Impact of sleep on monocytes and infection

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1. Introduction

'Sleep helps healing' says a proverb; the following work is dedicated to this proverb.

1.1. The immune system

The immune system is a protection mechanism that can be found in almost every multicellular creature [Kayser and Biron 2016]. The main function is the protection against pathogens and harmful infections. In order to cope with this complex task, the immune system consists of different components. The first barrier for pathogens are special tissues, such as skin and also the intestine and lung epithelium, they provide a physical barrier for pathogens and prevent them from entering the body. When these are transcended, the cellular and chemical components of the immune system enter into force. The first line of defense is the innate immune system, which is antigen-unspecific. It includes the complement system and the phagocytic cells such as dendritic cells (DCs), monocytes, neutrophils, and macrophages [Fearon and Locksley 1996] [Hoffmann et al. 1999]. Second in line is the adaptive immune system, which is highly specific. The main cell types of adaptive immunity are T- and B-cells, that can identify specific pathogens and build up a life time memory [Fearon and Locksley 1996] [Hoffmann et al. 1999].

1.1.1. The innate immune system

The capability of uptake and destruction of pathogens is a critical function of several innate immune cells, which thus provide a first line of defense against endogenous infections. Professional phagocytes, such as neutrophils, DCs, macrophages, and monocytes, engulf large particles, such as microorganisms. Phagocytes need to identify pathogens, to provide effective protection. The outstanding mechanism of the innate immune system are the pattern recognition receptors (PRRs) which can be found on the cell surface or on intracellular cell compartments [Medzhitov and Janeway 1997]. The counterparts of PRRs are the pathogen-associated molecular patterns (PAMPs). PAMPs are highly conserved molecules that can be found on the surface of microorganisms [Takeda et al. 2003]. The most acquainted ones are lipopolysaccharides (LPS), a part of the outer membrane of gram-negative

bacteria, bacterial lipoproteins, and lipoteichoic acids, flagellin or non-methylated CpG in the DNA of bacteria and viruses. In particular for viruses, double-stranded and single-stranded viral RNA represents important ligands for PRRs [Heil et al. 2004] [Matsumoto et al. 2003].

All PRRs are able to detect microorganisms. Upon activation, they can lead to several reactions with the goal to destroy the invading pathogen. They can activate the complement system, lead to phagocytosis, kick off a pro-inflammatory pathway, or induce apoptosis [Diebold et al. 2004] [Lund et al. 2004]. These mechanisms allow the cells of the innate immune system to identify and clear a broad range of pathogens, but they are not able to activate a pathogen-specific immune response or form an immunologic memory. This is the part of the adaptive immune system. The importance of the innate immune system becomes obvious when considering that primitive organisms like insects survive completely without an adaptive immune system. Their immune functions depend highly on their phagocytes [Ribeiro and Brehélin 2006].

1.1.2. Phagocytes

A particularly important group of immune cells are phagocytes with their ability to recognize, engulf, and destroy pathogens. Thereby, phagocytes like DCs, neutrophils (polymorph nuclear cells, PMNs), macrophages, and monocytes, are the first line of defense against invading pathogens [Pasquevich et al. 2015] [Autenrieth et al. 2012] [Bieber and Autenrieth 2015].

1.1.2.1. Monocytes

Monocytes are precursors and effector cells at the same time [Yona and Jung 2010]. In mice, they can be identified by their expression of CD115 and CD11b. Furthermore, monocytes can be divided into two major subgroups, classical monocytes $Ly6C^{hi}CX3CR1^{lo}CCR2^{+}CD62L^{+}$ and non-classical-monocytes $Ly6C^{lo}CX3CR1^{hi}CCR2^{-}CD62L^{-}$ [Yona and Jung 2010] [Ginhoux and Jung 2014] [Auffray et al. 2009]. The same subgroups can be found in humans and mice. Classical and non-classical monocytes in human and mice share their main functions, half-live, and site of generation, but they differ in numbers and marker expression. Human monocytes subsets are characterized by their

expression of CD14 and CD16. Classical human monocytes are identified as $CD14^{+}$ and non-classical monocytes are identified as $CD14^{lo}CD16^{+}$ [Gautier et al. 2009].

Strongly discussed are the intermediate monocytes that can be found in humans. They are also called double positive monocytes as they are described as $CD14^{++}CD16^{+}$ $CCR2^{+}CX3CR1^{++}CCR5^{+}$. It is not yet clear if they are one subgroup with distinct functions or just transitional state from classical to non-classical monocytes [Thomas et al. 2017] [Yang et al. 2014] [Ziegler-Heitbrock 2007] [Merino et al. 2011] [Wong et al. 2011].

Tab.1: Monocyte subsets

Table by Ginhoux and Jung, 2014 [Ginhoux and Jung 2014].

Mouse monocyte subset	Human counterpart	Precursors	Site of generation	Half-life	Survival factors	Main functions
$LY6C^{hi}CCR2^{+}$ $CD62L^{+}CX_3CR1^{mid}$	$CD14^{+}$	MDPs and cMoPs	Bone marrow	20 hours	Unknown	Classical monocytes that are the precursors of peripheral mononuclear phagocytes
$LY6C^{low}CD43^{+}CX_3CR1^{hi}$	$CD14^{low}CD16^{+}$	$LY6C^{hi}$ monocytes	Circulation	5 days	CSF1R, NUR77 and CX_3CR1	Reside in lumen and survey endothelial integrity

Classical $Ly6C^{hi}$ monocytes are so-called inflammatory monocytes. In the case of an infection, these cells get recruited to the affected tissue, where they can further develop into macrophages or monocyte-derived DCs [Geissmann et al. 2003] [Palframan et al. 2001] [Serbina et al. 2003] [Sunderkötter et al. 2004]. But classical monocytes are not just precursors; they also play an important role in immune defense. As phagocytic effector cells, they can take up pathogens, destroy them, or deliver them to the lymph nodes (LNs). Monocytes also contribute to steady-state surveillance in various tissues [Rodero et al. 2015]. Furthermore, classical $Ly6C^{hi}$ monocytes are able to produce cytokines and therefore modulate the immune response. The typical cytokines that can be produced by monocytes are IL-1, IL-12, and TNF [Geissmann et al. 2003] [Randolph et al. 1999] [Bieber and Autenrieth 2015]. Their importance for immune defense against infection is further indicated by monocytosis, the increase of circulating classical monocytes during systemic or chronic infection

[Geissmann et al. 2003] [Sunderkötter et al. 2004] [Yrlid et al. 2006] [Tacke et al. 2007].

The non-classical Ly6C^{lo} monocytes differ from classical Ly6C^{hi} monocytes in several points. First, in marker expression: They miss the typical markers of classical monocytes (Ly6C, CCR2 and CD62L) but express CX_3CR_1, a fractalkine receptor, instead. Second, the life expectancy: The half-life of non-classical monocytes is five days, which is more than five times the half-life of a classical monocyte. Third, precursors: Non-classical monocytes develop mostly from classical monocytes outside of the bone marrow (BM). Fourth, in their tasks: They fulfill deviating functions in immunity. Classical monocytes are called inflammatory monocytes due to their important role in immune defense. Non-classical monocytes are also called "patrolling" monocytes. They patrol the endothelial barrier of blood vessels, for pathogens and dead cells [Geissmann et al. 2003]. In case of an infection or tissue damage, they recruit neutrophils to the place of the infection [Auffray et al. 2007]. Non-classical monocytes have also been shown to be involved in wound-healing through the expression of the vascular endothelial growth [Arnold et al. 2007] [Dal-Secco et al. 2015]. Fifth, in their role as precursor cells: For classical monocytes, it has been shown that they can give rise to non-classical monocytes, monocyte-derived DCs, and monocyte-derived macrophages [Ginhoux and Jung 2014]. Whereas it is shown, that non-classical monocytes do not differentiate into DCs but it is possible that they develop into resident macrophages [Jakubzick et al. 2008a]. Interestingly, a big difference in composition of these two subgroups in humans and mice has been described. Typically, in human classical monocytes are more common than non-classical, making up 85-95% of monocytes, whereas non-classical monocytes just make up 5-15%. In mice, the composition of classical and non-classical monocytes is much more balanced. Here each subgroup makes up 50% of monocytes [Gautier et al. 2009] [Strauss-Ayali et al. 2007]. Regardless of the difference in frequency, the gene expression profiles of monocyte subgroups are well conserved between mice and humans [Ingersoll et al. 2010] [Ingersoll et al. 2011].

1.1.2.1.1. Development of monocytes

Monopoiesis, the development of monocytes, starts in the BM where they arise from hematopoietic stem cells (HSCs) [Liu and Nussenzweig 2010]. HSCs have a self-renewal capacity and they are the origin of many immune cells [Bryder et al. 2006]. In the case of monocyte development, HSCs evolve into granulocyte–macrophage progenitors (GMP). GMP themselves give rise to monocyte and dendritic cell progenitors (MDPs) [Fogg et al. 2006] [Varol et al. 2007]. MDPs are the last precursors in this line that can give rise to DCs and monocytes. To generate monocytes, MDPs develop into common monocyte progenitors (cMoPs). cMoPs can than progress further into monoblasts, which give rise to promonocytes [Pasquevich et al. 2015]. Promonocytes are the direct precursors of Ly6C^{hi} classical monocytes. The classical monocytes can now leave the BM and circulate through the bloodstream. They themselves are also precursors for Ly6C^{lo} monocytes, monocyte-derived DCs, and monocyte-derived macrophages [Ginhoux and Jung 2014]. Alternatively, Ly6C^{lo} non classical monocytes can also develop directly from cMoPs. It is believed that these cells than stay in the BM [Ginhoux and Jung 2014].

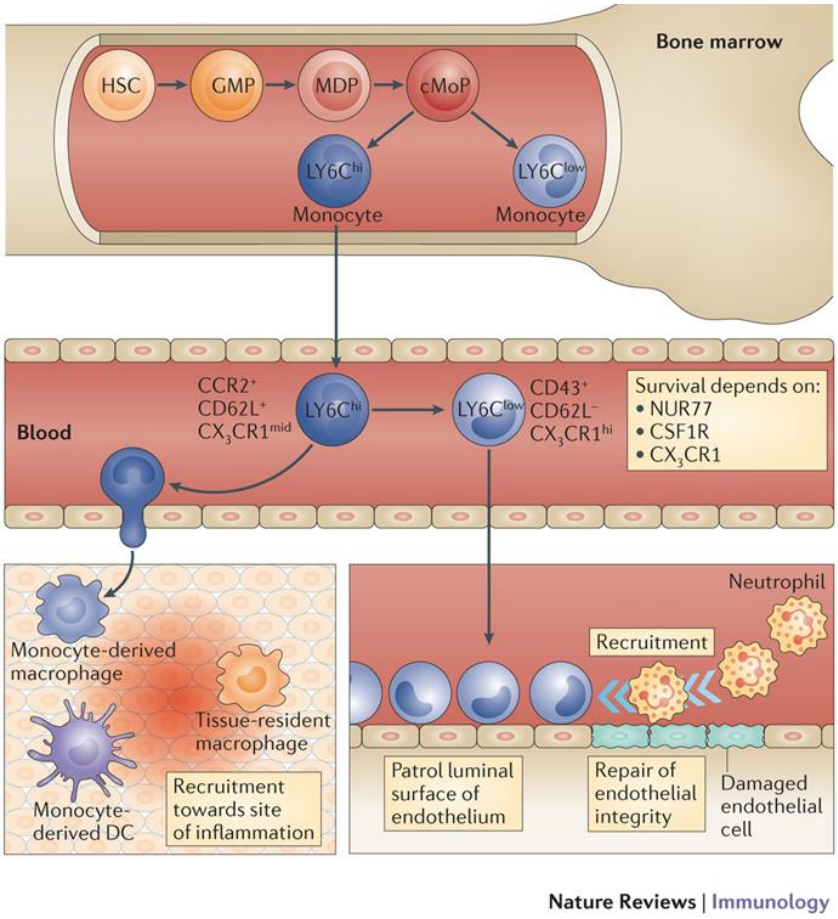

Fig. 1.1: Development of monocytes

Illustration by Ginhoux and Jung, 2014 [Ginhoux and Jung 2014].

1.1.2.1.2. Monocytes' migration

Monocytes arise in the BM but they can be found in the blood as well as in various organs and tissues. To get there, they first have to leave the BM, typically this is the case for classical monocytes. It has been demonstrated that they emigrate the BM in a C-C chemokine receptor type 2 (CCR2)-dependent manner [Serbina and Pamer 2006]. In CCR2 deficient mice monocytes accumulate in the BM. Even under inflammatory conditions, the monocytes do not leave the BM in these mice, which clearly demonstrates the importance of CCR2 [Serbina and Pamer 2006] [Scott and Flynn 2002]. The CCR2 and its ligands chemokine C-C motif ligand 2 (CCL2) or CCL7 (chemokine C-C motif ligand 7) are therefore responsible for the homeostasis of monocytes [Tsou et al. 2007]. After the release from the BM, monocytes have to find their way from the blood into different tissues, this is called homing. There is strong evidence, that CCR2 beyond emigrating of the BM also is involved in the homing of classical monocytes as well as in the infiltration of classical monocytes to the side of infection [Nahrendorf et al. 2007] [Serbina et al. 2008] [Serbina et al. 2003]. A strong indicator for CCR2 dependent trafficking of monocytes is the presence of CCL2 in sites of inflammation [Martin et al. 2006]. Given that CCL2 is a main ligand of CCR2 it seems logical that the interaction between these two is important for monocytes trafficking.

Furthermore, it was shown that CCR2-dependent migration of monocytes is required for the recruitment of monocytes from the blood to the gut during colitis [Platt et al. 2010] as well as for trafficking to the brain during an infection with the West Nile virus [Lim et al. 2011].

For non-classical monocytes, different chemokine receptors seem to be important for their migration. The main one known today is C-X3-C chemokine receptor 1(CX3Cr1), which is strongly expressed on non-classical monocytes. It binds to chemokine C-X-C motif ligand 1 (CX3CL1), which can be found on endothelial cells [Arnold et al. 2007]. But the role of CX3C1 is not completely understood yet, as studies also suggest that CX3CR1 is a survival factor and important for non-classical monocyte homeostasis [Jakubzick et al. 2008b] [Landsman et al. 2009].

Despite their differences, classical and non-classical monocytes also share similarities. This is especially the case for their migration behavior as they are both found to crawl or roll along blood vessels before extravasation [Gerhardt and Ley 2015] [Moore et al. 2013]. An important factor for crawling is the lymphocyte function-associated antigen 1 (LFA1) [Woollard and Geissmann 2010]. Interestingly LFA1 is expressed by classical and non-classical monocytes [Shi and Pamer 2011] [Auffray et al. 2007] [Carlin et al. 2013]. LFA1 has been in the center of many studies revealing that it can bind to several ligands, including ICAM1, ICAM2, ICAM3, and JAM-A [Fougerolles et al. 1993] [Marlin and Springer 1987] [Ostermann et al. 2002]. Especially ICAM1 and ICAM2 seem to be important for the attachment of monocytes to the epithelium, as blocking of these adhesion molecules leads to a reduction of crawling and transmigration of monocytes. Beside these results, it does not seem to be that simple as many other molecules also play a role in monocyte adhesion or extravasation including integrins, immunoglobulin superfamily, selectins, and chemokine receptors [Gerhardt and Ley 2015]. More research is needed to fully understand the complex network of monocyte migration.

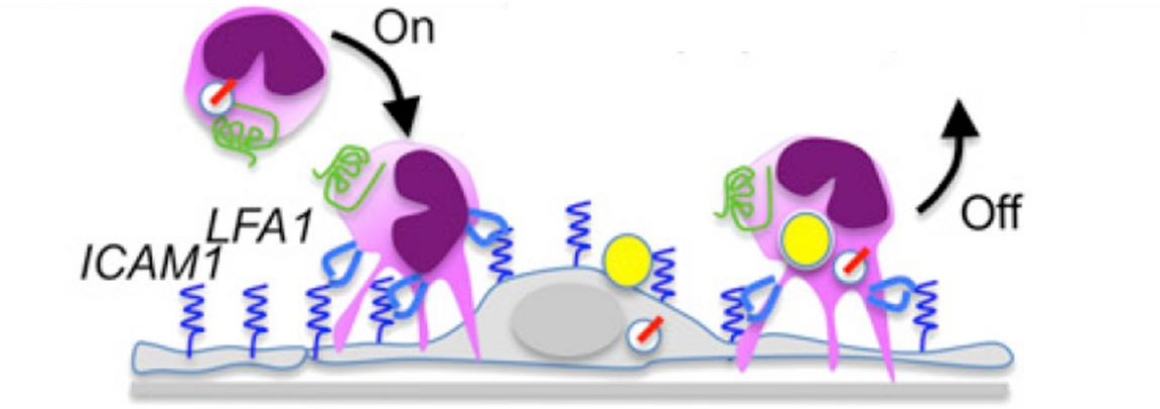

Fig. 1.3: Schematic model of the interaction of monocytes with the endothelium in a steady state

Illustration by Carlin *et al.* 2013 [Carlin et al. 2013].

1.1.2.2. Dendritic cells

Dendritic cells (DCs) belong to the group of antigen-presenting cells (APCs). They have first been discovered in the skin in 1868 by Paul Langerhans, but it took until 1973 until they were first described by Ralph M. Steinman [Steinman and Cohn 2007] [Banchereau and Steinman 1998]. They are the link between the innate and the adaptive immune system, as their main task is the initiation and regulation of immune responses and formation of an immunological memory. There are many subsets of DCs in a variety of tissues but still, most DCs in mice can be identified as $CD11c^{+}MHCII^{+}$ cells [Bell et al. 1999] [Hart 1997] [Steinman 1991]. The development of DCs starts in the BM. They derive from the same precursors as monocytes, $lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ hematopoietic stem cells (LSKs), common myeloid progenitor (CMP), and MDP. MDPs are the last shared precursors for monocytes and DCs. Due to Fms-like tyrosine kinase 3 ligand (Flt3L), MDP can give rise to common DC progenitor (CDP) and further to pre-DCs. Pre-DCs migrate out of the BM and then develop into DCs. Alternatively, CDP can already leave the BM and develop into plasmacytoid dendritic cells (pDCs) [Pasquevich et al. 2015] [Bieber and Autenrieth 2015]. The development of monocytes and DCs is a sensitive balance that can be effected by infection as shown by Pasquevich *et al.*

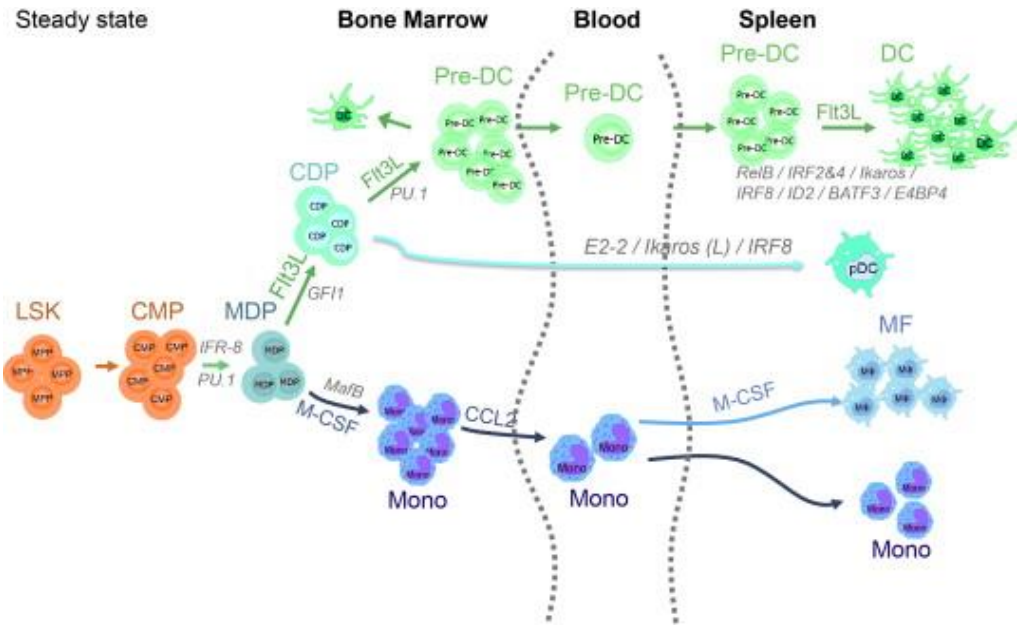

Fig. 1.3: Development of DCs and monocytes in steady state

Illustration by Pasquevich *et al.* 2015 [Pasquevich et al. 2015].

1.1.2.3. Neutrophils

Neutrophils or polymorph nuclear cells (PMNs) also develop in the bone marrow from hematopoietic stem cells (HSCs). They have a short live-time from 5 to 90 hours [Tak et al. 2013]. PMNs can be found in the bloodstream, from where they are able to migrate into various tissues [Witko-Sarsat et al. 2000] [Nathan 2006]. Neutrophils are the first phagocytes to migrate towards sites of infection in a CXCL1-dependent manner [Summers et al. 2010] [Yoo et al. 2011]. Key functions are the phagocyte microbes and the killing of pathogens. They can kill pathogens by the production of reactive oxygen species (ROS), antimicrobial peptides, or by the expulsion of their nuclear contents which leads to the formation of a neutrophil extracellular trap [Mayadas et al. 2014].
In humans, they account for up to 70% of all circulating leukocytes, and not surprisingly, they play a major role in host defense against bacteria, fungi, and protozoa infection [Edwards 2005]. It was believed that neutrophils are only present during the acute phase of the immune response and their only function is to kill by phagocytosis but more recent studies have shown that they play a bigger role in the immune defense. Neutrophils have been found to influence the immune landscape due to interaction with macrophages, dendritic cells, and also cells of the adaptive immune response [Kolaczkowska and Kubes 2013].
Neutrophils in mice can be characterized by the expression of Ly6G and CD11b.

1.1.2.4. Macrophages

Macrophages were discovered in 1884 by the zoologist Élie Metchnikoff [Cavaillon 2011]. Nowadays, macrophages are known to be central players in innate and adaptive immunity [Varol et al. 2015]. They develop from specific monocyte-progenitors [Ginhoux and Jung 2014]. Macrophages can engulf and digest large particles like cellular microbes, cancer cells, or diseased cells. Unlike PMNs that can mostly be found in the blood, macrophages are found in essentially all tissues, for example in the lung and the LP [Varol et al. 2015]. Due to their occurrence in such a variety of sites, macrophages can be found in

different forms and subtypes. They can be identified by their specific expression of CD14, CD40, CD11b, CD64, and F4/80 [Khazen et al. 2005]. Even as macrophages derive from monocytes they differ in many points as recently highlighted by Jung [Jung 2018]. He describes them as hare and tortoise, with monocytes being the hare, short-lived and fast, whereas the macrophages are the tortoise with a longer life span but less mobile.

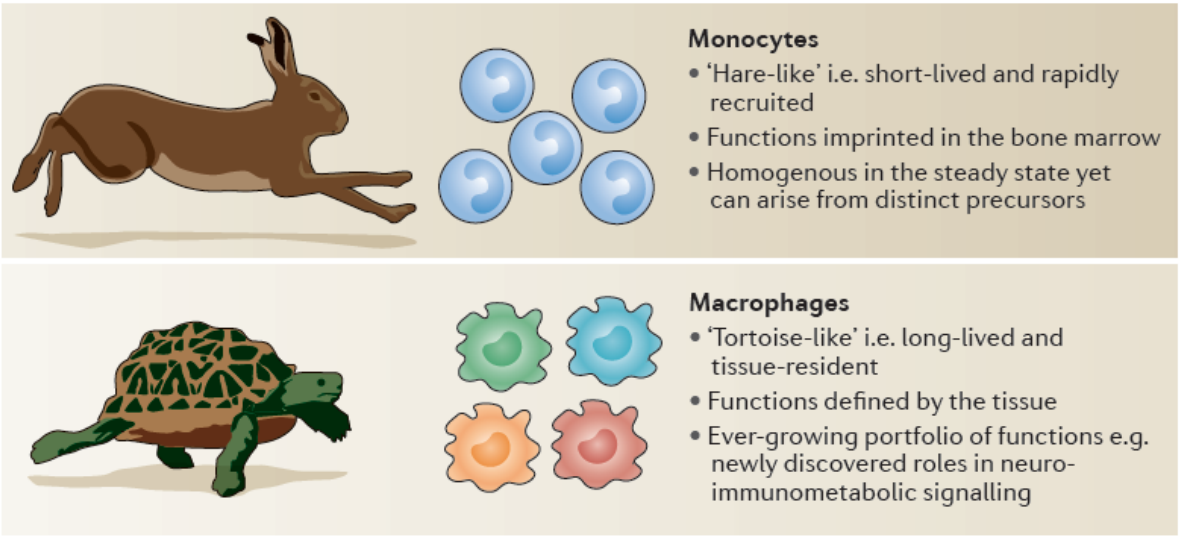

Fig. 1.4: Monocytes and macrophages comparison

Illustration by Jung, 2017 [Jung 2018].

1.1.3. Reactive oxygen species

Phagocytes must be able to efficiently kill engulfed pathogens. This task is taken over by reactive oxygen species (ROS). Several molecules can be determined as ROS, for example, oxygen, superoxide, hydrogen peroxide, and hydroxyl radical [Nathan and Cunningham-Bussel 2013]. Nevertheless, ROS is always produced by reduction of oxygen by oxygenase like NADPH oxidase (NOXs). The production of ROS can be initiated by chemokines, phagocytosis, and granulocyte-macrophage colony stimulating factor (GM-CSF), all of which have important roles in immune signaling [Nathan and Cunningham-Bussel 2013]. ROS, is a major contributor to the first line of defense of the innate immune system against pathogens. The assumption is supported by the immune deficiency that can be observed in patients with defects in ROS production [Fang 2004].

1.2. The marginal pool

Most immune cells have the ability to migrate. That means very often, that they leave the bloodstream in order to migrate into various tissues, which is achieved by a reversibly adhesion to the endothelium of the blood vessels [Nazziola and House 1992] [Schmidt et al. 1990] [Mayrovitz 1992]. This has been described as 'rolling ' or 'crawling' and is mediated by adhesion molecules such as ICAM-1, vascular cell adhesion molecule 1 (VCAM-1), CX3Cr1, and E-selectin [Allan and Rothwell 2001] [Ban 1994] [Bjerknes et al. 1986]. Rolling of immune cells can be found for different organs including lymphoid and non-lymphoid organs, Peyer's patches, spleen, lung, and the skin. This whole process of adherence and rolling plays an important role in immune cell immigration, but it can also lead to the formation of the so-called marginal pool [Hogg 1987] [Anderson et al. 1991] [Peters et al. 1985]. This second intravascular compartment has been found for granulocytes, B and T lymphocyte, NK-cells, as well as monocytes [Williams et al. 1987] [Klonz et al. 1996]. It means that cells of the immune system stay attached to the endothelium and therefore cannot be found in the peripheral blood or in tissues. Especially for monocytes it was shown that the marginal pool can be blocked by aL integrin antibodies, which leads to a 50% increase of non-classical monocytes [Auffray et al. 2007].

Therefore, the marginal pool is a possible explanation for quick alterations of cell number after stress or as an effect of the circadian rhythm [Druzd et al. 2017]. These alterations are often too quick to be caused by the release of cells from the BM, lymphoid organs, or cell migration [Nieman et al. 1992] [Gabriel et al. 1992]. Unfortunately, there are very few studies dedicated to this topic. One reason why the marginal pool is not studied more intensely could be because of technical challenges to isolate the cells of this second intravascular compartment as shown in the study by Klonz *et al.* from 1996. They analyzed the marginal pool in rats and to do so they first had to remove all peripheral blood from the animal by extensive perfusing of the rats with 100 ml cell-free medium. Second, they performed an additional perfusion with 200 ml cell-free medium to isolate cells of the marginal pool [Klonz et al. 1996]. Even though the studies on the marginal pool are very limited the marginal pool itself could play

an important role in immune function. It regulates the cell numbers in the blood as well as in various organs. Furthermore, the marginal pool contains functional cells that are able to influence the immune response of the body [Klonz et al. 1996].

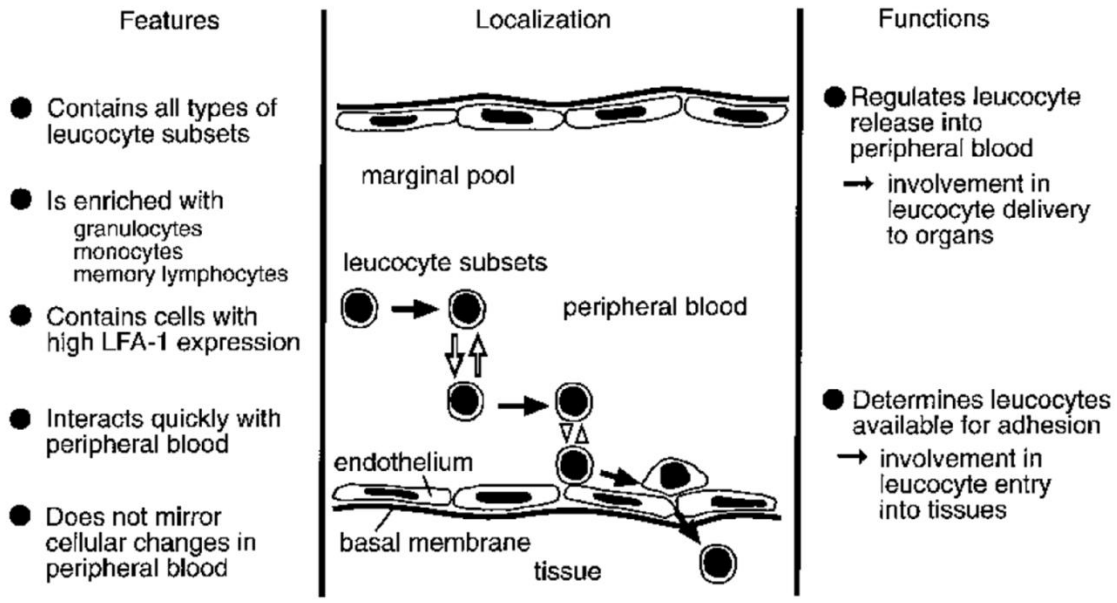

Fig. 1.5 The marginal pool

Illustration by Klonz *et al.* 1996 [Klonz et al. 1996].

1.3. Sleep

Sleep is an essential behavior that can be observed in all animals [Kayser and Biron 2016]. Although sleep behavior is an universal behavior and humans spend more than 30% of their life sleeping, little is known about the purpose of sleep or its importance for various bodily functions including immunity [Ganz 2012] [Besedovsky et al. 2012] [Irwin 2015]. Sleep consists of different stages which first were identified as non-rapid-eye-movement sleep and rapid-eye-movement sleep [ASERINSKY and KLEITMAN 1953]. Today sleep is being divided into five different stages [Rechtschaffen and Kales 1968]. See tab. below:

Tab.1.2: Sleep stages [Ganz 2012]

Stage		State
Non-rapid-eye-movement	Stage 1	Transitional state between wakefulness and sleep
	Stage 2	Asleep but easily arousable
	Stage 3	Slow-wave sleep Deep sleep Fast and slow brain waves
	Stage 4	Slow-wave sleep Deep sleep Primarily slow brain waves
Rapid-eye-movement	Stage 5	Can include dreaming state

The first sleep phase, the non-rapid-eye-movement (NREM) sleep, can be divided into four different stages. The first two are characterized as so-called light sleep. Stage 1 is the transition state between wakefulness and sleep. In stage 2, the sleeper is already unaware of the surrounding but can be woken up easily. Within the NREM the deep sleep stages are situated. Deep sleep or slow-wave sleep (SWS) can be detected via the electroencephalogram (EEG) on the basis of their low-frequency, high-amplitude waves of brain activity. Stage 3 and 4 represent the SWS. In these stages it is difficult to wake up a sleeping person [Frisk and Nordström 2003] [Zisapel 2007]. SWS has been linked to physiological repair and immune function [Hardin 2009].

The last stage is the rapid-eye-movement (REM), this stage can be identified based on the typical ragged eye movements. REM is the sleep stage in which dreaming occurs.

These five stages fulfill cycles, five to six cycles can occur per night. A typical cycle lasts for 90 minutes. Although the sleep patterns are species-specific, they are comparable with regards to NREM sleep that accounts for most of the sleep in all animal species and in humans [Lorton et al. 2006]. Fig. 1.6 illustrates a normal sleep pattern over one night in humans.

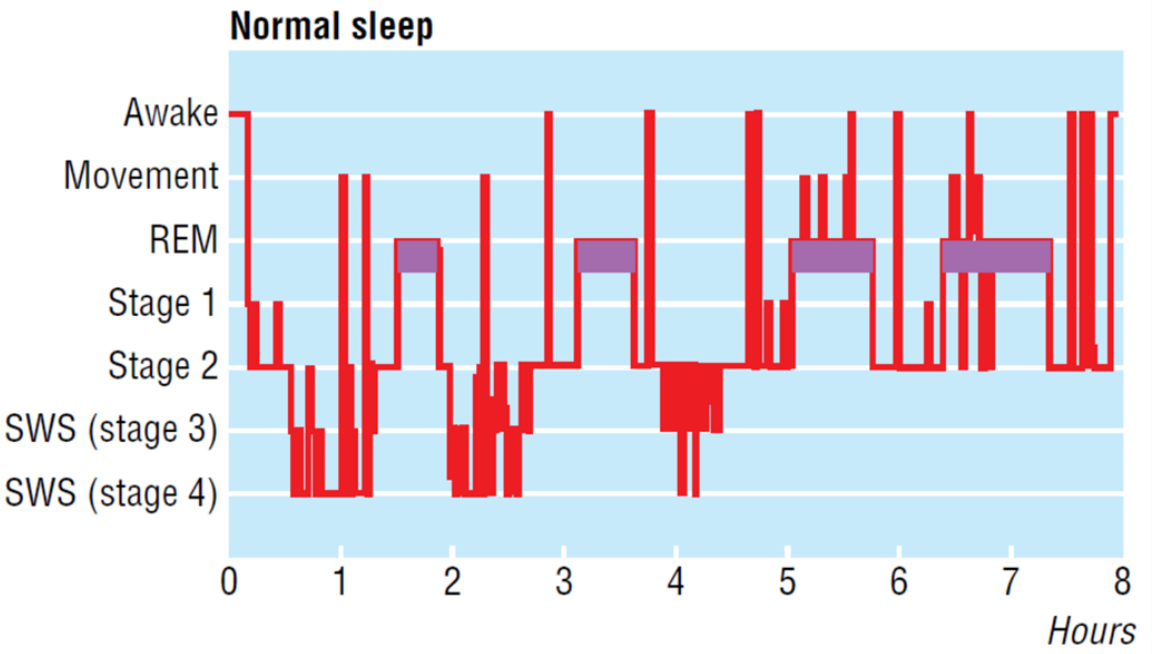

Fig 1.6: Sleep stages
Illustration by Zeman *et al*. [Zeman et al. 2004]. Depicted is the normal cyclical of sleep stages over the course of the night. Purple bars indicate rapid-eye-movement (REM) sleep.

1.3.1. The sleep and the circadian rhythm

The circadian rhythm and sleep are inextricably linked as sleep occurs in a sleep-wake cycle of 24 hours (h). This cycle controls nearly all body functions, including the temperature, hormonal secretion, metabolism, and immunity [Blask 2009]. The system is controlled by the central pacemaker, the suprachiasmatic nucleus (SCN). The SCN is located in the hypothalamus and synchronizes the biological clock that can be found in every body cell. The decisive factor for this machinery is light. A reduction of the light intensity leads to the production of melatonin.

Melatonin itself is a sleep-promoting factor, that can be found in day-active animals [Bollinger et al. 2010] [Zisapel 2007]. Recent studies have shown a strong impact of the circadian rhythm on immune cells [Scheiermann et al. 2013]. Fig. 1.3 summarizes the effect of the circadian rhythm on different immune cells in humans. T-helper (Th) and cytotoxic T-cells (CTL) were found to have a maximum cell count in the blood at nighttime. Whereas epinephrine levels and cell counts of proinflammatory monocytes, effector cytotoxic T-cells, and cytotoxic natural killer cells have a daytime maxima [Lange et al. 2010]. The interaction between the central SCN clock and immune cells is not fully understood yet, but it is assumed to work via humoral and neural signals [Nader et al. 2010].

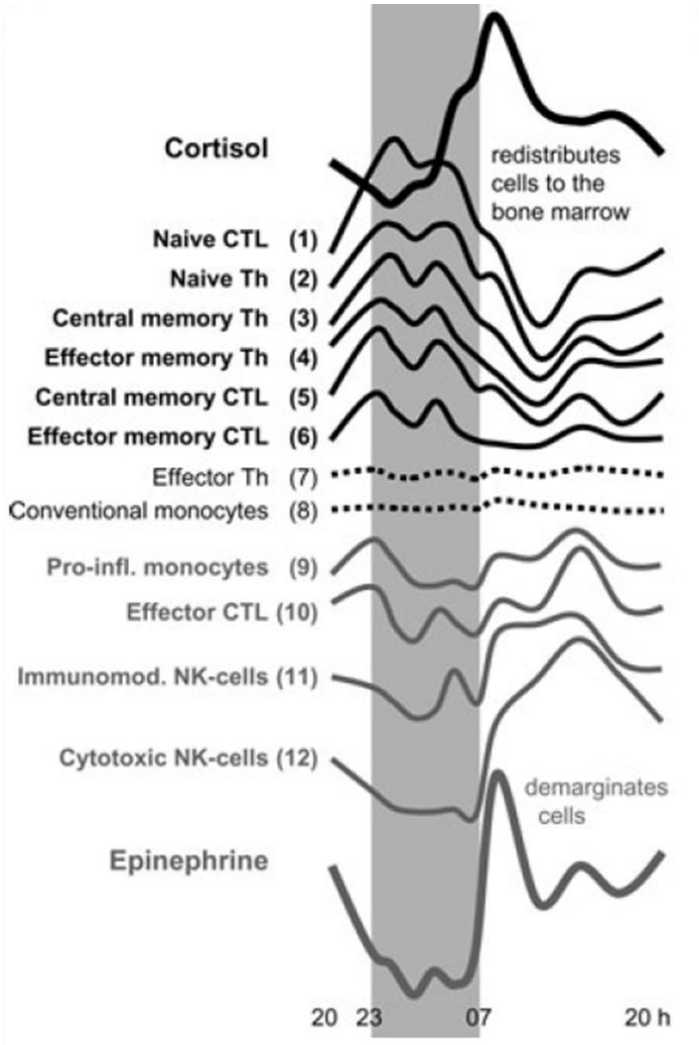

Fig. 1.7: Leukocytes and their rhythms over 24 h in healthy young men

Adopted from Lange *et al*. [Lange et al. 2010]. This figure shows a variety of immune cells as well as cortisol levels and epinephrine levels over a 24 h cycle. Grey areas represent the sleep time.

1.3.2. Sleep and the immune system

The assumption that 'sleep helps healing' can be supported by several studies, that found a link between sleep and the immune system. In the last decades it could be shown that sleep has an impact on the humoral and cellular immunity as well as on memory formation of the immune system. One of the key communication agents of the immune system are cytokines. Many types of cells, including neurons and immune cells, have receptors for various cytokines. Consequently, cytokines have been associated with sleep in animals and humans, including interleukins (ILs) 1, 2, 4, 6, 8, 10, 13, and 18; interferons α, β, and γ; transforming growth factor; and tumor necrosis factor (TNF)-α [Opp 2006] [Imeri and Opp 2009], see Tab. 1.2. In particular, IL-1 and TNF-α have been shown to correlate with sleep behavior. Neurons in the hippocampus, hypothalamus, brain stem, and cortex have receptors that allow them to interact with IL-1 and TNF-α [Kapsimalis et al. 2008] [Imeri and Opp 2009] [Allan and Rothwell 2001] [Ban 1994]. It was also found that the administration of IL-1 and TNF-α can affect sleep in different species. Depending on the given doses IL-1 and TNFα can lead to an increase NREM sleep in low doses and reduced it in higher doses [Opp 2006] [Imeri and Opp 2009] [Baracchi and Opp 2008].

Tab: 1.3: Influence of cytokines on sleep [Ganz 2012]

[Ranjbaran et al. 2007]

Cytokines that enhances sleep	Cytokines that inhibits sleep
IL-1(low doses)	IL-1(high doses)
IL-2	IL-4
IL-8	IL-10
IL-18	IL-13
TNF-α (low doses)	TNF-α (high doses)
IFN-γ	TGF-β

A variety of studies have investigated the impact of natural variations in sleep time on immune functions. Therefore, participants were grouped according to

their sleep duration. Three groups were formed: Short sleepers with less than 6 hours sleep per night, normal sleepers with 7 to 9 hour per night, and long sleepers with more than 10 hours per night. Cohen *et al.* compared short sleepers and normal sleepers [Cohen et al. 2009]. They exposed both groups to rhinoviruses and monitored them for 5 days afterwards. They found that short sleepers have an elevated risk of getting a cold. Other studies have also shown that short sleepers are associated with a higher risk of diabetes [Gottlieb et al. 2005], coronary heart disease and cardiovascular disease [Ikehara et al. 2009]. They were also found to have occurrence of inflammatory diseases like diabetes or myocardial infarction [Liu et al. 2013] [Ruesten et al. 2012] [Leproult et al. 2014] [Phillips et al. 2015] . On the other hand, long sleepers (>10 hours per night) have been also found to have an increase in cardiovascular disease. Particularly worrying are the results of studies that have investigated the continuous interference of the sleep cycle as found in night-shift workers. This group has been linked to an increased risk for cancer, diabetes, and obesity [Viswanathan and Schernhammer 2009] and a variety of inflammatory diseases. This data show that sleep is one key factor for general health maintenance with clinical relevance [Opp and Krueger 2015] [Flynn-Evans et al. 2013] [Rabstein et al. 2014] [Sigurdardottir et al. 2013] [Knutsson and Kempe 2014]. But not just the sleep duration is important, a poor sleep quality has also been linked to health risks and can lead to an increase in circulating white blood cells (WBC) especially in women [Obayashi et al. 2016].

To further investigate the connection between sleep and the immune system, two approaches can be taken. To analyze the influence of sleep on the immune system, sleep manipulation such as sleep deprivation can be used. In these studies, human volunteers or animals were deprived of sleep; in connection different immune parameters such as cellular count, antibody, and cytokine responses were measured. In other settings, sleep manipulation was combined with immune changes such as infection or vaccination [Kapsimalis et al. 2008]. It is important to keep in mind that different techniques of sleep deprivation and different time spans are used. In animal models for example, most studies that investigated the effect of sleep used paradoxical sleep

deprivation for at least 24 h by the multiple platform method [Nunes et al. 1994]. In the multiple platform method animals are placed on a small platform in a water tank. If the animals fall asleep and thereby performing uncontrolled movements, the animals fall into the water and are then put back onto the platform [Machado et al. 2004]. This technique is used for long-term sleep deprivation up to several days but it cannot foreclose all sleep stages.

A different way to prevent sleep is called gentle handling. In this approach animals are placed in a box that allows a good view of animals at all time, and a person has to watch the behavior. In case animals show signs of fatigue enrichments are put in the box. Hereinafter the order of this enrichment can be changed to distract the animal. If this procedure fails to prevent the animal from sleep, the animal is carefully touched. This prevents all sleep stages, but can hardly be applied for more than 12 h. Gentle handling is used to analyze the acute effect of sleep loss without being stressful for the animals [Rolls et al. 2015]. The different methods lead to different findings.

Nevertheless, these experiments revealed a strong impact of sleep on different immune functions.

With regard to humoral immunity, several studies suggest an increased secretion of IL-6, TNF-a [Späth-Schwalbe et al. 1991], IL-1, and IL-2 [Vgontzas et al. 2004] due to sleep deprivation.

In the case of memory formation, studies from Lange *et al.* and Spigel *et al.* showed a lower antibody response towards vaccination after sleep deprivation. This was shown for influenza [Spiegel et al. 2002] vaccination as well as hepatitis A [Lange et al. 2003] [Lange et al. 2011]. In the study from Lange *et al.* 2011, participants were vaccinated against hepatitis A. This vaccination is carried out in three steps over 16 weeks. The night after the vaccination the participants were either subjected to sleep or wakefulness. They found that sleep doubled the frequency of Ag-specific Th-cells and Ag-specific IgG1.

In the case of cellular immunity, studies performed in humans have mainly focused on blood cell analysis, and revealed an increase in numbers of white blood cells, monocytes, neutrophils, and lymphocytes during sleep deprivation

or sleep restriction [Born et al. 1997] [Dinges et al. 1994] [Christoffersson et al. 2014] [Lasselin et al. 2015]. In the study from Born *et al*. 1997, they analyzed the impact of the circadian rhythm and sleep on WBCs. The participants were divided into two groups: The WS-WS-group (WS = with sleep) with two night's regular wake-sleep (dashed lines). And the WW-WS-group (WW = with wakefulness) in which the subjects were sleep deprived in the first night and recovered sleep in the second night (solid lines). In particular they found that monocytes and natural killer (NK) cells decrease during sleep phase. Under sleep manipulation this natural peak disappears, see Fig. 1.8.

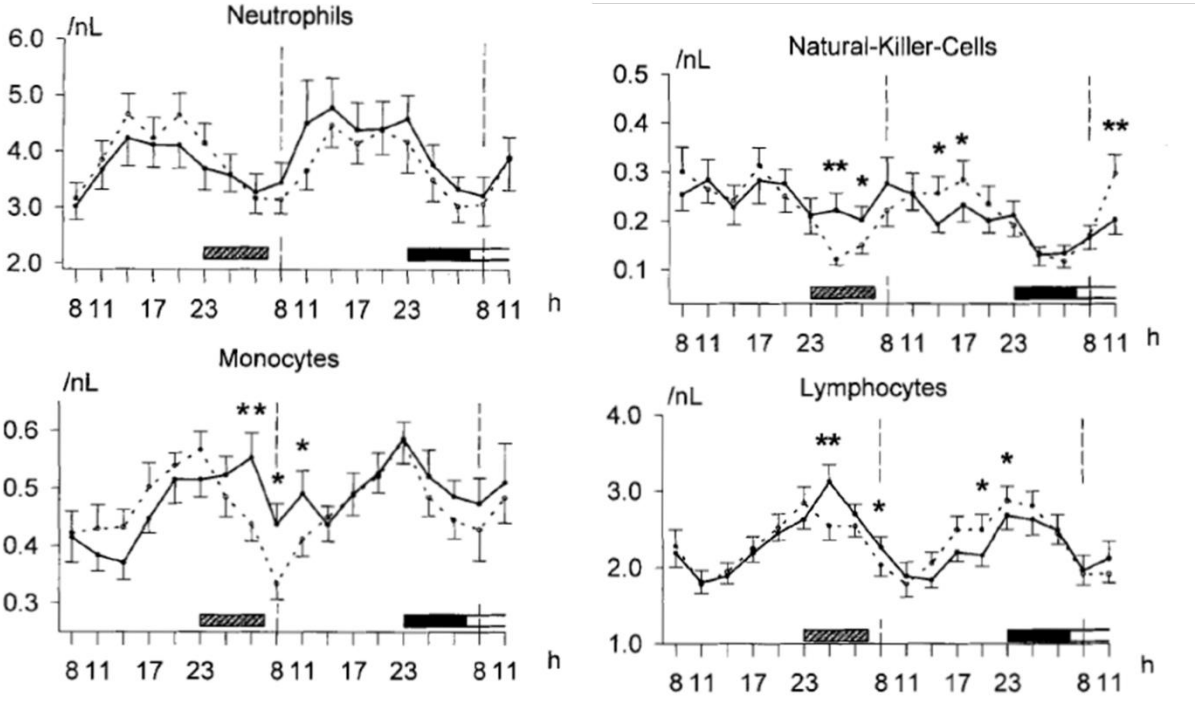

Fig.1.8: The impact of the circadian rhythm and sleep on WBCs

Adopted from Born *et al.* 1997 [Born et al. 1997]. The illustration shows the mean (+SEM) counts of peripheral blood neutrophils, monocytes, NK cells, and lymphocytes. WS-WS-group: two nights regular wake-sleep (dashed lines). WW-WS-group: one night of SD and one night of sleep (solid lines).

Not many studies exist regarding sleep deprivation and infection in humans due to ethical reasons but it was reported that humans with a self-reported sleep deprivation suffer more often under gingival inflammation. These kinds of infections are associated with an increased risk of cardiovascular diseases [Carra et al. 2017]. Sleep deprivation is also associated with decreased insulin

sensitivity and centripetal distribution of fat, pointing toward a possible health hazard due to sleep restriction [Bernardi Rodrigues et al. 2016].

In animal models, results are more contradictory on the effects of sleep on leukocyte counts in blood and tissues. This is mainly due to different timespans and different techniques of sleep deprivation as described above. Guariniello *et al.* perform experiments using paradoxical sleep deprivation (PSD) for 72 h induced by the multiple platform method. They found that PSD decreased the cellularity of the bone marrow and peripheral blood. In the BM, hematopoietic stem cell as well as the number of granulocytes and monocytes were decreased. In the blood, neutrophils and monocytes were increased [Guariniello et al. 2012]. Different approaches used paradoxical sleep deprivation for 24 and 96 h or chronic sleep restriction (SR) for 21 days in rats. With the result that PSD for 24 h increases neutrophils, PSD for 96 h increases complement C3 and corticosterone levels. Chronic sleep restriction for 21 days leads to a decrease in spleen weight, total leukocytes, and lymphocytes [Zager et al. 2007].

In a later study PSD was associated with reduction of circulating lymphocytes but no alteration was found on other immune sites [Zager et al. 2012].

On the other hand, it could be shown that sleep deprivation for several days in rats increases circulating phagocytic cells, mainly neutrophils in blood, lung, liver, and intestinal tissues. This was combined with blood monocytosis and reduced circulating lymphocyte counts [Everson 2005] [Everson et al. 2008].

Pointing towards a supportive effect of sleep on immunity are several studies that associate prolonged sleep manipulations with a higher mortality rate.

Most of these investigations were performed in rodents and using the PSD.

Mice were subjected to PSD for up to 72 h and showed increased death rates following malaria infection. 48 h of sleep rebound (RB) were necessary to achieve survival rates similar to animals without sleep manipulation [Lungato et al. 2015]. In the case of a sepsis sleep deprivation leads to increased mortality in mice [Friese et al. 2009]. It was also demonstrated that long term SD can lead to a sepsis infection by translocation of normally harmless gut bacteria [Everson and Toth 2000]. With respect to infection and acute total sleep deprivation only

one animal study exists so far and its main focus is more on the effect of the infection on sleep than other way round. Anyway, these experiments were performed in rabbits used gentle handling for 4 h to achieve sleep deprivation. Animals were intravenously infected either before or after SD with *Escherichia coli.* In this study no differences were found for WBC numbers or the positive blood cultures between rabbits that slept for 4 h or those that were kept awake [Toth et al. 1995a].

This study suggests a negative or neutral effect of sleep deprivation on the immune system and therefore demonstrates the importance of sleep for the immune system [Ibarra-Coronado et al. 2015].

But in contrast to this, other studies found that employed brief acute sleep manipulations demonstrated enhancing [Mullington et al. 2000] [Renegar et al. 1998], suppressive [Brown et al. 1989], or no [Toth and Rehg 1998] effects of sleep loss on anti-viral or anti-bacterial host defense.

These studies show that our understanding of sleep loss and its impact on the immune system is still quite incomplete. Fig 1.8 illustrates the complex interaction of sleep and immunity. Still our understanding is rudimentary and in a society with a growing number of shift workers and people with sleep disorders we have to improve our knowledge.

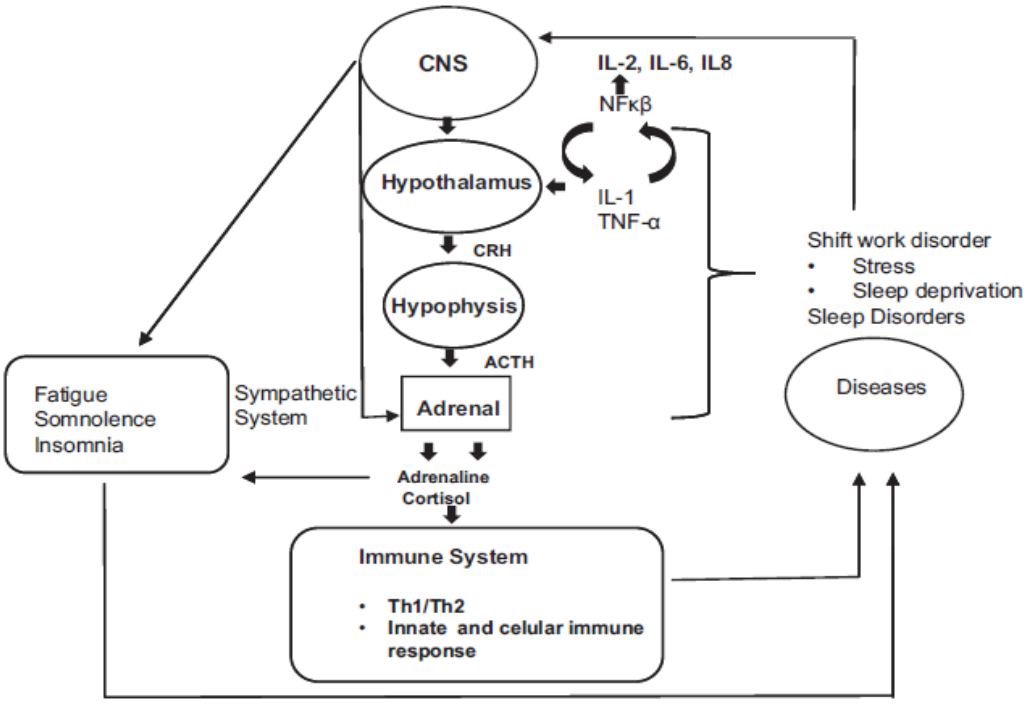

Fig.1.9: Theoretical model of human network of sleep and immunity
Illustration by Oliveira de Almeidaa and Malheiro [Almeida and Malheiro 2016].

1.3.3. Infections and sleep

Another approach to understand the underlying connection between sleep and the immune system is to study how the immune system influences the sleep behavior. This approach is very auspiciously as already Hippocrates over 2000 years ago observed an increased need for sleep during acute infections [Opp and Krueger 2015] [Prather et al. 2012] [Besedovsky et al. 2012]. And this 2000-year-old observation was confirmed by several studies, that found an increased feeling of sleepiness in humans with infections, especially for virus infections such as rhinovirus infection [Drake et al. 2000], mononucleosis [Guilleminault 1986], and HIV infection [Darko et al. 1995] [Norman et al. 1992]. Modern studies have analyzed the sleep of different model organisms including humans during different types of infections. Clear changes in sleep pattern were found in animals and humans during acute infections [Toth 1995b].

Most studies have been performed in rabbits. They show first an increase in slow-wave-sleep (SWS) followed by a decrease in SWS, after microbial inoculation [Toth and Krueger 1988].

Interestingly, different microorganisms have different effects on sleep behavior. Gram-negative bacteria, for instance, induce increased sleep behavior faster but for a shorter period than gram-positive bacteria or fungi do [Toth and Krueger 1989]. Looking at parasites, an infection with protozoan *Trypanosoma brucei brucei* needs several days before changes in sleep behavior can be found. An increased sleep behavior can be detected at the same time as the first fevers occur [Toth et al. 1994].

With regard to viruses, studies using an influenza virus could show extended sleep in mice [Fang et al. 1995] [Toth 1995b] and rabbits [Kimura-Takeuchi et al. 1992a]. Noteworthy, killed bacteria, as well as bacterial components, can still lead to changes in sleep behavior [Johannsen et al. 1990] [Krueger et al. 1982] [Krueger et al. 1986] [Masek et al. 1975], whereas killed viruses don't promote sleep anymore [Fang et al. 1995] [Kimura-Takeuchi et al. 1992a] [Toth et al. 1995a]. But double-stranded RNA (dsRNA), a main identifying feature of virus infections, induces sleep [Majde et al. 1991] [Kimura-Takeuchi et al. 1992b].

Despite this difference, in several animal models as well as in humans, infection whether virus or bacteria, microbial inoculation increases the amount of non-rapid-eye-movement (NREM) sleep as well as the intensity of SWS, but repressed REM [Toth and Krueger 1988] [Toth and Krueger 1989] [Baracchi et al. 2011] [Imeri and Opp 2009]. It seems that NREM sleep is beneficial for the recovery from infections [Toth et al. 1993] [Opp and Toth 2003]. Decades of research have clearly shown a strong connection between sleep and the immune system. But until today the findings are often contradictory and mechanisms remain unclear and so the question marks in Fig. 1.9 from 1995 still remain unanswered.

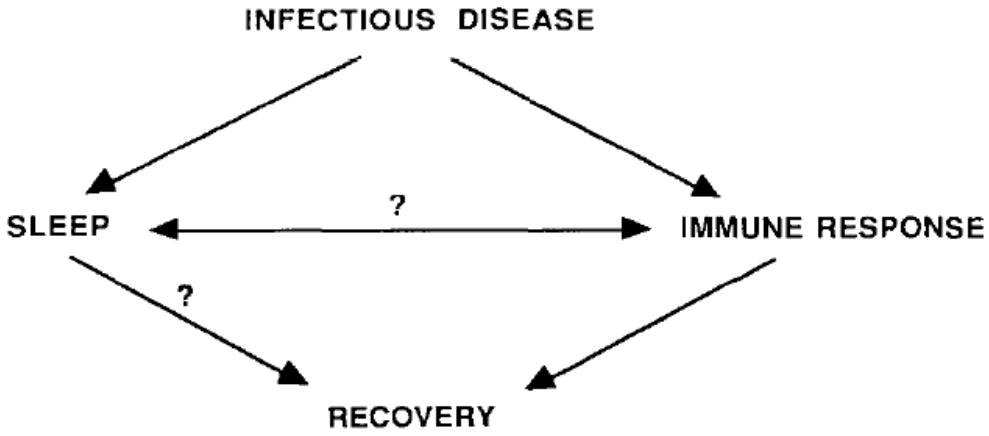

Fig. 1.9: Sleep and immunity

The relation between sleep, disease, and immune response by Toth 1995 [Toth 1995b]. Many key questions remain to be answered until today.

1.4. *Yersinia enterocolitica*

The genus *Yersinia* consists of three human- and rodent-pathogenic species: *Yersinia pestis, Yersinia pseudotuberculosis,* and *Yersinia enterocolitica.*

These are rod-shaped, gram-negative bacteria with a facultative anaerobic lethargy. Their optimal growth temperature is 27 °C to 30 °C [Cornelis 1998a]. Infection with *Yersinia enterocolitica* (YE) causes in most cases a self-limiting acute or chronic infection of the digestive tract. In rare cases, an infection with YE can lead to systemic manifestation, such as sepsis [Cover and Aber 1989].

YE is mostly absorbed via contaminated food. Once in the body, YE migrates into lymphoid tissues where it uses its ability of immune inversion. This allows the bacteria to resist the non-specific immune responses by circumvent phagocytosis through PMNs and monocytes. Electron microscopy shows that bacteria predominantly appears in extracellular space [Autenrieth and Firsching 1996] [Devenish and Schiemann 1981] [Hanski et al. 1989] [Shepel et al. 2001]. As many other pathogens, YE has different strains with different virulence. The different virulence factors are either coded on bacterial chromosome [Revell and Miller 2001] or on the 70 kb virulence plasmid pYV (plasmid *Yersinia* virulence) [Cornelis et al. 1998b] [Hu et al. 1998] [Perry et al. 1998] [Snellings et al. 2001]. Virulence factors include a type III secretion system and various effector proteins, called Yops (Yersinia outer proteins) [Cornelis 1998a]. YE is distinguished based on the biochemical and serological features in six biotypes (1A, 1B, 2, 3, 4, and 5) as well as 60 serotypes [Wauters et al. 1987] [Bottone 1999].

1.5. Aim of the work

This work is dedicated to the proverb 'Sleep helps healing'. Sleep behavior can be found through the entire animal kingdom which indicates the importance of sleep. But to this day our understanding of sleep remains incomplete. The growing field of Psychoneuroimmunology has taken on the tasks to understand the effects of sleep on the immune system. Also, previous work has shown that sleep has a supportive effect on bodily functions and immunity, but we still miss a deeper understanding of the effect that sleep has on innate immune cells and their function upon bacterial infection.

As no study has yet combined sleep with the analyses of DCs, PMNs and monocytes and their tissue distribution as well as their functions upon bacterial infection.

Further, it remains unclear how regular sleep promotes host defense against bacterial infections and whether such an immune-supportive function could stem from acute effects of sleep on innate immunity.

To illuminate the effect of acute sleep on the innate immune system in different tissues and relating to Infections, mice experiments were performed.

In these experiments, the animals were exposed to 6 h of sleep or 6 h of enhanced wakefulness by gentle handling. In connection, numbers and functions of different phagocytes were measured in blood, and also in various tissues. Because a number of studies have described that sepsis changes sleep parameters [Baracchi et al. 2011] [Ibarra-Coronado et al. 2015] and that conversely sleep impacts sepsis outcome [Friese et al. 2009], the well-established sepsis model *Yersinia enterocolitica* (Ye) was used [Autenrieth et al. 2010] to examine the link between sleep and infection. Ye is particularly well suited for infections in the mouse model as it is a natural pathogen of humans and can provoke sepsis [Autenrieth and Autenrieth 2008]. With these tools this work investigates the impact of sleep on innate immune phagocytes and hypothesizes that any effect of sleep on this first line of host defense would automatically also influence the course of a bacterial infection.

2. Materials and Methods

2.1. Materials

2.1.1. Experimental animals

All animal experiments were performed according to the German Animal Protection Law with permission from the Regierungspräsidium Tübingen. Permit Number: M11/14.

C57BL / 6JolaHsd mice were purchased from Janvier (St Berthevin Cedex, France). CX3CR1$^{GFP/GFP}$ and CX3CR1$^{GFP/-}$ mice (B6.129P-CX3CR1tm1Litt/J) [Jung et al. 2000] with a genetic C57BL/6 background were found under specifically pathogen-free (SPF) conditions in the animal facility of the University of Tübingen Germany. In the same animal facility B6.129S4-Ccr2tm1Ifc/J (CCR2$^{-/-}$) were housed [Boring et al. 1997]. ICAM-1$^{-/-}$ mice were kindly provided by Dr. rer. physiol. Britta Engelhardt from the Theodor Kocher Institut, University of Bern, in Switzerland. Mice used for experiments were on average 6-12 weeks old. All animals got water and were feed ad libitum. Animals were adapted to the 12 h light/dark cycle starting at 7:30 am for at least 2 weeks. All mice in the experiments were sacrificed using CO_2.

2.1.2. Bacterial strains

In this work, the virulent wild type strain *Yersinia enterocolitis* WA-314 (WAP) Serotype O: 8 (pYV +), clinically isolated [Heesemann et al. 1983], was used for intravenous (iv) infecting and killing assays.

2.1.3. Antibodies / fluorescent dyes

Tab. 2.1: Cell viability dyes

Name	Company
Zombie NIR™	Biolegend
7-Aminoactinomycin D (7-AAD)	Applichem
Aqua Live Dead (ALD)	Invitrogen

Tab. 2.2: Antibodies

Antigen	Label	Company	Clone	Isotype
Annexin-V	FITC	eBioscience		
CD3	FITC	eBioscience	145-2C11	hamster IgG1
	FITC	Miltenyi	145-2C11	hamster IgG1
CD4	eFluor450	eBioscience	RM4-5	rat IgG2a
CD8a	PE	eBioscience	53-6.7	rat IgG2a
	PE	Miltenyi	53-6.7	rat IgG2a, k
	BV 785	Biolegend	53-6.7	rat IgG2a
CD11b	APC	Biolegend	M1/70	rat IgG2b, k
	APC-efluor 780	eBioscience	M1/70	ratIgG2b
	Alexa Fluor 700	Biolegend	M1/70	rat IgG2b, k
	BV 605	Biolegend	M1/70	rat IgG2b, k
CD11c	PE/Dazzle 594	Biolegend	N418	hamster IgG
	PE	Miltenyi	N418	hamster IgG
	PE	eBioscience	N418	hamster IgG
	APC	Miltenyi	N418	hamster IgG
	Alexa Fluor 700	Biolegend	N418	hamster IgG
CD14	FITC	BD	rmC5-3	rat IgG1, k
CD19	FITC	Miltenyi	6D5	ratIgG2a
CD24	Pacific Blue	Biolegend	M1/69	rat IgG2b, k
CD45	PerCP/Cy5.5	Biolegend	30-F11	rat IgG2b, k
	PerCP	Biolegend	30-F11	rat IgG2b, k
CD45.2	PerCP/Cy5.5	Biolegend	104	mouse IG2a, k
CD45R (B220)	BV 650	Biolegend	RA-36B2	rat IgG2a, k
CD64	PE	Biolegend	X54-5/7.1	mouse IgG1, k

CD103	PE	BD	M290	rat IgG2a, k
	PE	Miltenyi	2E7	hamster IgG
	APC	Biolegend	2E7	hamster IgG
CD115	PE	eBioscience	AFS98	rat IgG2a, k
	APC	eBioscience	AFS98	rat IgG2a, k
	BV 605	Biolegend	AFS98	rat IgG2a, k
F4/80	PE	eBioscience	BM8	rat IgG2a
	BV 421	Biolegend	BM8	rat IgG2a
GR-1 (Ly6G/ Ly6C)	FITC	BD	RB6-8C5	rat IgG2b
	FITC	Miltenyi	RB6-8C5	rat IgG2b
	eFluor450	eBioscience	RB6-8C5	rat IgG2b
Ly6C	PE-Cy7	Biolegend	HK1.4	rat IgG2c
Ly6G	FITC	Biolegend	1A8	rat IgG2a, k
	BV 421	BD	1A8	rat IgG2a, k
	BV 510	Biolegend	1A8	rat IgG2a, k
MHCII	PerCP	Biolegend	M5/114.15.2	rat IgG2b
	BV 711	BD	M5/114.15.2	rat IgG2b
	BV711	Biolegend	M5/114.15.2	rat IgG2b, k
NK1.1	FITC	eBioscience	PK136	mouse IgG2a, k
Sca-1 (Ly6A/E)	FITC	eBioscience	D7	rat IgG2a, k
	BD Horizon V500	BD	D7	rat IgG2a, κ
TER-119	FITC	Miltenyi	Ter-119	rat IgG2b, k

Tab. 2. 3: Reagents

Reagent	Company
Ampuwa®	Fresenius Kabi Deutschland GmbH
Bovine Serum Albumin (BSA), Fraction V	Biomol
BD™ CompBeads	BD
CD11c MicroBeads	Miltenyi Biotec GmbH
Collagenase CLS II	Biochrom
Collagenase CLS IV	Biochrom
DNAse I	Sigma-Aldrich
DTT (1,4 – Dithiothreitol)	Roth
(1x) Dulbecco's PBS w/o Mg^{2+}/Ca^{2+}, liquid	Gibco® by Life Technologies
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Hanks buffered salt saline (HBSS)	Biochrom AG
HEPES Buffer Solution (1 M)	Gibco® by Life Technologies
Isopropanol	Merck KGaA
Liberase TL	Roche
Lung Dissociation Kit, mouse	Miltenyi Biotec GmbH
Natriumazid NaN_3 tablets	Merck
RPMI 1640 Media w/o 2 g/L $NaHCO_3$, w/o L-Glutamine, low endotoxin	Biochrom
Trypanblau	Sigma-Aldrich
2', 7'-Dichlorofluorescein diacetate reagent (DCFD)	Sigma-Aldrich

Tab. 2.4: Buffers

Buffer	Composition
Erythrocyte lysis buffer	4.15 g NH_4CL 0.5 g $KHCO_3$ 1.85 g Na_2 EDTA, add 500 ml H_2O
Lamina propria digestion medium	RPMI 1640 10 % FBS 10 mM HEPES 0.03 mg/ml DNAse I 0.12 mg/ml Collagenase II
Spleen digestion medium	RPMI 1640 2 % FBS (v/v) Colagenase Typ IV 1 mg/ml DNase I 100 µg/ml
Lymph nodes digestion medium	RPMI1640 2 % FBS (v/v) Liberase Typ TL 125 µg/ml DNase I 100 µg/ml
FACS buffer	1x PBS w/o Mg^{2+}/Ca^{2+} 1 % FBS 0.019 % NaN_3, 2 mM EDTA
LB- medium	Yeast extract (5 g/l) Trypton (10 g/l) Sodium chloride (0.5 g/l), add 1 l, pH 7.5
MACS®- buffer	PBS w/o Mg^{2+} und Ca^{2+} 2 mM EDTA 0.5 % BSA (v/v)
Müller-Hinton (MH)- agar plates	Yeast extract (5 g/l) Trypton (10 g/l) Natriumchlorid (0,5 g/l) Bacto Agar (15 g/l), add 1 l, pH 7.5

PBT	PBS 0.1 % Tergitol 0.1% BSA
Pre-digestion medium	RPMI 10 mM HEPES 5 mM EDTA 5 % FBS 1 mM DTT

Tab. 2.5: Consumables

Article	Company
Combitips	Eppendorf
Disposable cuvettes	Sarstedt
Easy strainer 40 µm, 70 µm, 100 µm sterile	Greiner Bio-one
Eppendorf tubes (0,5 ml, 1,5 ml, 2 ml)	Eppendorf
FACS tubes	BD Biosciences
gentleMACS™ C Tubes	Miltenyi Biotec GmbH
Greiner culture tubes, round bottom	Greiner Bio-one
Gloves	Paul Hartmann AG
MACS LS Columns	Miltenyi Biotec GmbH
Petri dish 60/15 mm; Petri dish 94/16 mm	Greiner Bio-one
Pipette tips	Sarstedt Ratiolab Greiner
Tubes (15 ml, 50 ml)	Greiner
Single injection cannula	Braun
96-well plate (v-bottom)	Greiner Bio-one

Tab. 2.6: Devices

Device	Company
Autoclave	Tuttnauer Systec
Centrifuge 5417R	Eppendorf
Eclipse TS100 Microscope	Nikon
Flow cytometer (FACS) Canto II	BD
Flow cytometer (FACS) Fortessa LSRII	BD
gentleMACS™ Octo Dissociator with Heaters	Miltenyi Biotec GmbH
Herasafe HS15	Kendro Laboratory Products
Incubator Kelvitron®	Heraeus Instruments
Innova 44 Incubator	New Brunswick Scientific
MACS Stand & Magnet	Miltenyi Biotech GmbH
Mouse injection chamber	Vetter
Multifuge3 S-R; Rotor 6445	Heraeus

Tab. 2.7: Software

Software	Company
Adobe Illustrator CS6	Adobe Systems GmbH
CorelDRAW® Graphic Suite X8	Corel Corporation
Diva	BD
FlowJo	TreeStar
Prism 7	GraphPad Software, Inc.
Microsoft Office programs	Microsoft

2.2 Methods

2.2.1 Gentle handling

To prevent all sleep phases without stressing the animal, mice were prevented from sleeping for 6 h by gentle handling, which is a well-established method [Rolls et al. 2015]. The procedure started at the onset of light, at 7:30 a.m. and lasted until 1:30 p.m. Whenever the animal adopted a sleeping posture, the nest has been disturbed, by gently touching the bedding underneath the animal or by providing new nesting material. Sleeping mice were left completely undisturbed during this time period. Both mouse groups were either sacrificed or infected at the same time to control for circadian influences [Nguyen et al. 2013].

2.2.2. Intravenous injection of mice

To mimic a sepsis mouse were infected after 6 h of sleep or wakefulness with the indicated amount of Ye WA-314 (serotype 0:8) in 200 µl PBS into the tail vein. The bacterial load in blood and spleen was obtained at different time points after plating serial dilutions of the cell suspensions on Müller-Hinton agar plates for 2 days at 27 °C. Mice were infected *iv* as described by Autenrieth previously [Autenrieth et al. 2012].

2.2.3. Lymphocyte isolation

2.2.3.1. Isolation of splenocytes

After removing the spleen from the dead mice, the organs were cut into small pieces followed by a digestion for 30 min at 37 °C in 2 ml spleen digestion medium. EDTA (0.1 ml, 0.1 M (pH 7.2)) was added and continued for 5 min on. Single-cell suspensions were achieved by filtration and erythrocytes were lysed with erythrocytes lysis buffer (15 ml erythrocytes lysis buffer/3min/room temperature (RT)). The total number of cells was determined using trypan blue exclusion. 1/30 of all cells were used for FACS staining.

2.2.3.2. Isolation of blood lymphocytes

Blood was collected from the heart of dead mice, using a syringe flushed with heparin. Erythrocytes were removed using the erythrocyte lysis buffer. Cells were incubated in 5 ml erythrocytes lysis buffer at RT. Subsequently, 5 ml of PBS with 2% FBS were added. This procedure was carried out 3 times. The total number of cells was determined using trypan blue exclusion. 200 µl blood was used for the flow cytometry analysis.

2.2.3.3. Isolation of cells from mesenteric and submandibular LN

To isolate cells from lymph nodes, all fat was removed from the lymph nodes. Afterwards, the lymph nodes were incubated for 20 min at 37 °C in 500 µl of digestion medium. After the digestion, the lymph nodes were pressed through a 70 µm nylon strainer to remove unwanted adipose tissue and achieve a single-cell solution. The total number of cells was determined using trypan blue exclusion. 50% of all cells were used for FACS staining.

2.2.3.4. Isolation of lamina propria

Lamina propria (LP) mononuclear cells were isolated from the small intestine (SI) of mice. SI was excised; all Peyer's patches and mesenteric fat tissues were removed. SI was opened longitudinally with scissors, cleaned in Petri dish containing PBS and 10% FBS, and cut into 0.5-1 cm long pieces. Then small intestine pieces were incubated for 20 min in a 37 °C waterbath in 20 ml pre-digestion medium to remove intestinal epithelial cells. Afterwards cell solution was shaken vigorously for 20 seconds and passed through a 100 µm nylon cell strainer over 50 ml Falcon tubes. The pieces were collected back in a new tube with 20 ml PBS and 5% FBS to wash away remaining epithelial cells. This step was performed twice, followed by a second pre-digestion. Subsequently, two more washing steps using PBS+5% FBS were performed. Pre-digested remaining pieces were incubated in 20 ml of RPMI and 5% FBS for 25 min by 37°C for the digestion. Finally, the pieces were digested in RPMI medium containing 0.12 mg/ml Collagenase II and 40 U/ml DNase I for 45 min at 37 °C. After incubation, the cell solutions were mixed vigorously for 15 seconds, passed through a 40 µm nylon cell strainer, and centrifuged for 10 min at 300xg. The cells were counted under a microscope using trypan blue. All cells were utilized for FACS staining.

2.2.3.5. Isolation of bone marrow

Bone marrow (BM) cells were harvested from femurs and tibias using PBS. Erythrocytes were removed using the erythrocyte lysis buffer. The cells were counted under a microscope using trypan blue and $3x10^6$ cells were used for FACS staining.

2.2.3.6. Isolation of lung

Isolation of lung cells was performed using the Lung Dissociation Kit together with the gentleMACS Dissociator according to the manufacturer's protocol (Miltenyi Biotec). The total number of cells was determined using trypan blue exclusion. 50% of all cells were used for FACS staining.

2.3. Flow cytometry

FACS buffer was used for all incubations and washing steps. First, dead cells were excluded by staining with aqua life dead or Zombi-NIR. Before staining with the antibodies, cells were incubated for 10 min at 4 °C with hybridoma supernatant from 2.4G2 cell line producing anti-FcgRII/III mAb. Extracellular staining was performed for 20 min at 4 °C. Samples were acquired for 6 to 16-color analysis using a Canto II or Fortessa LSR II flow cytometer. A total of $5 \times 10^5 – 2 \times 10^6$ cells were acquired.

2.3.1. Reactive oxygen species (ROS) detection

For ROS detection splenocytes and blood cells were extracellularly stained as described above, followed by 20 min with 2', 7'-Dichlorofluorescein diacetate reagent (DCFD), washed, and immediately analyzed by flow cytometry.

2.4. Statistics

Statistical significance was determined using a two-tailed t-test. For experiments with more than two investigated groups, statistical significances were calculated by using one-way analysis of variance (ANOVA) followed by Sidak Multiple Comparison Test. Statistical analysis of survival was performed using the log-rank test. All statistical tests were conducted with GraphPad Prism 6.0 software.

3. Results

Parts of this work are published under "Sleep enhances numbers and function of monocytes and improves bacterial infection outcome in mice" in Brain Behavior and Immunity [Hahn et al. 2020].

3.1. Impact of sleep on phagocytes in blood and spleen

To examine the influence of sleep on phagocytes, mouse experiments were performed using wild-type (WT) C57BL / 6JolaHsd mice.

The experiments were designed with 2 different groups of mice: 'sleeping' mice or the sleep group, those mice were allowed to sleep for 6 h after the onset of light. The other group was the 'waking' mice or the wake group; those mice were kept awake by gentle handling for 6 h after the onset of light.

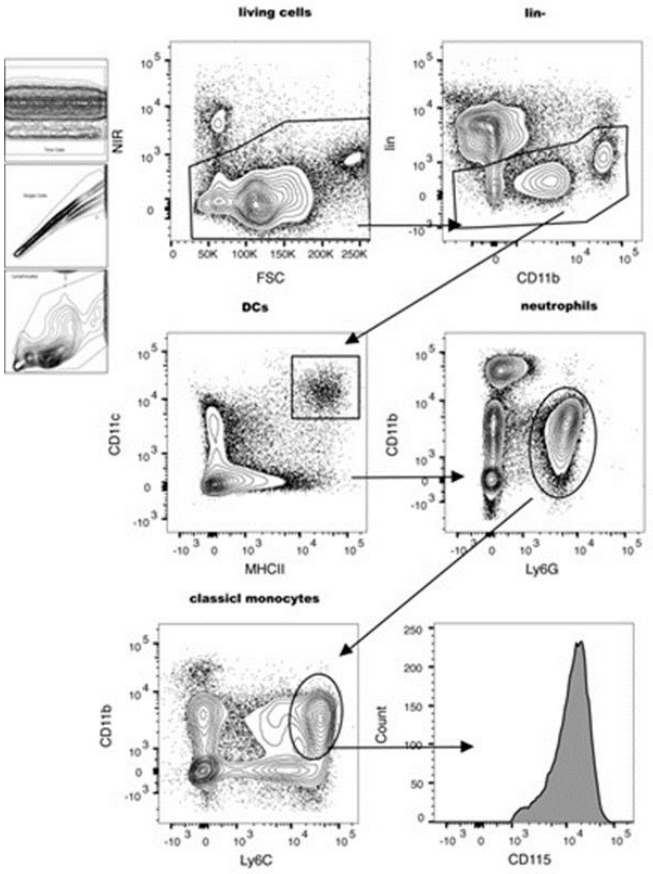

Fig. 3.1 Flow cytometry gating strategy of phagocytes

All populations were gated as follows: Singlets/leukocytes/living cells/lineage (CD3, Ter119, NK1.1, CD19 $^{-}$). Proceeding from that population, PMNs were gated as Ly6G^{+}. The remaining cells were gated for DCs defined as CD11c^{+}MHCII^{+} population. Subsequently, classical monocytes were selected according to Ly6C^{hi}CD11b^{+}CD115^{+} expression. Gating strategy was identical for blood (shown here) and spleen.

All experiments started at the beginning of the resting phase of the animals, which was controlled by the onset of light at 7:30 a.m. Both groups were always sacrificed at the same time to control the influence of the circadian rhythm [Scheiermann et al. 2012] [Nguyen et al. 2013]. This experimental setting allowed to investigate the influence of sleep on immune cells. Different innate immune cell populations were analyzed using multicolor flow cytometry.
In a first step, the total cell count of splenocytes and the white blood cell counts (WBC) were identified and compared, as well as the frequencies and numbers of neutrophils (PMNs), dendritic cells (DCs), and classical monocytes (Fig. 3.2 and 3.3).

Figure 3.1 shows the gating strategy applied for spleen and blood samples. All populations were gated as follows: In an initial step, only single cells were selected and starting from that all leukocytes were gated. In connection, all dead cells were excluded as well as all lineage$^+$ (CD3, Ter-119, NK1.1, CD19) cells. Within the remaining cells, PMNs (Ly6G$^+$) and DCs (CD11c$^+$MHCII$^+$) were identified and excluded from further analysis. Classical monocytes were characterized by their co-expression of CD11b, CD115, and high levels of Ly6C.

For splenic cells, the experiments showed that the overall cell count differed between the sleep and the wake mice groups. This difference was significant and composed of a 1.25-fold increase of cells in the sleep group as compared to the wake group (Fig. 3.2a). Neutrophils remained unaffected, as the data revealed no difference between the two groups with regard to the frequency and cell count of neutrophils (Fig. 3.2b). By contrast, a significant decrease of the DC-frequency was observed in the 'sleeping' group compared to 'wake' group. This was not the case for the cell count of DCs (Fig. 3.2c). The analysis of the classical monocytes demonstrated a strong impact of sleep on this type of cells. The abundance of classical monocytes was strongly increased by the factor of 2-3 regarding the frequency and number of cells (Fig. 3.2d).

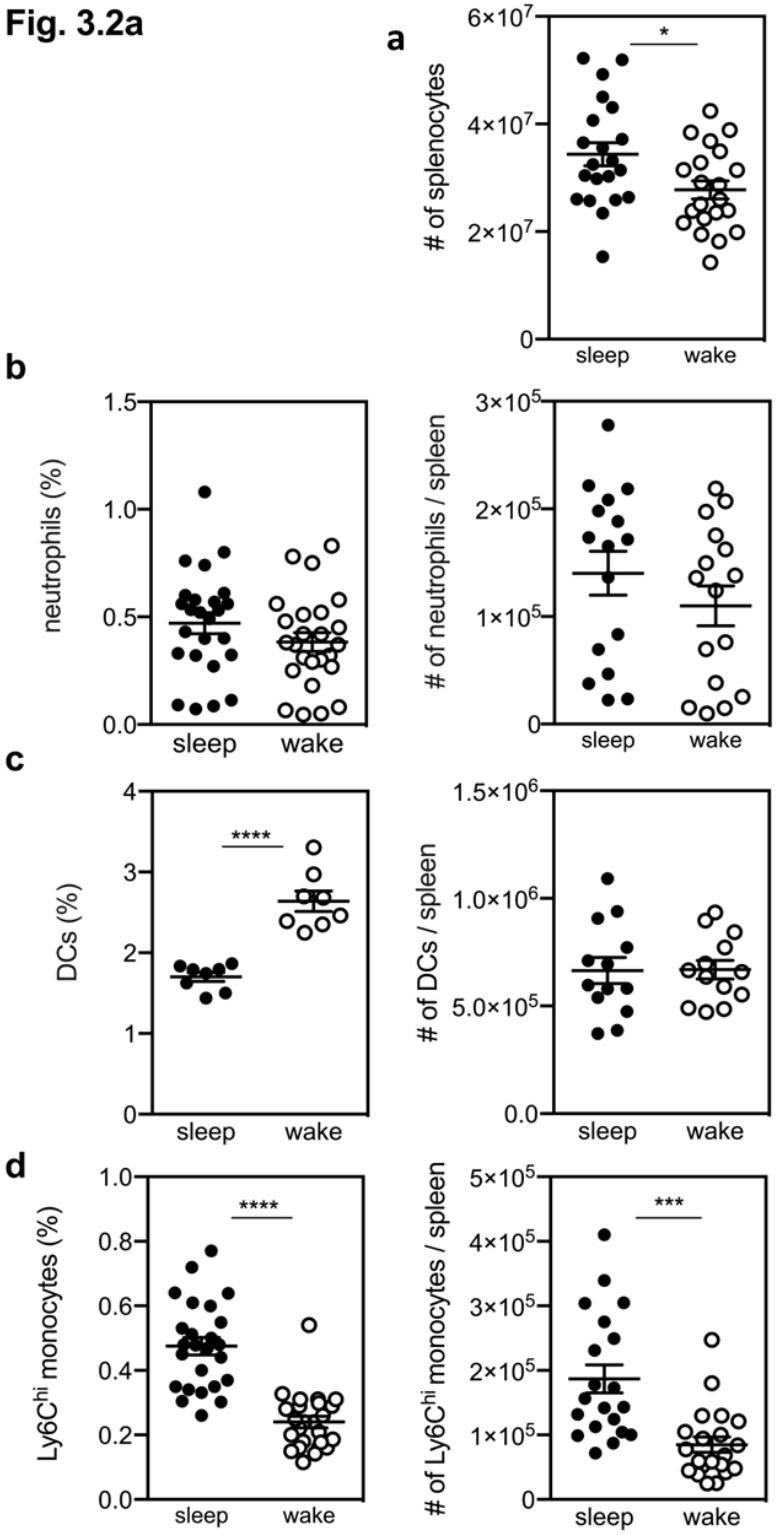

Fig. 3.2: Sleep increases splenic monocytes.

For 6 h, mice were either allowed to sleep ('sleep') or not ('wake'). (a) Number of living cells per spleen. (b) Graphs show the frequency and numbers of splenic neutrophils. (c) Graphs show the frequency and numbers of splenic DCs. (d) Graphs show the frequency and numbers of splenic classical monocytes ($Ly6C^{hi}CD11b^{+}CD115^{+}$). Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

The analysis of blood samples revealed an even stronger effect of sleep on immune cells. The WBC counts were doubled in 'sleeping' mice as compared to 'waking' mice (Fig. 3.4a). Neutrophils were again unaffected by sleep (Fig. 3.4b). In this blood DCs showed no changes in their frequency, but a slightly higher DC-count could be detected in the 'sleeping' group (Fig. 3.4c). Again, the classical monocytes were mostly affected by sleep. Classical blood-monocytes were dramatically increased in 'sleeping' as compared to 'waking' mice. In terms of frequency, there was a 3.3-fold increase in Ly6C^{hi}CD11b^{+} monocytes observed. The numbers of classical monocytes from 'sleeping' mice were enhanced by the factor of 6.7 as compared to those of 'waking' mice (Fig. 3.4d). These findings led to a focus on monocytes in the following experiments. To demonstrate the strong impact of sleep on classical monocytes, Figure 3.3 shows comparative countour plots of the different mice groups. For analysis of the classical monocytes, PMNs and DCs have been excluded. The countour plots clearly demonstrate the increase of classical monocytes upon sleep.

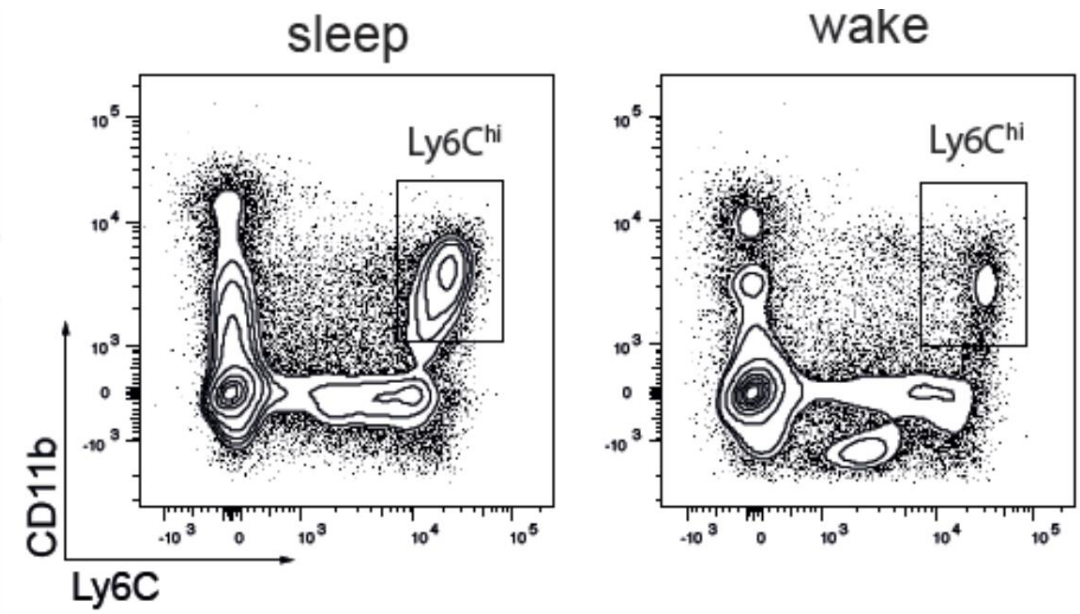

Fig. 3.3 Sleep increases blood monocytes shown as countour plots

For 6 h, mice were either allowed to sleep ('sleep', left plot), or not ('wake', right plot). Flow cytometry countour plots show blood classical monocytes (Ly6C^{hi}CD11b^{+}CD115^{+}).

Fig. 3.4

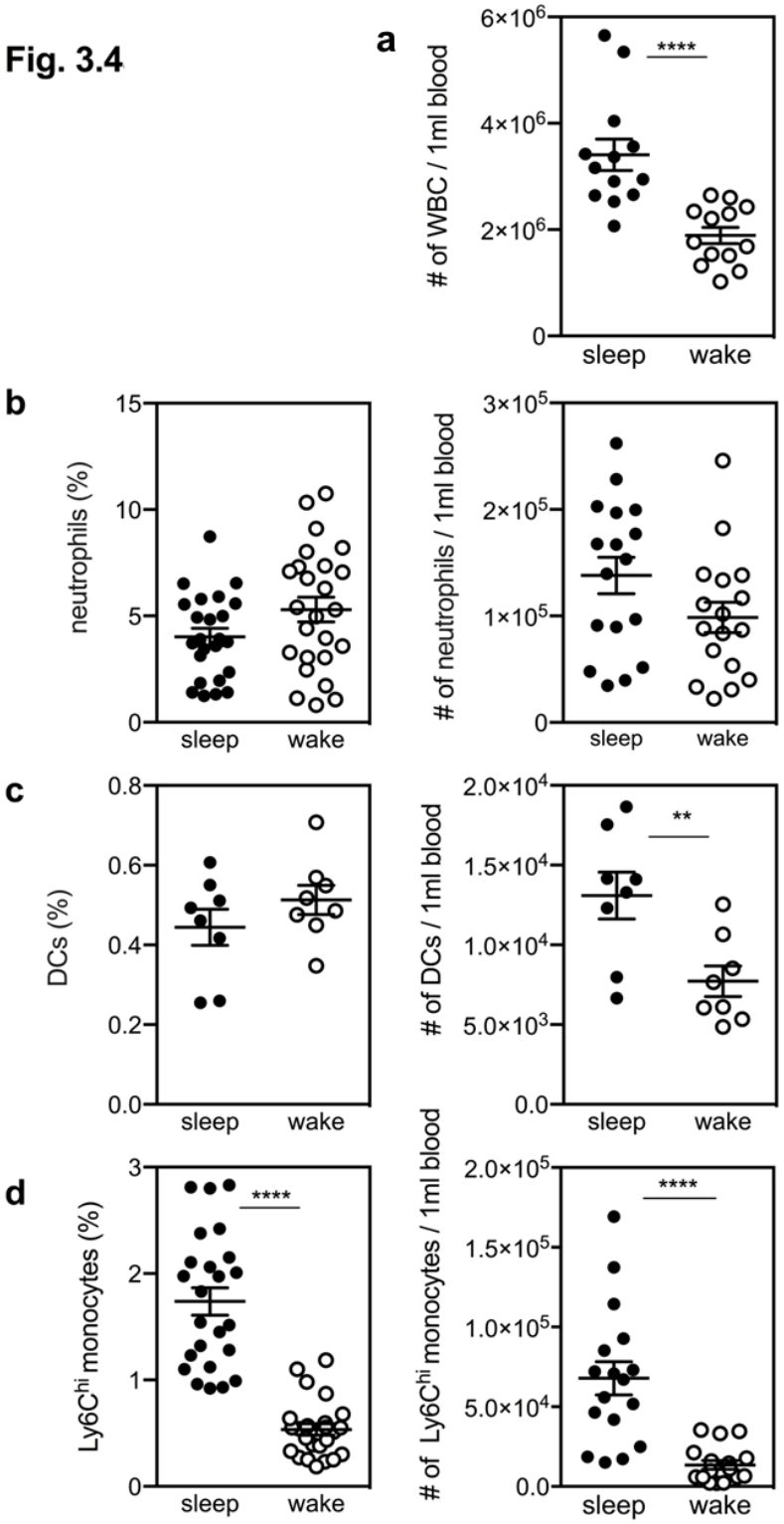

Fig. 3.4 Sleep increases blood monocytes

For 6 h, mice were either allowed to sleep ('sleep', filled circles) or not ('wake', open circles). (a) # of WBC 1 ml blood. (b) Graphs show the frequency and numbers of neutrophils in 1ml blood. (c) Graphs show the frequency and numbers in 1ml blood of DCs. (d) Graphs show the frequency and numbers of classical monocytes ($Ly6C^{hi}CD11b^{+}CD115^{+}$) in 1 ml blood. Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

3.2. Comparable corticosterone serum levels in sleep and wake mice

Gentle handling is a well-known method to prevent all sleep stages without inducing stress for the animals [Rolls et al. 2015] [Oyanedel et al. 2015]. On the other hand, Dhabhar *et al.* has shown that the stress hormone corticosterone can lead to reduction of blood monocytes [Dhabhar et al. 2012] which is the observed sleep phenotype. To ensure that the animals were not stressed during gentle handling the corticosterone levels in the blood serum of both mice groups were measured. Both groups showed the same corticosterone levels in the blood serum. Furthermore the corticosterone levels were within the normal range for this time period and this mouse strain [Parrillo and Fauci 1979] [Bowers et al. 2008] (Fig. 3.5).

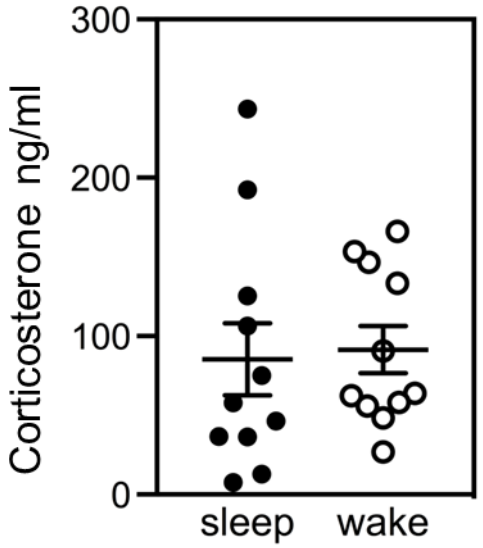

Fig. 3.5: Corticosterone levels in plasma
For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). Blood was taken and corticosterone levels in plasma were analyzed by ELISA. Every circle represents one mouse. Data represent the mean ± SEM (Student's t-test).

3.3. Impact of sleep on cells derived from classical monocytes

Previous experiments have shown that sleep has the strongest impact on classical monocytes, so the focus of the following work was to understand the underlying mechanism. Classical monocytes are not just important phagocytes they are also precursors of non-classical monocytes and macrophages. A strong reduction of these cells upon sleep would explain the observed phenotype. Therefore, non-classical monocytes and macrophages were investigated.

The gating strategy for macrophages and non-classical monocytes is shown in figure 3.5. As described before, doublet cells, dead cells, as well as all lineage$^+$ (CD3, Ter-119, NK1.1, CD19) cells were excluded. Macrophages were identified by their expression of F4/80$^+$ and CD11b$^+$. To allow a clear gating on non-classical monocytes, cells identified as macrophages were excluded from further analysis. Non-classical monocytes were identified by an expression of Ly6C^{lo} CD11b$^+$CD115$^+$ and CD62L$^-$.

The experiments clearly illustrate that sleep has a similar effect on non-classical Ly6C^{lo} monocytes as it has on classical Ly6C^{hi} monocytes.

In the spleen, non-classical Ly6C^{lo} monocytes were increased upon sleep, in frequency as well as in cell number. Their numbers were doubled in 'sleeping' mice as compared to 'waking' mice (Fig. 3.6a). Macrophages displayed increasing numbers upon sleep but there were no changes in frequency of respective cells (Fig. 3.6b).

Analysis of blood samples confirmed the previous findings. In blood non-classical Ly6C^{lo} blood-monocytes displayed a rise of the sleep group as compared to the wake group. This rise could be observed in frequency as well as in cell numbers. The number of non-classical Ly6C^{lo} monocytes was again doubled upon sleep (Fig. 3.6c). However, blood macrophages showed a reduction in their frequency upon sleep, but no significant changes in cell numbers (Fig. 3.6d). Nevertheless, these experiments demonstrate that sleep not only has an impact on classical Ly6C^{hi} monocytes but also on non-classical Ly6C^{lo} monocytes.

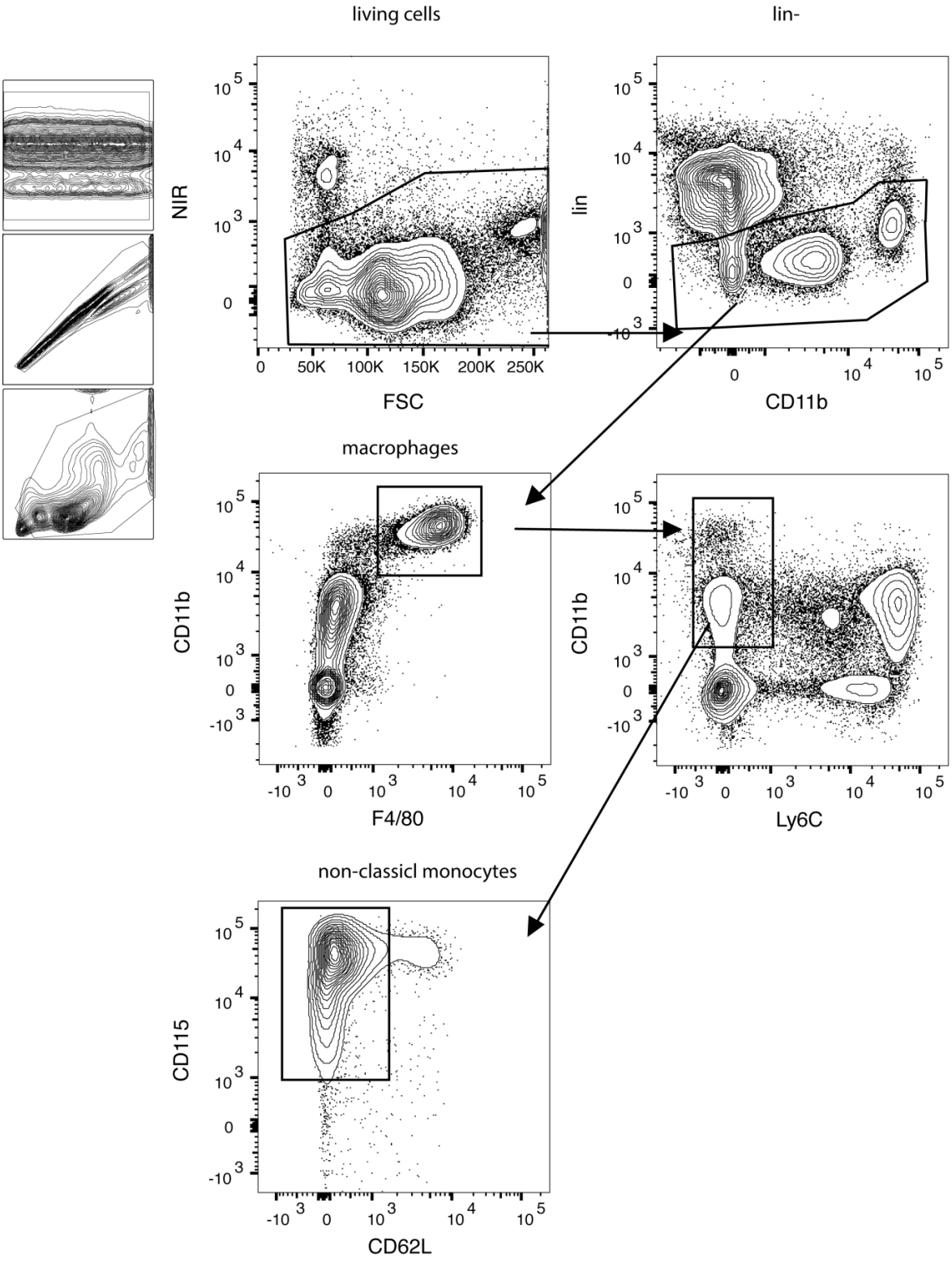

Fig. 3.5: Flow cytometry gating strategy for macrophages and non-classical monocytes

All populations were gated as follows: Singlets/leukocytes/living/lineage$^-$ (CD3, Ter119, NK1.1, CD19). Macrophages were gated as CD11b^{hi}F4/80$^+$ population and excluded from further analysis. The remaining non-classical monocytes were gated as Ly6C^{lo}CD11b$^+$CD115$^+$ and CD62L$^-$. Gating strategy was identical for blood (shown here) and spleen.

Fig. 3.6a

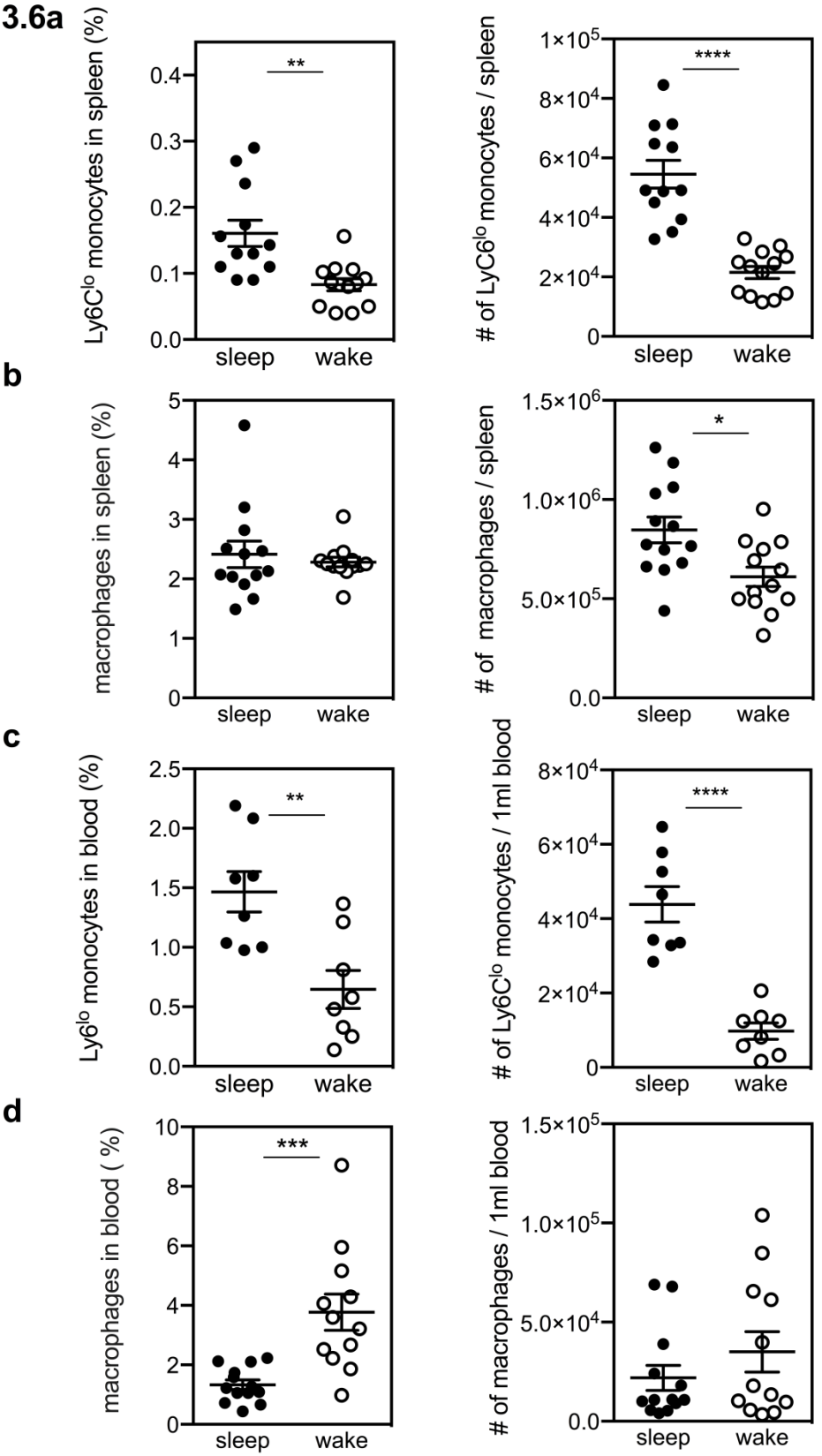

Fig.3.6: Sleep increases non-classical monocytes

For 6 h, mice were either allowed to sleep ('sleep', filled circles) or not ('wake', open circles). (a) Graphs show the frequency and numbers of splenic non-classical monocytes ($CD11b^{+}Ly6C^{lo}CD115^{+}$). (b) Graphs show the frequency and numbers of splenic macrophages ($CD11b^{+}F4/80^{+}$). (c) Graphs show the frequency and numbers

of 1ml blood for non-classical monocytes ($CD11b^+Ly6C^{lo}CD115^+$). (d) Graphs show the frequency and numbers of 1 ml blood for macrophages ($CD11b^+F4/80^+$). Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****)).

3.4. Impact of sleep on monocyte-precursors

After it has been shown that sleep not only affects classical $Ly6C^{hi}$ but also non-classical $Ly6C^{lo}$ monocytes, the question emerged, whether this sleep-induced rise in monocyte counts might stem from changes in myelopoiesis or the release of monocytes from the bone marrow (BM). To answer this question, the BM of 'sleeping' and 'waking' mice was compared. The gating strategy is shown in figure 3.7.

Bone marrow samples were analyzed by PD. Dr. Stella Autenrieth [Pasquevich et al. 2015].

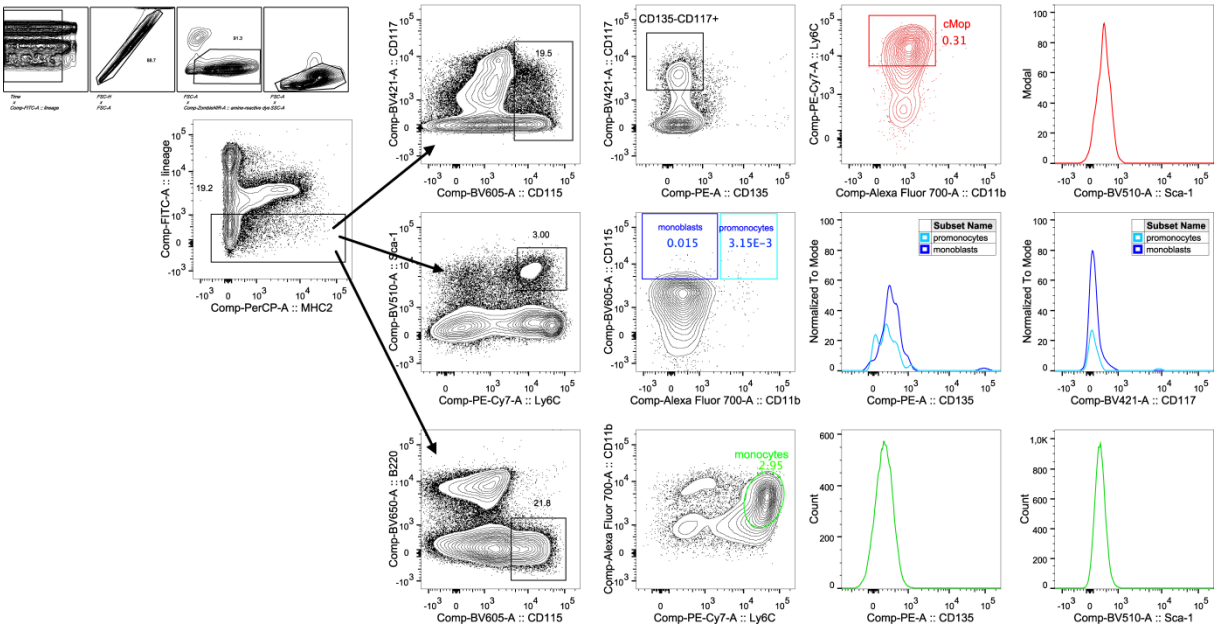

Fig. 3.7: Flow cytometry gating strategies of monocytes and their progenitors in the bone marrow

All populations were gated as follows: Time/singlets/living/FSCxSSC/lineage (CD3, Ter119, $NK1.1^-$). From that common monocyte progenitors (cMoPs) were gated as $M\text{-}CSFR^+c\text{-}kit^+Flt3^-Ly6C^+CD11b^-$ (upper panel: red). cMoPs are found to be $Sca\text{-}1^-$. Monoblasts were gated as $Sca\text{-}1^+Ly6C^+CD11b^-M\text{-}CSFR^+$ and pro-monocytes as $Sca\text{-}1^+Ly6C^+CD11b^+M\text{-}CSFR^+$ (middle panel: blue). Both cell populations neither express Flt3 nor c-kit. BM monocytes were further gated as $M\text{-}CSFR^+B220^-CD11b^+Ly6C^{hi}$ (lower panel: green) and do not express Flt3 and Sca-1.

Fig. 3.8

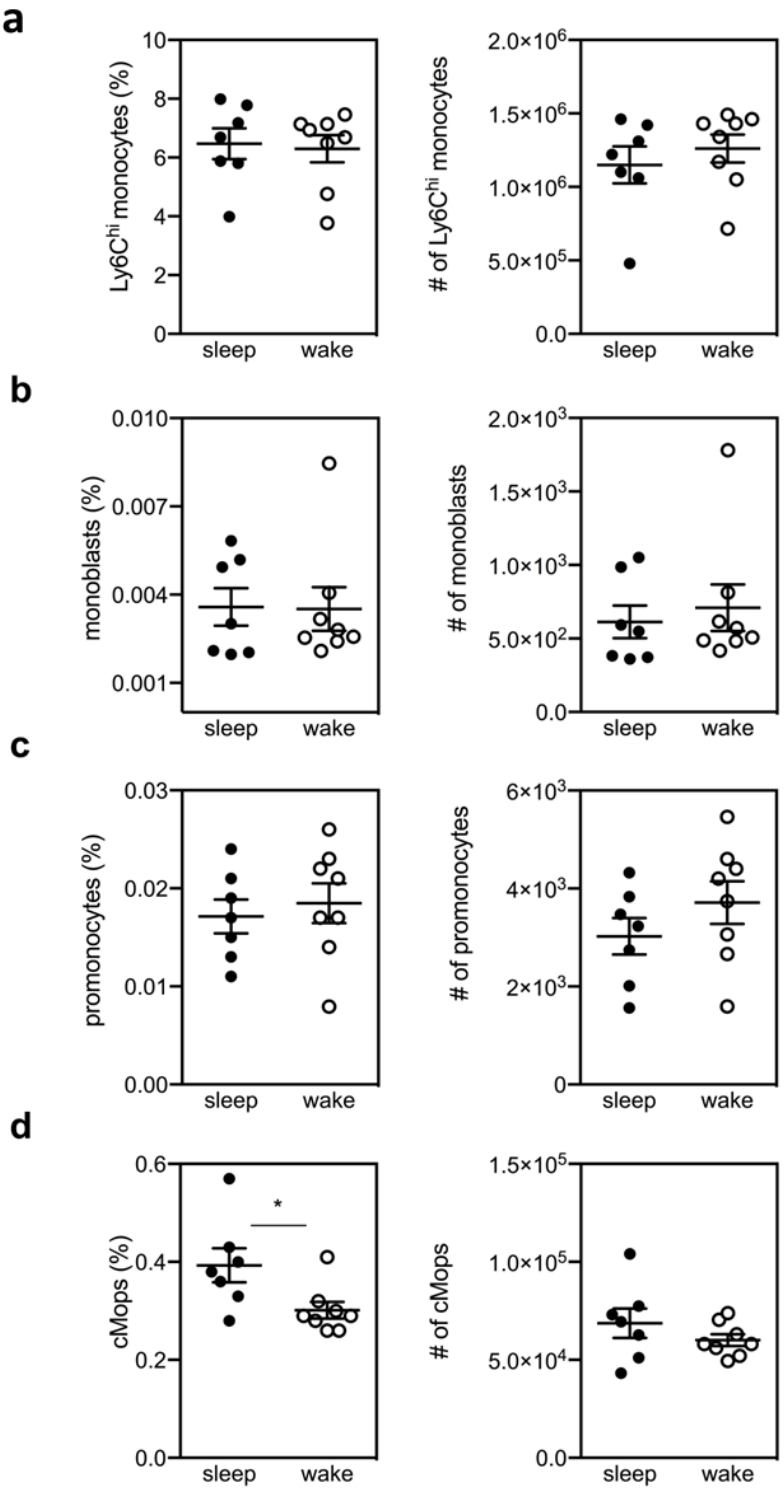

Fig. 3.8 Sleep does not lead to changes in myelopoiesis or the release of monocytes from the bone marrow

For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). (a) Graphs show the frequency and numbers classical monocytes ($CD11b^{+}Ly6C^{hi}CD115^{+}$) in the BM. (b) Graphs show the frequency and numbers of monoblasts in the BM (c) Graphs show the frequency and numbers of promonocytes in the BM. (d) Graphs show the frequency and numbers of cMoPs in the BM. Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

To be able to make a statement regarding the release of monocytes, their frequency as well as their cell numbers were analyzed in the Bone marrow (Fig. 3.7a). There was no influence upon sleep on monocyte-frequency or cell numbers, and therefore no impact of sleep on the release of monocytes from the BM.

For the analysis of myelopoiesis, different monocyte precursors were considered. The direct precursors of monocytes are monoblasts, their frequency and their cell numbers unchanged between the 'sleeping' and 'waking' mice (Fig. 3.7b). Pro-monocytes are the direct precursors of monoblasts. They were also completely unaffected by sleep (Fig. 3.7c).

Other precursors are the common monocyte progenitors (cMoPs), these cells showed an increase upon sleep in their frequency but not in their actual number (Fig. 3.7d).

This leads to the suggestion that the sleep-induced rise of monocytes cannot be explained by myelopoiesis or by the release of monocytes from the bone marrow.

3.5. Impact of sleep on monocytes in various tissues and their migration

Another possible explanation for the strong increase of monocytes upon sleep would be a shift of migration of monocytes. In this case monocytes would immigrate from other organs like lymph nodes (LNs), the gut lamina propria or the lung into blood and spleen. Therefore, a closer look at these organs was taken. Lymph nodes such as mesenteric and submandibular lymph nodes were analyzed. Submandibular lymph nodes showed a similar picture as blood and spleen, an increase of classical monocytes upon sleep. But this could just be found in the cell count and not in the frequency of classical Ly6C^{hi} monocytes. However, mesenteric lymph nodes (MLN) showed the contrasting effect, with a decrease of classical Ly6C^{hi} monocytes in their frequency and their numbers.

Fig. 3.9

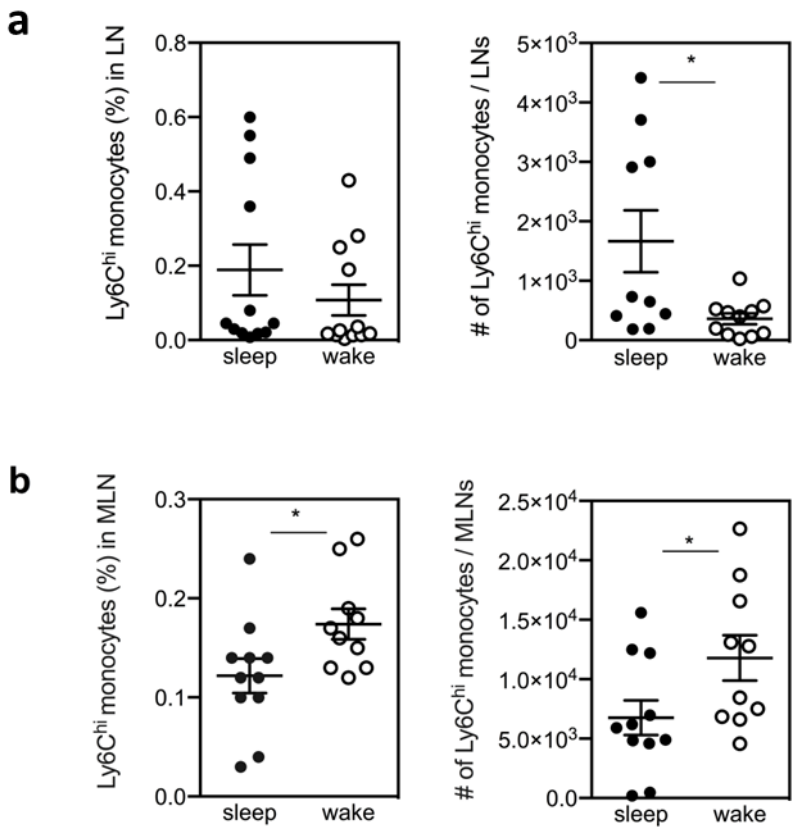

Fig. 3.9 Different impact of sleep on different lymph nodes

For 6 h, mice were either allowed to sleep ('sleep', filled circles) or not ('wake', open circles) (a) Graphs show the frequency and numbers of classical Ly6C^{hi} monocytes in submandibular lymph nodes. (b) Graphs show the frequency and numbers of classical Ly6C^{hi} monocytes in Mesenteric nodes. Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*)).

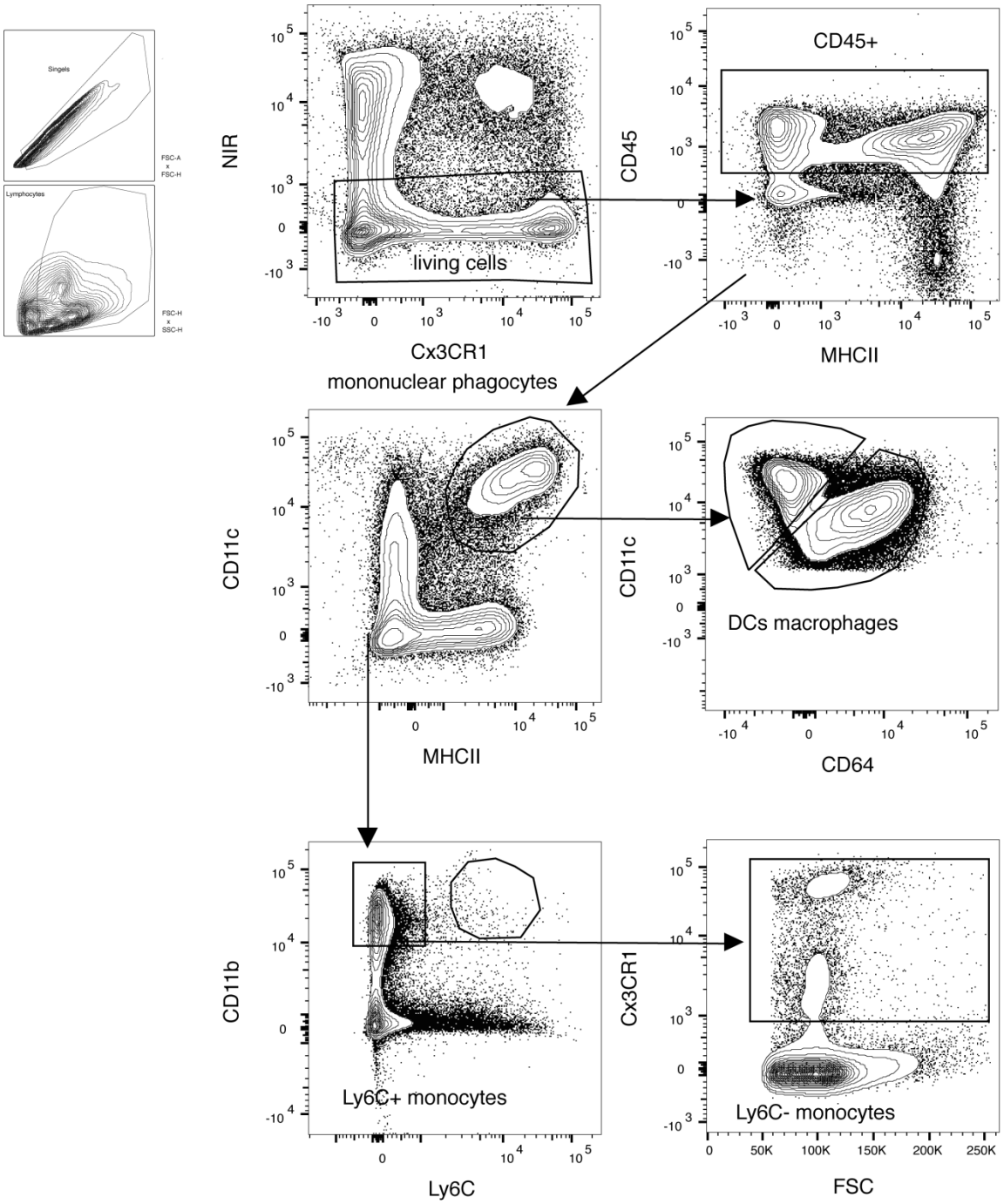

Fig. 3.10: Flow cytometry gating strategies of monocytes and macrophages in the lamina propria (LP)

$CX_3CR1^{gfp/-}$ were used to analyze the cellular composition of the lamina propria. All populations were gated as follows: Singlet s/lymphocytes/ living cells/ $CD45^+$. Starting from this population, mononuclear phagocytes were gated as $CD1c^{hi}MHCII^{hi}$. Next, macrophages ($CD64^+$) and DC ($CD64^-$) were gated. All mononuclear phagocytes were excluded from further analysis. Classical monocytes are found to be $CD11b^+$ and $Ly6C^+$. It was also gated on cells with a strong expression of CD11b. From there, non-classical monocytes were identified as CX_3CR1^+ population.

Fig. 3.11

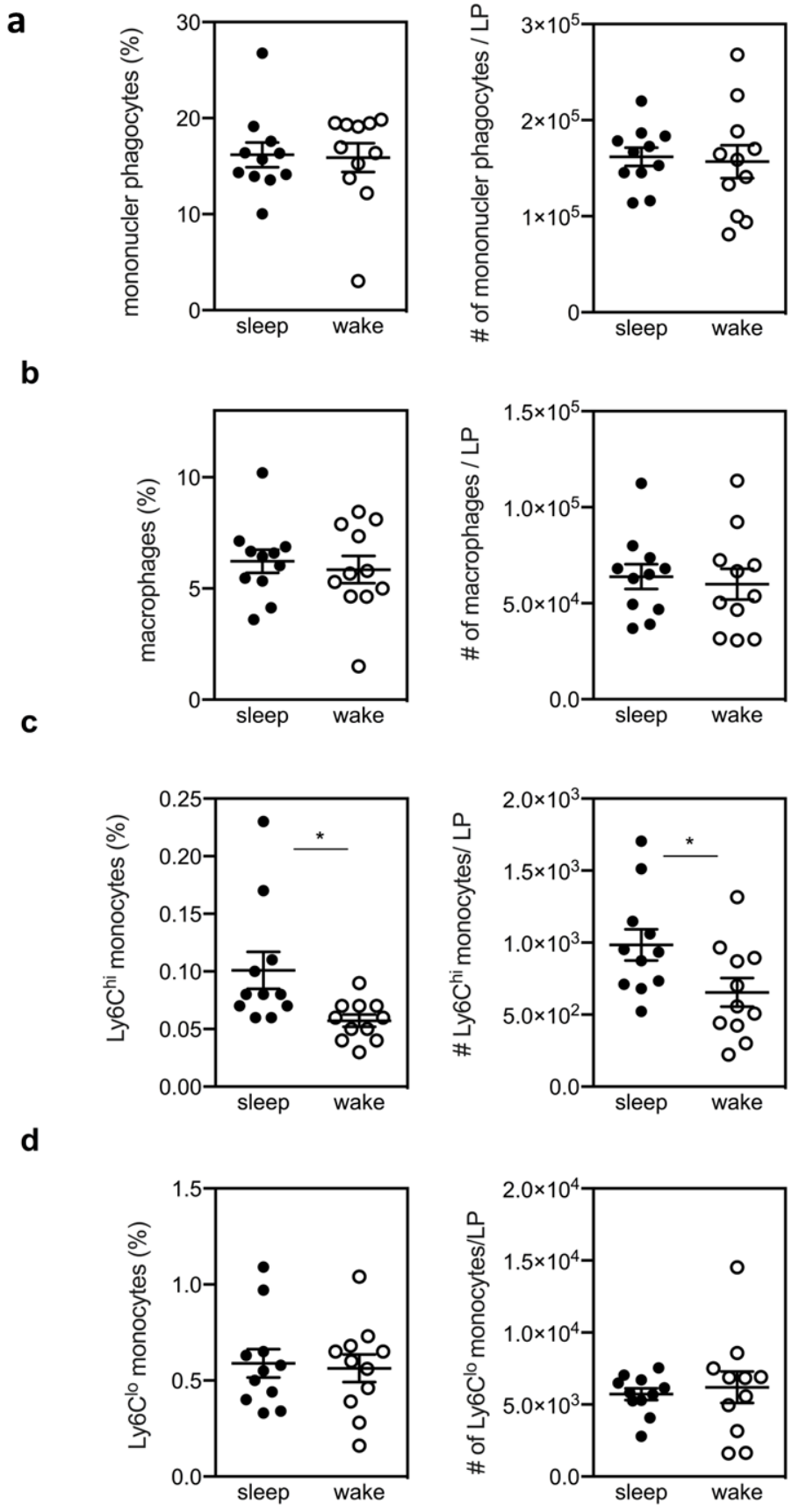

Fig. 3.11: Impact of sleep cellular composition of the lamina propria

For 6 h, $CX_3CR1^{GFP/-}$ mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). (a) Graphs show the frequency and numbers of mononuclear phagocytes in the LP ($CD11c^+MHCII^+$). (b) Graphs show the frequency and numbers of macrophages in the LP ($CD11c^+MHCII^+CD64^+$). (c) Graphs show the frequency and numbers of classical monocytes in the LP ($CD11b^+Ly6C^{hi}$). (d) Graphs show the frequency and numbers of non-classical monocytes in the LP ($CD11b^+Ly6C^{lo}CX_3CR1^+$). Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

The isolation of cells from the gut lamina propria can be a challenging task. As the viability of cells can be low, it is critical to remove dead cells from the analysis. Another problem is the impurities caused by epithelial cells. Therefore, it is essential to exclude these cells. That can be done by the expression of CD45, as CD45 is not expressed on epithelial cells. For the experiments with the lamina propria, $CX_3CR1^{GFP/-}$ mice were used to analyze the cellular composition of innate immune cells. In these mice, the fluorochrome green fluorescent protein (GFP) is expressed on CX_3CR1 receptors. The use of these mice allowed a better and clearer gating for non-classical monocytes in the LP. $Ly6C^{lo}$ non-classical monocytes were identified by their expression of CX_3CR1. As macrophages in the lamina propria also express CX_3CR1, a gating strategy had to consider that. Therefore, mononuclear phagocytes, which include macrophages, were gated first and then removed from further analysis. The data revealed that sleep has slight influence on the cellular composition of innate immune cells in this organ. The main population of innate immune cells, the mononuclear phagocytes, were unaffected by sleep (Fig. 3.11a). No changes occurred in this group, neither in frequency nor in cell numbers. Mononuclear phagocytes consist of macrophages and DCs (data not shown). Besides, LP-derived macrophages were unaffected by sleep as well, regarding frequency as well as cell count (Fig. 3.11b). For the rather small population of $Ly6C^{hi}$ classical monocytes, it could be demonstrated that sleep again leads to an increase in the sleep group as compared to the wake group. This result could be seen in frequency as well as in the cell count of $Ly6C^{hi}$ classical monocytes (Fig. 3.11c). Sleep did not modify frequency or cell number of $Ly6C^{lo}$ non-classical monocytes in the LP (Fig. 3.11d).

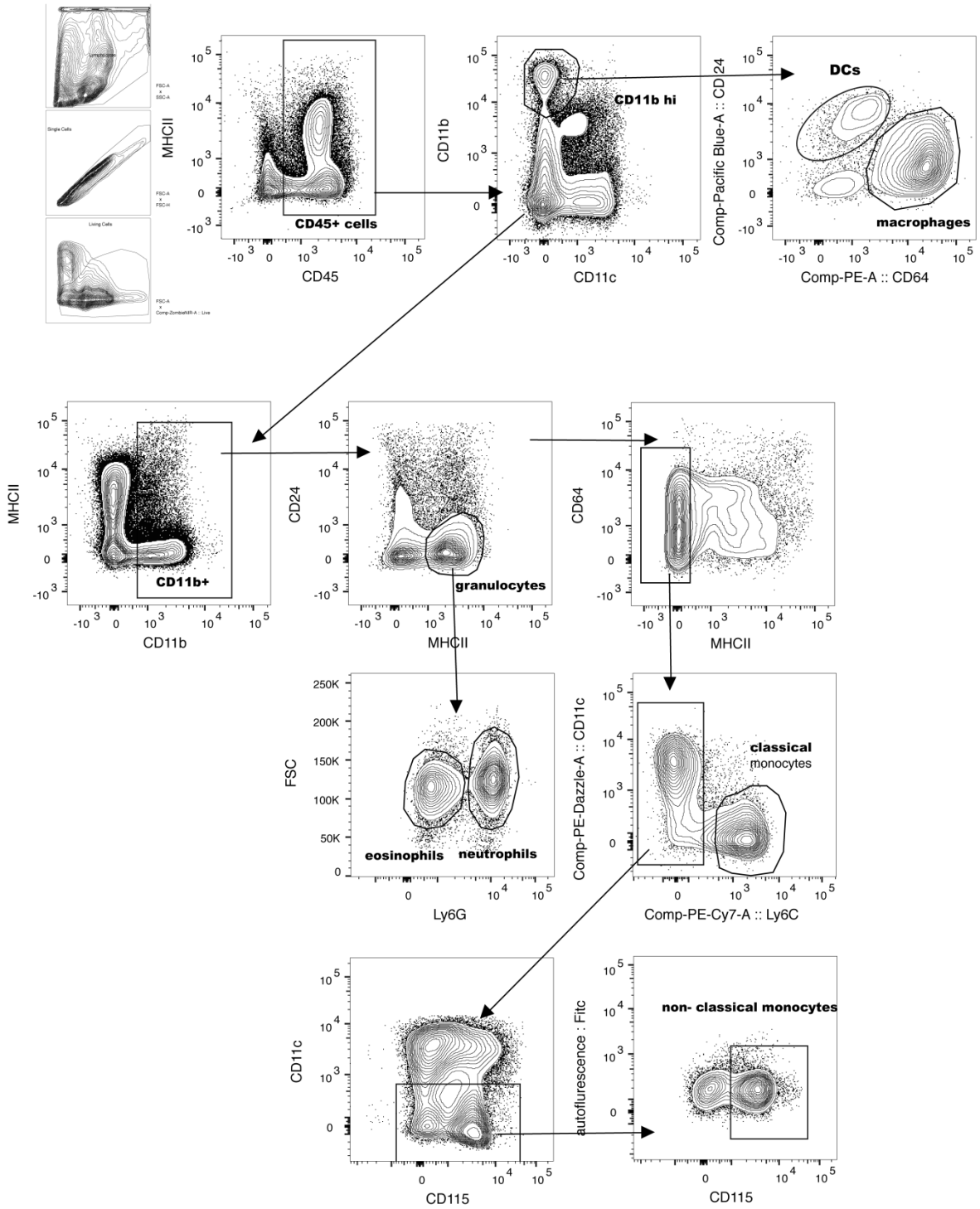

Fig. 3.12: Flow cytometry gating strategies of cellular composition of the lung

All populations were gated as follows: Singlets/lymphocytes/living cells/CD45^{+}. Starting from this population, mononuclear phagocytes were gated as CD11b^{hi}. From there macrophages (CD64^{+}) and DC (CD24^{+}) were gated. All mononuclear phagocytes were excluded from further analysis. Next, only CD11b^{+} cells were selected. From there, granulocytes were identified by MHCII-expression and further divided into eosinophils and neutrophils. All granulocytes were excluded from further analysis. In the next step, classical monocytes are found to be CD11b^{+} and Ly6C^{+}. It was also gated on cells with a strong expression of CD11b. From there, non-classical monocytes were identified as CD11c^{-} and CD115^{+}.

Fig. 3.13

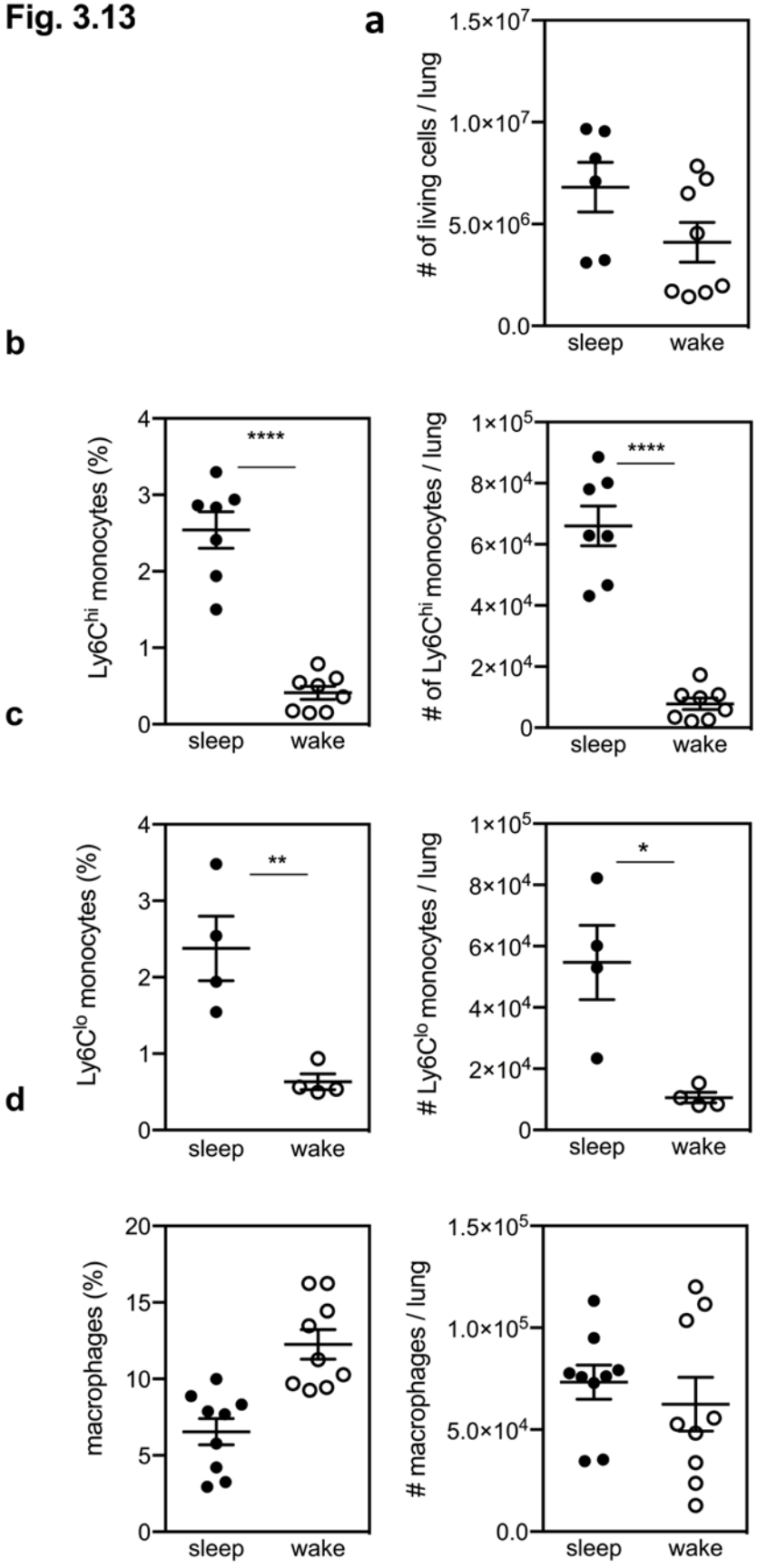

Fig. 3.13: Sleep increases classical and non-classical monocytes in the lung
For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). (a) Count of living cells per lung. (b) Graphs show the frequency and numbers

of classical monocytes ($Ly6C^{hi}CD11b^{+}$). (c) Graphs show the frequency and numbers of $Ly6C^{lo}$ non-classical monocytes ($Ly6C^{lo}CD11b^{+}CD115^{+}$). (d) Graphs show the frequency and numbers of lung macrophages ($CD11b^{hi}$, $CD64^{+}$). Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

The isolation of cells from the lung goes ahead with the same difficulties as those from LP, low viability of cells and possible contaminations by epithelial cells. Therefore, it was again critical to take that into account when designing a gating strategy. Dead cells were identified using Zombie NIR and epithelial cells were identified using CD45. To facilitate the gating on monocytes, a negative selection of DCs, macrophages, and granulocytes was done prior to analysis of monocytes.

The analysis of lung samples unveiled that sleep has a strong impact on monocyte populations. The number of living cells was not significantly altered upon sleep but revealed a tendency (Fig. 3.13a). The frequency and the cell count of $Ly6C^{hi}$ classical monocytes were more than doubled in 'sleeping' mice when compared to 'waking' (Fig. 3.13b). $Ly6C^{lo}$ non-classical monocytes were also increased in 'sleeping' mice when compared to 'waking' mice (Fig. 3.13c). By contrast, lung macrophages were not affected by sleep (Fig. 3.13d). These findings indicate that an enhanced rate of monocytes in the blood of 'sleeping' mice also facilitated their redistribution to peripheral tissues.

To continue the investigation of monocyte trafficking, further experiments were performed using $CCR2^{-/-}$ mice. CCR2 is a crucial factor for monocyte chemotaxis. It regulates monocyte release from the bone marrow and enables extravasation into various tissues. It is differentially expressed on classical ($CCR2^{+}$) and non-classical monocytes ($CCR2^{-}$). The effects of sleep on monocytes in $CCR2^{-/-}$ mice, which have a normal monocyte development, but disturbed migration in response to CCL2, were analyzed. Although the number of monocytes in the spleen and blood of these mice was already low due to reduced egress from the BM [Serbina and Pamer 2006] 'sleeping' mice still showed enhanced monocyte numbers as compared to 'waking' mice (Fig. 3.14). These data showed that the sleep-induced rise in blood and splenic monocytes are independent of CCR2 signaling.

Fig. 3.14

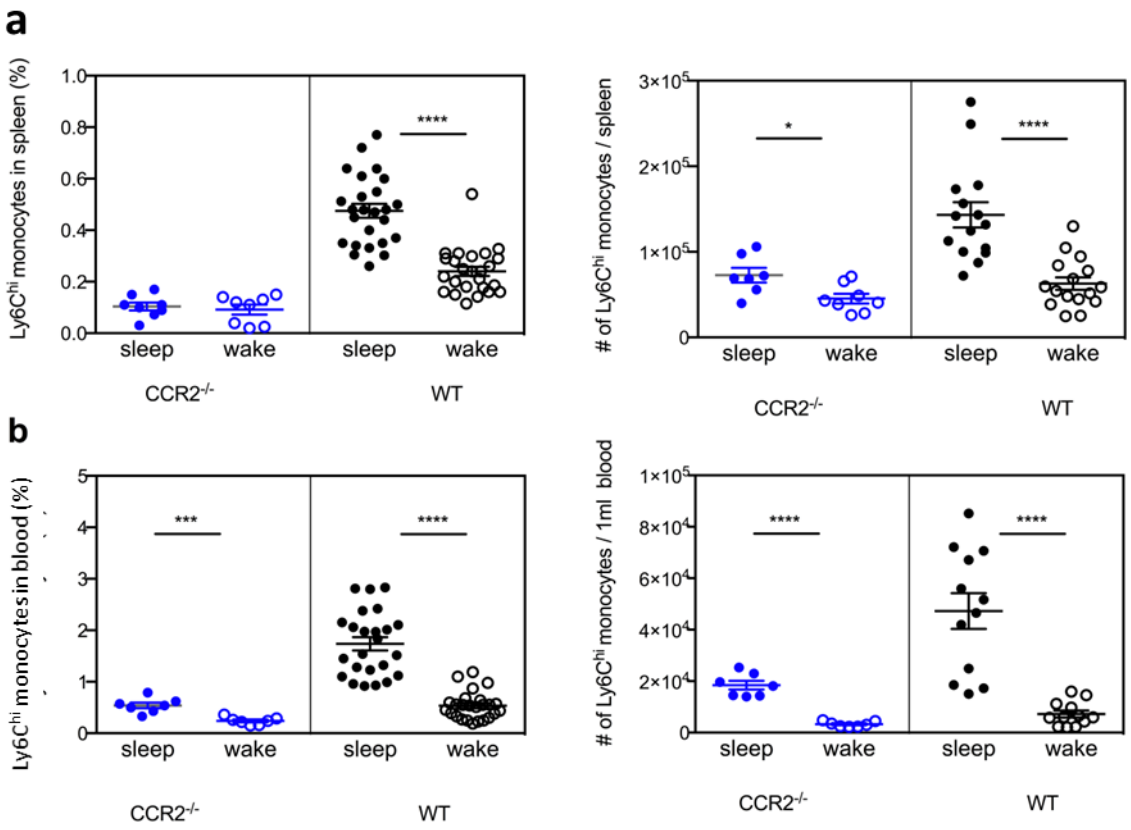

Fig. 3.14: Monocyte increase up on sleep in CCR2-independent
For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). Blue circles represent CCR2$^{-/-}$ mice, black circles wild type (WT) mice. (a) Graphs show the frequency and numbers of splenic classical monocytes (Ly6C^{hi}CD11b^{+}). (b) Graphs show the frequency and numbers of classical monocytes (Ly6C^{hi}CD11b^{+}) in 1ml blood. Every dot represents 1 mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

3.6. Impact of sleep on cell death of monocytes

In a next step, it was ruled out that the observed changes were due to cell death, apoptosis or necrosis upon sleep deprivation in the wake group. Therefore, the numbers of dead monocytes (Zombie NIR^{+}), apoptotic monocytes (Annexin-V^{+}), and necrotic monocytes (7-AAD^{+}and Annexin-V^{+}) were analyzed (Fig. 3.15). The data revealed no increase in the amount of dead, apoptotic, or necrotic monocytes in blood and spleen of 'waking' mice. This leads to the conclusion that the sleep-induced rise in monocytes is not due to enhanced cell death in the wake mice group.

Fig. 3.15

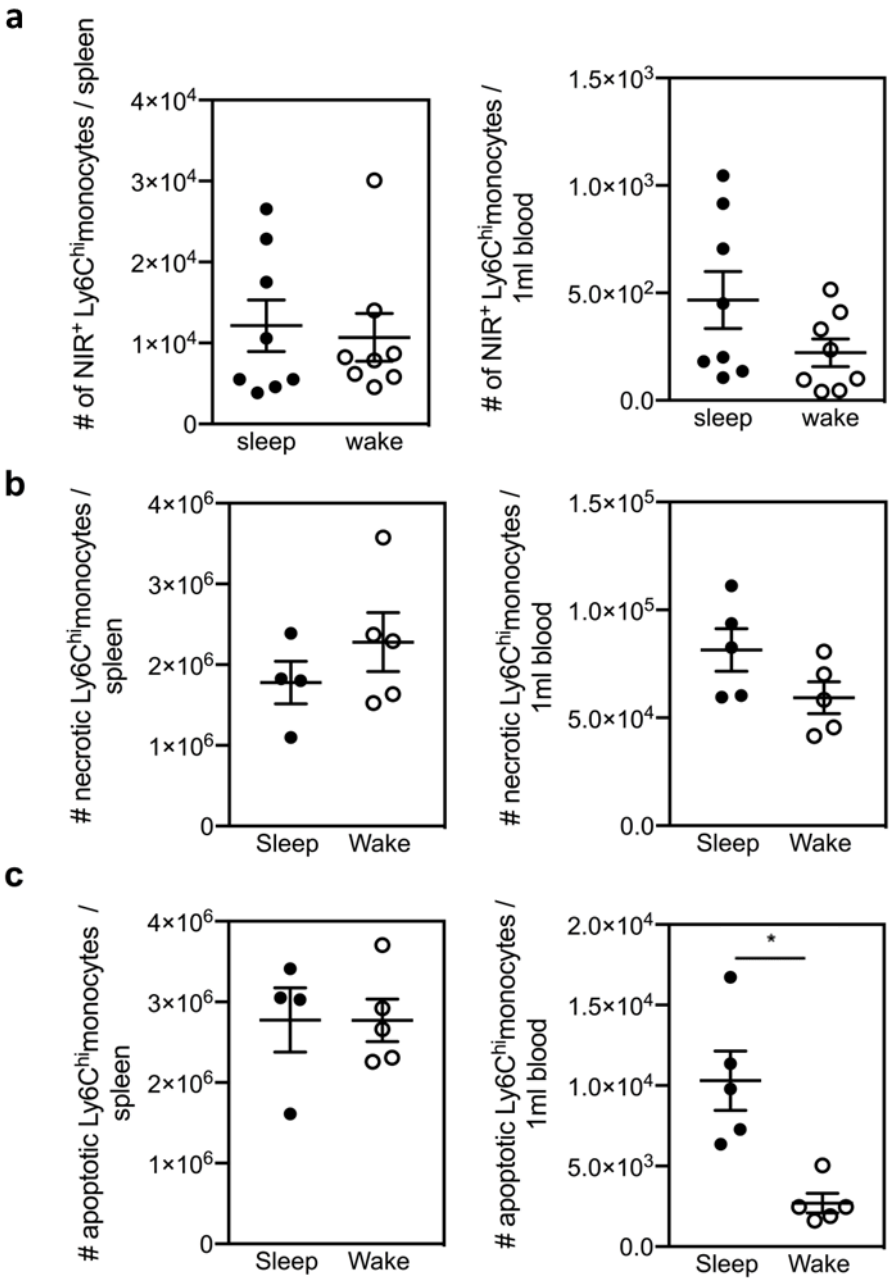

Fig. 3.15: Sleep has no impact on cell death, necrosis, or apoptosis regarding classical monocytes

For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). (a) Graphs show the numbers of NIR$^+$ classical monocytes in spleen and blood (11b$^+$Ly6C^{hi}NIR$^+$).(b) Graphs show the numbers of necrotic classical monocytes in spleen and blood (11b$^+$Ly6C^{hi} Annexin-V$^+$7AAD$^+$). (c) Graphs show the numbers of apoptotic classical monocytes in spleen and blood (11b$^+$Ly6C^{hi} Annexin-V$^+$)Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****)).

3.7. Long-lasting effect of sleep on monocytes

To examine whether the effect of sleep on monocytes is a short-term or long-term effect, mice were allowed to recover for various times after gentle handling and were compared to mice without sleep manipulation. All mice that were compared to each other were sacrificed at the same time, to exclude effects derived from the circadian system.

More precisely, mice were assigned into 7 groups: In group 1, mice were allowed to sleep undisturbed for 6 h. Mice belonging to group 2 were kept awake for 6 h. Group 3 mice were kept awake first for 6 h and then left undisturbed for 24 h. All mice in groups 1-3 were sacrificed at 1:30 pm.

Group 4 mice had slept for the usual 6 h plus additional 4 h. Group 5 mice were kept awake for 6 h and then left undisturbed for 4 h. Groups 4-5 were analyzed at 5:30 pm. Group 6 mice slept for the usual 6 h plus additional 6 h. Group 7 mice were kept awake for 6 h and then left undisturbed for 6 h. Groups 6-7 were analyzed at 7:30 p.m. (Tab. 3.1)

Tab. 3.1 Groups for recovery experiments

Group	6 h sleep or wake	Recovery time	Time of sacrifice
1	sleep	0 h	1:30 p.m.
2	wake	0 h	1:30 p.m.
3	wake	24 h	1:30 p.m.
4	Sleep	4 h	5:30 p.m.
5	Wake	4 h	5:30 p.m.
6	Sleep	6 h	7:30 p.m.
7	wake	6 h	7:30 p.m.

Fig. 3.16

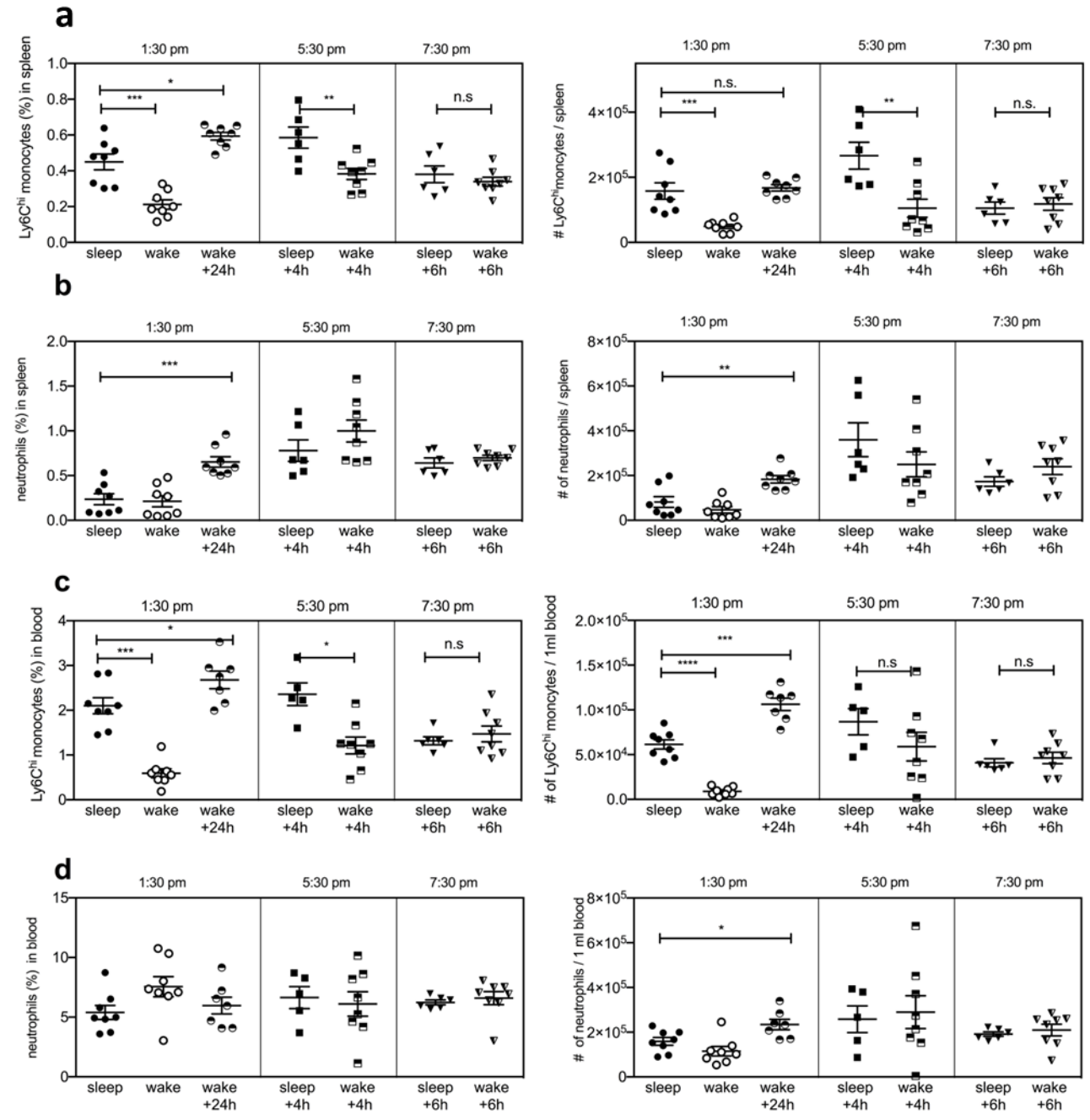

Fig. 3.16: Sleep leads to long-lasting changes in cell composition in spleen and blood
Mice were kept awake or subjected to sleep for 6 h and then were allowed to sleep for the indicated times afterwards (sleep; wake + xh). The time depicted above the graphs represents the time during the day the mice were sacrificed. (a) Graphs show the frequency and numbers of Ly6C^{hi} monocytes in the blood. (b) Graphs show the frequency and numbers of neutrophils (CD11b^{+}Ly6G^{+}) in the blood. (c) Graphs show the frequency and numbers of splenic Ly6C^{hi} monocytes. (d) Graphs show the frequency and numbers of splenic neutrophils (CD11b^{+}Ly6G^{+}). Every dot represents 1 mouse. Data represent the mean ± SEM (ANOVA followed by Tukey's Multiple Comparison Test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***),or $p < 0.0001$ (****)).

As described above, sleep had a strong impact on Ly6C^{hi} monocytes directly after 6 h of sleep or 6 h of enhanced wakefulness in the blood (group 1 and 2). After 4 h recovery time, Ly6C^{hi} monocytes in 'sleeping' mice were still increased in frequency as well as in their total numbers as compared to 'waking' mice (group 4 and 5). After another 2 h of recovery time the groups, 6 and 7, were not distinguishable anymore. Surprisingly after 24h of recovery time mice that were exposed to enhance wakefulness showed a higher frequency of monocytes than mice without sleep manipulation, group1 and 3 (Fig. 3.16a). As expected, sleep had no impact on neutrophil-frequency and total number directly after 6 h of sleep or 6 h of enhanced wakefulness. But unexpectedly after 24 h of recovery time blood neutrophils showed an increase in group 3 as compared to group 1. This phenomenon could be detected for the frequency and the cell count of blood neutrophils (Fig. 3.16b).

Similar findings were made in the splenic cell population. Splenic Ly6C^{hi} classical monocytes showed the typical rise in the sleep group as compared to the wake group, directly after 6 h of sleep or enhanced wakefulness. This rise lasted for 4 h. After 6 h of recovery time, the groups were not distinguishable. But after 24 h of recovery time, an increase of Ly6C^{hi} classical monocytes could be observed. Group 3 mice that were kept awake for 6 h and then were left undisturbed for 24 h, showed a higher frequency and number of classical monocytes in their spleens than mice without sleep manipulation (Fig. 3.16c). The splenic neutrophils were unaffected by sleep right after 6 h of sleep or enhanced wakefulness when comparing group 1 and 2. In addition, 4 h recovery time also leads to no differences between the groups 4 and 5. 6 h of recovery time also shows no effect on groups 6 and 7. But interestingly, the number of splenic neutrophils was elevated in the group 3, mice with 6 h of enhanced wakefulness followed by 24 h of recovery time, as compared to group1, mice without sleep manipulation (Fig. 3.16d). These results demonstrated that sleep has long-lasting effects on classical monocytes and neutrophils in the blood and spleen of mice.

3.8. Sleep and the marginal pool

The marginal pool describes the binding of Ly6C^{hi} classical monocytes and Ly6C^{lo} non-classical monocytes to the epithelium. If sleep would lead to release of monocytes from the marginal pool, this would be one possible explanation for the sleep-mediated increase in monocytes described above.

To examine the involvement of marginal pool binding of Ly6C^{hi} classical monocytes, transgene mice models defect for ICAM-1 were used (ICAM-1^{-} mice). These mice have no functioning ICAM-1 receptor anymore, which is normally expressed on epithelial cells and the key mediator for the binding of Ly6C^{hi} classical monocytes to the epithelium. Ly6C^{hi} classical monocytes express LFA-1 which is a ligand for the ICAM-1 receptor [Carlin et al. 2013]. Therefore, these mice were used for sleep experiments following the usual protocol.

Fig. 3.17

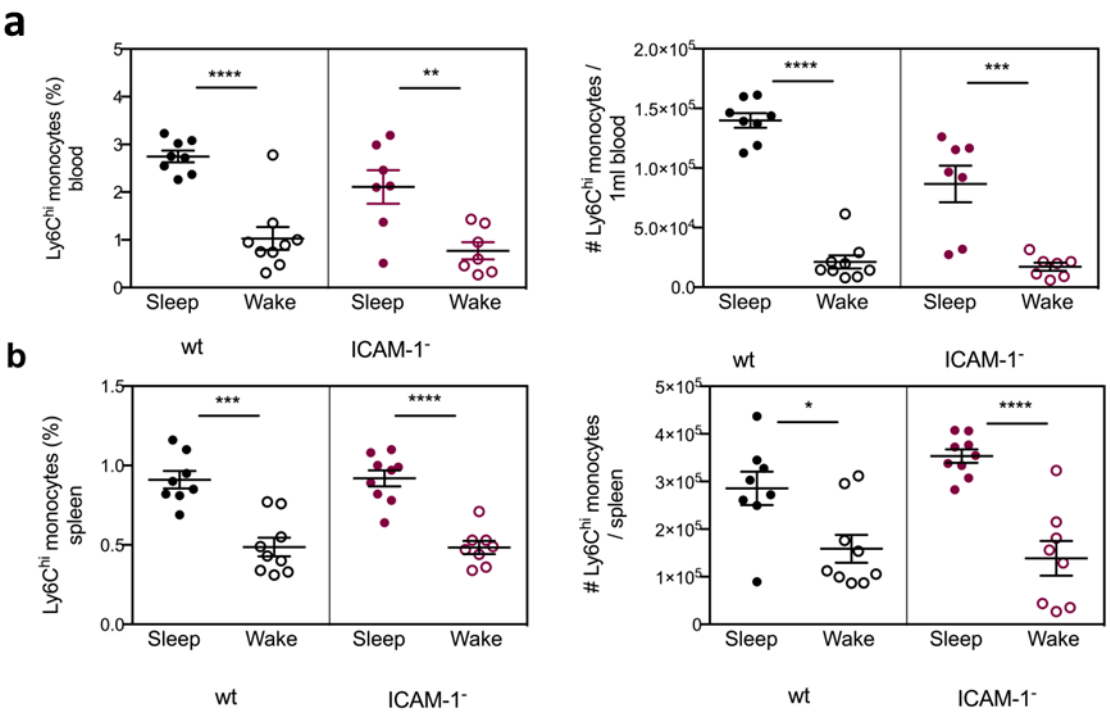

Fig. 3.17: Ly6C^{hi} classical monocytes increase upon sleep is ICAM-1 independent
For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). Black circles represent WT mice, purple circles ICAM-1$^{-/-}$ mice. (a) Graphs show the frequency and numbers of Ly6C^{hi} classical monocytes (CD11b^{+}Ly6C^{hi}CD115^{+}) in the blood of WT and ICAM-1$^{-/-}$mice. (b) Graphs show the frequency and numbers of splenic Ly6C^{hi} classical monocytes (CD11b^{+}Ly6C^{hi}CD115^{+}) of WT and ICAM-1$^{-/-}$mice. Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****))

Analysis of blood samples taken from ICAM-1^{-} mice revealed a sleep-mediated increase of Ly6C^{hi} classical monocytes. This was observed for the frequency as well as for the number of respective cells. Hence, these effects were equal to those that could be found in WT mice, suggesting that there are no changes induced by ICAM-1^{-} (Fig. 3.17a) The same effect could be observed for splenic samples taken from ICAM-1$^{-/-}$ mice (Fig. 3.17b).

To investigate the possible connection between the marginal pool and the increase of Ly6C^{lo} non-classical monocytes upon sleep, $CX_3CR1^{GFP/-}$ and $CX_3CR1^{GFP/GFP}$ mice were used. Homozygote $CX_3CR1^{GFP/GFP}$ mice have a GFP-tag on every CX_3CR1 receptor, which leads to a loss of function. By contrast, the heterozygote animals still own functioning CX_3CR1 receptors. These mice were selected for the investigation of the marginal pool regarding Ly6C^{lo} non-classical monocytes because it has been described that CX_3CR1 is the key mediator for the binding of Ly6C^{lo} non-classical monocytes to the marginal pool. CX_3CL1 is expressed on the epithelium and Ly6C^{lo} non-classical monocytes can bind to it via CX_3CR1 [Arnold et al. 2007].

In the following experiments WT, $CX_3CR1^{GFP/-}$, and $CX_3CR1^{GFP/GFP}$ mice were analyzed. First of all, the obtained data revealed that WT and $CX_3CR1^{GFP/-}$ mice reacted similar to sleep or enhanced wakefulness. This was the case for all considered cell types in blood and spleen (Fig 3.18). In the case of $CX_3CR1^{GFP/GFP}$ mice, the data showed that the loss of function had no consequences on the rise of Ly6C^{hi} classical monocytes upon sleep. This could be observed for the frequency and number of blood Ly6C^{hi} classical blood-monocytes and also for the frequency and number of splenic classical monocytes (Fig 3.18 a+c). For the Ly6C^{lo} non-classical monocytes, the situation was different as WT and $CX_3CR1^{GFP/-}$ mice showed a rise in Ly6C^{lo} non-classical monocytes in 'sleeping' mice as compared to 'waking' mice. This increase could not be seen in $CX_3CR1^{GFP/GFP}$ mice. Blood Ly6C^{lo} non-classical monocytes of $CX_3CR1^{GFP/GFP}$ mice were unaffected by sleep, in frequency and cell count (Fig. 3.18b). The same was observed for splenic Ly6C^{lo} non-classical monocytes in mice with the loss-of-function mutation. Sleep did not lead to the typical increase of cells (Fig. 3.18d).

Fig. 3.18

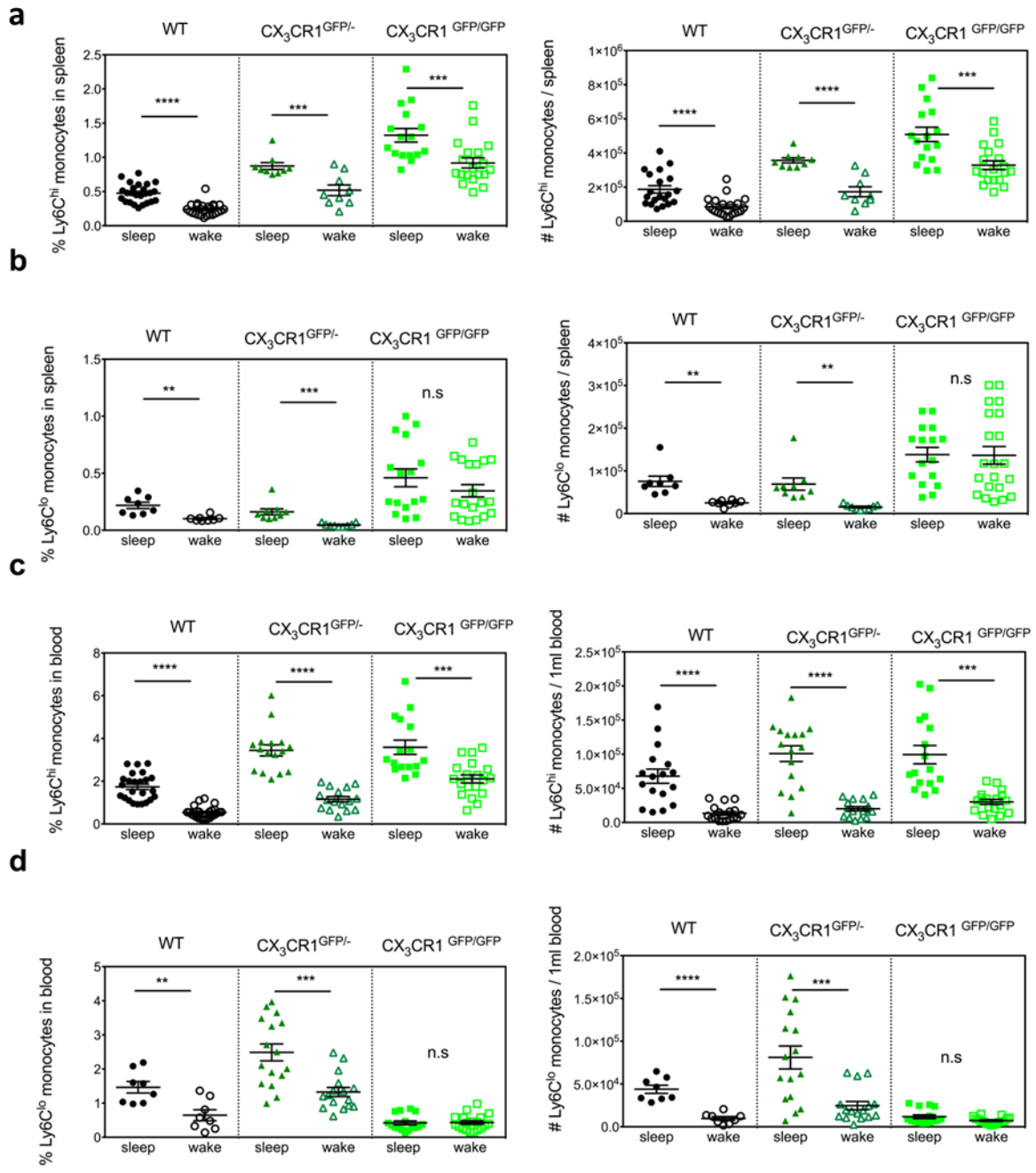

Fig. 3.18: Ly6C^{lo} non-classical monocytes increase up on sleep is CX$_3$CR1 dependent
For 6 h, mice were either allowed to sleep ('sleep', filled symbols), or not ('wake', open symbols). Black represents WT mice, dark green CX$_3$CR1$^{GFP/-}$ mice and light green CX$_3$CR1$^{GFP/GFP}$ mice. (a) Graphs show the frequency and numbers of Ly6C^{hi} classical monocytes (CD11b$^+$Ly6C^{hi}CD115$^+$) in the blood of WT CX$_3$CR1$^{GFP/-}$ and CX$_3$CR1$^{GFP/GFP}$ mice. (b) Graphs show the frequency and numbers of Ly6C^{lo} non-classical monocytes (CD11b$^+$Ly6C^{lo}CD115$^+$) in the blood of WT CX$_3$CR1$^{GFP/-}$ and CX$_3$CR1$^{GFP/GFP}$ mice. (c) Graphs show the frequency and numbers of splenic Ly6C^{hi} classical monocytes (CD11b$^+$Ly6C^{hi}CD115$^+$)of WT CX$_3$CR1$^{GFP/-}$ and CX$_3$CR1$^{GFP/GFP}$. (d) Graphs show the frequency and numbers of splenic Ly6C^{lo} non-classical monocytes (CD11b$^+$Ly6C^{lo}CD115$^+$) in WT CX$_3$CR1$^{GFP/-}$ and CX$_3$CR1$^{GFP/GFP}$ mice. Every dot represents one mouse. Data represent the mean ± SEM (Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****))

3.9. Impact of sleep on antimicrobial activity and systemic infection

The previous findings led to a strong interest regarding the impact of sleep on antimicrobial activity and systemic bacterial infection. To investigate if sleep is important for the antimicrobial activity of phagocytes, reactive oxygen species (ROS) production by blood phagocytes was analyzed. Upon bacterial uptake by phagocytes ROS mediate intracellular bacterial killing. Flow cytometry analysis revealed that the ROS production by PMNs and classical monocytes was significantly increased in 'sleeping' as compared to 'waking' mice (Fig. 3.19a). To address if the sleep-mediated increase of monocyte numbers and antimicrobial activity of phagocytes have an impact on pathogen defense, infection experiments were performed. Mice were infected intravenously with the model organism *Y. enterocolitica*, to mimic a sepsis infection. The infection was performed directly after 6 h of sleep or enhanced wakefulness. In the further course of the experiment blood and spleen of this animal were tested for their bacterial burden. Already 30 min post infection the bacterial burden in the blood of 'sleeping' mice was reduced by factor 2.7 compared to 'waking' mice (Fig. 3.19b). A similar effect was observed one day post infection in the spleen (Fig. 3.19c). Three days post infection the number of *Ye* colony forming units in the spleen of 'sleeping' mice was reduced by factor 7 compared to 'waking' mice (Fig. 3.19d). This observation was accompanied by a less pronounced splenomegaly (Fig. 3.19e). In addition, survival experiments following infection were performed. In the survival experiments, mice were infected as described above. The weight of every mouse was determined at the start of the experiment, hereinafter every mouse was weighed every 12 h. A mouse was sacrificed when its bodyweight dropped by 20%. The data from these experiments demonstrate a significantly enhanced survival rate in 'sleeping' mice when compared to 'waking' mice after *Ye* infection. In summary, the combined enhancing effects of 6 h sleep on monocyte numbers and on antimicrobial activity of phagocytes seems to acutely boost innate immune defense against systemic bacterial infection and thus benefit survival (Fig. 3.19f)

Fig. 3.19

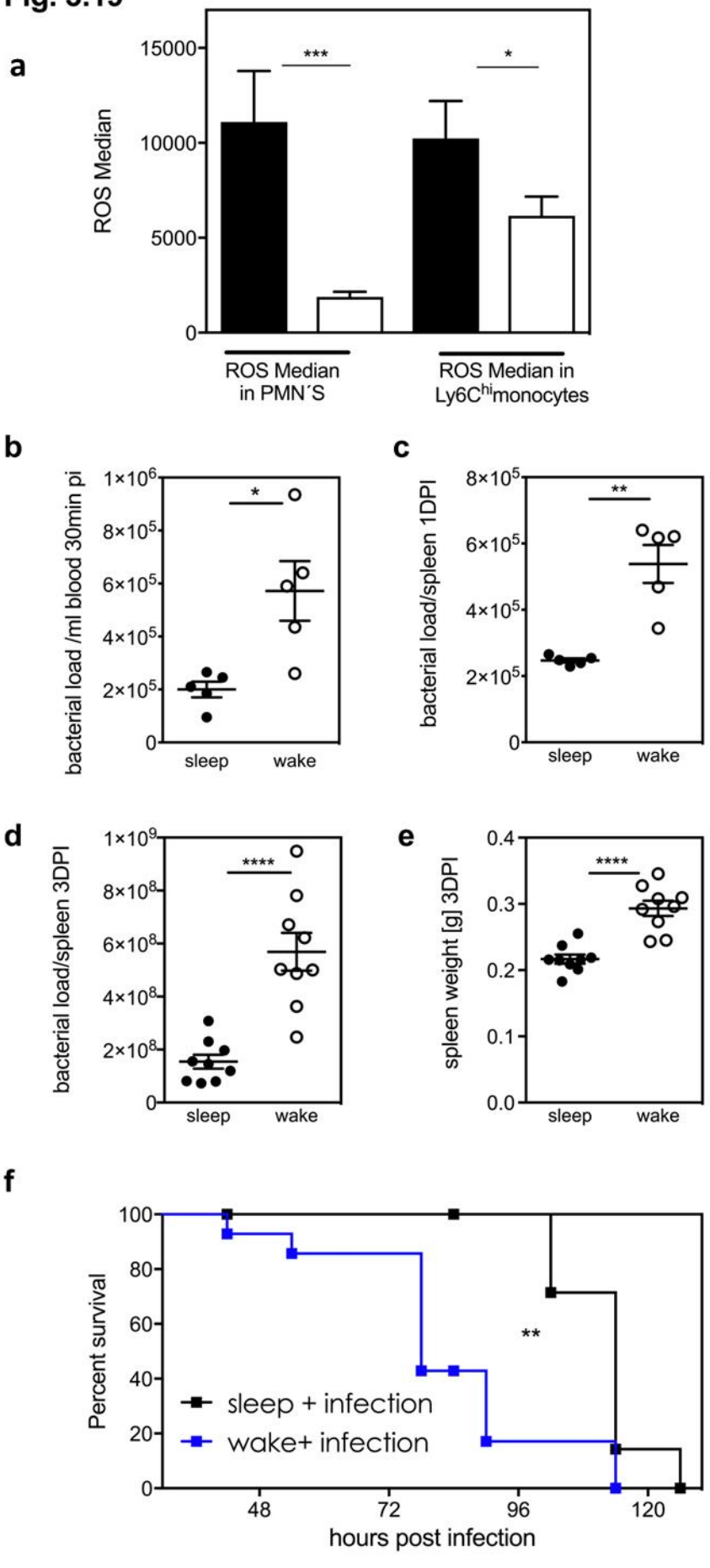
a
ROS Median
15000
10000
5000
0
***
*
ROS Median in PMN´S
ROS Median in Ly6C^hi monocytes
b
bacterial load /ml blood 30min pi
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
*
sleep
wake
c
bacterial load/spleen 1DPI
8×10^5
6×10^5
4×10^5
2×10^5
0
**
sleep
wake
d
bacterial load/spleen 3DPI
1×10^9
8×10^8
6×10^8
4×10^8
2×10^8
0
****
sleep
wake
e
spleen weight [g] 3DPI
0.4
0.3
0.2
0.1
0.0
****
sleep
wake
f
Percent survival
100
80
60
40
20
0
**
sleep + infection
wake+ infection
48
72
96
120
hours post infection

Fig. 3.19: Sleep boosts defense against systemic bacterial infection

For 6 h, mice were either allowed to sleep ('sleep', filled circles), or not ('wake', open circles). And either directly analyzed or intravenously infected with $5x10^8$ (b) or $5x10^4$ (c-e) *Yersinia enterocolitica*. (a) ROS production of blood PMNs and monocytes was analyzed by flow cytometry. (b) Graphs show the bacterial load (CFU) 30 min post infection in the blood. (c), Graphs show the bacterial load (CFU) 1 day post infection in the spleen. (d) Graphs show the bacterial load (CFU) 3 days post infection in the spleen. (e) Graphs show the spleen weight 3 dpi. (f) Graphs show the percent survival after an infected with *Yersinia enterocolitica*. Data show the mean ± SEM (n=5; Student's t-test; $p < 0.05$ (*), $p < 0.01$ (**) or, $p < 0.001$ (***)) (a – e). (f) Survival of mice treated and infected as described above was monitored until 6 dpi (n=7-14; Log-rank (Mantel-Cox) test, $p < 0.01$ (**)).

4. Discussion

The mutual influence of sleep and immunity has been in the center of many studies over the past decades. Several of them describe a strong impact of sleep on the immune system [Besedovsky et al. 2012] [Bryant et al. 2004] [Besedovsky et al. 2019]. Beyond this, it was found that infections modulate sleep behavior in animals and humans. This modulation was independent of species and type of infection [Toth 1995b] [Imeri and Opp 2009] [Ibarra-Coronado et al. 2015]. Despite these efforts, our understanding of the interplay between sleep and immunity is still incomplete. The underlying mechanism remains unknown as well as the effect of sleep on the innate immune system and infectious diseases. In particular, the impact of sleep on innate immune cells is very critical as they are the first line of defense against invading pathogens. A weakening of this defense would lead to an increased risk of infections and worse outcome. To address the question, whether sleep is necessary for a fully functioning innate immune system and therefore immune defense in the context of infection, mice experiments were performed. In these experiments, mice were either exposed to 6 hours of extended wakefulness or were allowed to follow their normal sleep behavior. Extended wakefulness was achieved by gentle handling. This technique is a suitable method to prevent all sleep phases without being stressful for the animals [Shi and Pamer 2011] [Oyanedel et al. 2015] [Rolls et al. 2015]. As it has been shown that the circadian rhythm has an impact on the immune system as well, it was important to collect all data at the same time [Nguyen et al. 2013] [Bellet et al. 2013] [Casanova-Acebes et al. 2013]. In this way, it was possible to investigate the effect of sleep on immune cells excluding those of the circadian rhythm.

The results clearly demonstrating a strong positive impact of sleep on immunity, mice following their normal sleep behavior were found to have more and more potent innate immune cells than mice that were exposed to 6 hours of extended wakefulness. Consequentially, that led to a weakened immune defense against bacterial infections.

4.1. Increased numbers and frequency of monocytes is due to sleep

Flow cytometry examination of the immune composition in blood and spleen revealed differences between the sleep and the wake group. Analyses of the blood cell composition showed, that the WBC were nearly double in mice with 6 h sleep when compared to mice with 6 h of enhanced wakefulness. In the case of neutrophils, the sleep and wake groups showed no differences, neither in frequency nor in cell count of neutrophils. With regards to DCs, the frequency of DCs was unchanged between the two groups. But the number of DCs was found to be elevated in the sleep group. A particularly strong effect of sleep was seen on the classical monocytes. The frequency of classical Ly6C^{hi} monocytes in the blood was more than doubled after 6 h of sleep. The same was seen for the cell count of Ly6C^{hi} monocytes, with an increase in the sleep group of over 100%. Thus, in the following we will describe this increase in frequency and count of cells due to sleep as the sleep phenotype.

Analysis of the spleen have shown a similar picture. Like in the blood, the overall cellularity of the spleen was elevated after 6 h of sleep. Even if the effect is not as big as in the blood, it was still significant. The neutrophils were again not affected by 6 h of sleep. DCs showed a decrease in frequency in the sleep group compared to the wake group, but no differences in cell count. Most affected by sleep were again the classical monocytes. Their frequency and numbers were also doubled by 6 h of sleep. These findings have shown that sleep is vital for a fully functional innate immune system. A lack of sleep for just 6 h led to changes in frequency and numbers of in particular classical monocytes in several immune compartments, whereas PMNs were unaffected. These findings are in contrast to previous studies in mice and rats that have shown an increase of neutrophils and monocytes in blood following prolonged sleep deprivation [Guariniello et al. 2012] [Everson 2005]. In these studies, sleep was prevented by the multiple platform method which leads to paradoxical sleep deprivation (PSD). Paradoxical sleep deprivation by the multiple platform method is different from the enhanced wakefulness used in this work. First, the multiple platform method cannot prevent all sleep phases, as sleep interruption occurs due to unconscious movements of the experimental animals. Gentle

handling has been used to achieve enhanced wakefulness preventing all sleep phases. Secondly, the time span: In the study from Everson *et al.*, they exposed the animal to PSD for up to 72h while the animals for this work were only exposed to enhanced wakefulness for 6 h. This difference may explain the different findings. The increase of innate immune cells found in these studies using the multiple platform method can be the result of an inflammatory response to oxidative stress, DNA, cell, and tissue damage [Toth et al. 1995a]. In previous animals studies that used similar techniques to apply brief acute enhanced wakefulness monocyte numbers were not assessed [Renegar et al. 1998] [Mullington et al. 2000] [Brown et al. 1989] [Toth and Rehg 1998] [Toth et al. 1995a] [Druzd et al. 2017]. With respect to studies in humans, our findings are contrary. Sleep studies in which participants were allowed to follow their normal sleep behavior during the night or were exposed to nocturnal wakefulness, found an increase of monocytes during nocturnal wakefulness [Born et al. 1997]. Interestingly, classical monocytes were unaffected but numbers of non-classical monocytes were increased in the blood of participants that had not slept during the night [Dimitrov et al. 2007]. It is important to keep in mind, that mice and humans have different sleep behavior with different sleep patterns. Humans have a monophasic sleep pattern, whereas mice have a polyphasic sleep pattern. So, a direct comparison of these two species is difficult. The question remains what led to the strong increase in classical monocytes in the sleeping mice? Possible explanations are:

Firstly: Mice in sleep group suffer from stress which leads to a reduction of monocytes.

Secondly: Sleep is a mediator in monocyte development and without sleep more monocytes differentiate into non-classical monocytes, DCs, or macrophages.

Thirdly: Sleep affects the monopoiesis.

Fourthly: Sleep is a survival factor for monocytes and a lack of sleep leads to the death of monocytes.

Fifthly: Sleep regulates monocyte migration and/or their adherence to the marginal pool.

4.2. Gentle handling does not cause stress in mice

Stress and the influence it has on the immune system have been in the center of many studies. And there is an undeniable connection between stress and immunity. Several studies could show that stress leads to a reduction of classical monocytes in mice [Dhabhar et al. 2012]. So, the assumption that gentle handling induces stress and therefore a reduction of monocytes in the wake group is reasonable. Yet the methods used in stress studies are very different from gentle handling, in such experiments animals are exposed to strong social or physical stress and that for a long period of time. For social stress, small male mice are placed in a cage with a bigger and dominate male that leads to continual battles between the mice. Another possibility to induce stress is to isolate mice from their social group and to put this animal in very small place, so that they cannot move at all, for example in a 50mL tube. These stressors are applied to the animal for several hours of several days [Bowers et al. 2008]. In our experimental setup the animal always remained in their social group. When male animal was used, they were brothers and were regularly examined for signs of fighting. During the 6 h of gentle handling, the animals were barely touched and the social groups were unchanged. Furthermore, gentle handling is a well-established way for sleep prevention without causing stress [Rolls et al. 2015].

Nevertheless, to ensure the mice in the wake group where not stressed by gentle handling the corticosterone levels in the serum of mice were measured. Corticosterone is a good indication for stress as it is one of the main stress hormones found in animals. The hormone levels in the serum of both mouse groups were comparable and no signified alteration of corticosterone in the wake group was detected. Furthermore, the corticosterone levels were within the normal range for this time period and this mouse strain [Parrillo and Fauci 1979] [Rolls et al. 2015] [Oyanedel et al. 2015]. These analyzes roll out stress as an explanation for the sleep-phenotype.

4.3. Sleep effect is not due to development of monocytes

As classical monocytes are precursors of different cell types there was the possibility that a lack of sleep would interfere with their normal development. To address this possibility, different progenies were analyzed in blood and spleen. Progenies of monocytes include DCs, non-classical monocytes, and macrophages [Jung 2018].

DCs were gated as $CD11c^+$ and MHC II^+ cells [Bell et al. 1999]. In case of splenic DCs the frequency was significantly reduced in the sleep group but no effect was found with regards to the count of DCs. This highlights that it is essential to check the frequency and the count, because the frequency is not a fix single value, it is depending on the overall cell composition. In the blood we found no changes in the frequency of DCs but we found the sleep phenotype in the DCs count, a significant increase in the sleep group.

Synoptic, the changes in the DCs population in blood and spleen cannot explain the strong increase of classical monocytes.

Also analyzed were macrophages as they develop from classical monocytes [Jung 2018]. Macrophages were identified as $CD11b^{hi}$ and $F4/80^+$ cells. In order to explain the sleep phenotype by the differentiation of monocytes, the number of macrophages in blood, spleen, and peripheral tissues like LP and lung should be greatly reduced in the sleep group.

But the results showed a different picture:

The frequency in splenic macrophages was identical in both groups but the number of splenic macrophages was increased in the sleep group, showing the sleep phenotype. For the blood the frequency of macrophages was strongly increased but no changes in the numbers of macrophages had been observed - again, indicating the importance of the measurement of the cell count.

With regard to the peripheral tissues, the lung and the LP was analyzed. No difference between mice with a normal sleep pattern and mice with 6 h of extended wakefulness could be found in the LP. For the lung an increase in frequency but not in the cell count of macrophages was found. To conclude,

the sleep-induced increase in monocytes is not due to differentiation into macrophages.

As the third possibility, classical monocytes can also give rise to non-classical monocytes [Ginhoux and Jung 2014]. These cells were analyzed in mice with undisturbed sleep for 6 h and mice with enhanced wakefulness for 6 h. The frequency and numbers of non-classical monocytes in blood, spleen, LP, and lung were determined.

Interestingly, the frequency and the count of non-classical monocytes were strongly increased in sleep mice for the blood, spleen, and the lung but not for the LP.

These findings show that the sleep phenotype is not due to decelerated differentiation from classical monocytes to DCs, macrophages, or non-classical monocytes up on sleep. But on the contrary non-classical monocytes show the same sleep phenotype as do classical monocytes: increase in frequency and cell count in mice with 6 h sleep.

4.4. Sleep effect on monopoiesis

Classical monocytes as well as non-classical have their origin in the bone marrow [Ginhoux and Jung 2014]. The consideration that sleep promotes myelopoiesis, or the release of monocytes from the BM was taken into account. To analyze if sleep promotes monocyte development, different monocyte precursors in the BM were measured. Cell types that were analyzed are monoblasts, pro-monocytes, and common monocyte progenitors (cMoPs). The direct precursors of monocytes are monoblasts, their frequency and their cell numbers were found to be identical between the sleep and the wake group. Next in line are pro-monocytes, their frequency and their cell numbers were also unaffected by sleep. With regard to cMoPs, they showed an increase upon sleep in their frequency but not in their numbers.

Although changes in the frequency of cMoPs were detected, they fail to explain the increase of classical and non-classical monocytes in the sleep group. To explain the strong increase of monocytes in blood and spleen after 6 h of sleep the effect on precursors is not strong enough.

To address whether the release of monocytes from the BM is responsible for the sleep phenotype, classical monocytes in the BM were analyzed. Their frequency and cell numbers were unaffected by 6 h of sleep when compared to 6 h of enhanced wakefulness. Therefore, the migration from monocytes out of the BM seems not to cause the sleep phenotype.

In addition to the analysis in WT mice, the same experiment was also performed in $CCR2^{-/-}$ mice. These mice were of special interest as CCR2 is a crucial factor for the release of monocytes from the bone marrow and enables their extravasation into various tissues. CCR2 is the main mediator for monocyte release from the BM. Serbina *et al*. have shown that in $CCR2^{-/-}$ mice the release from monocytes out of the BM is strongly reduced even under inflammatory circumstances. So, the overall number of monocytes in these mice is already reduced [Serbina et al. 2008]. Should the sleep phenotype be due to a promoting effect of sleep on egress from monocytes out of the BM in $CCR2^{-/-}$ mice this phenotype should be absent.

Animals in the sleep or wake group should show the same number of monocytes in blood and spleen, but this was not the case. As expected, the CCR2$^{-/-}$ mice had reduced numbers of monocytes in blood and spleen, there was still an increase in monocytes in the sleep group when compared to the wake group.

Not many studies exist that have analyzed the effect of sleep on the development of hematopoietic cells or monocyte progenitors in the BM. In the study from Guariniello *et al*. they found reduced numbers of progenitors for all hematopoietic cells including monocytes in the mouse BM after 72h of PSD [Guariniello et al. 2012]. Our finding seems to contradict that. But it must be taken into account that PSD for 72h and enhanced wakefulness for 6 h are two different physiological states as described above. A second study by Rolls *et al.* investigated the effect of sleep on HSCs showing that sleep is necessary for the homing of HSCs [Rolls et al. 2015].

4.5. Sleep and cell death

Another interesting possibility for the sleep phenotype is increased cell death due to enhanced wakefulness. This was taken into account as a study by Everson *et al*. has shown an increase in cell death due to long term sleep deprivation [Everson et al. 2014].

To rule out cell death as the reason for the sleep phenotype, we analyzed dead monocytes, necrotic monocytes, as well as apoptotic monocytes in the spleen of sleep and wake mice. The frequencies in all cases were identical between both groups. But the count of dead monocytes, necrotic monocytes, as well as apoptotic monocytes was increased in the sleep group. This is the opposite of the finding of that of Everson *et al.*, again indicating the fundamental difference between long term sleep deprivation and enhanced wakefulness. About the reason for the increase in dead monocytes in the sleep group it can only be speculated. Wang *et al*. have shown a link between the circadian system and cell death. By silencing Clock gene expression, they found an increase in apoptosis as well as cycle arrest [Wang et al. 2016].

4.6. Long-lasting effect of sleep

As stress, differentiation into progenies, and monopoiesis have been ruled out as possible reasons for the sleep phenotype, another question comes up: How long does the sleep phenotype last?

With the exception of the infection experiments, all mice were sacrificed directly after 6 h of sleep or gentle handling. The question was: How long does it take until both groups are indistinguishable again?

In order to answer this question, recovery sleep experiments were performed. In these experiments mice were allowed to sleep for 4h, 6 h, or 24h after gentle handling and were compared to mice directly after 6 h of sleep or enhanced wakefulness.

Here, it is important to be aware that due to recovery time of 4h and 6 h mice were sacrificed at different times and therefore exposed to possible effects of the circadian rhythm [Nguyen et al. 2013] [Scheiermann et al. 2012] [He et al. 2018].

The results showed that enhanced wakefulness even when applied just once for 6 h has a long-lasting effect on immune cell composition.

At first, it seemed like both mouse groups are the same after 6 h of recovery time, but after 24h of recovery time increases of WBC [Hahn et al. 2020], blood monocytes and splenic neutrophils were observed in the wake group, which is a reversal of the sleep phenotype.

Whether it is an inflammatory reaction, as occurs in long-term sleep deprivation [Guariniello et al. 2012], or overcompensation of the sleep phenotype remains unanswered.

4.7. Sleep and monocyte migration

Monocytes and their progenies are migrating cells. Classical monocytes, non-classical-monocytes, and macrophages can be found in various tissues and different lymphatics; they reach this distribution by migrating through the body.

To address the question, if sleep influences the migration of monocytes or their progenies, different organs were analyzed for frequencies and cell count of classical, non-classical monocytes and macrophages.

In the case of lymph nodes, mesenteric and submandibular lymph nodes were examined. Classical monocytes in the MLN were found to be reduced in the sleep group; this is in clear contrast to the sleep-phenotype of an increase of monocytes due to sleep, but the reduction is too small to explain the sleep-phenotype found in blood and spleen. Classical monocytes in the SLN showed a slight increase due to sleep and therefore the sleep-phenotype. These results point out that the sleep-phenotype is not due to migration of classical monocytes to LN.

Macrophages in particular are found in immune infiltrated tissues such as the lamina propria and the lung epithelium. Cells in the LP seem to be unaffected by sleep or enhanced wakefulness for 6 h, as mononuclear phagocytes, macrophages and non-classical monocytes are at the same level in both groups. Classical monocytes in LP were found to express the sleep-phenotype again.

With respect to the lung epithelium, a non-significant increase of livings cells in the lung in the sleep group was observed. Classical as well as non-classical monocytes, were found to show a strong increase in their frequency and their cell numbers upon 6 h of sleep. Macrophages displayed no significant difference between the two groups. However, it could be shown that the frequency of alvecular macrophages is increased in mice with 6 h of enhanced wakefulness but not the cell count [Hahn et al. 2020].

These findings indicate that the sleep-phenotype is not due to changes in migration of classical monocytes or their progenies. To explain the sleep phenotype by emigration from blood and spleen, less classical monocytes or their progenies must be found in peripheral organs. However, this is not the case. On the contrary, the sleep-phenotype could be observed in the lung, too.

Of course, not all possible migration targets could be analyzed.

So, to further investigate the role of migration for the sleep phenotype it was necessary to analyze the key molecules involved in monocyte traffic and their

crawling behavior along blood vessel before extravasation [Gerhardt and Ley 2015] [Moore et al. 2013], which can lead to the formation of the marginal pool [Klonz et al. 1996].

For classical monocytes, although the underlying mechanisms for their migration are not yet fully understood [Thomas-Ecker et al. 2007], it is known that CCR2 plays a key role for monocyte migration as well as their release from the BM [Bain and Mowat 2014] [Kehrl 1998] [Ingersoll et al. 2011].
Hence the results from the $CCR2^{-/-}$ mice can also be consulted here. As discussed above $CCR2^{-/-}$ mice were used for sleep experiments and showed a reduced sleep-phenotype. The number of classical monocytes in this mouse was lower than in WT mice, as expected. Up on sleep an increase of classical monocytes was found, compared to the wake group. The increase was not as strong as in the WT mice indicating that the sleep phenotype is partly CCR2 dependent.
For the crawling behavior along blood vessels before the extravasation of classical monocytes, other factors are involved [Gerhardt and Ley 2015] [Moore et al. 2013]. This is mediated by LFA1 [Woollard and Geissmann 2010] which is found to be expressed on classical and non-classical monocytes [Shi and Pamer 2011] [Auffray et al. 2007] [Carlin et al. 2013]. LFA1 and its ligands, ICAM1, ICAM2, ICAM3, and JAM-A [Fougerolles et al. 1993] [Marlin and Springer 1987] [Ostermann et al. 2002], were therefore of special interest.
In particular, ICAM1 and ICAM2, as Gerhart *et al.* has demonstrated, are important for the attachment of monocytes to the epithelium [Gerhardt and Ley 2015]. To examine the involvement of the marginal pool binding of $Ly6C^{hi}$ classical monocytes, transgene mice models defect for ICAM-1 were used ($ICAM\text{-}1^{-}$ mice). These mice have no functioning ICAM-1 receptor, which is normally expressed on epithelial cells and the key mediator for the binding of $Ly6C^{hi}$ classical monocytes to the epithelium. $Ly6C^{hi}$ classical monocytes express LFA-1 which is a ligand for the ICAM-1 receptor [Carlin et al. 2013]. Therefore, these mice were used for sleep experiments following the usual protocol.

The experiments revealed that ICAM-1-deficient mice show the same sleep phenotype as WT mice, a strong increase in classical monocytes due to sleep, clearly showing that the sleep phenotype is not ICAM-1 dependent.

In a different approach, LFA-1 was targeted directly, blocking the receptor *in vivo* with an anti-LFA-1 antibody. In this setting, mice were injected with the anti-LFA-1 antibody or with the isotype control and compared to each other and to sleep mice without injection. Sleep mice could not be injected as this would disturb the sleep behavior massively. It was found that there was no significant difference between mice injected with anti-LFA-1 antibody or with the isotype control [Hahn et al. 2020]. These results again do not suggest a connection between the sleep phenotype for classical monocytes and the LFA-1 receptor.

For non-classical monocytes, CX_3CR1 has been described as the key factor for their crawling behavior [Arnold et al. 2007]. So $CX_3CR1^{GFP/-}$ and $CX_3CR1^{GFP/GFP}$ mice were used to address the question, if the sleep effect on non-classical monocytes is CX_3CR1dependend. In homozygote $CX_3CR1^{GFP/GFP}$ mice the CX_3CR1 receptor is not functional, by contrast, the heterozygote animals still own functioning CX_3CR1 receptors [Arnold et al. 2007]. The same protocol was used as for the WT mice with 6 h of sleep and 6 h enhanced wakefulness. The experiments revealed that heterozygote animals $CX_3CR1^{GFP/-}$ still show the sleep phenotype for classical and non-classical monocytes, just like the WT mice. In contrast, in the experiments using the homozygote animals, $CX_3CR1^{GFP/GFP}$, no difference between sleep and wake mice could be observed with regards to non-classical monocytes. Classical monocytes in these mice were still increased upon sleep like in WT mice.

This finding suggests that the CX_3CR1 receptor is involved in the increase of non-classical monocytes due to sleep. But it is necessary to keep in mind that CX_3CR1 is also known to be a survival factor and important for non-classical monocyte homeostasis [Jakubzick et al. 2008b] [Landsman et al. 2009]. More research is needed to fully understand the role of CX3CR1 receptor in migration of non-classical monocytes.

4.8. The marginal pool

The marginal pool has not been in the center of many studies but it presumably plays an important role in immune cell migration. It has been described by Klonz *et al.* as a second intravascular compartment [Klonz et al. 1996] in which PMNs, lymphocytes, but also monocytes can be found [van Furth and Sluiter 1986]. When immune cells migrate through the body, they leave the blood stream. This goes along with the reversible adhesion to the endothelium called "rolling "or "crawling" which can lead to the formation of the marginal pool [Hogg 1987] [Anderson et al. 1991] [Peters et al. 1985]. Cells that stay attached to the endothelium cannot be found in the blood, lymphatic organs or tissues. Therefore, the marginal pool could explain the quick changes of immune cell numbers as found in the circadian rhythm or after stress [Druzd et al. 2017]. These changes cannot be explained by the release of cells from the BM, or migration, as they occur too quickly [Nieman et al. 1992] [Gabriel et al. 1992].

Crawling is dependent on adhesion molecules [Nazziola and House 1992] [Schmidt et al. 1990] [Mayrovitz 1992]. So far described amongst others are ICAM-1, VCAM-1, CX_3CR1, and E-selectin [Allan and Rothwell 2001] [Ban 1994] [Bjerknes et al. 1986] . For classical monocytes, a key mediator of this reversible adhesion of classical monocytes to endothelium is the LFA-1 on the lymphocytes and ICAM-1 on the vascular endothelium [Auffray et al. 2007] [Carlin et al. 2013] [Sumagin et al. 2010]. Recruitment and release of classical monocytes to the marginal pool could explain the sleep phenotype. This presumption was supported by increasing evidence of sleep as well as circadian regulation of adhesion molecule expressions like ICAM-1, VCAM-1 and E-selectin and monocyte binding to the vessel wall [Qin and Deng 2015] [Scheiermann et al. 2012]. Two different approaches were taken to address the question, if the sleep phenotype is dependent on the ICAM-1-LFA-1 axis: experiments using ICAM-1^{-} mice and the experiments blocking the LFA-1 receptor (see 4.7).

Despite these indications for a connection between the sleep phenotype and the ICAM-1-LFA-1 axis, the finding in this work cannot support this. As ICAM-$1^{-/-}$

mice and blocking the LFA-1 receptor could not prevent the sleep phenotype (see 4.7).

Non-classical monocytes also contribute to the marginal pool. Auffray *et al.* found a 50% increase of non-classical monocytes, after blocking the marginal pool with aL integrin antibodies [Auffray et al. 2007]. CX_3CR1seems to be the key factor for the binding of non-classical monocytes to the endothelium. As expected, the experiments with $CX_3CR1^{GFP/GFP}$ mice showed no sleep phenotype for non-classical monocytes (see 4.7). This indicates a connection between sleep and the marginal pool for non-classical monocytes.

In summary, it could be demonstrated that the marginal pool can explain the sleep phenotype for non-classical monocytes but not yet for classical monocytes. While the ICAM-1-LFA-1 axis is not involved other receptors still could be. A possible candidate could be CXCR4 as Zhao *et al.* have shown that this chemokine receptor is regulated by Clock genes [Zhao et al. 2017]. Also, sleep can support the expression of adhesion molecules on monocytes and the endothelium [He et al. 2018] [Rolls et al. 2015]. Furthermore, Scheiermann *et al.* showed that ß-adrenoreceptors are involved in endothelial oscillations of adhesion-molecule expression [Scheiermann et al. 2012] and Dimitrov *et al.* showed that this is sleep-dependent [Dimitrov et al. 2019].

These findings are promising and indicating a connection between the marginal pool of classical and non-classical monocytes. It would be interesting to see sleep experiments combining the analysis of ß-adrenoreceptors with monocyte counts.

4.9. Sleep and the circadian rhythm

Sleep and the circadian rhythm are strongly linked together so it should always be considered that they influence each other. The circadian rhythm has been found to be involved in a lot of body functions from temperature to hormonal secretion, from metabolism to immunity [Blask 2009]. The circadian system is regulated by so-called Clock genes. Mice which are deficient in the genes lose their circadian rhythm. One of these genes is Arntl. $Arntl^{-/-}$ mice are arrhythmic, meaning they show no circadian rhythm [Bunger et al. 2000]. This makes $Arntl^{-/-}$ mice very interesting for sleep experiments. If these mice would show no sleep phenotype any more it would be another clear link between sleep and the circadian system. This would indicate that sleep is necessary for the occurrence of the circadian rhythm. The increase of classical monocytes after 6 h of sleep is in line with their peak in the circadian rhythm in the middle of the resting period [Nguyen et al. 2013]. And indeed $Arntl^{-/-}$ mice showed no sleep phenotype when used in sleep experiments [Hahn et al. 2020]. In these experiments, the usual sleep protocol of 6 h of sleep and 6 h of enhanced wakefulness was used. $Arntl^{+/+}$ mice were used as a control. They showed the sleep phenotype with regards to numbers of classical monocytes in the blood. Whereas $Arntl^{-/-}$ showed no significant differences between wake and sleep mice [Hahn et al. 2020]. Furthermore, it could be demonstrated that the Arntl expression of monocytes from WT mice differs between the sleep and wake group with a significant higher gene expression in mice with 6 h of enhanced wakefulness compared to sleep mice. The Arntl expression in wake mice was similar to these of the start of the resting period [Hahn et al. 2020].

These findings are in line with the work of Nguyen *et al*. where they showed a decrease in the expression of Arntl in blood for classical monocytes at the middle of the resting phase [Nguyen et al. 2013]. The middle of the resting phase is equal to the sleep mice group as they had 6 h of sleep and the resting phase is twelve hours long.

This supports the idea of a close connection between the circadian rhythm and sleep, with sleep being imperative for the circadian rhythm in monocytes.

4.10. Sleep and infection

The next step to understand what the previous finding mean for the immune function was to connect them with infections. The connection between sleep and immunity has been in the center of many studies, mostly addressing the influence of infection on sleep [Besedovsky et al. 2019] and demonstrating a clear change in sleep behavior under infection [Toth and Krueger 1988]. But just a few studies have addressed the question if sleep is beneficial for infection outcome using sleep deprivation or enhanced wakefulness. It was shown by Toth *et al.* that rabbits with a natural robust early sleep response to bacterial infection showed a better survival than rabbits with reduced sleep, demonstrating the positive effect of sleep on infection [Toth et al. 1993]. And Preston *et al.* even demonstrated an evolutionary connection between sleep and infection, showing that an evolutionary increase in mammalian sleep durations is strongly associated with increased numbers of immune cells in the blood and reduced parasitic burden [Preston et al. 2009].

Nguyen *et al.* analyzed the connection between the circadian rhythm and infection. They found that the monocyte recruitment to the site of infection is different during the rest period and active period, with higher monocytes numbers during the resting phase. This leads to an improved bacterial clearance after infection in the resting phase [Nguyen et al. 2013].

Previous results in this work had shown a strong increase of, in particular, monocytes due to sleep. The assumption that this increase of monocytes is consequential for the immune defense upon infection is supported by studies that described the importance of monocytes for infection extermination [Pasquevich et al. 2015] [Autenrieth et al. 2012].

In a next step the activity of cells was analyzed. Therefore, the ROS production by PMNs and monocytes were measured, revealing a strong difference in the ROS production between the sleep and the wake group. PMNs isolated from the blood of mice in the sleep group were found to produce up to five times more ROS then the PMNs isolated from mice in the wake group. And in the case of classical monocytes, cells obtained from mice in the sleep group produced significantly more ROS than those cells obtained from the wake group which is

supported by Christoffersson *et al.* They could show similar results in humans [Christoffersson et al. 2014]. This improved production of ROS due to sleep is important, as ROS is essential for bacterial killing after phagocytosis. And it was shown that an increased ROS production by PMNs reduces the bacterial load in systemic infections [Autenrieth et al. 2012].

The combination of more immune cells combined with a higher ROS production should have a positive effect on infection outcome. To test this, mice were intravenously infected with a lethal dose of *Yersinia enterocolitica* to mimic a sepsis. The bacterial burden in the blood and spleen was determined at different times throughout the infection, as well as the survival time. Experiments showed a vast increase of the bacterial burden in the blood and spleen in the wake mice group followed by a reduced survival time for mice with 6 h of enhanced wakefulness.

Our data demonstrates that sleep is not just necessary for the homeostasis of innate immune cells but also important for their function. And these findings are supported by analysis of circadian rhythm and infection. These studies have demonstrated that a higher number of classical monocytes in the blood at the time of i.p. infection with *Listeria monocytogenes* was associated with facilitated CCR2-CCL2 signaling-dependent monocyte recruitment to the inflamed peritoneum which has led to a better immune response indicated by improved bacterial clearance [Nguyen et al. 2013]. Moreover, Toth LA *et al.* could show that rabbits with robust early sleep responses to bacterial infection showed a better survival than rabbits with reduced sleep, also indicating the importance of sleep for bacterial clears during infections [Toth et al. 1993].

We here extend these findings showing that sleep contributes to these rhythms by enhancing numbers of classical and non-classical monocyte in various tissues as well as their antimicrobial activity. Furthermore, sleep also promotes the antimicrobial activity of PMNs. This supports the assumption that sufficient sleep is essential for a fully functional innate immune system and prevents from infection. This is in line with in human studies by Patel *et al.* [Patel et al. 2012] and Cohen *et al.* [Cohen et al. 2009]. Both were able to show that humans with little sleep or poor sleep quality are more susceptible to infections.

5. Summary

5.1. Summary in English

Sleep has been linked to several vital body functions from memory and metabolism to immunity. The results in this work – showing that sleep in mice has a profound effect on innate immune cells and their ability to fight bacterial infection – support these findings. For the experiments two mice groups were used, sleep mice with 6 h of normal sleep were compared to mice with 6 h of enhanced wakefulness. 6 h of sleep increases the overall cellularity in the blood and spleen of WT mice, in particular the number and frequency of monocytes. This is described in this work as the sleep phenotype, a strong increase in frequency and count of monocytes due to sleep. This phenotype was found for classical monocytes and non-classical monocytes. The increase of monocytes is not due to stress or the progression into macrophages or DCs. Furthermore, no effect of sleep was found on monocytes precursors or their release from the BM. Interestingly, the increase of monocytes in blood and spleen did not lead to a reduction of monocytes in Lymph Nodes, the LP, or the lung. On the contrary, monocyte numbers as well increase in the lung after 6 h of sleep. Furthermore, the sleep phenotype is independent of the ICAM-1-LFA-1 axis and just partially dependent on CCR2. Both are very important factors for the migration of classical monocytes and their contribution to the marginal pool. However, it could be shown that for non-classical monocytes Cx3CR1 is involved in the sleep phenotype. Beyond this, it was demonstrated that the sleep phenotype for classical monocytes is dependent on the Clock gene Arntl, indicating a strong connection between the circadian system and sleep. With regards to immune cell function, sleep increases the ROS production of PMNs and classical monocytes. Consequential this leads to better bacterial clearance and improved survival time upon infection.

Taken together sleep is imperative for immune functions and monocyte numbers are highly affected by sleep as well as their ability to fight a bacterial infection. Even though the exact mechanism is not yet fully understood there are strong indications for a connection to the circadian system.

5.2. Zusammenfassung in Deutsch

Schlaf wird mit mehreren lebenswichtigen Körperfunktionen in Verbindung gebracht – von Gedächtnisbildung und Stoffwechsel bis hin zur Immunität. Die Ergebnisse dieser Arbeit zeigen eine tiefgreifende Wirkung von Schlaf auf das angeborene Immunsystem. 6 Stunden Schlaf bei Mäusen erhöht die Gesamtzellularität in Blut und Milz von WT-Mäusen, insbesondere die Anzahl und den prozentualen Anteil von Monozyten. Dieser durch Schlaf verursachte Anstieg wird in dieser Arbeit als Schlafphänotyp bezeichnet und wurde sowohl für klassische Monozyten als auch nicht-klassische Monozyten nachgewiesen. Dieser Anstieg ist nicht auf Stress oder die Weiterentwicklung zu Makrophagen oder DCs zurückzuführen. Darüber hinaus wurde keine Auswirkung des Schlafes auf Vorläufer der Monozyten oder deren Freisetzung aus dem Knochenmark gefunden. Interessanterweise führte der Anstieg der Monozyten in Blut und Milz nicht zu einer Verringerung der Monozyten in den Lymphknoten, der Lamina propria oder der Lunge. Die Zahl an Monozyten steigt im Gegenteil nach 6 Stunden Schlaf auch in der Lunge an. Darüber hinaus ist der Schlafphänotyp unabhängig von der ICAM-1-LFA-1-Achse und nur teilweise von CCR2 abhängig. Beide sind sehr wichtige Faktoren für die Migration klassischer Monozyten und ihren Beitrag zum Marginal Pool. Es konnte jedoch gezeigt werden, dass für nicht-klassische Monozyten Cx3CR1 am Schlafphänotyp beteiligt ist. Ferner wurde gezeigt, dass der Schlafphänotyp für klassische Monozyten vom Clock-Gen Arntl abhängt, was auf eine starke Verbindung zwischen dem circadianen System und dem Schlaf hinweist. In Bezug auf die Funktion der Immunzellen erhöht der Schlaf die ROS-Produktion von PMNs und klassischen Monozyten. Folglich führt dies zu einer besseren bakteriellen Bekämpfung und einer verbesserten Überlebenszeit nach der Infektion. Zusammengenommen ist der Schlaf für die Immunfunktionen unerlässlich. Die Monozytenzahlen werden stark vom Schlaf beeinflusst und es besteht die Fähigkeit, eine bakterielle Infektion zu bekämpfen. Auch wenn der genaue Mechanismus noch nicht vollständig erklärt werden kann, gibt es starke Hinweise auf eine Verbindung zum circadianen System.

6. Abbreviations

7-AAD	7-Aminoactinomycin D
APC	Allophycocyanin
APC	Antigen presenting cells
BD	Becton Dickinson
BM	Bone marrow
BV	Brilliant Violet
CFU	Colony-forming units
CCL	chemokine C-C motif
CMP	common myeloid progenitor ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDP	common DC progenitor
cMoP	common monocyte progenitor
CXCL	chemokine C-X-C motif ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cell
DNA	deoxyribonucleic acid
dpi	days post infection
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immuno-sorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

Flt3	Fms-like tyrosine kinase 3
Flt3L	Fms-like tyrosine kinase 3 ligand
GMP	granulocyte-macrophage progenitor
h	hour
HIV	Humanes Immundefizienz-Virus
HSC	hematopoietic stem cell
IL	Interleukin
IFN-γ	Interferon-γ
i.v.	intravenously
LIN	Lineage
LFA-1	eucocyte function associated molecule 1
LN	Lymph node
LP	Lamina Propria
LPS	Lipopolysaccharid
LSK	lin-Sca-1+c-Kit+ hematopoietic stem cells
MACS	Magnetic-activated cell separation
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex II
MDP	macrophage and dendritic cell progenitor
NK cells	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBS	phosphate buffered saline

pDC	plasmacytoid dendritic cells
PRRs	Pattern recognition receptors
REM	Rapid eye movement
ROS	Reactive oxygen species
SD	Sleep deprived
#	Cell count
%	Frequency

7. Literature

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9. Curriculum Vitae

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Career

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October 2005-March 2011	Study of biology at the CAU-Kiel

Scientific publications

Publications

Sleep enhances numbers and function of monocytes and improves bacterial infection outcome in mice

Julia Hahn, Manina Günter, Juliane Schuhmacher, Kristin Bieber, Simone Pöschel, Monika Schütz, Britta Engelhardt, Henrik Oster, Christian Sina, Tanja Lange, Stella E. Autenrieth

Mononuclear phagocytes contribute to intestinal invasion and dissemination of Yersinia enterocolitica.

Drechsler-Hake D, Alamir H, Hahn J, Günter M, Wagner S, Schütz M, Bohn E, Schenke-Layland K, Pisano F, Dersch P, Autenrieth IB, Autenrieth SE