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

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ABSTRACT

Sleep strongly impacts both humoral and cellular immunity; however, its acute effects on the innate immune defense against pathogens are unclear. Here, we elucidated in mice whether sleep affects the numbers and functions of innate immune cells and their defense against systemic bacterial infection. Sleep significantly increased numbers of classical monocytes in blood and spleen of mice that were allowed to sleep for six hours at the beginning of the normal resting phase compared to mice kept awake for the same time. The sleep-induced effect on classical monocytes was neither caused by alterations in corticosterone nor myelopoiesis, bone marrow egress or death of monocytes and did only partially involve Gαi-protein coupled receptors like chemokine receptor 2 (CCR2), but not the adhesion molecules intercellular adhesion molecule 1 (ICAM-1) or lymphocyte function-associated antigen 1 (LFA-1). Notably, sleep suppressed the expression of the clock gene Arntl in splenic monocytes and the sleep-induced increase in circulating classical monocytes was abrogated in Arntl-deficient animals, indicating that sleep is a prerequisite for clock-gene driven rhythmic trafficking of classical monocytes. Sleep also enhanced the production of reactive oxygen species by monocytes and neutrophils. Moreover, sleep profoundly reduced bacterial load in blood and spleen of mice that were allowed to sleep before systemic bacterial infection and consequently increased survival upon infection. These data provide the first evidence that sleep enhances numbers and function of innate immune cells and therewith strengthens early defense against bacterial pathogens.

1. Introduction

Sleep is assumed to 'help to heal' and, indeed, several lines of evidence support a bidirectional connection between sleep and the immune system (Besedovsky et al., 2019; Toth, 1995). Microbial inoculation increases the amount and intensity of sleep and, specifically, of non-rapid eye movement sleep (Baracchi et al., 2011; Toth, 1995). At the same time, such studies also suggest that non-rapid eye movement sleep is beneficial for the recovery from infection in particular following bacterial challenges (Opp and Toth, 2003). In keeping with such a notion, sleep has a regulatory effect on the host defense system, as it impacts numbers, function, and tissue distribution of white blood cells (WBCs) (Besedovsky et al., 2019; Bryant et al., 2004). Accordingly, in prospective analyses of healthy subjects, sleep duration was found to be associated with reduced pneumonia incidence (Patel et al., 2012). In the case of bacterial infection, innate immune cells, especially phagocytes like monocytes and neutrophils (polymorphonuclear cells, PMNs), represent the first line of defense against invading pathogens. Within

Abbreviations: WBCs, white blood cells; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; Ye, *Yersinia enterocolitica*; PTx, Pertussis toxin; BM, bone marrow; LN, lymph node; cMops, monocyte progenitors; GαiPCRs, Gαi protein-coupled receptors; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; WT, wild type; CCR2, chemokine receptor 2

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minutes to hours they recognize, engulf, and destroy bacteria and thus reduce the risk and severity of infections (Autenrieth et al., 2012; Bieber and Autenrieth, 2015; Pasquevich et al., 2015). In healthy humans, sleep acutely decreases blood numbers of monocytes and PMNs, but increases their capacity to produce reactive oxygen species (ROS) and pro-inflammatory cytokines following stimulation with bacterial components (Born et al., 1997; Christoffersson et al., 2014; Dimitrov et al., 2015). To our knowledge, only one animal study has so far assessed the acute effect of sleep versus sleep deprivation on anti-bacterial innate immunity. In that study rabbits were intravenously infected with *Escherichia coli* before or after 4 h of sleep or sleep deprivation. As expected, non-rapid eye movement sleep increased in response to sleep deprivation and/or *Escherichia coli*, yet disease severity in terms of fever and WBC or blood PMN counts remained unchanged (Toth et al., 1995). In addition to these experiments employing acute sleep manipulations, longer durations of sleep restriction or sleep deprivation quite consistently increase blood monocytes and PMNs in humans, mice, and rats (e.g., (Everson et al., 2008; Guariniello et al., 2011; Lasselin et al., 2015; Zager et al., 2012)) and impair anti-bacterial host defense in mice and rats (Everson and Toth, 2000; Friese et al., 2009).

Although these studies in sum gave insights into supportive effects of sleep on innate immunity, none combined the analyses of phagocyte numbers, tissue distribution, and their functions upon bacterial infection. Thus, it remains unclear how regular sleep promotes host defense against bacterial infections and whether such an immunosupportive function could stem from acute effects of sleep on innate immunity.

In the current study in mice, we therefore analyzed acute effects of sleep on innate immune phagocytes and hypothesized that any effect of sleep on this first line of host defense would consequently also influence the overall course of a bacterial infection. We subjected mice to 6 h of sleep or wakefulness during the first half of their regular rest phase and then measured numbers and function of classical ($Ly6C^{hi}CD11b^{+}CD115^{+}$) monocytes (Ginhoux and Jung, 2014) and $Ly6G^{+}CD11b^{+}$ PMNs in the blood, and also in various tissues like the spleen, bone marrow (BM), lung, intestine and lymph nodes. Monocytes and PMNs are produced in the BM and migrate via the blood to the tissues involving chemokines (e.g., CCL-2, CXCL-8), respective Gαi protein coupled chemokine receptors (e.g., CCR2 on monocytes or CXCR1/CXCR2 on PMNs (de Oliveira et al., 2016)), as well as adhesion molecules (e.g. lymphocyte function-associated antigen 1 (LFA-1) on PMNs and $Ly6C^{hi}$ monocytes or the cognate ligand intercellular adhesion molecule 1 (ICAM-1) on endothelial cells (Nourshargh and Alon, 2014; Nourshargh et al., 2010)). Previous studies in animals revealed that changes in circulating monocyte and PMN numbers during the 24 h sleep-wake cycle involve these chemokines, chemokine receptors and adhesion molecules (Nguyen et al., 2013; He et al., 2018). The role of sleep in this context, however, is presently unclear.

Because a number of studies have described that sepsis changes sleep parameters (e.g., (Baracchi et al., 2011)) and that, conversely, sleep impacts sepsis outcome (Friese et al., 2009), we decided to use the well-established sepsis model *Yersinia enterocolitica* (Ye) (Autenrieth et al., 2010). Ye is a Gram-negative extracellular rod, causing enteritis and enterocolitis in humans and mice with the ability to disseminate from the intestine and provoke sepsis (Autenrieth and Autenrieth, 2008). Thus, we assessed bacterial load and survival of mice that were allowed to sleep or not for 6 h before a lethal systemic Ye infection to elucidate the acute effects of sleep on anti-bacterial immune defense.

2. Materials and methods

2.1. Mice

Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Permit Numbers: M11/14). All efforts were made to minimize suffering of the animals.

Female C57BL/6JRj mice were purchased from Janvier (St Berthevin Cedex, France); ICAM-1^{null} ($Icam1^{tm1Alb}$) mice, generated by deletion of the entire coding region of the ICAM-1 gene were described before (Bullard et al., 2007), $CCR2^{-/-}$ ($Ccr2^{tm1Ifc}$) (Boring et al., 1997), and $Arntl^{-/-}$ mice (B6.129-$Arntl^{tm1Bra}$ (009100, Jackson Labs) were bred under specific pathogen-free conditions and an equal amount of male and female mice was used. Mice used for experiments were between 6 and 10 weeks of age, were housed in groups of 4–5 animals on a 12:12-h light: dark cycle at 22°C with free access to food and water. Where appropriate, animals were randomly assigned to interventions. Experiments were performed in a blinded fashion. Mice were sacrificed using CO_2.

2.2. Gentle handling

Mice were kept awake for 6 h by gentle handling. Whenever the animal adopted a sleeping posture, we disturbed the nest, gently touched the bedding underneath the animal or provided new nesting material. Gentle handlings started at the onset of light, at 7:30 a.m. (ZT0) and lasted until 1:30 p.m. (ZT6). 'Sleep' mice were left completely undisturbed during this time period. Both mouse groups were sacrificed or infected directly after 6 h of gentle handling or sleep at 1:30 p.m. to control for circadian influences (Nguyen et al., 2013). Plasma samples were taken at the same time.

To analyze the effect of chemokine receptors on monocyte migration during sleep, mice were injected intraperitoneally with 90 μg/kg bodyweight pertussis toxin (PTx) (Sigma Aldrich), which blocks chemokine receptor signaling, in 100 μl PBS (Gibco) 48 h before sleep or wakefulness. To assess the role of the adhesion molecule LFA-1 200 μg per mouse mAb rat-anti-mouse LFA-1 or the specific isotype rat IgG2b (both from Bio X-Cell) as control were injected in 200 μl PBS 48 h and 24 h before wakefulness.

2.3. Blood, tissue and cell preparation

Blood was taken from the heart with a syringe containing heparin (Braun). 200 μl blood was used for the flow cytometry analyses. To remove erythrocytes cells were lysed 3 times with lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM NaEDTA; (Sigma-Aldrich)) for 5 min at room temperature.

Spleen tissues were cut into small pieces and digested for 30 min at 37°C in 2 ml RPMI 1640 (Biochrom) + 2% FBS (Sigma) medium containing collagenase (1 mg/ml; type IV; Sigma-Aldrich) and DNase I (100 μg/ml, Roche). EDTA (0.1 ml, 0.1 M (pH 7.2); Roth) was added and mixing continued for 5 min. Single-cell suspensions were made by pipetting the digested organs. Undigested fibrous material was removed by filtration and erythrocytes were lysed with lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM NaEDTA) for 3 min at room temperature.

BM cells were harvested from femurs and tibias by flushing the bones with PBS. Fibrous material was removed by filtration through a 40 μm cell strainer and erythrocytes were lysed with lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM NaEDTA) for 3 min at room temperature.

Isolation of lung cells was performed using the lung dissociation kit together with the gentle MACS dissociator according to the manufacturer's protocol (Miltenyi Biotech).

The number of cells was determined either by trypan blue exclusion using a Neubauer cell counting chamber or by flow cytometry.

2.4. Flow cytometry

FACS buffer (PBS containing 1% FBS (Sigma-Aldrich) and 0.09% NaN_3 (Sigma-Aldrich)) was used for all incubations and washing steps

unless stated otherwise. Before staining, cells were incubated for 15 min at 4°C with hybridoma supernatant from 2.4G2 cell line producing anti-FcγRII/III mAb. Cells were stained with FITC-conjugated lineage antibodies (CD3 (145-2C11), CD19 (6D5), Ter-119 (Ter-119), NK1.1 (PK136); Miltenyi), CD4-eFluor450 (L3T4; eBiosciences), CD11b-Alexa Fluor700 (M1/70; BioLegend), CD11b-APC-eFluor780 or CD11b-BrilliantViolet605 (M1/70; eBioscience), CD11c-PE/Dazzle594 (N418; BioLegend), CD115-PE (AFS98; eBioscience), CD45-PerCP (30F-11; BioLegend), Ly6C-PE/Cy7 (HK1.4; BioLegend), Ly6G-BrilliantViolet510 or Ly6G-FITC (1A8; BioLegend), MHCII-BrilliantViolet711 or MHCII-PerCP (M5/114.15.2 BioLegend), CD64-PE (X54-5/7, BioLegend), CD24-Pacific blue (M1/69, BioLegend), CD135-PE (A2F10, Miltenyi), CD172a-APC (P84, BioLegend), CD45R-BrilliantViolet650 (RA-36B2, BioLegend), CD117-BrilliantViolet421 (2B8, BioLegend) for 20 min at 4°C. To exclude dead cells, Zombi Aqua or Zombi-NIR fixable viability kits (BioLegend) were used. For apoptotic cell analysis cells were incubated after extracellular staining using Annexin V-FITC (eBioscience) and 7-AAD (Biomol) in Annexin V binding buffer (eBioscience) for 15 min at room temperature.

Samples were acquired for 6 to 14-colour analysis using a Canto-II or LSRFortessa flow cytometer with DIVA software (BD Biosciences) and further analyzed using FlowJo 10 software (TreeStar Inc). A total of 500,000–2,000,000 cells were acquired.

2.5. Monocyte isolation and quantitative RT-PCR

For sorting of monocytes $CD11b^+$ cells were enriched using the CD11b Microbeads Kit (Miltenyi Biotec) according to themanufacturer's protocol. $CD11b^+$ cells were stained with Ly6C-PE/Cy7 (HK1.4; BioLegend) and CD11b-APC-eFluor780 (M1/70; eBioscience) antibodies for 20 min at 4°C. Thereafter, cells were incubated with 7-AAD (Biomol) for 10 min at 4°C to exclude dead cells. $Ly6C^{hi}CD11b^+7\text{-}AAD^-$ cells were then sorted on a FACS AriaIIIu cell sorter (BD Biosciences). Sort purity was above 93%.

RNA was isolated from 30,000 to 100,000 sorted monocytes using the RNeasy Plus Micro Kit (Qiagen). Reverse transcription was carried out using QuantiTect Reverse Transcription Kit (Qiagen), and quantitative PCR reactions were performed on LightCycler 480 II real-time PCR detection system (Roche) using QuantiFast SYBRGreen PCR Kit (Qiagen). Reactions were run in duplicates. Cycling conditions were as follows: 95°C for 5 min for enzyme activation followed by 50 cycles of 95°C for 10 sec and 60°C for 1 min. The relative expression level of mRNAs was calculated using the comparative cycle threshold method (2-ΔΔCT) with 36B4 as an internal control. Primers used for qRT- PCR analysis were:

Mm_36B4_forward 5′-GAGACTGAGTACACCTTCCCAC-3′
Mm_36B4_reverse 5′-ATGCAGATGGATCAGCCAGG-3′
Mm_Arntl_forward 5′-AGAGGCGTCGGGACAAAATGAACAG-3′
Mm_Arntl _reverse 5′-AACAGCCATCCTTAGCACGGTGA-3′

2.6. Reactive oxygen species (ROS) detection

WBCs were stained for flow cytometry as described above. 3×10^6 cells were incubated for 20 min at 37°C with 80 μM 2′, 7′-dichlorofluorescein diacetate reagent (DCFD, Sigma-Aldrich), washed and analyzed by flow cytometry.

2.7. Infection of mice

After 6 h of sleep or wakefulness mice were infected with the indicated amount of Ye WA-314 (serotype 0:8); (Heesemann et al., 1983) 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.

2.8. Statistics

For statistical analyses of two or more groups, we used Student's *t*-test or ANOVA followed by Tukey's test. The statistical evaluation of different cell populations obtained from the same measurement was performed using multiple t-tests with Holm-Sidak correction for multiple comparisons. In cases variances were not homogeneous, the data were analyzed by the non-parametrical Mann-Whitney *U* test. Two-way ANOVA was applied to the time-course analysis and comparison of different mouse strains or PTx treatment. Statistical analysis of survival was performed using the log-rank test. Differences with P-values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

3. Results

3.1. Sleep increases monocyte numbers in blood and spleen

To examine the influence of sleep on innate immune phagocytes, such as monocytes and PMNs, WT mice were allowed to sleep for 6 h ('sleep' mice) and compared to mice that were kept awake by gentle handling ('awake' mice) for the same time. The experiments started at the onset of light (7:30 am) at the beginning of the mouse resting phase when blood monocyte numbers start to increase (Nguyen et al., 2013). Both groups were sacrificed at the same time to control for the influence of the circadian system (Keller et al., 2009; Nguyen et al., 2013; Scheiermann et al., 2012). Diverse innate immune cell populations from various compartments were analyzed by flow cytometry (gating see Fig. S1).

WBC counts were doubled in 'sleep' compared to 'awake' mice (Fig. S2A). In addition, the frequency of classical monocytes (gated as $Ly6C^{hi}CD11b^+CD115^+$) in blood was dramatically increased by a factor of 2.5 in 'sleep' compared to 'awake' mice (Fig. 1A and B), whereas the frequency of blood PMNs ($Ly6G^+CD11b^+$) was slightly reduced (Fig. 1A; C). Consequently, blood numbers of classical monocytes and PMNs from 'sleep' mice were 5.3-fold and 1.5-fold higher, respectively, compared to those of 'awake' mice (Fig. 1B and C). Similar results were obtained for the spleen with a 1.2-fold increase in the splenocyte numbers (Fig. S2B) and a 2-fold increase in monocyte frequencies and numbers in 'sleep' compared to 'awake' mice (Fig. 1D). There was no difference observed in splenic PMN frequencies, whereas their total number was 1.5-fold higher in 'sleep' compared to 'awake' mice (Fig. 1E). Thus, sleep influences the number of monocytes in blood and spleen.

3.2. Sleep does not change cell death, myelopoiesis or differentiation of monocytes, but facilitates their rhythmic trafficking to the circulation

There are many possible biological mechanisms underlying the different monocyte numbers in blood and spleen in 'sleep' and 'awake' mice. The hormone corticosterone is known to reduce blood monocyte numbers, e.g., during acute stress (Dhabhar et al., 2012). However, similar corticosterone plasma levels within the normal range (Kopp et al., 2006) were detected in both 'sleep' and 'awake' mice (Fig. S3) indicating that corticosterone is not responsible for the sleep-induced increase in monocyte numbers. Moreover, this result shows that, in line with previous findings (Rolls et al., 2015), gentle handling for 6 h does not evoke marked stress effects in mice.

Next, we ruled out changes in cell death (Everson et al., 2014), as the frequency of $7AAD^+$ and Annexin V^+ monocytes in these compartments was similar in both groups of mice, corresponding to an increased number of dead $7AAD^+$Annexin V^+ monocytes in 'sleep' compared to 'awake' mice (Fig. 2A). The sleep-induced higher

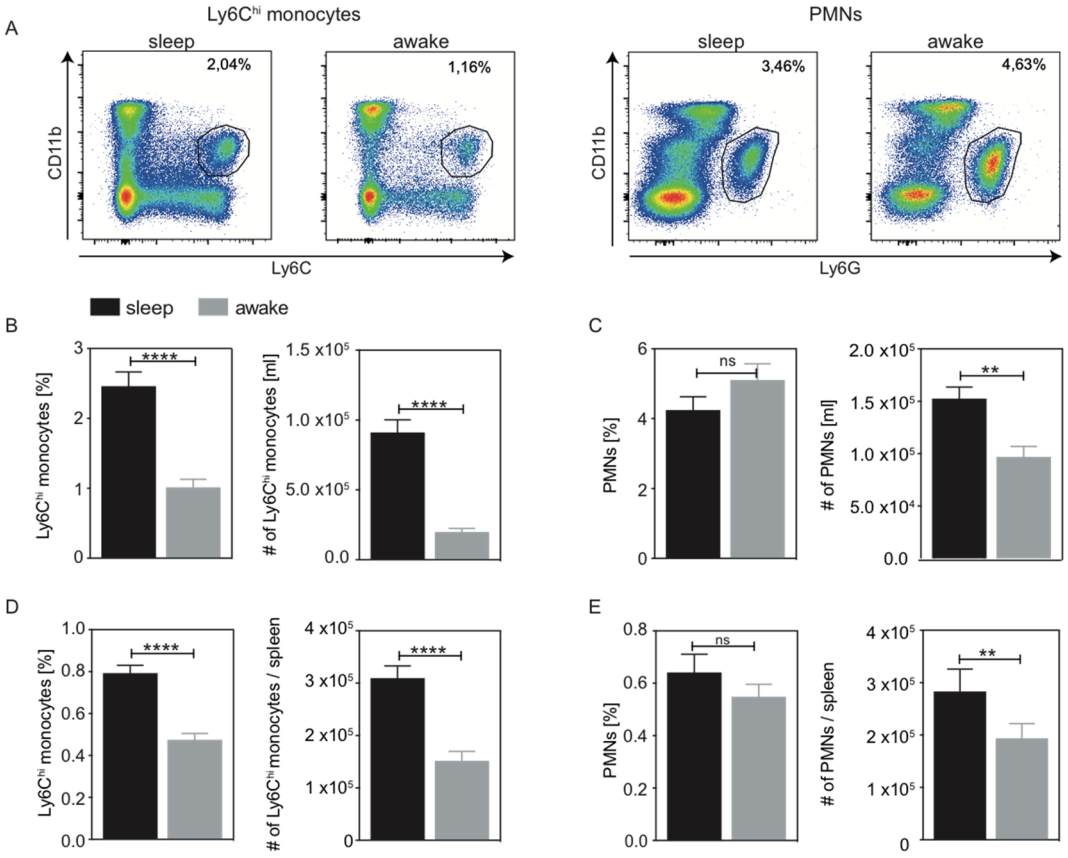

Fig. 1. Sleep increases monocytes in blood and spleen. Mice were allowed to sleep ('sleep', black bars) or not ('awake', grey bars) for 6 h. (A) Flow cytometry pseudocolor plots show classical Ly6C^{hi} monocytes (Ly6C^{hi}CD11b^{+}) and polymorphonuclear neutrophils (PMNs; gating see Fig. S1) in the blood. Graphs show the frequencies (% of white blood cells) and numbers of blood and spleen monocytes (B & D) and PMNs (C & E). Data represent the mean ± SEM from 6 experiments with 3–5 mice/group (multiple t-tests with Holm-Sidak correction; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****), ns: not significant).

monocyte counts might stem from changes in myelopoiesis or the release of monocytes from the BM. However, comparable frequencies and numbers of monocytes and monocyte progenitors (cMops) (Hettinger et al., 2013) were detected in BM of 'sleep' and 'awake' mice (Fig. 2B and C, for gating strategy, see Fig. S1).

Sleep could also impact the migration of monocytes from the circulation to peripheral organs like lymph nodes (LNs), the lung or the intestine. However, this is not the case because the monocyte numbers in these compartments were either unchanged (mesenteric LNs, gut lamina propria) or significantly increased (lung, submandibular LNs) in 'sleep' compared to 'awake' mice (Figs. S4 and 2D). By contrast, the frequency and number of PMNs in the lung were slightly increased or comparable in both groups of mice, respectively (Fig. 2E).

Another possible explanation is a change in the differentiation potential of monocytes into macrophages. Although the frequency of alveolar and interstitial macrophages in the lung was lower in 'sleep' compared to 'awake' mice, the macrophage numbers were comparable in both groups (Fig. 2F and G). Thus, the sleep-induced higher monocyte numbers are not due to a defect in the differentiation potential into macrophages.

Recent studies have demonstrated rhythmic trafficking of monocytes from the BM to the blood (Nguyen et al., 2013). Thus, we speculated that the difference in monocyte numbers observed in the blood of 'sleep' and 'awake' mice was due to the trafficking of monocytes into the circulation during the resting phase. Indeed, we observed an increase of WBCs and monocyte numbers from the beginning of the resting phase to 6 h later in 'sleep' mice, whereas the number of WBCs and monocytes were even reduced over this time period in 'awake' mice (Fig. 2H). Thus, sleep is a prerequisite for the circadian rhythm-regulated increase of leukocytes/monocytes in the circulation.

Furthermore, to examine whether the effect of sleep on monocyte numbers is also evident in 'awake' mice upon recovery sleep, mice were allowed to sleep for 24 h after 6 h of gentle handling and compared to mice without sleep manipulation. All mice were sacrificed at 1:30 pm. The frequencies and numbers of blood monocytes in mice, which were kept awake for 6 h and then left undisturbed for 24 h, were significantly increased compared to 'awake,' but also to 'sleep' mice (Fig. 2I). These results show that recovery sleep can normalize or even boost the regular monocyte increase in blood monocytes.

3.3. The sleep-induced rise in blood monocytes is partially dependent on chemokine receptor signaling and on clock genes function

Next we addressed the impact of chemokines on the sleep-induced increase of circulating monocytes. Most chemokine receptors act specifically through PTx-sensitive $G_{\alpha i}$ components of G protein-coupled receptors ($G_{\alpha i}$PCRs) (Kehrl, 1998). Thus mice were injected with PTx intraperitoneally 48 h and 24 h before sleep or wakefulness to prevent their extravasation. PTx-treated 'sleep' as well as 'awake' mice revealed a 2.8-fold increase in WBC numbers compared to PBS-treated mice (Fig. S5), demonstrating that inhibition of $G_{\alpha i}$PCRs in general increases the dwelling time of WBCs in the blood. Upon PTx treatment, the monocyte frequency in 'sleep' compared to 'awake' mice was increased by 1.7-fold, which is a 50% reduction compared to non-treated animals (Fig. 3A). Similar results were observed for monocyte numbers with a 2-fold increase in PTx-treated, but a 4-fold increase in vehicle-treated 'sleep' compared to 'awake' mice (Fig. 3A). A general increase of WBC numbers upon PTx treatment was observed (Fig. S5A). These data indicate that the chemokine-chemokine receptor axis is partially involved in the sleep-induced increase in circulating monocytes. In contrast,

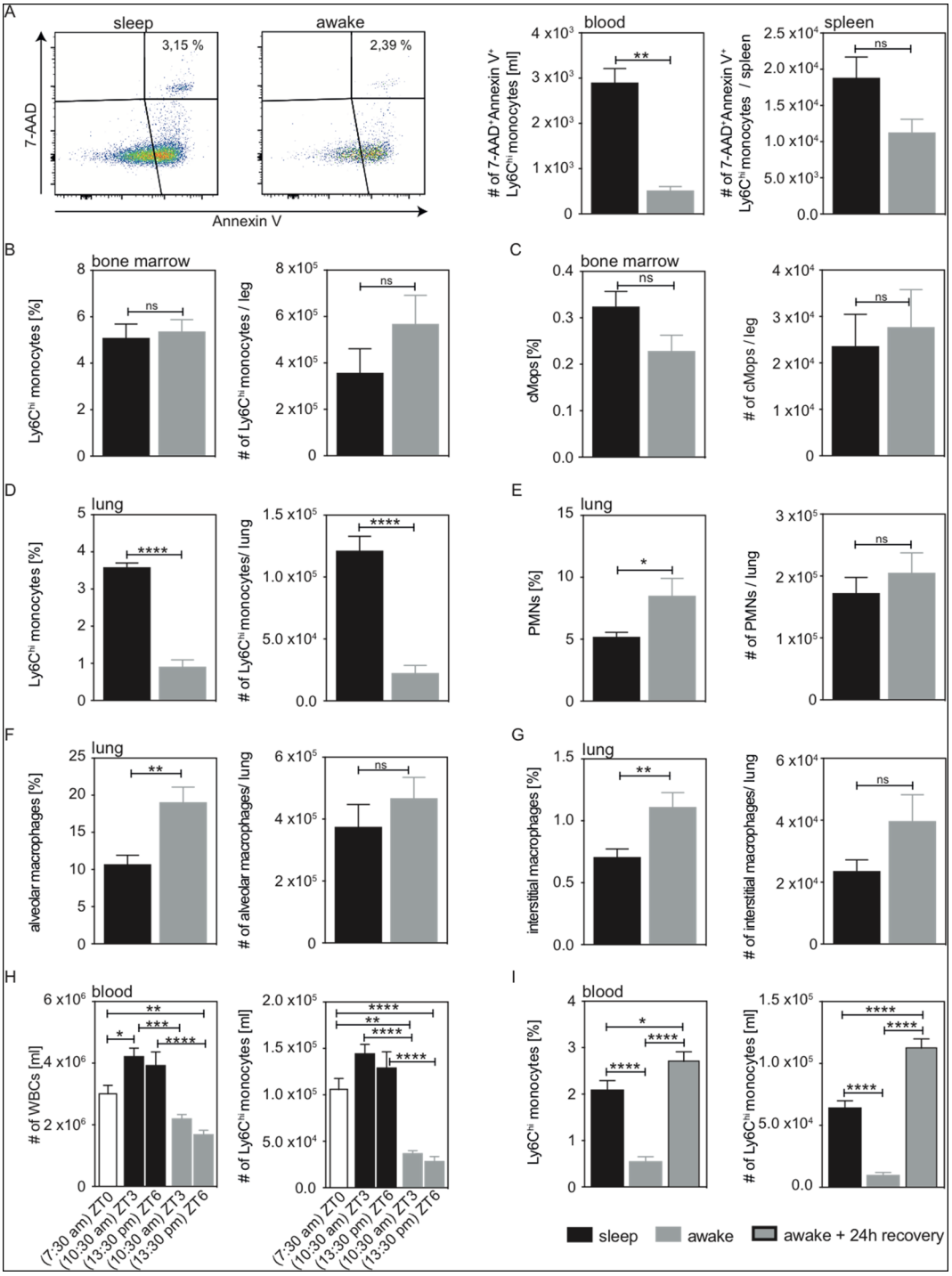

Fig. 2. Sleep effects on cell death, migration or differentiation of monocytes. Mice were allowed to sleep ('sleep', black bars) or not ('awake', grey bars) for 6 h (A-H) and left undisturbed for 24 h for recovery sleep (I). (A) Flow cytometry pseudocolor plots show one representative staining of blood Ly6C^{hi} monocytes for 7-AAD and Annexin V with the corresponding frequency of 7-AAD$^+$Annexin V$^+$ cells. Graphs show the numbers of 7-AAD$^+$Annexin V$^+$Ly6C^{hi} monocytes in the blood (left) and spleen (right) (A), the frequencies and numbers of Ly6C^{hi} monocytes (B) and their progenitors cMops (C) in the bone marrow, of Ly6C^{hi} monocytes (D), and PMNs (E) in the lung, alveolar (F) and interstitial (G) macrophages in the lung. Graphs show the numbers of WBCs and Ly6C^{hi} monocytes at baseline (starting at the onset of sleep or wakefulness; white bar) and after 3 h and 6 h of sleep or wakefulness (H) and the numbers of WBCs and blood Ly6C^{hi} monocytes 6 h after sleep or wakefulness followed by 24 h normal sleep cycle (24 h recovery) (I). Data represent the mean ± SEM from 1 experiment with 5 mice/group (A), 3 experiments with 4–5 mice/group (B - H) or 2 experiments with 3–4 mice/group (I) ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns: not significant).

sleep had no impact on the numbers of PMNs in circulation upon block of $G_{\alpha i}$PCRs (Fig. S5B).

CCR2 is a $G_{\alpha i}$PCR that is highly expressed on classical Ly6C^{hi} monocytes. It is of utmost importance for monocyte intravasation from the BM and extravasation to various tissues and also for their rhythmic trafficking (Nguyen et al., 2013). Therefore, we analyzed the effects of sleep on blood monocytes in CCR2$^{-/-}$ mice. These mice generally have a lower number of blood Ly6C^{hi} monocytes as they reside in the BM (Fig. 3B), which is consistent with published reports (Tsou et al., 2007). The difference in frequency of monocytes between "sleep" and "awake"

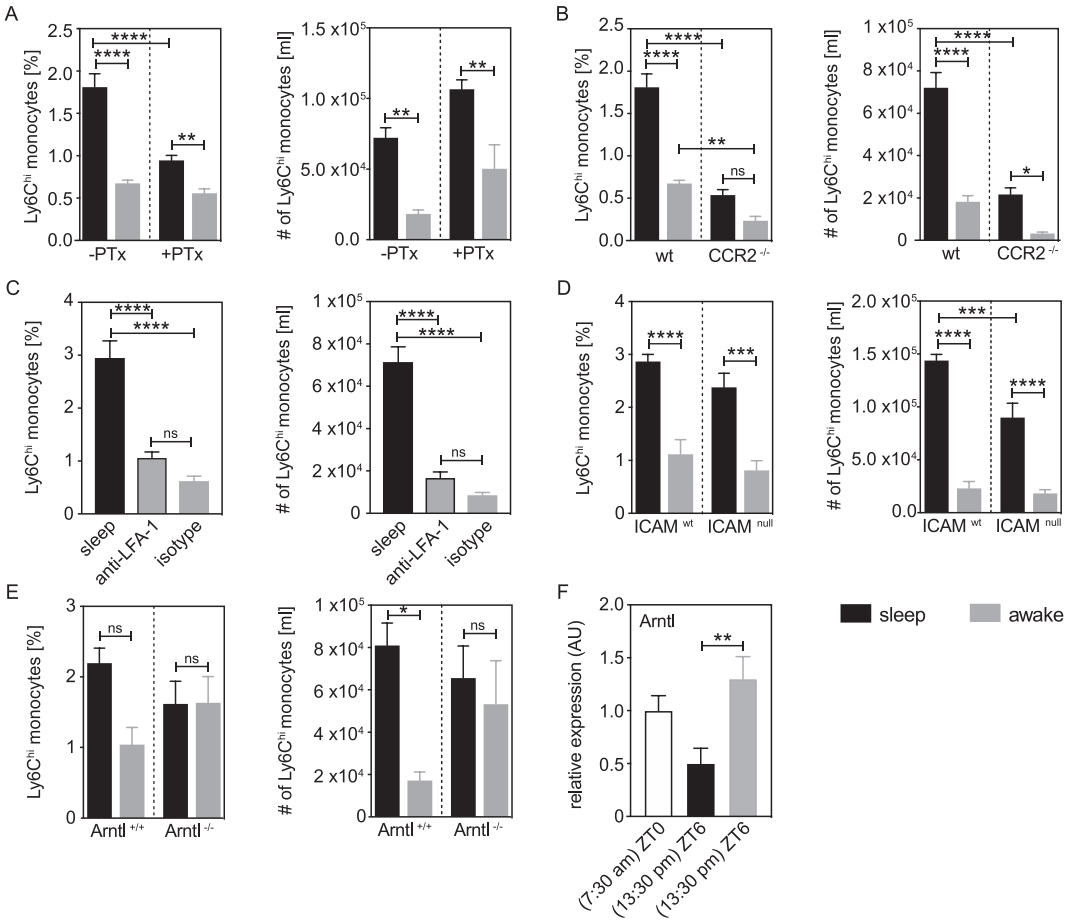

Fig. 3. Mechanisms involved in sleep-induced monocyte trafficking. WT, CCR2$^{-/-}$ (B), ICAM-1^{null} (D), Arntl$^{-/-}$ (E) mice or WT mice treated intraperitoneally either with PTx or PBS (A), or with anti-LFA-1 or isotype control antibodies (C) were allowed to sleep ('sleep', black bars) or not ('awake', grey bars) for 6 h. Graphs show the frequencies and numbers of blood Ly6C^{hi} monocytes (A-E) or the relative expression of Arntl in monocytes sorted from 'sleep' or 'awake' mice (F). Data represent the mean ± SEM from 2 experiments with 3–5 mice/group (A, B, D, F), 3 experiments with 3–5 mice/group (C) or 1 experiment with 3–5 mice/group (E) ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), ns: not significant).

conditions was significantly lower and therefore not significant in CCR2-deficient mice compared to WT mice (Fig. 3B left panel). In contrast, the monocyte numbers of "awake" mice were still significantly reduced compared to "sleep" mice (Fig. 3B right panel). Since sleep results in 2.6 times more WBCs in CCR2-deficient mice, but only 1.6 times more WBC in WT mice (Fig. S5C), this could be a possible explanation for the statistically significant differences observed in the frequencies and numbers of monocytes in CCR2-deficient mice. These data show that the sleep-induced increase in blood Ly6C^{hi} monocytes is partially dependent on CCR2 signaling.

We next tested the importance of the ICAM-1-LFA-1 axis in the sleep-induced trafficking of Ly6C^{hi} monocytes using either a blocking anti-LFA-1 antibody that was administered one day before and just before 6 h of gentle handling or using ICAM^{null} mice. The frequency and numbers of blood Ly6C^{hi} monocytes in 'awake' mice was similar upon treatment with the LFA-1 antibody and the corresponding isotype control antibody and 3-fold less than those of 'sleep' mice (Fig. 3C). Moreover, ICAM^{null} mice revealed similar results than ICAM$^{+/+}$ littermates regarding the frequency and numbers of blood Ly6C^{hi} monocytes upon sleep and wakefulness, respectively (Fig. 3D). In contrast, the frequencies and numbers of PMNs were increased upon wakefulness in anti-LFA-1-treated mice compared to the isotype control mice and in ICAM^{null} mice or compared to the sleep group (Fig. S5D-E). These data show that the ICAM-1-LFA-1 axis mediates binding of PMNs to the endothelium upon wakefulness but is dispensable for monocyte adhesion.

As circadian rhythms regulate blood numbers of Ly6C^{hi} monocytes involving BMAL1 and CCR2 (Nguyen et al., 2013), we tested whether circadian clock dysfunction affects sleep's effects on monocyte numbers using Bmal1-deficient mice (*Arntl*$^{-/-}$). Again, Ly6C^{hi} monocyte frequencies and numbers were increased by 2 to 4-fold in 'sleep' compared to 'awake' control *Arntl*$^{+/+}$ mice (Fig. 3E). This sleep effect was completely abrogated in *Arntl*$^{-/-}$ mice (Fig. 3E), clearly demonstrating the role of clock genes in sleep-induced changes in blood Ly6C^{hi} monocytes. No changes were observed for PMNs in 'sleep' compared to 'awake' control *Arntl*$^{+/+}$ or *Arntl*$^{-/-}$ mice (Fig. S5F).

To address whether sleep regulates clock genes, we investigated the Arntl expression in sorted monocytes from the spleen of 'sleep' and 'awake' mice. As shown in Fig. 3F, Arntl expression in monocytes from 'sleep' mice is significantly less than in that of 'awake' mice. More precisely, Arntl expression decreases from ZT0 to ZT6 in splenic monocytes from 'sleep' mice, which is associated with an increase in splenic monocytes (see Fig. 2H with the comparison ZT0 and ZT6), whereas Arntl expression increases from ZT0 to ZT6 in splenic monocytes from 'awake' mice which is correlated with lower numbers (Fig. 3F). We conclude that the lack of a functional clock in Arntl mutants prevents these mice from sleep-induced monocyte migration to the periphery, suggesting that sleep regulates clock genes and the circadian system and thereby the rhythmic migration of monocytes.

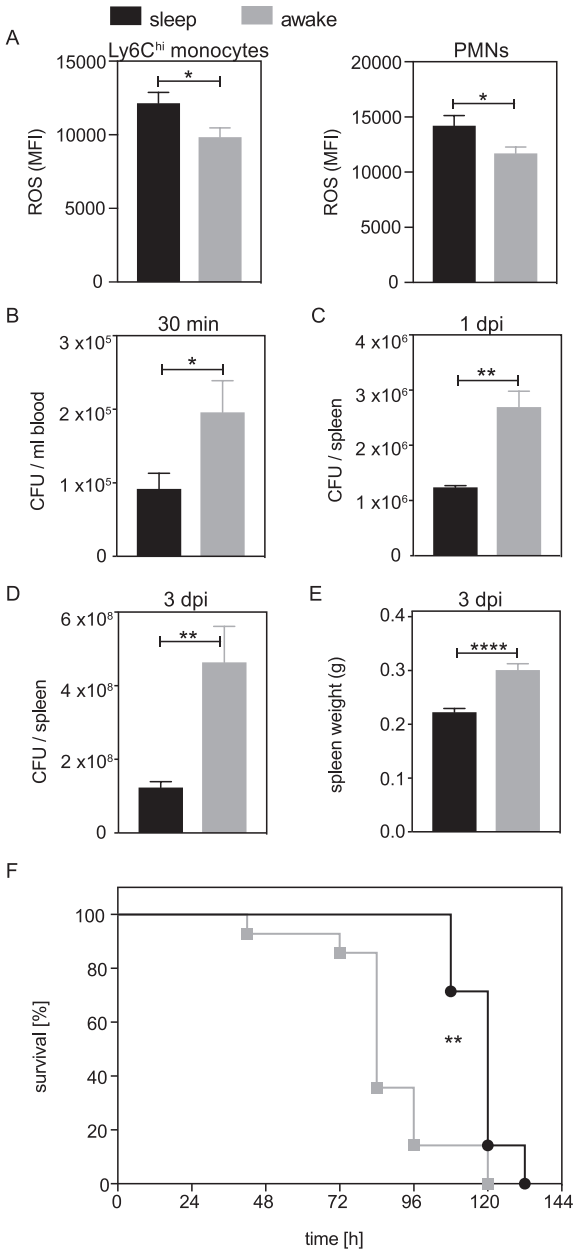

Fig. 4. Sleep boosts defense against systemic bacterial infection. Mice were allowed to sleep ('sleep', black bars) or not ('awake', gray bars) for 6 h and were either directly analyzed for ROS production (A) or intravenously infected with 5×10^8 (B) or 5×10^4 *Yersinia enterocolitica* (Ye; C - F). (A) ROS production of blood classical monocytes and PMNs was analyzed by flow cytometry. (B-D) Graphs show the bacterial load (colony forming units, CFU) 30 min (B), 1 day (C) and 3 days (D) post infection (dpi) and the spleen weight at 3 dpi (E). (F) Survival of mice treated and infected as described above was monitored until 6 dpi. Data from one out of three (A) or data from one (C) to two (B, D-E) independent experiments with 4–5 mice per group are presented as mean ± SEM. (F) Kaplan-Maier curve with data from one to two independent experiments with 7 mice per group. Statistical significance ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) was assessed using two-tailed Student's *t*-test (A – E) and log-rank (Mantel-Cox) test comparing the entire survival curves (F).

3.4. Sleep boosts antimicrobial activity of phagocytes and defense against systemic bacterial infection

Upon bacterial uptake by phagocytes ROS mediate intracellular bacterial killing. To investigate whether sleep is important for the antimicrobial activity of phagocytes, we analyzed ROS production by blood monocytes and PMNs. Flow cytometry analysis revealed that ROS production by PMNs and classical Ly6C^{hi} monocytes in the blood was significantly increased 1.6-fold in 'sleep' compared to 'awake' mice (Fig. 4A). These data show that sleep enhances the antimicrobial activity of PMNs and monocytes.

To address whether sleep-mediated increases of monocyte numbers and antimicrobial activity of phagocytes have an impact on pathogen defense, mice were infected intravenously with a lethal dose of the model organism Ye directly 6 h after sleep or wakefulness. Already 30 min post infection the bacterial burden in the blood of 'sleep' mice was 2-fold lower than that of 'awake' mice (Fig. 4B). A similar effect was observed one day post infection in the spleen (Fig. 4C). Three days post infection the number of Ye colony forming units in spleen of 'sleep' mice was already 3.8-fold lower than that of 'awake' mice (Fig. 4D). This was accompanied by a more pronounced splenomegaly (Fig. 4E) and a more rapid mortality (Fig. 4F) in 'awake' compared to 'sleep' mice after Ye infection.

In summary, the combined enhancing effects of 6 h sleep on monocyte numbers and on antimicrobial activity of phagocytes seem to acutely boost innate immune defense against systemic bacterial infection and thus benefit survival.

4. Discussion

Here, we show that sleep in mice is essential for a fully functional innate immune response. 6 h of sleep enhanced frequencies and numbers of classical Ly6C^{hi} monocytes in blood and spleen, supported rhythmic trafficking of monocytes, and increased ROS production by monocytes and PMNs, as well as bacterial clearance and sepsis survival following systemic Ye infection. In the present study, we employed gentle handling as a suitable method to prevent sleep in the absence of stress (Kopp et al., 2006; Rolls et al., 2015) and, accordingly we did not observe changes in corticosterone levels. Moreover, data from 'sleep' and 'awake' mice were collected at the same clock time to control for possible circadian influences on innate immune cell numbers and functions (Bellet et al., 2013; Casanova-Acebes et al., 2013; He et al., 2018; Nguyen et al., 2013).

The reported acute effects of sleep on monocytes seem to differ from those that can be observed on the long term. Recently, chronic sleep fragmentation for several weeks was shown to induce hematopoiesis leading to increased Ly6C^{hi} monocyte numbers and thereby accelerated atherosclerosis (McAlpine et al., 2019). Moreover, studies in mice and rats employing prolonged sleep deprivation protocols showed an increase of blood monocytes and PMNs in 'awake' mice (e.g. (Everson, 2005; Guariniello et al., 2011)). However, the rise in innate immune cells in these studies presumably reflected an inflammatory response to oxidative stress, DNA, cell and tissue damage, which emerges only after several days of sleep deprivation (Everson et al., 2014). We also observed increases in WBCs and blood monocytes with 24 h recovery after 6 h of gentle handling. However, it remains to be elucidated whether these delayed increases in innate immune cells reflect the beginning of an inflammatory response due to sleep loss or, alternatively, an overcompensation following recovery sleep.

To our knowledge acute effects of sleep on monocyte numbers have so far only been assessed in healthy humans. In contrast to the present findings in mice, in humans, a decline in blood monocyte counts during nocturnal sleep was observed (Born et al., 1997), which reflected a selective decrease in non-classical monocytes, whereas classical monocytes were unaffected by sleep (Dimitrov et al., 2007). Consequently, 24-hour rhythm analyses of blood monocyte subsets in humans showed a decrease during sleep for non-classical monocytes (Dimitrov et al., 2015). This pattern overall diverges from findings in mice displaying a circadian increase during the rest period only for classical monocytes (Nguyen et al., 2013) and a sleep-dependent rise in classical monocytes as shown in the present study. It should be noted that mice

are polyphasic sleepers whereas humans are monophasic sleepers which may also explain species differences. Recently, the molecular differences in the circadian regulation of murine and human leukocyte subsets were uncovered using humanized mice. Thus, murine and human leukocytes show opposing circadian oscillations in blood numbers that involve ARNTL, ROS and chemokine receptors in the same *in vivo* environment of humanized mice (Zhao et al., 2017), indicating major species differences in circadian immune regulation.

The acute rise in blood monocytes during 6 h of sleep in our study was not caused by increased cell death in 'awake' mice as it was the case for long term sleep deprivation (Everson et al., 2014). We even found the opposite meaning less dead monocytes in 'awake' mice compared to 'sleep' mice (Fig. 2A), which we cannot explain. Possibly this is due to circadian regulation of cell death which was shown to be increased in cells upon silencing of clock genes (Wang et al., 2015). Moreover, sleep did not impact monopoiesis (Fig. 2C), monocyte content in the BM (Fig. 2B) or the emigration of monocytes to peripheral tissues (Figs. 2D and S4). Lastly, we ruled out that sleep loss affects the differentiation of monocytes into their progeny, as macrophage numbers were comparable in the lung of 'sleep' and 'awake' mice (Fig. 2F and G). We observed an increase in the frequency, but not in numbers, of tissue resident macrophages of 'awake' compared to 'sleep' mice. In fact, tissue macrophages can proliferate (Garbi and Lambrecht, 2017; Schyns et al., 2019) which could possibly explain the observed difference of tissue macrophages in sleep and awake mice.

Hence, sleep must have affected monocyte trafficking, which involves various selectins, chemokine receptors, integrins, and their vascular ligands. In mice, the circadian peak in blood monocyte counts in the middle of the rest period seems to stem from enhanced monocyte release from the BM (Nguyen et al., 2013) and/or from reduced endothelial adhesion of monocytes in postcapillary venules. Our findings indicate that sleep is a prerequisite for the circadian increase in monocyte counts. This is due to the fact that sleep leads to a 5-fold higher number of monocytes in the blood, whereas the circadian rhythm only doubles the number of monocytes (Nguyen et al., 2013). Moreover, the sleep-dependent increase in blood monocyte numbers was abrogated in Arntl-deficient mice. As also shown in the paper by Nguyen et al. (2013) with blood monocytes, Arntl expression decreases from ZT0 to ZT6 in the splenic monocytes of sleep mice, which is associated with an increase in splenic monocytes (see Fig. 2H with the comparison 7:30 a.m. (ZT0) and 1:30 p.m. (ZT6)). In contrast, Arntl expression increases in the splenic monocytes of 'awake' mice and their number is lower (see Fig. 3F). This suggests that sleep regulates the clock genes. Our data with Arntl-deficient mice (Fig. 3E), which show that the number of blood monocytes does not change compared to ZT0 (see also Fig. 2H), i.e. the number of monocytes neither increases as it is the case with 'sleep' mice, nor is it reduced as it is the case with 'awake' mice, fit to this. We conclude that the lack of a functional clock in Arntl mutants prevents these mice from sleep-induced monocyte migration to the periphery, suggesting a role of the circadian system in regulating the rhythmic migration of monocytes by sleep. However, it should be noted that in contrast to our approach, Nguyen et al. used the monocyte-specific deletion of Arntl which rules out bystander effects of Arntl in other cells than monocytes.

The mechanisms underlying the steady-state emigration of monocytes to peripheral tissues are not completely understood (Thomas-Ecker et al., 2007), but CCR2 (Bain and Mowat, 2014) and other molecules like VCAM-1, ICAM-1, ICAM-2 on endothelial cells or the β2-integrins LFA-1, Mac-1 and VLA-4 on leukocytes (He et al., 2018) were shown to be involved. As demonstrated in this study, sleep effects on blood monocyte numbers seem to be, at least partially, dependent on G_{α}iPCRs and in particular CCR2 that is essential for monocyte trafficking (Kehrl, 1998).

Presumably sleep attenuates endothelial adhesion of monocytes or their accumulation in a second intravascular compartment, i.e., the marginal pool (Klonz et al., 1996; van Furth and Sluiter, 1986). Classical monocytes seem to contribute to the marginal pool, as adhesion to capillaries and postcapillary venules was described for this subsets in the steady state (Auffray et al., 2009; Carlin et al., 2013; Dal-Secco et al., 2015; Rodero et al., 2015). A key mediator of this reversible adhesion of monocytes (but not of PMNs) to uninflamed endothelium is the LFA-1 that binds to ICAM-1 on the vascular endothelium (Auffray et al., 2009; Carlin et al., 2013; Sumagin et al., 2010). Endothelial ICAM-1 expression as well as monocyte binding to the vessel wall are indeed reduced during the rest period compared to the active period in mice (Scheiermann et al., 2012). However, our results argue against a role for the ICAM-1-LFA-1 axis in sleep-induced rise in blood monocytes. These data correspond to findings from He et al. demonstrating the importance for these molecules in the rhythmic trafficking for the vast majority of leukocytes to the circulation except for classical monocytes (He et al., 2018). We thus can only speculate about additional molecules apart from CCR2 that are involved in the sleep-induced increase in blood monocytes and whether our findings reflect a supporting effect of sleep on the in phase expression of adhesion molecules on monocytes and the endothelium (He et al., 2018; Rolls et al., 2015). Possible additional factors involved could be G_{α}sPCRs like e.g. adrenoreceptors or other chemokine receptors like CXCR4, which is regulated by clock genes (Zhao et al., 2017). Signaling through β-adrenoreceptors was shown to be involved in the endothelial oscillations of adhesion-molecule expression (Scheiermann et al., 2012) and is clearly dependent on sleep (Dimitrov et al., 2019), indicating the possibility of a sleep-clock-immune axis.

Upon damage or infection classical monocytes are rapidly recruited to sites of inflammation. Circadian analysis revealed that a 2-fold increase in circulating classical monocytes during the rest compared to the active period improves bacterial clearance after i.p. infection with *Listeria monocytogenes*, because it facilitates monocyte recruitment to the inflamed peritoneum (Nguyen et al., 2013). Herein, we extend these findings showing that sleep is a prerequisite for this rhythm by distinctly enhancing classical monocyte numbers in blood and their steady-state trafficking to spleen, sLNs, and lung. Yet sleep is not just necessary for the regulation of monocyte numbers but also important for their function. Sleep acutely enhanced ROS production by monocytes and also by PMNs which is in line with previous findings in humans (Christoffersson et al., 2014). ROS production is essential for bacterial killing after phagocytosis. The importance of monocytes for infection eradication has already been described (Autenrieth et al., 2012; Pasquevich et al., 2015). Moreover, increased ROS production by PMNs reduces the bacterial load in the spleen upon various systemic infections (Autenrieth et al., 2012). Thus, it is not surprising that in 'sleep' mice a 5-fold increase in blood numbers of classical monocytes combined with enhanced phagocytic ROS production at the time of Ye injection renders these mice more resistant to the bacterial infection. Of note, bacterial load in blood of 'sleep' mice was reduced already 30 min after intravenous inoculation of Ye, demonstrating that sleep acutely strengthened host defense in this compartment. However, we cannot rule out that other innate immune mechanisms like the complement system are involved in enhanced bacterial killing upon sleep. Our findings support the assumption that sufficient sleep is essential for a fully functional innate immune system and in this way protects from infectious diseases (Patel et al., 2012). Indeed, rabbits with robust early sleep responses to bacterial infection showed a better survival than rabbits with reduced sleep, suggesting that sleep quality affects infection outcome (Toth et al., 1993). In summary, our data demonstrate that sleep acutely enhances phagocyte numbers and function, which is a prerequisite for (1) rhythmicity of monocytes and (2) a fully functional innate immune response against bacterial infections.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.01.001.

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