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Full Length Article

Effects of sleep on the splenic milieu in mice and the T cell receptor repertoire recruited into a T cell dependent B cell response

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ABSTRACT

Sleep is known to improve immune function ranging from cell distribution in the naïve state to elevated antibody titers after an immune challenge. The underlying mechanisms still remain unclear, partially because most studies have focused on the analysis of blood only. Hence, we investigated the effects of sleep within the spleen in female C57BL/6J mice with normal sleep compared to short-term sleep-deprived animals both in the naïve state and after an antigen challenge. Lack of sleep decreased the expression of genes associated with immune cell recruitment into and antigen presentation within the spleen both in the naïve state and during a T cell dependent B cell response directed against sheep red blood cells (SRBC). However, neither T cell proliferation nor formation of SRBC-specific antibodies was affected. In addition, the T cell receptor repertoire recruited into the immune response within seven days was not influenced by sleep deprivation. Thus, sleep modulated the molecular milieu within the spleen whereas we could not detect corresponding changes in the primary immune response against SRBC. Further studies will show whether sleep influences the secondary immune response against SRBC or the development of the B cell receptor repertoire, and how this can be compared to other antigens.

1. Introduction

Sleep supports the protective function of the immune system (Besedovsky et al., 2011). Individuals who sleep less than 6 h a day are more prone to pulmonary infections (Patel et al., 2012; Shakkottai et al., 2018) and immune-related cardiovascular disease (Wang et al., 2016). Furthermore, the need for sleep is increased during infections (Imeri and Opp, 2009; de Almeida and Malheiro, 2016; Asif et al., 2017). Accordingly, the effect of sleep on the distribution of immune cells, cytokine levels, and the immune response against pathogens was studied in humans and a variety of animal models (reviewed in (Besedovsky et al., 2019)) – covering both effects on the innate and adaptive immune system (Hahn et al., 2020). Human studies revealed reduced levels of leucocytes circulating in the blood during sleep versus nocturnal wakefulness (Bonacho et al., 2001; Born et al., 1997; Esquifino et al., 2004; Dimitrov et al., 2009). These findings suggest that sleep recruits T and B lymphocytes as well as antigen presenting cells into secondary lymphoid organs (SLOs) such as lymph nodes and spleen. This recruitment enhances the chance of interaction between lymphocytes and antigen presenting cells which is essential for antigen presentation and, subsequently, for the successful initiation of an adaptive immune response (Besedovsky et al., 2019). This conclusion is supported by studies that investigated the outcome of acute sleep deprivation (SD) on an immune response in humans which revealed that SD, in comparison with undisturbed sleep, reduced both numbers of circulating antigen-specific T cells and antibody titers in the blood following vaccination against hepatitis A,

Abbreviations: BCZ, B cell zone; CCL, C–C motif ligand; CCR, C–C motif receptor; CD, cluster of differentiation; CIITA, class II major histocompatibility complex transactivator; CXCL, C-X-C motif ligand; FDR, false discovery rate; GC, germinal center; IL, interleukin; IFN, interferon; MHC-II, major histocompatibility complex II; SLO, secondary lymphoid organ; SD, sleep deprivation; SRBC, sheep red blood cells; TCR, T cell receptor; TCR-R, T cell receptor repertoire; TCZ, T cell zone.

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hepatitis B, influenza, and H1N1 (swine flu) viruses, respectively (Spiegel et al., 2002; Lange et al., 2011; Lange et al., 2003). However, in order to elucidate the molecular mechanisms by which sleep influences a T cell dependent B cell response, immediate effects in the SLOs need to be investigated because it is here where the T cell response is initiated and their interaction with B cells takes place (Besedovsky et al., 2019; Kurosaki et al., 2015). Since it is difficult to conduct such investigations in humans, we set out to close this gap by inducing a T cell dependent B cell response in mice which allows to focus on the events within SLOs.

We first determined the effect of sleep compared to a period of SD on the molecular milieu of lymph nodes and spleen focusing on genes relevant for T cell function such as migration, antigen presentation and differentiation. Subsequently, we induced a T cell dependent B cell response in the spleen prior to undisturbed sleep and a period of SD, respectively. This kind of immune response takes effect in most of today's successfully applied immunization protocols. We chose SRBC as antigen because they provide a large number of antigenic epitopes which increases the chance to reveal even subtle effects caused by SD (Stamm et al., 2013; Textor et al., 2018). In addition, the different steps of this immune response are well known so that the time points for analysis after injection of SRBC can easily be determined, for example after 6h (i.e. during antigen uptake), 12h (i.e. during antigen presentation), 72h (i.e. during T cell proliferation), and 96h (i.e. during germinal center (GC) development). Throughout the immune response, T cell proliferation and GC formation were monitored. In addition, we determined the influence of sleep on the T cell receptor (TCR) repertoire by deep sequencing of the CDR3β region and identified antigen-specific clonotypes by differential gene expression analysis (Textor et al., 2018; Robinson et al., 2009).

Our results show that sleep alters the molecular composition of SLOs. However, although SD reduced the expression of several genes involved in T cell function, neither the T cell proliferation and the serum level of SRBC-specific antibodies nor the TCR repertoire (TCR-R) recruited into the immune response seemed to be altered.

2. Material & methods

2.1. Mice and activity measurement

8 to 12-week old female C57BL/6J mice (Charles River) were kept at constant temperature (20.0 ± 0.5°C) and humidity (50–60%) and had *ad-libitum* access to standard food and water. The mice were group-housed (4-5 animals per cage) under a 12:12h dark:light (50 lux) cycle. Activity was measured in single-cages as described previously (Husse et al., 2017). Animal experiments were carried out either at the University of Tuebingen (SLO comparison) or the University of Luebeck (Immunization) in accordance with the German Law on Animal Welfare and ethically approved by the Animal Research Ethics Board of the respective Ministry of Environment (Tuebingen, permit no. M11/14; Luebeck, permit no. 72-5/15 and 25-3/18). Male mice were excluded from our study as housing-conditions especially in connection with SD sometimes increased aggressive behavior between male littermates and hence stress, which was supposed to be excluded in our study. Also, the TCR-R in female individuals is broader compared to males and hence more suited to study the subtle effects of SD (Fink, 2019).

2.2. Immunization and sleep deprivation

For immunization, SRBC (Labor Dr. Merk, Ochsenhausen, Germany) were washed and injected into the tail vein at a concentration of 10^9 SRBC in 200μl PBS (Stamm et al., 2013). Injections took place about 1.5h prior to the switch to light cycle under dim red light to not disturb the circadian rhythm of the mice. Control mice were injected with PBS only. Both groups were allowed to recover until the end of the dark cycle. Subsequently, half of each the SRBC- and PBS-injected mice were kept awake by gentle handling for the first 6h of the light period as previously described (Husse et al., 2017).

2.3. Corticosterone level assessment

To assess corticosterone levels in the least stressful way, groups of 5 mice each were put into fresh cages after the injection. Feces were collected from the cage bedding after the mice were euthanized for organ harvest or transferred into new cages 6h, 9h and 12h later. Corticoid extraction was performed from the total sample of one cage (Abraham et al., 2016; Cavigelli et al., 2004).

2.4. Tissue isolation and blood processing

Mice were sacrificed by exposure to an overdose of inhaled isoflurane followed by total blood withdrawal 7 or 13h post injection (*p.i.*) or 3, 4, 7 and 10 days *p.i.*. Mesenteric lymph nodes and spleen were snap frozen and stored at −80°C until further analysis. Full blood was harvested by heart puncture and allowed to clot. Serum was then separated by centrifugation at 2000×*g* for 15min. Individual serum samples of SRBC-injected mice at 10d *p.i.* (n = 10) were pooled for reference serum (RS). Individual serum samples (n = 6) of naïve mice were pooled for normal mouse serum (NMS).

2.5. ELISA for identification of SRBC-specific IgG antibodies

SRBC suspension ($1x10^8$/0.05ml PBS) was added to flat bottom 96-well microtiter plates (Maxisorp 446612, Nunc). Plates were incubated overnight at 4°C and subsequently washed. Non-specific binding sites were blocked with 1% skim milk in PBS for 1h at room temperature. Sample sera of mice including RS and NMS were added and the plate was incubated for 1h at room temperature. HRP-conjugated rabbit-anti-mouse IgG (H + L) (1:500, 210-120-02, BioFX Laboratories) was then added and the plate was incubated 1h at room temperature in the dark. TMB substrate (Invitrogen) was added and incubated for 10–15min. The color reaction was stopped by addition of 2M H_2SO_4. Detection was conducted at 405nm using a microtiter plate reader. Relative IgG was calculated as quotient of optical density values (OD_{sample}-OD_{MNS})/(OD_{RS}-OD_{MNS}).

2.6. Histological analysis

Cryosections (12μm thickness) were stained by immunohistochemistry using a monoclonal biotinylated antibody (B220 for B cells; BD Biosciences) to visualize B cell zones (BCZs) of the spleen as described previously (Stamm et al., 2013). To visualize proliferating cells and thereby GCs, we stained for Ki-67 (TEC-3; DakoCytomation), also described previously (Barthelmann et al., 2012). Digital images were taken using Axiophot Microscope and AxioCam (Carl Zeiss, Jena, Germany). Analyses were performed with ImageJ (National Institutes of Health).

2.7. RNA isolation and quantification

Five splenic cryosections (12μm) were lysed in 0.7ml lysis buffer (Analytik Jena), total RNA was extracted with the innuPREP RNA mini kit (Analytik Jena) and treated with DNase I (Sigma-Aldrich). RNA quantity was determined using the Quantus fluorometer (Promega Biosystems, Sunnyvale, CA).

2.8. cDNA synthesis and real-time RT-PCR

Messenger RNA expression levels were measured by quantitative real-time RT-PCR (qPCR) using the SDS ABI 7000 or SDS ABI 7900 system (Applied Biosystems, Foster City, CA, USA). First, 800ng of total RNA were translated into cDNA using 200ng of random primer (Promega), 0.01M DTT, 1μl reaction buffer, 0.5mM dNTP (each obtained from Promega), and 100U reverse transcriptase Superscript II RNase H Minus (Invitrogen Life Technologies) in a total volume of 20μl. Samples were

incubated at 42°C for 50min. Primer and probe sequences as well as gene accession numbers are provided on request. Relative abundances of target gene transcripts in a given sample were first calculated as differences in CT compared with the geomean of four independent housekeeping genes, β-*ACTIN*, *GAPDH*, *MLN51* and *HPRT* (delta-CT), and then relative to control (delta-delta-CT).

2.9. CDR3 sequence analysis of the TCRβ-chain

Total RNA was extracted as described above and TCRβ-chain transcripts were amplified independently in a two-step reaction according to the manufacturer's protocol (iRepertoire; patent no. 7,999,092). Gene-specific primers targeting each of the V and J genes were used for reverse transcription and first-round PCR (OneStep RT-PCRMix; Qiagen). In addition to a nested set of gene-specific primers, sequencing adaptors A and B for Illumina paired-end sequencing were added during second-round PCR (Multiplex PCR Kit; Qiagen). PCR products were run on a 2% agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen). The obtained TCRβ libraries were quantified using the PerfeCTa-NGS-Quantification Kit according to manufacturer's protocol (Quantabio) and sequenced using the Illumina MiSeq Reagent Kit v2 300-cycle (150 paired-end read; Illumina), gaining an average of $\approx 1.8 \times 10^6$ reads per sample. The number of unique amino acid sequences ranged from approximately 50,000 to 110,000. Throughout this paper, different nucleotide sequences coding identical amino acid sequences are treated as equal. CDR3β identification, clonotype clustering, and correction of sequencing errors such as removal of nonfunctional CDR3β sequences were performed using ClonoCalc software (Fähnrich, 2017). Also, we considered only sequences that were detected at least two times. For simplicity, in this paper the term 'clonotype' refers to a set of T cells with identical CDR3β-chain. Since we abstained from using naïve control groups also for the 7d time point, the control groups from 3d and 4d were pooled and used as overall control for all time points. For the differential gene expression analysis (see below), all naïve mice were pooled into one control group independent of their belonging to the "sleep" or "awake" condition. Via differential gene expression analysis, we identified 47 CDR3β sequences whose frequency changed significantly in response to immunization. In an additional step, 3 sequences were excluded, because they were only expanded in the control group. Hence, the immunization effect on the remaining 44 clonotypes was analyzed. Raw data are provided on request.

2.10. Statistics

GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for layout and statistical testing of the qPCR data. Data sets consisting of two groups were analyzed for statistical differences with Student's t-test when data displayed normal distribution and variance homogeneity and Welch's correction when variances were unequal. P-values less than 0.05 were regarded as significant and asterisked. False discovery rate (FDR) correction was performed using the Benjamini-Hochberg method (Benjamini et al., 2001). Data sets interrogating interactions between sleep (deprivation) and immunization were analyzed by two-way ANOVA, with significant ($p < 0.05$) main effects for sleep indicated by $, interactions were resolved for sleep and indicated by § when single comparison was significant. The analysis of the CDR3β sequence was performed using the R platform for statistical computing (R Core Team, 2018). We quantified the similarity of the repertoires of different mice by analyzing the clonal overlap measured by the Jaccard-Index. Since the application of inferential statistics (p-values, confidence intervals) is problematic due to multiple dependencies between the calculated indices of each subgroup, we restricted our analysis here on descriptive considerations. As a further parameter, we investigated the amino acid sequence length and the V and J usage of the CDR3β repertoire. Here we considered the three segments which were most frequently detected among the top100 clonotypes in naïve mice. Note that the determination of the number of considered segments as three is arbitrary, but the observed dynamics turned out to be quite robust in view of variations of this parameter (data not shown). Due to obvious deviations from the normal distribution the Scheirer-Ray-Hare-test was used for multivariate statistics (Sokal and Biometry, 2012; Dytham, 2011). Hypotheses concerning only one experimental parameter were tested using the Mann-Whitney-U-test. Unless specified otherwise, p-values less than 0.05 were considered statistically significant. To identify those T cell clones which expand in response to immunization, we fitted generalized linear models to the distribution of scaled copy numbers. After this, the CDR3β sequences were tested for differential gene expression using a likelihood-ratio test. The calculations were performed using the edgeR software (Robinson et al., 2009). In this analysis, only those clonotypes were included which were detected in at least three quarters of the immunized mice. In order to reduce the effect of unspecific proliferation phenomena or PCR artefacts, the threshold of significance for the likelihood-ratio test was determined as 0.005 (Textor et al., 2018).

2.11. Study design

Animals were assigned randomly to the experimental groups and data was processed randomly. An appropriate sample size was computed when the study was designed.

3. Results

3.1. Sleep deprivation dampens the expression of genes involved in T cell recruitment into the spleen

To get a general idea about whether sleep influences the steady-state milieu within the spleen, we investigated the expression profile of a selected set of genes relevant for compartment organization, homoeostasis and adaptive immune responses in the spleen after 6h of SD in naïve mice compared to naïve mice with undisturbed sleep. After SD, mRNA expression levels of *ccl19, cd44*, *cd62l* (Fig. 1A), and *cd86* were reduced (Fig. 1B). Additionally, we found reduced expression of *il-10* (Fig. 1C). For comparison, also mesenteric lymph nodes were investigated (Supplement Fig. A1) where we found reduced expression levels of *ccl19* and *ccr7, cd62l, cxcl13, cd11c,* and *cd86* after SD.

For investigating the impact of sleep on an immune response following an antigen challenge encountered prior to the sleep or SD phase, the spleen is particularly suitable. Antigens reach the spleen via the blood which ensures that the time course of antigen accumulation, uptake and presentation after intravenous injection is very predictable. One antigen for which the time course of accumulation and uptake in splenic compartments as well as the subsequent immune response are well described, are SRBC (Stamm et al., 2013; Textor et al., 2018). SRBC are well suited to determine sleep-induced alterations of the T cell response because they (i) are non-replicating and thus antigen uptake is timely restricted, (ii) do not require adjuvant and thus all T cell clones recruited into the immune response will be antigen-specific, and (iii) provide a large number of antigenic epitopes to which a great number of T cell clones can respond to, increasing the chance to detect even small effects within the investigated parameters (Stamm et al., 2013; Textor et al., 2018).

3.2. Sleep deprivation dampens immunization-induced changes in gene expression

To ensure that neither the injection procedure as such nor the injection of a large amount of SRBC disturbs the physiological sleeping pattern of the animals, we recorded activity patterns of mice prior and after the injection of either PBS or SRBC. Activity patterns of all mice were normal (Supplement Fig. A2), indicating that neither injection nor SD induces significant levels of stress. This conclusion was supported by determining

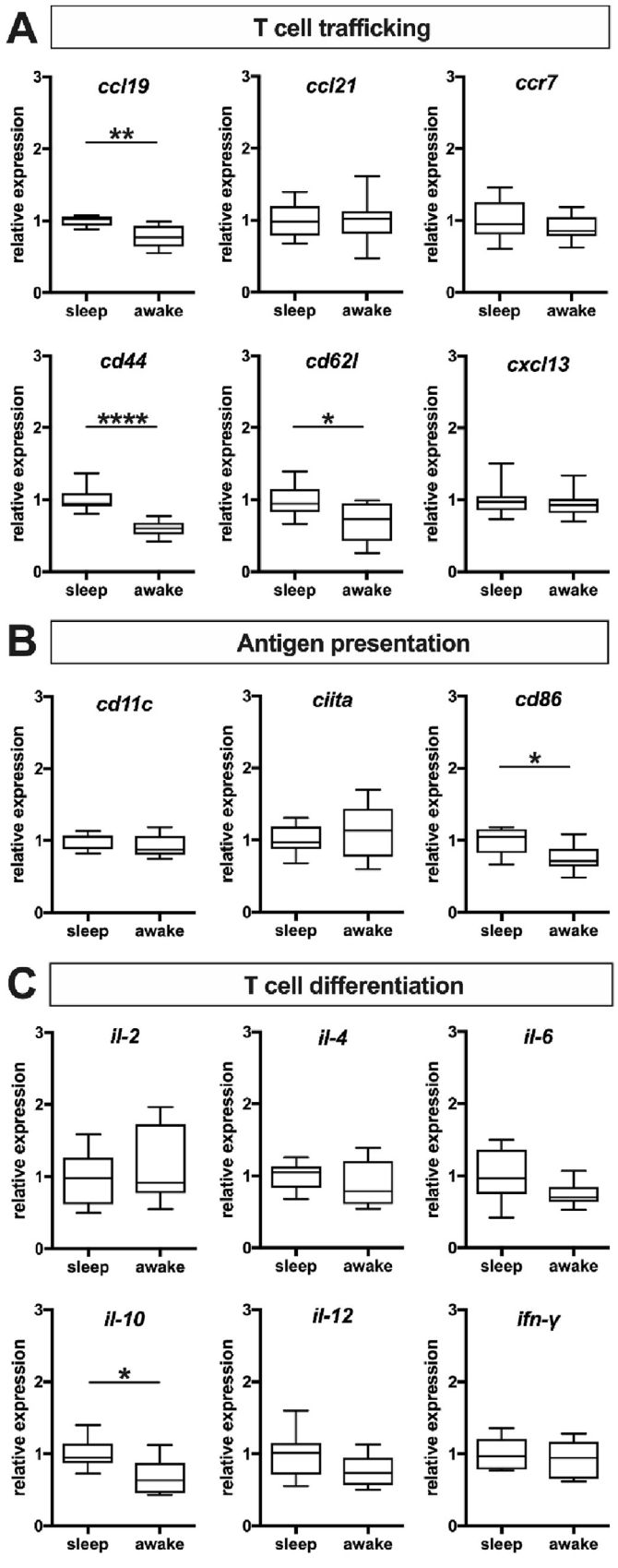

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Fig. 1. Sleep deprivation reduces expression of selected genes in spleen. Naïve, 12-week old C57BL/6J mice were either allowed to sleep normally ("sleep", n = 10) or subjected to 6h of SD at the beginning of the sleeping phase ("awake", n = 10). Gene expression levels of the spleen taken directly subsequent to the 6h of sleep or SD were assessed by qPCR and normalized to the "sleep" group. Boxplots show medians and interquartile ranges; whiskers indicate minima and maxima of one single experiment. P-values obtained by Welch's test for comparison of two samples with unequal variance are displayed as * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Although the changes in gene expression were moderate and remained within the two-fold range, the reduction in *ccl19* and *cd44* expression remained significant after FDR correction for multiple testing of all genes.

corticosterone excretion in feces (Supplement Fig. A3), showing that even the maximal corticosterone level per mouse did not exceed values of unstressed control animals reported by others (Touma et al., 2008; Razzoli et al., 2018).

To determine the effect of sleep on the outcome of an antigen challenge encountered precedent to the sleep or SD phase, we first analyzed gene expression at several time points after antigen injection (Fig. 2 and Supplement Table A.1): after 6h (i.e. during antigen uptake), 12h (i.e. during antigen presentation), 72h (i.e. during T cell proliferation), and 96h (i.e. during GC development).

The initiation of an immune response was reflected by the rise of *ciita* and *cd86* expression after 12h in immunized animals with normal sleep which further increased until 72h. As expected from previous SRBC immunization studies, cytokine expression also peaked at this time point, with only *il-4* and *il-10* staying elevated until 96h, while *ifn-γ* expression was reduced compared to PBS-injected animals. This indicates the Th2 nature of the immune response which is known to be elicited by immunization with high dosages of SRBC (Lagrange et al., 1974; Lagrange et al., 1975; Hurtrel et al., 1992). Remarkably, all immunization-induced expression changes were dampened in SRBC-injected mice inflicted with SD (Fig. 2).

3.3. Sleep deprivation does not alter T and B cell proliferation during the SRBC response

To assess if the differences in gene expression levels induced by SD over the time course of the immune response impact T and B cell proliferation, we determined the number of proliferating, i.e. Ki-67 positive cells, within the T cell zones (TCZs) of the spleen 3d, 4d, 7d, and 10d after immunization (Fig. 3A). As expected from results of previous investigations (Stamm et al., 2013), we found a rise in proliferating cells at 4d compared to naïve mice that vanished until 7d (Fig. 3B). However, no differences were detected between sleep-deprived and normal sleep mice. All immunized animals developed GCs from 4d onward (Fig. 3C), but GCs of sleep-deprived mice differed neither in size nor in number (not shown) from GCs of mice with normal sleep. This finding was supported by the analysis of the serum levels of SRBC-specific antibodies which were similar in sleep-deprived mice and animals with normal sleep 7d and 10d after immunization, respectively (Fig. 3D).

3.4. Sleep deprivation does not alter TCR-R changes induced by an immune response

To ascertain whether the T cell clones recruited into the response against SRBC are influenced by SD, we performed deep sequencing analysis of the CDR3β region of the TCR in splenic cryosections 3d, 4d, and 7d after injection of SRBC. Since only few T cell clonotypes expand in response to a given antigen while the bulk of the TCR-R pool remains unaffected, we did not only analyze the total pool of clonotypes determined, but also ranked all clonotypes detected within the spleen by their

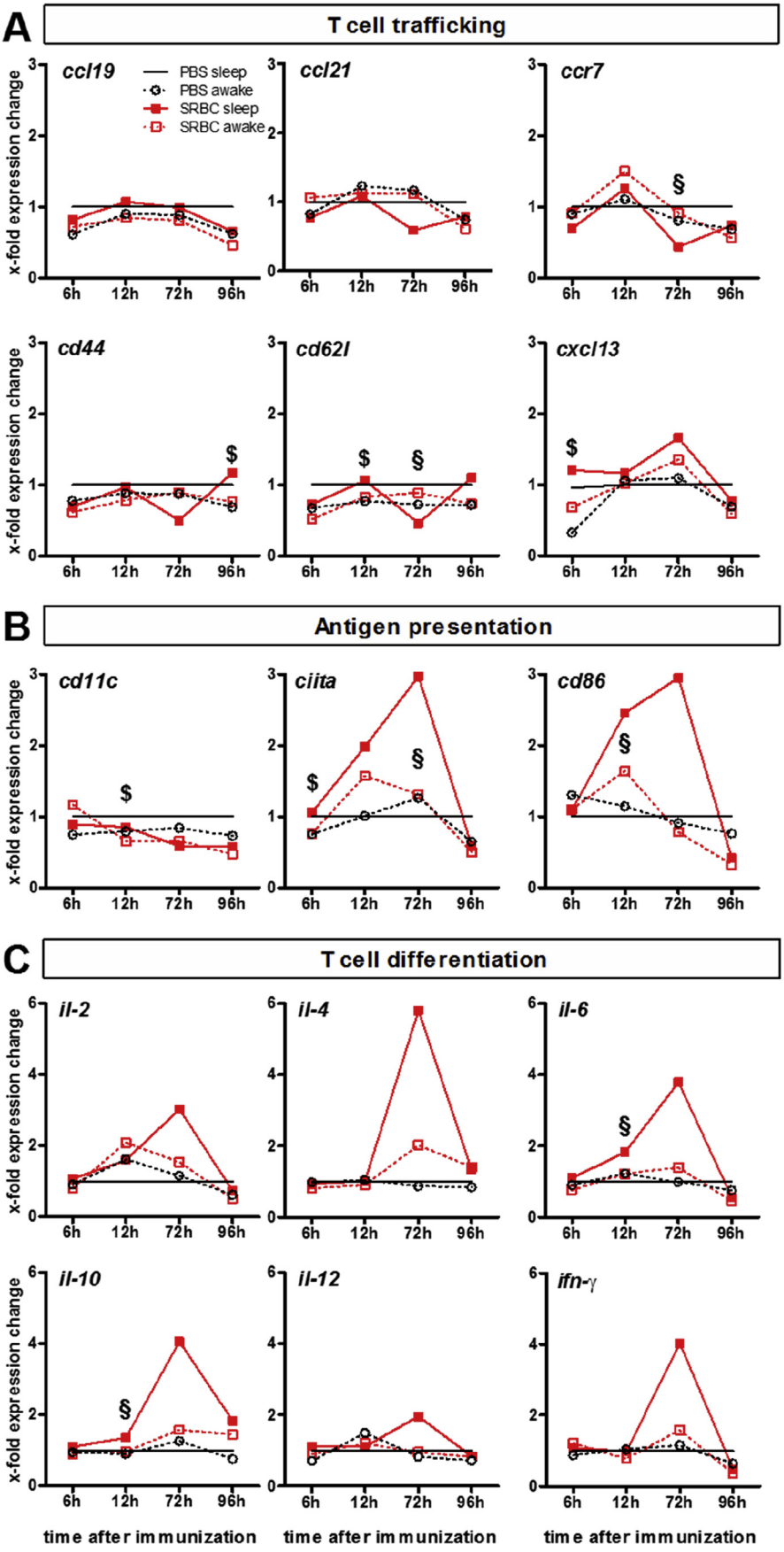

Fig. 2. Sleep deprivation dampens immunization-induced changes in gene expression levels in the spleen. 12-week old C57BL/6J mice were injected either with PBS or SRBC 1.5h prior to the sleeping phase and were either allowed to sleep normally ("sleep") or subjected to 6h of SD at the beginning of the sleeping phase ("awake"). Gene expression levels of the spleen were assessed by qPCR and normalized to the "PBS sleep" group at: 6h (i.e. directly after the SD phase); 12h (i.e. after the 12h light = sleeping phase), 72h (i.e. during T cell proliferation), and 96h (i.e. during GC development). Displayed are means (n = 5) for each group and time point and significant sleep effects obtained by two-way ANOVA with Sidak's correction for multiple comparisons: § indicates a significant difference between naïve and immunized mice when significant interactions were resolved for sleep or SD. $ indicates significant main effect for sleep or SD. In order to improve clarity, standard deviations are omitted (and p-values given in Supplement Table A.1).

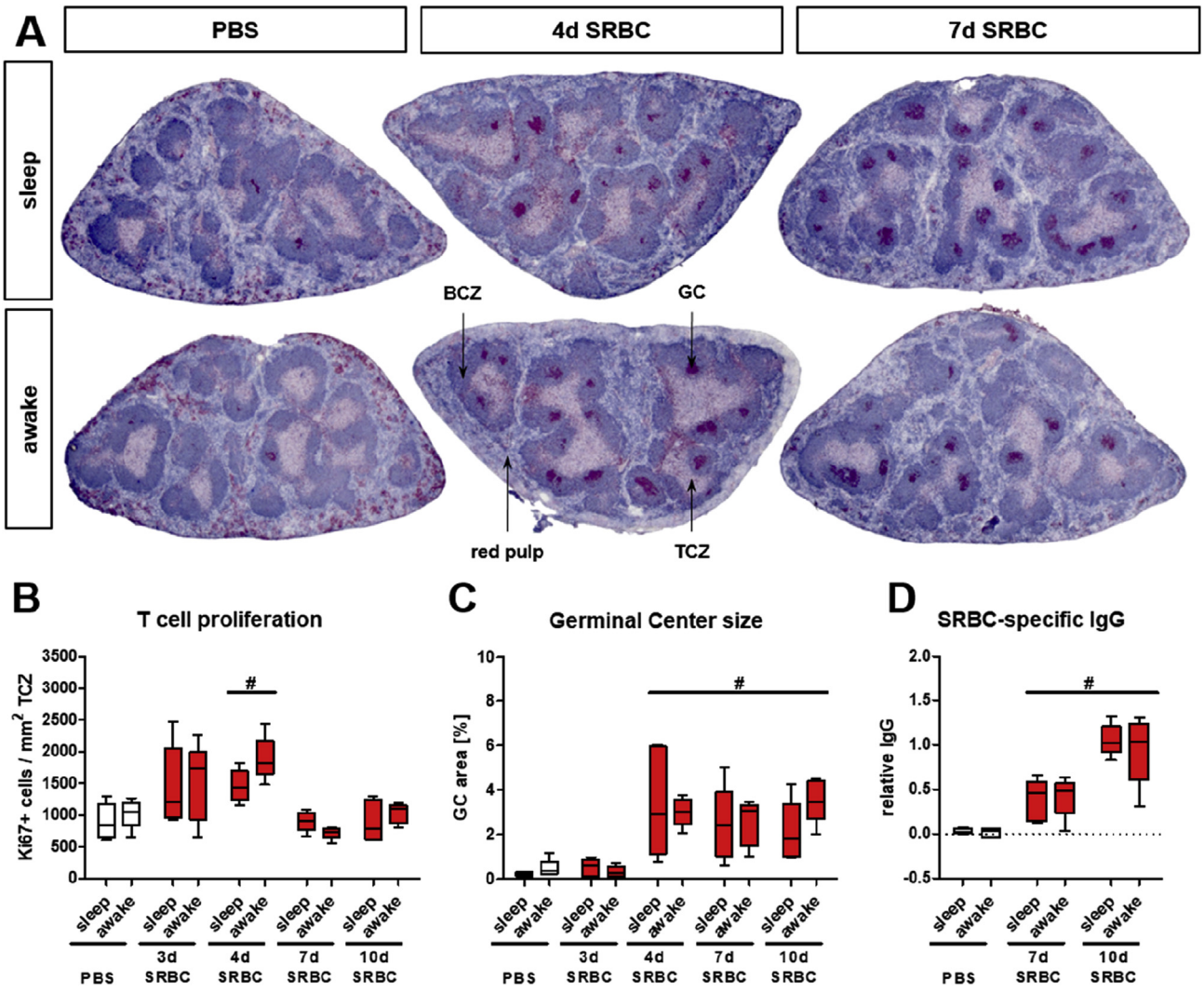

Fig. 3. Sleep deprivation does not alter T and B cell proliferation and SRBC-specific antibody production. (A) Cryosections representative for the indicated group stained for B cells (blue, B220) and proliferating cells (red, Ki-67). Images were taken at 1.25-fold magnification. Quantification of **(B)** T cell proliferation assessed by determining Ki-67 positive cells within the TCZ, **(C)** GC size assessed by measuring the area of clustered Ki-67 positive cells within the BCZ, and **(D)** SRBC-specific IgG levels in blood assessed by ELISA (n = 5 per group and time point). Boxplots show medians and interquartile ranges (whiskers indicate minima and maxima). Data were analyzed by two-way ANOVA with Bonferroni post hoc testing (# indicates significant immunization effect compared to the respective naïve group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

frequency (copy number) and analyzed these fractions separately, i.e. the top4000, top1000, top500, and top100 (Fig. 4). This increases the chance of detecting changes within the TCR-R even if they occur only within a particular fraction.

The clonal overlap between animals measured by the Jaccard-Index was substantially reduced 3d and 4d after immunization, especially within the fractions of numerically dominant clonotypes (Fig. 4A). This indicates that in each animal mainly different T cell clonotypes responded to SRBC. Furthermore, the mean length of the CDR3β amino acid sequences was slightly increased in immunized groups, reaching significance for the top100 fraction at 3d and both the top500 and top1000 fraction at 4d after immunization, whereas the mean length in the total fraction remained nearly unaffected (Fig. 4B). Next, we examined the V and J usage by considering those three V and J segments which are numerically dominant in naïve mice (V12–2, V16, V19 and J1-1, J2-4, J2-7, respectively) and analyzed whether they would be displaced by the different segments of clonotypes responding to SRBC. Indeed, the percentage of top3 V segments was reduced in immunized animals for all fractions at every time point with the exception of the top100 at 4d and 7d after immunization. Furthermore, in contrast to the Jaccard-Index and the mean CDR3β amino acid length, the top3 V segment usage and top3 J segment usage within the total TCR-R were also significantly reduced at 7d after immunization (Fig. 4C, Supplement Fig. A4). However, although we were able to clearly demonstrate the effects of an immune response within the TCR-R up to 7 days after immunization, none of the investigated parameters displayed any differences between mice with normal sleep and SD.

To investigate if there are clones shared between animals (so-called "public" clones) and if those are influenced by sleep, we performed differential gene expression analysis (Fig. 5A). Visualization of their proportion among all sequences (clonotypes including their copy numbers) demonstrated that SRBC-specific clonotypes were increased at 3d (4-fold) and 4d (6.5-fold) after immunization and decreased at 7d but still being 2-fold above baseline level (Fig. 5B). Again, no differences were found between normal sleep and sleep-deprived animals.

Taken together, deep sequencing clearly revealed immunization effects in various parameters characterizing the TCR-R obtained from splenic sections. However, we did not detect any effects of sleep on the investigated parameters.

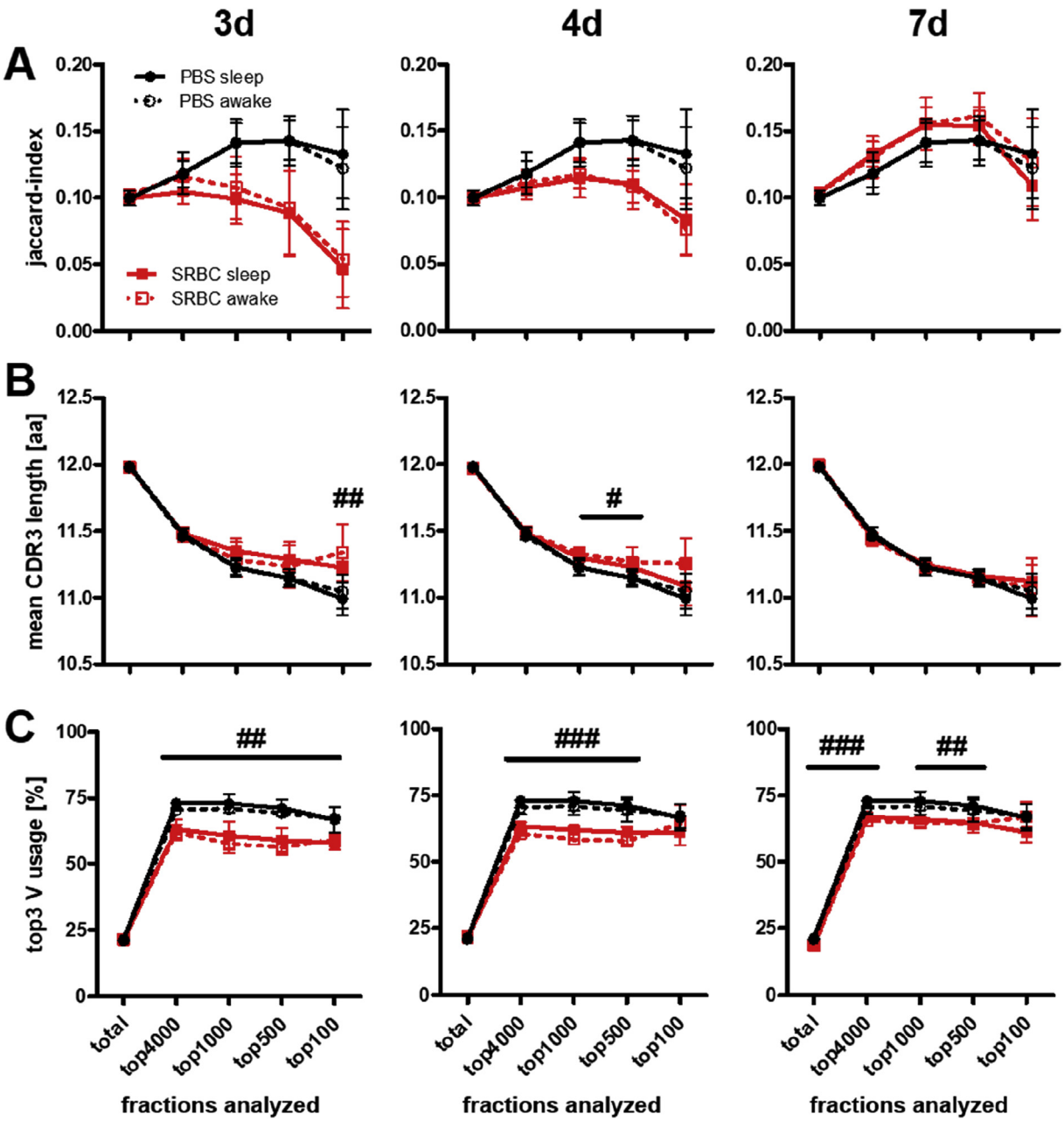

Fig. 4. Sleep deprivation does not alter the TCR-R recruited into an immune response. (A) Jaccard-Index indicating clonal overlap (1 = 100%; 0 = 0%), **(B)** CDR3β sequence length (number of amino acids, aa), and **(C)** Percentage of those three V segments (top3: V12–2, V16, V19) which are most common among the numerically dominant clonotypes in naïve mice 3, 4, and 7d after injection of PBS or SRBC into mice without ("sleep") and with SD ("awake"). The clonotypes are grouped according to their frequency (copy number): total, all clonotypes displayed (on average 70,000); top4000, only the 4000 clonotypes with the highest copy number; top1000, top500 and top100. Data are given as means with standard deviation (n = 10 for PBS-injected groups and n = 5 per SRBC-injected group; p-values obtained by Scheirer-Ray-Hare-test for immunization effects are displayed as #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$). Correction for multiple testing was performed using Holm's method. Analyses were performed on splenic cryosections.

4. Discussion

The aim of this study was to elucidate the impact of sleep on a T cell dependent B cell response against an antigen encountered directly prior to the sleep phase. Therefore, we focused on the analysis of mice SLOs as the T cell dependent B cell response takes place in these organs. The effect of sleep was determined by comparing control mice (with undisturbed sleep) to mice subjected to acute SD for 6h right at the beginning of the sleep phase. As neither the period of SD led to increased levels of corticosterone (Supplement Fig. A3) nor did the injection of SRBC disturb the sleep pattern (Supplement Fig. A2), the changes observed in the parameters analyzed are most likely caused by the lack of sleep rather than representing non-specific stress-related consequences of animal handling during antigen injection and SD (Renegar et al., 1998; Lungato et al., 2012). In order to analyze the effect of SD and not the effect of stress, male mice were excluded from our study as housing-conditions especially in connection with SD sometimes increased aggressive behavior between male littermates and hence stress. This can be seen as a limitation of this study and will be investigated in further studies.

The genes investigated in order to monitor T cell function included (i) *ccl19, ccl21* and their receptor *ccr7* that recruit T cells and dendritic cells into the TCZ (Baekkevold et al., 2001), (ii) *cd44* and *cd62l* that mediate T

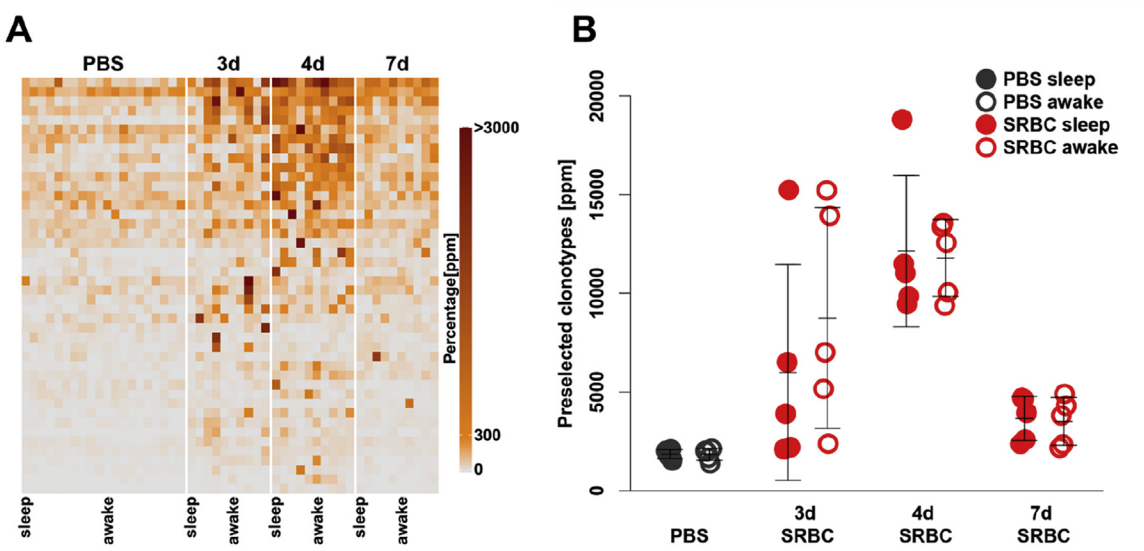

Fig. 5. Sleep deprivation does not alter the number of SRBC-specific clonotypes shared by the majority of immunized animals. (A) Heatmap of the expression levels of the 44 clonotypes identified as overexpressed in SRBC-immunized mice (n = 30) as compared to naïve mice (n = 20). **(B)** Proportion of the 44 clonotypes among the total number of clonotypes (parts per million) in control animals and 3, 4, and 7d after immunization ("sleep": normal sleep; "awake": SD). Whiskers indicate mean and standard deviation (n = 10 for PBS-injected groups and n = 5 per SRBC-injected group). At each time point the percentage of the SRBC-specific clonotypes was tested for SD effects using the Mann-Whitney-U-test.

cell extravasation into SLOs (Gonda et al., 2005) and (iii) *cxcl*13, an important factor for follicle organization that regulates the recruitment of follicular T helper cells into the GC as well as their interaction with B cells (Crotty, 2011). Furthermore, we investigated *cd11c* as marker for dendritic cells, the MHC-II transactivator (*ciita*) as master regulator of MHC-II expression and *cd86* (expressed by B cells, dendritic cells, and macrophages) as costimulatory ligand to assess antigen presentation and T cell activation (Dimitrov et al., 2007; Xiao et al., 2015). Finally, we determined the mRNA expression of a set of cytokines (*il-2*, *il-4*, *il-6*, *il-10*, *il-12* and *ifn-γ*) that has been shown to regulate T cell differentiation (Besedovsky et al., 2019; Qi, 2016). Our analysis of expression changes of these genes in spleen and lymph node of naïve mice immediately after a 6h-period of normal sleep or SD indicates that sleep might impact T cell trafficking into as well as antigen presentation within these SLOs (Fig. 1, Supplement Fig. A1). However, the effects observed were small. Therefore, in a reanalysis we pooled the data derived from the gene expression analysis of the experiment on naïve SLO comparison (Fig. 1, Supplement Fig. A1) and the corresponding 6h time point of the immunization time course experiment (Fig. 2) for both organs. Analysis of this data set confirmed the significant reduction in expression levels of *ccl19, cd44, cd62l*, and *cxcl13* as well as *ciita* and *cd86* in sleep-deprived mice (Supplement Fig. A5). This indicates that already short-term SD leads to molecular alterations within SLOs with the potential to influence lymphocyte functions, supporting the hypothesis that sleep promotes both the recruitment of immune cells into SLOs and antigen presentation (Bonacho et al., 2001; Born et al., 1997; Esquifino et al., 2004; Dimitrov et al., 2009). Furthermore, expression of *il-6* and *il-10* was significantly reduced in lymph node and spleen of sleep-deprived animals which corresponds to the majority of human studies showing reduced IL-6 serum levels after early night SD (Redwine et al., 2000). A study investigating the influence of circadian rhythm and clock genes on parameters influencing T cell function found the expression levels of CCR7 within the lymph node to be regulated by the circadian rhythm (high around night onset) whereas that of CCL19 stayed unaltered (Druzd et al., 2017). We observed that both *ccr7* and *ccl19* mRNA expression is dampened by SD, suggesting that circadian rhythm and sleep, although using the same pathway, influence lymphocyte migration differently. Taken together, our results indicate that sleep promotes immune cell trafficking into and antigen presentation within lymph node and spleen, thereby supporting comparable conclusions from human studies (Bonacho et al., 2001; Born et al., 1997; Esquifino et al., 2004; Dimitrov et al., 2007; Lange et al., 2003).

Surprisingly, although the expression of genes involved in immune cell migration, antigen presentation, and T cell differentiation was dampened in sleep-deprived mice after immunization with SRBC, T cell proliferation turned out not to be affected (Fig. 3). The inability to see any effects of SD might be due to the fact that potential changes are too subtle to be detected by analyzing proliferation of the total T cell population. We addressed this issue by taking a closer look at the TCR-R to follow T cell clonotypes that were recruited into the immune response (Fig. 4, Supplement Fig. A4). By grouping the different clonotypes according to their frequency we were able to characterize three phases of the immune response to SRBC. First, three days after immunization the expanding clonotypes were distinct among individuals as shown by the decreased Jaccard-Index of the dominant clones between the different animals (reflecting a reduced overlap). This is characteristic for a so-called "private" immune response where in each animal mainly different clonotypes respond to the antigen (Greiff et al., 2015). Those "private" clonotypes are known to have more nucleotide additions in their underlying sequence (Venturi et al., 2006) which is in line with our finding of an increase in mean CDR3β amino acid length after immunization. Second, one day later we detected ongoing proliferation (Fig. 3), but the Jaccard-Index was less reduced. This could be explained either by an expansion of shared SRBC-specific clonotypes ("public") leading to an increase of the Jaccard-Index, or by dilution of the responding TCR-R due to emigration of SRBC-specific T cells (mainly "private" clonotypes, reducing the Jaccard-Index) and immigration of antigen-unspecific T cells (increasing the Jaccard-Index) into the spleen (Textor et al., 2018). At the first sight, expansion of "public" clonotypes seems to be a reasonable explanation because the "public" clonotypes identified by differential gene expression analysis in the present study increased from day three to four after immunization (Fig. 5). However, they can only represent a minor contribution: If the "public" clonotypes would represent a major population within immunized animals, their TCR-Rs should show more overlap (many "public" clonotypes in all animals) than the TCR-Rs of immunized and naïve mice (many "public" clonotypes in immunized animals only). Therefore, the TCR-Rs of immunized animals should reveal a higher Jaccard-Index (more overlap) than that of

immunized and naïve animals (less overlap). However, this is not the case (Supplement Fig. A.6). Thus, the similar overlap of TCR-Rs among immunized animals and between immunized and naïve animals strongly suggests that the increase of the Jaccard-Index at 4d after immunization is mainly caused through dilution by naïve T cells. Third, although both Jaccard-Index and mean CDR3β amino acid length were back to normal at 7d after immunization, clonotypes with the top3 V and J segments were still displaced by responding ones, clearly indicating that the immune response is still ongoing (Stamm et al., 2013). Additionally, this shows that the top3 V and J segment analysis is a very sensitive tool to trace late phases of ongoing immune responses.

Despite our ability to characterize the TCR-R recruited into a splenic T cell dependent B cell response in detail, we did not detect any effect of SD. Similarly, neither T cell proliferation and GC size nor SRBC-specific antibody formation was affected by SD. How can this be reconciled with well documented observations that sleep strengthens the immune system and is essential for human health (Lange et al., 2019)? First, studies in humans reporting a decrease in the number of antigen-specific T cells and in the titer of specific antibodies used either a whole night (Benedict et al., 2007; Lange et al., 2011; Lange et al., 2003) or multiple episodes of SD (Spiegel et al., 2002). Thus, 6h of SD as used in the present study might be too short to result in measurable effects. However, increasing the period of SD easily leads to induction of stress in mice so that it becomes impossible to separate both factors. Second, in order to induce a T cell dependent B cell response by SRBC, a high dosage of antigen has to be applied (Stamm et al., 2013) and its huge amount available in the spleen could still be sufficient to induce a normal immune response masking possible dampening effects of SD (ceiling effect).

Future studies will show whether the investigation of single organ compartments (TCZ, BCZ, and GCs), or the analysis of a secondary instead of a primary immune response will reveal the molecular mechanisms by which sleep supports the function of the immune system. Because most human studies are based on Th1 responses, the study of another antigen which elicits a Th1 response might be of use. In addition, it might well be that the beneficial role of sleep is mediated via the B cell receptor repertoire which might be able to influence both glycosylation patterns and affinity of antibodies and thereby their function (Lange et al., 2019).

5. Conclusions

Our study suggests that already short-term sleep deprivation decreased the expression of genes important for immune cell recruitment and antigen presentation within spleen and lymph nodes of mice. Even though we were not able to detect further effects of sleep regarding proliferation, antibody production or the T cell receptor repertoire, we are convinced that the analysis of the T cell receptor repertoire in more detail will contribute to further elucidating the effects of sleep on the different phases of immune responses.

Author's contribution

CT conducted experiments, assembled the figures and wrote the manuscript draft, MM conducted the NGS analyses and gave critical advise, KK and RP conducted experiments and gave critical advice, LKS discussed the data, JH and SEA conducted sleep experiments, CEK conducted sleep experiments and intravenous injections, HO gave critical advice and enabled sleep experiments, AS assembled figures, organized experiments and revised the manuscript and JW organized experiments, wrote and revised the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100082.

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