# Effects of sleep and sleep-related hormones on T-cell migration

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To my family.

*A mi familia*.

## Statement of contributions

Some of the research presented in this thesis has been published as a scientific paper under the title "*Sleep promotes T-cell migration towards CCL19 via growth hormone and prolactin signaling in humans*", in Brain, Behavior, and Immunity, Volume 118, May 2024, with co-authors Nicolas D. Lutz, Robert Hübener, Stoyan Dimitrov, Tanja Lange, Jan Born, and Luciana Besedovsky. I, Estefanía Martínez Albert, hereby declare the following contributions to this research:

The conceptualization and design of the study was developed by Luciana Besedovsky with contributions from Tanja Lange and Jan Born. I established and implemented the immunological methodologies utilized in this study, especially the Transwell® migration assay, which was previously not established in the lab. Stoyan Dimitrov assisted with the establishment of the assays involving flow cytometry. Luciana Besedovsky supervised all experiments. I was involved in the organization and data collection process of *Experiment I*, including preparation of experimental protocols, data acquisition, and coordination of experiments in the sleep laboratory with polysomnographic measurements, collection of blood samples throughout the night and day, processing of blood samples in the wet laboratory for hormonal analyses and flow cytometry. The flow cytometry experiments were mainly performed by myself. I created the study's database for the sleep, immune, endocrine, behavioral, and actigraphy data. Robert Hübener was involved in the execution of the elaborate experiments of *Experiment I*, which could not be performed by one person alone due to their complexity. Nicolas D. Lutz assisted with the coordination of the sleep experiments in *Experiment I*. I collected all the data for *Experiment II* and *Experiment III.* Additionally, I performed the majority of the data analysis, including analysis of flow cytometry data, statistical analyses, and (re-)scoring of polysomnography data. Nicolas D. Lutz helped with the sleep analyses. I was furthermore responsible for creating visualizations to represent the data

collected during the study. This included designing graphs, charts, and other graphical representations to illustrate the findings. I wrote the initial draft of the paper, incorporating findings from the data analysis, interpretation, and relevant literature, with contributions from Luciana Besedovsky who refined the content and ensured accuracy. Some parts of the original draft and revised manuscript are included in this dissertation. I revised the manuscript at various stages of development addressing comments and suggestions from co-authors. I contributed intellectually to the project by offering insights, interpretations, and hypotheses based on the data and literature review. I engaged in discussions with co-authors to refine our understanding of the research findings and their implications. In summary, while this research project was a collaborative effort involving multiple authors, my contributions encompassed almost all stages of the research process with major contributions.

## Contents

## Abbreviations

AASM: American academy of sleep medicine

ACTH: adrenocorticotropic hormone

ALDO: Aldosterone

ANS: Autonomic nervous system

APCs: Antigen presenting cells

BFI: Brief Fatigue Inventory

CCC: Cation-chloride cotransporter

CCL19: C-C motif chemokine ligand 19

CCL21: C-C motif chemokine ligand 21

CCL5: RANTES, regulated on activation, normal T cell expressed and secreted chemokine

CCR5: C-C motif chemokine receptor 5; CCL5 receptor

CCR7: C-C motif chemokine receptor 7; CCL19 and CCL21 receptor

CD: cluster of differentiation

CD62L: L-selectin

CORT: Cortisol

CRH: Corticotropin-releasing hormone

D-MEQ: Morningness-Eveningness Questionnaire

DC: Dendritic cell

ECG: electrocardiogram

EDTA: Ethylenediaminetetraacetic Acid

EEG: electroencephalogram

ELISA: Enzyme-Linked Immunosorbent Assay

EMG: electromyogram

EOG: electrooculogram

FSC-A: Forward scatter area

GH: Growth hormone

$GH_b$: Growth hormone blocker

GHRH: Growth hormone releasing hormone

GPCR: G-protein coupled receptor

HEVs: High endothelial venules

HPA: Hypothalamic-pituitary-adrenal

Hz: Hertz

ICAM-1: intercellular adhesion molecule 1

Ig: Immunoglobulins

IGF-1: Insulin-like growth factor 1

IFN-γ: Interferon-gamma

IL: Interleukin

IL-2: Interleukin 2

IL-2R: Interleukin 2 receptor

KCC: K-Cl cotransporter

LFA-1: Lymphocyte function-associated antigen

LMM: Linear mixed models

LN: Lymph node

MALT: mucosa-associated lymphoid tissue

MDBF: Mehrdimensionaler befindlichkeitsfragebogen

MHC: Major histocompatibility complex

Na-Hep: Sodium heparin

NCC: Na-Cl cotransporter

NKCC: Na-K-Cl cotransporter

NREM: non-Rapid Eye Movement

OD: Optical density

PANAS: Positive and Negative Affect Schedule

PBS: Phosphate buffer saline

PMBC: Peripheral blood mononuclear cells

PMN: Polymorphonuclear leukocytes

PNI: Psychoneuroimmunology

PRL: Prolactin

$PRL_b$: Prolactin blocker

PSQI: Pittsburgh Sleep Quality Index

REM: Rapid eye movement

rpm: Revolutions per minute

SD: Standard deviation

SEM: Standard error of mean

SF-A: Schlaffragebogen A

SLO: Secondary lymphoid organ

SSC-A: Side scatter area

SSS: Stanford Sleepiness Scale

SST: Somatostatin

SWS: Slow wave sleep

$T_{CM}$: Central memory T cells

TCR: T-cell receptor

$T_{TE}$: Terminally differentiated effector T cells

$T_{EM}$: Effector memory T cells

TEM: transendothelial migration

$T_N$: Naïve T cells

TRH: Thyrotropin-releasing hormone

VCAM-1: vascular cell adhesion molecule 1

WNK1: with-no-lysine kinase 1

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# 1. Introduction

We spend approximately one-third of our lives sleeping (Zielinski et al., 2016). It is widely recognized that sleep can be affected by illness, and it is a common practice to seek adequate sleep as a supportive measure for recovery from disease states. Sleep has a pivotal role in regulating the immune system, influencing the body's ability to defend itself against infections (Besedovsky et al., 2019; Opp & Krueger, 2015). Experimental studies demonstrate a twofold increase in antigen-specific antibody and T-cell responses in individuals who slept after vaccination compared to individuals who stayed awake, underscoring the significant impact of sleep on adaptive immunity (Lange et al., 2011). The distribution of immune cells between blood and lymphoid tissue in humans is intricately influenced by the circadian system and sleep patterns. Notably, sleep actively reduces T-cell numbers in blood, suggesting a potential mechanism for sleep's impact on vaccination responses by fostering T-cell migration and homing to lymph nodes (LNs) (Besedovsky et al., 2016; Born et al., 1997). However, this process has so far been largely ignored as possible target of sleep. Furthermore, the pro-inflammatory endocrine milieu during sleep has been associated with enhanced adaptive immune responses (Besedovsky et al., 2012), suggesting a connection between sleep, hormonal balance, and immune function. Therefore, the experiments conducted in the context of this thesis aimed at investigating the role of sleep and sleep-associated hormones on the migration potential of T cells towards LNs.

## 1.1. The immune system: the innate and the adaptive immune system

The immune system keeps us healthy by fighting against pathogens that threaten our body. It is divided into two parts according to the speed and specificity of the reaction: the innate and the adaptive arms of the immune system (Abbas et al., 2021). Although they are sometimes

considered two separate entities, they work together. The initial line of defense against pathogens involves specialized tissues, such as the skin, intestine, and lung epithelium, which create a physical obstacle, preventing the entry of pathogens into the body. Once these barriers are breached, the cellular and chemical elements of the immune system, including neutrophils, macrophages, monocytes, the complement system, cytokines, and acute phase proteins come into action. Combined, this genetically inherited line of defense represents the "innate immune system" (Delves & Roitt, 2000a). The "adaptive immune system" develops throughout an organism's life after pathogen exposure. This response consists of antigen-specific reactions through T lymphocytes and B lymphocytes. One of the hallmarks of the adaptive response is that it has memory, so that subsequent exposure leads to a more vigorous and rapid response (Sperling & Bluestone, 1998).

The distinctive feature of the adaptive immune system is the utilization of antigen-specific B and T cells, which become activated after the surface receptors of these cells bind to an antigen. An antigen is a molecule or molecular structure that is recognized by the immune system as foreign or non-self. Antigens can trigger an immune response in the body, leading to the production of antibodies or the activation of immune cells to defend against potential threats like viruses, bacteria, fungi, or other pathogens. Initially, antigen-presenting cells (APCs) (e.g., dendritic cells, DCs) present the antigen to a T cell via major histocompatibility complexes (MHCs), whereas B cells recognize the non-processed intact antigen in its native conformation (Delves & Roitt, 2000b). These steps usually take place within the specialized environment of lymphoid tissue (Ruddle & Akirav, 2009). Upon recognition of the antigen, the APCs are activated and undergo changes in their functional and phenotypic characteristics, differentiating into effector T cells with specific functions. After this stage, the effector response takes place, either through activated T cells that leave the lymphoid tissue to reach the disease site where they carry out different effector functions, or through the antibody release by activated B cells

(i.e., plasma cells) (LeBien & Tedder, 2008; Quinn et al., 1998). Thus, humoral immunity (i.e., immunity involving soluble molecules like antibodies) depends on B cells whereas cellular (i.e., cell-mediated) immunity depends predominantly on T cells. In humoral defense, when B cells encounter specific antigens, they differentiate into plasma cells, which produce large quantities of antibodies specific to that antigen. Antibodies have several functions, including neutralizing pathogens, enhancing phagocytosis, and activating the complement system. Cell-mediated immunity focuses on eliminating infected cells and controlling the spread of intracellular pathogens involving the activation of T cells (Marshall et al., 2018).

### 1.1.1. T lymphocytes

T lymphocytes originate from hematopoietic stem cells in the bone marrow, which produce progenitor T cells. These progenitor cells migrate and maturate in the thymus (Kumar et al., 2018). The process of developing functional mature T lymphocytes occurs step by step, leading to the formation of a T-cell receptor (TCR) complex. This process selectively chooses T lymphocytes with the correct affinity for self-antigens linked with MHC molecules, known as positive selection. In this process, cells predisposed to autoimmunity undergo apoptosis (negative selection) (Klein et al., 2014).

When released from the thymus, mature undifferentiated T cells (naïve T cells; $T_N$) migrate to secondary lymphoid organs (SLOs), where they become activated if the antigen specific for their TCR is presented by APCs (Gebhardt et al., 2011). When T cells encounter an antigen in SLOs, they proliferate from their naïve or resting state to become effector cells that carry out effector functions (Katz, 1977). Two signals, the first coming from the TCR and the second from the co-stimulation, are required for the cells to be activated and become ready to mount an immune response. According to the receptor molecules expressed on the lymphocyte

surface, called cluster of differentiation (CD), T cells can be divided into different T-cell subpopulations with a unique phenotype and different functional characteristics (Mahnke et al., 2013). Two of these surface molecules are the glycoproteins CD4 and CD8, which are co-receptors of the TCR. T cells can overall be divided into T helper cells, expressing the CD4 molecule, and cytotoxic T cells, which express the CD8 molecule (Lucas & Gribben, 2021). The main function of T helper cells is the secretion of cytokines, such as interleukin (IL)-4, IL-6, and interferon-gamma (IFN-γ), which stimulate the production of antibodies by B lymphocytes and the microbicide activities of macrophages. These are the two main mechanisms for the elimination of micro-organisms that are extracellular or live in phagocytic vesicles (Raphael et al., 2015). Cytotoxic T cells lyse cells that have foreign antigens on their membrane, eliminating cells infected by viruses and bacteria, as well as neoplastic cells (Wong & Pamer, 2003).

Immunocytochemistry methods allow to distinguish between the different classes of T cells because of the surface markers they present. In addition to the molecules CD4 and CD8, human T cells can be further divided into four subsets based on the expression of three surface molecules: the C-C motif chemokine receptor 7 (CCR7), L-selectin (CD62L), and the high-molecular-weight isoform of CD45 (called CD45RA). There is an overlap between the expression of CD62L and CCR7 on T cells (Wherry et al., 2003). Both surface molecules are involved in the homing of lymphocytes to lymphoid tissues, such as LNs. CCR7 and its ligands play a fundamental role in guiding different subsets of T cells and antigen-presenting DCs to migrate towards LNs (Förster et al., 2008) (*see section 1.1.2.*), which is why $CCR7^+$ cells (i.e., cells that express CCR7 on their membrane) routinely travel to LNs. CD62L promotes the initial attachment of T cells to high endothelial venules (HEVs) in LNs, facilitating their entry into the lymphoid tissues (Miyasaka, 1998). CD45RA is a specific isoform of the CD45 antigen, also known as leukocyte common antigen, critical to the proper function of the TCRs (Courtney

et al., 2019). In the context of T cells, CD45RA is often used as a marker to distinguish between two major subsets: T cells expressing CD45RA ($CD45RA^+$) and T cells without expressing this protein ($CD45RA^-$). These subsets represent different stages of T-cell differentiation and the expression or not of CD45RA helps distinguish between resting (naïve) or terminal effector ($CD45RA^+$), and memory ($CD45RA^-$) T cells (Cossarizza et al., 1996; Tian et al., 2017). According to the expression or lack of it of these surface markers, T cells are divided as follows (***Figure 1***) (Mahnke et al., 2013; Sallusto et al., 2004):

- Naïve T cells ($T_N$): T cells expressing CD45RA and CCR7/CD62L ($CD45RA^+CCR7^+CD62L^+$).
- Central memory T cells ($T_{CM}$): T cells lacking CD45RA but expressing CCR7/CD62L ($CD45RA^-CCR7^+CD62L^+$).
- Effector memory T cells ($T_{EM}$): T cells lacking CD45RA and CCR7/CD62L ($CD45RA^-CCR7^-CD62L^-$).
- Terminally differentiated effector T cells ($T_{TE}$): T cells expressing CD45RA but lacking CCR7/CD62L ($CD45RA^+CCR7^-CD62L^-$).

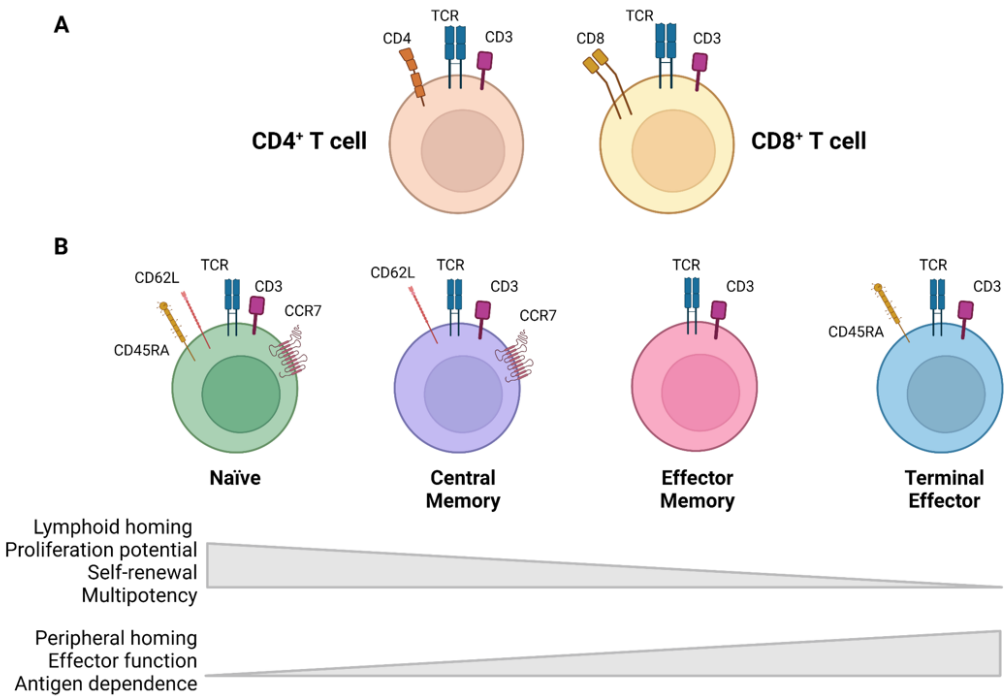


**Figure 1. T-lymphocyte subpopulations according to their surface phenotype.**

**(A)** All T cells express the surface molecule CD3 and the T cell receptor (TCR). T cells can be divided into $CD4^+$ T cells, which secrete cytokines to stimulate macrophages and antibody production by B-cells after activation, and $CD8^+$ T cells, which lyse infected or neoplastic cells after activation. **(B)** According to the expression of CCR7, CD45RA, and CD62L, T cells can be divided into naïve ($T_N$), central memory ($T_{CM}$), effector memory ($T_{EM}$), and terminally differentiated effector T cells ($T_{TE}$). $T_N$ and $T_{CM}$ present high LN homing potential due to the expression of CCR7 and CD62L, whereas $T_{EM}$ and $T_{TE}$ present a more pronounced effector function. Adapted from Mahnke et al. 2013. Created with BioRender.com.

#### 1.1.1.1. Naïve T cells ($T_N$)

$T_N$ are cells that have not (yet) encountered their cognate antigen. Naïve $CD4^+$ and $CD8^+$ T cells leave the thymus and enter the circulation in a quiescent state ($G_0$ of the cell cycle). They continuously recirculate between SLOs and blood via the lymphatic system (van den Broek et al., 2018). $T_N$ are activated in SLOs after contact with an APC, where they receive specific and co-stimulatory signals, resulting in a cascade of phosphorylations and dephosphorylations that

induce the expression of several genes, like *IL-2* (interleukin 2) and its receptor, *IL-2R*. The autocrine secretion of IL-2 by the T cell provides the initial signal that allows the cell to enter the cell cycle and proliferate, creating after 4 or 5 days an expanded clone, and differentiating into effector T cells (Peterson & Maltzman, 2019). Following antigen clearance, almost all the effector cells die, but some will differentiate into memory T cells, which will provide an effective defense against frequent pathogenic micro-organisms in the environment after reencounter (Lefrançois, 2006).

#### 1.1.1.2. Central ($T_{CM}$) and Effector ($T_{EM}$) Memory T cells

Memory T cells result from the clonal expansion and differentiation of antigen-specific T cells that ultimately persist for an extended period, often for years or even decades. The primary function of memory T cells is to provide a faster and enhanced immune responses upon re-exposure to the same type of antigen compared to the initial immune response (Sprent & Surh, 2011). More than twenty years ago, the concept of cellular immunological memory was redefined by the division of circulating memory T cells into two subsets according to their migration potential, effector function, and proliferative capacity. These subpopulations were defined as Central Memory T cells ($T_{CM}$) and Effector Memory T cells ($T_{EM}$) (Sallusto et al., 1999). $T_{CM}$ are able to migrate to lymphoid organs, where they can be re-stimulated by antigens. They are also present in blood but are relatively deficient in non-lymphoid tissues (Mueller et al., 2013). On the other hand, $T_{EM}$ have lost the ability to home to LNs, however, they home to peripheral non-lymphoid tissues and present rapid effector functions (Mahnke et al., 2013). They express tissue homing receptors associated with inflammation (i.e., CCR5 and CXCR3) and display more readily effector functions than $T_{CM}$ (Gray et al., 2018; Martin & Badovinac, 2018).

#### 1.1.1.3. Terminally differentiated Effector T cells ($T_{TE}$)

$T_{TE}$ no longer home to SLOs. Instead, $T_{TE}$ cells are designed to exert their functions at the site of infection or inflammation, primarily in peripheral tissues. $T_{TE}$ cells express markers of senescence and display the shortest telomeres among T cells (Henson & Akbar, 2009). They can be subdivided into three types: Effector $CD8^+$ T cells, or cytotoxic cells, which recognize and eliminate target cells that are infected by a pathogen or have become cancerous; effector $CD4^+$ $T_{H1}$ cells, which stimulate cellular immune responses participating in the macrophage activation; and effector $CD4^+$ $T_{H2}$ cells, which stimulate humoral immune response promoting B-cell proliferation and antibody production (Kaech et al., 2002; Wan & Flavell, 2009).

### 1.1.2. Lymph nodes (LNs) and transendothelial migration

The lymphatic system is a complex network of vessels that spans the entire body and is responsible for transporting lymph, which contains antigens and APCs (Grant et al., 2020). It is functionally organized into two types of lymphoid organs: primary or central lymphoid organs (i.e., thymus and bone marrow), which provide the environment for lymphocyte maturation, and SLOs, which provide the environment for lymphocytes to interact with each other, or with APCs and other accessory cells, and to encounter antigens. The SLOs comprise the spleen, the mucosa-associated lymphoid tissues (MALTs), and the LNs (Boehm et al., 2012).

The human body contains approximately 500 to 600 LNs distributed throughout the body to provide region-specific immune responses (Moore Jr. & Bertram, 2018). LNs are strategically located in the network of lymphatic vessels at anatomical sites where it is easier to receive immune signals from the body (***Figure 2A***). $T_N$ search for their cognate antigen during

frequent visits to LNs. Each LN is equipped with blood vessels and lymphatic vessels, which allow lymphocytes to enter and exit, respectively. The LNs are specially designed to retain antigens as lymph passes through them and provide an ideal environment for communication between immune cells. Each LN is divided into compartments with important functions for enabling communication between lymphocytes (***Figure 2B***). The cortex, which is the outer layer, contains the B-cell areas or follicles. The paracortex or middle area is rich in T cells and DCs (Fossum & Ford, 1985). Finally, the medulla contains B and T cells, and macrophages, and serves as a site for immune cell interaction, antibody production, antigen presentation, and lymphocyte trafficking, contributing to the overall function of LNs in immune surveillance and immune responses. T cells enters the LN through the specialized blood vessels HEVs (Girard & Springer, 1995) and move around within the T-cell area interacting with APCs, and finally leave the node via efferent lymphatic vessels to re-enter the circulation (Mondino et al., 1996). These migratory patterns give APCs, T cells, and B cells many opportunities to interact.

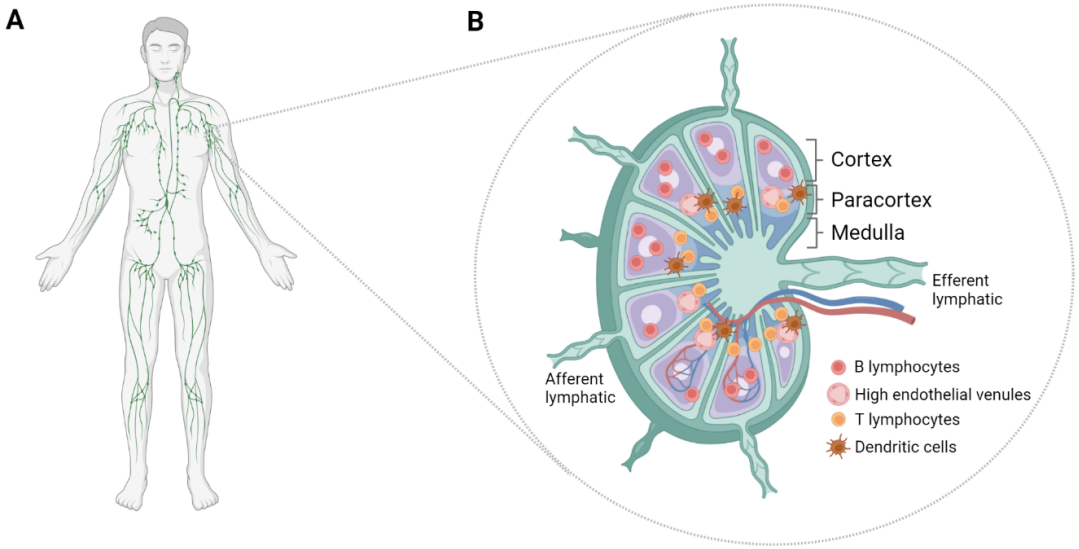


**Figure 2. Human lymphatic system.**

**(A)** Schematic distribution of the lymphatic vessels and LNs throughout the human body. **(B)** LN architecture: the lymph enters from afferent lymph vessels into the LNs and exits through efferent vessels. Three main regions can be distinguished in LNs, the cortex (B-cell area), the paracortex (T-cell area, where circulating lymphocytes enter the LNs through the specialized blood vessels HEVs and

where T cells interact with DCs), and the medulla (contains B cells, T cells, and macrophages). Created with BioRender.com.

In order to perform their defensive functions, T lymphocytes need to circulate constantly throughout the body (Butcher & Picker, 1996; von Andrian & Mackay, 2000). Lymphocyte trafficking requires specific molecules that are capable of enabling cell interactions (i.e. adhesion molecules) on the one hand and of directing cell movements to the tissues (i.e. chemokines) on the other (Alberts et al., 2002).

Adhesion molecules are found on the surface of leukocytes, endothelial, and other cells and enable the adhesion of leukocytes to other cells, which allows communication between them, an indispensable phenomenon in the generation of the immune response and cytotoxicity phenomena (Etzioni, 1996).

The main families of adhesion molecules are selectins, integrins, and the superfamily of immunoglobulins (Ig). CD62L is a selectin highly expressed in $T_N$ and $T_{CM}$, as mentioned in the previous section. CD62L plays a central role in immune cell trafficking and homing to SLOs facilitating the initial tethering and rolling of lymphocytes along the endothelial walls of blood vessels (Miyasaka, 1998). Integrins are the principal receptors to bind the extracellular matrix and the actin cytoskeleton. The superfamily of Ig involved in adhesion, including the molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which are expressed on endothelial cells and act as receptors for integrins, has functions in leukocyte adhesion to endothelial cells, thus facilitating extravasation and migration into tissues (Carlos & Harlan, 1994).

Chemokine attraction is an essential phenomenon in the migration of lymphocytes from blood vessels to lymphoid tissues and to sites of inflammation in response to a chemical

(chemokine) gradient (Griffith et al., 2014). Chemokines are classified into different families based on the arrangement of cysteine residues in their amino acid sequences. The main chemokine families include CXC-chemokines (with two cysteines separated by one amino acid), CC-chemokines (with two adjacent cysteines), CX3C-chemokines (with three amino acids between the first two cysteines), XC-chemokines (that lack the first and the third cysteines of the motif), and CX-chemokines (non-classical chemokines). Chemokines are named according to a systematic nomenclature that includes the subfamily designation explained above, followed by the letter L (denoting "ligand"), and a number according to when the gene was first isolated. Chemokines exert their function by binding to cell receptors. Chemokine receptors are named according to the predominant type of chemokine they bind (i.e., CC, CXC, CX3C, or XC), followed by the letter R (denoting "receptor"), and a number reflecting the order of their discovery. Chemokine receptors belong to the vast family of G-protein coupled receptors (GPCRs), which are seven transmembrane receptors that bind extracellular ligands and consequently initiate intracellular signaling (Hughes & Nibbs, 2018). Receptor specificity is complex but, usually, specific receptors bind only one type of ligand and the particular receptor expressed on a cell determines which tissue a cell will migrate to. For example, cells expressing C-C motif chemokine receptor 7 (CCR7) (i.e., $T_N$ and $T_{CM}$) migrate to LNs because its ligands, C-C motif chemokine ligand 19 (CCL19) and C-C motif chemokine ligand 21 (CCL21) are constitutively expressed in HEVs and the stromal cells of the draining LNs (Stein & Nombela-Arrieta, 2005; Yan et al., 2019). Gene knockout mice lacking CCR7 or CCR7 ligands show marked impairment of T-cell migration into lymphoid organs indicating that CCR7 signaling is indispensable for T-cell recruitment *in vivo* (Förster et al., 2008). CCL19 binds with higher affinity than CCL21 to human CCR7 and appears to be more potent at low concentrations (Bardi et al., 2001). Antigen-experienced T cells like $T_{EM}$ and $T_{TE}$ do not express CCR7 (Hamann et al., 2006). In contrast, they express the C-C motif chemokine receptor 5 (CCR5), the most relevant known receptor for CCL5 (also called RANTES, which stands for

"regulated on activation, normal T cell expressed and secreted"). CCL5 induces migration and recruitment of T cells and other leukocytes to inflammatory sites (Marques et al., 2013).

As mentioned above, SLOs are crucial for initiation and execution of adaptive immune responses as they provide a setting for interactions between $T_N$ and APCs recruited from local infected or inflamed tissues (Colbeck et al., 2017). Therefore, the process of T-cell migration from the blood to LNs (also known as homing or transendothelial migration) is essential (***Figure 3***). This process involves the following steps (Butcher, 1991; Ley et al., 2007):

1. Rolling: Lymphocyte tethering and rolling is initiated by the contact between lymphocyte surface receptors, such as CD62L, and adhesion molecules on the HEV's endothelium. This causes the activation of the endothelial cells of the local blood vessels and consequently their expression of adhesion molecules, such as P- and E-selectins, and the integrins ICAM-1 and VCAM-1.
2. Adhesion: The interaction between a chemokine and its receptor leads to an intracellular signaling event, which activates adhesion molecules of the integrin family on the lymphocyte surface, such as lymphocyte function-associated antigen 1 (LFA-1). The expression of these molecules results in increased adhesion of lymphocytes to endothelial cells. Thus, the initial contact between the two cells is no longer random and transient but becomes strong enough to establish a firm interaction between lymphocytes and endothelium.
3. Extravasation and migration: After firm adhesion, the lymphocyte extends pseudopodia (cellular protrusions) that help it to push between the endothelial cells. This process, known as diapedesis or transmigration, allows the lymphocyte to cross the endothelial barrier and enter the LN parenchyma. This involves dynamic adhesion phenomena that allow cell movement into other tissues (Nourshargh & Alon, 2014; von Andrian & Mempel, 2003).

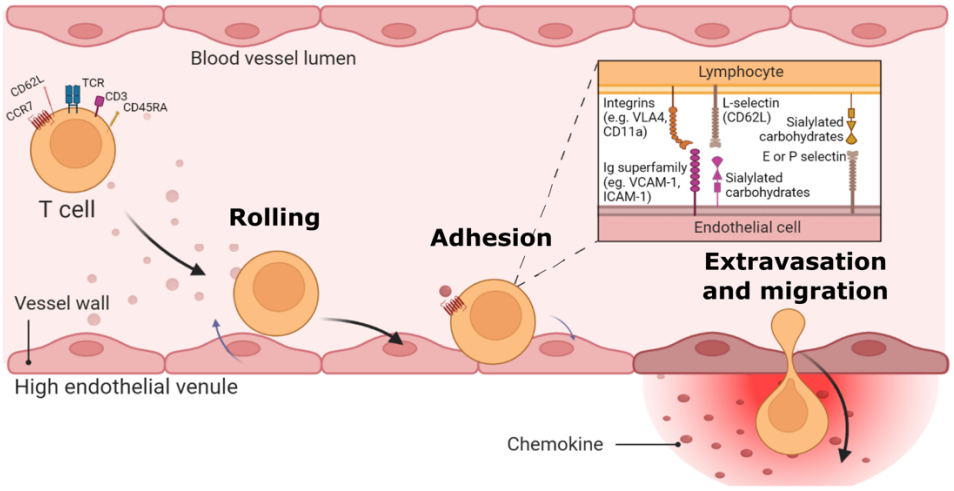


**Figure 3. Transendothelial migration of T lymphocytes.**

Transendothelial T-cell migration in high endothelial venules (HEVs) is a critical process that occurs within LNs and is vital for the immune response. It is a carefully orchestrated process that consists of several steps: 1. Rolling: T cells begin to roll along the surface of the endothelium. During rolling, T cells interact with endothelial cells through T-cell surface receptors, such as CD62L; 2. Adhesion: activated T cells undergo a transition from rolling to firm adhesion. This transition involves the interaction of integrins on the T-cell surface with their corresponding ligands on the endothelial cells; 3. Extravasation and migration: during this step, the T cell crosses the endothelial barrier and enters the LN parenchyma. Adapted from Ley et al., 2007. Created with BioRender.com.

## 1.2. Sleep

Sleep is a fundamental biological phenomenon that holds significant importance for human well-being and health. Despite its apparent simplicity, sleep involves a complex interplay of physiological processes that impact various aspects of our physical and mental health. On average, we spend about one third of our lifetime sleeping (Savage & West, 2007).

Although the full extent of the functions of sleep is not completely understood, sleep research has provided valuable insights into the roles that sleep plays in various physiological and cognitive processes. Sleep is crucial for memory consolidation (Diekelmann & Born, 2010) and is linked to brain plasticity (Weiss & Donlea, 2022). It is a time of cellular repair and growth

(Everson et al., 2014) and plays a role in regulating metabolism and energy balance (Schmid et al., 2015). Additionally, sleep is essential for emotional well-being and regulation (Vandekerckhove & Wang, 2018) and helps regulate body temperature (Szymusiak, 2018). Furthermore, it is involved in the regulation of various hormones (Kim et al., 2015) (*see section 1.4*). In addition to these points, one of the key functions of sleep, and the most important one for this thesis, is the effect of sleep on the immune system, which is explained in detail in *section 1.4*.

Polysomnography, which is considered the "gold standard" technique for measuring sleep, allows to define the primary stages of sleep and wakefulness, each marked by specific patterns of brain activity and physiological responses. Polysomnography measurements include electroencephalogram (EEG) to measure bran activity, electromyogram (EMG) to measure muscle tone, and electrooculogram (EOG) to measure eye movements (Kryger et al., 2011).

During sleep, two main states are observed: Non-Rapid Eye Movement (NREM) sleep and Rapid Eye Movement (REM) sleep. According to the more ancient classification by Kales & Rechtschaffen (Kales & Rechtschaffen, 1968). NREM sleep is divided into four different stages, S1-S4, each defined by specific polysomgrographic measures (***Figure 4A***). In a more recent classification system from the American Academy of Sleep Medicine (AASM), NREM is divided into the three stages N1-N3 (Iber, 2007). The initial stage, S1 (or N1 according to AASM), occurs primarily at the onset of sleep, and serves as a transitional stage between wakefulness and sleep. It represents a light sleep stage characterized by reduced muscle activity and slow eye movements. People in this stage can be easily awakened. After S1, a person enters stage 2, S2 (or N2), marked by reduced heart rate, breathing rate, and the occurrence of sleep spindles and K complexes, which are specific patterns of brain activity. Sleep spindles manifest as 12- to 14-Hz synchronized EEG waveforms. With the onset of S2 the arousal threshold increases and, as the stage progresses, high-voltage slow-wave activity increases to the point of

becoming stage 3, S3. Stages 3 and 4 of NREM sleep (stage N3 in the AASM classification) are referred to as deep sleep or slow-wave sleep (SWS). SWS is characterized by high amplitude (>75 μV) and slow (0.5–2 Hz) EEG waves, called delta waves. In contrast, REM sleep exhibits a distinct EEG pattern characterized by low voltage and rapid frequencies, accompanied by skeletal muscle atonia and episodes of rapid eye movements (Berry, 2012).

Each sleep stage serves distinct functions that contribute to overall health and well-being: S2 supports memory consolidation and cognitive processing (Antony et al., 2019; Ruch et al., 2012). During SWS, physical restoration, hormone regulation, memory consolidation, and potential cognitive benefits, including improved problem-solving, are prominent (Diekelmann & Born, 2010; Hilditch & McHill, 2019; Payne, 2011). REM sleep plays a role in emotional regulation, cognitive functions, and memory consolidation, particularly for procedural and emotional memories (Peever & Fuller, 2017).

The sleep cycle progresses through these stages in a cyclical manner and can be graphically represented in a hypnogram, a visual record of an individual's sleep stages and patterns throughout a period of time (***Figure 4B***). A complete sleep cycle typically lasts around 90 to 110 minutes and consists of several cycles throughout the night. The distribution of NREM and REM sleep within each cycle changes as the night progresses. Early in the night, deep sleep stages are more predominant, while REM sleep becomes more prominent in the later cycles, especially toward morning.

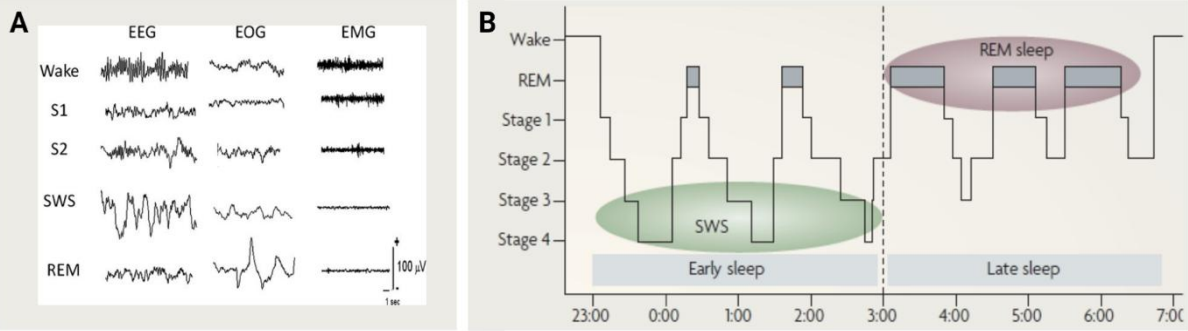

**Figure 4. Human EEG waveform patterns in each sleep stage and standard nocturnal sleep hypnogram.**

(**A**) EEG waveform patterns characterizing the individual sleep stages. (**B**) Generalized sleep architecture during the course of one night.

EEG: electroencephalogram; EOG: electrooculogram; EMG: electromyogram; S1: stage 1; S2: stage 2; SWS: slow wave sleep; REM: rapid eye movement. Modified from Lan et al., 2015, and Payne, 2011.

## 1.3. Sleep-dependent hormones

The endocrine system governs the secretion of essential hormones that regulate diverse physiological processes. Key components of this system include glands such as the hypothalamus and the pituitary gland, which comprises the adenohypophysis (or anterior pituitary) and the neurohypophysis (or posterior pituitary). The anterior pituitary synthesizes and secretes several hormones, including growth hormone (GH) and prolactin (PRL).

GH synthesis and secretion are regulated by hypothalamic hormones: growth hormone releasing hormone (GHRH) stimulates GH production and release, while somatostatin (SST) inhibits it. GH plays various roles in the body, including promoting growth and development, stimulating protein production, influencing fat distribution, regulating glucose metabolism through the production of insulin-like growth factor 1 (IGF-1), promoting bone growth, and stimulating the immune system (Murray et al., 2015).

PRL synthesis by lactotrophs is stimulated by thyrotropin-releasing hormone (TRH) and inhibited by dopamine. Additionally, PRL has a negative feedback mechanism on its own production by stimulating the release of dopamine in the hypothalamus. The primary functions of PRL include milk production and the development of mammary glands within breast tissues. However, it also exerts effects on other targets important for the reproduction of the mammalian

species. Furthermore, PRL plays a crucial role in regulating humoral and cellular immune responses in both physiological and pathological states (Freeman et al., 2000).

The mineralocorticoid hormone aldosterone (ALDO) is produced by the adrenal glands, specifically in the adrenal cortex. The renin-angiotensin-aldosterone system plays a crucial role in regulating ALDO production (Ames et al., 2019). ALDO influences water and salt regulation in the body, regulates blood volume and electrolyte balance, contributing to the maintenance of blood pressure, influences neurogenesis, and modulates the immune system (Katsu & Baker, 2021).

Cortisol (CORT), a glucocorticoid produced by the adrenal cortex, is a key player in the body's response to stress (Charmandari et al., 2005). It is linked to the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine system that mediates the effects of stressors by regulating several physiological processes, including metabolism, immune responses, and the autonomic nervous system (ANS) (Smith & Vale, 2006; Spencer & Deak, 2017). It comprises, besides the hypothalamus and the pituitary gland, the adrenal glands, situated on top of the kidneys. When the body encounters a stressor, the hypothalamus releases corticotropin-releasing hormone (CRH). CRH signals the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH travels to the adrenal cortex and induces the secretion of glucocorticoids, such as CORT. CORT helps the body cope with stress by regulating metabolism and having anti-inflammatory and immunosuppressive effects. Receptors in the hypothalamus and the pituitary gland sense when CORT levels in blood are high, triggering a negative feedback mechanism that shuts down the stress response (Sheng et al., 2021).

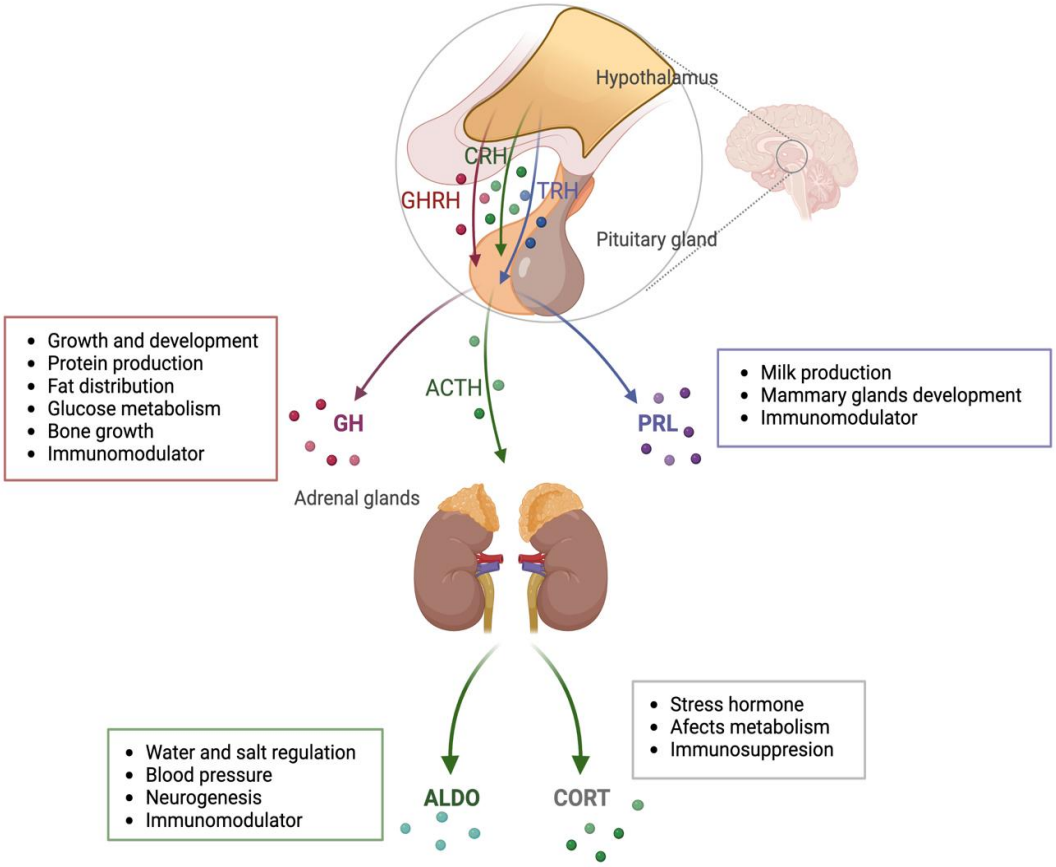


**Figure 5. Main functions and secretory pathways of growth hormone, prolactin, aldosterone, and cortisol.**

Among other hormones, the anterior pituitary gland secretes growth hormone (GH) through growth hormone releasing hormone (GHRH), and prolactin (PRL) through thyrotropin-releasing hormone (TRH). The adrenal cortex produces aldosterone (ALDO), a process regulated primarily through the renin-angiotensin-aldosterone system, and cortisol (CORT), regulated through the hypothalamic-pituitary-adrenal (HPA) axis, involving corticotropin-releasing hormone (CRH) and adrenocorticotropin hormone (ACTH). These hormones contribute to various regulatory processes within the body. Created with BioRender.com.

## 1.4. The relationship between sleep, the immune, and the endocrine system

Psychoneuroimmunology (PNI) is a young multidisciplinary field of study that explores the intricate connections between psychological processes, the nervous system, the immune

system, and the endocrine system. It seeks to unravel the complex interplay between these systems, shedding light on how thoughts, emotions, and behaviors can influence immune responses. Among various human behaviors, PNI investigates the dynamic interactions between sleep and immune responses, as well as the role of the endocrine system in mediating these interactions (Tausk et al., 2008).

Sleep plays a significant role in modulating the immune system. Research has shown that both the quantity and quality of sleep can influence immune responses, affecting the body's ability to defend against infections, regulate inflammation, and promote overall immune system health (Irwin, 2015). Specifically, sleep is a profound regulator of adaptive immunity (Besedovsky et al., 2019), as evidenced, e.g., by experimental studies in humans. These studies revealed that individuals who slept after vaccination exhibited a twofold increase in antigen-specific antibody and T-cell responses compared to those who stayed awake at night (Lange et al., 2011; Lange et al., 2003). These findings are further supported by broader observational studies across larger populations, indicating a close link between short habitual sleep duration and diminished responsiveness to vaccinations (Prather et al., 2012). However, the underlying mechanisms that drive the impact of sleep on the development of adaptive immunity remain poorly understood.

It is important to distinguish the role of sleep in the regulation of immunity from the influence of the circadian system. The circadian system synchronizes internal biological rhythms with external environmental cues, primarily the light-dark cycle, through internal biological clocks, like the suprachiasmatic nuclei located in the hypothalamus, and peripheral clocks (Scheiermann et al., 2013). When comparing the effect of a regular 24-hour sleep-wake cycle on certain immune parameters to a 24-hour cycle of continuous wakefulness on the same parameters, some studies revealed that certain aspects of immunity are mainly influenced by circadian processes, while other immune measures are primarily governed by sleep. In humans,

the distribution of immune cells between the blood, lymphoid tissue, and non-lymphoid tissue is dependent on combined influences from the circadian system and sleep (Besedovsky et al., 2012). Regardless of nocturnal sleep, the numbers of granulocytes, leukocytes, monocytes, and lymphocytes peak in the evening and then decline to reach a minimum in the morning (Born et al., 1997). On top of this rhythm, sleep actively reduces $CD4^+$ and $CD8^+$ T-cell numbers, as well as their subpopulations ($T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$) in blood when compared to the respective time point during a nocturnal vigil (Besedovsky et al., 2016). This fact has been taken to hypothesize, as an underlying mechanism of the effect of sleep on vaccination, that sleep fosters the extravasation of T cells and their subsequent homing to LNs, considering that a higher number of T cells recruited to LNs is linked to a more robust adaptive immune response (Pulendran & Ahmed, 2006). Studies in animals have demonstrated a decline in lymphocyte count in LNs (Ruiz et al., 2017) and a reduction in the mRNA expression levels of genes related to immune cell recruitment into LNs (i.e., *CCL19*, *CCR7*, *CD62L*) in mice subjected to sleep deprivation (Tune et al., 2020). However, there is currently a lack of evidence supporting the idea that sleep indeed enhances lymphocyte migration towards LNs in humans.

An important mediator of the effects of sleep on the immune system is the endocrine system. During sleep, blood levels of CORT drop (Steiger, 2002) while mediators that promote cell growth, such as GH and PRL, show a steep increase in their blood levels (Sadamatsu et al., 1995; Spiegel et al., 1995). In contrast, prolonged sleep loss might disrupt the balance of the HPA axis, leading to elevated CORT levels and impaired immune function. This imbalance, together with alterations in cytokine production, could foster a pro-inflammatory state in the body which, combined with a compromised immune system, would increase susceptibility to infections (Sternberg, 2006).

Studies suggest that during the early part of nocturnal sleep, which is when SWS is dominant, cell-mediated immunity is optimally promoted (Besedovsky et al., 2019). This is

attributed to the pro-inflammatory endocrine milieu present during SWS, where glucocorticoid release is suppressed, and GH and PRL release are promoted (Lange et al., 2010; Westermann et al., 2015). These hormones boost immune cell activation, proliferation, T-cell migration, differentiation, and the production of pro-inflammatory cytokines (Haus, 2007; Kelley et al., 2007), and, thus, eventually aid the initiation of adaptive immune responses (***Figure 6***).

The association between SWS and the pro-inflammatory endocrine milieu was also present during a vaccination study, where high SWS activity (SWA) as well as an increase in GH and PRL, and a decrease in CORT during the night following hepatitis A vaccination, was correlated with the percentage of antigen-specific $CD4^+$ T cells measured up to one year later (Lange et al., 2003), suggesting that GH and PRL might act as adjuvants that boost adaptive immunity. Furthermore, it has been shown that a specific enhancement of slow oscillations by auditory closed-loop stimulation during sleep, a technique that involves delivering auditory stimuli during specific sleep stages, in healthy participants, intensified the hormonal milieu characteristic of SWS and reduced lymphocytes counts (Besedovsky et al., 2017), likely reflecting a redistribution of these cells to lymphoid tissues.

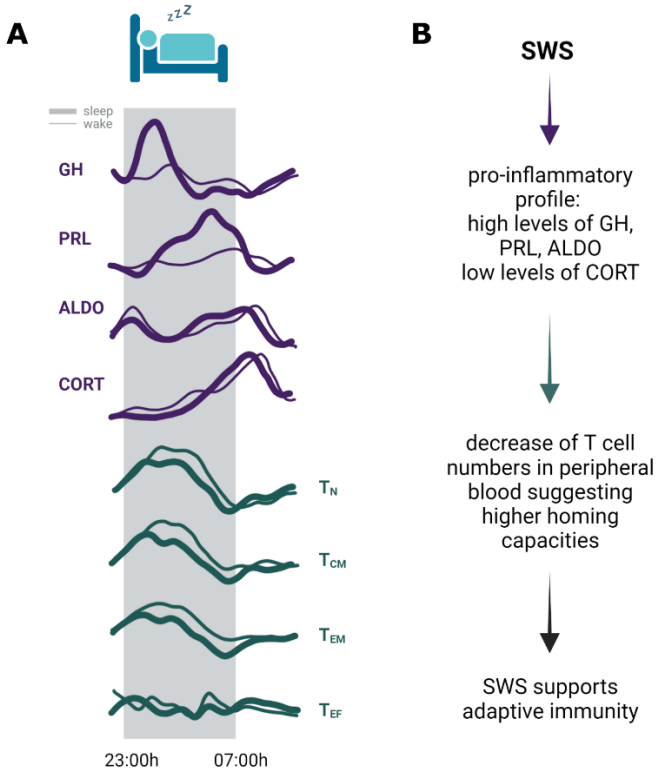


**Figure 6. Hormonal profile and T-cell numbers in blood during nocturnal sleep.**

(**A**) Difference in hormonal plasma levels (GH, PRL, ALDO, and CORT) and T-cell subsets ($T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$) during a 24h sleep-wake cycle (sleep: thick line; wake: thin line. Gray area represents sleep time). (**B**) Potential mechanism explaining the supporting role of sleep on adaptive immunity: During slow wave sleep (SWS) there is a pro-inflammatory profile characterized by high levels of GH, PRL, and ALDO, and low levels of CORT, which is thought to mediate the decrease of T-cell numbers in blood and, therefore, to increase the migration of the cells to LN, which supports adaptive immunity. Adapted from Lange et al., 2010. Created with BioRender.com.

# 2. Aim of the work and hypotheses

There are hints that sleep supports the homing of T cells to LNs, which could explain the beneficial effect of sleep on the adaptive immune response. Nevertheless, functional assays that prove this hypothesis in humans were lacking so far. For that reason, the primary aim of this project was to investigate the effects of sleep on the migration potential of T cells towards LNs. To this aim, the migration of the main human $CD4^+$ and $CD8^+$ T-cell subpopulations (i.e. $T_N$, $T_{CM}$, $T_{EM}$, $T_{TE}$) towards the chemokine CCL19, which is important for attracting T cells to LNs (*Experiment I*), was measured in healthy participants during a normal 24-h sleep-wake cycle and during a 24-h wakefulness condition.

To determine if the potential effect of sleep on T-cell migration is specific to CCL19, it was also in the scope of this thesis to investigate whether sleep affects the migration of T cells towards the chemokine CCL5, which serves a very different function than CCL19 as it is involved in regulating recruitment of T cells to inflammatory sites.

In addition, it is important to characterize in more detail the mediators responsible for the impact of sleep on distinct T-cell subsets. Thus, another aim of this work was to investigate the effects of sleep-dependent hormones on the migration potential of T cells as potential hormone mediators of the sleep effects. In order to do this, T-cell migration towards CCL19 was measured after the incubation of the cells with sleep-like concentrations of the hormones GH and PRL, which were strongly affected by sleep in *Experiment I* (*Experiment II*). Furthermore, to determine if the endocrine constellation present during sleep is responsible for enhancing the migratory potential, T cells were incubated with plasma from sleeping participants and receptor-blockers of the mentioned hormones (*Experiment III*).

Together, the combined hypotheses of this thesis are:

I. T cells exhibit a stronger directed migration towards CCL19 when donors are normally sleeping compared to when they are sleep deprived.

II. The effect of sleep on T-cell migration is specific to the migration of T cells towards CCL19 and it does not affect the migration towards the chemokine CCL5.

III. T cells incubated with sleep-like concentrations of GH and PRL display a stronger directed migration towards CCL19 compared to the migration of T cells incubated with phosphate buffer saline (PBS) as a control.

IV. T cells incubated with plasma from sleeping donors display a stronger directed migration towards CCL19 compared to T cells incubated with plasma from donors who spent 24 h awake.

# 3. Materials and methods

## 3.1. Transwell® migration assay

To assess the migration of T cells, Transwell® assays were performed in the experiments of this thesis. A Transwell® assay, also known as Boyden chamber assay, cell migration or cell invasion assay, is a technique employed to study the movement of cells through a porous membrane. It measures the migratory capability of cells in response to or in the absence of a chemoattractant. This assay involves two chambers separated by a porous membrane that permits cellular transmigration (Boyden, 1962). The selection of the membrane pore size depends on the size of the cell to be analyzed. Initially, cells are placed in the upper chamber within a medium and then migrate vertically through the pores in the membrane into the lower compartment. The lower compartment contains a medium with chemoattractants or an increased serum content, facilitating cellular attraction and movement. A concentration gradient of chemoattractant is established between the two chambers, maintained by surface tension, which prevents the chemoattractant from readily diffusing into the upper chamber (Kramer et al., 2013) (***Figure 7***).

It is also possible to measure the spontaneous migration of cells in a Transwell® assay. It refers to the movement of cells through the porous membrane of the insert without the presence of any external chemotactic or migratory cues. In other words, cells move on their own accord without any specific signaling factors guiding their migration. In experimental settings, spontaneous migration is often considered a baseline or control measurement. It allows researchers to distinguish between cells that are truly responding to an attractant and those moving due to inherent cellular processes.

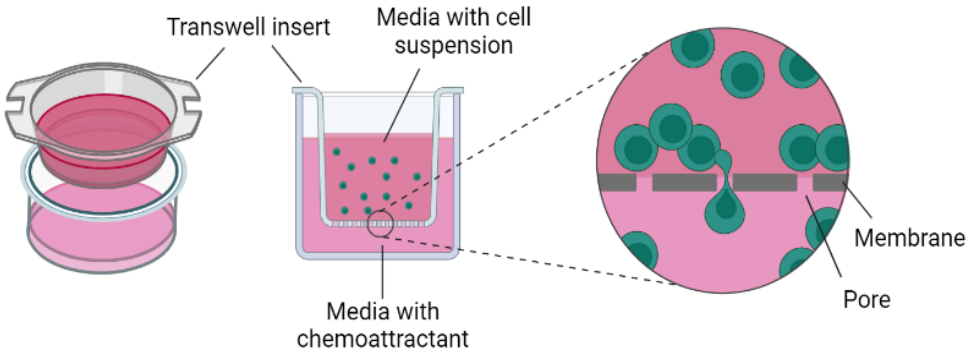


**Figure 7. Diagram of the Transwell® cell migration assay.**

Media with cell suspension is added into a Transwell® insert with a porous membrane. A chemoattractant is added into the bottom chamber to form a chemotactic gradient. Cells sense the chemotactic gradient and migrate through the pores of the Transwell® membrane. Created with BioRender.com.

There are several standardized protocols explaining how to perform a typical Transwell® assay, but its optimization is required to set up well organized and reproducible studies. There are several important considerations to keep in mind to ensure the accuracy, reliability, and success of the assay, which are described in the following.

### 3.1.1. Selecting the right Transwell® permeable support system

The choice of pore size depends on the type of cells under study. If the pore size is too small, migration may be limited because cells simply cannot adjust their morphology enough to pass through the pores. On the other hand, if the pores are too large, cells are more likely to fall through the pores generating inaccurate migration assay results. According to Corning's "Transwell® permeable supports selection and use guide", the appropriate pore size for a chemotaxis study, specifically for transepithelial migration, are typically 3, 5, or 8 µm. For the study of T-cell migration present in this thesis, a pore size of 5 µm was used.

Proper controls are also essential for understanding and validating the results. In a chemotaxis assay, these controls may include wells without chemoattractant to assess the spontaneous migration of the cells.

### 3.1.2. Quantification of migrated cells

Choosing an appropriate method to quantitate the number of migrating cells is an important step. A commonly employed technique includes delicately swabbing the upper side of the permeable insert to remove cells that have not migrated, followed by fixing and staining the migrated cells. The cells are then counted using a microscope (Pijuan et al., 2019). However, this method does not allow discrimination among different leukocytes. For this reason, in the present thesis, a flow cytometry-based Transwell® assay was used that combined the migration assay and its quantification by staining both, the cell fraction in the upper chamber (non-migrated cells) and the cell fraction in the lower chamber (migrated cells), with fluorescence-labeled monoclonal antibodies, which are then analyzed by a flow cytometer (Kränkel et al., 2011).

### 3.1.3. Optimizing seeding concentration

Determining the optimal cell density to use in the upper chamber to avoid overcrowding or sparse cell distribution is another important factor. If too few cells are used, there will not be enough cells to get accurate counts due to the small sample size. On the other hand, using too many cells can oversaturate the pores, leading to inaccurate results.

Before determining the optimal cell concentration, it was necessary to decide which type of sample preparation to use. Typically, Transwell® assays are performed using peripheral blood mononuclear cells (PBMCs). However, the goal was to use whole blood (WB), because

this has several advantages. WB represents the natural physiological state and includes all components of blood, including leukocytes, erythrocytes, platelets, and plasma. This allows for a comprehensive analysis of immune cells and their interactions within the context of the bloodstream (Basu & Kulkarni, 2014). Likewise, WB retains the cellular interactions present in the physiological environment, which can be critical for studying cell-cell communication and immune cell functionality (Forlin et al., 2023). Furthermore, using WB requires minimal processing, making it suitable for rapid assays. In contrast, use of PBMC is labor-intensive and requires several methodological steps that must be strictly followed, which adds more complexity to the experimental design and widens the potential for methodological errors. PBMC processing for lymphocyte isolation also requires a higher amount of blood than when using WB. Due to the multiple blood draws on the participants during the whole experiment setup in the current study, it is important to minimize the amount of blood used as much as possible. For these practical and methodological reasons, the first step for the assay's optimization was to compare WB vs PBMC as a sample source.

While several groups in the past have tried to use WB as the source of polymorphonuclear leukocytes (PMN), such as granulocytes, in a Transwell® assay successfully, there is no detailed and established protocol for the procedure. Some studies have even found that PMNs in the WB preparations exhibited greater chemotactic responses than the isolated PMNs, suggesting that the isolation of PMN could lead to injury of the cells, or that there were factors present in the WB preparations that enhanced the ability of the PMN to respond to the chemoattractants (Rice & Bignold, 1992).

In order to compare both cellular sources, a series of test experiments was performed seeding PBMCs or WB in the upper chamber. First, for assessing the optimal concentration of WB to add, different dilutions of WB in RPMI media were examined (undiluted WB, 1:2, 1:4, and 1:10 dilution). All the tests were conducted in duplicates. The concentration that allowed

optimal migration and proper differentiation of T cells was 1:4. Afterwards, a comparison between WB and PBMCs was made. According to the manufacturer, the optimal concentration of PBMCs to seed in a Transwell® assay is 2-5x$10^5$ cells/ml. PBMCs were isolated using standard protocols (Kleiveland, 2015). All the tests were conducted in duplicates. There was no difference in T-cell migration between WB and PBMCs in any of the tests performed, which indicated that WB could be used as a source of lymphocytes for future experiments.

### 3.1.4. Optimizing chemoattractant concentration

It is important to perform a serial dilution of the chemoattractant to select the optimal concentration. CCL19 and CCL5 are two chemokines widely used in chemotaxis assays, but their optimal concentration depend on the type of experiment performed. To titrate the optimal chemokine concentration, tests were performed using concentrations of 100 ng/ml, 200 ng/ml, 400 ng/ml, and 600 ng/ml. All tests were performed in duplicates and included a negative control. The concentration that ensured optimal T-cell migration was found to be 400 ng/ml for CCL19 and 200 ng/ml for CCL5.

### 3.1.5. Optimizing migration time

There are many factors that influence the time required for a cell to migrate through the pores of a permeable support. Some of these include chemoattractant type/concentration, cell biology, and barrier thickness/concentration. Therefore, it is critical to optimize the migration time for the specific conditions. Typically, an incubation time of 2 h to 4 h is sufficient to achieve the desired results. In a battery of tests, the optimal incubation time was determined by testing the following times: 30 min, 1 h, 2 h, 4 h, and 6 h. All the tests were conducted in duplicates under consistent incubation conditions of 37 ºC and 5% $CO_2$. The results indicated that the optimal incubation time, which yielded better migration indexes for both chemokines, was 4 h.

## 3.2. Experiment I

### 3.2.1. Participants

Fourteen young healthy participants, 7 women and 7 men, were included in this randomized, within-subject, *in vivo* experiment (mean ± SD age: 23.9 ± 3.37 years). None of the participants had a medical history of any relevant chronic disease or psychological disorders, and the absence of acute illness was confirmed through physical examination and routine laboratory investigation. They were non-smokers, did not suffer from known sleep disturbances, and were not taking any medication at the time of the experiments, except for oral contraceptives in female participants.

The participants were synchronized by daily activities and nocturnal rest. They had a regular sleep-wake rhythm and were required to fill in a sleep diary and wore an actigraph (MotionWatch 8, ©CamNtech Ltd, Cambridgeshire, UK) during the week preceding the study and until the end of the experimental session. Additional inclusion criteria comprised age between 18 to 30 years, no alcohol or drug dependence, a body-mass index between 20-25 $kg/m^2$, no shift work or intercontinental flights within the last 6 weeks, and no blood or plasma donation within the last 4 weeks. They were asked to not ingest caffeine or alcohol and not take naps during the days of the experimental sessions. All participants spent one adaptation night in the laboratory in order to become accustomed to the experimental setting. The study was approved by the Ethics Committee of the University of Tübingen, and all participants gave written informed consent prior to participation in accordance with the Declaration of Helsinki.

### 3.2.2. Experiment design

Participants spent two 24-hour experimental sessions in the sleep laboratory. On experimental nights, they arrived at the laboratory at 20:30 h for the preparation of blood sampling and polysomnographic recordings, and stayed until 22:30 h the next day. The Sleep condition included a regular sleep-wake cycle (participants were allowed to sleep for an eight-hour period starting at 23:00 h (lights off ± 15 minutes)) whereas in the Wake condition, participants remained awake throughout the 24 h experimental period. During this time, they were allowed to watch TV, listen to music and talk to the experimenter at dim room light (approximately 2 lx measured at the eye level). On the Wake condition, participants stayed awake in bed in a half-supine position between 23:00 h and 07:00 h. There were four weeks of separation between each experimental session and the order of conditions was balanced across participants. In order to control for the influence of the menstrual hormones, both conditions took place in the same phase of the menstrual cycle in the female participants.

To analyze lymphocyte phenotyping and assess T-cell migration, blood samples were obtained every 4 hours from 22:00 h (baseline) until 18:00 h the following day. Additionally, hourly blood collections were conducted from 22:00 h to 7:00 h during the experimental nights and at 22:00 h on the subsequent day for hormone concentration measurement. During the time in between blood sampling, the participants remained in the laboratory under experimenter supervision to ensure consistent meals, tasks, and physical activity. They received meals at 08:00 h, 12:00 h, and 18:15 h. Following the final blood sampling at 22:00 h, the participants left the laboratory and had a regular night of sleep at home. The following day, they resumed their normal activities and returned to the laboratory for an additional blood sampling at 20:00 h to assess whether any effect of the sleep manipulation persisted after recovery sleep (***Figure 8***). Blood was collected through an intravenous forearm catheter connected to an extended thin tube. This setup facilitates blood collection from an adjacent room without disrupting the

participant's sleep. Blood samples were processed immediately after collection. To prevent clotting during the experimental period, approximately 800 ml of saline solution was infused.

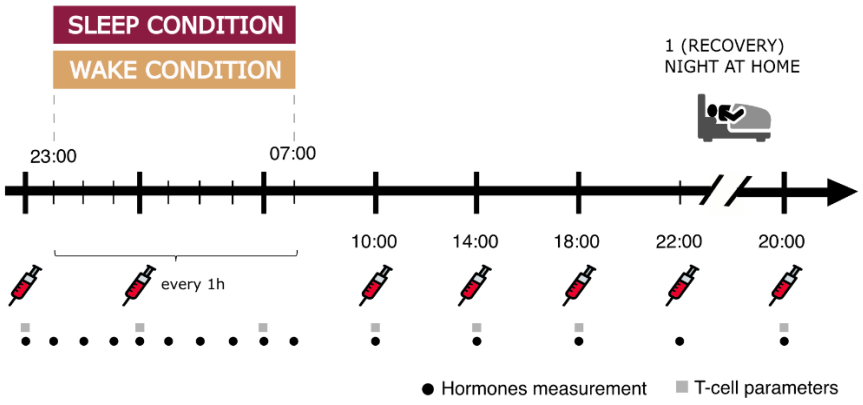


**Figure 8. Experiment I design.**

Healthy participants underwent two 24 h sessions in the sleep laboratory with repeated blood sampling to assess T-cell parameters (represented by gray squares) and hormones (represented by black circles). During the Sleep condition, participants had 8 h of nocturnal sleep, while in the Wake condition, they remained awake throughout the 24 h period. They spent the following night at home before returning the next day to the laboratory for an additional blood sampling at 20:00 h. Adapted from Martinez-Albert et al., 2024.

### 3.2.3. Polysomnography and sleep EEG analyses

Sleep was determined from polysomnographic recordings (according to the international 10-20 system) from electrodes attached at F3 and F4 (frontal), C3 and C4 (central), and O1 and O2 (occipital) positions, and referenced online against the mean of the two mastoids (A1, A2). EOG, EMG (electrodes positioned on the chin) and electrocardiogram (ECG) were also recorded with bipolar montages. The recordings were digitized at a sampling rate of 500 Hz using a BrainAmp MR plus system (Brain Products, Germany). EEG and EOG data were filtered at a frequency of 0.16 Hz (high-pass) and 30 Hz (low-pass) and EMG data at a frequency of 5.31 Hz (high-pass) and 90 Hz (low-pass). In addition, a notch filter was applied

at 50 Hz. Sleep stages were determined offline and manually for subsequent 30-seconds recording epochs following standard criteria (Kales & Rechtschaffen, 1968).

### 3.2.4. Questionnaire assessment tools employed

To account for potential covariates that could influence the results, participants filled in the following questionnaires during the adaptation and/or experimental nights:

- Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989): a self-rated questionnaire, which assesses retrospectively (for the last four weeks) different aspects of sleep (i.e. subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction).
- Stanford Sleepiness Scale (SSS) (Hoddes et al., 1972): evaluates a person's subjective level of sleepiness at a given moment. The scale consists of seven points, ranging from 1 ("Feeling active and vital; alert; wide awake.") to 7 ("Almost in reverie; sleep onset soon; lost struggle to remain awake.").
- Morningness-Eveningness Questionnaire (D-MEQ) (Horne & Ostberg, 1976): this scale assesses individual differences in morningness and eveningness in human circadian rhythms. The respondents are asked to indicate when, for example, they would prefer to wake up or start sleep, rather than when they actually do.
- Schlaffragebogen A (SF-A) (Görtelmeyer, 2011): used for the quantitative and qualitative description and evaluation of sleep behavior and sleep experience related to the previous night.
- Mehrdimensionaler Befindlichkeitsfragebogen (MDBF) (Steyer et al., 1994): a multidimensional questionnaire to determine subjective well-being. It contains a total

of 24 items such as “I feel good” or “I feel balanced”, which are rated on a five-point response scale with the endpoints 1 (“not at all”) and 5 (“very much”).

- Positive and Negative Affect Schedule (PANAS) (Watson et al., 1988): a scale consisting of different words that describe feelings and emotions used to measure positive and negative affect.
- Brief Fatigue Inventory (BFI) (Mendoza et al., 1999): a short scale used to assess quickly the severity of fatigue. Participants rate each item on a 0-10 numeric scale, with 0 meaning “no fatigue” and 10 meaning “fatigue as bad as you can imagine”.

### 3.2.5. Measurement of T-cell subpopulations

The absolute counts of total $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells as well as their naïve ($T_N$, $CD45RA^+CD62L^+$), central memory ($T_{CM}$, $CD45RA^-CD62L^+$), effector memory ($T_{EM}$, $CD45RA^-CD62L^-$), and terminal effector ($T_{TE}$, $CD45RA^+CD62L^-$) subsets were determined using a “lyse no-wash” flow cytometry procedure. Briefly, 90 µl of WB sampled in a sodium-heparin S-monovette® (Sarstedt, Nümbrecht, Germany) was immunostained with anti-CD3/BV510, anti-CD4/BV421, anti-CD8/PerCP, anti-CD45RA/AF700, and anti-CD62L/FITC (Catalogue numbers: 317332, 357424, 980916, 304120, 304838; all from BioLegend, San Diego, CA, US)), in Trucount™ tubes (BD Biosciences, San Jose, CA, US). Following a 15 minutes incubation in the dark at room temperature, 900 µl of FACS lysing solution (BD Biosciences) was added and the cells were incubated for another 15 minutes. After this time, the samples were stored at 4 ºC and measured within the next 24 h. At least 10,000 $CD3^+$ cells were acquired on a LSRFortessa using DIVA Software (BD Biosciences). The gating strategy used to determine the different T-cell populations is shown in ***Figure 9***. The absolute number of cells per µl was then calculated using the following formula: cells/µl =

(acquired cell events in the respective gate) x (number of beads per tube) / ((acquired bead events) x (sample volume [µl])).

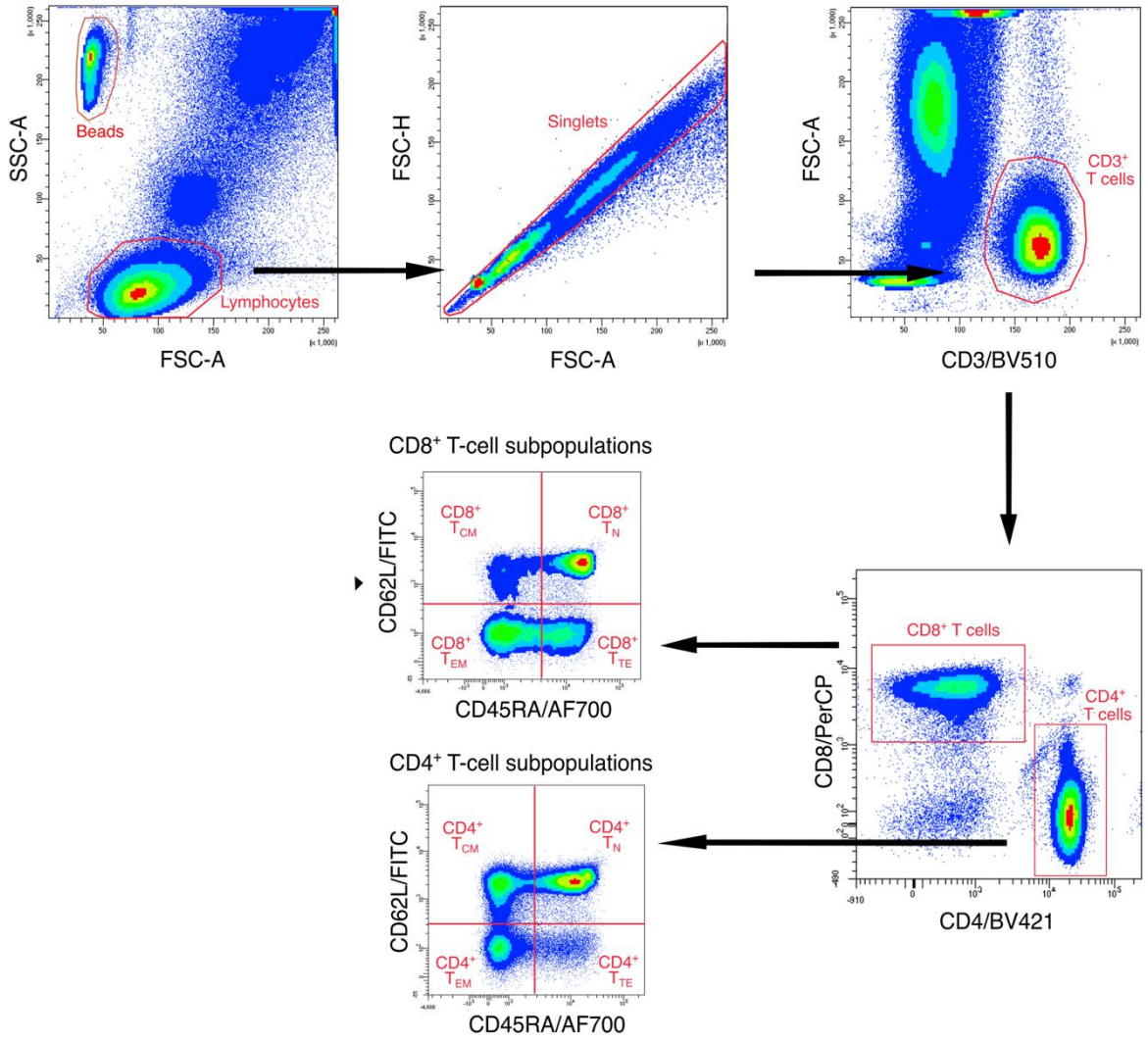


**Figure 9. Flow cytometry gating strategy for T lymphocytes.**

Representative density plots showing the gating strategy used for defining the different T-cell subpopulations. From left to right on the above panel, the lymphocyte gate (on the forward scatter area [FSC-A]/ side scatter area [SSC-A] plot), the FSC-A/FSC height (FSC-H) duplet exclusion, and the gating of $CD3^+$ cells are shown. On the lower panel (from right to left), proceeding from the $CD3^+$ T cells population, the $CD8^+$ or $CD4^+$ T cells, and the subsequent gating of their populations based on the antibodies CD62L and CD45RA are shown.

### 3.2.6. Transwell® migration assays

Migration assays were performed using 5-µm pore-sized polycarbonate membrane Transwell® inserts (Corning Incorporated, Tewksbury, MA, US). Briefly, 200 µl of a 1:4 dilution of the participant's fresh WB sampled in a sodium-heparin (Na-Hep) S-monovette® and media RPMI 1640 (Gibco, Life Technologies Corporation, Carlsbad, CA, US) were added to the upper chamber. In the lower chamber, RPMI with Recombinant Human CCL19 (400 ng/ml; BioLegend), Recombinant Human CCL5 (200 ng/ml; BioLegend), or RPMI alone (for assessment of spontaneous migration) were added in a total volume of 600 µl. After 4 h of incubation at 37 °C and 5% $CO_2$, 100 µl of the liquid from both, the upper and lower chambers, were collected and immunostained with anti-CD3/BV510, anti-CD4/BV421, anti-CD8/PerCP, anti-CD45RA/AF700, and anti-CD62L/FITC (all from BioLegend). After 15 minutes of incubation at room temperature in the dark, 900 µl of FACS lysing solution (BD Biosciences) was added and the cells were incubated for another 15 minutes. After this time, the samples were centrifuged for 5 minutes at 500 g and 21 ºC, and 700 µl of the supernatant was discarded. Then, the samples were stored at 4 ºC and measured within the next 24 h on a BD LSRFortessa. To calculate a migration index, the number of cells that had migrated to the lower chamber as well as the number of cells in the upper chamber (i.e., the number of non-migrated cells) were counted and used in the following calculation: [number of migrated cells / number of non-migrated cells] x 100. Targeted migration towards CCL19 and CCL5 was calculated by subtracting their migration index from the RPMI control.

### 3.2.7. Hormones assays

Blood for measuring the concentration of GH, PRL, and CORT was sampled in a Lithium-Heparin S-monovette® and stored at 4 ºC until the end of the experimental session for their assay. The tubes were centrifuged and the levels of these hormones were measured using an

automated immunoassay process (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, US) at the central laboratory unit of the Universitätsklinikum Tübingen. In this system, participants' samples were collected and mixed with specific reagents containing antibodies designed to selectively bind to the target hormone. Following incubation and the formation of immune complexes, solid-phase separation and washing procedures removed unbound substances. The remaining immune complexes were detected using a signal-producing system. Calibration standards with known hormone concentrations assisted in creating a calibration curve for accurate quantification. The lower limit of detection was 0.05 µg/l for GH, 0.3 µg/l for PRL, and 5.5 nmol/l for CORT. CORT levels were measured to exclude that this stress hormone was affected by the sleep manipulation.

Regarding the assessment of ALDO levels, blood was sampled in a Ethylenediaminetetraacetic Acid (EDTA) S-monovette® and centrifuged immediately at 3,000 g and 4 ºC for 15 minutes to obtain EDTA plasma that was then stored at -80 °C until assay. ALDO concentration was measured using a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kit (DRG Instruments GmbH, Marburg, Germany), which is based on the principle of competitive binding. A monoclonal antibody directed towards a unique antigenic site of the ALDO molecule was immobilized on a microtiter plate provided in the kit. Standards and samples were added to the plate. The microplate was first incubated on a plate shaker for 1 h at room temperature at ~500 rpm. The ALDO in the sample competes with the added enzyme conjugate (ALDO conjugated to horseradish peroxidase) for binding to the coated antibody. The plate was washed with wash solution provided in the kit to remove all unbound substances and incubated with the substrate solution containing 3,3',5,5'-tetramethylbenzidine. After 30 minutes incubation on a plate shaker at room temperature, stop solution was added to stop the colorimetric reaction. The optical density (OD) of the resulting yellow product was then measured at 450 nm using an LB 942-TriStar2S microplate reader

(Berthold, Bad Wildbad, Germany). The intensity of the color is inversely proportional to the concentration of the analyte in the sample. A standard curve is constructed by plotting OD values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

## 3.3. Experiment II

Seven healthy participants, 4 women and 3 men (mean ± SD age: 26.4 ± 3.04 years), took part in this *in vitro* experiment. None of the participants had a medical history of any chronic disease or mental disorder, and acute illness was excluded by physical examination and routine laboratory investigation. All participants had a regular sleep/wake rhythm, did not take any medications at the time of the experiment (except for oral contraceptives in female participants), and were nonsmokers. All of them gave written informed consent prior to participation in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the University of Tübingen.

The participants' blood was sampled using a Na-Hep S-monovette® and then incubated with sleep-like concentrations of GH and PRL (GH: 10 ng/ml, Gibco, Life Technologies Corporation, Carlsbad, CA, US; PRL: 20 µg /L, Sigma-Aldrich, Saint Louis, MO, US), defined by the physiological sleep concentrations of the hormones seen in previous studies (Dimitrov et al., 2004; Lange et al., 2006), or with PBS (as a "no hormone" control condition) for 2 h at 37 ºC and 5% $CO_2$. The blood was sampled at 18:00 h because it has been shown in our own results and previous literature (Lange et al., 2006) that the blood concentration of these hormones is low at this time of the day. The rationale behind choosing a time when hormone levels are low is to isolate or minimize the influence of the blood donors' own hormones on the experiment.

The duration of incubation with the hormones was determined in previous experiments where 6 durations were studied (5 minutes, 30 minutes, 1 h, 2 h, 3 h, and 4 h). Among these times, 2 h was the optimal incubation time in order to see an effect on the migration. After the incubation time, a Transwell® chemotaxis assay in the presence or absence of the chemokine CCL19 was performed using the same procedure as in *Experiment I*. A summary of the different conditions measured in *Experiment II* is shown in ***Figure 10A***.

## 3.4. Experiment III

Three healthy donors, 1 woman, 2 men (mean ± SD age: 25 ± 2 years) were additionally recruited for this *plasma* experiment. None of them had a medical history of any chronic disease or mental disorder, and acute illness was excluded by physical examination and routine laboratory investigation. They had a regular sleep/wake pattern, did not take any medications at the time of the experiment (except for oral contraceptives in female participants), and were nonsmokers. All of them gave written informed consent prior to participation in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the University of Tübingen.

On the day of the experiment, freshly sampled WB from the donor participants was sampled at 18:00 h in a Na-Hep S-monovette®. This blood was then incubated with plasma collected from seven participants during the sleep condition or during the wake condition of the *in vivo* experiment (*Experiment I*) to investigate whether the effects of sleep on T-cell migration are mediated by endocrine factors. In order to collect the plasma during the *in vivo* experiment, additional blood was sampled at 02:00 h in BD Vacutainer® heparin tubes (BD Biosciences) and centrifuged at 2,000 g, 4 ºC for 10 minutes for isolation of plasma. The plasma was then aliquoted and stored at -80 ºC until the day of the experiment.

To study if the CCL19-directed migration was affected by the presence of GH or PRL in the plasma, blood was also incubated with plasma collected in the sleep condition + Human GH Receptor Antibody (1 ug/ml) or plasma collected in the sleep condition + Human PRL Receptor Antibody (400 ng/ml) (both from R&D Systems, Minneapolis, MN, US). The blood was incubated with the plasma mixtures in a 1:4 dilution for 2 h at 37 ºC and 5% $CO_2$. Afterwards, a Transwell® chemotaxis assay was performed using the same procedure as for the *in vivo* and *in vitro* experiments (***Figure 10B***). Experiments were performed in duplicates.

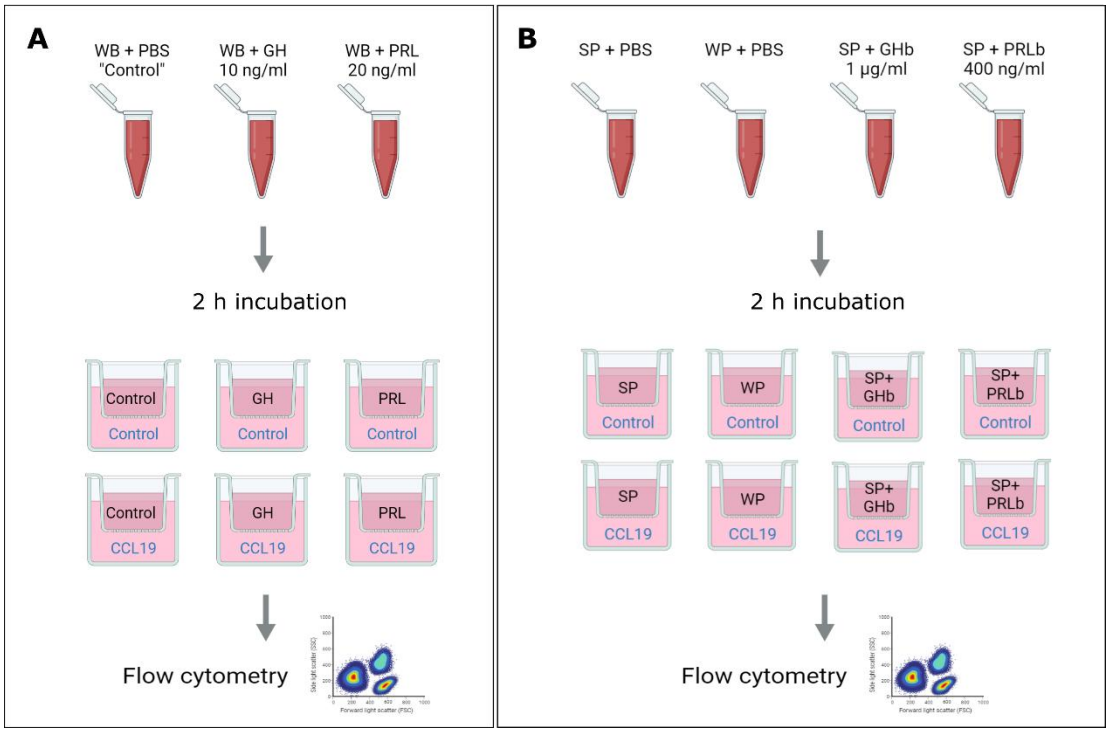


**Figure 10. Diagram of the Experiment II and Experiment III.**

(**A**) Experiment II: Whole blood (WB) from seven healthy participants was incubated for 2 h with PBS ("control condition"), or sleep-like concentrations of GH or PRL (upper chamber of the Transwell® inserts). Afterwards, spontaneous and CCL19-directed T-cell migration were measured using a Transwell® migration assay and flow cytometry. (**B**) Experiment III: Whole blood from healthy donors was incubated during 2 h with plasma of seven participants of the *in vivo* experiment. The blood was incubated with plasma isolated during the sleep condition (SP), plasma isolated during the wake condition (WP), SP + GH receptor blocker ($GH_b$) or SP + PRL receptor blocker ($PRL_b$) (upper chamber of the Transwell® insert). Afterwards, spontaneous and CCL19-directed T-cell migration were measured using a Transwell® migration assay and flow cytometry. Created with BioRender.com.

## 3.5. Quantification and statistical analysis

All statistical analyses were performed using IBM SPSS Statistics, version 26 (IBM Corp., Armonk, N.Y., US). To examine the influence of sleep on T-cell numbers and migration, linear mixed models (LMM) analyses were applied with "Condition" (wake vs. sleep), "Time" (22:00 h, 02:00 h, 06:00 h, 10:00 h, 14:00 h, 18:00 h, and 20:00 h of the subsequent day), and "Condition x Time" as fixed effects, and "participant" as random effect. All models included the baseline measurement (at 22:00 h) as a covariate. To evaluate the impact of sleep on hormone concentration, comparable LMM with "Condition" (wake vs. sleep), "Time" (22:00 h – 07:00 h, 10:00 h, 14:00 h, 18:00 h, 22:00 h, and 20:00 h of the subsequent day), and "Condition x Time" as fixed effects, "Participant" as random effect, and with the baseline measure as a covariate were performed. Additionally, to investigate the acute effects of sleep on hormone levels, a model categorizing time points into Early (22:00 h-03:00 h) and Late night (04:00 h-07:00 h) was employed. The "Early/late" factor was introduced as the effects of sleep on some of the hormone levels are expected mainly during the early night. For the analyses of *Experiment II* and *Experiment III*, paired-samples t-tests tests were applied to analyze differences between conditions. The data was found to follow a normal distribution, as confirmed by the Kolmogorov-Smirnov test.

# 4. Results

## 4.1. Effects of sleep on the number of different T-cell subpopulations in blood (Experiment I)

To examine the effects of sleep vs wakefulness on the absolute count of circulating T cells, 14 healthy participants (50% females) spent two 24-hour sessions in the sleep laboratory, separated by at least four weeks. In one condition, participants were allowed to sleep for eight hours at night, while in the other condition, participants spent the 24-hour session continuously awake. Blood was collected every 4 hours within the 24-hour sessions as well as after an additional night at home (serving as a recovery night in the Wake condition). The blood was processed immediately and labeled with the respective antibodies for the determination of the absolute counts of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells, as well as their subsets (i.e., $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$).

Compared with nocturnal wakefulness, sleep reduced the number of total $CD3^+$, $CD4^+$ and $CD8^+$ T cells, as well as the counts of all $CD4^+$ and $CD8^+$ T-cell subsets during the night except for the $T_{TE4}$ population (***Figure 11, Table 1***).

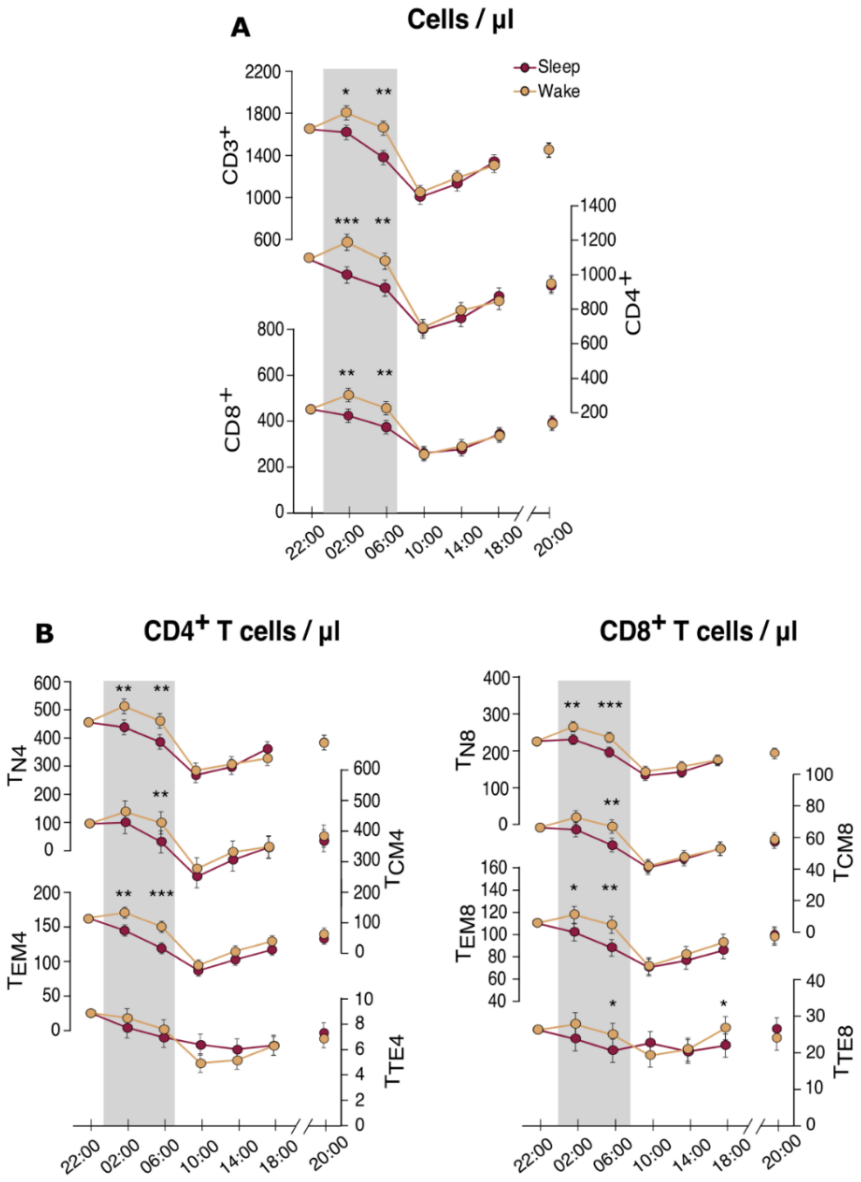


**Figure 11. Effects of sleep on the absolute counts of circulating T cells.**

(**A**) Estimated marginal means ± SEM of the number of circulating total $CD3^+$, $CD4^+$, and $CD8^+$ T cells and (**B**) their $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$ $CD4^+$ and $CD8^+$ subsets during a regular sleep-wake cycle (red circles) and during continuous wakefulness (yellow circles). Gray area indicates time in bed. n = 14. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between conditions. See Table 1 for overall results of the LMM analyses. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells; LMM: linear mixed model. Adapted from Martinez-Albert et al., 2024.

**Table 1. Results of LMM analyses for absolute counts of circulating T-cell subsets in the *in vivo* sleep experiment.**

| | Main effect of Condition | Main effect of Time | Condition x Time interaction |
|---|---|---|---|
| $CD3^+$ | **P = 0.005** | **P < 0.001** | **P = 0.027** |
| $CD4^+$ | **P = 0.008** | **P < 0.001** | **P = 0.038** |
| $CD8^+$ | **P = 0.030** | **P < 0.001** | P = 0.060 |
| $T_{N4}$ | **P = 0.032** | **P < 0.001** | **P = 0.027** |
| $T_{CM4}$ | **P = 0.004** | **P < 0.001** | P > 0.1 |
| $T_{EM4}$ | **P < 0.001** | **P < 0.001** | P > 0.1 |
| $T_{TE4}$ | P > 0.1 | **P = 0.008** | P > 0.1 |
| $T_{N8}$ | **P < 0.001** | **P < 0.001** | P > 0.1 |
| $T_{CM8}$ | **P = 0.026** | **P < 0.001** | P > 0.1 |
| $T_{EM8}$ | **P = 0.003** | **P < 0.001** | P > 0.1 |
| $T_{TE8}$ | P > 0.1 | **P = 0.002** | **P = 0.019** |

P < 0.05 are shown in bold. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells; LMM: linear mixed model. Adapted from Martinez-Albert et al., 2024.

## 4.2. Effects of sleep on T-cell migration (Experiment I)

To study the effects of sleep on human T-cell migration, blood was collected during both experimental sessions, every 4 hours from 22:00 h (baseline) until 18:00 h of the next day, and at 20.00 h the following day after a recovery sleep night to measure the migration of T cells in the absence (to assess spontaneous migration) or presence of the chemokines CCL19 or CCL5.

### 4.2.1. Effects of sleep on T-cell migration towards CCL19

Compared to continuous wakefulness, sleep selectively promoted the migratory potential of various T-cell subpopulations. Specifically, sleep increased the directed migration towards the chemokine CCL19 of total $CD3^+$, $CD4^+$, and $CD8^+$ T cells, as well as $T_{N4}$ and $T_{N8}$ (***Figure 12***, see ***Table 2*** for results of LMM analyses) with a significant difference during the day after the

experimental night at 10:00 h and at 14:00 h. The effect did not reach significance in $T_{CM4}$ and $T_{CM8}$ subsets. The migration towards CCL19 remained unaffected by the sleep manipulation in $T_{EM}$ and $T_{TE}$.

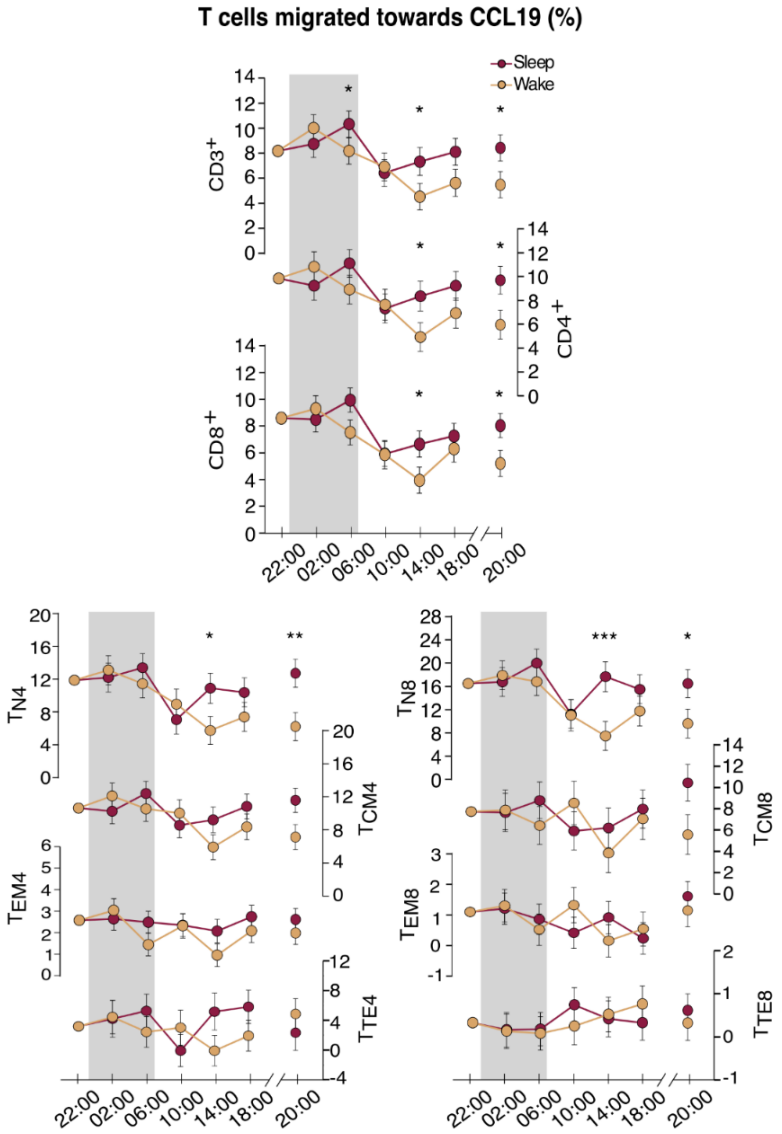


**Figure 12. Effects of sleep on CCL19-directed migration of various T-cell subsets.**

Estimated marginal means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells and their $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$ $CD4^+$ and $CD8^+$ subsets in the presence of the chemokine CCL19 during a regular sleep-wake cycle (red circles) and during 24 hours of continuous wakefulness (yellow circles). Gray area indicates time in bed. n = 14. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between conditions. See Table 2 for overall results of the LMM analyses. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector

memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells; LMM: linear mixed model. Adapted from Martinez-Albert et al., 2024.

**Table 2. Results of linear mixed models analyses for spontaneous, CCL19-directed, and CCL5-directed migration of various T-cell subsets in the *in vivo* sleep experiment.**

| | Spontaneous migration | | | CCL19-directed migration | | | CCL5-directed migration | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cond. main effect | Time main effect | Cond. x Time inter. | Cond. main effect | Time main effect | Cond. x Time inter. | Cond. main effect | Time main effect | Cond. x Time inter. |
| $CD3^+$ | **P = 0.010** | P > 0.1 | P = 0.082 | **P = 0.004** | **P = 0.001** | P > 0.1 | P > 0.1 | **P = 0.041** | P > 0.1 |
| $CD4^+$ | **P = 0.013** | P > 0.1 | P = 0.068 | **P = 0.014** | **P = 0.012** | P > 0.1 | P > 0.1 | **P = 0.046** | P > 0.1 |
| $CD8^+$ | **P < 0.001** | P > 0.1 | P > 0.1 | **P = 0.015** | **P < 0.001** | P > 0.1 | P > 0.1 | **P = 0.018** | P > 0.1 |
| $T_{N4}$ | **P = 0.005** | P > 0.1 | P > 0.1 | **P = 0.015** | **P = 0.006** | P = 0.062 | P > 0.1 | P = 0.053 | P > 0.1 |
| $T_{CM4}$ | P = 0.060 | P = 0.067 | P > 0.1 | P = 0.066 | P = 0.052 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 |
| $T_{EM4}$ | P > 0.1 | P = 0.087 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P = 0.081 | P > 0.1 |
| $T_{TE4}$ | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 |
| $T_{N8}$ | **P < 0.001** | P > 0.1 | P > 0.1 | **P = 0.004** | **P = 0.005** | P > 0.1 | P > 0.1 | **P = 0.017** | P > 0.1 |
| $T_{CM8}$ | **P = 0.047** | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 |
| $T_{EM8}$ | P > 0.1 | P = 0.065 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 |
| $T_{TE8}$ | **P = 0.002** | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | P > 0.1 | **P < 0.001** | P > 0.1 |

$P < 0.05$ are shown in bold. Cond.: Condition; Inter.: Interaction. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells. Adapted from Martinez-Albert et al., 2024.

### 4.2.2. Effects of sleep on spontaneous T-cell migration

Sleep also increased the spontaneous migration (i.e., the migration in the absence of added chemokines) of total $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Again, these differences between conditions were still present even after a full night of recovery sleep. (***Figure 13***, ***Table 2***). Within the eight subpopulations measured (i.e., $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$ $CD4^+$ and $CD8^+$ T cells), sleep increased the spontaneous migration of $T_{N4}$, $T_{N8}$, $T_{CM8}$, and $T_{TE8}$. Importantly, for $T_{N4}$, $T_{N8}$, and $T_{TE8}$, the reduction in spontaneous migration after sleep deprivation was still present

even after a night with an 8-hour recovery sleep period, demonstrating the persistent nature of the effect.

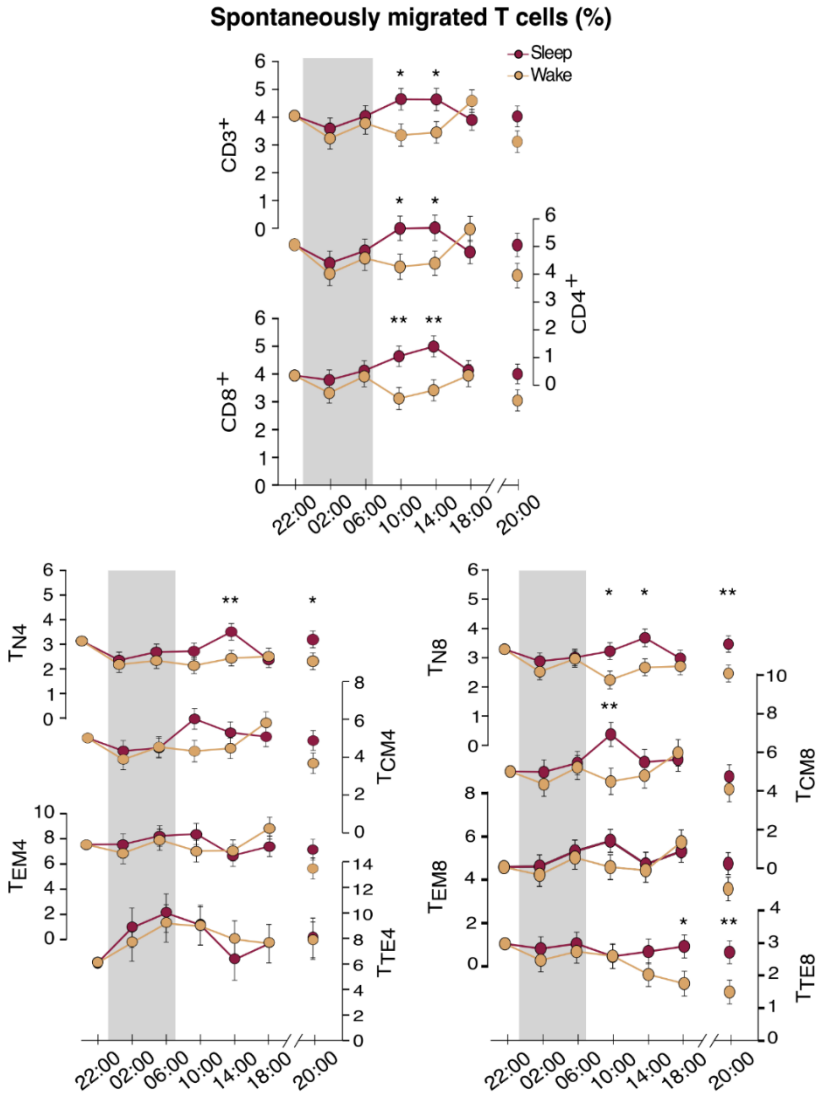


**Figure 13. Effects of sleep on spontaneous T-cells migration.**

Estimated marginal means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells and their $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$ $CD4^+$ and $CD8^+$ subsets in the absence of any exogenous chemokine (i.e., spontaneous migration) during a regular sleep-wake cycle (red circles) and during 24 hours of continuous wakefulness (yellow circles). Gray area indicates time in bed. n = 14. *p < 0.05, **p < 0.01 for pairwise comparisons between conditions. See Table 2 for overall results of the LMM analyses. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells; LMM: linear mixed model. Adapted from Martinez-Albert et al., 2024.

### 4.2.3. Effects of sleep on T-cell migration towards CCL5

Regarding the migration towards the chemokine CCL5, which promotes lymphocyte infiltration to sites of inflammation, there was no difference between the conditions in any of the T-cell subsets studied (***Figure 14, Table 2***).

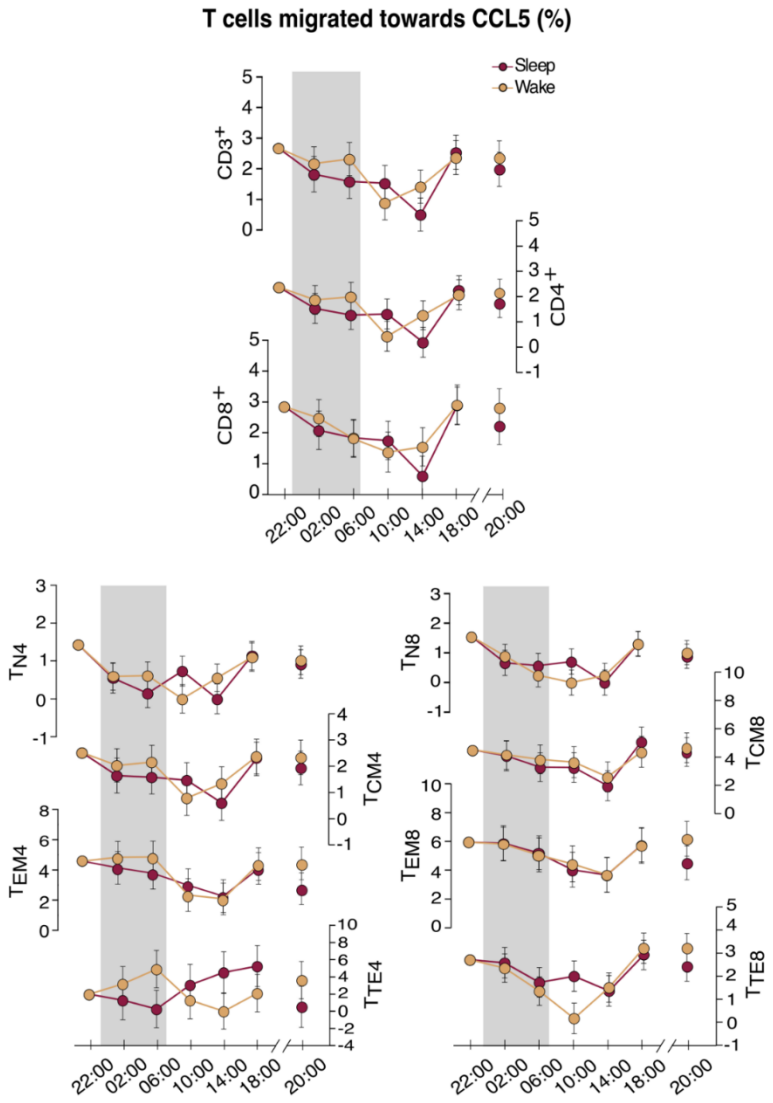


**Figure 14. Effects of sleep on CCL5-directed migration of various T-cell subsets.**

Estimated marginal means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells and their $T_N$, $T_{CM}$, $T_{EM}$, and $T_{TE}$ $CD4^+$ and $CD8^+$ subsets in the presence of the chemokine CCL5 during a

regular sleep-wake cycle (red circles) and during 24 hours of continuous wakefulness (yellow circles). Gray area indicates time in bed. n = 14. See Table 1 for overall results of the LMM analyses. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{EM4}$: effector memory $CD4^+$ T cells; $T_{TE4}$: terminally differentiated effector $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells; $T_{EM8}$: effector memory $CD8^+$ T cells; $T_{TE8}$: terminally differentiated effector $CD8^+$ T cells; LMM: linear mixed model. Adapted from Martinez-Albert et al., 2024.

## 4.3. Effects of sleep on hormone levels (Experiment I)

To measure levels of the immune-modulating hormones GH, PRL, ALDO, and CORT, blood was collected every hour from 22:00 to 7:00 h, thereafter every 4 hours until 22:00, and again following a night at home.

Levels of PRL and GH were increased in the Sleep condition compared to the Wake condition (p-values PRL: 0.021 and 0.024; p-values GH: 0.093 and 0.020, for the "Condition x Time" and "Condition x Early/late" interaction respectively). ALDO and CORT were not affected by sleep when the LMM factors were defined as "Condition x Time" interaction (ALDO, p-value = 0.563; CORT, p-value = 0.728) nor "Condition x Early/late" interaction (ALDO, p-value = 0.756; CORT, p-value = 0.340) (***Figure 15***).

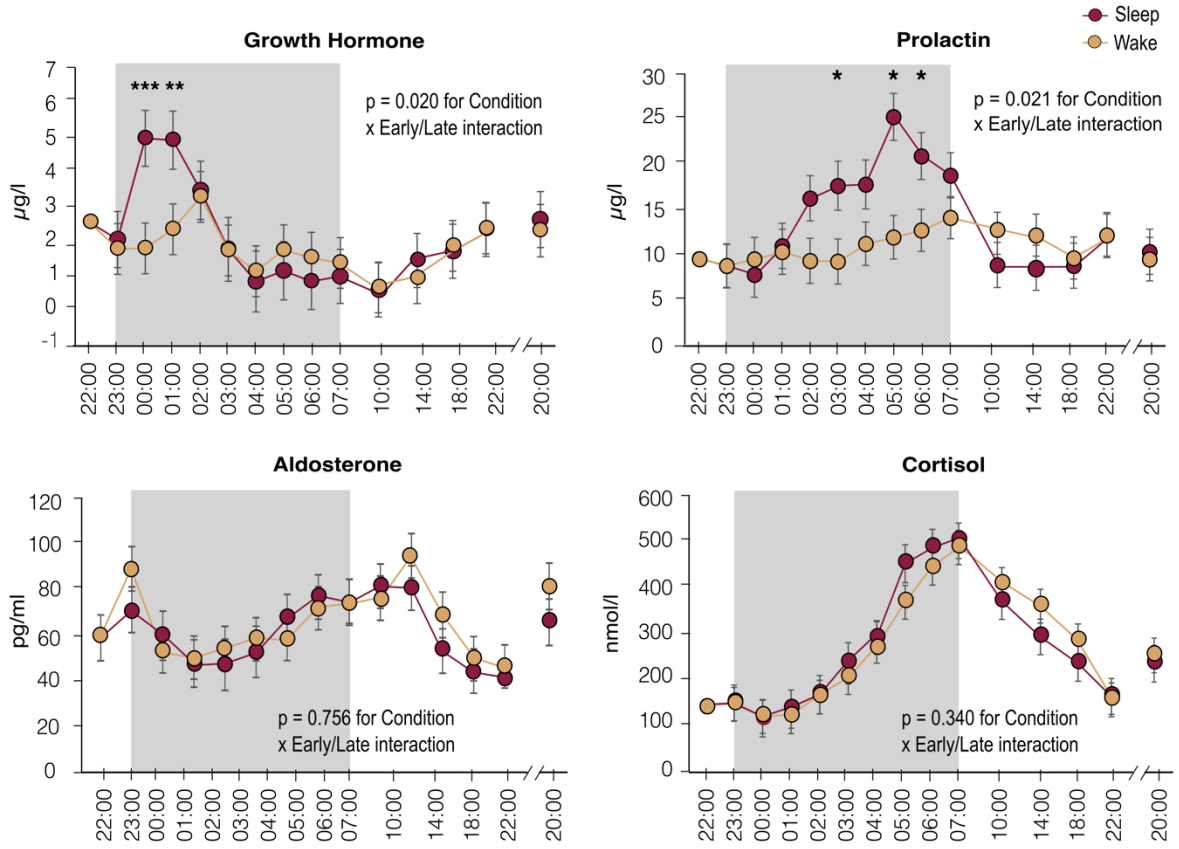


**Figure 15. Effects of sleep on GH, PRL, ALDO, and CORT levels.**

Estimated marginal means ± SEM of GH, PRL, ALDO, and CORT levels during a regular sleep-wake cycle (red circles) versus 24-hours of continuous wakefulness (yellow circles). Gray area indicates time in bed. n = 14. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparison between conditions.

## 4.4. Effects of sleep-like concentrations of GH and PRL on T-cell migration (Experiment II)

Hormonal changes hallmarking sleep have been considered a candidate mechanism for mediating the effects of sleep on peripheral immune functions (Besedovsky et al., 2019). In subsequent *in vitro* experiments, the effects of GH and PRL were examined as potential endocrine mediators of the sleep effect on T-cell migration, because these hormones were affected by the sleep manipulation in *Experiment I*. We focused on $T_N$ and $T_{CM}$ subsets, which

recirculate through LN and showed robust migration towards CCL19 (see ***Figure 12***). GH specifically increased the migration of total $CD3^+$ and $CD4^+$ T cells, as well as $T_{N4}$ and $T_{N8}$ towards CCL19 compared to a PBS control. PRL also promoted T-cell migration towards CCL19 with even broader effects than those of GH, including effects on all the subsets studied (***Figures 16*** *and* ***17, Table 3***).

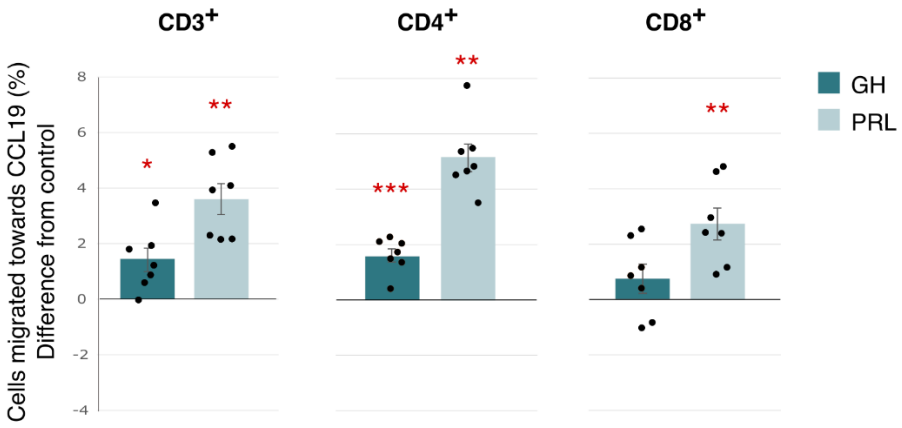


**Figure 16. Effects of GH and PRL on $CD3^+$, $CD4^+$, and $CD8^+$ T-cell migration towards CCL19.**

Means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the presence of the chemokine CCL19 (CCL19-directed migration) after incubating the cells with GH (dark green) and PRL (light green). Values are indicated as difference from the PBS control. n = 7. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between the control and the respective hormone (paired t tests) (see also Table 3).

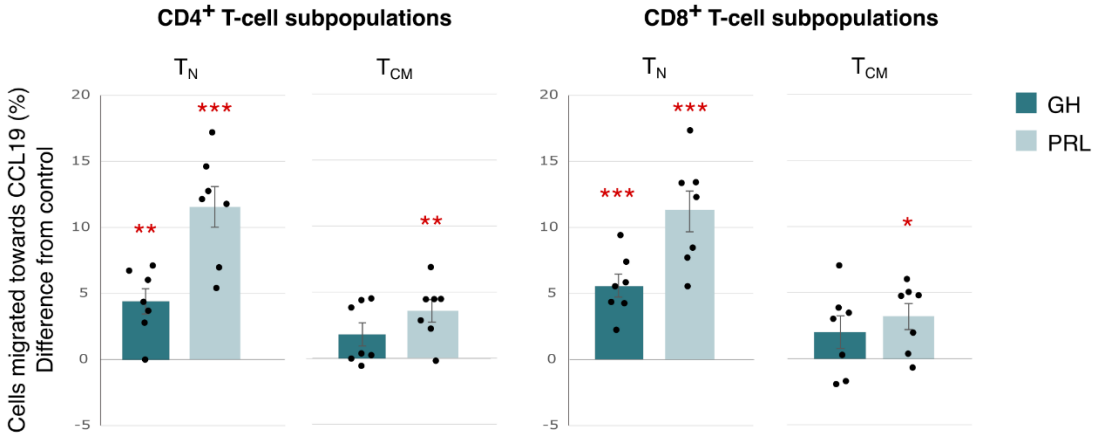

**Figure 17. Effects of GH and PRL on the migration of $T_N$ and $T_{CM}$ subsets of $CD4^+$ and $CD8^+$ T cells towards CCL19.**

Means ± SEM of the percentage of migrated $CD4^+$ and $CD8^+$ $T_N$ and $T_{CM}$ in the presence of the chemokine CCL19 (CCL19-directed migration) after incubating the cells with GH (dark green) and PRL (light green). Values are indicated as difference from the PBS control. n = 7. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between the control and the respective hormone (paired t tests) (see also Table 3).$T_N$: naïve T cells; $T_{CM}$: central memory T cells.

**Table 3. Percentage of different T-cell subpopulations migrated towards the chemokine CCL19 (mean ± SEM) after incubation with sleep-like concentrations of GH or PRL compared to their migration when incubating with a PBS control.**

| | Control | GH | PRL |
|---|---|---|---|
| $CD3^+$ | 6.68 ± 1.17 | 8.21 ± 1.28* | 10.41 ± 1.01** |
| $CD4^+$ | 7.53 ± 0.97 | 9.14 ± 0.94*** | 12.67 ± 0.57*** |
| $CD8^+$ | 7.30 ± 1.67 | 8.05 ± 1.69 | 10.03 ± 1.65** |
| $T_{N4}$ | 7.74 ± 1.66 | 12.14 ± 2.13** | 19.32 ± 1.73*** |
| $T_{CM4}$ | 8.85 ± 1.27 | 10.65 ± 1.04 | 12.43 ± 1.03** |
| $T_{N8}$ | 11.08 ± 1.68 | 16.65 ± 1.95*** | 22.29 ± 1.61*** |
| $T_{CM8}$ | 4.98 ± 0.80 | 6.98 ± 1.73 | 8.16 ± 1.24* |

n = 7. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between Control and the different hormone conditions (paired t tests). Control = PBS, GH = 10 ng/ml, PRL = 20 µg/l. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells.

None of the hormones of interest affected the spontaneous migration of T cells (***Figures 18** and **19, Table 4***).

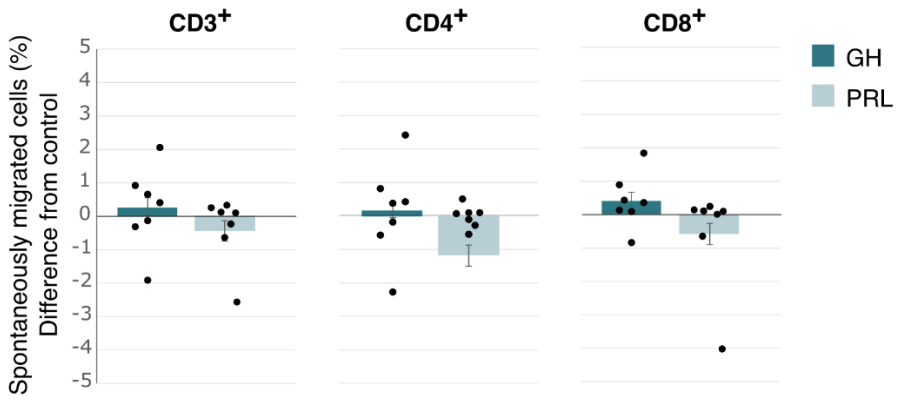


**Figure 18. Effects of GH and PRL on spontaneous migration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells.**

Means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the absence of chemoattractants after incubating the cells with GH (dark green) and PRL (light green). Values are indicated as difference from the PBS control. n = 7.

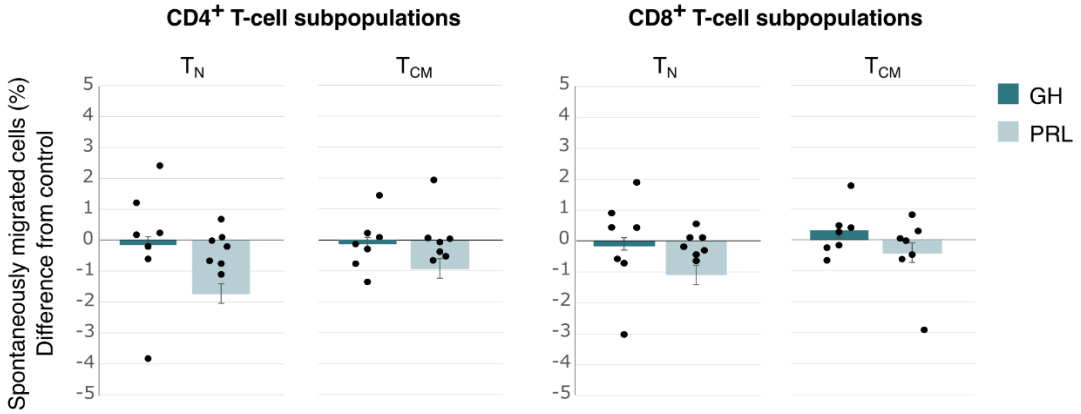


**Figure 19. Effects of GH and PRL on spontaneous migration of $T_N$ and $T_{CM}$ subsets of $CD4^+$ and $CD8^+$ T cells.**

Means ± SEM of the percentage of migrated $CD4^+$ and $CD8^+$ $T_N$ and $T_{CM}$ T cells in the absence of chemoattractants after incubating the cells with GH (dark green) and PRL (light green). Values are indicated as difference from the PBS control. n = 7. $T_N$: naïve T cells; $T_{CM}$: central memory T cells.

**Table 4. Percentage of different T-cell subpopulations migrated in the absence of a chemotactic stimulus (spontaneous migration) (mean ± SEM) after incubation with sleep-like concentrations of GH or PRL compared to their migration when incubating with a PBS control.**

| | Control | GH | PRL |
|---|---|---|---|
| $CD3^+$ | 4.52 ± 0.69 | 4.76 ± 0.45 | 3.57 ± 0.23 |
| $CD4^+$ | 5.06 ± 0.82 | 5.21 ± 0.26 | 3.87 ± 0.26 |
| $CD8^+$ | 3.49 ± 0.49 | 3.91 ± 0.37 | 2.91 ± 0.25 |
| $T_{N4}$ | 4.25 ± 0.97 | 4.17 ± 0.55 | 2.53 ± 0.29 |
| $T_{CM4}$ | 5.49 ± 0.71 | 5.38 ± 0.58 | 4.57 ± 0.39 |
| $T_{N8}$ | 3.62 ± 0.84 | 3.53 ± 0.55 | 2.53 ± 0.49 |
| $T_{CM8}$ | 4.67 ± 0.36 | 4.94 ± 0.29 | 4.27 ± 0.23 |

n = 7. Control = PBS, GH = 10 ng/ml, PRL = 20 µg/l. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells.

## 4.5. Effects of endocrine factors on T-cell migration (Experiment III)

To confirm that the impact of sleep on T-cell migration observed in the *Experiment I* was indeed mediated by endocrine factors, and specifically by GH and PRL, whole blood from healthy donors was incubated with plasma that had been previously collected at 2:00 h during the Sleep and the Wake condition of *Experiment I*. In addition, in separate tubes, blockers of the hormones mentioned above were added to the plasma from the Sleep condition. Whole blood from the donor participants was freshly collected at 18:00 h, when endogenous concentrations of the hormones of interest are low.

Incubation with the plasma derived from the Sleep condition specifically increased the CCL19-directed migration of total $CD3^+$, $CD4^+$, and $CD8^+$ T cells (***Figure 20, Table 5***) as well as of all subsets of interest (i.e., $T_{N4}$, $T_{N8}$, $T_{CM4}$, and $T_{CM8}$) (***Figure 21, Table 5***) when compared with plasma derived from the Wake condition. Adding antagonists of GH and PRL to the "sleep plasma" significantly decreased the migration towards CCL19 compared to incubation with the sleep plasma without antagonists in total $CD3^+$, $CD4^+$, $CD8^+$ T cells, $T_{N4}$, and $T_{N8}$.

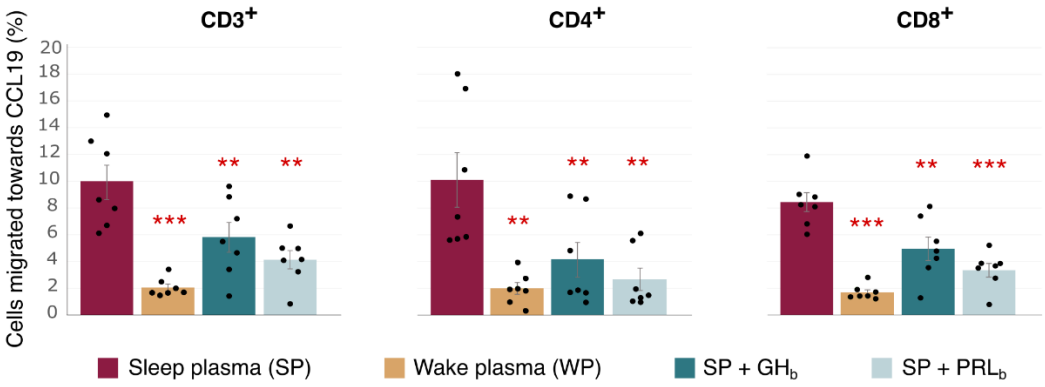


**Figure 20. Effects of plasma collected during sleep vs. wakefulness and of hormone receptor blockers on the migration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells towards CCL19.**

Means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the presence of the chemokine CCL19 after incubation with plasma from sleeping participants ("sleep plasma", SP, red bars), from awake participants ("wake plasma", WP, yellow bars), sleep plasma plus a growth hormone receptor blocker ($SP+GH_b$, dark green bars), or sleep plasma plus a prolactin receptor blocker ($SP+PRL_b$, light green bars). n = 7. **p < 0.01, ***p < 0.001 for pairwise comparisons between conditions (paired t tests).

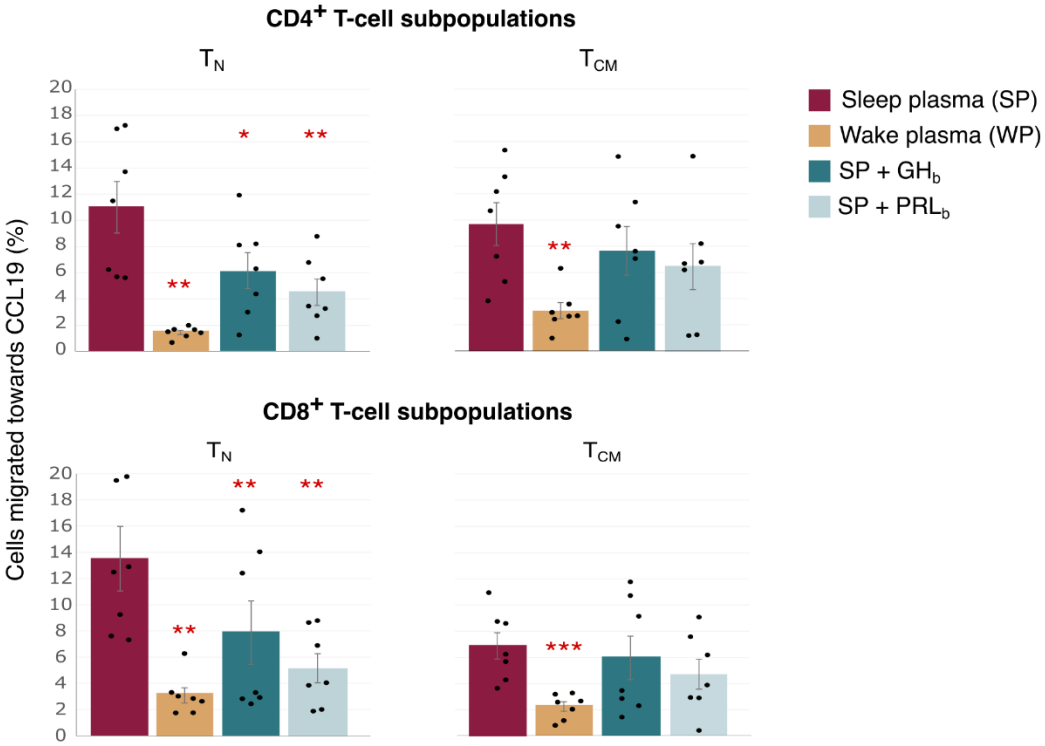

**Figure 21. Effects of plasma collected during sleep vs. wakefulness and of hormone receptor blockers on the migration of $T_N$ and $T_{CM}$ subsets of $CD4^+$ and $CD8^+$ T cells towards CCL19.**

Means ± SEM of the percentage of migrated $CD4^+$ and $CD8^+$ $T_N$ and $T_{CM}$ cells in the presence of the chemokine CCL19 after incubation with plasma from sleeping participants ("sleep plasma", SP, red bars), from awake participants ("wake plasma", WP, yellow bars), sleep plasma plus a growth hormone receptor blocker (SP+$GH_b$, dark green bars), or sleep plasma plus a prolactin receptor blocker (SP+$PRL_b$, light green bars). n = 7. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between conditions (paired t tests). $T_N$: naïve T cells; $T_{CM}$: central memory T cells.

**Table 5. Percentage of different T-cell subpopulations migrated towards the chemokine CCL19 (mean ± SEM) after incubation with plasma obtained during the Sleep condition (SP), plasma obtained during the Wake condition (WP), and "sleep plasma" plus hormone receptor blockers.**

| | **SLEEP plasma** | **WAKE plasma** | **SP + $GH_b$** | **SP + $PRL_b$** |
|---|---|---|---|---|
| $CD3^+$ | 9.91 ± 1.28 | 2.06 ± 0.25*** | 5.81 ± 1.11** | 4.14 ± 0.68** |
| $CD4^+$ | 10.09 ± 2.04 | 1.99 ± 0.44** | 4.12 ± 1.29** | 2.67 ± 0.83** |
| $CD8^+$ | 8.43 ± 0.71 | 1.66 ± 0.21*** | 4.94 ± 0.87** | 3.34 ± 0.51*** |
| $T_{N4}$ | 11.00 ± 1.96 | 1.33 ± 0.16** | 6.17 ± 1.36* | 4.51 ± 1.01** |
| $T_{CM4}$ | 9.69 ± 1.63 | 3.08 ± 0.61** | 7.64 ± 1.85 | 6.44 ± 1.75 |
| $T_{N8}$ | 13.51 ± 2.46 | 3.01 ± 0.58** | 7.87 ± 2.42** | 5.20 ± 1.11** |
| $T_{CM8}$ | 6.87 ± 1.00 | 2.25 ± 0.36*** | 5.95 ± 1.66 | 4.71 ± 1.14 |

n = 7. *p < 0.05, **p < 0.01, ***p < 0.001 for pairwise comparisons between "Sleep plasma" and other conditions (paired t tests). SP = Sleep plasma; $GH_b$ = Growth hormone blocker, $PRL_b$ = prolactin blocker. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells.

In contrast to the CCL19-directed migration, incubation of the cells with the plasma from sleeping participants did not affect the spontaneous migration in any of the T-cell subsets studied (***Figures 22*** and ***23, Table 6***).

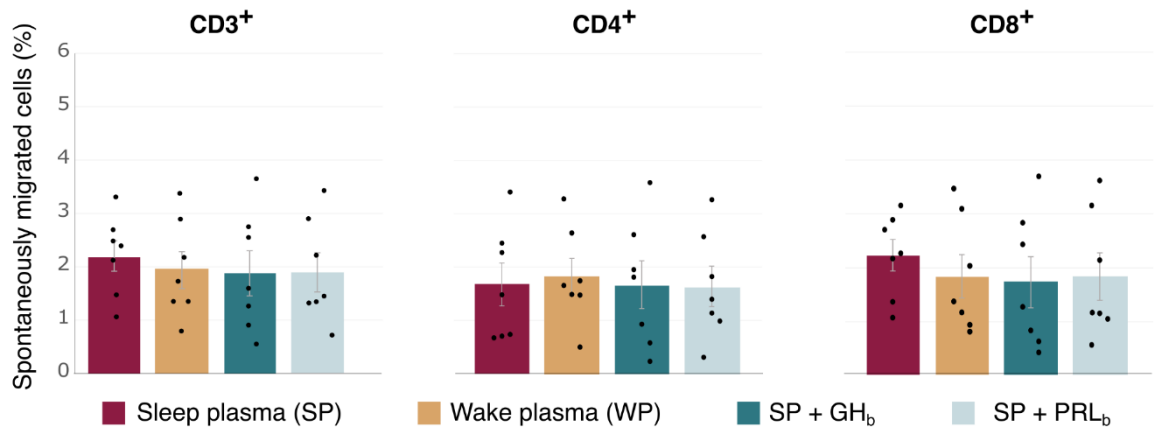


**Figure 22. Effects of plasma collected during sleep vs. wakefulness and of hormone receptor blockers on the spontaneous migration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells.**

Means ± SEM of the percentage of migrated $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the absence of chemoattractants after incubation with plasma from sleeping participants ("sleep plasma", SP, red bars), from awake participants ("wake plasma", WP, yellow bars), sleep plasma plus a growth hormone receptor blocker ($SP+GH_b$, dark green bars), or sleep plasma plus a prolactin receptor blocker ($SP+PRL_b$, light green bars). n = 7.

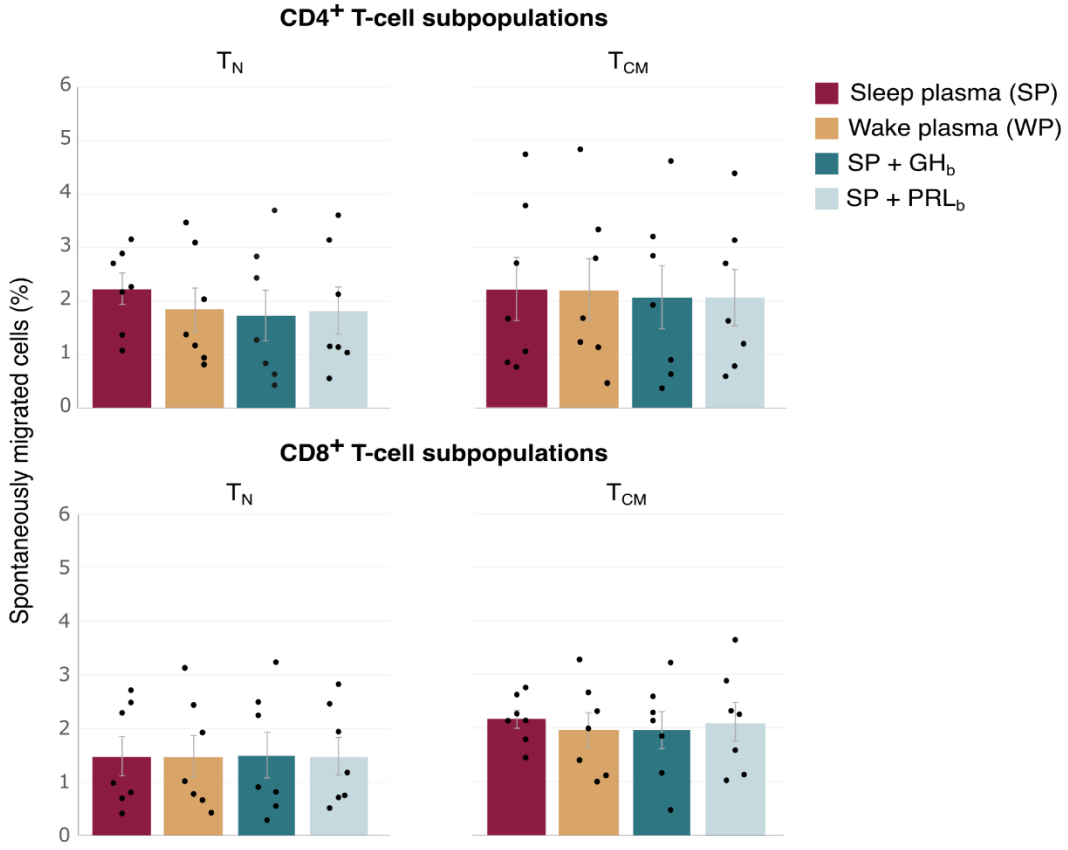

**Figure 23. Effects of plasma collected during sleep vs. wakefulness and of hormone receptor blockers on the spontaneous migration of $T_N$ and $T_{CM}$ subsets of $CD4^+$ and $CD8^+$ T cells.**

Means ± SEM of the percentage of migrated $CD4^+$ and $CD8^+$ $T_N$ and $T_{CM}$ T cells in the absence of chemoattractants after incubation with plasma from sleeping participants ("sleep plasma", SP, red bars), from awake participants ("wake plasma", WP, yellow bars), sleep plasma plus a growth hormone receptor blocker (SP+$GH_b$, dark green bars), or sleep plasma plus a prolactin receptor blocker (SP+$PRL_b$, light green bars). n = 7. $T_N$: naïve T cells; $T_{CM}$: central memory T cells.

**Table 6. Percentage of different T-cell subpopulations migrated in the absence of a chemotactic stimulus (spontaneous migration) (mean ± SEM) after incubation with plasma obtained during the Sleep condition (SP), plasma obtained during the Wake condition (WP), and "sleep plasma" plus hormone receptor blockers.**

| | **SLEEP plasma** | **WAKE plasma** | **SP + $GH_b$** | **SP + $PRL_b$** |
|---|---|---|---|---|
| $CD3^+$ | 2.20 ± 0.28 | 1.93 ± 0.34 | 1.87 ± 0.28 | 1.89 ± 0.11 |
| $CD4^+$ | 1.67 ± 0.40 | 1.82 ± 0.33 | 1.66 ± 0.44 | 1.63 ± 0.38 |
| $CD8^+$ | 2.23 ± 0.29 | 1.84 ± 0.40 | 1.73 ± 0.47 | 1.82 ± 0.43 |
| $T_{N4}$ | 1.53 ± 0.24 | 1.61 ± 0.39 | 1.45 ± 0.34 | 1.41 ± 0.29 |
| $T_{CM4}$ | 2.22 ± 0.59 | 2.21 ± 0.57 | 2.08 ± 0.59 | 2.07 ± 0.52 |
| $T_{N8}$ | 1.48 ± 0.36 | 1.49 ± 0.38 | 1.51 ± 0.42 | 1.49 ± 0.35 |
| $T_{CM8}$ | 2.16 ± 0.17 | 1.97 ± 0.32 | 1.96 ± 0.34 | 2.11 ± 0.35 |

SP = Sleep plasma; $GH_b$ = Growth hormone blocker, $PRL_b$ = prolactin blocker. $T_{N4}$: naïve $CD4^+$ T cells; $T_{CM4}$: central memory $CD4^+$ T cells; $T_{N8}$: naïve $CD8^+$ T cells; $T_{CM8}$: central memory $CD8^+$ T cells.

# 5. Discussion

For several decades, numerous studies have focused on the intricate relationship between sleep and immunity. Research conducted during this period has provided substantial evidence that disruptions in sleep can have significant effects on the adaptive immune system, such as reduced responses to vaccination, altered immune cell function, and altered inflammatory signaling, potentially impacting the body's vulnerability to infectious diseases (Garbarino et al., 2021; Ibarra-Coronado et al., 2015; Irwin, 2023; Schmitz et al., 2022). The scientific community dedicated to sleep research has increasingly focused on identifying the underlying biological mechanisms driving these effects. Despite these efforts, our comprehension of the dynamic interplay between sleep and immunity remains incomplete.

Several studies found that sleep compared to nocturnal wakefulness reduces the number of T cells in blood, with the cells potentially relocating to LNs where they encounter foreign antigens, triggering adaptive immune reactions (Lange et al., 2022). However, the exact destination of the cells as well as how these effects are mediated are still unknown. To answer these questions, experiments with healthy human participants were performed in the context of this thesis. In these experiments, each participant was exposed to a regular sleep-wake cycle in one condition and to 24 hours of wakefulness in another condition to investigate the role of sleep in the migration of T cells towards the LN-homing chemokine CCL19. Furthermore, consecutive experiments were carried out in which human T cells were incubated with GH and PRL, before measuring their migration towards CCL19, in order to decipher whether the sleep effect on T-cell migration was indeed mediated by the levels of GH and PRL that are characteristic of sleep.

Sleep compared to nocturnal wakefulness specifically promoted the migration of various T-cell subsets towards the chemokine CCL19, which is essential for LN homing and, thus, for the initiation and maintenance of adaptive immune responses. The CCL19-directed migration of several T-cell populations was affected by sleep, especially $CD3^+$, $CD4^+$, $CD8^+$, $N_4$ and $N_8$ T cells. Other groups have seen similar results in animals, where sleep-deprived mice presented a decreased expression of the genes *CCL19*, *CCR7*, and *CD62L*, crucial for immune cell homing, and lower lymphocyte counts in LNs (Ruiz et al., 2017; Tune et al., 2020). Sleep also increased the migration towards CCL19 of $T_{CM4}$ and $T_{CM8}$ populations, however without reaching statistical significance. This could be explained by the lower expression of CCR7 that these subsets present in comparison to naïve populations (Besedovsky et al., 2014). Finally, sleep did not affect the CCL19-directed migration in $T_{EM}$ and $T_{TE}$ subsets, which typically exhibit limited migration to LNs (Mahnke et al., 2013). Together, these findings suggest a role of sleep in promoting the migration of T cells to LNs.

Changes in hormonal activity during sleep have been proposed as a potential factor influencing peripheral immune functions (Besedovsky et al., 2019; Rolls et al., 2015). In *Experiment I*, GH and PRL were shown to be released in a strongly sleep-dependent manner, as demonstrated in previous studies (Besedovsky et al., 2012; Lange et al., 2010). The lack of effect of sleep on ALDO levels is in contrast with previous studies (Charloux et al., 2001; Charloux et al., 1999) and is likely due to the lower sampling rate in *Experiment I* compared to the previous studies. GH and PRL serve as pro-inflammatory signals, facilitating immune cell activation, proliferation, differentiation, and the production of pro-inflammatory cytokines (Haus, 2007; Kelley et al., 2007), thereby aiding the initiation of adaptive immune responses. In *Experiment II*, it was demonstrated that sleep-like concentrations of GH and PRL additionally promoted CCL19-directed migration. The effect of PRL was observed in all the T-

cell subsets studied. Notably, the impact of PRL on LN-homing had not been recognized previously, except in one animal study where PRL treatment increased $CD4^+$ and $CD8^+$ T-cells in axillary and inguinal mammary gland-draining LNs (Dill & Walker, 2017). These findings unveil an acute effect of physiological PRL levels on the homing potential of T cells to LNs, revealing a new role of PRL in promoting adaptive immune responses in humans. Sleep-like concentrations of GH enhanced the CCL19 directed-migration on all the subsets studied except for total $CD8^+$ T cells and $T_{CM}$. GH effects on T-cell migration have been widely studied in animals. *In vivo* experiments showed that GH favors the trafficking of naïve $CD4^+$ T cells to peripheral LNs in mice treated with GH intrathymically, and that GH-transgenic mice exhibit higher numbers of T lymphocytes in LNs as compared to wild-type controls (Smaniotto et al., 2010; Smaniotto et al., 2004).The results of this thesis significantly expand these animal experiments by demonstrating that physiological, sleep-like concentrations of GH rapidly enhance the LN homing potential of human T cells and their $T_N$ subsets.

The results of *Experiment III* demonstrate that the promoting effects of sleep on CCL19-directed T-cell migration can be mimicked *ex vivo* by a short-term incubation of T cells with plasma collected from sleeping participants. The effect of the plasma collected from the sleeping participants was reduced after addition of GH and PRL antagonists, suggesting that these two hormones mediated the sleep effects. Taken together, these series of experiments suggest that the effects of sleep on T-cell migration towards CCL19 were mediated by GH and PRL (***Figure 24***).

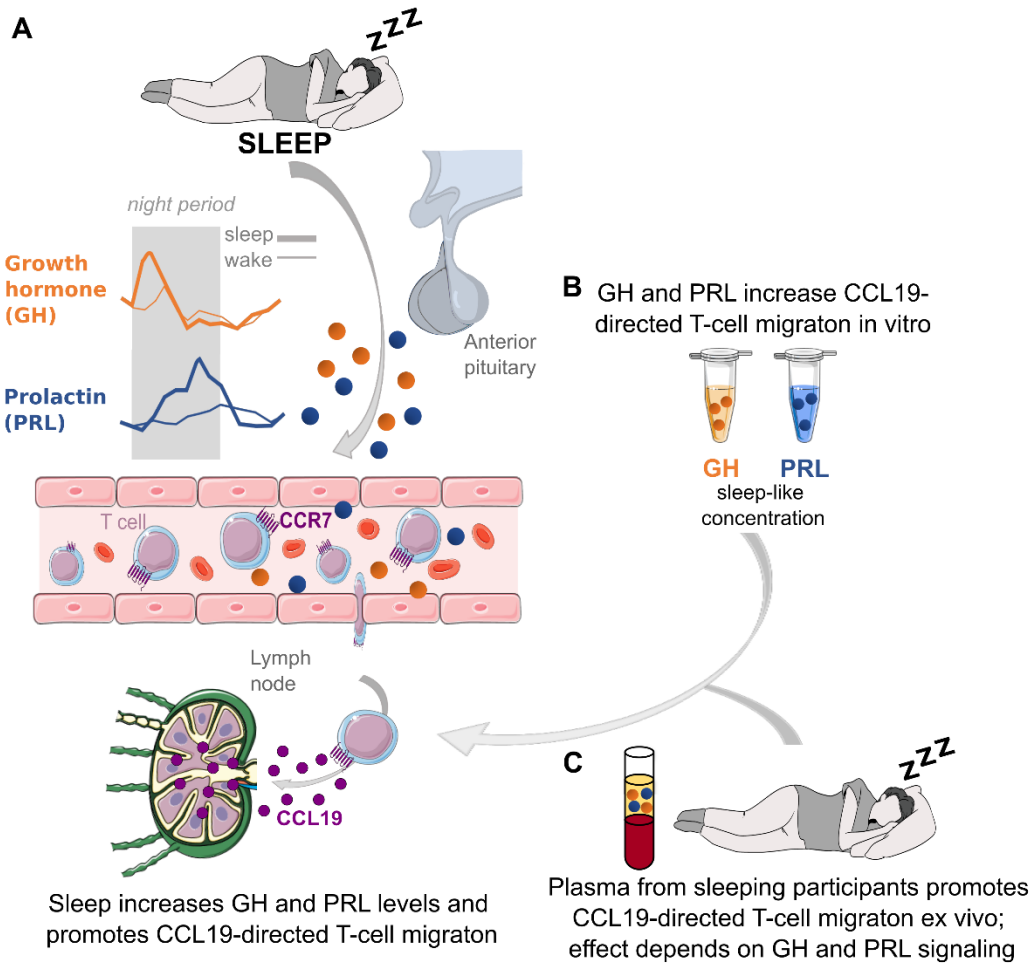


**Figure 24. Sleep enhances CCL19-directed T-cell migration via GH and PRL signaling.**

(**A**) **Concept**: Sleep promotes the migration of $T_N$ and $T_{CM}$ towards CCL19, an essential LN-homing chemokine. The receptor of this chemokine, CCR7 is highly expressed on these T-cell subsets. Nocturnal sleep is characterized by a specific hormonal milieu consisting of high levels of GH and PRL. These hormones act as mediators of the effects of sleep on T-cell migration. (**B**) *In vitro*, sleep-like concentrations of GH and PRL enhanced the migration of T cells towards CCL19. (**C**) Plasma from sleeping participants promoted CCL19-directed T-cell migration compared to incubation of the cells with plasma from awake participants. The effect depended on GH and PRL as adding antagonists of these hormones diminished the effect.

These findings lay the groundwork for forthcoming investigations into the molecular mechanisms underlying the effects of sleep on T-cell migration. One interesting molecule could be With-no-lysine kinase 1 (WNK1). Cell migration is a complex phenomenon characterized by cellular polarization, necessitating the dynamic regulation of receptors and adhesion

molecules. Additionally, it involves the coordinated generation and degradation of gradients of second messengers to facilitate the process effectively (Sánchez-Madrid & del Pozo, 1999). In particular, the migration of T cells induced by chemokines is distinguished by the polarization of the cell, resulting in the formation of a pseudopodium at the forefront of the moving cell. Within this structure, there exists a densely packed and remarkably dynamic network of branched actin filaments, which plays a vital role in facilitating the migration process (Dupré et al., 2015). This mechanism is in part controlled by cation-chloride cotransporters (CCCs). CCCs regulate intracellular chloride ($Cl^-$) concentrations in two manners: promoting the entrance of $Na^+$, $K^+$, and $Cl^-$ ions (through Na-K-Cl or Na-Cl cotransporters, NKCCs or NCCs, respectively) or the exit of $K^+$ and $Cl^-$ ions (through K-Cl cotransporters, KCCs) (Bhandage & Barragan, 2021). WNK1 is a serine-threonine kinase that phosphorylates KCC to inhibit its activity (Mercado et al., 2016), as well as NKCC and NCC to enhance their activity (Anselmo et al., 2006; Chávez-Canales et al., 2014), promoting the uptake of $Na^+$, $K^+$ and $Cl^-$ ions (Shekarabi et al., 2017). This results in ion and water influx, which is required for actin polymerization and T-cell migration (Boer et al., 2022). Recent research has shown that WNK1 is also expressed in T cells and is required for the homing of $CD4^+$ and $CD8^+$ T cells to LNs via CCR7 signaling (Köchl et al., 2016). Furthermore, it has been reported that WNK1 expression in the rat dorsal raphe neurons is higher during sleep than during wakefulness (Kim et al., 2018). Given the extensive cross-talk between the neural and the immune system, it could be possible that sleep also increases the expression of WNK1 in human T cells, leading to an accumulation of $Na^+$, $K^+$, and $Cl^-$ ions necessary for cell actin polymerization and migration.

Moreover, WNK1 is also related to GH and PRL. Similarly than with GH, sleep deprivation decreases blood levels of IGF-1 (Chennaoui et al., 2014), the principal mediator of GH which plays a crucial role in promoting cell growth and differentiation (Laron, 2001). It has

been shown that IGF-1 induces phosphorylation of WNK1, enhancing its activity (Cheng & Huang, 2011; Vitari et al., 2004). Additionally, rats treated with GH showed an increase in the phosphorylation of the co-transporter NKCC, one of the targets of WNK1 (Dimke et al., 2007). Likewise, PRL treatment in rats induced increased phosphorylation of the co-transporter NCC, another target of WNK1 (Rojas-Vega et al., 2015). Unfortunately, none of these studies measured the phosphorylation of WNK1. Future studies are required to clearly elucidate the exact mechanisms that promote the enhanced T-cell migration caused by sleep, whether sleep directly affects the expression of WNK1 and its function, whether sleep-like concentrations of GH and PRL increase phosphorylation of WNK1 and/or its targets, or both.

In contrast to CCL19-directed migration, the migration towards CCL5 remained unaffected by sleep in all the subsets studied. While CCL19 regulates migration to and within LN, CCL5 attracts T cells to sites of inflammation. The migration towards CCL5 was measured to investigate whether the impact of sleep on T-cell migration is specific to CCL19 or extends to chemokines in general. It has been shown that the chemotactic response to CCL5 varies among the different lymphocyte subsets (Stanford & Issekutz, 2003). Whereas human T cells with memory phenotype respond better to this inflammatory chemokine (Oppermann, 2004), naïve T-cells' transendothelial migration is less affected by the presence of CCL5 (Ding et al., 2000). The receptors for CCL5 (CCR1, CCR3, CCR4, and CCR5) are expressed across naïve, memory, and effector T cells, although not all receptors are uniformly expressed within each of these populations. CCL5 exhibits the highest affinity for CCR5 (Aldinucci et al., 2020) which is present on memory and effector T cells, but not on naïve T cells (Fukada et al., 2002; Qin et al., 1998; Sallusto et al., 1997). In contrast to CCR5 and CCR3, CCR1 and CCR4 are expressed on both naïve and memory T cells (Rabin et al., 1999; Song et al., 2005). This different expressions might explain why the directed migration towards CCL5, although observed in all

studied T-cell populations, was less pronounced in $T_N$ cells, which only express the receptor with lower CCL5 affinity.

Previous studies examining the role of sleep in CCL5 signaling focused on the chemokine concentration. In a recent study, a decrease in *CCL5* expression and in protein abundance as well as a downregulation of CCR5, one of the receptors of CCL5, has been shown in mice lung transcripts after 6 h of sleep deprivation, (Taylor et al., 2023). In humans, individuals with chronic insomnia disorder presented lower CCL5 levels in serum compared to good sleepers (Xia et al., 2021). Monocytes from patients with severe obstructive sleep apnea displayed a higher migration towards CCL5 measured with a Transwell® assay when the cells were incubated under hypoxia conditions compared to normoxia (Chuang et al., 2019). In certain diseases, the receptors for CCL5 are up-regulated, rendering T cells more responsive to CCL5 (Jo et al., 2003; Schaller et al., 2008; Wakugawa et al., 2001). However, an up-regulation of these receptors was not expected in our study sample.

In *Experiment I*, it was shown than sleep also enhanced the spontaneous migration (i.e., migration of the cells in the absence of a chemokine) of several T-cell populations. Spontaneous migration can arise due to various factors, including cell motility, interactions with the extracellular matrix, and intrinsic cellular processes (Gu & Rajewsky, 2008). This inherent migratory pattern enables T cells to explore a wide area in what may appear to be a disorderly manner (Miller et al., 2003; Textor et al., 2011). The primary purpose of T-cell motility is to search for their cognate peptide presented by APCs. However, the occurrence of these cognate APC within the pool of presenting cells is extremely rare, and there are no external cues available to assist the T cell in locating them (Krummel et al., 2016). One possible physiological significance of the effect of sleep on spontaneous T-cell migration could be to enhance the

likelihood to encounter their cognate antigens, since this is one of the purposes of the random walk pattern migration of T cells (Miller et al., 2003). The incubation of T cells with GH, PRL, or plasma from sleeping individuals did not affect spontaneous migration. This suggests that factors mediating the effects of sleep on spontaneous T-cell migration might not be present in the plasma.

It is important to mention that the effect of sleep on spontaneous and CCL19-directed migration of T cells persisted after a night of recovery sleep demonstrating the persistent nature of the effect. Other studies have shown that a single night of recovery sleep frequently fails to restore immune parameters alterations induced by short-term sleep deprivation (Besedovsky et al., 2019).

As demonstrated in prior research (Besedovsky et al., 2016; Born et al., 1997), the reducing effect of sleep on the number of circulating T cells becomes evident early in the night (i.e., at 2:00 h). Initially, this appears contradictory to the observed effects of sleep on the T-cell migratory potential, which appeared to emerge with some delay. However, this inconsistency in timing likely arises because sleep, by promptly enhancing T-cell extravasation during the night, leaves only those T cells in the circulation (and for these analyses) with diminished migratory potential. Consequently, this effect tends to obscure the true extent of the effect of sleep versus wakefulness on the migration potential at night. This explanation is supported by the earlier mentioned finding that incubation of T cells (collected at 18:00 h) with plasma obtained as early as 2:00 h at night from sleeping participants indeed facilitated T-cell migration.

# 6. Outlook and conclusion

## 6.1. Outlook

The work described in this thesis contributes to understand the complex relationship between sleep and the immune system, demonstrating that sleep selectively promotes the LN homing potential of T cells by increasing GH and PRL levels, thus revealing a fundamental mechanism underlying the supporting effect of sleep on adaptive immunity.

While the experiments carried out in the context of this thesis were not specifically designed to differentiate between effects of sleep manipulation on females versus males, future investigations could consider sex differences. In this thesis, the experiments were conducted during the same phase of the menstrual cycle in females to mitigate variability in sex hormones but lacked the statistical power to specifically investigate sex differences. For this reason, future studies should be designed to specifically examine sex differences in the influence of sleep on immune cell migration.

Additionally, the mechanisms underlying the observed changed in T-cell migration warrant further investigation. Future studies could delve deeper into the molecular pathways involved in mediating the effects of sleep on immune cell trafficking, potentially identifying novel therapeutic targets for immune-related disorders. One of these potential targets could be the molecule WNK1, which has been associated with T-cell migration via CCL19 and sleep (see *Discussion* section). Another possible molecular target mediating this effect could be CCR7, the receptor for CCL19 expressed on T cells, because the effects of sleep were specific to CCL19.

Furthermore, the experimental design used in the context of this thesis employed 24-hours of continuous wakefulness. This study design is useful to investigate the active role of sleep for the immune system compared with a condition without sleep. Nevertheless, this type

of sleep deprivation is not totally comparable to the more chronic forms of sleep deficiency occurring in the general population. Thus, future studies investigating how more prolonged and partial sleep deprivation affects T-cell migration are necessary.

Finally, translating these finding into clinical practice holds promise for improving patient outcomes. Investigating the efficacy of interventions aimed at improving sleep quality in individuals with compromised immune function could have significant implications for public health.

## 6.2. Conclusion

While the hypothesis that sleep enhances T-cell migration to LN has persisted for over two decades (Born et al., 1997), evidence supporting this concept in humans has been lacking so far. Collectively, the experiments conducted in this thesis provide compelling evidence that sleep specifically enhances the LN homing capacity of T cells by elevating levels of GH and PRL, thereby uncovering a fundamental mechanism underlying the supportive role of sleep in adaptive immunity. Furthermore, there are recent advances in knowledge regarding the impact of the CCL19-CCR7 axis on host immune responses (Yan et al., 2019). It has been shown that during some infections, CCR7 expression on T cells is decreased and, therefore, the cells present a less efficient chemotactic response to CCL19 (Kim et al., 2016; Potsch et al., 1999). Knowing that sleep specifically enhances the migration of T cells towards CCL19, something as “simple” as having sufficient sleep during an infection could promote the migration of T cells to LNs, an essential step for effective immune defense, resulting thus in a less severe presentation of the disease.

Findings uncovering acute effects of GH and PRL in physiological concentrations on CCL19-directed T-cell migration also have potential clinical implications. Thus, these

hormones could be considered as novel adjuvants for enhancing immune responses post-vaccination, especially in older individuals, who typically exhibit reduced levels of these hormones (Van den Berghe et al., 1998), along with impairments in sleep (Gulia & Kumar, 2018; Li et al., 2018), and vaccine-driven immune responses (Hainz et al., 2005; Murasko et al., 2002).

# 7. Summary

Sleep is a fundamental aspect of human life, occupying approximately one-third of our time. Its importance in maintaining overall health and well-being is widely recognized, including its role in regulating the immune system. Experimental studies demonstrate that individuals who sleep after vaccination exhibit significantly enhanced antigen-specific antibody and T-cell responses compared to those who remain awake, emphasizing the impact of sleep on immune defense mechanisms. The mechanisms behind this effect of sleep are still unclear. Based on the consistent finding that sleep reduces T-cell numbers in the bloodstream, one hypothesis is that sleep fosters T-cell migration to lymph nodes (LNs), where immune responses are coordinated. The effects of sleep on the endocrine system may mediate the impact of sleep on immune functions. During sleep, hormonal changes occur, with high growth hormone (GH) and prolactin (PRL) levels promoting a pro-inflammatory environment conducive to immune activation and response. This dissertation aimed to investigate the role of sleep in T-cell migration to LNs, which is crucial for adaptive immune responses. The experiments conducted within the scope of this dissertation sought to measure the migration of several T-cell subsets during normal sleep and nocturnal wakefulness, focusing on the LN-homing chemokine CCL19. Additionally, a second aim was to understand the role of the sleep-regulated hormones GH and PRL in potentially mediating the effects of sleep on T-cell migration by conducting experiments employing hormone concentrations comparable to those occurring under sleep conditions. It was hypothesized that sleep induces stronger T-cell migration towards CCL19 mediated by the sleep-related hormones GH and PRL.

To explore the effect of sleep on T-cell migration, three experiments were conducted within the context of this thesis. In *Experiment I*, participants underwent two 24-hour sessions in a sleep laboratory, with one session involving uninterrupted sleep and the other continuous wakefulness. Blood samples were collected regularly to assess T-cell counts and migration

patterns. Sleep was found to decrease the total number of circulating T cells and several subsets. Sleep also selectively enhanced the migration of various T-cell subsets towards the LN-homing chemokine CCL19, without affecting migration towards CCL5, a chemokine involved in inflammation. *Experiment II* explored the influence of the sleep-associated hormones GH and PRL on T-cell migration, revealing that GH and PRL increased migration towards CCL19. *Experiment III* confirmed that plasma from sleeping individuals enhanced T-cell migration towards CCL19, and this effect was mitigated by blockers of GH and PRL. These findings suggest a role for GH and PRL in mediating the effects of sleep on T-cell behavior.

Future research may delve into molecular pathways involved in these sleep effects, potentially identifying therapeutic targets for immune-related disorders. Translating these findings into clinical practice could lead to interventions aimed at improving sleep quality in individuals with compromised immune function, possibly improving patient outcomes and enhancing immune responses, particularly post-vaccination.

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