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der Universität zu Lübeck
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vertreten in der Technisch-Naturwissenschaftlichen Fakultät
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Effects of Sleep on Immune Cell Parameters

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Universität zu Lübeck
– Aus der Technisch-Naturwissenschaftliche Fakultät –

vorgelegt von
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Bulgarien

Lübeck 2008

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Tag der mündlichen Prüfung: **03. 04. 2008**

Zum Druck genehmigt. Lübeck, den **03. 04. 2008**

Prof. Dr. rer. nat. Enno Hartmann

– Dekan der Technisch-Naturwissenschaftliche Fakultät –

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Abbreviations

ANOVA – Analysis Of Variance

APCs – Antigen-Presenting Cells

DCs – Dendritic Cells

EEG – Electroencephalogram

GH – Growth Hormone

GR – Glucocorticoid Receptor

IFN-γ – Interferon-γ

Ig – Immunoglobulin

IL – Interleukin

MHC – Major Histocompatibility Complex

MR – Mineralcorticoid Receptor

NK – Natural Killer

PDC – plasmacytoid DCs

PMA – Phorbol Myristate Acetate

pre-mDC – pre-myeloid DCs

REM – Rapid Eye Movement

SWS – Slow Wave Sleep

Tc cells – Cytotoxic T cells

Th – T helper

Th1 – T helper 1

Th2 – T helper 2

TNF-α – Tumor Necrosis Factor-α

Treg cells – Regulatory T cells

Introduction

Sleep is a fundamental biological process with consequences on human health. It is a common assumption that lack of sleep lowers resistance to infections, such as common cold. In addition, epidemiological studies show that poor sleep is a predictive factor for mortality, which can be due to a compromised immune system (Dew et al., 2003). This view is reinforced by experimental evidence of reduced antibody titers in response to hepatitis A and influenza A virus vaccines after experimental sleep loss (Lange et al., 2003; Spiegel et al., 2002). Three studies are reported here which identify relevant underlying mechanisms by which sleep can ease the immune responses. First we analyzed cytokine balance between T helper 1 (Th1) and T helper 2 (Th2) cells in healthy men during regular nocturnal sleep and while remaining awake. Because nocturnal sleep is characterized by highest levels of generally immunosupportive hormones such as growth hormone (GH) and prolactine, and lowest levels of immunosuppressive hormones such as cortisol, in a second in-vitro study we tried to identify a possible endocrine mediation of the effects of sleep on the Th1/Th2 cytokine balance. Since, T cell activity critically depends on antigen-presenting cells (APCs) that can drive T cells toward type 1 or type 2 responses we were finally interested in cytokine profile and cell migration of specific subsets of APC precursors in peripheral blood as primary targets of sleep-associated changes toward enhanced adaptive immunity. Together our data support the hypothesis that sleep can facilitate immune defense. This role of sleep can be achieved (i) by high levels of immunosupportive and low levels of immunosuppressive hormones, (ii) by high levels of proinflammatory type I cytokines and (iii) by influences on cell migration, facilitating the extravasation of dendritic cells (DC) precursors.

Sleep

Polisomnographic recording of sleep

Depending on the brain activity as measured with an electroencephalogram (EEG) Rechtschaffen and Kales (1968) distinguished 5 different sleep stages in sleep architecture that cyclically succeed each other during the course of nocturnal sleep (Fig.1, top). Each stage of sleep is characterized by a particular pattern of brain waves. Stage 1 is a transition between wakefulness and sleep, and is characterized by mixed frequency waves (alpha and theta waves). During stage 2, eye movements stop, and brain activity slows. Stages 3 and 4 together are known as slow wave sleep (SWS), and are characterized by very slow delta waves. During

these stages, there is no eye movement or muscular activity. The final stage is rapid eye movement (REM) sleep. During this stage, breathing becomes rapid and shallow, and the eyes move back and forth quickly. The first period of REM sleep usually begins about 70-90 minutes after falling asleep; as the night progresses, each successive REM stage increases in length, and the duration of the other stages decreases.

Neuro-endocrine activity during sleep

Sleep is characterized by a specific regulation of the endocrine and autonomic nervous system, and thereby sleep is supposed to exert a systemic control over immune function. Characteristic time courses during sleep have been documented for the release of the hormones of the pituitary, including cortisol, GH and prolactin and for the activity of the autonomic nervous system (Fig. 1, bottom).

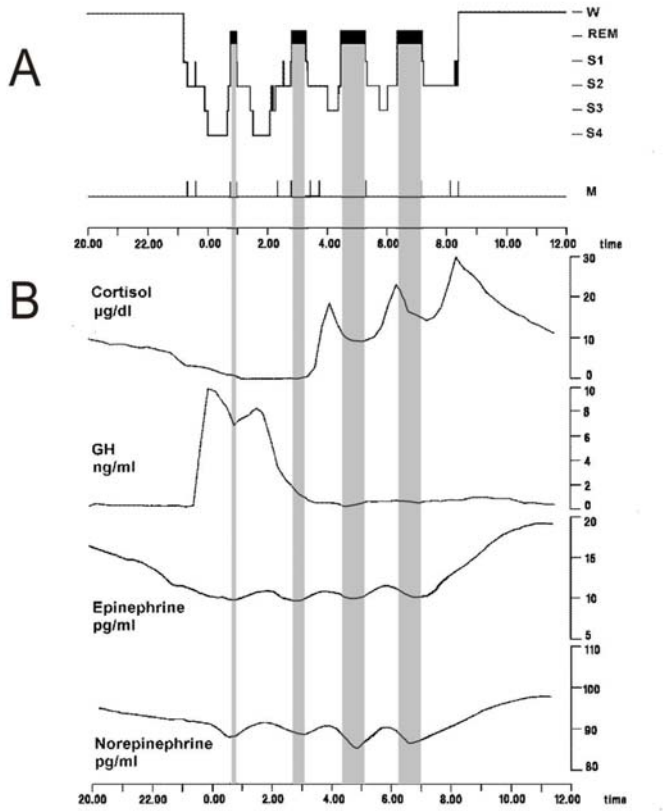


Figure 1.
(A) Representative profile of nocturnal sleep from a healthy young man. Abscissa indicates time of day, ordinate indicates sleep stage (W, wake; REM, rapid eye movement sleep; S1, S2, S3, S4, sleep stages 1-4; M, movements). S3 + S4 represents the time in slow wave sleep (SWS). Note that the first half of sleep is dominated by SWS, the second half by REM sleep. (B) Profiles of plasma concentrations of cortisol, growth hormone (GH), epinephrine, and norepinephrine. Minimum plasma concentrations of cortisol and maximum concentrations of GH occur during early sleep. Catecholamine concentrations are reduced during sleep and, in particular, during REM sleep.

Early sleep and particularly SWS inhibits secretory activity of the pituitary adrenal system so that plasma cortisol concentrations reach nadir values (Bierwolf et al., 1997; Born & Fehm, 1998) during this time. This inhibition coincides with the circadian minimum of the

pituitary/adrenocortical secretion. Simultaneously SWS-rich early sleep has a profoundly stimulating influence on the secretion of GH (Van & Copinschi, 2000) and also of prolactin (Spiegel et al., 1995; von Treuer et al., 1996). Also, during SWS sympathetic activity is decreased, while parasympathetic output appears to be enhanced. In the second half of the night when REM sleep predominates, cortisol secretion is high whereas GH concentrations are typically not detectable. Compared to non-REM sleep, REM sleep is characterized by a further depression of sympathetic tone. Accordingly, plasma catecholamine concentrations have been found to be even lower than during SWS.

Immune system

The immune system is typically divided into two categories - innate and adaptive. Innate immunity is the first line of defense against infection, protecting the host during the development of adaptive immunity and critically affecting the nature of the adaptive response. Immune responses are mediated by leucocytes, which derive from precursors in the bone marrow. A haematopoietic stem cell gives rise to the lymphocytes responsible for the adaptive immunity, and also to myeloid lineages that participate in both innate and adaptive immunity.

Cells of the immune system
Lymphocytes

The two major subgroups of lymphocytes – B and T lymphocytes are the only cells capable of specifically recognizing antigen. B lymphocytes or B cells are selected in the bone marrow, while the precursors of T lymphocytes or T cells originate from the bone marrow and migrate into the thymus. B cells, when activated differentiate into plasma cells that secrete antibodies, and into memory B cells, which are responsible for a long time antibody response. Antibodies help defend against pathogens by activating complement, serving as opsonins as well as agglutination and neutralizing antigens. There are different classes of antibodies, which differ in their structure and function: Immunoglobulin (Ig)M, IgD, IgG, IgA and IgE. IgM antibodies prevail during primarily infection, while IgG antibodies are the predominant class after secondary response. IgA prevents colonization of mucosal areas by pathogens and IgE is involved in allergy reactions. IgG antibodies can be further divided in humans into following subclasses: IgG1, IgG2, IgG3 and IgG4. The four IgG subclasses differ from each other with respect to their effector functions. In humans, antibody responses to viral and bacterial protein

antigens are mainly restricted to IgG1 and IgG3, which are characterized with high efficiency of opsonization and activation of complement system.

T cells fall into two functional classes that detect peptide antigens derived from different type of pathogens. Peptides from intracellular pathogens such as viruses or transformed cells are carried to the cell surface by Major Histocompatibility Complex (MHC) I molecules and presented to $CD8^+$ T cells. $CD8^+$ T cells differentiate in so-called cytotoxic T cells or Tc. Tc cells are known for their ability to kill virally infected and malignantly transformed cells, which are found in the body. Peptides derived from ingested extracellular pathogens such as extracellular bacteria or toxins are carried to the cell surface by MHC II molecules and presented to $CD4^+$ T cells or T helper cells (Th). These cells can differentiate into two functional types: Th1 and Th2. Th1 cells releasing mainly IFN-γ, aside from other cytokines including tumor necrosis factor-α (TNF-α) and tumor necrosis factor-β (TNF- β), become activated in response to intracellular viral and bacterial challenges and support various cellular (type 1) responses, including activation of macrophage and cytotoxic T cells. Th2 cells, which are characterized by production of IL-4 as well as IL-5, IL-10, and IL-13, tend to drive humoral (type 2) defense, which combat against extracellular pathogens via stimulating mast cells, eosinophils and B cells and inducing the production of weakly opsonizing antibodies such as IgG2 and IgG4 (human) and IgG1 and IgG3 (mouse) as well as IgA and IgE. However, Th1 cells, via production of IFN-γ, can also contribute to humoral immunity by induction of the strongly opsonazing antibodies IgG1 and IgG3 (human).

Regulatory T cells (Treg cells), formerly known as suppressor T cells, are crucial for the suppression of immune responses of other cells. Two major classes of $CD4^+$ Treg cells have been described, including the naturally occurring Treg cells and the adaptive Treg cells. Naturally occurring Treg cells (also known as $CD4^+CD25^+FoxP3^+$ Treg cells) arise in the thymus, whereas the adaptive Treg cells (also known as Tr1 cells) may originate during a normal immune response. Naturally occurring Treg cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3. The major roles of Treg cells is to suppress auto-reactive T cells that escaped the process of negative selection in the thymus and to shut down T cell mediated immunity towards the end of an immune reaction.

Natural killer (NK) cells are the third lymphocyte type, which in contrast to B and T cells recognize the foreign antigens in a non-specific manner. NK cells constitute a major component of the innate immune system and have the ability to both lyse target cells (tumors and virally infected cells) and provide an early source of immunoregulatory cytokines. The majority (~90%) of human NK cells have low-density expression of CD56 ($CD56^{dim}$) and

express high levels of Fcγ receptor III ($CD16^{bright}$), whereas ~10% of NK cells are $CD16^{-}$ $CD56^{bright}$. These two NK cell subsets have unique functional attributes and, therefore, distinct role in human immune response. The $CD16^{+}CD56^{dim}$ NK cell subset is more naturally cytotoxic against target cells than the $CD16^{-}CD56^{bright}$ NK cell subset. In contrast, $CD16^{-}$ $CD56^{bright}$ NK cells have the capacity to produce cytokines, express CD62L and CCR7 homing receptors, recirculate through lymph nodes and can have immunomodulatory role in lymphoid tissue.

Antigen presenting cells and their precursors in blood

Although almost every cell in the body can present antigen to $CD8^{+}$ T cells (via MHC class I molecules), the term APS is often limited to those specialized cells that can prime T cells. These cells are often referred to as professional APS. There are three main types of professional APS: macrophages, B cells and DCs. Macrophages, typically express low levels of costimulatory molecules and can not prime naïve T cells without activation. B cells can very efficiently present the antigen to which their antibody is directed, but are inefficient APC for most other antigens. Of the different types of APC, DCs are the most effective because of their widespread localization at all sites of antigen entry and their strong expression of MHC class II and costimulatory molecules (Langenkamp et al., 2000). For example, in the skin, DCs are called Langerhans cells. After uptake of antigen, they migrate with the afferent lymph to the draining lymph node, where they prime the naïve T cells through direct cell-cell contact and production of important regulatory cytokines, such as IL-12. However, IL-12 is produced only transiently after DC maturation, and its local availability therefore depends on a continuous migration of DC precursors from peripheral blood into the tissue (Sallusto, 2001). The major DC precursors in blood are monocytes and pre-DCs.

Monocytes represent by far the most abundant DC precursors in peripheral blood. However, they are relatively early precursors because they still maintain potential differentiation to either macrophages or DCs and, in vitro, require extensive maturation before developing typical DC features (Fagnoni et al., 2001). In peripheral blood, monocytes are identified by expression of CD14 and divide into at least 2 subsets according to CD16 expression, ie, the ordinary $CD14^{+}CD16^{-}$ monocytes and the rare $CD14^{dim}CD16^{+}$ monocytes accounting for only 10% to 15 % of all monocytes. The $CD14^{dim}CD16^{+}$ monocytes have been shown to exhibit distinct phenotype and function and were labelled proinflammatory based on

their higher expression of proinflammatory cytokines, such as TNF-α and higher potency in antigen presentation.

Pre-DCs relate much more closely to mature DCs than to monocytes (Fagnoni et al., 2001) and divide into at least 2 subsets with different functional capacities, ie, pre-mDC and plasmacytoid DCs (PDC), which are distinguished in human blood by a myeloid (CD11c, CD33) or plasmacytoid (CD123) marker. Pre-mDC represent 0.5% to 1.0 % and PDC only 0.2% to 0.5% of circulating mononuclear cells. Both DC precursors differ fundamentally in their migratory behavior and cytokine secretion pattern. While pre-mDC are characterized mainly by production of IL-12, a key factor in the induction of Th1-mediated responses, PDC start upon viral infection to produce large amounts of interferon-α (IFN-α), ie, up to 200 to 1000 times more than other blood cells after viral challenge (Liu, 2005).

Granulocytes

Granulocytes are produced in bone marrow and are released into the blood. They are characterized by the presence of granules in their cytoplasm and are typically divided to neutrophil granulocytes, eosinophil granulocytes and basophil granulocytes. Neutrophil granulocytes, which are the most abundant type of leucocytes in blood, are recruited to sites of inflammation to form the first line of defense. Eosinophil granulocytes and basophil granulocytes play a role in allergic responses.

Cytokines

Cytokines are a group of proteins and peptides that are used in organisms as signaling compounds. They are produced by a wide range of cell types and have often been broadly classified as monokines (produced by cells of the monocyte lineage) or lymphokines (produced by the cell of lymphocyte lineages). Other names include chemokines (cytokines with chemotactic activities), and interleukins (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (*pleiotropy*). Cytokines are *redundant* in their activity, meaning similar functions can be stimulated by different cytokines. Many characteristic features of cytokines are shared by two other groups of protein mediators – growth factors and hormones.

Cytokines, growth factors and polypeptide hormones all function as extracellular signal molecules featuring fundamentally similar mechanisms of action. This conclusion is supported by the finding that receptors for several cytokines and hormones (i.e. IL-2, IL-4, IL-5, IL-6, GH, prolactin etc.) show several common structural features (Teglund et al., 1998; Bazan, 1989). Structural analysis of cytokines themselves has made it possible to group many cytokines within "families". These are IFN-α/β family, IL-2/IL-4 family, TNF family, IL-6/IL-12 family, IL-10 family, chemokines family etc. Cytokines can also functionally divide into two main groups: type 1 cytokines (IFN-γ, IL-12 etc.), which activate Th1, Tc and macrophages to stimulate cellular immunity and inflammation and type 2 cytokines (IL-4, IL-10, IL-13 etc.), which stimulate antibody production by B cells, respectively. An excessive production of either cytokine type leads to inflammation and tissue damage on the one hand and to susceptibility to infection and allergy on the other hand (Lucey et al., 1996). To prevent overactivity, the type 1/type 2 cytokine balance is tightly regulated not only by cross-inhibition but also via a supra-ordinate neuroendocrine control (Romagnani, 1996).

Regulation of immune functions by sleep – present state of knowledge

There is an increasing body of evidence pointing to a reciprocal relationship between sleep and immunity, for review see (Bryant et al., 2004; Marshall & Born, 2002; Benca & Quintas, 1997). Epidemiological studies indicate that either a shorter or a longer than normal duration of sleep might be related to mortality (Dew et al., 2003; Kripke et al., 2002). Long lasting experimental sleep loss had a highly detrimental effect to the health of experimental animals (Everson, 1993). Direct evidence that sleep supports immune function and recovery from infection has been provided by human studies using vaccination as an experimental model of infection. Thus, one night of total sleep deprivation or a week of partial sleep deprivation, compared with normal sleep, reduced antibody titers in response to hepatitis A and influenza A virus vaccines, respectively (Lange et al., 2003; Spiegel et al., 2002). The effect of sleep on the immune system could be conveyed, eg, by effects of sleep on the redistribution of circulating immune cells and their cytokine production. Thus, in comparison with wakefulness, regular nocturnal sleep generally reduces the number of leukocytes in blood, most likely reflecting an extravasation of these cells to extravascular lymphoid tissue (Dickstein et al., 2000; Born et al., 1997; Dinges et al., 1994; Engeset et al., 1977). In mice, the maximum of T cell counts in peripheral blood is followed by the maximum of these cells in spleen with a delay of two hours (Ottaway & Husband, 1992). Transferred to findings in

humans, the maximum of T cells in spleen would therefore occur in the second half of nocturnal sleep.

Sleep-immune interaction appears to rely critically on specific signal molecules, such as the proinflammatory cytokines IL-1, IL-6 and TNF-α, that are partially controlled by sleep and, in addition, themselves exert regulatory influences on both immune and sleep functions (Majde & Krueger, 2005; Vgontzas et al., 2004; Vgontzas et al., 2005). Petrovsky & Harrison found a circadian peak of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1 and IL-12 as well as of the ratio of IFN-γ/IL-10 production in whole blood samples during nocturnal sleep. This peak was completely abolished following administration of cortisone acetate at 21:00 h in the preceding evening, suggesting that suppression of endogenous cortisol release during early sleep plays a mediating role for this increase in pro-inflammatory cytokine production. For IL-6, a sleep-wake associated release has been demonstrated, with increased IL-6 expression in blood and brain during the sleep period, i.e., in rats during daytime and in humans during the night (Seres et al., 2004; Sothern et al., 1995; Redwine et al., 2000; Guan et al., 2005). On the other hand, sleep deprivation experiments proved, that increased cytokine production – at least for IL-2 – is dependant by sleep. For example, Born at al. (1997) has found increased IL-2 production in whole blood samples after in-vitro stimulation with PHA in sleep condition compared to nocturnal wakefulness. Conversely, influences of the immune system on sleep by cytokines play a role in sleep induction during infections but also in physiological sleep regulation (Krueger et al., 1999). Thus, IL-1 and TNF are part of a complex biochemical cascade regulating sleep (Krueger et al., 2001).

Hypotheses

The purpose of the three studies reported in this thesis is to discover relevant immunological parameters as primary targets of sleep induced changes toward enhanced adaptive immunity. In the first experiment, we tested the hypothesis that nocturnal sleep could ease immune responses by shifting the balance between Th1 and Th2 cytokine activity towards Th1 dominance. The subsequent study 2 addressed the question, whether high levels of prolactin and GH and low levels of cortisol might have mediated the shift towards type 1 cytokines in the sleep condition as observed in the first study. Because of the key role of APCs in T cell regulation, in the third study we focused on specific subsets of APC precursors in peripheral blood and their cytokine production as primary targets of sleep induced changes toward enhanced adaptive immunity. In summary, our data support the idea that sleep can facilitate

immune responses by allowing type 1 cytokine responses and by facilitating accumulation of DC precursors in lymph nodes.

Experiment I: Sleep associated regulation of T helper 1/T helper 2 cytokine balance in humans

Published as:
Dimitrov,S., Lange,T., Tieken,S., Fehm,H-L., Born,J. (2004) Sleep associated regulation of T helper 1/T helper 2 cytokine balance in humans. *Brain Behav Immun. 2004, 18(4):341-8.*

Summary

We compared the Th1/Th2 cytokine balance in healthy men during regular nocturnal sleep (between 23:00 and 07:00 h) and while remaining awake during the same nocturnal interval, in a within-subject crossover design. Blood was collected every 2 hours. Production of T cell derived cytokines – IFN-γ, IL-2, IL-4, and TNF-α – was measured at the single cell level using multiparametric flow cytometry. Compared with wakefulness, early nocturnal sleep induced a shift in the Th1/Th2 cytokine balance towards Th1 activity. During this early half of the night, sleep was not only dominated by SWS but showed also the typical strong increases of prolacin and GH and low concentrations of cortisol.

Introduction

Immune responses against foreign pathogens and abnormal self responses involve complex cellular and humoral components acting both locally and systemically. Sleep is characterized by a specific regulation of the endocrine and autonomic nervous system, and thereby sleep is supposed to exert a systemic control over immune function. Recent human studies have provided first evidence for a facilitating influence of regular sleep on the primary immune response, as indicated by an enhanced antibody response to different vaccines (Spiegel et al., 2002; Lange et al., 2003). The mechanism of this influence needs to be specified. A factor critical for the development of an effective immune response is the cytokine balance between T helper 1 (Th1) and Th2 cells, determining the selection of the effector mechanisms, i.e., of type 1 or type 2 immunity. Th1 cells releasing mainly interferon-γ (IFN-γ), aside from other cytokines including interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α), become activated in response to intracellular viral and bacterial challenges and support various cellular (type 1) responses, including macrophage activation and antigen presentation. In contrast, the cytokines characteristic of Th2 immunity, IL-4 as well as IL-5, IL-10, and IL-13,

tend to drive humoral (type 2) defense via stimulating mast cells, eosinophils and B cells against extracellular pathogens. Predominance of type 2 cytokines, as observed for example in aged humans, has been associated with an inferior response to vaccination (Ginaldi et al., 1999). On the other hand, findings by Petrovsky & Harrison (Petrovsky & Harrison, 1997; Petrovsky & Harrison, 1998) suggest that nocturnal sleep could favor a shift towards Th1 mediated immune defense. They found a circadian peak of the ratio of IFN-γ/IL-10 production in whole blood samples during nocturnal sleep (around 03:00 h). This peak was completely abolished following administration of cortisone acetate at 21:00 h in the preceding evening, suggesting that suppression of endogenous cortisol release during early sleep plays a mediating role for this Th1 shift. However, sleep – particularly the slow wave sleep (SWS) dominant early part of nocturnal sleep – not only suppresses the release of glucocorticoids but also promotes the release of growth hormone (GH) and prolactin e.g., (Spiegel et al., 1995; Sadamatsu et al., 1995; Born & Fehm, 1998) which appear to support Th1 cell mediated immunity (Mellado et al., 1998; Chikanza, 1999; Matera et al., 2000). Also, the study of Petrovsky & Harrison (1997) did not aim at dissecting effects of the circadian rhythm from those of sleep. Here we tested the hypothesis that sleep shifts the Th1/Th2 cytokine balance towards Th1. For this purpose, we compared the ratio of IFN-γ/IL-4 expressing T helper cells in healthy men during sleep and while remaining awake at night. In addition we monitored several candidate hormones that could mediate a shift towards Th1 cytokines.

Methods

Subjects

Fourteen physically and mentally healthy men participated in the study (mean age 24.4 yrs, range 21.2–30.1 yrs). They were non-smokers, had normal body weight, did not suffer from sleep disturbances, and were not taking any medication at the time of the experiments (including over the counter medications such as aspirin). The recruitment procedure included posting advertisements in local buildings in the vicinity of the university. None of the subjects had a medical history of any relevant chronic disease or psychological disorders. Acute illness was excluded by physical examination and routine laboratory investigation, including chemistry panel, C-reactive protein <6 mg/ml, and white blood cell (WBC) count <9/µl.

The men were synchronized by daily activities and nocturnal rest. They had a regular sleep-wake rhythm for at least 6 weeks before the experiments. None of the subjects had worked on a night shift the 3 months before the experiments, and all were used (on work

days) to going to bed before midnight and to getting up around 07:00 h on the next day. During the week preceding the study they were required to turn off the lights for nocturnal sleep between 23:00 and 23:30 h, to get up by 07:00 h the next morning, and not to take any naps during the day. The presence of signs of sleep disturbances (including hints at apnea and nocturnal myoclonus) was excluded by interview. All subjects were adjusted to the experimental setting by spending an adaptation night in the laboratory before the experiment proper. Adaptation included the placement of a venous catheter for blood collection and attachment of electrodes for sleep recordings. The study was approved by the Ethics Committee of the University of Lübeck. All men gave written informed consent, and were paid for participation 120 Euro.

Experimental design and procedure

Experiments were performed according to a within-subject cross-over design. Each man participated in two experimental conditions, a night of sustained wakefulness and a night of regular sleep. Both experimental sessions for a subject were separated by at least one week, and the order of conditions was balanced across subjects.

On experimental nights, subjects arrived at the laboratory at 20:00 h for preparing standard sleep recordings and blood supply. They were offered a light snack at 21:00 h and thereafter went to bed. On the nights of regular sleep lights were turned off at 23:00 h to enable sleep till 07:00 h in the morning. On the waking nights subjects stayed awake in bed in a half-supine position between 23:00–07:00 h. During the time they were watching TV, listening to music and talking to the experimenter at normal room light (about 300 lx).

On both conditions, blood was sampled every 2 hours, between 21:00–07:00 h. For blood sampling the forearm catheter was connected to a long thin tube enabling blood collection from an adjacent room without disturbing the subject's sleep. To prevent clotting, 200 ml of saline solution were infused throughout the night. The same volume was also infused during the wake nights. Blood was collected for a leukocyte differential count, lymphocyte phenotyping, determination of cytokine production and concentration of different hormones.

Dependent measures, sleep and assays

Sleep

Sleep stages were determined off-line from polysomnographic recordings by two experienced experimenters following standard criteria (Rechtschaffen & Kales, 1968). For each night, sleep onset (with reference to lights off at 23:00 h), total sleep time, and percentage of total sleep time spent in the different sleep stages (wake, stages 1, 2, 3, and 4 and rapid eye movement (REM) sleep) were determined. SWS was defined by the sum of stage 3 and 4 sleep. Latencies of sleep stage 2, SWS, and REM sleep were assessed with reference to sleep onset. In addition, the percentage of time spent in the different sleep stages was determined separately for the early and late half of sleep.

Lymphocyte counts and phenotyping

For determination of WBC subset counts, blood samples were collected in ethylenediamine-tetraacetic acid (EDTA) tubes (Sarstedt, Nümbrecht, Germany). The total number of WBCs and a leukocyte differential count was determined automatically by means of a Technicon H1 counter (Technicon, Basingstoke, U.K.). For lymphocyte phenotyping blood was collected in sodium-heparin vacutainers (Becton Dickinson, San Jose, CA). Percentages of CD3+ (T cells), CD4+ (T helper cells) and CD8+ (T suppressor/cytotoxic cells) were determined in whole blood by FACS analysis with MultiTEST reagent (CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC, Becton Dickinson, San Jose, CA). At least 10,000 T cells were collected for analysis. The absolute counts of CD3+, CD4+, and CD8+ T cells were estimated on the basis of lymphocyte counts obtained by the hemogram.

Flow cytometric assay of cytokine production

Multiparametric flow cytometry was used for evaluation of cytokine production and phenotyping at a single cell level (Maino & Picker, 1998). With this method, intracellular cytokine production is measured following in-vitro activation of the cells and disruption of cytokine excretion (thereby preventing also confounding influences of surface and soluble extracellular receptors). For measuring intracellular cytokine production blood was sampled in sodium-heparin vacutainers and kept at room temperature until activation in the morning. Antibodies and reagents were purchased from Becton Dickinson, and the procedure was carried out according to the manufacturer's instructions. Briefly, whole blood was diluted 1:1 with RPMI 1640 medium (containing penicillin, streptomycin, glutamine, non essential amino acids and sodium pyruvate) in Falkon tubes (Becton Dickinson, San Jose, CA) and activated

with PMA and Ionomycin (Sigma-Aldrich Co., St. Louis, MO) at final concentrations of 25 ng/ml and 1 μg/ml, respectively. For stopping cytokine excretion Brefeldin A (Sigma-Aldrich Co., St. Louis, MO) was added at a final concentration of 10 μg/ml. Samples were incubated at 37 °C in 5 % CO_2 for 4 hours. Samples incubated only in the presence of Brefeldin A were used as unstimulated control.

Cells were labeled at room temperature for 20 min using directly conjugated antibodies CD3/PerCP and CD8/APC. Red blood cells were lysed with 2 ml lysing solution for 10 min. After centrifugation for 5 min at 500 g and removing of supernatant, probes were permeabilised with FACS permeabilizing solution for 10 min. After washing with CellWash + 0.5% FCS, probes were incubated with intracellular cytokines IFN-γ/FITC, IL-4/PE and TNF-α/FITC, IL-2/PE (Becton Dickinson, San Jose, CA) for 30 min. Isotype control antibodies were used to discriminate between positive and negative cells. After washing, cells were resuspended in PBS and analyzed immediately.

Flow cytometric analysis was performed on a FACSCalibur using CellQuest Software. A minimum of 10,000 cells in the CD3+ region were counted in each case. The typical forward and side scatter gate for lymphocytes together with a CD3+ gate were set to exclude any contamination. The threshold for cytokine positivity was set using isotype control antibodies. Results for cytokine positive cells were expressed as percentage of the respective subpopulation: CD3+/CD8+ (T suppressor/cytotoxic cells) and CD3+/CD8-. The latter subpopulation was considered CD3+/CD4+ (T helper cells).

Hormone assays and sIL-2 receptor concentration

Blood was centrifuged immediately after sampling and the serum was stored at –70 °C until analysis. The hormones determined and respective assays were as follows: GH (by RIA, Diagnostic Products Corporation, Los Angeles, CA, sensitivity 0.9 ng/ml, intra- and interassay coefficient of variation (CV) <5.9 and 8.3 %, respectively); prolactin (by ELISA, Diagnostic Systems Laboratories, Sinsheim, Germany, sensitivity 0.14 ng/ml, intra- and interassay CV <8.2 and 9.4 %); cortisol (by EIA, Diagnostic Systems Laboratories, Sinsheim, Germany, sensitivity 0.1 μg/dl, intra- and interassay CV <5 and 12 %), thyroid stimulating hormone (TSH, Lumitest TSH, Brahms Diagnostica, Berlin, Germany, sensitivity 0.03 mU/L, intra- and interassay CV <3 and 10.9 %), and melatonin (by RIA, Diagnostic Systems Laboratories, Sinsheim, Germany, sensitivity 0.75 pg/ml, intra- and interassay CV <8 and 10 %). Also soluble IL-2 receptor concentrations were determined (Human IL-2sRα Quantikine

ELISA Kit, R&D Systems, Mineapolis, USA, sensitivity 10 pg/ml, intra- and interassay CV <8 %). All measurements from an individual were performed in duplicate in the same assay.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was based on repeated measures ANOVA with Sleep/Wake and Time as factors. Measures before the nocturnal rest period (i.e., at 21:00 and 23:00 h) were used as covariates. Post hoc contrasts were applied to analyze differences at particular time points. Degrees of freedom were adjusted, where appropriate using the Greenhouse-Geisser procedure. A p-value <0.05 was considered significant.

Results

Sleep and lymphocyte subset counts

Table 1 summarizes the subject's sleep parameters for the sleep condition. Wake during the night, SWS and REM sleep were typical for laboratory condition. As expected, SWS predominated during the early part whereas REM sleep dominated during the late half of nocturnal sleep.

Table 1

Sleep parameters during whole night and first and second half of the night of sleep condition[a]

Parameter	Mean	SEM	Mean	SEM	Mean	SEM
	Total sleep		First half		Second half	
W (%)	5.7	±1.6	3.5	±1.8	7.8	±2.7
S1 (%)	4.4	±0.8	3.1	±0.7	7.6	±1.5
S2 (%)	52.6	±2.0	48.2	±1.8	54.9	±2.7
SWS (%)	18.4	±1.6	33.0	±2.5	4.3	±1.3
REM (%)	18.9	±0.9	12.2	±0.9	25.4	±1.6
Total sleep time (min)	468.6	±4.1				

[a]Time spent awake (W), in sleep stages 1, 2 (S1, S2), slow wave sleep (SWS) and rapid eye movement sleep (REM) is indicated as percentage of total sleep time.

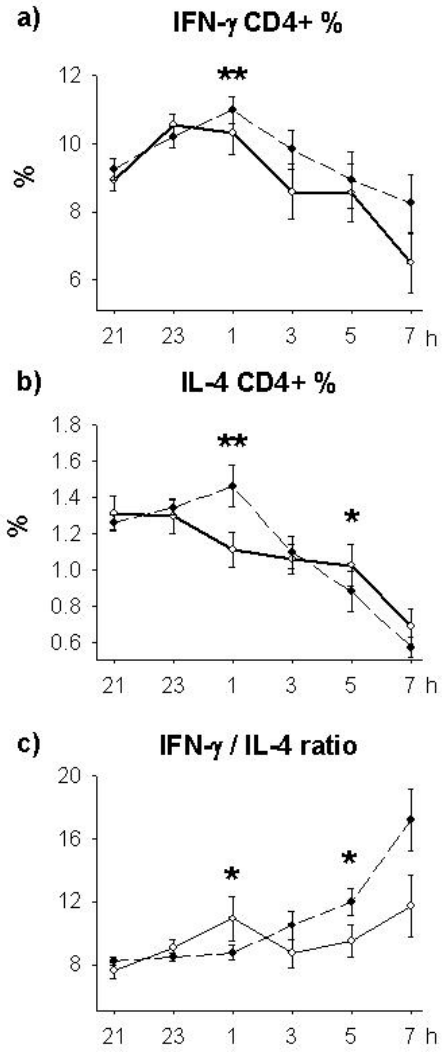


Figure 1.
Mean ±SEM percentages of CD4+ cells producing (a) IFN-γ, (b) and IL-4, and c) the ratio of IFN-γ/IL-4 producing CD4+ cells during regular nocturnal sleep (solid lines) and while subjects remained awake (dashed lines). Values are adjusted to the mean of first two time points as derived from covariance analysis. **, $p<0.01$; *, $p<0.05$ for pairwise comparison at particular time points between both conditions.

Total lymphocyte counts as well as total T cell counts showed the expected circadian dynamic, with increasing numbers during the early night which after peaking around 01:00 h decreased again towards the end of the night ($p<0.05$, for respective main effect of Time). Compared with counts during waking, sleep decreased the number of lymphocytes ($F(1,13)=5.4$, $p<0.05$), with this effect being most pronounced in the measurements at 03:00 h and 05:00 h (averaging 2.139±0.161 /nl vs. 2.391±0.145 /nl, $p<0.01$). During this time, sleep also reduced significantly the average numbers of circulating T cells (CD3+ 1.666±0.133 /nl vs. 1.825±0.128 /nl), T helper cells (CD4+ 1.039±0.087 /nl vs. 1.143±0.074 /nl) and T suppressor/cytotoxic cells (CD8+ 0.624±0.064 /nl vs. 0.679±0.062 /nl; $p<0.05$, for all comparisons).

Intracellular production of cytokines by T helper cells (CD4+)

Statistical results did not depend on whether absolute numbers or percentages of CD4+ cells producing, respectively, IFN-γ and IL-4 were analyzed. Therefore, and in light of the sleep dependent changes in absolute numbers of CD4+, the following report concentrates on per cent values for the respective CD4+ populations. The proportion CD4+ cells producing IFN-γ slightly increased during the early night and, thereafter declined ($p<0.001$, for effect of Time).

Compared to wakefulness sleep slightly reduced the percent of IFN-γ producing CD4+ cells. While this effect did not reach significance in the overall ANOVA it did so in the comparisons at single time points at 01:00 h ($p<0.01$, Figure 1a). The proportion of IL-4 producing CD4+ also declined towards the end of the night ($p<0.001$). In comparison with the waking condition, sleep decreased the proportion of CD4+ producing IL-4 during the early night and increased it during the late night ($F(3,39)=7.2$, $p<0.01$ for Sleep/Wake x Time, for tests at 01:00 and 05:00 h, $p<0.01$ and $p<0.05$, respectively, Figure 1b).

Sleep also distinctly influenced the ratio of IFN-γ to IL-4 secreting CD4+ cells indicating Th1/Th2 balance. As a result of the stronger sleep associated decrease in IL-4 than IFN-γ secreting CD4+ cells, the IFN-γ/IL-4 ratio of CD4+ was increased during the early part of the night when subjects slept as compared with sustained wakefulness. In contrast, during the late night, sleep gradually decreased the IFN-γ/IL-4 ratio of CD4+ ($F(3,39)=4.9$, $p<0.05$). In tests at single time points the decreasing influence of early sleep and the increasing influence of late sleep were significant at 01:00 h and at 05:00 h, respectively (Figure 1c).

There was a slight trend towards an increased percentage of CD4+ producing TNF-α throughout the sleep period as compared with the wake condition ($F(1,12)=3.2$, $p<0.1$). For IL-2 producing CD4+ no effects of sleep were revealed.

Intracellular production of cytokines by T suppressor/ cytotoxic cells (CD8+)

Sleep also influenced the percentage of CD8+ cells producing IFN-γ and TNF-α. Both subsets after an early peak declined gradually during the night ($p<0.001$). TNF-α secreting CD8+,

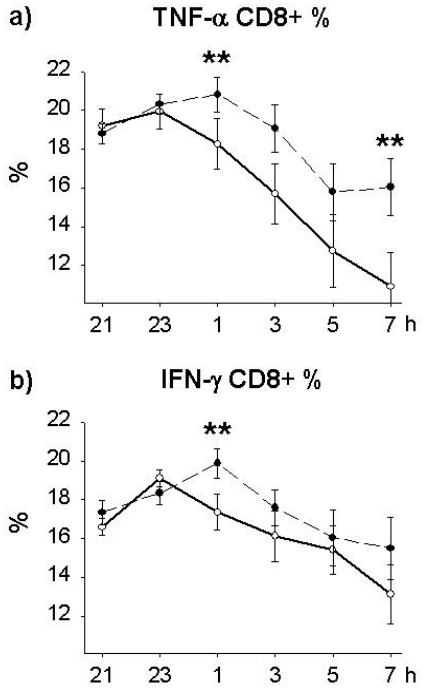


Figure 2.

Mean ±SEM percentages of CD8+ cells producing (a) TNF-α and (b) IFN-γ during regular nocturnal sleep (solid lines) and while subjects remained awake (dashed lines). Values are adjusted to the mean of first two time points as derived from covariance analysis. **, $p<0.01$ for pairwise comparison at particular time points between both conditions.

indeed, displayed the strongest change after sleep. Compared to wakefulness, during sleep this population was continuously decreased throughout the night ($F(1,12)=21.8$, $p<0.001$, Figure 2a). Significance ($p<0.01$) for this sleep associated decrease in TNF-α producing CD8+ was revealed for the measurements at 01:00 and 07:00 h. The decreasing effect of sleep on IFN-γ secreting CD8+ was more transient and in comparison with a wake condition reached significance ($p<0.01$) at 01:00 h only (Figure 2b).

Plasma hormone and sIL-2 receptor concentrations

Compared with nocturnal waking, regular sleep was, as expected, characterized by a distinct pattern of endocrine changes, summarized in Figure 3. Plasma concentrations of prolactin were strongly enhanced during sleep throughout the night as compared to the wake condition ($F(1,12)=59.8$, $p<0.001$) with this difference being significant at each single time point. Concentrations of GH were increased selectively during early sleep ($F(3,39)=8.9$, $p<0.01$), with about fourfold higher concentration at 01:00 h during sleep than during wakefulness ($p<0.001$). On the other hand, the nocturnal rise in TSH plasma levels observed in the wake condition was completely suppressed by sleep ($F(1,12)=18.1$, $p<0.001$).

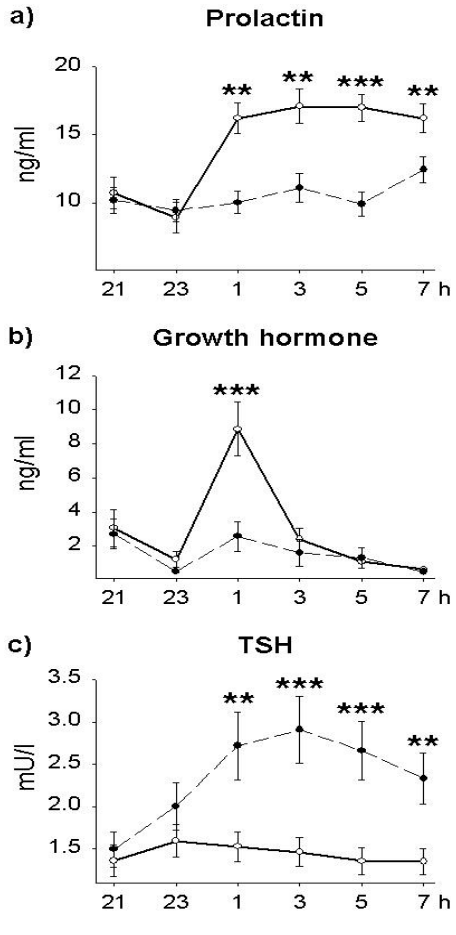


Figure 3.
Mean ±SEM plasma concentrations of (a) prolactin, (b) growth hormone (GH) and (c) thyroid stimulating hormone (TSH) during regular nocturnal sleep (solid lines) and while subjects remained awake (dashed lines). ***, $p<0.001$; **, $p<0.01$ for pairwise comparison at particular time points between both conditions.

Changes in concentrations of cortisol and melatonin, although pointing towards the expected decreasing influence of sleep, were too small and variable to reach significance. Effects of sleep on sIL-2 receptor concentration were also not significant.

Discussion

This study was based on the hypothesis that sleep induces a shift in the Th1/Th2 cytokine balance towards Th1 predominance. The data partly confirmed this hypothesis. Compared with sustained wakefulness, nocturnal sleep acutely suppressed IL-4 producing CD4+, i.e., Th2 cells during the early night. Since a similar sleep associated decrease in IFN-γ producing CD4+, i.e., Th1 cells, was less clear, the Th1/Th2 balance as indicated by the ratio of IFN-γ/IL-4 producing CD4+ cells, was revealed to be significantly enhanced by sleep. However, the effect was of moderate size only, and limited to early sleep, dominated by SWS. During the late part of sleep when REM sleep is dominant, the effect reversed so that at 05:00 h the ratio of IFN-γ/IL-4 producing CD4+ cells was significantly lower during sleep than wakefulness.

These findings complement previous observations by Petrovsky & Harrison (1997, 1998), who reported strong diurnal rhythms in the in-vitro production of IFN-γ and the Th2 cell cytokine IL-10 (in mitogen stimulated whole blood samples), with peaks at about 23:00 h for IFN-γ and at about 22:00 h for IL-10. The difference in circadian phase of the two cytokines leads to a shift of the Th1/Th2 cytokine balance, as indicated in that study by the ratio of IFN-γ/IL-10 production, towards Th1 during the early night. Although here we did not analyze circadian rhythms, the decline observed independently of sleep and wakefulness for the production of all cytokines of interest very likely reflects this type of influence. While Petrovsky & Harrison (1997) demonstrate a circadian peak in the Th1/Th2 cytokine balance during the early night, the present comparison between subjects awake and asleep indicates an additive effect of sleep strengthening Th1 dominance during the early night. Th1 dominance should lead to facilitated cell mediated immune response against intracellular bacteria and viruses e.g., (Alexander et al., 1999; Karupiah, 1998). In contrast, Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 stimulate various aspects of humoral defense, such as a B cell switch to the production of IgG1 and IgE, and, thereby, these cytokines are known to be involved also in some forms of allergic responses (Wierenga et al., 1990; Rothman, 1993; Hopkin, 1997). On this background, the relative increase of IL-4 secreting CD4+ cells observed in our subjects when waking might well be a factor contributing to increased IgE production and

allergic responses to cedar pollen that have been found in atopic patients after a single night of sleep deprivation (Kimata, 2002). On the other hand, the relatively small size of the effect of sleep (averaging 24 % at 01:00 h, Figure 1a) as well as the fact that sleep also decreased the percentage of IFN-γ CD4+ cells to a small extent could be taken to generally question the biological relevance of the effect of sleep. This, in fact, remains to be proven in a direct test.

Th1/Th2 polarization takes place once a naïve T cell upon antigen recognition enters into proliferating cycles. Sleep and sleep stages can gain influence on this process through their specific regulation of immunoactive hormones, such as GH and prolactin (Anisman et al., 1996). Prolactin supports Th1 differentiation and cooperates with IL-12 in the T cell production of IFN-γ (Gala, 1991; Chikanza, 1999; Matera et al., 2000; Matera & Mori, 2000). Likewise, GH administration in mice has been found to shift cytokine production (following immunization with HIV-1-gp120) from Th2 to Th1 predominance with lower IL-4 and higher IFN-γ activity (Mellado et al., 1998). Conversely, low levels of endogenous GH and prolactin have been found to be associated with increased Th2 cell numbers (Hall et al., 2002). The present data confirm strong increases in the release of GH and prolactin during sleep, which are known to be most pronounced during periods of SWS (Spiegel et al., 1995; Van Cauter et al., 1998). During early SWS-rich sleep, these increases might synergistically act to bias the Th1/Th2 balance acutely towards Th1 predominance. Whether the shift reflects changes in the production pattern or differential migration of T helper cells cannot be answered. Also, it is presently unclear whether the sleep related suppression of TSH further adds to the Th1 shift. The suppression of cortisol release during the early night as well as the enhanced nocturnal level of melatonin may play a permissive role for the observed Th1 dominance(Petrovsky & Harrison, 1997; Kuhlwein & Irwin, 2001). The relative increase of Th1 compared to Th2 cytokine activity during SWS rich early sleep fits a number of previous observations suggesting a similar association between sleep, SWS and Th1/Th2 cytokine balance. For example, elderly people not only suffer from severe disturbances of sleep and SWS, but also display a decrease of GH and prolactin release in conjunction with a cytokine shift towards Th2 (Van Cauter et al., 2000; Lio et al., 2000). Remarkable in this context are recent findings by Redwine and coworkers (Redwine et al., 2003) who found in chronic alcoholic patients with prominent deficits in SWS a persistently low ratio of IFN-γ/IL-10, indicating a shift towards Th2 dominance. Further confirmation of these relationships is now needed from experiments manipulating individual expression of SWS and associated hormonal secretion.

A finding of the present study diverging from previous observations (Born et al., 1997; Irwin et al., 1996) is the absence of any effect of sleep on intracellular production of T cell

derived IL-2, although this might reflect methodological differences. In those studies sleep as compared to sleep deprivation increased IL-2 production, determined in-vitro in whole blood samples after 48 h mitogen stimulation. However, this type of stimulation is probably associated with an acute upregulation of various receptors including those for GH, prolactin and catecholamines on the surface of T cells (Dardenne et al., 1994; Dardenne et al., 1998; Ramer-Quinn et al., 1997). Thus, mediators present in the whole blood sampled during sleep, may have induced via their own receptors increased release of IL-2. Here, we used multiparametric flow cytometry to evaluate cytokine production at the single cell level. Unlike the in-vitro determination of cytokine production in whole blood samples, this method involves adding of the secretion inhibitor Brefeldin A, which stops de-novo secretion of surface molecules and excretion of all molecules, including local hormones and cytokines. Thus, the acute number of cells producing the cytokine of interest is measured. However, this method of intracellular cytokine detection may not mimic what occurs in-vivo since it involves artificial stimulation of the cells to produce cytokine and addition of a blocking agent to prevent secretion.

Though unexpected, the most robust effect of sleep in the present study was a decrease in T suppressor/cytotoxic (CD8+) cells producing TNF-α. These data complement previous findings indicating a suppressing influence of sleep on TNF-α in LPS stimulated whole blood samples which targets monocyte derived TNF-α (Uthgenannt et al., 1995). Since among CD8+ cells TNF-α is produced primarily by effector and memory cells, the sleep induced decrease in CD8+ cells producing this cytokine pertained most likely to this subset rather than to naive CD8+ cells (Hoflich et al., 1998). This view is corroborated by the finding of a parallel, though smaller, sleep induced decrease in CD8+ producing IFN-γ, as the combination of these two cytokines is typical for effector and memory T cells (Hamann et al., 1997). While a change in the pattern of cytokine production cannot be excluded, the sleep induced decrease in TNF-α producing CD8+ is more likely to reflect migratory changes due to differential accumulation of these cells in extravascular tissues (von Andrian & Mackay, 2000). However, the role of reduced recirculation of these cells in the context of sleep mediated immune functions remains to be specified.

Experiment II: A regulatory role of prolactin, growth hormone and corticosteroids for human T-cell production of cytokines

Published as:
Dimitrov,S., Lange,T., Fehm,H-L., Born,J. (2004) A regulatory role of prolactin, growth hormone and corticosteroids for human T-cell production of cytokines. *Brain Behav Immun. 2004, 18(4):368-74.*

Summary

This study addressed the question, whether high levels of prolactin and GH and low levels of cortisol might mediate the shift towards type 1 cytokines observed during the first part of the sleep. Whole blood was sampled from 15 healthy humans in the morning after regular sleep and was activated in-vitro with ionomycin and phorbol myristate acetate (PMA). The morning blood is typically characterized by medium levels of prolactin, low levels of GH and peak concentrations of cortisol. Exposure of the whole blood to prolactin, GH, glucocorticoid receptor (GR) antagonist RU-486, or mineralcorticoid receptor (MR) antagonist spironolactone led to an increase in IFN-γ producing T-cells. These results confirmed our hypothesis that enhanced prolactin and GH concentrations as well as low cortisol level during early sleep synergistically act to enhance Th1 cytokine activity.

Introduction

Accumulating evidence suggests that the immunological effects of sleep in humans are wide-ranging, and not restricted to one function of the immune system (Rogers et al. 2001; Born, 1998). A clinically important component often overlooked in this relationship is the T helper1 (Th1)/T helper 2 (Th2) balance. Both branches support different functions of defense. Th1 responses include cell mediated reactions that are important for dealing with cellular pathogens, whereas Th2 responses regulate production of antibodies in response to extracellular pathogens and mediate allergic processes (Romagnani, 1999). Moreover effects of IFN-γ, a major Th1 cytokine, and IL-4, a major Th2 cytokine, are antagonistic. Patients with insomnia were found to display a shift towards Th2 dominance (Sakami et al., 2003). Petrovsky and Harrison (1997) demonstrated that the early period of nocturnal sleep is

characterized by a substantial shift in this balance towards predominant Th1 cytokines. Recently, we extended these results, separating effects of the circadian rhythm from those of sleep and showed that the early part of sleep, in which slow wave sleep (SWS) predominates, itself can induce a significant shift towards enhanced Th1 cytokine activity (Dimitrov et al., 2004). The shift disappeared and was even replaced by an opposite shift towards Th2 cytokine activity during late sleep which is dominated by rapid eye movement (REM) sleep and contains only negligible amounts of SWS. Early sleep and particularly SWS inhibits secretory activity of the pituitary adrenal system so that plasma cortisol concentrations reach nadir values during this time (Bierwolf et al., 1997; Born & Fehm, 1998). Simultaneously SWS-rich early sleep has a profoundly stimulating influence on the secretion of GH (Van & Copinschi, 2000) and also of prolactin (Spiegel et al., 1995; von Treuer et al., 1996). Data from different groups showed that prolactin and GH specifically influence the cellular arm of immune defense involving Th1 cytokines, although many of these studies did not directly assess T-cell function but focused on macrophage activity (Takagi et al., 1998; Majumder et al., 2002; Edwards, III et al., 1992; Kelley et al., 1992). With regard to corticosteroids evidence suggests that glucocorticoids alter cytokine production from a Th1 to a Th2 pattern e.g., (Agarwal & Marshall, Jr., 2001; Visser et al., 1998). However most of the studies used rather high concentrations of the hormones of interest or in the case of corticosteroids the highly potent and selective glucocorticoid agonist dexamethasone. Since GH, prolactin and cortisol are the main hormones the secretion of which is regulated during sleep and SWS we sought to dissect the effects of each of these hormones on the production of T-cell cytokines with the focus on Th1/Th2 cytokines. We evaluated in-vitro cytokine production in the absence or presence of prolactin and GH at concentrations within the normal physiological range occurring during sleep. Additionally, we examined the role of nadir cortisol concentrations during early nocturnal sleep for cytokine production by blocking the effects of cortisol in the morning blood samples, known to contain high amounts of endogenous corticosteroids. For this purpose, the glucocorticoid receptor (GR) antagonist RU-486 and the mineralocorticoid receptor (MR) antagonist spironolactone were used taking into account that cortisol exerts influences via both corticosteroid receptors (de Kloet et al., 1998; White et al., 1997).

Methods

Subjects

Fifteen physically and mentally healthy men aged 20-35 yrs participated in the study. They were non-smokers, did not suffer from sleep disturbances or allergies, and were not taking any medication at the time of the experiments. Acute and chronic illness was excluded by medical history, physical examination and routine laboratory investigation, including C-reactive protein < 6 mg/l, and white blood cell (WBC) count < 9 /µl. The study protocol was approved by the local ethics committee and written informed consent was obtained from all participants.

Antibodies and reagents

Recombinant prolactin and GH, anti-prolactin polyclonal Ab (AF682), GR antagonist RU-486 and MR antagonist spironolactone were purchased from Sigma-Aldrich (St. Louis, MO). Anti-GH monoclonal Ab (5801) was provided from Oy Medix Biochemica Ab (Kaunianinen, Finland) and was used after dialyzing in PBS buffer to remove azide.

Cell activation

Whole blood was obtained by venipuncture and collected in heparinized blood collecting tubes (Becton Dickinson, San Jose, CA) in the morning after regular sleep. The blood was diluted with RPMI 1640 medium (supplemented with penicillin, streptomycin, glutamine, non essential amino acids and sodium pyruvate) in Falkon tubes (Becton Dickinson, San Jose, CA) and incubated for 6 hours at 37°C and 5 % CO_2 in the absence or presence of prolactin, prolactin antibody, GH, the GR antagonist RU-486, or the MR antagonist spironolactone. 1 µg/ml ionomycin, 10 µg/ml Brefeldin A together with two concentrations of PMA (8 and 25 ng/ml, all reagents Sigma-Aldrich Co., St. Louis, MO) was added for the last 4 hours of stimulation. The two concentrations of PMA fall in the range of PMA dosage used for standard stimulation to induce cytokine activity (Picker et al. 1995; McNerlan et al. 2002)

GH and prolactin were used at a final concentration of 20 ng/ml. In some experiments prolactin was neutralized by the addition of 1 µg/ml anti-prolactin antibody. Results from pilot experiments showed that anti-prolactin antibody at a final concentration of 1 µg/ml together with 4 µg/ml anti-GH antibody completely blocked the proliferation of lactogen

dependent Nb2 cell line in the presence of human serum containing natural concentration of GH and prolactin.

The binding of cortisol to GR or MR was blocked by the addition of 8.3 μM RU-486 and spironolactone, respectively. This concentration is about 10fold higher than the morning blood cortisol level, and our pilot study results showed complete inhibition of cortisol.

Flow cytometric assay of cytokine production

Antibodies and reagents were purchased from Becton Dickinson, and the procedure was carried out according to the manufacturer's instructions. Cells were labeled using directly conjugated antibodies CD3/APC and CD8/PerCP at room temperature for 20 minutes. Red cells were lysed with 2 ml lysing solution for 10 minutes. After centrifugation for 5 minutes at 500 g and removing of supernatant, probes were permeabilized with FACS permeabilizing solution for 10 minutes. After washing with CellWash + 0.5% FCS, probes were incubated with intracellular cytokine-antibodies IFN-γ/FITC, IL-4/PE and TNF-α/FITC, IL-2/PE for 30 minutes. Isotype control antibodies were used to discriminate between positive and negative cells. After washing, cells were resuspended in PBS and analyzed immediately.

Flow cytometric analysis was performed on a FACSCalibur using CellQuest Software. At least 10,000 cells in the CD3+ region were counted in each case. The typical forward and side scatter gate for lymphocytes together with the CD3+ gate were set to exclude any contamination. Threshold for cytokine positivity was set using isotype control. Results for cytokine positive cells were expressed as percentage of the respective subpopulation: CD3+CD8+ (T suppressor/cytotoxic cells) and CD3+CD8-. The latter subpopulation was considered CD3+CD4+ (T helper cells).

Hormone assays and statistical analysis

For hormone assays blood was sampled in serum tubes. The blood was immediately centrifuged and the serum was frozen at –20°C until analysis. The hormones determined and respective assays were as follows: GH (by RIA, Diagnostic Products Corporation, Los Angeles, CA, sensitivity 0.9 ng/ml, intra- and interassay coefficient of variation (CV) < 5.9 and 8.3 %, respectively); prolactin (by ELISA, Diagnostic Systems Laboratories, Sinsheim, Germany, sensitivity 0.14 ng/ml, intra- and interassay CV < 8.2 and 9.4 %, respectively); cortisol (by EIA, Diagnostic Systems Laboratories, Sinsheim, Germany, sensitivity 0.1 μg/dl,

intra- and interassay CV < 5 and 12 %, respectively). Data are presented as means ± SEM. Paired-samples t-test was applied to analyze differences.

Results

Influences on the IFN-γ/IL-4 balance

We first examined the effects of the different cultural conditions on IFN-γ and IL-4 cytokine producing CD4+ cells. Effects were much more pronounced and reached significance mainly after stimulation with 8 ng/ml PMA. At this level of PMA stimulation, neutralizing prolactin through prolactin antibody distinctly decreased IFN-γ producing CD4+ cells ($t(15)=3.37$, $p<0.01$). Addition of extra prolactin versus prolactin antibody significantly increased the same population ($t(15)=2.84$, $p<0.01$). GH alone, GH plus RU-486, RU-486, and spironolactone distinctly enhanced IFN-γ+CD4+ cells after low PMA activation (see Figure 1a for significances). With the higher 25 ng/ml dose of PMA stimulation, the only effect reaching significance was an increase in the IFN-γ+CD4+ population following RU-486 ($t(15)=2.12$, $p<0.05$, data not shown). IL-4 producing CD4+ cells were not consistently influenced (Figure 1b), except for a decrease in this cell population following administration of GH plus RU-486 with the higher dose of PMA ($t(15)=2.71$, $p<0.05$).

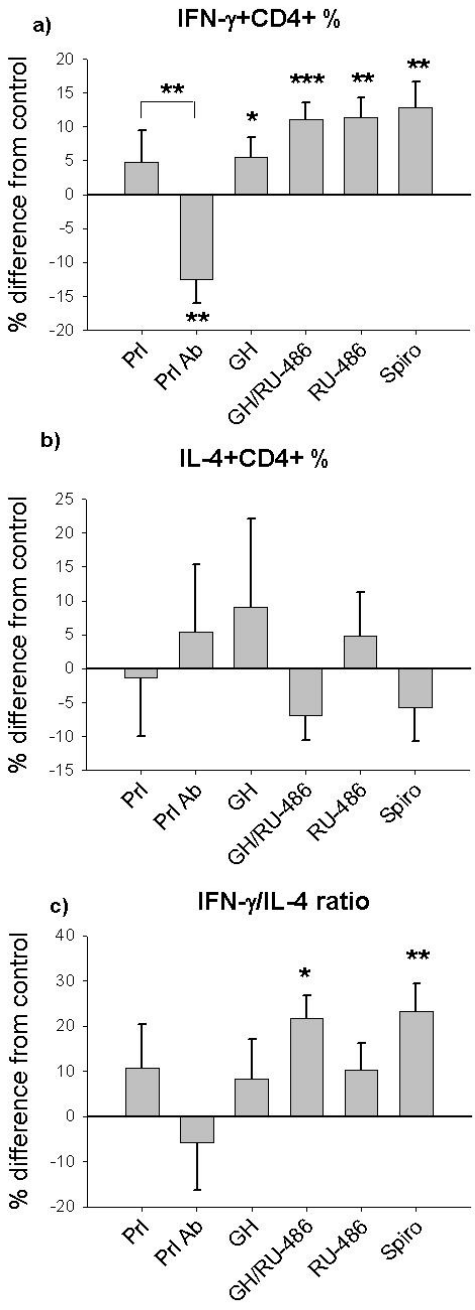


Figure 1.

Effects of prolactin (Prl) anti-prolactin antibody (Prl Ab), growth hormone (GH), the GR antagonist RU-486, and the MR antagonist spironolactone (Spiro) on percentages of (a) IFN-γ+CD4+, (b) IL-4+CD4+, and (c) the ratio of IFN-γ/IL-4 producing CD4+ cells after incubation with ionomycin and PMA at a dose of 8 ng/ml. The data are presented as percentage of changes relative to the stimulated control. The mean absolute values (±SEM) in the stimulated control were for (i) IFN-γ producing CD4+ cells: 14.5 ± 1.7 %; (ii) IL-4 producing CD4+ cells: 0.83 ± 0.1 %; (iii) IFN-γ/ IL-4 ratio: 21.6 ± 2.2 %. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$ for pairwise comparison between different cultural conditions and stimulated control.

Because the relative balance of Th1 and Th2 cytokines, rather than the absolute concentration of each type of cytokine, may be more relevant for the balance of cell mediated and humoral components of the host immune response *in vivo*, the ratio of IFN-γ to IL-4 producing CD4+ cells was determined. Although, all substances (except prolactin antibody) on average had an increasing influence on this ratio (at 8 ng/ml PMA), only the effects of spironolactone and of RU-486 in combination with GH reached significance (Figure 1c). No effects on the Th1/Th2 balance were observed with the higher dose of PMA.

Influences on TNF-α and IL-2 producing CD4+ cells

In light of the rather consistent hormonal effects on the production of IFN-γ we extended testing to other pro-inflammatory cytokines. Again, most consistent effects were obtained with the lower concentration of PMA. At this PMA concentration, prolactin versus prolactin antibody increased TNF-α+CD4+ cells ($t(15)=2.53$, $p<0.05$), but left IL-2 producing CD4+ cells unaffected (see Figure 2 for significances). Both TNF-α and IL-2 producing CD4+ cells were significantly increased by incubation with RU-486, and spironolactone. Moreover, the percentage of IL2+CD4+ cells was increased also by RU-486 plus GH (Figure 2). Effects at the higher PMA dose were limited to a rather strong decrement in TNF-α producing CD4+ cells after incubation with GH ($t(15) = 3.40$, $p<0.01$).

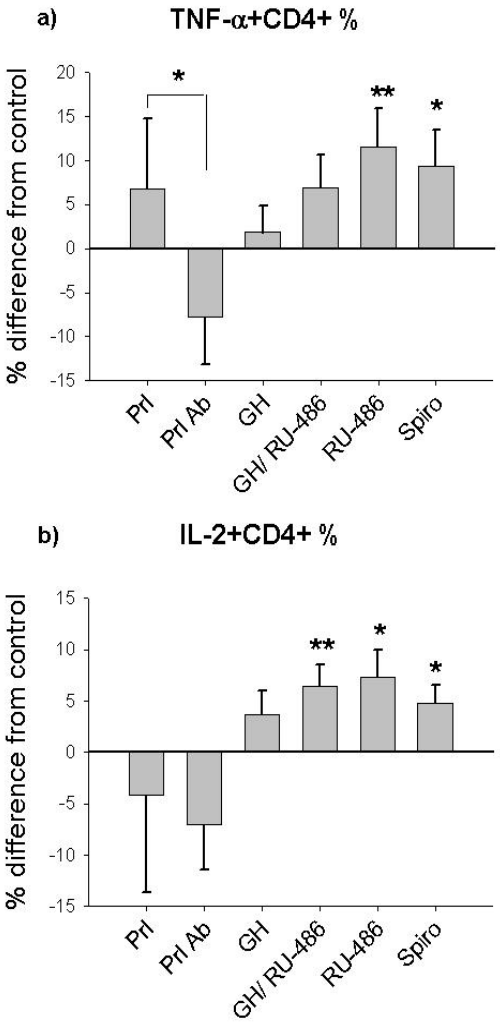
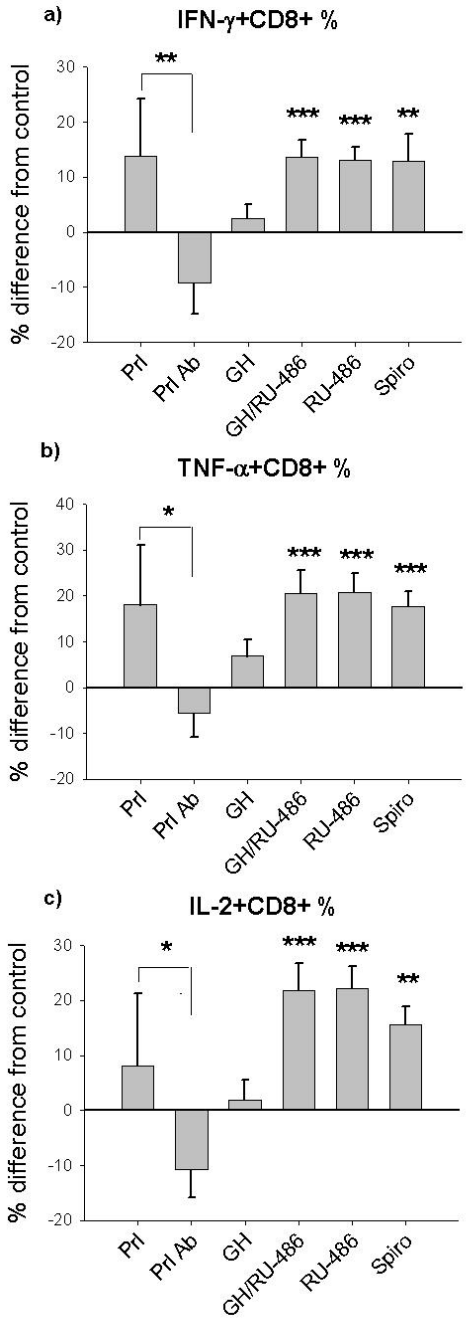


Figure 2.
Effects of prolactin (Prl) anti-prolactin antibody (Prl Ab), growth hormone (GH), the GR antagonist RU-486, and the MR antagonist spironolactone (Spiro) on percentages of (a) TNF-α+CD4+, and (b) IL-2+CD4+ cells after incubation with ionomycin and PMA at a dose of 8 ng/ml. The data are presented as percentage of change relative to the stimulated control. The mean absolute values (±SEM) in the stimulated control were for (i) TNF-α producing CD4+ cells: 28.3 ± 3.1 %; (ii) IL-2 producing CD4+ cells: 36.2 ± 3.9 %. **, $p<0.01$; *, $p<0.05$ for pairwise comparison between different cultural conditions and stimulated control.

Intracellular production of cytokines by CD8+ (T suppressor/ cytotoxic cells)

In the presence of 8 ng/ml PMA, stimulation with prolactin (vs. prolactin antibody) distinctly increased all cytokines analyzed in the CD8+ population, i.e. IFN-γ, TNF-α, and IL-2 (see Figure 3 for significances). The proportion of CD8+ cells producing IFN-γ, TNF-α and IL-2 markedly increased also in the presence of RU-486, spironolactone and RU-486 plus GH. With the higher PMA concentration again only GH was effective, however decreasing the percentages of IFN-γ+CD8+ and TNF-α+CD8+ cells ($t(15)=2.14$, $p<0.05$ and $t(15)=3.05$, $p<0.01$, respectively).



Plasma hormone concentrations

Hormone concentrations were evaluated in the same blood as sampled for cytokine measurements. GH concentrations were bellow the detection level of the assay (0.9 ng/ml) in all subjects. Cortisol concentrations ranged between 10.3 and 29.6 μg/dl (mean: 20.19 μg/dl) and prolactin concentrations ranged between 11.0 and 35.2 ng/ml (mean: 21.31 ng/ml).

Figure 3.
Effects of prolactin (Prl) anti-prolactin antibody (Prl Ab), growth hormone (GH), the GR antagonist RU-486, and the MR antagonist spironolactone (Spiro) on percentages of (a) IFN-γ+CD8+, (b) TNF-α+CD8+, and (c) IL-2+CD8+ cells after incubation with ionomycin and PMA at a dose of 8 ng/ml. The data are presented as a percentage of the change relative to the stimulated control. The mean absolute values (±SEM) in the stimulated control were for (i) IFN-γ producing CD8+ cells: 14 ± 2.6 %; (ii) TNF-α producing CD8+ cells: 28 ± 2.4 %; (iii) IL-2 producing CD8+ cells: 14.5 ± 1.8 %. ***, p<0.001; **, p<0.01; *, p<0.05 for pairwise comparison between different cultural conditions and stimulated control.

Discussion

This study aimed at examining in-vitro the role of sleep associated hormonal regulation on the production of T-cell cytokines. The focus was on influences of prolactin, GH, and corticosteroids on the Th1/Th2 cytokine balance. Our data point to most prominent influences on this balance by corticosteroids, since the MR antagonist spironolactone was the only agent which itself significantly shifted the Th1/Th2 balance towards Th1 dominance. The GR antagonist RU-486 induced a similar shift which, however, reached significance only in combination with GH. Notably both MR and GR antagonists did not affect the number of IL-4 producing CD4+ cells, so that the change in the Th1/Th2 cytokine balance was due solely to the distinct increase in IFN-γ producing CD4+ cells. These results confirm previous reports in humans, indicating that in the T-cell population corticosteroids induce an in-vitro shift in the Th1/Th2 cytokine balance toward a predominant Th2 response, primarily through inhibition of Th1 cytokines (Agarwal & Marshall, Jr., 2001).

Our data are novel in that they show a prominent role for MR in the mediation of corticosteroid effects which are similar in direction and amplitude to those mediated by GR. In many previous studies, a corticosteroid modulation of Th1 versus Th2 responses was evaluated mostly using the potent glucocorticoid dexamethasone, which has significant limitations, since dexamethasone binds selectively GR. In contrast, depending on the presence or absence of the enzyme 11β-hydroxysteroid dehydrogenase in the respective tissue, cortisol can bind MR with even higher affinity than GR (de Kloet et al., 1998; Edwards et al., 1996). So far, there are only few hints suggesting that MR is specifically involved in the regulation of immune functions. Blockade of MR by spironolactone was found to reverse an inhibition of monocyte IL-1ra secretion by cortisol (Sauer et al., 1996). Recently, Vedder et al. (2003) reported an increase in the secretion of TNF-α in PBMC from healthy humans after in vitro administration of spironolactone. Extending these observations, here we found that spironolactone like RU-486 enhances the production of TNF-α as well as IFN-γ and IL-2, both in CD4+ and CD8+ T-cells, suggesting that MR and GR synergistically act to mediate a suppression of proinflammatory responses via cortisol.

The other major endogenous ligand of MR is the mineralocorticoid aldosterone. The release of aldosterone is modulated by sleep and circadian rhythm (Charloux et al., 2001), and the mineralocorticoid appears to exert also some immunological influences, e.g., to reduce circulating lymphocytes, NK cells and monocytes (Miller et al., 1994). However, aldosterone concentrations in peripheral blood are 1000fold lower than cortisol levels (Charloux et al.,

2001) and hyperaldosteronism in chronic heart failure is paralleled by an increase rather than decrease in TNF-α (Anker & Rauchhaus, 1999). On this background, we suppose that the effects of spironolactone in the present experiments reflect predominantly blocking of actions of cortisol rather than aldosterone.

Unlike cortisol secretion, which is suppressed during early SWS-rich sleep, the secretion of prolactin and GH is stimulated during this period, and reduced during morning wakefulness. The comparison of changes after prolactin and anti-prolactin antibody indicated a stimulating effect of this hormone on IFN-γ and TNF-α producing CD4+ and CD8+ cells as well as on the production of IL-2 by CD8+ cells. These finding corroborate a number of previous studies likewise pointing to an increasing influence of prolactin on the production of Th1 cytokines (for a review, see (Matera et al., 2000). Like prolactin, adding GH at concentrations comparable with the SWS related increase in endogenous GH increased IFN-γ producing CD4+ cells in the morning blood samples. This result also fits several previous in-vivo studies in which GH enhanced Th1 cytokine production (Takagi et al., 1998; Mellado et al., 1998). However, contrasting with those observations in-vivo, in our in-vitro experiments we did not find evidence for a decreasing influence of GH on the production of IL-4. This failure might be taken to argue that GH, in fact, primarily acts to enhance the Th1 response which in-vivo, but not in-vitro, can act secondarily to suppress Th2 cytokine activity. The Th1 enhancing effect of GH although somewhat weaker resembled that of prolactin, which is not unexpected since both hormones act via receptors of the same cytokine/hemopoetin receptor superfamily, sharing binding affinity and a similar intracellular protein cascade during transduction (Matera et al., 2000; Yu-Lee, 2002).

To mimic sleep associated hormonal changes and their influence on cytokine production in-vivo we activated whole blood with PMA and ionomycin in the absence or presence of GH, prolactin, prolactin antibody, GC and MC receptor antagonists. However this in-vitro model has obvious limitations. Thus, it does not take into account any alterations in immune cell trafficking induced by sleep and hormonal secretion (Born et al., 1997; Dhabhar, 2002; Dhabhar et al., 1994; von Andrian & Mackay, 2000). A further unanalyzed variable of possible relevance in our in-vitro model concerns the expression of GH, prolactin, GC and MC receptors which is known to be increased during activation. Also the use of PMA deserves critical consideration since it represents a rather non-physiological stimulus of cytokine production. Surprisingly, here with the higher concentration of 25 ng/ml PMA results were distinctly less clear than with the lower 8 ng/ml PMA concentration. We suppose that the maximal stimulation induced with the higher PMA concentration is associated with a

ceiling effect reducing any physiological modulation of the response. Also, unexpectedly, at high PMA concentrations GH inhibited rather than increased the number of CD8+ cells producing TNF-α and IFN-γ, which might likewise point to the presence of non-physiological conditions. This conclusion, however, is challenged by the fact that we observed a similar decrease in TNF-α and IFN-γ producing CD8+ in conjunction with high blood GH concentrations during sleep in-vivo (Dimitrov et al., 2004). These issues are clearly in need of further exploration using also blood drawn during SWS with blocking of GH and prolactin or adding of cortisol, and with stimuli of cytokine production (like Staphylococcal enterotoxin B) that are more natural than PMA. Nevertheless, overall the present data lend themselves to conclude that the sleep induced increase in prolactin and GH in combination with the circadian nadir of cortisol during early sleep in humans are of sufficient amplitude to explain an increase in Th1 cytokine activity during this period.

Experiment III: Number and function of circulating human antigen-presenting cells regulated by sleep

Published as:
Dimitrov,S., Lange,T., Nohroudi,K., Born,J. (2007) Number and function of circulating human antigen-presenting cells regulated by sleep. *Sleep*, 30(4): 401-411

Summary

Here we focused on specific subsets of APC precursors in peripheral blood and their cytokine production as primary targets of sleep induced changes toward enhanced adaptive immunity. We examined circulating numbers of several DC precursors, i.e. $CD14^{+}CD16^{-}$ and $CD14^{dim}CD16^{+}$ monocytes, myeloid dendritic cell precursors (pre-mDC) and plasmacytoid dendritic cells (PDC) together with two key cytokines produced by the DCs, i.e. IL-12 and IFN-α, in healthy humans during a regular sleep-wake cycle and during 24 hours of sustained wakefulness. Compared with wakefulness, sleep induced throughout the night a striking increase in the number of pre-mDC producing IL-12, which is a main inducer of Th1 responses. In addition, sleep reduced circulating numbers of PDC and $CD14^{dim}CD16^{+}$ monocytes probably due to extravasation.

Introduction

Available data point to an enhancing role of sleep for immune defense (Bryant et al., 2004; Marshall & Born, 2002; Benca & Quintas, 1997). Experimental sleep loss induced acutely after vaccination reduced antibody titers in response to influenza A and hepatitis A virus vaccines (Spiegel et al., 2002; Lange et al., 2003). In the latter study by Lange et al, sleep was deprived on the first night, ie, within 24 hours, after vaccination (taking place in the morning at 9:00 AM). This observation indicates an influence at a rather early stage in the formation of an adaptive immune response that could be conveyed, eg, by effects of sleep on the redistribution of circulating immune cells and their cytokine production. In fact, in comparison with wakefulness, regular nocturnal sleep generally reduces the number of leukocytes in blood but increases their numbers in lymphatic tissues (Dickstein et al., 2000; Born et al., 1997; Dinges et al., 1994; Engeset et al., 1977). Moreover, sleep increases the production of T cell-derived interleukin-2 (IL-2) and activity of other type 1 cytokines

supporting adaptive immune defense (Born et al., 1997; Dimitrov et al., 2004). Overall these studies suggest a fine-tuned orchestration of the distribution and cytokine production of T cells through sleep processes, as well as by supraordinate circadian oscillators, whereby part of these functions becomes subjected to a 24-hour oscillation independently of sleep (Haus & Smolensky, 1999; Petrovsky, 2001).

During an immune response, T cell activity critically depends on antigen-presenting cells (APCs) that can drive T cells toward type 1 or type 2 responses. However, despite this key role of APCs in T cell regulation and recent observations indicating that sleep increases type 1 cytokine activity in an undifferentiated monocyte population (Lange et al., 2006), so far there are no studies that have focused on specific subsets of APC precursors in peripheral blood as primary targets of sleep-associated changes toward enhanced adaptive immunity.

Of the different types of APC, dendritic cells (DCs) are the most effective because of their widespread localization at all sites of antigen entry, their strong expression of MHC class II and costimulatory molecules, and their production of important regulatory cytokines, such as interleukin-12 (IL-12), in response to different types of antigens (Langenkamp et al., 2000). However, IL-12 is produced only transiently after DC maturation, and its local availability therefore depends on a continuous migration of DC precursors from peripheral blood into the tissue (Sallusto, 2001). The major DC precursors are monocytes and pre-DCs.

Monocytes represent by far the most abundant DC precursors in peripheral blood. However, they are relatively early precursors because they still maintain potential differentiation to either macrophages or DCs and, in vitro, require extensive maturation before developing typical DC features (Fagnoni et al., 2001). In peripheral blood, monocytes are identified by expression of CD14 and divide into at least 2 subsets according to CD16 expression, ie, the ordinary $CD14^{+}CD16^{-}$ monocytes and the rare $CD14^{dim}CD16^{+}$ monocytes accounting for only 10% to 15 % of all monocytes (Passlick et al., 1989).

Pre-DCs relate much more closely to mature DCs than to monocytes (Fagnoni et al., 2001) and divide into at least 2 subsets with different functional capacities, ie, pre-myeloid DCs (pre-mDC) and plasmacytoid DCs (PDC), which are distinguished in human blood by a myeloid (CD11c, CD33) or plasmacytoid (CD123) marker. Pre-mDC represent 0.5% to 1.0 % and PDC only 0.2% to 0.5% of circulating mononuclear cells. Both DC precursors differ fundamentally in their migratory behavior and cytokine secretion pattern. While pre-mDC are characterized mainly by production of IL-12, a key factor in the induction of Th1-mediated responses, PDC start upon viral infection to produce large amounts of interferon-α (IFN-α), ie, up to 200 to 1000 times more than other blood cells after viral challenge (Liu, 2005).

Whereas effects of sleep and circadian rhythm have been investigated in undifferentiated monocyte populations (Born et al., 1997; Haus & Smolensky, 1999), here we aimed for the first time at dissociating such influences in specific subpopulations of DC precursors in blood from healthy human donors. The number of circulating monocyte subpopulations ($CD14^+CD16^-$ and $CD14^{dim}CD16^+$), pre-mDC, and PDC together with 2 key cytokines produced by these DC precursors (IL-12 and IFN-α) were examined during a regular sleep-wake cycle and a 24-hour period of continuous wakefulness. Natural killer (NK) and T cell counts known to display circadian peaks during daytime and nighttime, respectively (Born et al., 1997), were also measured. Since numbers and cytokine production of DC precursors can be affected by immunoactive hormones (such as growth hormone [GH], prolactin, cortisol, and catecholamines) known to be specifically regulated by sleep (Shodell et al., 2003; Steppich et al., 2000; Born & Fehm, 1998), we also monitored plasma concentration of these hormones to reveal hints at a possible endocrine mediation of the effects of sleep.

Methods

Subjects

Twenty-seven physically and mentally healthy men participated in the study (mean age 24 years, range 18-30 years, mean body mass index 23 kg/m^2, range 20-26 kg/m^2). They were nonsmokers, did not suffer from sleep disturbances, and were not taking any medication at the time of the experiments. None had a medical history of any relevant chronic disease or mental disorder. Acute illness was excluded by physical examination and routine laboratory investigation, including chemistry panel, C-reactive protein concentration < 6 mg/L, and a white blood cell (WBC) count < 9000/μL.

The men were synchronized by daily activities and nocturnal rest. They had a regular sleep-wake rhythm for at least 6 weeks before the experiments. During the week preceding the study, they were required to turn off lights for nocturnal sleep between 11:00 PM and 11:30 PM, to get up by 7:00 AM the next morning, and not to take any naps during the day. The presence of signs of sleep disturbances, including apnea and nocturnal myoclonus, was excluded by interview and by recordings during a separate adaptation night. All subjects were adjusted to the experimental setting by spending at least 1 adaptation night in the laboratory that took place before the experiment proper and included the attachment of all electrodes for

sleep recordings. Written informed consent was obtained by each participant, and the study was approved by the local ethics committee.

Experimental Design and Procedure

Experiments were performed according to a within-subject cross-over design. Each man participated in 2 experimental conditions, each starting at 8:00 PM and ending 24 hours later on the next day. One condition ("Sleep") included a regular sleep-wake cycle while, in the other, ("Wake") subjects remained awake continuously throughout the 24-hour experimental period. Both experimental sessions for a subject were separated by at least 4 weeks, and the order of conditions was balanced across subjects. To exclude possible psychological effects deriving from anticipating a night of wakefulness or of regular sleep, subjects were generally kept misinformed about the actual condition established on a particular night until 11:00 PM, ie, the time lights were turned off on the sleep condition.

On experimental nights, subjects arrived at the laboratory at 6:00 PM for preparing standard polysomnographic recordings and blood sampling. In the sleep condition, sleep was allowed between 11:00 PM (lights off) and 7:00 AM in the morning. In the wake condition, subjects stayed awake in bed during this period in a half-supine position, watching TV, listening to music, and talking to the experimenter at normal room light (about 300 lux). For both sessions, standardized meals were provided at appropriate times for breakfast (8:00 AM), lunch (12:00 PM), and dinner (6:00 PM).

On both conditions, blood was sampled first at 8:00 PM, then every 1.5 hours between 11:00 PM and 8:00 AM, and every 3 hours between 8:00 AM and 8:00 PM the next day. Blood was sampled via an intravenous forearm catheter that was connected to a long thin tube and enabled blood collection from an adjacent room without disturbing the subject's sleep. To prevent clotting, about 700 mL of saline solution were infused throughout the 24-hour experimental period. The total volume of blood sampled during a session was 250 mL. Blood samples were always processed immediately after sampling.

Sleep and sleep stages were determined off-line from polysomnographic recordings following standard criteria (Rechtschaffen & Kales, 1968). For each night, sleep onset (with reference to lights off at 11:00 PM), total sleep time, and the time as well as the percentage of total sleep time spent in the different sleep stages were determined. Sleep stages are wake, non-rapid eye movement (NREM) sleep stages 1, 2, 3, and 4, slow-wave sleep (SWS, equal to the sum of stage 3 and 4 sleep), and rapid eye movement (REM) sleep.

Determination of Absolute Counts of Monocytes, DC Precursors, and NK Cell Subpopulations

Identification of cell subsets, based on a flow cytometry "lyse no-wash" procedure, was performed first to identify pre-mDC as $CD14^{-}CD16^{-}HLA\text{-}DR^{+}CD33^{+}$ cells, as previously described (Ma et al., 2004). (In some of the samples, pre-mDC were identified in parallel with $Lin^{-}HLA\text{-}DR^{+}CD11c^{+}$ cells, which yielded essentially identical results; $r = 0.9$, $p < .001$, for cross-correlation). Using antibodies (CD14/F, CD16/F, CD3/PE, CD16/PE, CD123/PE, CD45/PerCP, HLA-DR/PerCP, CD33/APC, CD56/APC, and HLA-DR/APC) from BD Biosciences (San Jose, Calif), we then additionally determined the number of circulating monocyte and NK cell subpopulations and PDC and T cells. For determination of absolute counts, 50 μL phosphate buffered saline solution of brightly fluorescent polystyrene beads (Fluoresbrite® YG Microspheres 6.00 μm, Polysciences Europe, Eppelheim, Germany) with a concentration of approximately1000 microspheres per μL was used. The exact concentration of the microspheres was obtained by comparison with tubes of known microsphere concentration (TruCOUNT Control, BD Biosciences, San Jose, Calif), and it was ensured that tube-to-tube variation during pipetation of the microspheres did not exceed 10%. Samples were mixed gently, and at least 100,000 leucocytes were acquired on a FACS Calibur for analysis using CellQuest Software (BD Biosciences; refer to Figure 1 for the gating strategy used to identify monocyte subpopulations, DC precursors, NK cell subpopulations, and T cells). The absolute number of the cells per microliter blood was calculated as previously described (Ma et al., 2004), using the following formula: Cells/μL = (acquired cell events in the respective gate) × (number of beads per tube)/(acquired bead events) × (sample volume [μL]).

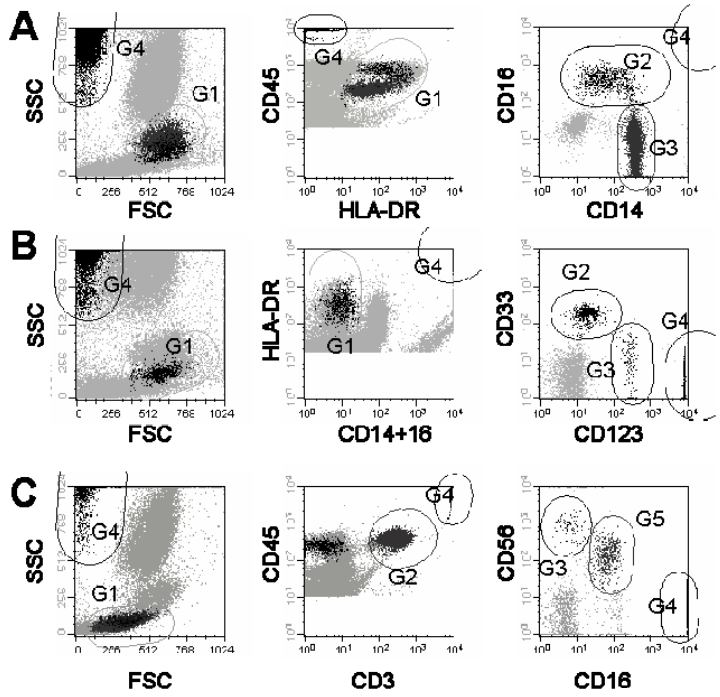


Figure 1—Gating strategy for identifying monocyte, dendritic cell (DC) and natural killer (NK) cell subpopulations by multiparametric flow cytometry. (A) $CD14^{dim}CD16^{+}$ vs $CD14^{+}CD16^{-}$ monocytes: A gate G1 is drawn around putative monocyte populations based on their (left panel) forward and side scatter (FSC and SSC) characteristics and expression of (middle panel) CD45 and HLA-DR antigen molecules. Gate G2 (right panel) defines $CD14^{dim}CD16^{+}$ monocytes, whereas gate G3 defines $CD14^{+}CD16^{-}$ monocytes. Note, NK cells, which are $CD14^{-}CD16^{+}$, do not falsely contribute to $CD14^{dim}CD16^{+}$ monocytes because they are HLA-DR negative and thus excluded from G1. (B) myeloid dendritic cell precursors (pre-mDC) and plasmacytoid dendritic cells (PDC): A gate G1 (middle panel) is drawn around putative $CD14^{dim/-}CD16^{dim/-}$HLA-DR^{+} dendritic cell precursors. Note (left panel), these cells, in comparison with lymphocytes and monocytes, have intermediate FSC and SSC characteristics. Gate G2 (right panel) defines $CD33^{+}CD123^{-}$ dendritic cell precursors (pre-mDC), whereas gate G3 defines $CD123^{+}CD33^{-}$ dendritic cells (PDC). (C) NK and T cells: Gate G1 (left panel) is drawn around putative lymphocyte populations based on their FSC and SSC characteristics. Gate G2 defines $CD3^{+}$ T cells, whereas gate G3 and G5 defines $CD16^{-}CD56^{bright}$ and $CD16^{+}CD56^{dim}$ $CD3^{-}$ NK cells, respectively. Gate G4 is drawn around the fluorescent beads to allow for measuring absolute counts of the monocyte, DC, and NK cell subpopulations.

Production of IL-12 and IFN-α in DC Precursors

The stimulated production of IL-12 in pre-mDC was evaluated at the single cell level by multiparametric flow cytometry, as previously described (Dimitrov et al., 2006; Lange et al., 2006). Blood was incubated with LPS at a final concentration of 20 ng/mL at 37 ºC in a 5% CO_2 atmosphere for 6 hours. For stopping cytokine excretion Brefeldin A (Sigma-Aldrich Co., St. Louis, MO) was added at a final concentration of 10 µg/ml the last 4 hours. Intracellular IL-12 staining was performed according to the manufacturer's instructions using antibodies (IL-12/PE, p40/p70, clone C11.5) and reagents from BD Biosciences (San Jose, CA). At least 1,000 $CD14^{-}CD16^{-}$HLA-$DR^{+}CD33^{+}$ cells (pre-mDCs) were acquired and

subsequently analyzed for the expression of IL-12 by a FACS Calibur using CellQuest Software (BD Biosciences). Results for cytokine positive cells were expressed as percentages of the total number of pre-mDCs.

The capacity of PDC to produce IFN-α was assessed by stimulating total blood with herpes simplex virus-1 (HSV-1). The whole blood assay is a common and effective method for analyzing production of IFNs and provides an appropriate model of the in vivo situation. After drawing samples, heparinized venous blood was diluted 1:1 with RPMI 1640 medium in 96-well U-bottom plates preloaded with UV-inactivated HSV-1 at a final concentration of 10^4 plaque forming units per milliliter. Culture supernatants were harvested after 24 hours of incubation at 37ºC and were stored at -80ºC until analysis. IFN-α concentrations were determined by human IFN-α multisubtype ELISA kit (R&D Systems, Minneapolis, Minn, sensitivity 40 pg/mL). IFN-α concentrations in unstimulated control (without HSV-1) were below the detection limit of the ELISA.

Hormone Assays

GH, prolactin, and cortisol were measured in serum using commercial assays (Immulite, DPC-Biermann GmbH, Bad Nauheim, Germany). Norepinephrine was measured in plasma by standard high-performance liquid chromatography.

Statistical Analysis

Statistical analysis was based on repeated-measures analysis of variance (ANOVA), including the factors "Sleep/Wake" (regular sleep-wake cycle versus 24-hour wakefulness); "Night/Day," reflecting average values for the nocturnal [11:00 PM-07:00 AM] and diurnal [8:00 AM-8:00 PM] periods; and "Time," representing the different time points of measurement. Posthoc contrasts were applied to analyze differences at particular time points. Because of the limited amount of blood available per subject, not all parameters were measured in the blood from 1 individual but, combinations of the parameters measured were distributed over different subgroups. For additional cross-correlation analyses, subpopulation counts and hormonal levels were expressed at each time point as percentages of the individual 24-hour mean. Pearson-moment correlation coefficients were calculated across all time points and across subjects without or with 3 or 6 hours of delay between subpopulation counts and

hormonal levels. To identify circadian rhythms, cosinor analysis was performed separately for the wake and sleep condition using Chronolab (Mojon et al., 1992).

Results

Sleep Decreases Selectively the Number of Circulating $CD14^{dim}CD16^{+}$ Monocytes

Total monocyte counts are summarized in Figure 2A. During continuous 24-hour wakefulness they showed a slight increase in the early night (11:00 PM - 2:00 AM), which was diminished when the subject slept. However, neither the circadian variation observed during constant wakefulness nor the effect of nocturnal sleep reached significance ($p > .11$).

Within the monocyte scatter gate, $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{-}$ cells were separated. Compared with continuous wakefulness, sleep distinctly decreased the number of circulating $CD14^{dim}CD16^{+}$ monocytes. The drop was pronounced during the whole sleep period, with minimum counts reached during the second half of the night ($F_{1,26} = 17.3$, $p < .001$ for Sleep/Wake × Night/Day, Figure 2B). Pairwise tests at single points in time revealed significance at 12:30 AM, 2:00 AM, 5:00 AM, and 6:30 AM ($p < .01$) and at 3:30 AM ($p < .05$). Cosinor analyses confirmed the absence of any circadian rhythm during continuous wakefulness ($p > .15$). Applied to the data of the sleep condition, a systematic diurnal fluctuation was revealed ($p < .001$), with the fitted cosine curve showing a mean level (mesor) of 57.0 ± 4.0 cells/μL, a (peak-to-trough) amplitude of 6.5 ± 1.5 cells/μL, and an acrophase (peak time) at 3:48 PM ± 68 min.

In contrast to $CD14^{dim}CD16^{+}$ monocytes, $CD14^{+}CD16^{-}$ monocyte counts were not influenced by sleep ($p > .20$, for respective main and interaction effects, Figure 2C). Cosinor analyses also did not provide any hint at the presence of a circadian rhythm in $CD14^{+}CD16^{-}$ monocyte counts during wakefulness or the regular sleep-wake cycle ($p > .30$).

The selective drop of $CD14^{dim}CD16^{+}$ monocyte counts during nocturnal sleep, with unchanged $CD14^{+}CD16^{-}$ counts, led to an imbalance between these populations with respect to the total number of circulating monocytes. Thus, the proportion of $CD14^{dim}CD16^{+}$ cells in the total pool of circulating monocytes was distinctly lowered during sleep compared to the wake condition ($F_{1,26} = 13.3$, $p < .001$ for Sleep/Wake × Night/Day, refer to Figure 2D for results from pairwise tests at single time points). The opposite held for the proportion of $CD14^{+}CD16^{-}$ cells, being higher during nocturnal sleep than wakefulness ($F_{1,26} = 14.5$, $p < .001$).

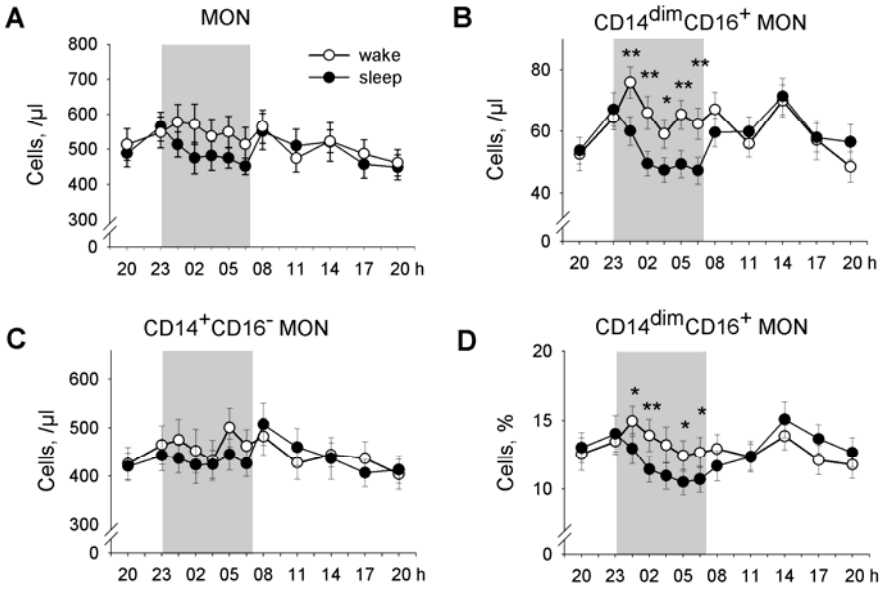


Figure 2—Sleep decreases circulating $CD14^{dim}CD16^{+}$ monocyte counts but does not affect $CD14^{+}CD16^{-}$ monocyte counts. Cells were enumerated in peripheral blood from healthy men during a regular sleep-wake cycle (sleep) and during 24 hours of continuous wakefulness (wake). (A) Absolute monocyte counts, (B) absolute counts and underneath (D) percentages (with reference to total monocyte counts) of $CD14^{dim}CD16^{+}$ monocytes, and (C) absolute count of $CD14^{+}CD16^{-}$ monocytes. Means (± SEM) are shown; n = 27. Shaded area indicates bed time. ** $p < .01$; *$p < .05$ for pairwise comparison between conditions at single time points.

Pre-mDC and PDC Counts Reveal Prominent Circadian Variations

Pre-mDC counts did not differ between sleep and wake conditions ($p > .22$, for respective comparisons). PDC counts showed a slight, marginally significant drop during the sleep period ($F_{1,26} = 4.4$, $p < .05$ for Sleep/Wake × Night/Day, Figure 3). However, cell counts for both populations revealed clear circadian variations that were independent of sleep ($p < .001$). Maximum counts of pre-mDC were generally reached in the middle part of the night (~2:30 AM), whereas PDC counts peaked in the morning hours (~7:00 AM). Results from cosinor analysis of these rhythms are summarized in the Table.

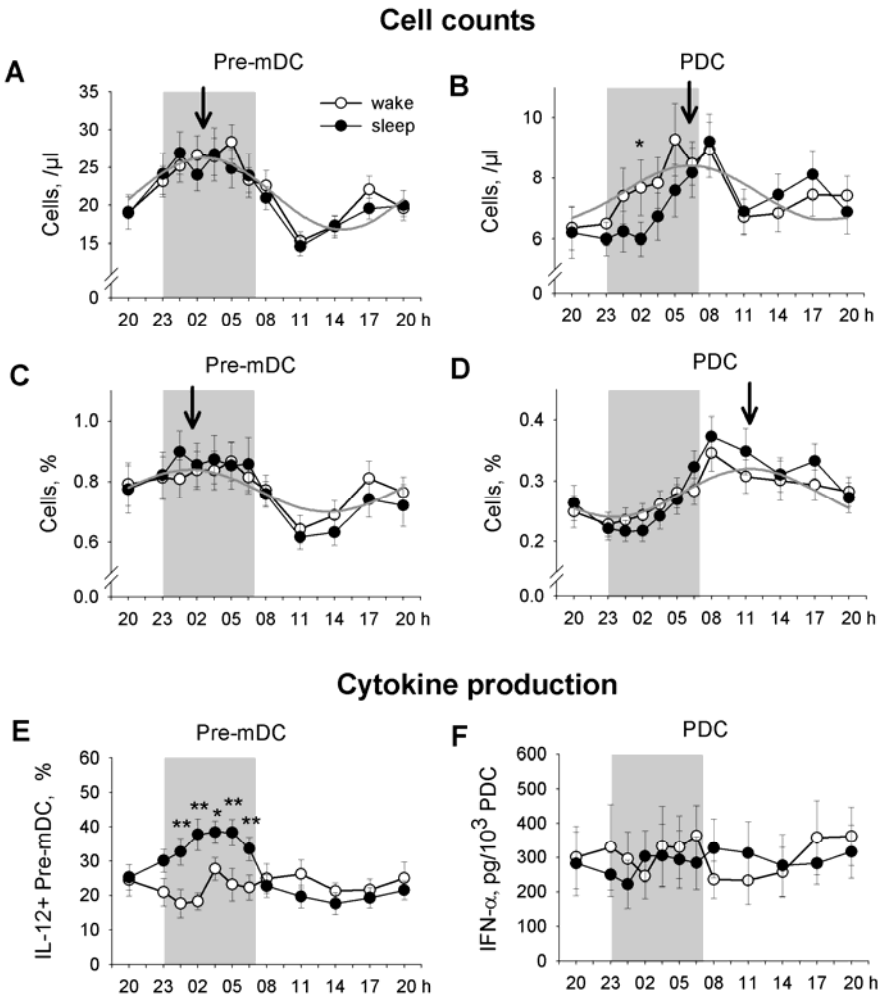


Figure 3—Sleep is associated with a slight reduction in circulating plasmacytoid dendritic cell (PDC) counts and a strong increase in myeloid dendritic cell precursors (pre-mDC) producing IL-12. Cells were analyzed by flow cytometry in peripheral blood from healthy men during a regular sleep-wake cycle (sleep) and during 24 hours of continuous wakefulness (wake). Absolute counts and percentages (with reference to mononuclear cell total count) of pre-mDC (A and C), and of PDC (B and D); n = 27. (E) The percentage of pre-mDC producing IL-12 after lipopolysaccharide (LPS) stimulation; n = 11. (F) Interferon (IFN)-α production of PDC, measured by ELISA in whole blood samples after herpes simplex virus (HSV)-1 stimulation, divided by PDC numbers; n = 14. Means (± SEM) are shown. Shaded area indicates bed time. Grey lines represent cosine curves fitted to data during continuous wakefulness to indicate significant circadian variation, with the arrow marking the peak time (acrophase). **p < .01; *p < .05 for pairwise comparison between conditions at single time points.

Table—Circadian Rhythms of pre-mDC and PDC Parameters as Described by Fitted Cosine Curves During 24-Hours of Continuous Wakefulness and a Regular Sleep-Wake Cycle[a]

Parameter	Mean level	Amplitude	Peak time, time ± min	p Value
Pre-mDC, /μL				
24-hour wakefulness	21.5 ± 1.5	4.8 ± 0.9	02:38 ± 29	< .001
Sleep-wake	20.9 ± 1.7	5.0 ± 1.1	02:06 ± 29	< .001
Pre-mDC, %[b]				
24-hour wakefulness	0.77 ± 0.05	0.07 ± 0.01	01:25 ± 60	< .02
Sleep-wake	0.76 ± 0.06	0.12 ± 0.01	01:58 ± 23	< .001
PDC, /μL				
24-hour wakefulness	7.5 ± 0.6	0.9 ± 0.2	06:19 ± 75	< .001
Sleep-wake	7.2 ± 0.7	1.0 ± 0.3	10:36 ± 50	< .002
PDC, %[b]				
24-hour wakefulness	0.28 ± 0.02	0.04 ± 0.01	11:24 ± 36	< .001
Sleep-wake	0.29 ± 0.02	0.07 ± 0.01	11:50 ± 27	< .001

[a]Data are presented as mean ± SEM and were derived by cosinor analysis; n = 27. Raw data were obtained in 27 men every 1.5 hours during the night and every 3 hours during daytime of a 24-hour interval. Pre-mDC refers to myeloid dendritic cell precursors; PDC, plasmacytoid dendritic cells.

[b]Values represent percentages with reference to the total number of mononuclear cells.

Sleep Increases Production of IL-12 by Pre-mDC, But Not IFN-α Production of PDC

In addition to cell counts, we measured production of 2 key cytokines by pre-mDC and PDC to characterize sleep-dependent functional changes in these populations. Intracellular IL-12 in pre-mDC was assessed by multiparametric flow cytometry in a whole blood culture system after stimulating the cells with lipopolysaccharide (LPS). Compared with the wake condition, sleep strikingly increased the percentage (with reference to total pre-mDC counts) of pre-mDC producing IL-12. The rise extended over the whole nocturnal sleep period and peaked around midnight ($F_{1,10} = 20.4$, $p < .001$ for Sleep/Wake × Night/Day; refer to Figure 3E for pairwise comparisons at single points in time).

IFN-α production by PDC was determined in supernatant after stimulation with HSV-1. Absolute IFN-α concentrations did not differ between the sleep and wake conditions ($p > .1$) but revealed a circadian rhythm with peak concentrations at approximately 10:00 AM ($p < .05$). However, this rhythm completely disappeared when IFN-α production was determined relative to the number of PDC stimulated with HSV-1 ($p > .41$, Figure 3F), indicating that changes in IFN-α primarily reflect changes in circulating PDC numbers. Also, there was no difference between the sleep and wake condition in the percentages of IFN-α producing PDC ($p > .49$).

NK and T cells

We contrasted changes in APC precursor counts with those of 2 lymphocyte subpopulations (NK and T cells), which, in previous studies, were revealed to be sensitive to effects of sleep and circadian rhythm. The average number of NK cells during the night was markedly lower in sleeping than waking subjects ($F_{1,26}$ = 11.8, $p < .002$ for Sleep/Wake × Night/Day, Figure 4A). In addition, cosinor analysis revealed the expected circadian fluctuation with peak NK cell counts at approximately 2:30 PM ($p < .001$, for both conditions). To clarify whether these changes differed for the major NK subpopulations, we enumerated cells for the $CD16^-CD56^{bright}$ and $CD16^+CD56^{dim}$ subsets of NK cells. As expected, counts of $CD16^+CD56^{dim}$ cells representing the predominant phenotype of NK cells, reflected the same sleep-dependent drop ($F_{1,13}$ = 6.6, $p < .02$) and the same circadian variation ($p < .001$, Figure 4B) seen for total NK cell counts. In contrast, $CD16^-CD56^{bright}$ counts remained uninfluenced by sleep ($p > .41$) or circadian rhythm ($p > .18$, Figure 4C).

T cell counts displayed a slight but consistent drop during nocturnal sleep, as compared with nocturnal wakefulness ($F_{1,26}$ = 6.2, $p < .05$ for Sleep/Wake × Night/Day, Figure 4D). Moreover, cosinor analysis revealed strong circadian variations with maximum values at approximately 2:00 AM at night on both conditions ($p < .001$).

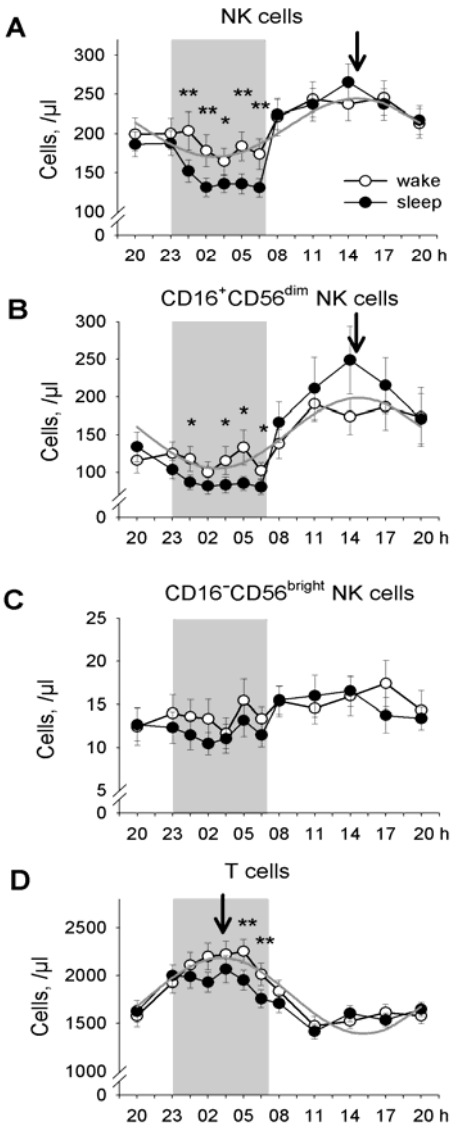


Figure 4—Sleep is associated with a decrease in circulating natural killers (NK) and T cells on top of distinct circadian rhythms. Cells were analyzed by flow cytometry in peripheral blood from healthy men during a regular sleep-wake cycle (sleep) and during 24 hours of continuous wakefulness (wake). (A) Absolute count of NK cells; n = 27. Within the NK cell population, the prevailing (> 90%) $CD16^+CD56^{dim}$ NK cells (B) and the $CD16^-CD56^{bright}$ subpopulation (C) were differentiated, with the latter subpopulation not influenced by sleep or circadian rhythm; n = 14 for both NK subpopulations. (D) Absolute count of T cells; n = 27. Means (± SEM) are shown. Shaded area indicates bed time. Grey line represents a cosine curve fitted to data of the wake condition to indicate significant circadian rhythm, with the arrow marking the peak time (acrophase). **p < .01; *p < .05 for pairwise comparison between conditions at single time points.

Sleep and Hormones

Polysomnographic recordings ensured that sleep in our subjects was typical for laboratory conditions. Mean (± SEM) sleep onset was 18.8 ± 2.7 minutes. Mean values were for sleep time 444.7 ± 8.9 minutes; time in stage 1 sleep, 32.1 ± 4.2 minutes, stage 2 sleep, 240.3 ± 8.6 minutes; SWS, 71.3 ± 5.0 minutes; and REM sleep, 78.7 ± 4.9 minutes. Latency (with reference to sleep onset) was 20.2 ± 2.0 minutes for SWS and 121.5 ± 10.5 minutes for REM sleep. In all subjects, SWS predominated during the early part of the night (50.5 ± 4.6 minutes vs 20.8 ± 3.4 minutes during the late night), whereas REM sleep dominated during the second half of the night (17.9 ± 2.4 minutes vs 60.8 ± 3.9 minutes, during early and late night, respectively; all differences $p < .001$).

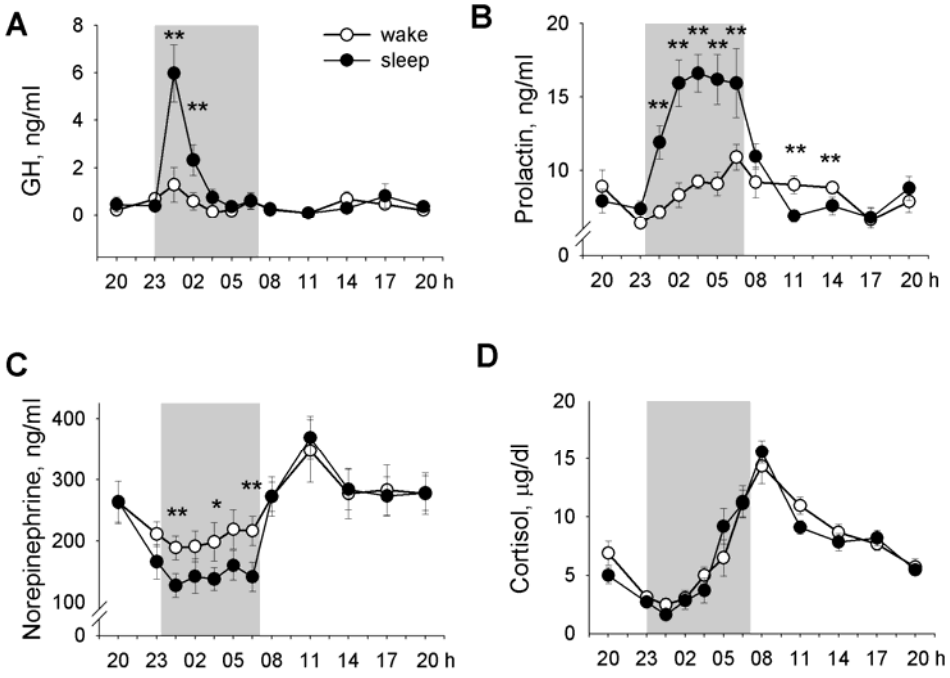


Figure 5—Sleep stimulates release of growth hormone (GH) and prolactin and reduces norepinephrine concentrations. Plasma concentrations of (A) GH, (B) prolactin, (C) norepinephrine, and (D) cortisol were measured during a regular sleep-wake cycle (sleep) and during 24 hours of continuous wakefulness (wake); n = 14. Shaded area indicates bed time. **p < .01; *p < .05, for pairwise comparison between conditions at single time points.

Compared with nocturnal wakefulness, regular sleep was, as expected, characterized by a distinct pattern of endocrine changes (summarized in Figure 5). Concentrations of GH were increased selectively during early sleep, between 12:30 AM and 2:00 AM ($F_{1,13} = 19.3$, $p < .001$, for Sleep/Wake × Night/Day, see Figure 5 for comparisons at single time points). Plasma concentration of prolactin were strongly enhanced during sleep throughout the night,

compared with the wake condition ($F_{1,13}$ = 38.9, $p < .001$). In contrast, norepinephrine concentrations showed a nocturnal drop ($p < .03$, for the circadian variation during wakefulness), which was amplified by sleep during the night ($F_{1,13} = 4.4$, $p < .05$, Sleep/Wake ×Night/Day). Cortisol concentrations did not differ between sleep and wake conditions ($p>.69$) but showed a pronounced circadian rhythm ($p < .001$), with a minimum at 12:30 AM and a maximum at approximately 8:00 AM.

Temporal Relationships Between Immune, Sleep and Hormonal Parameters

We calculated cross-correlation functions between immune cell counts and cytokine production on the one hand and sleep and hormonal parameters on the other hand. These analyses did not reveal hints at a systematic relationship between the time spent in SWS or REM sleep during the whole night or during the first or second half of the night and the various immune parameters of interest ($p > .1$, for all correlations).

However, cell counts and cytokine production were specifically linked to hormonal changes (summarized in Figure 6). GH concentrations during sleep were correlated (at a 0 time lag) positively with the numbers of pre-mDC ($r = .41$, $p < .001$) and of T cells ($r = 0.36$, $p < .001$). High prolactin concentrations during sleep were associated (with 0 time lag) with low $CD14^{dim}CD16^{+}$ monocyte ($r = -0.27$, $p < .001$) and NK cell counts ($r = -0.42$, $p < .001$, Figure 6). Changes in norepinephrine concentrations during sleep were positively correlated with NK cell numbers ($r = 0.50$, $p < .001$) and numbers of $CD14^{dim}CD16^{+}$ monocytes, with the latter relationship reaching a maximum at a phase delay of 3 hours for $CD14^{dim}CD16^{+}$ monocyte count ($r = 0.31$, $p < .001$, Figure 6). Norepinephrine also correlated negatively with pre-mDC ($r = -0.49$, $p < .001$) and T cell counts ($r = -0.54$, $p < .001$). In the wake condition, these correlations were weaker, although, overall, the pattern was similar. Cortisol concentrations were generally negatively correlated with counts of pre-mDC and T cells and positively with NK cells counts. These correlations were maximal when cell counts were measured with a delay of 3 hours with reference to cortisol concentrations (pre-mDC - $r = -0.63$, $p < .001$; T cells - $r = -0.75$, $p < .001$, NK cells $r = 0.43$, $p < .001$, Figure 6).

The production of IL-12 by pre-mDC during sleep was linked to high levels of prolactin ($r = 0.47$, $p < .001$) and low levels of norepinephrine ($r = -0.37$, $p < .001$) and, with a delay of 3 hours, with low levels of cortisol ($r = -0.53$, $p < .001$, Figure 6). This relationship was not present in the wake condition ($r < .09$).

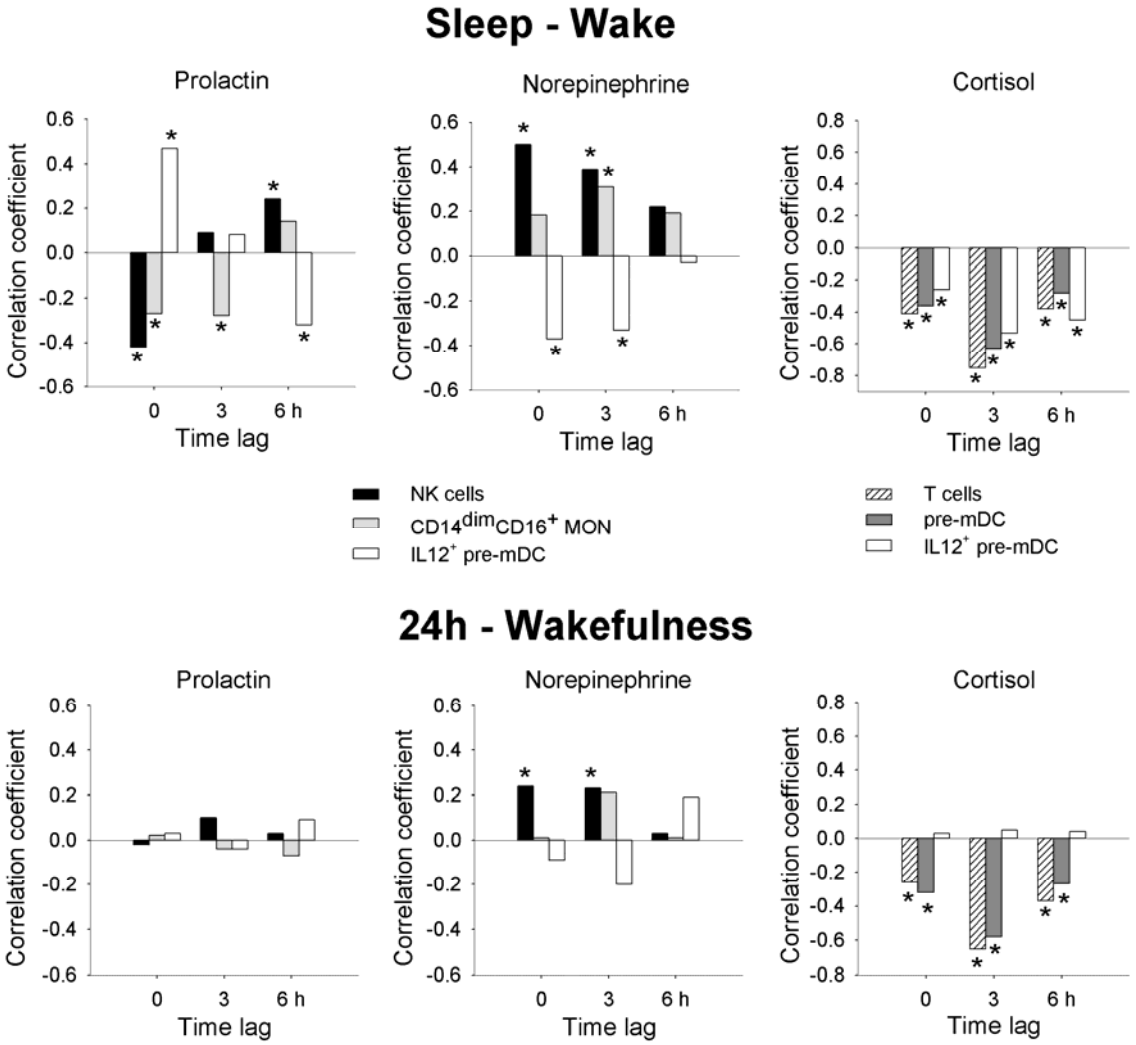


Figure 6—Cross-correlations between plasma prolactin (left), norepinephrine (middle), and cortisol concentrations (right) and, respectively, relevant mononuclear cell counts and percentage of myeloid dendritic cell precursors (pre-mDC) producing interleukin (IL)-12. Correlations were calculated separately for the condition of (top) a regular sleep-wake cycle and (bottom) 24 hours of continuous wakefulness. A time lag (x-axis) indicates that changes in cell counts followed with a delay of, respectively, 3 or 6 hours, the changes in hormonal concentration. Note, only highly significant correlation coefficients are marked by asterisk: *p < .001.

Discussion

Our data indicate that sleep, beyond circadian rhythm, specifically affects the redistribution of DC precursor subpopulations and their ability to produce cytokines. In comparison with continuous 24-hour wakefulness, sleep was associated with a striking increase in the number of pre-mDC producing IL-12, a cytokine centrally involved in initializing a Th1 cell-mediated immune response (Kalinski et al., 1999). Because IL-12 was measured after in-vitro stimulation with LPS, the increase reflects the capacity of these cells to respond with IL-12 to antigen but not the spontaneous IL-12 production by these cells, which, in the circulation, is

negligible (~0.1 % of monocytes). Additionally, sleep led to slight decreases in numbers of circulating PDC and T cells but did not affect production of IFN-α by PDC. Sleep, however, substantially decreased the number of circulating $CD14^{dim}CD16^{+}$ monocytes together with $CD16^{+}CD56^{dim}$ NK cell counts. Independent of sleep, numbers of circulating pre-mDC and PDC showed clear-cut circadian rhythms, reaching peak values, respectively, in the middle of the night and in the morning hours. Our finding of a profound sleep-dependent increase in type 1 cytokine activity specific to pre-mDC, ie, a subset of DC precursors most closely linked to mature APC in lymphoid tissue, in combination with changes in numbers of PDC and distinct monocyte subsets, provides novel evidence for a supporting role sleep plays in launching an effective T cell-mediated immune response.

In previous studies, variations of monocyte absolute counts across the 24-hour period were less regular than those of the other major leukocyte populations (Haus & Smolensky, 1999). Our data add to this picture inasmuch as the increase in monocyte counts observed in the late evening, as well as the reducing influence of sleep on nocturnal monocyte counts, unlike in some foregoing investigations (Born et al., 1997), here did not reach statistical significance. However, the population assigned as monocytes on FSC/SSC dot plot is heterogeneous and includes at least 2 monocyte and 2 DC precursor subpopulations (Fagnoni et al., 2001), each of which turned out to be specifically affected by sleep and circadian oscillators. Differentiating $CD14^{+}CD16^{-}$ monocytes, representing the largest monocyte subset, from $CD14^{dim}CD16^{+}$ monocytes revealed that sleep selectively and strongly reduced blood counts only of the latter $CD14^{dim}CD16^{+}$ population, representing 10% to 15 % of the total monocytes. $CD14^{dim}CD16^{+}$ monocytes differ functionally from $CD14^{+}CD16^{-}$ monocytes in their greater ability to invade tissues under noninflamed conditions (Geissmann et al., 2003). The sleep-dependent reduction in circulating numbers of these cells could indicate that sleep supports this function by facilitating their extravasation but could also reflect increased margination of the cells.

Along with the decrease in $CD14^{dim}CD16^{+}$ monocytes, sleep boosted a nocturnal decrease in the number of $CD16^{+}CD56^{dim}$ NK cells, representing the most frequent NK cell type in blood. In contrast, the small fraction of immunoregulatory $CD16^{-}CD56^{bright}$ NK cells, which recirculate across the lymph nodes (Cooper et al., 2001), did not exhibit sleep-dependent or circadian variations. The common decrease in numbers of $CD14^{dim}CD16^{+}$ monocytes and $CD16^{+}CD56^{dim}$ NK cells during nocturnal sleep might reflect a particular sensitivity of these cells that are $CD62L^{-}$ to the influence of catecholamines. Previous studies have demonstrated a catecholamine-dependent mobilization of $CD14^{dim}CD16^{+}$ monocytes and

$CD16^{+}CD56^{dim}$ NK cells in response to physical and psychological stressors, probably from the marginal pool (Steppich et al., 2000; Bosch et al., 2005; Benschop et al., 1996). Corroborating this view, our findings not only replicate previous observations that sleep amplifies the nighttime decrease in norepinephrine concentration (Dodt et al., 1997), but also show that $CD14^{dim}CD16^{+}$ monocyte and $CD16^{+}CD56^{dim}$ NK cell numbers were distinctly correlated with norepinephrine concentrations, particularly in the sleep condition.

Numbers of pre-mDC and PDC, as well as T cell counts, revealed prominent circadian rhythms. Sleep slightly modulated the rhythm of PDC and T cells by decreasing their nocturnal counts, with the latter finding replicating previous observations (Born et al., 1997). The pronounced circadian variation in T cell counts, which correlated negatively and with a time lag of 3 hours to the fluctuations in cortisol concentrations, can be ascribed to the suppressing influence of cortisol, which is known to decrease, with a delay of about 3 hours, the number of these cells in blood (Ottaway & Husband, 1994; Kronfol et al., 1997). Whether these mechanisms can be generalized to pre-mDC counts showing a circadian variation remarkably similar in phase to that of T cells, remains to be investigated. The variation in T cell numbers reflects a redistribution of the cells between blood, bone marrow, and lymphoid tissues. In mice, the circadian maximum of T cell counts in peripheral blood is followed by a maximum of these cells in spleen with a delay of 2 hours (Ottaway & Husband, 1994). Complementing the sleep-dependent decrease in T cells in peripheral blood, animal and human studies have provided evidence for a sleep-dependent accumulation of T cells in lymphoid tissues (Dickstein et al., 2000; Engeset et al., 1977), and this could also hold for PDC (Liu, 2005; von Andrian & Mempel, 2003).

The central finding of this study is that sleep strikingly increases pre-mDC producing IL-12, which is a main inducer of the Th1 response (Kalinski et al., 1999). In comparison with wakefulness, sleep almost doubled the percentage of pre-mDC producing IL-12 after stimulation with LPS, predictably increasing the efficacy of emerging T cell responses (Trinchieri, 2003). In fact, the increase in $IL\text{-}12^{+}$ pre-mDC observed here during sleep, which amounted to 166% of the level during wakefulness, was distinctly stronger and more consistent than sleep-dependent increases in type 1 cytokine activity revealed in foregoing studies in T cells and monocyte populations.(Dimitrov et al., 2004; Petrovsky & Harrison, 1997; Lange et al., 2006) For the production of IL-12 in an undifferentiated sample of monocytes, a sleep-dependent increase of comparable size (~170 % compared to levels during nocturnal wakefulness) was revealed in 1 of these studies (Lange et al., 2006), but this increase was remarkably variable compared with the increase in $IL\text{-}12^{+}$ pre-mDC observed

here, supporting the view that sleep does not affect the population of monocytes in a global way. Notably, within the set of DC precursors examined here, the enhancing effect of sleep was found to be specific to that subpopulation most representative for mature APC function in lymphoid tissue (Fagnoni et al., 2001; O'Doherty et al., 1994).

Among the endocrine signals possibly mediating this effect on pre-mDCs, basically all of the hormones measured here have to be considered. Catecholamines via β_2-adrenergic receptors, as well as glucocorticoids via the classic glucocorticoid receptors, potently inhibit production of IL-12 (Elenkov et al., 1996; Blotta et al., 1997; Panina-Bordignon et al., 1997). Here, both norepinephrine and cortisol reached lowest concentrations during the night with the decrease in norepinephrine being most pronounced when subjects slept. In the sleep condition, the decrease in the concentration of these hormones was clearly correlated with increasing numbers of IL-12^+ pre-mDC, which concurs with the view that the sleep-wake–dependent regulation of these hormones is critically involved in mediating the effect of sleep on IL-12. However, the number of IL-12^+ pre-mDC was also correlated with concentrations of prolactin and, to a lesser degree, of GH, which both showed the well-known increase during sleep (Spiegel et al., 1995; Van & Copinschi, 2000). The correlation with prolactin concentrations ($r = 0.47$) was roughly similar to that observed in a previous study in our lab (Lange et al., 2006) for the production of IL-12 in an undifferentiated sample of monocytes (amounting to $r = 0.33$; calculated with the same algorithm), which suggests a comparable sensitivity to prolactin in these populations. Triggering the monocytes prolactin receptor at concentrations close to the upper limit of the physiologic range significantly amplified the LPS-stimulated synthesis of IL-12 and TNF-α in these cells through induction of nuclear factor-κB and IFN regulatory factor-1 (Brand et al., 2004). Moreover, blocking of sleep-associated prolactin with anti-prolactin antibody decreased the percentage of LPS-induced IL-12^+ monocytes in blood sampled from sleeping subjects (Lange et al., 2006). In combination, these findings support the view that sleep-dependent release of prolactin belongs to the primary factors promoting synthesis of IL-12 in DC precursors.

In contrast to IL-12 production by pre-mDC, production of IFN-α by PDC remained entirely unaffected by sleep or circadian rhythm. The circadian changes observed here in blood concentrations of IFN-α could be reduced to corresponding changes in the number of PDC representing the main source of this cytokine (Liu, 2005). Thus, immediate antiviral effects of IFN-α do not appear to be enhanced by sleep.

Focusing on DC precursor cell numbers and function, our study indicates a decreasing influence of sleep on circulating $CD14^{dim}CD16^+$ monocyte numbers that likely reflect

increased margination of these cells due to reduced catecholaminergic activity during sleep, keeping these cells in a state of rest. Mobilization of these cells during wakefulness in parallel with $CD16^{+}CD56^{dim}$ NK cells presumably strengthens innate immune defense against potential tissue damage and infection. On the other hand, sleep induced a striking increase in IL-12 producing pre-mDCs, accompanied by slight decreases in PDC and T cell counts likely reflecting increased migration to lymphoid tissues. The enhancing effect of sleep on $IL\text{-}12^{+}$ pre-mDC, being robust and specific to this DC precursor subpopulation, makes likely that APC function represents a key target for the regulatory effect of sleep on immune function. The changes indicate a supportive influence of sleep on Th1 mediated adaptive immune responses that eventually explains observation of previous studies, where sleep after vaccination increased the specific IgG1 and IgG3 antibody response to the viral vaccines (Spiegel et al., 2002; Lange et al., 2003), as well as more general clues to a supportive function of sleep for adaptive immunity (Bryant et al., 2004). The extent of the sleep-dependent increase in $IL\text{-}12^{+}$ pre-mDC encourages attempts to exploit this effect for clinical purposes.

General Discussion

The purpose of the three experiments reported in this thesis is to discover relevant immunological parameters as primary targets of sleep induced changes toward enhanced adaptive immunity. In the first experiment, we tested the hypothesis that nocturnal sleep could ease immune responses by shifting the balance between Th1 and Th2 cytokine activity towards Th1 dominance. The data partly confirmed this hypothesis. As a result of the stronger sleep associated decrease in IL-4 than IFN-γ secreting $CD4^+$ cells, the IFN-γ/IL-4 ratio of $CD4^+$ was increased during the early part of the night when subjects slept as compared with sustained wakefulness. However, the effect was of moderate size only, and was replaced by Th2 dominance during late sleep. During the early half of the night, sleep was not only dominated by SWS but showed also the typical strong increases of prolacin and GH and low concentrations of cortisol. Experiment 2 addressed the question, whether these high concentrations of prolactin and GH as well as a decrease in cortisol are responsible for the shift towards Th1 cytokines during this time. Exposure in vitro of morning blood to prolactin, GH, glucocorticoid receptor antagonist RU-486, or mineralcorticoid receptor antagonist spironolactone led to an increase in IFN-γ, TNF, and IL-2 producing T-cells, whereas no effects on IL-4 $CD4^+$ cells were observed. These results suggest that enhanced prolactin and GH concentrations as well as low cortisol levels during early nocturnal sleep synergistically act to enhance Th1 cytokine activity. The effect of cortisol on Th1 cytokines pointed at a strong involvement of mineralcorticoid receptors, in addition to glucocorticoid receptor signaling, in the regulation of the type 1/type 2 balance. Since, T cell activity critically depends on APCs that can drive T cells toward type 1 or type 2 responses, in the third experiment we were finally interested in the cytokine profile and cell migration of specific subsets of APC precursors in peripheral blood as primary targets of sleep-associated changes toward enhanced adaptive immunity. The main finding of this study is that in comparison with continuous 24-hour wakefulness, sleep was associated with a striking increase in the number of pre-mDC producing IL-12, a cytokine centrally involved in initializing a Th1 cell-mediated immune response (Kalinski et al., 1999). Additionally, sleep led to slight decreases in numbers of circulating PDC and T cells but did not affect production of IFN-α by PDC. Sleep, however, substantially decreased the number of circulating $CD14^{dim}CD16^+$ monocytes, probably due to extravasation. In summary, our data support the idea that sleep can facilitate immune responses by allowing type 1 cytokine responses and by facilitating extravasation of DC precursors.

Our findings of a sleep associated shift in the Th1/Th2 balance towards Th1 activity during early sleep complement previous observations by Petrovsky & Harrison (1997, 1998), who reported strong diurnal rhythms in the in-vitro production of IFN-γ and the Th2 cell cytokine IL-10 (in mitogen stimulated whole blood samples), with peaks at about 23:00 h for IFN-γ and at about 22:00 h for IL-10. The difference in circadian phase of the two cytokines leads to a shift of the Th1/Th2 cytokine balance, as indicated in that study by the ratio of IFN-γ/IL-10 production, towards Th1 during the early night. Also, production of other proinflammatory cytokines like IL-1, IL-2 and TNF-a show their highest levels in the first half of the night (Petrovsky, 2001). Although here we did not analyze circadian rhythms, the decline observed independently of sleep and wakefulness for the production of all cytokines very likely reflects this type of influence. While Petrovsky & Harrison (1997) demonstrate a circadian peak in the Th1/Th2 cytokine balance during the early night, the present comparison between subjects awake and asleep indicates an additive effect of sleep strengthening Th1 dominance during the early night.

The unique endocrine setting during nocturnal sleep is an ideal prerequisite for the support of immune cells, which possess receptors for steroid and peptide hormones. In search of potential neuroendocrine mechanisms underlying the sleep-induced shift in the type 1/type 2 cytokine balance, we measured plasma concentrations of several immunoactive hormones with characteristic secretory patterns during sleep. Our data confirm strong increases in the release in prolactin and GH, which are known to be most pronounced during periods of SWS. During early SWS-rich sleep, this increases might synergistically act to bias the Th1/Th2 balance acutely towards Th1 predominance. Indeed, exposure in-vitro of morning blood to prolactin and anti-prolactin antibody indicated stimulating effect of this hormone on IFN-γ and TNF-α producing $CD4^+$ and $CD8^+$ cells as well as on the production of IL-2 by $CD8^+$ cells. These findings corroborate a number of previous studies likewise pointing to an increasing influence of prolactin on the production of Th1 cytokines, for a review see (Matera et al., 2000). Like prolactin, adding GH at concentrations comparable with the SWS related increase in endogenous GH increased IFN-γ producing $CD4^+$ cells in the morning blood samples. This result also fits several previous in-vivo studies in which GH enhanced Th1 cytokine production (Takagi et al., 1998; Mellado et al., 1998). The Th1 enhancing effect of GH although somewhat weaker resembled that of prolactin, which is not unexpected since both hormones act via receptors of the same cytokine/hemopoetin receptor superfamily, sharing binding affinity and a similar intracellular protein cascade during transduction (Matera et al., 2000; Yu-Lee, 2002).

Unlike prolactin and GH, which peak during early SWS-rich sleep, the secretion of cortisol is suppressed during this period and reaches maximum concentrations after morning awakening. Our data point to prominent influences of corticosteroids on the Th1/Th2 cytokine balance, since in-vitro exposure of morning blood to GR antagonist RU-486 and MR antagonist spironolactone significantly shifted this balance towards Th1 dominance. Notably both MR and GR antagonists did not affect the number of IL-4 producing $CD4^+$ cells, so that the change in the Th1/Th2 cytokine balance was due solely to the distinct increase in IFN-γ producing $CD4^+$ cells. These results confirm previous reports in humans, indicating that in the T-cell population corticosteroids induce an in-vitro shift in the Th1/Th2 cytokine balance toward a predominant Th2 response, primarily through inhibition of Th1 cytokines (Agarwal & Marshall, Jr., 2001). Our data are novel in that they show a prominent role for MR in the mediation of corticosteroid effects, which are similar in direction and amplitude to those mediated by GR. So far, there are only few hints suggesting that MR is specifically involved in the regulation of immune functions. Blockade of MR by spironolactone was found to reverse an inhibition of monocyte IL-1ra secretion by cortisol (Sauer et al., 1996). Recently, Vedder et al. (2003) reported an increase in the secretion of TNF-α in PBMC from healthy humans after in vitro administration of spironolactone. Extending these observations, here we found that spironolactone like RU-486 enhances the production of TNF-α as well as IFN-γ and IL-2, both in $CD4^+$ and $CD8^+$ T-cells, suggesting that MR and GR synergistically act to mediate a suppression of proinflammatory responses via cortisol.

The finding from the third study that sleep was associated with a striking increase in the number of pre-mDC producing IL-12 is of particular interest because this cytokine is centrally involved in initializing a Th1 cell-mediated immune response (Kalinski et al., 1999). In comparison with wakefulness, sleep almost doubled the percentage of pre-mDC producing IL-12 after stimulation with LPS, predictably increasing the efficacy of emerging T cell responses. In fact, the sleep induced increase observed here in $IL\text{-}12^+$ pre-mDC appeared to be distinctly stronger than sleep-dependent increases in type 1 cytokine activity in T cells and as revealed in foregoing studies in undifferentiated monocyte populations (Lange et al., 2006). Notably, within the set of DC precursors examined here, the enhancing effect of sleep was found to be specific to that subpopulation most representative for mature APC function in lymphoid tissue.

Among the endocrine signals possibly mediating this effect on pre-mDCs, basically all of the hormones measured here have to be considered. The number of $IL\text{-}12^+$ pre-mDC was correlated with concentrations of prolactin and, to a lesser degree, of GH, which both showed

the well-known increase during sleep (Spiegel et al., 1995; Van & Copinschi, 2000). The correlation with prolactin concentrations ($r = 0.47$) was roughly similar to that observed in a previous study in our lab (Lange et al., 2006) for the production of IL-12 in an undifferentiated sample of monocytes (amounting to $r = 0.33$; calculated with the same algorithm), which suggests a comparable sensitivity to prolactin in these populations. Triggering the monocytes prolactin receptor at concentrations close to the upper limit of the physiologic range significantly amplified the LPS-stimulated synthesis of IL-12 and TNF-α in these cells through induction of nuclear factor-κB and IFN regulatory factor-1 (Brand et al., 2004). Moreover, blocking of sleep-associated prolactin with anti-prolactin antibody decreased the percentage of LPS-induced IL-12^+ monocytes in blood sampled from sleeping subjects (Lange et al., 2006). In combination, these findings support the view that sleep-dependent release of prolactin belongs to the primary factors promoting synthesis of IL-12 in DC precursors. Additionally, catecholamines via β_2-adrenergic receptors, as well as glucocorticoids via the classic glucocorticoid receptors, potently inhibit production of IL-12 (Elenkov et al., 1996; Blotta et al., 1997; Panina-Bordignon et al., 1997). Both norepinephrine and cortisol reached lowest concentrations during the night with the decrease in norepinephrine being most pronounced when subjects slept. In the sleep condition, the decrease in the concentration of these hormones was clearly correlated with increasing numbers of IL-12^+ pre-mDC, which concurs with the view that the sleep-wake–dependent regulation of these hormones is critically involved in mediating the effect of sleep on IL-12.

Th1 dominance should lead to facilitated cell mediated immune response against intracellular bacteria and viruses e.g., (Alexander et al., 1999; Karupiah, 1998). In contrast, Th2 cytokines such as IL-4, IL-5, and IL-13 stimulate various aspects of humoral defense, such as a B cell switch to the production of IgG2, IgG4 and IgE, and, thereby, these cytokines are known to be involved also in some forms of allergic responses (Wierenga et al., 1990; Rothman, 1993; Hopkin, 1997). On this background, the relative increase of Th1 compared to Th2 cytokine activity during SWS rich early sleep fits a number of previous observations suggesting a similar association between sleep, circadian rhythm, SWS and Th1/Th2 cytokine balance. For example, nocturnal sleep is characterized by the peak of delayed type hypersensitivity (DTH) reactions and graft rejections (Haus & Smolensky, 1999). Also, sleep compared to nocturnal wakefulness enhances the specific, Th1 dependant IgG1 and IgG3 antibody response to the viral vaccines (Spiegel et al., 2002; Lange et al., 2003). Opposite, increased IgE production and allergic responses to cedar pollen have been found in atopic patients after a single night of sleep deprivation (Kimata, 2002). In addition, chronic sleep

deficits in insomnia (Sakami et al., 2003), alcoholism (Redwine et al., 2003), stress and in the course of aging (Glaser et al., 2001) is associated with a shift of the type 1/type 2 cytokine balance towards type 2 activity.

A further result of our sleep deprivation studies is, that sleep compared to nocturnal wakefulness acutely reduced numbers of PDC and $CD14^{dim}CD16^{+}$ monocytes as well as T cell counts with the latter finding replicating previous observations (Born et al., 1997). The variation in T cell numbers reflects a redistribution of the cells between blood, bone marrow and lymphoid tissues. In mice the circadian maximum of T cell counts in peripheral blood is followed by a maximum of these cells in spleen with a delay of two hours (Ottaway & Husband, 1994). Complementing the sleep dependent decrease in T cells in peripheral blood, animal and human studies have provided evidence for a sleep dependent accumulation of T cells in lymphoid tissues (Dickstein et al., 2000; Engeset et al., 1977) and this could hold also for PDC (Liu, 2005; von Andrian & Mempel, 2003). Differentiating $CD14^{+}CD16^{-}$ monocytes, representing the largest monocyte subset, from $CD14^{dim}CD16^{+}$ monocytes revealed that sleep selectively and strongly reduced blood counts only of the latter $CD14^{dim}CD16^{+}$ population, representing 10-15 % of the total monocytes. $CD14^{dim}CD16^{+}$ monocytes differ functionally from $CD14^{+}CD16^{-}$ monocytes in their greater ability to invade tissues under non-inflamed conditions (Geissmann et al., 2003). The sleep-dependent reduction in circulating numbers of these cells could indicate that sleep supports this function by facilitating their extravasation, but could also reflect increased margination of the cells. These possibilities, however, should be investigated in sleep deprived animals.

In conclusion, the data can be summarized as follows: sleep can facilitate immune defense (i) by high levels of immunosupportive and low levels of immunosuppressive hormones, (ii) by high levels of proinflammatory type I cytokines and (iii) by influences on cell migration, facilitating the extravasation of DC precursors and T cells. The findings eventually explain observation of previous studies, where sleep after vaccination increased the specific IgG1 and IgG3 antibody response to the viral vaccines (Spiegel et al., 2002; Lange et al., 2003), as well as more general clues to a supportive function of sleep for adaptive immunity (Bryant et al., 2004).

Summary

Sleep is commonly viewed as a restorative process that influences the homeostatic regulation of the autonomic, neuroendocrine, and immune systems. Two key vaccination experiments have shown that regular sleep in the nights following vaccination, as compared to sustained wakefulness leads to a significantly higher antibody titter, supporting the notion that sleep plays an important role in the formation of adaptive immunity and immunological memory. Three studies are reported here which identify relevant underlying mechanisms by which sleep can ease the immune responses. Experiment I showed that early nocturnal sleep is associated with a shift in the Th1/Th2 cytokine balance towards increased Th1 activity, as indicated by an increased ratio of IFN-γ/IL-4 producing T helper cells. During this early half of the night, sleep was not only dominated by slow wave sleep but showed also the typical strong increase of prolactin and GH. In-vitro results of experiment II confirmed our hypothesis that increased prolactin and GH concentrations as well as low cortisol level, which characterize early nocturnal sleep synergistically act to enhance Th1 cytokine activity. Finally, Experiment III has found that sleep induced throughout the night a striking increase in the number of pre-mDC producing IL-12, which is a main inducer of Th1 responses. These studies together identify type1/type2 cytokine balance as a basic target of sleep that can effectively enhance adaptive immune responses. The unique endocrine setting during nocturnal sleep, i.e. high levels of immunosupportive and low levels of immunosuppressive hormones is an ideal prerequisite for the support of type 1 cytokines and eventually explains observation of previous studies, where regular sleep increased the specific IgG1 and IgG3 antibody response to the viral vaccines, as well as more general clues to a supportive function of sleep for adaptive immunity.

Zusammenfassung

Schlaf wird gemeinhin als Prozess der Erholung angesehen, der die homöostatische Regulation von autonomem, neuroendokrinem und Immunsystem beeinflusst. Zwei Schlüsselexperimente haben gezeigt, dass regulärer Nachtschlaf nach einer Impfung im Vergleich zu nächtlicher Wachheit zu einem signifikant erhöhten Antikörpertiter führt. Dies unterstützt die Annahme, dass Schlaf eine wichtige Rolle für adaptive Immunfunktionen und das immunologische Gedächtnis spielt. Die vorliegende Arbeit umfasst drei Studien, die relevante zugrunde liegende Mechanismen identifizieren, durch die Schlaf die Immunantwort begünstigen kann. Experiment I zeigt, dass früher nächtlicher Schlaf mit einer Verschiebung des Th1/Th2-Zytokin-Gleichgewichts zu erhöhter Th1-Aktivität einhergeht, wie von einem erhöhten Anteil an IFN-γ/IL-4-produzierenden T-Helfer-Zellen angezeigt wird. Während dieser frühen Nachthälfte wurde der Schlaf dabei nicht nur von Deltaschlaf dominiert, sondern zeigte auch den typischen starken Anstieg von Prolaktin und GH. Die in-vitro-Ergebnisse von Experiment II bestätigten unsere Hypothese, dass erhöhte Prolaktin- und GH-Konzentrationen sowie niedrige Kortisolpegel, die den frühen nächtlichen Schlaf charakterisieren, synergistisch die Th1-Zytokin-Aktivität steigern. Schließlich zeigte sich in Experiment III, dass Schlaf über die Nacht hinweg einen auffallenden Anstieg in der Anzahl der prä-mDC-Zellen bewirkt. Diese Zellen produzieren IL-12, das die Th1-Antwort hauptsächlich herbeiführt. Zusammengenommen identifizieren diese Studien das Typ1/Typ2-Zytokin-Gleichgewicht als grundlegendes Wirksystem des Schlafs, das adaptive Immunantworten effektiv verbessern kann. Die einzigartigen endokrinen Bedingungen während des Nachtschlafs, d.h. die hohen Konzentrationen von immunsupportiven und die niedrigen Spiegel von immunsuppressiven Hormonen, stellen eine ideale Voraussetzung für die Unterstützung von Typ1-Zytokinen dar. Dieser Zusammenhang erklärt darüber hinaus Beobachtungen aus früheren Studien, in denen Schlaf nach einer Impfung die spezifische IgG1- und IgG3-Antikörper-Antwort auf virale Impfstoffe verstärkte, und weist auf die unterstützende Funktion des Schlafs in adaptiven Immunprozessen hin.

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Thank you

I am deeply grateful to many people for their help and support in the last five years.

I would especially like to thank my advisor Prof. Dr. Jan Born for his generous time and commitment. Throughout my doctoral work he encouraged me to develop independent thinking and research skills and assisted me with scientific writing.

Special thanks to my adviser in immunology Prof. Dr. Jürgen Westermann for his support, guidance and helpful suggestions.

I owe a special note of gratitude to my coworker Tanja Lange who played an extremely important role in my research for the last five years.

I want to acknowledge Drs. Christian Schultz and Antje Müller for allowing me to use their labs and equipment for the Flow Cytometry work. Particular thanks also go to our four laboratory technicians: Christiane Otten, Heidi Ruf and Ingrid von Lützau.

Without my colleagues I would be lost, thank you all for scientific discussions, your critical views, and help in general. Many thanks to them: Manfred, Mathias, Lisa and Roumen; Ulli, Björn, Steffen and Kerstin; Anja, Ines, Susi, Christian, Spyros, Malte and Rosi.

I want to thank my wife Zornitsa and my parents for their love, support, encouragement and understanding in dealing with all the challenges I have faced.

Finally, I thank God. I believe nothing good comes from man without the blessing of God. I also see God's hand in the creation that we study.

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Oct 1993 – July 1998
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Research Experience

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Research associate, Department of Neuroendocrinology, University of Lübeck, Lübeck, Germany (research adviser: Dr. Jan Born):

- Effects of sleep deprivation on the adaptive immune response following vaccination, assessed by evaluating endpoint parameters (e.g. antibody titers, IgG-subclasses, antigen specific cells and their cytokine profile)
- Detailed analysis of number and function of immune cell subpopulations during a regular sleep-wake cycle and during 24 hours of sustained wakefulness

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Predoctoral Research associate, Immunologist, Central laboratory of immunology, National center of infectious and parasitic diseases, Sofia, Bulgaria (research adviser: Dr. Hristo Taskov):

- Detailed phenotypic analysis of T subpopulations in HIV infected patients through the course of HAART therapy. Measurement of antigen specific $CD8^+$ T cells in HIV infected patients
- Detecting of intracellular cytokines and calcium through flow cytometry

Laboratory Skills

Molecular biology techniques

- Cell culturing
- DNA, RNA isolation
- Gel electrophoresis techniques
- FPLC, HPLC
- Southern and western blotting
- Polymerase chain reaction (PCR)
- Microarray techniques

Immunological techniques

- Flow cytometry – intracellular cytokines, immunophenotyping
- Magnetic activated cell sorting (MACS)
- Enzyme linked immunosorbent assay techniques (ELISA)
- Enzyme linked immuno spot techniques (ELISPOT)

Physiological techniques

- Sleep stage scoring
- Radioligand-binding techniques
- Chemotaxis assays

Scientific Interests

- Psychoneuroimmunology (PNI)
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- Significance of these interactions in health and disease

List of own original articles generated during the thesis work

- **Dimitrov S**, Lange T, Westermann J, Born J (2007) Differential circadian regulation of migratory behavior of T-cell subpopulations via cortisol and catecholamines. (in preparation).

- Benedict C, **Dimitrov S**, Marshall L, Born J (2007) Sleep enhances serum interleukin-7 concentrations in humans. Brain Behav. Immun. May 22, [Epub ahead of print].

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Abstracts at international congresses

- **Dimitrov S**, Born J (2007) Number and function of circulating human immune cells regulated by sleep, 65th APC Meeting, 07.03-10.03.2007, Budapest, Hungary
- **Dimitrov S**, Lange T, Born J (2007) Differential circadian regulation of migratory behavior of lymphocyte and monocyte subpopulations via ß2-adrenoceptors, 6th GEBIN Meeting, 05.03-07.03.2007, Freiburg, Germany
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- **Dimitrov S**, Lange T, Fehm HL, Born J (2004) Physiological concentrations of growth hormone and cortisol modulate T helper1/T helper 2 in-vitro cytokine production in human T cells, 11th PNIRS Meeting, 26.05-29.05.2004, Titisee, Germany
- **Dimitrov S**, Lange T, Fehm HL, Born J (2003) Prolactin, growth hormone and corticosteroid receptor antagonists modulate T helper1/T helper 2 in-vitro cytokine production in human T cells, 3th GEBIN Meeting, 30.09-02.10.2003, München, Germany
- Lange T, **Dimitrov S**, Fehm HL, Born J (2003) Sleep modulates T helper 1/T helper 2 cytokine balance in humans, 3th GEBIN Meeting, 30.09-02.10.2003, München, Germany