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Sleep deprivation alters neutrophil functions and levels of Th1-related chemokines and CD4$^+$ T cells in the blood

Elias A. Said[1] · Mohammed A. Al-Abri[2] · Iman Al-Saidi[1] · Mohammed S. Al-Balushi[1] · Jumaa Z. Al-Busaidi[1] · Iman Al-Reesi[1] · Crystal Y. Koh[1] · Mohamed A. Idris[1] · Ali A. Al-Jabri[1] · Omar Habbal[3]

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Abstract

Purpose The state of knowledge about the effect of sleep deprivation on the immune system is scarce and conflicting. It would therefore be useful to investigate the consequences of sleep deprivation on the immune system. We have studied the effect of sleep deprivation on the changes in neutrophil functions, and the ex vivo proliferative pattern of CD4$^+$ T lymphocytes in relationship with blood cytokine and chemokine levels due to the crucial role of these cells in mounting potent immune responses.

Methods Healthy volunteers were followed for 3 weeks. They had normal sleep in weeks 1 and 3 and they were sleep-deprived on week 2, sleeping < 6 h per 24 h, a pattern similar to sleep behaviors of many chronically sleep-deprived individuals. We assessed the levels of Th1/Th2 and inflammatory cytokines and chemokines, CD4$^+$ T cells, and the NADPH oxidase activation and phagocytic functions in neutrophils.

Results Our results suggest that sleep deprivation leads to a decreased neutrophil capacity to phagocytose bacteria and activate NADPH oxidase ($p < 0.05$). Sleep deprivation was associated with a potential increase in CXCL9 levels and decrease in CXCL10/CXCL9 and CCL5/CXCL9 ratios ($p < 0.05$). Furthermore, our results suggest that the decrease in CD4$^+$ T cell due to sleep deprivation was not associated with changes in their proliferation as observed by Ki67 levels, but rather, it correlated with changes in CXCL10/CXCL9 ratio ($p < 0.05$).

Conclusions Sleep deprivation may lead to a decreased phagocytosis and NADPH oxidase activity in neutrophils and a decrease in the levels of CD4$^+$ T cells which is related to changes in the Th1-related chemokine balance.

Keywords Neutrophils · Chemokines · CXCL9 · CXCL10 · CCL5 · CD4$^+$ T cells · Sleep deprivation

Introduction

Over the last 30 years, the modern lifestyle has led to a constant decrease in average nocturnal sleeping time from 7 h per night to less than 6 h [1]. According to the Centers for Disease Control and Prevention (CDC), around 35.2% of adults do not get enough sleep [2]. Moreover, in industrialized countries, more than 50% of the population reports regularly getting insufficient sleep [3].

Sleep deprivation may harm host defense mechanisms [4] and impact the capacity to mount potent responses [3]; therefore, changes in the components of the immune system and their responses are potentially associated with sleep deprivation. Current information about changes in the immune system in sleep-deprived individuals is extremely limited and the results of the different studies are controversial, highlighting the necessity for new studies to clarify this relationship adequately.

An increase in the neutrophil count during sleep deprivation has been reported [3, 5]. Moreover, sleep deprivation has been shown to lead to a decrease in neutrophil superoxide production [3] and phagocytic activity [6]. In contrast, the

✉ Elias A. Said
elias.said@gmail.com

[1] Department of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, P.O. Box: 35, 123 Muscat, Oman

[2] Department of Clinical Physiology, Sultan Qaboos University Hospital, P.O. Box: 38, 123 Muscat, Oman

[3] Department of Human & Clinical Anatomy, College of Medicine and Health Sciences, Sultan Qaboos University, P.O. Box: 35, 123 Muscat, Oman

study of Christoffersson et al. showed no changes in the phagocytic activity of neutrophils [3]. In these studies, volunteers were subjected to sleep deprivation for 1 [3] or 2 [6] days only.

Sleep deprivation may also be associated with changes in $CD4^+$ T cells levels and activation. Although a study showed that $CD4^+$ T cell levels decrease after sleep deprivation [4], a more recent study reported that $CD4^+$ T cell levels increase 24 h after sleep deprivation and decrease at day 4 post sleep deprivation [7]. Another study has shown that sleep deprivation affects the sleep-dependent functional rhythm of natural regulatory $CD4^+$ T cells (nTreg) and leads to a decrease in non-Treg cell ($CD4^+CD25^-$ T cells) proliferation [8]. In both studies, volunteers were subjected to sleep deprivation for 1 day only [7, 8].

In addition, cytokine levels may be affected by sleep deprivation. In a study in which volunteers were subjected to 5 days of sleep deprivation, there was a general decrease of IL-2 production, and a decrease in the IL2/IL4 ratio upon in vitro stimulation of lymphocytes with phytohemagglutinin (PHA) [9]. Furthermore, the presence of associations between sleep deprivation and changes in the levels of cytokines in the blood has also been reported. Some studies showed that IL-6 levels were elevated in the blood because of sleep deprivation [10, 11]. On the contrary, another study reported a decrease in IL-6 levels in the blood upon sleep deprivation [12]. Moreover, the levels of TNF-α in the blood were also shown to be increased in sleep-deprived individuals [11]. In these studies, volunteers were subjected to sleep deprivation during 1 day only [10–12]. Additionally, circadian misalignment was found to be a reason for the increase in plasma levels of inflammation-related molecules such as TNF-α, IL-10, and C-reactive protein (CRP) [13].

Neutrophils and $CD4^+$ T cells are essential in fighting infections; moreover, $CD4^+$ T cells play a central role shaping the responses induced in other immune cells [14–16]. The majority of the information about the effect of sleep deprivation on the immune system was provided by studies involving acute sleep deprivation, while chronic partial sleep restriction is an increasingly more recognized form of sleep loss [2]. Therefore, in this study, we included for the first time volunteers who were subjected to sleep deprivation for 1 week, preceded and followed by a week of normal sleep. We tested the hypothesis that the NADPH oxidase activity and phagocytic functions in neutrophils are affected by sleep deprivation. We also sought to describe the percentages and the ex vivo proliferative pattern of $CD4^+$ T cells that may also be affected because of the changes in the blood levels of Th1/Th2 and inflammatory cytokines and chemokines. This method mimics the actual behavior of sleep-deprived individuals who have less nocturnal sleep hours [1, 3, 17].

Methods

Study population

This study included 8 healthy adults volunteers (4 males, 4 females; BMI > 18.5 and < 24.9 kg/m^2; Table 1), which is comparable with the number of donors included in similar studies [8, 9]. The volunteers underwent full polysomnography to exclude obstructive sleep apnea syndrome (OSAS). The apnea-hypopnea index (AHI) for all volunteers was < 5. All participants denied suffering from any inflammation, infection, autoimmune diseases, and psychiatric or sleep disorders and they were not taking any medication.

Procedure of sleep deprivation

Week 1 (W1, normal sleep from day 1 to day 7). The participants had regular sleep hygiene (nocturnal sleep of 7–8 h) and avoided unnecessary awakening from sleep (Fig. 1). Week 2 (W2, sleep deprivation from day 8 to day 14). The participants were asked to reduce their nocturnal sleep to 5 h/day and avoided any daytime napping (Fig. 1). Week 3 (W3, normal sleep from day 15 to day 21). Sleep conditions were similar to W1 (Fig. 1).

All participants were supplied with sleep diary and actigraphy watch (Somnowatch, Somnomedics, Germany). The data were analyzed by software (Somnowatch, Somnomedics, Germany). Actigraphy data were analyzed after each week separately.

Collection and preparation of blood samples

At the end of each week, 20 ml blood was collected in EDTA tubes (BD, UK) and 10 ml in plain tubes (BD, UK) from each volunteer (Fig. 1). The time of collection was the same for all volunteers. To collect sera, plain tubes were centrifuged for 10 min at 500*g*. The supernatant was then harvested and centrifuged again at 1140*g* for 5 min. Plasma was collected from the EDTA tubes by centrifugation. Then, the plasma was centrifuged at 1140*g* for 5 min. Sera and plasma were then stored at − 80 °C.

Table 1 Characteristics of the volunteers enrolled in the study

Participants	
N (M/F)	Average age
8(4/4)	26 ± 8.25

N number, *M* male, *F* female. Average age ± standard deviation

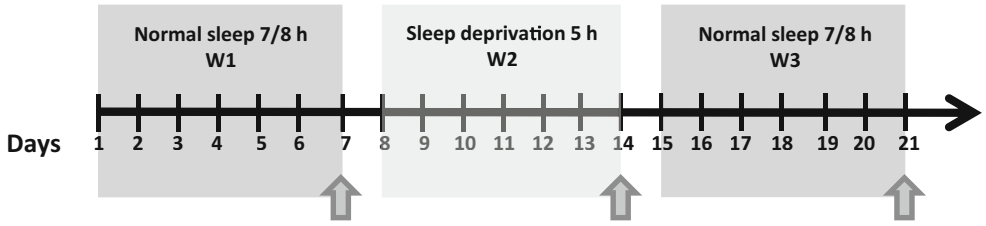

Fig. 1 Sleep deprivation protocol. Healthy volunteers ($n = 8$) were subjected to a week of normal sleep (nocturnal sleep of 7–8 h; W1), then a week of sleep deprivation (nocturnal sleep of 5 h/day; W2), followed by a week of normal sleep (nocturnal sleep of 7–8 h; W3). The gray arrows indicate the time of blood collection

Peripheral blood mononuclear cells (PBMCs) were obtained using histopaq-1077-based density gradient (Sigma-Aldrich, Germany). Blood was diluted (*v*/*v*) with PBS (Ambion, USA), and layered on the histopaq-1077, then centrifuged at 500*g* for 30 min. PBMC layer was washed three times with PBS, and then stored at − 80 °C.

Polymorphonucler cells (PMN) were collected by a double density gradient method using histopaq-1119 (Sigma-Aldrich, Germany) and histopaq-1077. Blood was diluted as mentioned above and layered on the histopaq-1077. The tubes were centrifuged for 30 min at 650*g*. The PMN layer was washed with PBS and thereafter was suspended in PBS.

Phagocytosis assay

PMN were added (10^6 cells/ml) in glass tubes. *Staphylococcus aureus* [in Hanks solution (Sigma-Aldrich, Germany); 0.6 OD at 600 nm as measured by Gene Quant spectrophotometer (Bioscience, USA)] was added, and the PMN + *S. aureus* mixture was supplemented with diluted patient serum (1:20 in PBS). The tubes were mixed well and centrifuged for 1 min at 200*g* before incubation for 20 min at 37 °C without disturbing the pellet. The supernatant was removed and the pellet was mixed. Smears were prepared on glass slides (MARINE FELD, Germany) and stained for 5 min with 10% Giemsa stain (Sigma-Aldrich, Germany) after fixing with 100% methanol (Sigma-Aldrich, Germany). The percentage of neutrophils, which were able to engulf the bacteria, was calculated microscopically using oil immersion (× 100). Cells that engulfed at least 3 bacteria were considered positive for phagocytosis.

NADPH oxidase activity test

Nitroblue-tetrazolium test (NBT) reagent (1 mg/ml; Sigma-Aldrich, Germany) was added to blood in glass tubes. Cells were stimulated using phorbol 12-myristate 13-acetate (PMA; 133 ng/ml; Sigma-Aldrich, Germany). The tubes were incubated for 10 min at 37 °C and then at room temperature for another 10 min. Cells were observed under the microscope after a blood smear was made as described above. The percentage of neutrophils with intra-cytoplasmic deposits of NBT-formazan was calculated.

Flow cytometry analysis

PBMCS were stained with anti-CD3-alexa700, anti-CD4-APC, and anti-CD8-PEcy5 (BD, USA) in PBS/2% FBS (Life Technologies, USA) then fixed with PFA 2% (Sigma-Aldrich, Germany). The intracellular staining with the anti-Ki67-FITC (BD, USA) antibody was performed using saponin (Sigma-Aldrich, Germany). FACS-Aria (BD, USA) was used for samples analysis.

Th1/Th2 and inflammatory cytokines and chemokines levels

The plasma sample was analyzed using Th1/Th2 cytokines, inflammatory cytokines, and chemokines cytometric beads array (CBA) kits (BD Pharmingen, San Diego). This allowed the investigation of the concentrations of IL-2, IL-4, IL-6, IL-5, IFN-α, TNF-γ, IL-1β, IL-10, IL-12, IL-8, RANTES, CXCL-9, CXCL-10, and CCL2. The experiments were performed following the manufacturer instructions. The samples were analyzed using FACS-Aria (BD, USA).

Statistical analysis

Wilcoxon test was used to investigate the significance of the differences in values between the different weeks. Spearman test was used to explore the significance of the correlations between two parameters. A p value < 0.05 was considered as significant. Data analysis was done using the Statistical Package for Social Sciences (SPSS ver.19), Prism and Microsoft Excel software.

Power analysis (1-β error probability) was performed with G*Power 3.1.9.2 software and power was > 0.8.

Results

Decrease in the phagocytic function and the NADPH oxidase activity in neutrophils during sleep deprivation

In order to assess the effect of sleep deprivation on neutrophil functions, we performed phagocytosis and NADPH oxidase activity assays. After a week of sleep deprivation (W2), we observed a ≈ 2-fold decrease in the percentage of neutrophils that were able to phagocytose bacteria ($p = 0.005$; Fig. 2a, b) compared with the initial week of normal sleep (W1). A ≈ 1.4-fold decrease was still observed after a week of normal sleep post sleep deprivation (W3) compared with W1 ($p = 0.012$; Fig. 2a, b). The percentage of phagocytosis was not significantly different when comparing W2 with W3 (Figs. 2a and 1b).

Furthermore, there was a ≈ 2-fold decrease in the percentage of neutrophils that were able to reduce NBT, a reaction that indicates NADPH oxidase activity, in W2 compared with W1 ($p = 0.004$) and this reduction was also observed in the third week as a ≈ 1.8-fold decrease was still detected at W3 compared with W1 ($p = 0.002$; Fig. 2c, d). The decrease in neutrophils with the oxidase activity was not significantly different between W2 and W3 (Fig. 2c, d).

Increased CXCL9 levels in the blood during sleep deprivation

To investigate the effect of sleep deprivation on the balance of Th1, Th2, and inflammatory cytokines and chemokines, the levels of 14 cytokines and chemokines were assessed in the plasma of the volunteers at W1, W2, and W3 (Fig. 3a, Table 2). We observed a ≈ 1.5-fold increase in the levels of

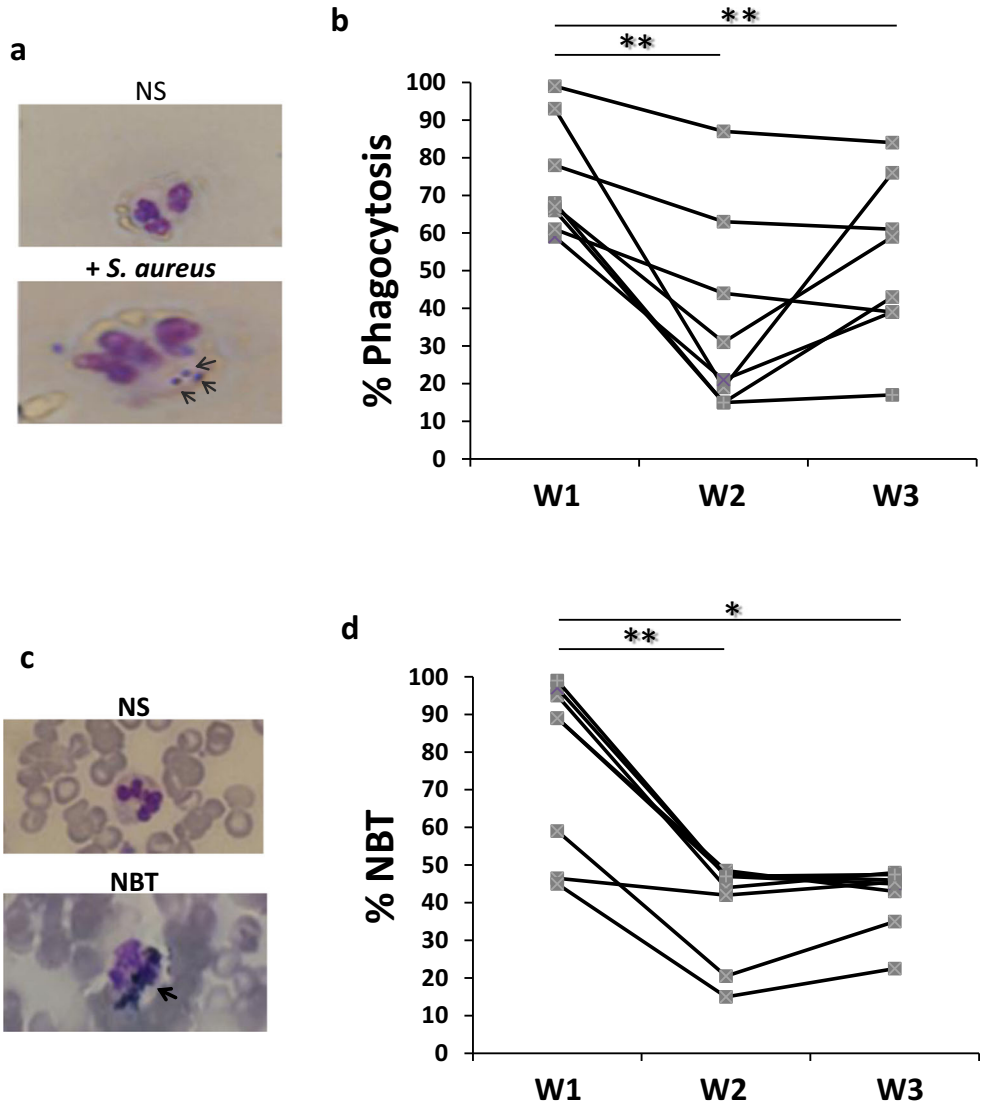

Fig. 2 The phagocytic function and the NADPH oxidase activation in neutrophils are decreased during sleep deprivation. **a** A representative image of the neutrophils when incubated with medium only (NS) or with *S. aureus*. The arrows indicate the bacteria (blue). **b** The percentage of neutrophils that were able to phagocyte bacteria at W1, W2, and W3 ($n = 8$). **c** A representative image of the neutrophils when incubated with medium only (NS) or with NBT. The arrows indicate the dark blue formazan crystals. **d** The percentage of neutrophils that were able to reduce NBT at W1, W2, and W3 ($n = 8$). *$p < 0.05$

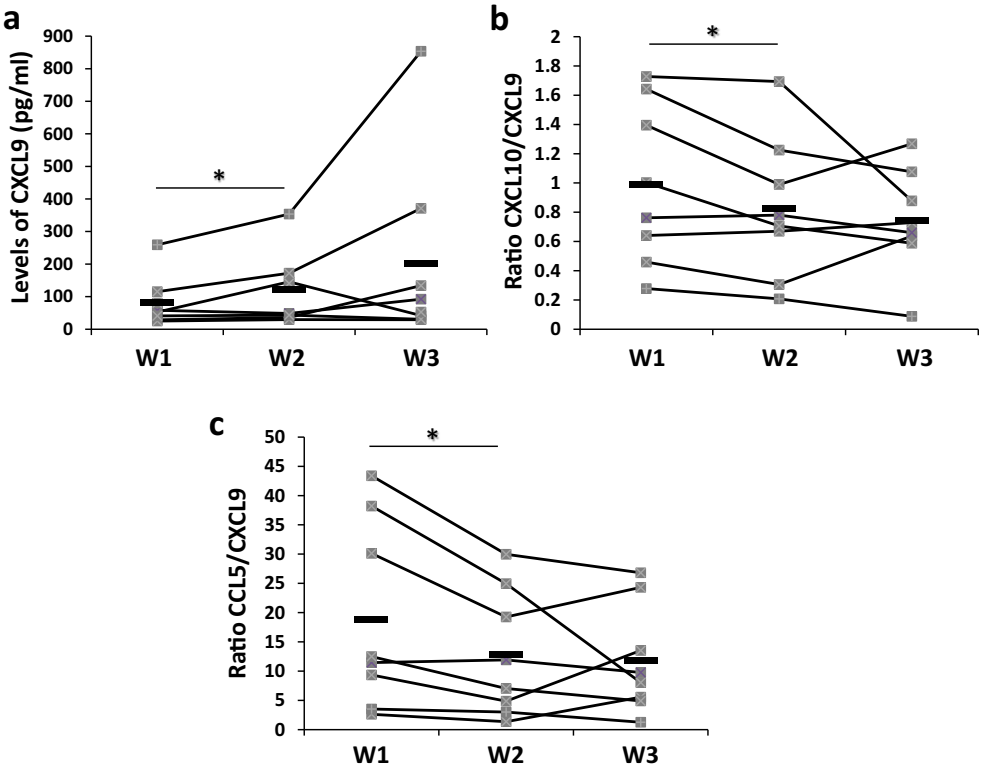

Fig. 3 Altered blood CXCL9 levels during sleep deprivation. CXCL9, CXCL10, and CCL5 levels were measured in the plasma using CBA kits at W1, W2, and W3 ($n = 8$). The samples were analyzed by flow cytometry. **a** The changes in CXCL9 levels. **b** The changes in the ratio CXCL10/CXCL9 levels. **c** The changes in the ratio CCL5/CXCL9 levels. *$p < 0.05$

CXCL-9, also known as monokine induced by gamma interferon (MIG), at W2 compared with W1 ($p = 0.028$; Fig. 3a), with no significant changes at W3 compared with W1 or W2 (Fig. 3a). No significant differences were found in the levels of IL-2, IL-4, IL-5, TNF-α, IL-10, IL-12, IL-8, RANTES, CXCL-9, CXCL-10, and CCL-2 when comparing W1, W2, and W3 (Table 2). Together with CXCL9, CXCL10 and CCL5 are considered Th1-related chemokines [18]. Interestingly, the ratio of CXCL10 or CCL5 to CXCL9 levels decreased at W2 compared with W1 ($p = 0.03$ and 0.019, respectively; Fig. 3b, c); such changes in the ratio were not observed for any other cytokines and chemokines measured.

Decrease in the percentage of CD4$^+$ T cells during sleep deprivation

We investigated the effect of sleep deprivation on the percentages of CD4$^+$ T cells among PBMCs. The percentage of CD4$^+$ T cells in the sleep-deprived volunteers showed ≈ 1.2-fold decrease in W2 compared with W1 ($p = 0.0017$; Fig. 4a), with no significant changes in W3 compared with W1 or W2 (Fig. 4a).

Moreover, to monitor whether sleep deprivation affects the proliferation of CD4$^+$ T cells, we measured the ex vivo levels of Ki67 [19], a nuclear protein expressed during cell

Table 2 Cytokine and chemokine levels

Cytokine (pg/ml)	Week 1	Week 2	Week 3	*p* value (W1/W2)	*p* value (W1/W3)	*p* value (W2/W3)
CCL-5	903.5 ± 393	783.1 ± 277.8	898.9 ± 425.9	0.1	0.8	0.2
CCL-2	50.2 ± 55.6	50.1 ± 40.6	55.9 ± 45.4	0.3	0.7	0.5
CXCL-10	54.6 ± 24.3	61.7 ± 28.5	71.7 ± 61.9	0.5	0.8	0.8
IL-8	59.3 ± 31.4	77.0 ± 37	77.5 ± 42.9	0.2	0.3	0.8
IL-2	1.9 ± 4.7	1.9 ± 5	2.1 ± 4.9	0.8	0.7	1
IL-4	10.4 ± 29.5	15.6 ± 46.2	13.6 ± 37.1	0.5	0.1	0.7
IL-5	1.3 ± 3.5	2.0 ± 5.6	1.7 ± 4.8	0.2	0.8	0.3
IL-10	5.0 ± 9.8	3.9 ± 10.7	4.6 ± 11.9	0.8	0.2	0.2
TNF-α	6.6 ± 18.6	7.7 ± 21.4	8.0 ± 21.2	0.2	0.2	0.7
IL-1β	3.3 ± 5.8	4.9 ± 9.8	3.6 ± 6.4	0.2	0.2	0.3
IL-6	0.6 ± 0.8	0.9 ± 1.1	0.4 ± 0.5	0.3	0.2	0.09

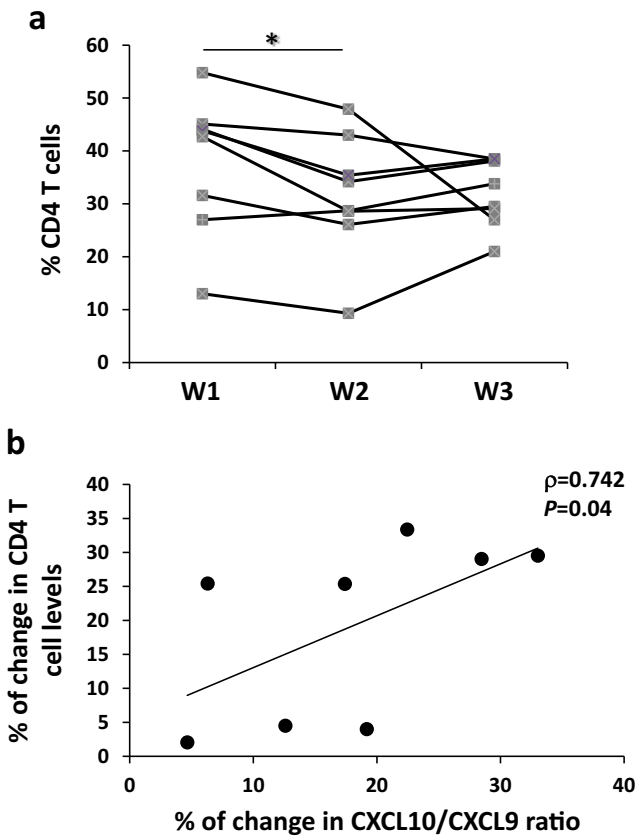

Fig. 4 The changes in CD4$^+$ T cell levels correlate with the changes in the ratio CXCL10/CXCL9 during sleep deprivation. PBMCs ($n = 8$) were stained with anti-CD3-alexa700, anti-CD4-APC, and anti-CD8-PEcy5 antibodies and then analyzed by flow cytometry at W1, W2, and W3. **a** The decrease in CD4$^+$ T cell levels. **b** The correlation of changes in CD4$^+$ T cell level changes with CXCL10/CXCL9 ratio changes. *$p < 0.05$

proliferation [20], in these cells. There were no differences in the levels of Ki67 expression in CD4$^+$ T cells (Table 3) in the sleep-deprived volunteers when comparing W1, W2, and W3.

Interestingly, the percentage of changes in the levels of CD4$^+$ T cells between W1 and W2 significantly correlated with that of the changes in the ratio CXCL10 to CXCL9 in the same period ($\rho = 0.742$, $p = 0.04$; Fig. 4b).

Discussion

Our results suggest that the phagocytic activity of neutrophils was decreased upon sleep deprivation. These results are supported by previous findings [6]. However, the 1-week recovery period (W3) after sleep deprivation in our study was not enough for the neutrophil functions to reach normal levels again, which may be due to the differences in the protocols used in both studies [6], as the volunteers in our study were exposed to a longer period of sleep deprivation. It is interesting to note that the method used in our study is a type of sleep restriction that mimics sleep behavior of many people in the community [1, 3, 17]. In contrast, another study did not find any changes in the capacity of neutrophils to phagocytose bacteria upon sleep deprivation [3], which could be due to the short period of sleep deprivation to which the volunteers were exposed to in that study. Additionally, our findings also suggest that the NADPH oxidase activation was reduced during sleep deprivation, which corroborates with the results of a previous study that showed decreased levels of reactive oxygen species production after 1 day of sleep deprivation [3]. The levels of NADPH oxidase activation remained low even after the recovery week (W3). It is noteworthy that similar defects in the phagocytic and NADPH activation functions in neutrophils were observed by our group as well as others in patients with obstructive sleep apnea (OSA), a disorder in which patients are sleep-deprived [19]. This highlights the role of sleep deprivation in the decrease of neutrophil functions in OSA. Nevertheless, the mechanisms that led to the decrease in neutrophil functions remain to be investigated. Neutrophils are short-lived cells and their half-life in the circulation is 6–8 h [21]. This indicates that the observed effects are lasting longer than this half-life, which suggests that these effects are due to factors that can affect the newly produced neutrophils.

We observed for the first time a potential deregulation in the balance of the Th1-related chemokines, CXCL9, CXCL10, and CCL5 [18], which was reflected by the increase of CXCL9 levels and the decrease of the CXCL10/CXCL9 and CCL5/CXCL9 ratios during the sleep deprivation period. CXCL9 is produced by a variety of cells including non-hematopoietic cells, e.g., endothelial cells, such as the brain endothelial cells [22, 23]. These cells are affected by sleep deprivation, which influences the expression of molecules related to the vascular endothelial function and inflammation in the cerebral microvessels [24]. It is then possible to hypothesize that the increased inflammation in the cerebral microvessels during sleep deprivation [24] is associated with an increase in the production of CXCL9 by the cells of these microvessels. Both CXCL9 and CXCL10 attract CXCR3$^+$ T cells, while CCL5 attracts CCR5$^+$ T cells. However, although CXCL9 and CXCL10 share the same receptor (CXCR3), the profile of the immune cells that are attracted by these chemokines may be different [25, 26]. For example, the absence of CXCL9 was associated with an increased accumulation of Th1 cells while the absence of CXCL10 was associated with the accumulation of Th17 cells [26]. Therefore, the changes in the balance between CXCL9, CXCL10, and

Table 3 Cell percentages and Ki67 expression

Ki67 CD4 T cells MFI	Week 1	Week 2	Week 3
	464 ± 141	443 ± 38.1	490 ± 107
p value	(W1/W2)	(W1/W3)	(W2/W3)
	0.7	0.2	0.6

W week

CCL5, during sleep deprivation, may have an impact on the profile of the cells that are involved in responding to these chemokines.

Furthermore, our study suggests that the percentage of $CD4^+$ T cells is decreased upon sleep deprivation and that this percentage returns to normal after the week of recovery (W3). These results are supported by a study that showed a decrease in $CD4^+$ T cell levels after 64 h of sleep deprivation [4]. In contrast, another study found that $CD4^+$ T cell levels increased 24 h after sleep deprivation and decreased at day 4 post sleep deprivation [7]. The difference in the findings might be due to the duration of sleep deprivation to which the volunteers were exposed to as in Wilder-Smith et al.'s study in which the volunteers were exposed to one night of sleep deprivation only [7]. The changes in $CD4^+$ T cell levels might not be related to a decrease in their proliferation capacity as no changes were observed in the level of Ki67, a nuclear protein expressed in proliferating cells. Interestingly, our results are the first to provide a potential explanation for the decrease in $CD4^+$ T cell levels during sleep deprivation as the percentage of changes in the levels of $CD4^+$ T cells showed a potential correlation with that in the ratio CXCL10/CXCL9. This shows that the changes in the levels of $CD4^+$ T cells and CXCL9 are significantly present and interrelated. It also suggests that the decrease in the percentage of $CD4^+$ T cells might be due to their increased migration to the sites where CXCL9 is produced. This hypothesis is supported by the fact that the pattern of the changes in the levels of $CD4^+$ T cells and CXCL9 was similar, as the levels of CXCL9 increased during sleep deprivation when $CD4^+$ T cells levels decreased and returned to normal during W3 when $CD4^+$ T cells levels were normal again. However, the balance between CXCL9 and CXCL10 may have a greater effect than CXCL9 alone on the changes in $CD4^+$ T cell levels. This may be explained by the fact that these two chemokines share the same receptor and therefore, the changes in the levels of one of them might be compensated, or interfered with, by the other [25, 26]. Of note, both the CXCL10/CXCL9 ratio and the percentages of $CD4^+$ T cells showed a similar pattern of change as they decreased upon sleep deprivation then reached normal levels in the week of recovery W3.

The levels of different hormones including cortisol are affected by sleep. Many of these hormones can affect the immune system with an important suppressive role for cortisol [27]. It is then tempting to hypothesize that the changes in the levels of these hormones are linked to the effects of sleep deprivation on the immune system that are observed in this study.

Considering the important role of $CD4^+$ T cells and neutrophils in fighting infections [14–16], our results imply that the defense mechanisms against infections are affected in sleep-deprived individuals, and therefore sleep-deprived individuals may have a higher susceptibility to infections. In fact, sleep-deprived patients have been found to be more vulnerable to respiratory infections [28, 29]. Moreover, $CD4^+$ T cells play a central role in the establishment of a proper immune response upon vaccination [14, 15, 30]; therefore, our results which are showing the decrease in the percentage of $CD4^+$ T cells upon sleep deprivation may explain the effect of sleep deprivation on the efficacy of vaccination [31, 32]. These hypotheses necessitate additional investigations.

Conclusion

Our results suggest that sleep deprivation affects the immune system by decreasing the phagocytosis and NADPH oxidase activity in neutrophils, changing the balance of the Th1-related chemokines, and decreasing the levels of $CD4^+$ T cells.

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Author contributions EAS, MAA, and OH designed the project, performed some experiments, and participated in data analysis and manuscript writing. IS, MSB, JZB, and IR performed experiments and participated in the manuscript writing. CYK, MAI, and AAJ participated in data analysis and writing the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Ethical considerations All volunteers were informed about this study and signed an informed consent form. This study was approved by the Ethical Committee of the College of Medicine and Health Sciences in the Sultan Qaboos University (SQU), Oman. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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