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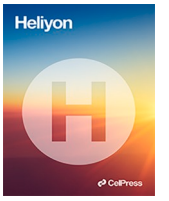

Research article

Altered neutrophil extracellular traps formation among medical residents with sleep deprivation

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ABSTRACT

Resident physicians on long-term night shifts often face sleep deprivation, affecting the immune response, notably neutrophils, vital to innate defense mechanisms. Sleep-deprived residents exhibit altered neutrophil counts and reduced phagocytosis and NADPH oxidase activity, critical to combating infections. Our study focused on neutrophil extracellular traps (NETs), a defense process against pathogens not previously linked to sleep loss. Results revealed that sleep-deprived residents exhibited a 19.8 % reduction in NET formation compared to hospital workers with regular sleep patterns ($P < 0.01$). Additionally, key NETs proteins, Neutrophil Elastase and Myeloperoxidase, were less active in sleep-deprived individuals (1.53mU; $P < 0.01$ and 0.95U; $P < 0.001$ decrease, accordingly). Interestingly, the ability to form NETs resumed to normal levels three months post-residency among pediatric residents. The causal relationship between reduced NETs due to sleep deprivation and the increased susceptibility to infections, as well as its implications for infection severity, is a critical area for further investigation.

1. Introduction

Sleep disorders are considered a public health epidemic, with about 30 % of the US adult population suffering from chronic sleep deprivation [1]. Sleep deprivation is particularly prevalent among shift workers, including medical professionals [2]. It is well-recognized that insufficient sleep has major deleterious effects on health [3,4]. Sleep deprivation has been associated with alterations of innate and adaptive immune parameters. There is growing evidence associating reduced sleep quality with increased risk of infection and poor infection outcome [5,6]. Furthermore, sleep deprivation can lead to complications including a variety of pathologies, like cardio-metabolic, neoplastic, autoimmune, and neurodegenerative diseases [7,8]. The alterations of the immune system that have been demonstrated in recent studies include increased numbers of total leukocytes and of specific cell subsets, mainly neutrophils, as well as, monocytes, B cells, CD4 T cells, and decreased circulating numbers and cytotoxic activity of natural killer cells [9–11]. In addition to alterations in the numbers of circulating neutrophils, it was recently demonstrated that sleep deprivation may lead to decreased neutrophil phagocytosis and NADPH oxidase activity [12].

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Neutrophils, the most abundant circulating leukocyte in the blood, are the first line of immune defense within the innate immune system [13]. They protect the host by numerous mechanisms, including phagocytosis, release of cytotoxic molecules (e.g., neutrophil elastase [NE] and myeloperoxidase [MPO]), and formation of neutrophil extracellular traps (NETs) (13). NETs are extruded by activated neutrophils and are composed of DNA fibers, histones, and antimicrobial proteins [14,15]. The clinical importance of NETs was first recognized in the context of chronic granulomatous disease, where congenital deficiency of NADPH oxidase impairs ROS production and NETs formation [16], resulting in severe, particularly fungal infections [17]. In addition, it was recently demonstrated that NETs are also formed in non-infectious conditions. Increased NET release and its delayed clearance has been associated with a wide variety of diseases, including inflammatory and autoimmune diseases [18], thrombosis-associated conditions [19] and cancer progression and dissemination [20].

We chose to focus our investigation on resident physicians involved in regular nightshift schedules that subject them to acute sleep deprivation and disruption [21]. Since their residency lasts for several years, their sleep deprivation is chronic [22], providing a unique population for testing sleep deprivation in a real-life setting. Herein, we demonstrate, for the first time, the effect of sleep deprivation during medical training on NETs formation and its possible impact on other essential neutrophil functions, such as NE and MPO enzymatic activities.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Institutional Review Board of the Tel Aviv Medical Center IRB0014-13. All study participants signed informed consent.

2.2. Study and control population

Sixteen healthy pediatric residents (ages 29–38, median 34) were recruited from the medical staff of the Tel Aviv Sourasky Medical Center, Tel Aviv, Israel, during the period 2021–2023. All residents underwent a brief health screening to confirm eligibility. This included self-reported absence of current illness, autoimmune/inflammatory/infectious (viral or bacterial) diseases, and medication use. These residents participated in a residency program lasting 4–4.5 years (median residency duration: 2.5 years). Throughout this period, they completed a demanding schedule of 6 or 7 nightshifts per month, each lasting 26 h. This chronic sleep deprivation serves as the focus of this study. Baseline assessments of the study parameters were conducted in the morning after a nightshift (NS) and again after 3 consecutive nights off-call (post NS). To further investigate recovery, follow-up blood samples were obtained from 5 residents that completed the residency program (Recovery). Recovery is defined as the three-month period following the completion of residency training. During this time, physicians were no longer exposed to the demanding schedules and sleep deprivation characteristic of residency, and self-reported experiencing regular sleep. A control group of fifteen healthy hospital workers (ages 33–46, median age 35.5 years) was recruited. Similar to the residents, these individuals underwent a health screening to confirm eligibility (absence of current illness, autoimmune/inflammatory/infectious diseases, and medication use). Importantly, the control group did not participate in nightshifts and self-reported no history of sleep deprivation.

3. Materials

All chemicals were obtained commercially: phosphate-buffered saline (Biological Industries), ethylene diamine tetra-acetic acid, bovine serum albumin, glucose, phorbol 12-myristate 13-acetate (PMA), and Triton X-100 (Sigma-Aldrich, MO, USA); poly-L-lysine solution (0.01 %) and buffered 4 % formaldehyde solution (Merck, NJ, USA).

3.1. Isolation of neutrophils

Neutrophil isolation was performed as previously described [23] using immunomagnetic negative selection with the EasySep Direct Human Neutrophil Isolation Kit (StemCell Technologies Inc.) according to the manufacturer's instructions. Briefly, peripheral blood samples (2–5 mL) collected in EDTA-coated vacutainer tubes (Greiner Bio-One) from healthy volunteers were mixed with an antibody cocktail (StemCell Technologies Inc.) conjugated to magnetic beads targeting all major leukocytes and erythrocytes except neutrophils. After a 5-min incubation, the sample tube was placed in the EasySep magnet (StemCell Technologies Inc.) for 10 min. This allowed bound cells to adhere to the magnet, enriching the neutrophil population in the supernatant. The neutrophil-enriched fraction was then centrifuged at $300\times g$ for 7 min at room temperature. Prior to the experiments neutrophil count were performed and absolute neutrophil counts were within normal range. Purity of the isolated neutrophils was assessed by cytospin and hematoxylin staining, revealing a yield of 98 %.

3.2. Neutrophil Elastase and Myeloperoxidase enzymatic activity

As previously described [23], enzymatic activity of both NE and MPO was measured. Briefly, 10^5 neutrophils were lysed in 0.2 % Triton X-100 solution.

3.3. Neutrophil elastase

Incubated with chromogenic peptide elastase substrate (final concentration 0.5 mM, stock 20 mM in DMSO, Calbiochem) for 90 min at 37 °C. Enzymatic activity measured at 415 nm using an iMark Microplate Absorbance Reader (Bio-Rad). Calibration curve established using purified NE (5–100 ng, Athens Research & Technology). Positive control: 10 ng purified NE. Negative control for each experiment: 10 ng purified NE with specific NE inhibitor IV (final concentration 100 μM, stock 20 mM in DMSO, Calbiochem).

3.4. Myeloperoxidase

Incubated with O-phenylenediamine (final concentration 50 μg/mL, stock 10 mg/mL in PBS, Sigma-Aldrich) and H_2O_2 (final concentration 1 mM, Sigma-Aldrich) for 20 min at room temperature. Enzymatic activity measured at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad). Calibration curve established using purified MPO (1–10 μg, Athens Research & Technology). Positive control: 2 μg purified MPO. Negative control for each experiment: 2 μg purified MPO with 4-aminobenzoic acid hydrazide (final concentration 5 mM, stock 0.5 M in DMSO, Cayman Chemicals), a specific MPO inhibitor.

3.5. Neutrophil activation

Building upon a previously described method [23], 2 x 10ˆ5 neutrophils were seeded onto coverslips pre-coated with poly-L-lysine. The cells were then activated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 3 h at 37 °C. PMA was prepared from a stock solution in DMSO (Sigma-Aldrich). Following activation, the cells were fixed using 4 % formaldehyde solution. As a control, an equal volume of DMSO, used to dissolve PMA, was added to RPMI medium, and the cells were incubated under the same conditions.

3.6. Immunofluorescent staining and NET quantification

Following activation, neutrophils were stained for visualization of NET formation.

Neutrophil Labeling: Cells were incubated with Sytox Green (Invitrogen) and Hoechst 33342 (Sigma-Aldrich) nuclear dyes according to the manufacturer's instructions. Briefly, this allows for differentiation between condensed and decondensed chromatin.

Image Acquisition and Analysis: Images were captured using an LSM700 Laser Scanning Confocal Fluorescence Microscope (Zeiss). For each sample, three regions of interest containing 100–200 cells were analyzed. NET formation was quantified manually based on established criteria. Neutrophils without NETs: Cells with compact DNA stained by both dyes (Sytox Green and Hoechst 33342). NETs-forming neutrophils: Cells with diffused DNA stained only by Sytox Green. The percentage of NET formation was calculated as the ratio of NETs-positive neutrophils to the total number of neutrophils (NETs-positive and -negative).

Average Cell Surface Area Analysis: Additionally, the average area of cells was calculated using Image J software. Briefly, we created a single channel (green) tiff file and changed the image type to 8-bit. Subsequently, we adjusted threshold to 35 and analyzed particles (size: 0.5-infinity, circularity: 0.0–1.0, show: overlay mask) and extracted total area. Then total cell area was divided by the number of cells which resulted in the average cell surface of each image. Finally, statistical analysis was applied to compare the average cell surface between the 3 conditions.

3.7. NE-DNA complex ELISA

ELISA for NE-DNA complexes was carried out as described previously with minor modifications [23]. In brief, 96-well plates (Corning Incorporated) were first coated with the rabbit monoclonal anti-human NE antibody (1 μg/mL; Abcam) and incubated overnight at 4 °C. Subsequently, 96-well plates were washed 3-times with PBS and incubated with blocking solution containing 1 % bovine serum albumin in PBS for 90 min at RT. Next, serum (final dilution at 1:10) was added to 96-well plates and processed with a limited 15-min DNase digestion, in order to shorten chromatin threads for a maximum binding between NE-antibody and NE-DNA from NETs, before overnight incubation at 4 °C. The following day, monoclonal mouse anti-human DNA antibody (Abcam) were applied to the wells for 90 min at RT, followed by Peroxidase AffiniPure Goat Anti-Mouse IgG (H + L) (Jackson Immuno Research Inc.) for 60 min at RT and then color development according to the manufacturer's instructions. OD was measured for each well at a wavelength of 415 nm, using 490 nm as reference iMark Microplate Absorbance Reader (Bio-Rad).

3.8. Statistical analysis

GraphPad Prism version 5 (GraphPad Software Inc., CA, USA) was used for statistical analysis. The data are presented as mean ± standard error of the mean. Statistical differences were determined by employing an ANOVA test with a Tukey *post-hoc* test. Statistical significance was determined at $^*P < 0.05$.

4. Results

4.1. Sleep deprivation alters NETs formation

In order to assess the effect of sleep deprivation on neutrophil functions, we examined NETs formation induced by PMA, the most

established *in-vitro* NETs inducer [24]. We first compared pediatric residents in the morning after a nightshift (NS) to hospital workers who do not participate in any nightshift schedules who served as the control group. Results revealed that sleep deprivation significantly reduced NET formation in residents following a night shift compared to hospital workers with regular sleep patterns. Residents following a night shift exhibited only 33.8 % ± 4.6 NET formation, which was significantly lower ($P < 0.01$) than the 53.6 % ± 4.0 observed in hospital workers. In addition, our results demonstrated significantly reduced total surface covered by DNA forming NETs among resident physicians the morning after a nightshift (97 $\mu m^2 \pm 18.9$) compared to hospital workers who served as controls (163.2 $\mu m^2 \pm 12.5$, $P < 0.05$) (Fig. 1A–D). Next, we examined NETs formation in resident physicians following at least 3 nights of self-reported good night sleep (post NT). In this condition, NETs formation was also found to be significantly decreased compared to controls (35.4 % ± 3.7, $P < 0.01$). Interestingly, there was no significant difference between medical residents following a 26-h nightshift, and 3 nights of proper sleep, demonstrating that some days of normal sleep could not restore the effect of continuous sleep deprived condition.

4.2. Sleep deprivation inhibits enzymatic activities of NE and MPO

Activities of NE and MPO are necessary for efficient NETs formation regardless of the inducer [15]. Additionally, we had previously shown that downregulation of NETs formation after stem cell transplantation was correlated with impaired activity of NE [25]. We next sought to investigate whether sleep deprivation has an impact on enzymatic processes that orchestrate NETs formation, including MPO and NE enzymatic activities. For this purpose, neutrophils obtained from all 3 described groups were examined. The results showed that the enzymatic processes of both NE (Fig. 1E) and MPO (Fig. 1F) were both inhibited in medical residents following a nightshift in comparison to control (6.03 ± 0.42 vs 7.56 ± 0.81 for NE, $P < 0.01$; and 0.61 ± 0.05 vs 1.56 ± 0.13 for MPO $P < 0.001$), providing a possible explanation for the mechanism of NETs formation inhibition. Finally, in order to assess spontaneous NET formation, we measured serum levels of NE-DNA complexes, a fragment released during NET formation, using NE-DNA ELISA in samples from controls and resident physicians. However, serum NE-DNA levels did not differ significantly between the three groups (control 0.20 nM ± 0.018 vs night shift 0.19 nM ± 0.025, $P = 0.69$) (Fig. 1G).

4.3. NETs formation recovers among resident physicians completing their training

Next, we examined NETs formation among 5 residents from the initial cohort, at least 3 months after completing their training. Our results demonstrated equal NETs formation compared to the control group (53.8 % ± 5.1 vs 53.6 % ± 4.0, $P = 0.89$) (Fig. 2A and D). Among those 5 participants, no statistically significant difference in NE and MPO activity was demonstrated compared to controls (7.1 ± 0.34 vs 7.56 ± 0.8 for NE, $P = 0.7$; and 1.02 ± 0.3 vs 1.56 ± 0.13 for MPO $P = 0.63$), (Fig. 2B and C).

5. Discussion

This study investigated the effects of sleep deprivation associated with residency training on neutrophil functions. Due to the constant exposure to nightshift schedules, medical residents, without exception, develop chronic sleep deprived condition. We found that medical residents were associated with decreased NETs formation. In addition, examination of NE and MPO enzymatic activities revealed a decrease that might explain the mechanism of NETs dysregulation. Interestingly, NETs formation recovered 3 months after pediatric residents completed the residency.

There is growing evidence associating reduced sleep quality with increased risk of infection and poor infection outcomes [5,6]. In this work we show that neutrophils of sleep deprived residence physicians had a decreased ability to form NETs after activation with PMA, mimicking an inflammatory challenge. We suggest that the decreased ability to form NETs following sleep deprivation might be one reason for higher risk of infections and for poor infection outcomes in individuals with reduced sleep quality.

Interestingly, our investigation of serum NE-DNA complex levels, a putative marker of spontaneous NET formation, revealed no significant differences between the groups. However, in the research period no infections disease or other inflammatory challenge was reported, providing a possible explanation to that result. In addition, it's important to acknowledge the limitations of NE-DNA ELISA. Spontaneous NETs could be primarily confined to tissues or rapidly eliminated by circulating nucleases like DNase1, making them difficult to detect in serum. Our study did not measure DNase activity, which could provide valuable insights into NET clearance mechanisms.

A recent study demonstrated that the circulating neutrophil population following sleep loss was relatively less mature (i.e., there was a significant decrease in the $CD16^{bright}/CD62L^{bright}$ neutrophil population) [26]. This shift in neutrophil populations into more immature cells was hypothesized to cause the reduced neutrophil capacity to produce reactive oxygen species in response to stimuli and possibly also contribute to the decreased NETs formation capacity.

Neutrophils are short-lived cells and their half-life in circulation is estimated to be less than 24h [27]. However, our findings showed that chronic sleep deprivation induced a long-lasting dysregulation of NETs formation (following 3 nights without working a nightshift), much longer than the neutrophil's half-life, suggesting that other factors may influence the newly produced neutrophils. The process might involve previously reported changes in circulating levels of hormones and inflammatory mediators during sleep deprivation (e.g., low levels of growth hormones, prolactin, and melatonin, and high levels of catecholamines, IL-1b, TNF-a, IL-6, and CRP) [28,29]. These mediators had also been described as influencing neutrophil functions [30–34], including NETs formation [35]. Taken together with the growing evidence associating reduced sleep quality with increased risk of infection and poor infection outcomes [5,6], the current study findings offer a possible explanation for this phenomenon by suggesting that sleep deprivation may

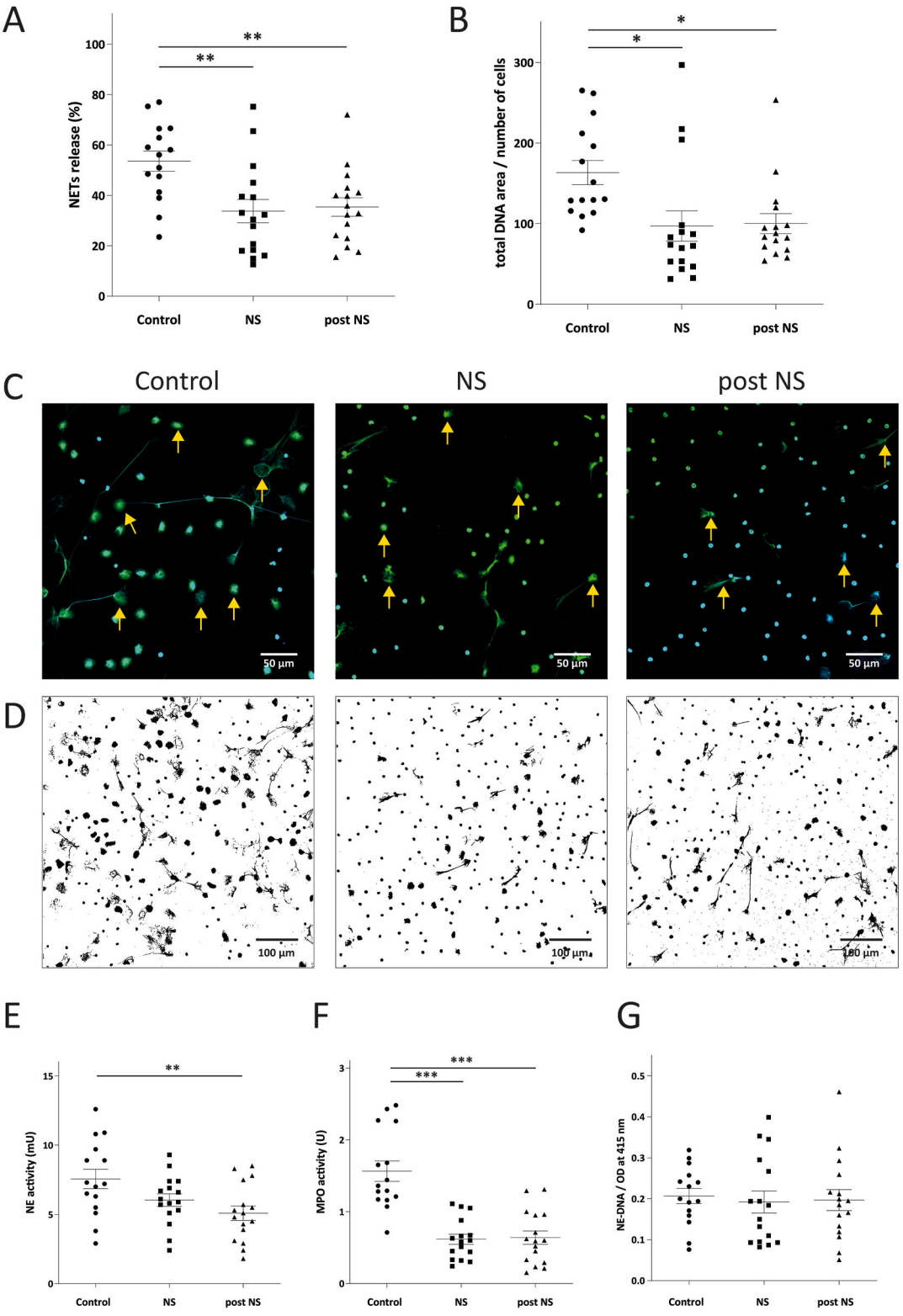

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Fig. 1. Inhibition of NETs formation by sleep deprivation. **(A)** Both pediatric residents in the morning after a nightshift (NS) (RP; 33.8 % ± 4.6), and resident physicians following 3 nights of self-reported good night sleep (post NT). (RB; 35.4 % ± 3.7) presented a significantly (**P < 0.01) reduced NETs formation compared to the control group (53.6 % ± 4.0). (**B**) The average DNA areas of samples obtained from residents after 26-h shift (NS; 97.1 ± 18.9) and following 3 nights of self-reported good night sleep (post NS) (RB; 100.0 ± 12.5) were significantly reduced compared to controls (163.2 ± 14.9) (*P < 0.05). (**C**) Representative images of immunofluorescent staining of DNA with Sytox green and Hoechst 33342. The yellow arrows represent NETs-forming neutrophils, (scale bar 50 μm). (**D**) Representative images of DNA area of cells, (scale bar 100 μm). **(E)** Residents after 26-h shift (NS) had significantly reduced NE activity compared to that of control group (**P < 0.01). Residents following 3 nights of self-reported good night sleep (post NS) showed decrease in NE activity, in comparison to control. The effect was not statistically significant (P = 0.054). **(F)** Both, NS and Post NS samples demonstrated a significantly reduced MPO activity compared to the control group (***P < 0.001). **(G)** NE-DNA ELISA assay showed no significant differences between the groups (control 0.20 nM ± 0.018 vs NS 0.19 nM ± 0.025, P = 0.69).

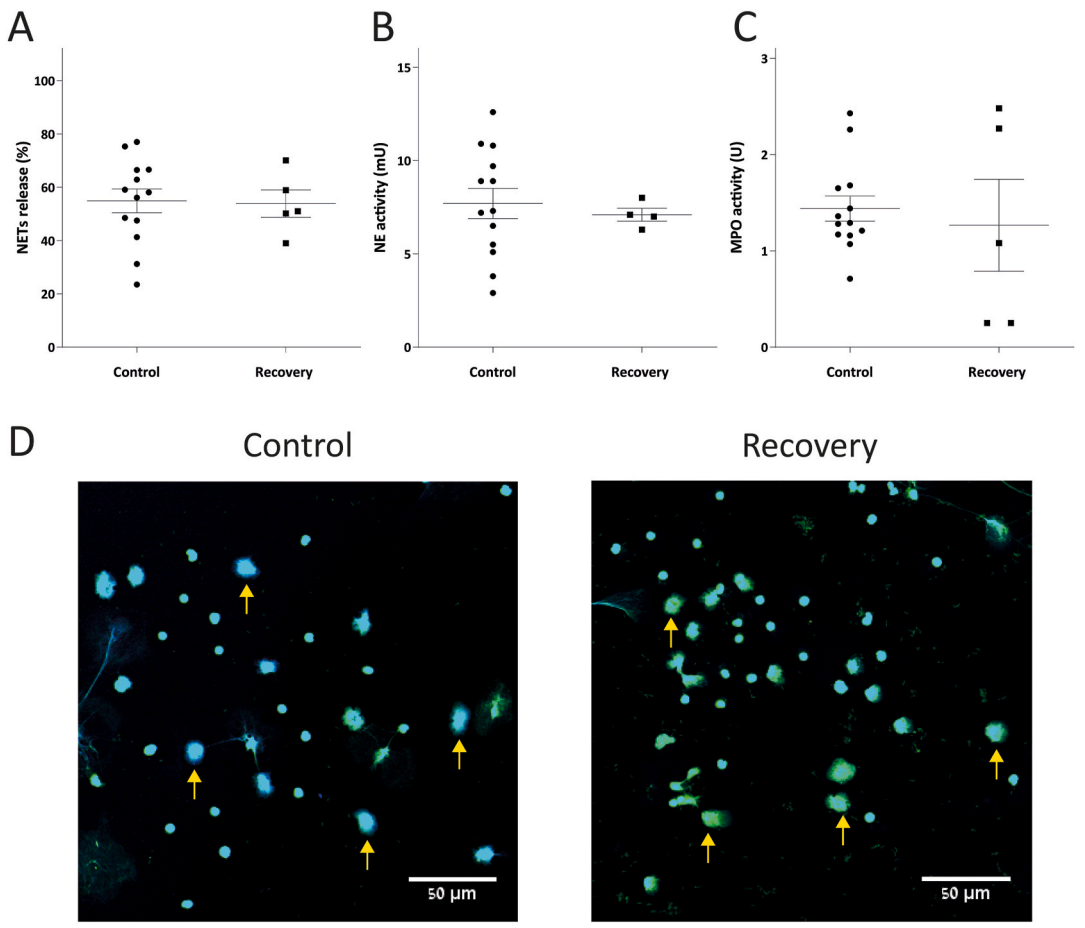

Fig. 2. NETs Formation recovers among residence completing their training (A–D) No differences were demonstrated between residents at least 3 months after completing their training (Recovery) and control group in (A) NETs formation, (53.8 % ± 5.1 vs 53.6 % ± 4.0, P = 0.89), (B) NE activity (7.1 ± 0.34 vs 7.56 ± 0.8, P = 0.7) and (C) MPO activity (1.02 ± 0.3 vs 1.56 ± 0.13 P = 0.63). (D) Representative images of immunofluorescent staining of DNA with Sytox green and Hoechst 33342. The yellow arrows represent NETs-forming neutrophils, (scale bar 50 μm).

increase vulnerability to infections through impaired NETs release.

The observed reduction in NET formation following sleep deprivation suggests a potential therapeutic role for NET inhibition in the management of autoimmune diseases. NETs are known to contribute to tissue damage and serve as a source of autoantigens in conditions like rheumatoid arthritis and systemic lupus erythematosus (SLE) [36,37]. However, evidence from other studies suggests an association between sleep deprivation and an increased risk of SLE [38]. This underscores the importance of a more nuanced understanding of NET regulation within the context of autoimmune diseases. There are limitations to this study. Besides sleep deprivation, residents were also exposed to stress, coffee consumption and additional factors which could have an important effect on neutrophils functions. However, investigating neutrophil function in residents after finishing their training and nightshift schedules provides a real life setting for recuperation. Additionally, we did not evaluate other neutrophil functions, such as antimicrobial activity or neutrophil maturation subset analysis. Assessing these aspects could provide further insights into the potential consequences of sleep deprivation-mediated NET inhibition. This remains an important area for future investigation. Another limitation includes its

relatively small sample size. Further research with a larger cohort is warranted to validate the findings.

In conclusion, there is accumulating evidence that sufficient sleep is one of the most important lifestyle factors influencing health, and that sleep deprivation has major deleterious effects [39,40]. Adding further support to these findings, the current study demonstrated that sleep deprivation resulted in decreased NETs formation and reduced activity of essential enzymes in neutrophils. Given that impaired NETs release might potentially expose medical residents to susceptibility to infections, the improvement of resident's working schedule to minimize short-term and potential long-term sequelae are long overdue.

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Data availability statement

The authors confirm that all the data supporting the findings of this study are available within the article. The raw data are currently not deposited in a publicly available repository. However, we acknowledge the importance of data sharing for scientific transparency and reproducibility. Therefore, the data are available upon reasonable request from the corresponding author (Dr. Rachel Shukrun, rachelzich@tlvmc.gov.il, Shukrun.rachel@gmail.com).

CRediT authorship contribution statement

Ronit Elhasid: Writing – review & editing, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Szilvia Baron:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Victoria Fidel:** Project administration, Formal analysis, Data curation. **Kira Kaganov:** Writing – review & editing, Data curation. **Rachel Shukrun:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Gemini in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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