Univerzita Karlova

Přírodovědecká fakulta

Studijní program: Biologie

Studijní obor: Fyziologie živočichů

Bc. Veronika Langová

Lokální steroidogeneze v periferních tkáních a její regulace

Local steroidogenesis in peripheral tissues and its regulation

Diplomová práce

Vedoucí diplomové práce: Ing. Peter Ergang, Ph.D.

Praha, 2018

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne:

8. 8. 2018

Podpis:

Poděkování

Na tomto místě bych ráda poděkovala zvláště svému školiteli, Ing. Peterovi Ergangovi, Ph.D., za trpělivé vedení mé práce, ochotu, vstřícnost, podporu, nadhled a cenné rady do současného i budoucího pracovního života.

Můj dík patří i ostatním členům oddělení Funkce epitelu Fyziologického ústavu Akademie věd ČR, v. v. i., kteří mi vždy byli ochotni poradit a pomoct, se kterýmkoli problémem a kteří mi nabídli přítomnost u mnoha zajímavých experimentů.

Zvláštní poděkování patří vedoucímu oddělení, panu prof. RNDr. Jiřímu Páchovi, DrSc., který mi umožnil vypracovat práci na svém oddělení, byl vždy vstřícný a ochotný věnovat mi čas a poskytnout konzultace, rady a odpovědi na veškeré mé otázky.

Mnohokrát děkuji také své rodině a svému partnerovi, Tomáši Kubáčovi, za podporu a zázemí, které mi po celou dobu studia poskytovali.

Abstract

The innate and adaptive immune processes are modulated by hormones including glucocorticoids and by microbiota. The exact mechanisms underlying the microbial and hormonal contributions to this control are not completely clear.

Present study is therefore focused to crosstalk between microbiota and *de novo* biogenesis or local regeneration of glucocorticoids. In particular, the study analysed the effect of commensal microbiota on expression of genes encoding steroidogenic enzymes (Star, Cyp11a1, Hsd3b1, Cyp21a1, Cyp11b1) and regeneration of glucocorticoids (Hsd11b1) in adrenal glands, colon, spleen and mesenteric lymph nodes using conventional and germ-free mice. The expression of all 5 components of steroidogenesis was identified only in the adrenal gland and colon, whereas the lymphoid organs expressed predominantly Star, Cyp11a1 and Hsd3b1 indicating the ability to produce only progesterone but not corticosterone. Microbiota decreased the expression of Star in all studied tissues but the expression of other genes was insensitive to microbiota or did not respond homogenously depending on the tissue and gene.

Hsd11b1 expression was upregulated by microbiota in the spleen but not in other tissues. Similarly, the *in vitro* treatment of immune cells isolated from mesenteric lymph nodes by microbial structures activated Toll-like receptor pathway but didn't affect the expression of Hsd11b1. In summary, microbiota seems to influence the biogenesis of glucocorticoids at the level of Star, the rate limiting link of steroidogenesis, whereas its effect of regeneration of glucocorticoids is less obvious.

Key words:

glucocorticoids, steroidogenesis, local regeneration, colon, spleen mesenteric lymph nodes, Star, Hsd11b1, microbiome

Abstrakt

Vrozené i adaptivní imunitní reakce jsou modulovány hormonálně, mimo jiné pomocí glukokortikoidů, a působením mikrobiomu, nicméně přesný mechanismus není zcela objasněn.

Tato studie se zabývá vlivem mikrobiomu na *de novo* biogenezi a lokální regeneraci glukokortikoidů. Konkrétním předmětem analýzy je vliv komenzálů na expresi genů kódujících steroidogenní enzymy (Star, Cyp11a1, Hsd3b1, Cyp21a1, Cyp11b1) a enzym zajišťující regeneraci glukokortikoidů (Hsd11b1) v nadledvinách, tračníku, slezině a mesenteriálních lymfatických uzlinách. Studie byla provedena na konvenčních a bezmikrobních myších.

Exprese všech 5 komponent *de novo* steroidogeneze byla pozorována pouze v nadledvinách a tračníku. V lymfatických orgánech byla pozorována především exprese Star, Cyp11a1 a Hsd3b1, naznačující pouze schopnost produkce progesteronu, nikoli jeho další konverzi na kortikosteron. Vlivem přítomného mikrobiomu došlo k poklesu exprese Star ve všech studovaných tkáních, exprese ostatních enzymů byla ovlivněna tkáňově specificky či ovlivněna nebyla.

Konvenční myši vykazovaly zvýšenou expresi Hsd11b1 ve slezině, v ostatních orgánech exprese ovlivněna nebyla. Exprese Hsd11b1 nebyla ovlivněna ani *in vitro* stimulací Toll-like receptorů v imunitních buňkách izolovaných z mesenteriálních lymfatických uzlin.

Z výsledků lze tedy předpokládat vliv mikrobiomu na biogenezi glukokortikoidů na úrovni exprese Star, kódujícího limitní enzym steroidogeneze, nicméně vliv na další kroky *de novo* steroidogeneze a lokální regeneraci je méně zřejmý.

Klíčová slova:

glukokortikoidy, steroidogeneze, lokální regenerace, tračník, slezina, mesenteriální lymfatické uzliny Star, Hsd11b1, mikrobiom

Table of contents

Abbreviation

5-HT – 5-hydroxytryptamine

AC – adenylate cyclase

ACTH - adrenocorticotropic hormone

AP1 – activator protein 1

APC – antigen presenting cell

ASK1 - apoptosis signal-regulating kinase 1

ATP – adenosine triphophate

BCR – B-cell receptor

BMPR – bone morphogenic protein receptor

cAMP – cyclic adenosine monophosphate

CAH - congenital adrenal hyperplasia

CBG – corticosteroid binding globulin

cDNA – complementary deoxyribonucleic acid

CNS – central neural system

COX2 – cyclooxygenase 2

CRE – cyclic adenosine monophosphate response element

CREB - cyclic adenosine monophosphate response element binding protein

CRH – corticotropin releasing hormone

CRHR1 - corticotropin releasing hormone receptor 1

CRHR2 - corticotropin releasing hormone receptor 2

CSF – cerebrospinal fluid

CYP11A1 - cholesterol side-chain cleavage enzyme

CYP11B1 - 11β-hydroxylase 1

CYP11B2 - 11β-hydroxylase 2

CYP17A1- 17α-hydroxylase 1

CYP21, HSD21 – 21-hydroxylase

CYP21A1 - 21α-hydroxylase 1

DAMP – damage-associated molecular pattern

DC – dendritic cell

DD – death domain

DHEA – dehydroepiandrosterone

DNA – deoxyribonucleic acid

dsRNA – double strand ribonucleic acid

ERK1 – extracellular signal-regulated kinase 1

ERK2 – extracellular signal-regulated kinase 2

FNEDA - fibronectin extra domain A

GADD45G - growth arrest and DNA damage inducible gamma

GALT – gut-associated lymphoid tissue

GATA – GATA binding factor

GATA3 – GATA binding factor 3

GATA4 – GATA binding factor 4

GC – glucocorticoid

GR – glucocorticoid receptor

GRE – glucocorticoid response element

HDL – high density lipoprotein

HMGB1 - high mobility group box 1 protein

HPA axis – hypothalamic-pituitary-adrenal axis

HSD3B1, 3βHSD – 3β-hydroxylase

HSD11B1 - 11β-hydroxysteroid dehydrogenase 1

HSD11B2 - 11β-hydroxysteroid dehydrogenase 2

HSP – heat shock protein

IBD – inflammatory bowel disease

IκBα - nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α

IFNα – interferon α

IFNγ – interferon γ

IgG – immunoglobulin G

IKKα - inhibitory κ B kinases α

IKKβ - inhibitory κ B kinases β

IL-1 – interleukin 1

IL-1β – interleukin 1β

IL-4 – interleukin 4

IL-7 – interleukin 7

IL-8 – interleukin 8

IL-10 – interleukin 10

IL-13 – interleukin 13

IRAK1 - interleukin-1 receptor-associated kinase 1

IRAK4 - interleukin-1 receptor-associated kinase 4

IRF3 - interferon regulatory factor 3

JAK – Janus kinase

JNK - c-Jun N-terminal kinases

KLF4 - krüppel-like factor 4

L. reuteri – Lactobacillus reuteri

LDL – low density lipoprotein

LPS - lipopolysaccharide

LRR domain – leucine rich repeat domain

MALT - mucosa-associated lymphatic tissue

MAMP – microbe-associated molecular pattern

MAPK - mitogen-activated protein kinase

MAP3K7, TAK1 - mitogen-activated protein kinase kinase kinase 7, transferring growth factor β activated kinase 1

MC – mineralocorticoid

MCR2 – melanocortin receptor 2

MEK1 - mitogen-activated protein kinase kinase 1

MEK2 - mitogen-activated protein kinase kinase 2

MKK3 - mitogen-activated protein kinase kinase kinase 3

MKK4 - mitogen-activated protein kinase kinase kinase 4

MKK6 - mitogen-activated protein kinase kinase kinase 6

MKK7 - mitogen-activated protein kinase kinase kinase 7

MKP - mitogen-activated protein kinase phosphatase

MLN – mesenteric lymph nodes

MR – mineralocorticoid receptor

mRNA – messenger ribonucleic acid

MRAP - melanocortin-2 receptor accessory protein

MyD88 - myeloid differentiation primary response 88

NADH - nicotinamide adenine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

NBFIB - nerve growth factor IB

NBRE - nerve growth factor IB response element

NFκB - nuclear factor κ-light-chain-enhancer of activated B cells

nGRE – negative glucocorticoid response element

PAMP – pathogen-associated molecular pattern

PGD2 – prostaglandine D2

PKA – protein kinase A

PKC – protein kinase C

PLA_2 – phospholypase A_2

PMA – phorbol myristate

PPARγ - Peroxisome proliferator-activated receptor

PRR – pattern recognition receptor

PRS – partial restraint stress

RIP1 - receptor-interacting serine/threonine-protein kinase 1

RONS – reactive oxygen and nitrogen species

rRNA – ribosomal ribonucleic acid

SCFA – short chain fatty acid

SEGRM - selective glucocorticoid receptor modulator

SF1 – steroidogenic factor 1

SF2, LRH1 – steroidogenic factor 2, liver receptor homologue 1

SOC1 – suppressor of cytokine signalling 1

ssRNA – single strand ribonucleic acid

StAR – steroidogenic acute regulatory protein

STAT1 - signal transducer and activator of transcription 1

STAT3 signal transducer and activator of transcription 3

STAT5 - signal transducer and activator of transcription 5

STAT6 - signal transducer and activator of transcription 6

SUMO – small ubiquitine-like molecule

TAB1 – transferring growth factor β activated kinase 1 binding protein 1

TAB2 - transferring growth factor β activated kinase 1 binding protein 2

TAB3 - transferring growth factor β activated kinase 1 binding protein 3

Tc – cytotoxic T-lymphocyte

TCR - T-cell receptor

TF – transcription factor

TGFβ – transferring growth factor β

Th – helper T-lymphocyte

Th2 – helper T-lymphocyte 2

Th17 – helper T-lymphocyte 17

TIR domain – Toll/interleukin 1 receptor domain

TJ – tight junction

TLR – Toll-like receptor

TNFα – tumour necrosis factor α

TNFAIP3 - tumour necrosis factor α-induced protein 3

TNFR1 – tumour necrosis factor receptor 1

TNFR2 – tumour necrosis factor receptor 2

TRADD - tumour necrosis factor receptor 1-associated death domain protein

TRAF6 – tumour necrosis factor receptor associated factor 6

Treg – regulatory T-lymphocyte

TRIF - Toll/interleukin 1 receptor domain-containing adapter-inducing interferon β

TyK2 – tyrosine kinase 2

VLDL – very low-density lipoprotein

UCN1 – urocortin 1

UCN2 – urocortin 2

UCN3 – urocortin 3

XIAP-X-linked inhibitor of apoptosis protein

Names of genes and mRNAs are capitalisated

1. Introduction

Every multicellular organism living in natural conditions continually communicates with omnipresent microorganisms. Diverse microbial strains inhabit all barriers separating inner environment of the organism from surroundings, while threatening host's homeostasis. Principles of innate and adaptive immunity consist of the ability to recognize own healthy cells and quickly and effectively destruct foreign or own unfunctional cells. However, an uncontrolled immune reaction can lead to a life-threatening condition, e.g. an anaphylactic shock. Thus, every immune reaction in healthy individuals is being controlled by a plethora of regulatory mechanisms. These include controlling of inflammation by steroid hormones.

A group of steroid hormones can be divided into 5 subgroups, glucocorticoids (GCs), mineralocorticoids (MCs), androgens, estrogens and progesterone. Steroid hormones are produced during a process called steroidogenesis. Systemically are steroid hormones produced in adrenal (GCs, MCs, androgens) and gonadal (androgens, estrogens, progesterone) glands. Besides the systemic steroidogenesis has been steroidogenesis recently observed also in other tissues including epithelial barriers, such as intestine, skin or lungs; immune and visceral organs, brain.

2. Glucocorticoids and their physiological role

GCs, MCs, androgens, estrogens and progesterone form a group of steroid hormones. MCs serve to maintain an electrolyte and fluid homeostasis. Androgens and estrogens function primarily as sex hormones. Progesterone act as a sex hormone, but also as a precursor of other steroid hormones. Major functions of GCs consist in the regulation of developmental, metabolic and immune processes and their relation to a stress reaction.

The main murine and rat GC is corticosterone, while in humans it is cortisol. Both molecules can be seen in the Fig. 1. In humans, corticosterone is utilized primarily for the synthesis of the mineralocorticoid aldosterone. Nevertheless, a small level of corticosterone also occurs in human plasma and a little higher in a cerebrospinal fluid (CSF). Higher level in CSF than in plasma may point to a regulatory function during a stress reaction (Raubenheimer et al. 2006).

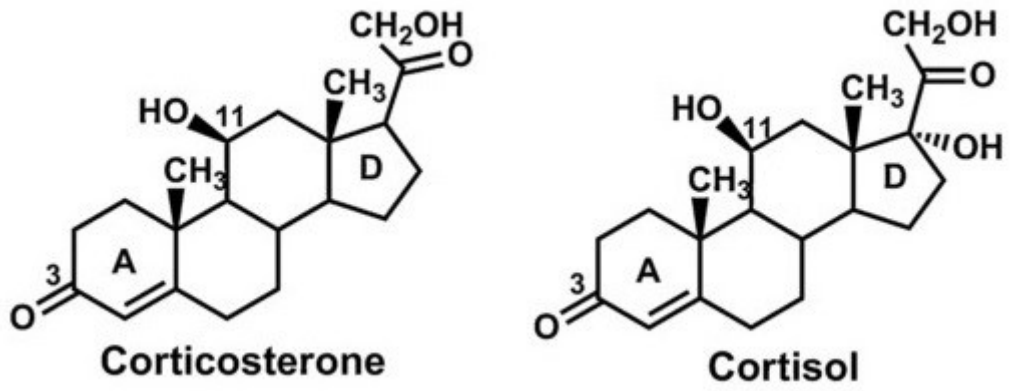

Figure 1: Two major mammalian glucocorticoids. Taken from *Baker et al. 2013.*

Due to a hydrophobic character, the GCs penetrate easily through membranes. On the other hand, the hydrophobicity complicates transport in the bloodstream. Therefore, the majority of GCs is transported in the bloodstream bound to corticosteroid binding globulin (CBG).

2.1 Regulation of a glucocorticoid response

GCs execute regulatory functions in almost every tissue. Due to a higher affinity, GCs preferentially interact with mineralocorticoid receptors (MRs) than with glucocorticoid receptors (GRs, NR3C1). It is assumed, that in a resting state, GCs bind only MRs, while during a stress reaction, when the level of GCs is high, both types of receptors are occupied (Reul & Kloet 1985).

In a resting state, the majority of cytosolic GRs is bound to chaperons, particularly heat shock proteins (HSPs). When linkage between GC and their receptors is formed, GR-HSP complex

dissociates. After that, the newly formed GC-GR complex moves from cytosol into the nucleus. The GC-GR complex can translocate into the nucleus either as monomer or homodimmer. The translocation into the nucleus regulates the expression of many proteins. Small amount of GR succeeds in a transition into the nucleus without GC. Besides that, incessant movements of molecules cause repetitive dissociations and reassemblies. These and analogous phenomena enable the presence of GR without GC in the nucleus and thus GC-independent regulation of expression (Fig. 2).

There are four possible mechanisms how GRs regulate gene expression. GRs bind to a specific promoter region of target gene on a deoxyribonucleic acid (DNA). Promoters of positively regulated genes or regulatory regions contain glucocorticoid response element (GRE), while negatively regulated genes are repressed via negative GRE (nGRE). Direct binding of GRs to GREs or nGREs leads to a phenomenon called a *cis*-regulation (*cis*-activation or *cis*-repression) of expression (Fig. 2). GR mostly interacts with transcription regulatory sites (John et al. 2011). In that case, activator protein 1 (AP1) can probably help remodelling a chromatin to make an accessible place for GR (Biddie et al. 2012). Cis-activation operates for example at the genes encoding growth arrest and DNA-damage-inducible protein (GADD45G) and krüppel-like factor 4 (KLF4), *cis*-repression at gene of inflammatory cytokine interleukin-1β (IL-1β) (Dostert & Heinzel 2004; Yang et al. 2017). GRs can also affect expression indirectly or in cooperation with other transcription factors via *trans*-regulation (*trans*-activation or *trans*-repression). *Trans*-regulation is provided by protein-protein interactions with other transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), AP1 and signal transducers and activators of transcription 3, 5 and 6 (STAT3, STAT5 and STAT6), whose function is facilitated or attenuated by binding of GR.

Group of *trans*-activated genes include the genes coding proteins participating in anti-inflammatory processes, like tumour necrosis factor α (TNFα)-induced protein 3 (TNFAIP3) or nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), while example of *trans*-repressed genes represent pro-inflammatory cytokines (Scheinman et al. 1995; Oh et al. 2017).

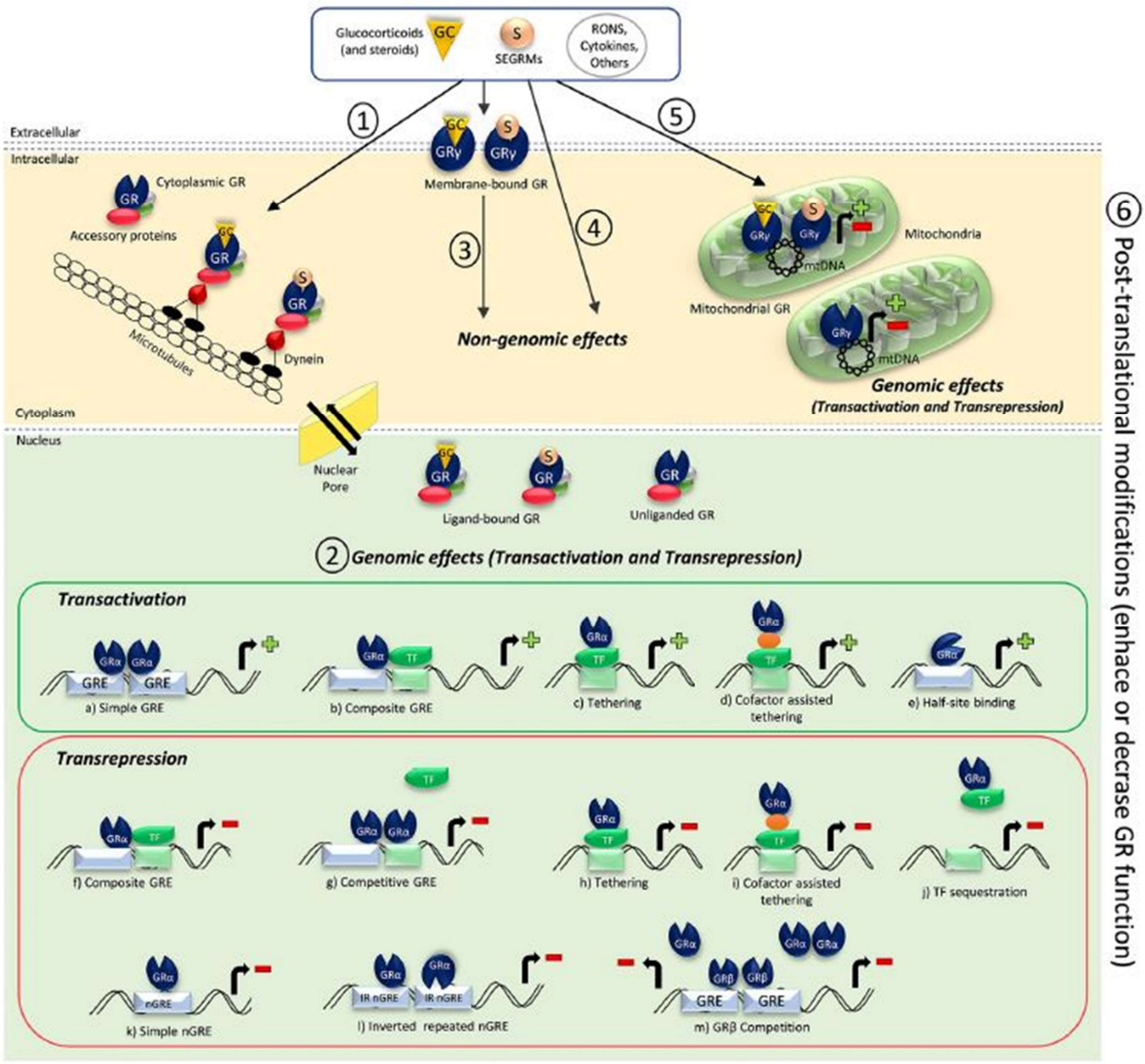

Figure 2: GC or other steroid hormones impact cells on severe levels. Direct interaction of GR GRE and nGRE motifs is also known as *cis*-regulation. Selective glucocorticoid receptor modulators (SEGRMs) are nonsteroidal molecules, which are able to interact with GR. Reactive oxygen and nitrogen species (RONS) and cytokines are able to trigger GC-independent effects of GR. Taken from *Scheschowitsch et al. 2017*. **Abbreviations:** GC – glucocorticoid**,** GR – glucocorticoid receptor, GRE – glucocorticoid response element, RONS – reactive oxygen and nitrogen species, SEGRM - selective glucocorticoid receptor modulator, TF – transcription factor

Some isoforms of GRs also execute non-genomic effects. In this case, GR function as a receptor trigerring signaling cascades in a cell. In most cases, membraneous types are responsible for non-genomic effects (Fig. 2).

All isoforms of GR origin from 1 gene constituted from 9 exons (Fig. 3). Alternative splicing and initiation of a translation enable expression of several variants. After translation, individual isoforms can be modified by phosphorylation, binding of small ubiquitine-like molecule (SUMO, SUMOylation) or ubiquitination, determining the localization of GRs in a cell and the GR lifespan.

The majority of GR is actually localized in cytosol, however, there are also evidences for nuclear and membraneous localization (Hollenberg et al. 1985; Pérez et al. 2013). Distinct representation of GR isoforms offers a possible explanation for differences in the sensitivity and reactivity of various tissues to GC (Oakley & Cidlowski 2011). Polymorphism in Nr3c1 gene is also partly responsible for variations in responses to GC treatment, which can lead to a GC dependence or resistance (Krupoves et al. 2011).

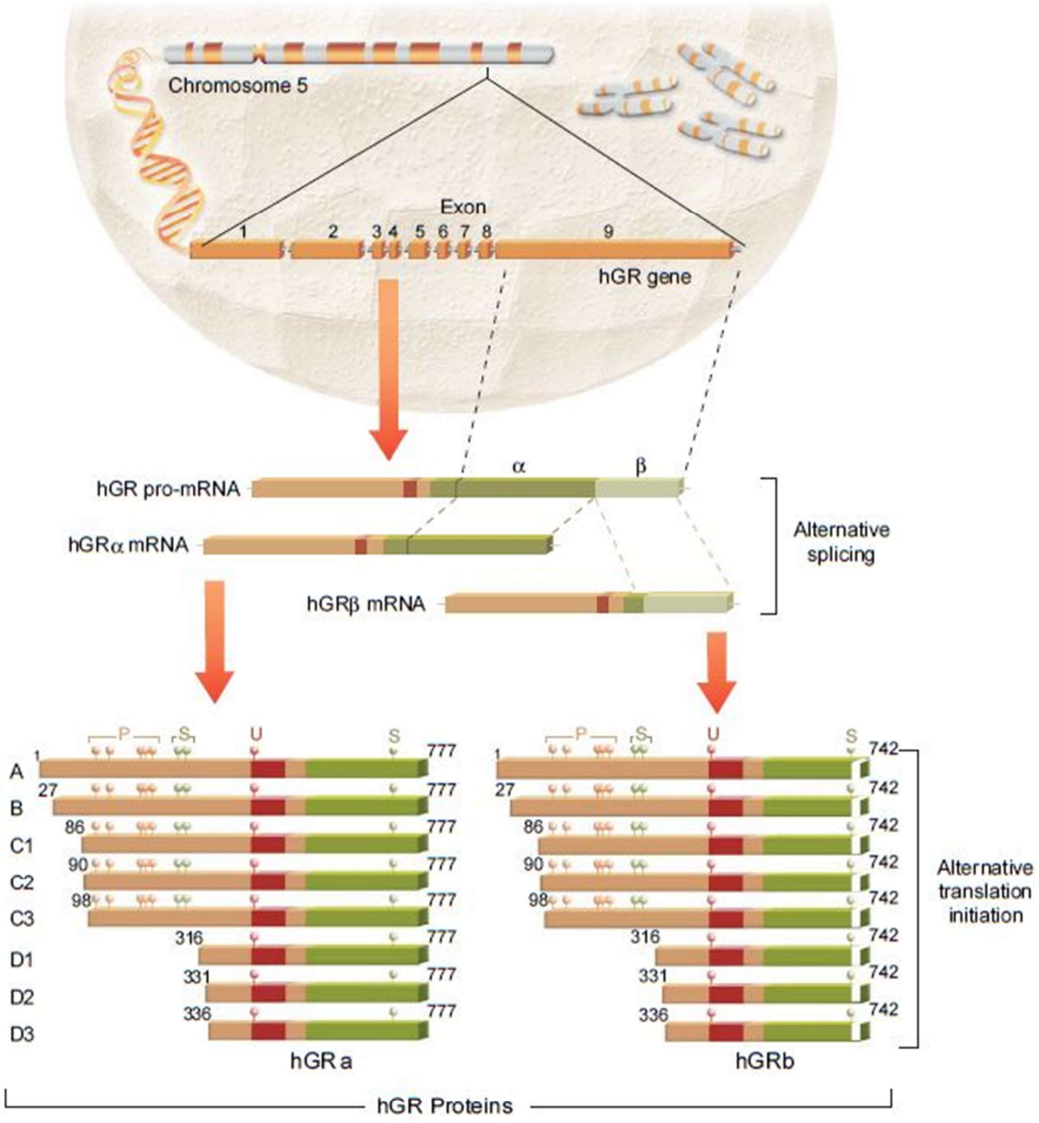

Figure 3: A plethora of GR isoforms is generated from 1 gene. Taken from *Lu & Cidlowski 2006.* **Abbreviations:** hGR – human glucocorticoid receptor, mRNA – messenger ribonucleic acid

3. Adrenal glands as a systemic source of glucocorticoids

Systemic production of GCs is localized into the cortex of adrenal glands, particularly into zona fasciculata and reticularis. Zona reticularis produces also androgens, zona glomerulosa MCs. Major signal triggering production and secretion of GCs is adrenocorticotropic hormone (ACTH), which binds to to melanocortine receptor 2 (MCR2). ACTH is released from the anterior pituitary during a stress reaction activating hypothalamic-pituitary-adrenal (HPA) axis.

However, adrenal glands also express receptors recognising microbial structures (for detail see chapter 5) indicating a possible direct immune activation of adrenal glands. Lack of these receptors affect production of GCs resulting in altered systemic immune reaction (Bornstein et al. 2004; Zacharowski et al. 2006).

3.1 *De novo* steroidogenesis

The biosynthetic pathway of steroid hormones is known as a steroidogenesis. The whole steroidogenesis contains several reaction steps provided by distinct enzymes (Fig. 4). All enzymes are members of a cytochrome P450 or dehydrogenase families.

Although, individual steroid hormones differ in a plethora of features, all have a common precursor – cholesterol.

3.1.1 Cholesterol – a precursor of steroid hormones

Cholesterol can be obtained from several possible sources. First one is an uptake from circulating low- or high-density lipoprotein particles (LDL, HDL). Cells can also use own lipid droplets containing cholesterol esters or utilize cholesterol stored in membranes. Finally, cholesterol can be *de novo* synthetized in endoplasmic reticulum (Balasubramaniam et al. 1977; Rainey et al. 1986; Lange 1991; Kraemer et al. 2007).

Next steps of steroidogenesis are localized into the endoplasmic reticulum or the inner membrane of mitochondria.

Transport of cholesterol through cytosol, but also membranes, requires auxiliary mechanisms. Transcellularly is cholesterol transported in vesicles, lipid droplets or bound to transport proteins (Londos et al. 1995; Heino et al. 2000).

Transporting cholesterol from the cytosol into the mitochondrion is provided by intermediate filaments, mostly by steroidogenic acute regulatory protein (StAR), but also e.g. vimentin (Shen et al. 2017). It is a crucial and a rate limiting step of a whole process. Correct delivery of StAR protein to mitochondrion is ensured by N-terminus mitochondrial targeting sequence. However, although the mitochondrial targeting sequence is localized on the N-terminus, its deletion doesn't affect transport a lot. Surprisingly is steroidogenesis more affected by mutations of the

C-terminus (Privalle et al. 1983; Clark et al. 1994; Arakane et al. 1996). Major transcription factor of StAR is a cyclic adenosine monophosphate response element (CRE), interacting with CRE binding sequence (CREB) on a DNA molecule.

3.1.2 Steroidogenic enzymes

Transporting cholesterol to the inner mitochondrial membrane leads to a next step (Fig. 4), which is provided by a cytochrome side-chain cleavage enzymatic complex (cytochrome P450scc, CYP11A1) localised in the inner mitochondrial membrane. Cytochrome P450scc includes a flavoprotein (reduced nicotinamide adenine dinucleotide-adrenodoxin reductase (NADH-adrenodoxin reductase)), a ferredox (adrenodoxin) and a cytochrome P450 (Müller et al. 2001). CYP11A1 reaction consists of 3 parts. C20 and C22 of the cholesterol molecule are being hydroxylated. In the first reaction 22R-hydroxycholesterol arises, second reaction produces 20α22R-dihydrocholesterol. After hydroxylation of a cholesterol molecule, a bond between C20 and C22 is being cleaved. Dissociation of 6C long side chain (isocaproid aldehyde) produces a 26C product - pregnenolone. After the StAR protein reaction, this is a second rate limiting step.

Human pregnenolone can be hydroxylated at C17 by 17α-hydroxylase (HSD17A, CYP17) (Fig. 4). The hydroxylation of C17 results in 17α-hydroxypregnenolone. CYP17 than continue with a lyase reaction producing dehydroepiandrosterone (DHEA). The lyase activity is downregulated in zona fasciculata, but not in zona reticularis of adrenal cortex.

Pregnenolone and 17-hydroxypregnenolone are then converted by 3β-hydroxysteroid dehydrogenase (HSD3B1) to progesterone and 17-hydroxyprogesterone (Fig. 4).

The enzyme 21-hydroxylase (HSD21A1, CYP21) then produces 11-deoxycorticosterone or 11-deoxycortisol (Fig. 4), which are final precursors for corticosterone and cortisol, respectively.

The last hydroxylation is mediated by the first isoform of 11β-hydroxylase (CYP11B1) (Fig. 4). The second isoform of CYP11B, the so-called aldosterone synthase, provides a conversion of corticosterone to aldosterone.

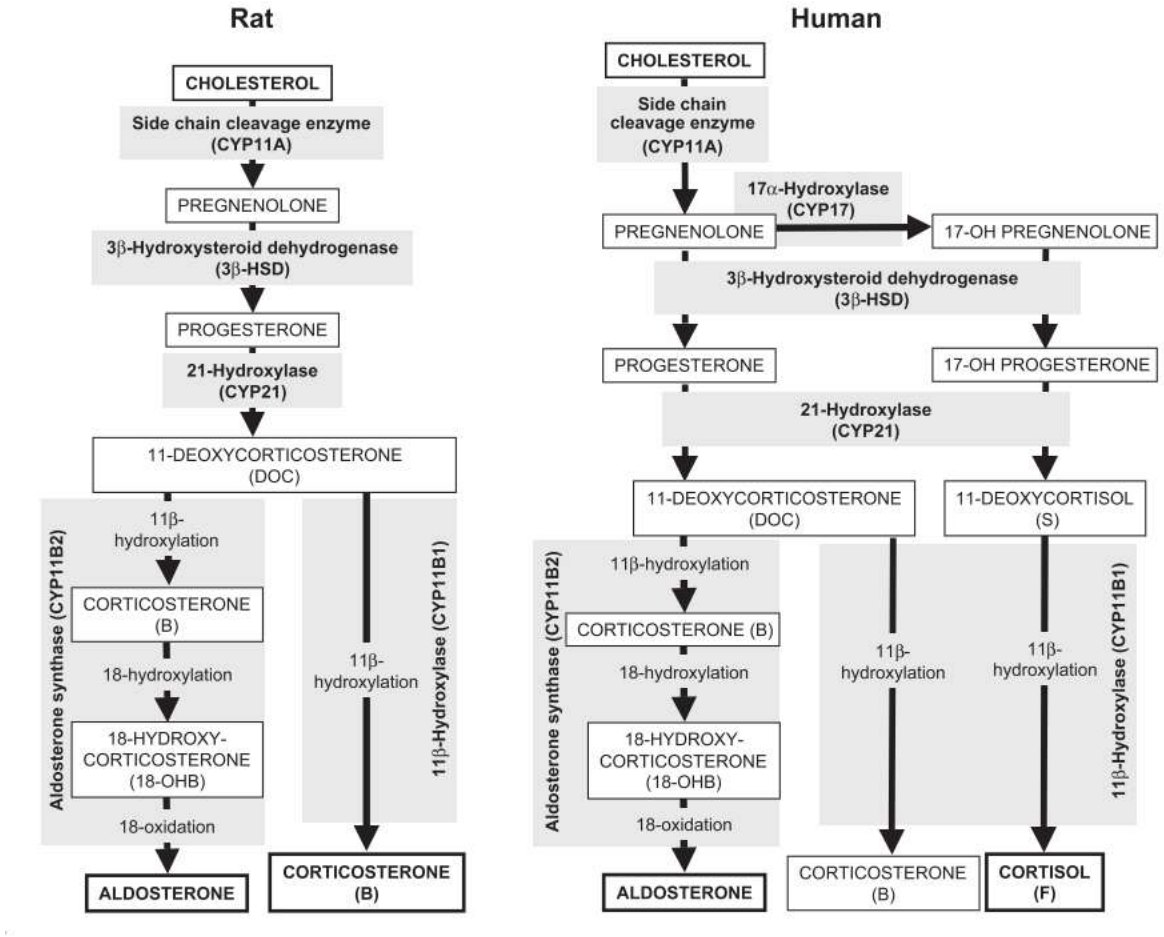

Figure 4: Scheme of steroidogenetic pathway leading to production of GCs and MCs. Taken from *Davies & Mackenzie 2003.* **Abbreviations:** 3βHSD - 3β-hydroxylase, CYP11A – cytochrome side-chain cleavage enzymatic complex, CYP11B1 and 2 - 11β-hydroxylase 1 and 2, CYP17 - 17α-hydroxylase, CYP21 - 21-hydroxylase

3.1.3 Regulation of *de novo* steroidogenesis

The signalling pathway leading to a systemic steroidogenesis is initiated by binding ACTH to MCR2, coupled with G-proteins activating adenylyl cyclase (AC). Activated AC than produces cyclic adenosine monophosphate (cAMP). Sufficient amount of cAMP triggers cAMP dependent pathways. The main signalling process leading to the expression of steroidogenic enzymes is a pathway via protein kinase A (PKA) resulting in phosphorylation and thus an activation of many proteins, including transcription factors of steroidogenic enzymes (Æsøy et al. 2002; Zhou et al. 2016). Another mechanism involved in the expression of steroidogenic enzymes in adrenals represents the protein kinase C (PKC) pathway, which reacts to angiotensin II and adenosine (Bird et al. 1996; Chen et al. 2010). In human, the PKC pathway seems to have distinguishable effects depending on a presence of other substances. The presence of angiotensin II results in the increased expression of CYP17 and HSD3B1, supporting the production of both, GC and aldosterone. However, a combination with forskolin

(activating AC), angiotensin II down-regulates the expression of CYP17, while still up-regulates the expression of HSD3B1, which probably supports the production of aldosterone to the detriment of cortisol (Bird et al. 1996). Angiotensin II has been also shown to activate the extracellular signal-regulated kinase (ERK) pathway, which is also involved in regulation of steroidogenesis. The ERK pathway can be triggered by the Janus kinase (JAK) pathway (Li et al. 2003).

In adrenals and other tissues is expression of steroidogenic enzymes, MCR2 and other proteins involved in a regulation of steroidogenesis, under the control of several transcription factors. One of the most important is CREB, which is localized in the nucleus, where it can be activated trough phosphorylation e.g. by PKA (Parker et al. 1996).

Another important transcription factor is steroidogenic factor 1 (SF1). SF1 is expressed in canonical steroidogenic organs, such as adrenal and gonadal glands and placenta. In a small amount, there is also expressed its second isoform, liver receptor homologue 1 (SF2, LRH1), in the adrenals. However, SF1 is irreplaceable with LRH1 in adrenal development (Zubair et al. 2006). SF1 probably interacts with other transcription factors including CREB, proteins from GATA family and CCAAT/enhancer binding proteins. Some evidences support the theory, that SF1 expression is under the control of GATA (Tevosian et al. 2015).

4. Extraadrenal sources of glucocorticoids

Despite adrenal glands, the biogenesis of corticosteroids has also been observed in other tissues like skin (Tiganescu et al. 2014; Vukelic et al. 2011), intestine (Noti et al. 2010; Wang et al. 2013), lungs (Hostettler et al. 2011; Simard et al. 2010), brain (Mackenzie et al. 2000; Ye et al. 2008), heart (Silvestre et al. 1998), thymus (Qiao et al. 2008). Though, extraadrenal sources of corticosteroids exist, they can never fully replace the adrenal one. The importance of systemic steroidogenesis can be proved in adrenalectomized mice, which are not able to resist inflammations (Cima et al. 2004). A low level of GCs in adrenalectomized individuals also indicate that a local steroidogenesis may not be mediated by systemic ACTH, even if it cannot be excluded that local ACTH system operates in some tissues, particularly in skin (Slominski et al. 1996). ACTH-independence is demonstrated by the absence of its receptors in the intestine (Mueller et al. 2007). Conversely, regulation of skin steroidogenesis shares some similarities with HPA axis (Slominski et al. 1996; Lytinas et al. 2003). Expression of POMC and production of ACTH have been observed in keratinocytes and melanocytes and increased after CRH treatment (Rousseau et al. 2008).

4.1 De novo steroidogenesis and its regulation in the intestine and lymphatic organs

4.1.1 Intestine

Intestinal tract represents a large surface containing diverse microbiome. Data from the Metagenomics of the Human Intestinal Tract indicates that human gut is able to host up to ten trillion microbial cells (www.metahit.eu). Thus, efficient, but well regulated, intestinal immune system is a necessity. Several cell types have been shown to produce GCs, probably to control immune functions. Simultaneously has been proven, that microbiome affects expression of some steroidogenic enzymes in intestine (Mukherji et al. 2013).

The expression of steroidogenic enzymes has been observed particularly in the epithelial cells from the crypts of small intestine (Cima et al. 2004; Mueller et al. 2007; Mukherji et al. 2013). Besides that, expression of steroidogenic enzymes has been observed in T-lymphocytes isolated from the small intestine (Cima et al. 2004) and in macrophages and T-lymphocytes from the large intestine (Noti et al. 2010). Expression of colonic epithelial cells is not clear, in some studies was detected (Noti et al. 2010), when is others not (Mukherji et al. 2013).

Intestinal isoform of steroidogenic factor slightly differs from the adrenals' one. There hasn't been observed any expression of Sf1 in the intestine, but Lrh1. In adrenals, PKA pathway leads to an increase of Sf1 expression, whereas intestinal LRH1 only vary between active and inactive state (Lee et al. 2006; Kulcenty et al. 2015). Dependence of intestinal inflammations relevancy

can be demonstrated by using Lrh1 $^{+/+}$ and Lrh1$^{+/-}$ mice, when Lrh1 $^{+/+}$ show milder course of inflammation with faster regenerative ability. The difference between Lrh1 $^{+/+}$ and Lrh1$^{+/-}$ mice consists of increased expression of Il-1β and Il-6 in Lrh1$^{+/-}$ individuals simultaneously with decreased expression of Cyp11a1 and Cyp11b1. A similar expression profile can be measured on patients with IBD. The level of Tnfα is also increased in human (Coste et al. 2007).

The activation of LRH1 requires phosphorylation of a hinge domain provided by ERK, which facilitates tethering of the hinge, ligand binding and DNA binding domains. ERK also activates SF1 by phosphorylation of its hinge domain, though targeted serine residues of LRH1 does not correspond to those of SF1. The ERK function can be triggered by PKC as a result of phorbol myristate (PMA) treatment (Lee et al. 2006). The PMA-mediated expression of steroidogenic enzymes have been observed in the intestine, but not the adrenals (Mueller et al. 2007). In contrast, SUMOylation inhibits activity of LRH1.

The intestinal epithelium displays also expression of proteins of CRH/ACTH regulatory pathway (Mahajan et al. 2014) but the local, intestinal, regulation of steroidogenic enzymes by ACTH has not been ascertained in isolated enterocytes. In contrast, the effect of cAMP-dependent PKA pathway influenced the enzymes of intestinal steroidogenesis but its effect has been opposite in the intestine compared to adrenal glands. In epithelial cells PKA pathway inhibited the expression of steroidogenic enzymes whereas it is associated with stimulation in adrenal glands (Mueller et al. 2007). It may reflect distinct regulation of transcription factors SF1 and LHR1.

It has been shown, that the expression of some steroidogenic enzymes in ileum is under the control of clock genes, whose expression is modulated by microbiome (Mukherji et al. 2013).

4.1.2 Lymphatic organs and immune cells

Lymphatic organs can be divided into two groups, primary and secondary. Primary lymphatic organs are responsible for differentiation and maturation of immune cells and include thymus and bone marrow. The thymus produces T-lymphocytes, while bone marrow B-lymphocytes and innate immune cells. Mature immune cells move into secondary lymphatic organs, spleen, and mucosa-associated lymphatic tissue (MALT) including lymphatic organs surrounding gut such as mesenteric lymph nodes (MLNs), Peyer's plates, caecum and intraepithelial immune cells, collectively called as gut associated lymphoid tissue (GALT). Centralisation in secondary lymphatic organs enables fast communication between immune cells and thus also efficient reaction to antigens followed by initiation of inflammation (Rescigno et al. 2001).

Some studies indicate, that GCs participates on selection, differentiation and apoptosis of T-lymphocytes and thus also on development of thymus (Brandt et al. 2007; Mittelstadt et al. 2011). Increasing expression of steroidogenic enzymes together with age supports theories suggesting involvement of GC in apoptosis of T-lymphocytes and age-related involution of thymus. Conversely to that, the steroidogenetic activity in thymic epithelial stromal cells decreases, which indicates distinct regulatory mechanisms between these two cell types (Qiao et al. 2008; Taves et al. 2016).

Spleen is the largest mammalian lymphatic organ. It can be divided into two parts, red and white pulp. Both pulps are amply vascularised. White pulp serves, analogically to lymphatic nodes, as a lymphatic tissue, which hosts B- and T-lymphocytes. Red pulp removes and destructs abraded or nonfunctional erythrocytes and provides for the lymphocytes an access from white pulp to blood and vice versa. Between the two pulps is an area containing B-cells, macrophages and dendritic cells (DC). T-cells represents a proven source of GC in the spleen (Mahata et al. 2014; Li et al. 2015). Expression of some steroidogenic enzymes have been found also in endothelial cells (Morohashi et al. 1999).

Expression of SF1 has been detected also in splenic endothelial cells, although it probably does not control the expression of steroidogenic enzymes (Morohashi et al. 1999). Nevertheless, in splenic endothelial cells act SF1 as a crucial transcription factor during a development, but the expression continues also in adult individuals. Mutation in Sf1 is related to maldevelopment and altered architecture of the spleen in mouse and complete asplenia in human (Zangen et al. 2014; Katoh-Fukui et al. 2017; Morohashi et al. 2017).

Th2 lymphocytes from MLN and spleen of mice infected with parasite have been observed to upregulate expression of proteins involved in synthesis and transport of cholesterol and the expression of Cyp11a1 coding a protein converting cholesterol into pregnenolone. None of mRNA of other steroidogenetic enzymes was detectable during that study. However, coexpression of Cyp11a1 and mRNAs of anti-inflammatory cytokines and subsequent suppression of Cyp11a1 and cytokine transcription by neutralizing antibody led authors to the presumption that pregnenolone produced in CYP11A1 reaction also acts as an anti-inflammatory agent. The assumption can be supported by the fact, that pregnenolone also inhibited proliferation of Th, Tc and B-lymphocytes (Mahata et al. 2014).

4.2 Local regeneration and its regulation

Considerable effect of GC on every tissue, including the brain, requires a precise regulation, which can't be ensured only by steroidogenesis as an only one regulatory mechanism. It has

been shown, that GC can circulate in the bloodstream also as biologically inactive forms. Human main active glucocorticoid is cortisol, which can be converted to inactive cortisone, its 11-oxoderivate. Similarly, corticosterone can be oxygenated to 11-deoxycorticosterone. Biologically inactive forms interact with neither GR nor MR due to their low binding affinity to receptors. The ratio between biologically active and inactive form varies during the day and between tissues (Morineau et al. 1997). The conversion of active GC to inactive one and vice versa is provided by 2 isoforms of 11β-hydroxysteroid dehydrogenase (11βHSD, HSD11B) (Fig. 5).

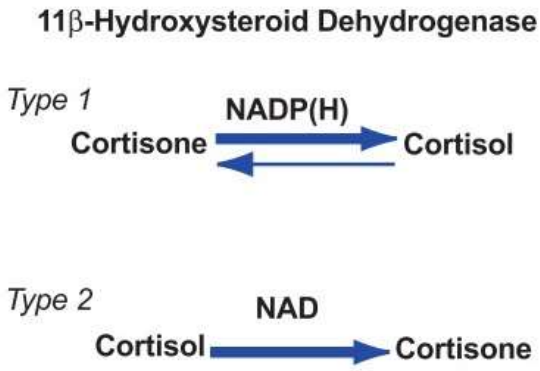

Figure 5: Local metabolism of glucocorticoids by two isoforms of HSD11B. As acceptors/donors of electrones are used $NADP^+(H)$ and NADH. Taken from *Tomlinson & Stewart 2001*. **Abbreviations:** NAD^+ - nicotinamide adenine dinucleotide, NADP(H) - nicotinamide adenine dinucleotide phosphate

HSD11B1 utilizes $NADP^+(H)$ as a cofactor and is able to work in both directions depending on redox potentials of $NADP^+$/NADPH regulated by hexose-6-phosphate dehydrogenase (Lavery et al. 2006).

HSD11B1 is expressed e.g. in heart (Mazancová et al. 2005; Small et al. 2005), vascular smooth muscle (Brem et al. 1995), adipose tissue (Mariniello et al. 2006), gonads (Leckie et al. 1998; Yong et al. 2002), spleen (Moore et al. 2000) bone (Cooper et al. 2018) and skin (Tiganescu et al. 2011).

Conversely to HSD11B1, which is able to catalyse oxidation and reduction, HSD11B2 works only as dehydrogenase, which oxidases corticosterone and cortisol to their 11-oxo derivates and thus decreases in the cell the glucocorticoid signal.

HSD11B2 is typical for the epithelia and placenta. It is assumed, that HSD11B2 is often situated to the target tissues of MCs. The colocalization of HSD11B2 and MR has been demonstrated in several epithelial tissues, e.g. in the intestine or kidney (Hirasava et al. 1997). Because of a higher affinity and plasma higher level of GC than MC, MR would primarily

interact with GC. It means to keep ion and water homeostasis it is desirable for the MR to bind aldosterone and not GC (Gomez-Sanchez & Gomez-sanchez 2015).

4.2.1 Intestine

Levels of HSD11Bs differ along the intestine. The activity of HSD11B2 is generally high in tissues expressing MR, such as colon, to maintain ion and water homeostasis (Rubis et al. 2006).

The expression of Hsd11b1 is detectable along the small intestine and rapidly rises after immune stimulation, e.g. with anti-CD3. The upregulated expression affects surroundings immune cells (Cima et al. 2004).

However, differences have been observed also between individual cell types. The expression of colonic Hsd11b1 is localized into intestinal immune cells (macrophages, lymphocytes, DCs and fibroblasts), but has not been observed in the epithelium (Whorwood et al. 1994; Vagnerová et al. 2005). Conversely, the expression of Hsd11b2 has been observed only in epithelial cells, not in immune cells or cells of lamina propria. Interestingly, Hsd11b2 is expressed more likely in the matured cells of intestinal surfaces, than in crypts (Whorwood et al. 1994; Pácha et al. 2002). The occurrence contrasts with enzymes of *de novo* steroidogenesis, which are mostly expressed in the epithelial crypts.

Levels of Hsd11b mRNA fluctuate during inflammations. It is assumed, that maintaining levels of active and inactive forms of GCs provides an effective mechanism to control adequate inflammatory reaction (Žbánková et al. 2007).

4.2.2 Lymphatic organs and immune cells

HSD11B1 seems to affect viability of immune cells and a development and function of lymphatic organs. As mentioned above, GC probably participates in development and involution of the thymus. The ability of Th and Tc cells to express Hsd11b1 indicates, that T-lymphocytes are able to control their own viability and regulate the process of selection. Conversely, presence of inactive GCs supports the production of antiapoptotic factors and receptor of interleukin 7 (IL-7), which supports proliferation. Thus, presence of inactive GCs seems to support proliferation and survival of T-lymphocytes (Zhang et al. 2005). This hypothesis is supported by results of study performed on young and adult mice proving age-dependent increase of *de novo* steroidogenesis, but also the regeneration (Qiao et al. 2008). However, in some cases can locally produced or regenerated GCs facilitate immune activation of T-cells, as seen after exposure to anti-CD3. Authors assume, that the effect depends on whether the activation is provided indirectly with help of APCs or directly via interaction of.

The activation via APC is presumed to be inhibited by GCs, while direct activation of lymphocytes with antibodies is intensified by GCs (Cima et al. 2004).

However, local regeneration of GCs also critically affects differentiation and maturation of innate immune cells, particularly monocytes. It seems, that human monocytes during physiological conditions don't express any isoform of Hsd11b. However, the expression rises during differentiation into macrophages, probably as a reaction to Th2 cytokines IL-4 and IL13. It is possible, that increased local regeneration of GCs serves as a self-control mechanism preventing excessive production of pro-inflammatory cytokines by macrophages (Thieringer et al. 2001).

Hsd11b1 is abundantly expressed also in spleen and MLNs and other components of GALT. The local regeneration seems to be a preferred mechanism used to generate GCs in lymph organs, rather than *de novo* steroidogenesis. The probable reason is a requirement of mechanism dynamically reacting to inflammatory reaction (Taves et al. 2016). The idea is supported by experimentally induced chronic inflammation during which expression of Hsd11b1 is upregulated in secondary lymphatic organs (Ergang et al. 2011).

More detailed examination of MLN showed, that during chronic inflammation rises the expression of Hsd11b1 only in the MLN cortex, but not in paracortex or in medulla (Ergang et al. 2017). The cortex hosts primarily B-cells supported by stromal cells, though DCs, epithelial cells and a smaller fraction of T-cells are also presented (Willard-Mack 2006). However, the fact, that the expression rose only in the cortex, but not in other anatomical compartments of MLN, led to the assumption that upregulation of Hsd11b1 by inflammation does no occur DCs and stromal cells (Ergang et al. 2017). Although, in another study, was the expression after activation with CD-3 observed in contrary only in stromal cells of immune organs, not in any immune cell type (Hennebold et al. 1996).

It has also been shown, that local regeneration support proliferation and survival of both, B- and T-cells via induction of antiapoptotic factors and receptors for interleukin 7 (IL-7), which is known to support proliferation (Zhang et al. 2005).

5. Implications of GCs and gut microbiota for immune functions

The immune system and systemic chronic inflammation are strongly shaped by hormonal milieu, especially glucocorticoids, and by gut microbiota. An immunosuppressive effect of GCs is used to treat skin, gut or lung inflammations like eczema, inflammatory bowel disease, asthma. The effect of GC on immune function is abundantly used in medicine since Hench's first observation of therapeutic effects of GC on rheumatoid arthritis (Hench et al. 1949).

Similarly, the gut microbiota and its metabolites have been shown to influence immune functions directly via bacterial translocation across the intestinal barrier or indirectly via interaction of bacteria or their metabolites with specific receptors localized on the cells of GI tract.

5.1 Intestinal microbiome

Intestinal and other surfaces are being settled by microbiota since the moment of passing the birth canal. Early settlement is crucial for morphological, but also behavioural development. Infants, which were born by caesarean section have later less fraction of commensals (bifidobacterial, *Bacteroides fragilis*) in the gut that vaginally delivered ones. Conversely, pathogenic strains (*Clostridium difficile*) tend to be increased after caesarean section (Penders et al. 2008).

Commensals can be beneficial to support digestion, production of vitamins and of substances toxic for pathogens, but also to protect epithelial barriers from lesion and increase of permeability, due to competition for space and resources with pathogens and from inadequate immune response to pathogens (Rakoff-Nahoum et al. 2004; O´Mahony et al. 2008; Stefka et al. 2014). On the other hand, pathogens danger is based on an infection, invading trough an epithelial barrier or a production of unknown or toxic substances.

Epithelial surfaces protect inner environment of organism as a mechanical barrier. Epithelial cells are being closely connected by tight junctions (TJ), which confine massive paracellular flow of liquid to the lumen, but also a free intrusion of microorganisms into the body. Thus, microbial structures can be recognised by receptors of immune cells belonging to GALT (Cebra et al. 1998). In severe cases microorganisms get into lymph and blood and interact with immune cells of peritoneal lymphatic organs, such as splenocytes (Zarember & Godowski 2002). Microbial structures can be also carried to immune organs by intestinal DCs after phagocytosis of microbes (Rescigno et al. 2001; Brandl et al. 2007).

Perforations of the barrier resulting in bacteraemia leading to extensive inner inflammations typically occur in Crohn's disease. A potential disruptor of TJs causing an protrusions of epithelial barrier during Crohn's disease is assumed to be the pro-inflammatory cytokine TNFα (Ma et al. 2004).

Crohn's disease has been traditionally treated by GCs. However, the treatment is not always effective, in some cases develops the resistance to GCs (Canani et al. 2006; Krupoves et al. 2011). Some studies have shown, that syndromes of inflammatory bowel diseases such as

Crohn's disease and ulcerative colitis can be more successfully treated using commensals compared to GCs' treatment (Sartor 2004; Canani et al. 2006; Ait-Belgnaoui et al. 2012).

Possibly, commensals modulate inflammation via controlling the proliferation and maturation of immune cells and production of cytokines. Commensal species *Bifidobacterium infantis* and *Lactobacillus reuteri* (*L. reuteri*) mediate DCs to produce transferring growth factor-β (TGF-β) and interleukin-10 (IL-10), which are known to support maturation of regulatory T lymphocytes (Treg). Also, applying *L. reuteri* as a treatment reduced production of TNFα (O´Mahony et al. 2008; Karimi et al. 2009).

Regulation by Tregs probably consists in a production of IL-10 supressing T helper cell 17 (Th17) mediated inflammations (Chaudhry et al. 2011; Lennon et al. 2014). The proliferation of Treg cells and production of IL-10 can be supported by short chain fatty acids (SCFA; acetic, propionic a butyric acids), which are produced during microbial fermentation processes (Smith et al. 2013).

5.2 Molecular mechanism of microbial recognition

Fast recognition of unknown substances is a necessity for successful immune reaction. Therefore, innate immune cells and epithelial cells play a crucial role. Neutrophils, basophils, eosinophils, natural killer cells, macrophages, but also DCs, epithelial cells and keratinocytes, express on their surfaces or in cytosol pattern recognition receptors (PRRs) (Miller 2009). PRRs interact with conserved microorganismal molecules, which are known as microbiota-associated molecular patterns (MAMPs), which can be divided into two groups. The first group includes molecules characteristic for commensals, second for pathogens (PAMPs). Interactions of PAMPs and PRRs initiate a signalling pathway activating expression of cytokines and other proteins involved in inflammation (Blander & Medzhitov 2006). Besides microbial structures, PPRs also react with some endogenous components, such as components of extracellular matrix or damaged cells. These endogenous particles are known as damaged-associated molecular patterns (DAMP).

5.2.1 Toll-like receptors

The MAMPs interact with receptors on the host cells, which belong to several families. The most known group of PRRs is the family of Toll-like receptors (TLRs) (Rakoff-Nahoum et al. 2004). Another considerable group of receptors recognising molecular patterns is a nucleotid binding oligomerization domain (NOD) family (Takeda & Akira 2005).

13 members of the TLR family have been discovered up to now. Common features of all TLR family are ligand binding leucin-rich repeat (LRR) domain, hydrophobic transmembrane

domain and Toll/interleukin-1 receptor (TIR) domain, which is named after homology with interleukin-1 receptor.

Even though, TLR contains a hydrophobic transmembrane domain, not every TLR is expressed on the cell surface. TLR 3, 7, 8, 9, 11, 12 and 13 are localized in endosomes or lysosomes (Matsumoto et al. 2003; Heil et al. 2003; Sturge et al. 2013; Song et al. 2015).

Each TLR recognizes a specific spectre of PAMPs and DAMPs, as summarized in Table 1.

TLR	PAMP	DAMP	Human	Rat	Mouse
1	di- ,triacylated lipopeptides		✓	✓	✓
2	lipoproteins, lipopeptides	VLDL	✓	✓	✓
3	dsRNA	HMGB1	✓	✓	✓
4	LPS	FNEDA, HMGB1, Tenascin-C	✓	✓	✓
5	Flagellin		✓	✓	✓
6	di- ,triacylated lipopeptides		✓	✓	✓
7	ssRNA		✓	✓	✓
8	unmethylated CpG DNA, dsRNA		✓	✓	✓
9	IgG-chromatin complex	DNA, HMGB1	✓	✓	✓
10	Lipopeptides		✓	✓	
11	Profilin			✓	✓
12	Profilin			✓	✓
13	23s rRNA			✓	✓

Table1: Table shows the ligand specificity of TLRs. It also points on TLR10, which is a pseudogene in a mouse as TLR11 in human. Human completely lack of TLR12 and TLR13. Data taken from *www.uniprot.com* database and *Piccinini & Midwood 2010.* **Abbreviations:** DAMP - damaged-associated molecular patterns, DNA – deoxyribonucleic acid, dsRNA – double strand ribonucleic acid, FNEDA - fibronectin extra domain A, IgG – immunoglobulin G, HMGB1 - high mobility group box 1 protein, LPS – lipopolysaccharide, rRNA – ribosomal ribonucleic acid, ssRNA – single strand ribonucleic acid, VLDL – very low-density lipoprotein

Recognition of a broad spectrum of PAMPs and DAMPs is not enabled only by the variety of TLR isoforms. Some isoforms can also form homo- or heterodimers or cooperate with other unrelated receptors. Recognition of multitudinous microbial components then results in a specific immune reaction.

5.2.2 Signalling pathways from Toll-like receptors to cytokines

After activation, each TLR couples with adapter proteins. Mainly used adapter protein are MyD88 and TIR-domain-containing adapter-inducing interferon β (TRIF).

MyD88 is an adapter molecule. Its TIR domain is able to couple with TIR domains of all TLR isoform, except for TLR3 (Yamamoto et al. 2002). Simultaneously, is MyD88 via its death domain (DD) able to interact with a DD of interleukin-1 receptor associated kinase 1 and 4 (IRAK1 and IRAK4) (Neumann et al. 2007). The complex can be phosphorylated by the IL-1 receptor-associated kinase M (IRAK-M) operating as a negative regulator of TLR signalling by trapping IRAK1 in the activated receptor complex and preventing downstream signalisation (Kobayashi et al. 2002). On the other hand, autophosphorylation and phosphorylation provided by IRAK4 enables a release of IRAK1 from the complex and coupling to ubiquitin ligase tumour necrosis factor receptor associated factor 6 (TRAF6). Activated TRAF6 ubiquitinates and thus activates the complex constructed from mitogen-activated protein kinase kinase kinase 7 (MAP3K7, TAK1) and TGFβ activated kinase 1, 2 and 3 (TAB1, TAB2 and TAB3). Activated complex than phosphorylates IκB kinase α and β (IKKα and IKKβ) and NFκB essential modulator (NEMO) complex. Phosphorylated complex of IKKα, IKKβ and NEMO phosphorylates NFκB inhibitors (IκBs), which remarks them for ubiquitination, release from a complex with NFκB and degradation. Finaly, NFκB can translocate into the nucleus (Fig. 6) (Qian & Cao 2013).

TAK1 protein also interacts with mitogen-activated protein kinase (MAPK), which than activates c-Jun N-terminal kinase (JNK). JNK phosphorylates c-Jun. C-Jun together with c-Fos act as transcription factors also known as AP1. AP1, together with NFκB than function as transcription factors of target genes (e.g. proinflammatory cytokines) (Fig. 6) (Qian & Cao 2013).

Forming a complex of TRIF, TRAF3, TRAF6 and receptor-interacting serine/threonine-protein kinase 1 (RIP1) represents an alternative pathway leading from TLR3 and TLR4 to activation of TAK1 and consequently to the activation of transcription factors NFκB, MAP kinase pathway and interferon regulatory factor 3 (IRF3) (Takeuchi et al. 2000).

These pathways lead to phosphorylation, dimerization and translocation of STAT1 to the nucleus, where they act as transcription factors regulating expression of cytokines. Beside the direct activation pathways involving MyD88 and TRIF, STAT1 can be also activated indirectly, via expression of cytokines, such as type 1 interferon (IFN1). The receptor of IFN1 can activate JAK, which than phosphorylates STATs (Rhee et al. 2003; Luu et al. 2014).

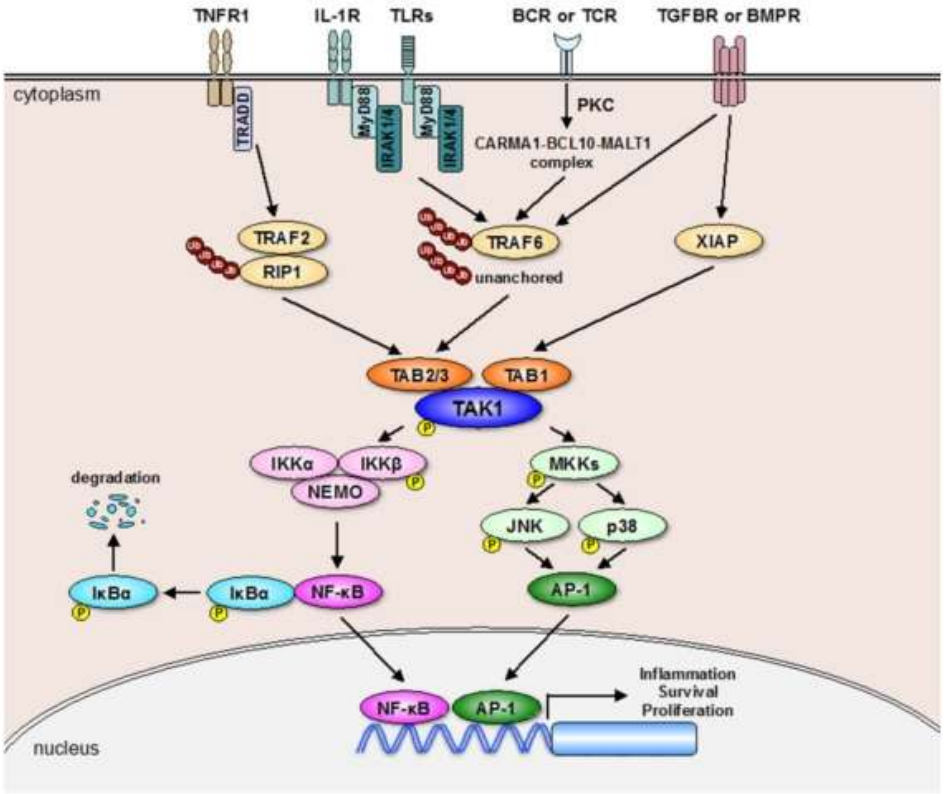

Figure 6: Figure shows signalling pathways leading from TLR to expression of cytokines. Pathways can be activated via interaction of cytokines, microbial structures, bone morphogenic protein (BMPR) or antigenes of B- and T-cell receptors with their receptors (BCR and TCR) expressed in immune cells. Receptors activate downstream pathways directly or undirectly, by interaction with adapter proteins. Next signallisation can activate expression of cytokines. It can be also activated an antiapoptotic pathway via X-linked inhibitor of apoptosis protein (XIAP). Taken from *Hirata et al. 2017.* **Abbreviations:** AP1 - activator protein 1, BCR – B-cell receptor, BMPR – bone morphogenic protein receptor, IκBα - nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α, IKK-α and β - nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α kinase α and β, IL-1R – interleukin 1 receptor, JNK – Janus kinase, MKKs - mitogen-activated protein kinase kinase, PKC – protein kinase C, TAB1, 2, 3 –transforming growth factor β-activated protein kinase 1-binding protein 1, 2, 3; TAK1 - transforming growth factor β-activated protein kinase 1, TGFBR - Transforming growth factor beta receptor 1, TNFR1 – tumour necrosis factor receptor 1, TCR - T-cell receptor, TLR – Toll-like receptor, TRADD – tumour necrosis factor receptor1-associated death domain protein, TRAF2 and 6 – tumour necrosis factor receptor-associated factor 3 and 6, RIP1 - receptor-interacting serine/threonine-protein kinase 1, XIAP - X-linked inhibitor of apoptosis protein

Both pathways, MyD88 and TRIF, can be suppressed by GC. The probable mechanism of inhibition of the level of IκB. There are two possibilities, GC suppress a degradation or trigger an expression of IκB. GC are also able to support expression of MAPK phosphatase (MKP), which in turn dephosphorylates and thus deactivates MAPKs (Chinenov & Rogatsky 2007; Vandevyver et al. 2012). GC are also able to suppress TLR function through upregulating expression of suppressor of the cytokine signalling 1 (SOC1), which than downregulates activation of STAT1, activated by TLR3 a 4 (Bhattacharyya et al. 2011).

Expression of intestinal TLRs oscillates with circadian rhythmicity, which depends on presence of commensal microbiome. Absence of microbiome results in loss of rhythmicity and simultaneous overall decrease in expression of Tlrs and also Irak4. Probably due to downregulated expression of these genes, thus also declines activity of IKKβ and JNK. In contrary to that, expression of steroidogenic enzymes and production of GCs in intestine increases (Mukherji et al. 2013).

5.3 Glucocorticoids during inflammation

As mentioned above, GCs display anti-inflammatory effects. This consists mainly of suppression of proinflammatory cytokines expression and enhancement of expression of the anti-inflammatory ones. However, recent studies indicate also the effect of cytokines on expression of the steroidogenic enzymes. Frequently studied are particularly the effects of pro-inflammatory cytokine TNFα. Possible mechanism, how TNFα affects expression of steroidogenic enzymes is through c-Jun and NFκB, which are able to suppress the expression and activity of LRH1 (Lan et al. 2007; Huang et al. 2014) (Fig. 7).

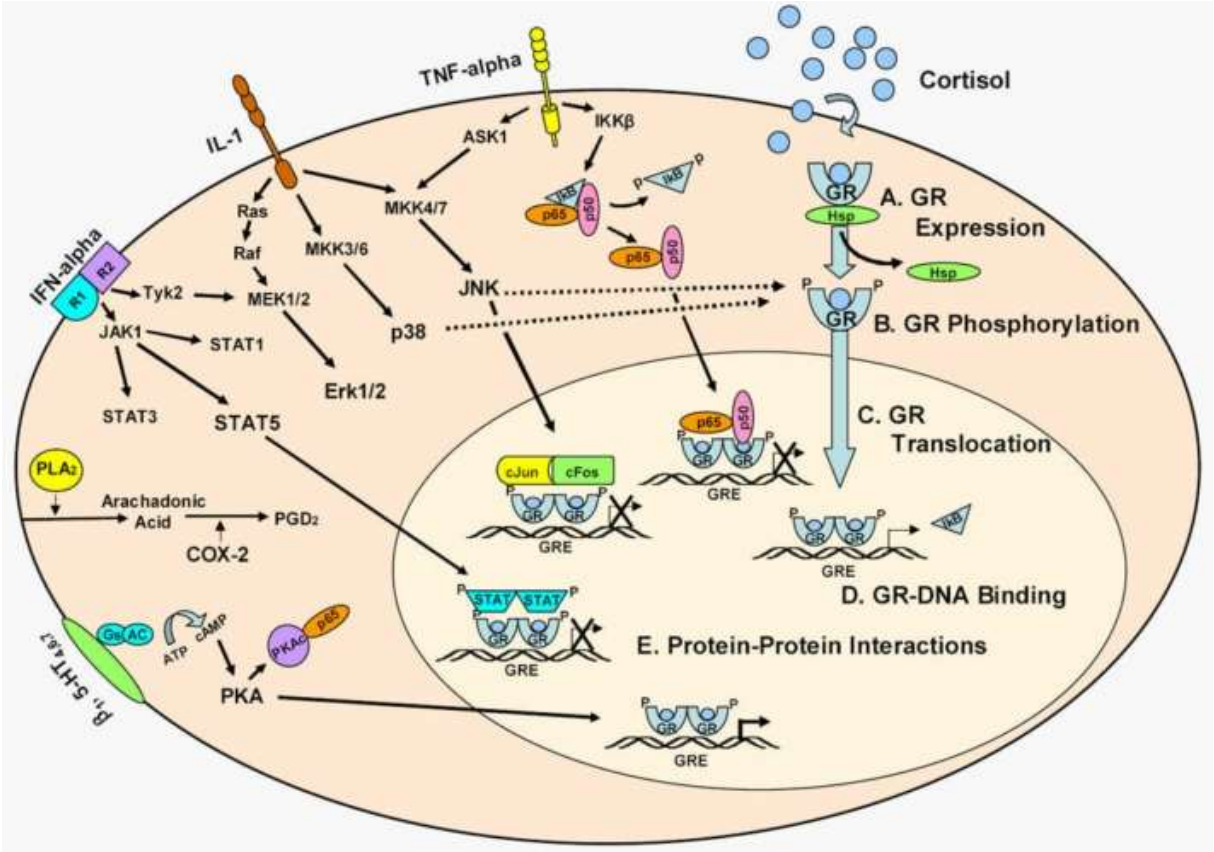

Figure 7: Regulation of a glucocorticoid response is mediated by cytokines and other substances mediating inflammation (e.g. 5-HT). Cytokines can via diverse signalling pathways inhibit or facilitate function of GCs on a genomic level (e.g. mitogen-activated protein kinase kinase kinase 3, 4, 6 and 7 (MKK3, 4, 6 and 7) pathway). They can also regulate via covalently modificate (phosphorylate) the GC-GR complex and thus regulate its translocation of into the nucleus (e.g. p38, JNK pathway). Taken from *Pace et al. 2007*. **Abbreviations:** 5-HT – 5-hydroxy tryptamine, ASK1 - apoptosis signal-regulating kinase 1, ATP – adenosine triphophate, cAMP – cyclic adenosine monophosphate, COX2 – cyclooxygenase 2, ERK1 and 2 - extracellular signal–regulated kinase 1 and 2, GR – glucocorticoid receptor, HSP – heat shock protein, IFNα – interferon α, IKK-β - inhibitory κ B kinase β, IL-1 – interleukin 1, JAK1 – Janus kinase 1, JNK - c-Jun N-terminal kinase, MEK 1 and 2 - mitogen-activated protein kinase kinase 1 and 2, MKK3, 4, 6 and 7 - mitogen-activated protein kinase kinase kinase 3, 4, 6 and 7, PGD_2 – prostaglandine D_2, PKA – protein kinase A, PLA_2 – phospholypase A_2, STAT 1, 3, 5 - signal transducer and activator of transcription 1, 3 and 5, TyK2 – tyrosine kinase 2

5.3.1 Intestine

Impaired or repressed local production of GCs often accompanies chronic inflammations, together with elevated level of TNFα (Huang et al. 2014). It is assumed, that TNFα has a dual effect on intestinal *de novo* steroidogenesis during inflammations. The effect is probably dose and duration dependent. During acute inflammatory reaction, TNFα and both its receptors (TNFR1, TNFR2) support the expression of mRNA of steroidogenic enzymes and thus the expression of steroidogenic enzymes resulting in increased level of intestinal GC (Noti et al. 2010; Huang et al. 2014). However, chronically increased level of TNFα mediates suppression

of expression of steroidogenic enzymes via TNFR1 (Huang et al. 2014). The expression of steroidogenic enzymes is probably mediated by altered activity or expression of LRH1 during inflammation (Noti et al. 2010; Huang, C. Lee, et al. 2014).

The expression of intestinal Hsd11b1 is upregulated during chronic inflammation in response to high levels of TNFα and IL-1β. Upregulated expression of Hsd11b1 can result in upregulated local regeneration of GCs Conversely, the expression of Hsd11b2 decreases during inflammation. Together with the anti-inflammatory activity of GCs, would such result suggest, that GCs regulate an expression of proinflammatory cytokines in a negative feedback loop (Vagnerová et al. 2005; Žbánková et al. 2007).

It is assumed, that GCs activate the expression of peroxisome proliferator-activated receptor γ (PPARγ) downregulating expression of proinflammatory cytokines, which can explain one of mechanisms, GCs downregulate inflammatory processes (Bouguen et al. 2015; Liu et al. 2015).

5.3.2 Lymphatic organs and immune cells

Splenocytes from animals experiencing relatively short (0.5-2h/day) repeated stressful situation (resulting in elevation of plasma level of GCs) display altered expression of pro- (IFNγ, IL-1, TNFα and IL-6) and anti-inflammatory (IL-10) cytokines. The production is shifted mostly in behalf of pro-inflammatory ones. Although, the balance between Th1 and 2 cytokines seems to be preserved. As a probable explanation has been assumed an impairment in a glucocorticoid sensitivity and amplified proliferation of splenocytes (Merlot et al. 2004; Avitsur et al. 2005). Nevertheless, prolonged repeated stress (12h/day) result in reduction of splenocytes and a shift of balance of Th lymphocytes in behalf of Th2 (Li et al. 2015).

Interestingly, the ability to control systemic inflammation by GCs during repeated stressful conditions seems to decline with age. Groups of young (2 months old) and older (13-15 months old) adult mice were repeatedly exposed aggressive individual. Repeated elevation of serum GCs resulted in higher expression of splenic TNFα and IL-6 in reaction to LPS. The effect was more consider in elder animals, pointing to age-related increasing resistance to GCs (Kinsey et al. 2008).

Chronically elevated GCs do not affect only the production of cytokines, but also expression of steroidogenic enzymes. Splenic Th cells from animals repeatedly exposed 12h/day to stress upregulated expression of Cyp11a. Interestingly, the upregulation of splenic, but probably also systemic, steroidogenesis depended on a presence of TLR9. Authors suggest, that TLR9 could be an important link between immune, humoral and neural system (Li et al. 2015).

It has also been shown, that presence of foreign antigens in plasma affects expression of steroidogenic enzymes in secondary lymph organs. Immunization of mice with bovine serum upregulates the activity of HSD3B1 and elevated local level of GCs, especially in lymph nodes, but also in spleen. The steroidogenesis was probably upregulated by IL-6 and TNFα (Mukhopadhyay & Bishayi 2009).

6. Aims of work

As mentioned above, every epithelial barrier permanently communicates with presented microbiome, composed from pathogenic, but also commensal strains. Sophisticated controlling mechanisms of immune reactions are thus a necessity. GCs probably regulate immune functions in a negative feedback loop, but in some conditions can also support it.

The data summarized in previous sections indicate that both, microbiota shaping and hormonal milieu, in particular, the glucocorticoids, are able to govern inflammation. However, there is only a limited knowledge about crosstalk between microbiota and glucocorticoid regeneration or *de novo* steroidogenesis, therefore the present study is focused on the effect of commensal microbiota on expression of enzymes responsible for *de novo* biogenesis and regeneration of glucocorticoids in colon, spleen, mesenteric lymph nodes and adrenal glands. Specifically, the diploma work tried to analyse the following questions:

1) Does the microbiome affect the expression of enzymes participating in *de novo* steroidogenesis of glucocorticoids in adrenal glands, colon, spleen and mesenteric lymphatic nodes?
2) Does the microbiome affect the local regeneration of glucocorticoids in adrenal glands, colon, spleen and mesenteric lymphatic nodes?
3) Does the *in vitro* stimulation of TLRs by microbial structures affect the expression of Myd88 in mesenteric lymph nodes?
4) Does the *in vitro* stimulation of TLRs by microbial structures affect the expression of Hsd11b1 in mesenteric lymph nodes?

7. Materials & methods

7.1 Animals

Adult germ free (GF) and conventional (CV) BALB/C male mice were obtained from Laboratory of Gnotobiology – Institute of Microbiology of the CAS, v. v. i. Trexler-type isolators were used for breeding GF animals. 4-5 animals kept in home cages received autoclaved and γ-irradiated (5,9 kGy for 30 min) standard pellet diet and tap water. The alternation of day and night was simulated by 12-h light/dark cycle. GF animals were controlled once per week to detect prospective microbial contaminations. All experiments accomplished with the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic.

7.2 Tissue sampling

Animals were anesthetised with isoflurane, blood collected by a cardiac punction and decapitated. Adrenal glands, colon, spleen and mesentheric lymphatic nodes were removed, snap frozen and stored in a liquid nitrogen.

7.3 Isolation of cells from MLN

Tissues were placed into the cold RPMI 1640 medium. MLN were pressed between glass slides to disengage to individual cells, two times washed with RPMI 1640 and sieved into culture plates. Cells were counted and diluted ($1,7E^6$ cells/0,5ml medium). 0,5µl of mixture of medium and PAMPs was added. Cell cultures were incubated at 37°C in atmosphere of 5% CO_2/air. After incubation cells were transferred into Eppendorf microtubes (Sigma-Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen until next experimental work. Before the next work were cells transferred into culture plates.

7.4 *In vitro* stimulation of TLRs with cocktail of PAMPs

The cocktail of PAMPs was prepared from lipopolysaccharide (LPS; 0,5µg/ml), zymosan (5µg/ml), Pam_3CSK_4 (synthetic bacterial lipopeptide; 50ng/ml), flagellin (50ng/ml) (activated TLR isoforms in Tab. 1) and added into each well of culture plates containing cells from MLN. Cells were collected after 0, 0.5h, 1h, 2h, 4h, 6h or 24h from adding of the cocktail of PAMPs.

7.5 Isolation of total RNA

Total RNA was isolated using a GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the original manufacturer's protocol. Homogenisation was performed using the UP50H ultrasonic homogeniser (Hielscher Ultrasonics, Teltow, Germany). Homogenised samples were transferred into filtering columns

and centrifuged (10 000g/1min). Filtrates were dehydrated by mixing with 70% ethyl alcohol of the same volume. Mixtures were transferred into nucleic acids binding collecting columns and centrifuged (10 000g/1min). Tubes were dried, washed with 350µl of Wash Solution 1 and centrifuged (10 000g/1min). Tubes were dried again, 500µl of Wash Solution 2 was added into collecting tubes and centrifuged (10 000g/1min). Collecting tubes were dried and centrifuged (10 000g/5min). Tubes were changed and columns with dry tubes were centrifuged (10 000g/5min). When tubes didn't contain any liquid, 20µl of the PCR water was added into columns and used for elution.

Samples were quantified by NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

7.6 Reverse transcription

The total RNA was used to prepare matrices for synthetization of first strands the cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) in a Mastercycler Eppendorf. As primers were used random hexamers. Samples were 5x diluted after the process.

7.7 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (Q-PCR) was performed on Viia 7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). As primers were used 5x Hot Firepol Probe Q-PCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia). To remark and detect cDNA were used TaqMan Assays (Life Technologies) figured in Tab. 2.

Gene of interest	Catalog number
Star	(Mm00441558_m1)
Cyp11a1	(Mm00490735_m1)
Hsd3b1	(Mm01261921_mH)
Cyp21a1	(Mm00487230_g1)
Cyp11b1	(Mm01204952_m1)
Hsd11b1	(Mm00476182_m1)
Myd88	(Mm00440338_m1)

Table 2: List of used TaqMan Assays.

To quantify the PCR products was used the standard curve method. As potential reference genes were chosen Hprt1 (Mm01545399-m1) and Tbp (Mm00446973_m1) in colon, Hprt1 and Ppib (Mm00478295_m1) in spleen, Hprt1 and Gapdh (cat. no. 4351309) in adrenal gland. The suitability of these genes as housekeeping genes was determined in previous experiments using

a large panel of 10 potential housekeeping genes and and the geNorm algorithm to determine the most stable reference genes.

Several genes of interest showed only a low level of expression in some tissues, which required the specific preamplification step of gene assays with TaqMan PreAmp Master Mix (Life Technologies), according to manufacturer's instructions.

7.8 Statistical analysis

7.8.1 Effect of microbiome on *de novo* steroidogenesis & the local regeneration

Outlier values were identified and rejected using the Dean's-Dixon's (DD) test. The Fisher's exact test was used to statistically analyzed variances of the data. Average values of mRNA levels were then analyzed using Student's t-test for data with equal or non-equal variances. The results are expressed as the mean ± SEM. As a threshold of statistical significance of all data was determined a $p<0.05$. Statistically significant data (labelled as *) were further analyzed for a $p<0.01$ (**) and $p<0.001$ (***).

7.8.2 Effect of the *in vitro* stimulation by pathogen associated molecule patterns on the expression of Myd88 and Hsd11b1 in mesenteric lymph nodes

Based on the DD test were rejected the outliers. The data were analyzed by two-way ANOVA (microbial status and time of treatment) followed by *post hoc* Fisher Least Significant Difference test. The results are expressed as the mean ± SEM. As a threshold of statistical significance of all data was determined a $p<0.05$. Statistically significant data (labelled as *) were further analyzed for a $p<0.01$ (**) and $p<0.001$ (***).

8. Results

8.1 Effect of microbiome on *de novo* steroidogenesis in adrenal glands

Statistical analyses confirmed, that microbiome significantly affected the expression of Star, the gene encoding the transporter of cholesterol to the mitochondria (Student's t-test: $p<0.05$).

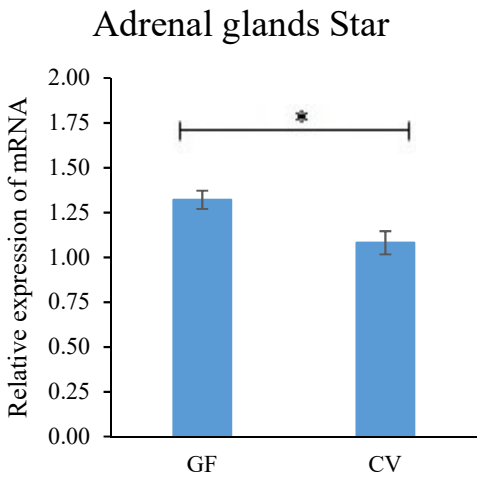

Figure 9: Effect of microbiome on the expression of Star in adrenal glands. The chart shows an arithmetic mean of mRNA and standard error of the mean (SEM). The significant difference between GF and CV is labelled as * ($p<0.05$).

Although, all steroidogenic enzymes were measured in adrenal glands, there has not been observed any effect of microbiome (Student's t-test: $p>0.05$), as seen in Fig. 10, a)-d).

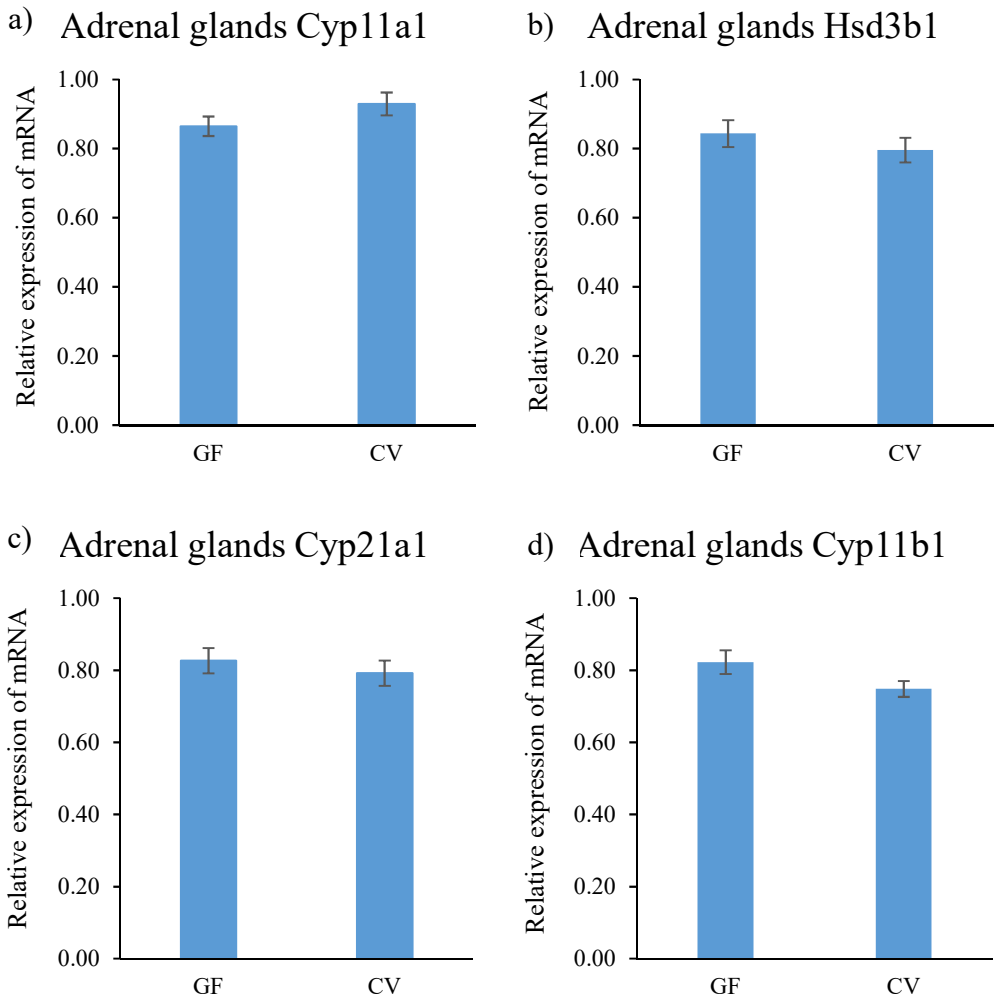

Figure 10: Effect of microbiome on expression of steroidiogenic eznymes in adrenal glands. The chart shows an arithmetic mean of mRNA and standard error of the mean (SEM).

8.2 Effect of microbiome on a local regeneration of GCs in adrenal glands

Effect of microbiome on expression of Hsd11b1, and thus the local regeneration of corticosterone from 11-dehydrocorticosterone, hasn't been observed in adrenal glands (Student's t-test: $p>0.05$) (Fig. 11).

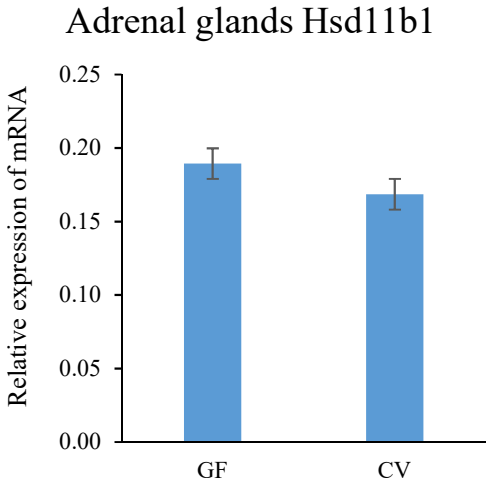

Figure 11: Effect of microbiome on expression of Hsd11b1 in adrenal glands. The chart shows an arithmetic mean of mRNA and standard error of the mean (SEM).

8.3 Effect of microbiome on expression of *de novo* steroidogenesis in colon

In contrast to the adrenal gland, we observed the significant effect of microbiome on the expression of Star in colon. GF mice had higher level of Star than CV animals (Student's t-test: $p<0.01$) (Fig. 12).

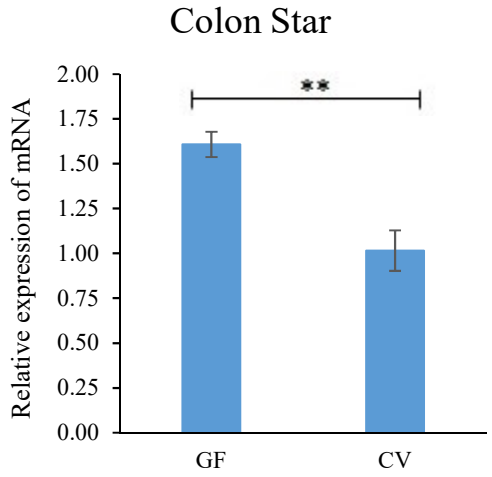

Figure 12: Effect of microbiome on expression of Star protein in colon. The chart shows an arithmetic mean of mRNA and SEM. The difference between GF and CV is labelled as ** ($p<0.01$).

Student's t-test has confirmed a significant difference in expression of Cyp11a1 (Student's t-test: $p<0.05$) and Cyp11b1 (Student's t-test: $p<0.05$) (Fig. 13 a), d)). Expression of Hsd3b1 and Cyp21a1 was not significantly affected by microbiome (Student's t-test: $p>0.05$).

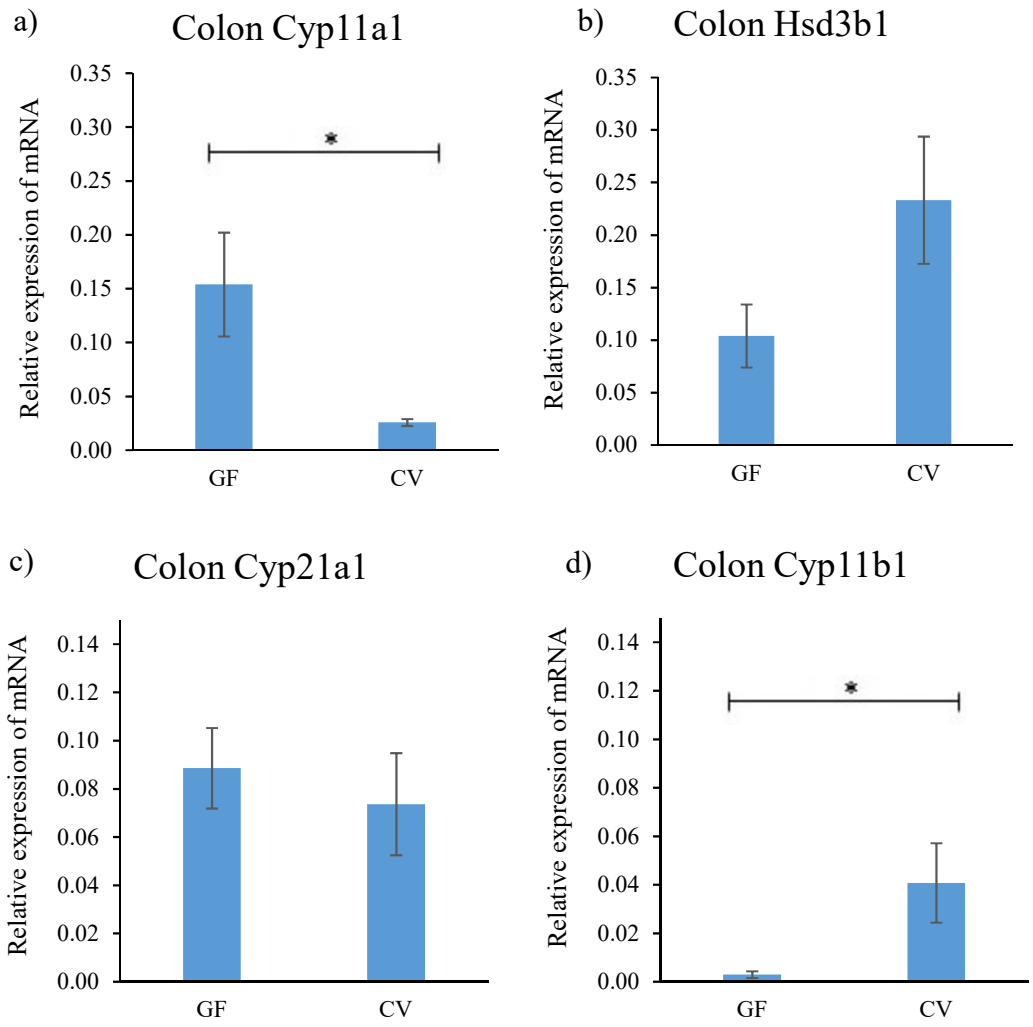

Figure 13: Effect of microbiome on expression of steroidogenic enzymes in colon. The chart shows an arithmetic mean of mRNA and SEM. The difference between GF and CV is labelled as * ($p<0.05$).

8.4 Effect of microbiome on a local regeneration of GCs in colon

The expression of Hsd11b1 didn't differ between GF and CV animals ($p>0.05$) (Fig. 14).

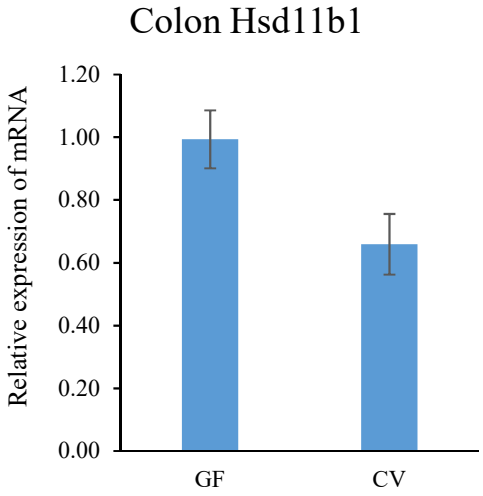

Figure 14: Effect of microbiome on expression of Hsd11b1 in colon. The chart shows an arithmetic mean of mRNA and standard error of the mean.

8.5 Effect of microbiome on *de novo* steroidogenesis in spleen

The expression of splenic Star was considerably lowered in the presence of microbiome ($p<0.01$) (Fig. 15).

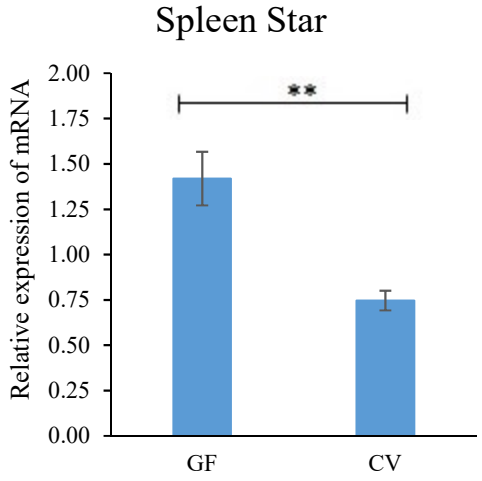

Figure 15: Effect of microbiome on expression of Star in spleen. The chart shows an arithmetic mean of mRNA and standard error of the mean. The difference between GF and CV is labelled as ** ($p<0.01$).

Of all steroidogenic enzymes, only expression of Cyp11a1 and Cyp21a1 was detectable in spleen. There was no difference in the expression of Cyp11a1 between GF and CV animals (Student's t-test: $p>0.05$), but the expression of Cyp21a1 was significantly lowered by microbiome (Student's t-test: $p<0.05$) (Fig. 16, a)-b)).

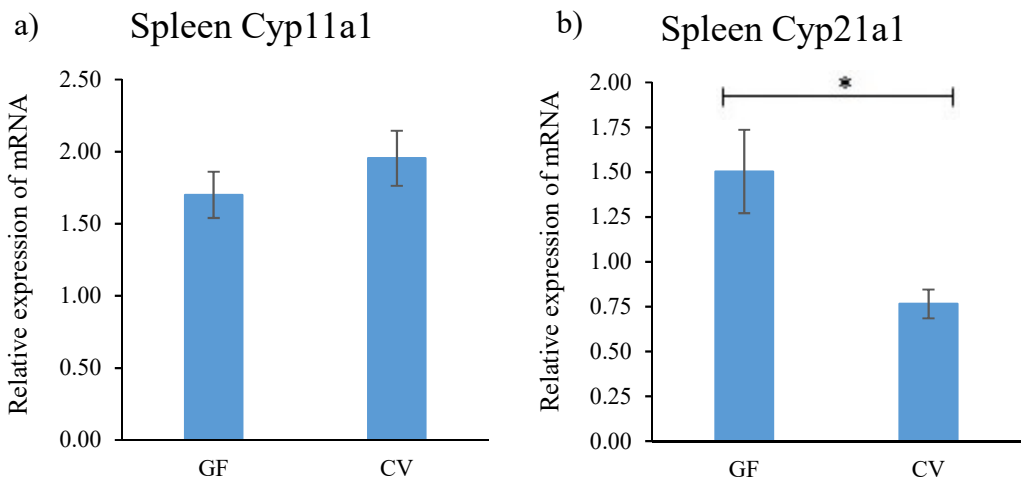

Figure 16: Effect of microbiome on expression of steroidogenic enzymes in spleen. The chart shows an arithmetic mean of mRNA and standard error of the mean. The difference between GF and CV is labelled as * ($p<0.05$).

8.6 Effect of microbiome on a local regeneration of GCs in spleen

The presence of microbiome elevated expression of mRNA splenic Hsd11b1 (Student's t-test: $p<0.001$), indicating considerably upregulated local regeneration in the spleen, as seen on Fig. 17.

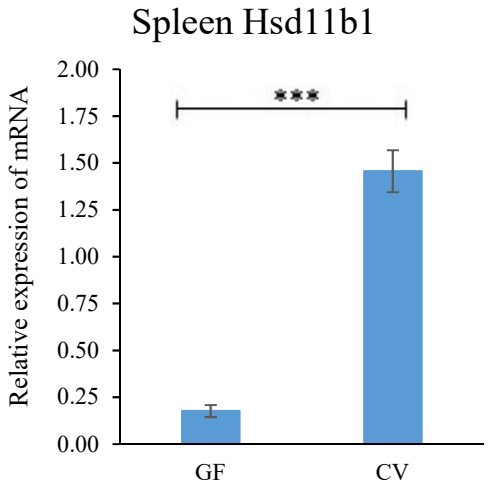

Figure 17: Effect of microbiome on expression of Hsd11b1 in spleen. Graph shows arithmetic mean of mRNA and SEM. The difference between GF and CV is labelled as *** ($p<0.001$).

8.7 Effect of microbiome on *de novo* steroidogenesis in mesenteric lymph nodes

Microbiome significantly lowered expression of Star in MLN (Student's t-test: $p<0.001$) (Fig.18).

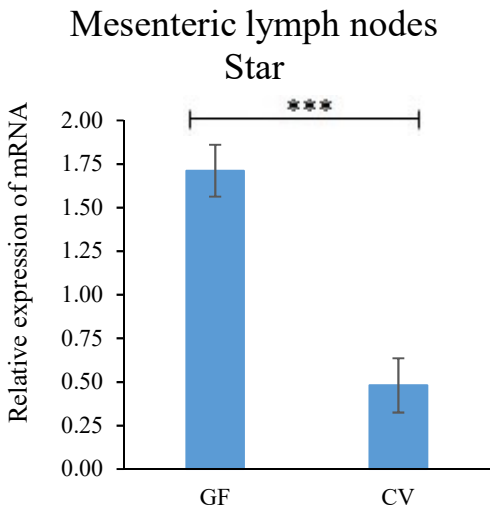

Figure 18: Effect of microbiome on expression of Star in MLN. The chart shows an arithmetic mean of mRNA and standard error of the mean. The difference between GF and CV is labelled as *** ($p<0.001$).

Any effect of microbiome on expression of Cyp11a1, Hsd3b1 and Cyp21a1 was not observed during a present study (Fig. 19 a)-c)) (Student's t-test: $p>0.05$). The expression of Cyp11b1 was under the limit of detection.

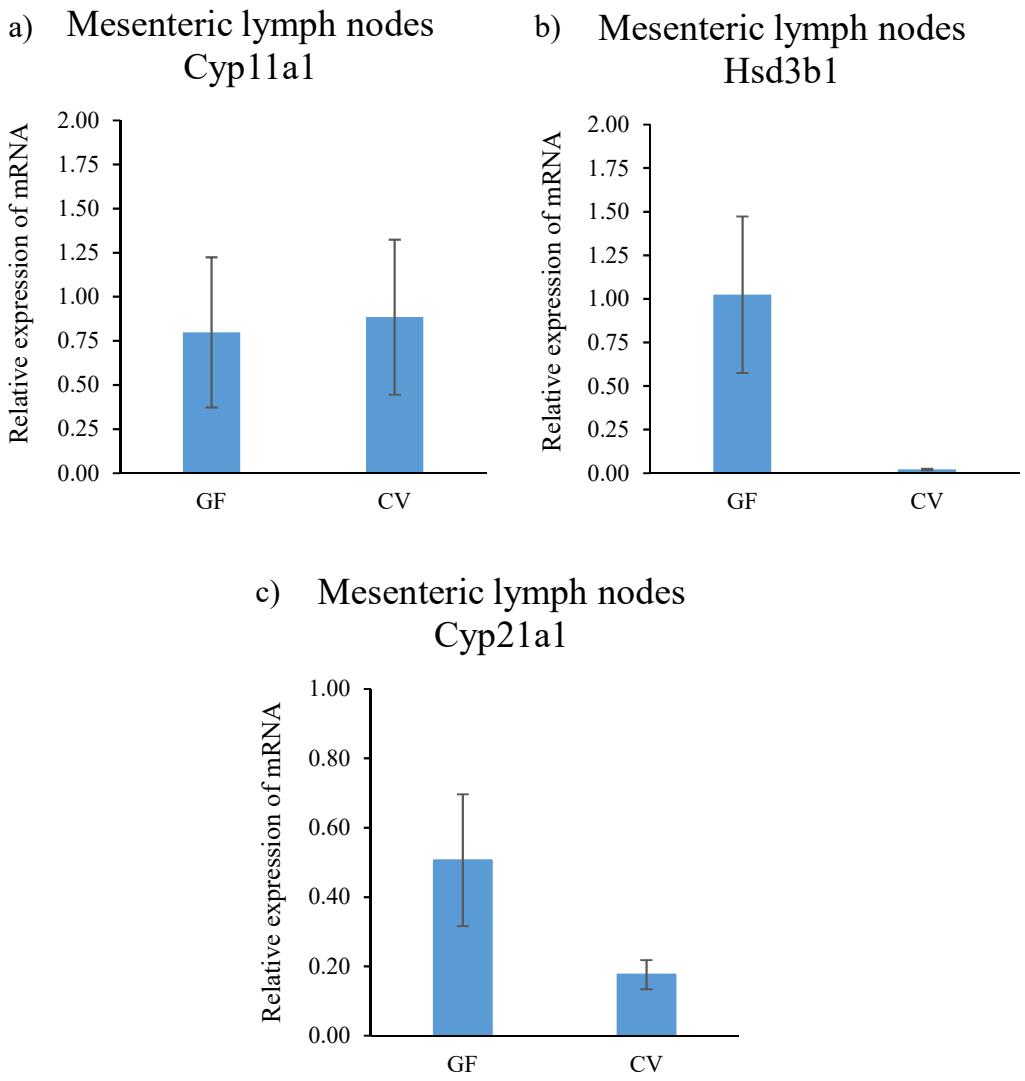

Figure 19: Effect of microbiome on expression of steroidogenic enzymes in MLN. The chart shows an arithmetic mean of mRNA and standard error of the mean.

8.8 Effect of microbiome on a local regeneration of GCs in mesenteric lymph nodes

Microbiome did not modulate the regeneration of GCs in MLN in present study (Student's t-test: $p>0.05$) (Fig. 20).

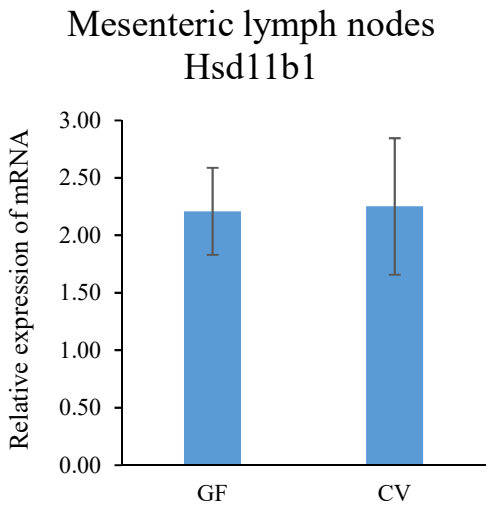

Figure 20: Effect of microbiome on expression of Hsd11b1 in spleen. The chart shows an arithmetic mean of mRNA and SEM.

8.9 Effect of the *in vitro* stimulation of TLRs by microbial structures on the expression of Myd88 in mesenteric lymph nodes

Two-way ANOVA showed a significant effect of microbiome ($F_{1, 24} = 4.449$, $p<0.05$) but not the time ($F_{6, 24} = 2.215$, $p>0.05$) on expression of Myd88 after exposure to microbial structures. The interaction between these two factors was significant ($F_{6, 24} = 4.4584$, $p<0.05$) ($p<0.05$). Significant interaction between GF and CV mice indicates that the upregulation of Myd88 transcript in GF was slower and reached the maximum value at t = 24 h, but the expression profile in CV mice reached maximum value much faster (t = 2 h) and that declined to the values similar as before TLR stimulation. Due to these two different time profiles we observed significant differences between GF and CV mice at times 2 ($p<0.05$), 6 ($p<0.05$) and 24 h ($p<0.001$), as seen in Fig. 21 and 22.

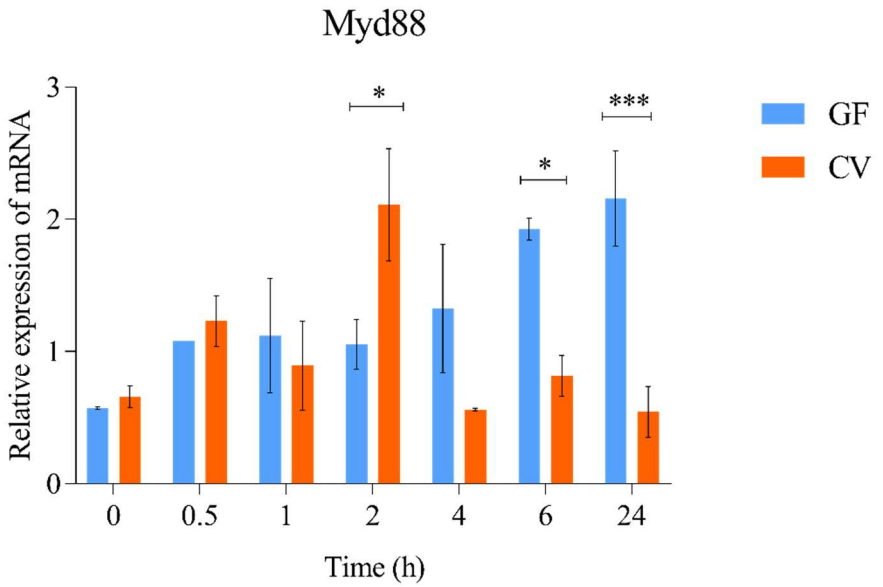

Figure 21: Effect of the in vitro stimulation of TLRs by microbial structures on the expression of Myd88 in mesenteric lymph nodes. The chart shows an arithmetic mean of mRNA and SEM. The difference between GF and CV is labelled as * ($p<0.05$) and *** ($p<0.001$),

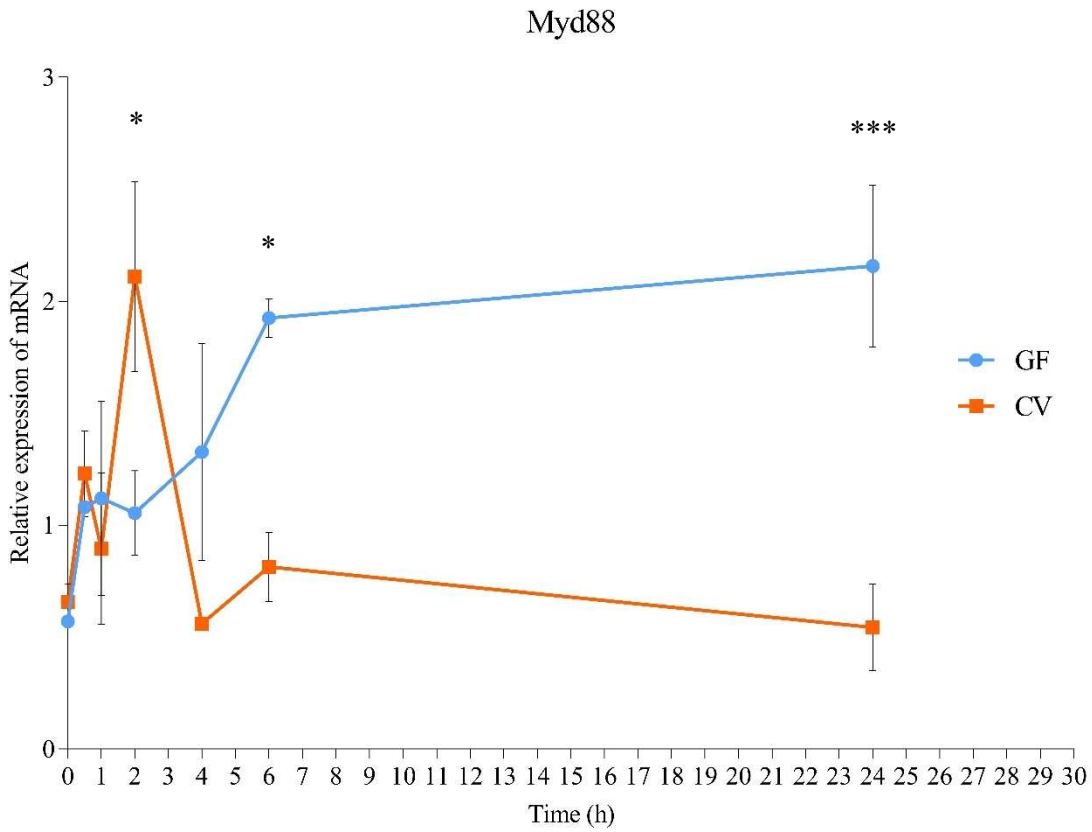

Figure 22: Effect of the in vitro stimulation of TLRs by microbial structures on the expression of Myd88 in mesenteric lymph nodes. The chart shows an arithmetic mean of mRNA and SEM. The difference between GF and CV is labelled as * ($p<0.05$).

8.10 Effect of the in vitro stimulation of TLRs by microbial structures on the expression of Hsd11b1 in mesenteric lymph nodes

Two-way ANOVA didn't reveal any significant effect of microbiome ($F(1, 28) = 0.9949$, $p>0.05$), time ($F(6, 28) = 0.8781$, $p>0.05$) or interaction between these two factors ($F(6, 28) = 1.185$, $p>0.05$) on the expression of Hsd11b1 between in distinct time points from immune stimulation (Fig. 23).

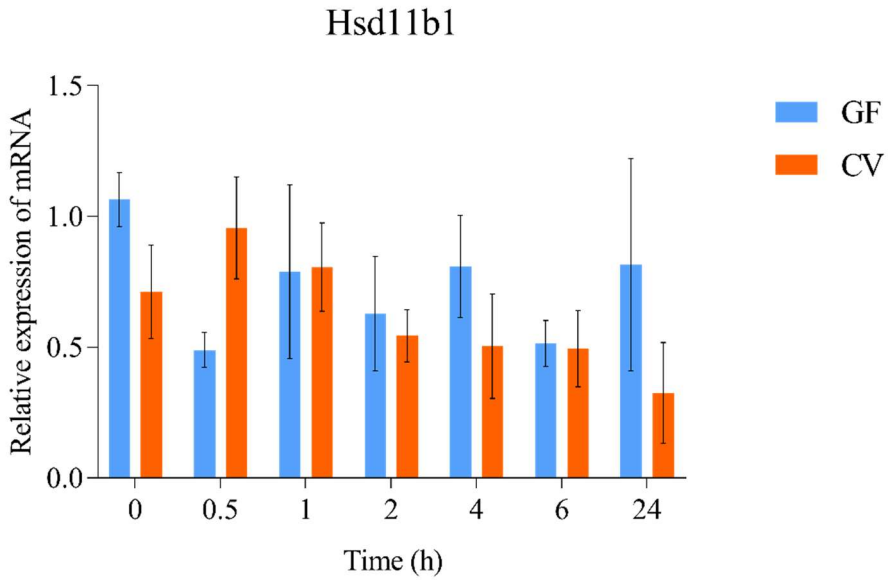

Figure 23 Effect of the in vitro stimulation of TLRs by microbial structures on the expression of Hsd11b1 in mesenteric lymph nodes. The chart shows an arithmetic mean of mRNA and SEM.

9. Discussion

Present experimental work revealed several differences in the biogenesis and regeneration of glucocorticoids between individual studied tissues of GF and CV animals, but also few similarities. The first similarity can be found in the gene encoding cholesterol transporter into mitochondria, Star. In all studied tissues the expression of Star was decreased in a case of presented microbiome. The expression was decreased in peripheral tissue directly exposed to microbiome (colon), in immune tissues (spleen and MLN), but also centrally in the tissues providing systemic steroidogenesis (adrenal glands).

The decrease in expression of Star was the only difference observed in adrenal glands of CV animals compared to GF ones. However, as a limiting step of steroidogenesis, the expression of StAR protein determines the production of all corticosteroids and thus affects the whole health condition and phenotype. Mutated Star can results in severe inflammations, an altered HPA axis reactivity, growth disorders and salt loss as seen in patients suffering from lipoid CAH (Sahakitrungruang et al. 2010). However, consequences of reduced expression of Star are probably not as severe as in the case of lipoid CAH, where the impaired function or expression is caused by a mutation. The severity of lipoid CAH can be also demonstrated in a mice model of disrupted Star, where the mutation is lethal (Carson et al. 1997). Although, a presence and a composition of microbiome may participate on the whole phenotype.

Conversely, the overexpression of Star has been observed in patients suffering from adrenocortical adenomas, resulting in Cushing's syndrome. The overproduction can be caused by a mutation in cAMP/PKA signalling pathway, which activates the expression of Star (Zhou et al. 2016).

On the other hand, an i*n vitro* study of macrophages has shown, that the overexpression of StAR can have beneficial effects in peripheral tissues. Macrophages overexpressing StAR protein displayed increased efflux of cholesterol and lowered intracellular lipid levels, decreased production of inflammatory substances and prolonged survival. Such condition can prevent the development of atherosclerosis (Ning et al. 2009).

In contrast to Star, the effect of microbiome on the expression of steroidogenic enzymes was not identical in the studied tissues. No effect of microbiome was observed in adrenal glands, however, adrenal glands are able to express other isoforms of steroidogenic enzymes (e.g. Hsd3b2) (Rainey & Nakamura 2008), which we have not studied. Therefore, I cannot exclude the possibility, that other isoforms of adrenal steroidogenic enzymes are more sensitive to microbiome. Future studies will be necessary.

The rate limiting enzyme of steroidogenesis, Cyp11a1 (Cima et al. 2014; Miller & Auchus 2011), was significantly upregulated by microbiota in the colon but not in adrenal gland and lymphoid organs. This finding is in agreement with the study of Mukherji et al. (2013). Comparing GF and CV individuals revealed an impaired circadian inhibitory mechanism controlling expression of Cyp11a1 in GF mice in the ileum. Exacerbated inhibition of the expression of Cyp11a1 is than associated with permanently increased production of GC (Mukherji et al. 2013).

Higher expression of Cyp11a1 has also been observed during food allergy reaction. CYP11A1 probably induces differentiation of Th lymphocytes into types 2 and 17, which in turn leads to expression of Th2 cytokines and IL-17 (Wang et al. 2013; Gelfand et al. 2014). Some authors indicate inhibitory effects of LRH1 on the expression of Cyp11a1 during a long term exposure to TNFα (Huang et al. 2014). These findings together support the hypothesis, that the production of GCs participates in control of inflammation.

On a regulation of expression of Cyp11a1 may also participate other transcription factors than LRH1. It has been previously shown, that the expression of Cyp11a1 can be also regulated by GATA4 a 3 transcription factors (Wang et al. 2013) at least in a basal condition.

In previous studies, the expression of intestinal Cyp11a1 was detected in animals housed under conventional conditions only after a strong activation of the immune system by anti-CD-3 (Cima et al. 2004). Such a result is not consistent with present results, because the expression of Cyp11a1 was detected also during a basal state independently on presented microbiome. These discrepancies might reflect the differences in the microbiome composition in our and Brunner laboratory breeding facilities.

The *de novo* steroidogenesis and the local regeneration was also measured in immune organs. In my study, which was performed in basal conditions, the expression of Cyp11a1 was detected in spleen as well as in MLN, but without any effect of microbiome. The expression has been shown also in previous study, when it increased during immune reaction. Simultaneously with the expression of Cyp11a1 rose also the product of CYP11A1 reaction, pregnenolone, which have been shown to control inflammatory processes (Mahata et al. 2014).

Another study shown, that the expression of splenic Cyp11a1 is associated with modulation of the balance between Th1 and Th2 cells (Oka et al. 2000). Thus, it is being offered a question, whether the expression differs during inflammation.

Present results confirm the ability of colon to express Hsd3b1, the second enzyme in the order of steroidogenesis (Cima et al. 2013). The expression was not modulated by the presence of microbiota. Similar to colon, adrenal expression of Hsd3b1 was not influenced by the

microbiota and concerning immune organs, in spleen the expression was below the detection limit in spleen but was detected in MLN of GF mice. Similar to our results previous study also didn't detect any Hsd3b1 transcript in spleen (Taves et al. 2016). These findings indicate that microbiota do not represent strong factor for regulation of Hsd3b1 expression.

However, Mukhopadhyay & Bishayi detected activity of HSD3B1 in spleen, lymph nodes and other lymphatic tissue. Particularly, they revealed a considerable increase of expression and activity after injection of *E.coli* cells and IL-6 (Mukhopadhyay & Bishayi 2009). A possible explanation is, that the expression of Hsd3b1 (and also Cyp11b1) is too low to be detected during basal conditions, but act as rate limiting enzyme during inflammations (as well as Cyp11b1). Taves also assumes that in lymphatic tissues of adult individuals are GCs preferentially regenerate rather than produced *de novo* (Taves et al. 2016).

The gene Cyp21a1, which encodes 21-hydroxylase, an enzyme catalyzing the conversion of progesterone to 11-deoxycorticosterone was expressed in all investigated tissues but predominantly insensitive to the presence of microbiota. Only in the splenic tissue we identified upregulated Cyp21a1 transcript in GF animals. The mechanism that underlie the microbiome effect in spleen is currently unknown. Only little is known about expression of Cyp21a1 in spleen. Previous study have shown, that the expression of Cyp21a1 changes during ontogeny being higher in adult individuals compared to neonatal ones, which, together with changes in expression of other steroidogenetic enzymes, result in age-related changes in production of GCs (Taves et al. 2016)

The final step of corticosteroid biogenesis is 11-hydroxylation of 11-deoxycorticosterone into corticosterone, which requires the enzyme 11-hydroxylase encoded by Cyp11b1. Our results showed strong expression of this enzyme in adrenal tissue and weaker in colon but the transcript was below the detection limit in spleen and MLN. Such result may indicate weak or absent production of final glucocorticoids corticosterone and cortisol in spleen and MLN in contrast to the possible biogenesis of pregnenolone and progesterone, whose biogenesis does not require 11- and 21-hydroxylation. This conclusion is supported by the finding of biogenesis of pregnenolone in immune cells (Mahata et al. 2014). During a study comparing adult and neonatal individuals, performed in conventional conditions, was not detected any expression in adult mice. However, the expression of Cyp11b1 exceeded expression of genes encoding other steroidogenic enzymes in neonatal individuals. Taves also suggests, that Cyp11b1 may act as one of rate limiting enzymes in lymphatic tissues in adult individuals (Taves et al. 2016).

In present study colonic expression of Cyp11b1 was elevated in CV individuals, conversely to Cyp11a1, which was significantly lowered in CV animals. Some studies emphasize

a common transcription regulator for Cyp11a1 and Cyp11b1, LRH1. Previous studies shown, that acute immune reaction activated expression of Lrh1. Overexpression of Lrh1 resulted probably also in increasing production of its protein, which elevated expression of both enzymes, Cyp11a1 and Cyp11b1 (Mueller et al. 2006; Atanasov et al. 2008). Simultaneously, the activity of LRH1, and thus also expression of both enzymes, depends on a cell cycle, when gene of both enzymes are mostly expressed during a G1 and S phase (Atanasov et al. 2008). CYP11A1 and CYP11B1 than may inhibit expression or activity of LRH1 in a negative feedback loop (Mueller et al. 2006). Theory of a negative feedback loop also corresponds with results of experiments on chronically ill animals. Some authors indicate possible inhibitory effects of LRH1 on Cyp11a1 and Cyp11b1 expression during a long term exposure to TNFα (Huang et al. 2014). Present results indicate a distinct mechanism regulating the expression of both enzymes during a long-term exposure to microbiome. On a regulation of expression may also participate other transcription factors than LRH1, at least in a basal condition. In previous studies, the expression of Cyp11a1 in convent animals was detected only in a reaction to an immune impulse, while the expression of Cyp11b1 was detected also during basal conditions (Cima et al. 2004). Such a result is not consistent with present results, because the expression of Cyp11a1 was detected also during a basal state independently on presented microbiome. However, results probably correspond in indication of distinct regulatory mechanisms of expression of Cyp11a1 and Cyp11b1.

Present results thus indicate a lower overall basal *de novo* production of steroid hormones in the colon of CV mice with possibly slightly altered composition of steroids, in behalf of GCs (which are generated in Cyp11b1 reaction) rather than other steroid hormones. However, no protein expression (only mRNA) and expression of second isoforms of steroidogenic enzymes was measured in a present study. If actually would be the composition of steroid hormones altered in colon in behalf of GCs, it would probably prevent excessive immune reactions in physiological conditions. Conversely, disrupted production of GCs is associated with intestinal inflammations such as Crohn's disease and ulcerative colitis (Coste et al. 2007).

Local production of GCs is determined not only by local *de novo* biogenesis but also by regeneration of active hormones from their 11-oxo derivatives due to reduction of –OH group at C_{11} catalysed by HSD11B1. We proved the expression of gene encoding this enzyme in all investigated tissues, but microbiota significantly modulated the expression of Hsd11b1 only in spleen, where GF animals showed significantly lower transcription Hsd11b1 that CV animals. The mechanisms that underlie the effect of microbiota on splenic Hsd11b1 are currently unknown. However, one possibility is likely. Cytokines are important modulators of this

enzyme (Thieringer et al. 2001) and their expression is modulated by microbiome (Ghosh et al. 2007). The reason why cytokines might upregulate splenic but not colonic or MLN Hsd11b1 is difficult to explain. Some but not all cytokines upregulate Hsd11b1 and it cannot be excluded that the spectrum of cytokines expressed in various lymphoid organs including colon with lymphoid follicles in the presence of MAMPs signals is different. However, this hypothesis will require further experiments. Previous study shown, that the expression of Hsd11b1 rises also during experimentally induced chronic inflammation in spleen as well as in MLN (Ergang et al. 2011). In agreement with the absence of any significant effect of microbiome on MLN we did not find any upregulation of Hsd11a1 by the cocktail of specific TLR ligands in *in vitro* experiment using immune cells isolated from MLN. Using expression of Myd88, we have demonstrated activation of TLR and translocation of the ligand signal into the cells, which was observed 2, 6 and 24 hours after ligand application. After the stimulation was in MLN measured also the expression of Hsd11b1 at the same time points as Myd88. Although previous studies indicated, that in MLN are GCs more likely regenerated than produced *de novo* to be able to dynamically modulate immune function (Taves et al. 2016), present study didn't show any difference in expression of Hsd11b1 after stimulation of TLRs.

10. Conclusion

Present study examined the putative interaction/crosstalk between biogenesis and regeneration of corticosteroids and microbiome. Using germ-free and conventional mice we studied the expression of enzymes of steroidogenesis and glucocorticoid regeneration in adrenal glands, colon and secondary lymphoid organs – spleen and MLN. It is well known that colon, spleen and MLN permanently communicating with microbiome or receive information about microbiome via APCs or directly, when microorganisms penetrate into the body and that adrenal glands also have the capability to sense these signals.

Based on the results I can answer previously asked questions:

1) Does the microbiome affect the expression of enzymes participating in *de novo* steroidogenesis of glucocorticoids in adrenal glands, colon, spleen and mesenteric lymphatic nodes?

The microbiome downregulated the expression of Star in all studied tissues. In colon was also downregulated the expression of Cyp11a1 and upregulated the expression of Cyp11b1 by microbiome. In spleen microbiome downregulated the expression of Cyp21a1.

2) Does the microbiome affect the local regeneration of glucocorticoids in adrenal glands, colon, spleen and mesenteric lymphatic nodes?

Microbiome affected, particularly upregulated, only the expression of Hsd11b1 in spleen. Expression in other tissues was not affected.

3) Does the *in vitro* stimulation of TLRs by microbial structures affect the expression of Myd88 in mesenteric lymph nodes?

The expression of Myd88 was upregulated after 2 hours and downregulated after 6 and 24 hours after stimulation compared to control cell culture.

4) Does the *in vitro* stimulation of TLRs by microbial structures affect the expression of Hsd11b1 in mesenteric lymph nodes?

The expression of Hsd11b1 in mesenteric lymph nodes was not affected by *in vitro* stimulation of TLRs.

11. Literature

Æsøy, R. et al., 2002. Activation of cAMP-dependent protein kinase increases the protein level of steroidogenic factor-1. *Endocrinology*, 143, pp.295–303.

Ait-Belgnaoui, A. et al., 2012. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology*, 37, pp.1885–1895.

Arakane, F. et al., 1996. Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action. *Proceedings of the National Academy of Sciences*, 93, pp.13731–13736.

Atanasov, A.G. et al., 2008. Cell cycle-dependent regulation of extra-adrenal glucocorticoid synthesis in murine intestinal epithelial cells. *FASEB Journal*, 22, pp.4117–4125.

Baker, M.E., Funder, J.W. & Kattoula, S.R., 2013. Evolution of hormone selectivity in glucocorticoid and mineralocorticoid receptors. *Journal of Steroid Biochemistry and Molecular Biology*, 137, pp.57–70.

Balasubramaniam, S., Goldstein, J.L. & Brown, M.S., 1977. Regulation of cholesterol synthesis in rat adrenal gland through coordinate control of 3-hydroxy-3-methylglutaryl coenzyme A synthase and reductase activities. *Proceedings of the National Academy of Sciences*, 74, pp.1421–1425.

Bhattacharyya, S. et al., 2011. Glucocorticoids target suppressor of cytokine signaling 1 (SOCS1) and type 1 interferons to regulate Toll-like receptor-induced STAT1 activation. *Proceedings of the National Academy of Sciences*, 108, pp.23–28.

Biddie, S.C. et al., 2012. Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Molecular Cell*, 43, pp.145–155.

Bird, I.M. et al., 1996. Differential control of 17α-hydroxylase and 3β- hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. *Journal of Clinical Endocrinology & Metabolism*, 81, pp.2171–2178.

Blander, J.M. & Medzhitov, R., 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature*, 440, pp.808–812.

Bornstein, S.R. et al., 2004. Impaired adrenal stress response in Toll-like receptor 2-deficient mice. *Proceedings of the National Academy of Sciences*, 101, pp.16695–16700.

Bouguen, G. et al., 2015. Intestinal steroidogenesis. *Steroids*, 103, pp.64–71.

Brandl, K. et al., 2007. MyD88-mediated signals induce the bactericidal lectin RegIIIγ and protect mice against intestinal Listeria monocytogenes infection. *Journal of Experimental Medicine*, 204, pp.1891–1900.

Brandt, J. Van Den et al., 2007. Enhanced glucocorticoid receptor signaling in T cells impacts thymocyte apoptosis and adaptive immune responses. *American Journal of Pathology*, 170, pp.1041–1053.

Brem, A.S. et al., 1995. Bidirectional activity of 11β-hydroxysteroid dehydrogenase in vascular smooth muscle cells. *Steroids*, 60, pp.406–410.

Canani, R.B. et al., 2006. Short- and long-term therapeutic efficacy of nutritional therapy and corticosteroids in paediatric Crohn's disease. *Digestive and Liver Disease*, 38, pp.381–387.

Carson, K.M. et al., 1997. Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proceedings of the National Academy of Sciences*, 94, pp.11540–11545.

Cebra, J.J. et al., 1998. Development and maintenance of the gut-associated lymphoid tissue (GALT): the roles of enteric bacteria and viruses. *Developmental Immunology*, 6, pp.13–18.

Chaudhry, A. et al., 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity*, 34, pp.566–578.

Chen, Y. et al., 2010. Protein kinase Cμ mediates adenosine-stimulated steroidogenesis in primary rat adrenal cells. *FEBS Letters*, 584, pp.4442–4448.

Chinenov, Y. & Rogatsky, I., 2007. Glucocorticoids and the innate immune system: crosstalk with the Toll-like receptor signaling network. *Molecular and Cellular Endocrinology*, 275, pp.30–42.

Cima, I. et al., 2004b. Intestinal epithelial cells synthesize glucocorticoids and regulate T-cell activation. *Journal of Experimental Medicine*, 200, pp.1635–1646.

Clark, B.J. et al., 1994. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse leydig tumor cells. *Journal of*

Biological Chemistry, 269, pp.28314–28322.

Cooper, M.S. et al., 2018. 11β-hydroxysteroid dehydrogenase type 1 activity predicts the effects of glucocorticoids on bone. *Journal of Clinical Endocrinology & Metabolism*, 88, pp.3874–3877.

Coste, A. et al., 2007. LRH-1-mediated glucocorticoid synthesis in enterocytes protects against inflammatory bowel disease. *Proceedings of the National Academy of Sciences*, 104, pp.13098–13103.

Davies, E. & Mackenzie, S.M., 2003. Extra-adrenal production of corticosteroids. *Clinical and Experimental Pharmacology and Physiology*, 30, pp.437–445.

Dostert, A. & Heinzel, T., 2004. Negative Glucocorticoid Receptor Response Elements and their Role in Glucocorticoid Action. *Current Pharmaceutical Design*, 10, pp.2807–2816.

Ergang, P. et al., 2017. Inflammation regulates 11β-hydroxysteroid dehydrogenase type 1 differentially in specific compartments of the gut mucosal immune system. *Steroids*, 126, pp.66–73.

Ergang, P., Bryndová, J. & Mikˇ, I., 2011. Upregulation of 11β-hydroxysteroid dehydrogenase 1 in lymphoid organs during inflammation in the rat. *Journal of Steroid Biochemistry and Molecular Biology*, 126, pp.19–25.

Gelfand, E.W., Wang, M. & Jia, Y., 2014. Targeting the steroidogenic pathway for threating and/or preventing allergic diseases. *National Jewish Health*, 2, pp.1–23.

Ghosh, T.K. et al., 2007. Toll-like receptor (TLR) 2-9 agonists-induced cytokines and chemokines: I. comparison with T cell receptor-induced responses. *Cellular Immunology*, 243, pp.48–57.

Gomez-Sanchez, E. & Gomez-sanchez, C.E., 2015. The multifaceted mineralocorticoid receptor. *Comprehensive Physiology*, 4, pp.965–994.

Heil, F. et al., 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Journal of Immunology*, 7, pp.2987–2997.

Heino, S. et al., 2000. Dissecting the role of the Golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface. *Proceedings of the National Academy of*

Sciences, 97, pp.8375–5380.

Hench, P.S. et al., 1949. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotrophic hormone on rheumatoid arthritis. *Proceedings of the Staff Meetings*, 24, pp.97–104.

Hennebold, J.O.N.D. et al., 1996. 11β-hydroxysteroid dehydrogenase modulation of glucocorticoid activities in lymphoid organs. *American Journal of Physiology*, 270, pp.1296–1306.

Hirasava, G. et al., 1997. Colocalization of 11β-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. *Journal of Clinical Endocrinology & Metabolism*, 82, pp.21–25.

Hirata, Y. et al., 2017. Post-translational modifications of the TAK1-TAB complex. *International Journal of Molecular Sciences*, 18, pp.1–18.

Hollenberg, S.M. et al., 1985. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature*, 318, pp.638–641.

Hostettler, N. et al., 2011. Local glucocorticoid production in the mouse lung is induced by immune cell stimulation. *European Journal of Allergy and Clinical Immunology*, 67, pp.227–234.

Huang, S.-C., Lee, C.-T. & Chung, B.-C., 2014a. Tumor necrosis factor suppresses NR5A2 activity and intestinal glucocorticoid synthesis to sustain chronic colitis. *Science Signaling*, 7, pp.1–12.

John, S. et al., 2011. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nature Genetics*, 43, pp.264–270.

Karimi, K. et al., 2009. Lactobacillus reuteri–induced regulatory T cells protect against an allergic airway response in mice. *American Journal of Respiratory and Critical Care Medicine*, 179, pp.186–193.

Katoh-Fukui, Y. et al., 2017. Mouse polycomb M33 is required for splenic vascular and adrenal gland formation through regulating Ad4BP/SF1 expression. *Blood*, 106, pp.1612–1621.

Kobayashi, K. et al., 2002. IRAK-M Is a negative regulator of toll-like receptor signaling. *Cell*, 110, pp.191–202.

Kraemer, F.B. et al., 2007. The LDL receptor is not necessary for acute adrenal steroidogenesis in mouse adrenocortical cells. *American Journal of Physiology. Endocrinology and Metabolism*, 292, pp.408–412.

Krupoves, A. et al., 2011. Variation in the glucocorticoid receptor gene (NR3C1) may be associated with corticosteroid dependency and resistance in children with Crohn's disease. *Pharmacogenetics and Genomics*, 21, pp.454–460.

Kulcenty, K., Holysz, M. & Trzeciak, W.H., 2015. SF-1 (NR5A1) expression is stimulated by the PKA pathway and is essential for the PKA-induced activation of LIPE expression in Y-1 cells. *Molecular and Cellular Biochemistry*, 408, pp.139–145.

Lan, H. et al., 2007. Cyclic AMP stimulates SF-1-dependent CYP11A1 expression through homeodomain-interacting protein kinase 3-mediated Jun N-terminal kinase and c-Jun phosphorylation. *Molecular and Cellular Biology*, 27, pp.2027–2036.

Lange, Y., 1991. Disposition of intracellular cholesterol in human fibroblasts. *Journal of Lipid Research*, 32, pp.329–339.

Lavery, G.G. et al., 2006. Hexose-6-phosphate dehydrogenase knock-out mice lack 11β-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation. *Journal of Biological Chemistry*, 281, pp.6546–6551.

Leckie, C.M., Welberg, L.A.M. & Seckl, J.R., 1998. 11β-hydroxysteroid dehydrogenase is a predominant reductase in intact rat Leydig cells. *Journal of Endocrinology*, 159, pp.233–238.

Lee, Y. et al., 2006. Phosphorylation of the hinge domain of the nuclear hormone receptor LRH-1 stimulates transactivation. *Journal of Biological Chemistry*, 281, pp.7850–7855.

Lennon, E.M. et al., 2014. Early life stress triggers persistent colonic barrier dysfunction and exacerbates colitis in adult IL-10-/- mice. *Inflammatory Bowel Disease*, 19, pp.712–719.

Li, H. et al., 2015. Toll-like receptor 9 is required for chronic stress-induced immune suppression. *Neuroimmunomodulation*, 21, pp.1–7.

Li, J. et al., 2003. Janus kinase 2 and calcium are required for angiotensin II-dependent activation of steroidogenic acute regulatory protein transcription in H295R human adrenocortical cells. *Journal of Biological Chemistry*, 278, pp.52355–52362.

Liu, W. et al., 2015. Voluntary exercise protects against ulcerative colitis by up-regulating glucocorticoid-mediated PPAR-γ activity activity in the colon in mice. *Acta Physiologica*, 215, pp.24–36.

Londos, C. et al., 1995. Perilipin: unique proteins associated with intracellular neural lipid droplets in adipocytes and steroidogenic cells. *Biochemical Society Transactions*, 23, pp.126–129.

Lu, N.Z. & Cidlowski, J.A., 2006. Glucocorticoid receptor isoforms generate transcription specificity. *TRENDS in Cell Biology*, 16, pp.301–307.

Luu, K. et al., 2014. STAT1 plays a role in TLR signal transduction and inflammatory responses. *Immunology and Cell Biology*, 92, pp.761–769.

Lytinas, M. et al., 2003. Acute stress results in skin corticotropin-releasing hormone secretion, mast cell activation and vascular permeability, an effect mimicked by intradermal corticotropin-releasing hormone and inhibited by histamine-1 receptor antagonists. *International Archives of Allergy and Immunology*, 130, pp.224–231.

Ma, T.Y. et al., 2004. TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 286, pp.367–376.

Mackenzie, S.M. et al., 2000. Expression of 11β-hydroxylase and aldosterone synthase genes in the rat brain. *Journal of Molecular Endocrinology*, 24, pp.321–328.

Mahajan, S. et al., 2014. Urocortin 3 expression at baseline and during inflammation in the colon: corticotropin releasing factor receptors cross-talk. *Peptides*, 54, pp.58–66.

Mahata, B. et al., 2014. Single-cell RNA sequencing reveals T helper cells synthesizing steroids de novo to contribute to immune homeostasis. *Cell Reports*, 7, pp.1130–1142.

Mariniello, B. et al., 2006. Adipose tissue 11β-hydroxysteroid dehydrogenase type 1 expression in obesity and Cushing's syndrome. *European Journal of Endocrinology*, 155, pp.435–441.

Matsumoto, M. et al., 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *Journal of Immunology*, 171, pp.3154–3162.

Mazancová, K. et al., 2005. 11β-hydroxysteroid dehydrogenase in the heart of normotensive

and hypertensive rats. *Steroid Biochemistry & Molecular Biology*, 94, pp.273–277.

Miller, L.S., 2009. Toll-like receptors in skin. *Advanced Dermatology*, 24, pp.71–87.

Mittelstadt, P.R., Monteiro, J.P. & Ashwell, J.D., 2011. Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. *Journal of Clinical Investigation*, 122, pp.2384–2394.

Moore, X.-L., Hoong, I. & Cole, T.J., 2000. Expression of the 11β-hydroxysteroid dehydrogenase 2 gene in the mouse. *Kidney International*, 57, pp.1307–1312.

Morineau, G. et al., 1997. Radioimmunoassay of cortisone in serum, urine, and saliva to assess the status of the cortisol-cortisone shuttle. *Clinical Chemistry*, 43, pp.1397–1407.

Morohashi, B.K. et al., 1999. Structural and functional abnormalities in the spleen of an mFtz-F1 gene–disrupted mouse. *Blood*, 93, pp.1586–1594.

Mueller, M. et al., 2007. Differential regulation of glucocorticoid synthesis in murine intestinal epithelial versus adrenocortical cell lines. *Endocrinology*, 148, pp.1445–1453.

Mueller, M. et al., 2006. The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine. *Journal of Experimental Medicine*, 203, pp.2057–2062.

Mukherji, A. et al., 2013. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell*, 153, pp.812–827.

Mukhopadhyay, R. & Bishayi, B., 2009. Particulate antigen induced immune activation influences steroidogenesis in murine lymphoid organs with parallel increase in circulating IL-6. *Indian Journal of Experimental Biology*, 47, pp.699–705.

Müller, J.J. et al., 2001. Adrenodoxin reductase-adrenodoxin complex structure suggests electron transfer path in steroid biosynthesis. *Journal of Biological Chemistry*, 276, pp.2786–2789.

Neumann, D. et al., 2007. The death domain of IRAK-1: an oligomerization domain mediating interactions with MyD88, Tollip, IRAK-1, and IRAK-4. *Biochemical and Biophysical Research Communications*, 354, pp.1089–1094.

Ning, Y. et al., 2009. Overexpression of mitochondrial cholesterol delivery protein, StAR, decreases intracellular lipids and inflammatory factors secretion in macrophages.

Atherosclerosis, 204, pp.114–120.

Noti, M. et al., 2010. Lipopolysaccharide induces intestinal glucocorticoid synthesis in a TNFa-dependent manner. *FASEB Journal*, 24, pp.1340–1346.

O´Mahony, C.O. et al., 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-κB activation. *Proceedings of the National Academy of Sciences*, 4, p.e1000112.

Oakley, R.H. & Cidlowski, J.A., 2011. Glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. *Journal of Biological Chemistry*, 286, pp.3177–3184.

Oh, K. et al., 2017. Anti-inflammatory chromatinscape suggests alternative mechanisms of glucocorticoid receptor action. *Immunity*, 47, pp.298–309.

Oka, H. et al., 2000. Breakdown of Th cell immune responses and steroidogenic CYP11A1 expression in CD4+ T cells in a murine model implanted with B16 melanoma. *Cellular Immunology*, 15, pp.7–15.

Pace, T.W.W., Hu, F. & Miller, A.H., 2007. Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. *Brain, Behavior, and Immunity*, 21, pp.9–19.

Pácha, J., Lisá, V. & Mikšík, I., 2002. Effect of cellular differentiation on 11β-hydroxysteroid dehydrogenase activity in the intestine. *Steroids*, 67, pp.119–126.

Parker, D. et al., 1996. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Molecular and Cellular Biology*, 16, pp.694–703.

Penders, J. et al., 2008. Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics*, 118, pp.511–521.

Pérez, M.H.A. et al., 2013. A membrane glucocorticoid receptor mediates the rapid/non-genomic actions of glucocorticoids in mammalian skeletal muscle fibres. *Journal of Physiology*, 20, pp.5171–5185.

Piccinini, A.M. & Midwood, K.S., 2010. DAMPening inflammation by modulating TLR signalling. *Mediators of Inflammation*, 2010, pp.1–21.

Privalle, C.T., Crivello, J.F. & Jefcoatet, C.R., 1983. Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Biochemistry*, 80, pp.702–706.

Qian, C. & Cao, X., 2013. Regulation of Toll-like receptor signaling pathways. *Annals of the New York Academy of Sciences*, 1283, pp.67–74.

Qiao, S. et al., 2008. Age-related synthesis of glucocorticoids in thymocytes. *Experimental Cell Research*, 314, pp.3027–3035.

Rainey, W.E. & Nakamura, Y., 2008. Regulation of the adrenal androgen biosynthesis. *Journal of Steroid Biochemistry and Molecular Biology*, 108, pp.281–286.

Rainey, W.E., Shay, J.W. & Mason, J.I., 1986. ACTH induction of 3-hydroxy-3-methylglutaryl coenzyme a reductase, cholesterol biosynthesis, and steroidogenesis in primary cultures of bovine adrenocortical cells. *Journal of Biological Chemistry*, 261, pp.7322–7326.

Rakoff-Nahoum, S. et al., 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. *Cell*, 118, pp.229–241.

Raubenheimer, P.J. et al., 2006. The role of corticosterone in human hypothalamic-pituitary-adrenal axis feedback. *Clinical Endocrinology*, 65, pp.22–26.

Rescigno, M. et al., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature*, 2, pp.361–367.

Reul, J.M.H.M. & Kloet, E.R.D.E., 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology*, 117, pp.2505–2511.

Rhee, S.H. et al., 2003. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. *Journal of Biological Chemistry*, 278, pp.22506–22512.

Rousseau, K. et al., 2008. Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis. *FASEB Journal*, 21, pp.1844–1856.

Rubis, B., Krozowski, Z. & Trzeciak, W.H., 2006. Arginine vasopressin stimulates 11beta-hydroxysteroid dehydrogenase type 2 expression in the mineralocorticosteroid target cells. *Molecular and Cellular Endocrinology*, 256, pp.17–22.

Sahakitrungruang, T. et al., 2010. Four patients carrying partial loss-of-function mutations in the steroidogenic acute regulatory protein (StAR). *Endocrine Research*, 95, pp.3352–3359.

Sartor, R.B., 2004. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: angibiotics, probiotics, and prebiotics. *Gastroenterology*, 126, pp.1620–1633.

Scheinman, R.I. et al., 1995. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. *Molecular and Cellular Biology*, 15, pp.943–953.

Scheschowitsch, K. et al., 2017. New insights in glucocorticoid receptor signaling-more than just a ligand-binding receptor. *Frontiers in Endocrinology*, 8, pp.19–21.

Shen, W. et al., 2017. Ablation of vimentin results in defective steroidogenesis. *Endocrinology*, 153, pp.3249–3257.

Silvestre, J.-S. et al., 1998. Myocardial production of aldosterone and corticosterone in the rat. *Journal of Biological Chemistry*, 273, pp.4883–4891.

Simard, M. et al., 2010. Expression of genes related to the hypothalamic-pituitary-adrenal axis in murine fetal lungs in late gestation. *Reproductive Biology and Endocrinology*, 8, pp.1–15.

Slominski, A. et al., 1996. The expression of proopiomelanocortin (POMC) and of corticotropin releasing hormone receptor (CRH-R) genes in mouse skin. *Biochimica et Biophysica Acta*, 1289, pp.247–251.

Small, G.R. et al., 2005. Preventing local regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 enhances angiogenesis. *Proceedings of the National Academy of Sciences*, 102, pp.12165–12170.

Smith, P.M. et al., 2013. The microbial metabolites, short chain fatty acids, regulate colonic Treg cell homeostasis. *Science*, 341, pp.569–573.

Song, W. et al., 2015. Structural basis for specific recognition of single-stranded RNA by Toll-like receptor 13. *Nature Structural & Molecular Biology*, 22, pp.782–787.

Stefka, A.T. et al., 2014. Commensal bacteria protect against food allergen sensitization. *Proceedings of the National Academy of Sciences*, 111, pp.13145–13150.

Sturge, C.R. et al., 2013. TLR-independent neutrophil-derived IFN-γ is important for host resistance to intracellular pathogens. *Proceedings of the National Academy of Sciences*, 110, pp.10711–10716.

Takeda, K. & Akira, S., 2005. Toll-like receptors in innate immunity. *International Immunology*, 17, pp.1–14.

Takeuchi, O. et al., 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Japanese Society for Immunology*, 12, pp.113–117.

Taves, M.D. et al., 2016. Lymphoid organs of neonatal and adult mice preferentially produce active glucocorticoids from metabolites, not precursors. *Brain, Behavior, and Immunity*, 57, pp.271–281.

Tevosian, S.G. et al., 2015. Adrenal development in ice requires GATA4 and GATA6 transcription factors. *Endocrinology*, 156, pp.2503–2517.

Thieringer, R. et al., 2001. 11β-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. *Journal of Immunology*, 167, pp.30–35.

Tiganescu, A. et al., 2014. Increased glucocorticoid activation during mouse skin wound healing. *Society for endocrinology*, 221, pp.51–61.

Tiganescu, A. et al., 2011. Localization, age- and site-dependent expression, and regulation of 11β-hydroxysteroid dehydrogenase type 1 in skin. *Journal of Investigative Dermatology*, 131, pp.30–36.

Tomlinson, J.W. & Stewart, P.M., 2001. Cortisol metabolism and the role of 11β-hydroxysteroid dehydrogenase. *Best Practice & Research: Clinical Endocrinology and Metabolism*, 15, pp.61–78.

Vagnerová, K. et al., 2005. Intestinal inflammation modulates expression of 11β-hydroxysteroid dehydrogenase in murine gut. *Journal of Endocrinology*, 191, pp.497–503.

Vandevyver, S. et al., 2012. Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation. *Journal of Clinical Investigation*, 122, pp.2130–2140.

Vukelic, S. et al., 2011. Cortisol synthesis in epidermis is induced by IL-1 and tissue injury. *Journal of Biological Chemistry*, 286, pp.10265–10275.

Wang, M. et al., 2013. The steroidogenic enzyme Cyp11a1 is essential for development of peanut-induced intestinal anaphylaxis. *Journal of Allergy and Clinical Immunology*, 132, pp.1174–1183.

Whorwood, C.B., Ricketts, M.L. & Stewart, P.M., 1994. Epithelial cell localization of type 2 11β-hydroxysteroid dehydrogenase in rat and human colon. *Endocrinology*, 136, pp.2533–2541.

Willard-Mack, C.L., 2006. Normal structure, function, and histology of lymph nodes. *Toxicologic Pathology*, 34, pp.409–424.

Yamamoto, M. et al., 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *Journal of Immunology*, 169, pp.6668–6672.

Yang, F. et al., 2017. Glucocorticoid Receptor: megatrans switching mediates the repression of an ERα-regulated transcriptional program. *Molecular Cell*, 66, pp.321–331.

Ye, P. et al., 2008. Effects of ACTH, dexamethasone, and adrenalectomy on 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) gene expression in the rat central nervous system. *Journal of Endocrinology*, 196, pp.305–311.

Yong, P.Y.K. et al., 2002. Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1. *Human Reproduction*, 17, pp.2300–2306.

Zacharowski, K. et al., 2006. Toll-like receptor 4 plays a crucial role in the immune–adrenal response to systemic inflammatory response syndrome. *Proceedings of the National Academy of Sciences*, 103, pp.6392–6397.

Zangen, D. et al., 2014. Testicular differentiation factor SF-1 is required for human spleen development. *Journal of Clinical Investigation*, 124, pp.2071–2075.

Zarember, K.A. & Godowski, P.J., 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *Journal of Immunology*, 168, pp.554–561.

Žbánková, Š. et al., 2007. 11β-hydroxysteroid dehydrogenase 1 and 2 expression in colon from patients with ulcerative colitis. *Gastroenterology*, 22, pp.1019–1023.

Zhang, T.Y. et al., 2005. The expression of 11β-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. *Journal of Immunology*, 174, pp.879–889.

Zhou, W. et al., 2016. Steroidogenic acute regulatory protein overexpression correlates with protein kinase A activation in adrenocortical adenoma. *Proceedings of the National Academy of Sciences*, 11, p.e0162606.

Zubair, M. et al., 2006. Differential gene dosage effects of Ad4BP/SF-1 on target tissue development. *Biochemical and Biophysical Research Communications*, 341, pp.1036–1045.

www.metahit.eu