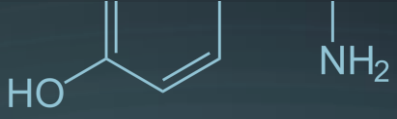
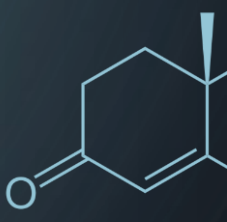
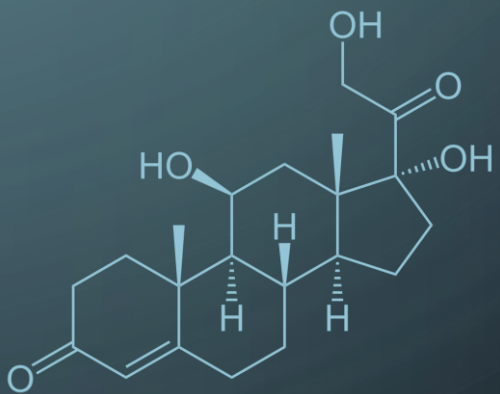
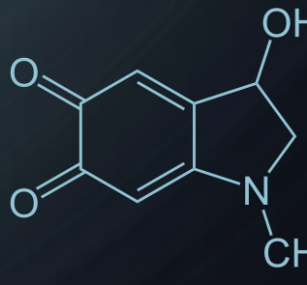


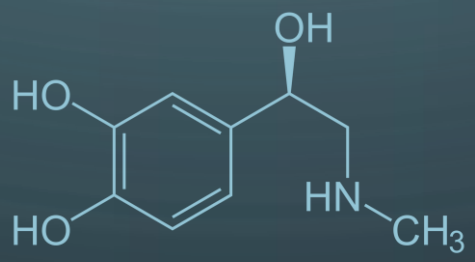
Stress hormone-induced immunomodulation and interplay between immune cells and bacteria in response to stress hormones in domestic pigs

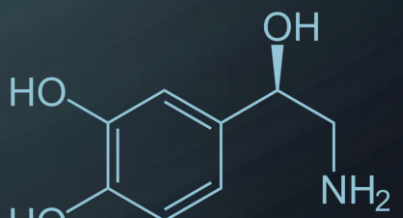
Lena Reiske











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Stress hormone-induced immunomodulation and interplay between immune cells and bacteria in response to stress hormones in domestic pigs

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3. Prüfer:	Prof. Dr. Ludwig E. Hölzle

FÜR PAPA

But then science is nothing but a series of questions that lead to more questions, which is just as well, or it wouldn't be much of a career path, would it?

Terry Pratchett

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AC	Adrenochrome
ACTH	Adrenocorticotropic hormone
ADR	Adrenaline
AHL	N-Acyl homoserine lactone
Ag.-exp.	Antigen-experienced
AI	Autoinducer
AP-1	Activator protein 1
APC	Antigen-presenting cell
AR	Adrenergic receptor
BW	Body weight
C/CORT	Cortisol
CA	Catecholamine
CD	Cluster of differentiation
CNS	Central nervous system
ConA	Concanavalin A
cpm	Counts per minute
CRF	Corticotropin-releasing factor
CTL	Cytotoxic T cell
CTRL	Control
CV	Coefficient of variance
DC	Dendritic cell
DHMA	3,4-dihydroxymandelic acid
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
K3 EDTA	Ethylenediaminetetraacetic acid tripotassium salt

LB	Lysogeny broth
LS-means	Least-square means
LSD	Least significant difference
ME	Metabolisable energy
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFAT	Nuclear factor of activated T-cells
NK cell	Natural killer cell
NQR	NADH:quinone oxidoreductase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein
PWM	Pokeweed mitogen
QS	Quorum sensing
REML	Restricted maximum likelihood
RIA	Radioimmunoassay
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
RT	Room temperature
S. Typhimurium	Salmonella enterica subspecies enterica serovar Typhimurium
SAM	Sympathetic-adrenal-medullary
SEM	Standard error of the mean
SNS	Sympathetic nervous system
SPRD	Spectral red
TCR	T cell receptor
T_H cell	T helper cell
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T cell
V. cholerae	Vibrio cholerae

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

Since the first description of a "general adaptation syndrome", nowadays known under the term "stress" by Hans Selye (1936), there has been extensive research regarding the biological mechanisms and mediators behind this phenomenon and its physiological and psychological consequences. Today, the definition of stress commonly includes the causal stimulus, called the stressor, the perception of the same by the central nervous system (CNS) and the successional physiologic reaction that is launched as a response to the stressor (Dhabhar and McEwen, 1997). Already in his pioneering publication, Selye described three phases, an acute phase, or "general alarm reaction", lasting from six to 48 hours, succeeded by a second stage where restrictions of physiologic functions, e.g. lactation and growth, occur, followed by a "resistance" of the animals. If stressor exposure continues over one to three months, the third stage is entered where resistance is lost and stress symptoms reoccur. Selye calls this the "phase of exhaustion". We now know that the physiologic reactions described here are caused by the release of so-called "stress hormones" upon activation of the sympathetic-adrenal-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis. After sensory information about a stressor reaches the CNS, the SAM axis is activated via the sympathetic nervous system, a part of the autonomic nervous system. Its preganglionic neurons leave the brain via the sympathetic trunk and sympathetic nerve fibres are spread universally throughout the body, including the adrenal gland (Elenkov et al., 2000). The adrenal medulla works as a modified sympathetic ganglion and releases the catecholamines (CAs) adrenaline (ADR) and noradrenaline (NA) into the blood stream upon activation by the preganglionic neuron (Silverthorn et al., 2016). In addition, NA is released directly from synaptic vesicles of postganglionic neurons into the different tissues since it serves as a neurotransmitter in almost all sympathetic nerve terminals. Due to this "hard-wiring" of the brain and the periphery, these processes take place within seconds after sensory perception of a stressor, whereas activation of the HPA axis takes a few minutes (Sapolsky et al., 2000). Here, the signalling process starts with the amygdala activating the neurons in the *paraventricular nucleus* of the hypothalamus (Herman et al., 2003), which react by secreting corticotropin-releasing factor (CRF) into the portal blood vessel system of the pituitary stalk. This network connects the hypothalamus with the posterior pituitary or neurohypophysis (Silverthorn et al., 2016). The pituitary reacts to CRF by secreting adrenocorticotropic hormone (ACTH) into the blood stream, which by this means reaches the cortex of the adrenal gland and

stimulates the biosynthesis of glucocorticoids (GCs). Originating from cholesterol, either cortisol (most mammals) or corticosterone (amphibians, reptiles, birds, rodents) is produced and released into the blood stream (Katsu and Iguchi, 2016). Almost all cells of the body express the GC receptor (GR) and most have at least one of the different CA receptors (Perez, 2006; Rosenfeld et al., 1988). Due to alternative splicing, there are several GR isoforms, which are all acting as a transcription factor and are therefore located intracellularly (Vandevyver et al., 2014). This mode of action causes further delay between the first perception of a stressor and the biological reaction to GCs, which can first be observed after about one hour (Sapolsky et al., 2000). Contrarily, adrenoceptors (ARs) are membrane-bound G protein-coupled receptors transducing the hormonal signal instantaneously into a cellular reaction upon CA binding by mechanisms involving phospholipase C or adenylyl cyclase. There are α1 and α2 as well as β ARs with three subclasses, respectively, whose differences in tissue distribution and ligand affinity are responsible for the multitude of possible CA effects (Perez, 2006; Strosberg, 1993). Both stress hormone classes can thus influence functions like glucose and lipid metabolism, blood pressure, lung ventilation, muscle perfusion, heart rate and many more to enable the body to react appropriately to the stressor (Antonelli et al., 2012; Ferrer-Lorente et al., 2005; Gordan et al., 2015; Jänig, 2006). Another important system immediately sensing and reacting to stress hormone secretion is the immune system (Elenkov et al., 2000; Sapolsky et al., 2000). The body's defence system against diseases, caused by e.g. pathogens or mutated body cells, consists of an innate and an adaptive arm, which are both further divided into a cellular and a humoral part (Murphy and Weaver, 2017). Both acute and chronic stress can influence the distribution and functionality as well as the lifespan of those different immune cell types. Generally, acute stress – which lasts minutes to hours and is mainly CA mediated – causes immune activation by enhancing both innate and adaptive immune responses, vaccine efficiency and anti-tumour immunity via leukocyte trafficking and cytokine secretion (Dhabhar, 2018). In the following phase, this increased immune reactivity is dampened by GC release to prevent overshooting inflammation (Dhabhar, 2018). If the stressful event continues or repeatedly recurs, stress can become chronic and cause detrimental outcomes like immunosuppression and dysregulation, resulting in increased susceptibility to infection and autoimmune reactions (Glaser and Kiecolt-Glaser, 2005). Independently of duration, also the individual coping strategy of an animal can result in a predominant activation of only one stress axis, especially in social stress scenarios (Koolhaas, 2008). Submissive animals with a reactive coping style often show signs of social defeat like passively crouching in corners and avoiding contact with dominant individuals accompanied by a marked increase of plasma GC

concentrations (Bohus et al., 1987; Henry, 1982; Holst, 1997; Veenema et al., 2005). Contrarily, subdominant animals with a proactive coping behaviour show increased activity, aggression and preparedness to fight combined with a predominant activation of the SAM axis (Koolhaas et al., 2007; Sgoifo et al., 1999). There is also some evidence for differential immune alterations depending on coping style and the endocrine responses relating thereto. A reactive coping style is for example associated with decreased lymphocyte proliferation or anti-tumour-immunity (Hardy et al., 1990; Vegas et al., 2006) while proactive animals show an upregulation of proinflammatory cytokines and reduced tumour growth (Kavelaars et al., 1999; Teunis et al., 2002). In pigs, a reactive coping style is associated with a shift from cellular to humoral immunity compared to proactive animals (Bolhuis et al., 2003; Hessing et al., 1994; Schrama et al., 1997).

In the last few decades, many studies focused on investigating the effects of different stress types (e.g. social, thermal or infectious) and stress durations (acute vs. chronic) as well as individual coping strategies (proactive vs. reactive) on the immune system and the underlying endocrine regulation. For a long time, the main focus lay on the anti-inflammatory effect of GCs, which can be used pharmacologically to treat allergies and autoimmune diseases (Coutinho and Chapman, 2011; Okano, 2009). After a natural elevation of blood GC concentrations, T and B lymphocyte numbers strongly decrease while neutrophil granulocyte numbers rise (Bilandzić et al., 2005; Engler et al., 2004; Zahorec, 2001). Functionally, GCs favour phagocytic functions of the innate immune system (Barriga et al., 2001; Forner et al., 1995; Ortega, 2003) and shift adaptive immunity from proinflammatory T helper (T_H) 1- to anti-inflammatory T_H2 responses (Almawi et al., 1999; Blotta et al., 1997; Elenkov, 2004; Engler et al., 2004; Gillis et al., 1979; Miyaura and Iwata, 2002).

When it comes to CAs, research has long focussed on their cardiovascular effects and the accompanying medical usefulness, while their impact on the immune system remains to be fully understood, especially in species other than laboratory rodents. Through binding to β2-ARs on immune and endothelial cells, CAs cause an elevation of monocyte, neutrophil granulocyte and natural killer (NK) cell numbers in the blood (Benschop et al., 1996; Dimitrov et al., 2010; Engler et al., 2004). These innate immune cells have phagocytic and cytotoxic functions and hence contribute to a rapid pathogen control as it may be necessary in a fight-or-flight situation with enhanced risk of injury and infection (Dhabhar, 2018; Dimitrov et al., 2010). Alongside with immune cell trafficking comes a modulation of different leukocyte functions through α- or β-AR binding. While it has been demonstrated that NK cell cytotoxicity is mostly hampered

via β2-ARs (Ben-Eliyahu et al., 2000; Rosenne et al., 2013; Shakhar and Ben-Eliyahu, 1998), especially T and B lymphocyte functionality can be either exacerbated or dampened, depending on AR ratio and extent of the CA elevation (Connor et al., 2005; Elenkov et al., 2000; Felsner et al., 1995; Hadden et al., 1970; Strahler et al., 2015).

But not only the tissues and cells of animals and humans are affected by the release of CAs. Due to the extensive distribution of noradrenergic nerve endings, NA can reach high local concentrations, accompanied by diffusion of the hormone over barriers to the outside world, like the epithelium of the oral cavity, intestine, lung or the skin (Eldrup and Richter, 2000; Furness, 2000; Purves and Williams, 2001). In stressful situations, CAs can also cross this border due to spillover from the blood circulation (Aneman et al., 1996; Purves and Williams, 2001). These niches are inhabited by – mostly commensal but also pathogenic – microbes and it comes as no surprise that many of them have evolved the ability to sense host CAs and other hormones (Lyte et al., 2011; Sandrini et al., 2015). It was even found that some bacterial species are able to produce CAs themselves (Asano et al., 2012; Malikina et al., 2010; Tsavkelova et al., 2000). NA can thus be used to gain iron, which is important for bacterial growth, as it forms complexes with the iron bound to transferrin, leading to its release (Miethke and Skerra, 2010; Sandrini et al., 2010; Schaible and Kaufmann, 2004). Additionally, many bacterial species are able to sense CAs by their quorum sensing (QS) systems (Clarke et al., 2006; Hegde et al., 2009; Sperandio et al., 2003). QS is a form of bacterial cell-to-cell communication through the secretion and sensing of microbial signal molecules, so-called autoinducers (Dyszel et al., 2010; Michael et al., 2001; Sun et al., 2004; Waters and Bassler, 2005). If this system is activated upon CA binding, it can lead to an increase of for example proliferation, motility or attachment to the epithelium and therefore also serves as a bacterial sensor for host stress, which is answered by increasing pathogenic traits (Bearson and Bearson, 2008; Freestone et al., 1999; Freestone et al., 2007; Halang et al., 2015; Lyte et al., 1997). Especially in the gut, where half of the entire NA amount of the body is located (Sandrini et al., 2015) and the microbial community is outstandingly big and diverse (Quigley, 2013), stress can thus have a substantial effect on the equilibrium of residing and invading bacteria and the risk of developing food-borne diseases like salmonellosis (Verbrugghe et al., 2012).

Salmonellosis is one of the most common causes of gastroenteritis globally and caused by bacteria of the *Salmonella* genus, most importantly by the serovars Typhimurium and Enteritidis of *Salmonella enterica ssp. enterica* (Hendriksen et al., 2011; Scallan et al., 2011). Since it is a zoonotic pathogen that can among others infect pigs and poultry, it is most

prevalently spread by eating contaminated meat or eggs (Boyen et al., 2008; Whiley and Ross, 2015). Especially porcine salmonellosis is difficult to eradicate since most pigs do not develop symptomatic infections or only mild symptoms, and therefore are usually not treated with antibiotics (Boyen et al., 2008; Helaine et al., 2014). Furthermore, *Salmonella* can persist chronically by hiding intracellularly in macrophages and lymphoid tissues (Eisele et al., 2013; Lathrop et al., 2015; Wood et al., 1989). In stressful situations, like transport to the slaughterhouse, those asymptomatic persisters get reactivated, leading to an increased shedding of the bacteria and increased meat contamination (Casanova-Higes et al., 2017; Verbrugghe et al., 2011; Verbrugghe et al., 2016). The mechanisms behind both stress-induced increase of primary infection and recrudescence of latent infections are far from being fully elucidated. Beside altered gut motility, mucus production and epithelial barrier function, CA sensing by *Salmonella* and a subsequent change in bacterial behaviour may be of crucial relevance (He et al., 2019; Konturek et al., 2011; Lyte et al., 2011).

Not only because of this zoonotic relationship between pigs and humans but also due to the many biological similarities between these species, the domestic pig represents a valuable animal model to take a closer look at the interplay of stress, the immune system and bacteria. To begin with, there are many anatomical consistencies: pigs have a similar size and body weight and the inner organs resemble the size of those of humans more closely than those of mice (Swindle et al., 2012; Tumbleson, 1986). Also, regarding the anatomy of immune organs, the pig resembles in many aspects the situation in humans, like for example the arrangement of lymphatic tissue in the nasopharynx (Horter et al., 2003), though there are also differences, most apparent in the inverse architecture of porcine lymph nodes (Gerdts et al., 2015). In terms of immune cell numbers and functionality, the porcine immune system shows more similarities to humans in more than 80% of analysed parameters whereas the murine immune system was only closer to that of humans in less than 10% (Dawson, 2012; Fairbairn et al., 2011; Meurens et al., 2012). The stress axes that impact immune functionality are also very similar between pigs and humans regarding the preferred GC (cortisol vs. corticosterone) and GC sensitivity as well as diurnal rhythmicity (Engert et al., 2018; Kanitz et al., 1999; Roth and Flaming, 1990; Ruis et al., 1997). Regarding the suitability of the pig as a model for gastrointestinal infections, it is also beneficial that both humans and pigs are omnivores with a correspondingly structured gastrointestinal tract (Heinritz et al., 2013; Roura et al., 2016; Zhang et al., 2013). As a practical issue, the pig's size and lifespan makes it possible to catheterize veins for repeated blood sampling over long periods of time.

In addition to being an excellent model for research in psychoneuroimmunology and infection immunology, the pig is interesting to study in its function as one of the most important farm animals. During the complete production cycle, pigs are repeatedly exposed to stress and risk of infection. Beginning from weaning at the age of three to four weeks and until slaughter at about six months, stressors like separation from the dam, regrouping, space limitation, transportation and changes in diet and temperature are common (Kick et al., 2011; von Borell, 2001). Previous studies have examined some of those stressors and their impact on the immune system. A decrease of lymphocytes and increase of neutrophils in the blood, resulting in a shift from adaptive to innate immunity, is a consistent finding over different stressors and age groups (Krebs and McGlone, 2009; McGlone et al., 1993; Salak-Johnson et al., 1996; Sutherland et al., 2009). Functionally, a lower lymphocyte proliferation and TNFα production but also an increased NK cell cytotoxicity and antibody response could be observed (Deguchi and Akuzawa, 1998; Grün et al., 2014; Hicks et al., 1998; Kanitz et al., 2004; Rudine et al., 2007; Tuchscherer et al., 2009). However, most studies did not measure plasma stress hormone concentrations and it can be assumed that most investigated stressors activate both HPA and SAM axis, making it impossible to discern GC and CA effects. Though few studies have examined the impact of GCs alone (Lo et al., 2005; Schwarz et al., 2005; Tuchscherer et al., 2016; Westly and Kelley, 1984), they have either used pharmacological doses or did not include important functional parameters and leukocyte subsets. The specific impact of CAs, however, has not been investigated at all in pigs. Studying the separate effects of cortisol, adrenaline and noradrenaline on porcine immune cell numbers and functions can thus contribute to basic science and help better understand and prevent stress-induced immunomodulation in livestock husbandry. Furthermore, to investigate the interplay of porcine immune cells and *Salmonella* under the influence of stress hormones has the potential to improve infection control, thus serving both animal welfare and public health.

1.1 Main research objectives and methodical approach

The main objective of the present doctoral thesis was to investigate the separate effects of cortisol, adrenaline and noradrenaline on the numbers of blood immune cell subsets and functionality of both innate and adaptive immunity in domestic pigs. As a second focus, the impact of catecholamine-treated *Salmonella* Typhimurium cultures on porcine immune cell functionality was assessed to contribute to a better understanding of a stress-related increased risk of infection. To address these topics, *in vitro* and *in vivo* experiments were designed,

resulting in three separate studies that are described in detail in the manuscripts included in this thesis. In general, male castrated fattening pigs, hybrids of the commercial breeds German Landrace and Pietrain, were used as experimental animals. All animals were surgically equipped with indwelling vein catheters (Kraetzl and Weiler, 1998) to enable blood sampling without endogenous stress-hormone release and to allow intravenous stress-hormone infusion. Analysis of the blood samples was performed using an automated haematological analyser and flow cytometry after staining with immunofluorescent monoclonal antibodies to delineate various immune cell subsets. For determination of plasma catecholamine concentrations, high performance liquid chromatography (HPLC) was used and cortisol was determined via radioimmunoassay (RIA). Functional assays included determination of plasma antibody concentrations via enzyme-linked immunosorbent assay (ELISA), flow cytometry-based analysis of phagocytosis and cytokine production and determination of lymphocyte proliferation was done measuring mitogen-induced uptake of tritiated thymidine. Differences between treatments were assessed statistically using linear mixed model analysis.

1.2 Overview of the included manuscripts

MANUSCRIPT I

Glucocorticoids and Catecholamines Affect in Vitro Functionality of Porcine Blood Immune Cells

Published in *Animals* **9**, 545 (2019)

Since information about cortisol impacts on porcine immune cell functionality is incomplete and the effects of catecholamines have not been investigated at all in pigs, the first study was designed as an *in vitro* experiment. The primary objective was to evaluate the effects of different doses of cortisol, adrenaline and noradrenaline on important porcine immune functions in a well-controlled environment and thus establish a basis for later *in vivo* investigations. In total, 32 barrows served as blood donors for *in vitro* testing. Pigs were individually penned and held under standard experimental conditions with twelve hours of light per day and concentrate feeding twice daily, with ad libitum access to hay and water. Blood was collected after feeding in the morning, followed by separation of peripheral blood mononuclear cells (PBMC). Upon addition of a wide range of concentrations of cortisol, adrenaline or noradrenaline, lymphocyte proliferation was determined via a ^{3}H-thymidine assay and the number of TNFα/IFNγ

producing immune cell subsets were assessed flow cytometrically by intracellular staining of the cytokines. Differences between treatments were verified by linear mixed model analysis.

MANUSCRIPT II

Intravenous Infusion of Cortisol, Adrenaline, or Noradrenaline Alters Porcine Immune Cell Numbers and Promotes Innate over Adaptive Immune Functionality

Published in *The Journal of Immunology* **204** (12), 3205-3216 (2020)

The aim of this study was to investigate the effects of elevated blood levels of one stress hormone at a time on both immune cell numbers and functionality in pigs. The 34 experimental animals were housed in individual pens with 14 hours light per day and standard feeding as in the first experiment. For this experiment, both cephalic veins were surgically cannulated to enable blood sampling alongside to infusion, which was carried out by automated infusion pumps. After an initial control phase, where all pigs received saline, the animals were infused with either cortisol, adrenaline, noradrenaline or saline for 48 hours. Stress hormones were applied in concentrations leading to plasma levels comparable to those occurring under mild stress. For the first time, the numbers of different leukocyte subsets were described in this detail by flow cytometric methods. Furthermore, lymphocyte proliferation, plasma antibody concentrations and number and activity of phagocytic cells were assessed, giving a valuable overview of the porcine immune system under the influence of a single stress hormone. This study was able to fill knowledge gaps about the effects of physiologically elevated cortisol concentrations and is the first report at all concerning particular adrenaline and noradrenaline impacts on the porcine immune system *in vivo*. Statistical differences between the treatments at different time points during and after infusion were proved with linear mixed models.

MANUSCRIPT III

Interkingdom Cross-Talk in Times of Stress: *Salmonella* Typhimurium Grown in the Presence of Catecholamines Inhibits Porcine Immune Functionality *in vitro*

Published in *Frontiers in Immunology* **11**: 572056 (2020)

After establishment of an *in vitro* model to assess porcine immune functionality upon addition of different substances in the first experiment, the objective of this study was to go one step further and assess the effects of catecholamine-treated *Salmonella* Typhimurium cultures on

porcine leukocytes. In total, 18 barrows were housed in single pens under standard conditions with 14 hours of light per day. The experimental design was chosen analogous to that of the first study, but this time cells were treated with supernatants from *S*. Typhimurium grown upon addition of adrenaline, noradrenaline or the adrenaline oxidation product adrenochrome. This is the first study to demonstrate effects of stress hormone-treated bacteria on mammalian immune cells, thus adding a new dimension to interkingdom-signalling. Differences between the supernatants were shown with linear mixed models.

1.3 References

Almawi, W.Y., Melemedjian, O.K., Rieder, M.J., 1999. An alternate mechanism of glucocorticoid anti-proliferative effect: promotion of a Th2 cytokine-secreting profile. Clinical Transplantation 13 (5), 365–374.

Aneman, A., Eisenhofer, G., Olbe, L., Dalenbäck, J., Nitescu, P., Fändriks, L., Friberg, P., 1996. Sympathetic discharge to mesenteric organs and the liver. Evidence for substantial mesenteric organ norepinephrine spillover. Journal of Clinical Investigation 97 (7), 1640–1646.

Antonelli, A., Torchio, R., Bertolaccini, L., Terzi, A., Rolfo, F., Agostoni, P., Gulotta, C., Brusasco, V., Pellegrino, R., 2012. Contribution of β-adrenergic receptors to exercise-induced bronchodilatation in healthy humans. Respiratory Physiology & Neurobiology 184 (1), 55–59.

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., Koga, Y., Sudo, N., 2012. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. American Journal of Physiology. Gastrointestinal and Liver Physiology 303 (11), G1288-95.

Barriga, C., Martín, M.I., Tabla, R., Ortega, E., Rodríguez, A.B., 2001. Circadian rhythm of melatonin, corticosterone and phagocytosis: effect of stress. Journal of Pineal Research 30 (3), 180–187.

Bearson, B.L., Bearson, S.M.D., 2008. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enterica* serovar Typhimurium. Microbial Pathogenesis 44 (4), 271–278.

Ben-Eliyahu, S., Shakhar, G., Page, G.G., Stefanski, V., Shakhar, K., 2000. Suppression of NK cell activity and of resistance to metastasis by stress: A role for adrenal catecholamines and β-adrenoceptors. Neuroimmunomodulation 8 (3), 154–164.

Benschop, R.J., Rodriguez-Feuerhahn, M., Schedlowski, M., 1996. Catecholamine-induced leukocytosis: early observations, current research, and future directions. Brain, Behavior, and Immunity 10 (2), 77–91.

Bilandzić, N., Simić, B., Zurić, M., Lojkić, M., 2005. Effect of ACTH administration on biochemical and immune measures in boars. Journal of Veterinary Medicine Series A 52 (9), 440–446.

Blotta, M.H., DeKruyff, R.H., Umetsu, D.T., 1997. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. Journal of Immunology 158 (12), 5589–5595.

Bohus, B., Benus, R.F., Fokkema, D.S., Koolhaas, J.M., Nyakas, C., van Oortmerssen, G.A., Prins, A.J., Ruiter, A.J. de, Scheurink, A.J., Steffens, A.B., 1987. Neuroendocrine states and behavioral and physiological stress responses. Progress in Brain Research 72, 57–70.

Bolhuis, J.E., Parmentier, H.K., Schouten, W.G.P., Schrama, J.W., Wiegant, V.M., 2003. Effects of housing and individual coping characteristics on immune responses of pigs. Physiology & Behavior 79 (2), 289–296.

Boyen, F., Haesebrouck, F., Maes, D., van Immerseel, F., Ducatelle, R., Pasmans, F., 2008. Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. Veterinary Microbiology 130 (1-2), 1–19.

Casanova-Higes, A., Andres-Barranco, S., Mainar-Jaime, R.C., 2017. Influence of on-farm pig *Salmonella* status on *Salmonella* shedding at slaughter. Zoonoses and Public Health 64 (5), 328–336.

Clarke, M.B., Hughes, D.T., Zhu, C., Boedeker, E.C., Sperandio, V., 2006. The QseC sensor kinase: a bacterial adrenergic receptor. Proceedings of the National Academy of Sciences of the United States of America 103 (27), 10420–10425.

Connor, T.J., Brewer, C., Kelly, J.P., Harkin, A., 2005. Acute stress suppresses pro-inflammatory cytokines TNF-alpha and IL-1 beta independent of a catecholamine-driven increase in IL-10 production. Journal of Neuroimmunology 159, 119–128.

Coutinho, A.E., Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Molecular and Cellular Endocrinology 335 (1), 2–13.

Dawson, H., 2012. A Comparative assessment of the pig, mouse and human genomes, in: McAnulty, P.A. (Ed.), The minipig in biomedical research, vol. 166. CRC Press/Taylor & Francis, Boca Raton, pp. 323–342.

Deguchi, E., Akuzawa, M., 1998. Effects of fighting after grouping on plasma cortisol concentration and lymphocyte blastogenesis of peripheral blood mononuclear cells induced by mitogens in piglets. The Journal of Veterinary Medical Science 60, 149–153.

Dhabhar, F.S., 2018. The short-term stress response - Mother Nature's mechanism for enhancing protection and performance under conditions of threat, challenge, and opportunity. Frontiers in Neuroendocrinology 49, 175–192.

Dhabhar, F.S., McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: A potential role for leukocyte trafficking. Brain, Behavior, and Immunity 11 (4), 286–306.

Dimitrov, S., Lange, T., Born, J., 2010. Selective mobilization of cytotoxic leukocytes by epinephrine. Journal of Immunology 184 (1), 503–511.

Dyszel, J.L., Smith, J.N., Lucas, D.E., Soares, J.A., Swearingen, M.C., Vross, M.A., Young, G.M., Ahmer, B.M.M., 2010. *Salmonella enterica* serovar Typhimurium can detect acyl homoserine lactone production by *Yersinia enterocolitica* in mice. Journal of Bacteriology 192 (1), 29–37.

Eisele, N.A., Ruby, T., Jacobson, A., Manzanillo, P.S., Cox, J.S., Lam, L., Mukundan, L., Chawla, A., Monack, D.M., 2013. *Salmonella* require the fatty acid regulator PPARdelta for the establishment of a metabolic environment essential for long-term persistence. Cell Host & Microbe 14 (2), 171–182.

Eldrup, E., Richter, E.A., 2000. DOPA, dopamine, and DOPAC concentrations in the rat gastrointestinal tract decrease during fasting. American Journal of Physiology. Endocrinology and Metabolism 279 (4), E815-22.

Elenkov, I.J., 2004. Glucocorticoids and the Th1/Th2 balance. Annals of the New York Academy of Sciences 1024, 138–146.

Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve - An integrative interface between two supersystems: The brain and the immune system. Pharmacological Reviews, 595–638.

Engert, L.C., Weiler, U., Pfaffinger, B., Stefanski, V., Schmucker, S.S., 2018. Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs. Developmental and Comparative Immunology 79, 11–20.

Engler, H., Dawils, L., Hoves, S., Kurth, S., Stevenson, J.R., Schauenstein, K., Stefanski, V., 2004. Effects of social stress on blood leukocyte distribution: The role of alpha- and beta-adrenergic mechanisms. Journal of Neuroimmunology 156 (1-2), 153–162.

Fairbairn, L., Kapetanovic, R., Sester, D.P., Hume, D.A., 2011. The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. Journal of Leukocyte Biology 89 (6), 855–871.

Felsner, P., Hofer, D., Rinner, I., Porta, S., Korsatko, W., Schauenstein, K., 1995. Adrenergic suppression of peripheral blood T cell reactivity in the rat is due to activation of peripheral alpha 2-receptors. Journal of Neuroimmunology 57 (1-2), 27–34.

Ferrer-Lorente, R., Cabot, C., Fernández-López, J.-A., Alemany, M., 2005. Combined effects of oleoyl-estrone and a beta3-adrenergic agonist (CL316,243) on lipid stores of diet-induced overweight male Wistar rats. Life Sciences 77 (16), 2051–2058.

Forner, M.A., Barriga, C., Rodriguez, A.B., Ortega, E., 1995. A study of the role of corticosterone as a mediator in exercise-induced stimulation of murine macrophage phagocytosis. The Journal of Physiology 488 (Pt 3), 789–794.

Freestone, P.P., Haigh, R.D., Williams, P.H., Lyte, M., 1999. Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. FEMS Microbiology Letters 172 (1), 53–60.

Freestone, P.P.E., Haigh, R.D., Lyte, M., 2007. Specificity of catecholamine-induced growth in *Escherichia coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica*. FEMS Microbiology Letters 269 (2), 221–228.

Furness, J.B., 2000. Types of neurons in the enteric nervous system. Journal of the Autonomic Nervous System 81 (1-3), 87–96.

Gerdts, V., Wilson, H.L., Meurens, F., van Drunen Littel-van den Hurk, S., Wilson, D., Walker, S., Wheler, C., Townsend, H., Potter, A.A., 2015. Large animal models for vaccine development and testing. ILAR Journal 56 (1), 53–62.

Gillis, S., Crabtree, G.R., Smith, K.A., 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. Journal of Immunology 123, 1624–1631.

Glaser, R., Kiecolt-Glaser, J.K., 2005. Stress-induced immune dysfunction: implications for health. Nature Reviews Immunology 5 (3), 243.

Gordan, R., Gwathmey, J.K., Xie, L.-H., 2015. Autonomic and endocrine control of cardiovascular function. World Journal of Cardiology 7 (4), 204–214.

Grün, V., Schmucker, S., Schalk, C., Flauger, B., Stefanski, V., 2014. Characterization of the adaptive immune response following immunization in pregnant sows (*Sus scrofa*) kept in two different housing systems. Journal of Animal Science 92 (8), 3388–3397.

Hadden, J.W., Hadden, E.M., Middleton, E., 1970. Lymphocyte blast transformation. I. Demonstration of adrenergic receptors in human peripheral lymphocytes. Cellular Immunology 1, 583–595.

Halang, P., Toulouse, C., Geißel, B., Michel, B., Flauger, B., Müller, M., Voegele, R.T., Stefanski, V., Steuber, J., 2015. Response of *Vibrio cholerae* to the catecholamine hormones epinephrine and norepinephrine. Journal of Bacteriology 197 (24), 3769–3778.

Hardy, C.-A., Quay, J., Livnat, S., Ader, R., 1990. Altered T-lymphocyte response following aggressive encounters in mice. Physiology & Behavior 47 (6), 1245–1251.

He, J., Guo, H., Zheng, W., Yao, W., 2019. Effects of stress on the mucus-microbial interactions in the gut. Current Protein & Peptide Science 20 (2), 155–163.

Hegde, M., Wood, T.K., Jayaraman, A., 2009. The neuroendocrine hormone norepinephrine increases *Pseudomonas aeruginosa* PA14 virulence through the las quorum-sensing pathway. Applied Microbiology and Biotechnology 84 (4), 763–776.

Heinritz, S.N., Mosenthin, R., Weiss, E., 2013. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutrition Research Reviews 26 (2), 191–209.

Helaine, S., Cheverton, A.M., Watson, K.G., Faure, L.M., Matthews, S.A., Holden, D.W., 2014. Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. Science 343 (6167), 204–208.

Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M.A., Jensen, A.B., Wegener, H.C., Aarestrup, F.M., 2011. Global monitoring of *Salmonella* serovar distribution from

the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. Foodborne Pathogens and Disease 8 (8), 887–900.

Henry, J.P., 1982. The relation of social to biological processes in disease. Social Science & Medicine 16 (4), 369–380.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., Cullinan, W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. Frontiers in Neuroendocrinology 24 (3), 151–180.

Hessing, M.J., Hagelsø, A.M., Schouten, W.G., Wiepkema, P.R., van Beek, J.A., 1994. Individual behavioral and physiological strategies in pigs. Physiology & Behavior 55 (1), 39–46.

Hicks, T.A., McGlone, J.J., Whisnant, C.S., Kattesh, H.G., Norman, R.L., 1998. Behavioral, endocrine, immune, and performance measures for pigs exposed to acute stress. Journal of Animal Science 76 (2), 474–483.

Holst, D., 1997. Social relations and their health impact in tree shrews. Acta Physiologica Scandinavica. Supplementum 640, 77–82.

Horter, D.C., Yoon, K.-J., Zimmerman, J.J., 2003. A review of porcine tonsils in immunity and disease. Animal Health Research Reviews 4 (2), 143–155.

Jänig, W., 2006. The integrative action of the autonomic nervous system: Neurobiology of homeostasis. Cambridge University Press, Cambridge, 610 pp.

Kanitz, E., Otten, W., Nürnberg, G., Brüssow, K.P., 1999. Effects of age and maternal reactivity on the stress response of the pituitary-adrenocortical axis and the sympathetic nervous system in neonatal pigs. Animal Science 68, 519–526.

Kanitz, E., Tuchscherer, M., Puppe, B., Tuchscherer, A., Stabenow, B., 2004. Consequences of repeated early isolation in domestic piglets (*Sus scrofa*) on their behavioural, neuroendocrine, and immunological responses. Brain, Behavior, and Immunity 18, 35–45.

Katsu, Y., Iguchi, T., 2016. Subchapter 95A - Corticosterone, in: Takei, Y., Ando, H., Tsutsui, K. (Eds.), Handbook of Hormones. Comparative endocrinology for basic and clinical research, First edition ed. Elsevier/AP, Oxford, 527-e95A-3.

Kavelaars, A., Heijnen, C.J., Tennekes, R., Bruggink, J.E., Koolhaas, J.M., 1999. Individual behavioral characteristics of wild-type rats predict susceptibility to experimental autoimmune encephalomyelitis. Brain, Behavior, and Immunity 13 (4), 279–286.

Kick, A.R., Tompkins, M.B., Almond, G.W., 2011. Stress and immunity in the pig. CAB reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 6, 1–17.

Konturek, P.C., Brzozowski, T., Konturek, S.J., 2011. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. Journal of Physiology

and Pharmacology: an official journal of the Polish Physiological Society 62 (6), 591–599.

Koolhaas, J.M., 2008. Coping style and immunity in animals: making sense of individual variation. Brain, Behavior, and Immunity 22 (5), 662–667.

Koolhaas, J.M., Boer, S.F. de, Buwalda, B., van Reenen, K., 2007. Individual variation in coping with stress: a multidimensional approach of ultimate and proximate mechanisms. Brain, Behavior and Evolution 70 (4), 218–226.

Kraetzl, W.D., Weiler, U., 1998. Erfahrungen mit einem implantierbaren Kathetersystem zur frequenten und chronischen Blutentnahme bei Schafen in Gruppenhaltung und bei säugenden Sauen. Tierärztliche Umschau, 567–574.

Krebs, N., McGlone, J.J., 2009. Effects of exposing pigs to moving and odors in a simulated slaughter chute. Applied Animal Behaviour Science.

Lathrop, S.K., Binder, K.A., Starr, T., Cooper, K.G., Chong, A., Carmody, A.B., Steele-Mortimer, O., 2015. Replication of *Salmonella enterica* serovar Typhimurium in human monocyte-derived macrophages. Infection and Immunity 83 (7), 2661–2671.

Lo, D.Y., Lee, W.M., Chien, M.S., Lin, C.C., Lee, W.C., 2005. Effects of dexamethasone on peripheral blood mononuclear cell phenotype in weanling piglets. Comparative Immunology, Microbiology and Infectious Diseases 28 (4), 251–258.

Lyte, M., Erickson, A.K., Arulanandam, B.P., Frank, C.D., Crawford, M.A., Francis, D.H., 1997. Norepinephrine-induced expression of the K99 pilus adhesin of enterotoxigenic *Escherichia coli*. Biochemical and Biophysical Research Communications 232 (3), 682–686.

Lyte, M., Vulchanova, L., Brown, D.R., 2011. Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell and Tissue Research 343 (1), 23–32.

Malikina, K.D., Shishov, V.A., Chuvelev, D.I., Kudrin, V.S., Oleskin, A.V., 2010. Regulatory role of monoamine neurotransmitters in *Saccharomyces cerevisiae* cells. Prikladnaia Biokhimiia i Mikrobiologiia 46 (6), 672–677.

McGlone, J.J., Salak, J.L., Lumpkin, E.A., Nicholson, R.I., Gibson, M., Norman, R.L., 1993. Shipping stress and social status effects on pig performance, plasma cortisol, natural killer cell activity, and leukocyte numbers. Journal of Animal Science 71 (4), 888–896.

Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V., 2012. The pig: a model for human infectious diseases. Trends in Microbiology 20 (1), 50–57.

Michael, B., Smith, J.N., Swift, S., Heffron, F., Ahmer, B.M., 2001. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. Journal of Bacteriology 183 (19), 5733–5742.

Miethke, M., Skerra, A., 2010. Neutrophil gelatinase-associated lipocalin expresses antimicrobial activity by interfering with L-norepinephrine-mediated bacterial iron acquisition. Antimicrobial Agents and Chemotherapy 54 (4), 1580–1589.

Miyaura, H., Iwata, M., 2002. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. Journal of Immunology 168 (3), 1087–1094.

Murphy, K.M., Weaver, C., 2017. Janeway's Immunobiology, 9th edition ed. GS Garland Science Taylor & Francis Group, New York, London.

Okano, M., 2009. Mechanisms and clinical implications of glucocorticosteroids in the treatment of allergic rhinitis. Clinical and Experimental Immunology 158 (2), 164–173.

Ortega, E., 2003. Neuroendocrine mediators in the modulation of phagocytosis by exercise: physiological implications. Exercise Immunology Review 9, 70–93.

Perez, D.M., 2006. The Adrenergic Receptors: In the 21st Century. Humana Press Inc, Totowa, NJ.

Purves, D., Williams, S.M. (Eds.), 2001. Neuroscience, 2nd ed. ed. Sinauer Associates, Sunderland, MA.

Quigley, E.M.M., 2013. Gut bacteria in health and disease. Gastroenterology & Hepatology 9 (9), 560–569.

Rosenfeld, P., van Eekelen, J.A.M., Levine, S., Kloet, E.R. de, 1988. Ontogeny of the Type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study. Developmental Brain Research 42 (1), 119–127.

Rosenne, E., Sorski, L., Shaashua, L., Neeman, E., Matzner, P., Levi, B., Ben-Eliyahu, S., 2013. *In vivo* suppression of NK cell cytotoxicity by stress and surgery in F344 rats: Glucocorticoids have a minor role compared to catecholamines and prostaglandins. Brain, Behavior, and Immunity 37, 207–219.

Roth, J.A., Flaming, K.P., 1990. Model systems to study immunomodulation in domestic food animals. Advances in Veterinary Science and Comparative Medicine 35, 21–41.

Roura, E., Koopmans, S.-J., Lallès, J.-P., Le Huerou-Luron, I., Jager, N.D., Schuurman, T., Val-Laillet, D., 2016. Critical review evaluating the pig as a model for human nutritional physiology. Nutrition Research Reviews 29 (1), 60–90.

Rudine, A.C., Sutherland, M.A., Hulbert, L., Morrow, J.L., McGlone, J.J., 2007. Diverse production system and social status effects on pig immunity and behavior. Livestock Science 111 (1), 86–95.

Ruis, M.A., Te Brake, J.H., Engel, B., Ekkel, E.D., Buist, W.G., Blokhuis, H.J., Koolhaas, J.M., 1997. The circadian rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress. Physiology & Behavior 62, 623–630.

Salak-Johnson, J.L., McGlone, J.J., Norman, R.L., 1996. In vivo glucocorticoid effects on porcine natural killer cell activity and circulating leukocytes. Journal of Animal Science 74, 584.

Sandrini, S., Aldriwesh, M., Alruways, M., Freestone, P., 2015. Microbial endocrinology: host-bacteria communication within the gut microbiome. The Journal of Endocrinology 225 (2), R21-34.

Sandrini, S.M., Shergill, R., Woodward, J., Muralikuttan, R., Haigh, R.D., Lyte, M., Freestone, P.P., 2010. Elucidation of the mechanism by which catecholamine stress hormones liberate iron from the innate immune defense proteins transferrin and lactoferrin. Journal of Bacteriology 192 (2), 587–594.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55–89.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States—major pathogens. Emerging Infectious Diseases 17 (1), 7–15.

Schaible, U.E., Kaufmann, S.H.E., 2004. Iron and microbial infection. Nature Reviews. Microbiology 2 (12), 946–953.

Schrama, J.W., Schouten, J.M., Swinkels, J.W., Gentry, J.L., Vries Reilingh, G. de, Parmentier, H.K., 1997. Effect of hemoglobin status on humoral immune response of weanling pigs differing in coping styles. Journal of Animal Science 75 (10), 2588–2596.

Schwarz, E., Saalmüller, A., Gerner, W., Claus, R., 2005. Intraepithelial but not lamina propria lymphocytes in the porcine gut are affected by dexamethasone treatment. Veterinary Immunology and Immunopathology 105 (1-2), 125–139.

Selye, H., 1936. A syndrome produced by diverse nocuous agents. Nature 138 (3479), 32.

Sgoifo, A., Koolhaas, J., Boer, S. de, Musso, E., Stilli, D., Buwalda, B., Meerlo, P., 1999. Social stress, autonomic neural activation, and cardiac activity in rats. Neuroscience & Biobehavioral Reviews 23 (7), 915–923.

Shakhar, G., Ben-Eliyahu, S., 1998. *In vivo* beta-adrenergic stimulation suppresses natural killer activity and compromises resistance to tumor metastasis in rats. Journal of Immunology 160 (7), 3251–3258.

Silverthorn, D.U., Johnson, B.R., Silverthorn, A.C., 2016. Human physiology: An integrated approach, Seventh edition, global edition ed. Pearson, Harlow, 40 pp.

Sperandio, V., Torres, A.G., Jarvis, B., Nataro, J.P., Kaper, J.B., 2003. Bacteria-host communication: the language of hormones. Proceedings of the National Academy of Sciences of the United States of America 100 (15), 8951–8956.

Strahler, J., Rohleder, N., Wolf, J.M., 2015. Acute psychosocial stress induces differential short-term changes in catecholamine sensitivity of stimulated inflammatory cytokine production. Brain, Behavior, and Immunity 43, 139–148.

Strosberg, A.D., 1993. Structure, function, and regulation of adrenergic receptors. Protein Science: A Publication of the Protein Society 2 (8), 1198–1209.

Sun, J., Daniel, R., Wagner-Döbler, I., Zeng, A.-P., 2004. Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. BMC Evolutionary Biology 4 (1), 1–11.

Sutherland, M.A., Bryer, P.J., Davis, B.L., McGlone, J.J., 2009. Space requirements of weaned pigs during a sixty-minute transport in summer. Journal of Animal Science 87 (1), 363–370.

Swindle, M.M., Makin, A., Herron, A.J., Clubb, F.J., Frazier, K.S., 2012. Swine as models in biomedical research and toxicology testing. Veterinary Pathology 49 (2), 344–356.

Teunis, M.A.T., Kavelaars, A., Voest, E., Bakker, J.M., Ellenbroek, B.A., Cools, A.R., Heijnen, C.J., 2002. Reduced tumor growth, experimental metastasis formation, and angiogenesis in rats with a hyperreactive dopaminergic system. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 16 (11), 1465–1467.

Tsavkelova, E.A., Botvinko, I.V., Kudrin, V.S., Oleskin, A.V., 2000. Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. Doklady biochemistry: proceedings of the Academy of Sciences of the USSR, Biochemistry Section 372 (1-6), 115–117.

Tuchscherer, M., Kanitz, E., Puppe, B., Tuchscherer, A., Viergutz, T., 2009. Changes in endocrine and immune responses of neonatal pigs exposed to a psychosocial stressor. Research in Veterinary Science 87 (3), 380–388.

Tuchscherer, M., Kanitz, E., Tuchscherer, A., Puppe, B., 2016. Effects of social support on glucocorticoid sensitivity of lymphocytes in socially deprived piglets. Stress: The International Journal on the Biology of Stress 19, 325–332.

Tumbleson, M.E. (Ed.), 1986. Swine in biomedical research. Plenum, New York, 698 pp.

Vandevyver, S., Dejager, L., Libert, C., 2014. Comprehensive overview of the structure and regulation of the glucocorticoid receptor. Endocrine Reviews 35 (4), 671–693.

Veenema, A.H., Sijtsma, B., Koolhaas, J.M., Kloet, E.R. de, 2005. The stress response to sensory contact in mice: genotype effect of the stimulus animal. Psychoneuroendocrinology 30 (6), 550–557.

Vegas, O., Fano, E., Brain, P.F., Alonso, A., Azpiroz, A., 2006. Social stress, coping strategies and tumor development in male mice: behavioral, neuroendocrine and immunological implications. Psychoneuroendocrinology 31 (1), 69–79.

Verbrugghe, E., Boyen, F., Gaastra, W., Bekhuis, L., Leyman, B., van Parys, A., Haesebrouck, F., Pasmans, F., 2012. The complex interplay between stress and bacterial infections in animals. Veterinary Microbiology 155 (2-4), 115–127.

Verbrugghe, E., Boyen, F., van Parys, A., van Deun, K., Croubels, S., Thompson, A., Shearer, N., Leyman, B., Haesebrouck, F., Pasmans, F., 2011. Stress induced *Salmonella* Typhimurium recrudescence in pigs coincides with cortisol induced increased intracellular proliferation in macrophages. Veterinary Research 42, 118.

Verbrugghe, E., Dhaenens, M., Leyman, B., Boyen, F., Shearer, N., van Parys, A., Haesendonck, R., Bert, W., Favoreel, H., Deforce, D., Thompson, A., Haesebrouck, F., Pasmans, F., 2016. Host Stress Drives Salmonella Recrudescence. Scientific reports 6, 20849.

von Borell, E.H., 2001. The biology of stress and its application to livestock housing and transportation assessment. Journal of Animal Science 79 (E-Suppl), E260.

Waters, C.M., Bassler, B.L., 2005. Quorum sensing: cell-to-cell communication in bacteria. Annual Review of Cell and Developmental Biology 21, 319–346.

Westly, H.J., Kelley, K.W., 1984. Physiologic concentrations of cortisol suppress cell-mediated immune events in the domestic pig. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) 177, 156–164.

Whiley, H., Ross, K., 2015. *Salmonella* and eggs: from production to plate. International Journal of Environmental Research and Public Health 12 (3), 2543–2556.

Wood, R.L., Pospischil, A., Rose, R., 1989. Distribution of persistent *Salmonella* Typhimurium infection in internal organs of swine. American Journal of Veterinary Research 50 (7), 1015–1021.

Zahorec, R., 2001. Ratio of neutrophil to lymphocyte counts—rapid and simple parameter of systemic inflammation and stress in critically ill. Bratislavske Lekarske Listy 102 (1), 5–14.

Zhang, Q., Widmer, G., Tzipori, S., 2013. A pig model of the human gastrointestinal tract. Gut Microbes 4 (3), 193–200.

CHAPTER 2

MANUSCRIPTS

2 MANUSCRIPTS

All manuscripts that were included in the present thesis were published in international peer-reviewed journals. Each manuscript is presented here in the published version. Text layout and formatting were adjusted to fit the layout of the thesis.

I Glucocorticoids and Catecholamines Affect in Vitro Functionality of Porcine Blood Immune Cells
Published in *Animals* **9**, 545 (2019)

II Intravenous Infusion of Cortisol, Adrenaline, or Noradrenaline Alters Porcine Immune Cell Numbers and Promotes Innate over Adaptive Immune Functionality
Published in *The Journal of Immunology* **204** (12), 3205-3216 (2020)

III Interkingdom Cross-Talk in Times of Stress: *Salmonella* Typhimurium Grown in the Presence of Catecholamines Inhibits Porcine Immune Functionality *in vitro*
Published in *Frontiers in Immunology* **11**: 572056 (2020)

MANUSCRIPT I

Glucocorticoids and Catecholamines Affect in Vitro Functionality of Porcine Blood Immune Cells

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Simple Summary: In modern livestock husbandry, animals may face stressful events like weaning, regrouping, or transportation, all of which can impair animal welfare and health. Research in model organisms has revealed that stress hormones, such as glucocorticoids and catecholamines, strongly modulate the immune system and thus the animals' ability to fight infections. In the pig, knowledge about this relationship is rare, and results from rodents cannot readily be transferred due to some physiological differences. Therefore, the effects of glucocorticoids and catecholamines on porcine immune cell proliferation and the production of the pro-inflammatory cytokine TNFα were investigated in an in vitro study. Blood was obtained from catheterized pigs to exclude pre-exposure to stress hormones. Glucocorticoids exerted inhibitory effects on both investigated immune functions. Catecholamines, on the other hand, showed diverse effects on lymphocyte proliferation and TNFα production of particular immune cell types. This suggests that studies from model species are not entirely transferrable to pigs. Future research should extend the preliminary findings on cytokine production and focus on the molecular mechanisms and health impacts of stress hormones in pigs.

Abstract: Stress hormones exert important modulating influences on the functionality of immune cells. Despite its major role as a livestock animal and its increasing use as an animal model, knowledge about this relationship in the domestic pig is rare. This study therefore aimed to characterize the effect of glucocorticoids and catecholamines on the proliferation and cytokine production of porcine peripheral blood mononuclear cells (PBMC). Blood was obtained from donor pigs equipped with indwelling catheters to exclude stress hormone exposition before in vitro testing. PBMC were stimulated in the presence of cortisol, adrenaline or noradrenaline at concentrations resembling low to high stress conditions. Proliferation was determined via ^{3}H-thymidine incorporation, and TNFα producers were quantified by intracellular cytokine staining. Cortisol led to a decrease in mitogen-induced lymphocyte proliferation and the number of TNFα producing cells. In contrast, catecholamines increased proliferation while exerting repressive or no effects on the number of cytokine producers. Remarkably, in concentrations presumably found in lymphatic tissue in stress situations, noradrenaline suppressed lymphocyte proliferation completely. The shown repressive effects might especially have implications on health and welfare in pigs. The obtained results provide a preliminary database for extended studies on the molecular mechanisms of glucocorticoid and catecholamine actions on porcine immune cells.

Keywords: pig; stress; immune system; cortisol; adrenaline; noradrenaline; catecholamines; lymphocytes; cytokines

1. Introduction

The physiological stress response enables the body to cope with threats via predominantly adaptive alterations in cardiac function, energy metabolism and the immune system [1–3]. However, if stress exposure lasts for a long time, it can negatively affect animal welfare and health. Chronically elevated levels of stress hormones, namely glucocorticoids (GCs) and the catecholamines (CAs) adrenaline (ADR) and noradrenaline (NA), contribute to an impaired immune function leading to increased risk of infection and reduced animal welfare [4,5]. Efforts to reduce the use of antibiotics in animal husbandry also require a well-functioning immune system and the prevention of stress-induced immunosuppression. For these reasons, it is of utmost importance to understand the actions of the particular stress hormones on different immune functions. So far, this topic has mostly been studied in humans and rodents. It was thus shown that GCs can inhibit important immune functions such as lymphocyte proliferation [6,7] and the production of pro-inflammatory cytokines like TNFα and IFNγ [8,9]. ADR and NA can exert effects similar to cortisol with lower proliferation [10] and cytokine production [11,12]. However, they may also lead to immune activation [13,14], depending on experimental conditions, such as dose or the timing of treatment [15].

In modern pig husbandry systems, animals face many potential stressors that can cause a release of GCs and CAs [5,16]. Cortisol (C), as the main GC in pigs, can thus be raised from basal levels of 20–30 ng/mL (8.3×10^{-8} M) to a plasma concentration of about 350 ng/mL (9.7×10^{-7} M) in highly stressful situations [17,18]. Using blood samples from catheterized pigs and thus avoiding a rapid CA release due to stressful sampling techniques, basal plasma ADR concentrations of approximately 180 pg/mL (10^{-9} M) and NA concentrations of around 325 pg/mL (2×10^{-9} M) were found [19]. In acute stress situations, plasma ADR concentrations can range between 700 pg/mL (1.5×10^{-9} M) and 100 ng/mL (5.5×10^{-7} M), while NA may reach levels between 1700 pg/mL (10^{-8} M) and 300 ng/mL (1.8×10^{-6} M) [20,21].

Even though the increase of GCs and CAs upon stressor exposure is well documented in pigs, only a few experiments have studied the functionality of immune cells under the influence of stress hormones in this important livestock species so far. It was shown, for example, that social isolation, weaning, restraint or regrouping led to an increase in endogenic cortisol production, thus resulting in the suppression of lymphocyte proliferation [16,17,22–24] and a reduced expression of pro-inflammatory cytokines [21,25,26]. However, it is likely that these immune-modulating effects cannot solely be attributed to cortisol, as a concurrent activation of the sympathetic nervous system (SNS) which leads to the secretion of ADR and NA is probable.

Studies that separately examine the effect of stress hormones in pigs are rare, and there are no studies on the specific effects of CAs on the functionality of porcine immune cells. It cannot readily be assumed that the effects of stress hormones observed in rodent studies are the same in pigs, as there are some important anatomical and physiological species differences. For example, the circadian rhythm of the plasma GC concentrations and blood immune cell numbers of rodents are opposite to that of pigs with regard to light and darkness [27–29]. Moreover, it is assumed that the porcine hypothalamus–pituitary–adrenal (HPA) axis is less sensitive than its rodent counterpart [30,31] while having ontogenetic similarities to humans [32]. Therefore, it would be premature to assume that findings from rodent studies are fully transferable to pigs. To get a better understanding of stress-induced immunomodulation in pigs, more studies are needed. A useful first approach is to examine the actions of the different stress hormones separately in a controlled in vitro environment, where conditions can be standardized and disruptive factors can be minimized compared to in vivo models.

The aim of the present study was thus to investigate the impact of different infra-to-supraphysiological concentrations of cortisol, adrenaline and noradrenaline on porcine lymphocyte proliferation in vitro. In addition, we also examined the effect of the three stress hormones on the number of TNFα producing immune cells among different leukocyte subsets.

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; ethical approval code: V324/15TH). In total, 32 castrated male pigs (German Landrace x Pietrain, 7–10 months old, body weight range 90–120 kg), divided into three consecutive experimental trials with 10–12 animals each, were available as blood donors for this study. Blood from each individual donor pig was used only once for each tested immunological parameter. The barrows were housed individually in pens (7 m²) with sight and tactile contact through the bars. Concentrate (1.3–1.5 kg/meal, ME 12 MJ/kg) was fed twice daily (0730 and 1500), and pigs had ad libitum access to water and hay. Pens were cleaned daily after feeding in the morning and littered with dust-free wood shavings. Light was turned on from 0630 until 2030. Since blood sampling methods including fixation by nose snare or obtaining blood at slaughter already resemble stressful conditions and thus compromise a controlled investigation

of defined hormone concentrations, pigs were equipped with indwelling vein catheters via *Vena cephalica* cannulation. Surgery was performed as published by Kraetzl and Weiler [33] with modifications described in Engert et al. [29] at least 14 d before sampling. All animals were thoroughly habituated to human handling to ensure stress free blood sampling via the vein catheters. Blood (10 mL per animal) was collected into lithium heparin tubes (Sarstedt, Nümbrecht, Germany) at 0830.

2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Porcine peripheral blood mononuclear cells (PBMC) were separated using LeucosepTM centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and Biocoll (density: 1.077 g/mL, Biochrom, Berlin, Germany) according to the manufacturer's protocol with the following modifications: After separation, cells were washed in PBS (Biochrom) supplemented by 2 mM EDTA (Sigma-Aldrich, Taufkirchen, Germany) and subsequently in RPMI 1640 supplemented by 5% inactivated fetal calf serum (FCS) and 50 µg/mL of gentamycin (all Biochrom). PBMC were then suspended in RPMI 1640 supplemented with 10% FCS and 50 µg/mL gentamycin, and cell concentration was measured using a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany).

2.3. Lymphocyte Proliferation Assay

Using the PBMC of 20 donor pigs from Trials 1 and 2, a mitogen-induced lymphocyte proliferation assay was performed as previously described [34], including a dilution series of each investigated hormone. In brief, 1.5×10^5 of PBMC were seeded per well and stimulated with 5 µg/mL concanavalin A (ConA) or 5 µg/mL pokeweed mitogen (PWM, both Sigma-Aldrich) of left without stimulation. Stimulated samples were left without hormones or additionally supplemented with either C, NA or ADR in final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M, covering miscellaneous possible plasma concentrations from calmness to high stress. All treatments were done in triplicates. A second experiment with the PBMC of 12 barrows from Trial 3 was conducted including only NA in concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M, resembling the presumed milieu around noradrenergic nerve endings in lymphatic tissues [35,36]. Cells were incubated at 39 °C and 5% CO2 for 48 h, after which 0.25 µCi ^{3}H-thymidine were added for a further 24 h. Cells were harvested on glass fiber filters (Sigma-Aldrich), and the incorporated amount of radioactivity was measured in counts per minute (cpm) by a liquid scintillation analyzer (PerkinElmer, Rodgau, Germany). For statistical analysis, the cpm of the unstimulated triplicates were subtracted from the stimulated ones to

obtain the Δcpm. In the NA high-dose experiment, cpm were used for data analysis, as the highest NA dose led to negative Δcpm values.

2.4. Intracellular Cytokine Staining

For the investigation of the effects of stress hormones on the number of immune cells producing pro-inflammatory cytokines, an intracellular staining technique was conducted with the blood of 23 pigs from Trials 2 and 3. After separation, 10^6 of PBMC were transferred into sterile polystyrene tubes and, after the addition of either stress hormone in high (10^{-6} M) or moderate (10^{-8} M) concentrations or no hormone at all, cells were either left unstimulated or stimulated with 5 µg/mL PWM, which was found best suitable to elicit TNFα production without overstimulation, ensuring a sufficient sensitivity to hormone effects in own preceding experiments. To inhibit the secretion of cytokines, 1 µg/mL of brefeldin A was added. Cells were incubated for 4 h (39 °C, 5% CO_2) and subsequently fixated with a formaldehyde buffer (PBS, 2mM EDTA, 0.5% FCS, 0.5% Roth-Histofix formaldehyde, Karl Roth GmbH, Karlsruhe, Germany) for 20 min at room temperature. Then, cells were permeabilized using a saponin buffer (PBS, 2mM EDTA, 0.5% FCS, 0.05% saponin) and stained (15 min, 6 °C) with the following antibodies: CD3ε-biotin (clone PPT3, Acris Antibodies, Herford, Germany) and streptavidin-V500, CD4-PerCP-Cy5.5 (clone 74-12-4), CD8α-AlexaFluor 647 (clone 76-2-11), IFNγ-PE (clone P2G10, all BD Biosciences, NJ, USA) and TNFα-PacificBlue (clone Mab11, Biolegend, San Diego, CA, USA). Afterwards, cells were washed in saponin buffer and resuspended in PBS + 1 % FCS. Analysis was performed using a FACSCanto II™ flow cytometer (BD Biosciences) with the software BD FACSDiva™ by evaluating the percentage of cytokine-producing cells per population (10^5 events/sample). Populations were differentiated based on surface marker expression into: Cytotoxic T cells (CTL; $CD3^+CD4^-CD8\alpha^{high}$, ~$10^4$ events), γδ T cells ($CD3^+CD4^-CD8\alpha^{-/low}$, ~$2 \times 10^4$ events), naive T helper (T_H) cells ($CD3^+CD4^+CD8\alpha^-$, ~10^4 events), antigen-experienced (Ag-exp.) T_H cells ($CD3^+CD4^+CD8\alpha^+$, ~10^4 events) and natural killer (NK) cells ($CD3^-CD4^-CD8\alpha^+$, ~10^4 events). Due to a high background of IFNγ in the unstimulated samples, only the number of total TNFα producers were investigated and used for statistical analysis. For technical reasons, the intracellular staining of monocytes was conducted with deep-frozen PBMC. Therefore, the PBMC of 6 animals of Trial 3 stored at −80 °C in DMSO (Sigma-Aldrich) were thawed in RPMI-10 at 37 °C and washed twice in RPMI-5 before determination of cell concentration. Stimulation was conducted analogous to the first trial but with 1 µg/mL lipopolysaccharide (LPS; Sigma-Aldrich) used as stimulant. Cells were then stained with the antibodies CD172a-PE (clone 74-

22-15A, BD Biosciences) and TNFα-PacificBlue (clone Mab11, Biolegend). 5×10^4 events per sample were recorded, and monocytes were defined as CD172a$^+$ cells (~2×10^3 events).

2.5. Statistical Analysis

Data were analyzed using SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA). We used the MIXED procedure of SAS with degrees of freedom determined by the Kenward–Roger method [37]. Linear mixed-effect models included the factor treatment (addition of no hormone or different concentrations of C, NA, or ADR) as a fixed effect and individual (1–20, 1–12, 1–23), sampling date, and trial (1–3), as well as their interactions, as random effects. Normality and variance homogeneity were confirmed by visually checking normal probability plots and plots of fitted values versus residuals [38]. If necessary, square root or logarithmic transformation was performed. For all comparisons, $p < 0.05$ was considered significant. All results are presented as LS-means + standard error of the mean (SEM).

3. Results

3.1. Lymphocyte Proliferation

To investigate stress hormone effects on lymphocyte proliferation, we tested a wide range of concentrations in a mitogen-induced proliferation assay. Compared to the hormone-free control, cortisol caused a significant reduction of lymphocyte proliferation in a dose-dependent manner. When PBMC were stimulated with ConA, this inhibitory effect occurred at a concentration of 10^{-8} M and higher, whereas the proliferation of PWM-stimulated PBMC was first inhibited upon addition of 10^{-7} M cortisol (Figure 1a,b). In contrast, catecholamines generally had an enhancing impact on lymphocyte proliferation, but the magnitude of the effect of adrenaline or noradrenaline action was dependent on CA dose and mitogen (Figure 1c–f). Noradrenaline increased ConA-induced proliferation in all tested concentrations (Figure 1c). An enhancing effect could also be observed on PWM-stimulated PBMC proliferation but at a lower magnitude and only for the highest tested concentration of 10^{-5} M. Similarly, adrenaline led to a higher proliferation of mitogen-stimulated PBMC, but, here, the effect was much more pronounced for PWM than for ConA. If stimulated with PWM, all investigated concentrations enhanced lymphocyte proliferation significantly (Figure 1f), while ConA-stimulated proliferation was enhanced only for 10^{-5} M ADR (Figure 1e).

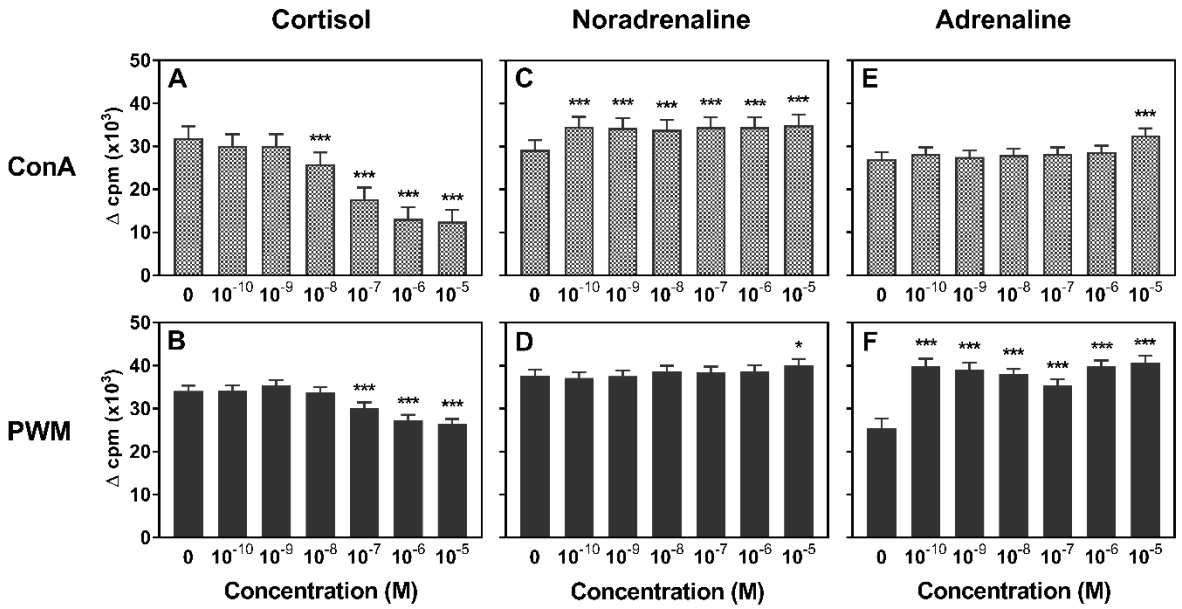

Figure 1. Lymphocyte proliferation after incubation with cortisol (A,B), noradrenaline (C,D) or adrenaline (E,F) (10^{-10}–10^{-5} M) and one of the mitogens concanavalin A (ConA) (A,C,E) or pokeweed mitogen (PWM) (B,D,F) in vitro (n = 20). Data are presented as lsmeans + standard error of the mean (SEM) of Δcpm (counts per minute) of the untransformed data. Asterisks indicate significant differences between treatment and hormone-free control (0): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p < 0.001$.

Beside production by the adrenal medulla and release into the blood stream, noradrenaline is also widely used as a neurotransmitter in the SNS. It can thus reach high local concentrations at sympathetic nerve endings, which are present in abundance in lymphoid tissues [28,29]. Therefore, a further experiment was conducted using higher NA concentrations (Fig. 2). Again, NA at concentrations of 10^{-6} and 10^{-5} M caused an increase of PWM-induced proliferation. A higher NA concentration of 10^{-4} M, however, led to a drastic reduction of cpm.

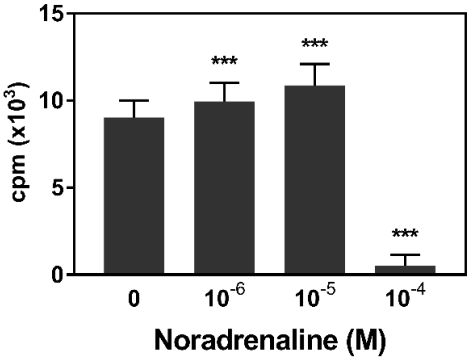

Figure 2. Lymphocyte proliferation after incubation with noradrenaline (10^{-6}–10^{-4} M) and the mitogen pokeweed mitogen in vitro (n = 12). Data are presented as lsmeans + SEM of cpm (counts per minute) of the untransformed data. Asterisks indicate significant differences between treatment and hormone-free control (0): * $p \leq 0.05$; ** $p \leq 0.01$; *** $p < 0.001$.

3.2. Intracellular Cytokine Staining

To get a more differentiated picture of the impact of stress hormones on immune cell activation, we used an intracellular staining technique which allowed us to quantify TNFα producers separately for different immune cell types. The results of linear mixed model analysis are shown in Table 1, and representative dot plots (i.e., antigen-experienced T_H cells) are shown in Figure 3. In all investigated leukocyte subsets except NK cells, cortisol at a concentration of 10^{-6} M decremented the number of TNFα producers (Table 1, Figure 3C), while lower cortisol concentrations of 10^{-8} M had no effect. For noradrenaline, on the other hand, neither of the tested concentrations had a significant impact on TNFα producing cells in any of the investigated cell types. Similar to cortisol, adrenaline reduced the number of cytokine-producing cells in some leukocyte populations. TNFα producers were reduced among γδ T cells and monocytes if ADR was added at a concentration of 10^{-6} M. The addition of ADR at the low concentration of 10^{-8} M had no significant effect on any of the investigated subsets.

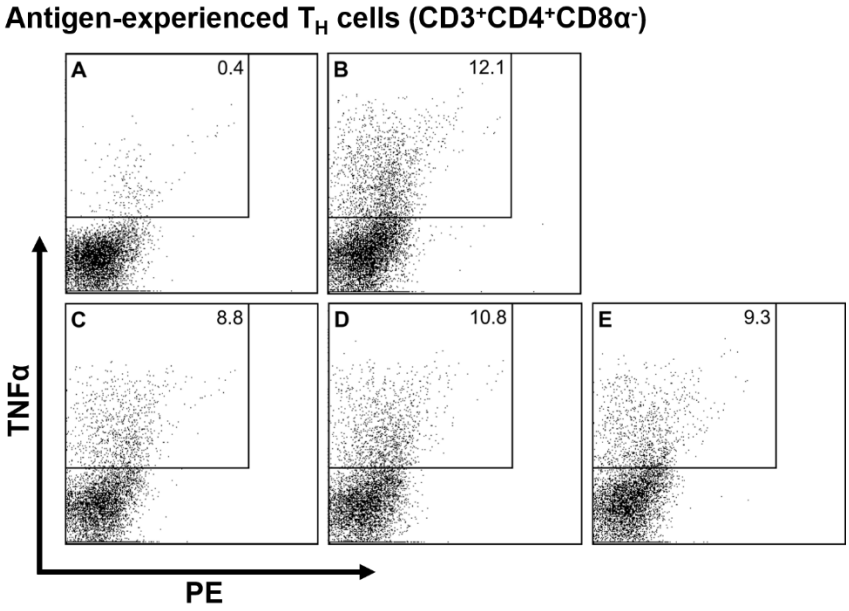

Figure 3. Representative plots of TNFα producers among antigen-experienced T helper (T_H) cells. Porcine peripheral blood mononuclear cells (PBMC) were stimulated with pokeweed mitogen and antigen-experienced T_H cells were discriminated based on surface marker expression. TNFα is plotted on the y axis against the PE channel on the x axis. TNFα-positive cells are shown in the rectangular gates, numbers in the corner indicate the percentage of TNFα producers among antigen-experienced T_H cells. Letters in the upper left corner indicate the treatment of the sample: A = No stimulation; B = Stimulated hormone-free control; C = Cortisol (10^{-6} M); D = Noradrenaline (10^{-6} M); E = Adrenaline (10^{-6} M).

Table 1. Frequency of TNFα producing cells (%) after stimulation in the presence of cortisol, noradrenaline or adrenaline.

Frequency (%)	Control	Hormone						Pooled SEM	Treatment *p*-Value
		Cortisol		Noradrenaline		Adrenaline			
		10^{-8} M	10^{-6} M	10^{-8} M	10^{-6} M	10^{-8} M	10^{-6} M		
Naive T_H cells †	1.37	1.31	1.07 ***	1.37	1.32	1.31	1.31	0.48	<0.001
Ag-exp. T_H cells	12.36	12.20	10.09 ***	12.48	11.92	11.89	11.60	1.99	<0.001
Cytotoxic T cells	2.42	2.46	1.93 ***	2.54	2.41	2.39	2.25	0.67	<0.001
γδ T cells ‡	1.34	1.31	1.08 ***	1.34	1.27	1.25	1.00 ***	0.08	<0.001
NK cells ‡	4.32	4.47	3.82	4.18	3.95	4.46	3.94	0.85	0.307
Monocytes †	26.35		22.15 ***		27.00		23.96 *	2.62	<0.001

Cells were stimulated with pokeweed mitogen (lines 1–5) or lipopolysaccharide (line 6). Data are shown as least-square means with pooled standard error of the mean (SEM). *p*-values indicate a significant effect of the treatment. Data that required †logarithmic or ‡ square root transformation are reported on the original scale after back transformation. Asterisks indicate a significant effect of the respective hormone treatment compared to the stimulated hormone-free control: * $p \leq 0.05$; *** $p < 0.001$.

4. Discussion

In this study, we found inhibitory as well as stimulatory effects of stress hormones on the proliferative capacity of porcine lymphocytes, depending on the hormone and concentration applied. This study also provides preliminary data on the effects of stress hormones on cytokine producing cells. Cortisol caused a significant reduction of lymphocyte proliferation in a dose-dependent manner, which is in accordance with results from social stress experiments. Deguchi and Akuzawa [17], for example, reported that, after regrouping, piglets showed elevated blood cortisol concentrations of 2×10^{-7} M accompanied by a reduced lymphocyte proliferation. In the present study, this immunosuppressive effect could be confirmed by in vitro cultivation with a similar amount of cortisol, proving the suitability of the chosen model. If stimulated with PWM in the presence of 10^{-8} M cortisol, proliferation was still on the same level as the hormone-free control. In mouse experiments, this concentration sufficed to inhibit lymphocyte functionality [39], which may be another hint that the porcine HPA axis is less GC-sensitive than their murine counterpart.

In contrast to cortisol, which takes a few minutes to rise and is responsible for the detrimental immune outcome in chronic stress situations, catecholamines are released into the blood circulation within seconds after a stressor [3,40]. As reviewed by Elenkov et al. [15], CAs can have inhibitory or stimulatory effects on immune cell functionality, depending on immune cell type, adrenoceptor (AR) type and abundance on these cells, as well as the localization and timing of the CA release. The immunomodulatory properties of ADR and NA were already investigated by Hadden et al. in the 1970s in an in vitro experiment on the phytohemagglutinin-induced proliferation of human lymphocytes [41]. Similar to the data presented here, NA had a β-AR-mediated inhibitory effect if 10^{-4} M were added, whereas lower concentrations of 10^{-7} M stimulated proliferation via α-ARs. An enhanced proliferation was also found in a study with murine B cells stimulated under the influence of 10^{-6}–10^{-5} M NA [13]. For adrenaline, Hadden et al. found no effect on lymphocyte proliferation and concluded that stimulating α- and inhibiting β-adrenergic actions nullified each other. In contrast, adrenaline also had an enhancing effect on proliferation in the present study, particularly distinct if PWM was used for stimulation. This seems to indicate that NA effects on proliferation might be mediated by similar mechanisms in human, murine, and porcine lymphocytes, while ADR seems to work differently in pigs, possibly caused by a shifted AR-ratio. In other species, stimulatory α_2-ARs on B and T cells are upregulated under certain disease states [42]. Future research into type and

quantity of ARs on porcine immune cells could reveal whether they express higher numbers of α_2-ARs than other species even under healthy conditions.

In order to get a more detailed picture on which cell types become activated or suppressed under the influence of stress hormones, we assessed cytokine production on the cellular level. In pigs, the T_H1/T_H2 paradigm is not very well investigated, and recent studies have indicated that some cytokine functions are different in pigs compared to other species. The classical T_H2 cytokine IL-4 does not fulfill this role in pigs, as it suppresses both T_H1 and T_H2 immunity including antibody secretion by B cells [43,44]. There are hints that instead of shifting the immune response from T_H1 to T_H2, GCs seem to be generally inhibitory in pigs [45]. Furthermore, IFNγ, which is usually increased in a T_H1 immune response, can be constantly produced in comparatively high concentrations in pigs [46] and is less sensitive to cortisol-mediated inhibition than other cytokines [47,48]. Because pre-tests did not reveal detectable IL-4 amounts upon mitogenic stimulation and IFNγ production was hardly overcoming background production, the effects of stress hormones on cytokine production of porcine PBMC were solely characterized by analysis of TNFα production in the present study. Though in varying amounts, this cytokine is produced by many porcine immune cell types, i.e., monocytes/macrophages, NK cells, γδ T cells, CTL and T_H cells, and is thus a good pan-marker of pro-inflammatory activation [43,49–51].

We discovered that the cortisol-mediated inhibition of immune cell activity did not only result in a reduced lymphocyte proliferation but also in lower numbers of cells producing TNFα in all investigated subsets except NK cells. This is in accordance with studies in humans and rodents, where GCs generally had a suppressive effect on the production of pro-inflammatory cytokines [8,9,52]. In the present study, cell populations of both innate and adaptive immune response were affected, which may have negative effects on the acute response to pathogens as well as memory formation.

While having dose-dependent inhibitory or stimulatory effects on proliferation, none of the tested concentrations of NA had a significant effect on the number of TNFα producers in any of the investigated subsets. Other studies have reported inconsistent results regarding the impact of NA on TNFα production. Some have found an increased number of TNFα producers in human lymphocytes [14], whereas others have observed a decrease of TNFα production in human whole blood cultures [53,54]. This again emphasises the diversity of possible CA actions, and with the present limited data, it would thus be premature to make conclusions about the underlying molecular mechanisms. However, some substantiated speculations about

possible pathways in comparison to literature can be made. Presuming that pigs, similarly to humans, have a low number of ARs on T_H cells in comparison to other immune cells [55], the absent responsiveness of the two T_H cell subsets toward both NA and ADR could be explained. Considering the high number of ARs on NK cells in other species, it is somewhat surprising that cytokine production of porcine NK cells was influenced by none of the stress hormones tested. In other species, NK cell activity is a very sensitive indicator of catecholamine action via β-adrenergic mechanisms [56] and GC-induced immunosuppression [57]. This discrepancy remains subject to future studies.

Interestingly, although PWM-induced proliferation increased significantly under ADR influence, the number of TNFα producers among γδ T cells and monocytes decreased if cultured with 10^{-6} M ADR, while other populations remained unaffected. These puzzling results might be explained by a possible particular action of ADR on regulatory T cells (Tregs). Using human PBMC from breast cancer patients, Zhou et al. [58] demonstrated that an in vitro culture in the presence of ADR resulted in an increased Treg proliferation. If porcine Tregs show the same effect under ADR treatment, their proliferation might also have been enhanced in the present study. As Tregs have an inhibitory effect, especially on the functionality of antigen presenting cells including monocytes [59], they might have hampered TNFα production in monocytes as well as their ability to induce cytokine production in other populations. To verify if Tregs are a special target of ADR action in the pig, studies investigating lymphocyte proliferation on the single cell level using fluorescent dyes, including markers for Foxp3 expression and the analysis of IL-10 concentration in cell culture supernatants, should be conducted. The inhibition of γδ T cells by ADR deserves special emphasis, as their numbers in porcine blood are higher than in mice and humans [60] and they are of great importance, especially in growing pigs [16]. The downregulation of pro-inflammatory cytokines in γδ T cells might therefore have implications for their own role in the early immune response to infections [60], as well as their regulatory function [61] on other immune cells in acute stress situations.

5. Conclusions

Especially in the light of growing public interest in animal welfare and stress assessment in livestock, this study contributes to a better understanding of stress-induced immunomodulation in pigs. The results provide further indications of the immunosuppressive effects of glucocorticoids on immune cell functionality found in previous studies in pigs and other

species. The observed impairment of both innate and adaptive immune cells might have implications on various functions like the elimination of infected cells by CTLs, the induction of B cells by T_H cells, or phagocytosis by macrophages. In addition, catecholamine-mediated inhibitory as well as stimulatory immunomodulation was shown for the first time in pigs, thus letting this serve as a preliminary work for the future assessment of molecular mechanisms of stress hormone actions in pigs. Beside further functional parameters, the number and distribution of the distinct glucocorticoid and adrenoceptor types on different immune cell populations or the effect of receptor blockers should be investigated.

Author Contributions: S.S., V.S., J.S. and L.R. conceived and designed research; L.R. conducted and performed experiments and analyzed data; S.S., and V.S. contributed materials and analysis tools; L.R. wrote and edited the paper; S.S., V.S. and J.S. reviewed the paper. V.S. and J.S acquired funding.

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References

1. Gordan, R.; Gwathmey, J.K.; Xie, L.-H. Autonomic and endocrine control of cardiovascular function. *World J. Cardiol.* **2015**, *7*, 204–214, doi:10.4330/wjc.v7.i4.204.
2. Kyrou, I.; Tsigos, C. Stress hormones: Physiological stress and regulation of metabolism. *Curr. Opin. Pharmacol.* **2009**, *9*, 787–793, doi:10.1016/j.coph.2009.08.007.
3. Dhabhar, F.S. The short-term stress response—Mother nature's mechanism for enhancing protection and performance under conditions of threat, challenge, and opportunity. *Front. Neuroendocrinol.* **2018**, *49*, 175–192, doi:10.1016/j.yfrne.2018.03.004.
4. Marsland, A.L.; Bachen, E.A.; Cohen, S.; Rabin, B.; Manuck, S.B. Stress, immune reactivity and susceptibility to infectious disease. *Physiol. Behav.* **2002**, *77*, 711–716.
5. De Groot, J.; Ruis, M.A.; Scholten, J.W.; Koolhaas, J.M.; Boersma, W.J.A. Long-term effects of social stress on antiviral immunity in pigs. *Physiol. Behav.* **2001**, *73*, 145–158, doi:10.1016/S0031-9384(01)00472-3.

6. Gillis, S.; Crabtree, G.R.; Smith, K.A. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J. Immunol.* **1979**, *123*, 1624–1631.

7. Strauss, G.; Osen, W.; Debatin, K.-M. Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs. *Clin. Exp. Immunol.* **2002**, *128*, 255–266, doi:10.1046/j.1365-2249.2002.01777.x.

8. Gutsol, A.A.; Sokhonevich, N.A.; Seledtsov, V.I.; Litvinova, L.S. Dexamethasone effects on activation and proliferation of immune memory T cells. *Bull. Exp. Biol. Med.* **2013**, *155*, 474–476.

9. Ashwell, J.D.; Lu, F.W.; Vacchio, M.S. Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.* **2000**, *18*, 309–345, doi:10.1146/annurev.immunol.18.1.309.

10. Felsner, P.; Hofer, D.; Rinner, I.; Porta, S.; Korsatko, W.; Schauenstein, K. Adrenergic suppression of peripheral blood T cell reactivity in the rat is due to activation of peripheral alpha 2-receptors. *J. Neuroimmunol.* **1995**, *57*, 27–34.

11. Connor, T.J.; Brewer, C.; Kelly, J.P.; Harkin, A. Acute stress suppresses pro-inflammatory cytokines TNF-alpha and IL-1 beta independent of a catecholamine-driven increase in IL-10 production. *J. Neuroimmunol.* **2005**, *159*, 119–128, doi:10.1016/j.jneuroim.2004.10.016.

12. Strahler, J.; Rohleder, N.; Wolf, J.M. Acute psychosocial stress induces differential short-term changes in catecholamine sensitivity of stimulated inflammatory cytokine production. *Brain Behav. Immun.* **2015**, *43*, 139–148, doi:10.1016/j.bbi.2014.07.014.

13. Kouassi, E.; Li, Y.S.; Boukhris, W.; Millet, I.; Revillard, J.P. Opposite effects of the catecholamines dopamine and norepinephrine on murine polyclonal B-cell activation. *Immunopharmacology* **1988**, *16*, 125–137.

14. Torres, K.C.L.; Antonelli, L.R.V.; Souza, A.L.S.; Teixeira, M.M.; Dutra, W.O.; Gollob, K.J. Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. *J. Neuroimmunol.* **2005**, *166*, 144–157, doi:10.1016/j.jneuroim.2005.06.006.

15. Elenkov, I.J.; Wilder, R.L.; Chrousos, G.P.; Vizi, E.S. The Sympathetic Nerve—An Integrative Interface between Two Supersystems: The Brain and the Immune System. *Pharmacol. Rev.* **2000**, *52*, 595–638.

16. Kick, A.R.; Tompkins, M.B.; Almond, G.W. Stress and immunity in the pig. *CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour.* **2011**, *6*, 1–17, doi:10.1079/PAVSNNR20110018.

17. Deguchi, E.; Akuzawa, M. Effects of fighting after grouping on plasma cortisol concentration and lymphocyte blastogenesis of peripheral blood mononuclear cells induced by mitogens in piglets. *J. Vet. Med. Sci.* **1998**, *60*, 149–153.

18. Li, L.-A.; Xia, D.; Bao, E.-D.; Wei, S.; Xiao, J.-S.; Bao, J.-W.; Chen, W.-H.; Chen, J.; Hartung, J.; Zhao, R.-Q. Erhualian and Pietrain pigs exhibit distinct behavioral, endocrine

and biochemical responses during transport. *Livest. Sci.* **2008**, *113*, 169–177, doi:10.1016/j.livsci.2007.03.008.

19. Rosochacki, S.J.; Piekarzewska, A.B.; Poloszynowicz, J.; Sakowski, T. The Influence of Restraint Immobilization Stress on the Concentration of Bioamines and Cortisol in Plasma of Pietrain and Duroc Pigs. *J. Vet. Med. Physiol. Pathol. Clin. Med.* **2000**, *47*, 231–242, doi:10.1046/j.1439-0442.2000.00284.x.
20. Althen, T.G.; Ono, K.; Topel, D.G. Effect of Stress Susceptibility or Stunning Method on Catecholamine Levels in Swine. *J. Anim. Sci.* **1977**, *44*, 985–989, doi:10.2527/jas1977.446985x.
21. Bacou, E.; Haurogné, K.; Mignot, G.; Allard, M.; de Beaurepaire, L.; Marchand, J.; Terenina, E.; Billon, Y.; Jacques, J.; Bach, J.-M.; et al. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. *Physiol. Behav.* **2017**, *169*, 1–8, doi:10.1016/j.physbeh.2016.11.012.
22. Kanitz, E.; Tuchscherer, M.; Puppe, B.; Tuchscherer, A.; Stabenow, B. Consequences of repeated early isolation in domestic piglets (*Sus scrofa*) on their behavioural, neuroendocrine, and immunological responses. *Brain Behav. Immun.* **2004**, *18*, 35–45.
23. Blecha, F.; Pollmann, D.S.; Nichols, D.A. Weaning pigs at an early age decreases cellular immunity. *J. Anim. Sci.* **1983**, *56*, 396–400.
24. Westly, H.J.; Kelley, K.W. Physiologic concentrations of cortisol suppress cell-mediated immune events in the domestic pig. *Proc. Soc. Exp. Biol. Med.* **1984**, *177*, 156–164.
25. Ciepielewski, Z.M.; Stojek, W.; Glac, W.; Wrona, D. Restraint effects on stress-related hormones and blood natural killer cell cytotoxicity in pigs with a mutated ryanodine receptor. *Domest. Anim. Endocrinol.* **2013**, *44*, 195–203, doi:10.1016/j.domaniend.2013.02.003.
26. Tuchscherer, M.; Kanitz, E.; Puppe, B.; Tuchscherer, A.; Viergutz, T. Changes in endocrine and immune responses of neonatal pigs exposed to a psychosocial stressor. *Res. Vet. Sci.* **2009**, *87*, 380–388, doi:10.1016/j.rvsc.2009.04.010.
27. Jozsa, R.; Olah, A.; Cornélissen, G.; Csernus, V.; Otsuka, K.; Zeman, M.; Nagy, G.; Kaszaki, J.; Stebelova, K.; Csokas, N.; et al. Circadian and extracircadian exploration during daytime hours of circulating corticosterone and other endocrine chronomes. *Biomed. Pharmacother.* **2005**, 59, S109–S116.
28. Ruis, M.A.; Te Brake, J.H.; Engel, B.; Ekkel, E.D.; Buist, W.G.; Blokhuis, H.J.; Koolhaas, J.M. The circadian rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress. *Physiol. Behav.* **1997**, *62*, 623–630.
29. Engert, L.C.; Weiler, U.; Pfaffinger, B.; Stefanski, V.; Schmucker, S.S. Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs. *Dev. Comp. Immunol.* **2018**, *79*, 11–20, doi:10.1016/j.dci.2017.10.003.
30. Griffin, J.F. Stress and immunity: A unifying concept. *Vet. Immunol. Immunopathol.* **1989**, *20*, 263–312.

31. Roth, J.A.; Flaming, K.P. Model systems to study immunomodulation in domestic food animals. *Adv. Vet. Sci. Comp. Med.* **1990**, *35*, 21–41.

32. Kanitz, E.; Otten, W.; Nürnberg, G.; Brüssow, K.P. Effects of age and maternal reactivity on the stress response of the pituitary-adrenocortical axis and the sympathetic nervous system in neonatal pigs. *Anim. Sci.* **1999**, *68*, 519–526, doi:10.1017/S1357729800050542.

33. Kraetzl, W.D., Weiler, U. Erfahrungen mit einem implantierbaren Kathetersystem zur frequenten und chronischen Blutentnahme bei Schafen in Gruppenhaltung und bei säugenden Sauen. *Tierarztl. Umsch.* **1998**, *53*, 567–574.

34. Schalk, C.; Pfaffinger, B.; Schmucker, S.; Weiler, U.; Stefanski, V. Effects of repeated social mixing on behavior and blood immune cells of group-housed pregnant sows (*Sus scrofa domestica*). *Livest. Sci.* **2018**, *217*, 148–156, doi:10.1016/j.livsci.2018.09.020.

35. Felten, D.L.; Felten, S.Y.; Bellinger, D.L.; Carlson, S.L.; Ackerman, K.D.; Madden, K.S.; Olschowki, J.A.; Livnat, S. Noradrenergic sympathetic neural interactions with the immune system: Structure and function. *Immunol. Rev.* **1987**, *100*, 225–260.

36. Bergquist, J.; Tarkowski, A.; Ewing, A.; Ekman, R. Catecholaminergic suppression of immunocompetent cells. *Immunol. Today* **1998**, *19*, 562–567.

37. Kenward, M.G.; Roger, J.H. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **1997**, *53*, 983–997.

38. Faraway, J.J. *Extending the Linear Model with R: Generalized Linear, Mixed Effects and Nonparametric Regression Models*; Chapman & Hall/CRC: Boca Raton, FL, USA, 2006.

39. Roess, D.A.; Bellone, C.J.; Ruh, M.F.; Nadel, E.M.; Ruh, T.S. The effect of glucocorticoids on mitogen-stimulated B-lymphocytes: Thymidine incorporation and antibody secretion. *Endocrinology* **1982**, *110*, 169–175, doi:10.1210/endo-110-1-169.

40. Sapolsky, R.M.; Romero, L.M.; Munck, A.U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* **2000**, *21*, 55–89, doi:10.1210/edrv.21.1.0389.

41. Hadden, J.W.; Hadden, E.M.; Middleton, E. Lymphocyte blast transformation. I. Demonstration of adrenergic receptors in human peripheral lymphocytes. *Cell. Immunol.* **1970**, *1*, 583–595.

42. Kohm, A.; Sanders, V.M. Norepinephrine: A messenger from the brain to the immune system. *Trends Immunol.* **2000**, *21*, 539–542.

43. Gerner, W.; Talker, S.C.; Koinig, H.C.; Sedlak, C.; Mair, K.H.; Saalmüller, A. Phenotypic and functional differentiation of porcine αβ T cells: Current knowledge and available tools. *Mol. Immunol.* **2015**, *66*, 3–13, doi:10.1016/j.molimm.2014.10.025.

44. Murtaugh, M.P.; Johnson, C.R.; Xiao, Z.; Scamurra, R.W.; Zhou, Y. Species specialization in cytokine biology: Is interleukin-4 central to the TH1–TH2 paradigm in swine? *Dev. Comp. Immunol.* **2009**, *33*, 344–352, doi:10.1016/j.dci.2008.06.014.

45. Skjolaas, K.A.; Grieger, D.M.; Hill, C.M.; Minton, J.E. Glucocorticoid regulation of type 1 and type 2 cytokines in cultured porcine splenocytes. *Vet. Immunol. Immunopathol.* **2002**, *87*, 79–87, doi:10.1016/S0165-2427(02)00122-8.

46. Amadori, M.; Cristiano, A.; Ferrari, M. Constitutive expression of interferons in swine leukocytes. *Res. Vet. Sci.* **2010**, *88*, 64–71, doi:10.1016/j.rvsc.2009.05.011.

47. Sutherland, M.A.; Niekamp, S.R.; Johnson, R.W.; van Alstine, W.G.; Salak-Johnson, J.L. Heat and social rank impact behavior and physiology of PRRS-virus-infected pigs. *Physiol. Behav.* **2007**, *90*, 73–81, doi:10.1016/j.physbeh.2006.08.029.

48. Skjolaas, K.A.; Minton, J.E. Does cortisol bias cytokine production in cultured porcine splenocytes to a Th2 phenotype? *Vet. Immunol. Immunopathol.* **2002**, *87*, 451–458, doi:10.1016/S0165-2427(02)00073-9.

49. Fairbairn, L.; Kapetanovic, R.; Sester, D.P.; Hume, D.A. The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. *J. Leukoc. Biol.* **2011**, *89*, 855–871, doi:10.1189/jlb.1110607.

50. Mair, K.H.; Essler, S.E.; Patzl, M.; Storset, A.K.; Saalmüller, A.; Gerner, W. NKp46 expression discriminates porcine NK cells with different functional properties. *Eur. J. Immunol.* **2012**, *42*, 1261–1271, doi:10.1002/eji.201141989.

51. Sedlak, C.; Patzl, M.; Saalmüller, A.; Gerner, W. CD2 and CD8α define porcine γδ T cells with distinct cytokine production profiles. *Dev. Comp. Immunol.* **2014**, *45*, 97–106, doi:10.1016/j.dci.2014.02.008.

52. Kunicka, J.E.; Talle, M.A.; Denhardt, G.H.; Brown, M.; Prince, L.A.; Goldstein, G. Immunosuppression by glucocorticoids: Inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell. Immunol.* **1993**, *149*, 39–49, doi:10.1006/cimm.1993.1134.

53. Maes, M.; Lin, A.; Kenis, G.; Egyed, B.; Bosmans, E. The effects of noradrenaline and alpha-2 adrenoceptor agents on the production of monocytic products. *Psychiatry Res.* **2000**, *96*, 245–253.

54. Van der Poll, T.; Jansen, J.; Endert, E.; Sauerwein, H.P.; van Deventer, S.J. Noradrenaline inhibits lipopolysaccharide-induced tumor necrosis factor and interleukin 6 production in human whole blood. *Infect. Immun.* **1994**, *62*, 2046–2050.

55. Maisel, A.S.; Harris, T.; Rearden, C.A.; Michel, M.C. Beta-adrenergic receptors in lymphocyte subsets after exercise. Alterations in normal individuals and patients with congestive heart failure. *Circulation* **1990**, *82*, 2003–2010, doi:10.1161/01.cir.82.6.2003.

56. Ben-Eliyahu, S.; Shakhar, G.; Page, G.G.; Stefanski, V.; Shakhar, K. Suppression of NK Cell Activity and of Resistance to Metastasis by Stress: A Role for Adrenal Catecholamines and β-Adrenoceptors. *Neuroimmunomodulation* **2000**, *8*, 154–164, doi:10.1159/000054276.

57. Irwin, M. Stress-induced immune suppression: Role of brain corticotropin releasing hormone and autonomic nervous system mechanisms. *Adv. Neuroimmunol.* **1994**, *4*, 29–47, doi:10.1016/S0960-5428(06)80188-9.

58. Zhou, L.; Li, Y.; Li, X.; Chen, G.; Liang, H.; Wu, Y.; Tong, J.; Ouyang, W. Propranolol Attenuates Surgical Stress-Induced Elevation of the Regulatory T Cell Response in Patients Undergoing Radical Mastectomy. *J. Immunol.* **2016**, *196*, 3460–3469, doi:10.4049/jimmunol.1501677.

59. Sojka, D.K.; Huang, Y.-H.; Fowell, D.J. Mechanisms of regulatory T-cell suppression—A diverse arsenal for a moving target. *Immunology* **2008**, *124*, 13–22, doi:10.1111/j.1365-2567.2008.02813.x.

60. Takamatsu, H.-H.; Denyer, M.S.; Stirling, C.; Cox, S.; Aggarwal, N.; Dash, P.; Wileman, T.E.; Barnett, P.V. Porcine gammadelta T cells: Possible roles on the innate and adaptive immune responses following virus infection. *Vet. Immunol. Immunopathol.* **2006**, *112*, 49–61, doi:10.1016/j.vetimm.2006.03.011.

61. Girardi, M. Immunosurveillance and immunoregulation by gammadelta T cells. *J. Investig. Dermatol.* **2006**, *126*, 25–31, doi:10.1038/sj.jid.5700003.

MANUSCRIPT II

Intravenous Infusion of Cortisol, Adrenaline, or Noradrenaline Alters Porcine Immune Cell Numbers and Promotes Innate over Adaptive Immune Functionality

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Abstract

Despite the importance of pigs (*Sus scrofa domestica*) in livestock production and their increasing role as a model organism for human physiology, knowledge about the porcine immune system under the influence of stress hormones is fragmentary. Exceptionally little is known about the effects of catecholamines. Therefore, the aim of this study was to examine the in vivo effects of adrenaline, noradrenaline, and cortisol on number and functionality of porcine blood immune cells. Castrated male pigs (n = 34) were treated with physiological doses of either adrenaline, noradrenaline, or cortisol via i.v. infusion for 48 h. Blood samples were collected before treatment (−24 h, −22 h, 0 h), during treatment (+2 h, +24 h, +48 h), and at 72 h postinfusion. Immune cell numbers and phagocytic activity were evaluated by flow cytometry and lymphocyte proliferation by ^{3}H-thymidine incorporation. Total IgG and IgM Ab levels were determined via ELISA. Pigs receiving cortisol showed strongly decreased adaptive immune cell numbers and increased neutrophils, accompanied by hampered lymphocyte proliferation but increased monocyte phagocytosis. Catecholamine effects on immune cell numbers were mostly similar to cortisol in direction but smaller in intensity and duration. Lymphocyte proliferation was inhibited after 2 h of noradrenaline infusion, and both catecholamines promoted monocyte and neutrophil phagocytosis. These findings indicate a shift from adaptive to innate immunity in stressful situations. This study is the first (to our knowledge) to systematically investigate specific glucocorticoid and catecholamine actions on the porcine immune system in this level of detail and confirms many similarities to humans, thus strengthening the pig as a human model in psychoneuroimmunology.

Key points

- Cortisol strongly decreases porcine adaptive immune cells and increases neutrophils
- Catecholamines exert acute effects on porcine immune cell numbers and function
- All stress hormones promote innate over adaptive immune functionality in pigs

Introduction

Stress is a biological process helping the body to cope with threats, like being attacked by a predator, by activating several neural and endocrine systems. The most important stress systems are the hypothalamic-pituitary-adrenal axis, leading to glucocorticoid (GC) release, and the sympathetic-adrenal-medulla axis, causing an increase in catecholamine (CA) levels. Besides their actions on other physiological systems like cardiovascular or respiratory function (1, 2), both hormone groups can affect numbers and distribution as well as functionality of different immune cell types. At the onset of a stressful situation, the first, quick reaction is the redistribution of immune cells within minutes. As reviewed by Dhabhar (3), the numbers of most immune cell subsets in the blood rise through mobilization from lymphoid organs and the marginated pool, an effect mostly caused by CAs. Subsequently, leukocytes leave the blood stream heading to sites of immune activation (e.g., an injury) or return to reservoir compartments, like the spleen. Through the action of GCs, blood immune cell numbers can decrease to values even below normal, potentially leading to immunosuppression. It was also shown that immune cell functionality is modulated in stress situations, resulting in an initial enhancement of functions like lymphocyte proliferation or antitumor immunity, followed by a decreased activation and, if stress becomes chronic, dysregulation (4–8). To understand this complex regulatory network, studies in traditional models like the laboratory mice were of great value, but because of differences (e.g., in size, diurnal rhythm, or nutrition) they may not always ideally represent human physiology. In recent years, large animal models like the domestic pig (*Sus scrofa domestica*) with a high similarity to humans have therefore gained importance (9–11), but there is still a lack of knowledge in many aspects, including the immune system. Although it is well established that stress does modulate the porcine immune system (12, 13), the underlying hormonal mechanisms are still poorly understood. Particularly, research is needed to clarify whether the action of the two major stress hormone classes, GCs and CAs, on immune cells is comparable between pigs and humans or rodent species. Most studies concerning pigs so far were focused on the predominantly negative effects of chronic stress mediated mostly by their main GC, cortisol (CORT), and found similarities to humans. For example, it was found that administration of adrenocorticotropic hormone, which triggers CORT release, caused an increase in neutrophil numbers but decreased lymphocyte and eosinophil numbers, NK cell cytotoxicity and lymphocyte proliferation (14, 15). The investigation of short-term stress reactions in pigs has long been neglected, and the isolated effects of CAs have not been investigated in in vivo studies so far. It was found in vitro that

CAs had rather contrary effects to CORT on lymphocyte functionality, indicated by an enhanced proliferation after mitogen stimulation (16). This finding is partly contradictory to previous studies in rodents (17) and requires further elucidation through in vivo studies. Furthermore, there are no studies in pigs regarding the impact of CAs on the number of blood immune cells and no valid information on their effects on innate immune functions like phagocytosis. Although pigs in modern intensive husbandry systems are often exposed to acute and chronic stressors like limited space, rehousing, and mixing (18–20), knowledge about the impact of both GCs and CAs is fragmentary, and studies in this field may help to promote animal welfare and health. Also, to further establish the pig as a human model in psychoneuroimmunology, it is necessary to differentiate between the particular effects of each stress hormone to compare the two species. The present study therefore investigated the in vivo effects of i.v. infusion of pigs with either CORT, adrenaline (ADR), or noradrenaline (NA) on both distribution and function of innate and adaptive immune cells.

Material and methods

Animals and surgery

All experimental procedures were approved by the local authority for animal care and use (Regional Council Stuttgart, Germany; V324/15TH) and conducted in accordance with the German Animal Welfare Act. Male castrated pigs (German Landrace × Pietrain, initial body weight of 80–100 kg), bred by the experimental unit of the University of Hohenheim "Unterer Lindenhof," were used in this study, which was conducted in three consecutive trials with 12 animals each. Because of medical conditions, two animals had to be excluded from the study, resulting in a final sample size of $n = 34$. The pigs were housed in individual crates (5.4 m^2) that enabled visual and tactile contact to other pigs. Pens were cleaned twice daily and littered with dust-free wood shavings after concentrate feeding (1.5 kg/meal, metabolizable energy 12 MJ/kg). Access to hay and water was provided ad libitum. Light was turned on at 06:30 h in the morning, 30 min before feeding, and turned off at 20:30 h. All animals were surgically equipped with two indwelling vein catheters by cannulation of the cephalic vein on both sides. The surgery was performed as described previously (21) with few modifications (11). Surgery was carried out at least 12 d before the beginning of the experiment, and animals were thoroughly habituated to human handling and manipulation at the catheters.

Experimental procedure

To evaluate the effects of stress hormones on the porcine immune system, animals were infused with either ADR, NA, CORT, or saline (control [CTRL]) via one of the catheters. Blood was collected using the second catheter. During the infusion period, the first catheter was elongated and attached to a hinge above the pen so that it was out of the pigs' reach. Thereby, animals could move in their pens without restraint. Animals were constantly monitored to ensure continuous infusion delivery. Infusion was carried out with infusion pumps (Eickemeyer, Tuttlingen, Germany) at a rate of 100 ml/h. On the first 2 d (CTRL period), all animals received 0.9% saline (B. Braun Melsungen AG, Melsungen, Germany), then they were randomly assigned to one of four treatment groups.

Eight animals served as the CTRL group and continuously received saline for further 2 d.

Nine animals were treated with CORT (Hydrocortison 100; Rotexmedica, Trittau, Germany) at a dosage of 140 μg/kg/h in saline. This dosage was found to result in plasma CORT concentrations of ∼60 ng/ml (22) and resembles a mild chronic stressor (14, 23).

Eight animals received saline with NA (arterenol; Sanofi-Aventis, Frankfurt am Main, Germany) at a dosage of 15 μg/kg/h. Because of the lack of investigations on the effect of CAs on the porcine immune system, we determined the dosage based on studies examining other parameters under NA infusion. The dosage used in the current study was earlier shown to produce typical NA-induced physiological alterations, such as elevated blood pressure or increased heart rate (24).

Nine pigs were treated with 3 μg/kg/h ADR (adrenalin 1:1000; Infectopharm, Heppenheim, Germany) added to the saline infusion. As with NA, we chose a dosage that led to a mild elevation of blood pressure, heart rate, and body temperature in previous studies, indicating a physiological effect (25).

Each of the three trials included animals of each treatment group.

Blood samples were drawn during infusion at −24 h, −22 h, 0 h, +2 h, +24 h, +48 h (relative to start of stress hormone phase), and 72 h postinfusion as illustrated in FIG. 1. Blood was transferred directly into lithium heparin tubes and K3 EDTA tubes (both Sarstedt, Nümbrecht, Germany) and immediately processed after each sampling. To take diurnal oscillation of hormones and immune cells into account, the −22 h sample was included. Comparisons

between −22 h and +2 h of the CTRL group as well as −24 and 0 h (all animals) confirmed no statistical differences between these time points.

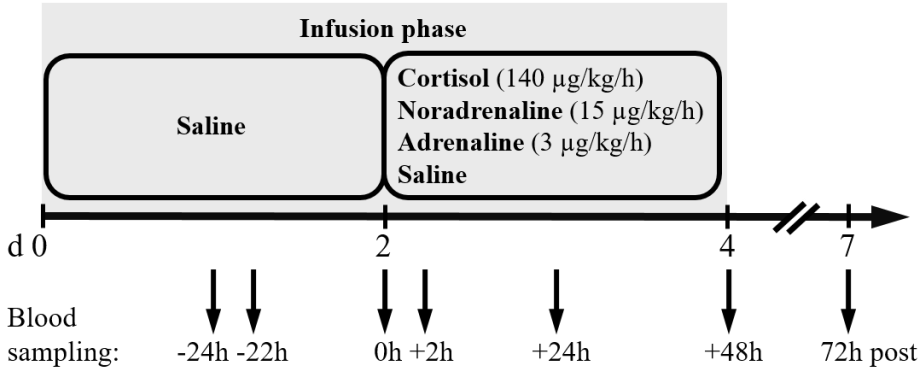

Figure 1: Time course of stress hormone infusion and blood sampling of male castrated pigs. Gray shading indicates the infusion phase, arrows symbolize time points of blood sampling. All pigs received saline for 48 h before stress hormone infusion or continued saline infusion for 48 h. Three subsequent experimental trials were conducted with a total of 34 pigs of which $n = 8$ were treated with saline or NA and $n = 9$ were treated with CORT or ADR.

Hormone determinations

CA. Plasma for the analysis of NA and ADR concentrations was obtained by centrifugation of EDTA (+0.001 pg/ml glutathione) blood (1000 × *g*, 4°C, 10 min, stored at −80°C until analysis). Samples were analyzed by HPLC with electrochemical detection. At the time points 0, 2, 24, and 48 h, all samples from all CA-treated pigs were analyzed, and the other samples were measured on a random basis (minimum $n = 3$ per treatment group and time point, see parentheses in TABLE I). The sample preparation with alumina extraction was adapted from the method first described by Anton and Sayre (26). In brief, 1 ml of plasma and 500 pg of an internal standard (dihydroxybenzylamine; Thermo Fisher Scientific, Darmstadt, Germany) were added to extraction tubes containing 20 mg of aluminum oxide previously activated with 600 µl of 2 M Tris/EDTA buffer (pH 8.7). Samples were thoroughly mixed in an overhead shaker for 10 min and centrifuged at 1000 × *g* for 1 min (4°C). Samples were washed three times with 1 ml of 16.5 mM Tris/EDTA buffer (pH 8.1), followed by centrifugation. The CAs were eluted by addition of 120 µl of eluting solution (Recipe, Munich, Germany), short mixing, and centrifugation at 1000 × *g* for 1 min (4°C). Aliquots of 50 µl were injected into the HPLC system (ISO-3100BM; Thermo Fisher Scientific) with electrochemical detector (Coulochem III, conditioning cell [model 50210A], analytical cell [model 5011A]; Thermo Fisher

Scientific). The potentials of the cells were set at 300, 50, and −250 mV. The system was equipped with the column Reprosil Pur 120 C18-AQ (4.6 mm × 75 mm) (A. Maisch, Ammerbuch, Germany). Cat-A-Phase II was used as the mobile phase, with a flow rate of 1.1 ml/min. Concentrations were evaluated by means of the internal standard method using peak areas. The system was prepared for the detection of high analyte concentrations, therefore all measured concentrations below 200 pg/ml were set to 150 pg/ml (NA) or 50 pg/ml (ADR) for statistical evaluation and values below the detection limit were set to 10 pg/ml. The intra-assay coefficients of variance (CV) were determined with biological samples spiked with 500 and 1000 pg/ml NA or ADR. They were 4.9 and 1.5% for NA, and 5.4 and 1.7% for ADR. The interassay CV were tested with biological samples approximately within the range of high and low control for the measured samples. The interassay CV were 27.5, 13.6, and 13.2% for NA (samples with 550, 1000, 2000 pg/ml) and 20.3 and 14.2% for ADR (samples with 400 and 650 pg/ml).

CORT. Plasma was obtained by centrifugation of Li-heparin-blood at 1000 × *g* at 4°C for 10 min and, until analysis, samples were stored at −20°C. For determination of CORT concentrations, a RIA was conducted after ethanolic extraction as described previously (27). As a tracer, 1.2-^{3}H-CORT (50 Ci/mmol; Hartmann Analytic, Braunschweig, Germany) was used. For calibration, a dilution series from 2 to 200 ng/ml CORT (Sigma-Aldrich) was prepared in charcoal-stripped plasma. Repeatability was determined with biological samples (25 and 40 ng/ml). The intra-assay CV was 6.55%, and the interassay CV was 9.98%.

Flow cytometry and hematology

Total WBC counts in EDTA blood were analyzed using an automated hematology analyzer (pocH 100-iV Diff; Sysmex, Norderstedt, Germany). To determine the relative numbers of various leukocyte subsets, heparinized whole blood was analyzed with three-color flow cytometry after immunofluorescent Ab staining. For a detailed description of the staining procedure, see Engert et al. (11). Briefly, cells were stained with combinations of the following mAbs: mouse anti-pig CD3ε (IgG1, clone PPT3, SPRD), mouse anti-pig CD4 (IgG2b, clone 74-12-4, FITC), mouse anti-pig CD8α (IgG2a, clone 76-2-11, FITC or PE), and mouse anti-pig CD172a (IgG1, clone 74-22-15, PE) (all SouthernBiotech, Birmingham, AL). Subsequently, cells were fixed and erythrocytes were lysed using BD FACS Lysing Solution (BD Biosciences, Heidelberg, Germany) and stored at 4°C until analysis (not exceeding 1 h). For flow cytometric determination (BD FACSCalibur; BD Biosciences), the software BD CellQuest Pro 6 (BD Biosciences) was used. Granulocytes were differentiated from PBMC based on size and

granularity and then further divided into neutrophils and eosinophils by the autofluorescent properties of eosinophils. Leukocytes were categorized by marker expression into the following subsets: $CD3^+CD4^+CD8\alpha^-$ (naive T_H cells), $CD3^+CD4^+CD8\alpha^+$ (Ag-experienced T_H cells), $CD3^+CD4^-CD8\alpha^{high}$ (CTL), $CD3^+CD4^-CD8\alpha^{-/dim}$ ($\gamma\delta$ T cells), $CD3^-CD8\alpha^+CD172a^-$ (NK cells), $CD3^-CD8\alpha^-CD172a^{high}$ (monocytes), $CD3^-CD8\alpha^-CD172a^{dim}$ (mainly dendritic cells [DC]), and $CD3^-CD8\alpha^-CD172a^-$ (mainly B cells). The gating strategy is illustrated in SUPPLEMENTAL FIG. 1. By combining flow cytometric analysis of relative cell numbers and hematologic total leukocyte count, the absolute cell number of each particular immune cell type was calculated.

IgG and IgM concentrations

Total IgG and IgM concentration in plasma was determined via ELISA as described previously (28). In brief, 96-well flat-bottom microtiter plates (Thermo Fisher Scientific) were coated with 200 ng/well goat anti-pig-IgG_{Fc} (Bethyl Laboratories, Montgomery, TX) or 1000 ng/well goat anti-pig IgM Ab (Bethyl). After incubation for 60 min at room temperature (RT), plates were blocked with 1% BSA (Roth, Karlsruhe, Germany) at 4°C overnight. Plasma was added at a dilution of 1:50,000 (IgG) or 1:15,000 (IgM) and incubated at RT for 60 min. After washing, HRP-conjugated goat anti-pig-IgG_{Fc} or -IgM (Bethyl) was added and incubated for 60 min at RT, and, after washing with PBS, tetramethylbenzidine (AppliChem, Darmstadt, Germany) was added. After 20 min at RT, the reaction was stopped with 2 M H_2SO_4 (Roth), and color formation was measured photometrically at 450 nm with a Power Wave X plate reader (Bio-Tek Instruments, Bad Friedrichshall, Germany). Intra-assay CV was 7.7% for IgG and 6.85% for IgM determination, and interassay CV was 17.2% for IgG and 19.6% for IgM.

Functional assays

Isolation of PBMC. For analysis of lymphocyte proliferation, PBMC were separated from heparinized whole blood by density centrifugation using Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany) modified after Grün et al. (20). Leucosep tubes were filled with 16 ml of Biocoll cell separation solution and overlaid with 16 ml of blood 1:2 diluted with PBS (Biochrom, Berlin, Germany). After centrifugation (11 min, 1000 × *g*, RT), the PBMC layer was transferred to a fresh Falcon Tube (Sarstedt), and cells were washed first with PBS + 1% EDTA (Sigma-Aldrich, Munich, Germany) and subsequently with RPMI-5 (RPMI-1640 supplemented with 5% FCS and 50 μg/ml gentamicin) by centrifugation for 10 min at 300 × *g* and RT. The cells were resuspended in RPMI-10 (RPMI-1640 + 10% FCS + 50 μg/ml

gentamicin) (all Biochrom), and cell concentration was determined with a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany).

Lymphocyte proliferation assay. For the assessment of lymphocyte proliferative capacity, a mitogen-induced ^{3}H-thymidine proliferation assay was performed as described previously (29) with a few modifications. PBMC of each animal were transferred into U-bottom 96-well cell culture plates (Neolab, Heidelberg, Germany) with 1.5×10^5 cells per well and were stimulated in triplicate with 5 µg/ml of the mitogens ConA or PWM (both Sigma-Aldrich) or left unstimulated. Cell culture plates were incubated for 48 h (39°C, 5% CO_2), after which 0.25 µCi of tritiated thymidine (PerkinElmer, Rodgau, Germany) was added and cells were incubated for further 24 h at the same conditions. Cells were harvested on glass fiber filters (Sigma-Aldrich) and dried overnight at RT. The incorporated radioactivity was analyzed with a Tri-Carb 2800 TR liquid scintillation analyzer (PerkinElmer) after addition of 3.6 ml of IrgaSafe Gold (PerkinElmer). The mean cpm was calculated from triplicates, and cpm of the unstimulated control was subtracted to get the Δcpm. Intra-assay CV was below 10%, and interassay CV (determined by using frozen porcine PBMC of one untreated animal) was <5% for PWM and <10% for ConA.

Phagocytosis assay. Number and efficiency of phagocytosing monocytes and neutrophil granulocytes was assessed using a phagocytosis kit (Phagotest; Glycotope Biotechnology GmbH, Heidelberg, Germany) with opsonized and FITC-labeled *Escherichia coli* bacteria according to the manufacturer's instructions except for a few modifications. Instead of using 100 µl of heparinized whole blood for all samples, we determined the number of neutrophils and monocytes with an automated hematology analyzer (pocH 100-iV Diff) and adjusted the applied blood volume to always contain 5×10^5 phagocytes in the assay. For fixation of cells and lysis of RBCs, BD FACS Lysing Solution (BD Bioscience) was used. To determine the frequency of phagocytosing cells, neutrophils were identified by their size and granularity before determining FITC-positive cells among all neutrophils. Because of the insufficient separability of monocytes from other PBMC via forward and side scatter, cells were gated for all PBMC, and monocyte numbers among PBMC were calculated using the flow cytometry data. As a measure for the amount of phagocytosed *E. coli* per neutrophil or monocyte, geometric mean fluorescence intensity of FITC was recorded.

Statistical analysis. For statistical analysis, we used the software SAS Version 9.4 (SAS Institute, Cary, NC). A linear mixed model analysis was performed using the MIXED procedure with "animal" included as a repeated factor to take the individual baseline of each animal into

account. The factors “treatment,” “trial,” as well as “treatment(time point)” were included as fixed effects, and “animal,” “dam,” “sire,” “pen,” and “time point × trial” were considered as random effects. The restricted maximum likelihood method was used to estimate variance components, and df were determined by the Kenward-Roger method (30). Normal distribution and variance homogeneity were confirmed visually with normal probability plots and plots of fitted values versus residuals (31). Differences between least square means (LS-means) of treatment groups at each time point were evaluated using the Fisher least significant difference test. All results are presented as LS-mean ± SEM, p values <0.05 are defined as statistically significant, and p values <0.1 are defined as a tendency.

Results

Plasma stress hormone concentrations

All hormones caused a significant enhancement of their plasma concentrations during the whole stress hormone infusion period (+2 h, +24 h, and +48 h) compared with the CTRL group at each respective time point (TABLE I). None of the treatment groups showed an enhanced concentration for those hormones they were not treated with. NA-treated pigs had decreased ADR levels at +24 h.

Table I. Plasma concentrations of CORT, NA, or ADR before, during, and after hormone infusion

Measured Hormone Concentration / Treatment Group	Time Point Relative to Start of Stress Hormone Infusion						
	-24h	**-22h**	**0h**	**+2h**	**+24h**	**+48h**	**72h After**
CORT ng/mL							
CTRL	26.12 ±1.74 (34)	18.88 ±1.74 (34)	24.32 ±1.74 (34)	23.97 ±3.27 (8)	30.36 ±3.22 (8)	24.92 ±3.22 (8)	30.43 ±3.22 (8)
CORT				57.47*** ±3.08 (9)	56.61*** ±3.03 (9)	49.88*** ±3.03 (9)	25.47 ±3.03 (9)
NA				16.66 ±3.27 (8)	30.36 ±3.21 (8)	24.54 ±3.21 (8)	26.88 ±3.21 (8)
ADR				15.99^{t} ±3.10 (9)	25.60 ±3.05 (9)	27.62 ±3.05 (9)	24.71 ±3.05 (9)
NA pg/mL							
CTRL	194.56 ±24.26 (12)	169.49 ±21.13 (12)	204.10 ±19.49 (26)	164.18 ±37.38 (3)	155.54 ±26.05 (6)	141.30 ±31.17 (3)	152.36 ±25.52 (6)
CORT				164.75 ±37.51 (3)	157.41 ±26.36 (6)	164.75 ±36.34 (3)	157.41 ±26.36 (6)
NA				5000.05*** ±771.60 (8)	6244.02*** ±932.78 (8)	5148.85*** ±769.41 (8)	192.36 ±28.74 (8)
ADR				169.21 ±24.81 (9)	177.26 ±25.21 (9)	172.21 ±24.49 (9)	177.81 ±25.29 (9)
ADR pg/mL							
CTRL	44.93 ±9.65 (12)	44.93 ±9.65 (12)	42.22 ±6.25 (26)	26.44 ±11.41 (3)	42.86 ±12.99 (6)	24.45 ±10.13 (3)	32.77 ±9.94 (6)
CORT				46.27 ±19.96 (3)	47.31 ±14.34 (6)	46.99 ±19.48 (3)	47.31 ±14.34 (6)
NA				11.32 ±3.08 (8)	14.12** ±3.75 (8)	14.18 ±3.77 (8)	38.72 ±10.28 (8)
ADR				738.75*** ±189.53 (9)	835.16*** ±209.27 (9)	400.69*** ±100.40 (9)	41.83 ±10.48 (9)

Data are expressed as LS-mean 6 SEM. Asterisks indicate significant differences to the CTRL group (continuous saline infusion) at each respective time point (***$p < 0.001$, **$p < 0.01$, $^{t}p < 0.1$). Numbers of measured samples are indicated in parentheses.

Leukocyte numbers and subsets

During the CTRL period, the investigated leukocyte subsets were similar to previous studies regarding numbers and diurnal pattern (11, 32, 33). In the stress hormone phase, all of the applied hormones caused changes in the number of distinct leukocyte subsets, as illustrated in FIG. 2. After 2 h, the number of lymphocytes decreased in CORT-treated animals and dropped even more drastically by almost 50% at 24 and 48 h. The NA-treated animals showed similarly decreased lymphocyte numbers after 2 h but returned to CTRL level from 24 h onwards. ADR treatment, in contrast, had no effect on lymphocyte numbers after 2 h, but they increased slightly after 24 h before returning to CTRL level by the end of infusion. Opposite to lymphocytes, neutrophil granulocytes were increased during the complete CORT infusion phase, reaching numbers more than twice as high as the CTRL before dropping below CTRL level after cessation of the infusion. Again, NA-treated animals showed a similarly directed

effect after 2 and 24 h but to a lesser extent with an elevation of ∼25%. ADR treatment had no effect on the number of neutrophil granulocytes. In contrast to their neutrophil counterpart, the numbers of eosinophil granulocytes were decreased at all time points during CORT infusion, most pronounced after 24 h, when numbers were only about one-third of those of the CTRL group. Again, NA exerted similar effects but only after 24 and 48 h of infusion, and numbers decreased less strongly to a level of about two-thirds of the CTRL group. ADR only had a weak reducing effect on the number of eosinophils, shown as a tendency after 24 h ($p = 0.08$) and 48 h ($p = 0.09$) of infusion. Monocyte numbers were not affected during CORT infusion but showed a tendency ($p = 0.09$) to increase after its end. NA infusion caused an increase of monocytes by ∼15% after 24 h. Similarly, ADR raised monocyte numbers after 24 h of infusion, which returned to CTRL level after 48 h and showed a tendency ($p = 0.06$) to decrease below CTRL 72 h after termination of the treatment. DC were decreased under the influence of CORT at all time points during and after infusion, dropping to almost half of the numbers of CTRL animals at 24 and 48 h. NA infusion also tended to decrease DC after 2 h ($p = 0.09$) and 48 h ($p = 0.09$) and led to a significant reduction 72 h after cessation of the infusion, whereas ADR only showed a tendency to decrease DC numbers after 48 h ($p = 0.07$). NK cells tended to be lower in CORT-treated animals compared with the CTRL group after 2 h ($p = 0.09$) of infusion and were significantly lower 72 h after termination of the treatment. Whereas NA left NK cell numbers unaffected, ADR caused a sharp peak in numbers after 2 h, reaching about twice the number of the CTRL group. B cell counts decreased in CORT-treated animals by ∼15% after 24 h and stayed significantly lower until 72 h after infusion, with a non–statistically significant decrease already being apparent at 2 h infusion time ($p = 0.09$). Meanwhile, neither NA nor ADR had an effect on the number of B cells. In CORT-treated animals, T cells decreased successively at 2, 24, and 48 h, reaching about half the numbers of the CTRL group at 48 h. The number of T cells in the NA group was lower after 2 h but not after 24 and 48 h. The slight initial decrease was also seen in the ADR group after 2 h, but in this group the T cell count was increased to ∼10% above CTRL after 24 h before returning to CTRL level at 48 h. For a more-detailed picture, we looked at stress hormone effects on some T cell subsets. Analogous to its effect on total T cells, CORT caused a prominent decrease in the numbers of all investigated subsets during the complete infusion phase and a return to CTRL level after termination. The only exceptions were naive T_H cells and $CD8^-$ γδ T cells, which were slightly increased 72 h after stop of infusion. The pattern observed in total T cell numbers in response to NA was reflected in all investigated T cell subsets, namely a decline after 2 h before returning to CTRL level from 24 h onwards. Like in CORT-treated animals, NA led to increased numbers

of naive T_H cells 72 h after the end of infusion. For ADR, the distinct T cell subsets showed a more differential picture: the decrease seen in total T cells after 2 h was also observed as a tendency in CTL ($p = 0.06$) and a significant decline in $CD8^-$ T_H and γδ T cell subsets. The increase in total T cells after 24 h was reflected by total T_H and γδ T cells and their $CD8^-$ subsets, whereas $CD8^+$ T_H and γδ T cells as well as CTL remained unaffected by ADR. Reflecting the changes of immune cell numbers, the neutrophil/lymphocyte ratio as well as T_H/CTL ratio was altered by stress hormone infusion. At all time points during infusion, CORT elevated the neutrophil/lymphocyte ratio with an almost 4-fold increase at 24 h. NA also exerted this effect but only after 2 h and to a lesser extent. The T_H/CTL ratio was elevated in CORT-treated animals after 24 h and in NA-treated animals after 2 h.

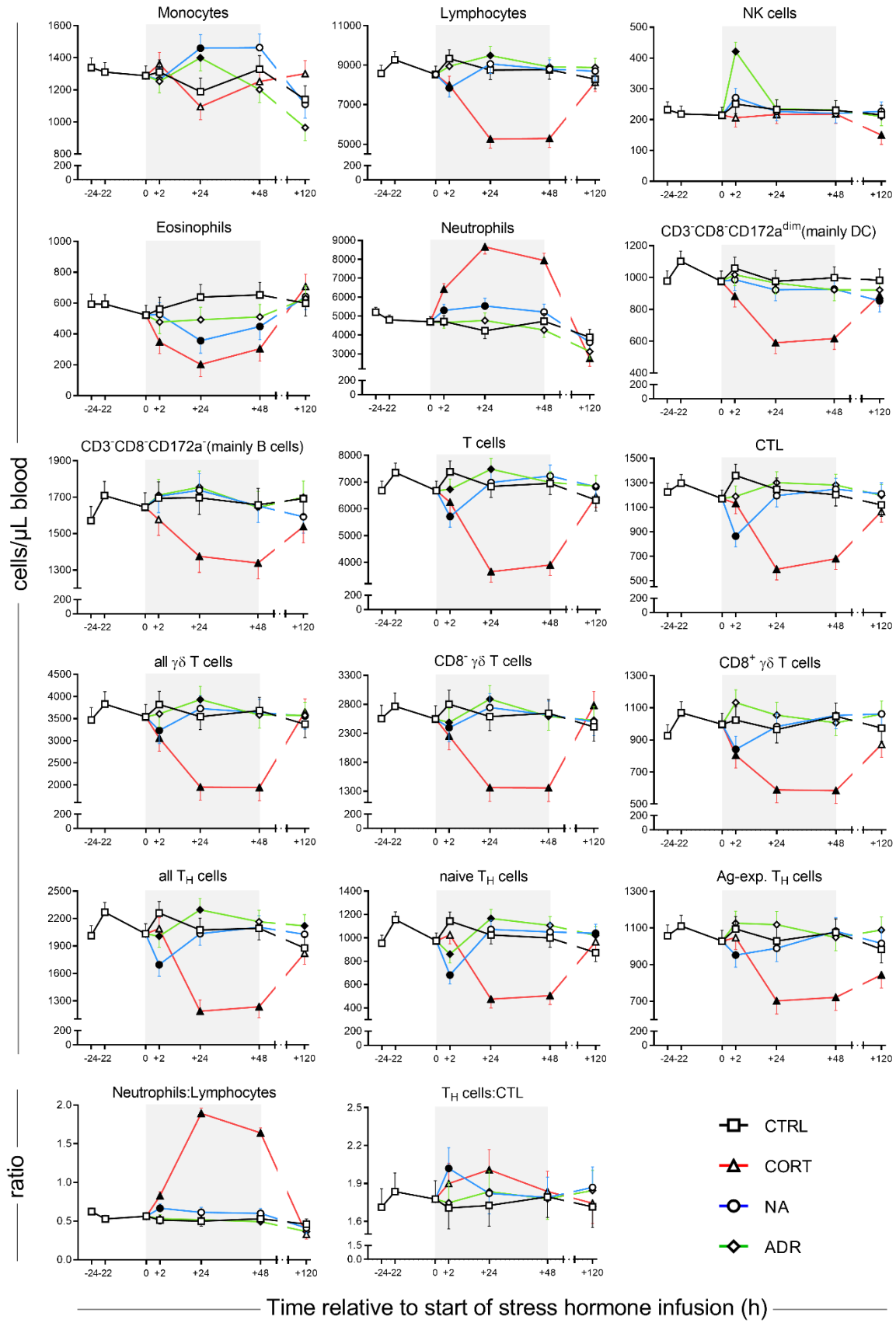

Figure 2: Immune cell numbers before, during (gray background), and after stress hormone infusion (100 ml/h) of male castrated pigs. Red lines: CORT (140 µg/kg/h); blue lines: NA (15 µg/kg/h); green lines: ADR (3 µg/kg/h); black lines: 0.9% saline (CTRL). Data were obtained from three subsequent

experimental trials with a total of 34 pigs of which $n = 8$ were treated with saline or NA and $n = 9$ were treated with CORT or ADR. Results are depicted as LS-mean ± SEM. Filled symbols indicate significant differences ($p < 0.05$) to CTRL group at the respective time point. Ag-exp., Ag-experienced.

IgG and IgM concentration

As described in TABLE II, none of the treatments caused any differences in IgG or IgM plasma concentrations during the whole course of the experiment.

Table II. Plasma concentrations of IgG and IgM before, during, and after hormone infusion

Measured Ig Class / Treatment Group	**Time Point Relative to Start of Stress Hormone Infusion**						
	-24h	**-22h**	**0h**	**+2h**	**+24h**	**+48h**	**72h After**
IgG (µg/mL)							
CTRL	6930.79 ±357.23	6684.59 ±357.06	6720.26 ±357.44	6972.84 ±390.52	6880.81 ±402.43	6781.89 ±402.43	7230.19 ±407.79
CORT				6897.91 ±384.97	7101.67 ±394.91	6977.23 ±394.91	6637.24 ±394.91
NA				6801.34 ±389.77	6784.93 ±402.43	6535.62 ±407.79	6923.97 ±407.79
ADR				6802.14 ±385.87	7382.8 ±396.33	7288.35 ±396.32	7379.67 ±396.32
IgM (µg/mL)							
CTRL	47.32 ±1.83	47.23 ±1.83	47.69 ±1.83	47.15 ±1.95	49.54 ±1.98	47.94 ±1.98	49.33 ±1.98
CORT				47.92 ±1.92	48.89 ±1.95	48.43 ±1.95	47.47 ±1.95
NA				48.69 ±1.94	48.14 ±1.96	47.60 ±1.96	48.01 ±1.96
ADR				47.31 ±1.93	48.22 ±1.95	48.66 ±1.95	49.85 ±1.95

Treatment groups received either saline (CTRL), 140 µg/kg/h cortisol (CORT), 15 µg/kg/h noradrenaline (NA) or 3 µg/kg/h adrenaline (ADR). Data are expressed as least-square means ± standard error of the mean (SEM), n = 34 for all time points. Statistical analysis revealed no significant differences between any of the treatments and the control group at all investigated time points.

Lymphocyte proliferation

To investigate the effect of stress hormone infusion on some functional parameters, we assessed lymphocyte proliferation (FIG. 3). CORT infusion led to a lower proliferation after 24 and 48 h if cells were stimulated with ConA, whereas PWM-stimulated proliferation was only significantly lower after 24 h and tended to be hampered after 48 h ($p = 0.09$). Already after 2 h of infusion, proliferation was decreased in lymphocytes from NA-treated animals independent of mitogen. At the later time points, no effects on proliferation were observed if pigs were infused with NA. No change in proliferation was observed in ADR-treated animals at any of the investigated time points.

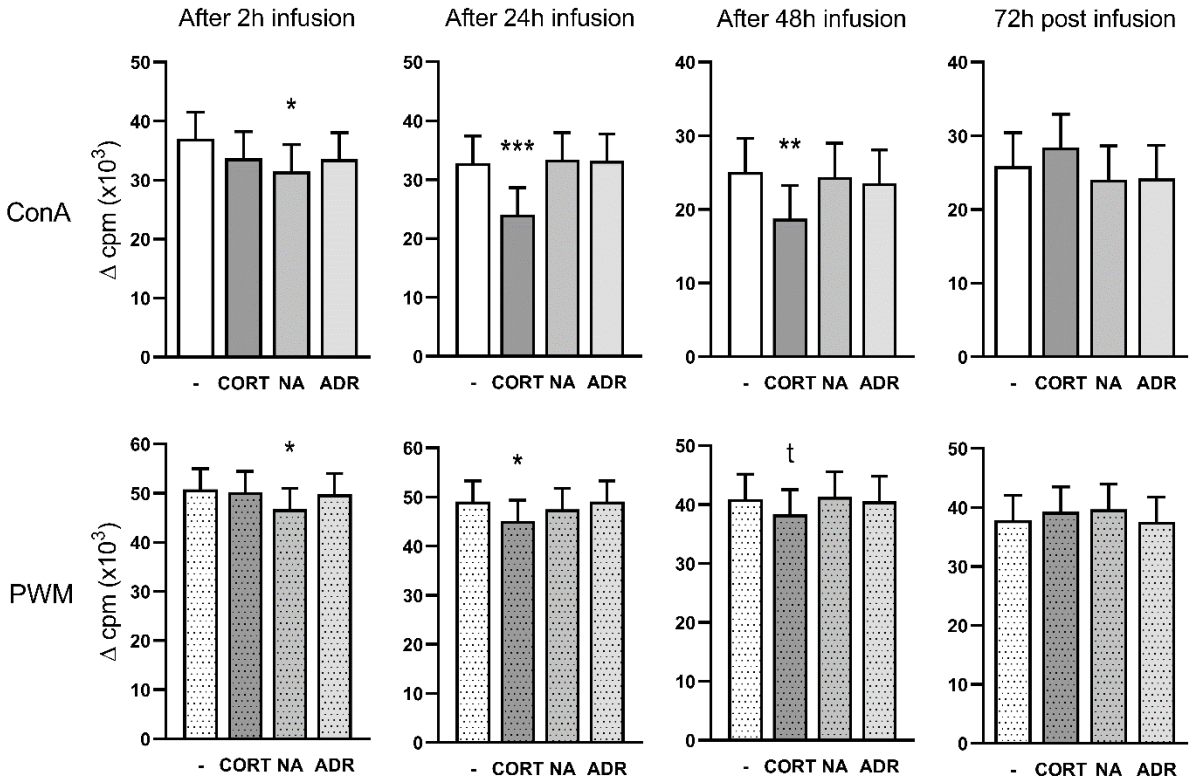

Figure 3: Effect of stress hormone infusion on mitogen-induced proliferation of porcine PBMC. Proliferation in shown in Δcpm after stimulation of PBMC with ConA (upper panel) or PWM (lower panel) after 2, 24, and 48 h stress hormone infusion (– = saline CTRL, CORT = 140 µg/kg/h CORT, NA = 15 µg/kg/h NA, ADR = 3 µg/kg/h ADR) as well as 72 h after end of infusion. Data were obtained from three subsequent experimental trials with a total of 34 pigs of which $n = 8$ were treated with saline or NA and $n = 9$ were treated with CORT or ADR. Results are presented as LS-mean + SEM, asterisks indicate significant differences to CTRL group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $^{t}p < 0.1$.

Phagocytosis

For a measure of innate immune reactivity, we conducted a whole blood phagocytosis assay. We found that the frequencies of phagocytosing monocytes showed only little variance in response to hormone infusion, depicted as a decline after 24 h in the CORT-treated animals. However, the phagocytic activity of monocytes was increased by all three hormones after 24 h of infusion and in CORT-treated pigs also after 48 h (FIG. 4). The frequencies of phagocytosing neutrophils remained constant during the whole observational period, reaching almost 100% in all treatment groups (FIG. 5). Similar to monocytes, neutrophil phagocytic activity was stimulated by stress hormones but only by CAs. After 24 h, NA caused enhanced phagocytic activity, which was also seen in ADR-treated animals as a tendency ($p = 0.09$). For NA, this

enhancement was still present after 24 h as a tendency ($p = 0.09$). CORT had no influence on phagocytosis by neutrophils at any investigated time point.

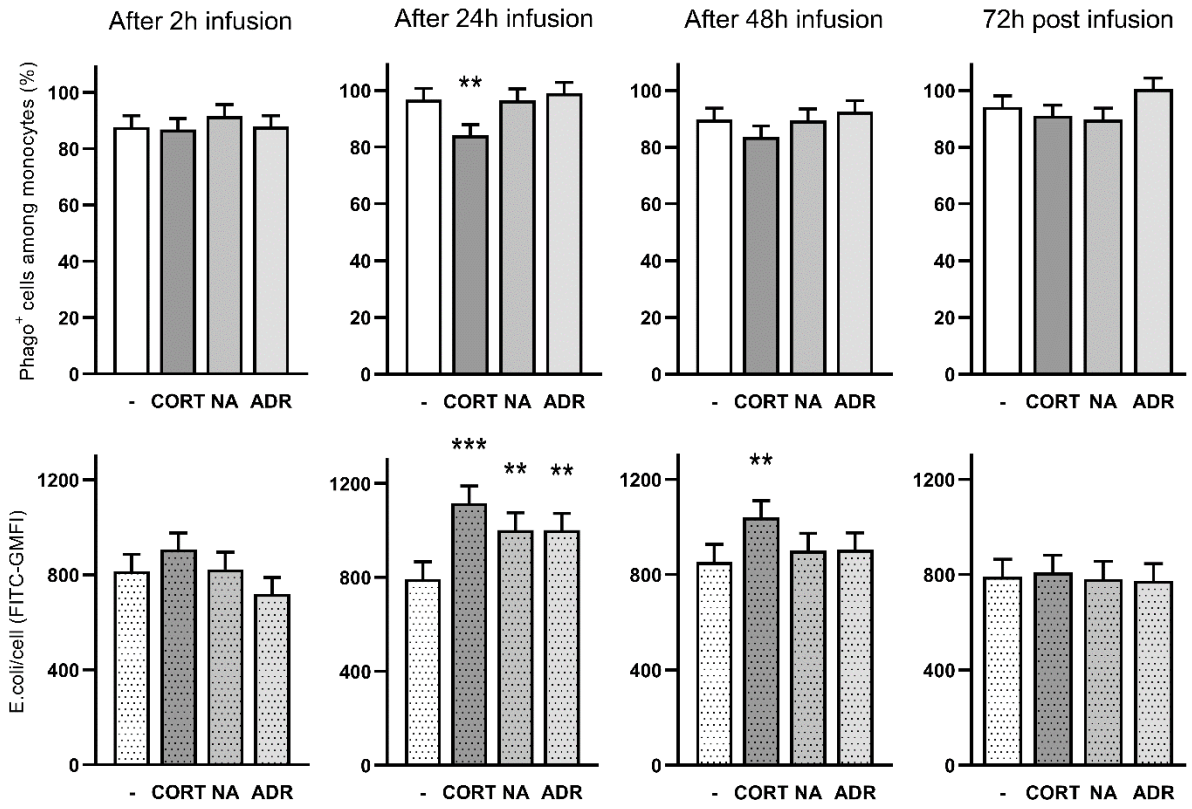

Figure 4: Effect of stress hormone infusion on phagocytic activity of porcine monocytes. Number of phagocytosing monocytes among all monocytes in percent (upper panel) and engulfed FITC-fluorescent particles per monocyte (expressed as geometric mean fluorescence [GMFI], lower panel) after 2, 24, and 48 h stress hormone (– = saline CTRL, CORT = 140 µg/kg/h CORT, NA = 15 µg/kg/h NA, ADR = 3 µg/kg/h ADR) as well as 72 h after end of infusion. Data were obtained from three subsequent experimental trials with a total of 34 pigs of which $n = 8$ were treated with saline or NA and $n = 9$ were treated with CORT or ADR. Results are presented as LS-mean + SEM, asterisks indicate significant differences to CTRL group. $^{**}p < 0.01$, $^{***}p < 0.001$.

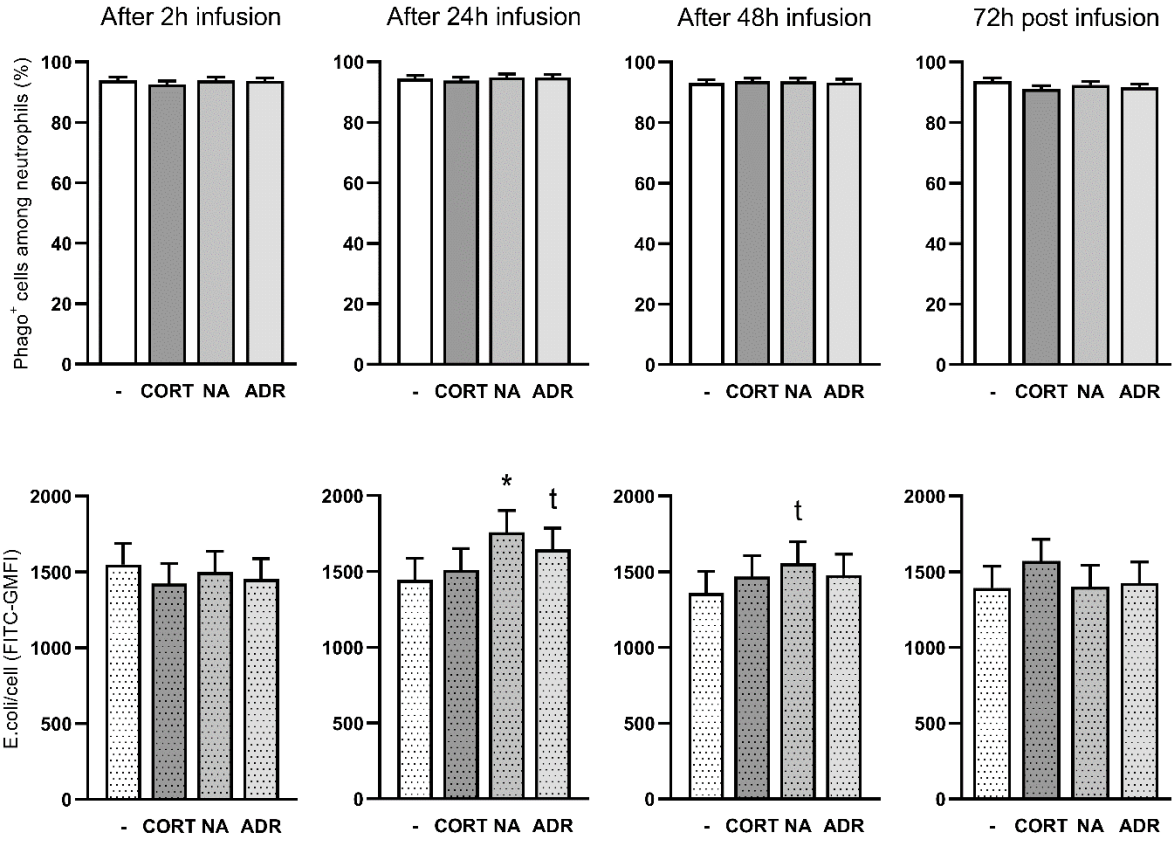

Figure 5: Effect of stress hormone infusion on phagocytic activity of porcine neutrophils. Number of phagocytosing neutrophil granulocytes among all neutrophils in percent (upper panel) and engulfed FITC-fluorescent particles per neutrophil (expressed as geometric mean fluorescence [GMFI], lower panel) after 2, 24, and 48 h stress hormone infusion (– = saline CTRL, CORT = 140 µg/kg/h CORT, NA = 15 µg/kg/h NA, ADR = 3 µg/kg/h ADR) as well as 72 h after end of infusion. Data were obtained from three subsequent experimental trials with a total of 34 pigs of which $n = 8$ were treated with saline or NA and $n = 9$ were treated with CORT or ADR. Results are presented as LS-mean + SEM, asterisks indicate significant differences to CTRL group. *$p < 0.05$, $^{t}p < 0.1$.

Discussion

In the current study, we investigated the effects of three important stress hormones on porcine immune cells separately via i.v. infusion. We demonstrated differences in the numbers of distinct immune cell subsets as well as selected innate and adaptive immune functions and found similarities to stress hormone effects on human immune cells. An overview of the main findings discussed in this article and their comparability to human studies is given in TABLE III.

Table III. Summary of main CORT, NA, and ADR effects on porcine blood immune cells and comparison to humans

Applied Hormone Investigated Parameter	**Effect**	**Comparison to Human Studies**
CORT		
Innate immune cell numbers	NK = Mono, Eos, DC ↓ Neutro ↑	 Eos ↓ ↑
Adaptive immune cell numbers	↓	↓
Lymphocyte proliferation	↓	↓
Neutrophil phagocytosis	=	↑
Monocytic phagocytosis	↑	↑
NA		
Innate immune cell numbers	NK, DC = Eos ↓ Mono (2h), Neutro ↑	 ↓ ↑
Adaptive immune cell numbers	B cells = All T cells ↓ (2h)	= ↓
Lymphocyte proliferation	↓ (2h)	↓
Neutrophil phagocytosis	↑	↑
Monocytic phagocytosis	↑	↑
ADR		
Innate immune cell numbers	Eos, Neutro, DC = Mono, NK ↑ (2h)	Eos ↓; Neutro ↑ ↑
Adaptive immune cell numbers	B cells, $CD8^+$ T cells = $CD8^-$ T cells ↓ (2h)	B cells ↑ ↓
Lymphocyte proliferation	=	↑
Neutrophil phagocytosis	(↑)	↑
Monocytic phagocytosis	↑	↑

Overview of the main findings of the current study and their comparability to humans as presented in the discussion.

Eos, eosinophils; Mono, monocytes; Neutros, neutrophils; ↓, decrease; ↑, increase; =, no effect.

Previous studies in pigs investigating the effects of GC administration often used synthetic analogs with a high immunosuppressive potential (34, 35). We intended to simulate a lifelike stress hormone elevation by using a moderate dosage of the natural GC in pigs, CORT. The desired plasma level of ~60 ng/ml was achieved with an average concentration of 55 ng/ml in the CORT-treated pigs, whereas both CTRL pigs and CA-treated pigs stayed at baseline levels of around 24 ng/ml. This is particularly important as it verifies that the application of neither of these hormones triggered the release of CORT. HPLC determinations of CAs confirmed this finding for plasma ADR and NA concentrations, therefore all detected effects can be solely attributed to the action of one particular hormone. Thus, it can be said that by infusion of CORT,

an endocrine situation similar to that of animals with a reactive coping style, which leads to an elevation of CORT without increases in CA levels, can be imitated (36). The obtained elevation of CORT concentrations resembles a physiologic stressor, and comparable levels were found in pigs exposed to, for example, shipping stress (23) or mixing (37, 38). Although having little effect on total leukocyte numbers, CORT treatment caused drastic changes in the numbers of innate and adaptive immune cell subsets. This is in accordance with other studies in pigs that produced comparable plasma CORT concentrations by injection of adrenocorticotropic hormone and observed no effect on total leukocytes while neutrophils increased and lymphocytes decreased, similar to the results presented in this article (14). The inverted course of blood numbers of these cell types is characteristic for stressful situations with elevated GC concentrations and has been described both in pigs (39) and humans (40), indicating once more the similarity of these species. In contrast, the neutrophilia caused by social stress in rats was shown to be exclusively CA induced (41). The decrease of porcine lymphocyte numbers involved all investigated subsets and could be caused either by apoptosis (42, 43) or by redistribution and migration to lymphoid organs and other tissues, especially the bone marrow (44–47). Because almost all subsets returned to baseline levels after infusion, the latter appears to be the predominant cause in this study. However, DC, B cell, and Ag-experienced T_H cell numbers did not fully regenerate after infusion, indicating partially hampered immune surveillance even after normalization of CORT levels. Opposed to the strong increase in neutrophil numbers, eosinophil granulocytes were reduced by CORT treatment. This negative correlation between CORT levels and eosinophil numbers has been known for a long time in humans (48) and was recently confirmed by our group for the domestic pig in a diurnal context (11). Stress is a provoking factor for several inflammatory diseases, and it was shown in mouse models of bronchial asthma that the numbers of eosinophils in bronchoalveolar lavage were increased after stress exposure (49). One possible explanation of the eosinopenia observed in the current study in porcine blood might thus be a redistribution to lung tissue. As reviewed by Kirschvink and Reinhold (50), the pig shows many similarities to humans regarding airway anatomy and eosinophilic inflammation after sensitization. Further studies tracking the redistribution of eosinophils from the blood to other organs in CORT-treated pigs may confirm a trafficking to the lung and increase the value of pigs as a model for human allergic asthma.

CORT infusion not only affected immune cell numbers in peripheral blood but also their functionality. Lymphocyte proliferation was reduced after 24 and 48 h of infusion, especially pronounced if stimulated with ConA. This finding confirms the results of an in vitro study with

CORT-treated lymphocytes recently published by our group, in which ConA-stimulated cells were more sensitive toward CORT-induced suppression than those stimulated with PWM (16). In a study on social stress in pigs, animals with higher plasma CORT concentrations likewise showed a lower ConA-induced proliferation, whereas PWM-induced proliferation was not affected (51). It was also shown in humans that ConA-stimulated lymphocytes were more susceptible to GC-mediated suppression of proliferation than PWM-stimulated samples (52–55). As ConA is presumed to better stimulate T cells (56, 57), whereas PWM activates both T and B cells with a preference for B cells at the used concentration of 5 mg/ml (58, 59), this might hint toward a lower sensitivity of porcine B cells to CORT compared with T cells. To validate this hypothesis, studies evaluating lymphocyte proliferation on a single-cell level using fluorescent dye and mAbs against B and T cell markers should be conducted to investigate the mitogen-specific differences in effect size observed in this study. However, regardless of the exact phenotype of the impaired lymphocytes, the results presented in this article show once again the immunosuppressive potential of CORT already seen in social stress experiments in pigs (37, 60, 61) as well as their similarity to humans regarding GC sensitivity (52, 62).

Whereas lymphocyte proliferation was decreased by CORT, neither total IgG nor total IgM plasma concentration was affected. This finding was not unexpected and is in accordance with previous stress studies in pigs (60, 63). As reviewed for example by Fleshner (64) and Cohen et al. (65), GCs do modulate Ab response, but this occurs mostly during primary and secondary immune response, whereas the degradation of circulating plasma Igs only occurs after longstanding GC elevation.

As a measure of the innate immune response, we investigated the phagocytic activity of monocytes and neutrophil granulocytes. The number of particles ingested per monocyte increased after both 24 and 48 h of CORT infusion. A stimulation of monocyte/macrophage phagocytosis by GCs has also been shown in mouse experiments both in vitro (66, 67) and in vivo (68, 69) and human monocyte-derived M2 macrophages in vitro (70). As reviewed by Ortega (71), neutrophils can react to GCs in various ways but mostly by an enhanced activity. However, porcine neutrophils showed no reaction to CORT treatment in the current study. A probable explanation for this finding may be a lower CORT sensitivity of neutrophils compared with monocytes because their GC receptors have a lower affinity and are less abundant than those found in PBMC of pigs (27).

Knowledge about the implications of CAs for the porcine immune system is extremely rare, and to our knowledge, this is the first study to examine them separately and with a controlled

dosage via i.v. administration. We thus decided to choose infusion dosages based on studies in the field of cardiovascular and blood circulation research, in which pigs are often used as a human model (72). The obtained plasma concentrations are within the range reported in the few studies that examined different stressors in pigs and in which plasma CA concentrations were determined, ranging between 1700 pg/ml and 300 ng/ml (NA) and 700 pg/ml and 100 ng/ml (ADR) (38, 73–75). Similar to CORT treatment, only the administered CA hormone increased and did not cause any elevations in the plasma concentrations of the other two hormones. The CA-treated animals are thus resembling similarities with the hormonal status of animals with a proactive coping style in stressful situations, as reviewed by Koolhaas et al. (76).

By looking into the numbers of different immune cell types in blood, many CA-induced changes could be demonstrated for the first time in pigs. Both CA hormones caused an increase in monocyte numbers, which has been described in humans as a consequence of demargination from the endothelium (71). A study in rats could demonstrate that CA-induced monocytosis is, at least in this species, mediated via β-adrenergic receptors (ARs) (41). Similar to CORT, NA induced an increase of neutrophils and decrease of eosinophils, although to a lesser extent. This has also already been described for CA-treated rats and humans (44, 77). In the current study, no changes in the numbers of these cell types occurred in ADR-treated animals, possibly portending species differences. In an early study in humans, both ADR and NA injection led to a decrease of eosinophil numbers, but in this study, the effect of ADR was six times as high as that of NA (78).

A strong increase in NK cell numbers is a well-described ADR effect in other species and attributed to their exceptionally high number of β-ARs (41, 77, 79–82). Although AR numbers on porcine immune cells remain to be explored, we could confirm this effect for the pig, indicating a similarly high number of β-ARs on porcine NK cells and thus giving further incidence for their suitability as a model species.

Not only the investigated innate immune cells but also some cell types of the adaptive immune system displayed CA-induced changes in numbers. As reviewed by Elenkov et al. (83), CAs generally cause lymphocytosis after ~30 min of treatment, followed by a decrease in numbers after 2 h, similar to the data reported in this study. The effect was only observed in T cells, whereas B cell numbers remained unaffected. This seems to be β-AR mediated because in humans, the diminishing effect on T cells was imitable by application of the β2-agonist isoproterenol (84). In van Tits et al. (84), there was also no impact on B cell numbers, further hinting at similar mechanisms in humans and pigs. Contrarily, an α-AR-mediated decrease of

T cells and a β-AR-mediated decrease of B cells was demonstrated in rats (41). Thus, additional studies are needed to verify the molecular mechanisms involved in porcine lymphocyte trafficking.

Notably, some immune cell types responded differently to NA and ADR. Whereas NA caused a transient decrease of all T cell subsets after 2 h, ADR only reduced $CD8^-$ T cell subsets after 2 h, followed by an increase after 24 h. Although this finding remains to be examined further, one could speculate on differences in AR subtype distribution because in other species, NA has a higher α-AR affinity than ADR, whereas β-AR sensitivity is higher for ADR (85).

Accompanying the changes in blood immune cell numbers, we observed some changes regarding their functionality. After 2 h, lymphocytes of NA- but not ADR-infused animals showed a reduced proliferation rate. Similar effects were obtained in studies in humans, in which NA caused a decreased mitogen-induced proliferation after 1 h of NA infusion (77), although not after 2 h in that case. Infusion with the β-AR agonist isoproterenol caused the same effect in another human study after 90 min (84). This suggests a β-AR-mediated inhibitory effect, which is also described in other studies, as reviewed by Nance and Sanders (86). Contrary to the findings in humans, a study with implantable retard tablets in rats found the α-AR to be responsible for ConA-stimulated inhibition (87). Similar to the data presented in this study, NA hampered proliferation whereas ADR did not. If combined with the β-AR blocker propranolol, both NA and ADR massively inhibited proliferation, whereas CA administration together with the α-antagonist phentolamine had no effect. However, it must also be taken into consideration that a shift in the ratio of different lymphocyte subsets with varying CA sensitivity also played a role in the observed inhibition of PBMC proliferation. In other species, NK cells have the highest number of β-ARs among lymphocytes, followed by B cells, CTL, and then T_H cells with the lowest number (88). As T cells decreased after 2 h of NA infusion and B cells did not, there was a relatively higher number of B cells in the stimulated PBMC and, thus, presumably a higher number of β-ARs. To verify these explanatory approaches, future studies examining receptor-specific agonists should be conducted, and the number of α- and β-ARs on porcine lymphocytes must be determined. To date, little is known about the AR expression on porcine immune cells because specific Abs to identify porcine ARs are not available yet. Notably, a recent study examining macrophage ARs on the mRNA level confirmed a high similarity with humans, at least for this cell type (89).

Similar to CORT, both ADR and NA promoted innate immunity by enhancing the phagocytic capacity of monocytes and, in addition, neutrophil granulocytes. This CA effect is in accordance

with findings in other species, as described in several reviews (71, 90, 91). There have only been a few studies examining the effect of increased stress hormones on phagocytic function in the pig. A decrease in the number of phagocytosing monocytes was observed in pigs subjected to a social stress experiment, in which it was accompanied by an increase of CORT, ADR, and NA plasma levels (38). However, neutrophils were not investigated in said study, and there is no information given about the efficacy of the phagocytosing cells. In another pig study, untrained and thus presumably more-stressed pigs subjected to a novel object test had a higher number of phagocytosing neutrophils accompanied by a higher efficacy than neutrophils of trained pigs. Because the experiment did not cause changes in CORT, the enhancement was supposedly caused by increased CA concentrations, which were not quantified in the study (92). To the best of our knowledge, the current study is the first to demonstrate stress hormone effects on the phagocytic function of porcine innate immune cells, in this level of detail, and for CORT, ADR, and NA separately. The results also show similarities to humans for this parameter, thus giving further indication of the pig's suitability as a human model in immunologic research.

Our study presents differential effects of the three main stress hormones on number and functionality of various porcine innate and adaptive immune cell populations. It is particularly noteworthy that we achieved physiologic stress levels of each individual hormone without triggering an endogenous release of the others. Although the numbers of most cell types returned to preinfusion levels after the end of the experiment, some effects appear to be longer lasting. We also observed functional alterations indicating a shift from adaptive toward innate immune functionality. A redistribution of immune cells to potentially endangered tissues like the skin combined with an enhanced efficacy of phagocytes may help animals cope with threats that would typically be accompanied by an increase of stress hormones, like being attacked by a predator. This study thus provides further evidence for an adjusting rather than a generally suppressive short-term response of the immune system to physiologic stress hormone levels. Taken together, our findings not only add to knowledge about the impact of stress on the pig for its own sake but also strengthen its status as a suitable human model.

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Disclosures

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References

1. Gordan, R., J. K. Gwathmey, and L.-H. Xie. 2015. Autonomic and endocrine control of cardiovascular function. World J. Cardiol. 7: 204–214.
2. Antonelli, A., R. Torchio, L. Bertolaccini, A. Terzi, F. Rolfo, P. Agostoni, C. Gulotta, V. Brusasco, and R. Pellegrino. 2012. Contribution of β-adrenergic receptors to exercise-induced bronchodilatation in healthy humans. Respir. Physiol. Neurobiol. 184: 55–59.
3. Dhabhar, F. S. 2018. The short-term stress response - Mother nature's mechanism for enhancing protection and performance under conditions of threat, challenge, and opportunity. Front. Neuroendocrinol. 49: 175–192.
4. Wiegers, G. J., G. Croiset, J. M. Reul, F. Holsboer, and E. R. de Kloet. 1993. Differential effects of corticosteroids on rat peripheral blood T-lymphocyte mitogenesis in vivo and in vitro. Am. J. Physiol. 265: E825–E830.
5. Ben-Eliyahu, S., G. Shakhar, G. G. Page, V. Stefanski, and K. Shakhar. 2000. Suppression of NK cell activity and of resistance to metastasis by stress: a role for adrenal catecholamines and β-adrenoceptors. Neuroimmunomodulation 8: 154–164.
6. Dhabhar, F. S., A. R. Satoskar, H. Bluethmann, J. R. David, and B. S. McEwen. 2000. Stress-induced enhancement of skin immune function: a role for gamma interferon. Proc. Natl. Acad. Sci. USA 97: 2846–2851.
7. Dhabhar, F. S., A. N. Saul, C. Daugherty, T. H. Holmes, D. M. Bouley, and T. M. Oberyszyn. 2010. Short-term stress enhances cellular immunity and increases early resistance to squamous cell carcinoma. Brain Behav. Immun. 24: 127–137.
8. Straub, R. H., and J. R. Kalden. 2009. Stress of different types increases the proinflammatory load in rheumatoid arthritis. Arthritis Res. Ther. 11: 114.
9. Gimsa, U., M. Tuchscherer, and E. Kanitz. 2018. Psychosocial stress and immunity-what can we learn from pig studies? Front. Behav. Neurosci. 12: 64.
10. Walters, E. M., K. D. Wells, E. C. Bryda, S. Schommer, and R. S. Prather. 2017. Swine models, genomic tools and services to enhance our understanding of human health and diseases. Lab Anim. (NY) 46: 167–172.

11. Engert, L. C., U. Weiler, B. Pfaffinger, V. Stefanski, and S. S. Schmucker. 2018. Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs. Dev. Comp. Immunol. 79: 11–20.

12. Martínez-Miró, S., F. Tecles, M. Ramón, D. Escribano, F. Hernández, J. Madrid, J. Orengo, S. Martínez-Subiela, X. Manteca, and J. J. Cerón. 2016. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet. Res. 12: 171.

13. Kick, A. R., M. B. Tompkins, and G. W. Almond. 2011. Stress and immunity in the pig. CAB Rev. Perspect. Agric. Vet. Sci. 6: 1–17.

14. Salak-Johnson, J. L., J. J. McGlone, and R. L. Norman. 1996. In vivo glucocorticoid effects on porcine natural killer cell activity and circulating leukocytes. J. Anim. Sci. 74: 584–592.

15. Wallgren, P., I. L. Wilén, and C. Fossum. 1994. Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. Vet. Immunol. Immunopathol. 42: 301–316.

16. Reiske, L., S. Schmucker, J. Steuber, and V. Stefanski. 2019. Glucocorticoids and catecholamines affect in vitro functionality of porcine blood immune cells. Animals (Basel). DOI: 10.3390/ani9080545.

17. Felsner, P., D. Hofer, I. Rinner, S. Porta, W. Korsatko, and K. Schauenstein. 1995. Adrenergic suppression of peripheral blood T cell reactivity in the rat is due to activation of peripheral alpha 2-receptors. J. Neuroimmunol. 57: 27–34.

18. de Groot, J., M. A. Ruis, J. W. Scholten, J. M. Koolhaas, and W. J. A. Boersma. 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiol. Behav. 73: 145–158.

19. Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. J. Anim. Sci. Biotechnol. 4: 19.

20. Grün, V., S. Schmucker, C. Schalk, B. Flauger, U.Weiler, and V. Stefanski. 2013. Influence of different housing systems on distribution, function and mitogen response of leukocytes in pregnant sows. Animals (Basel) 3: 1123–1141.

21. Kraetzl, W. D., and U. Weiler. 1998. Erfahrungen mit einem implantierbaren Kathetersystem zur frequenten und chronischen Blutentnahme bei Schafen in Gruppenhaltung und bei säugenden Sauen. Tierärztl. Umsch. 53: 567–574.

22. Weiler, U., S. Finsler, and R. Claus. 2003. Influence of cortisol, gonadal steroids and an energy deficit on biochemical indicators of bone turnover in Swine. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 50: 79–87.

23. McGlone, J. J., J. L. Salak, E. A. Lumpkin, R. I. Nicholson, M. Gibson, and R. L. Norman. 1993. Shipping stress and social status effects on pig performance, plasma cortisol, natural killer cell activity, and leukocyte numbers. J. Anim. Sci. 71: 888–896.

24. Regueira, T., B. Bänziger, S. Djafarzadeh, S. Brandt, J. Gorrasi, J. Takala, P. M. Lepper, and S. M. Jakob. 2008. Norepinephrine to increase blood pressure in endotoxaemic pigs is associated with improved hepatic mitochondrial respiration. Crit. Care 12: R88.

25. Marsh, J. W., J. G. Drougas, J. K. Wright, W. C. Chapman, Y. T. Becker, S. E. Barnard, K. L. Donovan, I. Feurer, M. Sika, K. T. Blair, et al. 1998. The effect of low dose epinephrine infusion on hepatic hemodynamics. Transplant. Proc. 30: 2306–2308.

26. Anton, A. H., and D. F. Sayre. 1962. A study of the factors affecting the aluminium oxide-trihydroxyindole procedure for the analysis of catecholamines. J. Pharmacol. Exp. Ther. 138: 360–375.

27. Engert, L. C., U. Weiler, V. Stefanski, and S. S. Schmucker. 2017. Glucocorticoid receptor number and affinity differ between peripheral blood mononuclear cells and granulocytes in domestic pigs. Domest. Anim. Endocrinol. 61: 11–16.

28. Grün, V., S. Schmucker, C. Schalk, B. Flauger, and V. Stefanski. 2014. Characterization of the adaptive immune response following immunization in pregnant sows (Sus scrofa) kept in two different housing systems. J. Anim. Sci. 92: 3388–3397.

29. Schalk, C., B. Pfaffinger, S. Schmucker, U. Weiler, and V. Stefanski. 2018. Effects of repeated social mixing on behavior and blood immune cells of group-housed pregnant sows (sus scrofa domestica). Livest. Sci. 217: 148–156.

30. Kenward, M. G., and J. H. Roger. 1997. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53: 983–997.

31. Faraway, J. J. 2006. Extending the Linear Model with R: Generalized Linear, Mixed Effects and Nonparametric Regression Models. Chapman & Hall/CRC, Boca Raton, FL.

32. Engert, L. C., U. Weiler, B. Pfaffinger, V. Stefanski, and S. S. Schmucker. 2019. Photoperiodic effects on diurnal rhythms in cell numbers of peripheral leukocytes in domestic pigs. Front. Immunol. 10: 393.

33. Engert, L. C., U. Weiler, V. Stefanski, and S. S. Schmucker. 2017. Data characterizing diurnal rhythms in the number of peripheral $CD8a^-$ and $CD8a^+$ γδ T cells in domestic pigs. Data Brief 16: 843–849.

34. Schwarz, E., A. Saalmüler, W. Gerner, and R. Claus. 2005. Intraepithelial but not lamina propria lymphocytes in the porcine gut are affected by dexamethasone treatment. Vet. Immunol. Immunopathol. 105: 125–139.

35. Lo, D. Y., W. M. Lee, M. S. Chien, C. C. Lin, and W. C. Lee. 2005. Effects of dexamethasone on peripheral blood mononuclear cell phenotype in weanling piglets. Comp. Immunol. Microbiol. Infect. Dis. 28: 251–258.

36. Hessing, M. J., A. M. Hagelsø, W. G. Schouten, P. R. Wiepkema, and J. A. van Beek. 1994. Individual behavioral and physiological strategies in pigs. Physiol. Behav. 55: 39–46.

37. Deguchi, E., and M. Akuzawa. 1998. Effects of fighting after grouping on plasma cortisol concentration and lymphocyte blastogenesis of peripheral blood mononuclear cells induced by mitogens in piglets. J. Vet. Med. Sci. 60: 149–153.

38. Bacou, E., K. Haurogné, G. Mignot, M. Allard, L. De Beaurepaire, J. Marchand, E. Terenina, Y. Billon, J. Jacques, J.-M. Bach, et al. 2017. Acute social stressinduced immunomodulation in pigs high and low responders to ACTH. Physiol. Behav. 169: 1–8.

39. Bilandzić, N., B. Simić, M. Zurić, M. Lojkić. 2005. Effect of ACTH administration on biochemical and immune measures in boars. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 52: 440–446.

40. Zahorec, R. 2001. Ratio of neutrophil to lymphocyte counts--rapid and simple parameter of systemic inflammation and stress in critically ill. Bratisl. Lek Listy 102: 5–14.

41. Engler, H., L. Dawils, S. Hoves, S. Kurth, J. R. Stevenson, K. Schauenstein, and V. Stefanski. 2004. Effects of social stress on blood leukocyte distribution: the role of alpha- and beta-adrenergic mechanisms. J. Neuroimmunol. 156: 153–162.

42. Stojek, W., A. Borman, W. Glac, B. Baracz-Jóźwik, B. Witek, M. Kamyczek, and J. Tokarski. 2006. Stress-induced enhancement of activity of lymphocyte lysosomal enzymes in pigs of different stress-susceptibility. J. Physiol. Pharmacol. 57(Suppl. 8): 61–72.

43. Cain, D. W., and J. A. Cidlowski. 2017. Immune regulation by glucocorticoids. Nat. Rev. Immunol. 17: 233–247.

44. Dhabhar, F. S., W. B. Malarkey, E. Neri, and B. S. McEwen. 2012. Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. Psychoneuroendocrinology 37: 1345–1368.

45. Cox, J. H., and W. L. Ford. 1982. The migration of lymphocytes across specialized vascular endothelium. IV. Prednisolone acts at several points on the recirculation pathways of lymphocytes. Cell. Immunol. 66: 407–422.

46. Fauci, A. S. 1975. Mechanisms of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and b lymphocytes to the bone marrow. Immunology 28: 669–680.

47. Stefanski, V., A. Peschel, and S. Reber. 2003. Social stress affects migration of blood T cells into lymphoid organs. J. Neuroimmunol. 138: 17–24.

48. Kothari, N. J., and J. C. Saunders. 1961. Effects of corticotrophin, hydrocortisone and methopyrapone on the circulating eosinophiles in man. Nature 191: 1105–1106.

49. Joachim, R. A., D. Quarcoo, P. C. Arck, U. Herz, H. Renz, and B. F. Klapp. 2003. Stress enhances airway reactivity and airway inflammation in an animal model of allergic bronchial asthma. Psychosom. Med. 65: 811–815.

50. Kirschvink, N., and P. Reinhold. 2008. Use of alternative animals as asthma models. Curr. Drug Targets 9: 470–484.

51. Tuchscherer, M., B. Puppe, A. Tuchscherer, and E. Kanitz. 1998. Effects of social status after mixing on immune, metabolic, and endocrine responses in pigs. Physiol. Behav. 64: 353–360.

52. Rupprecht, R., N. Wodarz, J. Kornhuber, B. Schmitz, K. Wild, H. U. Braner, O. A. Müller, and P. Riederer. 1990–1991. In vivo and in vitro effects of glucocorticoids on lymphocyte proliferation in man: relationship to glucocorticoid receptors. Neuropsychobiology 24: 61–66.

53. Blomgren, H., and B. Andersson. 1976. Steroid sensitivity of the PHA and PWM responses of fractionated human lymphocytes in vitro. Exp. Cell Res. 97: 233–240.

54. Heilman, D. H. 1972. Failure of hydrocortisone to inhibit blastogenesis by pokeweed mitogen in human leucocyte cultures. Clin. Exp. Immunol. 11: 393–403.

55. Heilman, D. H., M. R. Gambrill, and J. P. Leichner. 1973. The effect of hydrocortisone on the incorporation of tritiated thymidine by human blood lymphocytes cultured with phytohaemagglutinin and pokeweed mitogen. Clin. Exp. Immunol. 15: 203–212.

56. Peacock, J. S., A. S. Colsky, and V. B. Pinto. 1990. Lectins and antibodies as tools for studying cellular interactions. J. Immunol. Methods 126: 147–157.

57. Weiss, A., R. Shields, M. Newton, B. Manger, and J. Imboden. 1987. Ligand receptor interactions required for commitment to the activation of the interleukin 2 gene. J. Immunol. 138: 2169–2176.

58. Greaves, M., and G. Janossy. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11: 87–130.

59. Mellstedt, H. 1975. In vitro activation of human T and B lymphocytes by pokeweed mitogen. Clin. Exp. Immunol. 19: 75–82.

60. Kanitz, E., M. Tuchscherer, B. Puppe, A. Tuchscherer, and B. Stabenow. 2004. Consequences of repeated early isolation in domestic piglets (Sus scrofa) on their behavioural, neuroendocrine, and immunological responses. Brain Behav. Immun. 18: 35–45.

61. Kanitz, E., M. Tuchscherer, A. Tuchscherer, B. Stabenow, and G. Manteuffel. 2002. Neuroendocrine and immune responses to acute endotoxemia in suckling and weaned piglets. Biol. Neonate 81: 203–209.

62. Roess, D. A., C. J. Bellone, M. F. Ruh, E. M. Nadel, and T. S. Ruh. 1982. The effect of glucocorticoids on mitogen-stimulated B-lymphocytes: thymidine incorporation and antibody secretion. Endocrinology 110: 169–175.

63. Tuchscherer, M., E. Kanitz, B. Puppe, and A. Tuchscherer. 2006. Early social isolation alters behavioral and physiological responses to an endotoxin challenge in piglets. Horm. Behav. 50: 753–761.

64. Fleshner, M. 2000. Exercise and neuroendocrine regulation of antibody production: protective effect of physical activity on stress-induced suppression of the specific antibody response. Int. J. Sports Med. 21 (Suppl. 1): S14–S19.

65. Cohen, S., G. E. Miller, and B. S. Rabin. 2001. Psychological stress and antibody response to immunization: a critical review of the human literature. Psychosom. Med. 63: 7–18.

66. Barriga, C., M. I. Martín, E. Ortega, and A. B. Rodriguez. 2002. Physiological concentrations of melatonin and corticosterone in stress and their relationship with phagocytic activity. J. Neuroendocrinol. 14: 691–695.

67. Chapman, K. E., A. Coutinho, M. Gray, J. S. Gilmour, J. S. Savill, and J. R. Seckl. 2006. Local amplification of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 and its role in the inflammatory response. Ann. N. Y. Acad. Sci. 1088: 265–273.

68. Barriga, C., M. I. Martín, R. Tabla, E. Ortega, and A. B. Rodríguez. 2001. Circadian rhythm of melatonin, corticosterone and phagocytosis: effect of stress. J. Pineal Res. 30: 180–187.

69. Forner, M. A., C. Barriga, A. B. Rodriguez, and E. Ortega. 1995. A study of the role of corticosterone as a mediator in exercise-induced stimulation of murine macrophage phagocytosis. J. Physiol. 488: 789–794.

70. Gratchev, A., J. Kzhyshkowska, J. Utikal, and S. Goerdt. 2005. Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand. J. Immunol. 61: 10–17.

71. Ortega, E. 2003. Neuroendocrine mediators in the modulation of phagocytosis by exercise: physiological implications. Exerc. Immunol. Rev. 9: 70–93.

72. Suzuki, Y., A. C. Yeung, and F. Ikeno. 2011. The representative porcine model for human cardiovascular disease. J. Biomed. Biotechnol. 2011: 195483.

73. Althen, T. G., K. Ono, and D. G. Topel. 1977. Effect of stress susceptibility or stunning method on catecholamine levels in swine. J. Anim. Sci. 44: 985–989.

74. Rosochacki, S. J., A. B. Piekarzewska, J. Połoszynowicz, and T. Sakowski. 2000. The influence of restraint immobilization stress on the concentration qf bioamines and cortisol in plasma of Pietrain and Duroc pigs. J. Vet. Med. A. Physiol. Pathol. Clin. Med. 47: 231–242.

75. Weiß, C. 2015. Stress- und Schmerzbelastung des Schweines bei Entnahme eines Tracheobronchialabstriches im Vergleich zum Nasentupfer und der Fixierung in der Oberkieferschlinge. Dissertation, Ludwig-Maximilians-Universität, München, Germany.

76. Koolhaas, J. M., S. M. Korte, S. F. De Boer, B. J. Van Der Vegt, C. G. Van Reenen, H. Hopster, I. C. De Jong, M. A. W. Ruis, and H. J. Blokhuis. 1999. Coping styles in animals: current status in behavior and stress-physiology. Neurosci. Biobehav. Rev. 23: 925–935.

77. Crary, B., M. Borysenko, D. C. Sutherland, I. Kutz, J. Z. Borysenko, and H. Benson. 1983. Decrease in mitogen responsiveness of mononuclear cells from peripheral blood after epinephrine administration in humans. J. Immunol. 130: 694–697.

78. Humphreys, R. J., and W. Raab. 1950. Response of circulating eosinophils to nor-epinephrine, epinephrine and emotional stress in humans. Proc. Soc. Exp. Biol. Med. 74: 302–303.

79. Maisel, A. S., T. Harris, C. A. Rearden, and M. C. Michel. 1990. Beta-adrenergic receptors in lymphocyte subsets after exercise. Alterations in normal individuals and patients with congestive heart failure. Circulation 82: 2003–2010.

80. Schedlowski, M., A. Falk, A. Rohne, T. O. Wagner, R. Jacobs, U. Tewes, and R. E. Schmidt. 1993. Catecholamines induce alterations of distribution and activity of human natural killer (NK) cells. J. Clin. Immunol. 13: 344–351.

81. Landmann, R. M., F. B. Müler, C. Perini, M.Wesp, P. Erne, and F. R. Bühler. 1984. Changes of immunoregulatory cells induced by psychological and physical stress: relationship to plasma catecholamines. Clin. Exp. Immunol. 58: 127–135.

82. Dimitrov, S., T. Lange, and J. Born. 2010. Selective mobilization of cytotoxic leukocytes by epinephrine. J. Immunol. 184: 503–511.

83. Elenkov, I. J., R. L. Wilder, G. P. Chrousos, and E. S. Vizi. 2000. The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52: 595–638.

84. Van Tits, L. J., M. C. Michel, H. Grosse-Wilde, M. Happel, F. W. Eigler, A. Soliman, and O. E. Brodde. 1990. Catecholamines increase lymphocyte beta 2-adrenergic receptors via a beta 2-adrenergic, spleen-dependent process. Am. J. Physiol. 258: E191–E202.

85. Padro, C. J., and V. M. Sanders. 2014. Neuroendocrine regulation of inflammation. Semin. Immunol. 26: 357–368.

86. Nance, D. M., and V. M. Sanders. 2007. Autonomic innervation and regulation of the immune system (1987-2007). Brain Behav. Immun. 21: 736–745.

87. Felsner, P., D. Hofer, I. Rinner, H. Mangge, M. Gruber, W. Korsatko, and K. Schauenstein. 1992. Continuous in vivo treatment with catecholamines suppresses in vitro reactivity of rat peripheral blood T-lymphocytes via alpha-mediated mechanisms. J. Neuroimmunol. 37: 47–57.

88. Marino, F., and M. Cosentino. 2013. Adrenergic modulation of immune cells: an update. Amino Acids 45: 55–71.

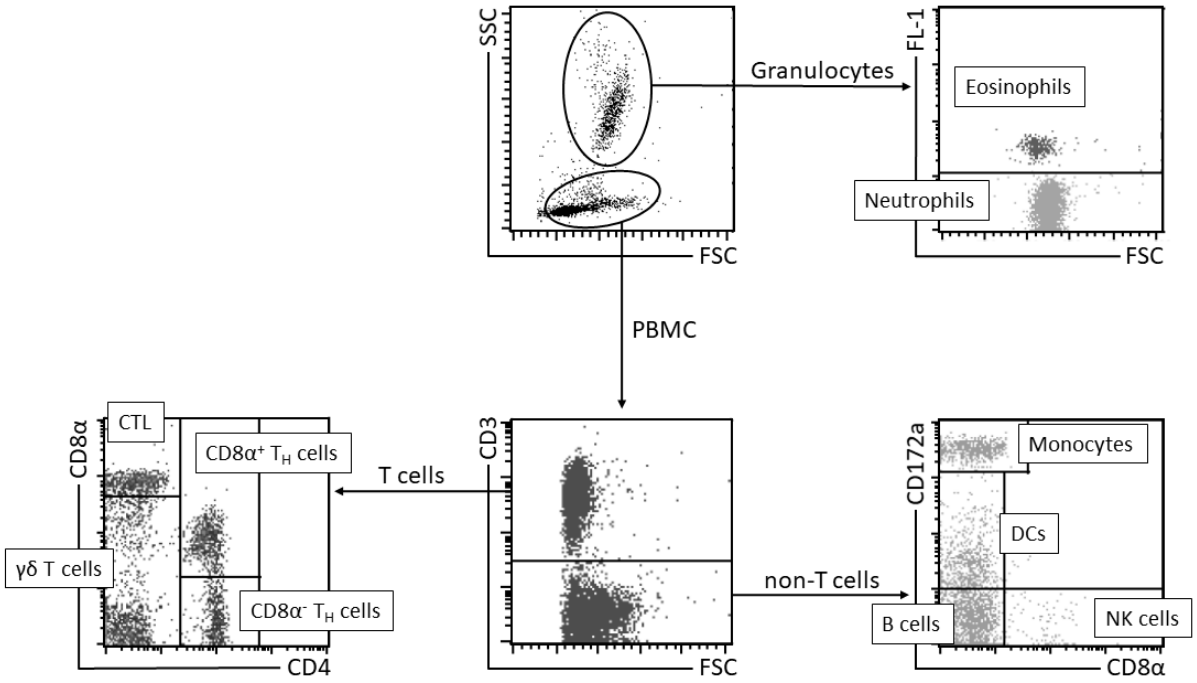
89. Bacou, E., K. Haurogné, M. Allard, G. Mignot, J.-M. Bach, J. Hervé, and B. Lieubeau. 2017. β2-adrenoreceptor stimulation dampens the LPS-induced M1 polarization in pig macrophages. Dev. Comp. Immunol. 76: 169–176.

90. Ortega, E., E. Giraldo, M. D. Hinchado, L. Martín, J. J. García, and M. De la Fuente. 2007. Neuroimmunomodulation during exercise: role of catecholamines as ‘stress mediator’ and/or ‘danger signal’ for the innate immune response. Neuroimmunomodulation 14: 206–212.

91. Nagatomi, R., T. Kaifu, M. Okutsu, X. Zhang, O. Kanemi, and H. Ohmori. 2000. Modulation of the immune system by the autonomic nervous system and its implication in immunological changes after training. Exerc. Immunol. Rev. 6: 54–74.

92. Lewis, C. R. G., L. E. Hulbert, and J. J. McGlone. 2008. Novelty causes elevated heart rate and immune changes in pigs exposed to handling, alleys, and ramps. Livest. Sci. 116: 338–341.

Supplementary material



Supplementary Fig. S1. Gating strategy for discrimination of immune cell subsets. Staining was performed with heparinized whole blood and combinations of fluorochrome-conjugated pig-specific mAbs against CD3ε, CD4 and CD8α (combination A), CD3ε, CD8α and CD172a (combination B) or without antibody addition. After red blood cell lysis, leukocyte subsets were determined by flow cytometry. Based on size and granularity, PBMC and granulocytes were discriminated using forward and side scatter. Subsequently, granulocytes were further divided into neutrophils and eosinophils by autofluorescence of eosinophils in the unstained sample. PBMC were differentiated into $CD3^-$ non-T cells and $CD3^+$ T cells. T cells were further subdivided into $CD3^+CD4^+CD8\alpha^-$ cells (naive T_H cells), $CD3^+CD4^+CD8\alpha^+$ cells (antigen-experienced T_H cells), $CD3^+CD4^-CD8\alpha^{high}$ cells (cytotoxic T cells) and $CD3^+CD4^-CD8\alpha^{-/low}$ cells (γδ T cells), within staining combination A. By staining with combination B, non-T cells were further divided into $CD3^-CD172a^-CD8\alpha^+$ cells (NK cells), $CD3^-CD172^-CD8\alpha^-$ cells (mostly B cells), $CD3^-CD172^{dim}CD8\alpha^-$ cells (mainly DCs) and $CD3^-CD172^{high}CD8\alpha^-$ cells (monocytes). Shown are exemplary dot plots of flow cytometric analysis of blood immune cells from pigs of the present study.

MANUSCRIPT III

Interkingdom Cross-Talk in Times of Stress: *Salmonella* Typhimurium Grown in the Presence of Catecholamines Inhibits Porcine Immune Functionality *in vitro*

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Abstract

In stressful situations, catecholamines modulate mammalian immune function, and in addition, they can be sensed by many bacteria. Catecholamine sensing was also found in the zoonotic gut pathogen *Salmonella* Typhimurium, probably contributing to the stress-induced increased risk of salmonellosis. Virulence traits such as proliferation and invasiveness are promoted upon bacterial catecholamine sensing, but it is unknown whether *S.* Typhimurium may also inhibit mammalian immune function in stressful situations. We thus investigated whether supernatants from *S.* Typhimurium grown in the presence of catecholamines modulate porcine mitogen-induced lymphocyte proliferation. Lymphocyte proliferation was reduced by supernatants from catecholamine-exposed *Salmonella* in a dose-dependent manner. We further examined whether adrenaline oxidation to adrenochrome, which is promoted by bacteria, could be responsible for the observed effect, but this molecule either enhanced lymphocyte functionality or had no effect. We could thereby exclude adrenochrome as a potential immunomodulating agent produced by *S.* Typhimurium. This study is the first to demonstrate that bacteria grown in the presence of catecholamine stress hormones alter their growth environment, probably by producing immunomodulating substances, in a way that host immune response is suppressed. These findings add a new dimension to interkingdom signaling and provide novel clues to explain the increased susceptibility of a stressed host to *Salmonella* infection.

Keywords

Salmonella Typhimurium, catecholamines, adrenaline, noradrenaline, adrenochrome, pig, stress, interkingdom-signalling, lymphocytes, immune function

Introduction

In acute stress situations, the mammalian body launches a rapid physiologic response, which enables it to cope with threats imposed on its health. In the course of such a "fight-or-flight" reaction, substantial amounts of stress hormones, particularly adrenaline (ADR) and noradrenaline (NA), can be released from the adrenal gland and at sympathetic nerve endings. These catecholamines (CAs) not only exert effects on blood circulation, respiration, energy metabolism, and many other functions supporting physical exertion (1–3), but also affect the immune system (4, 5). The long-held view of general immunosuppression by stress hormones was increasingly challenged in recent years, as especially CA actions are rather diverse and dose-dependent, including both inhibiting and enhancing actions (5–9). In some organs, such as the spleen or the gut, stress-related CA release can lead to local concentrations of up to 10^{-4} to 10^{-3} M (10, 11), which is much higher than in the blood, where levels are between 10^{-9} and 10^{-6} M (12, 13). This is caused by NA discharge from synaptic vesicles at noradrenergic nerve endings (10, 11, 14). In the gut and other tissues with contact to the external world via epithelial surfaces, CAs can even cross the epithelial border and interact with microorganisms living in those ecological niches (15–18). In the colon, NA can reach a concentration of about 50 ng/g luminal content (14).

In the last two decades, more and more studies in the field of microbial endocrinology emerged, investigating the cross-talk between the endocrine and nervous system of host species and microorganisms inhabiting or invading them. A plethora of microorganisms exist naturally as commensals, e.g., in the gut, oral cavity, and on the skin (19–21). It is therefore no surprise that both parties evolved mechanisms to communicate with each other via mammalian hormones and hormone-like microbial molecules, with mutual benefits supporting symbiosis. However, many pathogens have been proven to sense stressful situations with high CA levels and exploit them by boosting virulence (22, 23). NA can be used by many bacterial species as an iron donor (24, 25) or activate quorum sensing–a bacterial cell-to-cell communication–by directly binding to QseC or QseE (26–28). Elevated ADR and NA concentrations can thus lead to an increased bacterial growth rate (29, 30), motility (26, 29), or attachment to epithelial surfaces (22)–in short, higher chances of infection. This interkingdom signaling works in both directions. Independently of host stress, bacteria produce molecules for interbacterial communication, some of which have a hormone-like side effect on host cells (31). For instance, many Gram-negative bacteria produce substances, which are chemically analogous to eukaryotic lipid hormones and can modulate host immune functions such as neutrophil chemotaxis and

lymphocyte proliferation (32–35). Moreover, some quorum-sensing molecules produced by several regular inhabitants of the gastrointestinal tract (GIT) probably act as agonists at adrenergic receptors (ARs) (36).

Regarding this intense cross-talk between kingdoms, it is conceivable that in stressful situations, pathogens not only modulate their own properties but may even actively manipulate immune cells to exploit a weakened host. Upon CA perception, they might react with the release of bacterial hormone-like molecules similar to the aforementioned ones. Furthermore, a microbial alteration of mammalian CAs might lead to the formation of an immunomodulating substance. CAs are vulnerable to oxidation (37), and in the presence of superoxide, the oxidation of ADR to adrenochrome (AC) is promoted (38). A boost of AC formation by superoxide-producing bacteria might cause immunomodulation as it was shown that AC can bind to β-ARs (39), which can be found on most immune cells (40). Indeed, it was demonstrated in *Vibrio cholerae* O395N1 that the bacterial Na^+-translocating NADH:quinone oxidoreductase (NQR) promoted the oxidation of ADR to AC by superoxide production (41). AC supported the pathogenicity of *V. cholerae* by stimulating its growth even stronger than ADR and in addition exerted immunomodulating effects by inhibiting tumor necrosis factor α (TNF-α) production in a human monocytic cell line (41). It can be hypothesized that *V. cholerae* is not the only gut pathogen capable of this reaction, and the promotion of AC formation may be a strategy also used by other bacteria to manipulate host immune functionality. An interesting candidate to test this hypothesis is the important zoonotic gut pathogen, *Salmonella enterica* ssp. *enterica* serovar Typhimurium (*S.* Typhimurium), which is common in domestic pigs (*Sus scrofa domestica*) and difficult to eradicate. It is known that stress has a negative impact on primary *Salmonella* infection in pigs and also on the recrudescence of asymptomatic latent infections, for example, by transportation to the slaughterhouse (42). The resulting bacterial shedding by slaughter pigs leads to increased carcass contamination and thus intensifies the risk of food-borne transmission to humans (43). However, despite the importance of this bacterial infection both from a veterinary and a medical point of view, the underlying mechanisms of these observations are still not sufficiently resolved. Because an enhanced motility and growth rate upon CA sensing have also been found in *Salmonella* (16, 26), studying interkingdom signaling is a promising approach to better explain the promotion of salmonellosis by stress.

The aim of the present study was thus to investigate whether *S.* Typhimurium grown in the presence of CAs has the potential to hamper porcine immune functionality. We examined the

effects of supernatants from *S.* Typhimurium cultures exposed to NA or ADR on lymphocyte proliferation and demonstrated an inhibitory effect. Furthermore, we investigated whether AC is the causative agent of this inhibition.

Materials and Methods

Animals and Sampling

To obtain blood for *in vitro* studies without stress hormone release during the sampling procedure, 37 castrated male pigs (German Landrace × Pietrain, age 7 months) with indwelling vein catheters were used in total. At least 14 days before the beginning of blood sampling, *Vena cephalica* cannulation was performed under generalized anesthesia. Surgery was performed as previously described (44) with few modifications (45). The barrows were housed individually in pens (5.4 m^2) with visual and tactile contact to their conspecifics. Pens were littered with dust-free wood shavings and cleaned every day after feeding. Light was on from 06:30 until 20:30. Pigs were fed hay *ad libitum* and concentrate (1.5 kg/meal, ME 12 MJ/kg) twice a day in the morning at 07:30 and in the afternoon at 15:00. To ensure blood sampling without disturbance of the animals, pigs were thoroughly habituated to human handling. Catheters were rinsed with heparinized saline (115 IU/mL; B. Braun Melsungen AG, Melsungen, Germany) every day during feeding in the morning. For blood collection via the catheters, 5 mL of blood was drawn and discarded before 10 mL blood per animal was collected into lithium heparin tubes (Sarstedt, Nümbrecht, Germany). Separation of peripheral blood mononuclear cells (PBMCs) from whole blood was performed with Leucosep™ Centrifuge Tubes (Greiner Bio-One, Frickenhausen, Germany) using Biocoll with a density of 1.077 g/mL (Biochrom, Berlin, Germany) as previously described (6). In brief, PBMCs were separated by a density gradient, and after two washing steps; cells were suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 50 µg/mL gentamycin (all Biochrom). Afterward, cell concentration was determined with a Z2 Coulter Counter (Beckman Coulter, Krefeld, Germany).

Preparation of Bacterial Supernatants

To acquire supernatants from bacteria grown *in vitro* in presence and absence of 0.1 mM ADR, 0.1 mM NA, or 0.02 mM AC (Sigma-Aldrich, Taufkirchen, Germany), *S. enterica* serovar *Typhimurium* Zoosaloral his^{-155}/ade^{-4} (*S.* Typhimurium; DSM-No: 11320), auxotroph for histidine and adenine was chosen. *S.* Typhimurium was first allowed to grow on LB agar overnight at 37°C [1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol)

NaCl, and 1.5% (wt/vol) bacto agar]. A single colony was used to inoculate 25 mL of LB medium [1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 1% (wt/vol) NaCl]. After incubation overnight at 37°C and 180 rpm shaking (Infors HT Ecotron), *S.* Typhimurium cells were harvested by centrifugation (3 min, 10,000 × *g*), washed, and resuspended in heat-treated serum-SAPI cultivation medium (29) to obtain an optical cell density at 600 nm of 2 (Diode Array HP 8462A, Hewlett Packard, Palo Alto, CA, United States). Heat-treated serum-SAPI cultivation medium contains SAPI solution [6.25 mM NH_4NO_3, 1.84 mM KH_2PO_4, 3.35 mM KCl, autoclaved; 1.01 mM $MgSO_4$, 2.77 mM glucose, 10 mM HEPES pH 7.5 sterile filtered (0.22 μm)], 30% (vol/vol) FCS (Sigma-Aldrich), which was heat inactivated at 55°C for 20 min prior to use and supplementation of 0.12 mM adenine monohydrochloride and 0.13 mM L-histidine. Serum-SAPI was used as it is the medium of choice for analysis of CA effects on bacteria (15, 29, 30). Cultivation medium was inoculated with the cell suspension to obtain an OD_{600} of 0.01. To triplicates of 20 mL inoculated serum-SAPI either 10^{-4} M ADR, 10^{-4} M NA, or 2×10^{-5} M AC (Sigma-Aldrich), or no further compound was added and incubated at 37°C and shaking (180 rpm). As control, cultivation medium without bacterial cells and without CAs or AC was also incubated under the same conditions. After 8 h of growth, when cells were in the exponential growth phase, cells were harvested by centrifugation (15 min, 7,000 rpm) and the supernatant was sterile filtered (0.22 μm), frozen in liquid nitrogen, and stored at -80°C. Cells were harvested for collection of supernatants at OD600 = 0.34 (no addition), 0.47 (ADR), 0.49 (NA), and 0.36 (AC).

Determination of CA Contents in Bacterial Supernatants via High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) with electrochemical detection was conducted to determine the concentration of CAs in bacterial supernatants grown in the presence of NA or ADR. The HPLC system (ISO-3100BM, Thermo Fisher Scientific) was connected to an electrochemical detector [Coulochem III, conditioning cell (model 50210A), analytical cell (model 5011A), Thermo Fisher Scientific]. The potentials of the cells were set at 300, 50, and -250 mV. The system was equipped with the column Reprosil Pur 120 C18-AQ (4.6 × 75 mm) (A. Maisch, Ammerbuch, Germany). Cat-A-Phase II was used as the mobile phase, with a flow rate of 1.1 mL/min. The sample preparation with alumina extraction were adapted from the method first described by Anton and Sayre (46). Bacterial supernatants were diluted (1:10,000 and 1:20,000) to be in the range of the applied calibration curve. In brief, 1 mL of sample and 500 pg of an internal standard (dihydroxybenzylamine; Thermo Fisher

Scientific, Darmstadt, Germany) were added to extraction tubes containing 20 mg aluminum oxide previously activated with 600 µL 2 M Tris/EDTA buffer (pH 8.7). Samples were thoroughly mixed in an overhead shaker for 10 min and centrifuged at 1,000 × *g* for 1 min (4°C). Samples were washed three times with 1 mL of 16.5 mM Tris/EDTA buffer (pH 8.1), followed by centrifugation. The CAs were eluted by addition of 120 µL eluting solution (Recipe, Munich, Germany), short mixing, and centrifugation at 1,000 × *g* for 1 min (4°C). Aliquots of 50 µL were injected into the HPLC system. The internal standard method using peak areas was applied to evaluate the concentration of the samples.

Lymphocyte Proliferation Assay

For investigation of lymphocyte proliferative capacity, a mitogen-induced lymphocyte proliferation assay was performed as previously described (47). In short, PBMCs were seeded into 96-well round-bottom cell culture plates (Neolab, Heidelberg, Germany) with 1.5×10^5 cells/well and either stimulated with 5 µg/mL concanavalin A (ConA) or 5 µg/mL pokeweed mitogen (PWM) (both Sigma-Aldrich) or left without stimulation. Subsequently, supernatants from the differently treated *S.* Typhimurium cultures were added in concentrations of either 5, 10, or 15% of the total cell culture volume. To guarantee similar growth conditions throughout the wells, pure serum-SAPI was applied to control wells as well as for volume compensation, resulting in 15% serum–SAPI–based additive in every well. Each treatment was done in triplicates. Cells were incubated at 39°C, and 5% CO_2 for 48 h before 0.25 µCi ^{3}H-thymidine/well (PerkinElmer, Rodgau, Germany) was added, followed by a further incubation for 24 h. PBMCs were harvested using glass fiber filters (Sigma-Aldrich), and the incorporated radioactivity was measured by a liquid scintillation analyzer (PerkinElmer). For each treatment, the mean of counts per minute (cpm) was calculated, and the mean cpm of the unstimulated control was subtracted to gain Δcpm.

HPLC analysis of the *Salmonella* supernatants showed that substantial amounts of CAs were still present in CA-treated cultures. We thus performed an additional experiment to ensure that probable bacterial effects were not in fact caused by CAs or by mere synergistic effects of bacterial products and CAs. Therefore, previously frozen PBMCs of three animals were thawed and seeded with 1.5×10^5 cells/well in 96-well round-bottom cell culture plates in RPMI 1640 supplemented with 10% FCS and 50 µg/mL gentamycin. Cells were incubated at 39°C and 5% CO_2 as described for the first experiment after adding one of the following treatments: PBMCs were either left unstimulated after addition of 15% serum-SAPI medium or stimulated with 5 µg/mL ConA. Stimulated cells were supplemented with one of the following additives: 15%

serum-SAPI alone, 15% serum-SAPI and 10^{-5} M NA, 15% serum-SAPI and 10^{-5} M ADR, 15% supernatants from *S.* Typhimurium grown without hormone, 15% supernatants from *S.* Typhimurium grown in the presence of 10^{-4} M NA or 10^{-4} M ADR, or 15% supernatants from *S.* Typhimurium grown without hormone with retrospective addition of 10^{-5} M NA or 10^{-5} M ADR.

In a third experiment, lymphocyte proliferation was assessed again as described previously but with addition of AC (Sigma–Aldrich). As the effective concentration (and the amount of presumed ADR oxidation in *Salmonella* cultures) was unknown, we investigated a wide range of concentrations (10^{-10} to 10^{-5} M). After addition of AC and stimulation with 5 μg/mL ConA or 5 μg/mL PWM, cells were incubated, and proliferation was determined as described above.

Statistical Analysis

For statistical analysis, we used the software SAS, version 9.4 (SAS institute Inc., Cary, NC, United States), applying the MIXED procedure. Degrees of freedom were determined with the Kenward–Roger method (48); normal distribution and variance homogeneity were confirmed visually by normal probability plots and plots of residuals versus fitted values (49). For estimation of variance components, we used the restricted maximum likelihood method. The models included the factors "treatment" and "trial," as well as their interaction as fixed effects and "sampling day" and "sampling day × treatment" as random effects. To take into account the individual level of the pigs, "animal" was included as a repeated effect. If data were not normally distributed, logarithmic or square root transformation was performed to attain normality. The results are presented as least square (ls)-means + standard error of the mean (SEM). Statistically significant differences were determined by Fisher's least significant difference test. Significance limits were set as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{t}p < 0.1$ (tendency).

Results

Supernatants From CA-Treated S. Typhimurium Cultures Inhibit Lymphocyte Proliferation

We first evaluated the effects of supernatants from *S.* Typhimurium cultures on lymphocyte proliferation. Compared to the media control, the addition of supernatants from hormone-free *Salmonella* cultures enhanced ConA-induced lymphocyte proliferation (FIGURE 1A). In comparison to supernatants from hormone-free *Salmonella* cultures, lymphocyte proliferation

was reduced significantly if 10% or 15% of supernatants from *Salmonella* grown in the presence of ADR or NA were added and already tended to be lower ($p = 0.053$) if 5% of supernatants from *Salmonella* grown in the presence of ADR were added. In PWM-stimulated PBMCs, already the addition of supernatants from hormone-free *Salmonella* cultures reduced proliferation compared to the media control (FIGURE 1B). But similar to ConA-stimulated cells, addition of 10% or 15% of supernatants from *Salmonella* grown in the presence of NA further reduced proliferation significantly. The addition of supernatants from *Salmonella* grown in the presence of ADR caused a less pronounced suppression of PWM-stimulated cells with a significant effect if 15% and a tendency ($p = 0.058$) if 10% were added.

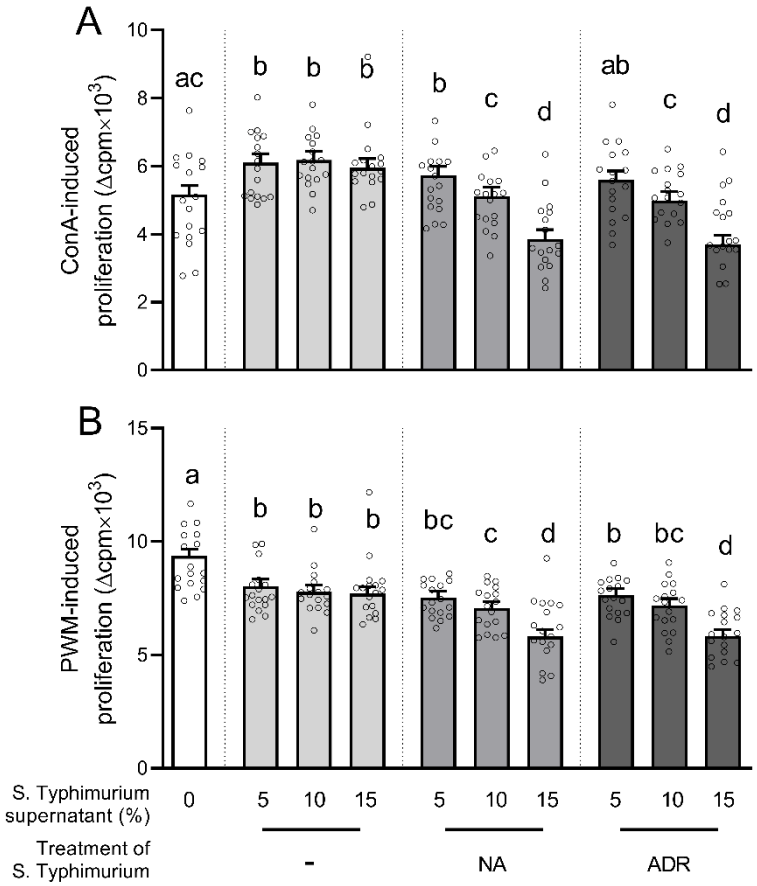

Figure 1. Lymphocyte proliferation after stimulation with either **(A)** concanavalin A (ConA) or **(B)** pokeweed mitogen (PWM), as well as addition of either serum-SAPI medium (white) or supernatants from *Salmonella* Typhimurium cultures grown for 8 h at 37°C without hormones (light gray) or in the presence of 10^{-4} M noradrenaline (NA; medium gray) or 10^{-4} M adrenaline (ADR; dark gray). Supernatants were added in concentrations of either 5%, 10%, or 15% of the cell culture volume as indicated on the *x* axis. Treatments that are statistically significant from each other are indicated by different letters on top of their bars, whereas bars that share a

common letter do not differ significantly. Data are presented as ls-means + SEM (bars) and single values of each animal (circles), $n = 16$.

Suppression of Lymphocyte Function Is Not Due to CA Action

Because CAs themselves are well-described to modulate immune cell functionality, we determined whether CAs were still present in *S.* Typhimurium cultures incubated for 8 h in the presence of either NA or ADR by HPLC analysis. Thereby, an ADR concentration of 19.67 µg/mL (1.07×10^{-4} M) was found, representing the same level as applied at the start of incubation (1×10^{-4} M). NA showed a slight decrease compared to the initial concentration of 1×10^{-4} M, but was still present in the supernatants at a concentration of 8.08 µg/mL (4.8×10^{-5} M). Thus, to verify that probable bacterial effects were not "ordinary" immunomodulating effects of CAs or caused by mere synergistic effects of bacterial products and CAs, we tested the effects of simultaneous addition of supernatant from *S.* Typhimurium grown without hormones and either NA or ADR in the same range as found within the culture supernatants tested in the initial experiment (cf. FIGURE 1).

As seen in FIGURE 2A, ConA-induced lymphocyte proliferation was significantly lower if supernatants from *Salmonella* grown in the presence of NA or ADR were added compared to supernatants from hormone-free *Salmonella* culture. Thus, the results presented above (cf. FIGURE 1) could be confirmed. Notably, in contrast to this effect, no suppression occurred on ConA-induced lymphocyte proliferation if supernatants from hormone-free *Salmonella* cultures were added simultaneously with ADR or NA (FIGURE 2A). Opposite to the effect of supernatants from *Salmonella* grown in the presence of NA or ADR, proliferation was slightly increased if cells were treated with NA ($p = 0.073$) or ADR ($p = 0.068$) alone (FIGURE 2B).

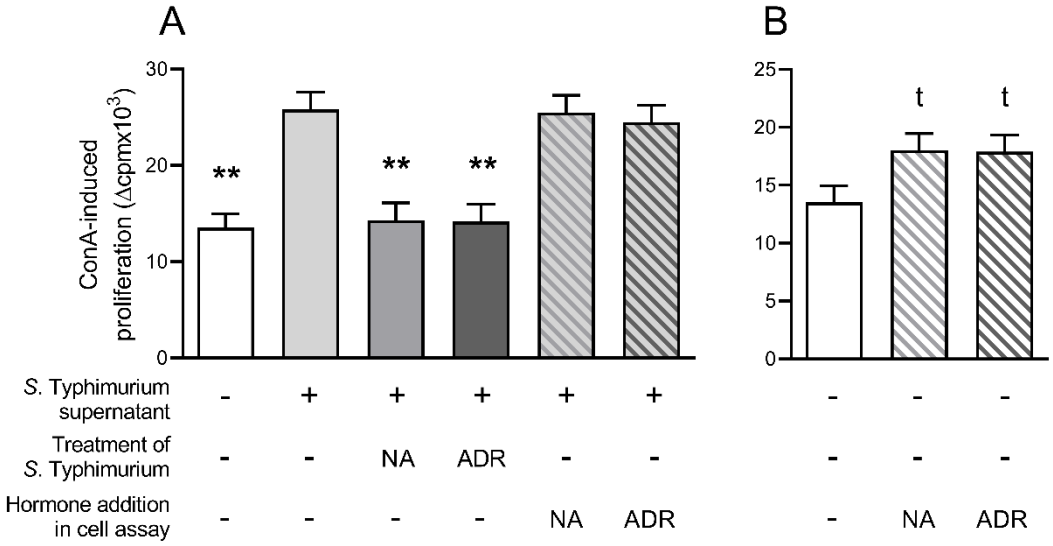

Figure 2. Lymphocyte proliferation after stimulation with 5 µg/mL concanavalin A and upon addition of 15% serum-SAPI (white), 15% supernatants from *Salmonella* Typhimurium cultures grown without hormones (light gray) or grown with either 10^{-4} M noradrenaline (NA; middle gray) or 10^{-4} M adrenaline (ADR; dark gray) for 8 h at 37°C, or addition of 15% supernatants from *S.* Typhimurium cultures grown without hormones simultaneous to catecholamine addition [10^{-5} M NA (light gray hatched in middle gray) or 10^{-5} M ADR (light gray hatched in dark gray)] **(A)**; or upon addition of 15% serum-SAPI without further additives (white) or additional supplementation with 10^{-5} M NA (white hatched in middle gray) or 10^{-5} M ADR (white hatched in dark gray) **(B)**. Data are presented as ls-means + SEM, $n = 3$. Asterisks and t in superscript indicate significant differences and tendencies compared to supernatants from hormone-free *Salmonella* culture **(A)** or the hormone-free control **(B)**, respectively.

The ADR Oxidation Product AC Is Not the Active Inhibitory Agent in Supernatants From CA-Treated Salmonella Cultures

To assess whether the oxidation of CAs by *Salmonella* might cause the observed suppressive effect of supernatants from CA-treated bacterial cultures, we performed the lymphocyte proliferation assay under the same conditions as in the first experiment (cf. FIGURE 1) but added AC instead of bacterial supernatants (FIGURES 3A,B). If PBMCs were stimulated with ConA, all tested concentrations led to an enhancement of proliferation compared to the AC-free control (FIGURE 3A), whereas no effect was observed upon stimulation with PWM (FIGURE 3B).

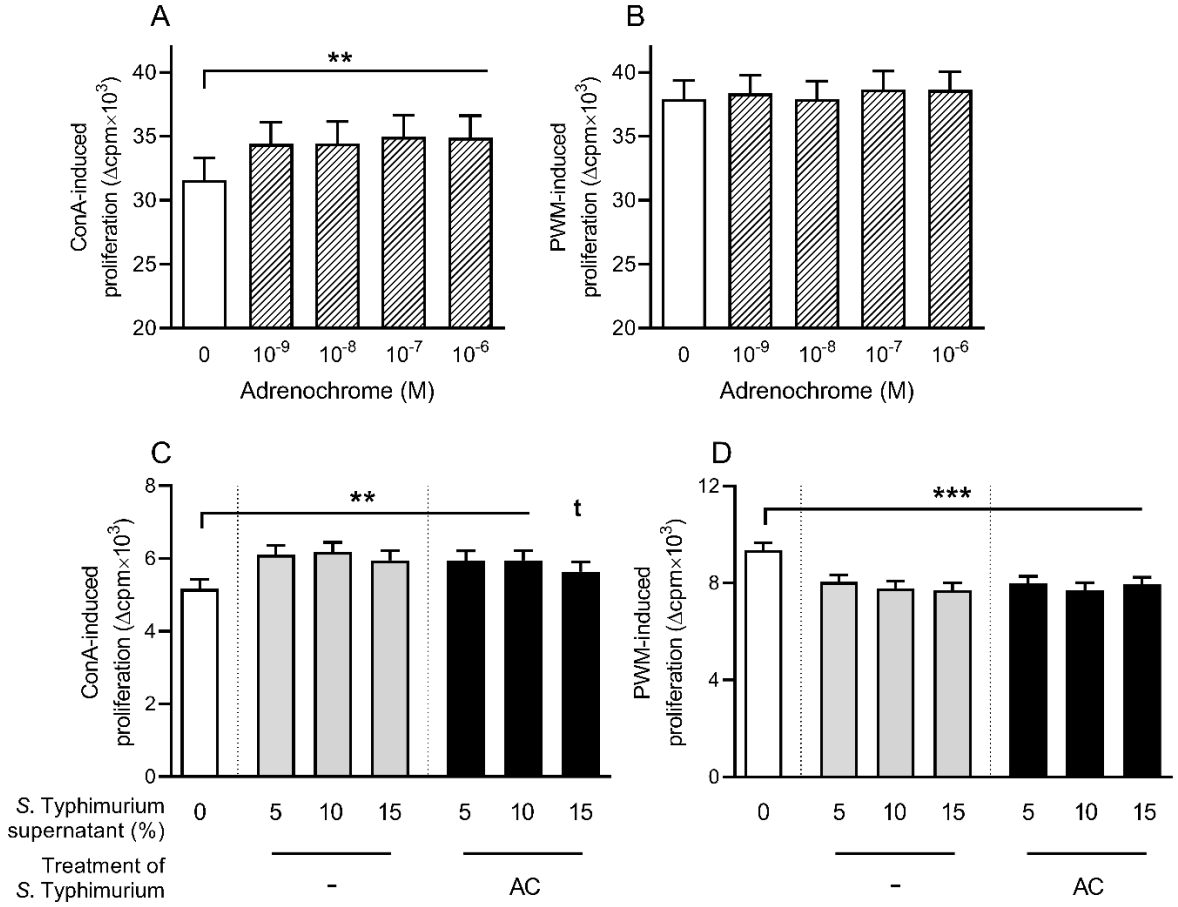

Figure 3. Lymphocyte proliferation upon addition of adrenochrome **(A,B)** or serum-SAPI medium or supernatants from *Salmonella* Typhimurium cultures grown without additive or in the presence of 2×10^{-5} M adrenochrome for 8 h at 37°C **(C,D)**, and stimulation with either 5 µg/mL concanavalin A [ConA; **(A,C)**] or 5 µg/mL pokeweed mitogen [PWM; **(B,D)**]. Significant differences are marked by asterisks, tendencies are indicated by a *t* in superscript. Data are presented as ls-means + SEM, $n = 19$ **(A,B)**, $n = 16$ **(C,D)**.

Because AC can also have a direct effect on bacteria, like in *V. cholerae* (41), we assumed that its effect on PBMCs might possibly be mediated indirectly, by modulating the behavior of *S.* Typhimurium upon sensing. In addition to the treatment of *Salmonella* cultures with NA or ADR, we thus also cultured *S.* Typhimurium with 2×10^{-5} M AC for 8 h at 37°C before centrifugation and microfiltration. If supernatant from these cultures was added to ConA-stimulated PBMCs, proliferation was enhanced compared to the serum–SAPI–control but not significantly different from the proliferation upon addition of supernatants from hormone-free *Salmonella* cultures (FIGURE 3C). If PWM was used, proliferation was lower than upon serum-SAPI addition and on the same level as with the supernatant from hormone-free *Salmonella* cultures (FIGURE 3D).

Discussion

The results of the present study indicate a close host–pathogen cross-talk in situations with elevated stress hormone levels in pigs. Based on pioneering work demonstrating the ability of many bacteria to increase pathogenicity in response to CAs (23, 50), we here show that interkingdom signaling also works the other way. Our data indicate that there is a direct action of CA-treated bacteria on host immune cells. Lymphocytes treated with cell-free supernatants from *S.* Typhimurium grown in the presence of ADR or NA showed a decreased proliferation, which is probably not the only hampered immune function. Future studies should investigate further important immune functions such as the production of pro-inflammatory cytokines, which are also involved in *Salmonella* control (51).

We demonstrate that the inhibition of lymphocyte proliferation does not simply reflect an immunomodulating effect of CAs, as retrospective addition of ADR or NA in combination with supernatant of non-treated *S.* Typhimurium did not inhibit mitogen-induced proliferation of porcine immune cells. This is also supported by our previous study, showing that under the same cell culture conditions, the sole addition of ADR or NA led to an increased lymphocyte proliferation instead of its reduction (6). This implies that the proposed immunosuppressive substance produced by CA-treated *S.* Typhimurium must be very potent if it even diminishes the enhancing effect of the CAs that were still present in the supernatants.

To the best of our knowledge, this is the first study to report that bacteria grown in the presence of stress hormones alter their growth environment—probably by producing immunomodulating substances—in a way that host immune response is impaired.

Based on own previous studies, AC was a promising candidate for the observed immunosuppression by *S.* Typhimurium. These experiments demonstrated that AC was formed during bacterial culture of *V. cholerae* (29, 41) upon ADR addition, and AC treatment of the human monocytic cell line THP-1 caused a hampered TNF-α production (41). Also, it is already known that AC can bind to ARs (39), which are present on all immune cells (4). We thus investigated whether this oxidation product of ADR may be responsible for the observed effects on porcine primary immune cells. However, AC either added directly to porcine lymphocytes or added to *S.* Typhimurium cultures did not decrease porcine lymphocyte functionality but instead had no effect or even increased it. Based on these results, it can be ruled out that AC is the immunomodulating substance responsible for the observed inhibition. Thus, *S.* Typhimurium must have produced different signaling molecule(s). At this point, it can

only be speculated as to what substance might be responsible for the findings by comparing the demonstrated effects with those attributed to already identified molecules that are produced by *S.* Typhimurium or other bacteria.

It was shown that NA triggers the release of autoinducers (AIs) in many Gram-negative bacteria including *Salmonella* (16). This group of quorum-sensing molecules not only enhances the growth and virulence of the bacteria themselves but may also influence the host immune system. The most prominently mentioned and potentially immunomodulatory AI in the literature is AI-3, which is also produced by *S.* Typhimurium (36, 52). Although the exact structure still remains unknown, it has an aminated aromatic compound and seems to have a high similarity to CAs because it can be blocked by α- and β-adrenergic antagonists (53–55), and both NA and AI-3 can bind to QseC (27). It is thus likely that AI-3 can bind to mammalian ARs. However, we have previously shown by *in vitro* culture with CAs that AR binding leads to increased proliferation of porcine PBMCs, contrary to the effects of supernatants from ADR- or NA-treated *Salmonella* presented here (6). Also, an α-adrenergic action of AI-3 is unlikely as binding to these receptors generally causes an enhanced immune functionality (4, 9). Nevertheless, it cannot be precluded at this point that AI-3 might specifically bind to β_2-ARs in mammalian immune cells, which are mostly immunosuppressive (56).

There is a second important AI molecule produced by *S.* Typhimurium in the exponential growth phase, named AI-2 (57). It plays a role in invasion and intracellular survival in macrophages (58, 59), but indications for a direct modulation of host immune cells have not been found so far. Whether this is a candidate for immunosuppression by *Salmonella* in a stressed host may be subject of future studies.

Another interesting class of bacterial hormone-like molecules is the lipophilic acyl homoserine lactones (AHLs). They are chemically analogous to eukaryotic lipid hormones and can either impair or exacerbate immune functions, depending on their concentration. It has even been shown that they have the ability to inhibit lymphocyte proliferation and TNF-α production in macrophages and T_H cells (32, 60, 61). Although this very much resembles the findings of the present study, an AHL production was so far not described in *Salmonella* species (62).

Also, it was shown that *S.* Typhimurium can deacylate the lipid A portion of their lipopolysaccharide, which results in a lower activation of Toll-like receptor 4 on antigen-presenting cells. As a consequence, the immune-activating intracellular nuclear factor κB signaling, as well as the release of pro-inflammatory cytokines, is hampered (63). It is

conceivable that the effects observed in the present study may at least partly be caused by an activation of this mechanism upon CA sensing of the bacteria.

Conclusively, this study added further novel clues to explain the increased susceptibility of a stressed host to infection. It has been shown earlier that stress has a negative impact on *Salmonella* recrudescence in pigs by increasing intracellular *Salmonella* proliferation in macrophages (64). A direct effect on invasiveness and intracellular survival rate of *S.* Typhimurium by binding of NA to the histidine kinase QseC was demonstrated in another study in mice (65). *S.* Typhimurium infection in calves was also aggravated by an increase of bacterial proliferation by NA, probably through acting as an iron donor for the bacteria (66). The present work shows for the first time that bacteria grown under the influence of NA or ADR are even able to hamper mammalian lymphocyte functionality. Thus, valuable information is added to the phenomenon of increased *Salmonella* susceptibility of stressed pigs. Pigs represent an important meat-producing agricultural species and are relevant carriers of the widely distributed zoonotic agent *S.* Typhimurium (67). At the same time, pigs are an excellent model for human salmonellosis because porcine nutritional physiology and gut anatomy as well as the immune system are very similar to that of humans (68–71). Upon this basic study, it is thus possible to make presumptions about effects of stress on the risk of salmonellosis in humans, i.e., increased risk of infection due to immunosuppression by CA-primed bacteria, while at the same time gaining knowledge about porcine immunology that may have impacts on pig husbandry and food hygiene at the slaughterhouse.

Data Availability Statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation. The animal study was reviewed and approved by the Regierungspräsidium Stuttgart.

Ethics Statement

The animal study was reviewed and approved by the Regierungspräsidium Stuttgart.

Author Contributions

VS and JS conceived and designed the study. VS, JS, SS, CT, and LR designed the experiments. CT produced bacterial supernatants. BP conducted the CA analyses. LR performed and SSS supervised the immunological experiments. LR analyzed and interpreted the data, and wrote

the original draft of the manuscript. VS, JS, SS, CT, and BP contributed to the manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

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.

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References

1. Gordan R, Gwathmey JK, Xie L-H. Autonomic and endocrine control of cardiovascular function. World J Cardiol. (2015) 7:204–14. doi: 10.4330/wjc.v7.i4.204
2. Kyrou I, Tsigos C. Stress hormones: physiological stress and regulation of metabolism. Curr Opin Pharmacol. (2009) 9:787–93. doi: 10.1016/j.coph.2009.08.007
3. Antonelli A, Torchio R, Bertolaccini L, Terzi A, Rolfo F, Agostoni P, et al. Contribution of β-adrenergic receptors to exercise-induced bronchodilatation in healthy humans. Respir Physiol Neurobiol. (2012) 184:55–9. doi: 10.1016/j.resp.2012.07.007
4. Kohm A, Sanders VM. Norepinephrine: a messenger from the brain to the immune system. Trends Immunol. (2000) 21:539–42.
5. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve–an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev. (2000) 52:595–638.
6. Reiske L, Schmucker S, Steuber J, Stefanski V. Glucocorticoids and catecholamines affect in vitro functionality of porcine blood immune cells. Animals. (2019) 9:545. doi: 10.3390/ani9080545
7. Kouassi E, Li YS, Boukhris W, Millet I, Revillard JP. Opposite effects of the catecholamines dopamine and norepinephrine on murine polyclonal B-cell activation. Immunopharmacology. (1988) 16:125–37.

8. Torres KC, Antonelli LR, Souza AL, Teixeira MM, Dutra WO, Gollob KJ. Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC. J Neuroimmunol. (2005) 166:144–57. doi: 10.1016/j.jneuroim.2005.06.006

9. Hadden JW, Hadden EM, Middleton E. Lymphocyte blast transformation. I. Demonstration of adrenergic receptors in human peripheral lymphocytes. Cell Immunol. (1970) 1:583–95.

10. Felten SY, Olschowka J. Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. J Neurosci Res. (1987) 18:37–48. doi: 10.1002/jnr.490180108

11. Bergquist J, Tarkowski A, Ewing A, Ekman R. Catecholaminergic suppression of immunocompetent cells. Immunol Today. (1998) 19:562–7.

12. Althen TG, Ono K, Topel DG. Effect of stress susceptibility or stunning method on catecholamine levels in swine. J Anim Sci. (1977) 44:985–9. doi: 10.2527/jas1977.446985x

13. Bacou E, Haurogné K, Mignot G, Allard M, de Beaurepaire L, Marchand J, et al. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. Physiol Behav. (2017) 169:1–8. doi: 10.1016/j.physbeh.2016.11.012

14. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, et al. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol. (2012) 303:G1288–95. doi: 10.1152/ajpgi.00341.2012

15. Lyte M, Ernst S. Catecholamine induced growth of gram negative bacteria. Life Sciences. (1992) 50:203–12.

16. Freestone PP, Haigh RD, Williams PH, Lyte M. Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. FEMS Microbiol Lett. (1999) 172:53–60. doi: 10.1111/j.1574-6968.1999.tb13449.x

17. Roberts A, Matthews JB, Socransky SS, Freestone PP, Williams PH, Chapple IL. Stress and the periodontal diseases: effects of catecholamines on the growth of periodontal bacteria in vitro. Oral Microbiol Immunol. (2002) 17:296–303. doi: 10.1034/j.1399-302X.2002.170506.x

18. Aneman A, Eisenhofer G, Olbe L, Dalenbäck J, Nitescu P, Fändriks L, et al. Sympathetic discharge to mesenteric organs and the liver. Evidence for substantial mesenteric organ norepinephrine spillover. J Clin Invest. (1996) 97:1640–6.

19. Kong HH. Skin microbiome: genomics-based insights into the diversity and role of skin microbes. Trends Mol Med. (2011) 17:320–8. doi: 10.1016/j.molmed.2011.01.013

20. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu W-H, et al. The human oral microbiome. J Bacteriol. (2010) 192:5002–17. doi: 10.1128/JB.00542-10

21. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell. (2006) 124:837–48. doi: 10.1016/j.cell.2006.02.017

22. Lyte M, Erickson AK, Arulanandam BP, Frank CD, Crawford MA, Francis DH. Norepinephrine-induced expression of the K99 pilus adhesin of enterotoxigenic Escherichia coli. Biochem Biophys Res Commun. (1997) 232:682–6. doi: 10.1006/bbrc.1997.6356

23. Freestone PP, Sandrini SM, Haigh RD, Lyte M. Microbial endocrinology: How stress influences susceptibility to infection. TrendsMicrobiol. (2008) 16:55–64. doi: 10.1016/j.tim.2007.11.005

24. Miethke M, Skerra A. Neutrophil gelatinase-associated lipocalin expresses antimicrobial activity by interfering with L-norepinephrine-mediated bacterial iron acquisition. Antimicrob Agents Chemother. (2010) 54:1580–9. doi: 10.1128/AAC.01158-09

25. Schaible UE, Kaufmann SH. Iron and microbial infection. Nat Rev Microbiol. (2004) 2:946–53. doi: 10.1038/nrmicro1046

26. Bearson BL, Bearson SM. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of Salmonella enterica serovar Typhimurium. Microb Pathog. (2008) 44:271–8. doi: 10.1016/j.micpath.2007.10.001

27. Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V. The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci USA. (2006) 103:10420–5. doi: 10.1073/pnas.0604343103

28. Hegde M, Wood TK, Jayaraman A. The neuroendocrine hormone norepinephrine increases Pseudomonas aeruginosa PA14 virulence through the las quorum-sensing pathway. Appl Microbiol Biotechnol. (2009) 84:763–76. doi: 10.1007/s00253-009-2045-1

29. Halang P, Toulouse C, Geißel B, Michel B, Flauger B, Müller M, et al. Response of Vibrio cholerae to the catecholamine hormones epinephrine and norepinephrine. J Bacteriol. (2015) 197:3769–78. doi: 10.1128/JB.00345-15

30. Freestone PP, Haigh RD, Lyte M. Specificity of catecholamine-induced growth in Escherichia coli O157:H7, Salmonella enterica and Yersinia enterocolitica. FEMS Microbiol Lett. (2007) 269:221–8. doi: 10.1111/j.1574-6968.2006.00619.x

31. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. (2005) 21:319–46. doi: 10.1146/annurev.cellbio.21.012704.131001

32. Telford G, Wheeler D, Williams P, Tomkins PT, Appleby P, Sewell H, et al. The Pseudomonas aeruginosa quorum-sensing signal molecule N-(3-Oxododecanoyl)-l-homoserine lactone has immunomodulatory activity. Infect Immun. (1998) 66:36–42.

33. Smith RS, Harris SG, Phipps R, Iglewski B. The Pseudomonas aeruginosa quorum-sensing molecule N-(3-Oxododecanoyl)homoserine lactone contributes to virulence and induces inflammation In Vivo. J Bacteriol. (2002) 184:1132–9. doi: 10.1128/jb.184.4.1132-1139.2002

34. Zimmermann S, Wagner C, Müller W, Brenner-Weiss G, Hug F, Prior B, et al. Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-Oxododecanoyl)-l-homoserine lactone. Infect Immun. (2006) 74:5687–92. doi: 10.1128/IAI.01940-05

35. Mota LJ, Cornelis GR. The bacterial injection kit: type III secretion systems. Ann Med. (2005) 37:234–49. doi: 10.1080/07853890510037329

36. Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci USA. (2003) 100:8951–6. doi: 10.1073/pnas.1537100100

37. Bors W, Michel C, Manfred S, Lengfelder E. The involvement of oxygen radicals during the autoxidation of adrenalin. Biochim Biophys Acta Gen Subj. (1978) 540:162–72. doi: 10.1016/0304-4165(78)90445-2

38. Alhasan R, Njus D. The epinephrine assay for superoxide: why dopamine does not work. Anal Biochem. (2008) 381:142–7. doi: 10.1016/j.ab.2008.06.030

39. Yates JC, Taam GM, Singal PK, Beamish RE, Dhalla NS. Protection against adrenochrome-induced myocardial damage by various pharmacological interventions. Br J Exp Pathol. (1980) 61:242–55.

40. Marino F, Cosentino M. Adrenergic modulation of immune cells: an update. Amino Acids. (2013) 45:55–71. doi: 10.1007/s00726-011-1186-6

41. Toulouse C, Schmucker S, Metesch K, Pfannstiel J, Michel B, Starke I, et al. Mechanism and impact of catecholamine conversion by Vibrio cholerae. Biochim Biophys Acta Bioenerg. (2019) 1860:478–87. doi: 10.1016/j.bbabio.2019.04.003

42. Verbrugghe E, Boyen F, Gaastra W, Bekhuis L, Leyman B, van Parys A, et al. The complex interplay between stress and bacterial infections in animals. Vet Microbiol. (2012) 155:115–27. doi: 10.1016/j.vetmic.2011.09.012

43. Verbrugghe E, Dhaenens M, Leyman B, Boyen F, Shearer N, van Parys A, et al. Host stress drives Salmonella recrudescence. Sci Rep. (2016) 6:20849. doi: 10.1038/srep20849

44. Kraetzl WD, Weiler U. Erfahrungen mit einem implantierbaren Kathetersystem zur frequenten und chronischen Blutentnahme bei Schafen in Gruppenhaltung und bei säugenden Sauen. Tierärztl Umsch. (1998) 53:567–74.

45. Engert LC, Weiler U, Pfaffinger B, Stefanski V, Schmucker SS. Diurnal rhythms in peripheral blood immune cell numbers of domestic pigs. Dev Comp Immunol. (2018) 79:11–20. doi: 10.1016/j.dci.2017.10.003

46. Anton AH, Sayre DF. A study of the factors affecting the aluminum oxidetrihydroxyindole procedure for the analysis of catecholamines. J Pharmacol Exp Ther. (1962) 138:360–75.

47. Schalk C, Pfaffinger B, Schmucker S, Weiler U, Stefanski V. Effects of repeated social mixing on behavior and blood immune cells of group-housed pregnant sows (Sus scrofa domestica). Livest Sci. (2018) 217:148–56. doi: 10.1016/j.livsci.2018.09.020

48. Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics. (1997) 53:983. doi: 10.2307/2533558

49. Faraway JJ. Extending the Linear Model With R: Generalized Linear, Mixed Effects and Nonparametric Regression Models. Boca Raton, FL: Chapman & Hall/CRC (2006).

50. Lyte M, Vulchanova L, Brown DR. Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell Tissue Res. (2011) 343:23–32. doi: 10.1007/s00441-010-1050-0

51. Dolowschiak T, Mueller AA, Pisan LJ, Feigelman R, Felmy B, Sellin ME, et al. IFN-gamma hinders recovery from mucosal inflammation during antibiotic therapy for Salmonella gut infection. Cell Host Microbe. (2016) 20:238–49. doi: 10.1016/j.chom.2016.06.008

52. Walters M, Sircili MP, Sperandio V. AI-3 synthesis is not dependent on luxS in Escherichia coli†. J Bacteriol. (2006) 188:5668–81. doi: 10.1128/JB.00648-06

53. Clarke MB, Sperandio V. Events at the host-microbial interface of the gastrointestinal tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on. Am J Physiol Gastrointest Liver Physiol. (2005) 288:G1105–9. doi: 10.1152/ajpgi.00572.2004

54. Bearson BL, Bearson SM, Lee IS, Brunelle BW. The Salmonella enterica serovar Typhimurium QseB response regulator negatively regulates bacterial motility and swine colonization in the absence of the QseC sensor kinase. Microb Pathog. (2010) 48:214–9. doi: 10.1016/j.micpath.2010.03.005

55. Yang Q, Anh ND, Bossier P, Defoirdt T. Norepinephrine and dopamine increase motility, biofilm formation, and virulence of Vibrio harveyi. Front Microbiol. (2014) 5:584. doi: 10.3389/fmicb.2014.00584

56. Fan X, Wang Y. b2 Adrenergic receptor on T lymphocytes and its clinical implications. Prog Nat Sci. (2009) 19:17–23. doi: 10.1016/j.pnsc.2008.10.001

57. Surette MG, Miller MB, Bassler BL. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc Natl Acad Sci USA. (1999) 96:1639–44. doi: 10.1073/pnas.96.4.1639

58. Choi J, Park J, Ryu S. Repressed Quorum sensing by overexpressing LsrR Hampers Salmonella evasion from oxidative killing within macrophages. J Microbiol Biotechnol. (2010) 20:1624–9.

59. Choi J, Shin D, Kim M, Park J, Lim S, Ryu S. LsrR-mediated quorum sensing controls invasiveness of Salmonella typhimurium by regulating SPI-1 and flagella genes. PLoS One. (2012) 7:e37059. doi: 10.1371/journal.pone.0037059

60. Ritchie AJ, Jansson A, Stallberg J, Nilsson P, Lysaght P, Cooley MA. The Pseudomonas aeruginosa quorum-sensing molecule N-3-(oxododecanoyl)-L-homoserine lactone inhibits T-cell differentiation and cytokine production by a mechanism involving an early step in T-cell activation. Infect Immun. (2005) 73:1648–55. doi: 10.1128/IAI.73.3.1648-1655.2005

61. Pritchard DI, Todd I, Brown A, Bycroft BW, Chhabra SR, Williams P, et al. Alleviation of insulitis and moderation of diabetes in NOD mice following treatment with a synthetic Pseudomonas aeruginosa signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone. Acta Diabetol. (2005) 42:119–22. doi: 10.1007/s00592-005-0190-2

62. Kendall MM, Sperandio V. Cell-to-cell signaling in E. coli and Salmonella. EcoSal Plus. (2014) 6:1–15. doi: 10.1128/ecosalplus.ESP-0002-2013

63. Kawasaki K, Ernst RK, Miller SI. 3-O-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of Salmonella typhimurium, modulates signaling through toll-like receptor 4. J Biol Chem. (2004) 279:20044–8. doi:10.1074/jbc.M401275200

64. Verbrugghe E, Boyen F, van Parys A, van Deun K, Croubels S, Thompson A, et al. Stress induced Salmonella Typhimurium recrudescence in pigs coincides with cortisol induced increased intracellular proliferation in macrophages. Vet Res. (2011) 42:118. doi: 10.1186/1297-9716-42-118

65. Moreira ML, Dorneles EM, Soares RP, Magalhães CP, Costa-Pereira C, Lage AP, et al. Cross-reactivity of commercially available anti-human monoclonal antibodies with canine cytokines: establishment of a reliable panel to detect the functional profile of peripheral blood lymphocytes by intracytoplasmic staining. Acta Vet Scand. (2015) 57:51. doi: 10.1186/s13028-015-0142-y

66. PullingerGD, Carnell SC, Sharaff FF, van Diemen PM, Dziva F, Morgan E, et al. Norepinephrine augments Salmonella enterica-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. Infect Immun. (2010) 78:372–80. doi: 10.1128/IAI.01203-09

67. Hald T, Wingstrand A, Swanenburg M, Altrock A, Thorberg BM. The occurrence and epidemiology of Salmonella in European pig slaughterhouses. Epidemiol Infect. (2003) 131:1187–203. doi: 10.1017/s0950268803001171

68. Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends Microbiol. (2012) 20:50–7. doi: 10.1016/j.tim.2011.11.002

69. Mair KH, Sedlak C, Käser T, Pasternak A, Levast B, Gerner W, et al. The porcine innate immune system: an update. Dev Comp Immunol. (2014) 45:321–43. doi: 10.1016/j.dci.2014.03.022

70. Zhang Q, Widmer G, Tzipori S. A pig model of the human gastrointestinal tract. Gut Microbes. (2013) 4:193–200. doi: 10.4161/gmic.23867

71. Roura E, Koopmans S-J, Lallès J-P, Le Huerou-Luron I, Jager ND, Schuurman T, et al. Critical review evaluating the pig as a model for human nutritional physiology. Nutr Res Rev. (2016) 29:60–90. doi: 10.1017/S0954422416000020

CHAPTER 3

GENERAL DISCUSSION

3 GENERAL DISCUSSION

Psychoneuroimmunology is the interdisciplinary study of the connection between psychological states and health, essentially mediated by endocrine, neuronal and immune mechanisms (Besedovsky and del Rey, 2007). Two central regulatory systems involved in the modulation of the immune system by physical or psychological stress are the HPA and SAM axes. The release of cortisol, adrenaline or noradrenaline can thus influence immune cell numbers and function as well as the development of inflammatory, autoimmune and infectious diseases (del Rey et al., 2008; Goldstein, 2010). Stress can either enhance or suppress immune functions, depending on duration and predominantly activated stress axis (Dhabhar, 2009; Koolhaas et al., 1999). The stress-induced increase of bacterial infections and the recrudescence of latent infections, for example known for *S.* Typhimurium, is not conclusively understood yet (Casanova-Higes et al., 2017; He et al., 2019; Konturek et al., 2011; Verbrugghe et al., 2012). In the endeavour to understand the complex interplay between stress hormones, the immune system and bacteria, large animal models with a high similarity to humans are of increasing importance but require further verification. The present thesis added many new insights to the knowledge about the impact of stress hormones on the immune system of the domestic pig. By examining the effects of cortisol, adrenaline and noradrenaline separately, their impact on porcine immune cell numbers and functionality was characterised on a high level of detail. In addition, indications of a new form of interkingdom communication under the influence of catecholamines were discovered, which presumably contributes to the increased susceptibility of stressed animals to *Salmonella* infection.

3.1 Main findings

To systematically assess the effects of cortisol, adrenaline and noradrenaline as the main stress hormones of the HPA- and the SAM axis, this project was partitioned into three studies, using both *in vitro* and *in vivo* approaches. To set the stage for a subsequent more in-depth analysis, a first experiment was designed based on the *in vitro* establishment of general principles and dose-response relationships of GC and CA application to porcine peripheral blood mononuclear cells (MANUSCRIPT I). Based on these findings, methods for functional immune assessment

were applied and extended under *in vivo* conditions, complimented by additional measurements like immune cell numbers and antibody concentrations (MANUSCRIPT II). Finally, the interplay of mammalian immune cells and *Salmonella* Typhimurium under the influence of stress hormones was explored using an experimental setting analogous to the first trial combined with bacteriological methods (MANUSCRIPT III).

3.1.1 Glucocorticoid effects on blood immune cell numbers and functionality

Since the Nobel price-awarded discovery of the therapeutic potential of glucocorticoids for the treatment of inflammatory diseases like allergies and autoimmune disorders by the mid of the 20th century, research long set a main focus on their clinical application (Hench et al., 1950; The Nobel Prize in Physiology or Medicine 1950). The underlying mechanisms of immunomodulation and the physiologic function of GCs in stressful situations, however, are still subject of intense scientific exploration. In pigs, many behavioural experiments have been conducted to study the consequences of stress on immunity. Many of them reported increased plasma cortisol concentrations and consequently attributed the observed immune effects to this stress hormone (Kick et al., 2011). However, although not intensively studied in pigs, it is most probable that the investigated stressors often likewise enhanced CA levels. To completely understand the impact of stress on immune functionality, it is important to dissect the actions of GCs and CAs. An elegant approach to this challenge is to iatrogenically increase stress hormone concentrations by administration of one hormone at a time, thus ensuring controllable and comparable blood levels. After injection of cortisol, CRF or ACTH, a decreased lymphocyte proliferation, cytokine production, NK cell cytotoxicity as well as an increased neutrophil:lymphocyte ratio was reported (Johnson et al., 1994; Otten et al., 2007; Otten et al., 2008; Salak-Johnson et al., 1996; Wallgren et al., 1994). Though it must not be forgotten that CRF can, in addition to the activation of the pituitary and subsequent cortisol synthesis in the adrenal cortex, also lead to CA release (Minton, 1994). Therefore, to study the distinct effects of CORT, it is advisable not to treat pigs with ACTH or CRF but with cortisol itself to prevent a distortion of results. Also, the blood sampling technique may skew the outcome of experiments since stressful conditions during blood collection like fixation via nose snare and vein puncture lead to rapid CA release (Dhabhar, 2018; Grouzmann et al., 2003; Sapolsky et al., 2000). To avoid these pitfalls, the present thesis relied on an innovative experimental design, using indwelling catheters for blood sampling and drug administration. By measurement of plasma hormone concentrations, it was demonstrated that the application of one stress hormone

never caused an increase of the other two. Also, these analyses confirmed that animals which received no hormone did not react with endogenous stress hormone release to the sampling procedure.

An inhibition of porcine lymphocyte proliferation after *in vitro* CORT treatment of thusly obtained PBMC (MANUSCRIPT I) could be repeated *in vivo* by intravenous administration of CORT at concentrations resembling mild physiologic stress (MANUSCRIPT II). Other experiments in pigs that obtained similarly elevated plasma cortisol concentrations are in agreement with this finding (Deguchi and Akuzawa, 1998; Kanitz et al., 2004; Tuchscherer et al., 2016; Wallgren et al., 1994). The minimal inhibitory concentration was comparable to human studies (Cupps et al., 1985; van den Brink et al., 1992) and confirms the pig as a "GC resistant" species like humans, in contrast to rodents, where lymphocyte functions are hampered already at lower GC concentrations and which are therefore deemed a "GC sensitive" species (Claman, 1972; Parrillo and Fauci, 1979; Roess et al., 1982; Westly and Kelley, 1984). This downregulation of lymphocyte function is achieved by direct gene regulation by the GR. Also, T cell receptor signalling is attenuated through the interaction of the GR with activating transcription factors like NF-κB, AP-1 or NFAT (Cain and Cidlowski, 2017; Petrillo et al., 2014; Tsitoura and Rothman, 2004). Furthermore, antigen presentation and activation of lymphocytes by release of TNFα and other proinflammatory cytokines by monocytes and dendritic cells are modulated by GCs (Cain and Cidlowski, 2017; Shodell et al., 2003; Szatmari and Nagy, 2008). In the present thesis, a downregulation of TNFα could also be demonstrated in almost every porcine leukocyte subset after *in vitro* addition of cortisol (MANUSCRIPT I). In contrast, innate immune function, portrayed by phagocytic efficiency of monocytes, was enhanced in cortisol-infused pigs (MANUSCRIPT II). A stimulation of phagocytic function under the influence of CORT was also found in humans (Barriga et al., 2001; Forner et al., 1995; Gratchev et al., 2005) and GCs were even found to prevent apoptosis in human neutrophils (Cox, 1995). This promotion of phagocytic activity by both GCs and CAs (Ortega et al., 2007) is highly valuable in stressful situations involving fighting or fleeing where an injury and subsequent bacterial contamination is likely to occur and phagocytes are the first cell types to keep a local infection at bay by fast, unspecific killing of pathogens (Lim et al., 2017).

For an efficient response to pathogens, also the distribution of immune cells in the body is essential. Under resting conditions, immune cells constantly circulate between their origins – mostly bone marrow and thymus –, blood and lymphatic vessels as well as the different non-

lymphatic organs (Dhabhar, 2002). A decrease or increase of certain immune cells in the blood as it occurs e.g. in infections but also in stressful situations, can thus indicate the release of naive cells from secondary lymphoid organs, death of circulating cells or their trafficking to different tissues (Dhabhar et al., 2012; Hermann et al., 1994; van Tits et al., 1990). While most studies both in pigs and in other species investigated blood immune cell numbers in a context of different stressors, thus mostly measuring the mixed effect of GCs and CAs, the present thesis was able to dissect the trafficking effects of CORT, ADR and NA by separate infusion (MANUSCRIPT II). The impacts of cortisol infusion on blood immune cell numbers of pigs very much resemble those reported for humans (Dale et al., 1975; Dhabhar et al., 2012; Kothari and Saunders, 1961; Zahorec, 2001). All lymphocytes belonging to adaptive immunity as well as dendritic cells and eosinophils showed a marked decrease, some reaching a nadir at numbers about half of those of the control group. At the same time, neutrophil numbers changed in the opposite direction, outranging control values by more than 100%. Under resting conditions, most neutrophils are attached to the endothelium, especially in the lung (Peters, 1998). The detachment of neutrophils from this marginated pool by GCs and CAs in a stressful situation contributes to the enhancement of innate immunity (Beis et al., 2018; Dale et al., 1975; Dhabhar et al., 2012; Fay et al., 2016). The decrease of adaptive immune cell types in the blood by cortisol may indicate a hampered immune surveillance (Dhabhar et al., 1996). However, it was also shown that a GC-induced redistribution from the blood to the skin enhanced local cell-mediated immunity (Dhabhar et al., 2000), which might contribute to the promotion of pathogen killing at a site of injury, as described for phagocytic innate immune cells.

Taken together, the findings in cortisol-infused pigs are very similar to those obtained in human studies and match the overall picture of a GC-induced shift from adaptive to innate and from T_H1 to T_H2 immunity (Ashwell et al., 2000; Elenkov and Chrousos, 1999; Leung and Bloom, 2003). Of note, the detection of classical T_H2 cytokines like IL-4, IL-5 or IL-13 in pigs is technically challenging and was not included in the present study but might be subject to future investigations with a methodological focus. Since cortisol infusion caused no changes in CA plasma concentrations, the obtained results also give valuable new insights into the immune reaction of animals with a reactive or passive coping style in stressful situations, as observed in submissive animals (Bohus et al., 1993; Henry, 1982; Holst, 1997; Koolhaas, 2008; Koolhaas and van Reenen, 2016; Stefanski, 1998).

3.1.2 Catecholamine actions on the immune system

The impact of catecholamines on immune and other body cells is mediated via α- and β-adrenergic receptors, which are embedded in the cell membrane (Perez, 2006). The distribution of the different subtypes varies depending on cell type but can also be modulated by up- or downregulation in different situations (Hadcock and Malbon, 1988; Krief et al., 1993; Schwinn, 1994; Thawornkaiwong et al., 2003). Most immune effects of catecholamines are mediated by the β2-AR which has predominantly inhibitory effects on functional parameters like proliferation and production of proinflammatory cytokines (Scanzano and Cosentino, 2015). Nevertheless, they can also enhance immune functionality via α-adrenergic stimulation (Hadden et al., 1970). The present study is the first to systematically investigate catecholamine effects on porcine immune cell numbers and functionality (MANUSCRIPT I & II). There are only few reports on stressor-associated increases of blood CA concentrations and associated immune modulations in pigs (Bacou et al., 2017b; de Groot et al., 2001; Kanitz et al., 2019; Ruis et al., 2001), but it has to be assumed that the reported immune effects may be caused by simultaneous CA- and GC release. By applying only NA or ADR, this interference was avoided in the present project. The effects of catecholamines on immune cell functionality found in this thesis show a differentiated picture of both immune enhancement and inhibition, depending on investigated cell type and time of stress hormone application in relation to cell stimulation. While mitogenic stimulation after catecholamine infusion caused a reduced lymphocyte proliferation (MANUSCRIPT II), *in vitro* addition at the time of stimulation caused the opposite effect (MANUSCRIPT I). This nicely shows that the timing of AR binding in relation to antigenic (or mitogenic) stimulation is crucial for the outcome of CA treatment on lymphocyte functionality. As reviewed by Sanders (2012), adrenergic receptor engagement before stimulation usually leads to reduced activation while CA binding during or after stimulation has either no effect or enhances lymphocyte function. The reduced proliferation in the *in vivo* trial might also in parts be explained by a redistribution phenomenon. In a human CA infusion study, a decreased lymphocyte proliferation was accompanied by an increased β-AR-density on immune cells and a redistribution of circulating lymphocytes to other tissues while naive splenic lymphocytes were released. These cells carry more β2-ARs, and are also more responsive toward β2-AR mediated inhibition (van Tits et al., 1990).

Similar to cortisol, both adrenaline and noradrenaline promoted innate immune function in pigs, as portrayed by a higher phagocytic activity of monocytes and neutrophils (MANUSCRIPT II).

Few other studies in pigs have investigated phagocytosis in pigs under the influence of stress hormones (Bacou et al., 2017b; Lewis et al., 2008), but none of them has portrayed their effects separately and investigated both number of active phagocytes and efficiency of the single phagocytic cell. This project thus adds new details and gives further evidence for a promotion of innate immune function described in other species also for the pig and increases its value as a human model.

Analogous to CORT, the impact of ADR and NA on immune cell numbers in porcine blood was analysed via separate infusion. The two catecholamines exerted mostly similar effects after 2h, where almost all T cell subsets decreased, an effect previously described in other species (Dhabhar et al., 2012). The short-term effects of CAs are generally biphasic with a fast increase of blood lymphocyte numbers within 30 min, which is primarily caused by detachment from vascular endothelia and release from the spleen (Benschop et al., 1996; Dhabhar et al., 2012). The subsequent drop in numbers is then due to redistribution to endangered tissues, like the skin or gut, and homing to lymphatic organs (Carlson et al., 1997; Suzuki and Nakai, 2017). Particularly noteworthy is the reaction of NK cells to adrenaline, which is directed in an opposite direction to the other lymphocytes. This has also been described in other species and is consistent with the elevation of other innate immune cells that are responsible for fast, unspecific pathogen control (Schedlowski et al., 1993; Schedlowski et al., 1996). In contrast to cortisol, where the reduction of immune cell numbers lasted the whole infusion period, lymphocytes returned to normal after 24h or showed even a temporary overshoot to levels above the control group. Consistent with the enhanced phagocytic efficiency, also the numbers of monocytes and neutrophils in the blood increased in CA-treated pigs.

In summary, the demonstrated catecholamine actions on the porcine immune system are diverse, but in comparison to cortisol, an enhancement of immune cell function and numbers seems to be prevail. Thus, the findings of the present thesis support the picture of an enhanced protection by increased immune functionality in fight-or-flight situations with acute catecholamine release (Dhabhar, 2018) also for the pig. Also, the data obtained here add new information about the immune reaction of animals with a proactive coping style, which is characterised by a high sympathetic reactivity and more aggressive behaviour (Holst, 1997; Kanitz et al., 2019; Koolhaas, 2008; Koolhaas and van Reenen, 2016; Stefanski, 1998).

3.1.3 Immunomodulation by catecholamine-primed bacteria

It has been known for a long time that the incidence and persistence of *Salmonella* infections is enhanced by stress (Miraglia and Berry, 1962; Previte et al., 1973). While this observation has long been attributed to a stress hormone-related impairment of immune competence, a further dimension of stress hormone action was discovered around the turn of the millennium. It was demonstrated that catecholamines can also be sensed by many microorganisms and their perception is answered by enhancement of pathogenic properties (Lyte et al., 1996; Lyte and Ernst, 1992). *Salmonella* Typhimurium also responds to CAs with increased growth and motility, which is mediated by both direct sensing of NA via QseC and the use of CAs as siderophores for iron acquisition (Bailey et al., 1999; Bearson and Bearson, 2008; Moreira et al., 2010; Pullinger et al., 2010). The present thesis now added a third dimension to this interkingdom cross-talk by demonstrating that *S.* Typhimurium grown in the presence of CAs can even inhibit host immune functionality (MANUSCRIPT III). Upon addition of supernatants of these bacterial cultures to porcine PBMC, a decrease of lymphocyte proliferation and numbers of TNFα producers was observed. The hampered TNFα production affected all investigated subsets, involving both cells of innate and adaptive immunity. It can therefore be assumed that important functions for an effective infection control, such as antigen presentation by DCs, monocytes and B cells and T_H cell help, connecting innate and adaptive immunity, as well as killing of infected cells by CTLs and NK cells are weakened by CA-treated *Salmonella* bacteria. The next step is now to identify the underlying mechanisms of this phenomenon. It was demonstrated in the present study that the suppressive effects were not caused by the CAs themselves, which remained in the supernatants at high concentrations after bacterial culture. Contrarily, the effects of the supernatants from CA-cultured *Salmonella* were directed in an opposite direction to those exerted by CAs under the same conditions (MANUSCRIPT I + III). It was further investigated whether the ADR oxidation product adrenochrome or bacterial conversion thereof might explain the findings. AC formation can be promoted by bacterial superoxide production and supports bacterial growth (Halang et al., 2015; Toulouse et al., 2019). Though it was already known that AC has an impact on mammalian cells by binding to adrenergic receptors (Yates et al., 1980), the present thesis found mild stimulating effects on porcine immune cells and could thus disqualify it as a possible immunosuppressing substance produced by CA-treated *Salmonella*.

At this point, it can only be speculated as to what mechanisms may cause these immunosuppressive effects of CA-primed *S.* Typhimurium. Possible candidates may be immune modulating bacterial communication molecules like AHLs and AIs (Freestone et al., 1999; Pritchard et al., 2005; Ritchie et al., 2005; Sperandio et al., 2003; Telford et al., 1998; Walters et al., 2006). They have been identified in microbiological studies independently of CA sensing but an enhancement of their production upon CA perception is conceivable. As discussed in MANUSCRIPT III, the effect of AIs are presumably different to those observed in the present thesis, but further studies are needed to disqualify them or prove this assumption wrong. The actions of AHLs, on the other hand, very much resemble those described here for supernatants from CA-treated *Salmonella* cultures, but based on current knowledge, these molecules are produced by many other gram-negative bacteria but not *Salmonella* (Kendall and Sperandio, 2014). For E. coli, it was found that it converts NA into 3,4-dihydroxymandelic acid (DHMA), which acts as a chemoattractant and promotes virulence factor expression and attachment to epithelia via QseC (Sule et al., 2017). Future studies may investigate whether *Salmonella* also produces DHMA and if this molecule has immunosuppressive properties. Another candidate molecule might be haemolysin E, which was found in *S.* Typhi after exposure to NA and ADR and its release could be inhibited by the β-AR blocker propranolol (Karavolos et al., 2011). Haemolysins serve the purpose of releasing iron from erythrocytes but also leukocytes by inducing pores in their cell membranes (Sritharan, 2006). The observed reduction of lymphocyte proliferation by supernatants of CA-treated bacteria in the present thesis might thus also be caused by leukocyte cell death if this molecule is produced by *S.* Typhimurium, too.

A proteome analysis of CA-treated *V. cholerae* revealed altered abundances of many proteins (Toulouse et al., 2019). Especially the increase of one protein, which is not characterised until now but probably mediates the release of other substances, may be of interest if it is also produced by *S.* Typhimurium. Future proteome analyses and liquid chromatography-mass spectrometry investigations regarding the supernatants of *Salmonella* grown with ADR or NA supplementation may help finding the proposed immunomodulating substances.

3.2 Implications for porcine health and animal welfare

The present thesis gives a detailed description of alterations in porcine blood immune cell numbers combined with innate and adaptive functional parameters under the influence of a

single stress hormone. It thus serves a dual purpose: first, it adds valuable information on the comparability of pigs and humans in the field of psychoneuroimmunology and strengthens the role of domestic pigs as a human relevant model. Second, the results create a solid base for a better understanding of porcine immunomodulations by GCs and CAs in stress situations and the associated different coping strategies and have the potential to improve animal welfare and health. An intact immune system is important to maintain healthy and productive animals and to reduce the risk of infectious diseases (Colditz, 2002). Though especially chronic stress is generally known to enhance the risk of infections, this phenomenon is not fully understood yet and it is important to have a closer look at the underlying hormonal mechanisms and the interplay of immune cells and bacteria. While a short-term adaptation of the immune system to stress is biologically useful, especially GCs have the potential to impair immune competence if plasma levels are elevated chronically. In the present thesis, most investigated immune functions were inhibited both *in vitro* and *in vivo* and the numbers of important specialised adaptive immune cells were drastically decreased. Even after cessation of cortisol infusion, the numbers of NK cells, DCs, B cells and antigen-experienced T_H cells were reduced, implying a possible longer-lasting effect on the important presentation of foreign antigens by innate immune cells, T_H cell mediation and amplification of the message and effective subsequent B cell activation. Also, as it was shown in earlier studies after ACTH administration (Salak-Johnson et al., 1996), the killing of infected cells by NK cells may be impaired and even more so if their numbers are decreased. To prevent these negative effects, pig husbandry systems should be designed to reduce stressors accompanied by chronic GC elevation, like housing in gestation crates (Grün et al., 2013; Grün et al., 2014) or repeated mixing of unfamiliar pigs (Deguchi and Akuzawa, 1998).

While GC administration had no effect on the degradation of circulating total IgG and IgM in the present project, it is known that they impair primary and secondary immune response to novel antigens (Cohen et al., 2001; Fleshner, 2000). It is therefore important to optimize management practices and handling of the animals to prevent elevated cortisol concentrations during vaccinations. Also, since cortisol concentrations in pigs show a diurnal peak in the morning (Ruis et al., 1997) this might not be the best time of day to carry out vaccinations and possibly other medical treatments. In human patients, surgery in the morning with elevated plasma cortisol levels is associated with a slower recovery and increased levels of

proinflammatory cytokines compared to surgery in the afternoon where cortisol levels are low (Kwon et al., 2019).

Catecholamines, on the other hand, exerted some interesting immunoenhancing effects on pigs, with increased lymphocyte proliferation (MANUSCRIPT I) and phagocytic function of both monocytes and neutrophils (MANUSCRIPT II). Also, the numbers of some adaptive immune cells were enhanced, especially after 24h ADR treatment (MANUSCRIPT II). This is in accordance with previous studies that found mostly beneficial effects of short-term stress and especially for CAs (Dhabhar, 2018). For example, it was demonstrated in other species that enhanced plasma CA concentrations can promote memory formation after vaccination (Dhabhar and Viswanathan, 2005). In practical pig husbandry, this knowledge may also be used to improve the efficiency of vaccinations. For example, CAs could be administered simultaneously with the vaccine or the timing of vaccinations could be adjusted to the natural diurnal peak of endogenous CAs in pigs, which appears to occur around noon and thus later than the cortisol peak in the morning (Hay et al., 2000). Furthermore, it was shown in human surgical patients, that a preoperative enhancement of plasma CAs has beneficial effects on wound healing after the operation through the adaptive redistribution of immune cells (Rosenberger et al., 2009). On the other hand, CAs also hamper NK cell activity and resistance to tumour metastases which is why β-AR antagonists are administered before tumour surgery (Ben-Eliyahu et al., 2000; Neeman et al., 2012). These findings might also be useful for surgical procedures in pigs, for example by applying CAs before the intervention. However, since CAs suppress NK cell activity, they should only be given in routine surgeries in young, healthy pigs, like cryptorchidectomy or umbilical hernia repair.

Furthermore, chronic SAM axis activation caused by management practices or housing conditions should be avoided, as this thesis provided indications for a detrimental effect on the defence against *S.* Typhimurium. The increased incidence of primary *Salmonella* infections and the recrudescence of latent asymptomatic infections remains to be fully understood and is subject of ongoing research efforts. Modulation of intestinal mucus production and peristaltic motility, immunomodulation by GCs and bacterial CA sensing have been found to contribute to the pathology (Berends et al., 1996; He et al., 2019; Konturek et al., 2011; Lyte et al., 2011; Silva-Herzog et al., 2015; Stapels et al., 2018; Verbrugghe et al., 2011; Verbrugghe et al., 2012; Verbrugghe et al., 2016).

The present thesis now added a new piece to this puzzle and may help tackle porcine and human salmonellosis as well as zoonotic transmission. The prevention of chronic stress, especially in

conjunction with repeated hierarchical fights, might not only contribute to a more potent immune response to *Salmonella* but also prevent CA-induced enhancement of *S.* Typhimurium pathogenicity and immunosuppression by CA-primed bacteria. This may even help to reduce the usage of antibiotics in pig husbandry as it is strived for in the endeavour to fight the development of antibiotic resistance (Laxminarayan et al., 2013; van Boeckel et al., 2015).

3.3 Suggestions for future research

While this project was able to present many new insights into the interplay of stress hormones, the porcine immune system and bacteria, it also raised new research questions that may be subject to future investigations. The numbers of blood immune cell subsets during GC or CA infusion were documented on a high level of detail but the origin of increased cell types as well as the fate of decreasing subsets remain unknown. Studies with labelled immune cells and histologic and flow cytometric analysis of lymph nodes, spleen, lung and bone marrow will give a detailed picture of underlying trafficking processes and homing sites. Especially the lung might be a tissue of interest to establish the pig as a model for asthma. It was shown that neutrophil asthma in humans is promoted by GCs (Saffar et al., 2011) and that the numbers of neutrophils in airway tissue are increased (Nguyen et al., 2005). Tracking of neutrophil migration in stress hormone treated pigs will show if this observation applies also to this species. Furthermore, studies with the same experimental setting but with multiple blood samplings during the first two hours would be of interest to validate if pigs like humans show an initial increase of blood lymphocyte numbers during CA treatment (Dimitrov et al., 2010; van Tits et al., 1990) to further verify the similarities between pigs and humans regarding immune cell trafficking.

The present work delivered valuable information about the isolated immune effects of ADR and NA, which is also interesting regarding the use of CAs in other research fields. The two CAs are often applied separately; for example ADR is used for haemostasis (Cartotto et al., 2000; Gacto et al., 2009) or resuscitation after cardiac arrest (Jacobs et al., 2011; Mauch et al., 2014) and NA is often applied via continuous infusion during surgery to counter the anaesthesia-induced drop of blood pressure (Hiltebrand et al., 2011; Regueira et al., 2008). The data presented in MANUSCRIPT II deliver a new perspective for possible side effects of such routine treatments on the immune system. However, in a natural stress situation, the

enhancement of only one of these CAs is rare and in fight-or-flight situations as well as in proactively coping animals, usually both ADR and NA are released (de Boer et al., 1990; Koolhaas et al., 1999). Therefore, follow-up experiments with pigs receiving both CAs via intravenous infusion or even all three stress hormones simultaneously will be of interest to simulate different biological stress situations.

Another important issue that should be explored in the future is the number and distribution of adrenergic receptors on the different porcine immune cell subsets. It is known from other species that there can be big differences, resulting in disparate effects of catecholamines on different immune cell types (Sanders et al., 2001). While the expression of the mostly suppressive β2-AR is most widely distributed among immune cells, the β1-, α1- and α2-ARs with predominantly stimulating effects can also be found (Cosentino et al., 2007; Jetschmann et al., 1997; Kavelaars, 2002). Beside the time of CA binding in relation to immune cell activation, the number and ratio of different ARs on a cell has a substantial impact on the resulting CA effect (Karaszewski et al., 1990; Kin and Sanders, 2006). Ligand binding studies to determine the AR distribution on porcine immune cell subsets would be of great interest to better explain the findings of the present thesis. However, due to the lack of pig-specific tools, this is not possible to date but investigations on the transcriptional level might give a first impression (Bacou et al., 2017a). Another possibility to address the differential effects of α- and β-ARs would be infusion studies with ADR or NA administration and the concurrent application of specific α- or β-adrenergic antagonists, like propranolol, butoxamine or phentolamine (Arai et al., 2013; Engler et al., 2004).

To investigate the impact of increased CA or GC plasma levels on the efficiency of vaccines, which is of high practical relevance in pig husbandry, follow-up studies should be conducted with either primary or secondary vaccination carried out during the stress hormone infusion phase. Since fixation of the pigs via nose snare often is necessary for intramuscular injection but is accompanied by CA release also in non-CA-treated pigs, oral vaccination might offer an alternative option.

Moreover, while castrated males have many advantages as experimental animals regarding the absence of confounding effects of sex hormones and the easy handling, it would be of interest to investigate GC and CA effects in entire males and sows for a better comparability to humans. Generally, androgens suppress T- and B- cell responses, while oestrogens only affect T cells. Furthermore, the GC response to stress is inhibited by androgens (Da Silva, 1999).

More research should also be conducted in order to further characterise the interplay of porcine immune cells and *Salmonella* under the influence of enhanced CA concentrations. In addition to the abovementioned further characterisation of the composition of the supernatants obtained from *S.* Typhimurium cultured with CAs, *in vivo* studies investigating the interkingdom communication in a natural setting will be of high value. Therefore, combining the intravenous infusion of CAs at doses high enough to cross the intestinal border with an intestinal loop technique (Boutrup et al., 2010) may bring interesting insights regarding the actual consequences of the cross-talk with intraepithelial and lamina propria lymphocytes.

3.4 Conclusion

The present thesis investigated the isolated effects of cortisol, adrenaline and noradrenaline on immune cell numbers and function in the domestic pig and thus contributed to closing major knowledge gaps. The effects of physiologically elevated cortisol concentrations on leukocyte subsets in the blood have been described in unprecedented detail and for the first time ever have the impacts of separately applied catecholamines been demonstrated. By validating its high similarity to humans also in the field of stress physiology, the present project established the pig as a model in psychoneuroimmunology research. The obtained results furthermore have the potential to increase animal welfare and health by demonstrating potential risks of immunosuppression by stress. For the first time in any species, this work provided evidence for a modulation of mammalian immune functionality by catecholamine-exposed bacteria, thus providing new explanatory approaches for a stress-induced increased susceptibility to bacterial infections.

3.5 References

Arai, M., Sato, T., Takeuchi, S., Goto, S., Togari, A., 2013. Dose effects of butoxamine, a selective β2-adrenoceptor antagonist, on bone metabolism in spontaneously hypertensive rat. European Journal of Pharmacology 701 (1-3), 7–13.

Ashwell, J.D., Lu, F.W., Vacchio, M.S., 2000. Glucocorticoids in T cell development and function. Annual Review of Immunology 18, 309–345.

Bacou, E., Haurogné, K., Allard, M., Mignot, G., Bach, J.-M., Hervé, J., Lieubeau, B., 2017a. β2-adrenoreceptor stimulation dampens the LPS-induced M1 polarization in pig macrophages. Developmental & Comparative Immunology 76, 169–176.

Bacou, E., Haurogné, K., Mignot, G., Allard, M., de Beaurepaire, L., Marchand, J., Terenina, E., Billon, Y., Jacques, J., Bach, J.-M., Mormède, P., Hervé, J., Lieubeau, B., 2017b. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. Physiology & Behavior 169, 1–8.

Bailey, M.T., Karaszewski, J.W., Lubach, G.R., Coe, C.L., Lyte, M., 1999. *In vivo* adaptation of attenuated *Salmonella* Typhimurium results in increased Growth upon exposure to norepinephrine. Physiology & Behavior 67 (3), 359–364.

Barriga, C., Martín, M.I., Tabla, R., Ortega, E., Rodríguez, A.B., 2001. Circadian rhythm of melatonin, corticosterone and phagocytosis: effect of stress. Journal of Pineal Research 30 (3), 180–187.

Bearson, B.L., Bearson, S.M.D., 2008. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of *Salmonella enterica* serovar Typhimurium. Microbial Pathogenesis 44 (4), 271–278.

Beis, D., Känel, R. von, Heimgartner, N., Zuccarella-Hackl, C., Bürkle, A., Ehlert, U., Wirtz, P.H., 2018. The role of norepinephrine and α-adrenergic receptors in acute stress-induced changes in granulocytes and monocytes. Psychosomatic Medicine 80 (7), 649–658.

Ben-Eliyahu, S., Shakhar, G., Page, G.G., Stefanski, V., Shakhar, K., 2000. Suppression of NK cell activity and of resistance to metastasis by stress: A role for adrenal catecholamines and β-adrenoceptors. Neuroimmunomodulation 8 (3), 154–164.

Benschop, R.J., Rodriguez-Feuerhahn, M., Schedlowski, M., 1996. Catecholamine-induced leukocytosis: early observations, current research, and future directions. Brain, Behavior, and Immunity 10 (2), 77–91.

Berends, B.R., Urlings, H.A.P., Snijders, J.M.A., van Knapen, F., 1996. Identification and quantification of risk factors in animal management and transport regarding *Salmonella* spp. in pigs. International Journal of Food Microbiology 30 (1-2), 37–53.

Besedovsky, H.O., del Rey, A., 2007. Physiology of psychoneuroimmunology: a personal view. Brain, Behavior, and Immunity 21 (1), 34–44.

Bohus, B., Koolhaas, J.M., Heijnen, C.J., Boer, O. de, 1993. Immunological responses to social stress: dependence on social environment and coping abilities. Neuropsychobiology 28 (1-2), 95–99.

Boutrup, T.S., Schauser, K., Agerholm, J.S., Jensen, T.K., 2010. Application of a pig ligated intestinal loop model for early *Lawsonia intracellularis* infection. Acta Veterinaria Scandinavica 52, 17.

Cain, D.W., Cidlowski, J.A., 2017. Immune regulation by glucocorticoids. Nature Reviews Immunology 17 (4), 233–247.

Carlson, S.L., Fox, S., Abell, K.M., 1997. Catecholamine modulation of lymphocyte homing to lymphoid tissues. Brain, Behavior, and Immunity 11 (4), 307–320.

Cartotto, R., Musgrave, M.A., Beveridge, M., Fish, J., Gomez, M., 2000. Minimizing blood loss in burn surgery. The Journal of Trauma 49 (6), 1034–1039.

Casanova-Higes, A., Andres-Barranco, S., Mainar-Jaime, R.C., 2017. Influence of on-farm pig *Salmonella* status on *Salmonella* shedding at slaughter. Zoonoses and Public Health 64 (5), 328–336.

Claman, H.N., 1972. Corticosteroids and lymphoid cells. The New England Journal of Medicine 287 (8), 388–397.

Cohen, S., Miller, G.E., Rabin, B.S., 2001. Psychological stress and antibody response to immunization: a critical review of the human literature. Psychosomatic Medicine 63 (1), 7–18.

Colditz, I.G., 2002. Effects of the immune system on metabolism: implications for production and disease resistance in livestock. Livestock Production Science 75 (3), 257–268.

Cosentino, M., Fietta, A.M., Ferrari, M., Rasini, E., Bombelli, R., Carcano, E., Saporiti, F., Meloni, F., Marino, F., Lecchini, S., 2007. Human CD4+CD25+ regulatory T cells selectively express tyrosine hydroxylase and contain endogenous catecholamines subserving an autocrine/paracrine inhibitory functional loop. Blood 109 (2), 632–642.

Cox, G., 1995. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. Journal of Immunology 154 (9), 4719–4725.

Cupps, T.R., Gerrard, T.L., Falkoff, R.J., Whalen, G., Fauci, A.S., 1985. Effects of in vitro corticosteroids on B cell activation, proliferation, and differentiation. Journal of Clinical Investigation 75 (2), 754–761.

Da Silva, J.A., 1999. Sex hormones and glucocorticoids: interactions with the immune system. Annals of the New York Academy of Sciences 876, 102-17.

Dale, D.C., Fauci, A.S., Guerry D, I.V., Wolff, S.M., 1975. Comparison of agents producing a neutrophilic leukocytosis in man. Hydrocortisone, prednisone, endotoxin, and etiocholanolone. Journal of Clinical Investigation 56 (4), 808–813.

de Boer, S.F., Slangen, J.L., van der Gugten, J., 1990. Plasma catecholamine and corticosterone levels during active and passive shock-prod avoidance behavior in rats: Effects of chlordiazepoxide. Physiology & Behavior 47 (6), 1089–1098.

de Groot, J., Ruis, M.A., Scholten, J.W., Koolhaas, J.M., Boersma, W.J.A., 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiology & Behavior 73, 145–158.

Deguchi, E., Akuzawa, M., 1998. Effects of fighting after grouping on plasma cortisol concentration and lymphocyte blastogenesis of peripheral blood mononuclear cells induced by mitogens in piglets. The Journal of Veterinary Medical Science 60, 149–153.

del Rey, A., Chrousos, G.P., Besedovsky, H.O. (Eds.), 2008. The hypothalamus-pituitary-adrenal axis. Elsevier, Amsterdam, London.

Dhabhar, F.S., 2002. Stress-induced augmentation of immune function—the role of stress hormones, leukocyte trafficking, and cytokines. Brain, Behavior, and Immunity 16 (6), 785–798.

Dhabhar, F.S., 2009. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16 (5), 300–317.

Dhabhar, F.S., 2018. The short-term stress response - Mother Nature's mechanism for enhancing protection and performance under conditions of threat, challenge, and opportunity. Frontiers in Neuroendocrinology 49, 175–192.

Dhabhar, F.S., Malarkey, W.B., Neri, E., Mcewen, B.S., 2012. Stress-induced redistribution of immune cells—from barracks to boulevards to battlefields: A tale of three hormones – Curt Richter Award Winner. Psychoneuroendocrinology 37 (9), 1345–1368.

Dhabhar, F.S., Miller, A.H., McEwen, B.S., Spencer, R.L., 1996. Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. Journal of Immunology 157 (4), 1638–1644.

Dhabhar, F.S., Satoskar, A.R., Bluethmann, H., David, J.R., McEwen, B.S., 2000. Stress-induced enhancement of skin immune function: A role for gamma interferon. Proceedings of the National Academy of Sciences of the United States of America 97 (6), 2846–2851.

Dhabhar, F.S., Viswanathan, K., 2005. Short-term stress experienced at time of immunization induces a long-lasting increase in immunologic memory. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 289 (3), R738-44.

Dimitrov, S., Lange, T., Born, J., 2010. Selective mobilization of cytotoxic leukocytes by epinephrine. Journal of Immunology 184 (1), 503–511.

Elenkov, I.J., Chrousos, G.P., 1999. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends in Endocrinology & Metabolism 10 (9), 359–368.

Engler, H., Dawils, L., Hoves, S., Kurth, S., Stevenson, J.R., Schauenstein, K., Stefanski, V., 2004. Effects of social stress on blood leukocyte distribution: the role of alpha- and beta-adrenergic mechanisms. Journal of Neuroimmunology 156 (1-2), 153–162.

Fay, M.E., Myers, D.R., Kumar, A., Turbyfield, C.T., Byler, R., Crawford, K., Mannino, R.G., Laohapant, A., Tyburski, E.A., Sakurai, Y., Rosenbluth, M.J., Switz, N.A., Sulchek, T.A., Graham, M.D., Lam, W.A., 2016. Cellular softening mediates leukocyte demargination and trafficking, thereby increasing clinical blood counts. Proceedings of the National Academy of Sciences of the United States of America 113 (8), 1987–1992.

Fleshner, M., 2000. Exercise and neuroendocrine regulation of antibody production: protective effect of physical activity on stress-induced suppression of the specific antibody response. International Journal of Sports Medicine 21 Suppl 1, S14-9.

Forner, M.A., Barriga, C., Rodriguez, A.B., Ortega, E., 1995. A study of the role of corticosterone as a mediator in exercise-induced stimulation of murine macrophage phagocytosis. The Journal of Physiology 488 (Pt 3), 789–794.

Freestone, P.P., Haigh, R.D., Williams, P.H., Lyte, M., 1999. Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. FEMS Microbiology Letters 172 (1), 53–60.

Gacto, P., Miralles, F., Pereyra, J.J., Perez, A., Martínez, E., 2009. Haemostatic effects of adrenaline-lidocaine subcutaneous infiltration at donor sites. Burns: Journal of the International Society for Burn Injuries 35 (3), 343–347.

Goldstein, D.S., 2010. Adrenal responses to stress. Cellular and Molecular Neurobiology 30 (8), 1433–1440.

Gratchev, A., Kzhyshkowska, J., Utikal, J., Goerdt, S., 2005. Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scandinavian Journal of Immunology 61 (1), 10–17.

Grouzmann, E., Cavadas, C., Grand, D., Moratel, M., Aubert, J.-F., Brunner, H.R., Mazzolai, L., 2003. Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. Pflugers Archiv: European Journal of Physiology 447, 254–258.

Grün, V., Schmucker, S., Schalk, C., Flauger, B., Stefanski, V., 2014. Characterization of the adaptive immune response following immunization in pregnant sows *(Sus scrofa*) kept in two different housing systems. Journal of Animal Science 92 (8), 3388–3397.

Grün, V., Schmucker, S., Schalk, C., Flauger, B., Weiler, U., Stefanski, V., 2013. Influence of different housing systems on distribution, function and mitogen-response of leukocytes in pregnant sows. Animals 3 (4), 1123–1141.

Hadcock, J.R., Malbon, C.C., 1988. Down-regulation of beta-adrenergic receptors: agonist-induced reduction in receptor mRNA levels. Proceedings of the National Academy of Sciences of the United States of America 85 (14), 5021–5025.

Hadden, J.W., Hadden, E.M., Middleton, E., 1970. Lymphocyte blast transformation. I. Demonstration of adrenergic receptors in human peripheral lymphocytes. Cellular Immunology 1, 583–595.

Halang, P., Toulouse, C., Geißel, B., Michel, B., Flauger, B., Müller, M., Voegele, R.T., Stefanski, V., Steuber, J., 2015. Response of *Vibrio cholerae* to the catecholamine hormones epinephrine and norepinephrine. Journal of Bacteriology 197 (24), 3769–3778.

Hay, M., Meunier-Salaün, M.C., Brulaud, F., Monnier, M., Mormède, P., 2000. Assessment of hypothalamic-pituitary-adrenal axis and sympathetic nervous system activity in pregnant sows through the measurement of glucocorticoids and catecholamines in urine. Journal of Animal Science 78 (2), 420–428.

He, J., Guo, H., Zheng, W., Yao, W., 2019. Effects of stress on the mucus-microbial interactions in the gut. Current Protein & Peptide Science 20 (2), 155–163.

Hench, P.S., Kendall, E.C., Slocumb, C.H., Polley, H.F., 1950. Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions. Archives of Internal Medicine 85 (4), 545–666.

Henry, J.P., 1982. The relation of social to biological processes in disease. Social Science & Medicine 16 (4), 369–380.

Hermann, G., Tovar, C.A., Beck, F.M., Sheridan, J.F., 1994. Kinetics of glucocorticoid response to restraint stress and/or experimental influenza viral infection in two inbred strains of mice. Journal of Neuroimmunology 49 (1-2), 25–33.

Hiltebrand, L.B., Koepfli, E., Kimberger, O., Sigurdsson, G.H., Brandt, S., 2011. Hypotension during fluid-restricted abdominal surgery: effects of norepinephrine treatment on regional and microcirculatory blood flow in the intestinal tract. Anesthesiology 114 (3), 557–564.

Holst, D., 1997. Social relations and their health impact in tree shrews. Acta Physiologica Scandinavica. Supplementum 640, 77–82.

Jacobs, I.G., Finn, J.C., Jelinek, G.A., Oxer, H.F., Thompson, P.L., 2011. Effect of adrenaline on survival in out-of-hospital cardiac arrest: A randomised double-blind placebo-controlled trial. Resuscitation 82 (9), 1138–1143.

Jetschmann, J.-U., Benschop, R.J., Jacobs, R., Kemper, A., Oberbeck, R., Schmidt, R.E., Schedlowski, M., 1997. Expression and *in vivo* modulation of α- and β-adrenoceptors on human natural killer (CD16+) cells. Journal of Neuroimmunology 74, 159–164.

Johnson, R.W., von Borell, E.H., Anderson, L.L., Kojic, L.D., Cunnick, J.E., 1994. Intracerebroventricular injection of corticotropin-releasing hormone in the pig: acute effects on behavior, adrenocorticotropin secretion, and immune suppression. Endocrinology 135 (2), 642–648.

Kanitz, E., Tuchscherer, M., Otten, W., Tuchscherer, A., Zebunke, M., Puppe, B., 2019. Coping style of pigs is associated with different behavioral, neurobiological and immune responses to stressful challenges. Frontiers in Behavioral Neuroscience 13, 173.

Kanitz, E., Tuchscherer, M., Puppe, B., Tuchscherer, A., Stabenow, B., 2004. Consequences of repeated early isolation in domestic piglets (*Sus scrofa*) on their behavioural, neuroendocrine, and immunological responses. Brain, Behavior, and Immunity 18, 35–45.

Karaszewski, J.W., Reder, A.T., Maselli, R., Brown, M., Arnason, B.G., 1990. Sympathetic skin responses are decreased and lymphocyte beta-adrenergic receptors are increased in progressive multiple sclerosis. Annals of Neurology 27 (4), 366–372.

Karavolos, M.H., Bulmer, D.M., Spencer, H., Rampioni, G., Schmalen, I., Baker, S., Pickard, D., Gray, J., Fookes, M., Winzer, K., Ivens, A., Dougan, G., Williams, P., Khan, C.M.A., 2011. *Salmonella* Typhi sense host neuroendocrine stress hormones and release the toxin haemolysin E. EMBO Reports 12 (3), 252–258.

Kavelaars, A., 2002. Regulated expression of α-1 adrenergic receptors in the immune system. Brain, Behavior, and Immunity 16 (6), 799–807.

Kendall, M.M., Sperandio, V., 2014. Cell-to-cell signaling in *E. coli* and *Salmonella*. EcoSal Plus 6 (1).

Kick, A.R., Tompkins, M.B., Almond, G.W., 2011. Stress and immunity in the pig. CAB reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 6, 1–17.

Kin, N.W., Sanders, V.M., 2006. It takes nerve to tell T and B cells what to do. Journal of Leukocyte Biology 79 (6), 1093–1104.

Konturek, P.C., Brzozowski, T., Konturek, S.J., 2011. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. Journal of Physiology and Pharmacology: an official journal of the Polish Physiological Society 62 (6), 591–599.

Koolhaas, J.M., 2008. Coping style and immunity in animals: making sense of individual variation. Brain, Behavior, and Immunity 22 (5), 662–667.

Koolhaas, J.M., Korte, S.M., Boer, S.F. de, van der Vegt, B.J., van Reenen, C.G., Hopster, H., Jong, I.C. de, Ruis, M.A.W., Blokhuis, H.J., 1999. Coping styles in animals: current status in behavior and stress-physiology. Neuroscience & Biobehavioral Reviews 23 (7), 925–935.

Koolhaas, J.M., van Reenen, C.G., 2016. Animal behaviour and well-being symposium: Interaction between coping style/personality, stress, and welfare: relevance for domestic farm animals. Journal of Animal Science 94 (6), 2284–2296.

Kothari, N.J., Saunders, J.C., 1961. Effects of corticotrophin, hydrocortisone and methopyrapone on the circulating eosinophiles in man. Nature 191 (4793), 1105–1106.

Krief, S., Lönnqvist, F., Raimbault, S., Baude, B., van Spronsen, A., Arner, P., Strosberg, A.D., Ricquier, D., Emorine, L.J., 1993. Tissue distribution of beta 3-adrenergic receptor mRNA in man. Journal of Clinical Investigation 91 (1), 344–349.

Kwon, Y.S., Jang, J.S., Hwang, S.M., Tark, H., Kim, J.H., Lee, J.J., 2019. Effects of surgery start time on postoperative cortisol, inflammatory cytokines, and postoperative hospital day in hip surgery: randomized controlled trial. Medicine 98 (24), e15820.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O., 2013. Antibiotic resistance—the need for global solutions. The Lancet Infectious Diseases 13 (12), 1057–1098.

Leung, D.Y.M., Bloom, J.W., 2003. Update on glucocorticoid action and resistance. The Journal of Allergy and Clinical Immunology 111 (1), 3-22.

Lewis, C.R.G., Hulbert, L.E., McGlone, J.J., 2008. Novelty causes elevated heart rate and immune changes in pigs exposed to handling, alleys, and ramps. Livestock Science 116 (1), 338–341.

Lim, J.J., Grinstein, S., Roth, Z., 2017. Diversity and versatility of phagocytosis: roles in innate immunity, tissue remodeling, and homeostasis. Frontiers in Cellular and Infection Microbiology 7, 191.

Lyte, M., Ernst, S., 1992. Catecholamine induced growth of gram negative bacteria. Life Sciences 50 (3), 203–212.

Lyte, M., Frank, C.D., Green, B.T., 1996. Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. FEMS Microbiology Letters 139 (2-3), 155–159.

Lyte, M., Vulchanova, L., Brown, D.R., 2011. Stress at the intestinal surface: catecholamines and mucosa-bacteria interactions. Cell and Tissue Research 343 (1), 23–32.

Mauch, J., Ringer, S., Spielmann, N., Weiss, M., 2014. Impact of catecholamines in cardiac arrest due to acute asphyxia—a study in piglets. Paediatric Anaesthesia 24 (9), 933–939.

Minton, J.E., 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. Journal of Animal Science 72 (7), 1891–1898.

Miraglia, G.J., Berry, L.J., 1962. Enhancement of salmonellosis and emergence of secondary infection in mice exposed to cold. Journal of Bacteriology 84 (6), 1173–1180.

Moreira, C.G., Weinshenker, D., Sperandio, V., 2010. QseC mediates *Salmonella enterica* serovar Typhimurium virulence *in vitro* and *in vivo*. Infection and Immunity 78 (3), 914–926.

Neeman, E., Zmora, O., Ben-Eliyahu, S., 2012. A new approach to reducing postsurgical cancer recurrence: Perioperative targeting of catecholamines and prostaglandins. Clinical Cancer Research: an official journal of the American Association for Cancer Research 18 (18), 4895–4902.

Nguyen, L.T., Lim, S., Oates, T., Chung, K.F., 2005. Increase in airway neutrophils after oral but not inhaled corticosteroid therapy in mild asthma. Respiratory Medicine 99 (2), 200–207.

Ortega, E., Giraldo, E., Hinchado, M.D., Martín, L., García, J.J., La Fuente, M. de, 2007. Neuroimmunomodulation during exercise: role of catecholamines as ‘stress mediator’ and/or ‘danger signal’ for the innate immune response. Neuroimmunomodulation 14 (3-4), 206–212.

Otten, W., Kanitz, E., Tuchscherer, M., Brüssow, K.-P., Nürnberg, G., 2008. Repeated administrations of adrenocorticotropic hormone during late gestation in pigs: maternal cortisol response and effects on fetal HPA axis and brain neurotransmitter systems. Theriogenology 69 (3), 312–322.

Otten, W., Kanitz, E., Tuchscherer, M., Puppe, B., Nürnberg, G., 2007. Repeated administrations of adrenocorticotropic hormone during gestation in gilts: Effects on growth, behaviour and immune responses of their piglets. Livestock Science 106 (2-3), 261–270.

Parrillo, J.E., Fauci, A.S., 1979. Mechanisms of glucocorticoid action on immune processes. Annual Review of Pharmacology and Toxicology 19, 179–201.

Perez, D.M., 2006. The Adrenergic Receptors: In the 21st Century. Humana Press Inc, Totowa, NJ.

Peters, A.M., 1998. Just how big is the pulmonary granulocyte pool? Clinical Science 94 (1), 7–19.

Petrillo, M.G., Fettucciari, K., Montuschi, P., Ronchetti, S., Cari, L., Migliorati, G., Mazzon, E., Bereshchenko, O., Bruscoli, S., Nocentini, G., Riccardi, C., 2014. Transcriptional regulation of kinases downstream of the T cell receptor: another immunomodulatory mechanism of glucocorticoids. BMC Pharmacology & Toxicology 15, 35.

Previte, J.J., Alden, J.C., Egbert, M., 1973. Comparative dynamics of *Salmonella* infection after primary and secondary challenge of mice exposed to 10 and 23 C. Infection and Immunity 8 (4), 597–603.

Pritchard, D.I., Todd, I., Brown, A., Bycroft, B.W., Chhabra, S.R., Williams, P., Wood, P., 2005. Alleviation of insulitis and moderation of diabetes in NOD mice following treatment with a synthetic *Pseudomonas aeruginosa* signal molecule, N-(3-oxododecanoyl)-L-homoserine lactone. Acta Diabetologica 42 (3), 119–122.

Pullinger, G.D., Carnell, S.C., Sharaff, F.F., van Diemen, P.M., Dziva, F., Morgan, E., Lyte, M., Freestone, P.P.E., Stevens, M.P., 2010. Norepinephrine augments *Salmonella enterica*-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. Infection and Immunity 78 (1), 372–380.

Regueira, T., Bänziger, B., Djafarzadeh, S., Brandt, S., Gorrasi, J., Takala, J., Lepper, P.M., Jakob, S.M., 2008. Norepinephrine to increase blood pressure in endotoxaemic pigs is associated with improved hepatic mitochondrial respiration. Critical Care 12 (4), R88.

Ritchie, A.J., Jansson, A., Stallberg, J., Nilsson, P., Lysaght, P., Cooley, M.A., 2005. The *Pseudomonas aeruginosa* quorum-sensing molecule N-3-(oxododecanoyl)-L-homoserine lactone inhibits T-cell differentiation and cytokine production by a mechanism involving an early step in T-cell activation. Infection and Immunity 73 (3), 1648–1655.

Roess, D.A., Bellone, C.J., Ruh, M.F., Nadel, E.M., Ruh, T.S., 1982. The effect of glucocorticoids on mitogen-stimulated B-lymphocytes: thymidine incorporation and antibody secretion. Endocrinology 110, 169–175.

Rosenberger, P.H., Ickovics, J.R., Epel, E., Nadler, E., Jokl, P., Fulkerson, J.P., Tillie, J.M., Dhabhar, F.S., 2009. Surgical stress-induced immune cell redistribution profiles predict short-term and long-term postsurgical recovery. A prospective study. The Journal of Bone and Joint Surgery. American volume 91 (12), 2783–2794.

Ruis, M.A., Te Brake, J.H., Engel, B., Ekkel, E.D., Buist, W.G., Blokhuis, H.J., Koolhaas, J.M., 1997. The circadian rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress. Physiology & Behavior 62, 623–630.

Ruis, M.A.W., Groot, J. de, te Brake, J.H.A., Dinand Ekkel, E., van de Burgwal, J.A., Erkens, J.H.F., Engel, B., Buist, W.G., Blokhuis, H.J., Koolhaas, J.M., 2001. Behavioural and physiological consequences of acute social defeat in growing gilts: effects of the social environment. Applied Animal Behaviour Science 70 (3), 201–225.

Saffar, A.S., Ashdown, H., Gounni, A.S., 2011. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. Current Drug Targets 12 (4), 556–562.

Salak-Johnson, J.L., McGlone, J.J., Norman, R.L., 1996. *In vivo* glucocorticoid effects on porcine natural killer cell activity and circulating leukocytes. Journal of Animal Science 74, 584.

Sanders, V.M., 2012. The beta2-adrenergic receptor on T and B lymphocytes: do we understand it yet? Brain, Behavior, and Immunity 26 (2), 195–200.

Sanders, V.M., Kasprowicz, D.J., Kohm, A.P., Swanson, M.A., 2001. Neurotransmitter receptors on lymphocytes and other lymphoid cells, in: Ader R, Felten D, Cohen N (Ed.), Psychoneuroimmunology. 3rd edn Vol. 2. Academic Press, San Diego, CA, pp. 161–196.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21, 55–89.

Scanzano, A., Cosentino, M., 2015. Adrenergic regulation of innate immunity: a review. Frontiers in Pharmacology 6, 171.

Schedlowski, M., Falk, A., Rohne, A., Wagner, T.O., Jacobs, R., Tewes, U., Schmidt, R.E., 1993. Catecholamines induce alterations of distribution and activity of human natural killer (NK) cells. Journal of Clinical Immunology 13 (5), 344–351.

Schedlowski, M., Hosch, W., Oberbeck, R., Benschop, R.J., Jacobs, R., Raab, H.R., Schmidt, R.E., 1996. Catecholamines modulate human NK cell circulation and function via spleen-independent beta 2-adrenergic mechanisms. Journal of Immunology 156 (1), 93–99.

Schwinn, D.A., 1994. Adrenergic Receptors: unique localization in human tissues, in: Bosnjak, Z.J., Kampie, J.P. (Eds.), Advances in pharmacology, vol. 31. Academic Press, San Diego, CA, pp. 333–341.

Shodell, M., Shah, K., Siegal, F.P., 2003. Circulating human plasmacytoid dendritic cells are highly sensitive to corticosteroid administration. Lupus 12 (3), 222–230.

Silva-Herzog, E., McDonald, E.M., Crooks, A.L., Detweiler, C.S., 2015. Physiologic stresses reveal a *Salmonella* persister state and TA family toxins modulate tolerance to these stresses. PloS one 10 (12), e0141343.

Sperandio, V., Torres, A.G., Jarvis, B., Nataro, J.P., Kaper, J.B., 2003. Bacteria-host communication: the language of hormones. Proceedings of the National Academy of Sciences of the United States of America 100 (15), 8951–8956.

Sritharan, M., 2006. Iron and bacterial virulence. Indian Journal of Medical Microbiology 24 (3), 163–164.

Stapels, D.A.C., Hill, P.W.S., Westermann, A.J., Fisher, R.A., Thurston, T.L., Saliba, A.-E., Blommestein, I., Vogel, J., Helaine, S., 2018. *Salmonella* persisters undermine host immune defenses during antibiotic treatment. Science 362 (6419), 1156–1160.

Stefanski, V., 1998. Social stress in loser rats: opposite immunological effects in submissive and subdominant males. Physiology & Behavior 63 (4), 605–613.

Sule, N., Pasupuleti, S., Kohli, N., Menon, R., Dangott, L.J., Manson, M.D., Jayaraman, A., 2017. The norepinephrine metabolite 3,4-dihydroxymandelic acid is produced by the commensal microbiota and promotes chemotaxis and virulence gene expression in enterohemorrhagic *Escherichia coli*. Infection and Immunity 85 (10).

Suzuki, K., Nakai, A., 2017. Control of lymphocyte trafficking and adaptive immunity by adrenergic nerves. Clinical and Experimental Neuroimmunology 8 (1), 15–22.

Szatmari, I., Nagy, L., 2008. Nuclear receptor signalling in dendritic cells connects lipids, the genome and immune function. The EMBO Journal 27 (18), 2353–2362.

Telford, G., Wheeler, D., Williams, P., Tomkins, P.T., Appleby, P., Sewell, H., Stewart, G.S.A.B., Bycroft, B.W., Pritchard, D.I., 1998. The *Pseudomonas aeruginosa* quorum-sensing signal molecule N-(3-oxododecanoyl)-l-homoserine lactone has immunomodulatory activity. Infection and Immunity 66 (1), 36–42.

Thawornkaiwong, A., Preawnim, S., Wattanapermpool, J., 2003. Upregulation of β1-adrenergic receptors in ovariectomized rat hearts. Life Sciences 72 (16), 1813–1824.

The Nobel Prize in Physiology or Medicine 1950. NobelPrize.org. Nobel Media AB 2020. Sat. 29 Feb 2020. https://www.nobelprize.org/prizes/medicine/1950/summary/.

Toulouse, C., Schmucker, S., Metesch, K., Pfannstiel, J., Michel, B., Starke, I., Möller, H.M., Stefanski, V., Steuber, J., 2019. Mechanism and impact of catecholamine conversion by *Vibrio cholerae*. Biochimica et Biophysica Acta. Bioenergetics 1860 (6), 478–487.

Tsitoura, D.C., Rothman, P.B., 2004. Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. Journal of Clinical Investigation 113 (4), 619–627.

Tuchscherer, M., Kanitz, E., Tuchscherer, A., Puppe, B., 2016. Effects of social support on glucocorticoid sensitivity of lymphocytes in socially deprived piglets. Stress: The International Journal on the Biology of Stress 19, 325–332.

van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences of the United States of America 112 (18), 5649–5654.

van den Brink, H.R., van Wijk, M.J., Bijlsma, J.W., 1992. Influence of steroid hormones on proliferation of peripheral blood mononuclear cells in patients with rheumatoid arthritis. British Journal of Rheumatology 31 (10), 663–667.

van Tits, L.J., Michel, M.C., Grosse-Wilde, H., Happel, M., Eigler, F.W., Soliman, A., Brodde, O.E., 1990. Catecholamines increase lymphocyte beta 2-adrenergic receptors via a beta 2-adrenergic, spleen-dependent process. The American Journal of Physiology 258 (1 Pt 1), E191-202.

Verbrugghe, E., Boyen, F., Gaastra, W., Bekhuis, L., Leyman, B., van Parys, A., Haesebrouck, F., Pasmans, F., 2012. The complex interplay between stress and bacterial infections in animals. Veterinary Microbiology 155 (2-4), 115–127.

Verbrugghe, E., Boyen, F., van Parys, A., van Deun, K., Croubels, S., Thompson, A., Shearer, N., Leyman, B., Haesebrouck, F., Pasmans, F., 2011. Stress induced *Salmonella* Typhimurium recrudescence in pigs coincides with cortisol induced increased intracellular proliferation in macrophages. Veterinary Research 42, 118.

Verbrugghe, E., Dhaenens, M., Leyman, B., Boyen, F., Shearer, N., van Parys, A., Haesendonck, R., Bert, W., Favoreel, H., Deforce, D., Thompson, A., Haesebrouck, F., Pasmans, F., 2016. Host stress drives *Salmonella* recrudescence. Scientific Reports 6, 20849.

Wallgren, P., Wilén, I.-L., Fossum, C., 1994. Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. Veterinary Immunology and Immunopathology 42 (3-4), 301–316.

Walters, M., Sircili, M.P., Sperandio, V., 2006. AI-3 synthesis is not dependent on luxS in *Escherichia coli*†. Journal of Bacteriology 188 (16), 5668–5681.

Westly, H.J., Kelley, K.W., 1984. Physiologic concentrations of cortisol suppress cell-mediated immune events in the domestic pig. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) 177, 156–164.

Yates, J.C., Taam, G.M., Singal, P.K., Beamish, R.E., Dhalla, N.S., 1980. Protection against adrenochrome-induced myocardial damage by various pharmacological interventions. British Journal of Experimental Pathology 61 (3), 242–255.

Zahorec, R., 2001. Ratio of neutrophil to lymphocyte counts—rapid and simple parameter of systemic inflammation and stress in critically ill. Bratislavske lekarske listy 102 (1), 5–14.

CHAPTER 4

SUMMARY

4 SUMMARY

Stress is a regular feature of human and animal life, characterised by the perception of a potentially harmful stimulus and the subsequent physiologic response to such a stressor. The two main endocrine systems involved in the regulation of this reaction are the hypothalamus-pituitary-adrenal (HPA) axis, leading to the synthesis of glucocorticoids like cortisol or corticosterone, and the sympathetic-adrenal-medullary (SAM) axis, whose activation is associated with the release of the catecholamines adrenaline and noradrenaline. These stress hormones modulate the function of many cells and tissues including the immune system. Although pigs (*Sus scrofa domestica*) in modern husbandry systems face many potential stressors during the whole production cycle, the consequences of elevated plasma stress hormone levels on porcine immune cell numbers and functionality are insufficiently resolved. While some research on glucocorticoid effects has been conducted, data on many parameters are still missing and so far, catecholamines have not yet been studied systematically in the pig. It is known that stress can negatively affect pigs' resistance to infections like salmonellosis, but the underlying mechanisms are still subject to intense research efforts, with new perspectives arising since the discovery of interkingdom-signalling and microbial catecholamine perception.

The aim of the present doctoral thesis was to determine the distinct effects of cortisol, adrenaline and noradrenaline on porcine immune cell functionality and the blood numbers of different leukocyte subsets. Furthermore, the interplay of porcine immune cells and *Salmonella* Typhimurium under the influence of catecholamines was investigated. Adult male castrated pigs were surgically equipped with indwelling catheters to enable stress-free blood collection and intravenous application of hormones.

In an initial experiment, the effects of *in vitro* stress hormone treatment on lymphocyte proliferation and the production of the proinflammatory cytokine TNFα were described. Cortisol reduced both proliferation and number of TNFα producers. Both catecholamines caused an increased lymphocyte proliferation at low concentrations whereas noradrenaline drastically decreased proliferation at high concentrations. While noradrenaline had no impact on TNFα producers, they were reduced in γδ T cells and monocytes upon adrenaline addition. Overall, the effects were comparable to humans in terms of direction and dose but there were

some disparities regarding adrenaline that require further investigations regarding the molecular mechanisms.

In the second part of the project, the impact of *in vivo* stress hormone administration on immune cell numbers and functionality was examined by infusion for 48h. Cortisol and noradrenaline led to a decreased lymphocyte proliferation but to a variable extent and all three hormones promoted phagocytic function of innate immune cells. Cortisol caused a marked increase of neutrophil numbers while almost all other cell types declined strongly. For most cell types, noradrenaline exerted similar effects but solely after 2h whereas cortisol-induced alterations lasted the whole treatment period. Adrenaline effects were mostly reduced to $CD8^-$ T cells, which were reduced at first but increased after 24h. A sharp peak in NK cell numbers after 2h adrenaline infusion is particularly noteworthy and resembles findings from rodent and human studies. Overall, both hormone groups led to a shift from adaptive to innate immunity, underpinning the picture of a promotion of fast and unspecific defence systems to respond to threats in stressful situations.

In a third study, *S.* Typhimurium was grown in the presence of catecholamines to determine the effects of supernatants from these cultures on porcine immune cell function. Both lymphocyte proliferation and TNFα production were hampered substantially, as opposed to the findings on catecholamine effects in the first experiment. It was demonstrated that these effects were not caused by catecholamines or their oxidation products and the formation of a so-far unknown immunosuppressive substance by catecholamine-primed bacteria was assumed. The results contribute to a better understanding of the increased susceptibility to infection in stressed animals and reveal a new dimension of cross-species communication.

Finally, the results of the present thesis were discussed regarding their comparability to studies in humans and rodents and previous stress experiments in pigs. Furthermore, the effects of acute and chronic stress as well as different coping styles that are characterised by a SAM or HPA predominance on animal welfare and pig health were discussed, based on the endocrine mechanisms investigated in the present thesis. Possible implications of enhanced glucocorticoid and catecholamine levels for practical pig husbandry were given. Lastly, suggestions for future research to further elucidate the impact of stress hormones on the porcine immune system and the interplay with pathogenic bacteria were made.

In summary, the present thesis presents many new findings and details regarding the modulation of porcine immune cell numbers and functionality by cortisol. For the first time, adrenaline and

noradrenaline effects on the immune system of domestic pigs were investigated separately and systematically, thus filling a major research gap. Furthermore, a new explanatory approach for stress-induced salmonellosis based on interkingdom-signalling was discovered. This dissertation therefore contributes to a better understanding of stress-induced immunomodulation in the pig as an important livestock species and also strengthens its role as a suitable large animal model in psychoneuroimmunology research.

CHAPTER 5

ZUSAMMENFASSUNG

5 ZUSAMMENFASSUNG

Stress ist ein regelmäßiger Bestandteil des Lebens von Menschen und Tieren, welcher durch die Wahrnehmung eines potenziell schädlichen Reizes und die anschließende physiologische Reaktion auf einen solchen Stressor gekennzeichnet ist. Die beiden wichtigsten endokrinen Systeme, die an der Steuerung dieser Stressreaktion beteiligt sind, sind die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA), die zur Synthese von Glukokortikoiden wie Cortisol oder Corticosteron führt, und die Sympathikus-Nebennierenmark-Achse (SAM), deren Aktivierung mit der Freisetzung der Katecholamine Adrenalin und Noradrenalin verbunden ist. Diese Stresshormone modulieren die Funktion vieler Zellen und Gewebe einschließlich des Immunsystems. Obwohl Schweine (*Sus scrofa domestica*) in modernen Haltungssystemen während des gesamten Produktionszyklus vielen potenziellen Stressoren ausgesetzt sind, sind die Folgen erhöhter Plasma-Stresshormonspiegel auf die Anzahl und Funktionalität der Immunzellen des Schweins nicht ausreichend geklärt. Zwar wurden einige Untersuchungen zu den Effekten von Glukokortikoiden durchgeführt, jedoch fehlen noch immer Daten zu vielen Parametern, und bis heute wurden Katecholamine beim Schwein noch nicht systematisch untersucht. Es ist bekannt, dass Stress die Widerstandsfähigkeit von Schweinen gegen Infektionen wie die Salmonellose negativ beeinflussen kann, aber die zugrundeliegenden Mechanismen sind noch immer Gegenstand intensiver Forschungsbemühungen. Dabei haben sich seit der Entdeckung des „Interkingdom-Signalling“ und der Wahrnehmung von Katecholaminen durch Mikroorganismen neue Perspektiven ergeben.

Ziel der vorliegenden Doktorarbeit war es, die unterschiedlichen Effekte von Cortisol, Adrenalin und Noradrenalin auf die Funktionalität von Schweineimmunzellen und die Zellzahlen verschiedener Leukozyten-Subpopulationen im Blut zu bestimmen. Darüber hinaus wurde das Zusammenspiel von Schweineimmunzellen und *Salmonella* Typhimurium unter dem Einfluss von Katecholaminen untersucht. Dafür wurden adulte Kastraten chirurgisch mit Venenverweilkathetern ausgestattet, um eine stressfreie Blutentnahme sowie intravenöse Hormonapplikation zu ermöglichen.

In einem ersten Experiment wurden die Auswirkungen einer *in vitro*-Zugabe von Stresshormonen auf die Lymphozytenproliferation und die Produktion des proinflammatorischen Zytokins TNFα beschrieben. Cortisol führte zu einer Reduktion sowohl

der Proliferation als auch der Anzahl von TNFα-Produzenten. Beide Katecholamine bewirkten eine erhöhte Lymphozytenproliferation bei niedrigen Konzentrationen, wohingegen Noradrenalin die Proliferation bei hohen Konzentrationen drastisch verringerte. Während Noradrenalin keinen Einfluss auf TNFα-produzierende Zellen hatte, waren sie nach Zugabe von Adrenalin unter den γδ-T-Zellen und Monozyten reduziert. Insgesamt waren die Hormoneffekte hinsichtlich Richtung und Dosis mit den beim Menschen beschriebenen vergleichbar, aber es gab einige Unterschiede bei Adrenalin, die weitere Untersuchungen hinsichtlich der zugrundeliegenden molekularen Mechanismen erforderlich machen.

Im zweiten Teil des Projekts wurden die Auswirkungen einer *in vivo*-Gabe von Stresshormonen auf die Anzahl und Funktionalität von Immunzellen mittels 48-stündiger Infusion untersucht. Cortisol und Noradrenalin führten zu einer verminderten Lymphozytenproliferation, jedoch in unterschiedlichem Ausmaß, und alle drei Hormone förderten die Phagozytosefunktion angeborener Immunzellen. Cortisol verursachte einen deutlichen Anstieg der Neutrophilenzahl, wohingegen fast alle anderen Zelltypen stark zurückgingen. Bei den meisten Zelltypen übte Noradrenalin ähnliche Effekte aus, jedoch nur nach 2 Stunden, wohingegen die Cortisol-induzierten Veränderungen die gesamte Behandlungsdauer anhielten. Die Adrenalin-Effekte waren größtenteils auf CD8-negative T-Zellen begrenzt, deren Anzahl zunächst reduziert, aber nach 24 Stunden erhöht war. Ein starker Anstieg der NK-Zellzahl nach 2-stündiger Adrenalin-Infusion ist besonders erwähnenswert und spiegelt Ergebnisse aus Nager- und Humanstudien wider. Insgesamt betrachtet führten beide Hormongruppen zu einer Verschiebung von adaptiver zu angeborener Immunität, wodurch das Bild einer Förderung schneller und unspezifischer Abwehrsysteme zur Reaktion auf Gefahren in Stresssituationen untermauert wird.

In einer dritten Studie wurden *S.* Typhimurium-Kulturen unter Zugabe von Katecholaminen angelegt, um die Wirkung von Überständen aus diesen Kulturen auf die Funktion von Schweineimmunzellen zu bestimmen. Sowohl die Lymphozytenproliferation als auch die TNFα-Produktion waren – im Gegensatz zu den Erkenntnissen über die Katecholamin-wirkungen aus dem ersten Experiment – deutlich verringert. Es konnte gezeigt werden, dass diese Effekte nicht durch Katecholamine oder deren Oxidationsprodukte verursacht wurden, sodass die Bildung einer bislang unbekannten immunsuppressiven Substanz durch Katecholamin-behandelte Bakterien angenommen wird. Die Ergebnisse tragen zu einem besseren Verständnis der erhöhten Infektionsanfälligkeit gestresster Tiere bei und zeigen eine neue Dimension der artübergreifenden Kommunikation auf.

Schließlich wurden die Ergebnisse der vorliegenden Arbeit hinsichtlich ihrer Vergleichbarkeit mit Studien an Menschen und Nagern sowie früheren Stressexperimenten an Schweinen diskutiert. Darüber hinaus wurden die Auswirkungen von akutem und chronischem Stress sowie unterschiedlicher Coping-Strategien, die sich durch eine SAM- oder HPA-Dominanz auszeichnen, auf das Tierwohl und die Schweinegesundheit auf Grundlage der in der vorliegenden Arbeit untersuchten endokrinen Mechanismen diskutiert. Es wurden mögliche Auswirkungen von erhöhten Glukokortikoid- und Katecholaminwerten auf die praktische Schweinehaltung aufgezeigt. Schließlich wurden Vorschläge für zukünftige Forschungsvorhaben gemacht, um den Einfluss von Stresshormonen auf das Immunsystem von Schweinen und die Wechselwirkungen mit pathogenen Bakterien weiter aufzuklären.

Zusammenfassend präsentiert die vorliegende Arbeit viele neue Erkenntnisse und Details zur Modulation der Anzahl und Funktionalität von Immunzellen des Schweins durch Cortisol. Erstmals wurden die Effekte von Adrenalin und Noradrenalin auf das Immunsystem von Hausschweinen separat und systematisch untersucht und damit eine große Forschungslücke geschlossen. Darüber hinaus wurde basierend auf dem Prinzip des Interkingdom-Signalling ein neuer Erklärungsansatz für die stressinduzierte Salmonellose entdeckt. Diese Dissertation trägt somit zu einem besseren Verständnis der stressbedingten Immunmodulation beim Schwein als wichtiges Nutztier bei und stärkt auch dessen Rolle als geeignetes Großtiermodell auf dem Gebiet der Psychoneuroimmunologie.

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Place, Date Signature

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PEER-REVIEWED ARTICLES

Reiske, L.; Schmucker, S.; Steuber, J.; Stefanski, V., 2019: Glucocorticoids and Catecholamines Affect in Vitro Functionality of Porcine Blood Immune Cells. *Animals* **9**, 545 (2019).

Reiske, L.; Schmucker, S.; Pfaffinger, B.; Weiler, U.; Steuber, J.; Stefanski, V.: Intravenous Infusion of Cortisol, Adrenaline, or Noradrenaline Alters Porcine Immune Cell Numbers and Promotes Innate over adaptive immune functionality. *The Journal of Immunology* **204** (12), 3205-3216 (2020).

Reiske, L.; Schmucker, S.; Steuber, J.; Toulouse, C.; Pfaffinger, B.; Stefanski, V.: Interkingdom Cross-Talk in Times of Stress: *Salmonella* Typhimurium Grown in the Presence of Catecholamines Inhibits Porcine Immune Functionality *in vitro. Frontiers in Immunology* **11**: 572056 (2020).

CONFERENCE PROCEEDINGS

Reiske, L.; Schmucker, S.; Toulouse, C.; Steuber, J.; Stefanski, V. (2019): Catecholamines and products from catecholamine-treated Salmonella Typhimurium cultures modulate porcine lymphocyte function in contrary ways. Tagung des Veterinärimmunologischen Arbeitskreis der DGfI, München, Germany

Reiske, L.; Schmucker, S.; Toulouse, C.; Steuber, J.; Stefanski, V. (2019): Immunomodulation by catecholamines and catecholamine-treated Salmonella enterica cultures in pigs (Sus scrofa). International Veterinary Immunology Symposium, Seattle, USA

Reiske, L.; Schmucker, S.; Stefanski, V. (2018): Stress hormones have implications on lymphocyte number and functionality in pigs. European Veterinary Immunology Workshop, Utrecht, Netherlands

Reiske, L.; Schmucker, S.; Stefanski, V. (2017): Stresshormone modulieren die Funktionalität von porcinen Immunzellen in vitro. Jahrestagung der DGfZ und GfT, Stuttgart, Germany

Reiske, L.; Schmucker, S.; Stefanski, V. (2017): Effects of stress hormones on lymphocyte proliferation in pigs (Sus scrofa). 9th GEBIN Educational Short Course, Münster, Germany


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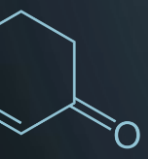
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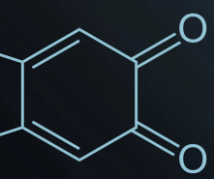
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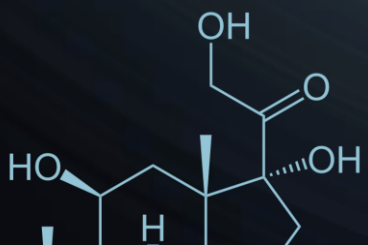
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