation and are aligned with previous reports showing a contribution of this cytokine to EAE[94] and brain ischemia upon injury[89,90].

Accordingly, a dual role for IL-17 is proposed: pro-cognitive at steady state and anti-cognitive in the context of neurodegeneration. This implies a hypothetic optimal meningeal IL-17 threshold below which cognitive deficits would appear and above which pathogenic neuroinflammation would be triggered[53]. Therefore, a tight control of meningeal IL-17 homeostasis seems required to maintain brain integrity and cognitive functions.

Building on these considerations, we proposed to investigate the underlying mechanisms that contribute to ensure meningeal IL-17-producing cells regulation. This control would optimize the levels of this cytokine necessary to guarantee optimal cognitive functions. Conversely, a dysregulation of these homeostatic mechanisms would result in an escalation of meningeal IL-17 production leading to neuroinflammatory and neurodegenerative events with harmful consequences for brain physiology.

Given their known important implications in the immune system and cognitive performance, we propose to explore a possible role for glucocorticoids, circadian rhythms and sleep on meningeal IL-17$^+$ immune cell regulation.

Thus, our main goals consist in:

1) Analyzing the impact of glucocorticoids on IL-17$^+$ immune cells upon *in vitro* and *in vivo* administration of the glucocorticoid receptor agonist dexamethasone.
2) Characterizing the diurnal oscillation of meningeal IL-17$^+$ immune cells in wild-type mice and in a neurodegenerative mouse model of Alzheimer's Disease (3xTg-AD).
3) Deciphering a potential influence of sleep for meningeal IL-17$^+$ cells immune cells resorting to an anesthetic procedure.

We believe that understanding the cellular and molecular mechanisms that regulate the homeostasis of the meningeal immune system will greatly advance our knowledge regarding the cascade of events that promote neuroinflammatory diseases.

3. Material and Methods

3.1 Mice

C57BL/6J mice were purchased from Charles River Laboratories (Spain). 5- to *24*-week-old (wo) males were used in all experiments. C57BL/6-129SvJ mice bearing three mutations (3xTg-AD) associated with familial AD (amyloid precursor protein [APPswe], presenilin-1 [PSEN1] and microtubule-associated protein tau [MAPT])[202] were purchased from the Jackson Laboratory and 30-wo females were used in the experiments. Transgenic mice overexpressing human alpha-synuclein (αSyn) under the Thy-1 promoter were generated on a mixed C57BL/6-DBA/2 background (Thy1-αSyn mice), as previously described[203]. Heterozygous female C57BL/6J-DBA/2 were bred with wild-type (WT) male B6D2F1 mice to generate homozygous male mice and their WT littermates analysed in this study at *24*-wo. Thy1-αSyn mice were kindly provided by Dr. Tiago Outeiro from Centro de Estudos de Doenças Crónicas (CEDOC). Mice were maintained at Instituto de Medicina Molecular (iMM) animal facility, housed under a 14h/10h light:dark cycle (light on for 14 hours from 7:00 a.m. to 9:00 p.m.) in specific pathogen–free conditions. For sleep studies, mice were housed in light-controlled cabinets under a reversed 24-hour dark:light cycle (light on for 12 hours from 8:00 p.m. to 8:00 a.m. as ZT0 to ZT12). Mice were maintained under these conditions for at least 3 weeks to habituate prior to experimentation. All mice were fed and watered ad libitum. The experiments were approved by the institutional Animal Welfare Body (ORBEA-iMM), submitted to the Direcção Geral de Alimentação e Veterinária (DGAV) for licensing and performed according to national and European regulations.

3.2 Cell isolation

Mice were euthanized with CO2 and transcardially perfused with cold 0.1 M phosphate-buffered saline (PBS), pH 7.4 (Corning Life Sciences). Meninges were dissected as described in the literature[58]. Briefly, surgical scissors were used to remove skull tops by making an incision along the parietal and squamosal bones. Dural meninges were carefully removed from the ventral and dorsal sides of the cranium and surfaces of the brain with forceps and collected in complete RPMI 1640 [+] L-Glutamine medium (10% fetal calf serum (FCS), 1% of sodium pyruvate, 1% of HEPES, 1% of MEM non-essential amino acid and 1% of penicillin and streptomycin) (ThermoFischer). Superficial cervical (scLNs) and/or peripheral lymph nodes, and the spleen were collected in complete RPMI medium. Cell suspensions were obtained by gently pressing meninges, scLNs and the spleen through a 70-µm nylon mesh cell strainer with a sterile plastic plunger. Cells suspensions were centrifuged at 1200 RPM for 8 min at 4°C. After discarding the supernatant, cell pellets were resuspended in complete RPMI medium. An additional Red Blood Cells lysis step was applied to spleen homogenates by resuspending and incubating the cells into a 1X solution of red blood cell (RBC) lysis buffer (Biolegend) for 2 min. All the organs were processed individually, except for meninges that were pooled from 2-4 mice in the indicated experiments.

3.3 *In vitro* dexamethasone treatment

Total γδ T cells ($CD3^+$ $TCR\delta^+$), or $CD27^+$ and $CD27^-$ γδ T cell subsets along with naïve $CD4^+$ $CD25^-$ αβ T cells were isolated from a pool of peripheral (superficial cervical, axillary, brachial and inguinal) lymph nodes and spleens of 8-wo WT male C57BL/6J mice. Cell suspensions were prepared as described above and negatively enriched in $CD3^+$ cells by magnetic cell sorting (MACS) using anti-biotin microbeads (Myltenyi biotec) and biotinylated anti-CD11b and anti-CD19 antibodies (Biolegend). The enriched fraction was incubated 20 min with purified anti-CD16/CD32 mAbs (Fc block) (ThermoFisher) and stained for 20 min on ice with anti-CD3, anti-TCRδ, anti-CD27, anti-CD4 and anti-CD25 antibodies and Live/Dead fixable dye (ThermoFisher) at $60x10^6$ cells/ml in fluorescence-activated cell sorting (FACS) buffer (0.1M PBS supplemented with 3% FCS and 2mM EDTA). Subsequentially, the indicated cell subsets were electronically sorted (purity>95%) on Aria cell sorters (BD Biosciences).

Sorted cells were stained with CellTrace™ Violet (CTV) Proliferation Kit (ThermoFisher) at a concentration of 1 million cells/ml with 5µM CTV in 37ºC pre-warmed 0.1M PBS for 20 min at room temperature. After washing, the cells were resuspended in complete RPMI (plus 0.1% of 2-Mercaptoethanol) (ThermoFisher) and poured in a 96 well U bottom plate previously coated with purified anti-CD3e antibody (3 µg/ml, 145-2C11, Biolegend) by a 2h incubation at 37ºC. Murine IL-1β (10 ng/ml, PeproTech), murine IL-23 (10 ng/ml, R&D system) and murine IL-7 (40 ng/ml, PeproTech) (when applicable) were added, along with the specified concentrations of Dexamethasone (D4902, Sigma-Aldrich) or the vehicle solution (ethanol absolute). Cells were incubated at 37°C in a 5% CO_2 environment for 20h or 72h, collected and analysed by flow cytometry.

3.4 *In vivo* dexamethasone treatment

8-wo WT male C57BL/6J mice were injected intraperitoneally (ip) at ZT0,5 with dexamethasone 21-phosphate disodium salt (200µg) (D1159, Sigma-Aldrich) or saline solution. Four hours later, the mice were sacrificed for *ex-vivo* analysis by flow cytometry.

3.5 Anesthetic protocol

8-wo WT male C57BL/6J mice on a reversed light:dark cycle were injected ip with a Ketamine (100 mg/kg, Imalgene® - Merial) and Xylazine (10mg/kg, Seton 20mg/ml) solution (K/X), or saline solution at ZT12.5. One third of the initial dose of K/X or saline was administered at ZT13.5. K/X injected mice were maintained on a warm pad and sacrificed at ZT14 for *ex vivo* analysis by flow cytometry.

3.6 Flow cytometry analysis

Cells were stimulated in complete RPMI medium with PMA (Phorbol 12-Myristate 13-Acetate; 50 ng/ml, P-1585, Sigma-Aldrich) and Ionomycin (1 µg/ml, I0634, Sigma-Aldrich) in presence of Brefeldin A (BFA) (10 µg/ml, B-6542, Sigma-Aldrich) for 3h-4h at 37°C 5% CO_2. Cells were transferred into 96-well V bottom plates and washed once in BFA-supplemented FACS buffer. For surface staining, cells were resuspended in BFA-supplemented FACS buffer containing purified anti-mouse CD16/CD32 mAbs (ThermoFisher) for 15 min and 20 min with antibodies against the indicated cell surface markers (**Table S1**) and Live/Dead Fixable Viability dye (ThermoFisher) on ice at $60x10^6$ cells/ml. For intracellular staining, cells were fixed in Fixation/Permeabilization buffer (ThermoFisher) supplemented with BFA for 30 min on ice. Cells were washed with FACS buffer and stored overnight at 4ºC. The day after, cells were washed twice in Permeabilization buffer (Thermofisher) and incubated in permeabilization buffer with Rat IgG (Sigma-Aldrich) and purified anti-CD16/CD32 mAbs (ThermoFisher) for 15 min on ice. Intracellular staining mix, containing the appropriate antibodies (**Table S1**) was added and the cells were incubated for 30 min on ice. Cells were washed first in Permeabilization buffer and then in FACS buffer and resuspended in FACS buffer. In the indicated experiments, early and late apoptotic cells were stained by incubating unfixed cells at 10.10^6 cells/ml with anti-Annexin-V antibody and 7-AAD in Annexin-V staining buffer (Biolegend) during 15 min at room temperature. FACS acquisition was performed on FACS Fortessa (BD Biosciences) cell analyser. The data was analysed using FlowJo software (BD biosciences) and the populations identified with the indicated gating strategy (**Figure S3**).

3.7 Statistical analysis

Statistical analysis and graphical representation of data were performed using GraphPad Prism 8.4.2 software (GraphPad Software Inc., San Diego, CA, USA). Sample data are represented as mean ± standard deviation (SD) of n independent experiments. An unpaired two-tailed Mann-Whitney test and one-way ANOVA with a Tukey's multiple comparisons test were used when applicable. Results were considered significant when $p < 0.05$ or for lower significance thresholds (*$p < 0,05$; **$p < 0,005$; ***$p < 0,001$; **** $p<0.0001$). Differences between populations are indicated in the figures when significant.

4. Results

4.1 IL-17 is supplied by an heterogenous and tissue-specific pattern of immune cells

IL-17 is a cytokine produced by multiple immune cell subsets throughout the body were exerts several immune regulatory functions. Interestingly, we found significant changes in the composition of IL-17$^+$ immune cells between meninges and lymphoid organs (scLNs and spleen). In accordance with a previous report from the host laboratory[53], we observed that meningeal IL-17 is mostly provided by γδ T cells in naïve WT mice (**Figure 4.5B**).

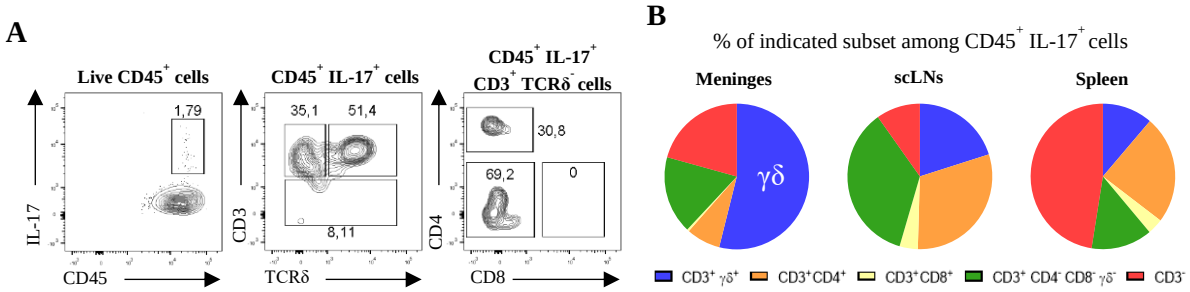

Figure 4.5 – IL-17 is mostly provided by γδ T cells in meningeal spaces at steady state in opposition to lymphoid organs. Cell suspensions were prepared from meninges, superficial cervical lymph nodes (scLNs), and the spleen of 12-week-old wild-type male C57BL6/J mice. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4, and CD8) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Identification strategy for IL-17 producing immune cells among meningeal cells. **(B)** Pie charts indicating the proportion of each cell subsets to IL-17$^+$ immune cell population in the indicated organs. Pie charts display the means values of data from 3 independent experiments with n=6 for meningeal spaces (pooled from 2-3 mice), and n=15 mice for scLNs or spleen.

By contrast, in the LNs IL-17 is mainly provided by other types of T cells, such as CD4$^+$ (T_H17), CD8$^+$ T cells and CD3$^+$ CD4$^-$ CD8$^-$ γδ$^-$ that can include NKT cells and MAIT cells; while in the spleen, it mostly comes from CD3$^-$ cells that can include ILC3s, NK cells, LTis and myeloid cells (**Figure 4.5B**). Hence, different tissues exhibit different immune populations responsible to produce IL-17 and an important contribution is provided by γδ T cells in the meningeal spaces.

4.2 γδ17 T cells are enriched for the expression of receptors sensing environmental and endogenous cues

Based on the dual role of IL-17 in the CNS, we searched for the underlying homeostatic mechanisms that could modulate the pattern of meningeal IL-17$^+$ immune cells at steady state. Considering the preponderant contribution of γδ T cells to IL-17 production in the meningeal spaces[53] (**Figure 4.5B**), we screened putative candidates that would enable γδ17 T cells to sense environmental and endogenous cues.

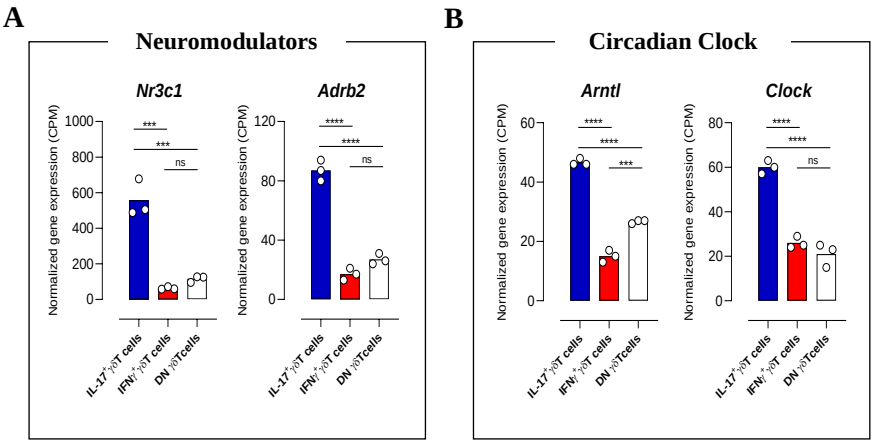

Figure 4.6 – γδ17 T cells are enriched for the expression of receptors sensing environmental and endogenous cues. IL-17$^+$, IFN-γ$^+$ and IL-17$^-$IFN-γ$^-$ (DN) γδ T cell subsets were sorted by FACS from peripheral LNs (superficial cervical, axillary, brachial and inguinal LN) and the spleen *Il17a*-GFP.*Ifnγ*-YFP reporter mice. Total RNA was extracted and analysed by RNA

sequencing (RNA-seq). Histograms depict the expression of **(A)** receptors of neuromodulators - namely glucocorticoids (Nr3c1) and catecholamines (Adrb2, Adrenergic Receptor Beta 2) and (**B**) master clock genes *Arntl* (encoding for BMAL1) and *Clock* (encoding for CLOCK) on IL-17$^+$, IFN-γ$^+$ and DN γδ T cells. CPM: Counts Per Million. Data are from 3 independent experiments. One-way ANOVA with a Tukey's multiple comparisons test. **** $p<0.0001$, *** $p<0.001$. [unpublished data from the host laboratory, by Tiago Amado and Anita Gomes].

The analysis of RNA sequencing (RNA-seq) data provided by the host laboratory (Tiago Amado, *unpublished data*), allowed us to observe a 2-4 fold increase in the expression of genes encoding for the glucocorticoid and β_2ARs (*Nr3c1* and *Adrb2*, respectively) by γδ17 T cells compared with the IFN-γ$^+$ and double negative (DN) subsets, isolated from a pool of peripheral LNs of *Il17a*-GFP.*IFN-γ*-YFP double reporter mice (**Figure 4.6A**). These data suggest that peripheral γδ17 T cells, and possibly meningeal γδ17 T cells, are prone to respond to glucocorticoids and catecholamines (adrenalin and noradrenaline) produced in a stress-context[107] or in a diurnal manner, driven by the circadian machinery[111].

Additionally, γδ17 T cells also displayed a higher expression of the master clock genes *Artnl* and *Clock* (**Figure 4.6B**), compared with IFN-γ$^+$ and the DN subsets. Supporting these results, an independent *in silico* analysis of γδ17 T Vγ6$^+$ cells transcriptional program has also pointed neuromodulators, hormones and circadian rhythm cues as new biological pathways involved in the maturation process of this cell subset[204]. Interestingly, the majority of meningeal γδ17 T cells are Vγ6$^+$ cells[53].

Altogether, we postulated that stress-related neuromodulators and/or internal circadian oscillators could shape the meningeal homeostasis of IL-17$^+$ immune cells.

4.3 Analysis of the impact of stress on IL-17-producing immune cells

4.3.1 *In vitro* glucocorticoid agonist dexamethasone promotes γδ17 T cell preferential survival and proliferation

Glucocorticoid release is a fundamental part of the stress response with powerful neuroimmunomodulatory functions[205]. To investigate a possible role of stress on the production of IL-17, we first investigated the impact of glucocorticoids in γδ T cells using an *in vitro* experimental approach. Due to the limited number of meningeal γδ T cells, we sorted total γδ T cells from LNs and spleen and cultured them for 72h with different concentrations of the high affinity synthetic glucocorticoid, dexamethasone.

We observed a drop in the total number of γδ T cells that received the highest concentration of dexamethasone (10^{-6}M), revealing a dose-dependent immunosuppressive effect mediated by GRs activation (**Figure 4.7A**). In addition, a ten-fold increase and two-fold decrease among the remaining live γδ T cells was observed for the proportions of IL-17$^+$ (**Figure 4.7B**) and IFN-γ$^+$ cells (**Figure S4**), respectively, although their individual cytokine production – IL17 and IFN-γ geomean fluorescence intensities, (gMFI) – were unaffected (**Figure 4.7B** and **Figure S4**). In this condition, we postulate that the substantial enrichment of IL-17$^+$ γδ T cells would be the result from a dexamethasone-induced apoptosis of other γδ T cells. Nonetheless, when γδ T cells were incubated with a lower concentration of dexamethasone (10^{-8}M), we observed a 2-fold increase in IL-17$^+$ cells (**Figure 4.7B)**, while the percentage of IFN-γ producers was similar (**Figure S4**), as well as the absolute cell numbers (**Figure 4.7A**). Therefore, the enrichment observed in γδ17 T cells after the dexamethasone treatment still takes place in the absence of apoptosis observed at high concentrations.

This first set of data suggests a differential effect of the dexamethasone treatment on γδ T cell subsets, promoting or depleting them, in a dose-dependent manner.

To confirm this differential effect, we sorted γδ T cells based on their expression of CD27, allowing to obtain populations enriched in IL-17 producers (γδ27$^-$ subset) and IFN-γ producers (γδ27$^+$ subset)[206]. These cells were cultured separately in the presence of 10^{-8} M of dexamethasone (optimal concentration, as defined in **Figure 4.7B**) for 20h to consider a possible early effect mediated by GRs stimulation. As a positive control, we also stimulated naïve CD4$^+$ T cells, a subset previously shown to be regulated by

glucocorticoid signalling[142]. To assess the effect of dexamethasone on survival and proliferation, we resorted to a Live-Dead staining and Cell Trace Violet (CTV) dilution method, respectively.

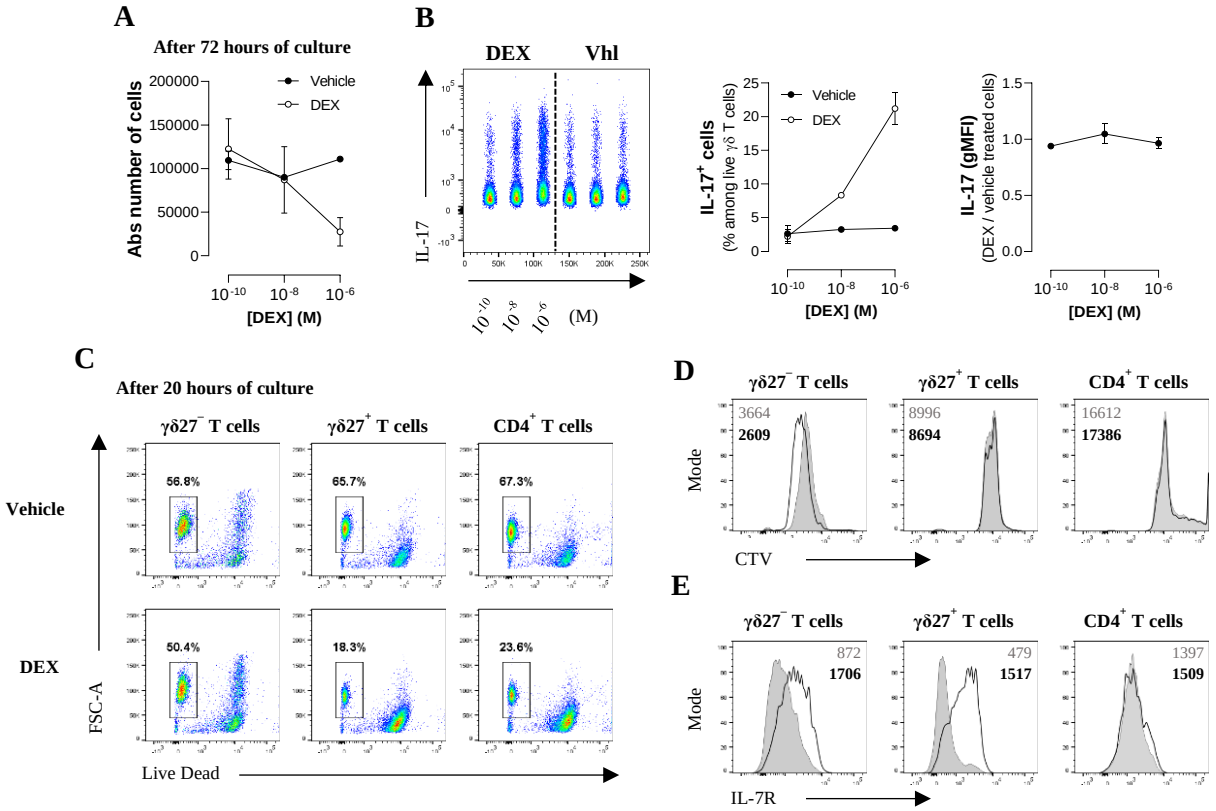

Figure 4.7 – *In vitro* dexamethasone treatment of γδ T cells favours the IL-17$^+$ cells survival and proliferation. Total γδ T cells were sorted by FACS from the peripheral LNs (superficial cervical, axillary, brachial and inguinal LN) and the spleen of 8-week-old wild-type male C57BL/6J mice. Cells were cultured in presence of anti-CD3e antibody (3 µg/ml, plate bound), IL-1β (10 ng/ml), IL-23 (10 ng/ml) and IL-7 (40 ng/ml); and treated with a dexamethasone (DEX) or the vehicle solution. **(A)** Total cells were counted after 72 hours of culture with different concentrations of dexamethasone (10^{-10} M, 10^{-8} M and 10^{-6} M), and analysed by flow cytometry for the expression of surface (CD3, TCRδ) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(B)** Representative dot plots displaying the IL-17$^+$ cells among 10000 dexamethasone or vehicle-treated live γδ T cells (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). CD27$^+$ or CD27$^-$ γδ T cells, and naïve CD4$^+$ T cells were treated with dexamethasone (10^{-8} M) or vehicle solution, in the culture conditions described above (without IL-7) for 20 hours. Total cells were collected and analysed by flow cytometry for their **(C)** survival (LiveDead dye), **(D)** proliferation (Cell Trace Violet dye dilution) and **(E)** their surface expression of the IL-7 receptor (IL-7R). **(D, E)** Empty dark histogram: dexamethasone-treated cells, Grey-filled histogram: vehicle-treated cells. Data extracted from three independent experiments. Mean ± SD.

Our results confirmed a cell subset-specific effect of dexamethasone with the induction of massive apoptosis of treated γδ27$^+$ cells and naïve CD4$^+$ T cells, while γδ27$^-$ T cell survival was unaffected (**Figure 4.7C**). Additionally, γδ27$^-$ T cells exhibited a higher proliferation only 20 hours after the dexamethasone treatment (**Figure 4.7D**).

Since glucocorticoids can modulate T cell homeostasis through a direct induction of IL-7R, promoting the survival, proliferation and differentiation of T cells[142], we analysed its surface expression as a functional read-out of GR activation[207]. We observed a 2-3 fold overexpression of the IL-7R in response to dexamethasone stimulation, particularly in the γδ T cell subsets (**Figure 4.7E**), while naive CD4$^+$ T cells significantly overexpressed IL-7R for higher concentrations of dexamethasone (10^{-6}M) (data not shown). These results validated the GR activation of IL-17$^+$ and IFN-γ$^+$ enriched cell subsets and suggest a higher affinity or more pronounced response of γδ T cells to dexamethasone, compared with αβ CD4$^+$ T cells.

Therefore, glucocorticoids can modulate the effector functions, proliferation and survival of peripheral γδ17 T cells.

4.3.2 *In vivo* dexamethasone administration does not alter meningeal IL-17$^+$ immune cells homeostasis

To test a possible *in vivo* glucocorticoid role in meningeal IL-17$^+$ cell homeostasis, we injected WT mice with dexamethasone or vehicle solutions in the beginning of their resting phase (lowest glucocorticoid level period) and analysed meninges, scLN and spleen after four hours by flow cytometry (**Figure 4.8A**).

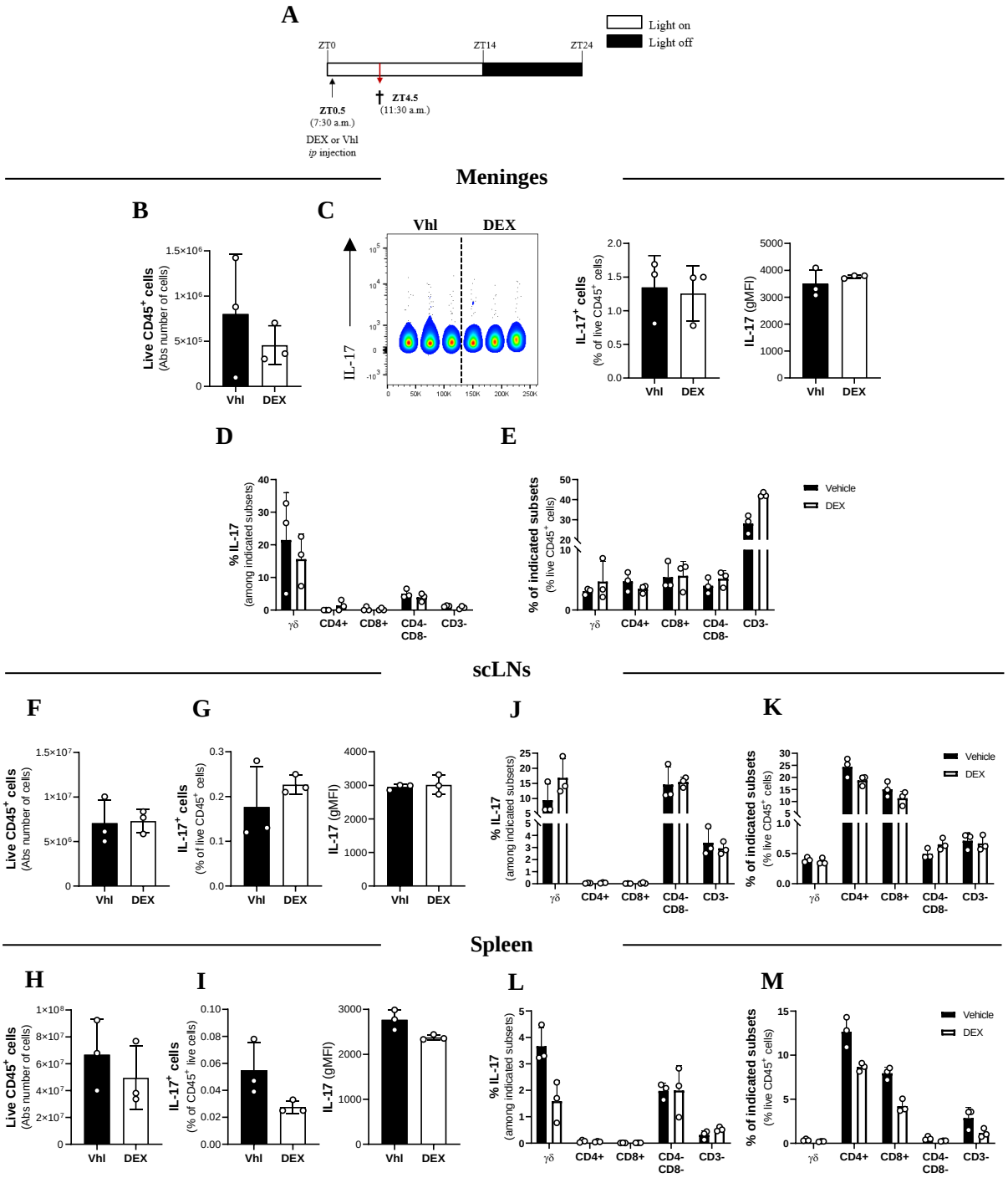

Figure 4.8 – *In vivo* dexamethasone administration does not alter meningeal IL-17$^+$ immune cells homeostasis. (A) Experimental procedure: 7-wo WT male C57BL6/J mice received an ip injection of dexamethasone (DEX, 200 µg) or vehicle saline solution at ZT0,5 (normal light-dark cycle), and were sacrificed 4 hours later. Cell suspensions were prepared from the indicated organs. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. Absolute numbers of live CD45$^+$ cells from the **(B)** meningeal, **(F)** scLNs and **(H)** spleen cell suspensions were calculated. **(C)** Smoothened dot plots depicting IL-17$^+$ cells among 900 live CD45$^+$ cells isolated from meninges of dexamethasone or vehicle-

treated animals (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for scLNs in **(G)** and spleen cell suspensions **(I)**. **(D, J, L)** Percentages of IL-17$^+$ cells among CD3$^+$γδ T cell, CD3$^+$CD4$^+$ T cell, CD3$^+$CD8$^+$ T cell, CD3$^+$CD4$^-$ CD8$^-$ T cell and non-T cell subsets; (**E, K, M**) and percentages of these cell subsets among total live CD45$^+$ cells. Data are from 1 independent experiment, n=3 mice per group. Mean ± SD.

We performed an extended analysis of cell functions (IL-17 and IFN-γ production), proliferation, survival and migration properties of a large panel of individual lymphocyte subsets. In these experimental settings, meninges did not display significant changes in the percentage of IL-17 among leukocytes (**Figure 4.8C**) or the different subsets (**Figure 4.8D**) after the dexamethasone treatment and only an increase in CD3$^-$ cells among total leukocytes could be perceived (**Figure 4.8E**).
However, a differential re-shaping of peripheral immune cells was observed in dexamethasone-treated animals. A decrease in the number of leukocytes, IL-17$^+$ cells and IL-17 expression could be observed in the spleen (**Figure 4.8H, I**), while in the scLNs we observed a slight increase of IL-17$^+$ cells (**Figure 4.8G**). Interestingly, the major difference in the percentage of IL-17 was found among γδ T cells from both organs (**Figure 4.8J, L**).
Similarly, dexamethasone did not impact the expression of IFN-γ in the meninges (**Figure S5A**), while a drop could be observed in the peripheral lymphoid tissues, particularly in the spleen (**Figure S5B, C**). Although not significant, the dexamethasone treatment also induced an overall reduction of the different IL-17 immune cell producers in the spleen of dexamethasone-treated animals (**Figure 4.8M**), suggesting that the limitation of splenic IL-17 production might be a consequence of a substantial cell apoptosis driven by the treatment. In the scLNs we only observed a drop in CD4$^+$ and CD8$^+$ T cells (**Figure 4.8K, M**), two subsets known to be regulated by glucocorticoids[140,142], suggesting that the IL-17$^+$ cell increase resulted from an enrichment in γδ17 T cells (**Figure 4.8J**). However, a recirculation of IL-17$^+$ cells between both lymphoid organs cannot be excluded.
These data indicate that early dexamethasone treatment has a dual role in the homeostasis of IL-17$^+$ cells in a lymphoid tissue-specific manner but does not affect meninges.

4.3.3 *In vivo* dexamethasone administration differentially affects IL-17 immune cell producer homeostasis in the scLNs

To further explore the underlying mechanisms of in vivo IL-17$^+$ cell modulation in the scLNs by the dexamethasone treatment, we performed an annexin V binding buffer and 7-AAD staining to identify early versus late (apoptotic) cells, a ki-67 staining to confirm the proliferative status and a RORγt staining to evaluate the differentiation of different cell subsets.
Due to incompatibility of intracellular cytokine staining with annexin V kit, we used the differential expression of the surface CD44 and CD45RB markers to segregate γδ T cells into subsets with IL-17A- or IFN-γ-secreting potential or DN among CD24$^-$ cells, as previously described[208]. According to what we previously described with the intracellular staining, we could observe an increase in the percentage of IL-17$^+$ committed γδ T cell subset ([d] CD44$^+$ CD45RB$^-$) and a concomitant decrease in IFN-γ$^+$ committed γδ T cell subsets ([b] CD44$^-$ CD45RB$^+$ and [c] CD44$^+$ CD45RB$^+$), while no differences were observed in the DN subset ([a] CD44$^-$ CD45RB$^-$) (**Figure 4.9A**).
As expected, we observed a 2-fold increase in the amounts of early and late apoptotic cells among IFN-γ committed and the DN subsets in sharp contrast with the IL-17 committed subset, whose apoptotic cell populations were unaffected by the dexamethasone treatment (**Figure 4.9C**). Besides γδ T cells, CD4$^+$ and CD8$^+$ T cells were the subsets more affected by the dexamethasone treatment (**Figure 4.9D**). Of note, an overexpression of IL-7R was observed in the populations more responsive to the dexamethasone treatment, i. e., γδ T cells, CD4$^+$ and CD8$^+$ T cells (**Figure S6**).
On the other hand, we detected an enrichment in Ki67$^+$ cells among the γδ T cells of dexamethasone treated mice (**Figure 4.9E**). Interestingly, these proliferative cells were enriched in RORγt-expressing cells, the master transcriptional factor controlling IL-17 production (**Figure 4.9F**).

These results validated our *in vitro* data by reporting a cell-differential pro-apoptotic or proliferative program downstream of the GR activation that favors the γδ17 T cell population.

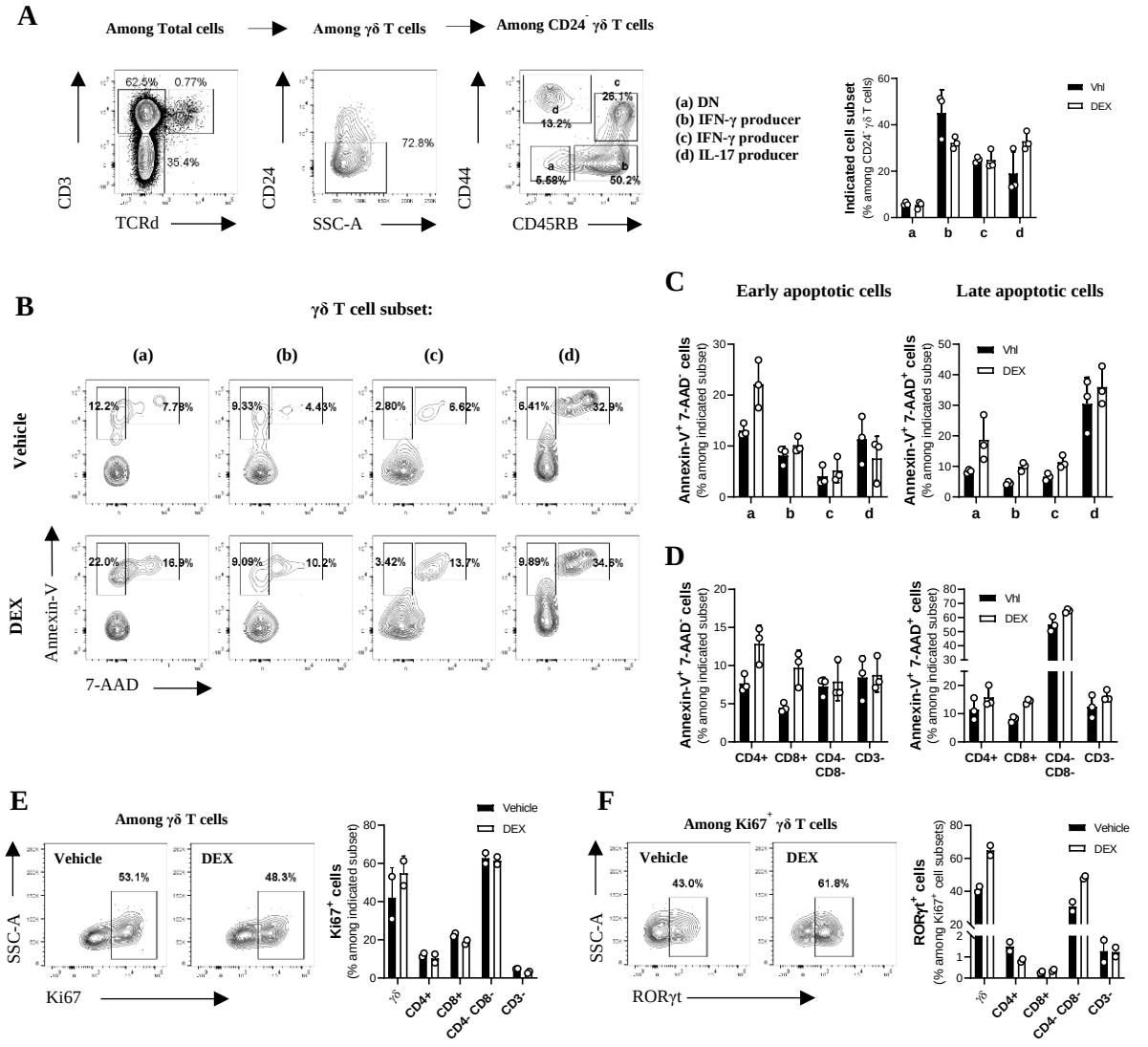

Figure 4.9 – *In vivo* dexamethasone administration differentially affects the γδ T cell homeostasis in the scLNs. 7-wo WT male C57BL6/J mice received an ip injection of dexamethasone (DEX, 200 µg) or vehicle saline solution at ZT0,5 (normal light-dark cycle), and were sacrificed 4 hours later. Cell suspensions were prepared from superficial cervical lymph nodes (scLNs) and were analysed by flow cytometry for the expression of surface (CD3, TCRδ, CD4, CD8, CD24, CD45RB, CD44, Annexin-V) markers and 7-AAD dye for the live/dead cell discrimination. **(A)** Identification (left) and quantification (right) of γδ T cell subsets as previously described[208]. **(B)** Representative contour plots of early (Annexin-V$^+$ 7-AAD$^+$) and late (Annexin-V$^-$ 7-AAD$^+$) apoptotic cells among indicated γδ T cell subsets isolated from the scLNs of dexamethasone or vehicle-treated animals. **(C)** Quantification of early and apoptotic cells among γδ T cell subsets **(D)** and other identified immune cell subsets. Cells were in parallel analysed for their expression of intracellular (Ki67, RORγt) markers. Live cells were gated using Live-Dead Fixable Viability Dye. **(E)** Representative contour plots of proliferative Ki67$^+$ cells among total γδ T cells in dexamethasone or vehicle-treated animals (left), and their quantification. **(F)** Representative contour plots of proliferative RORγt$^+$ cells among Ki67$^+$ γδ T cells in dexamethasone or vehicle-treated animals (left), and their quantification. Data are from one independent experiment, n=2-3 mice per group. Mean ± SD.

4.4 Putative circadian control of meningeal immune cells in pathophysiological conditions

4.4.1 Meningeal IL-17$^+$ immune cells exhibit a diurnal oscillatory pattern in the meninges of wild type mice

Considering the expression of circadian clock genes in γδ17 T cells (**Figure 4.6B**), we questioned a possible time-of-the-day regulation of IL-17$^+$ immune cells in the meninges that could optimize the IL-17 levels required to perform cognitive tasks dependent on short-term memory.

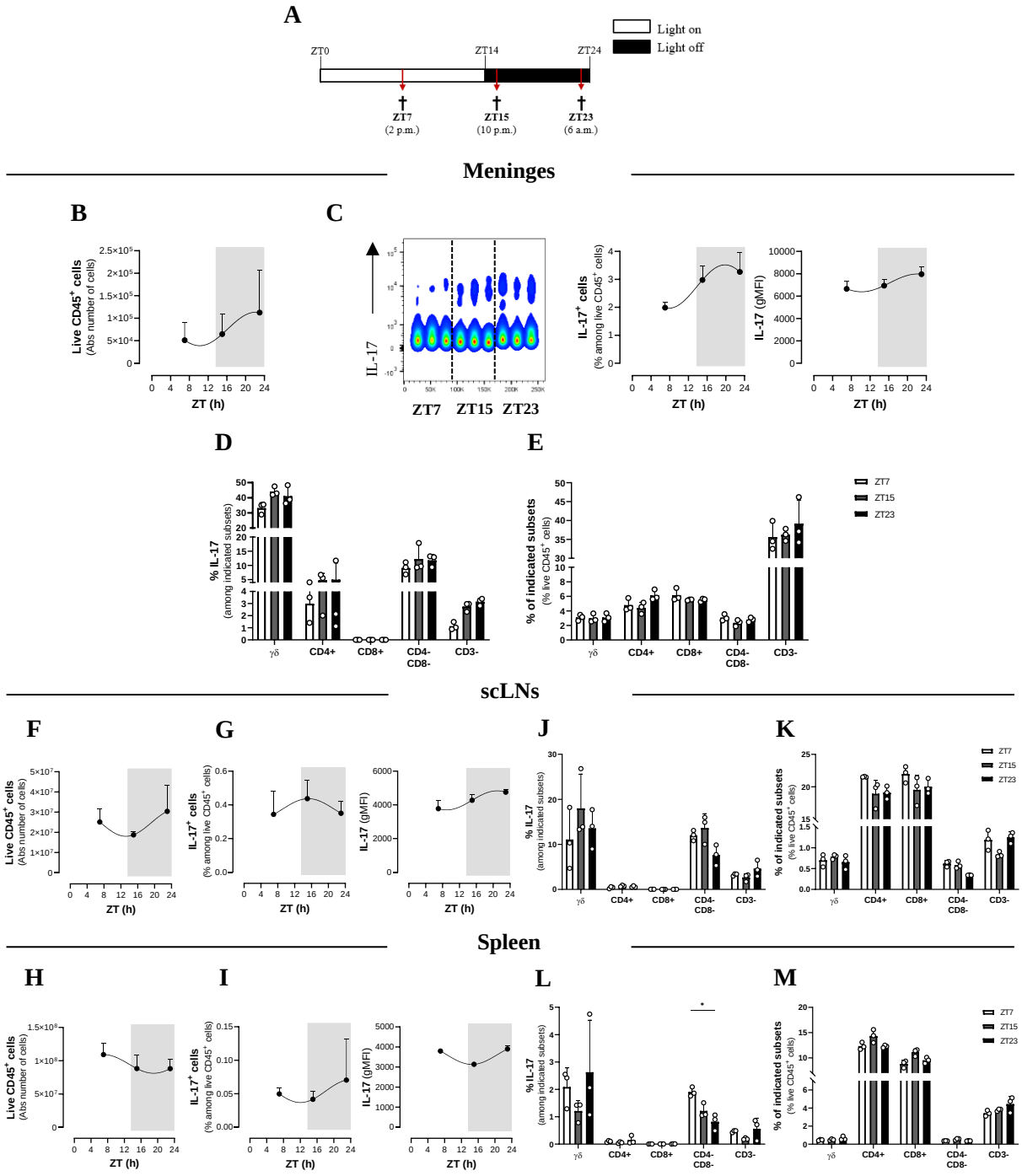

Figure 4.10 – The proportion of IL-17$^+$ immune cells exhibit a diurnal oscillatory pattern in the meninges of wild-type mice. (A) Experimental procedure: 17-wo WT male C57BL/6J mice were sacrificed at ZT7, ZT15, and ZT23 (normal light:dark cycle) within the same day. Cell suspensions were prepared from the indicated organs. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. Absolute numbers of live CD45$^+$ cells from **(B)** meningeal, **(F)** scLNs and **(H)** spleen cell suspensions were calculated. **(C)** Smoothened dot plots depicting IL-17$^+$ cells among 2000 live CD45$^+$ cells isolated from meninges at the indicated ZT (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for (**G**) scLNs and (**I**) spleen cell suspensions. **(D, J, L)** Percentages of IL-17$^+$ cells among CD3$^+$γδ T cell, CD3$^+$CD4$^+$ T cell, CD3$^+$CD8$^+$ T cell, CD3$^+$CD4$^-$ CD8$^-$ T cell and non-T cell subsets; and **(E, K, M)** percentages of these cell subsets among total live CD45$^+$ cells. Data are from 3 independent experiments, with n=3 individual measures per timepoint for meninges (obtained by pooling meningeal spaces of 3-4 mice), and n=3 mice per timepoint for the scLNs or spleen. Mean ± SD. A non-linear Cosinor regression was applied in **(B, F, G, J, K)** for the diurnal oscillation modelling. Mann-Whitney test, * P<0,05.

For this, we analysed the meninges, scLN and spleen of WT mice kept under a normal light:dark cycle by flow cytometry at 3 timepoints: ZT7 (2 p.m.), ZT15 (10 p.m.) and ZT23 (6 a.m.) (**Figure 4.10A**).
First, we found an increase in the absolute number of meningeal leukocytes during the night (**Figure 4.10B**). Interestingly, the percentage of IL-17$^+$ cells exhibited a diurnal oscillatory pattern by fluctuating ±50% in the meninges with a bathyphase (the lower part of the circadian pattern) during the day (ZT7) and an acrophase (the highest part of the circadian pattern) during the night (ZT19), while IL-17 gMFI remained stable (**Figure 4.10C**). Dissecting the individual subset contribution to IL-17 oscillation, we observed an overall increase in the percentage of IL-17$^+$ cells among the different considered subsets (exception for CD8$^+$ T cells), particularly in γδ T cells and CD3$^-$ cells (**Figure 4.10D**). Additionally, we observed an increase in CD3$^-$ cells and CD4$^+$ T cells at ZT23 (**Figure 4.10E**).
To then test a possible cell recirculation that could contribute to the diurnal oscillation of IL-17$^+$ cells in the meningeal spaces, we analysed the scLNs that were already described to participate in the continuous drainage of immune cells from the meningeal spaces[38], as well as the spleen. Consistent with previous findings, we observed an oscillation in the number of leukocytes that peaked during the night in the LNs[133,209] (**Figure 4.10F**) and a decrease in the spleen (**Figure 4.10H**). Interestingly, compared to the meningeal spaces, no fluctuation seems to occur in the percentage and gMFI of IL-17 from scLN (**Figure 4.10G**) or spleen (**Figure 4.10I**), suggesting that the meningeal oscillation is not the result of a recirculation through scLNs and spleen. Noteworthy, we found that meningeal IFN-γ$^+$ cells also exhibit a diurnal oscillation in the meninges with an acrophase at ZT23 (***Figure S7*A**), while no differences were found in the peripheral lymphoid tissues over our time course analysis (***Figure S7*B, C**).
Altogether, these data suggest a circadian control of immune cell functions in the meninges, featuring an increase of IL-17$^+$ and IFN-γ$^+$ cells during the active phase of the animal.

4.4.2 Meningeal IL-17$^+$ immune cells do not exhibit a diurnal oscillatory pattern in 3xTg-AD mouse model

Considering that neurodegenerative diseases are generally associated with circadian disturbances[161] and the meningeal IL-17 accumulation at AD onset (Brigas, H. et al., under review), we next hypothesized that early impairments in the circadian rhythms could lead to a dysregulation of IL-17-producing cell homeostasis, in the context of AD. To follow up on this hypothesis, we used the triple-transgenic mouse model of AD (3xTg-AD), a progressive model of the disease, to characterize a possible dysregulation of the diurnal oscillation of IL-17$^+$ cells in the meningeal spaces. These mice co-express three mutations associated with familial AD[202] (**Figure 4.11A**) and, in females, the onset of disease occurs at 5-6-months old (mo) with mild cognitive impairments, while the neuropathology and severe memory deficits are established at 8 mo[202,210–212]. Here, we analysed 7.5 mo 3xTg-AD females and their age- and sex-matched control at 2 different timepoints: ZT7 (2 p.m.) and ZT19 (2 a.m.) (**Figure 4.11B**), that would correspond to meningeal IL-17$^+$ cells bathyphase and acrophase, respectively, according to our previous results.
As previously described (Brigas, H. et al., *under review*), we observed a 40-50% increase in IL-17$^+$ cells in meninges (**Figure 4.11C**), scLNs (**Figure 4.11I**) and spleen (**Figure 4.11K**) of AD transgenic mice compared with the WT controls. Unexpectedly, in the meningeal spaces, we found no differences in the number of leukocytes between the two timepoints (**Figure 4.11B**), nor an oscillation of IL-17$^+$ cells in WT and AD mice (**Figure 4.11C**). Contrary to what was previously observed (**Figure 4.10**), a decrease in the IL-17 expression (**Figure 4.11C**) and IL-17$^+$ cell percentage specifically among γδ T cells was found during the night in WT and AD mice (**Figure 4.11D, F**). In the peripheral lymphoid tissues, and again contrary to was previously observed (**Figure 4.10**), we did not find an increase in the number of leukocytes in the scLNs (**Figure 4.11H**) or a decrease in the spleen (**Figure 4.11J**) during the night, both in WT and AD mice. No significant changes in the percentage of IL-17$^+$ cells in the scLNs and spleen between the two timepoints in WT and AD mice were noticed (**Figure 4.11I, K**).

Of note, meningeal IFN-γ^+ cells also exhibited no diurnal oscillation, while a decrease in IFN-γ^+ expression in meninges was observed during the night in both WT and AD mice (**Figure S8A**). The same results were found in the spleen of WT and AD mice during the night (**Figure S8C**), while both parameters remained stable in the scLNs (**Figure S8B**).

The absence of differences and inconsistency compared with the previously results obtained impaired us to draw any particular conclusions at this point.

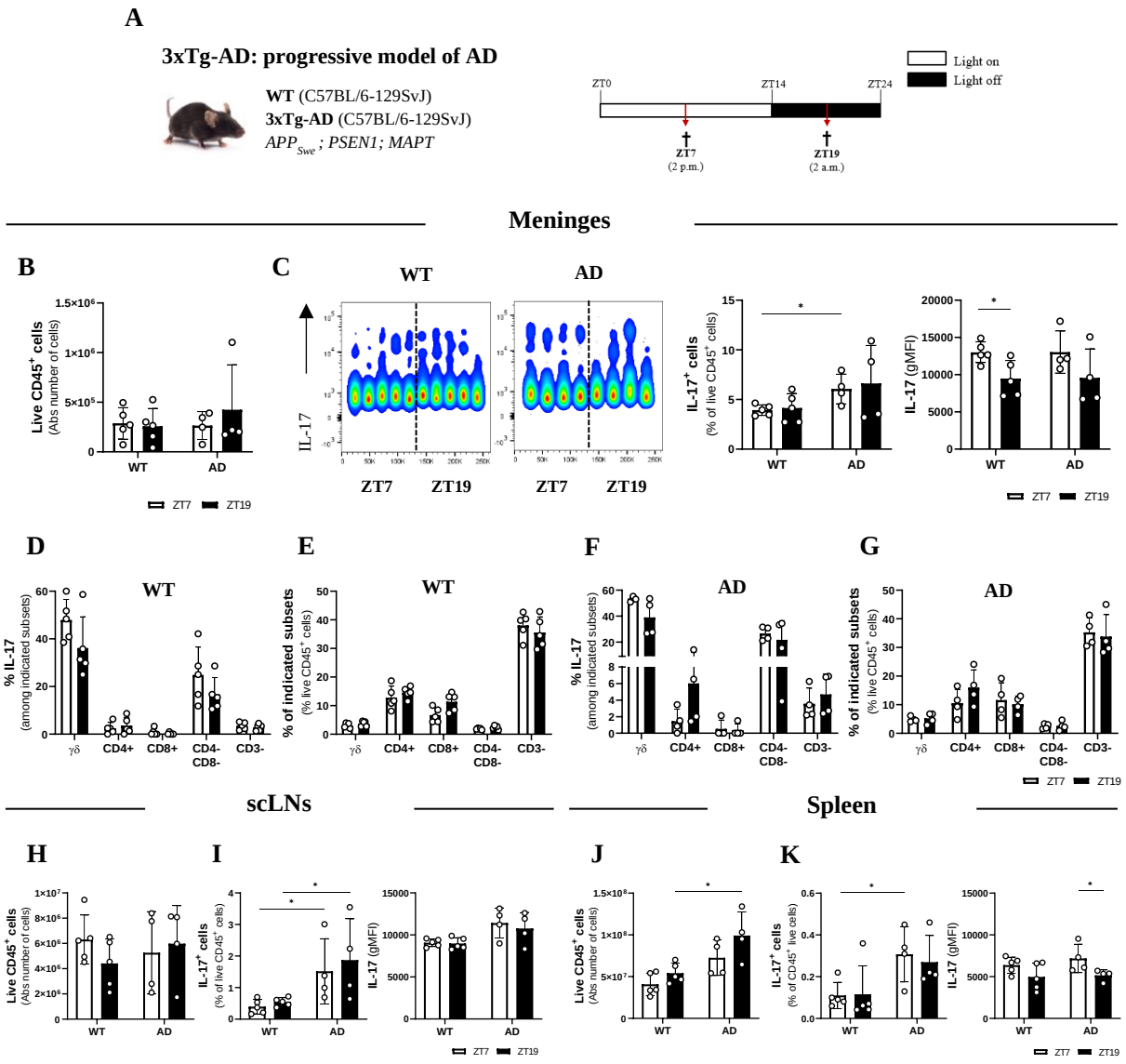

Figure 4.11 – The proportion of IL-17$^+$ immune cells in the meninges of a 3xTg-AD mice do not display a diurnal oscillatory pattern. (A) Experimental procedure: 7-month-old WT and 3xTg-AD female C57BL/6J-129SvJ mice were sacrificed at ZT7 and ZT19 (normal light:dark cycle). Cell suspensions were prepared from the indicated organs. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. Absolute numbers of live CD45$^+$ cells from **(B)** meningeal, **(H)** scLNs and **(J)** spleen cell suspensions were calculated. **(C)** Smoothened dot plots depicting IL-17$^+$ cells among 500 live CD45$^+$ cells isolated from meninges at the indicated ZT (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the (**I**) scLNs and (**K**) spleen cell suspensions. Percentages of IL-17$^+$ cells among CD3$^+$γδ T cell, CD3$^+$CD4$^+$ T cell, CD3$^+$CD8$^+$ T cell, CD3$^+$CD4$^-$ CD8$^-$ T cell and non-T cell subsets in meninges of **(D)** WT and **(F)** 3xTg-AD mice. Percentages of indicated cell subsets among meningeal live CD45$^+$ cells in **(E)** WT and **(G)** 3xTg-AD mice. Data are from 1 independent experiment, with n=4-5 mice per group and per timepoint. Mean ± SD. Mann-Whitney test, * P<0,05.

4.5 Analysis of the impact of sleep on IL-17-producing immune cells

4.5.1 Anesthetized mice exhibit a decrease in meningeal IL-17$^+$ immune cells

Besides circadian disturbances, AD patients also suffer sleep perturbations[213]. Thus, given our inconclusive observations gathered from **Figure 4.11**, we next hypothesized that sleep could also regulate the homeostasis of meninges IL-17 immune cell producers.

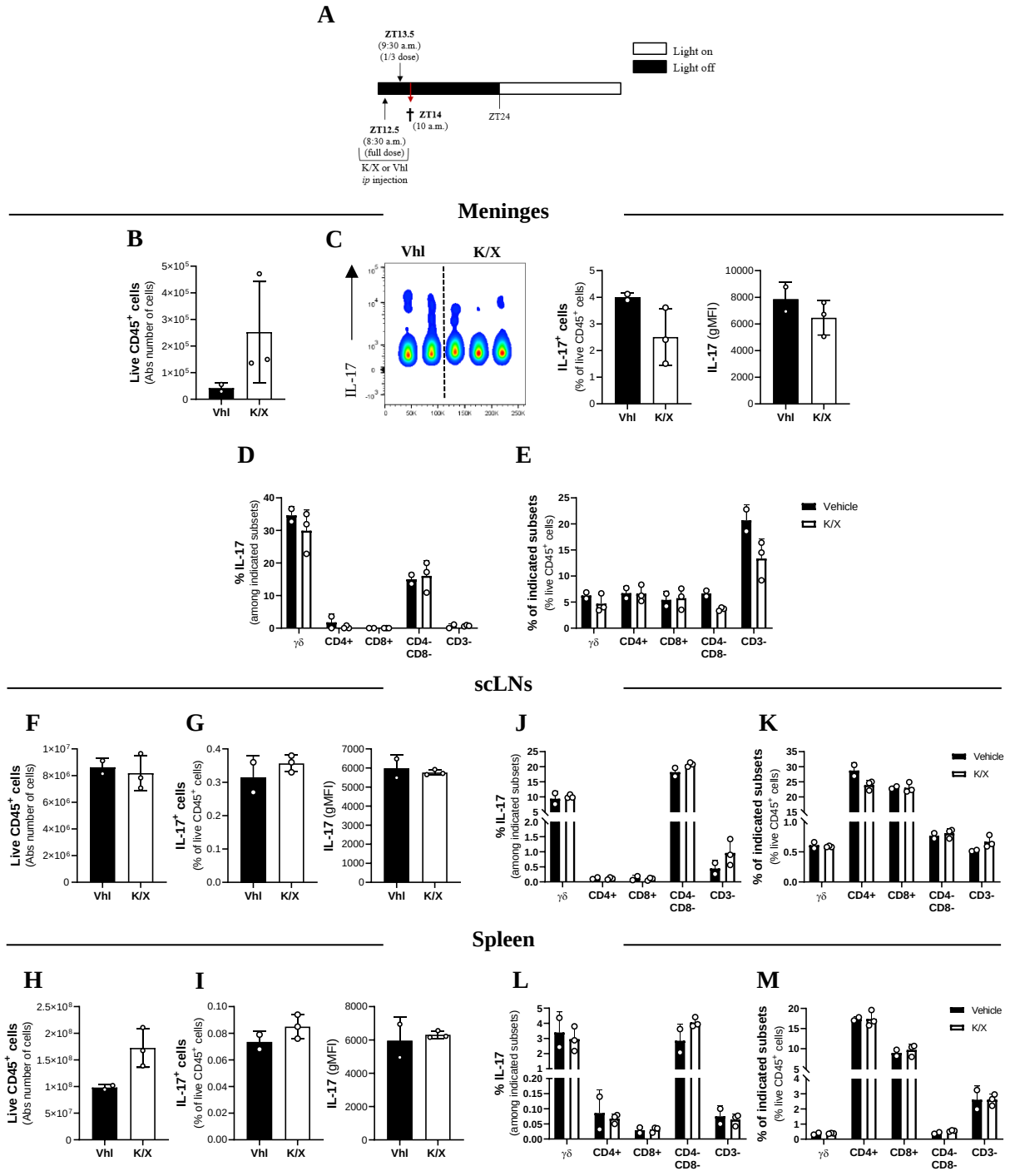

Figure 4.12 – Meningeal IL-17$^+$ immune cells proportion is reduced after a ketamine/xylazine-induced anesthesia. (A) Experimental procedure: 9-wo WT male C57BL6/J mice received an ip injection of ketamine (100 mg/kg)/ xylazine (10 mg/kg) (K/X) or vehicle saline solution at ZT12,5 (8.30 a.m.) (inverted light-dark cycle). After one hour, the anesthesia was prolonged by the injection of 1/3 of the initially administered dose of K/X. Mice were analysed at ZT14 (10 a.m.), and cell suspensions were prepared from the indicated organs. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. Absolute numbers of live CD45$^+$ cells from **(B)** meningeal, **(F)** scLNs and **(H)** spleen cell suspensions were

calculated. **(C)** Smoothened dot plots depicting IL-17$^+$ cells among 1000 live CD45$^+$ cells isolated from meninges of the awake (vehicle-treated) or anesthetized (K/X-treated) animals (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the scLNs in (**G**) and spleen cell suspensions **(I)**. **(D, J, L)** Percentages of IL-17$^+$ cells among CD3$^+$γδ T cell, CD3$^+$CD4$^+$ T cell, CD3$^+$CD8$^+$ T cell, CD3$^+$CD4$^-$ CD8$^-$ T cell and non-T cell subsets; **(E, K, M)** and percentages of these cell subsets among total live CD45$^+$ cells. Data are from 1 independent experiment, n=2-3 per group. Mean ± SD.

To test this hypothesis, we injected WT mice with an anesthetic solution of Ketamine/Xylazine in the beginning of the dark phase that reproduces the main features of sleep[185]. In these conditions, the control mice were at their awake phase (since mice are nocturnal) and were injected with a saline solution (vehicle). The mice were sacrificed one hour and a half later (**Figure 4.12A**).

Unexpectedly, the number of meningeal leukocytes was strongly increased after only one and a half hour of anesthesia, revealing a major influence of the sleep state in meningeal immunity (**Figure 4.12B**). Interestingly, a 2-fold reduction in the percentage of IL-17$^+$ cells and IL-17 expression, compared with vehicle-injected mice was observed (**Figure 4.12C**). Accordingly, a ±30% decrease in the overall percentages of γδ T cells, CD4$^-$ CD8$^-$ and CD3$^-$ among total leukocytes was observed in anesthetized mice (**Figure 4.12E**), which might account for the observed reduction in IL-17$^+$ cells, given their important contributing for IL-17 production (**Figure 4.12D**). Although no changes in the number of leukocytes were observed in the scLNs (**Figure 4.12F**), an increase was observed in the spleen (**Figure 4.12H**). Of note, no other changes were observed regarding the percentage of IL-17$^+$ cells, IL-17 expression, or its cellular contributors in the scLNs (**Figure 4.12G, J**) and spleen (**Figure 4.12I, L**), between vehicle and anesthetized mice. As observed with IL-17$^+$ cells, a decrease in IFN-γ$^+$ cells took place specifically in the meningeal spaces of anesthetized mice (**Figure S9A**), with no significant changes in the periphery (**Figure S9B, C**).

These findings suggest that sleep is a modulator of the cellularity and the cytokine content in the meningeal spaces and might represent another important mechanism contributing to brain homeostasis.

5. Discussion

IL-17 is a cytokine with critical pleiotropic implications in pathophysiology, namely in the meninges, being pro-cognitive at steady state[53] and anti-cognitive in the context of neurodegeneration (Brigas, H. et al., *under review*). Based on this intriguing background, we decided to drive an exploratory study to unravel possible homeostatic mechanisms implicated in the regulation of meningeal IL-17.

To do so, we screened putative candidates from the transcriptomic landscape of γδ17 T cells (Tiago Amado, *unpublished data*) that highlighted hormones and neuromodulators, such as glucocorticoids and catecholamines, and circadian rhythm cues, results further supported by data from the literature[204]. Thus, we postulate that these biological processes would be crucial for γδ17 T cell homeostasis. This data should be validated resorting to reverse transcription polymerase chain reaction (RT-PCR) techniques.

Due to technical limitations, it was not possible to sort enough γδ17 T cells from the meninges to run functional assays. This notwithstanding, and as a proof of concept, we could isolate γδ17 T cells from LNs and spleen to perform *in vitro* cell cultures and analyze the impact of glucocorticoids on these cells. We observed that glucocorticoid stimulation increased γδ17 T cells in a dose-dependent manner and decreased IFN-γ$^+$ cells at high concentrations, specifically by promoting γδ27$^-$ T cells survival, proliferation and the overexpression of IL-7R. Interestingly, IL-7 signalling is particularly important to the activation of γδ cells competent to produce IL-17 through a STAT3 mediated process[214]. However, considering that γδ27$^-$ increased proliferation occurred in culture settings were no IL-7 was present, a glucocorticoid-mediated expansion of γδ17 T cells by an IL-7R-independent mechanism also takes place. Additionally, IL-7 signaling does not account for the enhanced survival of γδ17 T cells, as previously demonstrated[214]. Therefore, the glucocorticoid regulation of γδ17 T cells is not exclusively dependent of IL-7R overexpression and the molecular response to GR stimulation still needs further investigation. Of note, the modulation of IL-17 *versus* IFNγ balance by glucocorticoids towards IL-17

production might also result from an increase in IFN-γ$^+$ cell apoptosis or a potential effect on γδ T cell differentiation. Nevertheless, in an *in vitro* culture setting, γδ17 T cells are highly responsive to glucocorticoid stimulation, as firstly predicted by the RNA-seq data.

Alongside the fundamental role of glucocorticoids in stress responses, a daily peak of glucocorticoids is observed in the beginning the active phase with important immunomodulatory functions[142]. Considering our *in vitro* data, this daily glucocorticoid peak could induce a response of meningeal IL-17 immune cell producers to prepare the organism for daily tasks requiring short-term working memory. On the other hand, acute stress response is associated with enhanced synaptic plasticity and facilitates spatial learning[149], two cognitive properties known to be modulated by IL-17$^+$ cells in the meningeal spaces[53].

An association between stress and IL-17 cytokine response signature was already proposed by a transcriptomic analysis of cortices from stressed animals[59]. To observe if glucocorticoids could influence IL-17 immune cell producer homeostasis *in vivo*, we performed a flow cytometry analysis of meninges, LNs and spleen from mice injected with dexamethasone 4h before. In these experimental settings, dexamethasone stimulation had no impact on meningeal IL-17$^+$ nor IFN-γ$^+$ cells, while impacting the peripheral IL-17$^+$ cell pool. Importantly, the direct induction of IL-7R on T cells by glucocorticoids here observed is responsible for the diurnal regulation of CXCR4 expression, a receptor critically involved in the redistribution of immune cells between LNs, spleen and blood[142]. Therefore, a possible effect of glucocorticoids on IL-17 immune cell producer recirculation between the scLNs and spleen might underly the differences observed in IL-17$^+$ cells. However, given our *in vitro* results and *in vivo* extended analysis in scLNs, a glucocorticoid-mediated increase in IL-17$^+$ cells might also result from an increased expansion, survival or differentiation of γδ17 T cells. Simultaneously, a massive apoptosis of splenic cells could have resulted in the observed IL-17$^+$ cells drop.

The unexpected absence of an effect of dexamethasone on the meningeal subsets might have several explanations. On one hand, 4h might not have been sufficient to observe an effect of glucocorticoids in the meningeal spaces. It was previously shown that a peak of IL-17 is observed 4h after acute stress induction, which induces a corticosteroid increase response within 15 minutes[215]. However, these responses were observed in the blood, meaning that the tissular responses might be delayed. Also, since the injection was intraperitoneal, the availability of the drug might be higher for the spleen and, in less extend, for the LNs, than for meninges. Additionally, IL-17 production in lymphoid tissues is mostly accounted by the Vγ4$^+$ γδ T cell subset[216], while in the meninges IL-17 is provided by Vγ6$^+$ γδ T cells. Therefore, and since our RNA-seq analysis was performed in γδ T cells isolated from peripheral LNs, this subset might be more prone to respond to glucocorticoids. To address these issues, further studies should include a later monitoring of meningeal IL-17$^+$ cells after dexamethasone injection and intracerebroventricular injections of dexamethasone to question a possible bioavailability issue. Another important pharmacological approach would be the injection of mifepristone, an antagonist of GRs, to evaluate the impact of the endogenous glucocorticoid secretion. Finally, to assess a glucocorticoid specific effect on IL-17 immune cell producers and its implications for brain cognitive functions, mice with a conditional deletion of *Nr3c1*-encoded GRs on *RORγt* expressing cells should be used. Additionally, acute stress induction studies resorting to mice restrainment, social isolation, cage switch or retro-orbital bleeding, amongst others[215] could help to clarify a possible effect of endogenous stress on the homeostasis of IL-17$^+$ cells in the meninges.

Another important part of the stress response that was not explored in this study is the SNS regulation of immune responses. The high expression of *Adrb2* by γδ17 T cells compared to their IFN-γ counterpart and DN cells (Tiago Amado, *unpublished data*), suggests another possible homeostatic mechanism exerting a modulation of meningeal IL-17$^+$ cells. Recent studies have shown that NK cells functional properties can be modulated by adrenaline and noradrenaline binding to β_2ARs expressed by these cells. Interestingly, while in early innate responses adrenergic signals negatively regulated IFN-γ responses and increased susceptibility to mouse cytomegalovirus (MCMV) infection in a cell-extrinsic manner[217],

adrenergic signalling was necessary to elicit NK adaptive responses in a cell-intrinsic manner and fundamental to control MCMV replication[218]. This differential control of immune responses through non-exclusive mechanisms emphasizes the complexity of stress responses on immune cell functions.

To further explore other possible mechanisms regulating the homeostasis of meningeal IL-17$^+$ cells, we investigated a potential diurnal control of this subset. We observed that IL-17$^+$ cells in the meningeal spaces from WT mice subjected to a normal light:dark cycle exhibited a diurnal oscillation that peaked during the active phase of the animal. Interestingly, considering the important role of IL-17 in short-term memory spatial working memory[53], a higher availability of the cytokine in the meningeal spaces during the active phase of the animal could be considered an evolutionary advantage, preparing the organism to perform cognitive tasks. Accordingly, in humans, a cognitive circadian control is observed with cognitively complex tasks requiring short-term memory peaking in the middle of the active phase[173].

Interestingly, IFN-γ^+ cells also exhibited a diurnal oscillation exclusively in the meninges that peaked during the active phase. This cytokine exerts a direct activation of the inhibitory prefrontal cortex (PFC) neurons supporting appropriate social behaviour, that is impaired in the absence of T cells or T cell-derived IFN-γ[59]. Therefore, a circadian control of IFN-γ^+ cells in the meningeal spaces would also be evolutionary advantageous to the organism to promote social interaction during their active phase.

Importantly, circadian cues have shown to control several aspects of IL-17 cell producers physiology in the gut, namely their trafficking[142], homeostasis in tissue and functions[168,169]. Mechanistically, a circadian regulation of T_H17 differentiation was shown to be exerted by NFIL3 direct repression of *Rorγt* transcription. REV-ERBα, whose expression is regulated by BMAL1/CLOCK, can directly repress *Nfil3* transcription, thereby promoting *Rorγt* transcription and increasing T_H17 frequencies[170]. We observed that meningeal IL-17$^+$ immune cells increase their expression of IL-17 during the night, suggesting that a diurnal control of *Rorγt* transcription might led to an *in situ* differentiation also in the meninges. Accordingly, no changes in IL-17$^+$ cells were observed in the scLNs or the spleen, indicating that this increase may not be due to a recirculation of IL-17-immune cell producers over 24h.

Considering the hormonal and neuroendocrine circadian control, the peak of glucocorticoids and catecholamines in the beginning of the awake phase might contribute for the diurnal oscillation here observed. From what was explored in this study, glucocorticoids can modulate two important IL-17 producers, γδ17 T cells and CD4$^+$ T cells. Even though we could not observe an effect *in vivo*, an endogenous glucocorticoid peak might influence the meningeal spaces more than 4h later and contribute to the IL-17$^+$ cell increase observed during the active phase of the animal, by affecting their proliferation, survival and differentiation. The injection of GRs antagonists would help to tackle this question.

Additionally, adrenergic cues are also associated with a rhythmic recruitment of immune cells to tissues that is achieved through a circadian expression of endothelial cell adhesion molecules and chemokines on non-hematopoietic cells and a differential responsiveness of chemoattractant receptors[132]. Important biological consequences of this immune control include overall survival in models of septic shock, sickle cell vaso-occlusion and bone marrow transplantation[209], as well as enhanced humoral immune responses during the active phase[133]. Although we found no evidences that IL-17$^+$ cell oscillation were the result of a recirculation, these cues are also involved in the modulation of the functional properties of NK cells[217,218], ILC2s[219], CD4$^+$ T cells[220], and other innate and adaptive immune cell responses. Consequently, a possible adrenergic control of IL-17 immune cell producers must be considered in future studies. For this, the use of pharmacological agonists of β_2ARs, such as clenbuterol, chemical denervation using 6-hydroxydopamine (6-OHDA) or adrb2$^{-/-}$ mice should be considered.

Of note, the data on diurnal oscillations result from only one independent experiment per timepoint, thus the observed pattern would need to be strengthened with more samples. Also, for a more accurate control of diurnal oscillations, a 12h:12h light:dark system should be used[221], instead of the 10h:14h currently settled in our animal house. It would also be important to assess IL-17 mRNA expression levels by RT-

PCR and perform IL-17 quantification studies in the meninges by enzyme-linked immunosorbent assay (ELISA) at different timepoints, since the methodology used in this study requires 3h-4h of cytokine stimulation in culture *ex vivo* during which cells continue their progression through the circadian cycle and might reach points in the cycle were the differences in cytokine expression are more attenuated[169]. Furthermore, it is important to mention that the involvement for the circadian rhythms can only be established by direct manipulation of intrinsic clock signals such as an ablation of the master circadian activator *Arntl*[222], extrinsic clock signals such as SCN ablation[223] or light:dark cues manipulation[224]. Such manipulations, specifically a conditional deletion of *Arntl* on *Rorγt* expressing cells, would allow us to prove a circadian control and further assess possible behaviour deficits induced by a putative circadian dysregulation of IL-17 immune cell producers. Thus, from a fundamental point of view, since we could not access to these tools, our study rather reveals diurnal – not circadian - oscillatory patterns. Given the important reports relating circadian and sleep perturbations with neurodegenerative diseases and the fact that increased levels of meningeal IL-17 contribute to the onset of AD (Brigas, H. et al., *under review*), we postulated that disturbances in diurnal oscillations could trigger the accumulation of this cytokine, thus contributing to disease progression. To tackle this question, we used a 3xTg-AD mice model that progressively reproduces the cognitive deficits and AD hallmarks[225]. These mice display abnormalities in their circadian rhythmicity, evidenced by a fragmented sleep-wake pattern with decreased activity levels during their active phase, increased activity during their resting phase[226] and alteration of circadian clock transcription patterns in the SCN[227]. Unexpectedly, we found no oscillation of IL-17$^+$ cells in the meninges of WT control mice in the two timepoints analysed, which were chosen based on the prediction that IL-17$^+$ cells would have their bathyphase in the middle of the resting phase and acrophase in the middle of the active phase, as previously observed. We also observed that IL-17 expression is reduced in the meninges and spleen during the night and no changes were found in the scLNs of both AD and their age- and sex-matched WT controls. The same phenotype was found for IFN-γ production. Several factors might have contributed to this phenomenon. Firstly, the genetic background of the mice used in both diurnal oscillatory experiments was different. To assess the diurnal oscillatory patterns in physiological contexts we used C57BL/6J mice, whereas in pathological contexts the AD mice and their recommended control have the C57BL/6-129SvJ background. Secondly, the sex of the mice was also different. In the first context we used males, while for the pathological implications the mice were females (according to Brigas, H. et al., *under review*). The circadian system response to light, its rhythm and amplitude has shown to be regulated by hormonal activation and therefore a sex dimorphism might explain the differential diurnal oscillation in IL-17$^+$ cells[228]. Furthermore, a differential effect of sex hormones in the innate and adaptive immune systems is well described[229]. For instance, estrogen impairments on the negative selection of B cells promotes the maturation of auto-reactive B cells[230], enhancing female's ability to produce antibodies[231] and mount a more effective response to pathogens, but also increasing the predisposition autoimmunity disorders[232]. On the other hand, testosterone has immunosuppressive effects that translate in the downregulation of NK cell responses[233] and reduced synthesis of TNFα and nitric oxide (NO) by DCs[234], while increasing the anti-inflammatory responses through IL-10 production[235]. Thirdly, the age of the mice was very different. 17 wo mice were used in physiological conditions, while the AD mice and their controls had 7,5 mo. Interestingly, both healthy and pathological brain ageing are associated with cognitive decline associated with a functional weakening or disruption of the circadian patterns[172] and a reduction of overall activity[236]. Therefore, one can postulate that circadian disruptions that occur during the natural ageing process might contribute to the development of neurodegenerative diseases. In fact, the C57BL/6-129SvJ mice with 7,5 mo already show accumulated levels of IL-17$^+$ cells in the meningeal spaces compared with the C57BL/6J indicative of a possible circadian disruption, although an impact of the genetic background and sex cannot be dismissed. Therefore, it would be interesting to analyse younger mice at the onset of cognitive decline (5-6 mo) or earlier to understand if the age difference would

translate oscillatory changes. Finally, due to technical limitations, only two timepoints were analysed. We might be observing two points that do not have differences, thus dismissing a potential oscillatory pattern. A much extensive analysis with middle timepoints is required to draw further conclusions.

As the transcriptomic analysis of cortices from sleep-deprived animals highlighted IL-17 regulated genes[59], we next questioned whether sleep could influence IL-17 immune cell producers in the meninges. Resorting to an anesthetic protocol shown to reproduce the main features of sleep[185], we observed an increase in the number of leukocytes present in meninges. Since meningeal lymphatics are a clearing route for CSF content[36,37], it is possible that during sleep meningeal lymphatic vessels act as a reservoir for different molecules and immune cells from the subarachnoid space and CSF[41]. Furthermore, we observed a decrease in the percentage of IL-17$^+$ cells and IL-17 expression among total leukocytes in the anesthetized mice. Since the percentage of IFN-γ^+ cells also decreased in the anesthetized mice, the effect of sleep on meninges might not be restricted to IL-17 immune cell producers. In turn, a global reduction in cytokine producers might be the consequence of the leukocyte influx observed during the night that creates a dilution effect. Interestingly, sleep restriction has been associated with increased levels of IL-1β, IL-6 and IL-17 in the blood[237] and multiple pathways can mediate the relationship between sleep and immune function. Growth hormones and melatonin secreted during sleep promote the secretion of IL-1[238], while ACTH and cortisol release, along with the sympathetic tone, will affect the production of T_H1 and T_H2 cytokines[239,240]. However, anesthetized mice display a decrease in the percentage of IL-17$^+$ cells specifically and exclusively in meninges, and not in the peripheral lymphoid organs, pointing at a local (rather than systemic) effect. Accordingly, the glymphatic flux increases during sleep[185] mediated by a circadian control[196] and a dysfunction of both pathways contributes to the pathophysiology of various CNS diseases, including AD[241]. Additionally, sleep disturbances increase IL-6 levels and induce microglia activation specifically in the mouse hippocampus. These effects result in a selective impairment of hippocampus-dependent learning and memory through neuroinflammation in the hippocampus[242]. In this context, and accordingly to our results, sleep disturbances may trigger an accumulation of meningeal IL-17 levels that further contributes to the onset of cognitive deficits and impaired synaptic plasticity observed in AD mice.

Of note, a possible effect of the anesthesia itself on IL-17 immune cell producers cannot be dismissed and might account for the differences observed. To control this variant, naturally sleeping mice should be used in further studies. Additionally, sleep deprivation models should also be included in the analysis. Last but not least, it is important to highlight that sleep and circadian rhythm are difficult to dissociated, thus we cannot exclude a combined impact of both parameters on the homeostasis of IL-17$^+$ cells in the meninges. By instance, the adrenergic tone is known to be regulated by circadian cues, rising during the active phase of the animal and being reduced during sleep[243]. Additionally, the administration of ketamine/xylazine reduces norepinephrine levels, demonstrating a circadian independent mechanism[185]. Therefore, if adrenergic inputs would contribute to the increase in meningeal IL-17 during the active phase of the animal, a combined role of the circadian rhythms and sleep would be responsible for such outcome. Further experiments should envision this hypothesis and employ the methodologies referred above.

Taken together, our results suggest that endogenous and environmental cues (stress, circadian rhythms and sleep) are valuable candidates to control the homeostasis of IL-17-producing immune cells in the meninges and open new lines of fundamental investigation. Importantly, all these factors are implicated in higher mental processes and neurodegenerative disorders. The modulation of these cues might give further insight into the cellular and molecular mechanisms that underlie learning and memory, with a biomedical potential for neurodegenerative diseases.

To conclude, this exploratory study is an important step towards the characterization of meningeal immune subset biology and laid the groundwork to advance our understanding of the mechanisms underlying modern neuroimmunology.

6. References

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Appendices

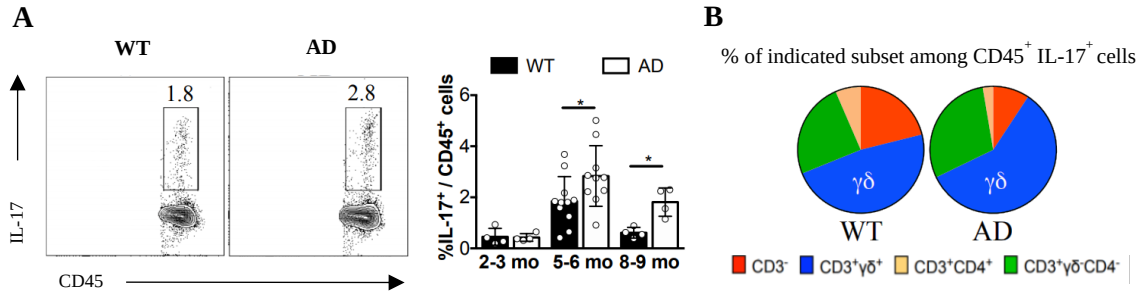

Figure S1 – Accumulation of IL-17$^+$ cells in the meninges at the onset of cognitive deficits of 3xTg-AD mice. Meningeal cell suspensions were prepared from 2-3, 5-6 and 8-9-month-old WT and 3xTg-AD female mice. Cells were analysed for the expression of surface (CD45, CD3, TCRδ and CD4) and intracellular (IL-17) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Representative contour plots displaying IL-17$^+$ cells among live CD45$^+$ cells in 5-6 month-old WT and 3xTg-AD mice (left), and their quantification through the disease progression (right). **(B)** Pie charts indicating the proportion of each cell subsets to the IL-17$^+$ immune cell population in the indicated organs. Pie charts display the mean values of data from 1-3 independent experiments with n=4-11 individual measures obtained by pooling meningeal spaces of 2 mice, per genotype and per age. Mean ± SD. Mann-Whitney test, * P<0.05. [Extracted from Brigas, H. et al., *under review*]

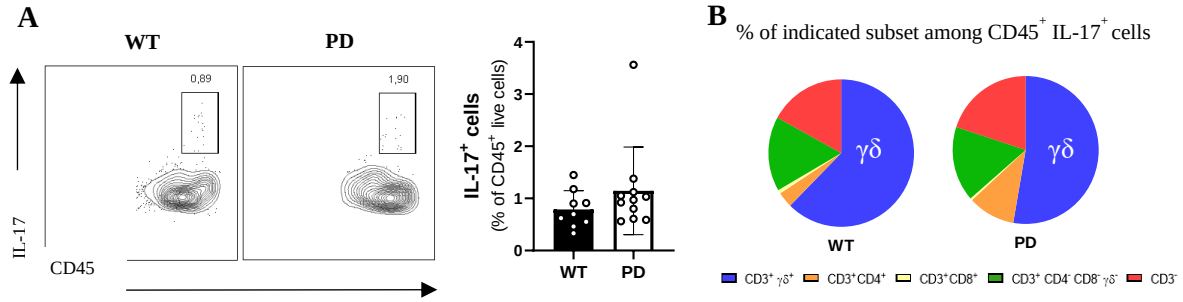

Figure S2 – Putative accumulation of IL-17$^+$ cells in the meninges of Thy1-αSyn PD mice. Meningeal cell suspensions were prepared from 6-month-old WT and Thy1-αSyn male mice. Cells were analysed for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IL-17) markers. **(A)** Representative contour plots displaying IL-17$^+$ cells among live CD45$^+$ cells in WT and PD mice (left), and their quantification (right). **(B)** Pie charts indicating the proportion of each cell subsets to the IL-17$^+$ immune cell population in the indicated organs. Live cells were gated using LiveDead Fixable Viability Dye. Data are from 4 independent experiments, n=9 WT mice and n=11 Thy1-αSyn mice. Mean ± SD.

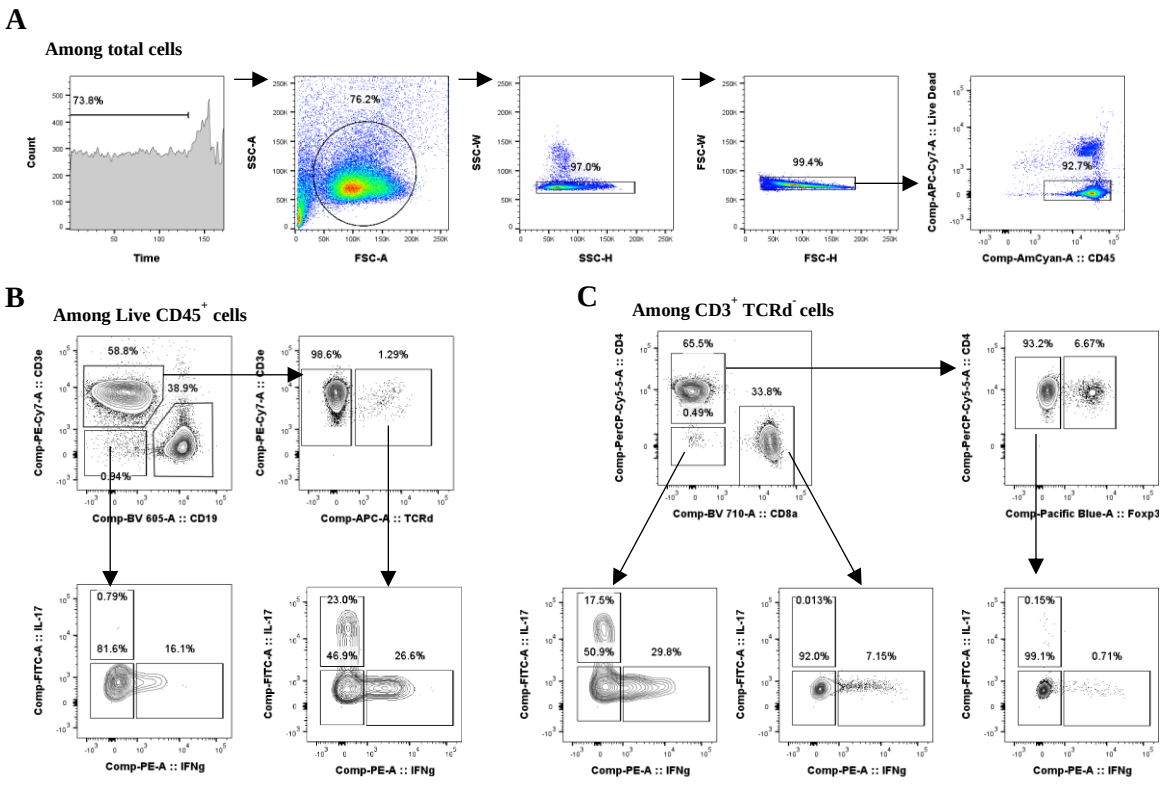

Figure S3 – Gating strategy used for the identification of immune cell subsets and their respective IL-17 and IFN-γ production by flow cytometry. (A) The identification live leukocytes was performed through the application of a time gating among total cells, followed by leukocyte selection, doublets exclusion, and the selection of $CD45^+$ among the LiveDead negative subset **(B)** Among live CD45+ cells, $CD3^-$ $CD19^-$ and $CD3^+$ $CD19^-$ immune cell subsets were identified. Within $CD3^+$ $CD19^-$, CD3+ $TCR\delta^+$ cells were identified. IL-17 and IFN-γ production was assessed within $CD3^-$ $CD19^-$ cells and CD3+ $TCR\delta^+$ cells. **(C)** Among live CD3+ $TCR\delta^+$ cells, $CD3^+$ $CD4^+$, $CD3^+$ $CD8^+$, and $CD3^+$ $CD4^-$ $CD8^-$ immune cell subsets were identified. IL-17 and IFN-γ production was assessed within the identified subsets.

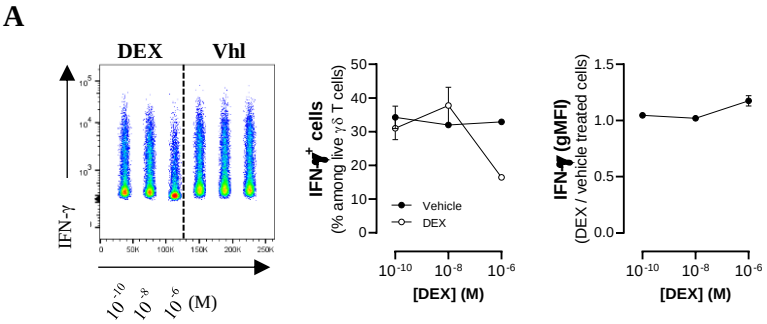

Figure S4 – *In vitro* dexamethasone treatment of γδ T cells inhibits the survival of $IFN\gamma^+$ cells at high dose. Total γδ T cells were sorted by FACS from the peripheral lymph nodes (superficial cervical, axillary, brachial and inguinal LN) and the spleen of WT C57BL/6J mice. Cells were cultured in presence of anti-CD3e antibody (3 µg/ml, plate bound), IL-1β (10 ng/ml), IL-23 (10 ng/ml) and IL-7 (40 ng/ml); and treated with a dexamethasone (DEX) or the vehicle solution. After 72 hours of culture with different concentrations of dexamethasone (10^{-10} M, 10^{-8} M and 10^{-6} M), cells were analysed by flow cytometry for the expression of surface (CD3, TCRδ) and intracellular (IFNγ) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Representative dot plots of two independent experiments displaying the $IFN\gamma^+$ cells among 10000 live γδ T cells from indicated samples (left), their quantification (middle), and the ratio of the geomean fluorescence intensity (gMFI) of dexamethasone- or vehicle-treated γδ T cell (right). Mean ± SD.

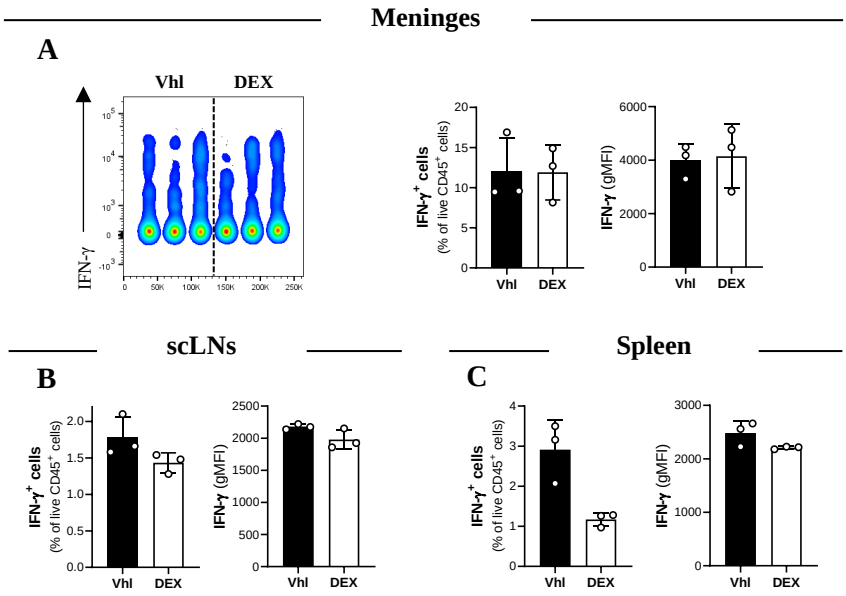

Figure S5 – *In vivo* dexamethasone administration does not alter meningeal IFN-γ⁺ immune cells homeostasis. Cell suspensions were prepared from the indicated organs of dexamethasone- or vehicle-treated C57BL/6J WT mice, and analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IFNγ) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Smoothened dot plots depicting IFNγ⁺ cells among 900 live CD45⁺ cells isolated from meninges of dexamethasone or vehicle-treated animals (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the scLNs in **(B)** and spleen cell suspensions **(C)**. Data are from 1 independent experiment, n=3 mice per group. Mean ± SD.

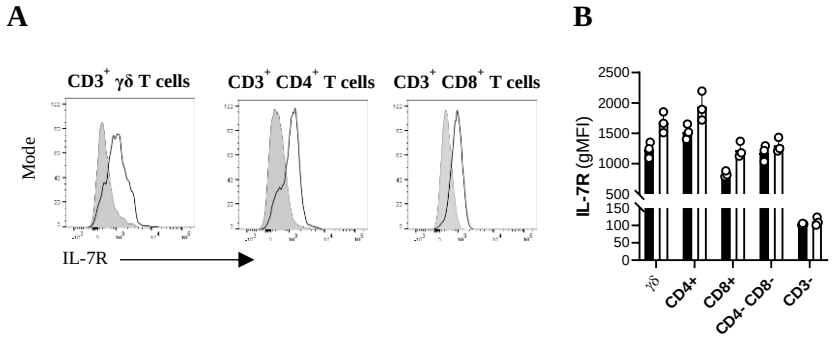

Figure S6 – *In vivo* dexamethasone administration drives the overexpression of IL-7 receptor by γδ, CD4⁺ and CD8⁺ T cells. Cell suspensions were prepared from superficial cervical lymph nodes (scLNs) of dexamethasone- or vehicle-treated C57BL/6J WT mice, and analysed by flow cytometry for the surface (CD3, TCRδ, CD4, CD8, IL-7R) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Representative histograms displaying the IL-7R expression by the indicated cell subsets isolated from dexamethasone- (empty dark) or vehicle- (grey-filled) treated animals. **(B)** Geomean fluorescence intensity (gMFI) for the associated IL-7R signal of CD3⁺γδ T cell, CD3⁺CD4⁺ T cell, CD3⁺CD8⁺ T cell, CD3⁺CD4⁻ CD8⁻ T cell and non-T cell subsets. Data are from one independent experiment. Mean ± SD.

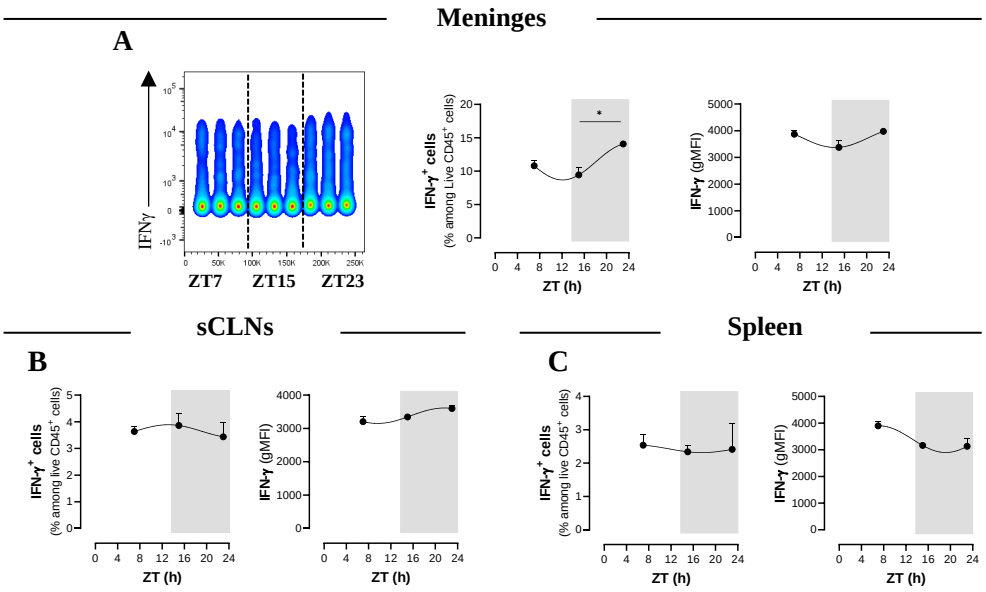

Figure S7 – The proportion of IFN-γ$^+$ immune cells exhibit a diurnal oscillatory pattern in the meninges of wild type mice. Cell suspensions were prepared from the indicated organs of WT C57BL/6J mice sacrificed at ZT7, ZT15, and ZT23 (normal light:dark cycle). Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IFNγ) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Smoothened dot plots depicting IFNγ$^+$ cells among 2000 live CD45$^+$ cells isolated from meninges at the indicated ZT (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the **(B)** scLNs and **(C)** spleen cell suspensions. Data are from 3 independent experiments, with n=3 individual measures per timepoint for meninges (obtained by pooling meningeal spaces of 3-4 mice), and n=3 mice per timepoint for scLNs or spleen. Mean ± SD. A non-linear Cosinor regression was applied in for the diurnal oscillation modeling. Mann-Whitney test, * P<0,05.

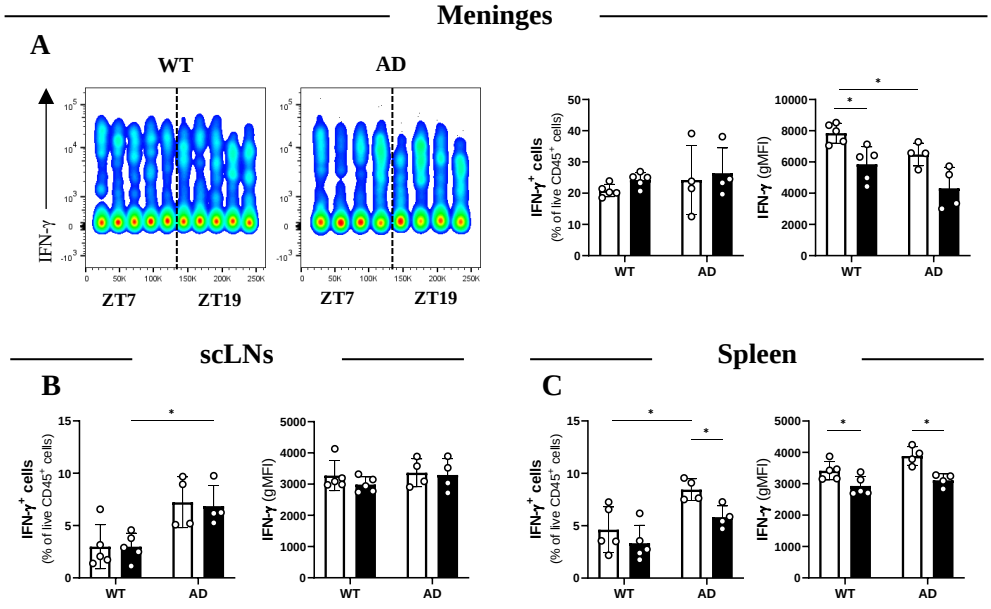

Figure S8 – The proportion of IFNγ$^+$ immune cells in the meninges of a 3xTg-AD mice do not display a diurnal oscillatory pattern. Cell suspensions were prepared from the indicated organs of 7-month-old WT and 3xTg-AD C57BL/6J-129SvJ female mice sacrificed at ZT7 and ZT19 (normal light:dark cycle). Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IFNγ) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Smoothened dot plots depicting IFNγ$^+$ cells among 500 live CD45$^+$ cells isolated from meninges at the indicated ZT (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the scLNs in **(B)** and spleen cell suspensions **(C)**. Data are from 1 independent experiment, with n=4-5 mice per group and per timepoint. Mean ± SD. Mann-Whitney test, * P<0,05.

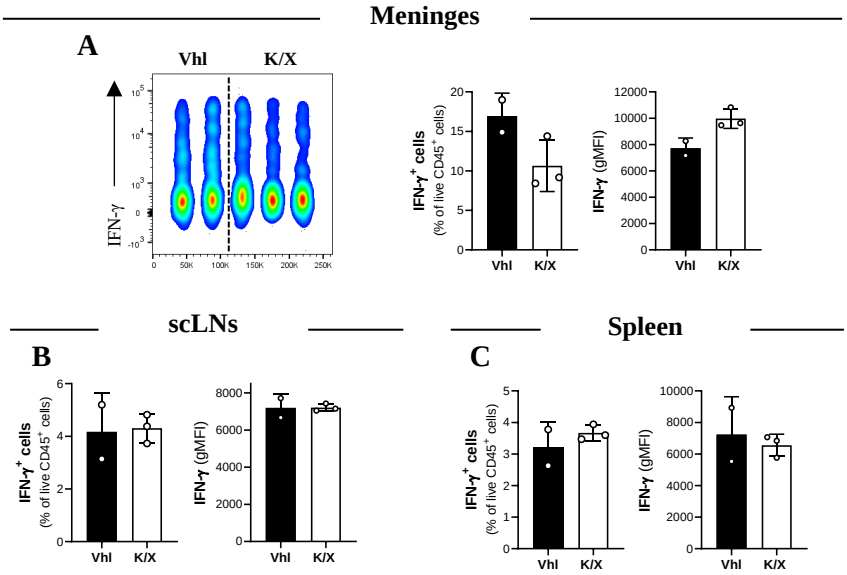

Figure S9 – Meningeal IFN-γ$^+$ immune cells proportion is reduced after a ketamine/xylazine-induced anesthesia. Cell suspensions were prepared from the indicated organs of anesthetized (K/X-treated) or awake (vehicle-treated) WT C57BL/6J mice. Cells were analysed by flow cytometry for the expression of surface (CD45, CD19, CD3, TCRδ, CD4 and CD8) and intracellular (IFNγ) markers. Live cells were gated using LiveDead Fixable Viability Dye. **(A)** Smoothened dot plots depicting IL-17$^+$ cells among 1000 live CD45$^+$ cells isolated from meninges of the anesthetized or awake animals (left), their quantification (middle), and their geomean fluorescence intensity (gMFI) (right). The same quantifications were made for the **(B)** scLN and **(C)** spleen cell suspensions. Data are from 1 independent experiment, n=2-3 mice per group. Mean ± SD.

Table S1 - List of antibodies used for FACS analysis.

Specificiy	Clone	Fluorochrome	Origin
anti-mouse CD3e	145-2C11	PE-Cy7	Biolegend
anti-mouse CD3e	17A2	BV711	Biolegend
anti-mouse CD3e	145.2C11	PerCp/Cy5.5	Biolegend
anti-mouse CD45	30-F11	BV510	Biolegend
anti-mouse CD4	GK1.5	PerCp/Cy5.5	Biolegend
anti-mouse CD4	GK1.5	PE-Cy7	ThermoFischer
anti-mouse CD8	53-6.7	PerCp/Cy-5.5	Biolegend
anti-mouse CD8	53-6.7	BV711	Biolegend
anti-mouse CD8	53-6.7	SB702	ThermoFischer
anti-mouse TCRγ/δ	GL3	APC	ThermoFischer
anti-mouse TCRγ/δ	GL3	BV421	Biolegend
anti-mouse TCRγ/δ	GL3	PE	Biolegend
anti-mouse CD19	6D5	BV605	Biolegend
anti-mouse IL-17A	TC11-18H10	FITC	Biolegend
anti-mouse IL-17A	TC11-18H10	APC	Biolegend
anti-mouse IFN-γ	XMG12	PE	Biolegend
anti-mouse IFN-γ	XMG1.2	APC	Biolegend
Anti-mouse RORγt	B2D	APC	ThermoFisher
Anti-mouse Ki67	16A8	BV605	Biolegend
Anti-mouse IL-7Rα	A7R34	PE	Biolegend
Anti-mouse CD44	IM7	BV711	Biolegend
Anti-mouse CD45RB	C363.16A	PE	Biolegend
Anti-mouse CD27	LG.3A10	PE	BD Biosciences
Anti-mouse CD27	LG.7F9	PE-Cy7	ThermoFisher
Anti-mouse CD25	PC61.5	APC	ThermoFisher