Dissertation

Glucocorticoid receptor-mediated responses to immune stress in pigs in the context of a natural gain-of-function substitution Ala610Val

By

Zhiwei Li

Institut für Tierwissenschaften

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Zhiwei Li

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Referent: Prof. Dr. Klaus Wimmers

Koreferentin: Prof. Dr. Dr. Helga Sauerwein

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Dedicated to my family

Abstract

Glucocorticoids (GCs) play vital roles in maintaining homeostasis of organisms during stress and are among the most potent anti-inflammatory drugs in medicine. GCs are also a trade-off factor between robustness and productivity in animal breeding since they support adaptations to adverse stimuli and regulate anabolism-based growth. GC functions are conveyed primarily by the glucocorticoid receptor (GR). Despite the importance of GCs and GR signaling, relevant knowledge in pigs is scant as compared to humans and model animals. This study aims to investigate the regulation, function, and underlying molecular basis of GR signaling in pigs in response to lipopolysaccharide (LPS)-induced immune stress, as well as to determine the impact of GR hypersensitivity caused by a natural Ala610Val substitution in this context. The research is anticipated to benefit our understanding of GR signaling in pigs and contribute to improved animal production, health, and welfare.

In the first approach **(manuscript 1)**, seven-week-old purebred German Landrace pigs (n = 36) were assigned into two experimental groups with an equal sex distribution. Pigs in the two groups received a bolus intramuscular injection of 60 μg/kg body weight (BW) dexamethasone (DEX) sodium phosphate and a corresponding volume of 0.9% saline respectively (T0-3 h), followed by an intraperitoneal injection of 100 μg/kg BW *Escherichia coli* LPS 3 hours later (T0). Blood samples were collected at T0-3 h, T0, T0+1 h, and T0+3 h to measure blood parameters involving neuroendocrine, metabolic, hematological, and inflammatory reactions. Sickness behaviors were monitored every 5 minutes over 4 hours post LPS treatment. This part discloses that DEX, as a potent GR agonist, alters levels of several blood parameters in a natural setting, including cortisol, adrenocorticotropic hormone (ACTH), glucose, red blood cells (RBCs), and white blood cells (WBCs). Furthermore, DEX mitigates LPS-induced physiological and behavioral disorders, as manifested by improved responses of cortisol, ACTH, glucose, lactate, triglycerides, RBCs, WBCs, platelets, interleukin 6 (IL-6), panting, and cyanosis. In the second part **(manuscript 2)**, PBMCs were collected from 24 German Landrace pigs (12 males, 12 females) at an average age of 170 days. PBMCs from each individual were divided into 4 groups and treated with either vehicle (control; CON), DEX (5 nM), LPS (10 μg/ml), or LPS+DEX for 2 hours. The resultant transcriptome responses were studied via mRNA sequencing and downstream differential expression analysis, k-means clustering, protein-protein interaction analysis, and functional enrichment analysis. This part shows that: 1) alongside pro-inflammatory responses, LPS initiates an anti-inflammatory program comprising genes involved in the inhibition of NF-κB and MAPK signaling and activation of

IL-10/STAT3 anti-inflammatory axis; 2) there is crosstalk between immune responses and GR signaling, which is evident in four aspects: constitutive inhibition of T cell signaling by DEX via a series of genes having no response to LPS; attenuated expression of LPS-induced inflammatory genes by DEX; diminished DEX actions by LPS paralleled by the regulation of genes implicated in cytokine and calcium signaling; and pro-inflammatory effects of DEX associated with genes related to the activation of Toll-like receptor (TLR), NF-κB, inducible nitric oxide synthase (iNOS), and IL-1 signaling. In the third work package **(manuscript 3)**, a total of 96 seven-week-old purebred German Landrace pigs were assigned into two experimental groups balanced for sex and GR genotypes (wild types (AlaAla), $GR_{Ala610Val}$ heterozygotes (AlaVal), and $GR_{Ala610Val}$ homozygotes (ValVal)). Pigs in the two groups were administrated intramuscularly with 60 µg/kg DEX sodium phosphate and a corresponding volume of 0.9% saline respectively (T0-3 h). Three hours later (T0) all pigs were administered intraperitoneally with 50 µg/kg BW LPS. Blood samples were collected at T0-3 h, T0, T0+1 h, T0+3 h, T0+6 h, and T0+24 h to examine neuroendocrine, metabolic, hematological, and inflammatory parameters. Feed intake in response to LPS challenge was recorded at T0+24 h and sickness behaviors were observed at a 5-minute interval during the 1st, 2nd, 3rd, 4th, and 24th hours after LPS administration. For all parameters, the GR genotype effect within each treatment group and treatment effect within each GR genotype were analyzed respectively. This part demonstrates that: 1) the $GR_{Ala610Val}$ affects baseline levels of several parameters such as cortisol, ACTH, triglycerides, granulocytes, and platelets; 2) the $GR_{Ala610Val}$ enhances the susceptibility of pigs to endotoxemia as evidenced by reduced feed intake and aggravated LPS-induced physiological and behavioral disorders involving blood urea nitrogen (BUN), triglycerides, platelets, IL-6, tumor necrosis factor α (TNF-α), panting, and vomiting; 3) the $GR_{Ala610Val}$ pigs, particularly heterozygotes, are more sensitive to DEX-mediated therapy during endotoxemia, as reflected by more pronounced improvement of physiological (cortisol, ACTH, IL-6, and TNF-α) and behavioral (panting) responses in these animals.

In summary, this study describes the impact of the GC and the hypersensitive $GR_{Ala610Val}$ on the biological reactions of pigs to LPS-induced immune stress and depicts the interplay of immune responses and GR signaling *in vivo* and *in vitro*. These data add information to the regulation, function, and underlying molecular basis of GR signaling in pigs and provide novel insights into the role of GR gain-of-function in coping with immune stress. These findings will benefit the health and welfare of pigs and improve the balanced breeding and application of GC-based drugs in this species.

Zusammenfassung

Glucocorticoide (GCs) spielen beim Erhalt der Homöostase in Stresssituationen eine wichtige Rolle und gehören zu den wirksamsten entzündungshemmenden Medikamenten in der Medizin. Das GC-System trägt aber auch zu einer gegenläufigen Abhängigkeit zwischen Robustheit und Produktivität in der Tierzucht bei, da einerseits Anpassungen an Belastung unterstützt werden und andererseits ein auf Anabolismus basierendes Wachstum reguliert wird. GC-Funktionen werden hauptsächlich durch den Glucocorticoid-Rezeptor (GR) vermittelt. Trotz der Bedeutung von GCs und der über GR vermittelten Signalwege sind relevante Zusammenhänge bei Schweinen im Vergleich zu Menschen und Modelltieren kaum bekannt. Das übergeordnete Ziel dieser Studie ist es, die Regulation, die Funktion und die zugrundeliegende molekulare Basis der GR-vermittelten Signalwege beim Schwein als Reaktion auf Lipopolysaccharid (LPS)-induzierten Immunstress zu untersuchen, sowie die Auswirkungen einer GR-Hypersensitivität, die durch eine natürliche Ala610Val-Substitution verursacht wird, in diesem Zusammenhang zu bestimmen. Es wird erwartet, dass die Forschung das Verständnis der GR-vermittelten Signalwege beim Schwein fördert und zu einer verbesserten Tierproduktion, Gesundheit und Wohlbefinden beiträgt.

Im ersten Ansatz (**Manuskript 1**) wurden sieben Wochen alte Schweine der Deutschen Landrasse (n = 36) in zwei Versuchsgruppen eingeteilt (18 männlich, 18 weiblich). Die Schweine in den beiden Gruppen erhielten eine intramuskuläre Bolusinjektion von 60 µg/kg Körpergewicht (KG) Dexamethason (DEX)-Natriumphosphat bzw. das entsprechende Volumen an 0,9%iger Kochsalzlösung (T0-3 h). Anschließend wurde 3 Stunden später eine intraperitoneale Injektion von 100 µg/kg KG *Escherichia coli* LPS verabreicht (T0). Blutproben wurden bei T0-3 h, T0, T0+1 h und T0+3 h gewonnen, um Blutparameter mit Bezug auf neuroendokrine, metabolische, hämatologische und inflammatorische Reaktionen zu messen. Die Krankheitssymptome wurden über 4 Stunden nach der LPS-Behandlung im 5-Minuten-Takt überwacht. Dieser Studienteil zeigt, dass DEX als potenter GR-Agonist die Werte verschiedener Blutparameter verändert, darunter Cortisol, adrenocorticotropes Hormon (ACTH), Glukose, rote Blutkörperchen (RBCs) und weiße Blutkörperchen (WBCs). Außerdem mildert DEX LPS-induzierte physiologische Auslenkungen und Krankheitsverhalten, was sich im verbesserten Profil von Cortisol, ACTH, Glukose, Laktat, Triglyceriden, Erythrozyten, Erythrozyten, Thrombozyten und Interleukin 6 (IL-6) und abgeschwächten Krankheitssymptomen, einschließlich Hecheln und Zyanose, zeigt. Im zweiten Teil (**Manuskript 2**) wurden mononukleäre Zellen des peripheren Blutes (engl., PBMCs) von 24

Schweinen der Deutschen Landrasse (n = 24) mit einem Durchschnittsalter von 170 Tagen gesammelt (12 männlich, 12 weiblich). PBMCs von jedem Individuum wurden in 4 Gruppen aufgeteilt und entweder mit Vehikel (Kontrolle; CON), DEX (5 nM), LPS (10 µg/ml) oder LPS+DEX für 2 Stunden behandelt. Die resultierende transkriptionelle Antwort auf die Behandlung wurde mittels mRNA-Sequenzierung und nachfolgender differentieller Expressionsanalyse, k-means Clustering, Protein-Protein-Interaktionsanalyse und funktioneller Anreicherungsanalyse untersucht. Dieser Studienteil zeigt, dass: 1) LPS neben pro-inflammatorischen Reaktionen eine anti-inflammatorische Antwort initiiert, die Gene umfasst, die an der Hemmung der NF-κB- und MAPK-Signalisierung und der Aktivierung der anti-inflammatorischen IL-10/STAT3-Achse beteiligt sind; 2) es einen Crosstalk zwischen Immunantwort und GR-vermittelten Signalwegen gibt. Beim Letzteren wurden vier Wechselwirkungen beobachtet, darunter (i) eine konstitutive Hemmung des T-Zell-Signalweges durch DEX über eine Reihe von Genen, die nicht auf LPS reagieren; (ii) eine abgeschwächte LPS-Induktion der Expression von entzündungsfördernden Genen durch DEX; (iii) eine gehemmte DEX-Wirkung durch den Einfluß von LPS auf die Regulierung von Genen, die in die Zytokin- und Kalzium-Signalkaskaden involviert sind; und (iv) pro-inflammatorische Effekte von DEX, die mit Genen verbunden sind, die mit der Aktivierung von Toll-like-Rezeptor (TLR), NF-κB, induzierbarer Stickoxid-Synthase (iNOS) und IL-1-Signalwirkung in Verbindung stehen. Im dritten Arbeitspaket (**Manuskript 3**) wurden insgesamt 96 sieben Wochen alte Schweine der Deutschen Landrasse in zwei Versuchsgruppen eingeteilt, die hinsichtlich des Geschlechts und der GR-Genotypen balanciert waren (Wildtypen (AlaAla), $GR_{Ala610Val}$-Heterozygoten (AlaVal) und $GR_{Ala610Val}$-Homozygoten (ValVal)). Die Schweine wurden durch eine intramuskuläre Applikation von 60 µg/kg DEX-Natriumphosphat, bzw. das entsprechende Volumen an 0,9%iger Kochsalzlösung, vorbehandelt (T0-3 h). Drei Stunden später (T0) wurden alle Schweinen mit LPS durch intraperitoneale Gabe von 50 µg/kg BW gechallenged. Blutproben wurden bei T0-3 h, T0, T0+1 h, T0+3 h, T0+6 h und T0+24 h entnommen, um neuroendokrine, metabolische, hämatologische und immunologische Parameter zu untersuchen. Die Futteraufnahme als Reaktion auf die LPS-Challenge wurde bei T0+24 h aufgezeichnet. Das Krankheitsverhalten wurde in 5-Minuten-Intervallen während der ersten, zweiten, dritten, vierten Stunde sowie der 24. Stunde nach der LPS-Gabe beobachtet. Für sämtliche Parameter wurde jeweils der Effekt des GR-Genotyps innerhalb jeder Behandlungsgruppe und der Behandlungseffekt innerhalb jedes GR-Genotyps analysiert. Dieser Studienteil zeigt, dass: 1) die $GR_{Ala610Val}$-Variante die Ausgangswerte mehrerer Parameter wie Cortisol, ACTH, Triglyceride, Granulozyten und Thrombozyten beeinflusst; 2)

die $GR_{Ala610Val}$-Variante die Anfälligkeit von Schweinen für Endotoxämie erhöht, was sich in einer reduzierten Futteraufnahme und LPS-induzierten physiologischen Auslenkungen, welche den Blut-Harnstoff-Stickstoff (BUN), Triglyceride, Thrombozyten, IL-6 und Tumor-Nekrose-Faktor α (TNF-α) betreffen, bzw. durch Krankheitssymptome wie Hecheln und Erbrechen, manifestiert; 3) die $GR_{Ala610Val}$-Variante, insbesondere in heterozygoter Ausprägung, empfindlicher auf die DEX-vermittelte Therapie während der Endotoxämie reagiert, was sich in einer deutlicheren Verbesserung der physiologischen (Cortisol, ACTH, IL-6 und TNF-α) und verhaltensbezogenen (Hecheln) Parameter bei diesen Tieren widerspiegelt.

Zusammenfassend beschreibt diese Studie den Einfluss von GC und des hypersensitiven $GR_{Ala610Val}$ auf die biologischen Effekte von LPS-induziertem Immunstress beim Schwein und beleuchtet die Wechselwirkung von Immunantwort und GR-vermittelten Signalwegen *in vivo* und *in vitro*. Die Ergebnisse dieser Studie liefern neue Informationen über die Regulation, Funktion und die zugrundeliegende molekulare Basis der GR-vermittelten Signalwege bei Schweinen und bieten neue Einblicke in die Rolle der GR-basierten *Gain-of-Function* bei der Bewältigung von Immunstress. Diese Erkenntnisse tragen zur Verbesserung der Gesundheit und des Wohlbefinden von Schweinen bei und liefern Grundlagen für eine balancierte Zucht und den Einsatz von GC-basierten Medikamenten bei dieser Spezies.

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1 Introduction

1.1 Introduction to glucocorticoids

The constant internal and external stimuli experienced by all living organisms result in a disruption to homeostasis [1]. The ability to maintain homeostasis and recover from the consequences caused by these stressors requires pleiotropic responses from multiple systems [2]. In animals, one of the most crucial responses to stress is activation of the hypothalamic-pituitary-adrenal (HPA) axis, which triggers the release of its main effectors, glucocorticoids (GCs) [3]. GCs facilitate stress responses and restore homeostasis due to various biological processes, including immune responses, energy stores, neural function, and cardiovascular output [4]. Therefore, GCs are important regulators for animal health and welfare in the context of stressful conditions. In humans and most domestic animals, the major endogenous GC is cortisol, as opposed to corticosterone in other species like rodents and poultry [5].

GCs are also widely employed to treat inflammation, allergies, autoimmune disorders, and cancer in humans [6], as well as being commonly prescribed drugs in veterinary medicine [5]. Since the first clinical application of GCs in the 1940s, scientists have successfully synthesized GC analogues such as prednisone, prednisolone, and dexamethasone (DEX), which are now commonly used due to their potent immunoregulatory properties [7].

1.1.1 Biosynthesis of glucocorticoids

GCs are synthesized in the *zona fasciculata* of the cortex of the adrenal glands, whereby cholesterol acts as a metabolic precursor [6]. The synthesis of cortisol involves a series of steroidogenic enzymes [5] (Fig. 1.1). Initially, intracellular cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane by steroidogenic acute regulatory (StAR) protein. The cholesterol side-chain cleavage enzyme (P450scc) then converts cholesterol to pregnenolone, which is then converted to 17-OH progesterone. This process occurs through the actions of 3β-hydroxysteroid dehydrogenase (3β-HSD) and steroid 17α-hydroxylase, with progesterone or 17-OH pregnenolone acting as the intermediate product. 17-OH progesterone is hydroxylated to 11-deoxycortisol by steroid 21-hydroxylase and then converted to cortisol by steroid 11β-hydroxylase. The cortex of the adrenal glands is additionally the site of aldosterone (mineralocorticoid) and dehydroepiandrosterone (DHEA) synthesis, which occur in the *zona glomerulosa* and the *zona reticularis*, respectively [6].

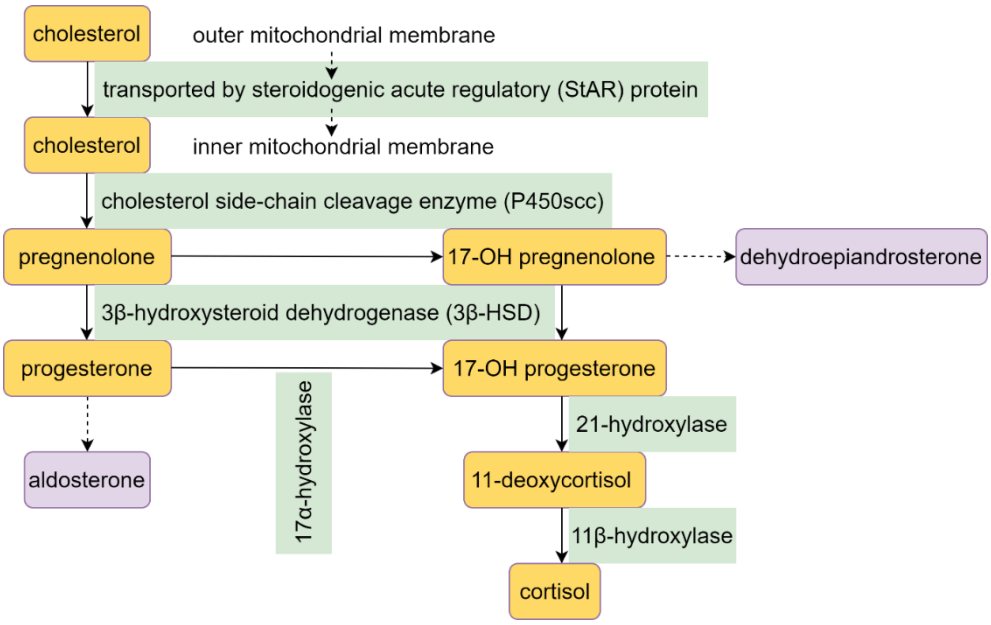

Figure 1.1. Biosynthesis of cortisol. Cholesterol is transported into the mitochondria of cells in the *zona fasciculata* of the cortex of the adrenal glands. The synthesis of cortisol then occurs following a series of steroidogenic enzymes. (Adapted from [5, 8]).

1.1.2 Hypothalamic-pituitary-adrenal axis and glucocorticoid production

The HPA axis controls the production of cortisol (Fig. 1.2). Under normal conditions, this process is regulated in a circadian- and ultradian-dependent manner [9]. In humans and pigs, physiological cortisol secretion peaks in the early morning, whilst the nadir value occurs at approximately midnight [9]. The HPA axis can be also activated by physiological or emotional stress [6]. Stress-induced HPA axis activation involves the initial stimulation of several afferent neural signaling pathways, which triggers the release of corticotropin-releasing hormone (CRH) and vasopressin (AVP) from the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) into the hypophyseal portal circulation. Subsequently, CRH and AVP bind to complementary receptors and initiate the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the general circulation. ACTH then acts on the adrenal cortex and stimulates cortisol production [10].

Cortisol regulates the activity of the HPA axis via a negative feedback mechanism (Fig. 1.2), which can occur in both a genomic and non-genomic manner. The former relies on the interaction between cortisol and the glucocorticoid receptor (GR), whereby cortisol inhibits the expression of proopiomelanocortin (POMC, a precursor of ACTH) in the anterior pituitary and represses the transcription of CRH and AVP in the hypothalamus [11]. The non-genomic regulation of the HPA axis occurs through the rapid inhibition of PVN activity by cortisol due

to the suppression of glutamate release [12], whilst the release of γ-aminobutyric acid (GABA) is promoted [13]. This feedback loop is essential to maintain sufficient baseline GC production and to terminate GC-mediated adaptive responses to stress.

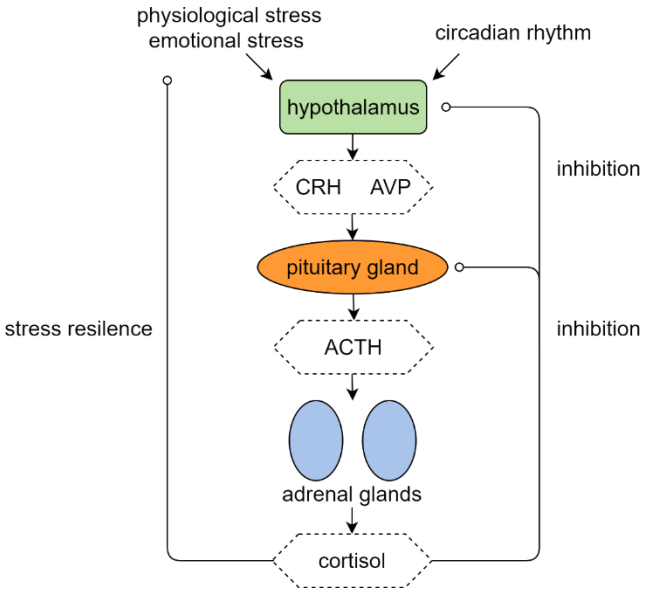

Figure 1.2. Regulation of cortisol release. Cortisol release is controlled by the HPA axis in a circadian rhythm or in response to stress. Cortisol enables animals to cope with stress through several mechanisms. Cortisol inhibits its own production via feedback inhibition of HPA axis activity. CRH, corticotrophin-releasing hormone; AVP, vasopressin; ACTH, adrenocorticotrophic hormone. (Adapted from [5, 11]).

1.1.3 Glucocorticoid bioavailability

Approximately 90% of endogenous GCs bind to plasma proteins in the bloodstream, of which 80-90% bind to the carrier protein known as corticosteroid-binding globulin (CBG), whilst approximately 10% bind to albumin [6]. The remaining unbound GCs (~10%) are biologically active as they can diffuse into target cells and maintain functionality [14]. GCs bind to CBG with high affinity and specificity. A key role of CBG is the transportation of GCs within the circulation, with the interaction between CBG and GCs being a crucial factor in stabilizing the pool of GCs in equilibrium with the free fraction and the targeted delivery of GCs to certain tissues and inflammatory sites [15]. In contrast, GCs have low affinity for albumin and bind non-specifically [16]. The clearance of GCs primarily occurs in the liver, where they are catabolized to water-soluble glucuronides and sulfates. These are then excreted in the urine [5].

GC bioavailability in the cytoplasm is dependent on the conversion between active and inactive forms, which is controlled by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and 2 (11β-HSD2). 11β-HSD1 converts inactive cortisone to active cortisol, whereas 11β-HSD2 catalyzes the reverse reaction [17]. Furthermore, 11β-HSD1 is abundant in tissues with high metabolic requirements, such as the liver and skeletal muscle. Alternatively, 11β-HSD2 is mainly located in tissues with high expression of the mineralocorticoid receptor (MR), such as the kidneys; here, it prevents non-selective MR activation caused by GCs [18]. The conversion from inactive cortisone to active cortisol enables spatial regulation of GC signaling in various tissues.

1.2 Glucocorticoid receptor and glucocorticoid signaling

GCs exert their functions through both non-genomic and genomic mechanisms [19], although genomic mechanisms are predominant, which are mediated by the GR through changes in the transcriptional regulation of target genes [20].

1.2.1 Glucocorticoid receptor structure and isoforms

The GR is a ligand-inducible transcription factor (TF) of the nuclear receptor superfamily. Specifically, it is encoded by the nuclear receptor subfamily 3 group c member 1 (*NR3C1*) gene and it is constitutively and ubiquitously expressed in the majority of tissues. The *NR3C1* gene comprises a total of 9 exons, with exon 1 generating the 5′-untranslated region (UTR), whilst exons 2-9 encode the functional domains. Alternative splicing at exon 9 of the GR transcript produces two GR splice variants: GRα and GRβ. The former variant is the predominant and active form [21]. However, GRβ cannot bind GCs due to the absence of helix 12 in the ligand-binding domain (LBD), and therefore, is regarded as a primary antagonist that counteracts GRα functions [22].

The GR protein consists of three functional domains: an N-terminal domain (NTD), a DNA-binding domain (DBD), and a C-terminal LBD. The DBD and LBD are linked by the hinge region [23], which also confers flexibility to the dimeric GR [24]. The NTD is the most variable domain and is susceptible to proteases; this is also where the N-terminal activation function 1 (AF1) is located, which interacts with basal transcription machinery and coregulators [25]. The DBD encompasses two highly conserved zinc fingers, each of which accommodates a zinc atom amidst four cysteine residues (Cys4-type). The first zinc finger contributes to the specific binding of the GR to target DNA sequences; the second zinc finger has an integral role in GR dimerization [26]. The LBD contains a central ligand-binding pocket composed of 12 α-helices and four β-sheets; this domain is vital for the specific binding of the GR to GCs. The LBD is

also involved in GR dimerization [27]. A second, ligand-dependent activation function (AF2) is located in the LBD and interacts with TFs, cochaperones, and coregulators [28]. Two nuclear localization signals (NLS) and one nuclear export signal (NES) are detected in the GR protein; these signals control GR translocation between the nucleus and cytoplasm [29, 30]. In addition, a nuclear retention signal (NRS) occupies the GR hinge region, which enhances GR function by inhibiting GR export from the nucleus [31].

1.2.2 Glucocorticoid receptor activation and translocation

When bioactive GCs are unavailable, the monomeric form of the GR is retained in the cytoplasm, involving a chaperone complex composed of heat shock proteins (e.g. Hsp90, Hsp70, and p23) and immunophilins (e.g. FK506-binding protein 51 (FKBP51), FKBP52, Cyp44, and PP5) [32]. The purpose of this multiportion complex is linked to maturation, ligand recognition, and translocation of the GR [33]. The first accessory protein to recognize and interact with the GR post-translation is Hsp70. Subsequently, Hsp40 provides a platform for interaction between Hsp90 and the GR-Hsp70 complex, which is assisted by an Hsp70-Hsp90-organising protein termed Hop [34, 35]. Following completion of the Hop-mediated binding of Hsp90 to the GR-Hsp70 complex, Hsp70 and Hsp40 detach from the chaperone complex and are replaced by p23 and FKPB51. This induces the maturation of the GR-chaperone complex to a conformation with high ligand affinity [36].

Upon binding to GCs, FKBP51 in the GR-chaperone complex is replaced by FKBP52, which causes GR reorganization and NLS exposure to initiate nuclear translocation [37]. Gene expression is regulated by the GR through various mechanisms within the nucleus. GR nuclear export is controlled by exportins and calreticulin binding to the NES [38]. The transport of GR between the cytoplasm and nucleus is important as it helps to regulate the potency of GR actions by adjusting the ratio of bioactive GR in the nucleus.

1.2.3 Genomic signaling mediated by glucocorticoid receptor

Within the nucleus, activated GR can positively or negatively coordinate target gene expression based on transactivation or transrepression mechanisms (Fig. 1.3). The best-established mechanism of transactivation is achieved by dimeric GR directly binding to the GC responsive element (GRE) in the promoter regions of target genes. Classic GRE comprises an inverted imperfect hexameric palindrome separated by a 3 bp spacer [39, 40]. The generally recognized GRE consensus sequence is 5′-AGAACAnnnTGTTCT-3′, where ‘n’ is any nucleotide [40]. The GR interacts with the GRE as a homodimer in a head-to-tail manner, whereby each half-

site of the GRE motif is bound by one receptor [6]. The interaction between the GR and GRE causes GR reorganization and the recruitment of coregulators and chromatin-remodeling complexes [41]. The variation in the GRE sequence makes GRE not only a simple site for GR binding but also an allosteric regulator controlling GR-mediated transcription, as it has been evidenced that even the smallest modification, such as a single base pair, in the GRE can stimulate distinct transcriptional procedures [42, 43].

Furthermore, certain genes accommodate the negative GC responsive element (nGRE). This differs from classical GRE, as consensus nGRE constitutes a palindromic sequence $CTCC(n)_{0-2}GGAGA$, where the spacer ranges from 0 to 2 nucleotides [44]. The transrepression mediated by the nGRE involves direct interaction with two GR monomers and requires the participation of corepressors NCoR1 and SMRT and histone deacetylases (HDACs) [44, 45]. In addition, activated monomeric GR can bind to the half-site of the consensus sequence of GRE [46]. This half GRE site is essentially a composite element with the binding site of another TF within the local vicinity; from this, the GR can regulate gene expression through positive or negative interactions with neighboring TFs [47].

As well as directly binding to target DNA, monomeric GR also regulates gene expression via protein-protein interactions with other DNA-bound TFs. This is referred to as tethering and is an important mechanism for the anti-inflammatory effects of GR [6]. Tethering is achieved without direct contact to DNA, and instead, relies on the recruitment of GR monomers to TFs that are bound to tethering sites lacking GRE [48]. Additionally, the GR physically interacts with other TFs prior to their binding to target DNA and causes their sequestration to obstruct TF-DNA binding. Impaired TF-DNA binding can also occur due to the GR competing with other TFs for contact with the overlapping binding site. Likewise, the GR can inhibit TF activity by recruiting cofactors that are required for TF-mediated transcription [49-51].

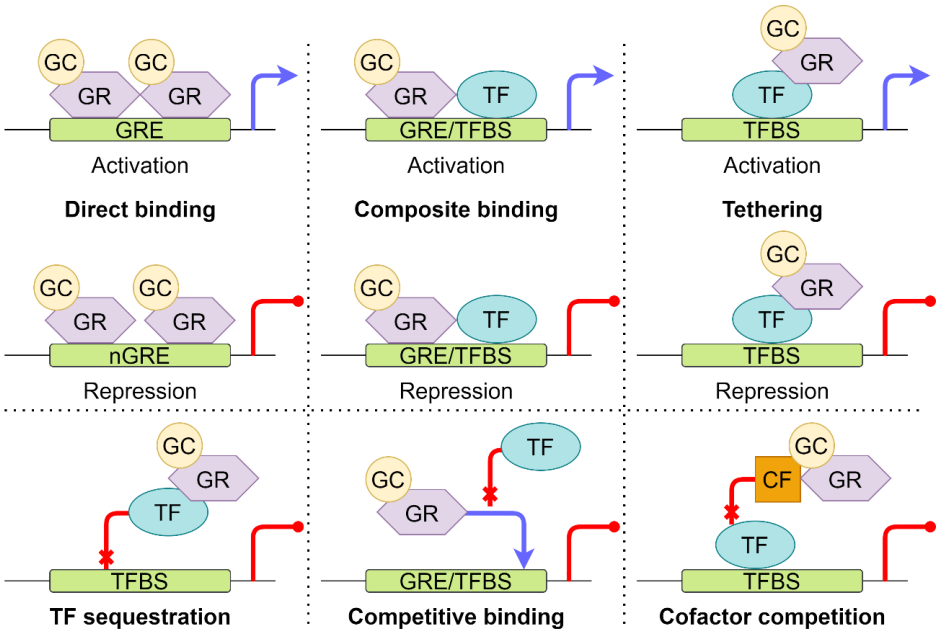

Figure 1.3. Genomic signaling mediated by GR. Activated GR translocates to the nucleus to regulate gene expression through transactivation or transrepression. GC, glucocorticoid; GR, glucocorticoid receptor; GRE, GC responsive element; TF, transcription factor; TFBS, transcription factor binding site; nGRE, negative GC responsive element; CF, cofactor. (Adapted from [52, 53]).

1.3 Glucocorticoid functions and their implications in pigs

GC functions aid the adaptive responses of organisms to stressors whilst also conveying beneficial or adverse effects during medical applications of GC-based drugs.

1.3.1 Immunomodulation by glucocorticoids

The most profound effect of GCs is immunomodulation, involving both innate and adaptive immune systems [54]. Immune stress and the resultant production of inflammatory cytokines activate the HPA axis and stimulate endogenous GC synthesis to prevent excessive immune responses [55]. Studies have shown that adrenalectomized animals exhibit elevated cytokine levels and increased mortality during infection, which is alleviated by GC administration [3]. In addition, the disruption of GR functions by mutations or inhibitors increases sensitivity to sepsis [56].

Administration of GCs leads to an increase in the total number of white blood cells (WBCs), which is partially attributed to the enhanced maturation of WBCs in the bone marrow [57]. The increase in total WBC count is also associated with an increase in circulating neutrophils as GCs inhibit neutrophil adhesion to endothelial cells, as well as migration to inflammatory sites,

whilst promoting their egress from the bone marrow [58, 59]. GCs also inhibit neutrophil apoptosis [60], yet conversely, they induce apoptosis in basophils and eosinophils [61].

GCs have been observed to play a dual role in the modulation of monocytes and macrophages. In particular, they limit the augmentation of inflammation by inhibiting the migration of pro-inflammatory monocytes, whilst simultaneously reducing the expression of various inflammatory mediators in activated monocytes and macrophages, such as cytokines, chemokines, and inducible nitric oxide synthase (iNOS) [62]. Additionally, GCs support the differentiation of anti-inflammatory monocytes and macrophages from naïve populations and increase their phagocytic activity to accelerate the clearance of harmful stimuli, including microbial agents and cellular debris. This then results in the resolution of inflammation [62, 63].

Dendritic cells (DCs) are equally affected by GCs, especially their maturation, survival, and function [64]. GCs restrict DCs' responsiveness to CD40L, a protein included in the tumor necrosis factor (TNF) superfamily; they also reduce DCs' capacity to stimulate T cells [65]. The resultant decrease in T cell activation is linked to the downregulation of co-stimulatory factors, including major histocompatibility complex class II (MHCII), CD86, and CD40 [66]. Moreover, GCs hinder lipopolysaccharide (LPS)-induced maturation of DCs, as well as LPS-induced generation of pro-inflammatory cytokines in DCs [67]. However, GCs stimulate the production of tolerogenic DCs, which triggers the development of regulatory T cells (Tregs) [68].

Another consequence of GC administration is a decrease in B cell count in the spleen and lymph nodes, alongside inhibited proliferation of B cell progenitors, and modified immunoglobulin proportions [69]. In particular, high-dose GCs result in boosted immunoglobulin catabolism and inhibited synthesis, causing a decline in the overall levels of immunoglobulins [64]. GCs also diminish the presence of B cell activating factor (BAFF) at both protein and mRNA levels [70]. This is significant as BAFF has a critical function in the survival, maturation, and immunoglobulin production of B cells [64].

T cell responses are also affected by GCs, as their responses are indirectly regulated by GCs due to the attenuation of innate immune cell functions, such as antigen presentation, co-stimulation, and cytokine release [71]. However, GCs primarily impact T cells in a direct manner; circulating T cell numbers are decreased partially by GCs facilitating their migration to the bone marrow and lymphoid tissues and by enhancing directed extravasation [72, 73]. In addition, GCs induce T cell apoptosis, yet findings have shown that B cells and natural killer

(NK) cells remain viable after treatment with GCs [74]. The susceptibility to GC-induced apoptosis varies among T cell subtypes, with $CD4^+CD8^+$ thymocytes being the most sensitive, which correlates with cell-specific differences in GR promoters [75]. Furthermore, GCs attenuate T cell receptor (TCR) signaling, as shown by a study where a single prenatal GC administration in mice caused lasting changes in the peripheral TCR repertoire [76]. GCs also inhibit T cell functions that are mediated by the TFs T-bet and GATA-3; T-bet regulates Th1 responses, whilst GATA-3 regulates Th2 responses [77-79]. Although GCs convey a general inhibitory effect on T cells, they simultaneously favor the responses of particular T cell subsets by differentially suppressing the reactions of Th1, Th2, and Th17 cells [71]. In contrast, GCs promote the number and functions of Tregs [64].

GCs can additionally regulate the transcription of immune mediators, based on two molecular mechanisms. Generally, researchers believe that GCs' anti-inflammatory effect is a result of the transrepression mediated by monomeric GR by tethering with other pro-inflammatory TFs, including NF-κB, AP-1, CREB, IRF3, NFAT, T-bet, GATA-3, and STAT proteins [80]. However, ample evidence has emerged showing that the transactivation dependent on dimeric GR is indispensable for GC-mediated anti-inflammatory actions, as demonstrated by the exacerbated LPS-induced lethality and disorders in GR^{dim} mice. In this context, GR dimerization is impaired but GR monomer-mediated action remains intact [81, 82]. GCs upregulate several immunoregulatory genes such as *TSC22D3* (encodes GILZ), *NFKBIA* (encodes IκBα), and *DUSP1* (encodes MKP-1), which subsequently counteracts the functions of NF-κB, AP-1, and MAPKs to block transactivation of inflammatory genes [71]. Additional GC-inducible anti-inflammatory molecules include ANXA1, ASBT, ADORA3, ADRB1, ANPEP, CD1d, CD163, CC10, CYP1A2, DOK-1, FPR, FOXP3, FCAR, IL-10, IL-1R2, IL-1RA, KLF2, LILRB1, MT1X, PAI-1, $p57^{Kip2}$, RASD1, RGS-2, SLAP, SLPI, S100A10, and TTP [33, 80, 83].

The GR dimer and GR monomer double control system ensures the effectiveness of GC-mediated immunomodulation but equally highlights the complexity. Consequently, the transcription of numerous immune regulators is significantly changed. At present, identified GC-repressed immune molecules include C-C motif chemokine ligand 2 (CCL2), CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL24, CCL26, CD2, CD28, CD137, cyclooxygenase 2 (COX-2), E-selectin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-11, IL-12, IL-13, IL-16, IL-18, IL-22, interferon α (IFN-α), IFN-β, INF-γ, iNOS, intercellular adhesion molecule (ICAM), metalloproteinase-9

(MMP-9), vascular cell adhesion molecule (VCAM), TNF-α, and thymic stromal lymphopoietin (TSLP) [64, 71, 83].

1.3.2 Other biological functions of glucocorticoids

GCs additionally affect metabolic homeostasis, such as the stimulation of gluconeogenesis, inhibition of protein synthesis, and regulation of lipid metabolism [5]. In the liver, GCs trigger gluconeogenesis by upregulating enzymes involved in the gluconeogenic pathway, such as glucose-6-phosphatase (G6Pase), fructose 1,6-bisphosphatase (FBPase), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), and phosphofructokinase 2 (PFK2) [84]. GC-mediated gluconeogenesis utilizes non-carbohydrate substrates as precursors, such as lactate, glycerol, and gluconeogenic amino acids, whilst increasing blood glucose concentration and further promoting glycogen deposition in the liver [84]. Comparatively, GCs inhibit uptake and utilization of glucose in skeletal muscles, and prevent insulin-stimulated glycogen synthesis; furthermore, they show a permissive effect on catecholamine-induced glycogenolysis. This is an important aspect of preserving sufficient circulating glucose, and thereby, is vital for optimal brain function during episodes of stress as glucose is the preferred energy source in the brain [85, 86]. The brain also relies on protein turnover to attain adequate fuel during stress; GCs inhibit protein synthesis but facilitate protein degradation, whereby amino acids are generated to be utilized as carbon sources for hepatic gluconeogenesis. During this process, urinary nitrogen increases due to amino acid mobilization [85, 87].

The effect of GCs on lipid metabolism is more complex. Firstly, GCs stimulate lipolysis, involving the hydrolysis of triglycerides to fatty acids and glycerol. Subsequently, the fatty acids are oxidized to provide energy, whilst the glycerol fuels hepatic gluconeogenesis. This metabolic conversion is crucial to manage stress as a result of fasting and starvation [5, 88]. GCs enable lipolysis partially by enhancing the expression of enzymes involved in the lipolytic pathway, such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MAGL). Moreover, GCs elevate cellular cAMP levels which enhance cAMP signaling; this is essential for the induction of adipocyte lipolysis [88, 89]. GCs also increase the expression of enzymes within the lipogenic pathway, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2), and lipin-1 [88, 90, 91]. GCs' influence on metabolism is indicative of their significance in anabolism-based growth of animals. However, this is associated with potentially unwanted side

effects, such as dyslipidemia, hyperglycemia, and osteoporosis, that can occur during long-term and high-dose GC therapy [41].

Further, the endocrine, cardiovascular, musculoskeletal, nervous, and immune systems are all affected by GCs. For example, the regulation of water and electrolyte balance, lung development and maturation, the maintenance of normal blood pressure and cardiac output, the preservation of gastric mucosal integrity, and the management of mood, behavior, and memory are all functional aspects of GCs [5, 87]. The pleiotropic effects of GCs on physiology render them an effective regulator of homeostasis despite various stressors, thereby influencing animal health and welfare [92].

1.3.3 Glucocorticoid receptor signaling and balanced breeding in pigs

As the human population continues to grow, the demand for animal-based food has equally intensified. Pigs are regarded as one of the most important livestock due to their extensive economic role in the alimentary industry, as well as local and international trade, especially as pork accounts for approximately 35% of global meat production [93]. The accelerated increase in demand for pork has escalated the selection of pig breeds for high production efficiency, with requirements such as high lean tissue growth rate, low backfat thickness, and low feed conversion ratio [94]. It has been well-documented that this selection is strongly paralleled by the reduction in HPA axis responsiveness and cortisol levels [95]. Furthermore, GR polymorphisms are closely associated with carcass composition and meat quality in pigs [96]. Therefore, GR signaling has a significant role in the targeted selection of pigs.

Meanwhile, directed selection towards favorable production traits has led to an impairment in the functional traits of animals, such as resistance to infections and adaptation to dynamic climatic conditions and breeding systems [95]. However, these functional traits have gradually become a specific breeding goal within the livestock industry and have consequently attracted growing interest from researchers. This is especially relevant in regard to the development of production systems towards high management efficiency and low production costs; therefore, animal densities have increased, and there has been insufficient management of individual animals, alongside increased risks of pathogen transmission and the emergence of more diversified breeding conditions [95]. These functional traits are also implicated in a particular characteristic of farm animals termed “robustness” [97]. GR signaling is important for animal robustness. As the final product of the HPA axis, GCs initiate adaptive responses to a range of challenges to maintain homeostasis associated with metabolic processes, immune responses,

and brain functions, whilst also coordinating behavioural and physiological actions to manage environmental changes [3, 98].

The current pig breeding industry tends to integrate genetic and genomic approaches to select animals with balanced robustness- and production-related traits, as this is anticipated to improve animal welfare and growth performance during stress, with limited impact on general production potential [95]. To achieve this, GR signaling has been prioritized. This is partially due to its governance of metabolic activities required for anabolism-based growth performance. GR signaling in animals is also essential for stress resilience to mitigate the negative effects of various stressors [95]. Proposals to enhance the robustness of pigs include breeding for a stronger HPA axis [95], yet the outcome of marker-assisted genetic selection is notoriously difficult to predict, particularly for robustness-related traits [97]. Therefore, the present study aims to improve the current comprehension of the actions and relevant mechanisms of GR signaling in pigs in the context of immune stress. This is expected to facilitate balanced breeding in pigs in terms of the trade-off between production and robustness.

1.3.4 Responses of pigs to glucocorticoids

As well as contributing to balanced animal breeding, GR signaling is a molecular basis for the application of GC-based drugs. Considering their distinct influence on the immune system, GCs are commonly employed to treat inflammatory diseases and immune disorders in pigs [99]. For instance, treatment with DEX reduces the porcine respiratory coronavirus-induced increase in T cell frequencies and cytokine production [100]. Another example is the use of methylprednisolone to improve ciprofloxacin therapy for pneumonia in pigs, which is reflected in the subsequent reduction in inflammatory cytokines and bacterial burden in the lungs [101]. GC-based agents can also aid the growth and health of pigs under stress conditions; in weanling pigs, DEX enhances the activity of digestive enzymes such as amylase and sucrase [102]. DEX is also used to control the post-weaning growth lag in early-weaned pigs, as this treatment is associated with reduced systemic and intestinal inflammation, inhibited muscle protein degradation, increased intestinal health, and improved dietary nutrient digestibility [103, 104]. In addition, GR signaling facilitates the intestinal metabolism of arginine and glutamine in weaned pigs [105].

Although GCs are used for a range of purposes in pigs, relevant scientific knowledge remains minimal and is usually transferred from studies involving humans and model animals [5]. Nonetheless, there is evidence to suggest that pigs have distinct responses to GCs. This species

is insensitive to DEX-mediated immunosuppression compared with cattle; intramuscular administration of 0.04 mg/kg BW DEX clearly and persistently affected antibody production and lymphocyte and neutrophil functions in cattle, whereas dosages as high as 2 mg/kg BW failed to induce these reactions in pigs [106-108]. Further, in pigs, intramuscular treatment of 6 mg/kg BW DEX induced only mild changes in neutrophil functions and temporary alterations to lymphocyte blastogenesis. However, antibody production remained unaffected [106]. Pigs' insensitivity to GCs has been verified by their reduced responsiveness to the lytic effect of prednisone on the thymus, lymph nodes, and lymphocytes compared with rodents at a corresponding dosage [109]. Additionally, pigs were less sensitive to prednisone suppression of cell-mediated immunity than humans [109].

This relative insensitivity to GCs may be partially explained by the observation of reduced systemic drug concentration. Following oral or intravenous administration of prednisone, pigs had a lower blood concentration than humans, which is associated with a higher volume of distribution, faster clearance, and diminished systemic availability of the drug [110]. Estimates have suggested that pigs require 10 to 30 times the human dose of prednisone to obtain a similar blood concentration [110]. This is corroborated by the finding that intravenous injection of DEX results in a higher volume of distribution (2.78 L/kg) and faster clearance (2.39 L/h kg) in pigs than other species. This includes dogs, cattle, horses, and humans, in which these two pharmacokinetic parameters for DEX are <2 L/kg and <0.6 L/h kg, respectively [99]. The relative resistance of pigs to GCs emphasizes the imminent need to reevaluate the regulation and function of GR signaling in this species from a more holistic perspective.

1.3.5 $GR_{Ala610Val}$ pigs as a natural model of glucocorticoid receptor hypersensitivity

GR responsiveness is influenced by genetic variations in the GR gene *NR3C1*, which affects GR signaling and biological features [111]. In humans, several GR mutations and polymorphisms have been determined to be functionally relevant, impacting GR responsiveness and HPA axis activity, as well as modifying the biological parameters involved in health and disease [112]. Furthermore, many artificial GR mutants have been established when studying the precise molecular basis of GR signaling, but most of these mutants induce GR loss of function and have been examined *in vitro* to test their impact on GR function. Their influence on organismal phenotypes under normal or stress conditions has yet to be fully established [113]. Compared with research focusing on humans and laboratory animals, this level of knowledge in farm animal species is scarce.

The results of a genome-wide association study enabled our group to identify a major quantitative trait locus (QTL) at the position of the *NR3C1* gene in different commercial pig breeds, which is strongly associated with decreased basal cortisol levels and adrenal weight [114]. Resequencing of the *NR3C1* coding region revealed a natural substitution of alanine to valine at helix 5 of the ligand-binding domain of the porcine GR (amino acid 610, $GR_{Ala610Val}$), induced by a single nucleotide polymorphism (SNP) c.1829C>T as the causal genetic variation for both parameters [114]. Further studies showed that the Ala610Val variation enhances GR ligand-binding affinity and transactivation activity [114, 115].

The phenotypic consequences of the hypersensitive $GR_{Ala610Val}$ were elucidated *in vivo*, evidencing the lack of a significant effect on the viability, growth (regarded as daily gain and feed intake), body composition (regarded as backfat thickness, area of *M. longissimus dorsi*, lean content, and carcass length), and several blood parameters (glucose, cholesterol, blood urea nitrogen (BUN), and WBCs) of the carriers, indicating a phenotypically normal status [116]. However, the $GR_{Ala610Val}$ pigs exhibited a compensatory reduction in HPA axis activity as manifested by decreased cortisol and ACTH levels, in addition to diminished adrenal gland weight and adrenal cortex size. Reduced HPA axis activity was also detected in the hypothalamus, where CRH and AVP expression decreased. In addition, the balance of GR hypersensitivity has been observed in the liver due to increased hepatic expression of CBG [116].

In pigs, peripheral blood mononuclear cells (PBMCs) carrying the $GR_{Ala610Val}$ substitution exhibit enhanced sensitivity to DEX-mediated suppression of concanavalin A (ConA)-stimulated cytokine production [116]. This implies that although $GR_{Ala610Val}$ carriers are essentially phenotypically normal under baseline conditions, the variant may affect the performance of pigs in response to either endocrine or immune stimuli. This hypothesis has been substantiated by an *in vivo* study that used $GR_{Ala610Val}$ pigs to show that the substitution strengthens the sensitivity of carriers to DEX-induced glucose increase [117]. In the same study, the intrinsic role of GR signaling in the regulation of immune responses was evidenced by DEX-induced alterations to the hepatic expression of several immune genes in the absence of an immune stimulus [117]. Thus, in light of the impact of $GR_{Ala610Val}$ on porcine responses to DEX and the key role of GR signaling in immune responses, this variant most likely exerts specific functions in situations of immune stress. Whilst this consequence is an important but vague issue in both humans and farm animals, pigs carrying the $GR_{Ala610Val}$ have served as a promising model to help further our evidence-based understanding of GR hypersensitivity.

1.4 Lipopolysaccharide-induced immune stress

LPS is an important component of the outer membrane of Gram-negative bacteria and pertains to pathogen-associated molecular patterns (PAMPs) that are crucial for the recognition of pathogens by immune cells [118].

1.4.1 Lipopolysaccharide recognition and signal transduction

Three structural components constitute LPS, including O-antigen, lipid A, and oligosaccharide core, with lipid A acting as the main stimulator of immune responses [119]. Host recognition of LPS is mainly moderated by the Toll-like receptor 4 (TLR4) along with several accessory molecules, including LPS-binding protein (LBP), CD14, and secreted glycoprotein myeloid differentiation 2 (MD-2). As a result, downstream NF-κB, MAPK, and IRF3 signaling pathways are activated and the production of inflammatory mediators ensues [118, 120]. LPS signaling conveyed by TLR4 is classified based on the level of dependence on myeloid differentiation primary response gene 88 (MyD88) [121]. Initially, circulating LPS is detected and recognized by LBP, which then transfers the LPS molecule to CD14. Subsequently, CD14 presents the LPS molecule to the MD-2/TLR4 complex, and the resultant binding induces the dimerization of the intracellular Toll/IL-1 receptor (TIR) domain of TLR4. This leads to the recruitment of adaptors MyD88 and TIR domain-containing adaptor protein (TIRAP) and further activates TNFR-associated factor 6 (TRAF6). This MyD88-dependent pathway then triggers the activation of NF-κB and MAPK signaling pathways and generates TNF-α, IL-1β, IL-6, and COX-2 [118, 120, 122]. Alternatively, a MyD88-independent pathway for the intracellular transduction of LPS-activated TLR4 signaling can be achieved based on the assembly of TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), and their subsequent interactions with TLR4. This pathway predominantly results in the activation of IRF3 and IRF7 and the release of IFN-β [120, 121, 123].

1.4.2 Susceptibility of pigs to lipopolysaccharide

Bacterial infections can seriously impair animal health and welfare and can cause disastrous economic losses [93]. Compared with other species such as poultry and rodents, pigs are more sensitive to LPS, which is partially reflected by a low lethal dose [124]. In pigs, 25 μg/kg BW LPS is considered to be high, and intravenous administration of 500 μg/kg BW LPS has the potential to induce 100% mortality [124, 125]. In contrast, intravenous administration of 2500 μg/kg BW LPS was not lethal in broiler chickens [126]. The lethal dose of intraperitoneal LPS injection in pigs is 5000 μg/kg BW, whereas the lethal dose in rats and mice falls between

20000-60000 and 25000-60000 μg/kg BW, respectively, and for chickens it was estimated to be ≥50000 μg/kg BW [127]. Similar findings were established in a study involving C57BL/6 mice, where intraperitoneal injection of 10000 μg/kg BW LPS was not lethal [128]. The greater susceptibility of pigs to LPS is also manifested by high responsiveness. A study determined that the response to LPS in rats and mice was in the dosage range of 1-1000 μg/kg BW, whereas LPS-sensitive species such as pigs, sheep, and primates responded to doses as low as 0.001 μg/kg BW [129]. Moreover, sheep, calves, and pigs have also developed shock, pulmonary hypertension, and increased-permeability lung edema in response to microgram quantities of LPS; in contrast, rodents were able to tolerate milligram quantities of LPS without obvious development of such symptoms [130].

Exposing pigs to LPS can trigger the production of various immune mediators, such as inflammatory cytokines (e.g. TNF-α, IL-1β, and IL-6), acute phase proteins (e.g. C-reactive protein (CRP), serum amyloid A (SAA), and haptoglobin), and arachidonic acid metabolites (e.g. prostaglandin E2 (PGE2) and thromboxane A2 (TXA2)). In particular, PGE2 and TXA2 are responsible for febrile responses and pulmonary hypertension, respectively [124]. LPS can additionally elicit a series of clinical symptoms in pigs, including reduced feed intake and the onset of sickness behaviors such as coughing, salivation, retching, vomiting, lethargy, somnolence, shivering, and cyanosis [124]. The evident sensitivity of pigs to LPS distinctly demonstrates the limitation and danger of using knowledge attained from studies involving other animal species to estimate porcine responses during endotoxemia. Therefore, in-depth studies are required to evaluate the efficacy of anti-inflammatory drugs and the crosstalk between GR and LPS signaling in pigs.

2 Objectives

This study aims to determine the regulation and mechanism of GR signaling in pigs, and the resultant biological consequences, in response to immune stress, whilst also deciphering the influence of GR hypersensitivity conferred by a natural Ala610Val substitution in this context. This is expected to benefit animal health and welfare and contribute positively to the modern porcine industry.

The objectives of this research include using DEX and LPS as agents to study GR signaling and immune stress. Compared with endogenous GCs, there are several advantages to synthetic GC analogues, such as increased potency for GR activation, reduced affinity to MR, and improved bioavailability [6]. DEX is one of the most commonly used GR agonists as it has a GC potency approximately 30 times greater than cortisol and does not bind to CBG; therefore, it is more biologically available [6, 131]. Regarding the selection of LPS in this study, it is amongst the most potent stimuli of the immune system, and consequently is an effective agent to investigate the regulation of immune signaling. Moreover, LPS elicits prolonged activation of the HPA axis and stimulates the release of GCs, deeming it a favored agent in research exploring the interplay of neuroendocrine and immune systems [3]. LPS is also widely preferred to establish an experimental endotoxemia model to investigate host responses to bacterial infection and to evaluate the efficiency of anti-inflammatory drugs. Compared with the use of bacteria, an LPS model is easier to manage and is regarded a more standardized and reproducible option [124, 132].

The study is further described as following:

Manuscript 1: GCs are commonly used to treat various inflammatory diseases and to enhance growth performance under stress conditions in pigs. However, comprehensive knowledge of GCs' performance in this species is limited in comparison to humans and model animals. As it is well established that pigs are relatively more sensitive to LPS and resistant to GCs, this investigation will focus on the effect of GCs on porcine responses to LPS. The first aim of this thesis is to examine the effect of LPS on the physiological and behavioral responses of pigs, as well as the therapeutic efficacy of DEX during endotoxemia in this species.

Manuscript 2: The molecular mechanisms that control the activation of the GR and immune signaling, along with their crosstalk, remain vague in pigs. The second aim of this thesis is to identify gene expression profiles and regulatory networks in porcine PBMCs in response to LPS and DEX stimulation. The purpose of this is to present a holistic insight into the molecular

foundation of the biological consequences following LPS and DEX treatment in pigs, whilst also creating a genomic landscape for the crosstalk between GR signaling and immune responses.

Manuscript 3: The GR is an integral part of achieving GC functions. In the porcine industry, GR signaling is an important target for the genetic selection of pigs to obtain a balance between production and robustness. Also, altered GR responsiveness caused by genetic variations has been linked to changes in health and disease status. The influences of genetic GR hypersensitivity on porcine responses to stress and GC therapy remain an obscurity, yet are considered important. Therefore, the third aim of this thesis is to determine the impact of GR hypersensitivity conferred by a natural Ala610Val substitution on porcine responses to LPS within and beyond the HPA axis, and to establish the efficiency of DEX therapy in this context.

3

Manuscript 1

Kinetics of physiological and behavioural responses in endotoxemic pigs with or without dexamethasone treatment

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Kinetics of physiological and behavioural responses in endotoxemic pigs with or without dexamethasone treatment

Zhiwei Li[1], Ellen Kanitz[2], Margret Tuchscherer[2], Armin Tuchscherer[3], Cornelia C. Metges[4], Nares Trakooljul[1], Klaus Wimmers[1], and Eduard Murani[1,*]

[1] Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[2] Institute of Behavioural Physiology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[3] Institute of Genetics and Biometry, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[4] Institute of Nutritional Physiology "Oskar Kellner", Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

***** Corresponding author.

Abstract

Although dexamethasone (DEX) is a widely used immunoregulatory agent, knowledge about its pharmacological properties in farm animals, especially pigs, is insufficient. Previous studies suggest that compared to other species, pigs are less sensitive to the immunosuppression conferred by DEX and more sensitive to the threat of bacterial endotoxins. However, there is a paucity of studies examining DEX immunomodulation in endotoxemia in this species. In this study, a porcine endotoxemia model was established by lipopolysaccharide (LPS) and the effect of DEX-pretreatment on the magnitude and kinetics of neuroendocrine, metabolic, hematologic, inflammatory, and behavioural responses were examined. DEX decreased cortisol, adrenocorticotropic hormone (ACTH), red blood cell, hemoglobin, hematocrit, and lymphocyte whereas glucose concentration was increased under both normal and endotoxemic conditions. By contrast, DEX decreased triglyceride, lactate, and IL-6 concentrations and increased platelet count only under an endotoxemic condition. DEX also reduced the frequency of sickness behaviour following LPS challenge. PCA showed that glucose and triglyceride metabolism together with red blood cell count mainly contributed to the separation of clusters during DEX treatment. Our study demonstrates that DEX protects pigs from inflammation and morbidity in

endotoxemia, in spite of their less sensitivity to DEX. Moreover, its considerable role in the regulation of the metabolic and hematologic responses in endotoxemic pigs is revealed for the first time.

Keywords: dexamethasone; lipopolysaccharide; pig; endotoxemia; physiological responses; sickness behaviour.

Introduction

Glucocorticoids (GCs) are a class of steroid hormones produced and secreted by the adrenal cortex as the final output of the neuroendocrine hypothalamus-pituitary-adrenal (HPA) stress axis [1]. They orchestrate many physiological activities, like metabolism and immune response, to maintain physiological equilibrium. GCs repress the expression of numerous inflammatory genes, including those encoding pro-inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-1, IL-6) via glucocorticoid receptor (GR) [2]. Pro-inflammatory cytokines released from activated monocytes and macrophages, e.g., in response to endotoxins, in turn strongly activate the HPA axis and ultimately stimulate the production of GCs to prevent overshooting of the inflammatory responses [3]. Owing to their potent anti-inflammatory action GCs became the most widely prescribed drugs in the world. A variety of compounds with GC activity has been developed and clinically used in humans in the therapy of various inflammatory and autoimmune diseases [1]. Likewise, GC-based drugs also play an irreplaceable role in veterinary medicine [4].

As one of the most potent GC drugs, dexamethasone (DEX) is extensively applied for the treatment of inflammatory and immunological dysfunctions [5, 6]. In addition, considering the comparatively rapid clearance in pigs [7], DEX seems to be a promising agent to protect pigs from the threat of systemic inflammation induced by various infections and to improve growth performance under different stress conditions like weaning by modulating the immune system [8, 9]. Although the pharmacological effects of DEX on inflammation were comprehensively studied in humans and model animals [2, 10, 11], related information in many important farm animals, especially pigs, is relatively insufficient [5]. Thus, GC therapy in most domestic animal species relies on clinical experience and knowledge from human medicine [4]. Compared to other mammalian species like cattle, pigs are relatively resistant to immunosuppression conferred by DEX manifested by less sensitivity and persistence of the response of antibody and immune cells to DEX in the latter [12, 13]. This relative resistance results, at least partly, from faster DEX metabolism in pigs compared to dogs, cattle, horses and humans [7]. Hence,

it is apparently inappropriate to use DEX in pigs based on the knowledge from other species including humans. In this context, the overall effect of DEX on general physiological indices includes not only immune/inflammatory, but also neuroendocrine, metabolic, and hematological variables that need comprehensive exploration to avoid unwanted side-effects.

Besides less DEX sensitivity pigs are more vulnerable to various inflammatory stimuli including bacterial endotoxins (e.g., lipopolysaccharide, LPS) [14]. Although LPS endotoxemia is the commonly used immune challenge in pigs [15], little is known about the anti-inflammatory effects of GCs in this context [5]. Given that pigs exhibit less sensitivity to DEX and conversely relatively greater sensitivity to endotoxins, it is imperative to reevaluate the specific effects of DEX on the endotoxin-induced inflammatory response in pigs.

As a synthetic hydrocortisone analogue, physiological action of DEX is tightly associated with feedback regulation of the hypothalamic-pituitary-adrenal gland (HPA) axis [1], which could be influenced by many factors like (epi)genetic make-up including genotype of GR and health condition, resulting in a large individual variation in therapeutic effects conferred by GCs [16-18]. DEX is widely used to examine individual differences in HPA feedback regulation under non-inflammatory condition (DEX suppression test) [16], but under inflammatory condition this test was so far limited to model animals [19]. However, HPA axis regulation in response to immune challenges is an important facet of stress resilience; a concept attracting increasing attention for its clear health benefits [20].

Therefore, the aim of the present study is to contribute to the knowledge base for therapeutic as well as the research application of DEX in pigs by exploring DEX action in a porcine endotoxemia model established by LPS. The effects of DEX under basal condition and on LPS-induced inflammatory responses were examined by multiple biomarkers. During this process, sickness behaviour and kinetics of neuroendocrine, hematological, metabolic, and inflammatory responses were also monitored.

Materials and methods

Animals, combined DEX/LPS challenge, and sampling

Seven-week-old (n = 36, 18 females and 18 males) purebred German Landrace pigs were used in this study. The experiment was performed in three replicates each consisting of 12 animals (6 males and 6 females). The pigs were born and reared at the experimental pig farm of the Leibniz Institute for Farm Animal Biology (FBN) (Dummerstorf, Germany) under standardized

conditions. Before entering the experiment their health status was visually approved by trained personnel. The following criteria were used for the visual approval: general condition, lethargy, refusal to eat, consistency of faeces, skin discolouration, coughing, lameness, swelling on the body or joints and abnormal behaviour. Two days before the experiment, the pigs were transferred to single pens (1.90 × 1.10 m^2) to facilitate observation of sickness behaviour during the challenge. Each pen was equipped with a feeder and a nipple waterer. One day prior to the experiment all pigs were weighed (mean ± SE = 13.6 ± 0.3 kg). The pigs were assigned into two experimental groups, each balanced for sex (3 males and 3 females in each group per replicate; in total 9 males and 9 females per group): 1. DEX which was given a bolus intramuscular injection of 60 µg/kg BW DEX sodium phosphate (Dexatat, aniMedica, Senden, Germany) at T0-3 h (three hours before the LPS challenge; ~8:00 a.m.). 2. Saline which was given a bolus intramuscular injection of the corresponding volume of sterile, endotoxin-free 0.9% saline at T0-3 h. At T0 (three hours post DEX/saline administration), all pigs were intraperitoneally injected with 100 µg/kg BW LPS (Escherichia coli O111: B4; Sigma-Aldrich, Taufkirchen, Germany) as previously described [21]. During the experiment (starting at T0-3 h) all animals were deprived of feed.

Blood samples were obtained via rapid (≤30s) anterior vena cava puncture at T0-3 h, T0, T0+1 h, and T0+3 h and collected into pre-chilled EDTA tubes. In addition, rectal temperature was measured at the same timepoints to monitor the febrile response.

Experimental animal use, care, handling and sample collection were performed under European Union and German legislation of animal protection. The experimental protocol and procedures were approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology and the State Mecklenburg-Western Pomerania (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3-1-024/16; approval date: 26 May 2016).

Measurement of neuroendocrine parameters

Plasma concentration of ACTH and cortisol were measured in duplicate using commercially available ELISA kits according to the manufacturer's recommendations (DRG Instruments GmbH, Marburg, Germany). The kits (EIA 3647 for ACTH and EIA 1887 for cortisol) used for the measurement of porcine plasma were validated in our previous study [18].

Measurement of biochemical parameters

Plasma concentration of BUN, triglyceride, and glucose were detected by a Fuji DriChem 4000i clinical chemistry analyzer (Scil, Viernheim, Germany). Plasma concentration of lactate, creatinine, and ALT were measured by an enzymatic-spectrophotometric assay using an ABX Pentra 400 instrument.

Measurement of hematological parameters

An aliquot of the blood samples was used to measure the hematological parameters including red blood cell count, hemoglobin, hematocrit, leukocyte count, lymphocyte proportion, and platelet count by the ABX Pentra 60 instrument (Axon Lab, Reichenbach/Stuttgart, Germany). Remaining blood samples were centrifuged at 4 °C and 2000 × *g* for 20 min to obtain the plasma samples [18] and collected plasma samples were stored at -80 °C for further use.

Measurement of inflammation-associated cytokines

Plasma concentration of inflammation-associated cytokines including IL-1β, IL-6, IL-10, and TNF-α were determined in duplicate using porcine cytokine magnetic bead panel kits according to the manufacturer's recommendations (Merck, Darmstadt, Germany). The data was collected by the MAGPIX® instrument (Merck).

Behavioural observation

The behaviour of each animal was observed using scan sampling [22] every 5 min over a period of 4 h after LPS application. At the time of observation, following symptoms of sickness were assessed for each animal: (1) panting (respiratory difficulties) (2) shivering (pigs laid on the floor or stood and displayed rapid, synchronous muscle contractions, frequently accompanied by piloerection) (3) vomiting (4) cyanosis (peripheral). Furthermore, (5) animal activity was characterized as motor active (walking, employment with pen equipment) or inactive (lying, sitting, standing without movement). All observations were carried out by a trained person who was blinded to the treatment of the piglets.

Statistical analysis

Prior to statistical analysis data distribution was tested to ensure approximate normal distribution. One animal (saline group) was excluded from the analysis due to uncertainty about the received treatment. Data on cytokine concentrations were log transformed to achieve approximate normality.

The effect of DEX on physiological responses to LPS represented by blood parameters was analysed using repeated measures ANOVA implemented in the mixed procedure in SAS/STAT software (version 9.4, SAS Inc., Cary, NC, USA). The main effects included in the model were treatment (DEX or saline), timepoint (T0-3 h, T0, T0+1 h, and T0+3 h, respectively), and their interaction, with the pig as experimental unit. The unstructured block diagonal covariance structure was used for the repeated measurement analysis on the same pig. In addition, the data were adjusted for the effects of glucocorticoid receptor genotype ($GR_{Ala610Val}$ [18], all three allele combinations), sex (male and female), and replicate (1-3) by fitting these as fixed effects in the model. Least-squares means (LS-means) and their standard errors (SE) were computed for the timepoint × treatment interaction and compared within timepoint and treatment, respectively, using the slice option. Tukey-Kramer adjustment was applied within slices.

The effect of DEX on LPS-induced sickness behaviour represented by frequencies of symptom occurrence was analysed by fitting a Poisson model using the GLIMMIX procedure in SAS/STAT software. The model included the fixed effects treatment, time (1st h, 2nd h, 3rd h, and 4th h after LPS application, respectively), genotype, sex, replicate (1-3) and their interactions. Additionally, LS-means and their standard errors were computed for each fixed effect in the models, and all pairwise differences between LS-means were tested using the Tukey-Kramer procedure.

The contribution of physiological parameters to the overall effect of DEX and LPS was determined by PCA using SIMCA 14.1 (Umetrics, Umea, Sweden). Parameters showing a significant difference between DEX and saline groups were included in the PCA analysis with the exception of hemoglobin and hematocrit, which overlapped with red blood cell count [23]. Body weight was included in the PCA analysis at T0-3 h to visualize the contribution of this intrinsic factor to the initial distribution of pigs.

Results

To comprehensively explore the effect of DEX on LPS-induced physiological and behavioural responses, pigs pretreated with DEX or saline were challenged with a dose of LPS that has been previously described [15] to induce systemic inflammation (Figure 1A). Results of statistical analysis are summarized in Table 1 and Table S1 for behavioural and physiological responses, respectively, and described in more detail below.

DEX diminished LPS-induced neuroendocrine response

Feedback regulation of the HPA axis during acute challenges is an important factor determining the extent of the physiological disturbances and duration of recovery [24]. Thus, we measured DEX-mediated suppression of the HPA axis in both non-inflammatory and endotoxemic pigs.

At T0, DEX almost totally blocked the production of cortisol and adrenocorticotropic hormone (ACTH) under non-inflammatory condition (Figure 1B, C). By contrast, LPS triggered pronounced cortisol and ACTH responses with dissociated kinetics in all challenged pigs (Figure 1B, C; Table S1). During inflammation, cortisol and ACTH concentrations in the DEX group were less than that in the saline group but still showed a notable increase compared with basal concentrations (Figure 1B, C; Table S1).

LPS caused an obvious febrile response in all pigs at T0+1 h, which continuously became stronger in the DEX group until at least T0+3 h (Figure 1D; Table S1). Conversely, the rectal temperature of saline-pretreated pigs decreased from T0+1 to T0+3 h (Figure 1D; Table S1).

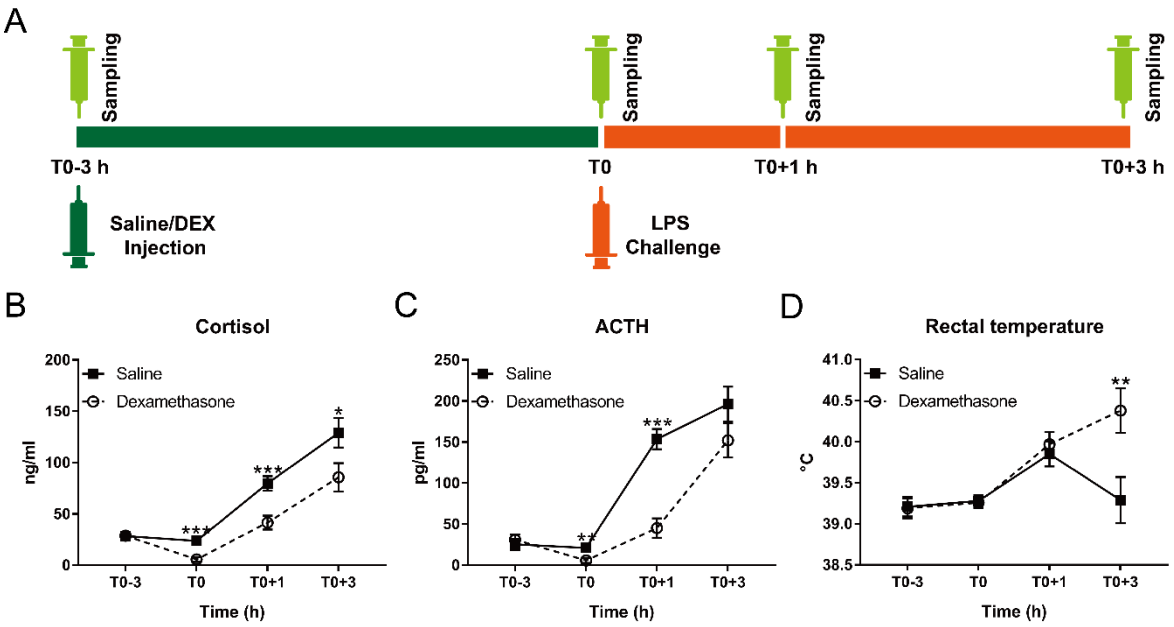

Figure 1. Effect of dexamethasone (DEX) on febrile and neuroendocrine responses following lipopolysaccharide (LPS) challenge. (A) Schematic diagram of experimental design; (B) cortisol; (C) adrenocorticotropic hormone (ACTH); and (D) rectal temperature. Data are presented as least-squares means ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DEX altered glucose and triglyceride metabolism during inflammation

Considering that the response of animals to infection is usually accompanied by anorexia and disturbance in metabolism, resulting in impeded growth and degraded product quality [25], we explored if DEX could improve metabolic health during the acute inflammatory response.

As expected, at T0, DEX elevated glucose concentration, but had no effect on triglycerides under non-inflammatory condition (Figure 2A, B). After LPS challenge, saline-pretreated pigs exhibited a significant decrease in glucose concentration from T0+1 to T0+3 h and increase in triglyceride from T0 to T0+3 h, which were prevented by the pretreatment of DEX (Figure 2A, B; Table S1).

Lactate concentration dropped significantly in both groups at T0+1h and raised again at T0+3h, reaching significantly greater concentrations in the saline-pretreated group (Figure 2C; Table S1).

In addition, LPS elevated the concentration of creatinine and blood urea nitrogen (BUN), which were not reversed by DEX (Figure 2D, E; Table S1). Both LPS and DEX showed no obvious effect on the concentration of alanine aminotransferase (ALT) (Figure 2F; Table S1).

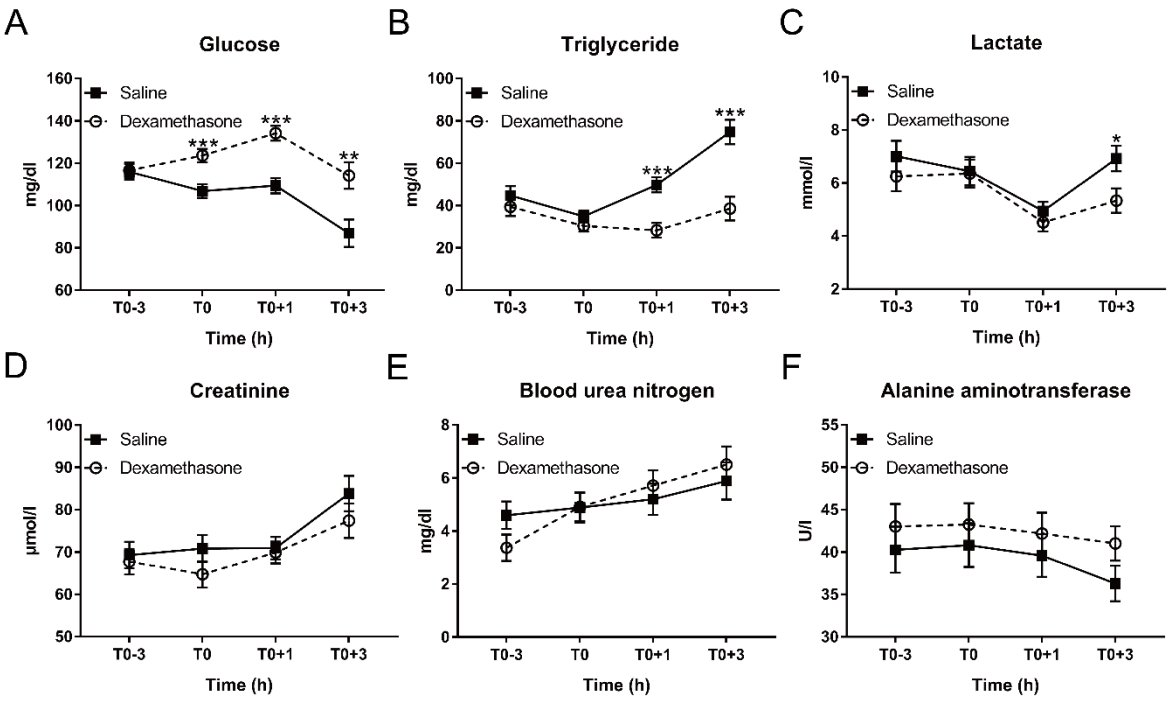

Figure 2. Effect of DEX on the kinetics of plasma biochemical parameters following LPS challenge. (A) Glucose; (B) triglyceride; (C) lactate; (D) creatinine; (E) blood urea nitrogen (BUN); and (F) alanine aminotransferase (ALT). Data are presented as least-squares means ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DEX blocked LPS-induced hematological response in varying degrees

Hematological disturbances are closely associated with the clinical situation and are also useful indicators for the poor growth performance in pigs [26]. Hence, we determined if DEX

contributes to hematological integrity in endotoxemic pigs. At T0, DEX reduced red blood cells, hemoglobin and hematocrit under non-inflammatory condition (Figure 3A-C). After LPS challenge, saline-pretreated pigs exhibited a significant increase in these variables at T0+3 h, which could be completely blocked by DEX-pretreatment (Figure 3A-C; Table S1).

In contrast, although DEX increased leukocytes at T0, it was unable to reverse LPS-induced leukocyte decrease (Figure 3D; Table S1). After LPS challenge, lymphocyte proportion in the saline group was increased at T0+1 h and returned to the baseline at T0+3 h (Figure 3E; Table S1). DEX reduced lymphocyte proportion under non-inflammatory condition; and this remained significantly less until T0+3 h (Figure 3E; Table S1).

Endotoxemia is frequently accompanied by severe thrombocytopenia [27]. We found that LPS decreased platelet number in a time-dependent manner, which could be partially reversed by DEX (Figure 3F; Table S1).

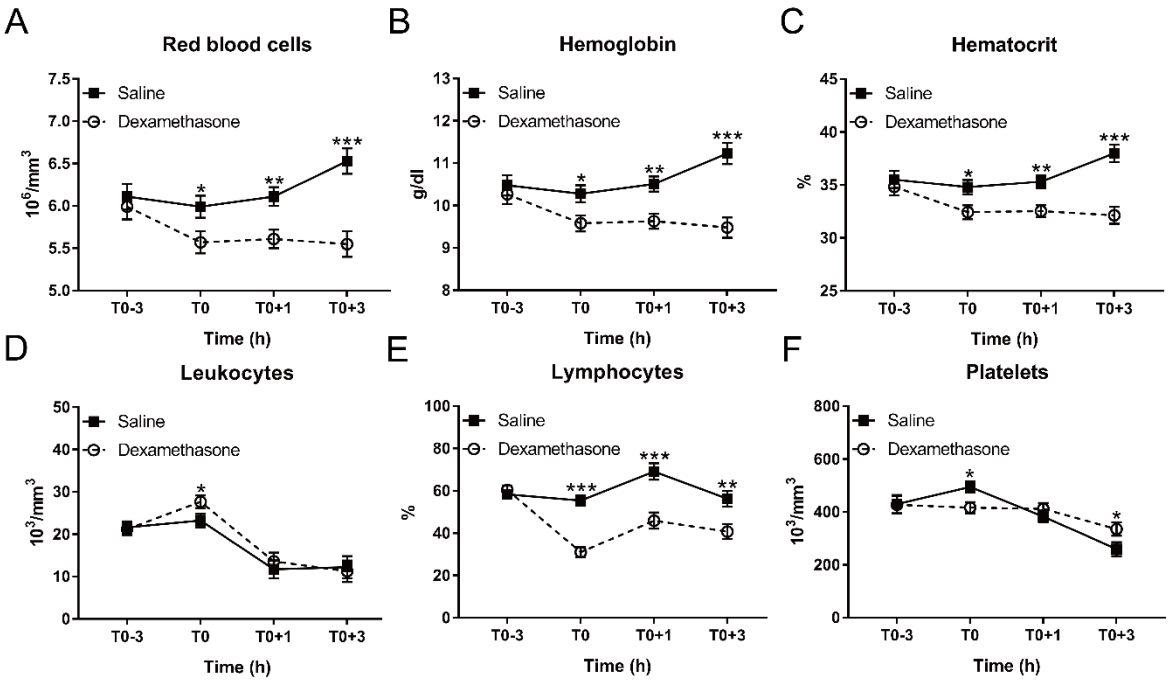

Figure 3. Effect of DEX on the kinetics of hematological parameters following LPS challenge. (A) Red blood cell count; (B) hemoglobin concentration; (C) hematocrit; (D) leukocyte count; and (E) lymphocyte proportion; (F) platelet count. Data are presented as least-squares means ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DEX relieved LPS-induced systemic inflammation

Excessive production of inflammation-associated cytokines is a hallmark of endotoxemia [28]. Therefore, we measured four cytokines including pro-inflammatory IL-1β, IL-6, TNF-α, and

their counterplayer IL-10 during the whole experiment. DEX showed no obvious effect on the production of these four cytokines under non-inflammatory condition (Figure 4A-D).

After LPS challenge, IL-1β and IL-6 were increased in a time-dependent manner in both groups until at least T0+3 h (Figure 4A, B; Table S1). Pretreatment by DEX did not alter the kinetics but reduced the concentration of IL-1β and IL-6 during inflammation: IL-6 concentration in the DEX group was significantly less than that in the saline group at T0+1 h; although the difference did not reach statistical significance, both IL-1β and IL-6 concentrations in DEX group were numerically less than that in saline group at T0+3 h (Figure 4A, B).

The kinetics of IL-10 and TNF-α was different from that of IL-1β and IL-6. Both peaked at T0+1h and declined from T0+1 to T0+3 h (Figure 4C, D; Table S1). Although showing no effect on the LPS-induced kinetics of TNF-α, DEX numerically reduced its concentration at both T0+1 h and T0+3 h (Figure 4D). Conversely, DEX showed no notable effect on IL-10 (Figure 4C).

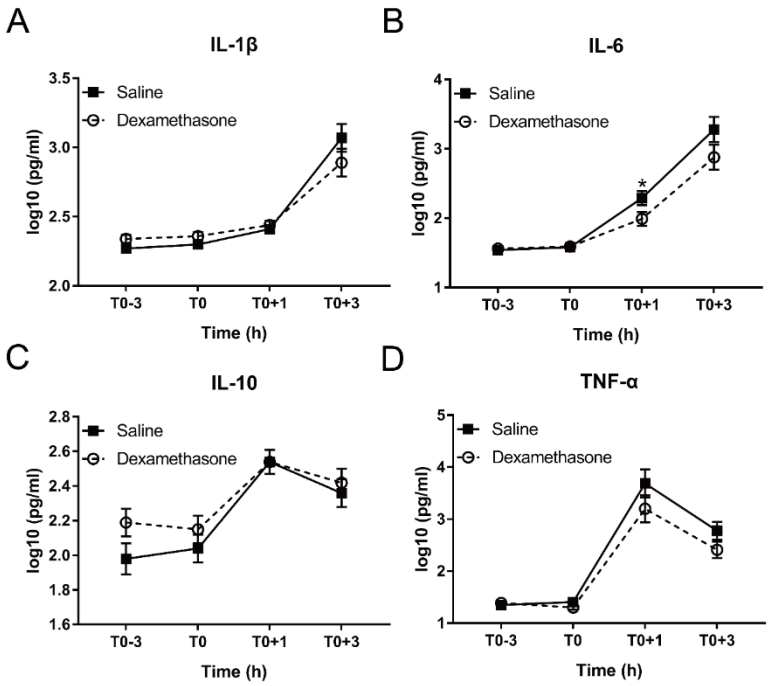

Figure 4. Effect of DEX on the inflammatory response following LPS challenge. (A) IL-1β; (B) IL-6; (C) IL-10; and (D) TNF-α. Data are presented as least-squares means ± SE. * $p < 0.05$.

DEX alleviated LPS-induced sickness symptoms

Elevated pro-inflammatory cytokines are associated with clinical symptoms and distinct behavioural changes, including lethargy and social withdrawal [29], summarily designated as sickness behaviour. To evaluate the effect of DEX on LPS-induced behavioural responses, the

frequency of five sickness symptoms over a period of 4 h post LPS challenge was recorded and summarized in Table 1.

Table 1. The frequency of sickness symptoms in pigs following LPS challenge.

Symptoms	Hour after LPS	Saline [1]	Dexamethasone [1]	*p*-value
Panting	1st hour	2.67 ± 0.79	1.08 ± 0.80	0.849
	2nd hour	6.29 ± 0.79	0.99 ± 0.80	< 0.001
	3rd hour	4.67 ± 0.79	0.96 ± 0.80	0.030
	4th hour	4.22 ± 0.79	0.68 ± 0.80	0.045
	Overall	4.46 ± 0.54	0.93 ± 0.61	< 0.001
Shivering	1st hour	2.22 ± 0.92	2.14 ± 0.94	1.000
	2nd hour	3.44 ± 0.92	6.47 ± 0.94	0.307
	3rd hour	4.33 ± 0.92	6.04 ± 0.94	0.896
	4th hour	3.44 ± 0.92	5.21 ± 0.94	0.879
	Overall	3.36 ± 0.63	4.97 ± 0.64	0.088
Vomiting	1st hour	0.78 ± 0.14	0.20 ± 0.15	0.099
	2nd hour	0.67 ± 0.14	0.43 ± 0.15	0.940
	3rd hour	0.11 ± 0.14	0.01 ± 0.15	0.990
	4th hour	0.01 ± 0.14	0.01 ± 0.15	1.000
	Overall	0.39 ± 0.08	0.16 ± 0.09	0.062
Cyanosis	1st hour	0.01 ± 0.64	0.01 ± 0.65	1.000
	2nd hour	1.39 ± 0.64	0.01 ± 0.65	0.789
	3rd hour	2.11 ± 0.64	0.01 ± 0.65	0.298
	4th hour	3.11 ± 0.64	0.38 ± 0.65	0.071
	Overall	1.65 ± 0.45	0.08 ± 0.46	0.024
Inactivity	1st hour	11.11 ± 0.29	10.42 ± 0.30	0.709
	2nd hour	11.72 ± 0.29	11.35 ± 0.30	0.985
	3rd hour	11.11 ± 0.29	11.39 ± 0.30	0.990
	4th hour	11.22 ± 0.29	11.29 ± 0.30	1.000
	Overall	11.29 ± 0.21	11.11 ± 0.21	0.548

[1] Least-squares means ± SE.

The most remarkable effect of DEX was pronounced reduction of panting. While in the saline group the frequency of panting peaked during the 2nd h, in the DEX group it was steadily decreasing and overall significantly less, particularly during 2nd, 3rd, 4th h post challenge ($p <$ 0.001, < 0.001, = 0.030, and = 0.045, respectively). In addition, in the DEX group also the overall occurrence of cyanosis was reduced. The frequency of cyanosis in both groups peaked during the 4th h post LPS challenge with a tendency for a reduced peak value in the DEX group

(3.11 ± 0.64 vs. 0.38 ± 0.65). Within the total time of observation, the average frequency of cyanosis in the DEX group was significantly less than that in the saline group ($p = 0.024$).

No statistically significant difference was observed in vomiting and inactivity between the two groups. DEX group showed a tendency for reduced vomiting and inactivity as manifested by the delayed arrival of peak and numerically decreased peak value. In saline and DEX groups, frequency of vomiting peaked during the 1st h and 2nd h post LPS challenge respectively with a numerically less peak value in the latter (0.78 ± 0.14 vs. 0.43 ± 0.15); similarly, the frequency of inactivity peaked during the 2nd h and 3rd h post LPS challenge respectively with a numerically less peak value in the latter (11.72 ± 0.29 vs. 11.39 ± 0.30).

Different from other symptoms, DEX group showed a tendency for greater shivering as manifested by the greater peak frequency and the average frequency compared to that in the saline group, but the difference did not reach statistical significance.

The overall effect of DEX on physiological responses in normal and endotoxemic pigs

Finally, we performed principal component analysis (PCA) to determine the contribution of physiological variables to the effect of DEX under both normal and endotoxemic condition. LPS shifted all challenged pigs regardless of pretreatment in the same direction featured by the increase in cortisol, ACTH, and IL-6, and the decrease in leukocytes and platelets, which exhibited a growing variation among individuals as indicated by the continuously decentralized symbols (Figure S1).

At the beginning of the experiment (T0-3 h), no discrimination was observed between saline and DEX groups (Figure 5A). At T0, DEX shifted pigs to the direction along the decrease in cortisol and ACTH and overall compressed the individual variation as reflected by the centralized symbols (Figure 5B).

During inflammation, a notable discrimination was observed between saline and DEX groups (Figure 5C, D). At T0+1 h, PC1 was mainly driven by an opposition between cortisol, ACTH, and lymphocytes (negatively correlated with DEX-induced shift), and leukocytes (positively correlated with DEX-induced shift), whereas PC2 was mainly driven by an opposition between red blood cells and triglycerides (negatively correlated with DEX-induced shift), and rectal temperature (positively correlated with DEX-induced shift) (Figure 5C). At T0+3 h, PC1 was mainly driven by an opposition between cortisol, ACTH, and IL-6 (negatively correlated with DEX-induced shift), and platelets (positively correlated with DEX-induced shift), whereas PC2

was mainly driven by an opposition between red blood cells, triglyceride and leukocytes (negatively correlated with DEX-induced shift), and rectal temperature and glucose (positively correlated with DEX-induced shift) (Figure 5D). During the whole inflammatory process, DEX consistently shifted pigs to the direction along the increase in glucose and the decrease in red blood cells and triglycerides (Figure 5C, D).

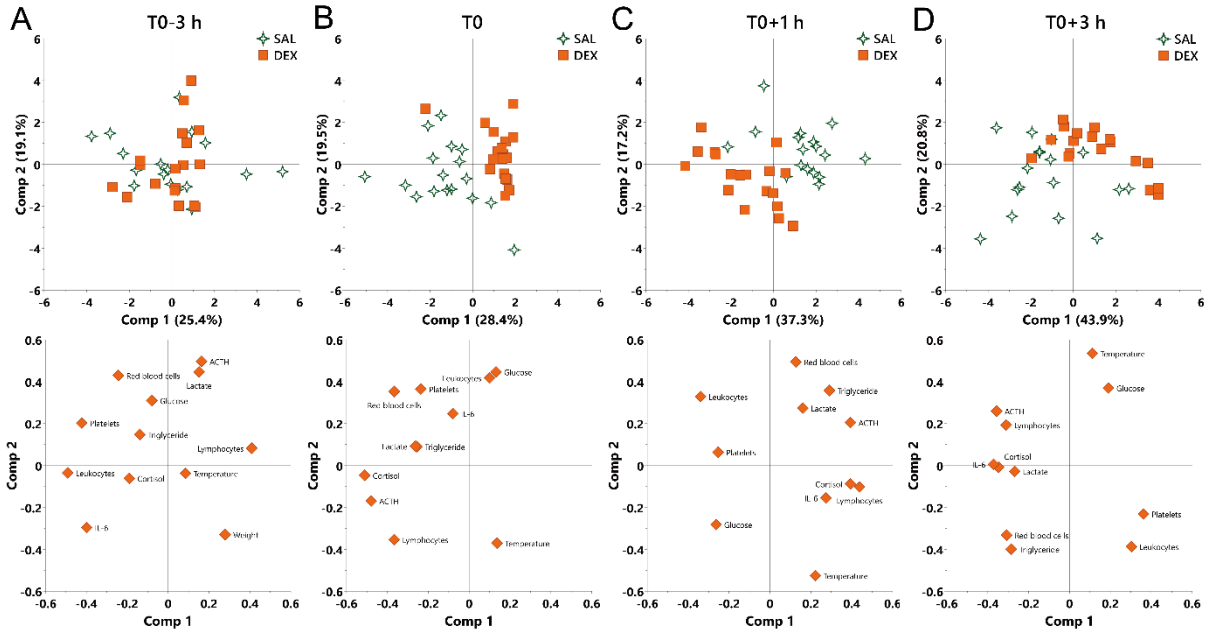

Figure 5. Principal component analysis (PCA) for the overall effect of DEX on pigs under normal and endotoxemic conditions. (A) T0-3 h, baseline; (B) T0, the effect of DEX under normal condition; (C) T0+1 h and (D) T0+3 h, the effect of DEX under endotoxemic condition. Loading of the parameters on component 1 and 2 are visualized below the PCA score plot to show the contribution of parameters to the group separation. SAL, saline; DEX, dexamethasone; Comp, component.

Discussion

To obtain a comprehensive overview of DEX effects in pigs we analyzed diverse biological responses in resting and LPS-challenged animals.

Compared to poultry and rodents, pigs are relatively sensitive to LPS so that doses in excess of 25 µg/kg BW are considered as high [14]. This may explain a decrease in rectal temperature from T0+1 to T0+3 h in the saline group since sometimes decrease in temperature could be observed in animals mounting strong inflammatory response [30].

Although DEX almost totally blocked the release of cortisol and ACTH at T0 due to the negative feedback of glucocorticoids on their own secretion [31], it only partially reduced LPS-

triggered neuroendocrine responses. In fact, pro-inflammatory cytokines induce tissue-specific glucocorticoid insensitivity by interfering with glucocorticoid receptor signaling [3]. While reduced glucocorticoid sensitivity in the HPA axis may serve as an adaptive mechanism facilitating stronger or prolonged glucocorticoid release, in peripheral tissues it has adverse effects and may lead to sepsis [1, 3]. We found that the kinetics of the cortisol response was dissociated from that of ACTH during the experiment. LPS-induced cytokines like IL-1β, IL-6, and TNF-α can either directly promote the release of cortisol by interacting with the adrenal gland, or function indirectly by interacting with hypothalamus and pituitary, resulting in the release of corticotropin-releasing hormone (CRH) and ACTH and thereby increase cortisol via activation of the HPA axis [32]. It appears that sensitivity to both, LPS and DEX, differs between the central branch of the HPA axis and the adrenal gland, thus pointing to different regulation of their responses.

Glucose was sharply reduced from T0+1 to T0+3 h accompanied by an increase in lactate, which may be explained by the anaerobic glycolysis during endotoxemia. It has been described that LPS promotes glycolysis via enhancing the expression and the activity of glycolysis-associated enzymes such as hexokinase, pyruvate kinase, and lactic dehydrogenase [33]. Then the product of glycolysis, pyruvate, could be converted to lactate due to the hypoxia which normally occurs in LPS-induced endotoxemia [34, 35]. Notably, a decrease in lactate was observed in both groups at T0+1 h. This may be associated with the removal of feed/anorexia during the experiment since a recent study demonstrated that circulating lactate could also be a primary source of carbon for the tricarboxylic acid cycle under fasting condition for hepatic gluconeogenesis [36]. Importantly, the subsequent rise in lactate at T0+3 h was blunted by DEX. This is a significant beneficial effect of DEX in endotoxemia, since lactate accumulation may lead to lactic acidosis, which is a serious complication in sepsis [1].

An obvious distinction in lipid metabolism was observed between saline and DEX groups. Whereas LPS has been shown to rapidly induce changes in hepatic lipid metabolism leading to hypertriglyceridemia [37], glucocorticoids including DEX induce lipolysis in adipose tissue [38], particularly during fasting [39].

An increase in creatinine and BUN was observed after LPS challenge, which was supported by similar studies using mouse or pig model [40, 41]. In the study using C57BL/6 mice, the increase in creatinine and BUN was partially inhibited by pretreatment of DEX 1 h before LPS injection [40]. However, in our experiment DEX showed no effect on LPS-induced increase in

creatinine and BUN, which may be associated with experimental factors and/or species differences.

We found ALT, the specific marker for hepatic injury, was slightly decreased at T0+3 h. This result is consistent with a previous observation that in pigs LPS increased the nonspecific hepatic injury marker aspartate aminotransferase (AST) and the ratio of AST/ALT at 3 h post challenge but showed no obvious effect on ALT concentration [42].

DEX decreased red blood cell related properties (number, blood hemoglobin content, and hematocrit) irrespective of the LPS treatment. Conversely, LPS increased these properties at T0+3 in saline-treated animals. This effect of DEX on red blood cells has not been characterized in pigs so far. Nevertheless, it is supported by the study of Sautron et al. [23], who observed a similar decrease in red blood cells following ACTH application, stimulating cortisol production. The mechanism behind this effect in pigs warrants further investigation. This could be related to the effect of glucocorticoids on fluid homeostasis or haemodynamics [4]. The decrease in the total number of leukocytes following LPS challenge might be, at least partly, associated with adhesion and migration of neutrophils [43]. Neutrophils are one of the most abundant leukocytes, which account for more than 40% of total leukocytes [44]. LPS could enhance the expression of endothelial adhesion molecules and thus promote the adhesion of neutrophils to endothelial cells and the transendothelial neutrophil migration, leading to the reduction of neutrophils in peripheral blood [43]. Although DEX could inhibit the migration of neutrophils by up-regulating annexin A1 via glucocorticoid-induced leucine zipper [45], it failed to reverse LPS-induced reduction in total circulating leukocytes. In contrast, DEX increased circulating leukocytes under the non-inflammatory condition, which may be explained by the increase in neutrophils, since the administration of glucocorticoids could increase neutrophils via enhancing neutrophil demargination from the endothelial layer and neutrophil release from the bone marrow [46].

We found that DEX blunted LPS-induced thrombocytopenia, a hallmark of sepsis and endotoxemia [47]. Platelet activation and local coagulation is a protective mechanism against endothelial dysfunction [48], and against pathogens serving their trapping and elimination [49]. However, exaggerated platelet activation is a major contributor to thrombocytopenia and may lead to disseminated intravascular coagulation, and ultimately to multiple organ failure [49]. Thus, prevention of thrombocytopenia is another important aspect contributing to the protective effect of DEX in endotoxemia, likely by inhibiting platelet aggregation [50].

So far, only a single study reported on DEX effects on inflammatory responses, including cytokine production, to LPS application in pigs [5]. However, the route of application (intravenous), dose of both LPS and DEX, application scheme of DEX, and LPS serotype (O55:B5) were different from the present study. Nevertheless, the results for IL-6, and IL-10 were similar. More specifically, whereas IL-6 was most potently reduced by DEX, while IL-10 activation was not influenced by DEX [5]. Similar to the study of Myers et al. [5], in our study TNF-α was also increased at 1 h post LPS challenge. However, the statistically significant effect of DEX on this increase was not observed in current study. A notable difference observed here is activation of IL-1β by LPS, which in the study of Myers et al. [5] was constitutively present and unaffected by both LPS and DEX. This dichotomy is likely explained by differences in LPS serotype and/or application.

Using PCA we found that LPS significantly shifted the clusters in a time-dependent manner regardless of the DEX treatment, which was mainly driven by the responses of neuroendocrine and immune systems, together with changes in platelet number. This observation is consistent with previous reports that LPS-induced endotoxemia is usually accompanied by systemic inflammation and thrombocytopenia [51, 52]. In addition, we found that the LPS-induced excessive physiological responses amplified the intrinsic individual variation which was manifested by dispersal of the clusters in a time-dependent manner. This observation is supported by previous studies likewise reporting tremendous variation among individuals in physiological responses to LPS [5, 53]. Similar to LPS, DEX also significantly shifted PCA clusters and this effect persisted both under inflammatory and non-inflammatory conditions. Notably, although LPS significantly affected triglyceride metabolisms, this was not the primary factor contributing to LPS-induced overall alteration. In contrast, the changes in glucose and triglycerides played an important role in DEX-induced overall shift during inflammation, implying that energy transformation may be an additional important factor for DEX-mediated protective effect in endotoxemic pigs [54].

Conclusion

Despite the less sensitivity of pigs to DEX the latter improves physiological and behavioural integrity in endotoxemic pigs. Our results suggest that glucose, lactate and triglyceride metabolism as well as red cell and platelet count play a notable role in the beneficial effect of DEX in this context. This study closes the knowledge gap in the application of DEX in pigs and presents the feasibility of glucocorticoids for the improvement of animal robustness in the face of immune stress.

Supplementary materials

Supplementary materials can be found at https://www.mdpi.com/1422-0067/20/6/1393/s1.

Author contributions

Conceptualization: E.M.; methodology: E.M., E.K., and M.T.; formal analysis: E.M., Z.L., E.K., M.T., and A.T.; investigation: Z.L., N.T., E.M., E.K., M.T., and C.C.M.; resources: C.C.M. and K.W.; data curation: E.M., Z.L., E.K., M.T., and A.T.; writing—original draft preparation: Z.L. and E.M.; writing—review and editing: Z.L., E.K., M.T., A.T., C.C.M., N.T., K.W., and E.M.; supervision: E.M. and K.W.; project administration: E.M.; funding acquisition: E.M.

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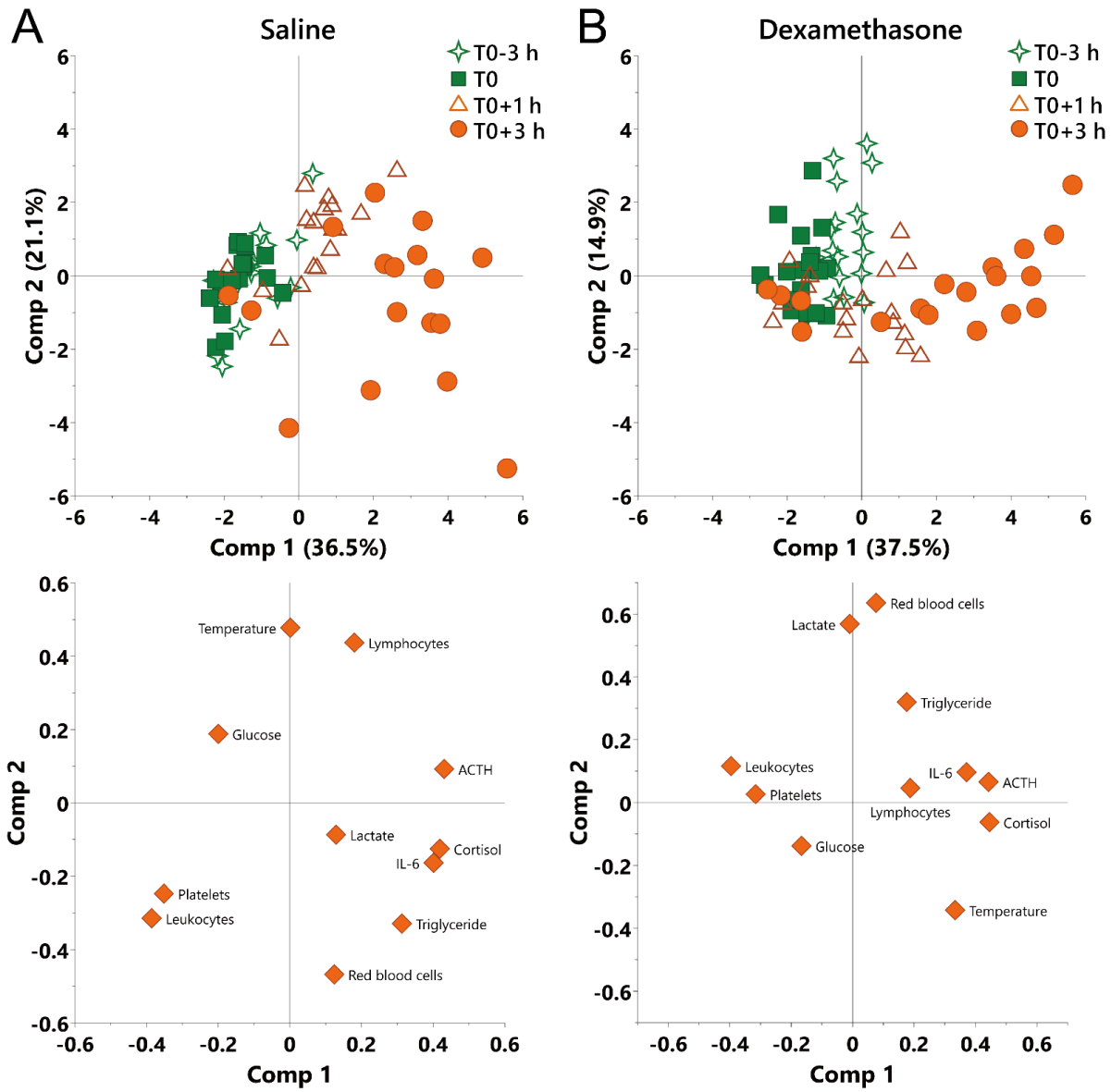

Figure S1. Principal component analysis (PCA) for time-depending overall effect of LPS on pigs with or without pretreatment of DEX. (A) Pigs pretreated with saline; (B) Pigs pretreated with DEX. Loading of the parameters on component 1 and 2 are visualized below the PCA score plot to show the contribution of parameters to the group separation. Comp, component.

Table S1. Overview of the effect of the time point and the treatment on studied parameters.

Parameter	Unit	*p*-value			LSM ± SE							
					Saline				Dexamethasone			
		Time	Treatment	T × T	T0-3 h	T0	T0+1 h	T0+3 h	T0-3 h	T0	T0+1 h	T0+3 h
RT	°C	<.0001	0.0622	0.0579	39.21 ± 0.12^{a}	39.28 ± 0.07^{a}	39.85 ± 0.15^{b}	39.29 ± 0.28^{ab}	39.19 ± 0.12^{A}	39.26 ± 0.07^{A}	39.97 ± 0.15^{B}	40.38 ± 0.27^{B}
Cortisol	ng/ml	<.0001	0.0019	<.0001	28.46 ± 3.09^{a}	23.68 ± 2.49^{a}	79.73 ± 6.99^{a}	129.11 ± 14.35^{b}	28.68 ± 3.00^{A}	5.70 ± 2.42^{B}	41.55 ± 6.79^{A}	85.70 ± 13.94^{C}
ACTH	pg/ml	<.0001	0.0013	<.0001	25.44 ± 6.50^{a}	21.23 ± 3.19^{a}	153.72 ± 12.27^{b}	196.29 ± 21.21^{c}	31.08 ± 6.31^{A}	5.97 ± 3.10^{B}	45.34 ± 11.92^{A}	152.22 ± 20.61^{C}
Glucose	mg/dl	<.0001	0.0004	0.0032	116.02 ± 3.80^{a}	106.90 ± 3.24^{a}	109.43 ± 3.62^{a}	86.96 ± 6.49^{b}	116.67 ± 3.69^{A}	123.67 ± 3.14^{A}	134.22 ± 3.51^{B}	114.22 ± 6.30^{A}
Triglyceride	mg/dl	<.0001	0.0004	0.0008	44.77 ± 4.44^{ab}	34.94 ± 2.79^{a}	49.83 ± 3.55^{b}	74.77 ± 5.76^{c}	39.46 ± 4.31^{A}	30.46 ± 2.71^{A}	28.41 ± 3.44^{A}	38.63 ± 5.60^{A}
Lactate	mmol/l	<.0001	0.1234	0.1982	7.02 ± 0.58^{b}	6.45 ± 0.54^{ab}	4.94 ± 0.35^{a}	6.93 ± 0.48^{b}	6.26 ± 0.57^{A}	6.36 ± 0.53^{A}	4.52 ± 0.34^{B}	5.34 ± 0.46^{AB}
Creatinine	µmol/l	<.0001	0.3560	0.3451	69.32 ± 3.13^{a}	70.87 ± 3.19^{a}	70.96 ± 2.66^{a}	83.84 ± 4.20^{b}	67.75 ± 3.04^{A}	64.75 ± 3.09^{A}	69.92 ± 2.57^{AB}	77.45 ± 4.08^{B}
BUN	mg/dl	<.0001	0.9858	<.0001	4.60 ± 0.52^{a}	4.89 ± 0.56^{a}	5.20 ± 0.59^{a}	5.89 ± 0.70^{b}	3.37 ± 0.50^{A}	4.92 ± 0.54^{B}	5.72 ± 0.57^{C}	6.51 ± 0.68^{D}
ALT	U/l	0.0005	0.3575	0.3645	40.29 ± 2.72^{a}	40.82 ± 2.59^{a}	39.59 ± 2.52^{a}	36.29 ± 2.09^{b}	43.03 ± 2.64^{A}	43.26 ± 2.51^{A}	42.20 ± 2.45^{A}	41.03 ± 2.02^{A}
RBC	10^{6}/mm^{3}	0.0038	0.0051	0.0012	6.11 ± 0.15^{a}	5.99 ± 0.13^{a}	6.11 ± 0.11^{a}	6.53 ± 0.15^{b}	5.99 ± 0.15^{B}	5.57 ± 0.13^{A}	5.61 ± 0.11^{A}	5.55 ± 0.15^{A}
Hemoglobin	g/dl	0.0111	0.0015	0.0011	10.48 ± 0.24^{a}	10.28 ± 0.20^{a}	10.51 ± 0.18^{a}	11.23 ± 0.25^{b}	10.27 ± 0.23^{B}	9.58 ± 0.19^{A}	9.63 ± 0.18^{A}	9.48 ± 0.24^{A}
Hematocrit	%	0.0047	0.0016	0.0013	35.53 ± 0.82^{a}	34.81 ± 0.68^{a}	35.32 ± 0.60^{a}	38.00 ± 0.83^{b}	34.82 ± 0.79^{B}	32.44 ± 0.66^{A}	32.54 ± 0.58^{A}	32.14 ± 0.80^{A}
Leukocytes	10^{3}/mm^{3}	<.0001	0.5934	0.0069	21.66 ± 1.41^{a}	23.26 ± 1.56^{a}	11.71 ± 2.10^{b}	12.21 ± 2.60^{b}	21.21 ± 1.36^{B}	27.68 ± 1.51^{C}	13.59 ± 2.04^{A}	11.26 ± 2.52^{A}
Lymphocytes	%	<.0001	<.0001	<.0001	58.41 ± 2.11^{a}	55.45 ± 2.42^{a}	69.15 ± 3.89^{b}	56.28 ± 3.67^{a}	60.47 ± 2.04^{B}	31.04 ± 2.34^{C}	45.98 ± 3.78^{A}	40.81 ± 3.56^{A}
Platelets	10^{3}/mm^{3}	<.0001	0.8278	0.0020	430.19 ± 34.24^{ab}	494.96 ± 21.58^{a}	383.07 ± 22.37^{b}	259.60 ± 26.38^{c}	427.68 ± 33.24^{AB}	416.90 ± 20.91^{A}	412.57 ± 21.68^{A}	335.90 ± 25.58^{B}
IL-1β	Log10, pg/ml	<.0001	0.8854	0.3932	2.27 ± 0.03^{a}	2.30 ± 0.03^{a}	2.41 ± 0.03^{b}	3.07 ± 0.10^{c}	2.34 ± 0.03^{A}	2.36 ± 0.03^{A}	2.44 ± 0.03^{B}	2.89 ± 0.10^{C}
IL-6	Log10, pg/ml	<.0001	0.0864	0.1743	1.54 ± 0.02^{a}	1.58 ± 0.02^{a}	2.29 ± 0.10^{b}	3.28 ± 0.18^{c}	1.56 ± 0.02^{A}	1.59 ± 0.02^{A}	1.99 ± 0.10^{B}	2.88 ± 0.18^{C}
IL-10	Log10, pg/ml	<.0001	0.2818	0.4156	1.98 ± 0.09^{a}	2.04 ± 0.08^{a}	2.54 ± 0.07^{b}	2.36 ± 0.08^{c}	2.19 ± 0.08^{AB}	2.15 ± 0.08^{A}	2.54 ± 0.07^{C}	2.42 ± 0.08^{B}
TNF-α	Log10, pg/ml	<.0001	0.1419	0.0530	1.35 ± 0.04^{a}	1.41 ± 0.05^{a}	3.69 ± 0.27^{b}	2.78 ± 0.17^{c}	1.39 ± 0.04^{A}	1.30 ± 0.04^{A}	3.20 ± 0.26^{B}	2.41 ± 0.16^{C}

Single underline highlights significant main effects at $p < 0.05$. Double underline highlights significance between saline and dexamethasone groups at the same time point ($p < 0.05$). Within each group, superscript highlights the significance among different time points; same superscript highlights non-significance ($p > 0.05$); different superscript highlights significance ($p < 0.05$); lowercase letter highlights the significance for saline group, capital letter highlights the significance for DEX group. T × T: time point-by-treatment interaction. LSM: least-squares means. RT: rectal temperature. ACTH: adrenocorticotropic hormone. BUN: blood urea nitrogen. ALT: alanine aminotransferase. RBC: red blood cells.

4

Manuscript 2

Transcriptome analysis of porcine PBMCs reveals lipopolysaccharide-induced immunomodulatory responses and crosstalk of immune and glucocorticoid receptor signaling

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4 Manuscript 2 published in Virulence

Transcriptome analysis of porcine PBMCs reveals lipopolysaccharide-induced immunomodulatory responses and crosstalk of immune and glucocorticoid receptor signaling

Zhiwei Li[1], Nares Trakooljul[1], Frieder Hadlich[1], Siriluck Ponsuksili[1], Klaus Wimmers[1], Eduard Murani[1,*]

[1] Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

***** Corresponding author.

Abstract

The current level of knowledge on transcriptome responses triggered by endotoxins and glucocorticoids in immune cells in pigs is limited. Therefore, in the present study, we treated porcine peripheral blood mononuclear cells (PBMCs) with lipopolysaccharide (LPS) and dexamethasone (DEX) separately or combined for 2 hours. The resultant transcriptional responses were examined by mRNA sequencing. We found that the LPS treatment triggered pronounced inflammatory responses as evidenced by upregulation of pro-inflammatory cytokines, chemokines, and related signaling pathways like NF-κB. Concurrently, a series of downregulated pro-inflammatory and upregulated anti-inflammatory molecules were identified. These are involved in the inhibition of TLR, NF-κB, and MAPK cascades and activation of signaling mediated by Tregs and STAT3, respectively. These findings suggested that LPS initiated also an anti-inflammatory process to prevent an overwhelming inflammatory response. The transcriptome responses further revealed substantial crosstalk of immune responses and glucocorticoid receptor (GR) signaling. This was apparent in four aspects: constitutive inhibition of T cell signaling by DEX through a subset of genes showing no response to LPS; inhibition of LPS-induced inflammatory genes by DEX; attenuation of DEX action by LPS paralleled by the regulation of genes implicated in cytokine and calcium signaling; and DEX-induced changes in genes associated with the activation of pro-inflammatory TLR, NF-κB, iNOS, and IL-1 signaling. Consequently, our study provides novel insights into inflammatory and GR signaling in pigs, as well as an understanding of the application of glucocorticoid drugs for the treatment of inflammatory disorders.

Keywords: transcriptome; porcine PBMCs; lipopolysaccharide; immune signaling; dexamethasone; glucocorticoid receptor signaling.

Introduction

Glucocorticoids (GCs) are considered to be the most potent and effective anti-inflammatory drugs in both human and veterinary medicine [1]. Immunomodulation by GCs is mediated primarily by the glucocorticoid receptor (GR), a ligand-inducible transcription factor (TF) of the nuclear receptor superfamily. GR signaling plays a vital role in many biological processes, such as cell proliferation and metabolic regulation [2]. It is generally believed that the anti-inflammatory action of GR is conferred by its monomeric form through transrepression of other, pro-inflammatory, TFs such as NF-κB, AP-1, IRF3, and T-bet. In contrast, GR dimer-dependent transactivation of genes involved in glucose and lipid metabolism is associated with undesirable side effects on metabolic homeostasis [2]. However, there is increasing evidence suggesting that the dimeric form is crucial in GR-mediated anti-inflammatory action, which is determined by a series of GC-inducible anti-inflammatory molecules, including TSC22D3, KLF2, and DUSP1 [3]. On the other hand, immune mediators like cytokines exert considerable influence on GR signaling [4, 5]. These findings emphasize the complexity and diversity of GR signaling and its function in controlling inflammation. It is vital that comprehensive research is undertaken to further explore the regulation of GR signaling and its crosstalk with immune pathways.

The current use of GC-based drugs in pigs relies mainly on findings from human studies, since knowledge of the effects of these drugs in farm animals is relatively lacking [6]. However, the distinct potency and pharmacokinetics of GC-based drugs, such as dexamethasone (DEX), in pigs, calls for deeper research in this field [7]. Moreover, pigs are comparatively more vulnerable to lipopolysaccharide (LPS) [8]. LPS administration induces a pronounced inflammatory response in pigs, alongside behavioral and physiological changes; most of these transformations are attenuated by co-administration of DEX [9, 10]. We have previously shown that short-term treatment by DEX regulates a large number of genes involved in inflammatory responses in the porcine liver even in the absence of immune stimuli [11]. This finding accentuates the substantial role of GCs and GC-based drugs in the immunomodulation in pigs. However, how acute activation of GR signaling by short-term exposure to GCs orchestrates responses in the presence and absence of immune stimuli in porcine immune cells is still poorly explored.

This study aims to investigate GR signaling and inflammatory responses, and to establish their interplay in porcine immune cells. To this end, porcine peripheral blood mononuclear cells (PBMCs) were treated with either vehicle (CON), DEX, LPS, or LPS+DEX for 2 hours to mimic acute inflammation and activation of GR signaling. The corresponding transcriptome responses were explored using mRNA sequencing; in addition, a range of different bioinformatics tools were employed to obtain a holistic overview of the events. The findings of this study will facilitate improved and informed application and development of GC-based drugs, and will also offer an insight into how stress – via the induction of natural GCs – modulates the immune system and influences animal health. These are important, foundational steps leading toward the successful application of the One Health concept [12].

Materials and methods

Sample collection

German Landrace pigs used to collect samples were raised until slaughter age (mean = 170 days) under standardized conditions at the experimental pig farm of the Leibniz Institute for Farm Animal Biology (Dummerstorf, Germany) in accordance with the German Law of Animal Protection.

PBMCs were isolated from whole blood as previously described [13]. Briefly, trunk blood was collected into pre-chilled tubes containing EDTA during exsanguination in the context of regular slaughter procedures, taking place in the morning. The blood samples were then centrifuged on a Histopaque-1077 density gradient (Sigma–Aldrich, Taufkirchen, Germany) to attain a layer of PBMCs, according to the manufacturer's instructions. The isolated PBMCs were stored in liquid nitrogen with 90% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany) and 10% DMSO until required.

In vitro LPS and DEX challenge

PBMCs taken from whole blood samples of 24 pigs (12 males, 12 females) were used for treatment assays as previously described with modifications [14]. First, cells were thawed and washed with RPMI 1640 medium (Biochrom, Berlin, Germany). Subsequently, cells were resuspended in cell culture medium (RPMI 1640 medium supplemented with 10% FBS, 2 mmol/l L-glutamine (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma–Aldrich, Taufkirchen, Germany)) and adjusted to 6×10^6 cells/ml. PBMCs from each individual were divided into four treatment groups and seeded in 24-well

plates at 3×10^6 cells/well, followed by overnight incubation at 37 °C with 5% CO_2. DEX and LPS stock solutions were prepared in ethanol (25 mM) and PBS (1 mg/ml), respectively and diluted in cell culture medium to the required concentration as needed. The four groups were treated with either vehicle (CON; cell culture medium + corresponding volume of ethanol + corresponding volume of PBS), DEX (Sigma–Aldrich; final concentration 5 nM (≈ 2 ng/ml) in cell culture medium + corresponding volume of PBS), LPS (*Escherichia coli* O111: B4; Sigma–Aldrich; final concentration 10 μg/ml in cell culture medium + corresponding volume of ethanol), or LPS (10 μg/ml) + DEX (5 nM), respectively. All the samples were treated for 2 hours at 37 °C with 5% CO_2. Afterward, cells were collected for RNA extraction.

RNA extraction and mRNA sequencing

Total RNA was extracted using TRI reagent (Sigma–Aldrich, Taufkirchen, Germany) and purified with the RNA Clean & Concentrator-25 Kit (Zymo Research, Freiburg, Germany), in accordance with the manufacturer's instructions. Genomic DNA was removed using the RNase-Free DNase Set (Qiagen, Hilden, Germany). Subsequently, the RNA integrity number (RIN) was assessed (mean ± SE = 8.66 ± 0.04) with the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano kit (Agilent Technologies, Waldbronn, Germany). Sequencing libraries were prepared using the TruSeq Stranded mRNA Sample Preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The quality of the DNA libraries was also determined by the Agilent Technologies 2100 Bioanalyzer and the Agilent DNA-1000 Chip kit (Agilent Technologies, Waldbronn, Germany). Concentration of the DNA libraries was quantified by the Qubit dsDNA HS assay kit (Invitrogen, Darmstadt, Germany). The cBot system (Illumina, San Diego, CA, USA) was used to generate clonal clusters, and sequencing was performed on the Illumina HiSeq 2500 sequencing platform with paired-end reads of 2 × 101 bp. The quality of pre- and post-processing data was assessed by the FastQC version 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw sequence files (fastq format) were preprocessed using TrimGalore version 0.5.0 to remove adapter-like sequences, and to trim low quality reads (Q-score < 20) and short reads (<30 bp). The resultant clean reads were then mapped to the reference genome Ssrofa11.1 (Ensembl release 98) using HISAT2 (version 2.1.0) [15]. Subsequently, the aligned reads were quantified using the HTSeq (version 0.11.2) [16]. The initial dataset contained 31,907 gene entries.

Differential expression analysis

Prior to the analysis, pre-filtering was carried out to remove genes associated with fewer than eight samples with normalized counts greater than or equal to 5; this retained 14,809 available genes from the initial 31,907 gene entries. Principal component analysis (PCA) was performed based on variance-stabilizing transformed (VST) counts of all analyzed genes. Four outlier samples were identified and omitted from further analyses. Cell type enrichment analysis was performed with the xCell webtool [17], using transcripts per million (TPM) of all filtered genes. A t-distributed stochastic neighbor embedding (tSNE) plot was generated using the R package Rtsne version 0.15 [18] based on the enrichment scores of 64 cell types obtained from the xCell.

Differential gene expression analysis was conducted using the R package DESeq2 version 1.28.1 [19]. Three factors were included in the design of the statistical model: sex (male and female), GR genotype (AlaAla, AlaVal, and ValVal) [11], and treatment (CON, DEX, LPS, and LPS+DEX). Treatment effect was analyzed using the Wald test in five pairwise comparisons: DEX and vehicle groups (DEX VS CON), LPS and vehicle groups (LPS VS CON), LPS+DEX and vehicle groups (LPS+DEX VS CON), LPS+DEX and DEX groups (LPS+DEX VS DEX), as well as LPS+DEX and LPS groups (LPS+DEX VS LPS). Genes with a false discovery rate adjusted *p*-value (*q*-values) < 0.05 were considered to be significantly differentially expressed. A volcano plot was made using the R package EnhancedVolcano version 1.6.0 [20] to illustrate differentially expressed genes (DEGs). The heat map was plotted using the R package pheatmap version 1.0.12 [21] based on the $\log_2$ fold change (LFC) of the analyzed genes. A Venn diagram was created using the TBtools toolkit [22].

Identification of functional modules and their hub genes

To study specific LPS and DEX functions in the context of their interplay, five modules (M1-M5) with different response patterns were identified based on significance and LFC. These modules comprised 4966 genes out of 8740 non-repetitive DEGs that were significantly regulated in at least one comparison. The criteria for defining each of the five functional modules are summarized in Table S2. Following this, k-means clustering of gene expression profiles within each module was performed using the R package ComplexHeatmap version 2.4.3 [23]. Cytokines, chemokines, and their receptors were identified using the ImmPort cytokine registry [24]. GR targets involved in immune responses were identified by comparing with a gene list comprising genes shared by three libraries: GR-regulated genes from the database of Ingenuity Pathway Analysis (IPA; Qiagen, Hilden, Germany); GR targets revealed by binding site profiling studies from Harmonizome [25]; and immune genes from InnateDB [26]. For each module, protein–protein interaction networks were constructed within each

module using the STRING database [27] and were then visualized using Cytoscape version 3.8.0 [28]. The top 30 hub genes displaying a high degree of connectivity were determined using the cytoHubba Cytoscape plugin [29]. Subsequently, the functional annotation of hub genes was performed with the ontology knowledgebase GO Biological Processes and Reactome Gene Sets using Metascape [30].

Functional enrichment analysis

Enrichment analysis of canonical pathways, diseases, biological functions, and upstream regulators was conducted using the IPA to uncover directional regulation of signaling, biological consequences, and upstream regulatory events. For this purpose, *q*-value and LFC calculated by different comparisons were used for different modules: LPS VS CON and DEX VS CON were used for M1 and M2, respectively, to illustrate the influence of LPS and DEX; LPS+DEX VS LPS was used for M3 to highlight the anti-inflammatory effect of DEX; LPS+DEX VS DEX was used for M4 to determine potential events induced by LPS that may be involved in impaired DEX effect; LPS+DEX VS CON was used for M5 to reveal consequences of the additive or synergistic effects of LPS and DEX. Terms with *p*-values < 0.05 and with absolute z-scores ≥ 2 were considered to be significantly enriched and directionally regulated. Enrichment analysis with ontology sources GO Biological Processes was conducted using the Metascape to complement IPA results. The R package ggplot2 version 3.3.2 [31] and GraphPad Prism 8.2.1 (GraphPad Software, Inc., San Diego, CA) were used to visualize the results.

Results

Differential expression analysis

After filtering, a total of 14,809 genes were retained for the differential expression analysis (Table S1). PCA revealed a primary separation of the samples by treatment type and a secondary separation of samples by sex (Figure 1A). Overall, the results showed that the treatment type shifted samples in the same direction regardless of sex (i.e. there is no obvious treatment by sex interaction). In contrast, cell type-specific responses were primarily due to the treatment, without an obvious effect of sex (Figure 1B). In addition, the cell-type enrichment scores indicate activation of regulatory T cell (Treg) signaling upon LPS stimulation (Figure 1C).

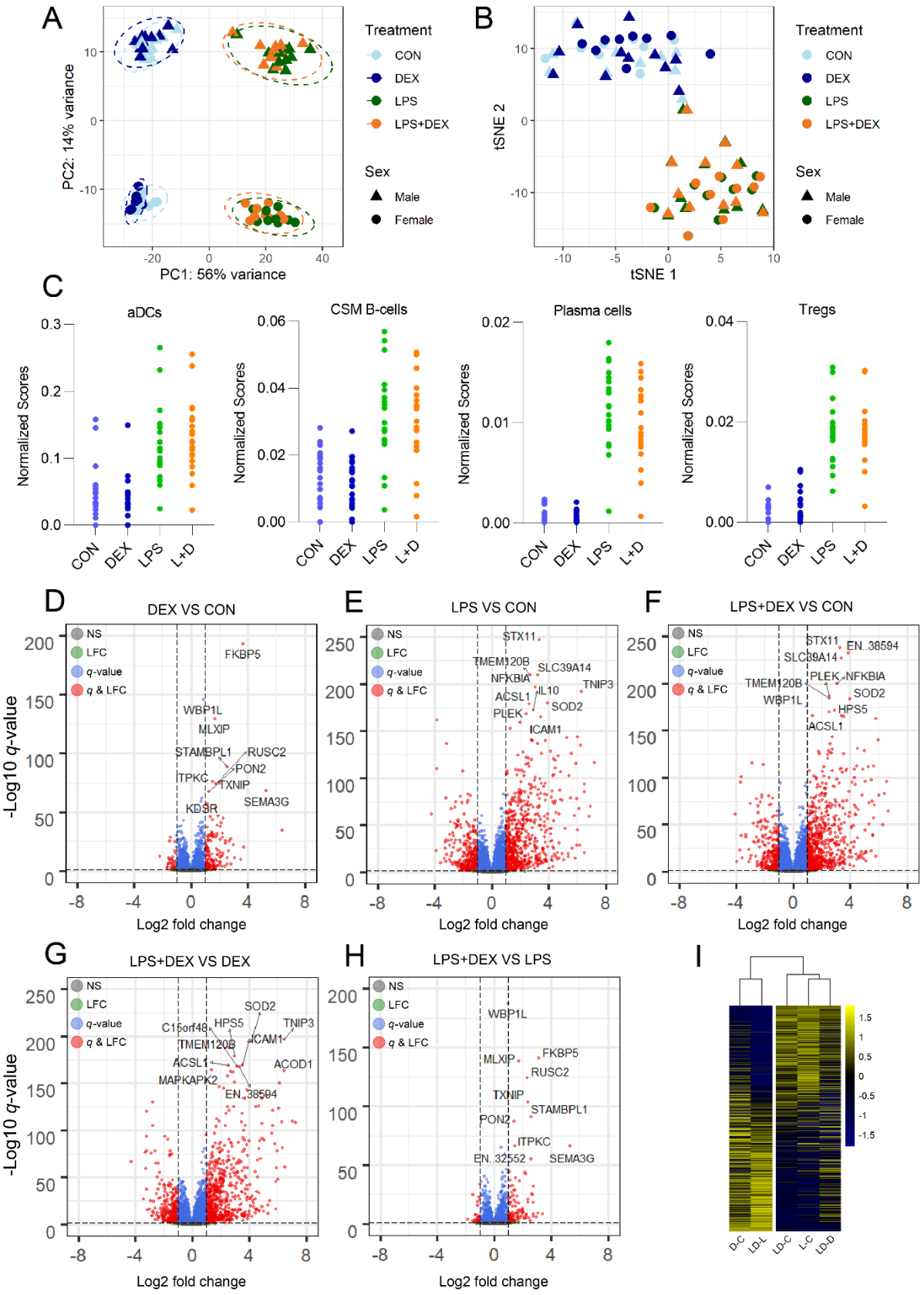


Figure 1. Differential expression analysis. (A) Principle component analysis of gene expression profiles using variance-stabilizing transformed (VST) counts of genes that passed filtering. (B) t-distributed stochastic neighbor embedding (t-SNE) plot of samples using enrichment scores

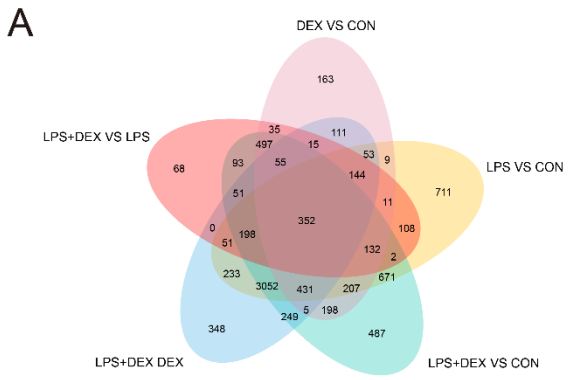
of 64 cell types generated by cell type enrichment analysis via xCell webtool. TPM (transcripts per kilobase million) of genes that passed filtering were used for cell type enrichment analysis. (C) Enrichment scores of four immune cell types generated by cell type enrichment analysis. (D-H) Volcano plots of pairwise comparisons DEX VS CON (D), LPS VS CON (E), LPS+DEX VS CON (F), LPS+DEX VS DEX (G), and LPS+DEX VS LPS (H). Top 10 most significant genes in each comparison were determined by *q*-value and highlighted in the plot. (I) Heatmap constructed using LFC of genes in matched comparisons. Values were centered in the row direction. Abbreviation: CON, control; DEX: dexamethasone; LPS, lipopolysaccharide; aDCs, activated dendritic cells; CSM B-cells, class-switched memory B cells; Tregs, regulatory T cells; EN..38594, ENSSSCG00000038594; EN..32552, ENSSSCG00000032552.

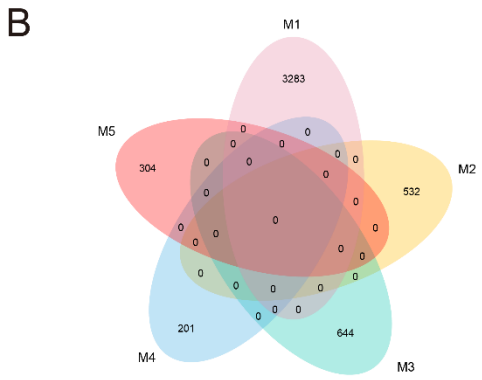
The five treatment comparisons (DEX VS CON, LPS VS CON, LPS+DEX VS CON, LPS+DEX VS DEX, and LPS+DEX VS LPS) yielded 2418 DEGs (1123 up- and 1295 downregulated), 6365 DEGs (3042 up- and 3323 downregulated), 6680 DEGs (3255 up- and 3425 downregulated), 5348 DEGs (2639 up- and 2709 downregulated), and 1812 DEGs (849 up- and 963 downregulated), respectively (Figure 1D-H; Table S1).

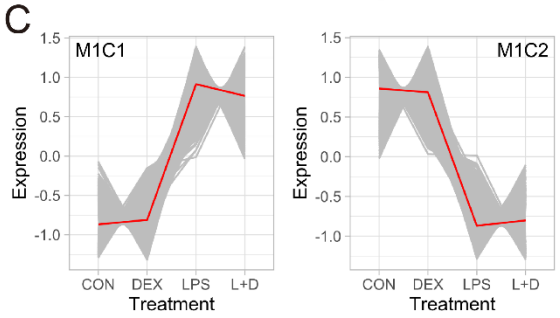
FKBP5, a co-chaperone of GR, showed the most potent responsiveness to DEX, whereas *STX11*, implicated in the transport of LPS-activated TLR4 (Toll-like receptor 4) to the plasma membrane, showed the most potent responsiveness to LPS (Figure 1D, E). Several negative regulators of inflammation, such as *TNIP3* (*ABIN3*), *NFKBIA*, *IL10*, *SOD2*, and *ACOD1*, were strongly upregulated by LPS (Figure 1E, G). Clustering based on the LFC of DEGs revealed approximately inverse directions of LPS and DEX effects (Figure 1I).

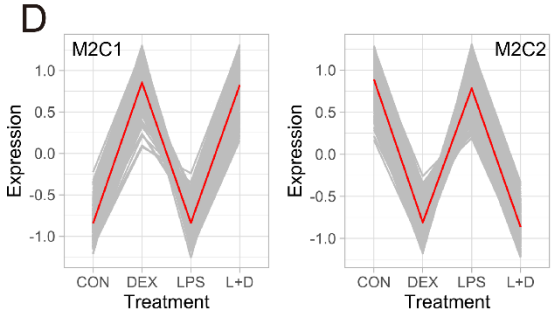
Distinct biological meanings of typical genes in functional modules

To study specific LPS and DEX functions in the context of their interplay, five functional modules (M1-M5; Table S2) with different response patterns were identified from DEGs that were significantly regulated by at least one stimulus. A total of 4966 genes, comprising almost all DEGs that were shared by all comparisons (350 out of 352, with the exception of *NIBAN2* and *TRIB3* without notable response patterns), could be assigned into a module (Figure 2A, B; Table S1).







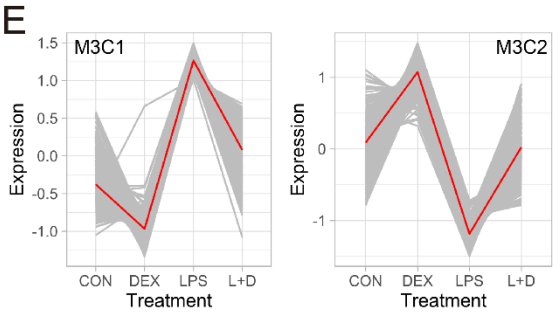


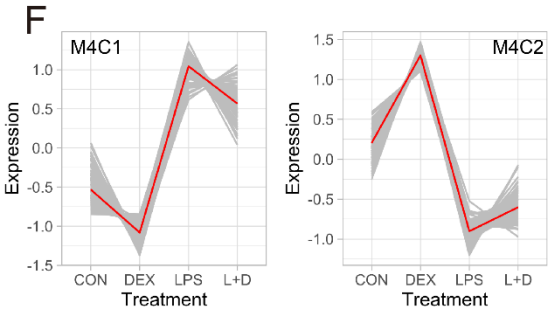
M1C1: CCL22, CCL5, CCR7, CD40, CSF2RB, CSF3, CXCL10, CXCL11 CXCL2, CXCL8, CXCL9, CXCR5, FASLG, GDF15, IFNAR1, IFNB1, IFNG, IFNGR2, IFNLR1, IL12B, IL15, IL19, IL21R, IL23A, IL23R, IL27 IL2RA, IL33, IL4R, IL7, MET, OSM, OSMR, RETN, TNFRSF1B, TNFSF13B, XCL1

M1C2: CCR2, CXCL14, LTBR, TGFBR1, TNFSF12, TNFSF13, XCR1

M2C1: BMF, CXCR4, FKBP5, HIP1, IL10RA, IL6ST, KIT, MEF2C, NET1, RORA, SLC9A1, VCL, ZBTB16

M2C2: CCR4, CCR6, CD40LG, DYRK2, IL16, IL6R, ING2, IRF2BP2, ITPR3, PDGFB, PLA2G4A, STAT6



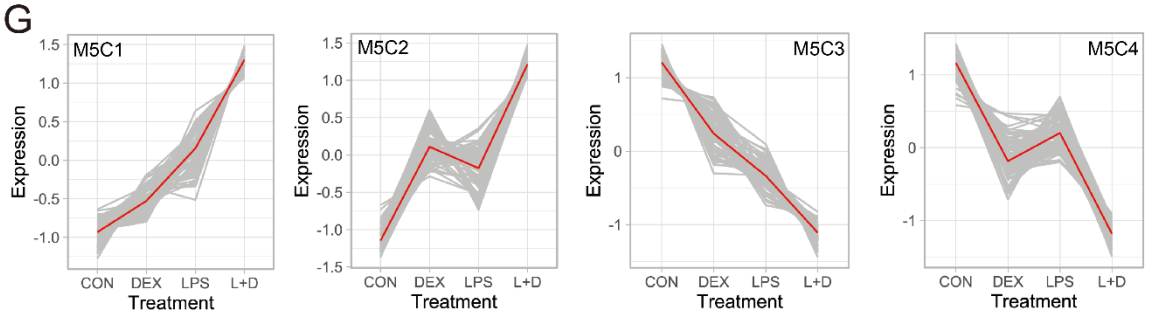


M3C1: ADM, BCL2L1, BCL3, BCL6, BIRC3, CASP7, CD38, CD47, CD48, CSF1, CSF2, EBI3, EZR, ID2, IER3, IL10, IL11, IL12A, IL6, ITGB1, LIF, LRR1, LTA, NUB1, PAFAH1B1, PDGFA, PTGS2, RACGAP1, RELB, RFX5, SMAD3, SMARCD2, TFRC, TGFB1, TGM2, TNFRSF4, TNFSF9, TRAF4, YWHAZ, ZFP36

M3C2: CCL14, CD163, DOK1, GADD45A, IL13RA1, IRS2, ITGB5, PATZ1, RFX2, THBD, TIMP4, TNFSF11, TSC22D3

M4C1: CCL2, CCL3L1, CCL4, CCL8, IL18, IL1A, JUNB, PHLDA1, RRAD, THBS1, TNF, TNFSF15, VEGFA

M4C2: FOXO3, HGF, RTN4



M5C1: GADD45B, IL10RB, PDGFRB, TNFSF8; **M5C2:** ATP2A2, BAG3, BTG1, CORO1C, DDIT4, DUSP1, IFNGR1, IL1R1, IL7R, PLAGL1, SGK1

M5C3: FLRT2, HES1, PLAU; **M5C4:** CMTM7, DDIT3, RARG, TGFBR2, TNFSF14

Figure 2. Functional modules and gene expression patterns. (A) Venn diagram illustrating unique and shared DEGs among five pairwise comparisons. (B) Venn diagram illustrating unique and shared DEGs among five functional modules. (C-G) K-means clustering of gene expression profiles of five functional modules M1 (C), M2 (D), M3 (E), M4 (F), and M5 (G). Cytokines, chemokines and their receptors (for M1-M5, identified using the ImmPort [24]) as well as GR targets implicated in immune responses (for M2-M5, in red, identified using the InnateDB [26], Harmonizome [25], and Ingenuity Pathway Analysis (IPA)) were indicated below corresponding modules.

The genes allocated to M1 (n = 3285) were significantly regulated by LPS but not by DEX. Inversely, genes in M2 (n = 532) were regulated by DEX but not by LPS. Genes in module M3 (n = 644) were oppositely regulated in LPS VS CON and LPS+DEX VS LPS, while the genes in M4 (n = 201) were regulated in the opposite direction by LPS (LPS VS CON and LPS+DEX VS DEX) and DEX VS CON. In addition, these genes showed no significant response in LPS+DEX VS LPS, which implies that DEX effect on their expression was blunted by the LPS co-treatment under the applied experimental conditions. In M5 (n = 304), it was found that the genes were affected by LPS and DEX in either an additive or synergistic way. In Figure 2 (C-G) functionally important members of the modules, including cytokines, chemokines, and their receptors (M1-M5), as well as the GR targets involved in immune responses (M2-M5) are displayed.

Besides a subset of pro-inflammatory cytokines and chemokines upregulated by LPS, module M1 also encompasses several positive regulators of immune response downregulated by LPS, including *CCR2*, *CXCL14*, *LTBR*, *TNFSF12*, *TNFSF13*, *XCR1*, *C5AR1*, *C5AR2*, *MAP3K3*, *MAP4K3*, *MAP4K5*, *FOS*, *IRF5*, *KLF6*, *TLR4*, *TLR5*, and *TLR8* (Figure 2C; Table S1). This finding suggests that parallel to the pro-inflammatory response LPS triggered a compensatory, homeostatic anti-inflammatory program. This proposition is corroborated by the upregulation of several negative regulators of immune response assigned to module M1, including *ANXA1*, *ANPEP*, *ACOD1*, *DUSP16*, *ETV3*, *IRF4*, *SOD2*, and *STAT3*, by LPS (Table S1).

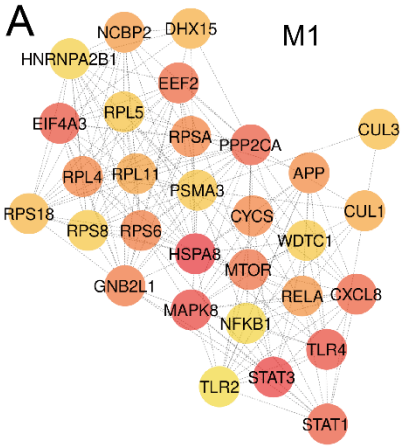
Module M2 comprised several immune genes, such as *IL16* and *CD40LG*, that were downregulated by DEX, but did not respond to LPS under our experimental condition (Figure 2D). The genes in module M3 characterize the anti-inflammatory function of DEX, which was indicated, for example, by the inhibition of pro-inflammatory *RELB* and *IL6* and upregulation of anti-inflammatory *ADORA3*, *CD163*, *DOK1*, and *TSC22D3* in LPS+DEX VS LPS (Figure 2E; Table S1). Unlike module M3, several cytokines, chemokines, and their mediators assigned

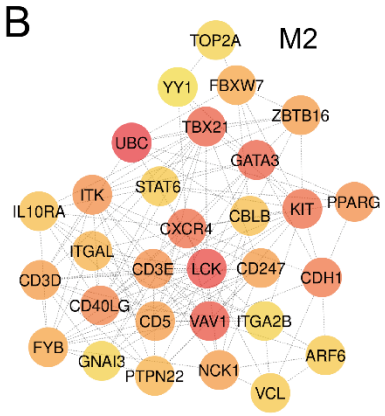
to module M4 were not efficiently regulated by DEX when co-treated with LPS; this included *TNF*, *IL1A*, *IL18*, *CCL2*, *CCL4*, *CCL8*, and *IRF3*. Thus, the genes in module M4 will allow a better understanding of the causes and consequences of reduced DEX sensitivity in the context of the pro-inflammatory response triggered by LPS (Figure 2F; Table S1).

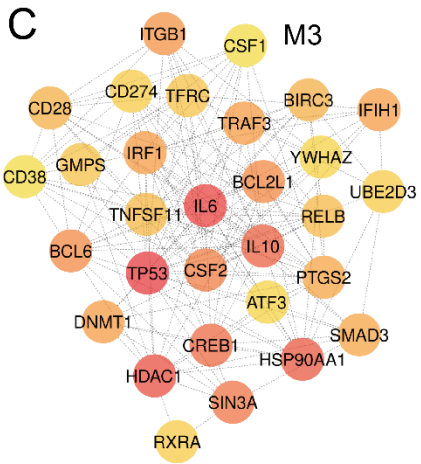
For the genes in M5 that showed an additive or synergistic effect of LPS and DEX, two main biological meanings can be deduced; firstly, these represent anti-inflammatory function of LPS as shown by the induction of the anti-inflammatory *TNFAIP3* and *DUSP1*, and inhibition of the pro-inflammatory *TNFSF14* (Figure 2G; Table S1); secondly, DEX also exhibits pro-inflammatory effects as evidenced by the activation of pro-inflammatory *IL1R1*, *IL1RAP*, *IRAK2*, *CD14*, *MYD88*, *CD80*, *TNFSF8*, *IL7R*, *JAK1*, and *STAT5B* and the inhibition of anti-inflammatory *NKIRAS1* and *NRROS* (Figure 2G; Table S1). These results will help to determine the priming effects of stress-induced GCs, which could subsequently enhance the vulnerability for subsequent inflammatory stimuli.

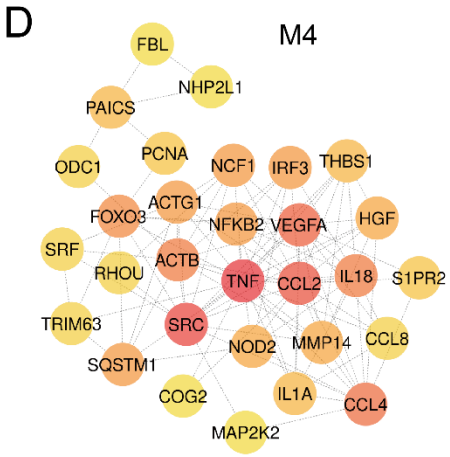
Typical genes of functional modules have high connectivity

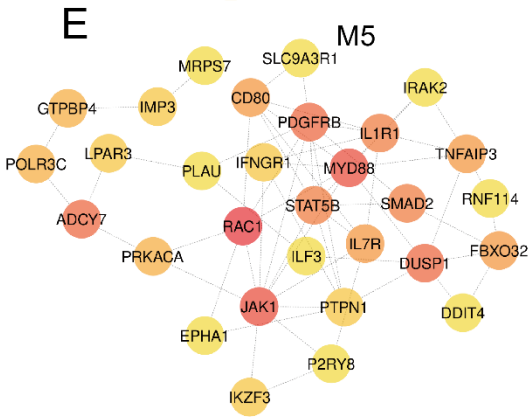
Protein–protein interaction networks were constructed within modules, which then allowed the identification of the top 30 hub genes that showed a high degree of connectivity (Figure 3). In line with LPS-induced inflammation, genes involved in NF-κB and MAPK cascades, such as *RELA*, *NFKB1*, *STAT1*, and *MAPK8*, were identified as hub genes in M1. Notably, the pleiotropic TF *STAT3* involved in the IL-10 mediated anti-inflammatory response was also included as a hub gene in M1 (Figure 3A). Functional annotation revealed that hub genes in this module were implicated in cellular responses to stress (Figure 3F). The most marked hub genes in M2 were associated with T cell functions, including *CD40LG*, *LCK*, *TBX21*, *GATA3*, *CD5*, *CD3D*, *CD3E*, and *CD247* (*CD3Z*) (Figure 3B; Figure 3F). In M3, DEX caused the downregulation of inflammation-related hub genes such as *RELB* and *IL6* (Figure 3C). Furthermore, *TNF*, *IL1A*, *IRF3*, *NFKB2*, and *MAP2K2* in M4 showed high connectivity, suggesting their fundamental role in counteracting the effects of DEX on pro-inflammatory responses (Figure 3D). Signaling by interleukins was enriched for both M3 and M4 (Figure 3F).





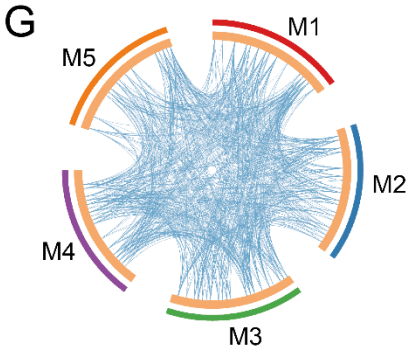






F

	Description	Count	Log10 (*q*)
M1	Cellular responses to stress	16	-14.72
M2	T cell activation	15	-14.09
M3	Signaling by interleukins	13	-10.94
M4	Signaling by interleukins	12	-9.61
M5	Regulation of innate immune response	9	-5.03



Description	Count	Log10 (*q*)
Signaling by interleukins	51	-47.95
Leukocyte differentiation	43	-33.47
Positive regulation of cytokine production	37	-28.53
Immune response-regulating signaling pathway	38	-23.67
Positive regulation of cell death	38	-22.66

Figure 3. Identification and annotation of hub genes of functional modules. (A-E) Protein–protein interaction (PPI) networks of top 30 hub genes with high connectivity within modules M1 (A), M2 (B), M3 (C), M4 (D), and M5 (E). PPI networks were constructed by the STRING resource within modules [27] and top 30 central elements of each network were identified and visualized by the Cytoscape plugin cytoHubba [28, 29]. Connectivity was correlated with color of circles where red indicates higher degree and yellow indicates lower degree. (F) The most significant biological function of hub genes of each module. (G) Overlap of functions of hub genes of different modules. The blue lines in the Circos plot linked genes annotated by the same functional term. Top five functional terms that were significantly enriched for all modules are shown on the right panel. For (F) and (G), annotation was performed with ontology sources GO Biological Processes and Reactome Gene Sets using the Metascape [30].

The hub genes in M5 evidence the previously discussed biological meanings of the additive or synergistic effects. This is shown by the involvement of *DUSP1* and *TNFAIP3* in the LPS-induced anti-inflammatory response and by *IL1R1*, *IRAK2*, *MYD88*, *CD80*, *IL7R*, *JAK1*, and *STAT5B* in the DEX-induced pro-inflammatory response (Figure 3E). These genes were enriched for functions related to regulation of innate immune response (Figure 3F). Certain functional themes were found to be enriched for the hub genes of all modules, such as signaling by interleukins and leukocyte differentiation (Figure 3G).

Canonical pathways, biological consequences, and upstream regulators of functional modules

The module M1 presents the activation of a series of pathways involved in the initiation, signal transduction, and effector stages of inflammation. This was consistent with the LPS-induced activation of the inflammatory response shown in the LPS VS CON comparison (Figure 4A; Table S3). Activation of the IRF and NF-κB signaling pathways suggested that both MYD88-dependent and -independent signaling were triggered by LPS. Activation of necroptosis signaling was also identified, which is in line with the upregulation of its key mediator *MLKL*. The substantial activation of immune responses by the identified genes was supported by the enrichment of GO terms linked with the regulation of the innate immune response (Table S4). In addition, a set of pathways involved in cytoskeletal reorganization were activated in M1 (Figure 4A). This is consistent with the predicted activation of IPA terms related to proliferation, maturation, survival, and viability of leukocytes (Figure 5A; Table S5). Despite the dramatic activation, rare pathways were inhibited in M1 such as anti-inflammatory PPAR signaling (Figure 4A). These results indicate a predominantly pro-inflammatory state in porcine

PBMCs triggered by LPS application. In M1, the predicted activation of upstream pro-inflammatory TFs by IPA, such as *IRF7*, *STAT1*, *NFKB1*, and *RELA*, corresponds to the observed upregulation of their expression (Figure 6A). Furthermore, several negative regulators of the immune response, such as *STAT3* and *NFKBIA* (z-score = 1.855) were predicted or tended to be activated for M1. This corresponded with their increased expression following LPS treatment, although *NFKBIA* itself was not assigned a module (Table S1; Table S6).

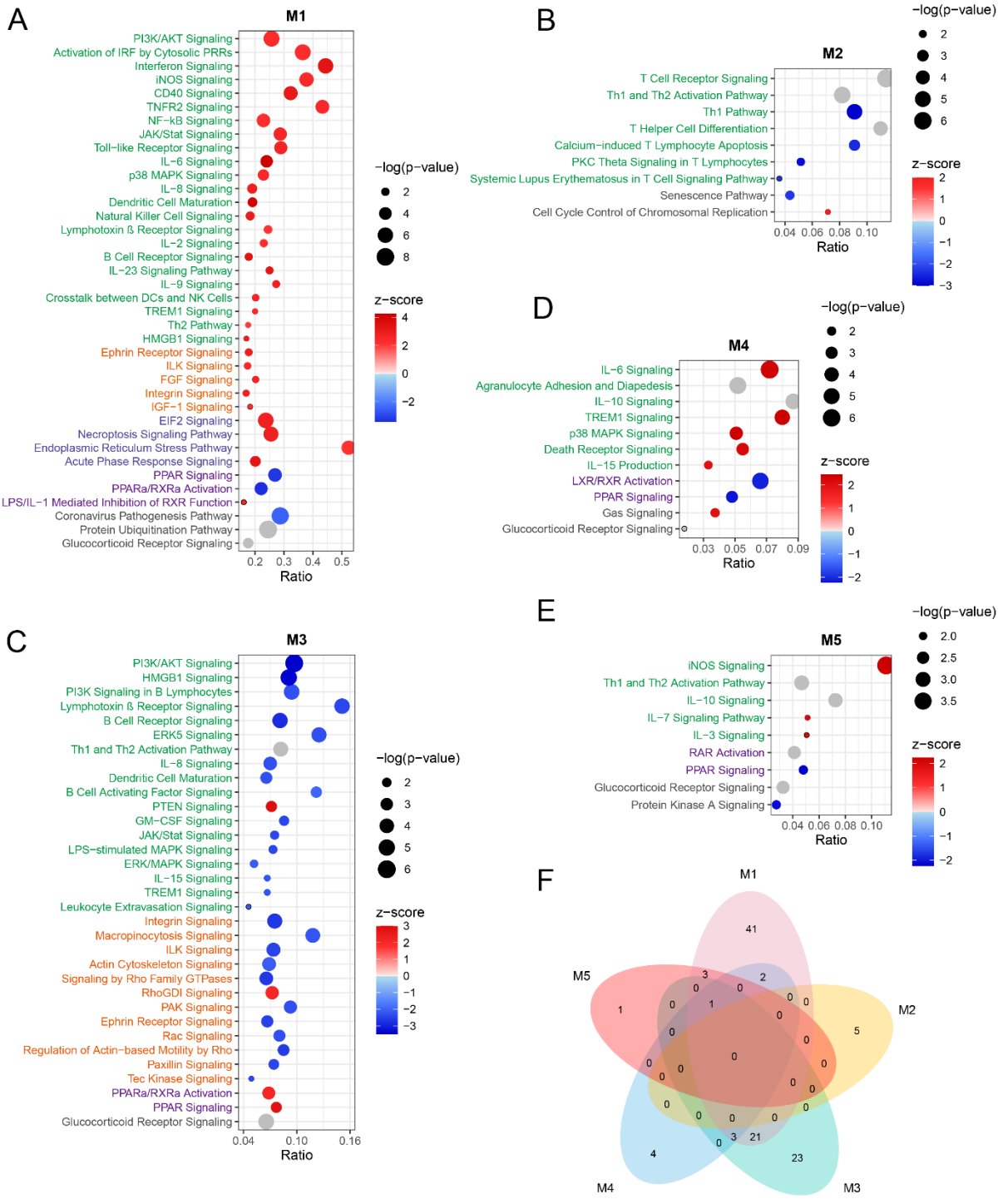


Figure 4. Canonical pathways enriched for functional modules. (A-E) Bubble diagram illustrating representative canonical pathways for modules M1 (A), M2 (B), M3 (C), M4 (D), and M5 (E). Enrichment was carried out with the IPA. The significance of terms was correlated with bubble size where large size indicates smaller *p*-values and all shown terms had *p*-values < 0.05. Enrichment z-scores were indicated by color of bubbles where red indicates z-score > 0 and blue indicates z-score < 0. Terms with unavailable z-scores were indicated in gray. The name of terms belonging to different categories was indicated in different color where green indicates immune response, orange indicates cytoskeleton and cell motility, blue indicates stress response and necroptosis, and purple indicates PPAR-related signaling. (F) Venn diagram illustrating unique and shared canonical pathways among five modules. Only terms with absolute z-scores ≥ 2 were used for the diagram.

The most notable insight from M2 (DEX VS CON) was the inhibition of T cell signaling in response to DEX (Figure 4B; Figure 5B). *CD40*, a key mediator conveying signals between T cells and other immune cells was predicted to be inhibited in M2 (Figure 6B). This is matched by the reduced expression of its ligand *CD40LG* (Figure 2D). As the M2 genes failed to respond to LPS in this particular study, these results suggest a constitutive inhibition of T cell function by DEX.

Many pathways and predicted upstream regulatory events enriched for M3 showed opposite directions compared with M1. This was seen for the inhibition of B cell receptor signaling (Figure 4C) and for the upstream regulator NF-κB (Figure 6C). The inhibition of B cell signaling was supported by the predicted inhibition of the biological consequence proliferation of B lymphocytes (Figure 5C). In module M3, DEX inhibited a set of pathways associated with cytoskeletal remodeling, suggesting that DEX might directly influence cytoskeleton-mediated immune cell function (e.g. phagocytosis and trafficking [32]) (Figure 4C). Furthermore, M3 highlighted the role of Rho family of GTPases, a type of well-known molecular switches, in controlling inflammation caused by DEX (Figure 4C). These results are supported by the enrichment of GO terms related to small GTPase mediated signal transduction and actin cytoskeleton organization (Table S4).

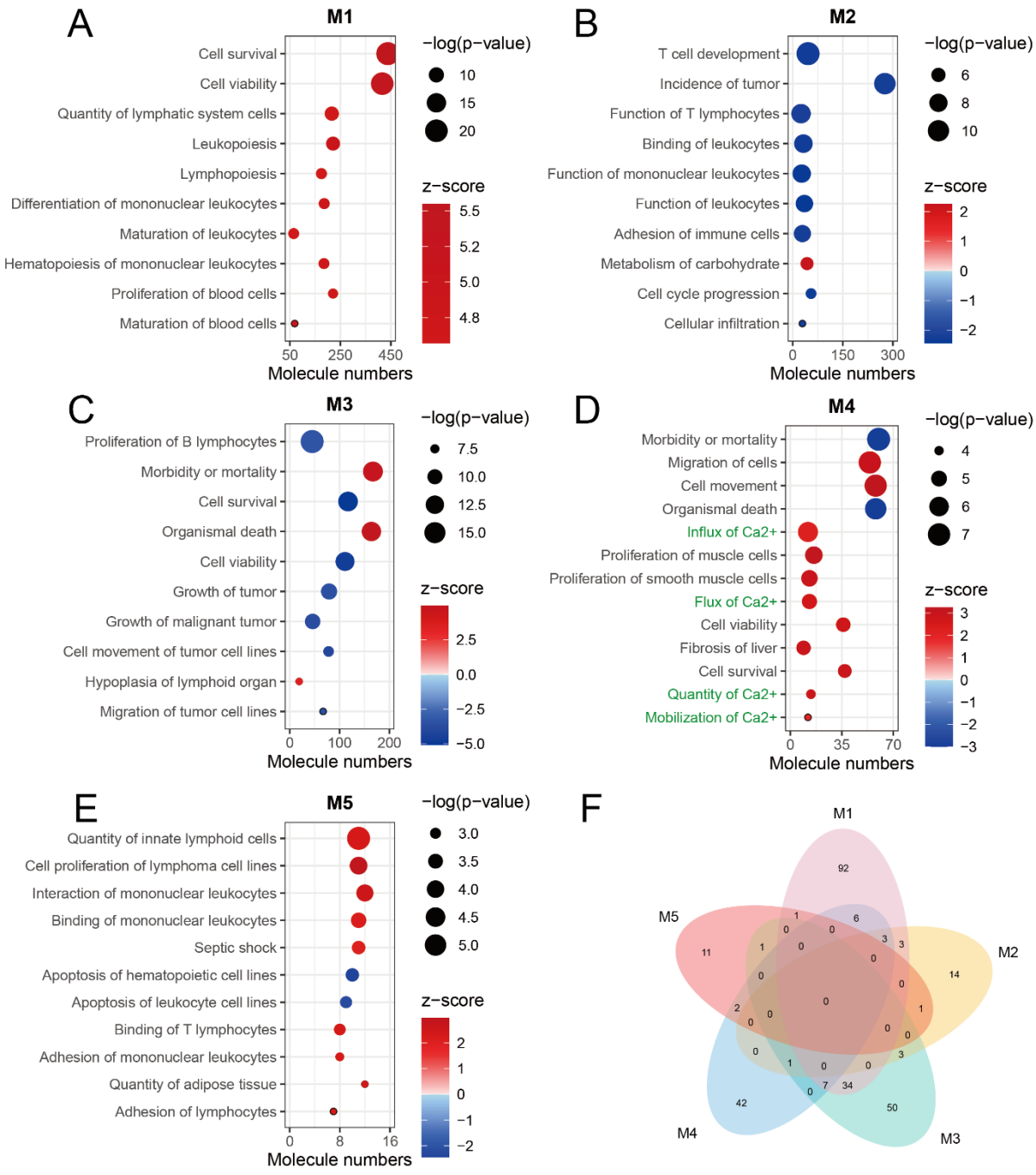


Figure 5. Biological consequences of functional modules. (A-E) Bubble diagram illustrating representative diseases or biological functions for modules M1 (A), M2 (B), M3 (C), M4 (D), and M5 (E). Enrichment was carried out with the IPA. The significance of terms was correlated with bubble size where large size indicates smaller *p*-values and all shown terms had *p*-values < 0.05. Enrichment z-scores were indicated by color of bubbles where red indicates z-score > 0 and blue indicates z-score < 0. Calcium-related terms in (D) were indicated in green. (F) Venn diagram illustrating unique and shared terms among five modules. Only terms with absolute z-scores ≥ 2 were used for the diagram.

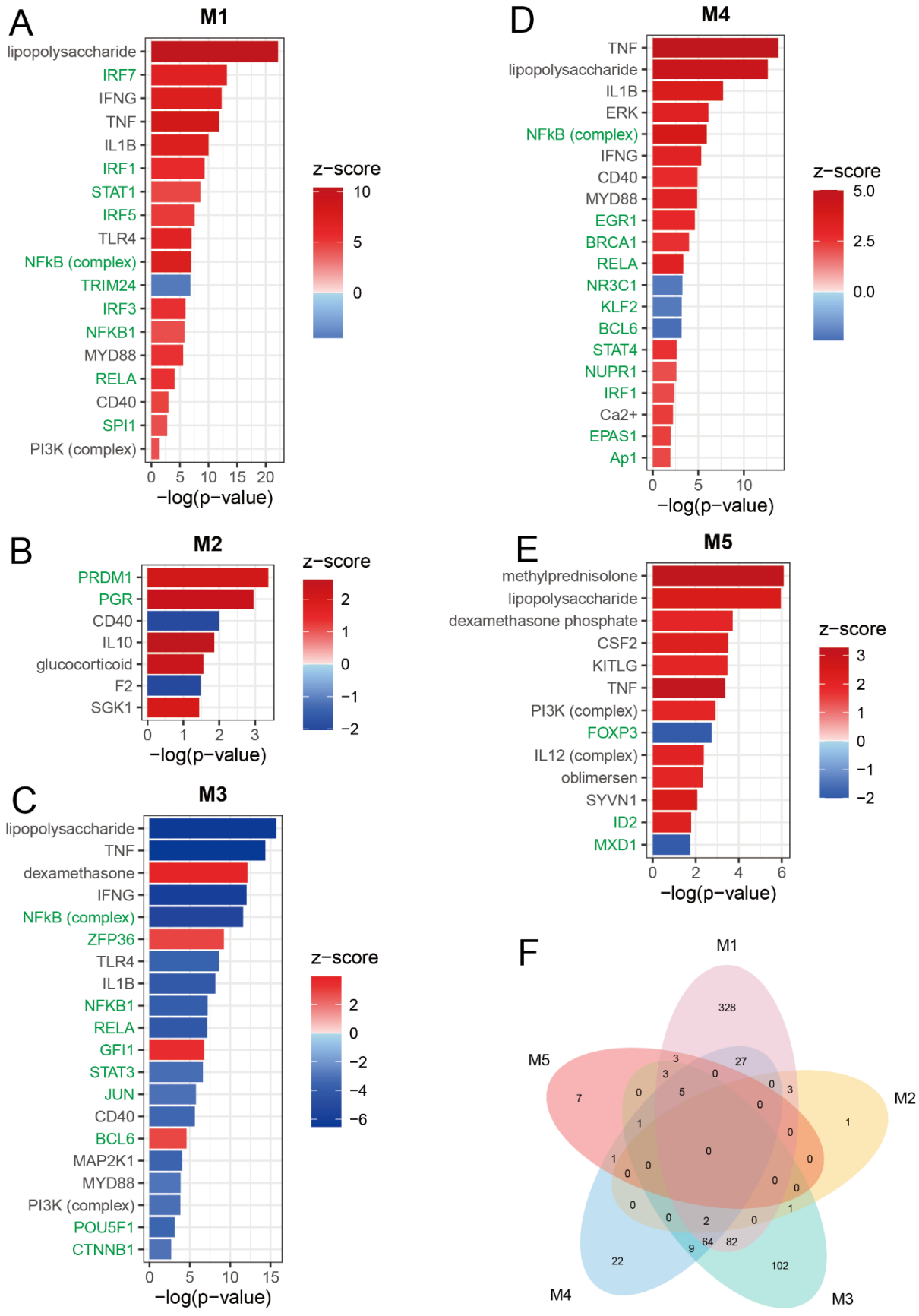


Figure 6. Potential upstream regulators enriched for functional modules. (A-E) Bar diagram illustrating representative upstream regulators for modules M1 (A), M2 (B), M3 (C), M4 (D),

and M5 (E). Enrichment was carried out with the IPA. Enrichment z-scores were indicated by color of bars where red indicates z-score > 0 and blue indicates z-score < 0. The name of transcription factors (TFs) was indicated in green. For M2, M4, and M5, all predicted upstream TFs were shown in the figure and for M1 and M3, only top 10 upstream TFs with high absolute z-scores were shown due to the large number of predicted terms. (F) Venn diagram illustrating unique and shared upstream regulators among five modules. Only terms with absolute z-scores ≥ 2 were used for the diagram.

The enrichment of p38 MAPK signaling in M4 (LPS+DEX VS DEX) was found by both IPA and GO analyses (Figure 4D; Table S4). Several biological consequences related to the influx of Ca^{2+} were predicted to be activated exclusively in M4 (Figure 5D, F; Figure S1A). These predictions were supported by the upregulation of *ORAI1* and the predicted activation of Ca^{2+} as an upstream regulator (Table S1; Figure 6D). *TNF* has been identified as the most prominent upstream regulator for M4, which corresponds with its upregulation and assignment to this module. *KLF2* was predicted to be inhibited, however it did not show a clear direction for any other modules (Figure 6D, F). *KLF2* is a GC-inducible anti-inflammatory TF that can reduce the LPS-stimulated cytokine production by inhibiting the transcriptional activity of NF-κB and AP-1 [33]. However, in our study, *KLF2* itself did not respond to DEX (Table S1). This observation, together with predicted inhibition of *KLF2* in M4, implies that the inability of DEX to activate *KLF2*-mediated transcriptional response contributes to the weakened DEX-responsiveness of a subset of genes.

In M5, strong activation of the iNOS signaling was predicted (Figure 4E). Cytokine-induced iNOS promotes the pathogenesis of septic shock due to excessive production of NO [34]. In combination with the predicted activation of septic shock (enriched only for M5), these results suggest that DEX-mediated pro-inflammatory action might result in severe pathological consequences, such as sepsis (Figure 5E, F; Figure S1B). In line with the downregulation of *PRKACA* that encodes the catalytic subunit α of protein kinase A (PKA), PKA signaling was predicted to be inhibited for M5 (Figure 4E). It could block NF-κB transcription via interaction with p65 and could potentially improve GR function in both ligand-dependent and -independent manners [5]. Thus, it was suggested that the pro-inflammatory action of DEX is linked to the impaired inhibition of NF-κB signaling and the alteration of GR function. This hypothesis was supported by the inhibition of *NKIRAS1* and by the enrichment of GR signaling (Table S1; Figure 4E). The pro-inflammatory effect of DEX could also be implicated in the dysfunction of Tregs since *FOXP3*, a critical TF controlling the development and function of Tregs [35]

was predicted to be inhibited (Figure 6E). However, in our study *FOXP3* itself was not regulated by the treatment at the transcriptional level. In addition, we observed that many pathways were shared by more than one module, in particular, PPAR signaling, which was enriched for all modules except M2 (Figure 4F).

Discussion

In this study, we identified extensive transcriptional responses as a result of both LPS and DEX applications. Based on these response patterns, five functional modules were established. Two major findings emerged from bioinformatic analysis of the modules; firstly, although LPS triggered predominantly pro-inflammatory responses, it concurrently induced an anti-inflammatory response. This study clearly depicted this anti-inflammatory feedback through a subset of LPS-repressed pro-inflammatory genes involved in cytokine and chemokine activities (*CCR2*, *CXCL14*, *LTBR*, *TNFSF12*, *TNFSF13*, and *XCR1*), complement system (*C5AR1* and *C5AR2*), TLR signaling (*TLR4*, *TLR5*, and *TLR8*), MAPK cascades (*MAP3K3*, *MAP4K3*, and *MAP4K5*), and regulation at the transcriptional level (*FOS*, *IRF5*, and *KLF6*). The downregulation of *TLR*s by LPS is described in a previous expression array study, where stimulation of porcine PBMCs by LPS for 24 hours represses expression of *TLR6* and *TLR8* [36]. In our study the expression of *TLR6* was not changed by LPS. Unlike the above *TLR*s, *TLR2* was upregulated by LPS and identified as a hub gene in M1. *TLR2* is a target of TF *RUNX1* [37], and the latter was also upregulated by LPS in our study. *RUNX1* is highly expressed in porcine PBMCs and is crucial for T and B cell development and activation [37]. Three *RUNX1* targets, including *TLR2*, *LCK*, and *VAV1*, were upregulated by LPS in porcine PBMCs after treatment for 6 hours [37]. Unlike *TLR2*, in our study *LCK* and *VAV1* were allocated to M2C2; they were repressed by DEX but did not respond to LPS under the applied experimental conditions.

Furthermore, this response comprised a series of LPS-induced anti-inflammatory molecules; in M1 this included *ANXA1*, *ANPEP*, *ACOD1*, *DUSP16*, *ETV3*, *IRF4*, *SOD2*, and *STAT3*, and outside of M1 it included *TNIP3*, *TNFAIP3*, *NFKBIA*, *IL10*, and *DUSP1*. Many of these genes suggest a negative regulation of the NF-κB cascade. *TNIP3* binds to TNFAIP3, a dual-function ubiquitin-editing enzyme, and inhibits NF-κB activation induced by TNF and IL-1 [38]. TNFAIP3 suppresses NF-κB activity through the removal of Lys-63-linked ubiquitin chains from, and/or adding degradation-inducing Lys-48 ubiquitin chains, to protein kinase RIPK1 upstream of IKK activation [39]. Upregulation of *ANXA1* and *NFKBIA*, negative regulators of NF-κB [2], provides additional evidence for the inhibition of NF-κB signaling. LPS-induced

upregulation of *SOD2* has been detected previously in porcine PBMCs [36]. *SOD2* belongs to the superoxide dismutase family that can inhibit lipid peroxidation-based release of inflammatory mediators such as prostaglandins, thromboxanes, and leukotrienes [40]. *SOD2* also hampers NF-κB activity and reduces TNF-α and IL-1β levels in LPS-activated microglia [41]. In addition to reducing the expression of positive mediators in MAPK signaling, LPS further upregulated the dual-specificity phosphatase 1 (*DUSP1*), which could inhibit inflammation through dephosphorylation and subsequent inactivation of MAPKs [2].

LPS-initiated immunosuppression might also be involved in the activation of IL-10/STAT3 signaling. IL-10 is a prominent anti-inflammatory cytokine with the ability to repress several LPS-inducible genes and antigen-presenting markers [42]. IL-10 stimulates activation of STAT3, which is necessary for the IL-10-mediated anti-inflammatory functions [43]. IL-10 can also promote the TLR-induced expression of *ZFP36* to reduce the mRNA stability of cytokines such as IL-1β and TNF-α; this is achieved by targeting AU-rich elements in the 3′ untranslated region [44]. The destabilization of mRNA by *ZFP36* could be enhanced by *DUSP1* via dephosphorylation of p38 MAPK. In turn, *DUSP1* could be induced by IL-10 [44]. Thus, the observed enhanced expression of *IL10*, *STAT3*, *DUSP1*, and *ZFP36* by LPS clearly supported the onset of this anti-inflammatory program. Furthermore, several negative mediators of the immune response downstream of the IL-10/STAT3 signaling were upregulated by LPS, such as *DUSP16* and *ETV3* [44].

An additional key theme in this study was the crosstalk between components of immune responses and GR signaling. This was shown through DEX-mediated constitutive inhibition of T cell signaling, DEX-mediated inhibition of inflammation, LPS interfering with the anti-inflammatory action of DEX, and DEX-mediated pro-inflammatory action.

Although GCs influence almost all immune cell types [45], genes that were shown to be significantly regulated by DEX but showed a lack of responsiveness to LPS were correlated with the inhibition of T cell functions; this was evidenced by canonical pathways and biological consequences enriched in M2. Accordingly, several hub genes involved in T cell functions were downregulated by DEX in M2, including *CD247*, *CD3D*, *CD3E*, *CD40LG*, *LCK*, and *TBX21*. Specifically, *CD247*, *CD3D*, and *CD3E* constitute part of the T cell receptor (TCR)-CD3 complex, which plays a vital role in the recognition of signals from antigen-presenting cells (APCs) [46]. The CD3 chains all possess immunoreceptor tyrosine-based activation motifs (ITAMs) that can be phosphorylated by *LCK*, a member of the Src family of protein tyrosine kinases. Consequently, this activates immune signaling [46]. Thus, inhibition of CD3 molecules

and *LCK* suggests DEX-mediated constitutive inhibition of TCR/CD3 signaling. The inhibition of T cell and APC engagement by DEX was corroborated by the downregulation of *CD40LG*. The T cell expression of *CD40LG* mediates immune responses by interacting with *CD40* expressed on APCs and B cells [47]. Therefore, inhibition of *CD40LG* may block signal transduction between T cells and other immune cells. *TBX21* is a lineage-specific TF expressed by Th1 cells; it was found to be downregulated by DEX in the current study. The inhibition of *TBX21* function by GCs occurs due to a reduction in mRNA and protein levels, but it is also a result of diminished binding of *TBX21* to DNA [45].

DEX-mediated inhibition of inflammation was evident in this study through the inhibition of pro-inflammatory genes, alongside increased expression of anti-inflammatory genes including *TSC22D3*, *ADORA3*, *CD163*, and *DOK1* [2]. *TSC22D3*, also known as *GILZ*, inhibits PI3K/AKT and MEK/ERK signaling through Ras and Raf-1 interactions [2]. Therefore, the upregulation of *TSC22D3* is consistent with significant inhibition of PI3K/AKT and ERK signaling as predicted by IPA. *TSC22D3* also inhibits NF-κB and AP-1 through the interplay with p65, and c-Fos and c-Jun subunits, respectively [2]. This is supported by the predicted inhibition of upstream regulators NF-κB and JUN in this study. *ADORA3* is another GC-dependent anti-inflammatory gene with possible PI3K and NF-κB interactions [48]. It is highly expressed in immune cells, including PBMCs; this distinguishes it as an important therapeutic target for many immune diseases [48]. Moreover, *DOK1*, an inhibitory adaptor protein with the ability to suppress MAPK cascades, was upregulated by DEX [49]. *CD163* exerts anti-inflammatory action by eliminating hemoglobin-haptoglobin complexes [50]. These findings demonstrate the fundamental role of DEX-inducible genes in terms of the anti-inflammatory action of DEX in porcine PBMCs.

We observed a distinct attenuation of the DEX-mediated anti-inflammatory effect due to LPS, which parallels the upregulation of *TNF*. Pretreatment with TNF-α was shown to reduce DEX-mediated inhibition of IL-6 in human whole-blood cell cultures [51]. TNF-α can impair GR-mediated transcriptional regulation by reducing the accessibility of the transcriptional cofactor p300 to GR; this was conveyed by NF-κB via sequestration of p300 [4]. The acetyltransferase activity of p300 is indispensable for GR-mediated transcription [52]. In addition, p300 can also serve as a scaffold for the recruitment of other cofactors involved in GR functions [53]. Access of GR to p300 facilitates the interaction between GR and the transcription initiation complex and ensures the transduction of GC signal to RNA polymerase II [54]. The cytokine *IL1A* may also counteract the DEX anti-inflammatory action, which could inhibit DEX-induced GR

translocation from cytoplasm to nucleus. Consequently, there would be decreased transcriptional activity of GR, paralleled by p38 MAPK activation [5]. Different from "master" cytokines *TNF* and *IL1A* initiating the earliest inflammatory response, the upregulation of "secondary" cytokines such as *IL6* and *IL10* by LPS was effectively inhibited by DEX in the present study [55].

LPS recognition by TLR4 activates two distinct downstream pathways, MYD88-dependent signaling which results in the activation of NF-κB and MAPK, and MYD88-independent signaling, which results in the activation of interferon regulatory factors *IRF3* and *IRF7* [56]. *IRF3*, showing high connectivity in M4, could potentially compete with GR for binding to its dual-function coregulator *GRIP1* [57]. This could result in decreased accessibility of *GRIP1* to GR. Certain p160 family members, such as *SRC1* and *RAC3*, act only as coactivators, while *GRIP1* also potentiates GR-mediated repression [58]. A previous study found that deficiency in *GRIP1* enhanced LPS-induced inflammation and reduced GR-mediated repression of NF-κB signaling in mice [59]. Moreover, activation domains (AD1 and AD2) of *GRIP1* have been shown to contribute to the recruitment of p300 [58]. These results suggest that the activation of MYD88-independent TLR4 signaling could possibly impair GRIP1-dependent GR-mediated anti-inflammatory action.

The results of this study indicated the potential involvement of Ca^{2+} signaling in the attenuation of the anti-inflammatory effect of DEX by LPS. This is supported for instance by the observed upregulation of *ORAI1* by LPS. ORAI1 is a subunit of the Ca^{2+} release-activated Ca^{2+} channel (CRAC) that plays a key role in the store-operated Ca^{2+} entry (SOCE) in T cells [60]. The induction of genes involved in Ca^{2+} pathways by LPS has been observed in porcine PBMCs [36]. However, the role of Ca^{2+} in the LPS-induced GC resistance has yet to be established. We hypothesize a mechanism involving reduced p300 availability caused by the activation of cyclic adenosine monophosphate (cAMP) signaling by Ca^{2+} and *ORAI1* [60]. Previous research has shown that activation of cAMP signaling can promote the degradation of p300 in human lung cancer cells [61]. On the other hand, Ca^{2+} positively regulates LPS-induced inflammation in macrophages in a dose-dependent manner, alongside ERK1/2 signaling activation [62]. Thus, Ca^{2+} signaling could also potentially promote LPS-induced diminishment of DEX effects, by regulating cytokine production. The direction of the regulation of genes in M4 meant that Ca^{2+} signaling was inhibited for DEX VS CON but not for LPS+DEX VS LPS. Essentially, DEX can reduce intracellular Ca^{2+} levels through a non-genomic action [63]. Thus, the enrichment

of Ca^{2+} signaling in M4 implies possible impairment of DEX-mediated non-genomic effects caused by LPS.

GCs are typically described as anti-inflammatory agents, but emerging evidence suggested that they also exert pro-inflammatory effects. However, the mechanisms involved are not yet well understood [64]. In this study, we identified a series of molecules regulated by DEX that are likely to be responsible for its pro-inflammatory actions. These molecules indicated activation of TLR, NF-κB, iNOS, and IL-1 signaling. These included for instance *CD14* and *MYD88*, which play a vital role in the recognition of LPS and activation of TLR4 signaling. *CD14* binds to the LPS-binding protein (LBP)/LPS aggregate and facilitates the LPS transfer to MD2/TLR4 complex. As a result, MYD88-dependent signaling and NF-κB cascade are activated [65]. The involvement of NF-κB was supported by the reduced expression of its inhibitors *NKIRAS1* and *NRROS*. *NKIRAS1* negatively regulates NF-κB activity by preventing degradation of IκBβ, the inhibitory IκB protein [66]. In turn, *NRROS* inhibits NF-κB activation mediated by TLR4 [67]. Furthermore, the nitric oxide synthase iNOS is inducible by inflammatory stimuli, which promotes NO production and NOX-mediated ROS generation to benefit the host defense [68]. Excessive NO production downstream of cytokine-induced iNOS promotes the pathogenesis of septic shock [34], which corresponds with the observed upregulation of *CD80* [69] by DEX in this study, and the predicted activation of septic shock for M5. In addition, the pro-inflammatory effect of DEX was supported by the upregulation of *IL1R1*, *IL1RAP*, and *IRAK2*, three key subunits of a functional complex essential for IL-1 signaling [70].

In this study, blood samples used to isolate PBMCs were collected from pigs at exsanguination after electrical stunning. It has been reported that *in vitro* bovine IFN-γ response to tuberculosis antigen can be inhibited when collecting blood at exsanguination after electrical stunning [71]. Nevertheless, here in porcine PBMCs, both *IFNG* expression and interferon signaling were highly activated by LPS. It should also be noted that frozen rather than fresh PBMCs were used in this study. Despite the wide application of frozen PBMCs in immune research [72, 73], the potential influence of cryopreservation should be considered when interpreting results of the current study.

Overall, our study provides a comprehensive overview of the responses triggered by LPS and DEX exposure in porcine PBMCs. Here, we show a novel analysis of the crosstalk between GR signaling and inflammatory pathways on a genome-wide scale in pigs. Regarding the mechanisms of pro-inflammatory pathways counteracting GR signaling, and the priming effects of DEX on pro-inflammatory genes, our findings have important implications for advancing

animal health and progressing the application of GC-based drugs. We also derived novel hypotheses based on this study, such as the role of calcium signaling in the impairment of DEX functions, which deserve further investigation in the future.

Data Availability Statement

The RNA-Seq dataset was submitted to the ArrayExpress repository (https://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-9808) at EMBL-EBI.

Supplementary materials

Supplementary materials can be found at https://doi.org/10.6084/m9.figshare.14138213.v1.

Author contributions

EM: Conceptualization and Funding acquisition. EM, ZL, NT: Methodology. ZL: Investigation and Formal analysis. NT, FH: Data Curation. FH: Software. SP: Resources. KW: Supervision. ZL, EM: Writing - Original Draft. All authors contributed to manuscript revision, read, and approved the submitted version.

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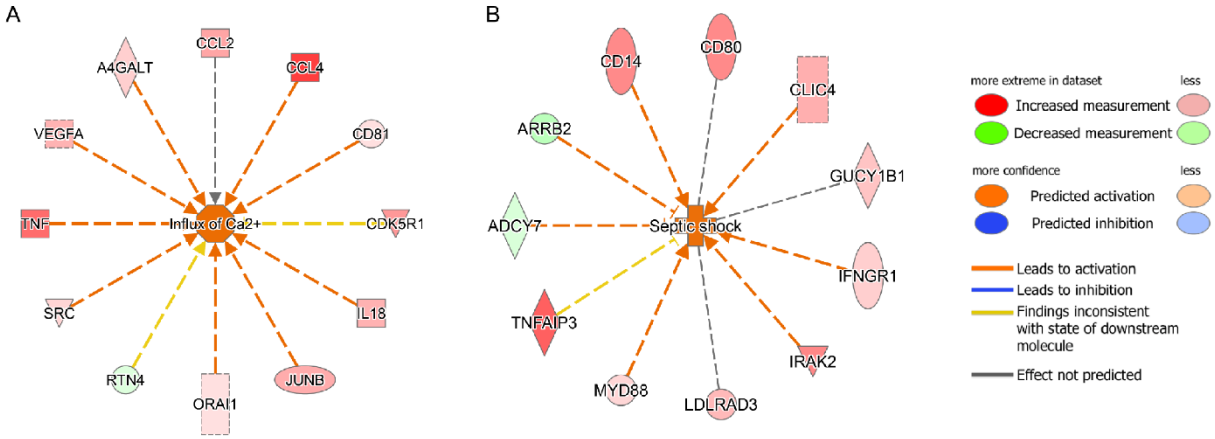

Figure S1. Representative biological consequences for modules M4 and M5. (A) Influx of Ca^{2+} was predicted to be activated only for module M4. (B) Septic shock was predicted to be activated only for module M5.

Table S1. Differential gene expression in treatment comparisons and assignment into functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

Table S2. Criteria for defining the five functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

Table S3. Canonical pathways enriched for functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

Table S4. GO biological processes enriched for functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

Table S5. Diseases and biological functions enriched for functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

Table S6. Upstream regulators enriched for functional modules. (https://doi.org/10.6084/m9.figshare.14138213.v1)

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Manuscript 3

A natural Ala610Val substitution causing glucocorticoid receptor hypersensitivity aggravates consequences of endotoxemia

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A natural Ala610Val substitution causing glucocorticoid receptor hypersensitivity aggravates consequences of endotoxemia

Zhiwei Li[1], Ellen Kanitz[2], Margret Tuchscherer[2], Armin Tuchscherer[3], Cornelia C. Metges[4], Nares Trakooljul[1], Klaus Wimmers[1], Eduard Murani[1,*]

[1] Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[2] Institute of Behavioural Physiology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[3] Institute of Genetics and Biometry, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

[4] Institute of Nutritional Physiology "Oskar Kellner", Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany

***** Corresponding author.

Abstract

Despite the crucial role of glucocorticoid receptor (GR) in proper immune responses, the effect of GR hypersensitivity on inflammation is rarely reported. To fill this knowledge gap, we exploited the natural gain-of-function substitution in the porcine glucocorticoid receptor ($GR_{Ala610Val}$) and challenged pigs carrying normal or hypersensitive GR using 50 µg/kg lipopolysaccharide (LPS) following pretreatment with either saline or single bolus of 60 µg/kg dexamethasone (DEX). The $GR_{Ala610Val}$ substitution reduced baseline cortisol, adrenocorticotropic hormone (ACTH), and triglyceride concentration and granulocyte proportion whereas baseline platelet counts were elevated. Val-carriers, i.e. AlaVal as well as ValVal pigs, showed less LPS-induced cortisol rise but the cortisol fold change was similar in all genotypes. Differently, ACTH response to LPS was most significant in $GR_{Ala610Val}$ heterozygotes (AlaVal). LPS-induced disorders, including sickness behaviors, anorexia, thrombocytopenia, cytokine production, and metabolic alterations were more intense in Val-carriers. On the other hand, Val-carriers were more sensitive to DEX effect than wild types (AlaAla) during endotoxemia, but not under unchallenged conditions. This is the first report

revealing aggravated responses to endotoxemia by GR gain-of-function. Together, these results imply that GR hypersensitivity is difficult to diagnose but may represent a risk factor for endotoxemia and sepsis.

Keywords: lipopolysaccharide; endotoxemia; HPA axis; dexamethasone; platelets.

Introduction

Glucocorticoids are a class of steroid hormones that play a key role in the adaptive response to stress including regulation of the immune system and metabolic homeostasis [1]. They are also widely used drugs for the treatment of numerous diseases, such as allergy, inflammation, and cancer [2]. Glucocorticoids exert their anti-inflammatory function by binding to glucocorticoid receptor (GR), a ligand-activated transcription factor belonging to the nuclear receptor superfamily [2].

The important role of GR in the control of inflammation was revealed by both gain-of-function and loss-of-function studies in mice. In these studies, on the one hand, deficiency in GR activity or dimerization aggravated lipopolysaccharide (LPS)-induced inflammation [3, 4]; on the other hand, artificial upregulation of GR expression in transgenic mice with increased GR gene dosage (YGR mice) or natural increase in GR expression and transactivation in SPRET/EI mice, blunted inflammatory response to LPS [5, 6]. Nevertheless, the effect of GR hypersensitivity on inflammation and on glucocorticoid therapy during inflammation is rarely reported in humans.

LPS-induced endotoxemia is commonly used to model systemic inflammation, sepsis, and cross-talk between the immune system and the hypothalamic-pituitary-adrenal (HPA) axis [7]. Previous studies revealed that mice poorly mimic human responses to endotoxemia, sepsis, and glucocorticoids [8-10]. Pigs are more suitable than rodents for the study of the crosstalk between immune and neuroendocrine systems not only because pigs possess high anatomical and physiological similarities to humans but also pigs and humans produce the same endogenous glucocorticoid, i.e. cortisol, with a similar circadian rhythm [11]. We previously described a unique natural Ala610Val substitution ($GR_{Ala610Val}$) in the GR ligand-binding domain in pigs caused by single nucleotide variant c.1829C>T [12], which induces GR hypersensitivity and compensatory reduction in HPA axis activity [13, 14]. Given that the effect of $GR_{Ala610Val}$ substitution is evident mainly within the limits of HPA axis and essentially without detectable alterations - and thus interference, in external phenotypic traits (viability, growth, and body composition) in a natural setting [14], it is a promising model to study the impact of GR

hypersensitivity on HPA axis regulation under stressed conditions and on stress-induced physiological responses. Moreover, although the hypersensitivity of $GR_{Ala610Val}$ is balanced, at least partly, by the regulation within GR signaling and HPA axis under unchallenged conditions [15], the direction towards which this balance will shift in response to stress (e.g. infection-induced inflammation) and to exogenous glucocorticoids, and how this shift affects host physiology needs to be investigated.

We thus aimed to determine the effect of the $GR_{Ala610Val}$ substitution on adaptive responses and on GR-mediated feedback regulation by exogenous glucocorticoids under stressed conditions modeled by LPS application. The overarching goal of the study was to provide insights into the consequences of genetically-based GR hypersensitivity for systemic inflammation and on glucocorticoid therapy. To this end, pigs with different GR genotypes were equally divided into two groups and pretreated with saline (SAL) and dexamethasone (DEX) respectively. Three hours later all pigs were challenged by LPS and monitored until 24 h post LPS administration. During the 27 h period, feed intake, sickness behaviors, together with parameters involved in neuroendocrine, inflammatory, metabolic, and hematological responses were measured in time budgets. Our results demonstrate for the first time that GR gain-of-function contributes to aggravated endotoxin-induced disorders.

Materials and methods

Animals, treatment, and sampling

Pigs used in this study were born and raised at the experimental pig farm of the Leibniz Institute for Farm Animal Biology (FBN) (Dummerstorf, Germany). Animal experiments were carried out under the European Union and German animal protection laws with the approval of the Animal Care Committee of the Leibniz Institute for Farm Animal Biology and the State Mecklenburg-Western Pomerania (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TSD/7221.3-1-024/16).

A total of 96 seven-week-old purebred German Landrace pigs including equal numbers of males and females for each of the three GR genotypes (wild types (AlaAla), $GR_{Ala610Val}$ heterozygotes (AlaVal), and $GR_{Ala610Val}$ homozygotes (ValVal)) were employed. The average weight of the pigs was 13.5 ± 0.3 kg. Their health status (general condition, lethargy, refusal to eat, consistency of feces, skin discoloration, coughing, lameness, swelling on the body or joints, and abnormal behavior) was visually examined by trained personnel before the start of the experiment.

The experiment was conducted in eight batches of 12 individuals each, as previously described with minor modifications [16]. In each replicate, pigs were randomly assigned into two groups (DEX group and SAL group), balanced for sex and GR genotypes within each group. Two days prior to the experiment, the animals were placed into individual pens (1.90 × 1.10 m^2) with *ad libitum* access to feed and water. One day before the experiment the animals were weighed to adjust the challenge doses to weight and the baseline feed intake was recorded. All experiments started at around 8:00 am to avoid variation due to circadian rhythms. At the beginning of the experiment (T_0-3 h), the DEX group received an intramuscular bolus of 60 µg/kg DEX sodium phosphate (Dexatat, aniMedica, Senden, Germany) and the saline group received a corresponding volume of 0.9% saline (sterile, endotoxin-free). Three hours later (T_0) 50 µg/kg LPS (LPS from Escherichia coli O111: B4; potency > 600000 EU/mg; Sigma-Aldrich, Taufkirchen, Germany) was administered intraperitoneally to all pigs. Blood samples (10 ml) were collected via rapid (≤30 s) anterior vena cava puncture at T_0-3 h, T_0, T_0+1 h, T_0+3 h, T_0+6 h, and T_0+24 h and transferred into pre-chilled EDTA tubes. At the same timepoints, rectal temperature was measured to evaluate the febrile response. Following the pretreatment feed was withdrawn and resupplied at T_0+6 h. Feed intake in response to LPS challenge was recorded at T_0+24 h. The schematic diagram of the experimental design was shown in Figure S1.

Genotyping of $GR_{Ala610Val}$

Genotype of $GR_{Ala610Val}$ was determined using custom designed KASP assay (Kompetitive allele specific PCR; LGC Genomics, Hoddesdon, United Kingdom) as previously described [15].

Measurement of blood parameters

Plasma samples were obtained by centrifugation of the blood samples at 4 °C, 2000 × g for 20 min and were stored at -80 °C for further analysis. Plasma cortisol (intra- and inter-assay CVs were 7.01% and 7.74% respectively) and adrenocorticotropic hormone (ACTH) (intra- and inter-assay CVs were 2.92% and 8.79% respectively) were measured using commercial ELISA kits (DRG Instruments GmbH, Marburg, Germany) following the manufacturer's instruction. Applicability of the kits was validated in our lab previously [14]. Plasma concentration of inflammatory cytokines including interleukin (IL)-10 (intra- and inter-assay CVs were 7.36% and 9.55% respectively), IL-1β (intra- and inter-assay CVs were 6.25% and 8.96% respectively), IL-6 (intra- and inter-assay CVs were 6.97% and 11.13% respectively), and tumor necrosis factor (TNF)-α (intra- and inter-assay CVs were 7.23% and 8.58% respectively) was measured

by MAGPIX® instrument (Merck, Darmstadt, Germany) using porcine cytokine magnetic bead panel kits (Merck, Darmstadt, Germany). Plasma metabolites including blood urea nitrogen (BUN), glucose, and triglycerides were assessed by Fuji DriChem 4000i clinical chemistry analyzer (Scil, Viernheim, Germany). Plasma concentration of free fatty acids (FFA) was measured by an enzymatic-spectrophotometric assay using the ABX Pentra 400 instrument (Horiba Medical, Montpellier, France). Hematological parameters including red blood cells (RBCs), leukocytes, lymphocytes, and platelets were counted in the whole EDTA blood using ABX Pentra 60 instrument (Axon Lab, Reichenbach, Germany).

Observation of sickness behaviors

The observation of sickness behavior was carried out using scan sampling during 1st, 2nd, 3rd, 4th, and 24th hours after LPS administration with a five-minute interval as previously described [17]. For all individuals, frequencies of symptom occurrence including activity, cyanosis, diarrhea, inactivity, panting, shivering, and vomiting were recorded. In detail, activity refers to walking and employment with pen equipment; inactivity refers to lying, sitting, and standing without movement; panting refers to respiratory difficulty; shivering refers to lying on the floor or standing and displaying rapid, synchronous muscle contractions, which is frequently accompanied by piloerection. A professional who was blinded to genotype and treatment of the experimental animals was responsible for performing all observations.

Statistical analysis

Data analysis was performed using SAS/STAT 9.4 (SAS Inc., Cary, NC, USA). Before analysis, distribution tests were carried out for all parameters. Data on inflammatory cytokines were log-transformed to ensure normal residuals. The principal component analysis (PCA) was performed using the FactoMineR package and visualized using the factoextra package in R software (version 3.6.1). Other data were visualized by GraphPad Prism 8.2.1 (GraphPad Software, Inc., San Diego, CA).

For feed intake, ANOVA implemented in the mixed procedure was applied. Fixed effects of genotype, sex, batch, and for feed intake after LPS challenge treatment and genotype × treatment interaction were included in the model. Body weight was included as covariate. Least-squares means (LSM) and standard errors (SE) of the genotype × treatment effect were calculated and compared within genotype and treatment, respectively, using the slice option.

For blood parameters, repeated measures ANOVA implemented in the mixed procedure was applied. To analyze the effect of $GR_{Ala610Val}$ substitution on baseline blood parameters, fixed effects of genotype, sex, and batch were included in the model. To analyze the effect of $GR_{Ala610Val}$ substitution on the magnitude of the responses to treatment, fixed effects of genotype, sex, batch, treatment × timepoint, and genotype × treatment × timepoint were included in the model. LSM and SE were computed for the genotype × treatment × timepoint interaction and compared within genotype × timepoint and treatment × timepoint, respectively, using the slice option. To analyze the effect of $GR_{Ala610Val}$ substitution on reactivity to treatment, the fold change of blood parameters relative to their baseline (T_0-3 h) was calculated for each of the remaining time points. The effect of genotype at each respective time point was analyzed using a model fitting fixed effects of genotype, sex, treatment, batch, and genotype × treatment interaction. LSM and SE for the genotype × treatment interaction were compared within genotype and treatment, respectively, using the slice option.

Data on sickness behaviors were analyzed by fitting a Gaussian model implemented in the mixed procedure. The model included the fixed effects of genotype, treatment, sex, batch, time (1st h, 2nd h, 3rd h, 4th h, and 24th h after LPS administration, respectively), and their interactions. LSM and SE were computed for the genotype × treatment × time interaction and compared within genotype × time and treatment × time, respectively, using the slice option. Tukey-Kramer adjustment was applied within slices. For all statistical tests the significance threshold was set at $p < 0.05$.

Results

The $GR_{Ala610Val}$ substitution affects several baseline blood parameters

The impact of $GR_{Ala610Val}$ on baseline concentration of examined parameters was analyzed in the whole cohort (n = 96) and summarized in Table S1. As expected, the $GR_{Ala610Val}$ substitution reduced baseline cortisol (genotype $p = 0.006$) and ACTH (genotype $p = 0.040$) concentration (Figure 1A, B), which confirms our previous finding that the Val variant induces a compensatory reduction in HPA axis activity. Platelet counts (genotype $p = 0.009$) were significantly increased and granulocyte proportion (genotype $p = 0.045$) was significantly reduced by $GR_{Ala610Val}$ (Figure 1C, D), but other baseline hematological, immune parameters, including inflammatory cytokines and numbers of leukocytes and lymphocytes, were not affected by the substitution (Table S1). Despite the crucial role of glucocorticoids in glucose metabolism and protein turnover, the $GR_{Ala610Val}$ substitution did not affect baseline glucose or

BUN concentration (Table S1). The $GR_{Ala610Val}$ substitution significantly reduced baseline triglyceride concentration (genotype $p = 0.016$; Figure 1E) but did not affect baseline FFA concentration (Table S1). The substitution showed no effect on feed intake (Figure 1F) and rectal temperature (Table S1) before challenge.

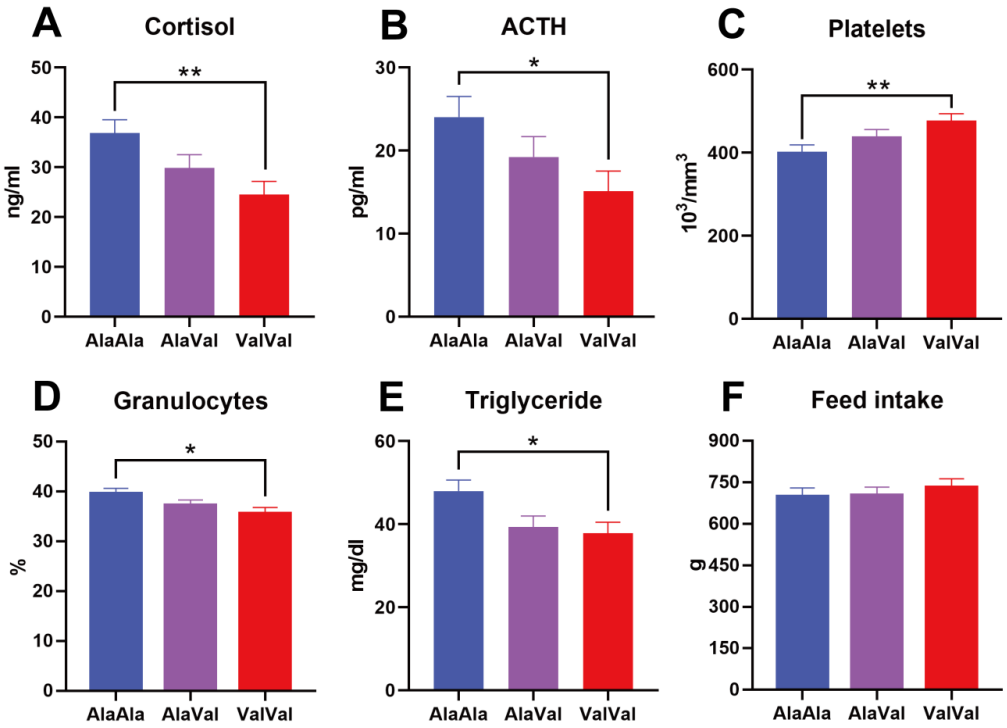

Figure 1. Effect of the $GR_{Ala610Val}$ substitution on baseline parameters. (A) Cortisol. (B) Adrenocorticotropic hormone (ACTH). (C) Platelets. (D) Granulocytes. (E) Triglyceride. (F) Feed intake. Data are presented as least-squares means ± standard errors. * $p < 0.05$, ** $p < 0.01$ (n = 32). GR, glucocorticoid receptor; AlaAla, wild types; AlaVal, $GR_{Ala610Val}$ heterozygotes; ValVal, $GR_{Ala610Val}$ homozygotes.

Enhanced responses to endotoxemia caused by $GR_{Ala610Val}$

The impact of $GR_{Ala610Val}$ on physiological and behavioural responses to LPS was examined in the saline pretreated group (n = 48). The results are summarized in Figures 2-4 and Tables S2-S6. The overview of the impact of fixed effects on blood parameters is shown in Table S2. Genotype ($p < 0.001$) and genotype × treatment × timepoint interaction ($p = 0.001$) significantly influenced cortisol concentration during the experiment.

The whole data of all measured parameters during the experimental period are summarized in Table S3. Figure 2 shows LPS-induced responses for parameters that were significantly affected by the $GR_{Ala610Val}$ during endotoxemia (cortisol, ACTH, platelets, and triglycerides). In addition,

responses that showed significant genotype differences when comparing treatments within genotype (IL-6, TNF-α, leukocytes, granulocytes, panting, vomiting, and BUN) as well as parameters that were tightly associated with aforementioned parameters (glucose and FFA) or showed distinct responding curves across genotypes (IL-1β and rectal temperature) are displayed. Some of the responses presented here show notable differences between genotypes, but did not reach statistical significance. In the following, these were referred throughout to as tendency, without application of a specific cut-off for the *p*-values.

Similar to the differences in baseline concentration, also during LPS challenge Val-carriers (AlaVal and ValVal) showed less cortisol rise compared to wild types (AlaAla) (T_0+24 h; Figure 2A). LPS-induced ACTH response was different from cortisol; AlaVal pigs tended to exhibit the highest ACTH response to LPS (Figure 2B).

LPS triggered production of pro-inflammatory cytokines, i.e. IL-1β, IL-6, and TNF-α, in all genotypes with a tendency towards greater concentration in Val-carriers (Figure 2C-E). With regards to blood cell counts, platelets showed the most notable genotype differences. In spite of a higher setpoint at baseline, platelet counts in Val-carriers dropped more sharply during LPS challenge, particularly at 3 h post LPS application (T_0+3 h) (Figure 2F). Also leukocyte numbers tended to decrease to a greater extent in response to LPS in Val-carriers until 6 h post application (T_0+6 h) (Figure 2G). The greater granulocyte proportion in wild type pigs tended to persist until T_0+3 h, but at T_0+6 h, when LPS induced the most distinct change in granulocyte proportion (i.e. increase), the genotype differences disappeared (Figure 2H). There was no clear tendency for the genotype effect on RBC-related parameters (Table S3). At 24 h post LPS application (T_0+24 h) cytokine concentrations and leukocytes largely returned to baseline, but platelets and RBC-related parameters remained below baseline with no obvious genotype differences in general (Figure 2; Table S3). Val-carriers tended to show stronger LPS-induced febrile response, particularly at T_0+6 h, but the genotype effect did not reach statistical significance (Figure 2I). Several sickness behaviors, including cyanosis, diarrhea, inactivity, panting, shivering, and vomiting, occurred in response to LPS (Table S4). Val-carriers tended to exhibit more frequent panting as compared with wild types (Figure 2J) and ValVal pigs tended to exhibit more frequent vomiting as compared with others (Figure 2K). There was no clear tendency for the genotype effect on other LPS-induced sickness behaviors (Table S5).

Feed consumption recorded until T_0+24 h decreased in response to endotoxemia, and tended to be reduced in ValVal pigs compared to other genotypes (Figure 3). In addition, BUN concentrations which gradually increased towards T_0+24 h, exhibited the greatest concentration

at T_0+24 h in ValVal pigs (Figure 2L). Similar to the effect at baseline, genotype had no obvious effect on glucose during LPS challenge (Figure 2N). FFA concentration peaked at T_0+6 h, where ValVal pigs tended to show the smallest concentration compared to other genotypes (Figure 2M). In contrast, triglycerides were affected by the $GR_{Ala610Val}$ substitution not only under unchallenged conditions but also during LPS challenge; at T_0+3 h there was a surge whereby ValVal pigs reached the greatest concentration, as opposed to their lowest baseline triglyceride concentration (Figure 2O).

For parameters exhibiting significant genotype differences already at baseline we calculated fold changes relative to baselines to evaluate the genotype effect on responsiveness to LPS. An overview of the impact of fixed effects on the fold change of blood parameters is shown in Table S6. During LPS challenge, genotype significantly influenced triglyceride fold change at T_0+3 h (p = 0.030) and platelet fold change at T_0+6 h (p = 0.048). Genotype × treatment interaction significantly influenced ACTH (p = 0.043) and platelet (p = 0.040) fold changes at T_0+3 h. Different from the absolute concentration, all genotypes showed a very similar cortisol fold change course in response to LPS (Figure 4A). This implies similar responsiveness at the adrenal level, with the glucocorticoid output fine-tuned to sensitivity of the respective allelic GR variant. For ACTH (Figure 4B), granulocytes (Figure 4D), and triglycerides (Figure 4E), similar patterns as described above for the analysis of absolute changes were observed. Notably, the LPS-induced relative changes in platelet counts revealed more pronounced genotype differences compared to absolute changes. With each copy of the Val variant the LPS-induced thrombocytopenia was incrementally aggravated (Figure 4C).

Taken together these results suggest enhanced responses to endotoxemia in Val-carriers. This conclusion is supported by the PCA; 95% confidence ellipses for the barycentres before and after LPS application are more widely separated for Val-carriers as compared to wild types, indicating LPS had more pronounced effects on Val-carriers (Figure S2).

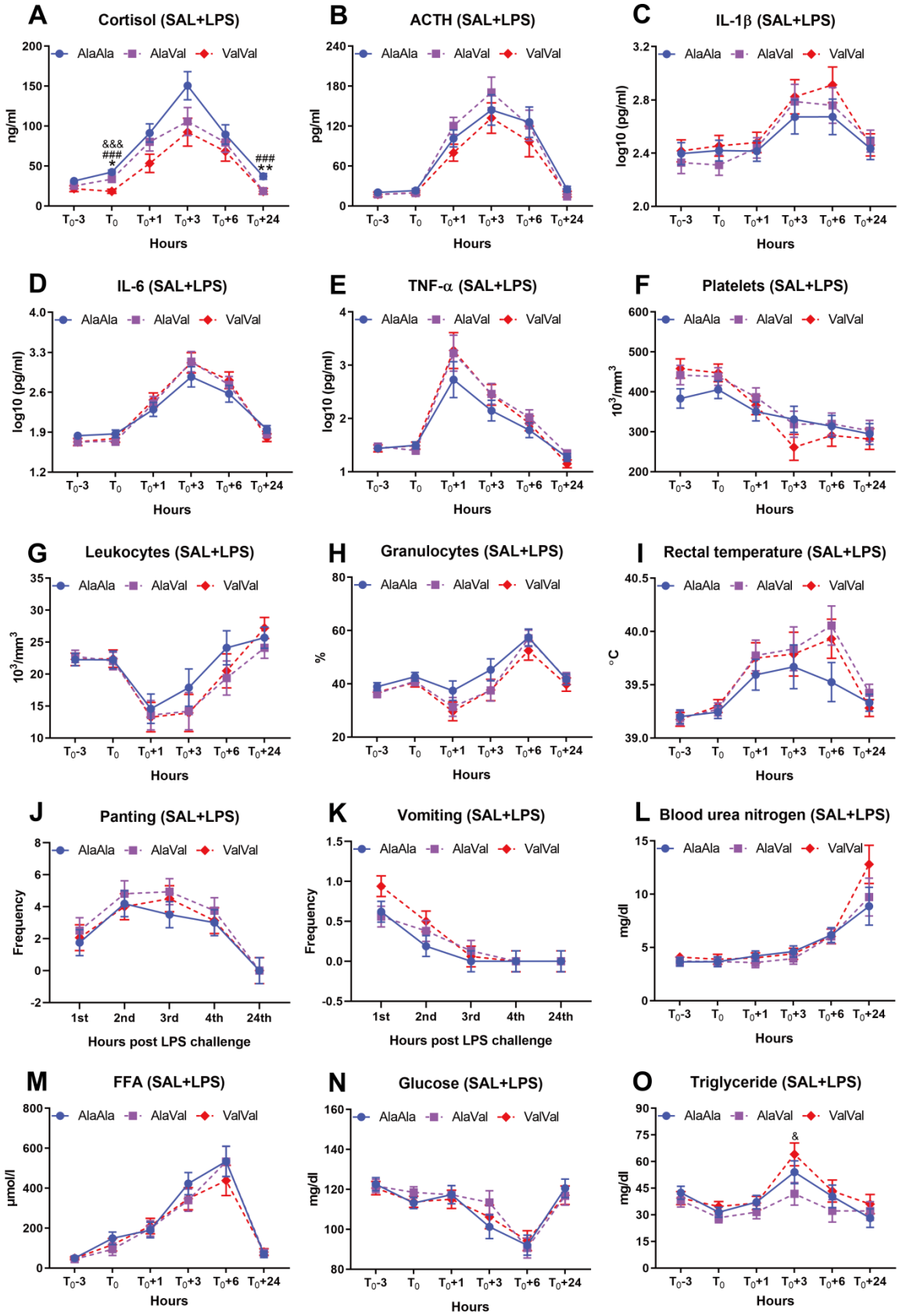

Figure 2. Effect of the $GR_{Ala610Val}$ substitution on the absolute level of physiological and behavioral parameters during lipopolysaccharide (LPS) challenge. (A) Cortisol. (B) ACTH. (C)

Interleukin (IL)-1β. (D) IL-6. (E) Tumor necrosis factor (TNF)-α. (F) Platelets. (G) Leukocytes. (H) Granulocytes. (I) Rectal temperature. (J) Panting. (K) Vomiting. (L) Blood urea nitrogen (BUN). (M) Free fatty acids (FFA). (N) Glucose. (O) Triglyceride. Data are presented as least-squares means ± standard errors. * indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ heterozygotes (AlaVal); # indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ homozygotes (ValVal); & indicates the significance between $GR_{Ala610Val}$ heterozygotes (AlaVal) and $GR_{Ala610Val}$ homozygotes (ValVal); one, two, and three symbols indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively (n = 16). SAL, saline.

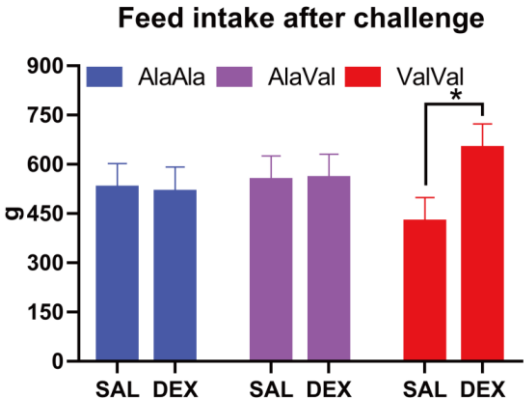

Figure 3. Feed intake of pigs carrying different $GR_{Ala610Val}$ substitution after LPS challenge with or without dexamethasone (DEX) pretreatment. Data are presented as least-squares means ± standard errors. * $p < 0.05$ (n = 16).

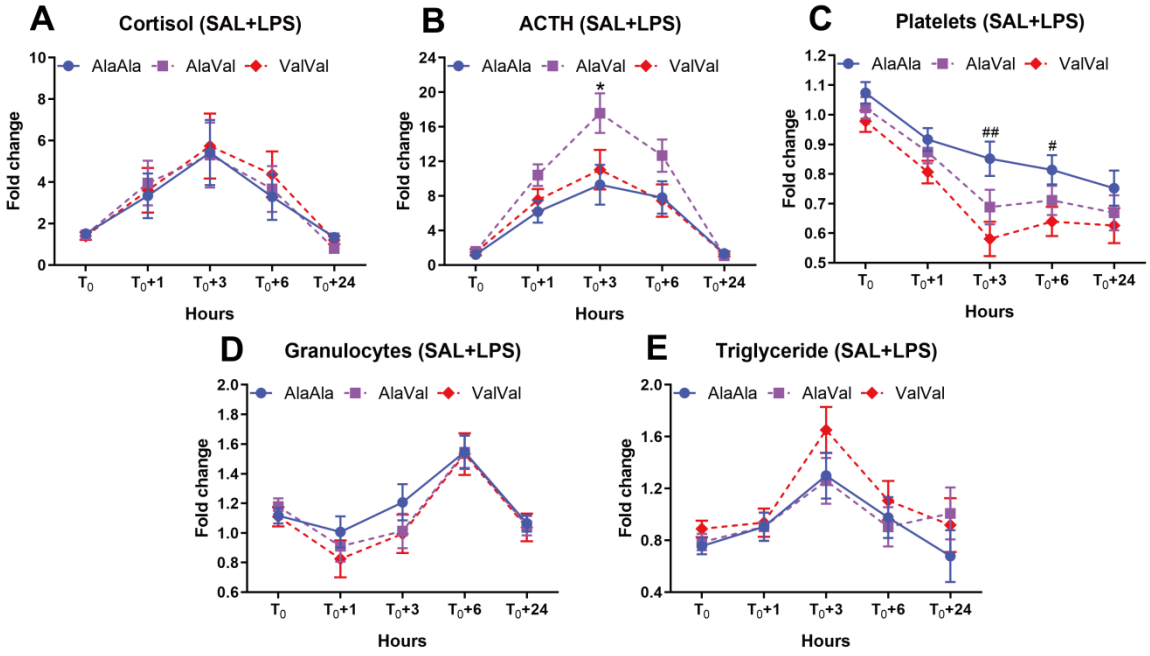

Figure 4. Effect of the $GR_{Ala610Val}$ substitution on the responsiveness of blood parameters during LPS challenge. (A) Fold change of cortisol. (B) Fold change of ACTH. (C) Fold change of platelets. (D) Fold change of granulocytes. (E) Fold change of triglyceride. Data are presented as least-squares means ± standard errors. * indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ heterozygotes (AlaVal); # indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ homozygotes (ValVal); one and two symbols indicate $p < 0.05$ and $p < 0.01$ respectively (n = 16).

$GR_{Ala610Val}$ substitution expands the difference between SAL+LPS and DEX+LPS groups by enhancing the sensitivity of pigs to LPS and/or DEX

GR-mediated feedback regulation during LPS-induced endotoxemia was examined by analyzing genotype differences within the DEX pretreated group (n = 48), and also by comparing differences in LPS-induced responses between SAL control and DEX pretreatment within genotypes.

DEX pretreatment influenced most of the analyzed parameters and responses, essentially as described previously [16]. As such, the effect of DEX was stronger before (T_0) than during LPS challenge. At T_0 $GR_{Ala610Val}$ genotype showed essentially no effect on the outcome of DEX treatment (Table S3). When analyzed across genotypes the differences after LPS challenge were generally modest, apart from parameters that were influenced by $GR_{Ala610Val}$ at baseline, e.g. cortisol (Figure 5A) or ACTH (Figure 5C). However, when the effect of DEX pretreatment on LPS-induced responses was analyzed within genotypes, distinct genotype-related differences could be discerned. As one might expect in view of the gain-of-function, differences in LPS-responses caused by DEX-pretreatment compared to saline control were more pronounced in Val-carriers pigs. Strikingly, for several parameters, including cortisol (Figure 5A, B), ACTH (Figure 5C, D), IL-6 (Figure 5E, F), TNF-α (Figure 5G, H), blood cell counts excluding platelets (Table S3), and panting (Figure 5I, J), AlaVal pigs showed the most marked difference between SAL+LPS and DEX+LPS. The separation between SAL and DEX within AlaVal genotype apparently results from a combination of a greater sensitivity to LPS as well as DEX, as exemplified by ACTH responses (Figure 2B, 5C, D).

While for platelet counts (Figure 6A), frequency of vomiting (Figure 6C), BUN (Figure 6E), and triglyceride concentration (Figure 6G), responses to LPS following DEX pretreatment showed similar patterns across genotypes, for these parameters ValVal pigs showed the highest response to LPS in the SAL group (Figure 2), leading to the most significant separation between

SAL and DEX in this genotype (Figure 6B, D, F, H). For platelet counts, DEX essentially restored genotype order observed at baseline (Figure 6A, B; Figure 7). Likewise, while ValVal pigs showed the most pronounced LPS-induced anorexia in the SAL group, they showed the highest feed intake post LPS-challenge when pretreated with DEX (Figure 3).

Altogether, these results on the one hand support greater sensitivity of Val-carriers to endotoxemia; on the other hand they suggest greater DEX sensitivity of AlaVal pigs during endotoxemia.

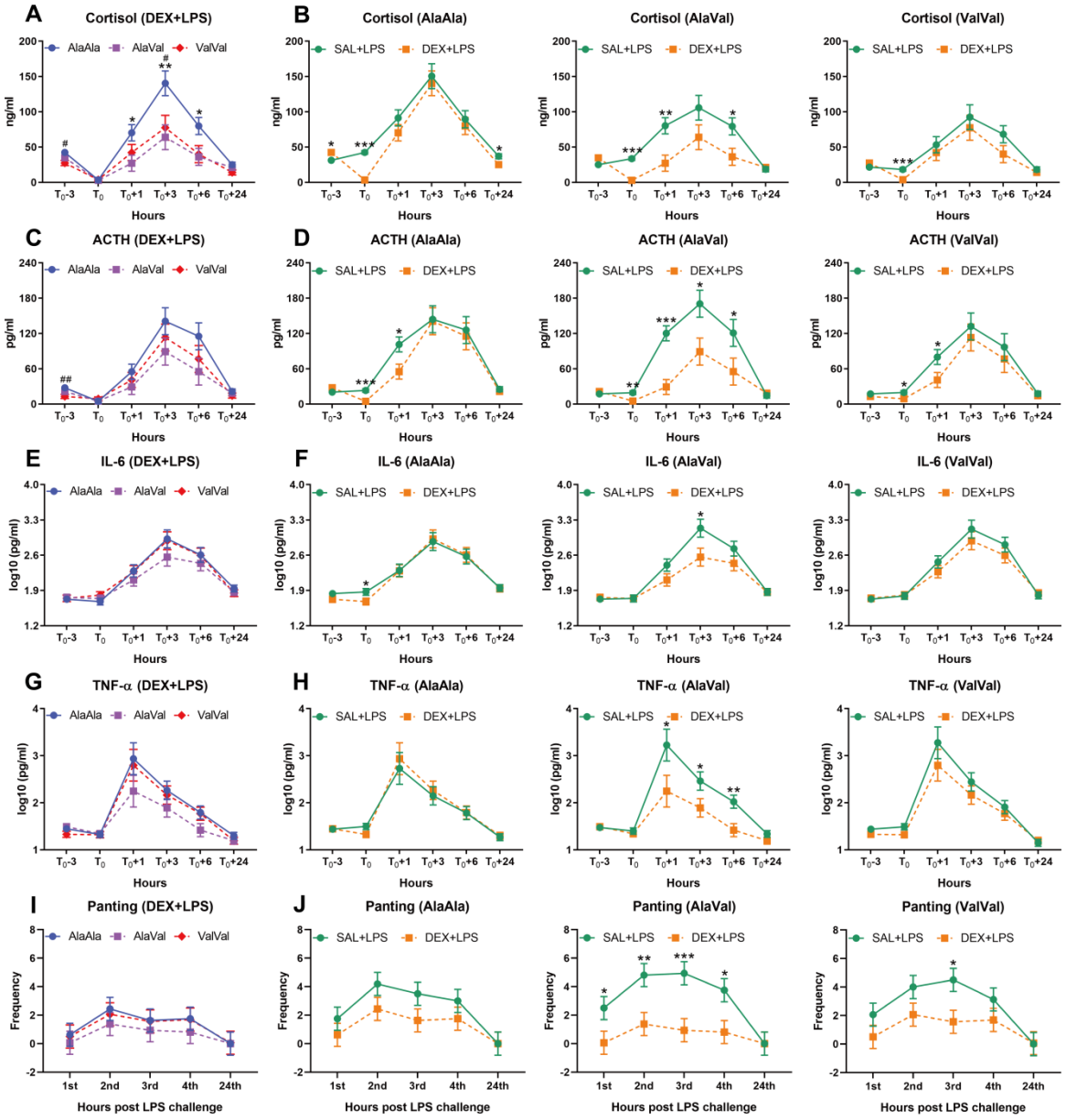

Figure 5. Effect of the $GR_{Ala610Val}$ substitution on parameters for which AlaVal pigs showed the most marked difference between SAL+LPS and DEX+LPS groups. (A, B) Cortisol. (C, D) ACTH. (E, F) IL-6. (G, H) TNF-α. (I, J) Panting. Data are presented as least-squares means ±

standard errors. Figures A, C, E, G, and I show direct genotype effect in endotoxemic pigs in response to DEX pretreatment; * indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ heterozygotes (AlaVal); # indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ homozygotes (ValVal); one and two symbols indicate $p < 0.05$ and $p < 0.01$ respectively (n = 16). Figures B, D, F, H, and J show the effect of DEX pretreatment in endotoxemic pigs within genotypes; * indicates the significance between SAL and DEX pretreated groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n = 16).

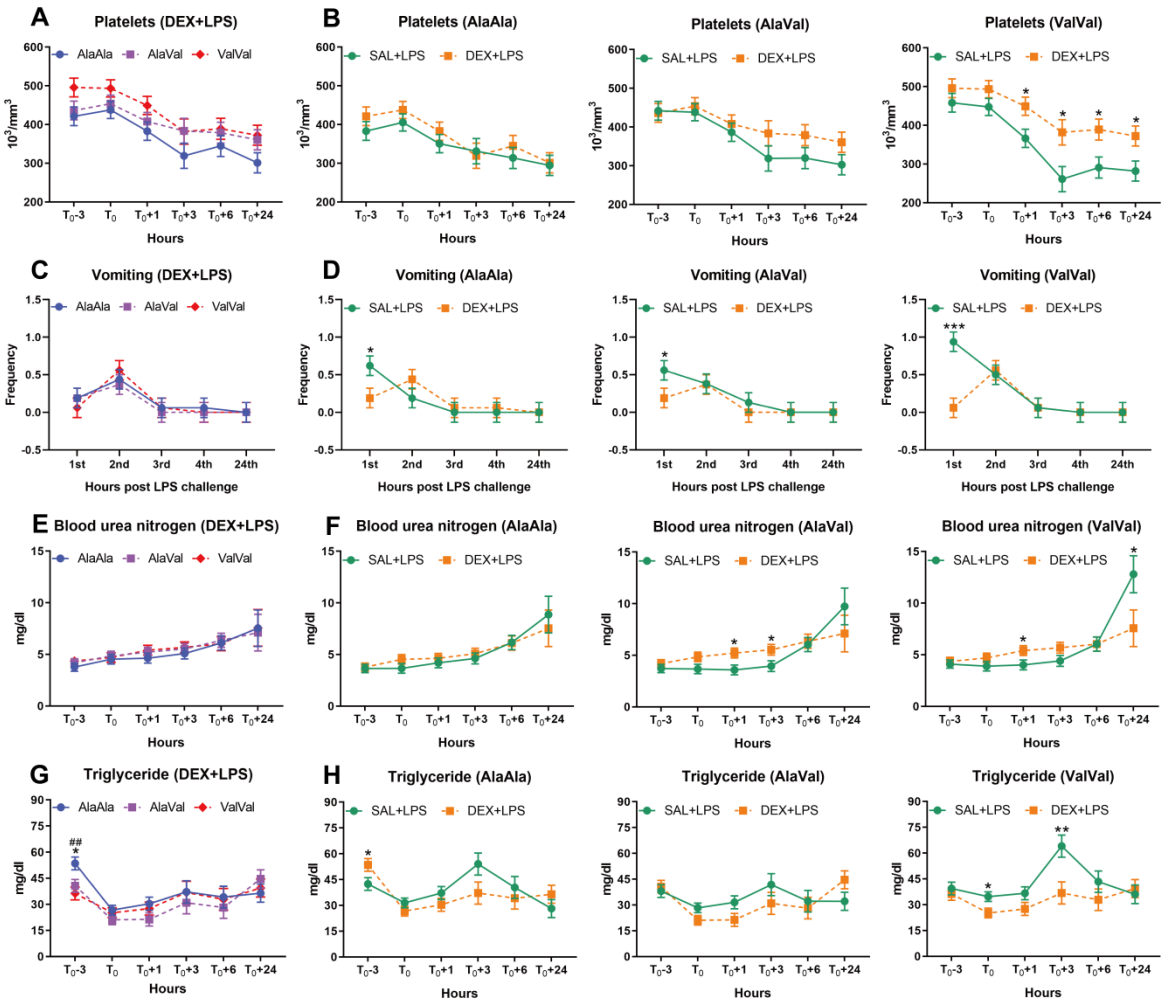

Figure 6. Effect of the $GR_{Ala610Val}$ substitution on parameters for which ValVal pigs showed the most marked difference between SAL+LPS and DEX+LPS groups. (A, B) Platelets. (C, D) Vomiting. (E, F) Blood urea nitrogen (BUN). (G, H) Triglyceride. Data are presented as least-squares means ± standard errors. Figures A, C, E, and G show direct genotype effect in endotoxemic pigs in response to DEX pretreatment; * indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ heterozygotes (AlaVal); # indicates the significance between wild types (AlaAla) and $GR_{Ala610Val}$ homozygotes (ValVal); one and two symbols indicate $p < 0.05$

and $p < 0.01$ respectively (n = 16). Figures B, D, F, and H show the effect of DEX pretreatment in endotoxemic pigs within genotypes; * indicates the significance between SAL and DEX pretreated groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n = 16).

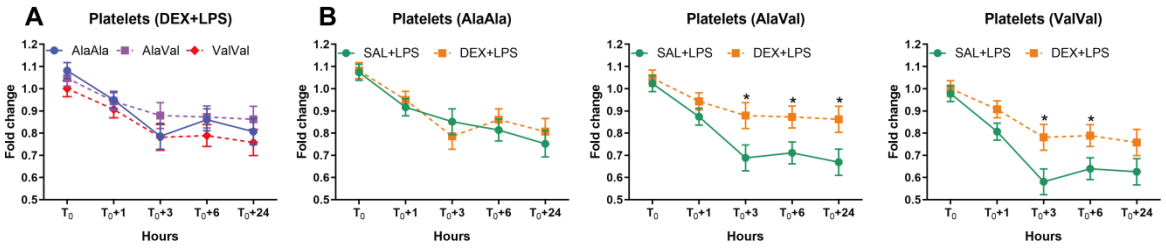

Figure 7. Effect of the $GR_{Ala610Val}$ substitution on platelet responsiveness of endotoxemic pigs in response to DEX pretreatment. (A) Direct genotype effect on the fold change of platelets in endotoxemic pigs in response to DEX. (B) Effect of DEX pretreatment on the fold change of platelets in endotoxemic pigs within genotypes. Data are presented as least-squares means ± standard errors. * indicates the significance between SAL and DEX pretreated groups; * $p < 0.05$ (n = 16).

Discussion

In our previous studies, we focused mainly on exploring the effect of $GR_{Ala610Val}$ on pig physiology under normal conditions, and on clarifying the onset and extent of reprogramming of HPA axis by $GR_{Ala610Val}$. Here we examined $GR_{Ala610Val}$ effects under challenging conditions, induced by LPS and DEX application, supposing that this will reveal impact of $GR_{Ala610Val}$ on other physiological systems, beyond HPA axis.

As expected, the most pronounced effects of $GR_{Ala610Val}$ occurred within HPA axis, particularly on cortisol production in terms of absolute concentrations. However, as clearly demonstrated by the analysis of relative response, activation of cortisol production by LPS was similarly strong in all three genotypes, so that the cortisol concentration showed more or less constant proportions among them throughout the experiment. Because it is unlikely that the cortisol production was at its maximum, at least not at all time points, this implies that the genotype differences in cortisol output were driven by GR-mediated feedback regulation rather than merely by different adrenocortical capacity. This implies that the phenotypic manifestations of $GR_{Ala610Val}$ discussed below are unlikely resulting from glucocorticoid insufficiency.

Though significant at baseline, effect of $GR_{Ala610Val}$ on ACTH during LPS challenge was less obvious, and showed a different pattern from cortisol, with AlaVal pigs exhibiting the strongest

response. Already our previous studies indicated that effects of $GR_{Ala610Val}$ on the adrenal gland and pituitary, respectively, might rely on different mechanisms [14]. Dissociation between adrenal and pituitary responses was likely propelled further by LPS as evidenced by a shift from central to local immune–adrenal regulation of glucocorticoid production in sepsis [18].

In the present study we explored and discovered for the first time an effect of $GR_{Ala610Val}$ on platelet counts. Noticeably, the genotype effect was dependent on the treatment. At baseline, Val-carriers featured greater platelet counts, with approximately additive differences. Glucocorticoids, both endogenous and exogenous, increase platelet counts and are therefore used to treat platelet related-disorders such as immune thrombocytopenia [19]. The mechanisms how glucocorticoids influence platelet counts are diverse and depending on the context. Under normal conditions the mechanism is not well understood, but the effect of $GR_{Ala610Val}$ on baseline platelet counts might be related to GR-mediated effects on proliferation and differentiation of megakaryocytes [20]. We found previously that $GR_{Ala610Val}$ influences the expression of *FAXDC2*, a gene involved in the regulation of megakaryopoiesis, however in the liver [15]; the effect on *FAXDC2* in bone marrow and on megakaryopoiesis has to be proven yet.

During LPS-challenge the platelet counts declined sharply in Val-carriers (below 60% in ValVal), while in wild types (AlaAla) the decline was slower and less dramatic, so that 3 h post LPS application the genotypes actually reversed order. Thrombocytopenia is a widely described hallmark of endotoxemia and sepsis, caused by multiple processes including immunothrombosis to prevent pathogen dissemination [21]. Excessive platelet activation contributes to sepsis complications, such as disseminated intravasal coagulation, and may ultimately lead to organ failure [22]. Consequently, the magnitude of the drop in platelet counts is a strong predictor of mortality in sepsis [21]. Activated platelets release a wide range of pro-inflammatory molecules, including IL-1β, and may directly, but also indirectly via interaction with leukocytes, contribute to the cytokine storm triggered by LPS [23]. Thus, the aggravated thrombocytopenia in Val-carriers might be both consequences, but also cause of their apparently enhanced response to endotoxemia. Another open question is whether just platelet counts, or also platelet function is influenced by $GR_{Ala610Val}$. Although there are only anecdotal reports on GR function in platelets [24], it has been reported that loading of platelet mediators contained in its granules happens in platelet-forming megakaryocytes [25]. Thus, besides platelet counts, $GR_{Ala610Val}$ might affect also platelet function based on the hypothetical effect on megakaryocytes.

DEX pretreatment largely normalized the course of platelet counts in Val-carriers. DEX pretreatment has been reported to inhibit LPS-induced release of platelet activating factor and thus thrombocytopenia [26] and accumulation of platelets in peripheral organs [27] in rodents. These mechanisms might also provide a basis for the effects of DEX observed here.

Another parameter that showed context-dependent genotype effect in the present study was triglyceride concentration. At baseline this was significantly reduced in Val-carriers. In our previous studies we found that baseline triglyceride concentrations tended to be reduced by $GR_{Ala610Val}$ [14, 15]. A tendency towards reduced triglycerides was observed also in the study of mice with a knock-in gain-of-function mutation in the glucocorticoid receptor GR_{M610L} [28]. This mouse model shares many molecular and phenotypic features with $GR_{Ala610Val}$ pigs, such as increased GR ligand affinity and markedly reduced adrenocortical activity and ACTH concentration [28]. Thus, the effect of GR-hypersensitivity on baseline triglyceride concentration appears to be quite weak, but genuine. In line with this, liver transcriptome signature of $GR_{Ala610Val}$ includes changes in expression of several glucose and also triglyceride-metabolism related genes under normal conditions, including for example downregulation of *GPAT3* encoding glycerol-3-phosphate acyltransferase 3, an enzyme involved in triglyceride synthesis [15]. The transcriptome signature of $GR_{Ala610Val}$, together with the reduced circulating triglycerides but normal glucose concentrations, indicates that under normal conditions metabolism might be shifted towards glucose utilization, rather than storage in the form of triglycerides.

Three hours post LPS application there was a temporary rise in triglyceride concentration that was highest in ValVal pigs, in spite of their lowest baseline concentration. This pattern is strikingly different from glucose and FFA. These two latter metabolic parameters gradually change with progressing feed deprivation towards T_0+6 h (decrease for glucose and increase for FFA, respectively), and return to baseline after refeeding at T_0+24 h. This suggests, that energy requirement is not the main driver of the surge of triglycerides and that the triglyceride surge most likely represents a defense response against LPS-induced toxicity, since triglycerides are able to bind and neutralize LPS [29].

For most of other responses, genotype differences appeared largely only when comparing treatments within genotype. Here, two different patterns emerged. The greatest separation between SAL+LPS and DEX+LPS in ValVal pigs occurred for traits for which ValVal pigs tended to show the strongest response to LPS among genotypes. This includes particularly traits that were affected by $GR_{Ala610Val}$ already at baseline, i.e. platelet counts and triglycerides. For

other traits showing this pattern, including feed intake, vomiting, and BUN, the aggravated response to LPS, and thus separation between SAL+LPS and DEX+LPS, might be explained by enhanced vulnerability of ValVal pigs to LPS. Elevated BUN concentration in ValVal might reflect more frequent vomiting and reduced feed intake in response to LPS, and thus greater protein catabolism to fuel hepatic gluconeogenesis. In addition, it might be also an indicator of more serious kidney damage by excessive platelet activation in ValVal pigs [23]. The more serious consequences of LPS-induced endotoxemia in Val-carriers is in stark contrast to findings in other models of GR gain-of-function examined for their response to LPS, such as YGR mice. Overexpression of GR in YGR mice was associated with less LPS-induced IL-6 production and increased survival rate after LPS injection [5]. Our findings argue against the view that enhanced GR signaling *per se* protects the organism from an overshooting immune response [5]. Pigs and rodents show distinct differences in LPS sensitivity [30], whereby pigs, similar to humans, are more sensitive compared to rodents. Thus, phenotypic consequences of GR gain-of-function are likely depending on the specific molecular and temporospatial context of the changes in GR signaling, including species-specific background. As mentioned in the introduction, pigs might provide a more suitable model to study the role of GR during endotoxemia or inflammation.

Traits related to neuroendocrine and immune cell responses showed a different pattern, with most pronounced separation between SAL+LPS and DEX+LPS in AlaVal pigs, which was partly attributable to their greater sensitivity to DEX during LPS challenge. Importantly, no differences in DEX sensitivity occurred at baseline. This implies that the adequate dose to reveal GR gain-of-function is depending on the state of the individual.

The stronger DEX suppression of neuroendocrine responses in endotoxemic AlaVal pigs is likely secondary to the stronger DEX suppression of cytokine response to LPS, e.g. of TNF-α or IL-6, whereby lowered concentration of the latter leads to reduced HPA-axis activation [31]. In addition, whereas the baseline genotypic differences appear mostly additive, the immune cell responses tend to be equally strong in AlaVal compared to ValVal. The tendency of $GR_{Ala610Val}$ for dominance, or even overdominance, for immune cell and DEX-responses, respectively, during LPS challenge is striking. This indicates that GR function might be modified by an intracellular interaction among the two allelic variants, which might be further intensified by DEX. The evidence that primarily the cytokine responses are more efficiently suppressed by DEX in AlaVal points to a potential mechanism that might involve oligomerization of GR. A widely discussed model of a functional relationship between oligomerization status of GR and

its regulatory properties suggests that GR monomers are involved primarily in the regulation of immune responses by transrepression, while GR dimers regulate metabolic responses by transactivation [32, 33]. This model provides foundation for the efforts to develop GR-targeting anti-inflammatory drugs with reduced unwanted side effects [34]. It is interesting to note that besides the canonical dimerization interface located in the DNA-binding domain a second dimerization interface has been proposed in the ligand binding domain (LBD), which among others involves regions close to $GR_{Ala610Val}$ [35]. Furthermore, LBD has been implied in the formation of higher order oligomers, predominantly tetramers [36], possessing enhanced regulatory activity [37]. Thus, the apparently enhanced DEX sensitivity of AlaVal pigs warrants further investigation, including genome-wide transcriptome studies of DEX responses in different cell types, and might provide novel insight into GR function.

It should be stressed here that a robust detection of phenotypic manifestations of $GR_{Ala610Val}$ is complicated by the outbred genetic background, which is a common obstacle in complex trait genetics. This problem is further compounded by high inter-individual variation which is typically observed in response to LPS-challenges [7].

Conclusion

Collectively our results provide convincing evidence for greater vulnerability to LPS-induced endotoxemia conferred by $GR_{Ala610Val}$ substitution and underscore the value of this animal model to explore GR function. An important implication of our study is that GR hypersensitivity is difficult to diagnose but represents a risk factor for systemic inflammation and sepsis.

Supplementary materials

Supplementary materials can be found at https://doi.org/10.1016/j.bbi.2020.08.009.

Author contributions

E. Murani designed research, administrated project, and acquired funding; E. Murani, E. Kanitz, and M. Tuchscherer contributed to methodology; Z.W. Li, N Trakooljul, E. Murani, E. Kanitz, M. Tuchscherer, and C.C. Metges performed research; E. Murani, Z.W. Li, E. Kanitz, M. Tuchscherer, and A. Tuchscherer performed data analysis and curation; K. Wimmers contributed to resources; Z.W. Li and E. Murani wrote the paper; Z.W. Li, E. Kanitz, M. Tuchscherer, A. Tuchscherer, C.C. Metges, N Trakooljul, K. Wimmers, and E. Murani reviewed and edited the paper; E. Murani and K. Wimmers supervised research.

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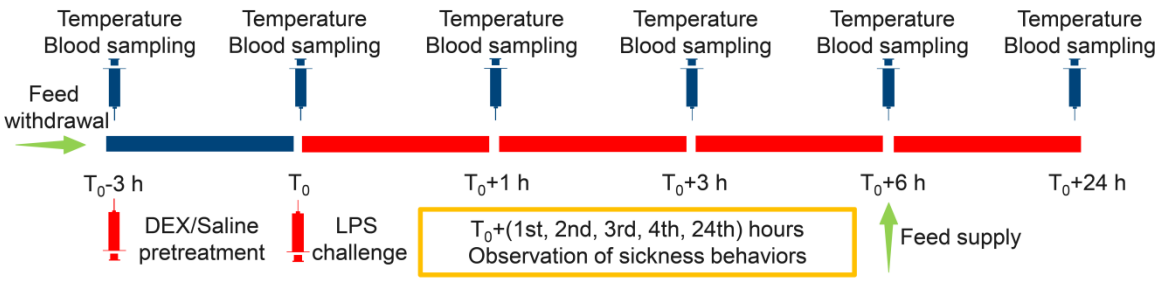

Figure S1. Schematic diagram of the experimental design.

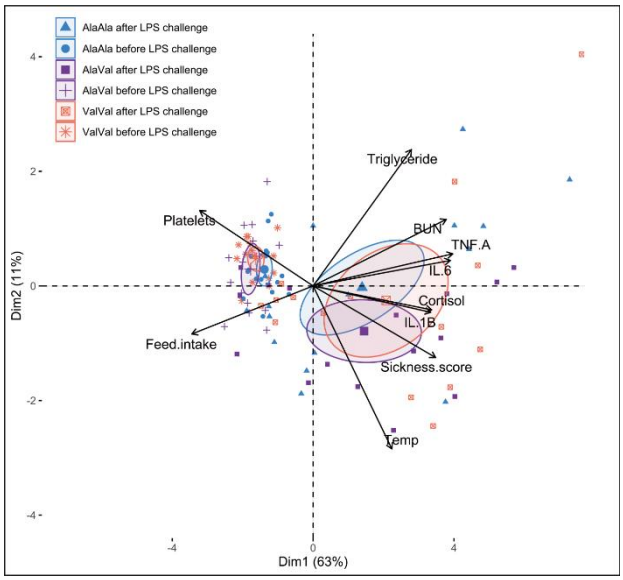

Figure S2. Overall Effect of LPS depending on genotypes. Parameters used for the principal component analysis (PCA) were shown in the figure. For parameters before LPS challenge, absolute values at T_0-3 h were used with the exception of feed intake, for which the percentage before LPS challenge was used. Before LPS challenge, the total sickness score for each animal was considered as 0 (the total sickness score was calculated as the sum of the frequency of cyanosis, diarrhea, inactivity, panting, shivering, and vomiting). For parameters after LPS challenge, TNF-α at T_0+1 h; cortisol, IL-6, triglyceride, and platelet at T_0+3 h; rectal temperature and IL-1β at T_0+6 h; BUN at T_0+24 h; total sickness score during the 3rd hours after LPS challenge; and feed intake percentage after LPS challenge were used. Black arrows indicate parameter loadings. 95% confidence ellipses for the group barycentres are graphed.

Table S1. Effect of $GR_{Ala610Val}$ genotype on baseline parameters.

Parameter	Unit	Genotype Effect	LSM ± SE		
			AlaAla	AlaVal	ValVal
ACTH	**pg/ml**	0.0403	24.04 ± 2.46^{a}	19.23 ± 2.46^{ab}	15.08 ± 2.46^{b}
Cortisol	**ng/ml**	0.0061	36.86 ± 2.67^{a}	29.87 ± 2.67^{ab}	24.49 ± 2.67^{b}
IL-10	**pg/ml**	0.6706	430.33 ± 87.44	255.03 ± 87.44	253.58 ± 87.44
IL-1β	**pg/ml**	0.8289	385.14 ± 62.05	263.38 ± 62.05	315.64 ± 62.05
IL-6	**pg/ml**	0.7206	93.66 ± 16.65	59.26 ± 16.65	62.77 ± 16.65
TNF-α	**pg/ml**	0.3138	35.78 ± 4.41	37.90 ± 4.41	35.78 ± 4.41
RBC	**10^6/mm^3**	0.7758	6.07 ± 0.09	6.15 ± 0.09	6.11 ± 0.09
HCT	**%**	0.9273	34.29 ± 0.40	34.08 ± 0.40	34.12 ± 0.40
HGB	**g/dl**	0.6434	10.90 ± 0.12	10.78 ± 0.12	10.76 ± 0.12
MCH	**pg/cell**	0.0568	18.04 ± 0.15	17.57 ± 0.15	17.66 ± 0.15
MCHC	**g/dl**	0.1307	31.84 ± 0.09	31.65 ± 0.09	31.57 ± 0.09
MCV	**µm^3**	0.0872	56.67 ± 0.37	55.49 ± 0.37	55.93 ± 0.37
RDW	**µm^3**	0.1301	15.93 ± 0.20	16.32 ± 0.20	16.49 ± 0.20
PCT	**%**	0.0133	0.32 ± 0.01^{a}	0.34 ± 0.01^{ab}	0.38 ± 0.01^{b}
PLT	**10^3/mm^3**	0.0085	402.22 ± 16.66^{a}	439.09 ± 16.66^{ab}	477.06 ± 16.66^{b}
LEU	**10^3/mm^3**	0.4098	22.70 ± 0.69	22.18 ± 0.69	21.40 ± 0.69
BAS	**10^3/mm^3**	0.8664	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
BAS	**%**	0.9618	0.47 ± 0.02	0.46 ± 0.02	0.47 ± 0.02
GRAN	**%**	0.0445	39.59 ± 1.06^{a}	37.25 ± 1.08^{ab}	35.61 ± 1.22^{b}
LYM	**10^3/mm^3**	0.9317	12.52 ± 0.40	12.57 ± 0.40	12.36 ± 0.40
LYM	**%**	0.3764	55.55 ± 1.08	56.71 ± 1.08	57.70 ± 1.08
BUN	**mg/dl**	0.4172	3.71 ± 0.28	3.95 ± 0.28	4.23 ± 0.28
FFA	**µmol/l**	0.5724	53.28 ± 5.08	45.97 ± 5.08	51.44 ± 5.08
GLU	**mg/dl**	0.6927	124.44 ± 2.33	124.41 ± 2.33	126.88 ± 2.33
TG	**mg/dl**	0.0163	47.97 ± 2.63^{a}	39.34 ± 2.63^{ab}	37.84 ± 2.63^{b}
Feed intake	**g**	0.7024	698.82 ± 31.06	702.07 ± 30.73	732.23 ± 30.81
RT	**°C**	0.6901	39.21 ± 0.04	39.26 ± 0.04	39.24 ± 0.04

Single underline highlights significant genotype effect at $p < 0.05$. Within a row, values not sharing a common superscript differ significantly at $p < 0.05$. ACTH, Adrenocorticotropic hormone; RBC, Red blood cells; HCT, Hematocrit; HGB, Hemoglobin; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; RDW, Red blood cell distribution width; PCT, Plateletcrit; PLT, Platelets; LEU, Leukocytes; BAS, Basophils; GRAN, Granulocytes; LYM, Lymphocytes; BUN, Blood urea nitrogen; FFA, Free fatty acids; GLU, Glucose; TG, Triglyceride; RT, Rectal temperature.

Table S2. Overview of the impact of fixed effects on the absolute level of parameters. (https://doi.org/10.1016/j.bbi.2020.08.009)

Table S3. Effect of $GR_{Ala610Val}$ genotype on the absolute level of parameters. (https://doi.org/10.1016/j.bbi.2020.08.009)

Table S4. Overview of the impact of fixed effects on sickness behaviors.

Effect	*p*-value						
	Activity	**Cyanosis**	**Diarrhea**	**Inactivity**	**Panting**	**Shivering**	**Vomiting**
Genotype	0.8011	0.2262	0.7630	0.7596	0.9940	0.9929	0.5263
Treatment	0.0877	0.3532	0.7952	0.0522	0.0008	0.8148	0.0673
Genotype × Treatment	0.5973	0.0647	0.7630	0.6225	0.5325	0.1624	0.4641
Sex	0.6118	0.5568	0.5194	0.5613	0.0033	0.9316	1.0000
Genotype × Sex	0.8634	0.8133	0.0163	0.8915	0.2429	0.4759	0.1653
Treatment × Sex	0.9453	0.6507	0.7202	0.9213	0.2621	0.3311	0.5164
Genotype × Treatment × Sex	0.5724	0.4139	1.0000	0.4909	0.0043	0.7355	0.6681
Time	<.0001	0.0077	0.0041	<.0001	<.0001	<.0001	<.0001
Time × Genotype	0.9706	0.3207	0.8885	0.9781	0.9979	0.9950	0.9579
Time × Treatment	0.1539	0.2642	0.7957	0.0976	0.0002	0.1883	0.0001
Time × Genotype × Treatment	0.8904	0.4007	0.7552	0.9481	0.9626	0.1298	0.8535
Time × Sex	0.8012	0.6759	0.5479	0.8271	0.0005	0.7137	0.9259
Time × Genotype × Sex	0.7530	0.2223	0.0435	0.6608	0.4288	0.2273	0.8266
Time × Treatment × Sex	0.9983	0.7983	0.2609	0.9872	0.2510	0.7146	0.5858
Time × Genotype × Treatment × Sex	0.7491	0.6853	0.9942	0.8343	0.0015	0.8925	0.2446
Batch	0.0251	0.5722	0.0482	0.0294	0.2185	0.4833	0.0415

Single underline highlights significant main effects at $p < 0.05$.

Table S5. Effect of $GR_{Ala610Val}$ genotype on sickness behaviors.

Symptom	LSM															SE
	1st hour			2nd hour			3rd hour			4th hour			24th hour			
	AlaAla	AlaVal	ValVal	AlaAla	AlaVal	ValVal	AlaAla	AlaVal	ValVal	AlaAla	AlaVal	ValVal	AlaAla	AlaVal	ValVal	
Activity (S)	1.88	1.81	2.19	0.38	0.50	0.50	0.63	0.69	0.62	0.94	0.94	1.13	0.25	0.25	0.19	0.46
Activity (D)	2.88	2.50	1.63	0.88	0.87	0.75	1.38	1.81	0.81	1.81	2.13	1.75	0.00	0.13	0.06	0.46
Cyanosis (S)	0.00	0.13	0.00	0.00	0.00	0.19	0.44	0.25	0.38	0.12	0.69	0.75	0.00	0.00	0.06	0.34
Cyanosis (D)	0.06	0.06	0.00	1.63	0.00	0.25	1.56	0.00	0.56	0.94	0.00	0.25	0.00	0.06	0.00	0.34
Diarrhea (S)	0.00	0.00	0.00	0.00	0.00	0.06	0.13	0.13	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.04
Diarrhea (D)	0.06	0.00	0.00	0.00	0.00	0.06	0.00	0.06	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.04
Inactivity (S)	9.94	10.06	9.81	11.56	11.38	11.44	11.38	11.31	11.31	11.06	10.94	10.81	0.75	0.75	0.81	0.46
Inactivity (D)	9.06	9.19	10.19	10.94	11.00	11.13	10.63	10.19	11.19	9.94	9.69	10.00	1.00	0.87	0.94	0.46
Panting (S)	1.75	2.50	2.06	4.19	4.81	4.00	3.50	4.94	4.50	3.00	3.75	3.13	0.00	0.00	0.00	0.81
Panting (D)	0.62	0.06	0.50	2.44	1.38	2.06	1.63	0.94	1.56	1.75	0.81	1.69	0.00	0.00	0.06	0.81
Shivering (S)	1.00	1.56	1.44	5.56	6.75	5.25	3.06	6.06	4.00	2.94	5.31	4.31	0.12	0.00	0.06	0.97
Shivering (D)	1.44	1.25	2.19	5.13	4.50	5.25	6.81	4.37	5.62	5.69	2.87	3.81	0.19	0.06	0.25	0.97
Vomiting (S)	0.62	0.56	0.94	0.19	0.38	0.50	0.00	0.13	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.13
Vomiting (D)	0.19	0.19	0.06	0.44	0.37	0.56	0.06	0.00	0.06	0.06	0.00	0.00	0.00	0.00	0.00	0.13

For each symptom, within a column, single underline highlights the significance between saline and dexamethasone groups at $p < 0.05$. (S), Saline pretreated group; (D), Dexamethasone pretreated group.

Table S6. Overview of the impact of fixed effects on the fold change of blood parameters.

Timepoint	Effect	*p*-value					
		ACTH	Cortisol	PCT	PLT	GRAN%	TG
T_0	Genotype	0.2378	0.9502	0.1755	0.0549	0.9915	0.0267
	Sex	0.9686	0.5459	0.0290	0.0276	0.8195	0.7698
	Treatment	0.0028	<.0001	0.4271	0.5446	<.0001	<.0001
	Batch	0.5279	0.9223	0.1123	0.1601	0.2676	0.2883
	Genotype × Treatment	0.4100	0.7189	0.9766	0.9686	0.5508	0.8426
T_0+1 h	Genotype	0.3895	0.6169	0.2152	0.1340	0.4033	0.2078
	Sex	0.6086	0.2714	0.9529	0.9400	0.1617	0.0215
	Treatment	<.0001	0.1653	0.0644	0.0336	0.0005	0.0091
	Batch	0.4467	0.9372	0.0418	0.2943	0.8934	0.3549
	Genotype × Treatment	0.0701	0.3214	0.8330	0.6800	0.2971	0.3478
T_0+3 h	Genotype	0.3400	0.3020	0.0628	0.0535	0.7316	0.0298
	Sex	0.0223	0.3416	0.5401	0.5409	0.0372	0.0246
	Treatment	0.0049	0.4030	0.0247	0.0251	0.0479	0.0018
	Batch	0.0039	0.8293	0.1734	0.2654	0.8907	0.1248
	Genotype × Treatment	0.0428	0.3528	0.0496	0.0396	0.1128	0.9157
T_0+6 h	Genotype	0.7554	0.3451	0.1017	0.0477	0.7934	0.2112
	Sex	0.0006	0.3234	0.3336	0.4786	0.0745	0.0003
	Treatment	0.0079	0.1398	0.0040	0.0041	0.8644	0.1310
	Batch	0.0066	0.6274	0.1677	0.2883	0.8240	0.0133
	Genotype × Treatment	0.0603	0.1257	0.4512	0.4422	0.6997	0.8633
T_0+24 h	Genotype	0.6490	0.2390	0.4252	0.2873	0.8474	0.0888
	Sex	0.5416	0.6531	0.1112	0.1086	0.3136	0.8801
	Treatment	0.8630	0.0332	0.0226	0.0105	0.2046	0.1991
	Batch	0.0017	0.1888	0.1608	0.0314	0.3677	0.2383
	Genotype × Treatment	0.6000	0.4525	0.5488	0.5038	0.6998	0.7523

Single underline highlights significant main effects at $p < 0.05$. ACTH, adrenocorticotropic hormone; TG, triglyceride; PCT, plateletcrit; PLT, platelets; GRAN, granulocyte.

6 General discussion

German Landrace pigs were used as the experimental subject *in vivo* and transcriptomic analysis was completed *in vitro* using porcine PBMCs. The results were interpreted to elucidate the potential influence on animal health, welfare, and breeding.

6.1 Reactions of pigs to DEX and LPS

A bolus intramuscular injection of 60 μg/kg BW DEX was administered to the experimental subjects to ascertain the effect of short-term activation of GR signaling on pig physiology *in vivo*. Notably, this dosage of intramuscular DEX was relatively lower than many other studies involving pig subjects, which tend to range from 0.2 mg/kg BW to 30 mg/kg BW [102, 104, 133]. As indicated in **manuscript 1**, without LPS challenge, there was no detectable effect of DEX treatment alone on IL-1β, IL-6, IL-10, and TNF-α concentrations; this suggests insubstantial changes in the function of immune cells in a natural setting. This finding is supported by previous studies whereby intramuscular injection of 2 mg/kg BW DEX failed to induce alterations in porcine neutrophil functions, including migration, cell cytotoxicity, and ingestion [106]. However, this dosage of DEX resulted in a significant change in the counts of neutrophils, lymphocytes, monocytes, and eosinophils [106]. This corresponds with the observations noted in **manuscript 1**, where DEX alone significantly increased WBC numbers but decreased lymphocyte proportions. The administration of DEX also reduced red blood cell (RBC)-related parameters including RBC counts, hemoglobin concentrations, and hematocrit. These results suggest that porcine sensitivity to GCs is context-dependent, as although the pigs were resistant to GC-mediated suppression of functions in immune cells, this species conveys sensitivity to GC-mediated changes in fluid homeostasis or hemodynamics.

An intraperitoneal injection was chosen for LPS challenge as this is deemed to produce a more appropriate reflection of the consequential sequence of events following LPS stimulation [124]. The study found that LPS triggered extensive reactions in pigs, including behavioral, neuroendocrine, metabolic, hematological, and inflammatory disorders. Therefore, the susceptibility of pigs to LPS is not only reflected by the agent's strength, as demonstrated by the low lethal dose [127], but also by the breadth of subsequent reactions involving multiple systems. This broad range of biological reactions may further contribute to the high mortality in pigs following LPS challenge [125]. For the parameters involving cytokines, triglycerides, and lactate, the effects of DEX are dependent on LPS treatment. These parameters were altered by DEX during endotoxemia but were unaffected in a natural setting, thereby indicating the

possible interplay between GR signaling and immune stimulation. As discussed in **manuscript 1**, both LPS and GCs independently regulate these parameters via different mechanisms. Furthermore, a previous study reported that, unlike the subjects treated with DEX alone, mouse macrophages treated with DEX following 3-hour LPS stimulation resulted in the GR binding to almost eight thousand new sites; this suggests that the coverage of GR binding is enhanced by the activation of immune signaling by adjusting the chromatinscape [134]. Hence, further research is required to establish whether significant DEX effects on the aforementioned parameters during endotoxemia are achieved directly by LPS-mediated modifications in GR signaling or indirectly by DEX-mediated changes in LPS signaling.

In **manuscript 1** and **manuscript 3**, the responses of ACTH and cortisol to LPS highlighted the development of GC resistance (GCR) in the HPA axis in endotoxemic pigs, as evidenced by the disrupted DEX-induced suppression of both parameters due to subsequent LPS administration. LPS-induced GCR was also observed in peripheral tissues (**manuscript 2**), where the effect of DEX on gene expression in M4 was attenuated by LPS. Immune stimulation can activate the HPA axis, yet it can also impair GC-mediated feedback inhibition of the axis [3]. For example, a study involving rats found that consecutive treatment with LPS eradicated DEX-mediated suppression of corticosterone secretion induced by acute photic stress [135]. This reduced feedback regulation in the HPA axis is believed to convey adaptive benefits during periods of infection as it enables prolonged GC release [3]. Conversely, reduced GC sensitivity in peripheral tissues is considered to facilitate sepsis. In sepsis patients, T cells show increased GRβ expression compared with healthy individuals [136], whereas neutrophils from sepsis patients exhibit decreased expression of GRα, which is negatively correlated to levels of IL-6 [137]. Similarly, GRβ expression in PBMCs from sepsis patients has been shown to be higher on hospital admission than on discharge, and treatment with septic serum attenuates the inhibitory effects of DEX on LPS-induced TNF-α production in PBMCs from healthy donors [138]. In **manuscript 1**, the lactate level in the LPS+SAL group increased significantly from T0+1 h to T0+3 h. This is significant as blood lactate is characterized as one of the best-validated biomarkers in sepsis, which is directly related to a poor prognosis and high risk of death [56]. The observed LPS-induced GCR in this study may partially explain the high susceptibility of pigs to LPS [124].

The ACTH and cortisol data also revealed the dissociation of their responses to LPS. This may be partially caused by the multipath regulation of cortisol production. As already mentioned in **manuscript 1**, apart from an ACTH-dependent route, LPS can induce GC production by

directly interacting with the adrenal gland. For instance, LPS stimulates cortisol release in human adrenocortical cells in a COX-2-dependent manner [139]. In a study using isolated rat left adrenal glands, LPS or IL-1β triggers corticosterone production in a dose-dependent manner [140]. In comparison to immune cells, human adrenocortical cells express multiple TLRs with a differential expression pattern and generate numerous inflammatory cytokines, including TNF-α, IL-6, and IL-8, in response to LPS [141]. In view of the substantial impact of cytokines on GR signaling [142], this cytokine production may further influence cortisol release at the adrenal level. Another plausible mechanism behind the dissociated ACTH and cortisol responses is tissue-specific sensitivity to GCs. Unlike certain brain regions and PBMCs, the pituitary is relatively insensitive to GC regulation [143]. However, several studies have suggested that the affinity and specificity of GR binding in GC-exposed adrenal tissue are compatible with those identified in WBCs [144]. The final possible cause of the dissociation is an LPS-induced shift from the central neuroendocrine to local immune-adrenal regulation of GC release, which has previously been observed in late sepsis [145]. Although the adrenal gland is responsive to LPS, this local immune-adrenal crosstalk, which affects LPS-induced adrenal inflammation and HPA axis activation, is driven by TLR signaling in immune cells that have been recruited to the adrenal gland, as opposed to TLR signaling in adrenocortical cells [145]. The findings in **manuscript 3** support the role of LPS in escalating dissociated ACTH and cortisol responses, as the data show that the $GR_{Ala610Val}$ affected baseline ACTH and cortisol in a similar dose-dependent manner, yet the effect on LPS-induced ACTH and cortisol responses differed considerably.

6.2 Implications of transcriptomic profiles following DEX and LPS stimulation

As indicated in **manuscript 2**, short-term stimulation of porcine PBMCs by DEX and LPS led to distinct responses at the transcriptomic level, with changes in gene expression profiles relating to various signaling pathways, biological functions, and regulatory networks. TLR4 recognition of LPS activated two downstream pathways: 1) MYD88-dependent signaling, which subsequently activated NF-κB and MAPK cascades, followed by cytokine release such as TNF-α and IL-6, and 2) TRIF-dependent signaling that activates IRF3 and IRF7 cascades with subsequent generation of type I IFNs [146]. In porcine PBMCs, LPS-initiation of both pathways was indicated by the activation of ingenuity pathway analysis (IPA) terms observed in M1, including Toll-like receptor signaling, NF-κB signaling, p38 MAPK signaling, IL-6 signaling, activation of IRF by cytosolic pattern recognition receptors, and interferon signaling. In addition, the application of DEX to porcine PBMCs seemed more effective as an inhibitor

of MYD88-dependent signaling, as LPS-induced expression of *IL6* was inhibited by DEX in M3, whereas LPS-regulation of *IRF7* only occurred in M1. The enrichment of interferon signaling for M1 but not M3, with the involvement of *IFI35*, *IFI6*, *IFIT1*, *IFITM3*, *IFNAR1*, *IFNB1*, *IFNG*, *IFNGR2*, *IRF9*, *ISG15*, *JAK2*, *MX1*, *OAS1*, *RELA*, *STAT1*, and *STAT2*, supports this hypothesis; these genes failed to respond to DEX under the experimental conditions.

Additionally, LPS provoked marked changes in other immunity-related regulation networks, which could explain LPS' substantial influence on pig physiology *in vivo*. As evidenced in M1, over 200 canonical signaling pathways were significantly enriched, with activation of 70 of these and inhibition of four. The majority of the affected pathways are related to immune responses, such as PI3K/AKT signaling [147], iNOS signaling [148], CD40 signaling [149], TNFR2 signaling [150], JAK/Stat signaling [151], lymphotoxin β receptor signaling [152], TREM1 signaling [153], and HMGB1 signaling [154]. Moreover, LPS administration resulted in the activation of a set of pathways related to cytoskeletal reorganization, cell development, migration, adhesion, proliferation, differentiation, and survival, such as ephrin receptor signaling [155], ILK signaling [156], FGF signaling [157], integrin signaling [158], and IGF-1 signaling [159].

Of particular notice was the finding that peroxisome proliferator-activated receptor (PPAR) signaling was among the few IPA terms inhibited by LPS in M1. PPARs are nuclear receptors that regulate metabolism and immunity; in addition, PPARs form heterodimers with retinoid X receptor (RXR) and control gene expression by binding to specific DNA elements [160]. Numerous cell types express PPARs, including T and B lymphocytes, monocytes, macrophages, platelets, DCs, epithelial cells, and endothelial cells. In these cases, PPAR activation can inhibit inflammatory mediators such as iNOS, cytokines, chemokines, and adhesion molecules [160]. Furthermore, PPARs also have an anti-inflammatory effect by altering the phenotypes and functions of immune cells. An example of this is PPARγ activation in human primary monocytes, which skews cell differentiation towards an anti-inflammatory phenotype [161]; in contrast, PPARγ-deficient macrophages exhibit impaired phagocytosis and increased production of inflammatory cytokines post-LPS treatment [162]. Thus, the inhibition of PPAR signaling by LPS in porcine PBMCs in this study likely contributes to the high susceptibility of pigs to endotoxemia [124]. In M4, PPAR signaling was inhibited in regard to the comparison of LPS+DEX VS DEX, suggesting the involvement of these receptors in diminished DEX functions caused by LPS. Additionally, liver X receptor (LXR) signaling may also correlate with the severity of LPS-induced disorders in pigs, based on the finding that the *NR1H3* gene

(encodes LXRα) and the IPA term LXR/RXR activation were inhibited by LPS. Previous studies have shown that LXR is an effective inhibitor of LPS-induced cytokine release in human monocytes, whilst mice lacking LXRs are highly susceptible to bacterial infection [163, 164].

PPAR signaling is also associated with DEX-mediated anti-inflammatory effects in pigs since it was among the minority of IPA terms that were activated by DEX in M3. Simultaneous activation of PPARα and GRα favors the inhibition of NF-κB-driven genes in a dose-dependent manner and additively suppresses cytokine production [165, 166]. Moreover, the combined activation of GR and PPARγ has been shown to alleviate skin inflammation to a greater extent compared with single activation [167]. Evidence shows that PPARα agonists attenuate the expression of GC-inducible genes, such as *TSC22D3* and *G6PC*, and impede DEX-induced glucose intolerance and hyperinsulinemia; this illustrates the potential of combining GR and PPAR agonists to treat inflammatory diseases to evade side effects [165]. Alongside PPAR, several other signaling pathways were shared by multiple modules: PI3K/AKT signaling, JAK/Stat signaling, lymphotoxin β receptor signaling, HMGB1 signaling, dendritic cell maturation, GM-CSF signaling, B cell receptor signaling, and IL-8 signaling (by M1 and M3); p38 MAPK signaling and IL-6 signaling (by M1 and M4); iNOS signaling (by M1 and M5); TREM1 signaling (by M1, M3, and M4). These findings may constitute the molecular basis of crosstalk between the GR and immune signalling in pigs.

Also in **manuscript 2**, a range of immunity-related GR targets were identified in M2 to M5, which support the substantial role of GR-mediated signaling pathways in the regulation of immune responses in pigs. Although this study found that genes in M1 were regulated by LPS but not DEX, many previously reported immunity-related GR targets, such as *ANXA1* (encodes annexin A1), were discovered in this module. These molecules are involved in the immune functions of pigs based on their positive or negative regulation by LPS in porcine PBMCs; therefore, their lack of responsiveness to DEX may somewhat explain the insensitivity of pigs to GC-mediated immunomodulation [106].

ANXA1 is a well-characterized GR dimer-dependent anti-inflammatory gene [33] that is primarily regarded as a molecule that is released from GC-stimulated cells [168]. *ANXA1* inhibits the activity of phospholipase A2, the main role of which is to stimulate the release of arachidonic acid by cleaving arachidonyl-containing phosphatides. Arachidonic acid can be metabolized via enzymatic reactions that are catalyzed by COX, producing inflammatory mediators such as prostaglandins and leukotrienes [33]. *ANXA1* also interacts with the p65 subunit of NF-κB, which hinders TF-DNA binding [169]. As described in **manuscript 2**, DEX

was unable to induce another GC-inducible anti-inflammatory gene, *KLF2*. Therefore, the absence of responses from these anti-inflammatory factors in the presence of DEX likely diminishes the effects of DEX on the transcription of a set of target genes, and additionally may contribute to the insensitivity of pigs to GC-mediated immunomodulation. Nevertheless, a relatively low dose of DEX (≈ 2 ng/ml) was used in this study, and thus, the exact role of these genes in pigs' distinct responses to GCs requires further validation.

In contrast, DEX successfully activated several other GR dimer-dependent anti-inflammatory factors, with *TSC22D3* (encodes GILZ) and *DUSP1* (encodes MKP-1) being among the most well-characterized genes [80]. MKP-1 reduces the activation of MAPK cascades by initiating the dephosphorylation of threonine and tyrosine residues in the kinases. It inhibits the function of all three classical MAPK members, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs, which is significant as these are closely related to the stimulation of pro-inflammatory TFs, such as NF-κB and AP-1, as well as the production of cytokines, chemokines, and other inflammatory mediators [170]. In this study, *DUSP1* was similarly induced by both DEX and LPS (LFC = 1.179 and 1.221, respectively) despite the administration of a lower dose of DEX compared with LPS (≈ 2 ng/ml VS 10 μg/ml). The protein glucocorticoid-induced leucine zipper (GILZ) is commonly used as a readout indicator of GR-mediated transactivation. As discussed in **manuscript 2**, GILZ can minimize inflammation by preventing the activation of PI3K/AKT, MEK/ERK, NF-κB, and AP-1 signalling pathways [33]. The results showing DEX-induced activation of these GR dimer-dependent anti-inflammatory factors in porcine PBMCs indirectly supports the emerging opinion that GR-mediated transactivation is indispensable, in terms of the anti-inflammatory effects of GCs [33, 80].

Researchers have proposed that the anti-inflammatory actions of GCs are mainly conferred by transrepression through interactions with other TFs, and require the participation of monomeric GR [80]. However, expanding evidence implies that GR dimer-dependent transactivation is fundamental for GC-mediated anti-inflammation. This evidence is particularly strong in studies involving GR^{dim} mice, which were constructed by introducing an A465T mutation into the DBD of mouse GR [171]. This mutation interrupts GR dimerization and GR-DNA binding, yet GR monomer-based functions remain unaffected, such as crosstalk with AP-1 and NF-κB [33]. Mice expressing this mutated GR are highly susceptible to LPS-induced inflammatory, metabolic, and behavioral disorders, and are more likely to develop cecal ligation and puncture (CLP)-induced sepsis [81, 172]. GR^{dim} mice also display enhanced LPS-induced lethality and

necroptosis in intestinal epithelial cells; however, circulating TNF concentration increases to the same extent due to LPS administration in both genotypes [82]. This has been corroborated by several other studies, where the introduction of GR^{dim} alters the responses of IL-6, IL-10, and IL-1β, but not TNF-α [81, 172]; this signifies that the regulation of TNF-α is independent of A465T-related GR dimerization. Also in **manuscript** 2, the dissociated responses of these cytokines to the crosstalk of DEX and LPS were evident, whereby DEX effectively suppressed LPS-induced *IL6* and *IL10* expression, yet suppression of *TNF* was diminished by the activation of LPS signaling.

Reports state that dimerization is not wholly eradicated in the GR^{dim} model, which may be due to other dimerization interfaces, such as that identified in the LBD [27]. Nevertheless, studies using GR^{dim} mice have confirmed that the GR monomer *per se* is insufficient for GC-mediated anti-inflammation. The required involvement of mechanisms beyond the GR monomer in regard to the anti-inflammatory actions of GCs is reflected in **manuscript 3**, where the AlaVal heterozygotes expressed the most pronounced DEX suppression of IL-6 and TNF-α. However, the molecular basis for this dominance is yet to be elucidated.

6.3 $GR_{Ala610Val}$ and balanced breeding for production and robustness in pigs

Manuscript 3 revealed that the $GR_{Ala610Val}$ led to a dose-dependent reduction in baseline cortisol and ACTH concentrations, indicative of diminished baseline HPA axis activity caused by adjusting GR hypersensitivity due to the Ala610Val variation. This adjustment was also illustrated by the reduced adrenocortical size and mRNA expression of CRH and AVP in the hypothalamus, alongside increased mRNA expression of CBG in the liver [116]. Other studies have found that the negative influence of $GR_{Ala610Val}$ on basal HPA axis activity, as determined by decreased cortisol concentrations, is evident in several commercial pig breeds, including German Landrace, German Large White, French Large White, and Pietrain × (German Large White × German Landrace) [114, 173]. This has also been found in pigs at one-week-old, one day prior to weaning at four weeks *post natum* (pn), one day after weaning, and at a peripubertal age (approximately 22 weeks pn) [116]. Therefore, the alteration of HPA axis activity caused by $GR_{Ala610Val}$ may have a genuine implication in the balanced breeding of pigs.

6.3.1 $GR_{Ala610Val}$ and genetic selection towards production-related traits

The genetic selection of pigs favoring domesticated phenotypes and high production usually results in diminished HPA axis activity [95]. In French Large White pigs, a comparison of progeny from sires born in 1977 with those born in 1998-2000 showed that HPA axis activity

had declined based on lowered cortisol production, as a result of breed selection for increased growth rate, feed efficiency, and carcass lean content [174]. This trend is consistent with findings showing that HPA axis activity and cortisol levels have mainly undesirable effects on the production traits of farm animals [95]. For example, the intensity of adrenal responses to ACTH in pigs, sheep, and chickens has been negatively associated with growth rate and feed efficiency [175-177]. In addition, the leanness of pigs can be negatively influenced by the production rate of cortisol [178]. Compared with European White-type breeds, Chinese Meishan pigs exhibit reduced growth rate and feed efficiency, as well as fattier carcasses, which has been linked to higher circulating cortisol levels [179, 180]. Accordingly, the stimulation of pigs by chronic-intermittent stress has been shown to elevate cortisol levels and impair weight gain [181]; meanwhile, treating pigs with DEX reduces weight and length gains and lean mass percentage, but supports fat deposition and protein catabolism [182].

Despite the observation of reduced baseline cortisol and ACTH levels of $GR_{Ala610Val}$ pigs in **manuscript 3**, their growth properties (e.g. daily gain and feed intake) and carcass composition (e.g. backfat thickness, area of *M. longissimus dorsi*, lean mass, and body length) remained generally similar to those of wild types. This was likely due to the balance between HPA axis hypoactivity and GR hypersensitivity in a natural setting [116]. Currently, genetic variability of the HPA axis has been associated with three main sources in farm animals: 1) adrenocortical responsiveness, 2) GC bioavailability, and 3) GR function [183], all of which are affected by $GR_{Ala610Val}$. However, minimal detection of changes in the growth and body composition of $GR_{Ala610Val}$ carriers has highlighted the complexity of associations between production traits and HPA axis activity, and has accentuated the challenge of predicting breeding outcomes based on the HPA axis alone. These results also underline the need to consider this specific GR variant and to integrate phenotypic approaches when using HPA axis responsiveness for the genetic selection of pigs towards favorable production traits.

6.3.2 $GR_{Ala610Val}$ and genetic selection for pigs with high robustness

The increased susceptibility of $GR_{Ala610Val}$ pigs to immune stress described in **manuscript 3** emphasizes a connotation of the Ala610Val variation in regard to the genetic selection of animals for enhanced robustness. Notably, the consequences of carrying the $GR_{Ala610Val}$ during endotoxemia affect not only parameters linked to the HPA axis and inflammation (such as cortisol, ACTH, and cytokines), but also sickness behaviors and parameters involving metabolism and hemodynamics (such as BUN, triglycerides, and platelets). In contrast to

observations of baseline production traits, the impact of $GR_{Ala610Val}$ on the adaptive responses of pigs under challenging conditions appears to be more extensive and profound.

Numerous studies have evidenced the positive associations between HPA axis responsiveness and robustness-related traits, including the viability of newborns and resistance to stressors [184]. The HPA axis is essential for intrauterine fetal homeostasis, parturition timing, and organ maturation, which are strongly associated with the postpartum viability of animals [184]. In addition, adrenal weight and fetal cortisol concentrations are positively correlated with estimated breeding values for piglet survival, which is feasibly due to improved fetal development and maturation [185, 186]. In another study, rats with reduced HPA axis activity, deemed by reduced blood corticosterone and ACTH levels alongside POMC mRNA abundance in the pituitary, showed increased abdominal temperature and aggravated dehydration, inflammation, and metabolic imbalance following heat stress [187]. Chickens with low basal corticosterone concentrations were compared with those with high basal corticosterone concentrations, whereby the latter were more resistant to *Eimeria necatrix* infection as evidenced by comparatively milder coccidiosis lesions and greater weight gain [188].

A study involving the genetic selection of French Large White pigs based on diverse HPA axis activity, according to plasma cortisol levels in response to ACTH administration, revealed that pigs with a high responsive HPA axis (HPA^{hi}) were more resilient to acute social stress caused by the placement of pigs in adjacent pens with unfamiliar conspecifics [189]. Compared with low responsive HPA axis (HPA^{lo}) pigs, HPA^{hi} subjects expressed increased RBC and WBC counts; this would likely enhance the oxygen-carrying capacity of RBCs and boost resistance to pathogens [190]. A genome-wide association study regarding HPA^{hi} and HPA^{lo} pigs indicated that the Ala610Val variation was the most significant causal mutation for reduced cortisol levels in HPA^{lo} pigs [173], further validating the meaningful impact of $GR_{Ala610Val}$ on the genetic selection of pigs towards high robustness. Correlating with observations made about HPA^{lo} pigs, as shown in **manuscript 3**, $GR_{Ala610Val}$ reduces baseline HPA axis activity and granulocyte counts. Also, $GR_{Ala610Val}$ carriers show decreased total WBC counts one day post-weaning [116]. However, further investigation is required to ascertain whether these changes in immune cells conclusively contribute to the high sensitivity of $GR_{Ala610Val}$ pigs to LPS, and whether the Val variant similarly impacts other components of the immune system.

The results from **manuscript 3** emphasize the involvement of the feedback regulation mediated by $GR_{Ala610Val}$ in the adaptions of pigs to immune stress. The hypersensitivity of $GR_{Ala610Val}$ and reduction in HPA axis activity are largely balanced at the baseline by adjusting GR signaling

within the HPA axis. Determining whether this balance will be maintained and what the relevant biological consequences will be beyond the HPA axis in a stressed setting are important. As represented by the lower absolute cortisol concentrations in $GR_{Ala610Val}$ pigs and the highly similar kinetics and magnitude of cortisol fold changes of three genotypes in response to LPS, the adrenal responsiveness and GR hypersensitivity induced by the Val variation can, nonetheless, become balanced when facing immune stress. However, this balance within the HPA axis cannot protect animals from the adverse effects of LPS to the same extent as wild types, as shown by the more severe LPS-induced behavioral and physiological disorders in Val carriers. Therefore, it is plausible that the physiological homeostasis at baseline induced by the adjustment of the HPA axis, fine-tuned to the reactivity of respective GR alleles, can be modified by external stimuli. This is indicative of potential gene-environment interaction regarding the effect of GR signaling on animal physiology [191, 192]. Overall, the compensatory reduction in HPA axis activity and aggravation of LPS-induced disorders caused by the hypersensitivity of $GR_{Ala610Val}$ are corroborative of the integral role of HPA axis responsiveness in animal robustness; alternatively, this could indicate the complex impact of enhanced GR signaling on animal adaptions to external stressors.

6.4 $GR_{Ala610Val}$ and assessment of stress in pigs

The existence of the Ala610Val variation in dominant commercial pig breeds [114, 173] and its impact on the baseline and stress-induced HPA axis responsiveness (as indicated in **manuscript 3**) mean that it could be a possible interference factor to monitor stress in pigs, using cortisol as a biomarker. The demanding selection of pigs towards favorable production traits jeopardizes the robustness of the animals, which then enhances their vulnerability to external stimuli. This is concerning as the intensifying farming system has generated additional stress in farm animals [193, 194].

Pigs experience numerous stressful events, including those triggered by human, social, metabolic, and immune factors. An example of this is the productive system, whereby pigs are subjected to human-controlled events such as weaning, transport, castration, snaring, sampling, tattooing, and regrouping, which can elicit undesirable behavioral and physiological consequences [194]. Metabolic stress can occur due to the mixing of low-ranking and high-ranking sows within the same pen, as access to feed is then limited in the low-ranking sows and decreased weight gain follows [195]. Metabolic stress caused by fasting results in a catabolic state as demonstrated by elevated FFA and β-hydroxybutyrate (BHB) levels and decreased glucose and insulin levels [196]. This catabolic state was detected in **manuscript 1** and

manuscript 3, where feed deprivation led to an increase in BUN and FFA levels; in contrast, reductions in glucose, lactate, and triglyceride levels were identified in the saline group from T0-3 h to T0.

Immune stress in pigs, such as exposure to pathogens, could result in the release of inflammatory cytokines and changes in immune cell counts and proportions [132, 197-199]. Furthermore, animal responses to immune stress progress beyond the immune system, including HPA axis activation, reduced feed intake and growth, increased inactivity and somnolence, and modified metabolites and microelements [198-201]. The various effects of immune stress on pigs have been corroborated by behavioral, neuroendocrine, hematological, and metabolic disorders induced by LPS, as described in **manuscript 1** and **manuscript 3**.

Nonetheless, other stressors can equally affect immune functions in pigs. Single social isolation has been linked with an increased ratio of $CD8^+$ cells, alongside reduced plasma TNF-α levels [202]. Immobilization stress has been shown to alter NK cell cytotoxicity and large granular lymphocyte counts [203]. Also, previous studies linked heat stress to significant changes in immune cell counts and cytokine responses in pigs [204-206]. These interactions between stressful events and the immune system may be attributed to GR signaling, as cortisol levels have been tightly associated with changes in immune cells and cytokines in the context of stress in pigs caused by single social isolation [202]. Indeed, the impact of GR signaling on porcine immune functions is reflected in **manuscript 3** by the divergent responses of pigs carrying different GR variants to LPS.

Due to the consequences of stressful events on animal health and growth, several approaches have been developed to assess animal stress using a panel of biomarkers, including those involved in the HPA axis (e.g. GCs), sympathetic-adrenal-medullary (SAM) axis (e.g. α-amylase and chromogranin A), hypothalamic-pituitary-gonadal (HPG) axis (e.g. testosterone), and the immune system (e.g. acute phase proteins, IgA, and IL-18). The most commonly used of these biomarkers are GCs (cortisol and corticosterone) [194, 207]. Blood, saliva, and feces or urine have been used as matrices to evaluate stress in pigs using cortisol. It was found that when female pigs were exposed to stressors such as confinement, snout roping, housing in crates, mating, and boars, plasma cortisol levels increased to varying degrees [208, 209]. Also in pigs, both urinary and salivary cortisol respond to ACTH administration. Urinary cortisol is associated with reduced stereotypies caused by housing stress, while salivary cortisol is stimulated by handling and transport stress [210-212]. Contrastingly, although hair cortisol is suitable to assess stress in various species [213, 214], its evidenced use in pigs remains vague.

It has been observed that repeated ACTH administration can lead to increased hair cortisol concentrations in cattle but not pigs [215].

Manuscript 3 highlights the significant reduction in absolute cortisol levels in $GR_{Ala610Val}$ carriers compared with wild types at baseline and during endotoxemia. However, LPS-induced behavioral and physiological disorders were found to be more severe in $GR_{Ala610Val}$ carriers. Therefore, the $GR_{Ala610Val}$ appears to disturb the evaluation of stress in pigs since the Ala610Val variant results in lower cortisol levels and a more pronounced state of stress. Differences in baseline cortisol levels were adjusted by calculating cortisol fold changes, yet the disturbance caused by the $GR_{Ala610Val}$ remained, as shown by all the genotypes presenting a similar cortisol fold change yet differing biological reactions. In the $GR_{Ala610Val}$ group, the obstruction to using cortisol as an assessor of animal stress was not limited to immune stress. Weaning is regarded as one of the most stressful events in a pig's life, whereby issues can lead to intestinal and immune dysfunctions and impairment of animal health, welfare, and growth [216]. One day post-weaning, cortisol levels were significantly lower in $GR_{Ala610Val}$ carriers than wild types, whereas most tested parameters remained the same among genotypes, with the exception of WBC count [116]. In addition, it is implied that the $GR_{Ala610Val}$ may affect free cortisol since it increases hepatic CBG expression [116]. In ewes suffering scrapie stress, there was no change in total cortisol concentrations, yet free cortisol levels increased due to the reduction in CBG affinity [217]. Integrative approaches should be developed involving the measurements of stress indicators and phenotypic consequences, in order to evaluate stress comprehensively in pigs.

6.5 Conclusion and perspectives

Several valuable findings emerged from this research: i) in pigs, immune stress triggered by LPS has an extensive influence on multiple systems and signaling pathways, which potentially harms animal health and welfare and restricts growth performance; ii) activation of GR signaling by DEX leads to the regulation of responses of various biological parameters and alters gene expression profiles in natural and stressed settings, providing potential leverage to improve animal robustness in the context of immune stress; iii) GR hypersensitivity caused by the Ala610Val substitution has a considerable influence on porcine responses to DEX and LPS, implying the involvement of this specific substitution in animal health and welfare and balanced breeding towards robustness and efficiency; iiii) there is substantial interaction between the GR and immune signaling in pigs at both physiological and molecular levels, as represented by the effects of DEX and $GR_{Ala610Val}$ on LPS-induced disorders, as well as the impact of LPS-

mediated activation and regulation of the HPA axis, and a series of coordinated genes and regulatory networks. Overall, the results from this study have expanded current knowledge concerning the regulation and effect of GR signaling in response to immune stress in pigs, whilst also disclosing the role of hypersensitive $GR_{Ala610Val}$.

However, there should be greater clarification of certain areas in future studies. Firstly, treatment with DEX before and after LPS stimulation was shown to trigger specific gene expression profiles involved in metabolic and immune processes in mouse macrophages [134]. In subsequent studies, it should be determined whether the timing of DEX treatment affects porcine responses when immune stress is involved. Secondly, several of this study's findings deserve further experimental verification, such as the role of iNOS signaling in the development of septic shock and the contribution of calcium signaling to LPS-induced attenuation of GC effects. Lastly, the $GR_{Ala610Val}$ study was the first of its kind to highlight the negative influence of GR hypersensitivity during inflammation. Nonetheless, the underlying molecular basis is less clear. Since the genotype difference is more pronounced following LPS stimulation than at baseline, it would be beneficial to investigate the effects of $GR_{Ala610Val}$ on the regulation of genes and pathways related to LPS recognition and downstream signal transduction. These future areas are expected to provide additional information regarding the performance of GR signaling in pigs and should pioneer the "One Health" concept that emphasizes the inextricable interconnections between humans and farm animals; consequently, optimal health and well-being outcomes can be pursued.

7 Summary

Due to the importance of GCs in the adaptive responses of animals to stress and the widespread application of GC-based drugs in veterinary medicine, understanding GR signaling is necessary to improve the health and welfare of pigs. In addition, GR signaling facilitates the trade-off between robustness and production in pigs, making it a desired target for genetic selection in animal breeding. However, the practical application of this phenomenon has been limited by the inadequate knowledge of the true performance of GR signaling in this species. Here, the integration of physiological, behavioral, and transcriptomic approaches has produced findings that could improve the current understanding of the regulation and influence of GR and immune signaling in pigs.

This study has shown the occurrence of LPS-induced physiological and behavioral outcomes in pigs and has determined the effect of DEX in pigs under normal conditions and during LPS-induced endotoxemia. Concurrently, the investigation into gene expression profiles and regulatory networks of porcine PBMCs in response to DEX and LPS was successful, and provided molecular insights into the mechanisms underlying the responses to immune stress and GC-mediated therapy in pigs. Finally, the significance of the natural Ala610Val substitution in the porcine GR was also explored in this context, which demonstrated that the hypersensitive $GR_{Ala610Val}$ boosts the sensitivity of pigs to both LPS and DEX. These results will help to ensure improved application of GC-based agents in pigs in the future and will aid balanced pig breeding in terms of robustness and production. In conclusion, this study will improve animal health, welfare, and productivity in the production system.

8 References

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Appendix I: List of abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotropic hormone
ADORA3	Adenosine A3 receptor
ADRB1	β1 adrenergic receptor
AF	Activation function
AGPAT2	1-acylglycerol-3-phosphate O-acyltransferase 2
AKT	Protein kinase B
Ala	Alanine
ALT	Alanine aminotransferase
ANPEP	Aminopeptidase N
ANXA1	Annexin A1
AP-1	Activating protein 1
ASBT	Apical sodium-dependent bile acid transporter
ATGL	Adipose triglyceride lipase
AVP	Vasopressin
BAFF	B cell activating factor
BHB	β-hydroxybutyrate
BUN	Blood urea nitrogen
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding globulin
CC10	Clara cell 10-kDa secretory protein
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
CON	Vehicle
ConA	Concanavalin A
COX-2	Cyclooxygenase 2
CREB	cAMP-response element-binding protein
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CYP1A2	Thymosin and β4 sulfoxide
DBD	DNA-binding domain
DCs	Dendritic cells
DEGs	Differentially expressed genes
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DOK-1	Docking protein 1
ERK	Extracellular signal-regulated kinase
FAS	Fatty acid synthase
FBPase	Fructose 1,6-bisphosphatase
FCAR	Receptor for Fc fragment of IgA

FFA	Free fatty acids
FGF	Fibroblast growth factor
FKBP	FK506-binding protein
FOXP3	Forkhead box P3
FPR	Formyl peptide receptor
G6Pase	Glucose-6-phosphatase
GABA	γ-aminobutyric acid
GATA-3	GATA binding protein 3
GCR	GC resistance
GCs	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPAT	Glycerol-3-phosphate acyltransferase
GR	Glucocorticoid receptor
GRE	GC responsive element
HDAC	Histone deacetylase
HMGB1	High mobility group box 1
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HSL	Hormone-sensitive lipase
Hsp	Heat shock protein
ICAM	Intercellular adhesion molecule
IECs	Intestinal epithelial cells
IFN-α	Interferon α
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL-1R2	Type 2 IL-1 receptor
IL-1RA	IL-1 receptor antagonist
ILK	Integrin-linked kinase
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
IκBα	Inhibitor of nuclear factor kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KLF2	Kruppel-like factor 2
LBD	Ligand-binding domain
LBP	LPS-binding protein
LILRB1	Leukocyte immunoglobulin like receptor B1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MD-2	Secreted glycoprotein myeloid differentiation 2
MEK	Mitogen-activated protein kinase kinase

MHCII	Major histocompatibility complex class II
MKP-1	MAPK phosphatase-1
MMP-9	Metalloproteinase-9
MR	Mineralocorticoid receptor
MSK1	Mitogen- and stress-activated protein kinase 1
MT1X	Methallothionein 1X
MyD88	Myeloid differentiation primary response gene 88
NCoR1	Nuclear receptor corepressor
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
nGRE	Negative GC responsive element
NK	Natural killer
NLS	Nuclear localization signal
NR3C1	Nuclear receptor subfamily 3 group c member 1
NRS	Nuclear retention signal
NTD	N-terminal domain
$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 1C
PAI-1	Plasminogen activator inhibitor 1
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PC	Pyruvate carboxylase
PCA	Principal component analysis
PEPCK	Phosphoenolpyruvate carboxykinase
PFK2	Phosphofructokinase 2
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinases
pn	Post natum
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPI	Protein-protein interaction
PVN	Paraventricular nucleus
QTL	quantitative trait locus
RASD1	Ras related dexamethasone induced 1
RBCs	Red blood cells
RDW	Red blood cell distribution width
RGS-2	Regulator of G-protein signaling 2
RT	Rectal temperature.
RXR	Retinoid X receptor
S100A10	S100 calcium binding protein A10
SAA	Serum amyloid A
SAM	Sympathetic-adrenal-medullary
SCD	Stearoyl-CoA desaturase
SEGRAs	Selective GR agonists
SLAP	Src-like-adaptor protein
SLPI	Secretory leukoprotease inhibitor
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor

SNP	Single nucleotide polymorphism
StAR	Steroidogenic acute regulatory
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
TF	Transcription factor
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF6	TNFR-associated factor 6
TRAM	TRIF-related adaptor molecule
Tregs	Regulatory T cells
TREM1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR domain-containing adaptor inducing IFN-β
TSLP	Thymic stromal lymphopoietin
TTP	Tristetraprolin
TXA2	Thromboxane A2
UTR	Untranslated region
Val	Valine
VCAM	Vascular cell adhesion molecule
WBCs	White blood cells

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Appendix III: Publications and proceedings

1. **Li Z**, Kanitz E, Tuchscherer M, Tuchscherer A, Metges CC, Trakooljul N, Wimmers K, Murani E: **Kinetics of physiological and behavioural responses in endotoxemic pigs with or without dexamethasone treatment**. *Int J Mol Sci* 2019, **20**(6).

2. **Li Z**, Kanitz E, Tuchscherer M, Tuchscherer A, Metges CC, Trakooljul N, Wimmers K, Murani E: **A natural Ala610Val substitution causing glucocorticoid receptor hypersensitivity aggravates consequences of endotoxemia**. *Brain Behav Immun* 2020, **90**:174-183.

3. **Li Z**, Trakooljul N, Hadlich F, Ponsuksili S, Wimmers K, Murani E: **Transcriptome analysis of porcine PBMCs reveals lipopolysaccharide-induced immunomodulatory responses and crosstalk of immune and glucocorticoid receptor signaling**. *Virulence* 2021, **12**(1):1808-1824.

4. **Li Z**, Kanitz E, Tuchscherer M, Tuchscherer A, Trakooljul N, Wimmers K, Murani E: **The natural Ala610Val substitution in the porcine glucocorticoid receptor enhances sensitivity to endotoxemia**. *Vortragstagung der DGfZ und GfT* 2019, B11. (Abstract and oral presentation; 11-12 September 2019, Gießen, Germany).

5. **Li Z**, Kanitz E, Tuchscherer M, Tuchscherer A, Trakooljul N, Wimmers K, Murani E: **The Ala610Val substitution in the glucocorticoid receptor enhances endotoxin-triggered immune stress**. *Book of Abstracts of the 71st Annual Meeting of the European Federation of Animal Science* 2020, 286. (Abstract and oral presentation; 1-4 December 2020, Virtual Meeting).