Diurnal and photoperiodic effects on the immune system and glucocorticoid signaling in domestic pigs

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

12L:12D	12 hours of light and 12 hours of darkness
16L:8D	16 hours of light and 8 hours of darkness
8L:16D	8 hours of light and 16 hours of darkness
ACTH	Adrenocorticotropic hormone
Ag	Antigen
Ag-exp.	Antigen-experienced
AIC	Akaike information criterion
ANOVA	Analysis of variance
AR(1)	First order autoregressive
BMAL1	Brain and muscle ARNT-like protein 1
BSA	Bovine serum albumin
BW	Body weight
CD	Cluster of differentiation
CEST	Central European Summer Time
CET	Central European Time
CI	95% confidence interval
CLOCK	Circadian locomotor output cycles kaput
CRF	Corticotropin-releasing factor
CRY	Cryptochrome
df	Numerator degrees of freedom, denominator degrees of freedom
FITC	Fluorescein isothiocyanate
FMO control	Fluorescence minus one control
GLMM	Generalized linear mixed model
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA axis	Hypothalamic-pituitary-adrenal axis
ID	Scaled identity
IgG	Immunoglobulin G
K3 EDTA	Ethylenediaminetetraacetic acid tripotassium salt
K_d	Dissociation constant

LD	Long day conditions
LED	Light-emitting diode
LMM	Linear mixed model
LSD	Least significance difference test
mAb	Monoclonal antibody
ME	Metabolizable energy
n	Sample size
NK cell	Natural killer cell
P	Probability value
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Pctl	25th–75th percentile
PE	Phycoerythrin
PER	Period
PI	Propidium iodide
PT	*Pars tuberalis*
PVN	*Paraventricular nucleus*
r	Pearson's correlation coefficient
REML	Restricted maximum likelihood method
REPL	Residual pseudo likelihood method
RIA	Radioimmunoassay
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT	Room temperature
SCN	S*uprachiasmatic nucleus*
SD	Short day conditions
SE	Standard error
SEM	Standard error of the mean
SPRD	Spectral red
Th cell	T helper cell
TierSchNutztV	Tierschutz-Nutztierhaltungsvordnung
WD	Working dilution
wk	Week

1 GENERAL INTRODUCTION

Biological timekeeping is an omnipresent adaptation of living organisms to the environment on earth and covers several orders of magnitude ranging from biological processes lasting microseconds to days up to years and the decades spanning a lifetime (Buonomano, 2007; Golombek et al., 2014). Thereby, distinct rhythmic changes occur within the different time scales. Biological rhythms with a period length of one day, i.e., 24 hours, in this thesis referred to as diurnal rhythms (from the Latin *diurnus*, meaning 'lasting a day'), as well as with a period length of one year, i.e., 365 days, commonly referred to as seasonal rhythms, are the most obvious and most intensively investigated among them (Buonomano, 2007; Helm et al., 2013). Diurnal and seasonal rhythms can be found in physiology and behavior of humans and animals (Bradshaw & Holzapfel, 2007; Golombek et al., 2014; Hut et al., 2013; Stevenson et al., 2015; Stothard et al., 2017; Yetish et al., 2015). Those rhythms adapt to certain external cues, called zeitgebers, such as light or darkness and feeding time in the case of diurnal rhythms (Challet, 2007; Mendoza, 2007) or photoperiod, i.e., the relative span of light per day, in the case of seasonal rhythms (Bradshaw & Holzapfel, 2007). However, if it is proven that diurnal or seasonal rhythmic processes continue to oscillate without any external stimuli, typically with period lengths slightly deviating from 24 hours or 365 days, they are specified as circadian or circannual rhythms, from the Latin *circa*, meaning 'about', *dies*, meaning 'day', and *annum*, meaning 'year' (Gwinner, 1981; Turek, 1985). This means that circadian or circannual rhythmicity is intrinsically regulated by a central pacemaker mechanism and represents evolutionary adaptation to live on the rotating earth (Bradshaw & Holzapfel, 2007; Hut et al., 2013; Panda et al., 2002).

The function of the central circadian pacemaker is relatively well understood and recently, the 2017 Nobel Prize in Physiology or Medicine was awarded for unveiling its basic molecular mechanism (Ledford & Callaway, 2017). In mammals, the master circadian clock is located in the *suprachiasmatic nucleus* (SCN) within the anteroventral hypothalamus of the brain and it regulates subordinated peripheral clocks in other bodily tissues and cells (Buijs et al., 2016; Welsh et al., 2010). Thereby, only the master clock in the SCN is entrainable by photic stimuli, i.e., the main zeitgeber 'light', via the retina to the solar day, but the molecular clock mechanism is the same between the master clock in SCN neurons and peripheral clocks (Buijs et al., 2016; Dibner et al., 2010). The clock mechanism

consists of interlinked autoregulatory transcriptional-translational feedback loops, which take about 24 hours to run (Partch et al., 2014). The core loop comprises four principal clock proteins, the two activators 'circadian locomotor output cycles kaput' (CLOCK) and 'brain and muscle ARNT-like protein 1' (BMAL1) and the two repressors period (PER) and cryptochrome (CRY), and its course is refined via secondary loop mechanisms including posttranslational modification of gene products (Albrecht, 2012; Partch et al., 2014; Zhang & Kay, 2010). In contrast, the molecular function of intrinsic circannual timing is still not unraveled, although the existence of a circannual pacemaker is generally postulated (Dupré & Loudon, 2007; Wood & Loudon, 2018). Moreover, recent research suggests that the mammalian circannual clock potentially resides in the *pars tuberalis* (PT) within the anterior pituitary (Lincoln et al., 2006; Wood et al., 2015; Wood & Loudon, 2018).

At least for circadian rhythmicity, it is known that time information can be transmitted from the central pacemaker to peripheral tissues via humoral and neural pathways (Kalsbeek et al., 2006). Among them, glucocorticoids are regarded as a main link from the master circadian clock towards peripheral clocks for several reasons (Haus, 2007; Le Minh et al., 2001; Pezük et al., 2012; Spencer et al., 2018). First, the rhythmic pulsatile excretion of glucocorticoids upon stimulation of the hypothalamic-pituitary-adrenal (HPA) axis is governed by the SCN (Haus, 2007). In detail, efferent neurons from the SCN to the *paraventricular nucleus* (PVN) within the hypothalamus promote the release of corticotropin-releasing factor (CRF) from PVN neurons into the hypophyseal portal system (Haus, 2007; Nader et al., 2010). Subsequently, the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the circulation is stimulated, which results in secretion of glucocorticoids from the adrenal cortex (Nader et al., 2010). Glucocorticoids act primarily through binding to glucocorticoid receptors (GR), which represent transcription factors altering gene expression via binding to glucocorticoid response elements (GRE) in the promoter regions of glucocorticoid sensitive genes (Vandevyver et al., 2014). Second, GR are present in virtually every cell type with the exception of the SCN itself (Balsalobre et al., 2000; Bookout et al., 2006; Rosenfeld et al., 1988). Finally, glucocorticoids were shown to be able to alter clock gene expression via GRE in peripheral tissues (Dickmeis, 2009; So et al., 2009; Torra et al., 2000; Yamamoto et al., 2005). Besides their importance in the circadian system to anticipate diurnal physiological demands, glucocorticoids are also essential for responses to unforeseen acute and chronic challenges, called stressors (Nader et al., 2010). Thereby, acute stress-induced glucocorticoids represent

an important survival mechanism in order to prepare the immune system against potential threats, whereas chronic stressor exposure is detrimental for immune function and health (Dhabhar, 2018). Stress-induced glucocorticoids, in turn, can affect circadian rhythms via the above mentioned pathways and hence can lead to circadian misalignment between the master clock and peripheral clocks (Koch et al., 2017; Spencer et al., 2018; Tahara et al., 2015). Moreover, circadian disruption itself represents a circadian stressor and, especially when chronic, circadian disruption is supposed to impair health, namely considered in a similar way as repeated stressor exposure (Koch et al., 2017; Spencer et al., 2018).

Circadian disruption may be caused by several reasons, predominantly investigated in humans and nocturnal rodents (Lunn et al., 2017). Modern life often entails uncoupling of rest-activity cycles from natural photoperiod due to electric lighting in the form of environmental light pollution, constant lighting, or insufficient daylight illumination (Lunn et al., 2017; Smolensky et al., 2015), due to food consumption at abnormal times of day (Asher & Sassone-Corsi, 2015), as well as due to sleep deprivation (Potter et al., 2016). Various health consequences were reported to be associated with circadian disruption, such as cardiovascular diseases, metabolic diseases, including diabetes and obesity, gastrointestinal disorders, cognition and mood disorders, cancer, as well as disrupted immune function in humans and nocturnal rodents (Castanon-Cervantes et al., 2010; Chiesa et al., 2015; Cuesta et al., 2016; Touitou et al., 2017). Furthermore, modern societies spend most of their time under artificial long photoperiods, i.e., in ‘eternal summer’, and seasonal disruption also is expected to be detrimental for health and well-being of humans and animals (Stevenson et al., 2015).

In animal husbandry, artificial light is widely used to influence maturation, fertility, growth, or behavior of different livestock species (Bessei, 2006; Chemineau et al., 2007; Grandin, 1990; Tucker & Ringer, 1982; Yang et al., 2016). Although domestication resulted in decreased seasonality of farm animals compared to their wild ancestors, animal production is subjected to more or less considerable seasonal fluctuations (Chemineau et al., 2007). Thus, artificial photoperiodic treatment is commonly used to control reproductive seasonality in livestock, i.e., increasing photoperiod supports fertility in long-day breeders, such as poultry (Sharp, 1993), rabbits (Szendrő et al., 2016), horses (Aurich, 2011), and cattle (Hansen, 1985), whereas decreasing photoperiod supports fertility in short-day breeders, such as sheep, goats (Chemineau et al., 1992), and domestic pigs (Claus & Weiler, 1985). Artificial long photoperiods are applied to enhance growth of livestock or to increase

milk yield (Bessei, 2006; Dahl et al., 2000; Martelli et al., 2015). Moreover, even extreme lighting schedules with nearly continuous lighting or intermittent lighting with several alternating light-dark phases during one day were shown to increase growth performance (Bessei, 2006; Bruininx et al., 2002; Duve et al., 2011). However, for animal welfare purposes, keeping farm animals in continuous light or darkness does not comply with current European and German legislation (Council Directive 98/58/EC, 1998; TierSchNutztV, 2006). Another field of application for artificial light in animal husbandry is the manipulation of animal behavior. Various artificial lighting conditions aim to stimulate feed intake, to influence rest-activity behavior in order to improve leg health, to avoid feather pecking, or to generally calm animals, by manipulating photoperiod, light intensity, or light spectrum (Archer, 2018; Bessei, 2006; Grandin, 1990; Lewis & Morris, 2000; Macmillan et al., 2018; Mohammed et al., 2010). Finally, it was shown that not only physiology of livestock is subject to seasonal variation but also farm animal health exhibits distinct seasonal fluctuation, e.g., in emergence of infectious diseases or mortality (Hertl et al., 2011; Koketsu, 2000; Lingaas & Rønningen, 1991).

Many of the above-mentioned diurnal and seasonal health-related problems in humans and animals seem to be associated with dysfunctions of the immune system, as diurnal and seasonal rhythms in the immune system are thought to assure temporally adjusted immune reactivity (Cermakian et al., 2013; Nelson & Demas, 1996). Diurnal and seasonal rhythms can be found in number and functionality of leukocytes in humans and animals (Cermakian et al., 2013; Dopico et al., 2015; Lange et al., 2010; Nelson & Demas, 1996; Scheiermann et al., 2013). Underlying mechanisms of rhythmicity in the immune system and potential associations with negative health consequences of circadian disruption are still not clearly defined. However, among other factors, glucocorticoids are thought to be important mediators of diurnal rhythmicity in the immune system, as glucocorticoids were shown to cause trafficking of immune cells between different tissues (Besedovsky et al., 2014a; Besedovsky et al., 2014b; Chung et al., 1986; Cox & Ford, 1982; Dimitrov et al., 2009; Fauci, 1975; Shimba et al., 2018). Moreover, underlying mechanisms driving seasonal changes in the immune system and potential effects on health remained largely unexplored until today. Besides other hormones, melatonin is expected to be involved in photoperiodic changes of immune function (Nelson & Demas, 1996; Stevenson & Prendergast, 2015).

So far, diurnal and seasonal rhythms in the immune system were mainly studied in humans and nocturnal rodents (Cermakian et al., 2013; Dopico et al., 2015; Lange et al.,

2010; Nelson & Demas, 1996; Scheiermann et al., 2013; Stevenson & Prendergast, 2015). Moreover, many common laboratory mice strains were shown to be melatonin-deficient (Ebihara et al., 1986; Goto et al., 1989; Kasahara et al., 2010; Vivien-Roels et al., 1998). Other model systems are rare in this field of science, but it has been mentioned that there is a need for a wider range of animal models, including long-lived seasonal species (Lunn et al., 2017; Stevenson et al., 2015). Hence, the moderately seasonal domestic pig (Chemineau et al., 2007) would represent a particularly suitable model species in chronoimmunology research. It exhibits many anatomical and immunological similarities to humans (Aigner et al., 2010; Gerdts et al., 2015; Meurens et al., 2012; Renner et al., 2016) and a genome sequence much more similar to humans than that of mice (Wernersson et al., 2005). Therefore, the pig is already regarded as highly suitable animal model in translational medicine (Aigner et al., 2010) and discussed as potential donor for human xenotransplantation (Cooper et al., 2016). Several transgenic pig models with specific gene knockouts are also available (Aigner et al., 2010; Qian et al., 2015), further increasing the potential future relevance of the pig as model species. Moreover, the domestic pig is diurnally active like humans (Ingram et al., 1980; Ingram & Dauncey, 1985; Takeishi et al., 2018) and its high blood volume enables frequent intra-individual blood collection. In addition, from a practical point of view, studying diurnal and seasonal rhythms in the porcine immune system could contribute to a better understanding of disease prevalence in pig husbandry and thus may result in improved management methods to enhance animal health and welfare.

1.1 Main research objective

To address these challenges mentioned above, the main objective of the present thesis was to investigate diurnal and photoperiodic effects on the immune system and glucocorticoid signaling as well as potential underlying endocrine, behavioral, and molecular mechanisms of these effects in domestic pigs.

This main approach was spread over separate studies, which are described in the included manuscripts of this thesis. An overview of the individual objectives and methodical approaches of each included manuscript are given in the next section.

1.2 Objectives and methodical approaches of included manuscripts

In general, the experimental approach of the present thesis was based on blood samples taken from surgically catheterized adult male castrated domestic pigs (Kraetzl & Weiler, 1998) held under specific lighting schedules. The blood samples were analyzed with different laboratory methods, including immunofluorescent antibody staining and flow cytometry for characterization of various porcine immune cell populations, automated hematological analyses, radioimmunological assessment of plasma cortisol concentration, and radioligand binding assays to determine GR number and affinity in immune cells. In addition, behavioral analyses based on video recordings were performed. Diurnal rhythms in the investigated variables were evaluated with cosinor analyses (Nelson et al., 1979) and potential associations between different investigated variables were assessed with linear mixed model analyses or generalized linear mixed model analyses.

MANUSCRIPT I

Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs

Published in *Developmental and Comparative Immunology* **79**, 11–20 (2018)

In order to provide the basis for chronoimmunological research with domestic pigs, the primary objective of this study was to characterize, for the first time with an immunofluorescence-based approach, diurnal rhythms in immune cell numbers of various immune cell populations in blood of domestic pigs held under standardized experimental conditions with 12 hours of light and 12 hours of darkness (12L:12D, lights on 07:00–19:00) and concentrate feeding two times a day (07:30 and 15:30). Blood samples were taken from 18 pigs every 2 hours over periods of up to 50 hours. Furthermore, the study aimed to assess underlying mechanisms of diurnal rhythmicity in immune cells by evaluating potential associations of immune cell numbers in porcine blood with different other variables, i.e., light, concentrate feeding, plasma cortisol concentration, hematocrit, and sampling, with linear mixed model analyses. Moreover, activity behavior was recorded in some animals of the study to verify the diurnally active behavioral pattern of this porcine model.

MANUSCRIPT II

Data characterizing diurnal rhythms in the number of peripheral CD8α$^-$ and CD8α$^+$ γδ T cells in domestic pigs

Published in *Data in Brief* **16**, 843–849 (2018)

The data presented in this manuscript are part of the study, which is described in MANUSCRIPT I. As specific physiologic functions of porcine CD8α$^-$ and CD8α$^+$ γδ T cells are still not clear (Talker et al., 2013), the results for these immune cell populations were presented separately. Thus, the objectives of this part of the study were to characterize diurnal rhythms in peripheral cell numbers of CD8α$^-$ and CD8α$^+$ γδ T cells in domestic pigs held under standardized experimental conditions (12L:12D, concentrate feeding twice daily) and to assess potential associations between cell numbers in porcine blood with the other investigated variables with linear mixed models. These data were also obtained from all investigated 18 pigs, which were sampled every 2 hours over periods of up to 50 hours.

MANUSCRIPT III

Photoperiodic effects on diurnal rhythms in cell numbers of peripheral leukocytes in domestic pigs

Published in *Frontiers in Immunology* **10**, 393 (2019)

The primary objective of this study was to investigate, for the first time in any species, the influence of photoperiod on diurnal rhythms in immune cell numbers of various leukocyte types in domestic pigs. The animals were held under standardized experimental settings either under long day conditions (LD) with a photoperiod of 16 hours per day (16L:8D, lights on 07:00–23:00, n = 9) or short day conditions (SD) with a photoperiod of 8 hours per day (8L:16D, lights on 07:00–15:00, n = 11) and fed concentrate twice daily (07:30 and 14:00). Blood samples were taken from all 20 pigs every 2 hours over periods of 50 hours. Moreover, the study also aimed to investigate photoperiodic effects on plasma cortisol concentration and activity behavior and to assess underlying mechanisms of diurnal rhythmicity in immune cells by evaluating potential associations of immune cell numbers in porcine blood with these and other variables, i.e., treatment, light, concentrate feeding, plasma cortisol concentration, relative activity behavior, hematocrit, and sampling with generalized linear mixed models.

MANUSCRIPT IV

Glucocorticoid receptor number and affinity differ between peripheral blood mononuclear cells and granulocytes in domestic pigs

Published in *Domestic Animal Endocrinology* **61**, 11–16 (2017)

The studies described in MANUSCRIPTS I–III found remarkable differences in diurnal rhythmicity as well as the association of plasma cortisol concentration with cell number in blood between neutrophils and most of the other investigated immune cell types. Therefore, to elucidate underlying molecular mechanisms, the objective of this study was to characterize, for the first time, GR number and affinity in peripheral blood mononuclear cells (PBMC) and granulocytes of domestic pigs. Blood samples of 6 domestic pigs were taken in the morning (08:00) and were examined with radioligand binding assays.

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2 MANUSCRIPTS

The following section contains the manuscripts, which were included in the present thesis. All manuscripts were published in international peer-reviewed journals. The manuscripts are presented within this thesis in the latest version of the text during the respective submission process. Text layout and formatting was adapted for the present thesis.

I **Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs**
Published in *Developmental and Comparative Immunology* **79**, 11–20 (2018)

II **Data characterizing diurnal rhythms in the number of peripheral CD8α⁻ and CD8α⁺ γδ T cells in domestic pigs**
Published in *Data in Brief* **16**, 843–849 (2018)

III **Photoperiodic effects on diurnal rhythms in cell numbers of peripheral leukocytes in domestic pigs**
Published in *Frontiers in Immunology* **10**, 393 (2019)

IV **Glucocorticoid receptor number and affinity differ between peripheral blood mononuclear cells and granulocytes in domestic pigs**
Published in *Domestic Animal Endocrinology* **61**, 11–16 (2017)

I

Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs

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Abstract

Diurnal rhythms within the immune system are considered important for immune competence. Until now, they were mostly studied in humans and rodents. However, as the domestic pig is regarded as suitable animal model and due to its importance in agriculture, this study aimed to characterize diurnal rhythmicity in porcine circulating leukocyte numbers. Eighteen pigs were studied over periods of up to 50 hours. Cosinor analyses revealed diurnal rhythms in cell numbers of most investigated immune cell populations in blood. Whereas T cell, dendritic cell, and eosinophil counts peaked during nighttime, NK cell and neutrophil counts peaked during daytime. Relative amplitudes of cell numbers in blood differed in T helper cell subtypes with distinctive differentiation states. Mixed model analyses revealed that plasma cortisol concentration was negatively associated with cell numbers of most leukocyte types, except for NK cells and neutrophils. The observed rhythms mainly resemble those found in humans and rodents.

Keywords: Diurnal rhythm; Immune system; Leukocyte; Cortisol; Activity behavior; *Sus scrofa domestica*

1. Introduction

Diurnal rhythms represent adaptations of living organisms to the alteration between day and night and are ubiquitous in the living world (Bell-Pedersen et al., 2005). They mainly result from synchronization to light or darkness (Challet, 2007) but can also be influenced by other external stimuli, called zeitgebers, such as feeding time (Patton and Mistlberger, 2013). Daily rhythmic variations can be found in many behavioral and physiological functions (Panda, 2016; Saper et al., 2005) as well as in the immune system (Carter et al., 2016; Cermakian et al., 2013; Curtis et al., 2014; Geiger et al., 2015; Labrecque and Cermakian, 2015). In this respect, diurnal variations in the cell numbers of different leukocyte subtypes in peripheral blood can be found (Ackermann et al., 2012; Born et al., 1997; Haus et al., 1983; Lasselin et al., 2015; Scheiermann et al., 2012). They seem to result from diurnal regulation of hematopoiesis and leukocyte trafficking between blood, the marginal pool, and different tissue sites (Besedovsky et al., 2014b; Druzd et al., 2014; Méndez-Ferrer et al., 2009; Scheiermann et al., 2012; Suzuki et al., 2016). All of these processes seem to be mediated by main endocrine mediators, such as glucocorticoids and catecholamines (Besedovsky et al., 2014b; Webster et al., 2002), adrenergic innervation into different tissues (Scheiermann et al., 2012; Suzuki et al., 2016), as well as to involve the master circadian clock in the *suprachiasmatic nucleus* of the brain (Curtis et al., 2014) and peripheral clocks

in immune cells (Bollinger et al., 2011; Nguyen et al., 2013). In general, diurnal rhythms in the immune system are thought to assure temporally adjusted reactivity and maintenance in immune defense (Cermakian et al., 2013). Chronic disruption of diurnal rhythmicity is associated with a dysregulation of the immune system (Castanon-Cervantes et al., 2010) and possibly with an increased risk for different diseases (Cermakian et al., 2013). Thus, diurnal immune rhythms potentially influence the onset and progression of infections or diseases (Bechtold et al., 2010; Edgar et al., 2016) and might also affect the outcome of therapeutic interventions like, e.g., vaccination or medication (Long et al., 2016; Smolensky and Peppas, 2007).

Until now, diurnal rhythms in the immune system were mainly studied in humans and nocturnal rodents (Scheiermann et al., 2013). Other model systems are rare in this field of science and for the domestic pig (*Sus scrofa domestica*) there are no studies showing diurnal rhythms in the cell numbers of various leukocyte subtypes so far. However, due to its high anatomical similarity to humans (Renner et al., 2016) and a genome sequence, which is more similar to humans than that of mice (Wernersson et al., 2005), the domestic pig is already regarded as highly suitable animal model (Aigner et al., 2010; Gerdts et al., 2015; Meurens et al., 2012). Moreover, the domestic pig is diurnally active like humans (Ingram et al., 1980; Ingram and Dauncey, 1985) and its high blood volume enables intraindividual blood collection, which altogether predisposes the domestic pig as valuable model for the analysis of diurnal immune rhythms and their underlying mechanisms as well as their influence on immune responses. In addition, from a practical point of view, high immune competence is important to prevent infectious diseases in pig stocks and to improve animal welfare (Colditz, 2002). The knowledge of factors potentially influencing this competence is therefore of high relevance for management in pig husbandry systems and veterinary interventions.

For these reasons, the aim of the present study was the characterization of diurnal rhythms in peripheral blood immune cell numbers in domestic pigs held under light-entrained conditions with concentrate feeding two times a day. This mimics usual practices in animal husbandry as well as life-habits of humans. We evaluated diurnal rhythmicity in immune cell numbers as well as activity behavior, plasma cortisol concentration, and hematocrit with cosinor analysis (Nelson et al., 1979). In addition, linear mixed model analysis was performed to assess potential associations between the investigated parameters.

2. Materials and methods

2.1. Animals and surgery

All procedures were conducted in accordance with the German Animal Welfare Act and approved by the local Animal Welfare Ethics Committee (Regional Council Stuttgart, approval number V309/13TH). A total of 18 castrated male pigs (Piétrain × German landrace, 6-month-old, body weight (BW) range 92 to 106 kg) were included in the study. The pigs were housed in a lightproof building of the experimental unit of the department Behavioral Physiology of Livestock at a constant ambient temperature of 21 ± 1°C. Animals were kept in individual pens (6.4 m^2 each) with sight and tactile contact to neighboring animals and had *ad libitum* access to hay and water. They were fed concentrate (1.2 kg/meal, ME 12 MJ/kg) twice daily at 07:30 h and 15:30 h. Pens were cleaned and littered with dust-free wood shavings daily after concentrate feeding in the morning. All pigs were maintained under a 12:12 light-dark cycle (lights on 07:00 h to 19:00 h) with on average 190 lx at pigs' eye level during the light phase (fluorescent tubes, Philips Master TL-D Super 80 58W/840, color temperature 4000 K) and 0 lx during the dark phase. All pigs were accustomed to the lighting and feeding regime for at least 8 weeks prior to the experiments and well habituated to human handling. To obtain blood samples without disturbing the animals, all pigs were surgically cannulated with indwelling vein catheters (*vena cava cranialis*) at least 2 weeks prior to sampling. The surgical procedure was conducted according to Kraetzl & Weiler (1998) with modifications. Anesthesia was introduced with a combination of ketaminhydrochloride (15 mg/kg BW intramuscular (i.m.), Ursotamin, Serumwerk Bernburg, Bernburg, Germany) and azaperone (2 mg/kg BW i.m., Stresnil, Sanochemia Pharmazeutika, Neufeld/Leitha, Austria) in all animals and general anesthesia was maintained with isoflurane inhalation (1.5 to 2.5%, Vetflurane, Virbac, Carros, France). Meloxicam (0.4 mg/kg BW i.m., Emdocam, Emdoka, Hoogstraten, Belgium) was used as analgesic. Catheters were exteriorized in the neck area and stored in a pouch fixed on the skin. Catheters were rinsed with heparinized saline (115 IU/ml, heparin sodium salt, Carl Roth, Karlsruhe, Germany) twice daily after concentrate feeding. All animals were weighed once per week (not during sampling periods) and health was monitored by daily measurement of rectal temperature.

2.2. Experimental protocol and sample processing

The study was subdivided into three different experimental trials (n = 6 each), which were conducted in spring 2014 and spring 2015 (Fig. 1). Blood sampling started at 10:00 h and was repeated every 2 hours in all trials. In the first two trials a total of 12 blood samples were taken until 08:00 h the following day (duration 22 hours each). The third trial included a total of 26 blood samples and sampling ended at 12:00 h on the second following day (duration 50 hours). Blood sampling at night was performed under dim light of averagely 7 lx at pigs' eye level, which was switched on and off for sampling (Philips energy-saving/LED bulbs 3W, color temperature 2700 K). Sampling all animals lasted not longer than 20 min in total per sampling. Animals were sampled in the same order each time. After discarding the heparinized saline solution from the catheters, 10 ml blood per sample was drawn. Subsequently the catheter was rinsed with approximately 10 ml heparinized saline (46 IU/ml) to keep the catheter patent and to compensate for the blood volume taken. Blood was transferred directly into lithium heparin tubes and K3 EDTA tubes (both Sarstedt, Nümbrecht, Germany). Blood samples were immediately processed after each sampling.

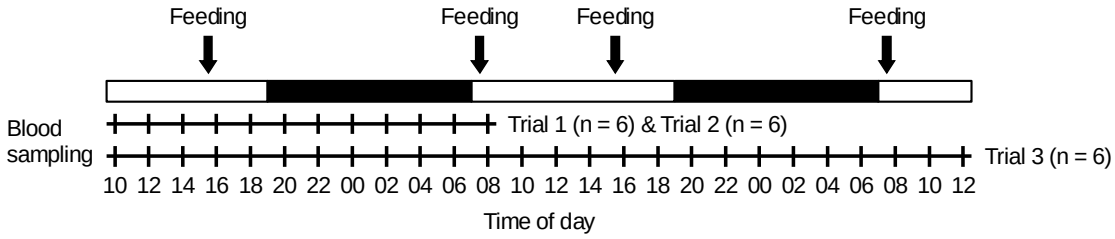

Fig. 1. Experimental protocol. White bars indicate light periods, black bars indicate dark periods, and arrows indicate concentrate feeding times. A total of 18 pigs were studied in three different experimental trials (n = 6 each) under identical experimental settings of 12:12 light-dark cycles. Blood sampling started at 10:00 h and was repeated every 2 hours. Twelve pigs were sampled for a total duration of 22 hours and 6 pigs were sampled for a total duration of 50 hours.

2.3. Hematology

To obtain total leukocyte counts and hematocrit, hematology measurements of K3 EDTA blood samples were carried out using an automated hematology analyzer (MEK-6108G, Nihon Kohden, Rosbach, Germany). All samples were measured in duplicate. The intra-

assay coefficient of variation for biological samples was 1.0% for leukocyte counts and 0.7% for hematocrit. Hematology analyses were finished within 60 min after blood sampling.

2.4. Flow cytometry

Specific immune cell populations were characterized using three-color flow cytometry with preceding immunofluorescent antibody staining of heparinized whole blood following an established standard protocol (Grün et al., 2013; Stefanski et al., 2005) with modifications. In detail, aliquots of 20 µl whole blood were incubated for 15 min at room temperature (RT) with different combinations of the following monoclonal antibodies (SouthernBiotech, Birmingham, AL, USA): SPRD-conjugated mouse anti-pig CD3ε (clone PPT3, IgG1, 0.1 mg/ml, working dilution (WD) 1:140), FITC-conjugated mouse anti-pig CD4 (clone 74-12-4, IgG2b, 0.5 mg/ml, WD 1:350), FITC-conjugated mouse anti-pig CD8α (clone 76-2-11, IgG2a, 0.5 mg/ml, WD 1:350), PE-conjugated mouse anti-pig CD8α (clone 76-2-11, IgG2a, 0.1 mg/ml, WD 1:350), and PE-conjugated mouse anti-pig CD172a (clone 74-22-15, IgG1, 0.1 mg/ml, WD 1:350). Then, erythrocytes were lysed and cells were fixed by incubation with BD FACS Lysing Solution (BD Biosciences, Heidelberg, Germany) for 10 min at RT, followed by two washing steps in phosphate-buffered saline supplemented with 2% fetal bovine serum and 0.1% NaN_3. The staining procedure was conducted immediately after blood sampling and completed within 90 min for all samples. All stained samples were maintained at 4°C until flow cytometric determination (BD FACSCalibur, BD Biosciences). Analysis was performed using the software BD CellQuest Pro 6 (BD Biosciences). At least 10,000 cells were analyzed per sample. Peripheral blood mononuclear cells (PBMC) and granulocytes were differentiated based on their forward and side scatter characteristics. As eosinophils exhibit bright autofluorescence (Weil and Chused, 1981), granulocyte subpopulations were further differentiated into non-fluorescent neutrophils and autofluorescent eosinophils by analyzing an unstained sample. Further discrimination of immune cell populations was carried out within PBMC. A specific characteristic of the porcine immune system is the occurrence of CD8α expression on porcine $CD4^+$ T cells, which is related to antigen (Ag) contact and indicates activated and memory $CD4^+$ T cells in pigs (Gerner et al., 2015). In accordance to this and based on previous research (Piriou-Guzylack and Salmon, 2008; Summerfield et al., 2003; Summerfield and McCullough, 2009; Talker et al., 2013), PBMC subpopulations were classified by the following combinations of surface marker expression: T cells ($CD3^+$), naive T helper (Th) cells ($CD3^+$ $CD4^+$ $CD8\alpha^-$),

Ag-experienced (Ag-exp.) Th cells ($CD3^+$ $CD4^+$ $CD8\alpha^+$), cytotoxic T cells ($CD3^+$ $CD4^-$ $CD8\alpha^{high}$), γδ T cells ($CD3^+$ $CD4^-$ $CD8\alpha^{-/dim}$), NK cells ($CD3^-$ $CD8\alpha^+$ $CD172a^-$), monocytes ($CD3^-$ $CD8\alpha^-$ $CD172a^{high}$), dendritic cells ($CD3^-$ $CD8\alpha^-$ $CD172a^{dim}$), and B cells ($CD3^-$ $CD8\alpha^-$ $CD172a^-$). The gating strategy is depicted in Supplementary Fig. S1. As it is not yet known whether porcine $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells display different functions (Talker et al., 2013), the results concerning these two subsets are presented separately in an associated *Data in Brief* article (Engert et al., submitted for publication). Absolute cell numbers were calculated by combining cell frequencies with total leukocyte counts.

2.5. Cortisol radioimmunoassay

Blood plasma was obtained from heparinized blood samples by centrifugation (15 min at 2000 × *g* at 4°C) within 45 min after blood sampling and stored at −80°C until measurement. Plasma cortisol concentrations were analyzed radioimmunologically with preceding ethanolic extraction. Measurements were carried out as previously described (Engert et al., 2017) with the modification that 80 pg of [1,2-^{3}H]cortisol (50 Ci/mmol, American Radiolabeled Chemicals, Saint Louis, USA) was used as tracer. The lower limit of quantification for routine measurements based on 50 µl ethanolic supernatant was 2 ng/ml. If concentrations were below the lower limit of quantification, the amount of ethanolic extract per determination was increased to 125 µl (linearity confirmed in preceding experiments). Precision was determined with spiked controls and revealed a mean recovery rate of 107%. Intra-assay and inter-assay variabilities for a biological sample were 7.3% and 13.1%, respectively.

2.6. Activity behavior

In the third trial, animal behavior was recorded with cameras, which were equipped with infrared technology for night records (VIEWEX-350/WS, Monacor International, Bremen, Germany). Recording started 2 hours before the first blood sampling and was continued until the last blood sampling. Data analysis was performed with the software The Observer XT 11 (Noldus, Oberreifenberg, Germany). Animal behavior was assessed by focal sampling and continuous recording and was divided into inactivity and activity. Resting of pigs in prone or lateral position was classified as inactivity, whereas all other behaviors were classified as activity. Relative activity behavior for every single animal was calculated as

proportion of time, in which the animal was active within each two-hour interval preceding the respective blood sampling.

2.7. Statistical analyses

Evaluation of diurnal rhythmicity was performed with R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). We used the package *cosinor* (Sachs, 2014) to carry out cosinor analysis (Nelson et al., 1979). Period length was set to 24 hours in all cosinor models. To obtain overall diurnal rhythmicity in each investigated variable, cosinor analyses were run with combined datasets of all animals. Subsequently, cosinor analyses were rerun for every single animal to obtain individual single diurnal rhythmicity (see Supplementary Table S1). The diurnal rhythms were characterized by mesor (average value of the fitted cosine function), amplitude (half the difference between maximum and minimum of the fitted cosine function), and peak time (time of the maximum of the fitted cosine function). Diurnal rhythms were considered significant if cosinor models revealed $P < 0.05$ for the amplitude. Peak times were calculated by the formula $-\Phi 24/(2\pi)$ using the phase shift Φ denoted by R and by setting 00:00 h (24 hours) as reference time.

Statistical comparisons of amplitudes and peak times were performed with IBM SPSS Statistics 22 (IBM Deutschland, Ehningen, Germany). Paired Student's *t*-tests (two-tailed) were used for pairwise comparisons. Normality of differences was confirmed by Shapiro-Wilk tests and quantile-quantile plots. For the comparison of more than two groups of variables, Gaussian distribution of data in all groups was analyzed by Shapiro-Wilk tests and quantile-quantile plots. If data were normally distributed, repeated measures ANOVA was used for comparisons. Greenhouse-Geisser correction was applied when assumption of sphericity was not met (checked by Mauchly's test). *Post hoc* tests with Bonferroni correction were used for subsequent multiple pairwise comparisons. If data were not normally distributed, Friedman test with subsequent Wilcoxon signed-rank tests with Bonferroni-Holm correction for *post hoc* analysis was performed.

To assess the potential association of light, concentrate feeding, plasma cortisol concentration, hematocrit, and repeated sampling with the cell numbers of different immune cell populations in porcine blood, IBM SPSS Statistics 22 was used to analyze the data of all animals (repeated measurements) with linear mixed models (LMM, MIXED command, Type III sums of squares, Satterthwaite's approximation to calculate denominator degrees of freedom). The restricted maximum likelihood method (REML) was used for parameter

estimation. The intercept, the factors light (off/on, indicating night or day) and concentrate feeding (yes/no, indicating the samplings just after feeding concentrate or not), as well as the covariates plasma cortisol concentration (ng/ml), hematocrit (%), and repeated sampling (1 to 26, indicating the ongoing number of samplings) were set as fixed effects. Repeated sampling was specified as repeated effect with animal identity as subject variable and the residual covariance structure was set as first order autoregressive (AR(1)) in all models. The strategy of backward model selection was used to identify relevant influencing random effects and the most appropriate model was selected by the lowest AIC. Model selection was performed for total leukocyte counts only. The final model structure was applied to all other immune cell populations to provide comparability among them. After testing several possible influencing effects, animal identity (n = 18), experimental trial (n = 3), and litter (n = 9) were finally set as random effects with a scaled identity (ID) covariance structure. Homoscedasticity and normality were confirmed by plotting residuals *versus* predicted values and by quantile-quantile plots of residuals, respectively. If necessary, logarithmic transformation was applied. Single outliers had to be excluded in three models due to technical difficulties in flow cytometric analysis (at most 1 value out of 300). Refer to Supplementary Table S2 for details. In all linear mixed models $P < 0.05$ was considered significant. Activity behavior was not included into mixed model analysis because of the limited number of animals, in which behavior was investigated.

3. Results

3.1. Domestic pigs exhibit diurnal rhythms in peripheral blood immune cell numbers

Within the main immune cell populations and investigated T cell subpopulations, absolute numbers in blood of NK cells, T cells, dendritic cells, monocytes, neutrophils, eosinophils, total Th cells, cytotoxic T cells, and γδ T cells exhibited overall diurnal rhythms as did the number of total leukocytes in blood, whereas B cell counts showed no overall diurnal rhythmicity (Table 1, Fig. 2A, B). When investigating Th cell subtypes with distinctive differentiation states, diurnal rhythmicity was only found in naive Th cell numbers but not in Ag-exp. Th cell numbers in blood (Table 1, Fig. 2C). When conducting individual single cosinor analyses, diurnal rhythmicity in the numbers of NK cells, T cells, eosinophils, cytotoxic T cells, γδ T cells, and naive Th cells in blood was found in all investigated animals (Supplementary Table S1). However, cell numbers of the other investigated cell

Table 1

Results of overall cosinor analyses with combined datasets of all animals

Variable	*P* [a]	n[b]	Mesor	Amplitude	Amplitude[c] [%]	Peak time[d]
Leukocytes [/µl]	< 0.001	18	16000.3 ± 139.9	696.7 ± 196.6	4.4 ± 1.2	20:44 ± 01:05
NK cells [/µl]	< 0.001	18	103.6 ± 2.5	32.8 ± 3.5	31.7 ± 3.4	10:55 ± 00:25
T cells [/µl]	< 0.001	18	7637.6 ± 67.7	685.1 ± 94.1	9.0 ± 1.2	23:14 ± 00:33
B cells [/µl]	0.510	18	1530.0 ± 25.7	23.9 ± 36.4	1.6 ± 2.4	19:57 ± 05:48
Dendritic cells [/µl]	0.005	18	998.9 ± 26.8	104.6 ± 37.5	10.5 ± 3.8	21:17 ± 01:24
Monocytes [/µl]	0.001	18	1044.3 ± 12.7	58.0 ± 18.1	5.6 ± 1.7	18:26 ± 01:10
Neutrophils [/µl]	< 0.001	18	3963.2 ± 53.0	412.7 ± 74.2	10.4 ± 1.9	12:47 ± 00:42
Eosinophils [/µl]	< 0.001	18	698.6 ± 13.3	226.2 ± 18.7	32.4 ± 2.7	20:47 ± 00:19
Total Th cells [/µl]	< 0.001	18	2256.9 ± 23.5	177.7 ± 32.6	7.9 ± 1.4	23:30 ± 00:43
Cytotoxic T cells [/µl]	< 0.001	18	1208.0 ± 21.2	223.1 ± 29.6	18.5 ± 2.4	23:49 ± 00:31
γδ T cells [/µl]	0.002	18	4183.7 ± 70.1	302.5 ± 97.5	7.2 ± 2.3	22:39 ± 01:16
Naive Th cells [/µl]	< 0.001	18	1275.0 ± 18.3	185.8 ± 25.5	14.6 ± 2.0	23:14 ± 00:32
Ag-exp. Th cells [/µl]	0.372	18	982.0 ± 12.1	15.4 ± 17.2	1.6 ± 1.8	07:28 ± 04:14
Activity behavior [%]	< 0.001	6	20.6 ± 1.5	18.4 ± 2.2	89.3 ± 10.6	14:14 ± 00:27
Cortisol [ng/ml]	< 0.001	18	15.5 ± 0.5	11.9 ± 0.7	77.0 ± 4.5	07:34 ± 00:13
Hematocrit [%]	< 0.001	18	32.9 ± 0.1	1.0 ± 0.1	3.1 ± 0.4	12:59 ± 00:33

Values are presented as mean ± SEM

[a] Significant diurnal rhythmicity at $P < 0.05$

[b] Number of animals in analyzed combined datasets

[c] Relative amplitude (amplitude/mesor)

[d] Time of day ± hh:mm

types in blood, including B cells and Ag-exp. Th cells, showed diurnal rhythms not in all but in a differing proportion of individual pigs (Supplementary Table S1).

To assess potential differences in the strength of diurnal oscillation, the relative amplitudes of cell numbers of different immune cell populations were compared by using the results of individual single cosinor analyses. In this respect, among Th cell subtypes with distinctive differentiation states in blood (Fig. 2D; $t(10) = 9.05$, $P = 3.94 \times 10^{-06}$), naive Th cell counts (95% confidence interval (CI) 13.2% to 15.8%) oscillated stronger than Ag-exp. Th cell

counts (CI 4.3% to 8.4%). Moreover, differences in the relative amplitudes of cell numbers in blood were also found for main immune cell types and the investigated T cell subpopulations (Supplementary Fig. S2A, B). To evaluate potential differences in the time

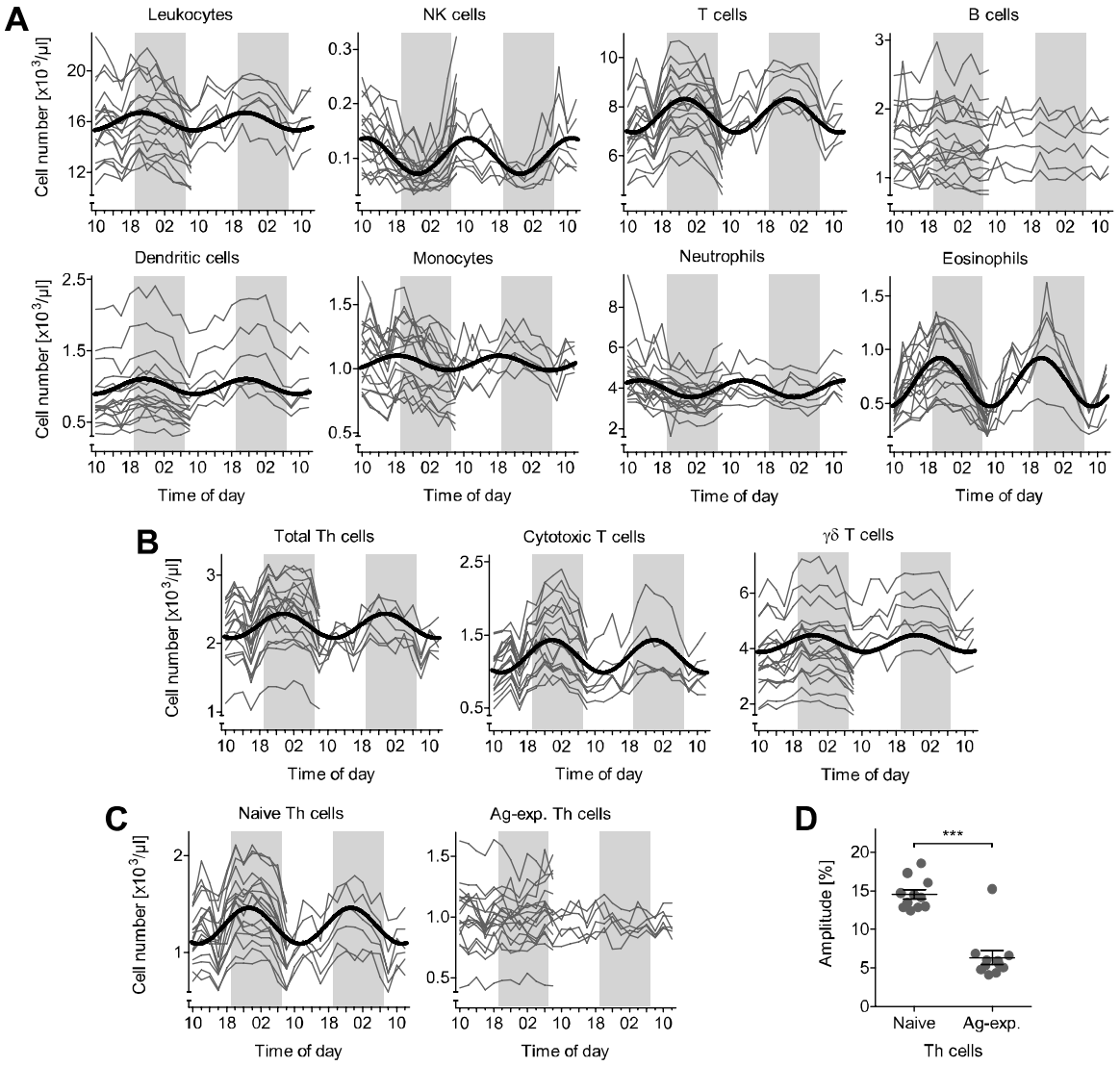

Fig. 2. Diurnal rhythms of the cell numbers of different immune cell populations in porcine blood. Shaded areas indicate lights off. Gray lines indicate measured values of each single animal in the study (n = 18), black curves correspond to the results of overall cosinor analyses with combined datasets of all 18 animals (significant diurnal rhythmicity at $P < 0.05$, refer to Table 1) for (**A**) main immune cell populations, (**B**) different T cell subpopulations, and (**C**) Th cell subtypes with distinctive differentiation states. (**D**) Relative amplitudes of the cell numbers of naive and Ag-exp. Th cells in porcine blood. The graph and statistical analysis only includes values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in both depicted Th cell subtypes (n = 11, mean ± SEM, individual values shown in gray, refer to Supplementary Table S1, see complete datasets in Supplementary Fig. S2C). Pairwise comparison was performed with two-tailed, paired Student's *t*-test, *** $P < 0.001$.

of the maximum in cell numbers of certain immune cell populations in blood, their peak times were compared. Among the main immune cell populations in blood (Fig. 3; $F(1.80,14.38) = 97.47$, $P = 6.76 \times 10^{-09}$), NK cell (CI 09:56 h to 11:48 h) and neutrophil counts (CI 11:08 h to 13:48 h) peaked during the light phase and thus exhibited the earliest peak time relative to the time of lights on, whereas dendritic cell (CI 21:15 h to 22:41 h), monocyte (CI 16:03 h to 20:11 h), and eosinophil counts (CI 20:47 h to 21:29 h) peaked later, at the end of the light phase or in the early dark phase. T cell counts (CI 22:38 h to 23:55 h) peaked during the dark phase and displayed the latest peak time. Among the different T cell subpopulations in blood (Fig. 3; $F(2,32) = 6.73$, $P = 0.004$), γδ T cell counts

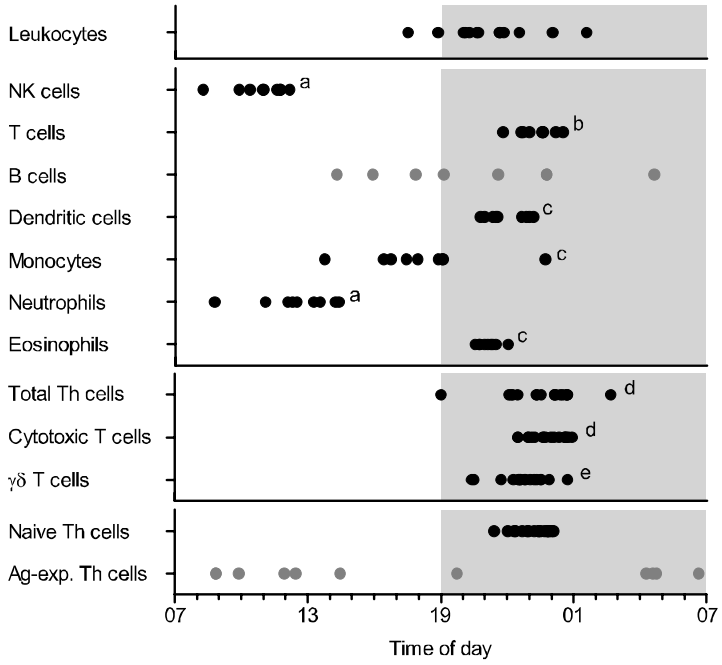

Fig. 3. Peak times of the cell numbers of different immune cell populations in porcine blood. Shaded area indicates lights off. The graph only includes values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in the respective immune cell populations (n = 8 to 18, refer to Supplementary Table S1). Gray points indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all 18 animals (significant diurnal rhythmicity at $P < 0.05$, refer to Table 1) in the respective cell types, which were excluded from statistical comparison. Multiple comparisons were performed within the main immune cell populations (NK cells, T cells, dendritic cells, monocytes, neutrophils, and eosinophils; n = 9) and within different T cell subpopulations (total Th cells, cytotoxic T cells, and γδ T cells; n = 17). For these cell types only the values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in all of the compared cell types, respectively, are depicted in the graph (see complete datasets in Supplementary Fig. S3). Comparisons were performed with repeated measures ANOVA followed by Bonferroni *post hoc* tests. Different letters indicate significant differences at $P < 0.05$.

(CI 22:08 h to 23:15 h) peaked earlier during the dark phase relative to the time of lights on than Th cell (CI 22:41 h to 00:24 h) and cytotoxic T cell counts (CI 23:21 h to 00:11 h). Whereas the numbers of naive Th cells in blood also peaked during the dark phase, Ag-exp. Th cell counts showed a wide distribution in peak times among individual pigs, as was also found for B cell counts (Fig. 3). Total leukocyte counts mainly peaked at the beginning of the dark phase in porcine blood (Fig. 3).

3.2. Parameters associated with peripheral blood immune cell numbers in domestic pigs

In addition to diurnal rhythmicity in peripheral blood immune cell numbers, the diurnal rhythms of activity behavior, plasma cortisol concentration, and hematocrit were investigated in the domestic pigs of the present study (Fig. 4). Overall cosinor analyses revealed that the calculated peak times of activity behavior and hematocrit were during the light phase (Table 1). The calculated peak time of plasma cortisol concentration was at the beginning of the light phase, coinciding with concentrate feeding in the morning (Table 1).

To assess potential associations of different parameters with immune cell numbers in porcine blood, namely light and concentrate feeding as main zeitgebers as well as cortisol as important endocrine mediator, linear mixed model analysis was performed (Table 2). In addition, hematocrit and sampling were included in the models to assess potential associations of diurnal changes in fluid balance and the experimental procedure itself with immune cell counts. As expected from cosinor analyses (refer to Table 1), the linear mixed

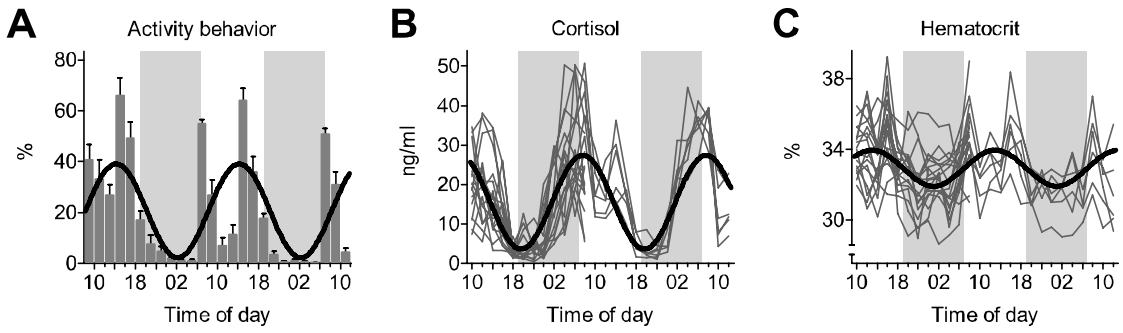

Fig. 4. Diurnal rhythms of activity behavior, plasma cortisol concentration, and hematocrit in domestic pigs. Shaded areas indicate lights off. Black curves correspond to the results of overall cosinor analyses with combined datasets of all animals (n = 6 to 18, significant diurnal rhythmicity at $P < 0.05$, refer to Table 1). (**A**) Bars represent the proportion of time, in which the animals were active within the two-hour interval preceding blood sampling (n = 6, mean ± SEM). Gray lines indicate measured values for (**B**) plasma cortisol concentration or (**C**) hematocrit of each single animal in the study (n = 18).

Table 2

Potential associations of various fixed effects with immune cell numbers in porcine blood[a]

Variable	Fixed effects: *P*-values and estimated associations[b]				
	Light (off)	Feeding (yes)	Cortisol	Hematocrit	Sampling
Leukocytes	1.18×10^{-07} ↑	3.44×10^{-26} ↓	5.36×10^{-04} ↓	1.03×10^{-08} ↑	0.019 ↓
NK cells	5.55×10^{-14} ↓	0.430 ↔	1.63×10^{-06} ↑	2.57×10^{-05} ↑	0.157 ↔
T cells	6.89×10^{-27} ↑	1.40×10^{-24} ↓	0.003 ↓	0.002 ↑	0.848 ↔
B cells	0.004 ↑	0.004 ↓	0.004 ↓	0.002 ↑	0.028 ↓
Dendritic cells	4.02×10^{-11} ↑	5.44×10^{-09} ↓	1.22×10^{-14} ↓	0.009 ↑	0.011 ↓
Monocytes	0.893 ↔	4.31×10^{-14} ↓	2.52×10^{-04} ↓	0.002 ↑	0.018 ↓
Neutrophils	1.28×10^{-06} ↓	0.007 ↓	0.916 ↔	2.33×10^{-05} ↑	0.014 ↓
Eosinophils	8.10×10^{-12} ↑	0.034 ↓	5.30×10^{-07} ↓	0.456 ↔	0.243 ↔
Total Th cells	8.44×10^{-18} ↑	1.05×10^{-26} ↓	0.001 ↓	8.80×10^{-04} ↑	0.193 ↔
Cytotoxic T cells	1.37×10^{-20} ↑	3.21×10^{-14} ↓	0.002 ↓	0.406 ↔	0.019 ↑
γδ T cells	1.28×10^{-22} ↑	1.41×10^{-09} ↓	2.54×10^{-04} ↓	0.002 ↑	0.093 ↔
Naive Th cells	2.17×10^{-27} ↑	3.17×10^{-28} ↓	1.06×10^{-08} ↓	0.043 ↑	0.884 ↔
Ag-exp. Th cells	0.754 ↔	8.22×10^{-14} ↓	0.276 ↔	1.04×10^{-05} ↑	0.003 ↑

[a] Potential associations were assessed by linear mixed model analyses: $y_{ij} = \mu + light_j + concentrate\ feeding_j + plasma\ cortisol\ concentration_{ij} + hematocrit_{ij} + sampling_j + animal\ identity_i + experimental\ trial_i + litter_i + \varepsilon_{ij}$. Thereby, y_{ij} represents the cell number/µl blood of the specific immune cell population in porcine blood for an animal *i* at sampling *j*, the intercept µ, the factors light (off/on) and concentrate feeding (yes/no), and the covariates plasma cortisol concentration (ng/ml), hematocrit (%), and sampling (1 to 12 in 12 animals or 1 to 26 in 6 animals) were set as fixed effects. The factors animal identity (n = 18), experimental trial (n = 3), and litter (n = 9) were set as random effects with a scaled identity (ID) covariance structure. Sampling was set as repeated effect with a first order autoregressive (AR(1)) residual covariance structure (ε_{ij}). Animal identity was set as subject.

[b] Estimated association: ↑ positive, ↓ negative, ↔ none (refer to Supplementary Table S2 for details)

model analyses revealed a positive association of the factor "lights off" with the cell numbers of most immune cell types in blood, whereas a negative association was found for NK cell and neutrophil counts. The lacking association of light with monocyte and Ag-exp. Th cell counts also confirmed cosinor results as the former peaked at the light-dark transition and the latter lacked overall rhythmicity. The factor concentrate feeding was negatively

associated with the cell numbers of almost all investigated cell types in blood, except for NK cells, for which no association was found. Plasma cortisol concentration was negatively associated with the cell numbers of most immune cell types in blood, except again for NK cells, for which a positive association was found, and except for neutrophils and Ag-exp. Th cells, for which no association was found. As one could expect, there was a positive association of hematocrit with the cell numbers of most immune cell types in blood, but interestingly, no association was found for eosinophils and cytotoxic T cells. Repeated blood sampling was not associated with the cell numbers of NK cells, T cells, eosinophils, total Th cells, γδ T cells, and naive Th cells in blood, whereas a negative association was found for the other investigated cell types, except for cytotoxic T cells and Ag-exp. Th cells, for which a positive association was found.

4. Discussion

The current study confirmed the occurrence of diurnal rhythms in the cell numbers of all investigated peripheral blood immune cell types in pigs, except for B cells and Ag-exp. Th cells. As blood immune cells oscillate according to the normal sleep-wake cycle of an organism (Lange et al., 2010), a comparison between data of the present study and results of other studies will be carried out in relation to the respective active or resting phase of the investigated species, with humans being diurnally active and rodents being nocturnally active. In this respect, analysis of activity behavior clearly showed that domestic pigs under the implied conditions are diurnally active as already shown by other studies (Ingram et al., 1980; Ingram and Dauncey, 1985). Consequently, porcine plasma cortisol concentrations were also found to be high in the morning and low in the evening in the present study, thus as expected (Désautés et al., 1999).

Like in humans, rodents (Born et al., 1997; Pelegrí et al., 2003), and miniature pigs (Tsutsumi et al., 1999; Tumbleson et al., 1972), total leukocyte counts in porcine blood peaked after the beginning of the resting phase in the present study. This rise in leukocyte numbers was mainly caused by an increase of total T cell counts, which again resembles findings in humans and rodents (Born et al., 1997; Dimitrov et al., 2007; Pelegrí et al., 2003). Investigating different T cell types in blood revealed that Th cell, cytotoxic T cell, and γδ T cell counts also peaked during the resting phase in pigs. These findings confirm studies in humans and rodents (Ackermann et al., 2012; Born et al., 1997; Dimitrov et al., 2009; Pelegrí et al., 2003; Suzuki et al., 2016), with the exception of one report showing higher

cytotoxic T cell and γδ T cell counts during the active phase in humans (Mazzoccoli et al., 2011). The reason for this discrepancy in findings in human studies is not clear. To our knowledge, the study of Mazzoccoli et al. (2011) is the only one investigating diurnal rhythms in peripheral γδ T cell counts in human blood so far. As γδ T cells are a major subset of up to 30% among all circulating lymphocytes in pigs (Takamatsu et al., 2006), this species seems to be a useful model to study mechanisms of diurnal rhythmicity in this cell type.

Glucocorticoids are regarded as a main link transmitting time information between the master circadian clock and peripheral clocks in different tissues (Dickmeis, 2009; Haus, 2007; Le Minh et al., 2001). In addition, there is some evidence that diurnal differences in the number of circulating T cells mainly result from glucocorticoid-mediated trafficking of these cells from blood to bone marrow (Besedovsky et al., 2014a; Besedovsky et al., 2014b; Chung et al., 1986; Cox and Ford, 1982; Fauci, 1975). This is also confirmed by studies on the effect of stress-induced glucocorticoid-release leading to redistribution of T cells from blood to tissue sites including the bone marrow (Dhabhar et al., 2012; Stefanski et al., 2003; Sudo et al., 1997). Accordingly, we found a negative association of plasma cortisol concentration with the cell numbers of total T cells and almost all T cell subpopulations in porcine blood. It was further shown in humans that the magnitude of diurnal oscillation of circulating T cells depends on their differentiation states, with naive T cells oscillating stronger than Ag-exp. T cells in human blood (Dimitrov et al., 2009). Interestingly, our results resembled these findings in humans. Naive Th cell counts in porcine blood displayed a pronounced oscillation and a negative association with plasma cortisol concentration, whereas Ag-exp. Th cell counts did not.

Like in humans and rodents (Dimitrov et al., 2007; Pelegrí et al., 2003), the peak of NK cell numbers in porcine blood was in the active phase. In accordance with findings in rodents (Dhabhar et al., 2012), plasma cortisol concentration was positively associated with NK cell number in porcine blood in the present study. In human blood, however, it was shown that catecholamines are responsible for elevations in the number of NK cells by presumably mobilizing them from the marginal pool (Benschop et al., 1994; Dimitrov et al., 2010; Schedlowski et al., 1996). There are only few studies investigating diurnal rhythms of catecholamines in pigs with conflicting results (Hay et al., 2000; Klemcke et al., 1989). Thus, further experiments should investigate the potential diurnal rhythmicity of catecholamines and their potential influence on NK cell numbers in porcine blood.

We did not find overall diurnal rhythmicity in the number of B cells in porcine blood, whereas in humans and rodents B cells were shown to peak during the resting phase (Ackermann et al., 2012; Born et al., 1997; Pelegrí et al., 2003). The reason for this discrepancy in findings is not yet clear. Analyzing B cell subtypes in porcine blood in more detail could possibly reveal diurnal rhythmicity. In general, underlying mechanisms for diurnal rhythms in B cell numbers in blood are not well described and therefore need further clarification. Nevertheless, plasma cortisol concentration was negatively associated with B cell number in blood in the present study, which resembles results in rodents (Dhabhar et al., 2012).

Within peripheral blood immune cells exhibiting Ag-presenting properties, dendritic cell counts peaked after the beginning of the resting phase, whereas the peak time of monocyte numbers accompanied the day-night transition in porcine blood. These results are in accordance with findings in humans (Axelsson et al., 2013; Dimitrov et al., 2007; Lasselin et al., 2015; Sennels et al., 2011), whereas in nocturnal rodents, monocyte counts were found to peak later, during the first half of the resting phase (Nguyen et al., 2013; Pelegrí et al., 2003). It is generally assumed that newly generated dendritic cells and monocytes migrate from the bone marrow through the bloodstream to different tissues (Auffray et al., 2009; Banchereau et al., 2000). However, diurnal regulation of these mechanisms has to be elucidated further. In the present study, plasma cortisol concentration was negatively associated with dendritic cell and monocyte counts in porcine blood. This result resembles findings concerning glucocorticoid-mediated changes of monocyte numbers in rodent blood (Dhabhar et al., 2012). However, the effect of glucocorticoids on dendritic cell counts is poorly investigated yet.

Blood neutrophil counts peaked at midday during the active phase in pigs. In human blood these cells peak during the active phase as well, although later in the day (Born et al., 1997; Haus et al., 1983; Sennels et al., 2011). Under steady-state conditions, newly generated neutrophils are released from the bone marrow into the circulation and undergo clearance in bone marrow, spleen, or liver after a short circulatory half-life of approximately 6 to 12 hours (Adrover et al., 2016; Silvestre-Roig et al., 2016). Thereby, granulopoiesis is directly linked to the neutrophil clearance rate (Stark et al., 2005) and both were shown to be diurnally regulated (Casanova-Acebes et al., 2013). In the present study, plasma cortisol concentration was not associated with the number of neutrophils in porcine blood, which resembles previous findings in rodents (Dhabhar et al., 2012) and which also applies to our previous

report of lower glucocorticoid receptor number and binding affinity in granulocytes compared to PBMC in domestic pigs (Engert et al., 2017). Interestingly, neutrophil counts in mice (Oishi et al., 2006) and total granulocyte counts in rats (Pelegrí et al., 2003) peak in the early resting phase in these nocturnal species. Thus, diurnal rhythms of neutrophil counts in blood might be regulated differently relative to rest and activity among different species.

The peak time of eosinophil counts was in the resting phase in porcine blood, like in humans and rodents (Born et al., 1997; Haus et al., 1983; Sennels et al., 2011). After egress from the bone marrow, eosinophils migrate with a circulatory half-life of approximately 12 to 18 hours (Foot, 1965; Steinbach et al., 1979) to mucosal tissues in the respiratory, gastrointestinal, and lower genitourinary tracts (Cara et al., 2000). It is well established that systemic glucocorticoids induce eosinopenia in blood (Kothari and Saunders, 1961). Accordingly, a negative association between plasma cortisol concentration and eosinophil counts was found in the present study.

Regarding the underlying mechanisms of diurnal rhythmicity, entrainment of the master circadian clock is mainly dependent on the synchronization by light (Panda et al., 2002) or darkness (Mendoza et al., 2007). Accordingly, mixed model results concerning the factor light resembled those of cosinor analyses. However, vertebrates can also adapt to other external stimuli, such as restricted feeding times (Damiola et al., 2000). In this respect, we found that concentrate feeding was negatively associated with cell numbers in all investigated cell types in porcine blood, except for NK cells. Postprandial differences in leukocyte counts in blood were also found in humans and decreases in monocyte and lymphocyte numbers are suggested to reflect trafficking of those cells to the gut in order to support local immune responses (Hansen et al., 1997). It is also remarkable that we found an additional cortisol peak, beside the morning peak, which coincided with afternoon concentrate feeding. Additional daily peaks in plasma cortisol concentration were already reported for pigs (Ingram and Dauncey, 1985) and meal-related cortisol peaks were also found in humans (Follenius et al., 1982; Hansen et al., 1997). They are thought to display the synchronizing role of meals on endogenous cortisol rhythms (Follenius et al., 1982). Based on these results, we conclude that light and concentrate feeding both acted as zeitgebers in the present study.

The analysis of a potential association of diurnal changes in fluid balance with immune cell counts showed that a higher hematocrit was generally accompanied by greater immune cell numbers in porcine blood, except for eosinophils and cytotoxic T cells. An increase in

hematocrit due to reduction of blood plasma volume and release of erythrocytes from the spleen is known to be caused by catecholamines (Allen and Patterson, 1995; Hannon et al., 1985). Thus, porcine hematocrit might have been elevated by increasing catecholamine concentrations in blood at the beginning of the active phase (Dimitrov et al., 2009; Linsell et al., 1985). Similar to plasma cortisol, we also found meal-related peaks in hematocrit, again emphasizing the role of concentrate feeding as potential zeitgeber in pigs. Notably, diurnal variations in hematocrit with a peak time during the active phase found in the present study were also described for humans (Pocock et al., 1989; Sennels et al., 2011). We found that repeated blood sampling was negatively associated with cell numbers in blood for several porcine immune cell types. Since acute sleep deprivation alters the cell numbers of different immune cell subsets in human blood at night (Born et al., 1997), disturbance of the sleep of pigs, although well habituated to the sampling procedure, might be a possible reason for these sampling-related effects rather than repeated blood collection itself.

To summarize, we confirmed the occurrence of diurnal rhythms in the cell numbers of various peripheral blood immune cell populations in domestic pigs, which mainly resembles studies in humans (Cermakian et al., 2013; Lange et al., 2010) and nocturnal rodents (Scheiermann et al., 2013) in respect to their rest and activity phases, except for monocytes, neutrophils, and γδ T cells. Whereas in porcine blood the peak time in the number of monocytes is similar to humans, that of γδ T cells is similar to rodents, and that of neutrophils is different from both species. The mixed model analyses showed that light and concentrate feeding should act as zeitgebers in pigs and that cortisol seems to be an important mediator of diurnal oscillation in immune cell numbers in porcine blood. In conclusion, the domestic pig represents a suitable diurnally active large animal model to study diurnal immune rhythms. Further investigations should focus on the particular mechanisms leading to diurnal rhythmicity in cell numbers of various peripheral blood immune cell populations and elucidate the role of light and concentrate feeding as zeitgebers in domestic pigs. In addition, the importance of diurnal immune rhythms for immune competence should be investigated for different porcine age groups as well as for domestic pigs held under different housing systems.

Author contributions

SSS conceived and designed the study. LCE and SSS designed experiments, performed research, analyzed and interpreted the data, and wrote the manuscript. UW conducted the

cortisol analyses and supervised surgery. BP conducted the behavioral analyses. VS assisted in experimental design and manuscript preparation.

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Declaration of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at …

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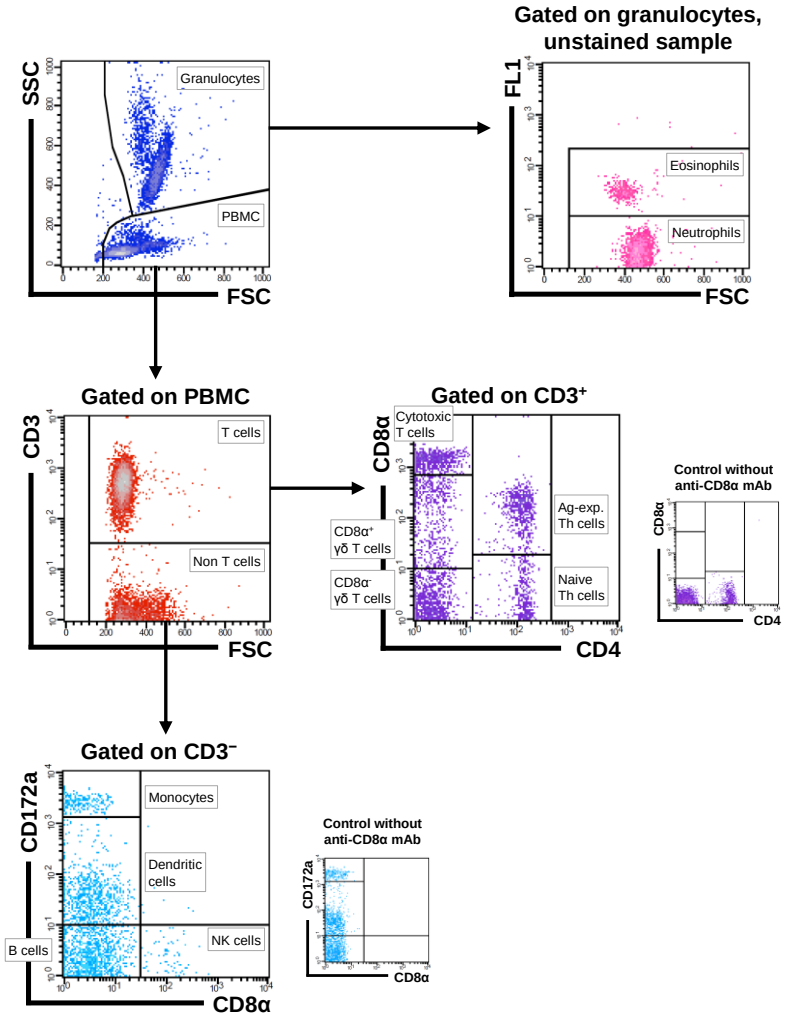
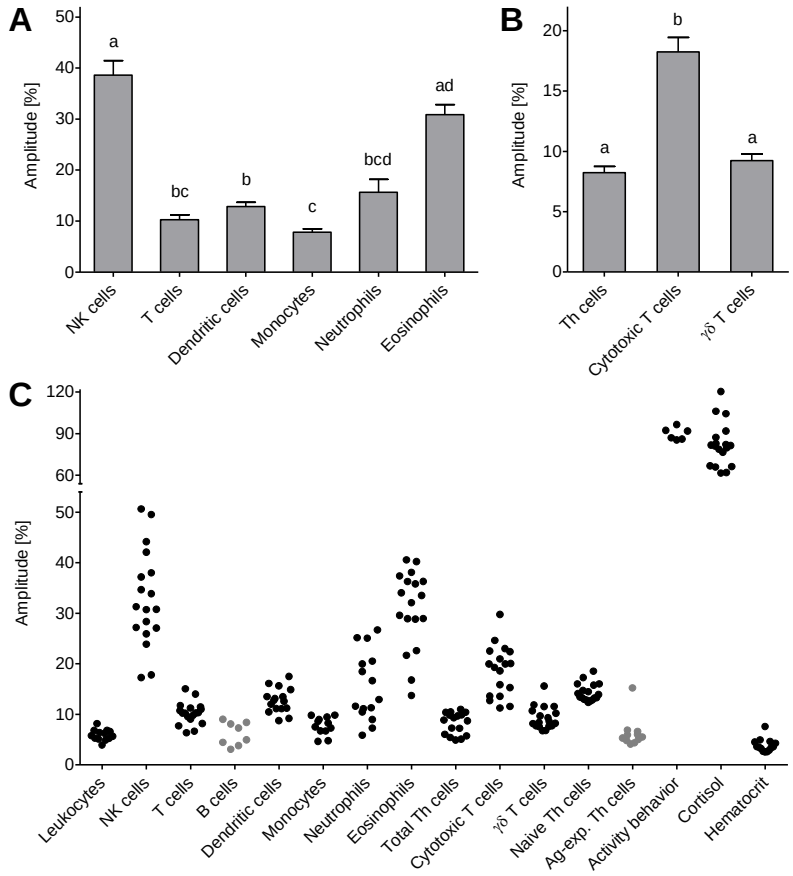
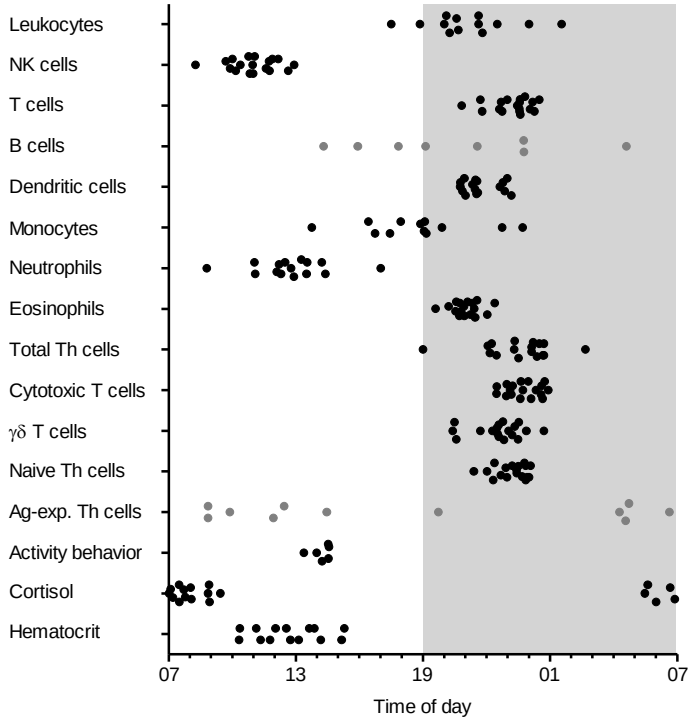
Supplementary material



Supplementary Fig. S1. Gating strategy for discrimination of porcine immune cells. Heparinized whole blood was either incubated with pig-specific fluorochrome-conjugated monoclonal antibodies (mAb) against CD3, CD8α, and CD4 (combination A) or against CD3, CD8α, and CD172a (combination B) or left without antibodies. After lysis of red blood cells, samples were analyzed by flow cytometry. Peripheral blood mononuclear cells (PBMC) and granulocytes were differentiated based on their forward and side scatter characteristics (upper left panel). In the unstained sample, granulocytes were further subdivided into neutrophils and eosinophils by plotting their forward scatter characteristics against the FL1 channel. Thereby, eosinophils were characterized by higher autofluorescence (upper right panel). The PBMC in either staining combination were initially differentiated into $CD3^+$ T cells and $CD3^-$ Non T cells (middle left panel). Subsequently, when stained with combination A, the $CD3^+$ T cells were further subdivided into $CD4^+/CD8\alpha^-$ naive and $CD4^+/CD8\alpha^+$ antigen-experienced (Ag-exp.) T helper (Th) cells, $CD4^-/CD8\alpha^{high}$ cytotoxic T cells, and $CD4^-/CD8\alpha^-$ and $CD4^-/CD8\alpha^{dim}$ ("$CD8\alpha^{+}$") γδ T cells (middle right panel). When stained with combination B, the $CD3^-$ Non T cells were further subdivided into $CD8\alpha^+/CD172a^-$ NK cells, $CD8\alpha^-/CD172a^{high}$ monocytes, $CD8\alpha^-/CD172a^{dim}$ dendritic cells, and $CD8\alpha^-/CD172a^-$ B cells (lower panel). For both combinations control stains without anti-CD8α mAb were included to verify precise gating of Ag-exp. Th cells and NK cells, respectively (small-sized purple and light blue panels).



Supplementary Fig. S2. Relative amplitudes of the cell numbers of different immune cell populations in blood, activity behavior, plasma cortisol concentration, and hematocrit in domestic pigs. (A+B) Both graphs only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in all of the compared cell types, respectively (n = 9 to 17, refer to Supplementary Table S1). Multiple comparisons within (A) main immune cell populations (n = 9, mean ± SEM) were performed with repeated measures ANOVA ($F(2.23,17.81) = 44.58$, $P = 7.01 \times 10^{-08}$; NK cells: 95% confidence interval (CI) 32.1% to 45.1%, T cells: CI 8.0% to 12.6%, dendritic cells: CI 10.9% to 14.8%, monocytes: CI 6.2% to 9.4%, neutrophils: CI 9.7% to 21.6%, eosinophils: CI 26.4% to 35.4%) followed by Bonferroni *post hoc* tests and within (B) different T cell subpopulations (n = 17, mean ± SEM) were performed with Friedman test ($\chi^2(2) = 25.53$, $P = 2.86 \times 10^{-06}$; Th cells: 25th to 75th percentile (Pctl) 5.9% to 10.1%, cytotoxic T cells: Pctl 13.6% to 21.7%, γδ T cells: Pctl 7.7% to 10.9%) followed by Wilcoxon signed-rank *post hoc* tests with Bonferroni-Holm correction. Different letters indicate significant differences at $P < 0.05$. (C) Relative amplitudes of all animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses (n = 6 to 18, refer to Supplementary Table S1). Gray points indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals (significant diurnal rhythmicity at $P < 0.05$, refer to Table 1 in main text).



Supplementary Fig. S3. Peak times of the cell numbers of different immune cell populations in blood, activity behavior, plasma cortisol concentration, and hematocrit in domestic pigs. Shaded area indicates lights off. The graph only includes values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in the respective variable (n = 6 to 18, refer to Supplementary Table S1). Gray points indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals (significant diurnal rhythmicity at $P < 0.05$, refer to Table 1 in main text).

Supplementary Table S1. Results of individual single cosinor analyses per animal

Variable	n[a]	Mesor	Amplitude	Amplitude[b] [%]	Peak time[c]
Leukocytes [/µl]	13	15549.5 ± 682.4	918.5 ± 63.9	5.9 ± 0.3	21:10 ± 00:35
NK cells [/µl]	18	105.5 ± 8.9	36.4 ± 4.8	32.8 ± 2.3	10:59 ± 00:16
T cells [/µl]	18	7423.5 ± 262.1	753.0 ± 46.8	10.2 ± 0.5	23:12 ± 00:14
B cells [/µl]	8	1428.7 ± 148.5	82.1 ± 10.3	6.1 ± 0.8	20:37 ± 01:40
Dendritic cells [/µl]	16	943.3 ± 104.9	117.5 ± 12.5	12.7 ± 0.6	21:44 ± 00:13
Monocytes [/µl]	12	1092.2 ± 53.4	82.9 ± 5.6	7.7 ± 0.5	18:44 ± 00:46
Neutrophils [/µl]	15	3932.0 ± 233.7	614.3 ± 91.0	15.5 ± 1.8	12:47 ± 00:28
Eosinophils [/µl]	18	678.5 ± 46.9	213.9 ± 21.5	30.9 ± 1.8	21:01 ± 00:09
Total Th cells [/µl]	17	2306.4 ± 104.4	189.4 ± 14.3	8.2 ± 0.5	23:32 ± 00:24
Cytotoxic T cells [/µl]	18	1252.0 ± 82.2	239.4 ± 26.9	18.6 ± 1.2	23:44 ± 00:11
γδ T cells [/µl]	18	3864.7 ± 273.6	357.1 ± 31.3	9.3 ± 0.5	22:35 ± 00:17
Naive Th cells [/µl]	18	1317.6 ± 72.3	189.1 ± 11.6	14.3 ± 0.4	23:11 ± 00:11
Ag-exp. Th cells [/µl]	11	1048.0 ± 68.0	67.8 ± 11.8	6.3 ± 0.9	07:29 ± 01:34
Activity behavior [%]	6	20.6 ± 1.6	18.5 ± 1.5	89.9 ± 1.8	14:12 ± 00:11
Cortisol [ng/ml]	18	14.4 ± 0.9	11.6 ± 0.7	82.0 ± 3.7	07:29 ± 00:16
Hematocrit [%]	14	33.1 ± 0.3	1.3 ± 0.1	3.8 ± 0.4	12:41 ± 00:26

Values are presented as mean ± SEM

[a] Number of animals out of 18 (out of 6 for activity behavior) with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses

[b] Relative amplitude (amplitude/mesor)

[c] Time of day ± hh:mm

Supplementary Table S2. Results of linear mixed model analyses[a]

Variable	Fixed effect	Estimate ± SE	*df* [b]	*F*	*P*
Leukocytes[c]	Intercept	9.2232 ± 0.0949	1,56.99	9926.25	1.26×10^{-65}
	Light (off)	0.0363 ± 0.0067	1,255.55	29.71	1.18×10^{-07}
	Feeding (yes)	-0.0824 ± 0.0068	1,226.78	145.34	3.44×10^{-26}
	Cortisol (per 1 ng/ml)	-0.0009 ± 0.0003	1,272.29	12.28	5.36×10^{-04}
	Hematocrit (per 1%)	0.0113 ± 0.0019	1,232.82	35.30	1.03×10^{-08}
	Sampling (per sample)	-0.0020 ± 0.0008	1,33.39	6.06	0.019
NK cells[c]	Intercept	3.1576 ± 0.4158	1,144.82	55.64	7.36×10^{-12}
	Light (off)	-0.2604 ± 0.0320	1,189.58	66.05	5.55×10^{-14}
	Feeding (yes)	0.0294 ± 0.0372	1,231.56	0.62	0.430
	Cortisol (per 1 ng/ml)	0.0061 ± 0.0012	1,199.99	24.42	1.63×10^{-06}
	Hematocrit (per 1%)	0.0437 ± 0.0102	1,256.93	18.38	2.57×10^{-05}
	Sampling (per sample)	0.0044 ± 0.0031	1,50.56	2.06	0.157
T cells[cd]	Intercept	8.5107 ± 0.1146	1,85.07	5920.73	1.98×10^{-80}
	Light (off)	0.1037 ± 0.0081	1,179.48	162.23	6.89×10^{-27}
	Feeding (yes)	-0.1072 ± 0.0092	1,210.78	136.04	1.40×10^{-24}
	Cortisol (per 1 ng/ml)	-0.0009 ± 0.0003	1,200.27	9.08	0.003
	Hematocrit (per 1%)	0.0079 ± 0.0025	1,233.90	9.62	0.002
	Sampling (per sample)	0.0002 ± 0.0008	1,34.13	0.04	0.848
B cells[d]	Intercept	983.54 ± 283.69	1,50.22	13.75	5.23×10^{-04}
	Light (off)	40.69 ± 13.90	1,181.17	8.57	0.004
	Feeding (yes)	-58.77 ± 20.41	1,272.90	8.29	0.004
	Cortisol (per 1 ng/ml)	-1.55 ± 0.53	1,154.79	8.61	0.004
	Hematocrit (per 1%)	16.91 ± 5.38	1,273.71	9.89	0.002
	Sampling (per sample)	-2.48 ± 1.12	1,117.69	4.95	0.028
Dendritic cells[c]	Intercept	6.2694 ± 0.2683	1,35.93	577.26	9.99×10^{-24}
	Light (off)	0.0922 ± 0.0131	1,181.28	49.45	4.02×10^{-11}
	Feeding (yes)	-0.0970 ± 0.0160	1,239.84	36.64	5.44×10^{-09}
	Cortisol (per 1 ng/ml)	-0.0043 ± 0.0005	1,179.90	70.74	1.22×10^{-14}
	Hematocrit (per 1%)	0.0116 ± 0.0044	1,263.02	7.01	0.009
	Sampling (per sample)	-0.0031 ± 0.0012	1,62.93	6.81	0.011
Monocytes[c]	Intercept	6.4517 ± 0.1848	1,90.24	1302.84	2.03×10^{-55}
	Light (off)	0.0018 ± 0.0132	1,209.22	0.02	0.893
	Feeding (yes)	-0.1206 ± 0.0150	1,236.87	64.62	4.31×10^{-14}
	Cortisol (per 1 ng/ml)	-0.0019 ± 0.0005	1,222.55	13.84	2.52×10^{-04}
	Hematocrit (per 1%)	0.0131 ± 0.0041	1,254.56	10.11	0.002
	Sampling (per sample)	-0.0032 ± 0.0013	1,55.62	5.98	0.018
Neutrophils[cd]	Intercept	7.7173 ± 0.1783	1,117.46	1968.74	3.14×10^{-75}
	Light (off)	-0.0761 ± 0.0153	1,261.39	24.58	1.28×10^{-06}
	Feeding (yes)	-0.0417 ± 0.0154	1,225.62	7.34	0.007
	Cortisol (per 1 ng/ml)	-0.0001 ± 0.0006	1,275.40	0.01	0.916
	Hematocrit (per 1%)	0.0184 ± 0.0043	1,233.10	18.65	2.33×10^{-05}
	Sampling (per sample)	-0.0052 ± 0.0020	1,29.69	6.87	0.014
Eosinophils[c]	Intercept	6.0953 ± 0.4001	1,186.44	262.28	2.11×10^{-37}
	Light (off)	0.2718 ± 0.0379	1,255.56	51.40	8.10×10^{-12}
	Feeding (yes)	-0.0818 ± 0.0383	1,220.56	4.55	0.034
	Cortisol (per 1 ng/ml)	-0.0074 ± 0.0014	1,275.38	26.38	5.30×10^{-07}
	Hematocrit (per 1%)	0.0079 ± 0.0105	1,233.42	0.56	0.456
	Sampling (per sample)	-0.0057 ± 0.0048	1,26.57	1.43	0.243

Supplementary Table S2. (*continued*)

Variable	Fixed effect	Estimate ± SE	*df* [b]	*F*	*P*
Total Th cells	Intercept	1254.27 ± 291.94	1,92.35	27.42	1.02×10^{-06}
	Light (off)	187.34 ± 19.72	1,192.54	90.28	8.44×10^{-18}
	Feeding (yes)	-288.08 ± 23.82	1,244.93	146.21	1.05×10^{-26}
	Cortisol (per 1 ng/ml)	-2.50 ± 0.76	1,193.55	10.75	0.001
	Hematocrit (per 1%)	21.95 ± 6.52	1,267.51	11.32	8.80×10^{-04}
	Sampling (per sample)	2.38 ± 1.81	1,68.85	1.73	0.193
Cytotoxic T cells[c]	Intercept	6.6837 ± 0.2564	1,88.96	750.26	3.95×10^{-45}
	Light (off)	0.1736 ± 0.0159	1,143.49	119.31	1.37×10^{-20}
	Feeding (yes)	-0.1698 ± 0.0210	1,245.48	65.07	3.21×10^{-14}
	Cortisol (per 1 ng/ml)	-0.0019 ± 0.0006	1,126.19	10.16	0.002
	Hematocrit (per 1%)	0.0047 ± 0.0057	1,279.61	0.69	0.406
	Sampling (per sample)	0.0033 ± 0.0014	1,59.37	5.79	0.019
γδ T cells	Intercept	2309.36 ± 657.79	1,52.16	17.10	1.29×10^{-04}
	Light (off)	435.90 ± 39.00	1,190.22	124.91	1.28×10^{-22}
	Feeding (yes)	-289.95 ± 45.99	1,235.96	39.75	1.41×10^{-09}
	Cortisol (per 1 ng/ml)	-5.61 ± 1.51	1,196.59	13.88	2.54×10^{-04}
	Hematocrit (per 1%)	39.30 ± 12.66	1,257.02	9.64	0.002
	Sampling (per sample)	-6.32 ± 3.69	1,56.65	2.93	0.093
Naive Th cells[c]	Intercept	6.6566 ± 0.1900	1,87.81	1355.50	3.65×10^{-55}
	Light (off)	0.1519 ± 0.0117	1,176.60	167.64	2.17×10^{-27}
	Feeding (yes)	-0.1935 ± 0.0155	1,257.59	155.40	3.17×10^{-28}
	Cortisol (per 1 ng/ml)	-0.0027 ± 0.0005	1,160.30	36.42	1.06×10^{-08}
	Hematocrit (per 1%)	0.0085 ± 0.0042	1,280.74	4.15	0.042
	Sampling (per sample)	-0.0001 ± 0.0010	1,85.27	0.02	0.884
Ag-exp. Th cells[c]	Intercept	6.2137 ± 0.1691	1,74.35	1433.09	2.44×10^{-50}
	Light (off)	-0.0035 ± 0.0111	1,204.36	0.10	0.754
	Feeding (yes)	-0.1039 ± 0.0131	1,242.59	62.82	8.22×10^{-14}
	Cortisol (per 1 ng/ml)	0.0005 ± 0.0004	1,210.23	1.19	0.276
	Hematocrit (per 1%)	0.0162 ± 0.0036	1,261.93	20.22	1.04×10^{-05}
	Sampling (per sample)	0.0032 ± 0.0011	1,67.99	9.36	0.003

[a] $y_{ij} = \mu + light_j + concentrate\ feeding_j + plasma\ cortisol\ concentration_{ij} + hematocrit_{ij} + sampling_j + animal\ identity_i + experimental\ trial_i + litter_i + \varepsilon_{ij}$; dependent variable: y_{ij} (cell number/µl blood) for animal *i* at sampling *j*; fixed effects: intercept µ, light (off/on), concentrate feeding (yes/no), plasma cortisol concentration (ng/ml), hematocrit (%), and sampling (1 to 12 in 12 animals or 1 to 26 in 6 animals); random effects (with scaled identity (ID) covariance structure): animal identity (n = 18), experimental trial (n = 3), and litter (n = 9); repeated effect (with first order autoregressive (AR(1)) residual (ε_{ij}) covariance structure): sampling; subject: animal identity

[b] Numerator degrees of freedom, denominator degrees of freedom

[c] Logarithmic transformation of data

[d] One single outlier (out of 300 measured values) excluded due to technical difficulties in flow cytometric analysis

II

Data characterizing diurnal rhythms in the number of peripheral CD8α⁻ and CD8α⁺ γδ T cells in domestic pigs

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Abstract

This data article is related to the original research article "Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs" of Engert et al. [1] and describes diurnal rhythms in the number of $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells in peripheral blood of domestic pigs. Blood samples were taken from 18 animals over periods of up to 50 hours and immune cell subtypes were determined by flow cytometry. Diurnal rhythmicity of cell numbers of γδ T cell subtypes was analyzed with cosinor analysis and different properties of rhythmicity (mesor, amplitude, and peak time) were calculated. In addition, associations between cell numbers of the investigated cell types in porcine blood with plasma cortisol concentration, hematocrit, and experimental conditions were identified with linear mixed model analysis.

Keywords: Diurnal rhythm; Immune system; Gamma-delta T cell; CD8 alpha; *Sus scrofa domestica*

Specifications Table

Subject area	Biology and Agricultural Science
More specific subject area	Porcine Immunology and Chronobiology
Type of data	Figures and tables
How data was acquired	Flow cytometry (BD FACSCalibur, BD Biosciences), cosinor analysis (R version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria), and linear mixed model analysis (IBM SPSS Statistics 22, IBM Deutschland, Ehningen, Germany)
Data format	Analyzed
Experimental factors	A total of 18 castrated male pigs (Piétrain × German landrace, 6-month-old) were held under a 12:12 light-dark cycle with *ad libitum* access to hay and water and concentrate feeding twice daily. Blood samples were taken every 2 hours over periods of up to 50 hours via indwelling vein catheters.
Experimental features	Heparinized whole blood samples were used to characterize diurnal rhythms in $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells of domestic pigs. The γδ T cell subtypes were characterized with fluorescent antibody staining and subsequent flow cytometric analysis.
Data source location	Experimental unit of the department Behavioral Physiology of Livestock, Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany
Data accessibility	Data are presented within this article and related to an original research article [1].

Value of the data

- The present data describe diurnal rhythms in the number of CD8α$^-$ and CD8α$^+$ γδ T cells in porcine blood and thus enhance knowledge about these specific porcine immune cell subtypes.
- The various properties of diurnal rhythmicity (mesor, amplitude, and peak time) in cell numbers of γδ T cell subtypes described here can be compared to data from other species as well as to other immune cell subtypes in domestic pigs.
- The association of CD8α$^-$ and CD8α$^+$ γδ T cell number in porcine blood with plasma cortisol concentration could contribute to future research about the effect of cortisol on circulating porcine γδ T cell numbers and its underlying mechanisms.

1. Data

In pigs and other livestock species γδ T cells are a major subset of up to 30% among all lymphocytes in blood with approximately one-third expressing CD8α [2]. The function of porcine CD8α$^-$ and CD8α$^+$ γδ T cells is not fully elucidated yet but studies imply potential functional differences between the two phenotypes [3–5]. The present data characterize diurnal rhythms in the cell numbers of these two subtypes of peripheral γδ T cells in domestic pigs (Fig. 1). A description of the different properties of rhythmicity (mesor, amplitude, relative amplitude, and peak time) is provided for overall cosinor analyses with combined datasets of all animals (Table 1) as well as for individual single cosinor analyses

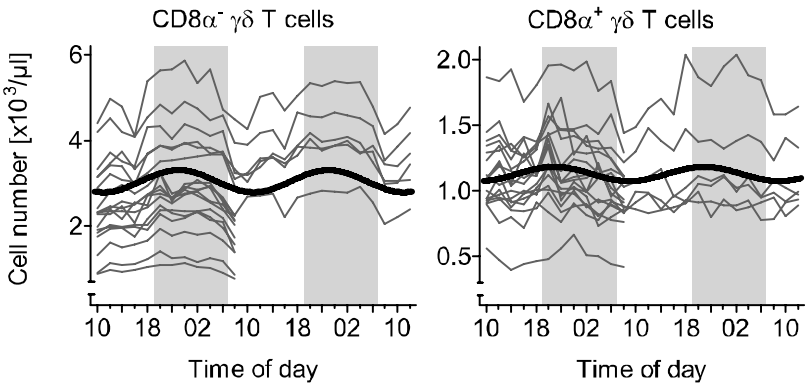

Fig. 1. Diurnal rhythms of the cell numbers of CD8α$^-$ and CD8α$^+$ γδ T cells in porcine blood. Shaded areas indicate lights off. Gray lines indicate measured values of each single animal in the study (n = 18), black curves correspond to the results of overall cosinor analyses with combined datasets of all 18 animals (significant diurnal rhythmicity at $P < 0.05$, refer to Table 1).

Table 1

Results of overall cosinor analyses for CD8α⁻ and CD8α⁺ γδ T cells with combined datasets of all animals

Variable	*P* [a]	n[b]	Mesor	Amplitude	Amplitude[c] [%]	Peak time[d]
CD8α⁻ γδ T cells [/µl]	0.004	18	3054.8 ± 63.6	255.3 ± 88.4	8.4 ± 2.9	23:00 ± 01:22
CD8α⁺ γδ T cells [/µl]	0.032	18	1128.9 ± 17.8	53.8 ± 25.0	4.8 ± 2.2	20:55 ± 01:48

Values are presented as mean ± SEM

[a] Significant diurnal rhythmicity at $P < 0.05$

[b] Number of animals in analyzed combined datasets

[c] Relative amplitude (amplitude/mesor)

[d] Time of day ± hh:mm

Table 2

Results of individual single cosinor analyses for CD8α⁻ and CD8α⁺ γδ T cells per animal

Variable	n[a]	Mesor	Amplitude	Amplitude[b] [%]	Peak time[c]
CD8α⁻ γδ T cells [/µl]	18	2752.7 ± 250.3	297.9 ± 25.4	11.3 ± 0.6	22:53 ± 00:14
CD8α⁺ γδ T cells [/µl]	12	1141.0 ± 96.1	95.3 ± 15.9	8.5 ± 1.2	21:31 ± 00:49

Values are presented as mean ± SEM

[a] Number of animals out of 18 with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses

[b] Relative amplitude (amplitude/mesor)

[c] Time of day ± hh:mm

performed per animal (Table 2). Both subtypes of γδ T cells exhibited diurnal rhythms in blood cell counts with mean peak times during the dark phase. Relative amplitudes did not differ (Fig. 2; $t(11) = 2.01$, $P = 0.070$) between CD8α⁻ γδ T cells (95% confidence interval (CI) 9.4% to 13.4%) and CD8α⁺ γδ T cells (CI 5.9% to 11.2%). Peak times also did not differ (Fig. 3; $t(11) = 1.50$, $P = 0.162$) between CD8α⁻ γδ T cells (CI 21:55 h to 23:19 h) and CD8α⁺ γδ T cells (CI 19:44 h to 23:18 h).

Linear mixed model analyses (Table 3) demonstrated that the cell numbers of CD8α⁻ and CD8α⁺ γδ T cells were positively associated with the factor light off and hematocrit but negatively associated with the factor concentrate feeding and plasma cortisol concentration. No association of the cell numbers of either γδ T cells subtypes was found with preceding sampling.

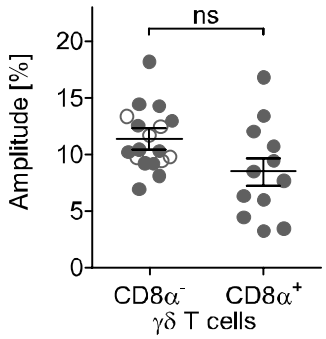

Fig. 2. Relative amplitudes of the cell numbers of CD8α⁻ and CD8α⁺ γδ T cells in porcine blood. The statistical analysis only includes values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in both depicted γδ T cell subtypes (n = 12 as mean ± SEM, the individual values included into comparison are shown as dots, refer to Table 2; the additional data values of complete datasets, which were not included into comparison, are shown as circles); ns $P \geq 0.05$.

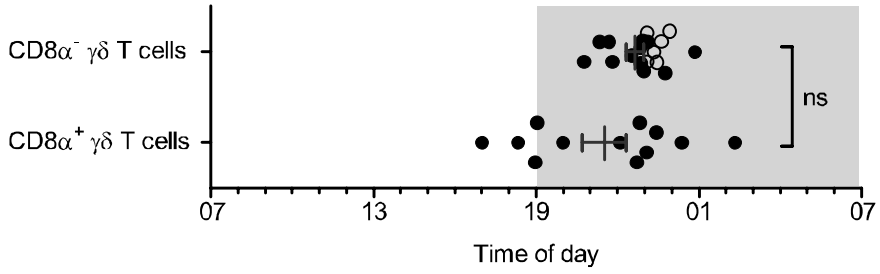

Fig. 3. Peak times of the cell numbers of CD8α⁻ and CD8α⁺ γδ T cells in porcine blood. The statistical analysis only includes values of animals with significant ($P < 0.05$) diurnal rhythm in individual single cosinor analyses in both depicted γδ T cell subtypes (n = 12 as mean ± SEM, the individual values included into comparison are shown as dots, refer to Table 2; the additional data values of complete datasets, which were not included into comparison, are shown as circles); ns $P \geq 0.05$.

2. Experimental design and methods

A detailed description of experimental design and methods used is provided in the accompanying research article [1]. Essential methodical information related to the present data is provided in the following sections.

Table 3

Results of linear mixed model analyses

Variable	Fixed effect	Estimate ± SE	*df* [a]	*F*	*P*	Dir.[b]
CD8α⁻ γδ T cells	Intercept	1689.13 ± 554.85	1,48.18	13.25	6.65×10^{-04}	
	Light (off)	359.32 ± 32.18	1,182.09	124.65	2.21×10^{-22}	↑
	Feeding (yes)	-233.96 ± 37.69	1,228.14	38.54	2.50×10^{-09}	↓
	Cortisol (per 1 ng/ml)	-3.88 ± 1.24	1,190.50	9.78	0.002	↓
	Hematocrit (per 1%)	25.57 ± 10.38	1,250.84	6.07	0.014	↑
	Sampling (per sample)	-3.33 ± 3.07	1,48.16	1.17	0.284	↔
CD8α⁺ γδ T cells[c]	Intercept	6.5272 ± 0.2184	1,56.23	943.00	7.99×10^{-37}	
	Light (off)	0.0574 ± 0.0127	1,190.18	20.36	1.12×10^{-05}	↑
	Feeding (yes)	-0.0538 ± 0.0157	1,248.30	11.65	7.50×10^{-04}	↓
	Cortisol (per 1 ng/ml)	-0.0016 ± 0.0005	1,186.34	10.64	0.001	↓
	Hematocrit (per 1%)	0.0125 ± 0.0043	1,269.78	8.42	0.004	↑
	Sampling (per sample)	-0.0021 ± 0.0011	1,75.66	3.49	0.066	↔

[a] Numerator degrees of freedom, denominator degrees of freedom

[b] Direction of estimated association: ↑ positive, ↓ negative, ↔ none

[c] Logarithmic transformation of data

2.1 Animals and surgery

All procedures were conducted in accordance with the German Animal Welfare Act and approved by the local Animal Welfare Ethics Committee (Regional Council Stuttgart, approval number V309/13TH). Eighteen castrated male pigs (Piétrain × German landrace, 6-month-old, weight range 92 to 106 kg) were included in the study and housed in a lightproof building (ambient temperature 21 ± 1°C). Animals were kept individually but had sight and tactile contact to neighboring animals. They had *ad libitum* access to hay and water and were fed concentrate (1.2 kg/meal, ME 12 MJ/kg) twice daily at 07:30 h and 15:30 h. All animals were maintained under a 12:12 light-dark cycle (lights on 07:00 h to 19:00 h). The average illuminance at pigs' eye level was 190 lx during the light phase (fluorescent tubes, 4000 K) and 0 lx during the dark phase. The animals were accustomed to the lighting

and feeding regime for at least 8 weeks prior to the experiments and well habituated to human handling. The pigs were surgically catheterized with indwelling vein catheters (*vena cava cranialis*) at least 2 weeks prior to sampling as previously described [1].

2.2 Experimental protocol and sample processing

The study was subdivided into 3 different experimental trials (n = 6 each, refer to Fig. 1 in the accompanying research article [1]). Blood sampling started at 10:00 h and was repeated every 2 hours in all trials. In the first 2 trials a total of 12 blood samples were taken until 08:00 h the following day (duration 22 hours each). The 3rd trial included a total of 26 blood samples and sampling ended at 12:00 h on the second following day (duration 50 hours). Blood sampling at night was performed under dim light, which was switched on and off for sampling (7 lx at pigs' eye level, 2700 K). Blood samples were immediately processed after each single sampling.

2.3 Flow cytometry

Heparinized whole blood (lithium heparin tubes, Sarstedt, Nümbrecht, Germany) was used to characterize immune cell subtypes by a three-color flow cytometric analysis as previously described [1]. Aliquots of 20 µl whole blood were incubated for 15 min at room temperature (RT) with different combinations of monoclonal antibodies (all obtained from SouthernBiotech, Birmingham, AL, USA). The characterization of CD8α$^-$ and CD8α$^+$ γδ T cells required SPRD-conjugated mouse anti-pig CD3ε antibody (clone PPT3, IgG1, 0.1 mg/ml, working dilution (WD) 1:140), FITC-conjugated mouse anti-pig CD4 antibody (clone 74-12-4, IgG2b, 0.5 mg/ml, WD 1:350), and PE-conjugated mouse anti-pig CD8α antibody (clone 76-2-11, IgG2a, 0.1 mg/ml, WD 1:350). Subsequently, the cells were incubated with BD FACS Lysing Solution (BD Biosciences, Heidelberg, Germany) for 10 min at RT, followed by two washing steps. The stained samples were maintained at 4°C until flow cytometric determination (BD FACSCalibur, BD Biosciences). At least 10,000 cells were analyzed per sample.

Flow cytometric data were processed using the software BD CellQuest Pro 6 (BD Biosciences). Initially, peripheral blood mononuclear cells (PBMC) and granulocytes were differentiated based on their forward and side scatter characteristics. According to previous research [3,6], T cells (CD3$^+$) were identified within PBMC by surface marker expression. Subsequently, CD8α$^-$ γδ T cells (CD3$^+$ CD4$^-$ CD8α$^-$) and CD8α$^+$ γδ T cells (CD3$^+$ CD4$^-$ CD8α^{dim}) were identified within T cells. The complete gating strategy within the present

study is depicted in Supplementary Fig. S1 in the accompanying research article [1]. Absolute cell numbers were calculated by combining cell frequencies with total leukocyte counts, which were obtained by an automated hematology analyzer (MEK-6108G, Nihon Kohden, Rosbach, Germany) measuring whole blood samples (K3 EDTA tubes Sarstedt, Nümbrecht, Germany).

2.4 Statistical analyses

Diurnal rhythms were assessed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Cosinor analysis [7] was carried out with the package *cosinor* [8]. As we were interested in diurnal rhythmicity according to the established 12:12 lighting regime, the period length was set to 24 hours in all cosinor models. At first cosinor analyses were run with combined datasets of all animals to obtain overall diurnal rhythmicity in the cell numbers of $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells in porcine blood. Then, cosinor analyses were rerun for every single animal to obtain individual single diurnal rhythmicity. Diurnal rhythmicity was characterized by mesor (average value of the fitted cosine function), amplitude (half the difference between maximum and minimum of the fitted cosine function), and peak time (time of the maximum of the fitted cosine function) and was considered significant if cosinor models revealed $P < 0.05$ for the amplitude. The peak times were calculated by the formula $-\Phi 24/(2\pi)$ using the phase shift Φ denoted by R and by setting 00:00 h (24 hours) as reference time.

Pairwise statistical comparisons were performed with IBM SPSS Statistics 22 (IBM Deutschland, Ehningen, Germany) using paired Student's *t*-tests (two-tailed). The normality of differences was confirmed by Shapiro-Wilk tests and quantile-quantile plots.

Associations of the cell numbers of $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells in porcine blood with the potential influencing variables light, concentrate feeding, plasma cortisol concentration, hematocrit, and repeated sampling were assessed with linear mixed models (IBM SPSS Statistics 22). A detailed methodical description of linear mixed model analysis and the data of the investigated explanatory variables can be found in the accompanying research article [1]. Homoscedasticity and normality were confirmed by plotting residuals *versus* predicted values and by quantile-quantile plots of residuals, respectively. If necessary, logarithmic transformation was applied. In all linear mixed models $P < 0.05$ was considered significant. After backward model selection to identify relevant random effects, the following model was applied:

$y_{ij} = \mu + light_j + concentrate\ feeding_j + plasma\ cortisol\ concentration_{ij} + hematocrit_{ij} + sampling_j + animal\ identity_i + experimental\ trial_i + litter_i + \varepsilon_{ij}$

Thereby, the dependent variable y_{ij} (cell number/µl blood) for an animal *i* at sampling *j* is explained by the fixed effects μ (intercept), *light* (off/on), *concentrate feeding* (yes/no), *plasma cortisol concentration* (ng/ml), *hematocrit* (%), and *sampling* (1 to 12 in 12 animals or 1 to 26 in 6 animals) as well as by the random effects *animal identity* (n = 18), *experimental trial* (n = 3), and *litter* (n = 9). The covariance structure for the repeated effect *sampling* was set as first order autoregressive (AR(1)) and for the random effects as scaled identity (ID). The variable *animal identity* designated the subjects in the analysis.

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Declaration of interest

The authors report no conflicts of interest.

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III

Photoperiodic effects on diurnal rhythms in cell numbers of peripheral leukocytes in domestic pigs

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Abstract

The photoperiod is known to modulate immune cell number and function and is regarded essential for seasonal disease susceptibility. In addition, diurnal variations in the immune system are regarded important for immune competence. Whereas few studies investigated the influence of season, none investigated the specific effect of the photoperiod on these diurnal immune rhythms until now. Therefore, the present study compared diurnal rhythms in cell numbers of peripheral leukocyte types in domestic pigs held either under long day conditions (LD) or short day conditions (SD). Cosinor analyses of cell numbers of various peripheral leukocyte subtypes investigated over periods of 50 hours revealed distinct photoperiodic differences in diurnal immune rhythms. Relative amplitudes of cell numbers of total leukocytes, NK cells, T cells, and monocytes in blood were higher under SD than LD. In addition, cell counts of total leukocytes, NK cells, T cells including various T cell subtypes, and eosinophils peaked earlier relative to the time of lights-on under SD than LD. In contrast, diurnal rhythms of neutrophil counts did not show photoperiodic differences. Mesor values did not differ in any leukocyte type. Generalized linear mixed model analyses revealed associations of leukocyte counts with plasma cortisol concentration and activity behavior in most investigated cell types. Moreover, the present study demonstrated photoperiodic effects on diurnal rhythms in plasma cortisol concentrations and activity behavior, which is in agreement with human and primate studies. The results of the present study imply stronger rhythmicity in leukocyte counts in general under SD. Common intrinsic mechanisms seem to regulate photoperiodic effects on diurnal rhythms in leukocyte counts, except for neutrophils, in domestic pigs. Our results reveal considerable insights into the regulation of immune rhythms in diurnally active species.

Keywords: photoperiod, season, diurnal rhythm, circadian rhythm, immune system, cortisol, activity behavior, swine

1 Introduction

Diurnal and seasonal rhythms are important characteristics of physiology and behavior in humans and animals (1–4). Thereby, the alteration between day and night regulates diurnal (i.e., 24 hour) rhythmicity via photic entrainment of the master circadian clock located in the *suprachiasmatic nucleus* (SCN) within the anteroventral hypothalamus of the brain (5–7). Correspondingly, seasonal rhythms are assumed to be mediated by an intrinsic circannual clock as well, potentially located in the *pars tuberalis* within the anterior pituitary (8–10),

with the relative span of light per day (i.e., long photoperiod during summer, short photoperiod during winter) serving as seasonal timer (4).

Beside differences in physiology and behavior, seasonal differences were also found in the incidence of disease and mortality in many species (11–19). In this respect, the photoperiod is regarded essential for seasonal disease susceptibility as it is known to modulate immune function (15, 20–23). Seasonal differences in the mammalian immune system were already described in humans and rodent models, whereas photoperiodic effects in particular were investigated in rodents only (21, 23–27). In addition to seasonal differences, diurnal variations in the immune system are well-documented in humans and rodents and are regarded important for immune competence due to timely orchestration of immune function (28–30). Only few studies investigated seasonal modulations of diurnal rhythms in the mammalian immune system (31–36) and to our knowledge, none investigated the specific effect of the photoperiod on diurnal immune rhythms until now. Moreover, whereas important mediators of diurnal rhythmicity in the immune system, such as glucocorticoids and the sympathetic nervous system, were already identified (37, 38), mechanisms driving seasonal changes in the immune system are not clearly defined yet, especially in diurnally active species.

Our group recently demonstrated the occurrence of diurnal rhythms in peripheral immune cell numbers in the diurnally active domestic pig (39), which is regarded as highly suitable model species as it provides great anatomical, physiological, and immunological similarity with humans (40). In addition, studying mechanisms of seasonal disease susceptibility in this species may result in improvement of animal health and welfare within pig husbandry systems.

The present study, therefore, investigated photoperiodic effects on diurnal rhythms in immune cell numbers of particular leukocyte types in domestic pigs. We assessed diurnal rhythmicity of cell numbers in various immune cell types as well as plasma cortisol concentration, activity behavior, and hematocrit of pigs held under two different lighting regimes with cosinor analysis (41) and performed generalized linear mixed model analysis to evaluate potential associations between the investigated variables.

2 Material and Methods

2.1 Animals, Experimental Conditions, and Surgery

All procedures were conducted in accordance with the German Animal Welfare Act and approved by the local Animal Welfare Ethics Committee (Regional Council Stuttgart, approval number V309/13TH). A total of 20 castrated male pigs (*Sus scrofa domestica*, Piétrain × German landrace, 7–8-month-old, body weight range 104–131 kg) were included in the study, subdivided into four different randomized experimental trials, which were conducted between April and May in 2015 and 2016. The pigs were housed in a lightproof building of the experimental unit of the department Behavioral Physiology of Livestock at a constant ambient temperature of 21 ± 1°C. The building is subdivided into two units, each equipped with an autonomous lighting system enabling different lighting regimes in parallel. Within every trial, one portion of animals was held under long day conditions (LD) with a photoperiod of 16 hours per day (16L:8D, lights on 07:00–23:00, n = 9 pigs) and the other portion of animals was held under short day conditions (SD) with a photoperiod of 8 hours per day (8L:16D, lights on 07:00–15:00, n = 11 pigs). The average illuminance was 190 lx at pigs' eye level during the light phase (fluorescent tubes, Philips Master TL-D Super 80 58W/840, color temperature 4000 K) and 0 lx during the dark phase. Allocation of pigs to LD or SD treatment was performed randomly and balanced for littermates. They were kept in individual pens (6.4 m^2 each) with sight and tactile contact to neighboring animals. All animals had *ad libitum* access to hay and water and were fed concentrate twice daily at 07:30 and 14:00 (1.1–1.2 kg/meal, ME 12 MJ/kg). Pens were cleaned and littered with dust-free wood shavings daily after concentrate feeding in the morning. All pigs were accustomed to the respective lighting and feeding regime for at least 4 weeks prior to the first sampling after being held for habituation under 12L:12D lighting conditions (lights-on at 07:00) for at least 6 weeks. Within this habituation period, time was switched from Central European Time (CET) to Central European Summer Time (CEST) at least 2 weeks prior to introduction of LD or SD. To enable blood collection without disturbing the animals, all pigs were well habituated to human handling and surgically catheterized with indwelling vein catheters (introduced into the *vena cava cranialis* via the *vena cephalica*) at least 2 weeks prior to the experiments as described previously (39). Catheters were rinsed with heparinized saline (115 IU/ml, heparin sodium salt, Carl Roth, Karlsruhe, Germany) twice a day after

concentrate feeding and all animals were weighed once per week (not during sampling periods). Health was monitored by daily measurement of rectal temperature.

2.2 Sampling Protocol and Sample Processing

From each pig, blood samples were taken every 2 hours, starting at 10:00 over periods of 50 hours (**Figure 1**, 26 blood samples per animal). At lights-off, the sampling procedure was performed under dim light of averagely 7 lx at pigs' eye level, which was switched on and off for sampling (Philips energy-saving/LED bulbs 3W, color temperature 2700 K). Sampling all animals lasted not longer than 20 min in total per sampling and animals were sampled in the same order each time. After discarding the heparinized saline solution from the catheters, 10 ml blood per sample was drawn. Subsequently, the catheter was rinsed with approximately 10 ml heparinized saline (46 IU/ml) to keep the catheter patent and to compensate for the blood volume taken. Blood was transferred directly into lithium heparin tubes and K3 EDTA tubes (both Sarstedt, Nümbrecht, Germany). Blood samples were immediately processed after each sampling.

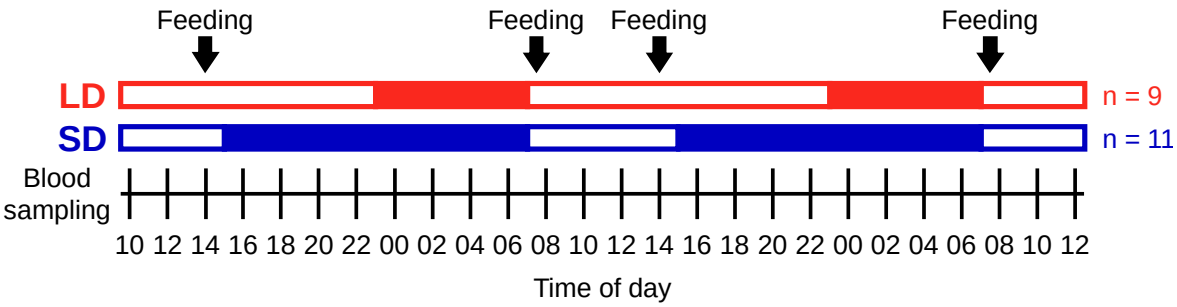

FIGURE 1 | Experimental protocol. Open bars indicate light periods, filled bars indicate dark periods, and arrows indicate concentrate feeding times. A total of 9 pigs were studied under long day conditions (LD, red) and a total of 11 pigs were studied under short day conditions (SD, blue) in 4 different randomized trials (n = 4 or 6 each). Blood sampling started at 10:00 and was repeated every 2 hours over periods of 50 hours in each trial.

2.3 Hematology

Total leukocyte counts and hematocrit were analyzed in K3 EDTA blood samples by an automated hematology analyzer (MEK-6108G, Nihon Kohden, Rosbach, Germany). All samples were measured in duplicate. The mean intra-assay coefficient of variation for biological samples was 1.1% for leukocyte counts and 1.0% for hematocrit. Validity was

verified by daily reference measurements of a hematology control (Para 12 Extend N, Streck, Omaha, NE, USA). Hematology analyses were finished within 60 min after blood sampling.

2.4 Flow Cytometry

Characterization of specific immune cell populations was performed using three-color flow cytometry with preceding immunofluorescent antibody staining of heparinized whole blood following an established standard protocol using different combinations of monoclonal antibodies (all SouthernBiotech, Birmingham, AL, USA) specific for porcine CD3ε (clone PPT3), CD4 (clone 74-12-4), CD8α (clone 76-2-11), and CD172a (clone 74-22-15) as described previously (39). All antibodies were titrated in preceding experiments for optimal staining. Controls included unlabeled cells, single antibody stains, and fluorescence minus one (FMO) controls. Identification of peripheral blood mononuclear cells (PBMC) and granulocytes was performed based on their forward and side scatter characteristics. Subsequently, non-fluorescent neutrophils were distinguished from autofluorescent eosinophils within granulocytes in an unstained sample (42). Further characterization of immune cells was carried out within PBMC based on surface marker expression following previous reports (43–47): T cells ($CD3^+$), naive T helper (Th) cells ($CD3^+$ $CD4^+$ $CD8\alpha^-$), Ag-experienced (Ag-exp.) Th cells ($CD3^+$ $CD4^+$ $CD8\alpha^+$), cytotoxic T cells ($CD3^+$ $CD4^-$ $CD8\alpha^{high}$), γδ T cells ($CD3^+$ $CD4^-$ $CD8\alpha^{-/dim}$), NK cells ($CD3^-$ $CD8\alpha^+$ $CD172a^-$), and monocytes ($CD3^-$ $CD8\alpha^-$ $CD172a^{high}$). The mean intra-assay coefficient of variation was 0.9% for PBMC, 2.1% for granulocytes, and 1.1% for T cells. Results concerning $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells found in the present study are presented in the Supplementary Material (Figures S4 and S5, Tables S1–S8) as it is not yet known whether the two investigated different phenotypic subsets of porcine γδ T cells also display different functions (44, 48).

2.5 Cortisol Radioimmunoassay

Blood plasma was obtained from heparinized blood samples by centrifugation (15 min at 2000 × g at 4°C) within 45 min after sampling and stored at −80°C until measurement. Plasma cortisol concentrations were analyzed radioimmunologically with preceding ethanolic extraction as reported previously (39, 49). Briefly, plasma samples were diluted 10-fold with 100% ethanol (Carl Roth), mixed, incubated for 5 min at 4°C, and centrifuged for 10 min at 2000 × g at 4°C to remove binding proteins. Aliquots of ethanolic supernatant were evaporated to dryness, resuspended in phosphate buffer, and analyzed in the RIA in duplicate. Therefore, a polyclonal antibody against cortisol-3-BSA (MBS316242,

MyBioSource, San Diego, CA, USA) at a final dilution of 1:112,000 in 0.1% BSA buffer was added and [1,2-^{3}H]-cortisol (50 Ci/mmol, American Radiolabeled Chemicals, Saint Louis, MO, USA) or [1,2,6,7-^{3}H]-cortisol (93 Ci/mmol, PerkinElmer, Boston, MA, USA) was used as tracer. Separation of bound/free was performed with dextran-coated charcoal (0.02% Dextran 70, Carl Roth; 0.2% Norit A, Serva Electrophoresis, Heidelberg, Germany) by centrifugation for 20 min at 2000 × g at 4°C. Supernatants were decanted to 5 ml Irga-Safe Plus (PerkinElmer) to determine radioactivity. Precision was determined with spiked controls and revealed a mean recovery rate of 105%. Intra-assay and inter-assay variabilities for biological samples were 2.7% and 8.3% (33.2 ng/ml) as well as 10.0% and 13.9% (16.2 ng/ml), respectively.

2.6 Activity Behavior

Animal behavior was recorded with cameras and analyzed as described previously (39). In brief, recording started 2 hours before the first blood sampling and was continued throughout each sampling period. The software *The Observer XT 11* (Noldus Information Technology, Wageningen, The Netherlands) was used to categorize animal behavior by focal sampling and continuous recording into inactivity or activity. Thereby, resting of pigs in prone or lateral position was classified as inactivity, whereas all other behaviors were classified as activity. Relative activity behavior was calculated for every single animal as proportion of time in which the animal was active within each two-hour interval preceding the respective blood sampling.

2.7 Statistical Analyses

Diurnal rhythmicity was assessed by cosinor analysis (41) using the package *cosinor* (50) in R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). Period length was set to 24 hours in all cosinor models. Overall diurnal rhythmicity was investigated by performing cosinor analyses with combined datasets of all animals per treatment group. Subsequently, individual diurnal rhythmicity was assessed by rerunning cosinor analyses for every single animal in the study (refer to Table S1 in Supplementary Material). Diurnal rhythms were characterized by mesor (average value of the fitted cosine function), amplitude (half the difference between maximum and minimum of the fitted cosine function), and peak time (time of the maximum of the fitted cosine function). In addition, the phase distribution, defined as standard deviation of mean peak times (51), was calculated from individual cosinor analysis results as a measure of peak time dispersal. Diurnal rhythms were

considered significant if cosinor models revealed $P < 0.05$ for the amplitude. Peak times were calculated by the formula $-\Phi 24/(2\pi)$ using the phase shift Φ denoted by R and by setting 00:00 (24 hours) as reference time. For visual evaluation of datasets, diurnal profiles are shown without superimposed cosine curves in the Supplementary Material (Figure S5).

Comparisons of mesor values, relative amplitudes, and peak times were performed with IBM SPSS Statistics 22 (IBM Deutschland, Ehningen, Germany). For pairwise comparisons the assumptions of normality and homogeneity of variances were checked with Shapiro-Wilk tests and Levene's tests, respectively. If data met these assumptions, they were compared with two-tailed, unpaired Student's *t*-tests. Otherwise, two-tailed unequal variance *t*-tests (lack of variance homogeneity) or two-tailed Mann-Whitney *U* tests (both assumptions violated) were used. In the case of paired Student's *t*-tests, normality of differences was confirmed by Shapiro-Wilk tests. For multiple comparisons, normality of data in all groups was analyzed with Shapiro-Wilk tests. If normal distribution was confirmed, repeated measures ANOVA with Mauchly's test to check sphericity was performed. Greenhouse-Geisser correction was applied if sphericity was not met. Bonferroni correction was used for multiple pairwise *post hoc* testing. If data were not normally distributed, Friedman test was performed. Subsequent Wilcoxon signed-rank tests were conducted either with Bonferroni-Holm correction or with Benjamini-Hochberg correction to compare five groups (52). Two-way factorial effects were investigated with 2 × 2 mixed factorial ANOVA (normality of data confirmed by Shapiro-Wilk tests) followed by inspection of simple main effects with the least significant difference (LSD) procedure if interactions were significant.

Potential associations between the cell numbers of the investigated immune cell populations in porcine blood with experimental factors and different intrinsic variables were assessed using IBM SPSS Statistics 22 to analyze the data of all animals (repeated measurements) with generalized linear mixed models (GLMM, GENLINMIXED command, Satterthwaite's approximation to calculate denominator degrees of freedom). The residual pseudo likelihood approach (REPL) was used for parameter estimation. In all models, the intercept, the factors treatment (LD/SD), light (off/on, indicating darkness or light), and concentrate feeding (yes/no, indicating the samplings at or just after concentrate feeding or not) as well as the covariates plasma cortisol concentration (ng/ml), relative activity behavior (%), hematocrit (%), and sampling (1–26, indicating the ongoing number of samplings) were set as fixed effects. Sampling was specified as repeated effect with animal identity as subject

variable and the residual covariance structure was set as first order autoregressive (AR(1)). The factor animal identity (n = 20) was set as random effect with a scaled identity (ID) covariance structure. The most appropriate random model term according to the lowest AIC was selected by exclusion of potential other effects, e.g., experimental trial (n = 4), dam (n = 10), or sire (n = 7), by backward model selection on total leukocyte counts only. The final model structure was applied to all other immune cell populations to achieve comparability among them. All models were calculated in six different specifications: Normal distribution with identity or log link functions, inverse Gaussian distribution with identity or log link functions, or gamma distribution with identity or log link functions. For each immune cell type, the most appropriate model structure was identified by evaluation of homoscedasticity and normality by plotting residuals *versus* predicted values and by quantile-quantile plots of residuals, respectively. In all models $P < 0.05$ was considered significant (refer to Table S2 in Supplementary Material for details).

3 Results

3.1 Diurnal Rhythms in Immune Cell Numbers Differ Between LD and SD in Porcine Blood

Overall cosinor analyses with combined datasets of all animals per treatment revealed diurnal rhythmicity ($P < 0.05$) in immune cell numbers in blood under both lighting regimes for total leukocytes, NK cells, T cells, monocytes, neutrophils, and eosinophils, as well as for the majority of investigated T cell subtypes, i.e., total Th cells, cytotoxic T cells, γδ T cells, and naive Th cells (**Table 1**, **Figures 2A–C**). Ag-exp. Th cell counts in porcine blood did not show overall diurnal rhythmicity ($P \geq 0.05$) under LD nor under SD (**Table 1**, **Figure 2C**). Individual cosinor analyses per animal revealed diurnal rhythms ($P < 0.05$) in the cell numbers of NK cells, T cells, eosinophils, cytotoxic T cells, γδ T cells, and naive Th cells in blood in all animals under both lighting regimes (Table S1 in Supplementary Material). The cell numbers of total leukocytes, monocytes, and total Th cells in blood exhibited diurnal rhythmicity ($P < 0.05$) in all animals held under SD but only in a proportion of animals under LD, whereas neutrophil counts exhibited diurnal rhythmicity ($P < 0.05$) in all animals held under LD and in a proportion of 91% of animals under SD (Table S1 in Supplementary Material). Finally, Ag-exp. Th cell numbers in blood oscillated diurnally ($P < 0.05$) only in a proportion of animals under both lighting regimes (Table S1 in Supplementary Material).

TABLE 1 | Results of overall cosinor analyses with combined datasets of all animals held under long day conditions (LD, n = 9) or short day conditions (SD, n = 11)

Variable	Treatment	*P*[a]	Mesor	Amplitude	Amplitude [%][b]	Peak time[c]
Leukocytes [/µl]	LD	<0.001	15577.8 ± 131.3	678.9 ± 181.2	4.4 ± 1.2	21:37 ± 01:04
	SD	<0.001	15156.1 ± 163.4	1303.6 ± 233.4	8.6 ± 1.5	19:20 ± 00:40
NK cells [/µl]	LD	<0.001	91.5 ± 3.2	31.8 ± 4.5	34.7 ± 4.9	12:59 ± 00:33
	SD	<0.001	93.1 ± 2.8	41.1 ± 3.8	44.1 ± 4.1	10:47 ± 00:22
T cells [/µl]	LD	<0.001	7548.4 ± 64.5	679.9 ± 88.3	9.0 ± 1.2	22:51 ± 00:32
	SD	<0.001	7283.3 ± 80.7	913.3 ± 111.6	12.5 ± 1.5	21:25 ± 00:29
Monocytes [/µl]	LD	0.006	941.7 ± 10.4	41.1 ± 14.9	4.4 ± 1.6	19:01 ± 01:21
	SD	<0.001	968.0 ± 19.9	116.4 ± 28.9	12.0 ± 3.0	17:43 ± 00:54
Neutrophils [/µl]	LD	0.001	3946.9 ± 81.3	390.6 ± 111.6	9.9 ± 2.8	11:48 ± 01:09
	SD	<0.001	3756.3 ± 58.5	440.8 ± 82.5	11.7 ± 2.2	13:51 ± 00:43
Eosinophils [/µl]	LD	<0.001	770.9 ± 16.9	242.4 ± 23.3	31.4 ± 3.0	21:57 ± 00:23
	SD	<0.001	658.7 ± 17.5	250.8 ± 24.7	38.1 ± 3.7	19:59 ± 00:23
Total Th cells [/µl]	LD	0.003	2252.7 ± 33.8	139.6 ± 46.3	6.2 ± 2.1	22:25 ± 01:21
	SD	<0.001	2187.3 ± 17.3	214.2 ± 24.1	9.8 ± 1.1	21:05 ± 00:27
Cytotoxic T cells [/µl]	LD	<0.001	1055.3 ± 20.5	143.6 ± 28.4	13.6 ± 2.7	00:27 ± 00:47
	SD	<0.001	1210.3 ± 20.5	208.6 ± 28.2	17.2 ± 2.3	22:17 ± 00:33
γδ T cells [/µl]	LD	<0.001	4249.4 ± 63.1	414.9 ± 86.6	9.8 ± 2.0	22:30 ± 00:51
	SD	<0.001	3908.1 ± 69.7	496.9 ± 96.7	12.7 ± 2.5	21:10 ± 00:46
Naive Th cells [/µl]	LD	<0.001	1216.3 ± 22.6	171.7 ± 31.0	14.1 ± 2.5	23:01 ± 00:44
	SD	<0.001	1271.9 ± 16.2	221.8 ± 22.5	17.4 ± 1.8	21:22 ± 00:24
Ag-exp. Th cells [/µl]	LD	0.204	1036.4 ± 22.5	39.9 ± 31.4	3.8 ± 3.0	13:10 ± 03:05
	SD	0.154	915.5 ± 8.8	17.7 ± 12.4	1.9 ± 1.4	13:32 ± 02:43
Cortisol [ng/ml]	LD	<0.001	15.4 ± 0.5	14.7 ± 0.8	95.6 ± 5.0	08:17 ± 00:12
	SD	<0.001	15.5 ± 0.5	15.3 ± 0.7	98.6 ± 4.6	06:31 ± 00:10
Activity behavior [%]	LD	<0.001	22.7 ± 1.2	16.1 ± 1.7	71.0 ± 7.3	14:59 ± 00:23
	SD	<0.001	24.7 ± 1.0	28.4 ± 1.4	115.2 ± 5.8	12:29 ± 00:12
Hematocrit [%]	LD	<0.001	34.2 ± 0.1	1.7 ± 0.2	4.9 ± 0.6	14:49 ± 00:27
	SD	<0.001	33.1 ± 0.1	1.6 ± 0.2	4.9 ± 0.6	13:42 ± 00:26

Values are presented as mean ± SEM.

[a] Significant diurnal rhythm at $P < 0.05$

[b] Relative amplitude (amplitude/mesor)

[c] Time of day ± hh:mm

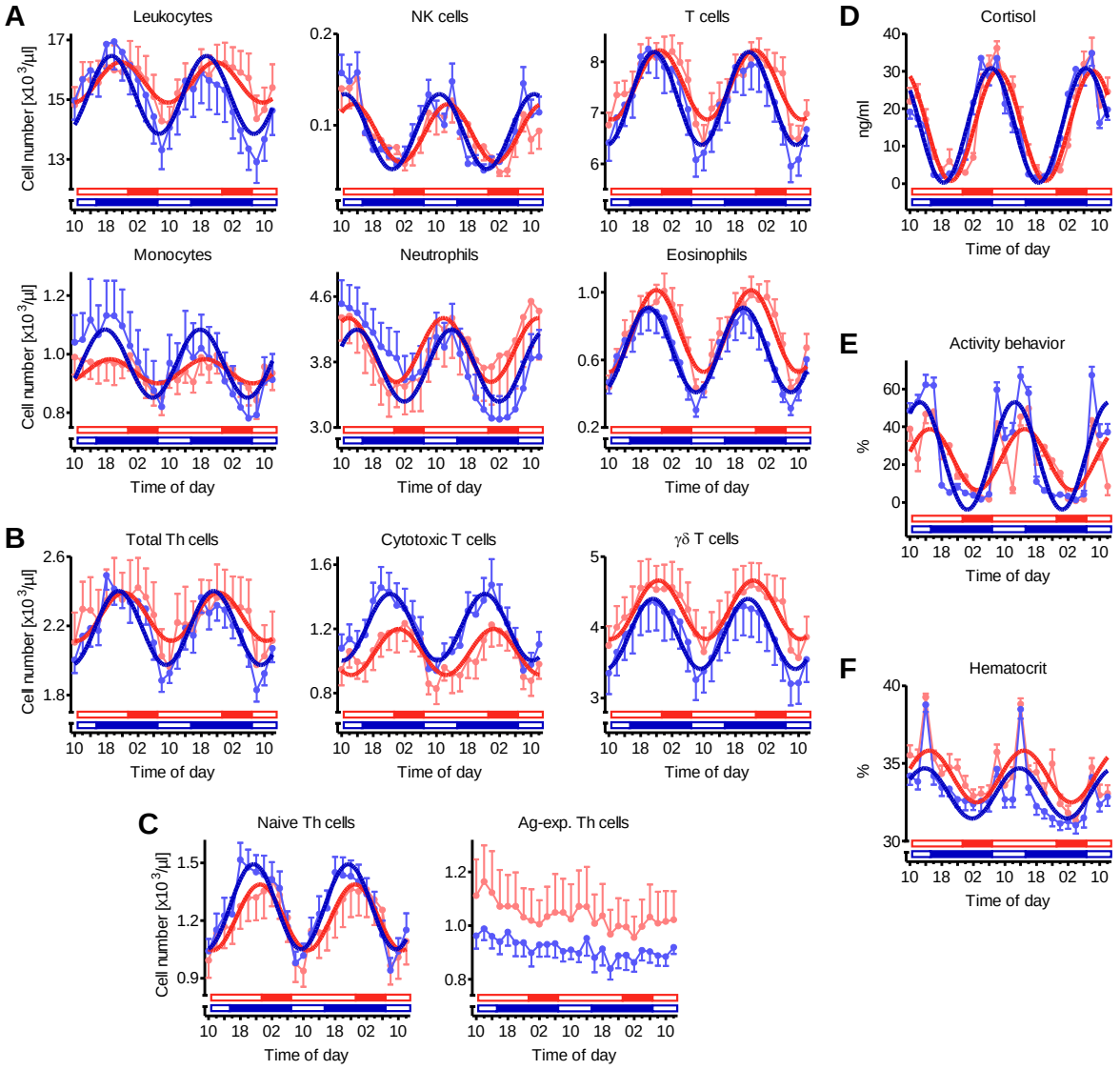

FIGURE 2 | Diurnal rhythms of the cell numbers of different immune cell populations in blood, plasma cortisol concentration, activity behavior, and hematocrit in domestic pigs. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. Open bars indicate light periods, filled bars indicate dark periods for the respective lighting regime. Measured values are shown in pale color per treatment (mean ± SEM, LD n = 9, SD n = 11). Dark curves correspond to the results of overall cosinor analyses with combined datasets of all animals per treatment (LD n = 9, SD n = 11, significant diurnal rhythm at $P < 0.05$, refer to **Table 1**). Diurnal rhythms in cell numbers in porcine blood are depicted for **(A)** main immune cell populations, **(B)** different T cell subpopulations, and **(C)** Th cell subtypes with distinctive differentiation states. In addition, diurnal rhythms in **(D)** plasma cortisol concentration, **(E)** activity behavior (values represent the proportion of time, in which the animals were active within the two-hour interval preceding blood sampling), and **(F)** hematocrit are shown.

To assess diurnal rhythmicity between treatments in more detail, the different properties of rhythmicity based on the results of individual cosinor analyses per animal (Table S1 in Supplementary Material) were analyzed. Thereby, mesor values of cell numbers in porcine blood were found not to differ ($P \geq 0.05$) between LD and SD in any investigated immune cell type (**Figures 3A–C**). This result was confirmed by calculation of mean values

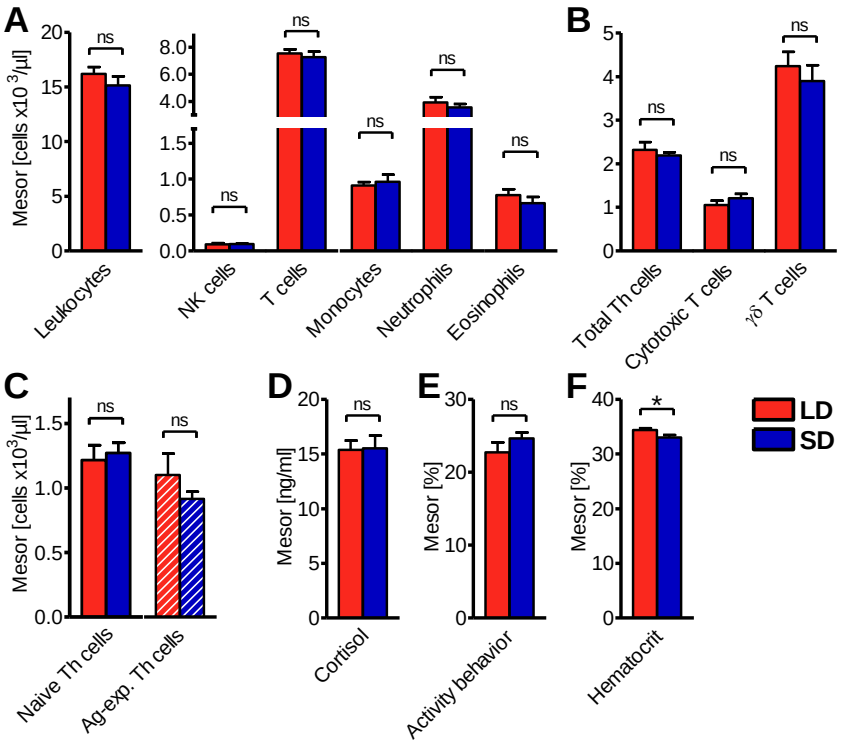

FIGURE 3 | Mesor values of the cell numbers of different immune cell populations in blood, plasma cortisol concentration, activity behavior, and hematocrit in domestic pigs. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue for **(A)** main immune cell populations, **(B)** different T cell subpopulations, **(C)** Th cell subtypes with distinctive differentiation states, **(D)** plasma cortisol concentration, **(E)** activity behavior, and **(F)** hematocrit. The graphs and statistical analyses only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses in the respective variable (mean ± SEM, LD n = 5–9, SD n = 7–11, refer to Table S1 in Supplementary Material). Hatched bars indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals per treatment (significant diurnal rhythm at $P < 0.05$, refer to **Table 1**). Pairwise comparisons were performed with two-tailed, unpaired Student's *t*-tests (total leukocytes, T cells, monocytes, neutrophils, eosinophils, γδ T cells, plasma cortisol concentration, hematocrit), two-tailed unequal variance *t*-test (activity behavior), or two-tailed Mann-Whitney *U* tests (NK cells, total Th cells, cytotoxic T cells, naive Th cells, Ag-exp. Th cells); refer to Table S5 in Supplementary Material for statistical details, *$P < 0.05$, ns $P \geq 0.05$.

of all 26 measurements per animal, which also did not differ between treatments (Figures S1A–C in Supplementary Material).

However, relative amplitudes in the cell numbers of total leukocytes, NK cells, T cells, monocytes, and naive Th cells in porcine blood were higher ($P < 0.05$) under SD than LD (**Figures 4A**, **C**). In contrast, no difference ($P \geq 0.05$) in the strength of diurnal oscillation was found between treatments in the cell numbers of neutrophils, eosinophils, total Th cells,

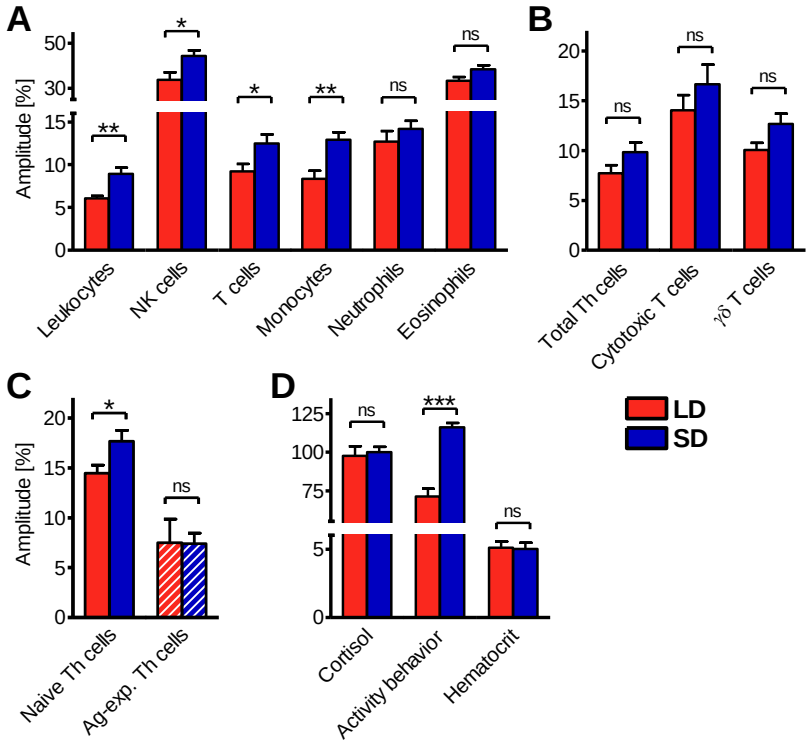

FIGURE 4 | Relative amplitudes of the cell numbers of different immune cell populations in blood, plasma cortisol concentration, activity behavior, and hematocrit in domestic pigs. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue for **(A)** main immune cell populations, **(B)** different T cell subpopulations, **(C)** Th cell subtypes with distinctive differentiation states, as well as **(D)** plasma cortisol concentration, activity behavior, and hematocrit. The graphs and statistical analyses only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses in the respective variable (mean ± SEM, LD n = 5–9, SD n = 7–11, refer to Table S1 in Supplementary Material). Hatched bars indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals per treatment (significant diurnal rhythm at $P < 0.05$, refer to **Table 1**). Pairwise comparisons were performed with two-tailed, unpaired Student's *t*-tests (NK cells, monocytes, neutrophils, total Th cells, cytotoxic T cells, γδ T cells, activity behavior, hematocrit) or two-tailed Mann-Whitney *U* tests (total leukocytes, T cells, eosinophils, naive Th cells, Ag-exp. Th cells, plasma cortisol concentration); refer to Table S6 in Supplementary Material for statistical details, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $P \geq 0.05$.

cytotoxic T cells, γδ T cells, and Ag-exp. Th cells in porcine blood (**Figures 4A–C**). Statistical comparisons revealed differences ($P < 0.05$) in the strength of diurnal oscillation among certain immune cell types within each treatment group, which were comparable between LD and SD, with the exception of monocytes (Figure S2 in Supplementary Material).

The cell numbers of most immune cell types in porcine blood peaked earlier ($P < 0.05$) under SD than LD relative to the time of lights-on at 07:00, i.e., total leukocytes, NK cells, T cells, eosinophils, cytotoxic T cells, γδ T cells, and naive Th cells (**Figure 5**). The peak times in cell numbers of monocytes, neutrophils, total Th cells, and Ag-exp. Th cells in porcine blood did not differ ($P \geq 0.05$) between the two lighting regimes (**Figure 5**). In general, individual peak times in cell counts seem to display greater dispersal under LD than SD in most cell types, except for Ag-exp. Th cells (Table S7 in Supplementary Material). In addition, statistical comparisons of peak times in cell numbers in blood between different leukocyte types within each treatment group revealed differences ($P < 0.05$), which were mainly comparable between LD and SD, with the exception of monocytes and neutrophils (Figure S3 in Supplementary Material).

3.2 Parameters Associated with Immune Cell Numbers in Porcine Blood

In addition to diurnal rhythmicity in immune cell numbers in blood, diurnal rhythms of plasma cortisol concentration, activity behavior, and hematocrit were confirmed ($P < 0.05$) with overall cosinor analyses in combined datasets of all animals per treatment (**Table 1**, **Figures 2D–F**). Furthermore, individual cosinor analyses revealed diurnal rhythms ($P < 0.05$) for these variables in almost all animals of the present study (Table S1 in Supplementary Material). The results of individual cosinor analyses revealed that the mesor of hematocrit, in contrast to plasma cortisol and activity behavior, was greater ($P = 0.036$) under LD than SD (**Figures 3D–F**) and that the relative amplitude of activity behavior, in contrast to cortisol and hematocrit, was higher under SD than LD (**Figure 4D**). All three variables peaked earlier under SD than LD (**Figure 5**) and activity behavior seems to exhibit greater peak time dispersal under LD than SD (Table S7 in Supplementary Material).

Potential associations of the experimental factors treatment, light, and concentrate feeding as well as the covariates plasma cortisol concentration, activity behavior, hematocrit, and sampling with the cell numbers of the various leukocyte types in porcine blood were assessed with generalized linear mixed models (**Table 2**). Concerning the factors treatment

and light, mixed model results resembled the findings of cosinor analyses. In accordance with the absent differences in mesor or mean values, we also found no association of immune cell numbers in porcine blood with LD or SD treatment. Cell numbers of immune cell types, which peaked in the dark phase or in the late light phase in porcine blood according to cosinor analyses, were found to be positively associated with lights-off, whereas NK cell and neutrophil counts, peaking during the early and mid-light phase, were found to be negatively associated with lights-off. Porcine monocyte and Ag-exp. Th cell counts were not

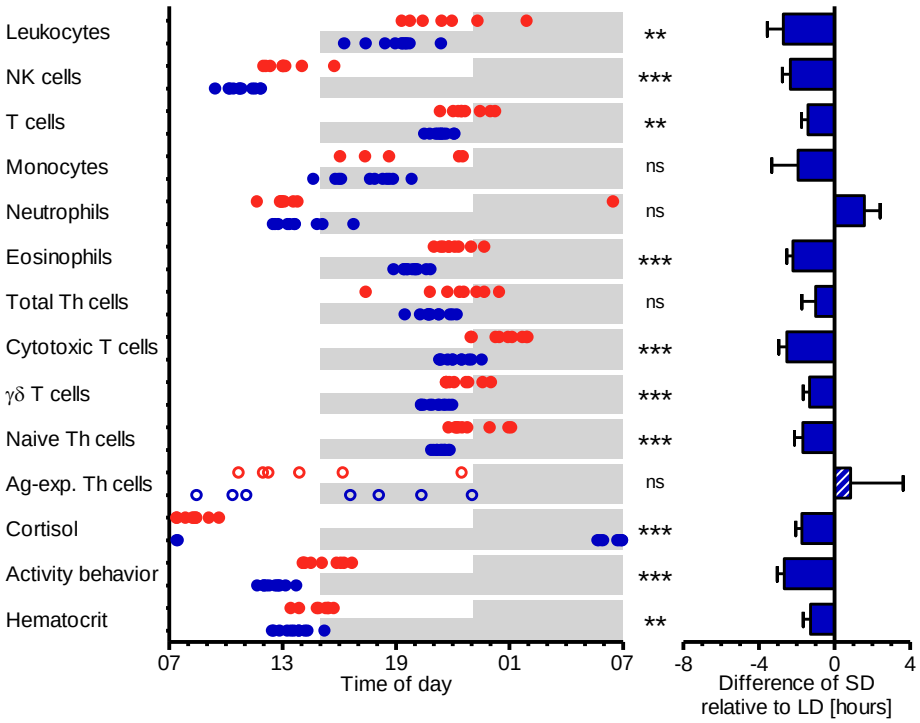

FIGURE 5 | Peak times of the cell numbers of different immune cell populations in blood, plasma cortisol concentration, activity behavior, and hematocrit in domestic pigs. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. Shaded areas indicate lights-off in the respective treatments. The graphs and statistical analyses only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses in the respective variable (LD n = 5–9, SD n = 7–11, refer to Table S1 in Supplementary Material). The bar graph on the right side illustrates the differences in peak times of SD relative to LD (mean ± SEM). Circles and the hatched bar indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals per treatment (significant diurnal rhythm at $P < 0.05$, refer to **Table 1**). Pairwise comparisons were performed with two-tailed, unpaired Student's *t*-tests (total leukocytes, NK cells, eosinophils, total Th cells, cytotoxic T cells, γδ T cells, Ag-exp. Th cells, plasma cortisol concentration, hematocrit), two-tailed unequal variance *t*-tests (T cells, monocytes, activity behavior), or two-tailed Mann-Whitney *U* tests (neutrophils, naive Th cells); refer to Table S7 in Supplementary Material for statistical details, ***$P < 0.001$, **$P < 0.01$, ns $P \geq 0.05$.

TABLE 2 | Potential associations of various fixed effects with immune cell numbers in porcine blood[a]

Variable	Fixed effects: Estimated associations[b] with significance levels[c]						
	Treatment (LD)	Light (off)	Feeding (yes)	Cortisol	Activity	Hematocrit	Sampling
Leukocytes	↔	↑ ***	↓ ***	↓ ***	↓ ***	↑ ***	↔
NK cells	↔	↓ ***	↓ *	↑ ***	↑ ***	↑ ***	↓ ***
T cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↔
Monocytes	↔	↔	↓ ***	↓ ***	↓ **	↑ ***	↓ **
Neutrophils	↔	↓ ***	↓ ***	↔	↑ **	↑ ***	↓ *
Eosinophils	↔	↑ ***	↓ ***	↓ ***	↓ ***	↑ ***	↔
Total Th cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↓ *
Cytotoxic T cells	↔	↑ ***	↔	↓ **	↓ ***	↑ **	↔
γδ T cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↔
Naive Th cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↔
Ag-exp. Th cells	↔	↔	↔	↑ *	↓ **	↑ ***	↓ **

[a] Potential associations were assessed by generalized linear mixed model analyses: $y_{ij} = \mu + treatment_i + light_{ij} + concentrate\ feeding_j + plasma\ cortisol\ concentration_{ij} + relative\ activity\ behavior_{ij} + hematocrit_{ij} + sampling_j + animal\ identity_i + \varepsilon_{ij}$. Thereby, y_{ij} represents the cell number/µl blood of the specific immune cell population in porcine blood for an animal *i* at sampling *j*, the intercept µ, the factors treatment (LD/SD), light (off/on), and concentrate feeding (yes/no) as well as the covariates plasma cortisol concentration (ng/ml), relative activity behavior (%), hematocrit (%), and sampling (1–26) were set as fixed effects. The factor animal identity (n = 20) was set as random effect with a scaled identity (ID) covariance structure. Sampling was set as repeated effect with a first order autoregressive (AR(1)) residual covariance structure (ε_{ij}). Animal identity was set as subject. Detailed model results can be found in Table S2 in Supplementary Material.

[b] Estimated association: ↑ positive, ↓ negative, ↔ none

[c] *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

associated with the factor light. No association of concentrate feeding with immune cell numbers in porcine blood was found for the major proportion of immune cell types, except for total leukocyte, NK cell, monocyte, neutrophil, and eosinophil counts, which were negatively associated with concentrate feeding. Whereas cell numbers in porcine blood for the most immune cell types were negatively associated with plasma cortisol concentration, positive associations were found for NK cell and Ag-exp. Th cell counts and no association was found for neutrophil counts. Furthermore, activity behavior was negatively associated

with cell numbers in blood in almost all investigated immune cell types, except for NK cells and neutrophils, for which positive associations were found. As expected, hematocrit was positively associated with the cell numbers in blood of all investigated immune cell types. Whereas the major proportion of investigated immune cell types showed no association between cell numbers in porcine blood and repeated sampling, a minor proportion was negatively associated with sampling. In addition, evaluation of experimental effects on the investigated covariates with mixed ANOVA revealed interaction effects of treatment and light for plasma cortisol concentration and activity behavior, whereas for hematocrit, there was a main effect of light only (**Figure 6**).

4 Discussion

The present study demonstrated that the photoperiod modulates diurnal rhythms in cell numbers of different immune cell types in blood of domestic pigs. Distinct photoperiodic differences were found in relative amplitudes and peak times but not in mesor or mean values of cell numbers in certain immune cell types in porcine blood. To our knowledge, this is the first study investigating the effect of different photoperiods on diurnal rhythms in peripheral

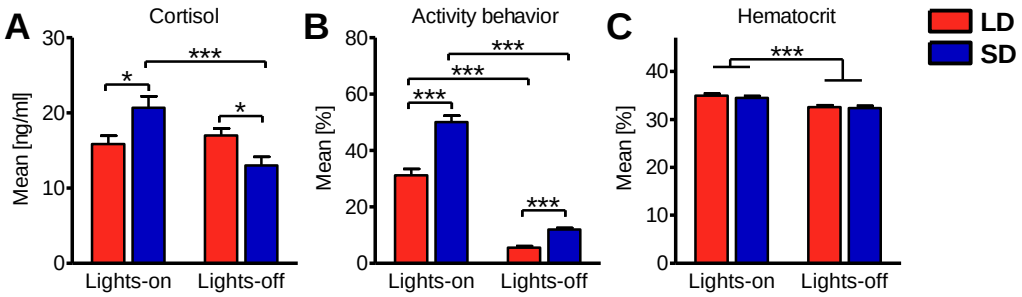

FIGURE 6 | Comparisons of mean values during lights-on or lights-off for the covariates plasma cortisol concentration, activity behavior, and hematocrit. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. The graphs and statistical analyses include values calculated from the 26 samplings per pig, subdivided into values during lights-on or lights-off for all animals per treatment (mean ± SEM, LD n = 9, lights-on = 18 samplings & lights-off = 8 samplings, SD n = 11, lights-on = 10 samplings & lights-off = 16 samplings). Mixed ANOVA with treatment (LD/SD) as between-subjects factor and light (on/off) as within-subjects repeated factor was performed for comparisons. If there was a significant interaction effect, simple main effects were analyzed with the least significant difference (LSD) procedure. Significant interactions between main effects were found for **(A)** plasma cortisol concentration and **(B)** activity behavior, whereas for **(C)** hematocrit, there was a significant main effect of light only; refer to Table S9 in Supplementary Material for statistical details, *** $P < 0.001$, * $P < 0.05$.

immune cell numbers within the same season in any species. We found diurnal rhythms in immune cell numbers in porcine blood under both lighting regimes for all investigated immune cell types except for Ag-exp. Th cells, which is consistent with our previous results for domestic pigs held under 12L:12D lighting conditions (39).

Interestingly, on individual level, higher proportions of animals held under SD than LD exhibited diurnal rhythms in cell numbers in several of the investigated leukocyte types in blood. In addition, several immune cell types oscillated stronger under SD than LD in the present study. This finding is in consistence with previous reports showing marked diurnal rhythmicity in leukocyte counts during winter months, i.e., under SD, and a weakening or loss of rhythmicity in summer months, i.e., under LD, in humans and nocturnal rodents (31, 33, 34). The present study also found a potentially greater dispersal of peak times under LD in most cell types. Thus, our results imply stronger rhythmicity in leukocyte counts in general under SD and weakened rhythmicity under LD. It was previously shown in mice that exposure to LD leads to weakened intercellular coupling within the SCN compared to SD, which is associated with greater dispersal of peak times in clock gene expression within the anterior SCN under LD (51). Further investigations should attempt to clarify if this mechanism is also present in domestic pigs and if it is directly linked to weakened rhythmicity and dispersed peak times in immune cell counts seen under LD.

Several leukocyte types peaked earlier relative to the time of lights-on under SD than LD. The consistency in effect direction for most investigated immune cell types displaying photoperiodic differences in relative amplitudes and peak times in cell numbers in porcine blood suggests that the experimental photoperiod acted on those immune cell types through presumably common intrinsic mechanisms. Moreover, it can be assumed that light or darkness (53) likely acted as main stimulus, called zeitgeber, on diurnal rhythmicity in those cell types. Notably, it is not known whether the time of lights-on or lights-off, mid-day, mid-night, the relative span of light or darkness per day, or a combination of these phenomena is most important for mediating photoperiodic effects on diurnal immune rhythms. The photoperiodic effect was particularly pronounced in total leukocytes, NK cells, and T cells including naive Th cells in the present study, which is in consistence with the above mentioned studies (31, 33, 34). In previous studies, it was shown that plasma cortisol and the rest-activity cycle represent main driving forces of diurnal rhythms in immune cell numbers in blood (37, 54). The results of generalized linear mixed model analyses of the present study support this concept, as those immune cell types, which exhibited marked

differences in relative amplitudes and peak times in cell numbers in porcine blood, were associated with plasma cortisol concentration and activity behavior, resembling previous results (55–58). Monocyte counts did not differ in peak times but oscillated stronger under SD in blood of domestic pigs. As only half of the pigs held under LD but two-thirds of pigs held under 12L:12D in our previous study (39) and all pigs held under SD exhibited diurnal rhythms in monocyte numbers in blood, photoperiod seems to be essential for diurnal rhythmicity in this cell type as well. Few investigated leukocyte types neither displayed differences in relative amplitudes nor in peak times of immune cell counts. Interestingly, diurnal rhythms in neutrophil numbers in porcine blood seem to be regulated differently from other immune cell types. Neutrophil counts were found not to be associated with plasma cortisol concentration, which is supported by other studies (39, 49, 55). As animals under both lighting regimes were fed simultaneously, feeding time potentially displays an important zeitgeber for this cell type (59). This hypothesis is supported by mixed model results revealing them as the only investigated cell type being associated with feeding while lacking association with plasma cortisol concentration. Notably, the association with feeding potentially involves activity behavior as mediating factor (57). Alternatively, the time of lights-on might be more important for diurnal rhythms in neutrophil counts than the photoperiod. The non-significant results for total Th cells might have been influenced by the proportion of Ag-exp. Th cells, which lack overall diurnal rhythmicity in the present study and under 12L:12D lighting conditions as well (39).

The present study also demonstrated photoperiodic effects on diurnal rhythms in plasma cortisol concentrations and activity behavior. Both variables exhibited earlier peak times relative to the time of lights-on under SD, emphasizing the importance of light or darkness as zeitgeber (53, 60). Our results for plasma cortisol concentration and activity behavior are in remarkable agreement with human and primate studies (61–63). Interestingly, the interaction effect of treatment with light in activity behavior seemed to result from the observed stronger diurnal oscillation under SD than LD. Thereby, pigs held under SD were more active during lights-on than animals held under LD, probably compensating for the shorter time span of lights-on. This effect was already described for pigs as well as in primate studies (63, 64).

In contrast to photoperiodic effects in peak times and relative amplitudes, no differences in mesor or mean values of cell numbers in porcine blood of any investigated leukocyte type were found in the present study. This result is in contrast to previous reports

demonstrating seasonal or photoperiodic differences in immune cell counts in mammals, with generally greater cell numbers in autumn, winter, or under SD (24, 26, 27, 31–34, 36, 65–68). There may be several possible reasons to explain this discrepancy. Some of the above mentioned studies sampled each subject or animal at a single point in time within 24 hours to compare different photoperiods or seasons (24, 26, 27, 65–68). It was pointed out by some authors that such single point measurements should be interpreted with caution as observed differences might not result from a change in mesor but rather from a shift in peak time or change in strength of diurnal oscillation (or both) between lighting regimes or seasons (69, 70). The experimental design used in the present study meets the recommendation of at least 12 time points per cycle across 2 full cycles for studying biological rhythms (71). Studies analyzing diurnal rhythms during different seasons showed differences in mesor values in leukocyte counts (31–34, 36). However, these studies evaluated the effect of season and not photoperiod in particular and investigated fewer time points across just 1 diurnal cycle. Thus, whether the differences in experimental settings between the above mentioned studies and the present study led to the observed discrepancy in mesor values cannot be answered yet. The crossbred domestic pigs used in the present study can enhance comparability to human studies compared to inbred models. Thereby, they reveal a high variability in total immune cell numbers between individuals, whereas the course of diurnal immune rhythms is highly comparable (refer to 39). Although photoperiodic differences were previously found with smaller numbers of hamsters (67), this inter-individual variability in pigs could have impeded the identification of significant effects in mesor or mean values. Further investigations using intra-individual approaches could elucidate this issue. Additionally, the above mentioned effect might have been enhanced by the use of castrated male pigs in the present study. It was suggested that gonadal hormones can play a modifying role in photoperiod-induced immune modulations, at least in seasonal breeding species (20, 21, 23, 72). Further investigations with gonad-intact domestic pigs could elucidate, whether gonadal hormones influence total leukocyte counts in this moderately seasonal breeding species (73). Apart from this, few studies investigating diurnal rhythms in rodents held under 12L:12D lighting conditions throughout the whole year found differences in mesor values of immune cell numbers (36, 70). These results in conjunction with the lacking photoperiodic effect on mesor values of leukocyte counts in the present study suggest that the proposed central circannual clock (8–10) and not the photoperiod might influence mesor values of leukocyte numbers in the pig.

Plasma cortisol concentration and activity behavior also did not exhibit photoperiodic differences in mesor or mean values in the present study. Whereas several studies investigating plasma cortisol concentration throughout the year in humans found higher diurnal mesor or mean values during the winter season (74–76), results of other human studies were inconsistent (77, 78). Remarkably, comparing our results with a primate study using the same experimental setting as we did reveals striking comparability in mesor or mean values of cortisol and activity behavior (63). Hematocrit was the only variable exhibiting a photoperiodic difference in mesor values, although mean values were not different and other studies also did not find seasonal or photoperiodic effects on hematocrit (24, 67, 79, 80).

In conclusion, the present study investigated, to our knowledge, for the first time in any species photoperiodic effects on diurnal rhythms in immune cell numbers in blood. Distinct photoperiodic differences in relative amplitudes and peak times in the cell numbers of certain leukocyte types in blood were found in domestic pigs, whereas there was no difference in mesor or mean values. Our results imply stronger rhythmicity in leukocyte counts in general under SD. Moreover, common intrinsic mechanisms seem to regulate photoperiodic effects on diurnal rhythms in cell counts of most porcine leukocyte types, except for neutrophils. Whether these differences influence disease susceptibility of domestic pigs cannot be answered yet. Human and primate studies using experimental settings similar to the present study, found comparable results for plasma cortisol concentration and activity behavior (61, 63), strengthening the importance of the domestic pig as suitable large animal model. Therefore, the domestic pig provides the opportunity to further elucidate the influence of environmental factors on diurnal and seasonal immune rhythms as well as the importance of these rhythms for immune competence and disease susceptibility in diurnally active species.

5 Author Contributions

SS conceived and designed the study. LE and SS designed experiments, performed research, analyzed and interpreted the data, and wrote the manuscript. UW conducted the cortisol analyses and supervised surgery. BP conducted the behavioral analyses. VS assisted in experimental design and manuscript preparation. All authors read and approved the submitted version of the manuscript.

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8 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9 Supplementary Material

The Supplementary Material for this article can be found online at …

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Supplementary Material

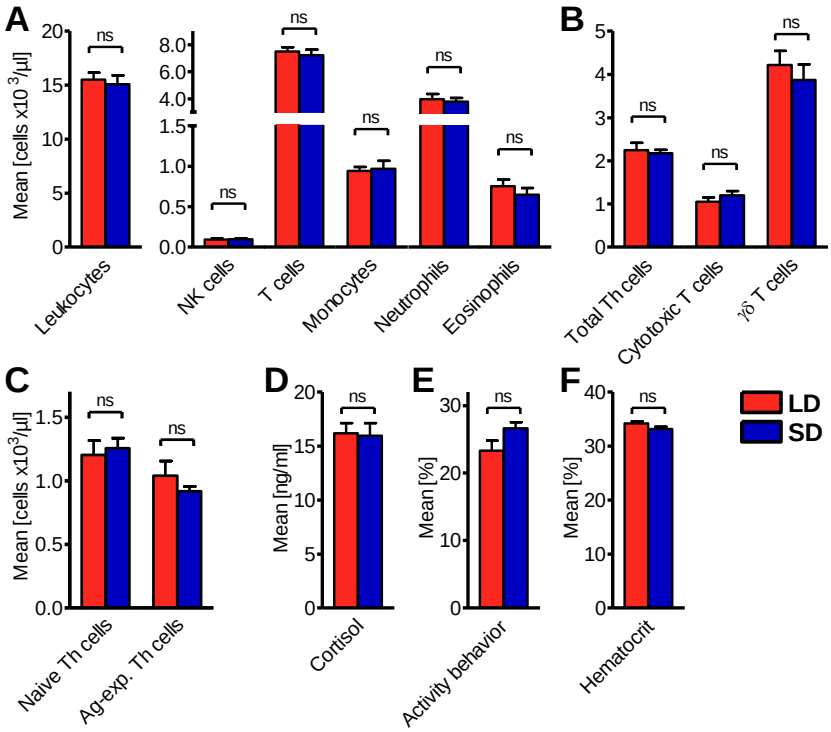

FIGURE S1 | Mean values of the cell numbers of different immune cell populations in blood, plasma cortisol concentration, activity behavior, and hematocrit in domestic pigs. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue for **(A)** main immune cell populations, **(B)** different T cell subpopulations, **(C)** Th cell subtypes with distinctive differentiation states, **(D)** plasma cortisol concentration, **(E)** activity behavior, and **(F)** hematocrit. The graphs and statistical analyses include values calculated from the 26 samplings per pig for all animals per treatment (mean ± SEM, LD n = 9, SD n = 11). Pairwise comparisons were performed with two-tailed, unpaired Student's *t*-tests (total leukocytes, T cells, monocytes, neutrophils, eosinophils, γδ T cells, plasma cortisol concentration, and hematocrit), unequal variance *t*-test (activity behavior), or two-tailed Mann-Whitney *U* tests (NK cells, total Th cells, cytotoxic T cells, naive Th cells, and Ag-exp. Th cells); refer to Table S8 in Supplementary Material for statistical details, ns $P \geq 0.05$.

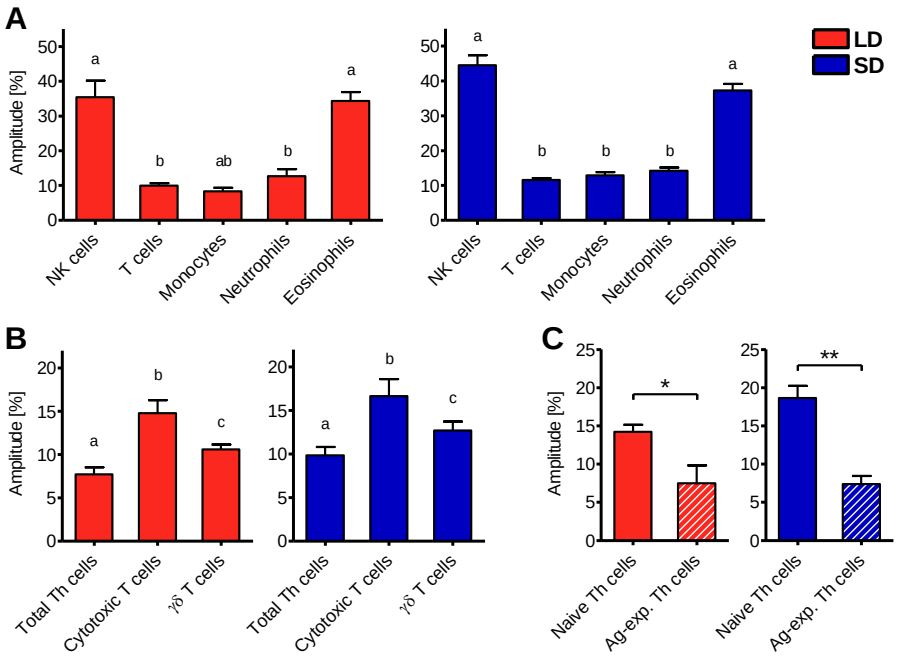

FIGURE S2 | Cell type comparisons of relative amplitudes of the cell numbers of different immune cell populations in porcine blood within treatments. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. The graphs and statistical analyses only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses in all compared cell types, respectively (mean ± SEM, LD n = 5–8, SD n = 7–11, refer to Table S1 in Supplementary Material). Hatched bars indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals per treatment (significant diurnal rhythm at $P < 0.05$, refer to Table 1 in main text). **(A)** Multiple comparisons within main immune cell populations for pigs held under LD (n = 5) were performed with Friedman test ($\chi^2(4) = 16.48$, $P = 0.002$; NK cells: 25th–75th percentile (Pctl) 27.5%–45.1%, T cells: Pctl 8.3%–11.2%, monocytes: Pctl 6.3%–10.4%, neutrophils: Pctl 9.7%–16.7%, eosinophils: Pctl 29.6%–39.7%) followed by Wilcoxon signed-rank *post hoc* tests with Benjamini-Hochberg correction and for pigs held under SD (n = 10) with repeated measures ANOVA with Greenhouse-Geisser correction ($F(1.99,17.92) = 85.21$, $P = 7.16 \times 10^{-10}$; NK cells: 95% confidence interval (CI) 37.9%–51.0%, T cells: CI 10.3%–12.8%, monocytes: CI 10.7%–15.1%, neutrophils: CI 12.1%–16.3%, eosinophils: CI 33.0%–41.5%) followed by Bonferroni *post hoc* tests. **(B)** Multiple comparisons within different T cell subpopulations for pigs held under LD (n = 8) were performed with repeated measures ANOVA ($F(2,14) = 19.51$, $P = 8.96 \times 10^{-05}$; total Th cells: CI 5.8%–9.6%, cytotoxic T cells: CI 11.2%–18.3%, γδ T cells: CI 9.3%–11.9%) followed by Bonferroni *post hoc* tests and for pigs held under SD (n = 11) with Friedman test ($\chi^2(2) = 9.46$, $P = 0.009$; total Th cells: Pctl 7.0%–12.1%, cytotoxic T cells: Pctl 10.4%–19.0%, γδ T cells: Pctl 10.8%–13.3%) followed by Wilcoxon signed-rank *post hoc* tests with Bonferroni-Holm correction. Different letters indicate significant differences at $P < 0.05$. **(C)** Pairwise comparisons between Th cell subtypes with distinctive differentiation states (LD n = 6, SD n = 7) were performed with two-tailed, paired Student's *t*-tests (LD: $t(5) = 2.66$, $P = 0.045$; naive Th cells: CI 11.9%–16.6%, Ag-exp. Th cells: CI 1.4%–13.6%; SD: $t(6) = 5.27$, $P = 0.002$; naive Th cells: CI 14.8%–22.5%, Ag-exp. Th cells: CI 4.8%–10.0%); ** $P < 0.01$, * $P < 0.05$.

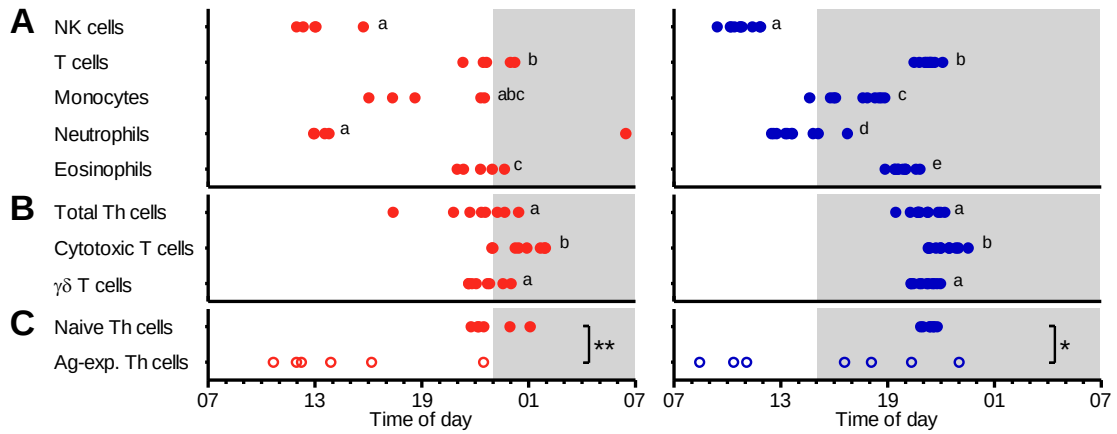

FIGURE S3 | Cell type comparisons of peak times of the cell numbers of different immune cell populations in porcine blood within treatments. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. Shaded areas indicate lights-off in the respective treatments. The graphs and statistical analyses only include values of animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses in all compared cell types, respectively (LD n = 5–8, SD n = 7–11, refer to Table S1 in Supplementary Material). Circles indicate missing diurnal rhythm in overall cosinor analyses with combined datasets of all animals per treatment (significant diurnal rhythm at $P < 0.05$, refer to Table 1 in main text). **(A)** Multiple comparisons within main immune cell populations for pigs held under LD (n = 5) were performed with Friedman test ($\chi^2(4) = 17.12$, $P = 0.002$; NK cells: 25th–75th percentile (Pctl) 12:10–14:24, T cells: Pctl 21:53–00:07, monocytes: Pctl 16:42–22:26, neutrophils: Pctl 09:42–13:41, eosinophils: Pctl 21:11–23:19) followed by Wilcoxon signed-rank *post hoc* tests with Benjamini-Hochberg correction and for pigs held under SD (n = 10) with repeated measures ANOVA ($F(4,36) = 302.35$, $P = 3.67 \times 10^{-27}$; NK cells: 95% confidence interval (CI) 10:13–11:19, T cells: CI 20:58–21:37, monocytes: CI 16:10–18:18, neutrophils: CI 12:53–14:48, eosinophils: CI 19:24–20:14) followed by Bonferroni *post hoc* tests. **(B)** Multiple comparisons within different T cell subpopulations for pigs held under LD (n = 8) and SD (n = 11) were performed with repeated measures ANOVA (LD: $F(2,14) = 10.92$, $P = 0.001$; total Th cells: CI 20:12–23:52, cytotoxic T cells: CI 23:38–01:39, γδ T cells: CI 21:47–23:18; SD: $F(2,20) = 10.67$, $P = 7.03 \times 10^{-04}$; total Th cells: CI 20:29–21:34, cytotoxic T cells: CI 21:41–22:41, γδ T cells: CI 20:43–21:31) followed by Bonferroni *post hoc* tests. Different letters indicate significant differences at $P < 0.05$. **(C)** Pairwise comparisons between Th cell subtypes with distinctive differentiation states (LD n = 6, SD n = 7) were performed with two-tailed, paired Student's *t*-tests (LD: $t(5) = 5.99$, $P = 0.002$; naive Th cells: CI 21:35–00:18, Ag-exp. Th cells: CI 10:03–19:05; SD: $t(6) = 2.94$, $P = 0.026$; naive Th cells: CI 21:04–21:41, Ag-exp. Th cells: CI 10:18–20:32); ** $P < 0.01$, * $P < 0.05$.

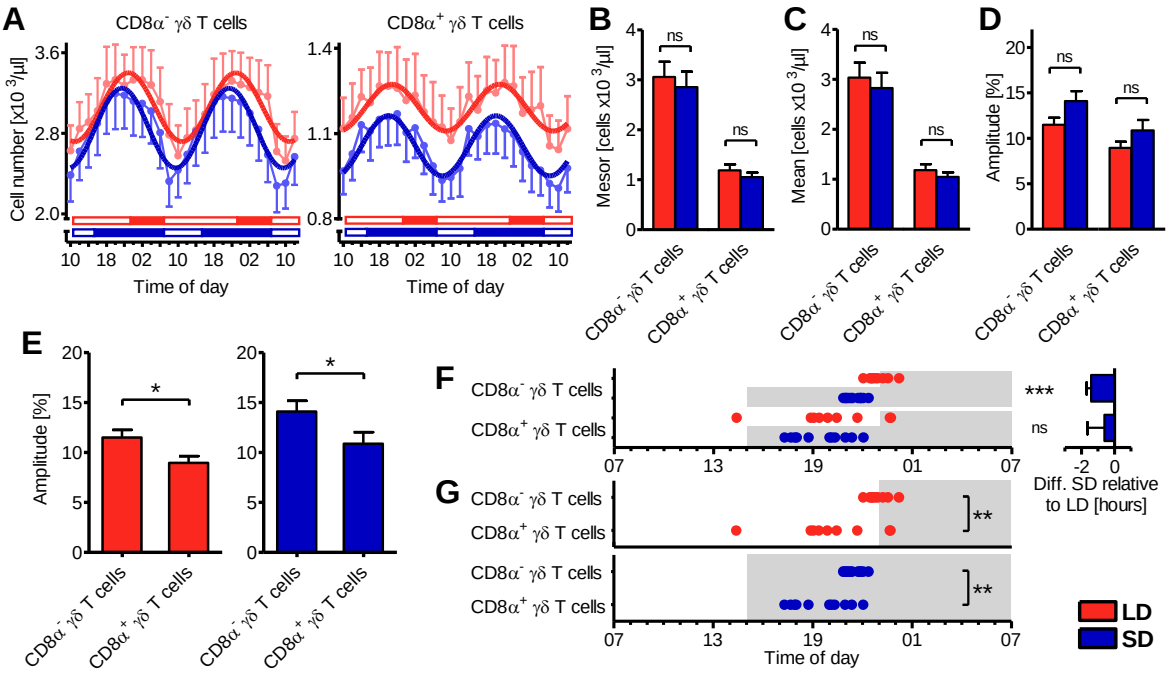

FIGURE S4 | Diurnal rhythms of the cell numbers of CD8α⁻ and CD8α⁺ γδ T cells in porcine blood. Values for pigs held under long day conditions (LD) are shown in red, values for pigs held under short day conditions (SD) are shown in blue. Shaded areas indicate lights-off in the respective treatments. **(A)** Open bars indicate light periods, filled bars indicate dark periods for the respective lighting regime. Measured cell numbers in porcine blood are shown in pale color per treatment (mean ± SEM, LD n = 9, SD n = 11). Dark curves correspond to the results of overall cosinor analyses with combined datasets of all animals per treatment (LD n = 9, SD n = 11, significant diurnal rhythm at $P < 0.05$, refer to Table S3 in Supplementary Material). **(B)** Mesor values calculated from individual cosinor analyses of all animals per treatment (all animals displayed significant diurnal rhythm at $P < 0.05$, refer to Table S1 in Supplementary Material), **(C)** mean values calculated from the 26 samplings per pig for all animals per treatment, and **(D)** relative amplitudes calculated from individual cosinor analyses of all animals per treatment were compared with two-tailed, unpaired Student's *t*-tests (mean ± SEM, LD n = 9, SD n = 11). **(E)** Cell type comparisons of relative amplitudes within treatments were performed with two-tailed, paired Student's *t*-tests (mean ± SEM, LD n = 9, SD n = 11; LD: $t(8) = 2.89$, $P = 0.020$; CD8α⁻ γδ T cells: 95% confidence interval (CI) 9.8%–13.3%, CD8α⁺ γδ T cells: CI 7.4%–10.5%; SD: $t(10) = 2.71$, $P = 0.022$; CD8α⁻ γδ T cells: CI 11.7%–16.5%, CD8α⁺ γδ T cells: CI 8.3%–13.5%). **(F)** Peak times calculated from individual cosinor analyses of all animals per treatment were compared with two-tailed, unpaired Student's *t*-tests (LD n = 9, SD n = 11). The bar graph on the right side illustrates the differences in peak times of SD relative to LD (mean ± SEM). (**G**) Cell type comparisons of peak times within treatments were performed with two-tailed, paired Student's *t*-tests (LD: $t(8) = 3.36$, $P = 0.010$; CD8α⁻ γδ T cells: CI 22:26–23:26, CD8α⁺ γδ T cells: CI 17:57–22:18; SD: $t(10) = 4.40$, $P = 0.001$; CD8α⁻ γδ T cells: CI 21:10–21:51, CD8α⁺ γδ T cells: CI 18:24–20:36); refer to Tables S5–S8 in Supplementary Material for statistical details, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P \geq 0.05$.

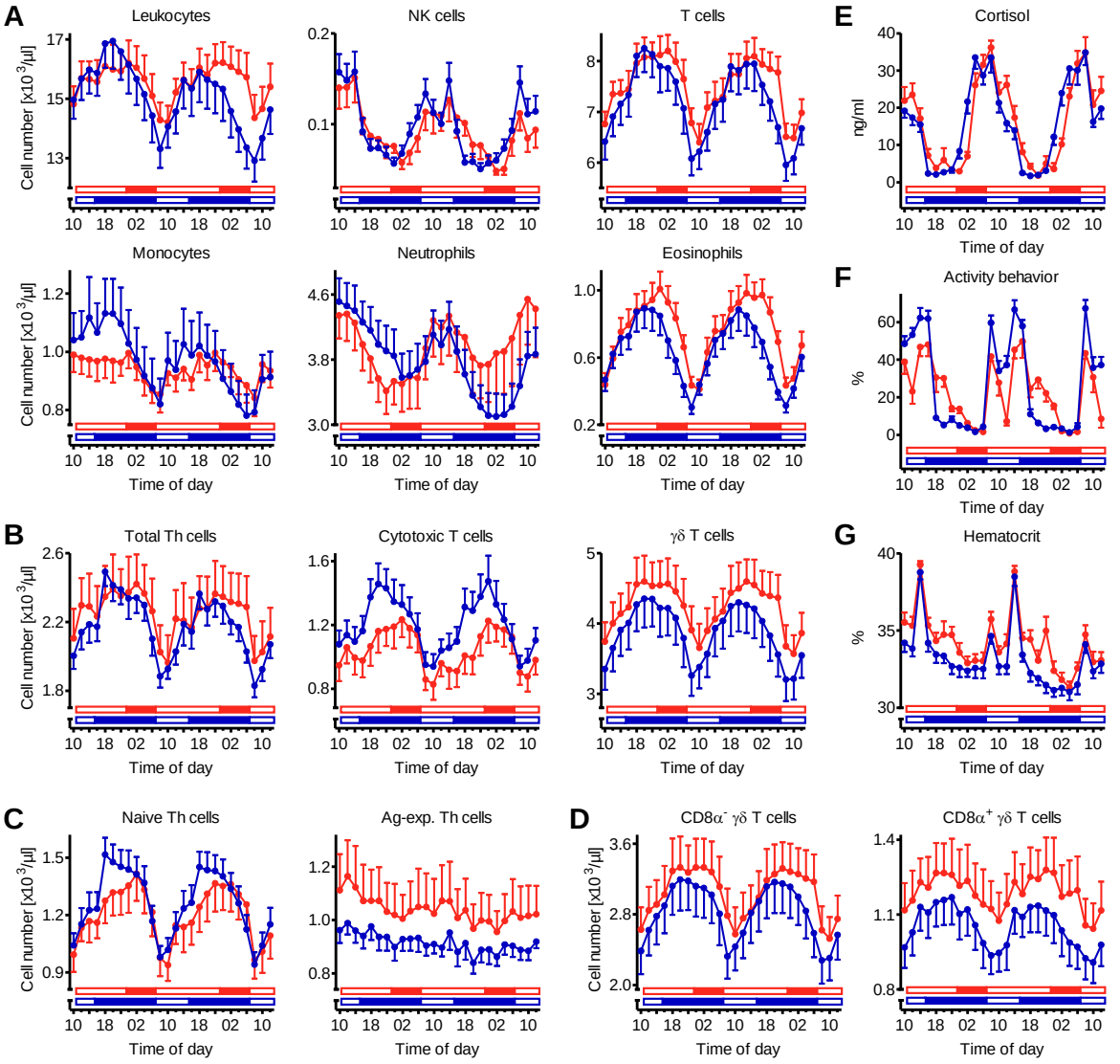

FIGURE S5 | Diurnal profiles (mean ± SEM) of the investigated variables in the study. Values for pigs held under long day conditions (LD n = 9) are shown in red, values for pigs held under short day conditions (SD n = 11) are shown in blue. For visual evaluation purposes, the figure illustrates the same datasets as Figure 2 in main text and Figure S4A in Supplementary Material just without the plotted cosine curves. Diurnal profiles in cell numbers in porcine blood are depicted for **(A)** main immune cell populations, **(B)** different T cell subpopulations, **(C)** Th cell subtypes with distinctive differentiation states, and **(D)** CD8α$^-$ and CD8α$^+$ γδ T cells. In addition, diurnal profiles in **(E)** plasma cortisol concentration, **(F)** activity behavior (values represent the proportion of time, in which the animals were active within the two-hour interval preceding blood sampling), and **(G)** hematocrit are shown.

TABLE S1 | Results of individual cosinor analyses per animal

Variable	Treatment	n[a]	Prop.[b]	Mesor	Amplitude	Amplitude [%][c]	Peak time[d]
Leukocytes [/µl]	LD	7	78%	16214.6 ± 615.3	979.7 ± 60.4	6.0 ± 0.3	21:43 ± 00:52
	SD	11	100%	15156.1 ± 819.3	1380.5 ± 178.1	8.9 ± 0.7	19:02 ± 00:24
NK cells [/µl]	LD	9	100%	91.5 ± 14.0	32.5 ± 7.0	33.6 ± 3.3	13:10 ± 00:23
	SD	11	100%	93.1 ± 11.3	41.8 ± 5.7	44.2 ± 2.6	10:50 ± 00:14
T cells [/µl]	LD	9	100%	7548.4 ± 308.1	698.6 ± 73.8	9.2 ± 0.9	22:46 ± 00:19
	SD	11	100%	7283.3 ± 406.1	920.2 ± 111.9	12.5 ± 1.1	21:22 ± 00:09
Monocytes [/µl]	LD	5	56%	915.5 ± 45.4	76.0 ± 8.1	8.3 ± 1.0	19:23 ± 01:19
	SD	11	100%	968.0 ± 99.1	126.9 ± 19.7	12.9 ± 0.9	17:28 ± 00:29
Neutrophils [/µl]	LD	9	100%	3946.9 ± 379.6	516.1 ± 88.4	12.7 ± 1.2	12:15 ± 00:45
	SD	10	91%	3592.5 ± 248.4	509.9 ± 51.1	14.2 ± 0.9	13:51 ± 00:25
Eosinophils [/µl]	LD	9	100%	770.9 ± 82.9	247.2 ± 23.1	32.8 ± 1.7	22:03 ± 00:17
	SD	11	100%	658.7 ± 86.5	252.9 ± 37.1	37.8 ± 1.8	19:51 ± 00:10
Total Th cells [/µl]	LD	8	89%	2318.1 ± 179.1	176.8 ± 21.3	7.7 ± 0.8	22:02 ± 00:46
	SD	11	100%	2187.3 ± 78.7	217.5 ± 23.3	9.8 ± 1.0	21:02 ± 00:15
Cytotoxic T cells [/µl]	LD	9	100%	1055.3 ± 102.5	149.6 ± 20.6	14.0 ± 1.5	00:42 ± 00:23
	SD	11	100%	1210.3 ± 101.0	211.2 ± 40.5	16.7 ± 2.0	22:11 ± 00:13
Total γδ T cells [/µl]	LD	9	100%	4249.4 ± 328.3	423.9 ± 40.6	10.1 ± 0.7	22:26 ± 00:18
	SD	11	100%	3908.1 ± 362.1	502.0 ± 69.5	12.7 ± 1.0	21:07 ± 00:11
Naive Th cells [/µl]	LD	9	100%	1216.3 ± 115.5	179.0 ± 21.9	14.5 ± 0.8	23:04 ± 00:25
	SD	11	100%	1271.9 ± 79.9	222.4 ± 15.8	17.7 ± 1.1	21:23 ± 00:06
Ag-exp. Th cells [/µl]	LD	6	67%	1107.6 ± 165.6	82.9 ± 27.8	7.5 ± 2.4	14:34 ± 01:45
	SD	7	64%	921.1 ± 56.0	67.5 ± 9.4	7.4 ± 1.1	15:25 ± 02:05
CD8α$^-$ γδ T cells [/µl]	LD	9	100%	3058.1 ± 305.3	345.4 ± 34.3	11.5 ± 0.8	22:56 ± 00:13
	SD	11	100%	2851.3 ± 311.0	399.5 ± 53.7	14.1 ± 1.1	21:30 ± 00:09
CD8α$^+$ γδ T cells [/µl]	LD	9	100%	1191.2 ± 120.6	104.5 ± 12.3	9.0 ± 0.7	20:07 ± 00:57
	SD	11	100%	1056.8 ± 92.5	114.7 ± 16.7	10.9 ± 1.2	19:30 ± 00:30
Cortisol [ng/ml]	LD	9	100%	15.4 ± 0.9	14.9 ± 1.2	97.6 ± 6.2	08:19 ± 00:15
	SD	11	100%	15.5 ± 1.1	15.6 ± 1.3	100.0 ± 3.4	06:34 ± 00:12
Activity behavior [%]	LD	9	100%	22.7 ± 1.4	16.6 ± 2.0	71.3 ± 5.1	15:12 ± 00:21
	SD	11	100%	24.7 ± 0.8	28.7 ± 1.3	116.1 ± 2.9	12:33 ± 00:11
Hematocrit [%]	LD	8	89%	34.4 ± 0.4	1.7 ± 0.1	5.1 ± 0.5	14:51 ± 00:17
	SD	11	100%	33.1 ± 0.4	1.7 ± 0.1	5.0 ± 0.5	13:36 ± 00:15

Values are presented as mean ± SEM.

[a] Number of animals out of 9 under long day conditions (LD) or out of 11 under short day conditions (SD) with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses

[b] Proportion of animals displaying diurnal rhythmicity

[c] Relative amplitude (amplitude/mesor)

[d] Time of day ± hh:mm

TABLE S2 | Results of generalized linear mixed model analyses[a]

Variable	Model term	Fixed coefficient ± SE	*df* [b]	*F*	*P*
Leukocytes[c]	*Corrected model* (Coefficient: Intercept)	9.2278 ± 0.0646	7,512.00	31.86	$<1.00 \times 10^{-36}$
	Treatment (LD)	0.0227 ± 0.0680	1,18.01	0.11	0.742
	Light (off)	0.0228 ± 0.0065	1,492.94	12.25	5.07×10^{-04}
	Feeding (yes)	-0.0233 ± 0.0066	1,445.61	12.34	4.89×10^{-04}
	Cortisol (per 1 ng/ml)	-0.0009 ± 0.0002	1,482.21	20.61	7.13×10^{-06}
	Activity (per 1%)	-0.0008 ± 0.0001	1,415.21	71.21	4.44×10^{-16}
	Hematocrit (per 1%)	0.0127 ± 0.0013	1,426.69	97.29	$<1.00 \times 10^{-36}$
	Sampling (per sample)	-0.0011 ± 0.0008	1,42.67	1.79	0.189
NK cells[c]	*Corrected model* (Coefficient: Intercept)	2.8140 ± 0.2831	7,512.00	82.19	$<1.00 \times 10^{-36}$
	Treatment (LD)	-0.1151 ± 0.1808	1,18.07	0.41	0.532
	Light (off)	-0.2433 ± 0.0313	1,399.94	60.57	6.13×10^{-14}
	Feeding (yes)	-0.0915 ± 0.0379	1,424.12	5.82	0.016
	Cortisol (per 1 ng/ml)	0.0083 ± 0.0010	1,339.90	75.02	2.22×10^{-16}
	Activity (per 1%)	0.0031 ± 0.0006	1,424.25	30.34	6.29×10^{-08}
	Hematocrit (per 1%)	0.0500 ± 0.0073	1,478.84	46.91	2.28×10^{-11}
	Sampling (per sample)	-0.0077 ± 0.0020	1,114.16	14.38	2.41×10^{-04}
T cells[c]	*Corrected model* (Coefficient: Intercept)	8.4669 ± 0.0768	7,512.00	51.15	$<1.00 \times 10^{-36}$
	Treatment (LD)	0.0485 ± 0.0711	1,18.05	0.46	0.504
	Light (off)	0.0712 ± 0.0084	1,490.35	71.68	2.22×10^{-16}
	Feeding (yes)	-0.0038 ± 0.0088	1,435.32	0.19	0.664
	Cortisol (per 1 ng/ml)	-0.0012 ± 0.0003	1,494.34	21.04	5.70×10^{-06}
	Activity (per 1%)	-0.0015 ± 0.0001	1,396.37	129.49	$<1.00 \times 10^{-36}$
	Hematocrit (per 1%)	0.0127 ± 0.0017	1,417.97	55.35	5.78×10^{-13}
	Sampling (per sample)	-0.0008 ± 0.0008	1,48.80	0.95	0.334
Monocytes[c]	*Corrected model* (Coefficient: Intercept)	6.2428 ± 0.1308	7,512.00	19.26	$<1.00 \times 10^{-36}$
	Treatment (LD)	0.0054 ± 0.1426	1,18.00	<0.01	0.970
	Light (off)	-0.0160 ± 0.0123	1,484.10	1.69	0.194
	Feeding (yes)	-0.0787 ± 0.0130	1,443.07	36.69	2.96×10^{-09}
	Cortisol (per 1 ng/ml)	-0.0018 ± 0.0004	1,491.91	21.83	3.84×10^{-06}
	Activity (per 1%)	-0.0006 ± 0.0002	1,409.84	9.54	0.002
	Hematocrit (per 1%)	0.0205 ± 0.0025	1,432.09	65.06	7.33×10^{-15}
	Sampling (per sample)	-0.0038 ± 0.0011	1,66.93	11.53	0.001
Neutrophils[d]	*Corrected model* (Coefficient: Intercept)	7.8673 ± 0.1102	7,512.00	13.63	3.33×10^{-16}
	Treatment (LD)	0.0461 ± 0.1197	1,17.89	0.15	0.705
	Light (off)	-0.0536 ± 0.0106	1,480.00	25.36	6.75×10^{-07}
	Feeding (yes)	-0.0687 ± 0.0109	1,460.62	39.54	7.49×10^{-10}
	Cortisol (per 1 ng/ml)	-0.0005 ± 0.0003	1,465.15	2.65	0.104
	Activity (per 1%)	0.0004 ± 0.0002	1,439.91	7.37	0.007
	Hematocrit (per 1%)	0.0133 ± 0.0021	1,452.83	40.63	4.53×10^{-10}
	Sampling (per sample)	-0.0041 ± 0.0020	1,28.65	4.30	0.047
Eosinophils[e]	*Corrected model* (Coefficient: Intercept)	-50.46 ± 105.91	7,71.39	20.40	8.99×10^{-15}
	Treatment (LD)	64.65 ± 82.36	1,14.54	0.62	0.445
	Light (off)	87.41 ± 15.94	1,286.45	30.06	9.20×10^{-08}
	Feeding (yes)	-49.10 ± 13.96	1,502.95	12.37	4.75×10^{-04}
	Cortisol (per 1 ng/ml)	-3.12 ± 0.45	1,251.12	47.45	4.52×10^{-11}
	Activity (per 1%)	-1.16 ± 0.22	1,425.58	29.22	1.08×10^{-07}
	Hematocrit (per 1%)	18.75 ± 2.78	1,346.86	45.39	6.72×10^{-11}
	Sampling (per sample)	2.55 ± 1.36	1,117.19	3.53	0.063

TABLE S2 | (*continued*)

Variable	Model term	Fixed coefficient ± SE	*df* [b]	*F*	*P*
Total Th cells[f]	*Corrected model* (Coefficient: Intercept)	1352.46 ± 202.29	7,82.56	32.69	$<1.00 \times 10^{-36}$
	Treatment (LD)	57.94 ± 177.13	1,18.06	0.11	0.747
	Light (off)	110.05 ± 21.39	1,449.23	26.46	4.03×10^{-07}
	Feeding (yes)	-7.59 ± 23.93	1,435.16	0.10	0.751
	Cortisol (per 1 ng/ml)	-3.00 ± 0.66	1,442.87	20.37	8.20×10^{-06}
	Activity (per 1%)	-3.38 ± 0.36	1,409.43	89.67	$<1.00 \times 10^{-36}$
	Hematocrit (per 1%)	28.33 ± 4.67	1,446.79	36.81	2.78×10^{-09}
	Sampling (per sample)	-3.59 ± 1.63	1,88.38	4.86	0.030
Cytotoxic T cells[d]	*Corrected model* (Coefficient: Intercept)	6.7845 ± 0.1351	7,512.00	38.51	$<1.00 \times 10^{-36}$
	Treatment (LD)	-0.1214 ± 0.1140	1,17.87	1.13	0.301
	Light (off)	0.1012 ± 0.0152	1,450.62	44.55	7.32×10^{-11}
	Feeding (yes)	-0.0046 ± 0.0164	1,434.37	0.08	0.780
	Cortisol (per 1 ng/ml)	-0.0012 ± 0.0005	1,461.24	6.77	0.010
	Activity (per 1%)	-0.0023 ± 0.0002	1,399.86	89.68	$<1.00 \times 10^{-36}$
	Hematocrit (per 1%)	0.0093 ± 0.0032	1,443.22	8.62	0.003
	Sampling (per sample)	-0.0020 ± 0.0012	1,84.02	3.12	0.081
Total γδ T cells[c]	*Corrected model* (Coefficient: Intercept)	7.7475 ± 0.1097	7,512.00	38.64	$<1.00 \times 10^{-36}$
	Treatment (LD)	0.1160 ± 0.1302	1,18.02	0.79	0.385
	Light (off)	0.0801 ± 0.0091	1,482.82	76.57	$<1.00 \times 10^{-36}$
	Feeding (yes)	-0.0027 ± 0.0097	1,425.04	0.08	0.782
	Cortisol (per 1 ng/ml)	-0.0013 ± 0.0003	1,492.50	21.84	3.82×10^{-06}
	Activity (per 1%)	-0.0012 ± 0.0001	1,383.31	68.81	1.78×10^{-15}
	Hematocrit (per 1%)	0.0141 ± 0.0019	1,409.08	56.13	4.23×10^{-13}
	Sampling (per sample)	-0.0005 ± 0.0009	1,48.30	0.38	0.542
Naive Th cells[c]	*Corrected model* (Coefficient: Intercept)	6.5871 ± 0.1243	7,512.00	62.26	$<1.00 \times 10^{-36}$
	Treatment (LD)	-0.0438 ± 0.1085	1,18.06	0.16	0.691
	Light (off)	0.1075 ± 0.0128	1,402.05	71.00	6.66×10^{-16}
	Feeding (yes)	-0.0054 ± 0.0148	1,405.23	0.13	0.717
	Cortisol (per 1 ng/ml)	-0.0032 ± 0.0004	1,364.31	67.52	3.77×10^{-15}
	Activity (per 1%)	-0.0024 ± 0.0002	1,386.15	118.91	$<1.00 \times 10^{-36}$
	Hematocrit (per 1%)	0.0174 ± 0.0029	1,442.55	36.68	2.97×10^{-09}
	Sampling (per sample)	-0.0007 ± 0.0009	1,74.23	0.57	0.454
Ag-exp. Th cells[c]	*Corrected model* (Coefficient: Intercept)	6.5419 ± 0.1031	7,512.00	8.32	1.22×10^{-09}
	Treatment (LD)	0.0783 ± 0.0927	1,18.06	0.71	0.409
	Light (off)	-0.0179 ± 0.0108	1,461.98	2.72	0.100
	Feeding (yes)	-0.0210 ± 0.0120	1,445.16	3.05	0.082
	Cortisol (per 1 ng/ml)	0.0007 ± 0.0003	1,461.39	3.89	0.049
	Activity (per 1%)	-0.0006 ± 0.0002	1,420.31	10.66	0.001
	Hematocrit (per 1%)	0.0099 ± 0.0023	1,451.34	17.73	3.08×10^{-05}
	Sampling (per sample)	-0.0028 ± 0.0008	1,97.76	10.53	0.002
CD8α$^-$ γδ T cells[c]	*Corrected model* (Coefficient: Intercept)	7.4562 ± 0.1299	7,512.00	45.25	$<1.00 \times 10^{-36}$
	Treatment (LD)	0.1072 ± 0.1597	1,18.02	0.45	0.511
	Light (off)	0.0948 ± 0.0099	1,464.60	91.01	$<1.00 \times 10^{-36}$
	Feeding (yes)	-0.0059 ± 0.0107	1,410.83	0.30	0.583
	Cortisol (per 1 ng/ml)	-0.0015 ± 0.0003	1,477.62	23.73	1.51×10^{-06}
	Activity (per 1%)	-0.0014 ± 0.0002	1,366.67	74.84	2.22×10^{-16}
	Hematocrit (per 1%)	0.0128 ± 0.0021	1,401.26	37.55	2.13×10^{-09}
	Sampling (per sample)	-0.0005 ± 0.0009	1,47.08	0.36	0.551

TABLE S2 | (*continued*)

Variable	Model term	Fixed coefficient ± SE	*df* [b]	*F*	*P*
CD8α$^+$ γδ T cells[f]	*Corrected model* (Coefficient: Intercept)	475.23 ± 135.74	7,81.83	16.38	2.65×10^{-13}
	Treatment (LD)	129.60 ± 142.34	1,18.02	0.83	0.375
	Light (off)	56.78 ± 12.87	1,465.45	19.47	1.27×10^{-05}
	Feeding (yes)	11.02 ± 14.08	1,440.03	0.61	0.434
	Cortisol (per 1 ng/ml)	-1.83 ± 0.40	1,469.84	20.93	6.10×10^{-06}
	Activity (per 1%)	-0.90 ± 0.21	1,410.79	18.43	2.20×10^{-05}
	Hematocrit (per 1%)	18.23 ± 2.75	1,440.69	43.93	9.95×10^{-11}
	Sampling (per sample)	-1.07 ± 1.05	1,82.33	1.04	0.310

[a] y_{ij} = μ + treatment_i + light_{ij} + $\text{concentrate feeding}_j$ + $\text{plasma cortisol concentration}_{ij}$ + $\text{relative activity behavior}_{ij}$ + hematocrit_{ij} + sampling_j + animal identity_i + ε_{ij}; dependent variable: y_{ij} (cell number/μl blood) for an animal *i* at sampling *j*; fixed effects: intercept μ, treatment (LD/SD), light (off/on), concentrate feeding (yes/no), plasma cortisol concentration (ng/ml), relative activity behavior (%), hematocrit (%), and sampling (1–26); random effect (with scaled identity (ID) covariance structure): animal identity (n = 20); repeated effect (with first order autoregressive (AR(1)) residual (ε_{ij}) covariance structure): sampling; subject: animal identity

[b] Numerator degrees of freedom, denominator degrees of freedom

[c] Gamma distribution with log link function

[d] Inverse Gaussian distribution with log link function

[e] Inverse Gaussian distribution with identity link function

[f] Normal distribution with identity link function

TABLE S3 | Results of overall cosinor analyses for CD8α⁻ and CD8α⁺ γδ T cells with combined datasets of all animals held under long day conditions (LD, n = 9) or short day conditions (SD, n = 11)

Variable	Treatment	*P*[a]	Mesor	Amplitude	Amplitude [%][b]	Peak time[c]
CD8α⁻ γδ T cells [/µl]	LD	<0.001	3058.1 ± 58.1	342.0 ± 79.5	11.2 ± 2.6	22:53 ± 00:57
	SD	<0.001	2851.3 ± 59.6	396.6 ± 82.5	13.9 ± 2.9	21:28 ± 00:50
CD8α⁺ γδ T cells [/µl]	LD	0.011	1191.2 ± 23.3	82.2 ± 32.4	6.9 ± 2.7	20:51 ± 01:33
	SD	<0.001	1056.8 ± 18.1	106.0 ± 25.6	10.0 ± 2.4	20:03 ± 00:55

Values are presented as mean ± SEM.

[a] Significant diurnal rhythm at $P < 0.05$

[b] Relative amplitude (amplitude/mesor)

[c] Time of day ± hh:mm

TABLE S4 | Summary of generalized linear mixed models for CD8α⁻ and CD8α⁺ γδ T cells[a]

Variable	Fixed effects: Estimated associations[b] with significance levels[c]						
	Treatment (LD)	Light (off)	Feeding (yes)	Cortisol	Activity	Hematocrit	Sampling
CD8α⁻ γδ T cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↔
CD8α⁺ γδ T cells	↔	↑ ***	↔	↓ ***	↓ ***	↑ ***	↔

[a] Refer to Table S2 in Supplementary Material for details.

[b] Estimated association: ↑ positive, ↓ negative, ↔ none

[c] *** $P < 0.001$

TABLE S5 | Statistical details for pairwise comparisons of mesor values of the investigated variables between animals held under long day conditions (LD) or short day conditions (SD)

Variable	Treatment	n[a]	Statistics	Mean difference ± SEM	CI[b] or Pctl[c]
Leukocytes [/µl] [d]	LD	7	$t(16) = 0.92$, $P = 0.369$	1058.4 ± 1145.1	CI 14709.0–17720.1
	SD	11			CI 13330.6–16981.7
NK cells [/µl] [e]	LD	9	$U = 43$, $Z = -0.49$, $P = 0.621$	−1.6 ± 17.8	Pctl 64.8–122.4
	SD	11			Pctl 66.9–123.6
T cells [/µl] [d]	LD	9	$t(18) = 0.50$, $P = 0.623$	265.1 ± 529.4	CI 6838.0–8258.9
	SD	11			CI 6378.5–8188.1
Monocytes [/µl] [d]	LD	5	$t(14) = -0.34$, $P = 0.736$	−52.5 ± 152.6	CI 789.4–1041.6
	SD	11			CI 747.2–1188.7
Neutrophils [/µl] [d]	LD	9	$t(17) = 0.80$, $P = 0.437$	354.4 ± 444.8	CI 3071.5–4822.3
	SD	10			CI 3030.5–4154.5
Eosinophils [/µl] [d]	LD	9	$t(18) = 0.92$, $P = 0.368$	112.2 ± 121.6	CI 579.9–962.0
	SD	11			CI 466.0–851.4
Total Th cells [/µl] [e]	LD	8	$U = 42$, $Z = -0.17$, $P = 0.869$	130.8 ± 177.4	Pctl 1903.8–2828.3
	SD	11			Pctl 2002.0–2327.7
Cytotoxic T cells [/µl] [e]	LD	9	$U = 33$, $Z = -1.25$, $P = 0.210$	−155.0 ± 145.2	Pctl 858.1–1112.1
	SD	11			Pctl 973.2–1541.7
Total γδ T cells [/µl] [d]	LD	9	$t(18) = 0.68$, $P = 0.503$	341.3 ± 499.0	CI 3492.3–5006.5
	SD	11			CI 3101.3–4714.9
Naive Th cells [/µl] [e]	LD	9	$U = 35$, $Z = -1.10$, $P = 0.271$	−55.5 ± 136.6	Pctl 977.5–1360.8
	SD	11			Pctl 998.9–1503.8
Ag-exp. Th cells [/µl] [e]	LD	6	$U = 13$, $Z = -1.14$, $P = 0.253$	186.4 ± 163.9	Pctl 907.0–1264.1
	SD	7			Pctl 840.9–1003.1
CD8α$^-$ γδ T cells [/µl] [d]	LD	9	$t(18) = 0.47$, $P = 0.645$	206.8 ± 441.3	CI 2354.0–3762.3
	SD	11			CI 2158.3–3544.3
CD8α$^+$ γδ T cells [/µl] [d]	LD	9	$t(18) = 0.90$, $P = 0.380$	134.5 ± 149.4	CI 913.1–1469.3
	SD	11			CI 850.6–1262.9
Cortisol [ng/ml] [d]	LD	9	$t(18) = -0.10$, $P = 0.921$	−0.1 ± 1.5	CI 13.4–17.4
	SD	11			CI 13.0–18.1
Activity behavior [%] [f]	LD	9	$t(13.19) = -1.24.$, $P = 0.236$	−2.0 ± 1.6	CI 19.6–25.9
	SD	11			CI 22.9–26.5
Hematocrit [%] [d]	LD	8	$t(17) = 2.28.$, $P = 0.036$	1.3 ± 0.6	CI 33.6–35.2
	SD	11			CI 32.1–34.0

[a] Number of animals out of 9 for LD or out of 11 for SD, which were included into pairwise comparisons (animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses, refer to Table S1 in Supplementary Material)

[b] 95% confidence interval

[c] 25th–75th percentile

[d] Unpaired Student's *t*-test (two-tailed)

[e] Mann-Whitney *U* test (two-tailed)

[f] Unequal variance *t*-test (two-tailed)

TABLE S6 | Statistical details for pairwise comparisons of relative amplitudes of the investigated variables between animals held under long day conditions (LD) or short day conditions (SD)

Variable	Treatment	n[a]	Statistics	Mean difference ± SEM [%]	CI[b] or Pctl[c] [%]
Leukocytes [/µl] [e]	LD	7	$U = 4$, $Z = -3.13$, $P = 0.002$	−2.9 ± 0.9	Pctl 5.5–6.8
	SD	11			Pctl 7.7–9.0
NK cells [/µl] [d]	LD	9	$t(18) = -2.54$, $P = 0.021$	−10.6 ± 4.2	CI 26.1–41.2
	SD	11			CI 38.3–50.1
T cells [/µl] [e]	LD	9	$U = 19$, $Z = -2.32$, $P = 0.020$	−3.3 ± 1.4	Pctl 7.2–11.2
	SD	11			Pctl 10.5–13.4
Monocytes [/µl] [d]	LD	5	$t(14) = -3.14.$, $P = 0.007$	−4.6 ± 1.5	CI 5.7–11.0
	SD	11			CI 11.0–14.9
Neutrophils [/µl] [d]	LD	9	$t(17) = -0.96$, $P = 0.348$	−1.5 ± 1.5	CI 9.9–15.6
	SD	10			CI 12.1–16.3
Eosinophils [/µl] [e]	LD	9	$U = 26$, $Z = -1.79$, $P = 0.074$	−5.0 ± 2.5	Pctl 27.9–35.4
	SD	11			Pctl 32.8–41.1
Total Th cells [/µl] [d]	LD	8	$t(17) = -1.61$, $P = 0.126$	−2.1 ± 1.3	CI 5.8–9.6
	SD	11			CI 7.7–12.0
Cytotoxic T cells [/µl] [d]	LD	9	$t(18) = -1.02$, $P = 0.320$	−2.6 ± 2.6	CI 10.6–17.5
	SD	11			CI 12.3–21.0
Total γδ T cells [/µl] [d]	LD	9	$t(18) = -2.02$, $P = 0.058$	−2.6 ± 1.3	CI 8.4–11.7
	SD	11			CI 10.4–15.0
Naive Th cells [/µl] [e]	LD	9	$U = 20$, $Z = -2.24$, $P = 0.025$	−3.2 ± 1.4	Pctl 12.6–16.6
	SD	11			Pctl 15.4–18.0
Ag-exp. Th cells [/µl] [e]	LD	6	$U = 16$, $Z = -0.71$, $P = 0.475$	0.1 ± 2.5	Pctl 4.0–12.3
	SD	7			Pctl 5.3–10.3
$CD8\alpha^-$ γδ T cells [/µl] [d]	LD	9	$t(18) = -1.87.$, $P = 0.078$	−2.6 ± 1.4	CI 9.8–13.3
	SD	11			CI 11.7–16.5
$CD8\alpha^+$ γδ T cells [/µl] [d]	LD	9	$t(18) = -1.34$, $P = 0.197$	−1.9 ± 1.4	CI 7.4–10.5
	SD	11			CI 8.3–13.5
Cortisol [ng/ml] [e]	LD	9	$U = 43$, $Z = -0.49$, $P = 0.621$	−2.4 ± 6.8	Pctl 84.7–108.7
	SD	11			Pctl 94.8–108.1
Activity behavior [%] [d]	LD	9	$t(18) = -8.03$, $P = 2.32 \times 10^{-07}$	−44.8 ± 5.6	CI 59.6–82.9
	SD	11			CI 109.6–122.6
Hematocrit [%] [d]	LD	8	$t(17) = 0.13$, $P = 0.902$	0.1 ± 0.7	CI 4.0–6.2
	SD	11			CI 4.0–6.0

[a] Number of animals out of 9 for LD or out of 11 for SD, which were included into pairwise comparisons (animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses, refer to Table S1 in Supplementary Material)

[b] 95% confidence interval

[c] 25th–75th percentile

[d] Unpaired Student's *t*-test (two-tailed)

[e] Mann-Whitney *U* test (two-tailed)

TABLE S7 | Statistical details for pairwise comparisons of peak times of the investigated variables between animals held under long day conditions (LD) or short day conditions (SD)

Variable	Treatment	n[a]	Statistics	Mean difference ± SEM [hh:mm]	CI[b] or Pctl[c] [time of day]	StdDev[g] [hh:mm]
Leukocytes [/µl] [d]	LD	7	$t(16) = 3.17$, $P = 0.006$	02:42 ± 00:51	CI 19:35–23:51	02:18
	SD	11			CI 18:08–19:55	01:20
NK cells [/µl] [d]	LD	9	$t(18) = 5.41$, $P = 3.85 \times 10^{-05}$	02:20 ± 00:26	CI 12:17–14:03	01:09
	SD	11			CI 10:19–11:21	00:46
T cells [/µl] [f]	LD	9	$t(11.33) = 4.03$, $P = 0.002$	01:24 ± 00:21	CI 22:02–23:29	00:57
	SD	11			CI 21:02–21:41	00:29
Monocytes [/µl] [f]	LD	5	$t(5.13) = 1.37$, $P = 0.229$	01:55 ± 01:24	CI 15:44–23:02	02:56
	SD	11			CI 16:23–18:33	01:37
Neutrophils [/µl] [e]	LD	9	$U = 27$, $Z = -1.47$, $P = 0.142$	−01:35 ± 00:50	Pctl 12:15–13:20	02:15
	SD	10			Pctl 12:44–14:54	01:20
Eosinophils [/µl] [d]	LD	9	$t(18) = 7.08$, $P = 1.34 \times 10^{-06}$	02:12 ± 00:19	CI 21:25–22:42	00:50
	SD	11			CI 19:28–20:13	00:33
Total Th cells [/µl] [d]	LD	8	$t(17) = 1.40$, $P = 0.179$	01:00 ± 00:43	CI 20:12–23:52	02:11
	SD	11			CI 20:29–21:34	00:48
Cytotoxic T cells [/µl] [d]	LD	9	$t(18) = 5.95$, $P = 1.26 \times 10^{-05}$	02:31 ± 00:25	CI 23:49–01:34	01:08
	SD	11			CI 21:41–22:41	00:45
Total γδ T cells [/µl] [d]	LD	9	$t(18) = 3.97$, $P = 8.92 \times 10^{-04}$	01:19 ± 00:20	CI 21:45–23:08	00:54
	SD	11			CI 20:43–21:31	00:35
Naive Th cells [/µl] [e]	LD	9	$U = 2$, $Z = -3.61$, $P = 3.08 \times 10^{-04}$	01:41 ± 00:26	Pctl 22:10–00:27	01:15
	SD	11			Pctl 21:09–21:36	00:19
Ag-exp. Th cells [/µl] [d]	LD	6	$t(11) = -0.30$, $P = 0.767$	−00:51 ± 02:47	CI 10:03–19:05	04:18
	SD	7			CI 10:18–20:32	05:32
CD8α⁻ γδ T cells [/µl] [d]	LD	9	$t(18) = 5.51$, $P = 3.12 \times 10^{-05}$	01:25 ± 00:16	CI 22:26–23:26	00:39
	SD	11			CI 21:10–21:51	00:30
CD8α⁺ γδ T cells [/µl] [d]	LD	9	$t(18) = 0.61$, $P = 0.547$	00:37 ± 01:01	CI 17:57–22:18	02:50
	SD	11			CI 18:24–20:36	01:38
Cortisol [ng/ml] [d]	LD	9	$t(18) = 5.60$, $P = 2.60 \times 10^{-05}$	01:44 ± 00:19	CI 07:45–08:52	00:44
	SD	11			CI 06:08–07:01	00:40
Activity behavior [%] [f]	LD	9	$t(12.23) = 6.86$, $P = 1.58 \times 10^{-05}$	02:39 ± 00:23	CI 14:25–16:00	01:02
	SD	11			CI 12:09–12:57	00:36
Hematocrit [%] [d]	LD	8	$t(17) = 3.32$, $P = 0.004$	01:16 ± 00:23	CI 14:11–15:32	00:48
	SD	11			CI 13:03–14:09	00:49

[a] Number of animals out of 9 for LD or out of 11 for SD, which were included into pairwise comparisons (animals with significant ($P < 0.05$) diurnal rhythm in individual cosinor analyses, refer to Table S1 in Supplementary Material)

[b] 95% confidence interval

[c] 25th–75th percentile

[d] Unpaired Student's *t*-test (two-tailed)

[e] Mann-Whitney *U* test (two-tailed)

[f] Unequal variance *t*-test (two-tailed)

[g] Standard deviation of mean peak times to define phase distribution

TABLE S8 | Statistical details for pairwise comparisons of mean values of the investigated variables between animals held under long day conditions (LD) or short day conditions (SD)

Variable	Treatment	n[a]	Statistics	Mean difference ± SEM	CI[b] or Pctl[c]
Leukocytes [/µl] [d]	LD	9	$t(18) = 0.40$, $P = 0.693$	429.9 ± 1071.9	CI 14032.5–17028.6
	SD	11			CI 13298.2–16903.2
NK cells [/µl] [e]	LD	9	$U = 43$, $Z = -0.49$, $P = 0.621$	−2.6 ± 18.4	Pctl 65.7–125.2
	SD	11			Pctl 68.8–126.9
T cells [/µl] [d]	LD	9	$t(18) = 0.53$, $P = 0.603$	276.7 ± 523.4	CI 6793.5–8202.4
	SD	11			CI 6327.8–8114.6
Monocytes [/µl] [d]	LD	9	$t(18) = -0.22$, $P = 0.826$	−26.2 ± 117.9	CI 826.8–1053.5
	SD	11			CI 747.2–1185.5
Neutrophils [/µl] [d]	LD	9	$t(18) = 0.42$, $P = 0.678$	195.0 ± 462.0	CI 3094.7–4855.8
	SD	11			CI 3160.1–4400.5
Eosinophils [/µl] [d]	LD	9	$t(18) = 0.91$, $P = 0.376$	108.0 ± 119.0	CI 565.7–941.5
	SD	11			CI 457.4–833.8
Total Th cells [/µl] [e]	LD	9	$U = 42$, $Z = -0.57$, $P = 0.569$	69.0 ± 176.1	Pctl 1873.5–2615.2
	SD	11			Pctl 1978.6–2314.6
Cytotoxic T cells [/µl] [e]	LD	9	$U = 33$, $Z = -1.25$, $P = 0.210$	−149.7 ± 142.4	Pctl 854.4–1098.3
	SD	11			Pctl 967.5–1522.5
Total γδ T cells [/µl] [d]	LD	9	$t(18) = 0.69$, $P = 0.496$	343.5 ± 494.7	CI 3466.4–4971.2
	SD	11			CI 3076.3–4674.3
Naive Th cells [/µl] [e]	LD	9	$U = 35$, $Z = -1.10$, $P = 0.271$	−53.3 ± 135.1	Pctl 969.4–1345.6
	SD	11			Pctl 983.1–1490.0
Ag-exp. Th cells [/µl] [e]	LD	9	$U = 37$, $Z = -0.95$, $P = 0.342$	122.3 ± 112.8	Pctl 836.7–1032.7
	SD	11			Pctl 825.4–999.1
$CD8\alpha^-$ γδ T cells [/µl] [d]	LD	9	$t(18) = 0.48$, $P = 0.640$	208.6 ± 437.9	CI 2333.4–3732.1
	SD	11			CI 2137.1–3511.3
$CD8\alpha^+$ γδ T cells [/µl] [d]	LD	9	$t(18) = 0.91$, $P = 0.374$	134.9 ± 148.1	CI 910.2–1461.9
	SD	11			CI 846.9–1255.3
Cortisol [ng/ml] [d]	LD	9	$t(18) = 0.16$, $P = 0.879$	0.2 ± 1.5	CI 14.1–18.3
	SD	11			CI 13.4–18.6
Activity behavior [%] [f]	LD	9	$t(13.42) = -1.89$, $P = 0.081$	−3.3 ± 1.8	CI 19.9–26.8
	SD	11			CI 24.6–28.6
Hematocrit [%] [d]	LD	9	$t(18) = 1.82$, $P = 0.085$	1.1 ± 0.6	CI 33.3–35.1
	SD	11			CI 32.2–34.1

[a] Data of all animals per treatment were included in comparisons.

[b] 95% confidence interval

[c] 25th–75th percentile

[d] Unpaired Student's *t*-test (two-tailed)

[e] Mann-Whitney *U* test (two-tailed)

[f] Unequal variance *t*-test (two-tailed)

TABLE S9 | Statistical details for mixed ANOVA investigating the effects of treatment (long day conditions (LD)/short day conditions (SD)) and light (on/off)[a]

Variable	Model term	Pairwise comparisons	Mean difference ± SEM	*Partial* η^2	*df* [b]	*F*	*P*
Cortisol [ng/ml]	Treatment			<0.01	1,18	0.77	0.785
	Light			0.48	1,18	16.53	7.25×10^{-04}
	Treatment × Light			0.63	1,18	30.44	3.08×10^{-05}
	Simple main effects:	LD – on *vs.* off	1.2 ± 1.2	0.05	1,18	0.96	0.341
		SD – on *vs.* off	−7.7 ± 1.1	0.74	1,18	51.02	1.18×10^{-06}
		On – SD *vs.* LD	4.9 ± 1.9	0.26	1,18	6.21	0.023
		Off – SD *vs.* LD	−4.0 ± 1.5	0.29	1,18	7.19	0.015
Activity behavior [%]	Treatment			0.78	1,18	64.46	2.33×10^{-07}
	Light			0.95	1,18	352.76	2.84×10^{-13}
	Treatment × Light			0.43	1,18	13.54	0.002
	Simple main effects:	LD – on *vs.* off	−25.7 ± 2.5	0.85	1,18	103.67	6.76×10^{-09}
		SD – on *vs.* off	−38.2 ± 2.3	0.94	1,18	280.29	2.02×10^{-12}
		On – SD *vs.* LD	18.9 ± 3.2	0.66	1,18	35.54	1.22×10^{-05}
		Off – SD *vs.* LD	6.4 ± 0.8	0.77	1,18	59.41	4.14×10^{-07}
Hematocrit [%]	Treatment			0.02	1,18	0.39	0.540
	Light			0.93	1,18	245.71	6.16×10^{-12}
	Treatment × Light			0.05	1,18	0.95	0.342

[a] 2 × 2 mixed factorial ANOVA with treatment (LD/SD) as between-subjects factor and light (on/off) as within-subjects repeated factor was performed on mean values calculated from the 26 samplings per pig, divided into values during lights-on or lights-off for all animals per treatment (LD n = 9, lights-on = 18 samplings & lights-off = 8 samplings, SD n = 11, lights-on = 10 samplings & lights-off = 16 samplings). If there was a significant interaction effect, simple main effects were analyzed with the least significant difference (LSD) procedure.

[b] Numerator degrees of freedom, denominator degrees of freedom

IV

Glucocorticoid receptor number and affinity differ between peripheral blood mononuclear cells and granulocytes in domestic pigs

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Abstract

The aim of the present study was to characterize the number and affinity of glucocorticoid receptors (GR) in peripheral blood mononuclear cells (PBMC) and granulocytes of domestic pigs because glucocorticoid signaling is considered important for animal health and welfare. To investigate GR binding characteristics in intact porcine immune cells, blood samples of six castrated male pigs were collected via indwelling vein catheters. Porcine PBMC and granulocytes were isolated using two-layer density gradients, followed by radioligand binding assays to determine the number of GR sites per cell and the dissociation constant K_d as a measure for GR binding affinity. The present study revealed a greater number of GR sites per cell ($P = 0.039$) in PBMC (mean ± SEM: 1953 ± 207 sites/cell) compared to granulocytes (1561 ± 159 sites/cell) in domestic pigs. Furthermore, porcine PBMC had a higher GR binding affinity than porcine granulocytes ($P = 0.003$) as the dissociation constant K_d of PBMC (1.8 ± 0.2 nM) was lower than that of granulocytes (3.5 ± 0.4 nM). Our results point to differences in underlying mechanisms of glucocorticoid signaling in different porcine leukocyte populations.

Keywords: Glucocorticoid receptor; Cortisol; Radioligand assay; Leukocyte; Granulocyte; *Sus scrofa domestica*

1. Introduction

Glucocorticoids are a central link between the neuroendocrine and the immune systems by influencing distribution and function of leukocytes [1,2]. Whereas their diurnal rhythmic release controls homeostasis of physiological functions on a daily basis, they are also known to be released in response to stressor exposure [3]. Especially when chronic, stress is associated with an impaired immune competence [4]. In modern pig husbandry, animals face many stressors, such as weaning, transportation, or repeated mixing of animal groups, and glucocorticoid signaling is therefore considered as important factor influencing animal health and welfare [5,6].

Glucocorticoids exert their immunomodulating effects via glucocorticoid receptors (GR) [1,7]. They act primarily through binding to cytosolic GR, which translocate into the nucleus after ligand binding and exert their influence on gene expression through binding as homodimers to glucocorticoid response elements (GREs) or via the interaction with other transcription factors [1]. Therefore, glucocorticoid signaling depends not only on available glucocorticoids but also on GR number and affinity. Differences in GR number or affinity

might therefore be related to variations found in glucocorticoid responsiveness among different leukocyte types [2,7]. So far, only a few studies directly compare GR binding characteristics in different leukocyte populations [8-11]. These studies suggest differences in the GR number between peripheral blood mononuclear cells (PBMC) and granulocytes in humans [8,9]. It should be mentioned that PBMC consist of lymphocytes, monocytes, and a minor proportion of dendritic cells, whereas the total population of granulocytes consists of primarily neutrophils and a minor proportion of eosinophils. Thereby, different leukocyte types have specific functions, e.g., neutrophils belong to the innate arm of the immune system and represent the first line of defense against pathogens, whereas T and B lymphocytes belong to the adaptive immunity with the capability of forming an immunological memory [12].

Until now, number and affinity of the GR have been characterized in various porcine tissues (e.g., preadipocytes, liver, kidney, different brain regions) as well as in porcine PBMC [13-15]. However, assessment of GR binding characteristics in other populations of porcine leukocytes remains limited. Therefore, the aim of the present study was to characterize GR number and affinity in PBMC and granulocytes of domestic pigs. This was carried out using radioligand binding assays to analyze GR binding characteristics in intact immune cells. Thus, not only the number of GR sites per cell was assessed but also the functioning of the GR in terms of receptor-ligand affinity.

2. Materials and methods

2.1 Animals and sampling

All procedures were conducted according to the ethical and animal care guidelines and approved by the local authority for animal care and use (Regional Council Stuttgart, Germany; V309/13TH). Six castrated male pigs (Piétrain × German Landrace, 10 to 11 months old, BW range 157 to 172 kg) were included in the study and were housed individually in the experimental unit of the department in pens, which were cleaned and littered daily with dust-free wood shavings. The pigs had *ad libitum* access to hay and water and were fed concentrate twice daily. The pigs were surgically catheterized with indwelling vein catheters (*vena cava cranialis*) to enable blood collection without disturbing the animals as previously described [16]. To circumvent anesthesia-related effects on glucocorticoid signaling, surgery was conducted at least 2 wk prior to probe sampling [17,18]. Blood samples (60 mL per animal) were collected into lithium heparin tubes (Sarstedt, Nümbrecht,

Germany) at 0800 h in the morning and processed immediately for analysis of GR number and affinity. Preceding leukocyte isolation, plasma was separated by centrifuging an aliquot of 1 mL of each blood sample for 10 min at 1000 × *g* at 4°C and stored at -80°C until cortisol analysis.

2.2 Isolation of PBMC and granulocytes

Using a two-layer density gradient, PBMC and granulocytes were isolated simultaneously from whole blood. The upper layer had a density of 1.077 g/mL (Biocoll separating solution, Biochrom, Berlin, Germany) and the lower layer was adjusted to a density of 1.097 g/mL by diluting 1.100 g/mL-Biocoll (Biochrom) with PBS (phosphate buffered saline, Biochrom). The heparinized blood (59 mL) was diluted 1:2 with PBS, carefully layered onto the gradient and centrifuged for 35 min at 500 × *g* at 20°C. Then, PBMC were collected from the top of the upper layer and granulocytes were collected from the top of the lower layer. Isolated cells were washed twice in PBS by centrifuging for 10 min at 300 × *g* at 20°C to remove endogenous cortisol molecules from the cell suspensions. Preceding flow cytometric experiments showed that this method leads to a cell purity of at least 95% for PBMC and granulocytes, respectively (data not shown). Finally, cells were resuspended in RPMI 1640 (Biochrom) without addition of any serum. Cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany). Cell viability (determined by PI staining and flow cytometric analysis) was at least 95% and did not differ significantly between PBMC and granulocytes.

2.3 Glucocorticoid receptor binding assay

Following leukocyte isolation, GR binding assays with intact cells were carried out according to Sartori et al. [19] with modifications. In detail, to support dissociation of bound endogenous cortisol from GR, suspensions of PBMC and granulocytes were preincubated in 50-mL tubes at a concentration of 6.25×10^6 cells/mL for 90 min at pig-specific physiological conditions of 39°C and 5% CO_2 [19]. Binding assays were carried out in duplicate. After preincubation, the cells were transferred to 96-well flat-bottom plates (1×10^6 cells/well) and incubated in a final volume of 200 µL/well under continuous shaking for 60 min at 39°C and 5% CO_2 in the presence of eight different concentrations (0.25, 0.5, 0.75, 1, 1.25, 2.5, 5, 10 nM) of [1,2,4,6,7-^{3}H]-dexamethasone (77 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) dissolved in PBS. Nonspecific binding was assessed at three different concentrations of [^{3}H]-dexamethasone (0.25, 2.5,

10 nM) with at least 200-fold excess of unlabeled dexamethasone (2 µM, Sigma-Aldrich, Munich, Germany) dissolved in RPMI 1640. Furthermore, blank controls were prepared without addition of dexamethasone. The incubation was slowed by placing the plates on ice. Free ligand was washed out by harvesting the cells with a cell harvester (Titertek instruments, Huntsville, AL, USA) with deionized water onto Whatman 934-AH glass microfiber filters (pore size 1.5 µm, Sigma-Aldrich). Filters were dried overnight at room temperature and then 3.6 mL Irga-Safe Plus (PerkinElmer, Groningen, The Netherlands) was added to each vial (Pico Prias Vial, PerkinElmer). Radioactivity was measured 24 h later using a Tri-Carb 2800TR liquid scintillation analyzer (PerkinElmer). The amount of inserted [^{3}H]-dexamethasone in each assay was determined by measuring duplicate aliquots of each concentration level directly in Irga-Safe Plus. Each assay was corrected for the inserted amount of radioligand for each concentration level and the rate of radioactive decay. Background noise for each sample was removed by including the blank controls into analysis. Binding data were analyzed using GraphPad Prism 5 (version 5.04, GraphPad Software, La Jolla, CA, USA) with nonlinear regression to reach greater accuracy than with Scatchard plot analysis [20]. The results are characterized by the number of GR sites per cell and the dissociation constant K_d, which specifies the binding affinity of the GR towards the ligand [^{3}H]-dexamethasone (lower K_d signifies higher affinity). Intra-assay variability was 9.6% for the number of GR sites per cell and 9.0% for the dissociation constant K_d.

2.4 Cortisol RIA

Plasma cortisol concentrations were analyzed radioimmunologically with preceding ethanolic extraction as previously described by Wesoly et al. [17] and Keane et al. [21] with modifications. Briefly, 50 µL plasma per blood sample was diluted 10-fold with 100% ethanol (Carl Roth, Karlsruhe, Germany), mixed, incubated for 5 min at 4°C, and centrifuged for 10 min at 2000 × *g* at 4°C to remove binding proteins. Then, the solvent in 50-µL aliquots of supernatant was evaporated under air in a vacuum dryer for 30 min at 55°C to dryness. The extracts were then resuspended in phosphate buffer to a volume of 100 µL and analyzed in the RIA in duplicate. Then, a polyclonal antibody against cortisol-3-BSA (MBS316242, MyBioSource, San Diego, CA, USA) at a final dilution of 1:112,000 in 0.1% BSA buffer was added and [1,2,6,7-^{3}H]-cortisol (93 Ci/mmol, PerkinElmer, Boston, MA, USA) was used as tracer. Separation of bound/free was performed with dextran-coated charcoal (0.02% Dextran 70, Carl Roth; 0.2% Norit A, Serva Electrophoresis, Heidelberg, Germany) by

centrifugation for 20 min at 2000 × *g* at 4°C. Supernatants were decanted to 5 mL Irga-Safe Plus to determine radioactivity (Tri-Carb 2800TR). Standards were prepared in acetic acid-treated and charcoal-stripped plasma with final concentrations ranging from 2 to 200 ng/mL cortisol (Sigma-Aldrich) and spiked controls (10, 20, and 40 ng/mL) were prepared in native pool plasma with low endogenous cortisol concentration. Precision was determined with spiked controls and revealed a mean recovery rate of 112%. All samples were analyzed in one single assay and intra-assay variability for a biological sample was 4.6%.

2.5 Statistics

IBM SPSS Statistics 22 (IBM Deutschland, Ehningen, Germany) was used for statistical analyses. As normality of differences and of single datasets was confirmed, paired Student's *t*-tests (two-tailed) were used for pairwise comparisons and Pearson's correlation coefficients were used to evaluate potential linear relationships. In all statistical analyses $P < 0.05$ was considered significant and $P < 0.10$ was considered as tendency.

3. Results

Pairwise comparisons revealed differences in GR binding characteristics between PBMC and granulocytes (Fig. 1). The number of GR sites per cell was greater in PBMC

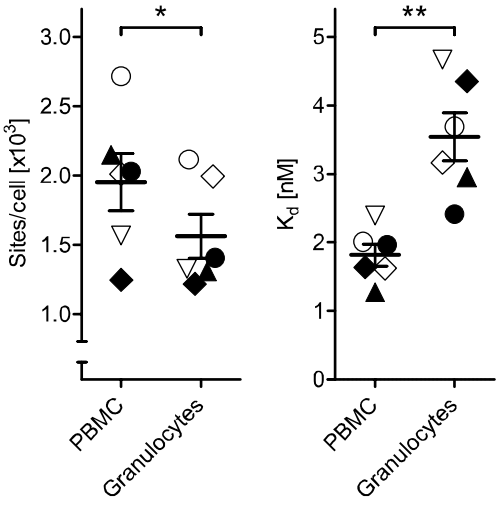

Fig. 1. Comparison of glucocorticoid receptor (GR) number and affinity between peripheral blood mononuclear cells (PBMC) and granulocytes in domestic pigs. The number of GR sites per cell was greater in PBMC compared to granulocytes (left panel). The GR binding affinity (specified by the dissociation constant K_d, lower K_d signifies higher affinity) was higher in PBMC compared to granulocytes (right panel). Data sets were analyzed using two-tailed, paired Student's *t*-tests (mean ± SEM and single values of each animal, n = 6). * $P < 0.05$, ** $P < 0.01$.

($t(5) = 2.776$, $P = 0.039$; 1953 ± 207 sites/cell (mean ± SEM); 95% confidence interval (CI): 1421 to 2484 sites/cell) compared to granulocytes (1561 ± 159 sites/cell; CI: 1153 to 1970 sites/cell). Furthermore, GR in PBMC had a higher affinity towards [^{3}H]-dexamethasone than granulocytes ($t(5) = -5.507$, $P = 0.003$) as the dissociation constant K_d was lower in PBMC (1.8 ± 0.2 nM; CI: 1.4 to 2.2 nM) than in granulocytes (3.5 ± 0.4 nM; CI: 2.6 to 4.4 nM).

The number of GR sites per PBMC tended to correlate with the number of GR sites per granulocyte (Fig. 2A upper panel; $r = 0.732$, $P = 0.098$). However, no linear relationship was found for GR binding affinity between granulocytes and PBMC (Fig. 2A lower panel; $r = 0.456$, $P = 0.363$). Furthermore, in both investigated leukocyte populations neither number of GR sites per cell (Fig. 2B upper panel; PBMC: $r = -0.201$, $P = 0.703$; granulocytes: $r = -0.707$, $P = 0.116$) nor GR binding affinity (Fig. 2B lower panel; PBMC:

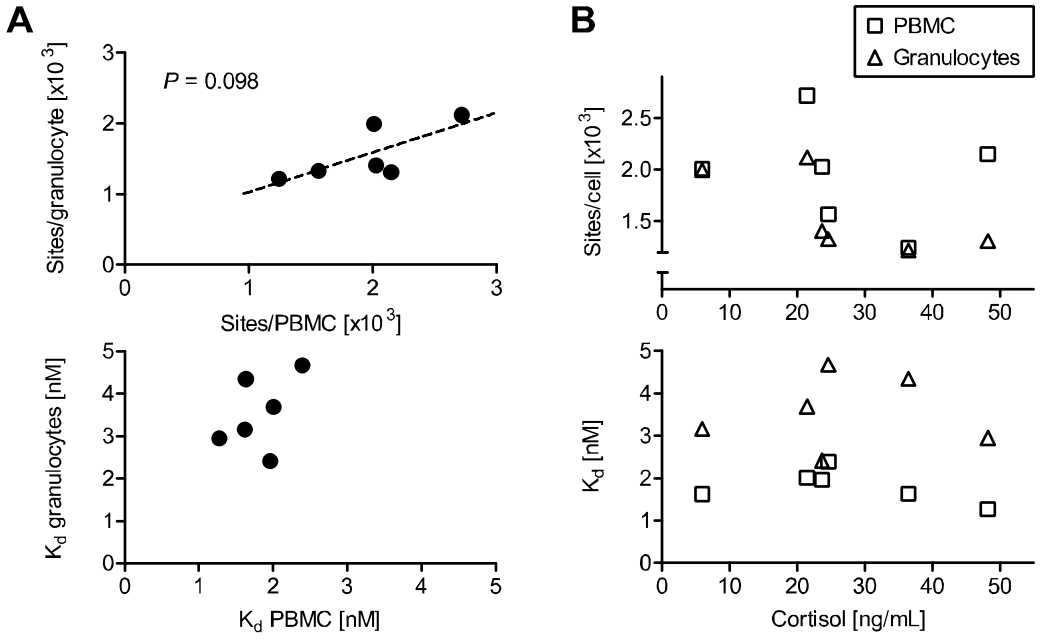

Fig. 2. Relationships of glucocorticoid receptor (GR) binding characteristics between leukocyte populations and with plasma cortisol concentration in domestic pigs. (A) There was a tendency for the GR number in granulocytes to be correlated with the GR number in peripheral blood mononuclear cells (PBMC; upper panel; $r = 0.732$, $P = 0.098$). The GR binding affinity (specified by the dissociation constant K_d, lower K_d signifies higher affinity) did not correlate between granulocytes and PBMC (lower panel; $r = 0.456$, $P = 0.363$). (B) Plasma cortisol concentration was not correlated with GR number (upper panel; PBMC: $r = -0.201$, $P = 0.703$; granulocytes: $r = -0.707$, $P = 0.116$) or GR binding affinity (lower panel; PBMC: $r = -0.431$, $P = 0.393$; granulocytes: $r = 0.053$, $P = 0.921$) in PBMC and granulocytes. Data sets were analyzed using Pearson's correlation coefficients (n = 6).

$r = -0.431$, $P = 0.393$; granulocytes: $r = 0.053$, $P = 0.921$) correlated with plasma cortisol concentration. There also was no linear relationship between GR number and affinity within the two investigated leukocyte populations (PBMC: $r = -0.057$, $P = 0.914$; granulocytes: $r = -0.178$, $P = 0.736$).

4. Discussion

This study investigated GR number and affinity simultaneously in PBMC and granulocytes in blood of domestic pigs with radioligand binding assays in intact immune cells. The GR interacts with many molecules in the living cell, such as heat-shock proteins and the microtubules of the cytoskeleton, before and after binding its ligand [22]. Thus, studying GR binding characteristics in intact immune cells at physiologic incubation temperatures mimics *in vivo* conditions more closely than using cytosolic extracts at low temperatures, as is usually done in binding studies investigating different tissues [14].

The present study revealed differences between the two investigated porcine leukocyte populations. The number of GR sites per cell was greater in PBMC compared to granulocytes, which resembles results found in human blood [8,9]. While a study in humans did not detect a difference in GR binding affinity between PBMC and granulocytes [9], GR in porcine PBMC exhibited higher affinity towards [^{3}H]-dexamethasone. Interestingly, GR in porcine leukocytes showed higher ligand binding affinity relative to human leukocytes [9,23], but this suits to the fact that domestic crossbred pigs have lower circulating cortisol concentrations compared to humans [24,25]. When this generally higher GR binding affinity is taken into account, the differences in K_d found in the present study are in a comparable order of magnitude with differences in K_d associated with substantial physiological effects found in humans [26,27].

There was a tendency for a correlation in the GR number between porcine granulocytes and PBMC, which corresponds to previous findings in humans [28,29]. This may point to individual differences in GR number among the animals. As breed-specific effects on plasma cortisol concentration are well described in domestic pigs as well [30], a potential genetic influence on glucocorticoid signaling might be assumed. As also found in other studies [28,29], there was no correlation of GR number or affinity with plasma cortisol concentration in the present study. This finding probably results from the time lapse of at least 30 min to approximately 4 h between glucocorticoid binding and the exertion of GR-mediated genomic effects [31,32], including the negative autoregulation of GR expression [33]. Time-

lagged correlation analyses relying on time-shifted measurement of plasma cortisol and GR binding characteristics should possibly demonstrate relationships between cortisol concentration in blood plasma and GR number or affinity in leukocytes in the future. The GR binding affinity did not correlate between the two investigated leukocyte populations and there was no linear relationship between GR number and affinity neither in PBMC nor in granulocytes. This is consistent with studies showing that GR binding affinity can be influenced in a cell type-specific manner by various biomolecules, such as insulin or cytokines [13,34].

The differences in GR number and affinity, found in the present study, point to enhanced glucocorticoid signaling in PBMC compared to granulocytes in domestic pigs. It is already known that the effect of glucocorticoids on leukocytes is cell type-specific [2,7]. High glucocorticoid concentrations mainly inhibit the function of or even induce apoptosis in most immune cell types [7]. However, they do not suppress the function of neutrophilic granulocytes and neutrophil survival is even found to be enhanced by glucocorticoid action [35,36]. Interestingly, this improved viability of neutrophils under glucocorticoid treatment in humans is linked to the higher expression of GRβ in these cells [37]. This protein isoform of the GR does not bind glucocorticoids and acts as an inhibitor of GRα, the transcriptionally active isoform [38]. Whereas this potential contribution of GRβ to glucocorticoid resistance is challenged by some studies with conflicting results [39], there is much evidence that this isoform plays an important role in the manifestation of various diseases related to glucocorticoid resistance [40-42]. As GRβ is present in domestic pigs as well [43], a possibly greater proportion of this isoform in porcine granulocytes could be responsible for reduced glucocorticoid signaling in porcine granulocytes compared to PBMC. But further experiments are needed to prove this assumption.

In conclusion, this study revealed that the number of GR sites per cell, as well as the GR binding affinity towards [^{3}H]-dexamethasone, is greater in PBMC compared to granulocytes in domestic pigs. Thus, this result points to differences in the underlying mechanisms of glucocorticoid signaling in these two leukocyte populations. These differences could potentially influence immune reactions triggered by infections or stressors in domestic pigs. Further experiments should determine the role of GRα and GRβ in regulating glucocorticoid-mediated effects on leukocytes in domestic pigs.

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3 GENERAL DISCUSSION

The present thesis revealed for the first time diurnal and photoperiodic effects on peripheral leukocyte numbers in various immune cell populations as well as glucocorticoid signaling in domestic pigs. Hence, this thesis project established an additional diurnally active mammalian model species in chronoimmunology research to study underlying mechanisms of diurnal and photoperiodic effects on the immune system and glucocorticoid signaling. As until now diurnal and photoperiodic or seasonal effects on the immune system were mainly studied in humans and nocturnal rodents (Cermakian et al., 2013; Dopico et al., 2015; Lange et al., 2010; Nelson & Demas, 1996; Scheiermann et al., 2013; Stevenson & Prendergast, 2015), the present project fulfilled the need for a wider range of animal models, particularly including long-lived seasonal species (Chemineau et al., 2007; Lunn et al., 2017; Stevenson et al., 2015). Moreover, conducting chronoimmunology research in the porcine species represents an innovative approach in agricultural science and provides entirely new opportunities to improve animal health and welfare.

3.1 Key findings

Initially, diurnal rhythms in the cell numbers of various peripheral leukocyte populations were characterized by cosinor analysis in domestic pigs held under 12L:12D lighting conditions with concentrate feeding twice daily during lights-on (MANUSCRIPTS I and II). The results concerning porcine CD8α$^-$ and CD8α$^+$ γδ T cells were reported separately in MANUSCRIPT II as the function of these different subsets is not fully elucidated yet (Sedlak et al., 2014; Sinkora & Butler, 2016; Talker et al., 2013). The diurnal immune rhythms described in MANUSCRIPTS I and II were found to be mainly comparable to humans and nocturnal rodents in relation to their respective active or resting phase, with the particular exception of neutrophils differing from both comparison species (Cermakian et al., 2013; Lange et al., 2010; Scheiermann et al., 2013). Furthermore, diurnal rhythmicity was demonstrated in activity behavior, plasma cortisol concentration, and hematocrit, confirming that domestic pigs are diurnally active under the applied experimental conditions mimicking usual practices in animal husbandry as well as life-habits of humans (Désautés et al., 1999; Ingram et al., 1980; Ingram & Dauncey, 1985; Sennels et al., 2011; Takeishi et al., 2018).

Thereby, potential associations between peripheral immune cell numbers and the experimental factors light and feeding as well as plasma cortisol concentration, hematocrit, and repeated sampling were demonstrated by linear mixed model analyses. In this respect, the negative associations between plasma cortisol concentration and cell number in porcine blood found for most of the investigated immune cell types resemble previous studies in humans and nocturnal rodents (Besedovsky et al., 2014a; Besedovsky et al., 2014b; Dhabhar et al., 2012; Dimitrov et al., 2009). These results underline the importance of glucocorticoid signaling in diurnal immune rhythms. Moreover, as strength of diurnal oscillation of glucocorticoids is linked with various health outcomes, i.e., lower oscillation is generally associated with poorer health in humans, particularly found in immune system-related variables (Adam et al., 2017), the present approach investigating diurnal rhythms in parallel in the immune system and in glucocorticoids creates an appropriate framework for further research in the porcine model species. Thus, due to the high comparability of the results described in MANUSCRIPTS I and II to results from humans and established rodent models, the domestic pig represents a suitable diurnally active large animal model in chronoimmunology research.

Subsequently, the focus of the present thesis project was directed to photoperiodic effects on diurnal rhythms in peripheral immune cell numbers in domestic pigs (MANUSCRIPT III). The investigation of animals either held under LD or SD revealed distinct photoperiodic differences in relative amplitudes and peak times of cell counts, whereas mesor values did not differ. It is remarkable, that this study is the first one investigating photoperiodic effects on diurnal rhythms on peripheral leukocyte numbers within the same season of the year in any species. The investigated diurnal rhythms were mainly comparable to the results reported in MANUSCRIPTS I and II, and again, with the noteworthy exception of neutrophils, mainly resembled human and rodent results in relation to their respective activity rhythms (Cermakian et al., 2013; Lange et al., 2010; Scheiermann et al., 2013). Moreover, associations of peripheral leukocyte numbers with potential influencing variables evaluated with generalized linear mixed model analyses mainly resembled the previous results under 12L:12D as well (MANUSCRIPTS I and II). However, as activity behavior was investigated in all animals in the photoperiodic study (MANUSCRIPT III), inclusion of this variable into the models presumably enhanced explanatory power and possibly caused slight differences in model results compared to MANUSCRIPTS I and II. In general, the results reported in MANUSCRIPT III indicate common intrinsic regulation of diurnal rhythms in most

investigated leukocyte types, except for neutrophils. In addition, these results (MANUSCRIPT III) also imply stronger diurnal rhythmicity of peripheral leukocyte numbers under SD and weakened rhythmicity under LD, which is comparable to results in humans and rodents under natural lighting conditions during different seasons of the year (Berger, 1980, 1981; Lévi et al., 1988). Moreover, an underlying mechanism for this phenomenon may potentially be inherent within the SCN as oscillations of electrical activity and clock gene expression in SCN neurons are more closely coupled under SD but phases, or simplified peak times, are more dispersed under LD (Buijink et al., 2016; Schaap et al., 2003; VanderLeest et al., 2007). Suspected neural or humoral pathways from this potential central nervous mechanism to the periphery remain to be investigated regarding diurnal rhythmicity in peripheral leukocyte number. In addition, the photoperiodic study described in MANUSCRIPT III further strengthens the importance of the domestic pig as suitable large animal model as results for plasma cortisol concentration and activity behavior strongly resemble results found in humans and rhesus macaques (Lemos et al., 2009; Wehr et al., 1993). In addition, this study emphasizes the importance of investigating photoperiodic effects on diurnal immune rhythms within the same season as a means of dissecting endogenous circannual from direct photoperiodic effects.

It is noteworthy that the overall statistical assessment of peak times and relative amplitudes in peripheral leukocyte numbers between the investigated immune cell types descripted in MANUSCRIPT I (Figs. 2D, 3, S2A, and S2B), MANUSCRIPT II (Figs. 2 and 3), and MANUSCRIPT III (Figs. S2, S3, S4E, and S4G) was a special approach to illustrate the potential importance of their relationship in peak time or oscillation strength to each other. This means that the chronological order in diurnal regulation of leukocyte trafficking is potentially important for immune function and immune regulation and that differences in oscillation strength might represent specific characteristics of their molecular properties in terms of their diurnal regulation. These comparisons will potentially support future investigations and interpretations of diurnal immune cell regulation.

Finally, the focus of the present thesis project was directed to underlying mechanisms of diurnal rhythms in the immune system. As distinct differences between neutrophils and most of the other investigated immune cell types concerning diurnal rhythmicity and association of peripheral cell numbers with plasma cortisol concentration were found (MANUSCRIPTS I–III), potential differences in glucocorticoid signaling between those leukocyte populations were assumed. Therefore, to gain better insight into underlying

mechanisms of the diurnal differences between these leukocyte populations, the present thesis project aimed to investigate the molecular basis of glucocorticoid signaling in those different leukocyte populations. As the population of granulocytes primarily consists of neutrophils and the major proportion of cell types within PBMC was negatively associated with plasma cortisol concentration (MANUSCRIPTS I–III), it was possible to address this objective by use of two-layer density gradients to separate PBMC and granulocytes simultaneously from fresh porcine blood samples. Notably, the study described in MANUSCRIPT IV characterized GR number and affinity in PBMC and granulocytes for the first time in the porcine species. Thereby, a greater number of GR sites per cell, resembling results from human studies (Armanini et al., 1985; Miller et al., 1998), and a higher GR binding affinity in PBMC compared to granulocytes in domestic pigs were found (MANUSCRIPT IV). These results point to differences in molecular mechanisms of glucocorticoid signaling in the different porcine leukocyte populations, i.e., a potentially greater glucocorticoid signaling capacity in PBMC compared to neutrophils. As diurnal rhythmicity in glucocorticoids is supposed to be a main link from the master circadian clock towards peripheral clocks (Haus, 2007; Le Minh et al., 2001; Pezük et al., 2012; Spencer et al., 2018), and especially influences diurnal immune function (Adam et al., 2017; Dumbell et al., 2016; Nader et al., 2010), these implied differences in glucocorticoid signaling (MANUSCRIPT IV) may potentially be connected to the found differences in diurnal rhythmicity between most investigated immune cell types and neutrophils reported in MANUSCRIPTS I–III.

3.2 Implications for immune function and health

Causes, underlying mechanisms, and consequences of diurnal and seasonal immune function and health are only partially understood so far. In particular, the detrimental consequences of circadian and seasonal disruption for health, including cardiovascular diseases, obesity, diabetes, cancer, and disrupted immune function (Cuesta et al., 2016; Touitou et al., 2017), are crucial issues of the era we live in (Lunn et al., 2017; Stevenson et al., 2015). Thus, to gain insight into underlying mechanisms of these interrelations, there is a need for model organisms, which are highly comparable to humans, in particular diurnally active model species. In addition, to gain knowledge about seasonal disruption, model organisms should show at least moderate seasonality, as even modern humans exhibit, e.g., moderately

seasonal reproduction (Martinez-Bakker et al., 2014). Therefore, the first investigation of diurnal and photoperiodic effects in a range of immunological, endocrinological, and behavioral variables (MANUSCRIPTS I–III) as well as potential underlying mechanisms of glucocorticoid signaling (MANUSCRIPT IV) in diurnally active and moderately seasonal domestic pigs (Chemineau et al., 2007; Ingram & Dauncey, 1985; Takeishi et al., 2018) contributes to gaining better insight into the interrelationship between circadian or seasonal regulation and health. In this respect, it has to be emphasized again that the investigation of photoperiodic effects on diurnal rhythms in peripheral leukocyte numbers (MANUSCRIPT III) is the first one in any species. Therefore, the knowledge in porcine chronoimmunology acquired within the present thesis project serves as solid basis for further research in this model organism to answer the open questions of our modern life mentioned above.

In this respect, research over the last few decades in other species, humans and mainly in nocturnal rodents, provided some mechanistic insights into diurnal rhythmicity in the immune system (reviewed in Arjona et al., 2012; Cermakian et al., 2013; Druzd et al., 2014; Lange et al., 2010; Scheiermann et al., 2013; Scheiermann et al., 2015; Scheiermann et al., 2018). Thereby, special emphasis was given to rhythmicity in peripheral leukocyte numbers, i.e., the interplay of hematopoiesis, trafficking, and clearance of immune cells (Scheiermann et al., 2015). There are detailed mechanistic findings about diurnal rhythms in trafficking of hematopoietic stem cells and innate immune cells from studies using nocturnal mouse models, mainly focusing on sympathetic nervous system regulation and adrenergic signaling (Casanova-Acebes et al., 2013; Katayama et al., 2006; Méndez-Ferrer et al., 2008; Scheiermann et al., 2012; Schloss et al., 2017; Winter et al., 2018) but innate immune cell regulation by clock genes was investigated in rodents as well (Hopwood et al., 2018; Keller et al., 2009; Nguyen et al., 2013; Pariollaud et al., 2018). However, more intensive studies of mechanisms in diurnal immune cell trafficking within the adaptive arm of the immune system were initially performed in human subjects with focus on glucocorticoid and mineralocorticoid signaling but also taking into account adrenergic regulation (Besedovsky et al., 2012a; Besedovsky et al., 2014a; Besedovsky et al., 2014b; Besedovsky et al., 2014c; Dimitrov et al., 2009; Dimitrov et al., 2010). In addition, adaptive immune cell regulation by clock genes and innate immune cell regulation was also addressed in humans (Bollinger et al., 2011; Dimitrov et al., 2007). In this regard, mechanisms of adaptive immune regulation were recently investigated in murine studies focusing either on adrenergic (Nakai et al., 2014; Suzuki et al., 2016), glucocorticoid (Shimba et al., 2018), and clock gene

regulation in lymph node trafficking (Druzd et al., 2017) or on clock gene regulation in T cell differentiation (Hemmers & Rudensky, 2015).

The preceding descriptions illustrate that research directions are slightly divergent between humans and rodent models so far, i.e., human studies mainly focused on glucocorticoid signaling and adaptive immunity, rodent studies on adrenergic signaling and innate immunity. Even one recent sophisticated approach using humanized mice to study underlying mechanisms of chronoimmunology stuck to this divergence as the compared murine leukocyte types were innate and the human cell types were mainly adaptive (Zhao et al., 2017). Moreover, it has to be noted that some species-specific differences in diurnal rhythmicity of leukocyte types potentially exist, e.g., neutrophils and lymphocytes oscillate nearly in parallel in rodents (Oishi et al., 2006; Pelegrí et al., 2003), more inhomogeneously in humans (Born et al., 1997; Haus et al., 1983; Sennels et al., 2011) and almost opposite in domestic pigs as described in MANUSCRIPTS I and III. However, it seems plausible that diurnal neutrophil regulation is dependent on meal timing, as indicated by the lack of a photoperiodic difference in peak times of neutrophil counts in porcine blood under experimental conditions with identical meal timing as described in MANUSCRIPT III. Moreover, as postprandial increases were reported for neutrophil counts in human blood (Hansen et al., 1997; Khan et al., 2016; Lippi et al., 2010; van Oostrom et al., 2003), species-specific diurnal regulation in those cells might, at least between humans and pigs, be explained by differences in experimental settings between species, with humans consuming 3 meals per day (Born et al., 1997), domestic pigs receiving 2 meals (morning and afternoon) under the present experimental conditions, and mice feeding *ad libitum* quite evenly distributed during their active phase but with more intensive feeding in the first half and at the very end of the active phase (Ho & Chin, 1988; Kohsaka et al., 2007). Correspondingly, meal-related cortisol peaks were found in the present thesis project (refer to MANUSCRIPT I) as well as in human studies (Follenius et al., 1982; Hansen et al., 1997). These peaks in plasma cortisol concentration, however, are unlikely to be the causing factor of an increase in neutrophil numbers in porcine blood around feeding times as glucocorticoid signaling seems to be reduced in neutrophils (MANUSCRIPT IV) and linear mixed models also showed no association of neutrophil counts with plasma cortisol concentration in porcine blood (MANUSCRIPTS I and III). As meal-intake is also associated with an increase of norepinephrine concentration in blood (Pirke et al., 1992; Welle et al., 1981), rather, catecholamines seem to induce the post-prandial increases in neutrophil numbers (Dhabhar

et al., 2012; refer to Hansen et al., 1997). However, causal relationships and underlying mechanisms of diurnal rhythmicity and potential species-specific or experimental condition-related differences cannot be derived yet and need further clarification.

Furthermore, the previously mentioned studies on underlying mechanisms of leukocyte trafficking focused on different tissue compartments, e.g., on lymph nodes in the recent murine studies or the blood stream in human studies. Thereby, recent human studies, investigating chemokine receptors, found some evidence for homing of lymphocytes to the bone marrow (Besedovsky et al., 2014a; Besedovsky et al., 2014b; Besedovsky et al., 2014c; Dimitrov et al., 2009). In studies using rodent models, there is a certain discrepancy regarding the investigated tissues. Whereas some studies clearly demonstrated synthetic glucocorticoid- and stress-induced homing of lymphocytes to the bone marrow and egress from lymph nodes (Chung et al., 1986; Cox & Ford, 1982; Fauci, 1975; Stefanski et al., 2003; Sudo et al., 1997), recent studies omitted investigation of the bone marrow. The latter showed glucocorticoid-induced homing to lymph nodes with knock-out mice (Shimba et al., 2018) or focused on adrenergic or clock gene regulated lymph node trafficking (Druzd et al., 2017; Nakai et al., 2014; Shimba et al., 2018; Suzuki et al., 2016). Thus, these studies are difficult to compare and the potential importance of different homing sites, including the bone marrow, has not been addressed with appropriate attention in recent research in model species so far. As a consequence, based on the high comparability between humans and domestic pigs in diurnal rhythms of peripheral leukocytes and glucocorticoid signaling (MANUSCRIPTS I, III, and IV), the domestic pig would be a particularly suitable model to further enlighten underlying mechanisms of diurnal immune rhythms by examining blood and many organ tissues in parallel in a more holistic approach.

3.3 Implications for animal husbandry and welfare

The detailed characterization of diurnal rhythmicity in the immune system in the porcine species (MANUSCRIPTS I–III) performed within the present thesis project provides new opportunities to improve animal health and welfare. High immune competence of farm animals is essential to prevent infectious diseases and parasitic infections and thus to improve animal welfare (Colditz, 2002). Thereby, a balanced relationship between energetic costs of immune responses and growth performance is considered important to support economic sustainability of livestock production (Pluske et al., 2018). Until now, most

strategies to ensure these requirements focused on housing systems (Grün et al., 2013; Grün et al., 2014; Millet et al., 2005; van de Weerd & Day, 2009), environmental hygiene (Chatelet et al., 2018), nutrition (Bauer et al., 2006; Heyer et al., 2015; Prunier et al., 2010), or genetic selection for immune traits (Clapperton et al., 2009; Flori et al., 2011; Hermesch et al., 2015). Thus, the present thesis extends these approaches by the fourth dimension, the time. New concepts can be developed to support animal health and welfare by means of optimizing housing environment and management strategies considering time of day and time of the year.

In this respect, different areas of animal husbandry might be optimized. To begin with, a time-controlled environment, i.e., lighting conditions and feeding times, could be provided. In more detail, different lighting regimes, light intensities, and light spectra, as well as different feeding times, feeding frequencies, and durations of access to food could be compared in view of improvement of immune function and health of livestock. However, it should be noted that maintaining an optimum level of immune function and health while optimizing animal performance by artificial lighting schedules potentially represents a conflict of aims. It was assumed that additional weight gain of livestock under artificial lighting schedules is supported by metabolic dysregulation triggered by circadian disruption (Russart & Nelson, 2018). Moreover, it was also suspected that using artificial lighting schedules could lead to seasonal disruption and thus could have negative health consequences in farm animals (Stevenson et al., 2015). Indeed, in light of the above mentioned detrimental effects of circadian and seasonal disruption on immune function and health (Cuesta et al., 2016; Touitou et al., 2017) and the clear evidence of tight coupling between porcine immune traits with time of day as well as photoperiod, provided in MANUSCRIPTS I–III, it is implied that there is an urgent need for further research in animal science.

Apart from that, time-controlled handling to support animal health and welfare should be taken into consideration. As diurnal differences in immune response to vaccination or infection were indicated by studies in humans and rodents (Edgar et al., 2016; Fortier et al., 2011; Kurupati et al., 2017; Long et al., 2016a, 2016b; Phillips et al., 2008; Tognini et al., 2017), vaccination timing in domestic pigs and other livestock species should be another area of future research, not only in relation to health maintenance but also in view of the forthcoming widespread adoption of immunologic castration (Mancini et al., 2017). Moreover, following recent research in chronopharmacology (Dallmann et al., 2016), timed

administration of drugs has the potential to support animal health and, thus, to possibly minimize the use of antibiotics in animal husbandry, a current issue for global health (Kuppusamy et al., 2018; Laxminarayan et al., 2013; Van Boeckel et al., 2015). Finally, optimized timing of events during animal handling representing potential stressors, such as weaning or transportation, could potentially attenuate some detrimental effects of such management measures or even support the assumed positive effects of short-term stressor exposure on health (Dhabhar, 2018). To conclude, these arguments indicate that chronobiology research in livestock is not even close to exhausting possibilities to improve animal health and welfare.

3.4 Methodological assessment

The present thesis project established the use of the domestic pig as additional animal model in chronoimmunology research. The experimental design used in the studies described in MANUSCRIPTS I and II (trial 3) as well as MANUSCRIPT III fully meets the recommendation of at least 12 samplings per cycle across 2 full cycles for studying biological rhythms (Hughes et al., 2017). In this regard, due to its high blood volume, the domestic pig provides the opportunity to investigate diurnal rhythms intra-individually with high frequent samplings, which contributes to reduce the number of animals necessary for experimental research. Thereby, the indwelling catheterization of the *vena cava cranialis* is a well-established surgical technique to enable blood collection without disturbance of the animals (Kraetzl & Weiler, 1998; Weiler et al., 2003).

Blood samplings during the dark phase of the experiments were performed under dim light of averagely 7 lx at pigs' eye level, switched on and off for sampling (MANUSCRIPTS I–III). Thereby, color temperature during the dark phase (2700 K, i.e., 'warm light') was lower than during the light phase (4000 K, i.e., 'white light', 190 lx). These light sources were chosen as higher color temperature is reported to suppress melatonin secretion and sleepiness or sleep to a greater extent than lower color temperature (Chang et al., 2015; Chellappa et al., 2011; Motamedzadeh et al., 2017). Notably, the illuminance of 7 lx is much brighter than natural moonlight with about 0.1 lx (Gaston et al., 2013; Kyba et al., 2017). Thus, to prevent possible effects of dim light at night on diurnal physiology of the pigs in future experiments, sampling under red light could be tested, as it does at least not inhibit nocturnal melatonin secretion (Figueiro & Rea, 2010). However, red light potentially influences cortisol

concentration and alertness in a similar way than blue light compared to darkness (Figueiro et al., 2009; Figueiro & Rea, 2010) and practicability of the potential poor visibility for experimental staff has to be taken into account. Moreover, the blood sampling technique could even be adopted to techniques used in human sleep laboratories (Besedovsky et al., 2014a) by lengthening of catheters and fixation above the animals as outlined earlier (Weiler et al., 2003), i.e., avoiding direct contact to the animals by sampling from an adjacent room. It would even be possible to perform automated sampling, which, however, requires technically extensive equipment (Marchant-Forde et al., 2012). In addition, dim light at night effects, mimicking nighttime light pollution (Lunn et al., 2017), could be investigated in particular to uncover detrimental health effects of modern life. In addition, light color or light intensity during the light phase could be modified in future experiments by testing, e.g., 'cold light' (> 5000 K), blue-enriched light or even full spectrum light, and by increasing light intensity to get closer to natural environmental conditions. The light intensity of 190 lx used in the experiments (MANUSCRIPTS I–III) was more than twice the value of 80 lx legally required in German pig husbandry (TierSchNutztV, 2006), but the illuminance on a cloudy day is at least 1,000 lx and in bright sunlight 100,000 lx (Gaston et al., 2013). In this regard, however, it was reported in a few studies that light intensity during the light phase does not affect porcine plasma melatonin concentration or sow performance (Canaday et al., 2013; Tast et al., 2001). Nevertheless, it should be emphasized again that the experiments using sampling under dim light during the dark phase described in MANUSCRIPTS I–III clearly demonstrate expectable outcomes in the investigated traits as outlined in the above sections.

The studies described in MANUSCRIPTS I–III revealed that the investigated domestic pigs were diurnally active under the applied experimental conditions, thus, providing comparability with human behavior (Lange et al., 2010). Notably, the investigated animals spent about 80% of the time lying, which is similar to group-housed fattening pigs (Martelli et al., 2015) and thus, rather mimicking modern sedentary lifestyles of humans (Booth et al., 2002). In addition, it was discussed that the porcine species in general is particularly adaptable concerning its rest-activity cycle (Ingram et al., 1980). This suggestion is also supported by research on the wild ancestor of the domestic pig, the wild boar (*Sus scrofa* L.), which is commonly known for displaying more or less nocturnal locomotor activity (Keuling et al., 2008). In fact, activity patterns of wild boars seem to be largely dependent on hunting pressure and human activity in their respective habitat. In regions with low hunting pressure and human activity, wild boars display higher locomotor activity during the

light phase compared to habitats with high hunting pressure and human activity (Keuling et al., 2008; Podgórski et al., 2013). Moreover, some authors state that diurnal activity is the natural behavior of wild boars (refer to Keuling et al., 2008). Moreover, it was assumed that even these wild animals could potentially be affected by circadian or seasonal disruption due to anthropogenic influences (Dominoni et al., 2016). Thus, further studies could investigate activity rhythms of pigs when fed during the dark phase and how this affects the porcine immune system. In addition, underlying mechanisms and consequences of circadian and seasonal disruption in the porcine species are completely unknown so far and should be examined in the future and compared to results from other species, in particular, with regard to immune function and health.

The domestic pigs used for the studies described in the present thesis (MANUSCRIPTS I–IV) were castrated males, which underwent surgical castration within their first week of life. The main advantage of the use of castrated males is easier handling at adult age compared to entire males. This intervention, however, can reduce direct comparability to human male subjects in such physiological contexts, in which gonadal steroids are important. As already described in MANUSCRIPT III, it is suggested that gonadal hormones play a modifying role at least in photoperiod-induced immune modulation (Nelson & Demas, 1996; Stevenson & Prendergast, 2015; Weil et al., 2015). A role of gonadal hormones in diurnal immune rhythms also seems plausible as potential interactions of glucocorticoids, sex hormones, and the immune system were described (Bereshchenko et al., 2018; Da Silva, 1999). In this respect, diurnal rhythms in plasma testosterone concentrations are well-described in humans (Tenover et al., 1988), whereas some literature is inconsistent concerning domestic pigs (refer to Bonneau et al., 1987). Thus, further studies on these issues are needed, i.e., future chronobiological research could be conducted in entire male as well as female pigs, at least to investigate the potential role of gonadal hormones in photoperiodic immune modulation. In addition, the relationship between biological sex of an organism, glucocorticoid signaling and circadian timing is largely unexplored but is proposed to be of distinct clinical relevance (Spencer et al., 2018).

Evaluating the experimental methods applied within the present thesis project reveals the manifold possibilities, which can be exploited when performing chronoimmunological and chronoendocrinological research with surgically catheterized domestic pigs. The blood samples frequently taken from the pigs can be investigated with various laboratory techniques. In doing so, the main limitation seems to be a lack of available immunological

tools in comparison with humans and rodent models (Dawson & Lunney, 2018). This issue becomes apparent in the fact that there is no mAb against the pan-B cell marker CD19 identified until today in the porcine species (Dawson & Lunney, 2018). Therefore, characterization of B cells in the experiments described in MANUSCRIPT I was performed as an exclusion procedure, i.e., immune cells being negative for CD3ε, CD8α, and CD172a within PBMC were termed B cells, as exclusion of total T cells ($CD3\varepsilon^+$), NK cells ($CD8\alpha^+$), dendritic cells ($CD172a^{dim}$), and monocytes ($CD172a^{high}$) from PBMC should solely leave the B cell population. However, some interference with possible weakly stained other immune cells within PBMC cannot be ruled out, i.e., this might have contributed to the lack of overall diurnal rhythmicity in peripheral B cell numbers described in MANUSCRIPT I. However, this lack of rhythmicity in the total B cell population could also likely be reasoned by some B cell subpopulations displaying opposing diurnal rhythms. Moreover, this explanation could also hold true for Ag-exp. Th cells, which did not show overall diurnal rhythmicity in peripheral cell numbers in the pigs as well (MANUSCRIPTS I and III), because it was shown that different subsets of human Ag-exp. T cells display differences in diurnal rhythms of cell counts (Dimitrov et al., 2009). Thus, there is an urgent need for further experiments utilizing new tools, which became recently available, to discriminate porcine B cell and Ag-exp. Th cell subsets, e.g., using mAb against CD2 and CD21 to discriminate between naive, primed, effector, and plasma B cells as well as against CD27 to discriminate central memory ($CD27^+$) from effector memory Th cells ($CD27^-$) within the Ag-exp. Th cell subset (Reutner et al., 2013; Sinkora & Butler, 2016). Moreover, current available tools could also shed light on diurnal trafficking in phenotypic subsets of other leukocyte populations, e.g., monocytes, dendritic cells, and cytotoxic T cells (Fairbairn et al., 2013; Gerner et al., 2015; Summerfield et al., 2015).

The cortisol radioimmunoassay used in the studies described in MANUSCRIPTS I, III and IV is a well-established method (Grün et al., 2013; Wesoly et al., 2016) whereby the preceding ethanolic extraction procedure (Keane et al., 1975) was successfully implemented for analyzing porcine plasma samples during the present thesis project to simplify and accelerate the protocol. Furthermore, the GR assay used for the experiments described in MANUSCRIPT IV should be evaluated as sensitive technique to determine GR number and affinity as many low concentrations of [^{3}H]-dexamethasone around the expected dissociation constant K_d were used so that curve fitting for nonlinear regression analysis should have led

to accurate results. Thus, the GR assay technique was refined compared to previous reports (Armanini et al., 1985; Sartori et al., 1998; Wassef et al., 1992).

Behavioral analysis was performed by video recording with infrared technology, which enabled recording during darkness (Hoy, 1998), and subsequent qualitative and quantitative personal assessment. The behavioral analyses described in MANUSCRIPTS I and III concentrated on evaluation of active or resting behavior of the domestic pigs. As video record-based analyses are time consuming in general, future chronobehavioral research in pigs could be automated, e.g., by ear tag transponders (Adrion et al., 2018) or even by software-based video tracking of animal behavior (Lind et al., 2005; Matthews et al., 2017). While video data records in conjunction with personal assessment generally offer the possibility to assess a wide range of specific behavioral traits in much more detail than automated methods, at least a combination of video data records with such automated methods, which are relatively easy to implement, could be beneficial.

3.5 Suggestions for future research

On the basis of the present thesis project diverse possibilities emerge for future research. In order to assess the functional basis of potential immune system-related health consequences of circadian disruption, it might be investigated if, besides the diurnal rhythms in peripheral leukocyte numbers described in the present thesis, diurnal differences are also apparent in functionality of the porcine immune system. As an examination of immune cell function requires more complex experimental laboratory settings, the number of samplings within one cycle has likely to be reduced drastically in comparison to the recommendations for chronobiological research of 12 samplings per cycle (Hughes et al., 2017). As a first step, future research on porcine immune function could concentrate on diurnal differences between the light phase and the dark phase. In addition, to decipher the importance of light and feeding as potential zeitgebers for diurnal rhythms in the cell numbers of different peripheral leukocyte types, especially neutrophils, the consequent approach might be an experimental setting varying feeding time (light or darkness, at various times of day), frequency (e.g., once, twice, three times daily), and duration of food availability (restricted or *ad libitum*), not only in domestic pigs but in other species as well. In this regard, as mentioned before, an influence of meal intake on specific peripheral leukocyte populations was already reported in humans (Hansen et al., 1997; Khan et al., 2016; Lippi et al., 2010;

van Oostrom et al., 2003). As another consequence of the successful establishment of the domestic pig as model species in chronoimmunology research described in the present thesis, future investigations might concentrate on circadian disruption and light pollution and their effects on porcine immune function and health, e.g., by use of 'chronic phase shift' or 'dim light at night' experiments (Castanon-Cervantes et al., 2010; Lunn et al., 2017). In addition, the importance of diurnal variations in the porcine immune system on disease susceptibility, e.g., on the occurrence of infections or allergies (Edgar et al., 2016; Nakao et al., 2015), might be investigated. Moreover, there is evidence of a bidirectional relationship between the immune system and central as well as peripheral timing mechanisms (Cavadini et al., 2007; Coogan & Wyse, 2008; Haimovich et al., 2010), which need further clarification whereby the diurnally active pig could also serve as suitable model. Moreover, as stress-induced glucocorticoids can affect circadian timing of peripheral clocks (Koch et al., 2017; Spencer et al., 2018; Tahara et al., 2015), stressor exposure at different times of the day, even in different settings examining acute or chronic stressor exposure, should be investigated in the porcine species to be able to derive consequences on diurnal immune regulation, function, and health in diurnally active species. Remarkably, to prove intrinsic circadian rhythmicity in the porcine immune system, experiments under constant conditions without zeitgeber exposure are necessary.

As the domestic pig is a long-lived and moderately seasonal species (Chemineau et al., 2007), it would also be an appropriate model for research on seasonal or circannual rhythms. In this respect, based on the results of the present thesis, it might be interesting to investigate diurnal rhythms in the porcine immune system in different seasons throughout the year, possibly even under different photoperiods in parallel, in order to dissect endogenous circannual from direct photoperiodic effects.

To further elucidate underlying mechanisms of diurnal rhythms in the immune system of domestic pigs, it might be essential to investigate a broader number of potentially influencing variables. First of all, melatonin might be examined in additional studies as this hormone, secreted during darkness, is regarded important to transmit photoperiodic information and known to influence the immune system (Carrillo-Vico et al., 2005; Zawilska et al., 2009). In addition, other hormones should be relevant for diurnal immune regulation in the domestic pig, such as catecholamines, in particular in regard to neutrophil trafficking as discussed above, aldosterone, prolactin, and growth hormone (Besedovsky et al., 2014c; Dimitrov et al., 2009; Kelley et al., 2007). Furthermore, as already described, the influence

of sex hormones on diurnal and seasonal or photoperiodic differences in the porcine immune system might be investigated with gonad-intact male and female pigs (Bereshchenko et al., 2018; Da Silva, 1999). In this respect, to elucidate signaling mechanisms of these different hormones, an investigation of the specific receptors would also be interesting. Thereby, the next obvious step is an assessment of diurnal differences in GR number and affinity in porcine leukocytes. Beyond that, it might be interesting to investigate the GR in specific leukocyte subpopulations with flow cytometry-based methods using specific antibodies or even fluorescence-stained dexamethasone (Dimitrov et al., 2009; Kowalik et al., 2013). Furthermore, clock gene expression in porcine immune cells is another field of research that could enlighten underlying mechanisms of diurnal immune regulation (Bollinger et al., 2011; Hopwood et al., 2018). In addition, behavioral and central nervous aspects of diurnal immune regulation should be taken into account. An important aspect of diurnal immune regulation in this regard is sleep (Besedovsky et al., 2012b; Besedovsky et al., 2017; Lange et al., 2010). However, its role in immune function of domestic pigs is not elucidated yet and needs further clarification. In addition, central circadian organization in the porcine species is not unraveled until now, so that investigation of spatiotemporal organization of the porcine SCN also might be beneficial for our understanding of diurnal rhythms in this species, also with regard to neural or humoral pathways potentially conveying greater phase distribution under LD than SD to immune rhythms (Brown & Piggins, 2009; Buijink et al., 2016).

Under practical aspects in animal husbandry, it is particularly interesting if time-controlled management measures could improve animal health and welfare. As the potential influences of diurnal and seasonal immune regulation on health in domestic pigs and other livestock species could not be answered yet, it is necessary to conduct further research in this regard. These issues should be investigated in laboratory settings at first and could later be transmitted to larger scales using field studies, e.g., to investigate different lighting and feeding schedules, different times of vaccination and medication, or diurnal differences in exposure to potential stressors in respect of diurnal immune function and health.

3.6 Conclusion

The present thesis revealed for the first time diurnal and photoperiodic effects on peripheral leukocyte numbers in various immune cell populations as well as glucocorticoid signaling in domestic pigs and uncovered potential underlying endocrine, behavioral, and molecular

mechanisms of these effects with experimental and statistical methods. Diurnal rhythms in cell numbers of the investigated immune cell populations in porcine blood mainly resembled results from humans and nocturnal rodents, with the remarkable exception of neutrophils. Furthermore, the investigated diurnal rhythms in activity behavior and plasma cortisol concentration confirmed that domestic pigs under the applied experimental conditions are diurnally active like humans. Linear mixed model analyses revealed associations of peripheral immune cell numbers with plasma cortisol concentration and activity behavior, which also resembles results from humans and rodents. In addition, the first study on photoperiodic effects on diurnal rhythms in peripheral immune cell numbers in any species conducted within the present thesis project revealed distinct photoperiodic differences in relative amplitudes and peak times of cell numbers in various leukocyte types, whereas mesor values did not differ. Moreover, these results imply stronger rhythmicity in general under SD than under LD. Thereby, common intrinsic mechanisms seem to regulate diurnal rhythms in peripheral immune cell numbers, except for neutrophils again. Finally, to investigate potential differences in diurnal regulation between neutrophils and the other investigated immune cell types, the molecular basis of glucocorticoid signaling was investigated in different leukocyte populations. Thereby, a greater number of GR sites per cell and a higher GR binding affinity in PBMC compared to granulocytes were found in the domestic pigs, pointing to differences in the molecular mechanisms of glucocorticoid signaling.

This means that the immune system and glucocorticoid signaling in domestic pigs are tightly regulated by light. In this regard, specific differences in regulation by light and underlying molecular mechanisms between leukocyte populations were demonstrated. Thus, the results of the present thesis contribute to a better understanding of health consequences caused by circadian and seasonal disruption due to our modern life. In this regard, the present thesis project fulfilled the need for a wider range of animal models in chronoimmunology research by establishing the diurnally active, moderately seasonal, and long-lived domestic pig as suitable large animal model. As a consequence, the domestic pig provides the opportunity to further elucidate the causes and underlying mechanisms of diurnal and seasonal immune function and health in clinical settings and in agriculture. Thereby, future studies might investigate diurnal differences in immune function, clarify the role of different zeitgebers on immune rhythms, and assess potential consequences of stressor exposure at different times of the day on the porcine immune system.

3.7 References

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4 SUMMARY

Physiology and behavior of humans and animals display pronounced diurnal and seasonal rhythmic variations. Diurnal rhythms are controlled by daylight and seasonal rhythms are adjusted by the photoperiod, i.e., the relative span of light per day. Modern human life and housing conditions of livestock are often directed against natural daylight conditions and thus, may entail circadian disruption causing misalignment between the central circadian pacemaker and peripheral tissues. Thereby, glucocorticoids are regarded as a main link between these compartments. Circadian disruption might be detrimental for health, in particular affecting immune function, which was mainly investigated in humans and nocturnal rodents but underlying mechanisms are not clearly defined yet.

Therefore, the main objective of the present thesis was to investigate diurnal and photoperiodic effects on the immune system and glucocorticoid signaling as well as potential underlying endocrine, behavioral, and molecular mechanisms of these effects in domestic pigs. Adult male castrated pigs, held under specific lighting schedules, were surgically catheterized to enable blood collection without disturbance of the animals.

Initially, domestic pigs were held under standard 12L:12D-lighting conditions and blood samples were taken every 2 hours over periods of up to 50 hours. Cosinor analyses revealed pronounced diurnal rhythmicity in peripheral leukocyte numbers of various immune cell populations. These rhythms were mainly comparable to results in humans and nocturnal rodents in relation to their respective rest-activity cycles, with the exception of porcine neutrophils differing from both species. Moreover, the investigated diurnal rhythms in activity behavior and plasma cortisol concentration confirmed that domestic pigs under the applied experimental conditions are diurnally active like humans. Linear mixed model analyses revealed associations of immune cell counts with plasma cortisol concentration, which also resembles results from humans and rodents.

Subsequently, photoperiodic effects on diurnal rhythms in peripheral immune cell numbers were investigated for the first time in any species. Domestic pigs were held either under long day conditions (LD) or under short day conditions (SD) and were sampled every 2 hours over periods of 50 hours. Distinct photoperiodic differences in relative amplitudes and peak times of cell counts in various porcine leukocyte types were found, whereas mesor values did not differ. Moreover, photoperiodic effects on diurnal rhythms in plasma cortisol

concentrations and activity behavior were found, which is in agreement with human and primate studies. Generalized linear mixed model analyses again revealed associations of leukocyte counts with plasma cortisol concentration and with activity behavior as well. In summary, the results imply stronger rhythmicity of peripheral immune cell numbers in general under SD than under LD. Common intrinsic mechanisms seem to regulate diurnal rhythms in peripheral leukocyte numbers in most immune cell types in domestic pigs, except for neutrophils again.

Finally, to investigate potential molecular differences in diurnal regulation between different immune cell types, glucocorticoid receptor (GR) number and affinity were examined in peripheral blood mononuclear cells (PBMC) and granulocytes of domestic pigs. Thereby, a greater number of GR sites per cell and a higher GR binding affinity in PBMC compared to granulocytes were found, pointing to differences in the molecular mechanisms of glucocorticoid signaling between leukocyte populations.

The results of the present thesis project are subsequently discussed in regard to specific implications for immune function and health as well as animal husbandry and welfare. Moreover, a methodological assessment of the approaches used within the thesis project was carried out and finally, suggestions for future research directions were given.

In conclusion, the present thesis revealed for the first time diurnal and photoperiodic effects on the immune system as well as glucocorticoid signaling in domestic pigs and uncovered potential underlying mechanisms of these effects. Hence, an additional diurnally active model species in chronoimmunology research was established. Moreover, conducting chronoimmunology research in the porcine species represents an innovative approach in agricultural science and provides entirely new opportunities to improve animal health and welfare. Thereby, future studies might investigate diurnal differences in immune function, clarify the role of different zeitgebers on immune rhythms, and assess potential consequences of stressor exposure at different times of the day.

5 ZUSAMMENFASSUNG

Physiologie und Verhalten von Menschen und Tieren unterliegen deutlich ausgeprägten diurnalen und saisonalen Schwankungen. Dabei werden diurnale Rhythmen über das Tageslicht und saisonale Rhythmen über die Photoperiode, also die Tageslänge, gesteuert. Unser modernes Leben und die Haltungsumwelt von Nutztieren stehen oft nicht im Einklang mit natürlichen Lichtbedingungen. Dadurch kann eine Entkopplung zwischen der sog. Inneren Uhr im Gehirn und peripheren Uhren in anderen Geweben entstehen. Glucocorticoide spielen dabei als Bindeglied eine wichtige Rolle. Diese Desynchronisation kann sich negativ auf die Gesundheit auswirken, besonders auf die Immunfunktion. Dies wurde bisher jedoch nur beim Menschen und bei nachtaktiven Nagern untersucht und mögliche Zusammenhänge sind bis jetzt nur unzureichend aufgeklärt.

Das Ziel der vorliegenden Arbeit war daher, diurnale und photoperiodische Einflüsse auf das Immunsystem und auf Glucocorticoide sowie zugrundeliegende endokrine, verhaltensbasierte und molekulare Wirkungsmechanismen beim Schwein zu untersuchen. Dafür wurden adulte Kastraten unter spezifischen Lichtbedingungen gehalten und die Blutprobenahme erfolgte über venöse Dauerkatheter, um die Tiere nicht zu stören.

Zunächst wurden Schweine unter standardisierten 12L:12D-Lichtbedingungen gehalten und alle 2 Std. über einen Zeitraum von bis zu 50 Std. Blutproben entnommen. Dabei wurden deutlich ausgeprägte diurnale Rhythmen in der Zellzahl verschiedener Leukozyten-Subpopulationen im peripheren Blut mittels Cosinoranalyse charakterisiert. Die Rhythmen waren zumeist vergleichbar mit Ergebnissen bei Menschen und Nagern im Verhältnis zum Aktivitätsrhythmus der jeweiligen Spezies. Eine Ausnahme stellten jedoch die neutrophilen Granulozyten dar, denn ihr Rhythmus war mit keiner der beiden genannten Spezies vergleichbar. Des Weiteren wurde durch die Untersuchung diurnaler Rhythmen im Aktivitätsverhalten und der Plasma-Cortisolkonzentration bestätigt, dass Schweine unter diesen experimentellen Bedingungen tagaktiv sind, also vergleichbar zum Menschen. Statistische Analysen zeigten Zusammenhänge zwischen der Immunzellzahl im Blut und der Plasma-Cortisolkonzentration. Dies bestätigt Ergebnisse von Menschen und Nagern.

Anschließend wurden – als weltweit erste Untersuchung überhaupt – photoperiodische Effekte auf diurnale Rhythmen in der Immunzellzahl im peripheren Blut untersucht. Dazu wurden Schweine entweder unter Langtag- (LD) oder Kurztagbedingungen (SD) gehalten

und alle 2 Std. über eine Dauer von 50 Std. beprobt. Dabei wurden bei verschiedenen Leukozyten-Subpopulationen deutliche Unterschiede in der relativen Amplitude und der Acrophase der Zellzahlen im peripheren Blut gefunden. Die Mesor-Werte unterschieden sich jedoch nicht. Des Weiteren wurden auch photoperiodische Effekte auf diurnale Rhythmen in der Plasma-Cortisolkonzentration und dem relativen Aktivitätsverhalten gezeigt, was mit bisherigen Studien bei Menschen und Primaten übereinstimmt. Statistische Analysen zeigten auch hier Zusammenhänge zwischen der Immunzellzahl im Blut und der Plasma-Cortisolkonzentration sowie dem Aktivitätsverhalten. Darüber hinaus deuten die Ergebnisse eine generell stärker ausgeprägte Oszillation der Immunzellzahlen im Blut unter SD als unter LD an. Ferner scheinen die diurnalen Rhythmen der Immunzellzahl im Blut bei den meisten porcinen Immunzelltypen über gemeinsame intrinsische Mechanismen reguliert zu werden, wobei die neutrophilen Granulozyten wiederum eine Ausnahme bilden.

Schließlich wurden zur Aufklärung möglicher molekularer Unterschiede in der diurnalen Regulation von Immunzellen, Anzahl und Affinität von Glucorticoidrezeptoren (GR) in peripheren mononukleären Zellen des peripheren Blutes (PBMC) und Granulozyten beim Schwein untersucht. Dabei wurde eine größere Anzahl und eine höhere Affinität der GR in PBMC im Vergleich zu Granulozyten festgestellt. Dies lässt auf Unterschiede in den molekularen Wirkungsmechanismen von Glucocorticoiden bei verschiedenen Leukozyten-Subpopulationen schließen.

Zusammenfassend werden die Ergebnisse der vorliegenden Arbeit im Hinblick auf ihre Bedeutung für die Immunfunktion und Gesundheit als auch für die Tierhaltung und das Wohlergehen der Tiere diskutiert. Zusätzlich wird die Arbeit umfassend methodologisch bewertet und es werden weiterführende Forschungsmöglichkeiten eröffnet.

In der vorliegenden Arbeit wurden erstmals diurnale und photoperiodische Einflüsse auf das Immunsystem sowie auf Glucocorticoide beim Schwein gezeigt und mögliche zugrundeliegende Wirkungsmechanismen charakterisiert. Damit wurde das Schwein als tagaktive Modellspezies in der chronoimmunologischen Forschung eingeführt. Darüber hinaus stellt diese Arbeit einen innovativen Ansatz in den Agrarwissenschaften dar und bietet somit ganz neue Möglichkeiten, um Gesundheit und Wohlergehen bei Nutztieren zu fördern. Zukünftige Studien könnten diurnale Unterschiede in der Immunfunktion untersuchen, die Rolle verschiedener Zeitgeber-Mechanismen im Immunsystem aufklären und mögliche Konsequenzen einer Stressoren-Einwirkung zu unterschiedlichen Tageszeiten erforschen.

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LIST OF PUBLICATIONS

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