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The Role of Toll-like Receptors in the Adrenal Gland

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Zusammenfassung

Sepsis entsteht durch eine Infektion, der oft eine unkontrollierte Entzündungsreaktion folgt und die letztendlich in Schock, Organversagen oder Tod münden kann. Jedes Jahr sterben allein in Deutschland bis zu 60.000 Menschen an einer Sepsis. Vieles weist darauf hin, dass die Sepsis in Zusammenhang mit dem Versagen der Hypothalamus-Hypophysen-Nebennierenrinden-Achse steht. Es ist bekannt, dass bei septischen Patienten die von den Nebennieren freigesetzten Glukokortikoide (Kortisol, Kortikosteron) eine wesentliche Rolle während der Unterdrückung einer exzessiven proinflammatorischen Reaktion spielen. Die Nebenniereninsuffizienz tritt in großer Anzahl bei Patienten im septischen Schock auf und ist verantwortlich für die erhöhte Sterblichkeit der an Sepsis Erkrankten. Im Rahmen des angeborenen Immunsystems spielen die sogenannten Toll-like Rezeptoren (TLRs) eine bedeutende Rolle bei der Erkennung von pathogen-assoziierten Molekülen. Interaktionen zwischen dem Immunsystem und dem endokrinen Stresssystem sind bekannt. Demzufolge ist eine gut aufeinander abgestimmte Antwort des adrenalen Systems und des Immunsystems für das Überleben während einer schweren Entzündungsreaktion von besonderer Bedeutung.

Die vorliegende Arbeit befasst sich mit der Rolle von TLR-2, TLR-4 und TLR-9 während adrenalem Stress. Die Ergebnisse zeigen, dass das Fehlen von TLR-2 oder TLR-4, aber nicht TLR-9 zur Änderung der Morphologie der Nebennieren, bezüglich ihrer Größe und zellulären Struktur, bei den Mäusen führt. Dennoch scheint diese Änderung den Phänotyp der TLR knock-out Mäuse nicht zu beeinträchtigen. Mäuse mit fehlendem TLR-2, 4 oder 9 sind unfähig, sich adäquat auf einen inflammatorischen Stress, der durch die entsprechenden Liganden Lipopolysaccharid (LPS, TLR-4), Lipoteichonsäure (LTA, TLR-2) oder Cytidin-Phosphat-Guanosin-Oligodeoxynucleotid (CpG-ODN, TLR-9) hervorgerufen wird, zu antworten. Diese verminderte adrenale Stressantwort scheint mit der Abschwächung der systemischen und auch der intra-adrenalen Zytokin-Expressionen einherzugehen.
Zusammenfassend deuten diese Ergebnisse darauf hin, dass TLR-2, 4 und 9 Schlüsselrezeptoren in der immun-adrenalen Antwort bei Entzündung und SIRS sind. Das enge funktionale Verhältnis zwischen den beiden Systemen sollte im Rahmen der Behandlung von entzündlichen Krankheiten, bei denen eine intakte adrenale Stressantwort erforderlich ist, berücksichtigt werden. Darüber hinaus könnten TLR Polymorphismen zu den zugrunde liegenden Mechanismen der beeinträchtigten adrenalen Stressantwort bei Patienten mit bakterieller Sepsis beigetragen haben.

Abstract

Sepsis is caused by infection and often followed by an overwhelming inflammatory response. This can lead to shock, organ failure and even death. Each year approximately 60,000 people die in Germany due to sepsis. There is good evidence that sepsis is associated with failure of the hypothalamic-pituitary-adrenal-axis. In patients with sepsis, glucocorticoids (e.g. corticosterone, cortisol) released from adrenal glands play an essential role in preventing an excessive pro-inflammatory response. Adrenal insufficiency occurs in a large number of patients with septic shock and is associated with an increased mortality. In the innate immune system, Toll-like receptors (TLRs) play a crucial role in its onset by recognizing pathogen-associated molecules. It is well known that there are interactions between the immune and endocrine stress systems; glucocorticoids and TLRs regulate each other in a bi-directional way. Therefore, a coordinated response of the adrenal and immune system is of vital importance for survival during severe inflammation.

This experimental study focuses on the role of TLR-2, TLR-4 and TLR-9 during adrenal stress. The results show that in mice, the absence of TLR-2 and TLR-4, but not TLR-9 leads to altered adrenal morphology, relating to size and cellular structure. However, this alteration does not appear to compromise the phenotype of TLR knock-out mice. Mice deficient of TLR-2, 4 and 9 are not able to respond adequately to inflammatory stress induced by their potential ligands lipopolysaccharide (LPS), lipoteichoic acid (LTA) or cytidine phosphate guanosine-oligodeoxynucleotides (CpG-ODN). This impaired adrenal stress response appears to be associated with a decrease in systemic and intra-adrenal cytokine expressions. Taken together, these results suggest that TLR-2, 4 and 9 are key players in the immuno-endocrine response during inflammation and SIRS. In conclusion, TLRs play a crucial role in the immune-adrenal crosstalk. This close functional relationship needs to be considered in the treatment of inflammatory diseases where an intact adrenal stress response is required. Furthermore, TLR polymorphisms could contribute to the underlying mechanisms of impaired adrenal stress response in patients with bacterial sepsis.

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Publications

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Abbreviations

ACTH	adrenocorticotropic hormone
BSA	bovine serum albumin
CARS	compensatory anti-inflammatory response syndrome
CD	cluster of differentiation
CpG-ODN	cytidine phosphate guanosine-oligodeoxynucleotides
CRH	corticotropin releasing hormone
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
GM-CSF	granulocyte macrophage-colony stimulation factor
GRADE	Grades of Recommendation, Assessment, Development and Evaluation
GSK3β	glycogen synthase kinase 3β
HPA-axis	hypothalamic-pituitary-adrenal-axis
HRP	horseradish peroxidase
ICU	intensive care unit
IFN	interferon
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
i.p.	intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MODS	multiple organ dysfunction syndrome
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-κB
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PI3K	phosphoinositide-3-kinase
PRR	pattern recognition receptor

RIA	radioimmunoassay
SD	standard deviation
SEM	standard error of means
SIRS	systemic inflammatory response syndrome
TAK-1	transforming growth factor-β-activated protein kinase-1
TIR domain	Toll/IL-1 receptor-containing domain
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
TRAF-6	TNF receptor associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β-mediated transcription factor
WT	wild-type

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1 Introduction

1.1 Background

Sepsis is one of the major clinical and economic burdens of the world and a leading cause of death in intensive care units (ICU). An epidemiological study of the United States showed that severe sepsis (sepsis with associated acute organ failure) affects approximately 750,000 people every year (1995) and accounts for more than 210,000 deaths annually (Angus et al. 2001). A study by Watson and his co-workers, based on the paediatric hospital discharge records in the US, revealed 42,365 cases of paediatric severe sepsis per year (Watson et al. 2003). Effective prevention and treatments are urgently needed, since the death rate from sepsis has climbed more than 90% over the last two decades (Murphy 2000). In the United States, the incidence of sepsis increased on average by 9% per year in the last 20 years (Martin et al. 2003). From an economic point of view, the annual costs caused by sepsis were estimated at $16.7 billion in the US, with the highest costs due to infants, non-survivors, ICU patients, surgical patients and those with failure of more than one organ (Angus et al. 2001).

A recent epidemiological study by Engel and co-workers has documented that 154,000 new cases of sepsis and severe sepsis occur annually in Germany, an incidence of 226 per 100,000 inhabitants. This equates to an estimated nation-wide prevalence of 12.4% for sepsis and 11.0% for severe sepsis. The mortality rates remain high. In Germany, severe sepsis and septic shock result in ICU mortality of 48.4% and a remaining hospital mortality of 55.2% (Engel et al. 2007). With more than 59,000 deaths, sepsis is the third most frequent cause of death in Germany, behind coronary artery disease and cancer. The economic burden of sepsis is considerable with average costs per patient of about €25,000 at daily costs of approximately €1,500. In Germany, €1.7 billion are spent on the intensive care of sepsis patients annually. This accounts for about 32% of the budget of intensive care medicine in Germany [source from German Competence Network Sepsis (SepNet) in newsletter no. 17 (Dec.2004): Gesundheitsforschung: Forschung für den Menschen. Sepsis fordert viel mehr Todesopfer als gedacht. From the German Ministry of Education and Science (BMBF)].
In sepsis, increases in rates of morbidity and mortality are due to aging populations. The widening use of aggressive and invasive medical procedures and the growth of antimicrobial resistance (caused by overuse of antibiotics) make the disease harder to manage (Alarcon et al. 2004;Birtles et al. 2004;Johnson et al. 2004;Thongpiyapoom et al. 2004). Sepsis can strike

anyone at anytime, developing from infections associated with for e.g. pneumonia, trauma, surgery, burns or cancer. In fact, deaths that are attributed to complications from pneumonia or cancer are often actually caused by severe sepsis. An epidemiological study by Williams revealed a prevalence of 16.4 cases of severe sepsis per 1000 cancer patients per year in the US. Furthermore, the mortality rate for cancer patients with severe sepsis was 37.8% (Williams et al. 2004b). Although gram-negative infections (e.g. *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa*) were predominant in the 1960s and early 1970s gram-positive infections (mainly *Staphylococci* and *Streptococci*) have increased in the past two decades and now account for about half of cases of severe sepsis (Bochud et al. 2001). In the study from Engel et al. (2007), among patients with a microbiologically proven infection, the numbers of gram-positive and gram-negative isolates were comparable (55.7 and 54.1%, respectively).

Adrenal insufficiency is recognized in 60% of patients with sepsis (Annane et al. 2006) and can be absolute or relative. Either of these conditions can be permanent or transient. Absolute adrenal insuffiency, as defined by low basal and stimulated cortisol level (usually basal and stimulated cortisol concentrations < 560 nM/l), seems to be extremely uncommon in critically ill patients (0-3%). In contrast, plasma cortisol levels are usually elevated in patients with septic shock. The blunted response to stimulation has been refered to as 'relative adrenal insufficiency' (Matot and Sprung 1998;Rothwell et al. 1991). This causes adrenergic receptor desensitization, a decreased number of both α- and β-adrenergic receptors. This is either related to sepsis itself or to the use of vasopressors for long periods of time. Relative adrenal insufficiency is probably the most common and most difficult to diagnose and treat in critically ill patients. During septic shock, replacement therapy with low 'physiological' doses of steroid (hydrocortisone and fludrocortisone) showed improved survival (Annane et al. 2002;Bornstein and Briegel 2003;Burry and Wax 2004). The main effect of low-dose glucocorticoids is due to the reversal of suppression of the hypothalamic-pituitary-adrenal (HPA)-axis. However, the efficacy of glucocorticoids is related to their endogenous catecholamine-enhancing effects (Williamson and Lapointe 2003).

There is evidence to suggest that intact adrenal stress response is very important for a host's defence to infections (Chrousos 1995;Webster and Sternberg 2004). Furthermore, there are bi-directional communications between the immune and endocrine systems. Cytokines produced by an inflammatory response cause changes in the endocrine system, which include

activation of the HPA-axis. Hormones produced in the endocrine system, especially glucocorticoids, affect the immune responses. In addition to hypothalamic hormones (corticotrophin releasing hormone (CRH) and vasopressin), inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α have been identified as important modulators of HPA-axis function (Bornstein and Chrousos 1999;Chrousos 1995). During inflammation, these cytokines are capable of maintaining high glucocorticoid output, suggesting a shift from neuro-endocrine to immune-endocrine regulation of the adrenal (Bornstein et al. 2004a). Therefore, a coordinated response of the adrenal and immune system is of vital importance for survival during severe inflammation (Galon et al. 2002;McEwen and Seeman 1999;Sapolsky et al. 2000). Moreover, there is evidence that impaired innate immune responses mediated by Toll-like receptors (TLRs) may lead to sepsis including multiple organ failure (Cook et al. 2004;Li 2004), immunodeficiency (Decker 2004), atherosclerosis (de and Pasterkamp 2003) in humans. The initial host defence against bacterial infection by the innate immune system essentially requires TLRs, which belong to family of pattern recognition receptors (PRRs). They are responsible for the detection and response to conserved microbial components by activating an inflammatory signal cascade.

This study investigates whether interactions between the innate immune system and the HPA-axis exist. Therefore, this study focuses on the signalling pathway of TLR-2, 4 and 9 in mediating responses of the adrenal stress system. The role of these three TLRs in the adrenal gland during development and progression of inflammatory conditions has not been investigated to date.

1.2 Aims of this Study

This thesis focuses on the following aims:

1. Expression of TLR-2, 4 and 9 in murine adrenal glands.
2. Expression pattern of these TLRs in adrenals after treatment with a non-lethal dose of their potential ligand lipopolysaccharide (LPS), lipoteichoic acid (LTA) or cytidine phosphate guanosine-oligodeoxynucleotides (CpG-ODN).
3. Alterations in adrenal morphology and function in TLR-2, 4 and 9 deficient mice.
4. Effects of the potential ligands on the release of adrenal corticosterone and adrenocorticotropic hormone (ACTH) in wild-type (WT) and TLR-2, 4 and 9 deficient mice.
5. Activation of the transcription factor NF-κB in murine adrenal glands after potential ligand stimulation.
6. Effects on several inflammatory cytokines during an intra-adrenal stress response induced by the potential ligands and in the absence of the respective TLR gene.

2 Review of the Literature

2.1 Innate Immune System

The immune system is a large and complex defence network of widely distributed elements in the body. The immune system can recognize, control and kill pathogens that gain successful entry into the body. There are two types of immunity: innate (natural) and adaptive (specific) immunity.

Although not pathogen specific, the innate immunity is largly responsible for containing and limiting the spread of infection. The first line of defence against invading microorganisms is the physical barriers, like skin or self-cleaning processes (e.g. sneezing and mucous flow) (Tizard 2000). The second line of defence is the inflammatory response, which results in increased vascular permeability and increased blood flow in affected tissues followed by an accumulation of leukocytes and certain soluble factors. Neutrophils, monocytes and natural killer cells migrate to affected areas guided by chemotactic factors, like complement factor C5a and IL-8 (Paape et al. 2002). The innate immunity distinguishes self from non-self (pathogens) through pattern recognition receptors (PRRs) by recognizing the conserved structures of invading microorganisms called pathogen-associated molecular patterns (PAMPs) (Aderem and Underhill 1999;Janeway, Jr. 1992). After recognition of transformed or infected cells, natural killer cells and T-cells secrete cytokines that play a critical role in coordinating the early defence by innate and later by the acquired immune system. In addition, evidence in the literature shows that the activation of innate immunity plays a key role in stimulating the adaptive immune response (Hoffmann et al. 1999).

Adaptive immunity develops later than the innate immunity and consists of lymphocytes activation and their products, including antibodies and cytokines (Vitetta et al. 1991). The important difference between innate and adaptive immune system is that the adaptive immune response is highly specific to pathogens. Moreover, the response improves with each successive encounter of the same pathogen due to the establishment of long-term memory of the antigen. The adaptive immunity can be divided into humoral and cell-mediated responses and becomes effective only after several days. This is the time required for lymphocytes to recognize specific antigens and to proliferate and differentiate into effector cells and finally to eradicate the pathogen. In contrast, the innate immunity which consists of antimicrobial

peptides, phagocytes, and complement system is activated immediately after infection and controls the replication of the pathogen (Medzhitov and Janeway, Jr. 2000).

2.2 PRRs and PAMPs

Innate immune responses are activated rapidly after the introduction of inflammatory stimuli to the host. Microbes contain highly conserved molecular structures called PAMPs, including cell wall components LPS (Poltorak et al. 1998b), LTA (Lien et al. 1999), peptidoglycan (Lien et al. 1999), mannan (starie-Dequeker et al. 1999), glycans (Ozinsky et al. 2000) as well as foreign DNA (Hemmi et al. 2000) and double-stranded ribonucleic acid (Alexopoulou et al. 2001). Other examples of PAMPs are bacterial lipoproteins and lipoarabinomannan of mycobacteria (Medzhitov and Janeway, Jr. 1997b). PAMPs are recognized as 'foreign' by specific PRRs which are preferentially expressed in monocytes and macrophages as well as in other cell types. The receptors can be structurally divided into those containing a leucine-rich repeat domain, a calcium-dependent lectin domain or a scavenger receptor protein domain. Furthermore, PRRs characterized so far include cluster of differentiation (CD)14 (Viriyakosol and Kirkland 1995), β2-integrins (CD11/CD18) (Ehlers 2000), C-type lectins (Keler et al. 2004;McGreal et al. 2004), macrophage scavenger receptors (Ramet et al. 2001;van der Laan et al. 1999) and complement receptors (Humbles et al. 2000). Functionally, they are divided into secreted or endocytic proteins or signalling molecules (Medzhitov and Janeway, Jr. 1997a). Secreted PRRs serve as opsonins and bind to the cell wall of a microbe, tagging it for recognition by the complement system. Endocytic PRRs are expressed on the surface of phagocytes and mediate phagocytosis and delivery of microbes to lysosomes for degradation. Signalling PRRs induce the expression of a variety of acute-phase reaction products. In mammals, PAMPs activate the production of bioactive lipids (e.g. platelet-activating factor) and cytokines (e.g. IL-1, IL-6 and TNF-α), which all are important in the response to infection (Hallman et al. 2001;Janeway, Jr. and Medzhitov 1998;Janeway, Jr. and Medzhitov 2002;Zhang and Ghosh 2001).

2.2.1 LTA

The cell wall of gram-positive bacteria is structurally simpler than the cell wall of gram-negative bacteria. No outer lipid layer exists and up to 90% of the cell wall of gram-positive bacteria may consist of peptidoglycan. LTA molecules are composed of a repeating glycerol phosphate backbone that is substituted with D-alanine, sugars (e.g. glucose) and a single lipid side chain, which intercalates into the cytoplasmic membrane (Fischer et al. 1990). The chemical structure of LTA derived from *Staphylococcus aureus* is shown in Fig. 1. LTA appears to be crucial for the vital function of the bacteria because their biosynthesis is not halted in the absence of phosphate. The biological functions of LTA include binding of metal cations by the negatively charged polysaccharide chains (Rose and Hogg 1995) and regulation of the activity of autolytic enzymes (Fischer 1988;Fischer 1994). To the host, they represent patterns for immune recognition similar to LPS. The amphiphlic LTA molecule resembles LPS, in that it forms micelles; it is lipid anchored and carries phosphate as well as repetitive subunits of carbohydrates.

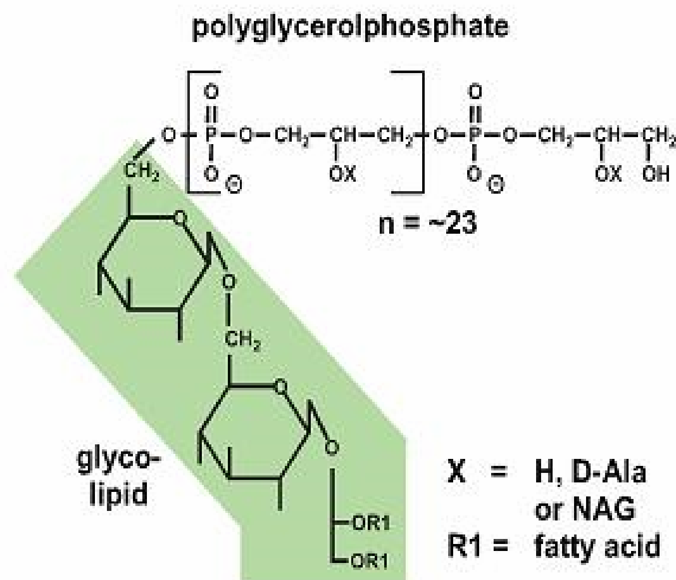


Figure 1: Schematic structure of LTA derived from *Staphylococcus aureus.* D-Ala, D-alanine; NAG, N-acetyl-glucosamine. Modified from http://www3.imperial.ac.uk/cmmi/research/angelikagrundling.

2.2.2 LPS

LPS represents the main outer membrane component of gram-negative bacteria and plays a key role in mediating severe gram-negative infection, sepsis and septic shock (Rietschel and Brade 1992;Rietschel et al. 1994;Schletter et al. 1995). LPS is now used as a synonym for endotoxin since it was discovered in the late 19th century by Richard Pfeiffer (Pfeiffer 1892). In contrast to secreted exotoxins of cholera bacteria, this heat stable toxin was found to be a constituent of the bacterial cell, thus Pfeiffer named it endotoxin. Although there is a great variation between endotoxins (derived from different bacterial serotypes), they all share a common structural. A schematic of the structure of LPS is shown in Fig. 2.

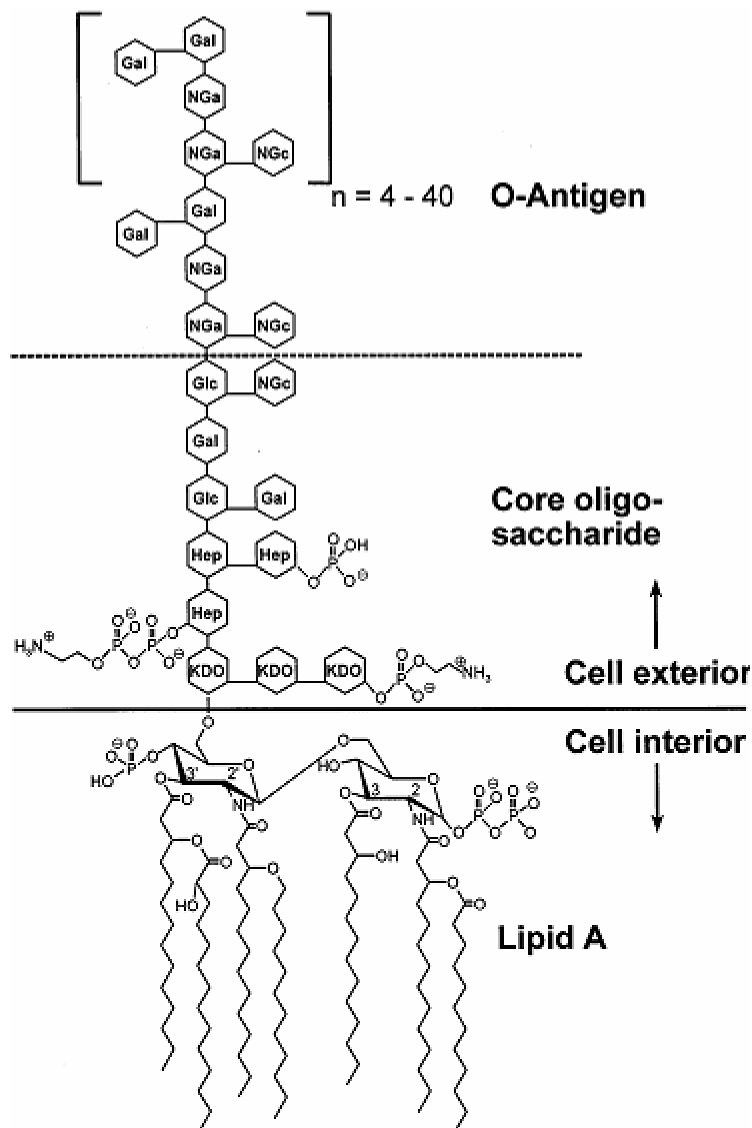


Figure 2: Schematic structure of LPS derived from *E. coli* (O111:B4). Hep, L-glycerol-D-manno-heptose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctonic acid; NGa, N-acetyl-galactosamine; NGc, N-acetyl-glucosamine (Magalhaes et al. 2007).

LPS consists of a sugar portion with varying length of polysaccharide chains. The sugar part is covalently linked to the hydrophobic part of LPS, lipid A, which anchors the LPS molecule to the outer membrane (Schletter et al. 1995). The complex structure of LPS can be further divided into the O-specific chain and lipid A. The O-specific chain is the part of LPS that shows the largest variation between species and the part that evokes a specific immune response. It stimulates the production of antibodies which are able to recognize that particular O-chain. The O-chain consists of repeated oligosaccharide units and is attached to the core. The core oligosaccharide is divided into outer (linked to the O-chain) and inner core (linked to lipid A). The core has a complex structure: It is joined to the lipid A region by 3-deoxy-D-manno-octulosonic acid, which is an eight carbon sugar. The core structure is conserved for each type of bacteria and its biological function is not clear. The structure of lipid A is more conserved than that of the core. The lipid A part is responsible for endotoxic properties, complement activation and B lymphocyte mitogenicity (Galanos et al. 1985).

2.2.3 CpG-DNA

CpG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide. ‘CpG’ stands for cytosine and guanine separated by a phosphate, which links the two nucleosides together in DNA. CpG dinucleotides are more frequent in the genomes of bacteria and viruses than of vertebrates. In addition, the cytosines in CpG dinucleotides are highly methylated in vertebrates. It can be noted that in mammals, 70-80% of CpG cytosines are methylated by DNA methyltransferases (Jabbari and Bernardi 2004). A decreased in CpG frequency and cytosine methylation leads to abrogation of the immunostimulatory activity in vertebrates. Therefore, unmethylated CpG sites can be used to detect DNA from pathogens.

There are two different subtypes of CpG-DNA: A/D-type CpG-DNA (CpG-A) and B/K-type CpG-DNA (CpG-B) (Klinman 2004). CpG-A is characterized by a phosphodiester backbone CpG motif and phosphorothioate-modified poly G stretches at the 5′ and 3′ ends. As a TLR-9 ligand, CpG-A strongly induces interferon (IFN)-α production from plasmacytoid dendritic cells (DC) and co-localizes with TLR-9, myeloid differentiation factor 88 (MyD88) and interferon regulatory factor (IRF)7 in endosomes in plasmacytoid DC (Hemmi et al. 2003;Krug et al. 2001). By contrast, CpG-A is rapidly transferred and degraded in the

lysosome in conventional DC and macrophages. However, when CpG-A is relocalized to the endosome in conventional DC using a cationic lipid, these cells can produce IFN-α as a consequence of activation of the MyD88-IRF7 pathway. On the other hand, CpG-B is phosphorothioate-modified throughout the sequence and is known to induce DC maturation and B cell proliferation. CpG-B poorly induces type I IFN, but induces inflammatory cytokines, also elicits IFN-α induction if it is manipulated to stay longer in the endosome of conventional DC (Honda et al. 2005). These findings suggest that retention of the CpG-DNA/TLR-9 complex in the endosome might cause the induction of robust IFN-α production.
Furthermore, several reports have shown that DNA without CpG motifs can be a biologically active ligand for TLR-9. Oligodeoxynucleotides containing nucleotide derivatives or the phosphorothioate backbone along with a bicyclic heterobase structure have immuno-stimulatory activity through TLR-9 (Kandimalla et al. 2005;Roberts et al. 2005;Vollmer et al. 2004). Thus, TLR-9 recognizes not just CpG motifs, but DNA itself with certain structures.

2.3 TLRs

TLRs are evolutionarily conserved and have been found in insects, plants and mammals. The discovery of TLRs was based on identification of Drosophila Toll, which was initially characterized as a developmental protein governing the formation of the dorsal-ventral axis in *Drosophila melanogaster* (Anderson et al. 1985;Hashimoto et al. 1988;Morisato and Anderson 1995). However, a subsequent study revealed that Drosophila Toll also plays a key role in triggering innate immune responses against fungal infection in adult flies (Anderson 2000;Belvin and Anderson 1996). The first characterized mammalian TLR (first named Toll and now termed TLR-4) was found in 1997 by Charles Janeway Jr. and his co-workers (Medzhitov et al. 1997). Since then, several proteins structurally related to TLR-4 were identified and named TLRs (Rock et al. 1998). To date, 11 TLRs in humans and 13 in mice have been identified (Akira 2004;Beutler 2004;Verstak et al. 2007). TLRs 1-9 are conserved in humans and mice. TLR-10 is present only in humans and TLR-11 is functional only in mice and is silenced by a stop codon in humans. The biological roles, expression patterns, ligands and modes of signalling of TLR-10, 12 and 13 remain to be defined. The known ligands recognized by these different receptors are summarized in Table 1.

TLR	Microbial Ligand	Endogenous Ligand
TLR-1/TLR-2	triacyl lipopeptides (bacteria and mycobacteria)	
TLR-2	PG (gram-positive bacteria); porins (*Neisseria*); lipoarabinomannan (mycobacteria); phospholipomannan (*Candida albicans*); glucoronoxylomannan (*Cryptococcus neoformans*); tGPI-mutin (*Trypanosoma*); haemagglutinin protein (Measles virus); HCMV; HSV1	necrotic cells (cellular injury); HSP60, HSP70 (cellular injury; controversial!); gp96 (cellular injury); HMGB1 (damaged cells); hyaluronate (degradation of extracellular matrix); fibronectin (release from cells in response to tissue damage); minimally modified LDL (atherosclerotic lesion); heparin sulphate (plasma membrane or extracellular matrix in injury or inflammation); HSPB8 (synovial tissue); αA crystallin
TLR-3	dsRNA (viruses)	dsRNA (necrotic cells)
TLR-4	LPS (gram-negative bacteria); glucuronoxylomannan (*Cryptococcus neoformans*); glucoinositolphospholipids (*Trypanosoma*); envelope proteins (RSV, MMTV)	same ligands as for TLR-2
TLR-5	flagellin (flagellated bacteria)	
TLR-6/TLR-2	diacyl lipopeptides (Mycoplasma); LTA (Group B *Streptococcus*); zymosan (*Saccharomyces cerevisiae*)	
TLR-7	ssRNA (RNA viruses)	ssRNA (dead or dying cells); purified snRNPs
TLR-8	ssRNA (RNA viruses)	ssRNA (dead or dying cells); purified snRNPs
TLR-9	CpG-DNA (bacteria and mycobacteria); haemozoin (*Plasmodium*); DNA (viruses)	DNA (dead or dying cells)
TLR-11	unknown component from uropathogenic bacteria; profiling-like molecule (*Toxoplasma gondii*)	

Table 1: Microbial and endogenous ligands of TLRs. HCMV, human cytomegalovirus; HMGB1, high-mobility group box 1; HSP, heat shock protein; HSPB8, heat shock 22kDa protein 8; HSV1, herpes simplex virus 1; LDL, low-density lipoprotein; LPS, lipopolysaccharide; LTA, lipotechoic acid; MMTV, mouse mammary tumor virus; PG, peptidoglycan; RSV, respiratory syncytial virus; snRNPs, small nuclear ribonucleoproteins; tGPI-mucin, glycosylphosphatidylinositol-anchored mucin-like glycoprotein. Information adapted from Akira et al. 2006;Marshak-Rothstein 2006;Tian et al. 2007.

All TLRs share the same structure: a ligand binding extracellular domain (ectodomain) consisting of leucine-rich repeats, a single transmembrane α-helix (transmembrane domain), and the cytoplasmic domain, known as the Toll/IL-1 receptor (TIR) domain, which couples downstream signal transduction to receptor engagement (see Fig. 3). A comparison of the sequences of the TLR members reveals five sub-families: TLR-2, 3, 4, 5, and 9. The TLR-2 subfamily is composed of TLR-1, 2, 6, and 10, while the TLR-9 subfamily is composed of TLR-7, 8, and 9 (Takeda et al. 2003). TLRs are PRRs that function as a CD14-associated signal inducers, helping cells to recognize and distinguish between pathogens. They also help to bridge innate and adaptive immunity by inducing various co-stimulatory and effector molecules (Zhang and Ghosh 2001). TLRs sense microbial products (PAMPs) as well as endogenous ligands (Tsan and Gao 2004).

Consistent with their role in pathogen recognition and host defence, TLRs are expressed on monocytes/macrophages, neutrophils, dentritic cells, intestinal epithelial cells and endothelial cells. These cell types are immediately accessible to microorganisms upon infection (Muzio et al. 2000). There are two different mechanisms by which activation of TLRs can contribute to host defence. First, activation of TLRs can directly mediate innate responses by regulating phagocytosis and triggering anti-microbial activity (Blander and Medzhitov 2004). Second, activation of TLRs can trigger the release of cytokines and the differentiation of immature to mature dendritic cells, enabling the innate immune system to instruct the adaptive immune response (Medzhitov and Janeway, Jr. 1997a).

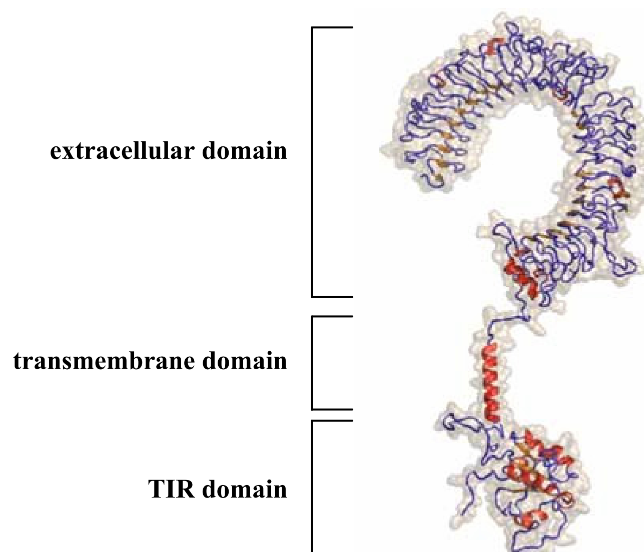


Figure 3: The structure of TLR-3. A diagram showing the extracellular, transmembrane and cytoplasmic domains of the receptor. Toll/IL-1–receptor-containing (TIR) domain.
Modified from: http://www.bio.indiana.edu/facultyresearch/faculty/Kao.html.

2.3.1 TLR-2

TLR-2 distinguishes a variety of microbial components such as lipoproteins from different pathogens, peptidoglycans and LTA from gram-positive bacteria (Takeda et al. 2003). Furthermore, TLR-2 has been shown to recognize LPS from non-enterobacteria such as *Leptospira interrogans*, *Phorphyromonas gingivalis* and *Helicobacter pyroli* (Hirschfeld et al. 2001;Smith, Jr. et al. 2003;Werts et al. 2001). This LPS structurally differ from the typical LPS of gram-negative bacteria (recognized by TLR-4) in the number of acyl chains in the lipid A component which presumably confers to differential recognition (Netea et al. 2002).
One aspect that could explain why TLR-2 recognizes a wide spectrum of microbial components is that TLR-2 forms heterophilic dimers with other TLRs such as TLR-1 and TLR-6, both are structurally related to TLR-2.
Studies on TLR-1 and TLR-6 deficient mice have shown the important role of these receptors in the recognition of structural differences between lipopeptides. Macrophages from TLR-6 deficient mice did not produce any inflammatory cytokines in response to lipopetides derived from gram-positive bacteria. However, these cells have normal production of inflammatory cytokines in response to lipopeptides derived from gram-negative bacteria (Takeuchi et al. 2001). In contrast, TLR-1 has an impaired response to wall fragments of gram-negative bacteria and a normal response to wall fragments of gram-positive bacteria (Takeuchi et al. 2002). Therefore, TLR-1 and TLR-6 associate with TLR-2 and participate in the recognition of different lipopetides.

2.3.2 TLR-4

TLR-4 was in fact the first TLR to be described and was originally named human Toll. LPS is an agonist for TLR-4. The discovery that this receptor is necessary for LPS signalling was confirmed in TLR-4 knock out (TLR-4$^{-/-}$) mice (Hoshino et al. 1999). The recognition of LPS by TLR-4 requires several additional molecules. LPS binds to LPS-binding protein, an acute phase protein which circulates in the bloodstream, there it recognizes and forms a high-affinity complex with the lipid A part of LPS (Schumann et al. 1990). LPS-binding protein helps LPS to dock on the receptor by binding and then forming a ternary complex with CD14, enabling LPS to be transferred to the receptor complex of TLR-4 (Viriyakosol and Kirkland 1995). The glycoprotein MD-2 acts as an extracellular adaptor protein in the activation of

TLR-4 by LPS and is necessary for LPS signalling (Shimazu et al. 1999;Yang et al. 2000). Visintin and colleagues demonstrated that LPS binds to MD-2 and induces subsequently TLR-4 signalling (Visintin et al. 2003). It has been demonstrated that MD-2 is indispensable for LPS responses, because MD-2 deficient mice are unresponsive to LPS (Nagai et al. 2002). In addition to recognizing LPS, TLR-4 mediates signals induced by exogenous compounds like taxol (Byrd-Leifer et al. 2001), several endogenous ligands such as heat shock proteins HSP60 and HSP70 (Gao and Tsan 2003), the extra domain A of fibronectins (Termeer et al. 2002), oligosaccharides of hyaluronic acid (Ohashi et al. 2000), heparin sulphate and fibrinogen (Johnson et al. 2002).

2.3.3 TLR-9

TLR-9 recognizes unmethylated CpG sites on DNA molecules and is hence the receptor for bacterial and viral CpG-DNA, as well as non-nucleic acids such as haemozoin from the malaria parasite (Akira et al. 2006;Hemmi et al. 2000). Upon *Mycobacterium tuberculosis* infection, TLR-9 cooperates with TLR-2 to induce innate and adaptive immune responses against the bacterium (Bafica et al. 2005). TLR-9 also plays an important role in the fight against infection with *Brucella* (Copin et al. 2007) and *Streptococcus pneumoniae* (Lee et al. 2007). Polymorphisms in TLR-9 have been reported to be associated with an increased susceptibility for *Helicobacter pylori* infection, suggesting that TLR-9 is involved in recognition and clearance of *Helicobacter* (Anderson et al. 2007). Genomes of DNA viruses often have unmethylated CpG motifs, and therefore act as a ligand for TLR-9. Mouse cytomegalovirus (Krug et al. 2004a), herpes simplex virus type 1 (Krug et al. 2004b) and type 2 (Lund et al. 2003) and adenovirus (Zhu et al. 2007) are recognized by TLR-9 on plasmacytoid DC, and the resulting activated plasmacytoid DC produce IFN-α and other cytokines.

TLR-9 is localized intracellularly in late endosomes or lysosomes, where it detects unmethylated CpG-DNA (Latz et al. 2004;Leifer et al. 2004). It has been suggested that the intracellular localization of TLR-9 is important to enable discrimination of foreign-derived DNA and self-DNA. Barton et al. (2006) demonstrated that when the transmembrane and cytoplasmic region of TLR-9 is replaced to that of TLR-4, the chimeric protein (TLR9N4C) is trafficked to the plasma membrane. Whereas TLR9N4C responds to CpG-DNA, it has no ability to sense viral DNA. More importantly, when TLR9N4C is expressed on the cell

surface of macrophages, these cells respond to self-DNA. As abnormal recognition of self-DNA is associated with the pathogenesis of autoimmune diseases, intracellular localization of TLR-9 might safeguard against contact with self-DNA.

2.4 TLR Signalling Pathways

Upon binding to specific ligands via pattern recognition, TLRs recruit and activate various downstream kinases such as IL-1 receptor-associated kinase (IRAK)-1, IRAK-4 and TNF receptor-associated factor (TRAF) family member-associated NF-κB activator binding kinase 1 (TBK1) via a specific set of adaptors. There are five Toll/IL-1 receptor-containing (TIR) domain-containing adaptors, namely myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR domain-containing adaptor protein inducing IFN-β-mediated transcription factor (TRIF), TRIF-related adaptor molecule (TRAM) and a sterile α- and armadillo-motif-containing protein (O'Neill and Bowie 2007). TLRs interact with their respective adaptor(s) via the homologous binding of their unique TIR domains present in both, the receptors and the adaptor molecules. Based on the specific adaptors recruited to TLRs, TLR signalling can be divided into two general pathways, namely MyD88-dependent and MyD88-independent pathway. The two distinct signalling pathways lead to the production of pro-inflammatory cytokines and type 1 IFN, respectively (O'Neill and Bowie 2007). Recently, a novel pathway of TLR signalling has been discovered, which involves phosphoinisitide-3-kinase (PI3K) and protein kinases B which is also called as Akt, leading to activation of nuclear factor-κB (NF-κB). An overview of the TLR signalling pathways is shown in Fig. 4.

2.4.1 MyD88-dependent Pathway

Upon recognition of PAMPs through TLRs, the adaptor molecule MyD88 interacts with the transmembrane receptor through the C-terminal TIR domain. MyD88 then recruits IRAK-4 (a serine threonine kinase) to TLRs through interaction of the death domain of both molecules and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Phosphorylated IRAK-1 associates with TRAF-6 (Cao et al. 1996;Wesche et al. 1997), leading to the activation of two

distinct signalling pathways. One pathway leads to activation of the transcription factor activator protein-1 (AP-1) through activation of mitogen-activated protein kinases (MAPKs). On the other pathway, the IRAK-1/TRAF-6 complex then activates transforming growth factor-β-activated protein kinase-1 (TAK-1) through a process involving the cytosol translocation of TAK-1 and two TAK-1-binding proteins (TAB) from membrane to cytosol and the ubiquitination of TRAF-6 (Jiang et al. 2002;Takaesu et al. 2001). Activated TAK-1 then enhances activity of the IκB kinase (IKK) complex as well as c-Jun N-terminal kinase (JNK)/p38 kinases (Wang et al. 2001). Once activated, the IKK complex induces phosphorylation and degradation of the inhibitor of NF-κB (IκB), which leads to subsequent translocation of NF-κB to the nucleus and finally transcription of pro-inflammatory genes (Akira and Takeda 2004;Dunne and O'Neill 2003;Janeway, Jr. and Medzhitov 2002).

As stated above, MyD88 is one of the five TIR domain-containing adaptors in TLR signalling (O'Neill and Bowie 2007) and plays a critical role in the signalling of all TLRs except TLR-3 (Janssens and Beyaert 2002;Medzhitov et al. 1998;Muzio et al. 1997).

2.4.2 MyD88-independent Pathway

In MyD88-deficient macrophages, TLR-4 activation of inflammatory cytokines does not occur. However, activation of NF-κB is observed with delayed kinetics (Kawai et al. 1999). This indicates that although TLR-4 activation of inflammatory cytokines depends on the MyD88-dependent pathway, a MyD88-independent component exists in TLR-4 signalling. Subsequent studies have shown that TLR-4 stimulation leads to activation of IRF-3 as well as the late phase of NF-κB activation in a MyD88-independent pathway (Kawai et al. 2001). TLR-4 activation of IRF-3 leads then to the production of IFN-β. A previous study proved that viral infection or double stranded RNA may activate IRF-3 (Yoneyama et al. 1998). Accordingly, the TLR-3-mediated pathway also activates IRF-3 and thereby induces IFN-β in a MyD88-independent way. Hence, TLR-3 and TLR-4 use the MyD88-independent signalling pathway to induce IFN-β.

TRIF has been demonstrated to be essential for TLR-3 and TLR-4 mediated MyD88-independent pathways. TRIF deficient mice generated by gene targeting showed no activation of IRF-3 and have impaired expression of IFN-β and IFN-inducible genes in response to TLR-3 and TLR-4 ligands (Diebold et al. 2004). IRF-3 binds to the IFN-stimulated response element and leads to induction of type-1 IFNs (Au et al. 1995).

Studies with TRAM deficient mice showed that TRAM is involved in TLR-4-mediated, but not TLR-3-mediated activation of IRF-3 and induction of IFN-β and IFN-inducible genes (Fitzgerald et al. 2003;Oshiumi et al. 2003;Yamamoto et al. 2003). Thus, TRAM is an adaptor molecule that provides specificity for the MyD88-independent pathway of TLR-4 signalling.

2.4.3 PI3K Properties in TLR-mediated Signalling

In vitro studies using pharmacological inhibitory compounds of PI3K, e.g. the fungal metabolite wortmannin or the synthetic inhibitor LY294002 suggested an involvement of PI3K in the modulation of innate immune responses, though the role of PI3K still remained controversial. Some studies showed direct or indirect TLR-mediated activation of IKK/NF-*κ*B signaling via PI3K/Akt. Inhibition of PI3K by pharmacological inhibitors or dominant negative PI3K constructs led to diminished NF-*κ*B activation and reduced inflammatory gene expression (Arbibe et al. 2000;Ojaniemi et al. 2003;Rhee et al. 2006). In contrast, others could show an enhancing effect of pharmacological inhibition of PI3K on pro-inflammatory gene expression, which was dependent on a number of signalling pathways such as p38, JNK, extracellular signal-regulated kinases and IKK (Aksoy et al. 2005;az-Guerra et al. 1999;Guha and Mackman 2002) Furthermore, several studies provide *in vivo* evidence for a down-regulatory role on innate immunity of PI3K in models of acute inflammation and sepsis, using the potent PI3K inhibitor wortmannin (Schabbauer et al. 2004;Williams et al. 2004a;Zhang et al. 2007).

The first genetic reports on the role of PI3K in the regulation of innate immune responses were published by the group of Fukao. It could be shown *in vitro* that ablation or down-modulation of PI3K activity had strong impact on the innate immune response in DCs. In particular, IL-12 expression upon PAMP stimulation (TLR-2, 4 and 9) was elevated in p85*α*-deficient cells (Fukao et al. 2002). Another study could show *in vivo* that p85*α* deficiency led to an enhanced response of gene-deficient mice to the TLR-5 agonist flagellin (Yu et al. 2006). These data indicate that PI3K is involved in the down-regulation of pro-inflammatory responses. More recently, Luyendyk and colleagues showed evidence for a role of PI3K in the modulation of the amplitude of inflammatory signals. Several pro-inflammatory signalling pathways activated by TLR-4, such as the MAP kinases p38, JNK and extracellular signal-regulated kinase 1 and 2 are affected by p85*α* deficiency in macrophages. Activation of

MAPKs upon LPS/TLR-4 activation was enhanced and persisted over a prolonged period of time in p85α-deficient macrophages, as compared to WT control cells (Luyendyk et al. 2008). Furthermore, one study found direct effects of Akt and glycogen synthase kinase 3β (GSK3β) on NF-κB activity (Martin et al. 2005). Downstream of Akt, GSK3β is a crucial target in the PI3K signalling cascade. GSK3β is important for a variety of cellular processes, such as glucose/glycogen metabolism, protein synthesis and apoptosis (Frame and Cohen 2001). Recently, Martin and colleagues proved that Akt-mediated GSK3β inhibition led to down-modulation of TLR-2, 4, 5 and 9 mediated inflammation both *in vitro* and *in vivo* (Martin et al. 2005).

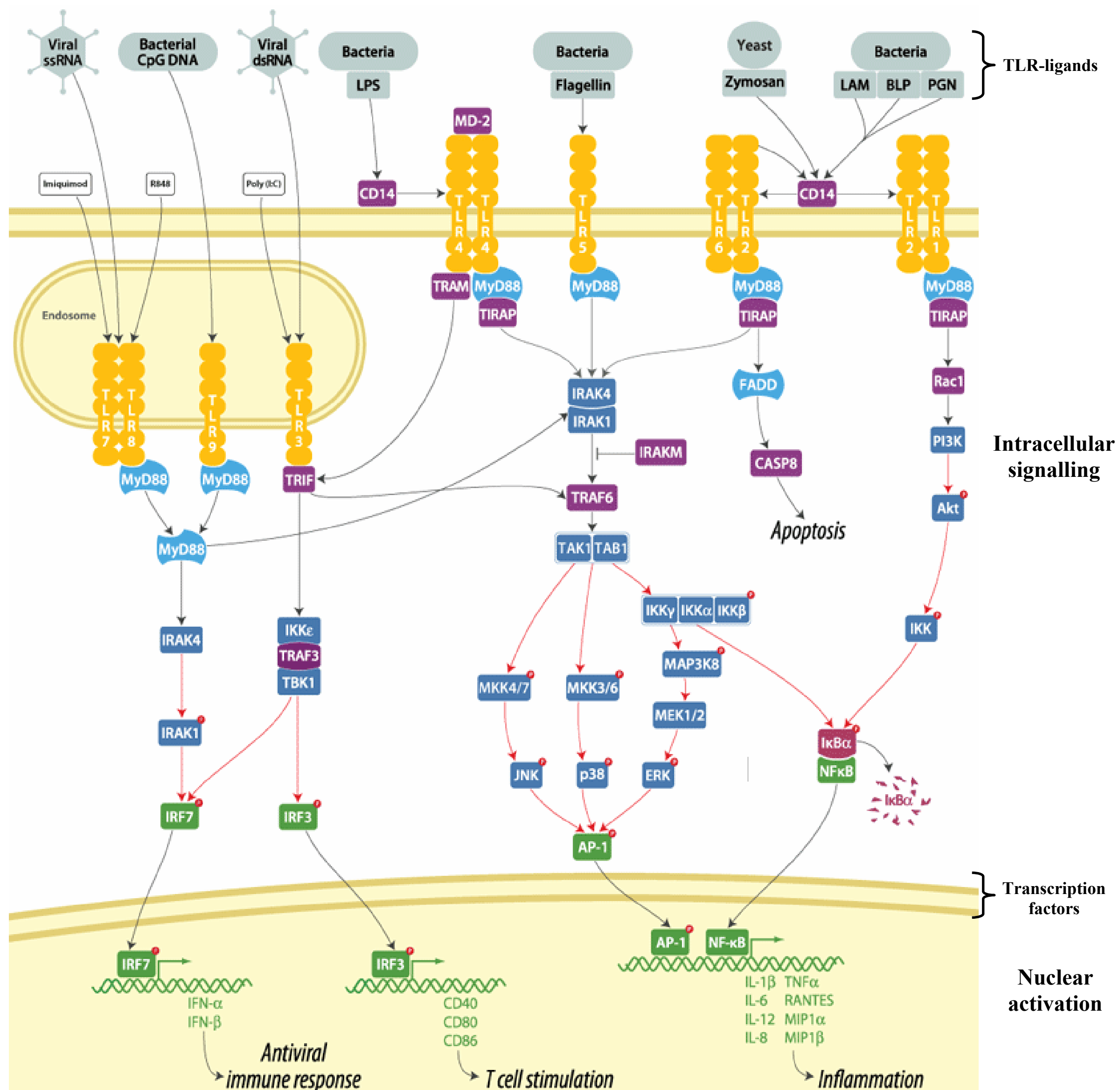


Figure 4: TLR signalling pathways. A schematic of TLR signalling initiated by various TLR ligands. On MyD88-dependent pathway, MyD88 serves as the central adapter protein associating with IRAK4 which in turn recruits and phosphorylates IRAK1. Following interaction with TRAF6, the activated IRAK complex phosphorylates TAB1 and TAK1, and activates the NF-κB and MAPK pathways. On MyD88-independent pathway, TLRs (only TLR-3 and TLR-4) interact with TRIF, thereby activating a complex of IKKε, TRAF3 and TBK1 that phosphorylates IRF3 and IRF7. A noveau discovered pathway of TLRs involving PI3K and Akt, which finally leads to activation of NF-κB.
Activation of IRF3 results in the induction of genes (CD40, CD80 and CD86) that stimulate T cell immunogenic responses. IRF7 promotes an antiviral immune response by the induction of IFN-α and -β gene expression. AP-1 and NF-κB mediate inflammatory responses through the expression of different cytokines (e.g. ILs, RANTES and TNF-α) and macrophage inflammatory proteins (MIP-1α and MIP-1β).

AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; IFN, interferon; IκB, inhibitor of NF-κB; IKK, IκB kinase; IRAK, IL-1 receptor-associated kinase; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinase; Mal, MyD88 adaptor-like protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MIP, macrophage inflammatory protein; MKK, MAP kinase kinase; MyD88, myeloid differentiation factor88; NF-κB, nuclear factor-κB; PI3K, phosphoinisitide-3-kinase; RANTES, regulated upon activation, normal T-cell expressed and secreted; TAB, TAK1-binding protein; TAK1, transforming growth factor-β-activated protein kinase 1; TBK1, TRAF-family member-associated NF-κB activator-binding kinase 1; TRAF6, TNF receptor-associated factor 6; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing INF-β-mediated transcription factor.
Obtained from http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue Analysis/ Signaling-Pathways/Toll-like-Receptor-TLR.html.

2.4.4 TLR Polymorphisms

Many years ago, the mouse strains C3H/HeJ and C57BL/10ScCr were found not to respond to bacterial endotoxin (Coutinho et al. 1977;Sultzer 1968). These mouse strains could tolerate high doses of LPS without manifesting any lethal effects. However, they were highly susceptible to gram-negative bacterial infection. Genetic studies identified a single LPS locus on chromosome 4, which was responsible for this hyporesponsiveness. In the late 90´s, this locus was mapped as the TLR-4 gene (Poltorak et al. 1998a;Poltorak et al. 1998b;Qureshi et al. 1999). The C3H/HeJ mouse strain has single co-dominant missense mutation on the third exon of the TLR-4 gene, which causes a replacement of proline with histidine at position 712 of the polypeptide. The strain C57BL/10ScCr is homozygous for a null mutation of TLR-4, which is caused by a deletion of about 75 kb on chromosome 4. The TLR-4 knock-out mouse generated by Hoshino and his co-workers had the same phenotype as naturally occurring TLR-4 mutant mice (Hoshino et al. 1999). The animals developed normally, however showed no response to LPS or synthetic lipid A. The hyporesponsiveness was later suggested to be due to a disruption of the TLR-4-mediated signalling pathway resulting from the inability of mutant TLR-4 to interact with the second messenger MyD88 (Rhee and Hwang 2000). In addition to interrupted LPS signalling, the 'loss-of-function' mutation in TLR-4 has been shown to be beneficial in preventing for instance neurodegeneration and artherogenesis (Kiechl et al. 2003;Lehnardt et al. 2003).

The importance of TLRs in human diseases has been identified in studies of polymorphisms in TLR genes. The first identified TLR polymorphism encodes a missense mutation Asp299Gly (an A →G substitution at nucleotide 896 from the start codon of the TLR-4 gene, which results in an aspartic acid - glycine substitution at position 299 of the amino-acid sequence) in TLR-4 (Arbour et al. 2000). Thus far, the most extensively studied polymorphism is the Asp299Gly mutation. The incidence of heterozygosity of the mutant allele in the Caucasian population is 9.4% (Feterowski et al. 2003). This polymorphism affects the extracellular domain of human TLR-4 and is associated with hyporesponsiveness to inhaled LPS or endotoxin (Arbour et al. 2000). While it was shown that the Asp299Gly polymorphism increased the susceptibility to gram-negative infections (Agnese et al. 2002;Lorenz et al. 2002), no correlation of this polymorphism to pre- or post-operative LPS-induced cytokine release could be demonstrated (Kumpf et al. 2006). Furthermore, during post-operative sepsis induced by mixed-bacterial infections, a correlation between the incidence of sepsis and mortality could not be demonstrated (Feterowski et al. 2003). Several

recent studies suggest an association between TLR-4 polymorphisms and the development and progression of atherosclerosis. The Asp299Gly mutation is associated with a reduced risk for carotid artery atherosclerosis (Kiechl et al. 2003), acute coronary events (Ameziane et al. 2003;Boekholdt et al. 2003) and asthma (Werner et al. 2003;Yang et al. 2004). There are other rare TLR-4 polymorphisms, which lead to missense mutations and influence meningococcal infections (Smirnova et al. 2003).

Among the TLRs, TLR-2 recognizes the broad variety of pathogens, including bacteria, parasites, viruses and fungi. Several studies described the existence of TLR-2 mutations in humans: The Arg753Gln polymorphism in TLR-2 is associated with a decreased response to bacterial peptides from *Borrelia burgdorferi* and *Treponema pallidum* (Schroder et al. 2005). This polymorphism may predispose people to staphylococcal infections (Lorenz et al. 2000) or to tuberculosis (Ben-Ali et al. 2004;Ogus et al. 2004). Schroder and co-workers reported a rate of 9.4 % heterozygosity for this Arg753Gln polymorphism (Schroder et al. 2003). Another mutation in the intracellular domain of human TLR-2 protein, the Arg677Trp polymorphism, is associated with lepromatous leprosy in the Korean population (Kang and Chae 2001;Kang et al. 2002).
Other subtypes, in particular TLR-9, have recently started to be characterized and their functional importance elucidated. Among these, the most studied is T1237C, a polymorphism located within the putative promoter region that may influence transcriptional regulation of the TLR-9 gene. The variation T1237C has been shown to be associated with an increased risk for asthma among European Americans (Lazarus et al. 2003) and preliminary data regarding a possible positive association with Crohn's disease was also reported (Torok et al. 2004). Three studies conducted in the UK, Korea and Hong Kong investigated possible associations of genetic variations of TLR-9 with systemic lupus erythematosus (De Jager et al. 2006;Hur et al. 2005;Ng et al. 2005), but they did not detect an association between TLR-9 gene variations and susceptibility to systemic lupus erythematosus. However in a recent study, Tao and co-workers found that the presence of a G1174A polymorphism is associated with an increased risk of systemic lupus erythematosus in the Japanese population. The C allele at position –1486 (T1486C) was also closely correlated with the risk of systemic lupus erythematosus. The combination of both alleles down-regulated the transcription of TLR-9 (Tao et al. 2007). Moreover, Bochud et al. showed that the polymorphisms G1174A and G1635A in TLR-9 are associated with rapid progression in human immunodeficiency virus (Bochud et al. 2007).

2.5 Sepsis

2.5.1 Definition of Sepsis

For many years clinicians used a variety of terms to describe illnesses associated with infection or what looked like infection. These terms included sepsis, septicaemia, bacteraemia, infection, sepsis shock, toxic shock, etc. Unfortunately, problems arose with the use of these terms: First, there were no strict definitions for the terms used and often these words were used incorrectly. Second, emerging evidence arose which led to the belief that systemic inflammation (rather than infection) was responsible for multiple organ failure. Since the 1992 consensus conference between the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM), new definitions and criteria for the diagnostics of sepsis were created. Although lacking specificity (particularly for paediatric patients) these definitions (see below) have enabled medical professionals to be unified in the definitions of such conditions and allowed comparisons of clinical data (Levy et al. 2003).

Infection

A host response to the invasion of microorganisms.

Bacteraemia

The presence of viable bacteria in circulating blood.

Systemic Inflammatory Response Syndrome (SIRS)

A systemic inflammatory response to a wide variety of severe clinical insults (e.g. infection, burns, trauma, etc.), manifested by two or more of the following conditions:

- Temperature > 38°C or < 36°C
- Heart rate > 90 beats/min
- Respiratory rate > 20 breaths/min or Pa CO_2 < 32 mm Hg
- White blood cell count > 12,000/mm^2, < 4000/mm^3, or 10% immature (band) forms.

Sepsis

Infection plus systemic manifestations of infection.

Severe sepsis

Sepsis associated with organ failure, hypoperfusion or hypotension. Hypoperfusion and perfusion abnormalities may include -but are not limited to- lactic acidosis, oliguria or an acute alteration in mental status.

Septic shock

A subset of severe sepsis and defined as sepsis associated with hypoperfusion (alterations), but with persistent hypotension even after suitable treatment (vasopressures or volumes).

Multiple organ dysfunction syndrome (MODS)

The presence of altered organ functions in an acutely ill patient, such that homeostasis can not be maintained without intervention. MODS may represent the final stage of sepsis.

2.5.2 Diagnosis and Therapy in Adults

The symptoms, which separate sepsis from severe sepsis and septic shock are not easily detected in ICU or even from a conceptual point of view (Bone 1996;Proulx et al. 1996). In 2001, the International Sepsis Definitions Conference of researchers and experts opted to increase the first of signs and symptoms of sepsis (Tab. 2), thus valuing the clinical experience of medical professionals (Levy et al. 2003).

Documented or suspected infection with some of the following conditions:
General variables:
Fever (core temperature > 38.3°C) Hypothermia (core temperature < 36°C) Heart rate >90 bpm or > 2 SD above the normal value for age Altered mental status Hyperglycaemia (plasma glucose > 110 mg/dl or 7.7 mM/l) in absence of diabetes
Inflammatory variables:
Leukocytosis (WBC > 12,000/µl) Leukopenia (WBC < 4,000/µl) Normal WBC count with > 10% immature forms Plasma C reactive protein > 2 SD above normal value Plasma procalcitonin > 2 SD above normal value

Haemodynamic variables:
Arterial hypotension (SBP < 90 mmHg, mean arterial pressure < 70, or SBP decrease > 40 mmHg in adults or < 2 SD below normal for age) Mixed venous oxygen saturation > 70% Cardiac index > 3.51 min^{-1} m^{-2}
Organ dysfunction variables:
Acute oliguria (urine output < 0.5 ml $kg^{-1}h^{-1}$ or 45 mM/l for at least 2 h) Coagulation abnormalities (international normalised ratio > 1.5 or activated partial thromboplastin time > 60 s) Thrombocytopenia (platelet count < 100,000/µl)
Tissue perfusion variables:
Hyperlactatemia (> 3 mmol/l) Decreased capillary refill or mottling

Table 2: Diagnostic criteria for sepsis. SBP, systolic blood pressure; SD, standard deviation; WBC, white blood cells. Modified from Levy et al. 2003.

Despite encouraging pre-clinical data, the majority of clinical trials in sepsis showed disappointing results (Polderman and Girbes 2004). One of the possible explanations for the failure of clinical trials (investigating sepsis) is that the current definitions, although important for clinical purposes, are too general and do not allow for precise characterization and staging of patients with sepsis. A possible approach to this problem is the development of a system for the stages of sepsis, which would allow classification of patients by both their ‘baseline risk’ of an adverse outcome and their potential to respond to therapy. This is why a new concept Predisposition-Infection-Response-Organ (PIRO) dysfunction is being proposed that can better characterize sepsis on the basis of predisposition factors, the nature of the underlying infection, the characteristics of the host response and the extent of resultant organ dysfunction (Levy et al. 2003). This means that beside the current criteria (see Tab. 2), genetic predisposition and specific response, as reflected by laboratory parameters, should be included in the diagnostics. Recently, the latest international guidelines for the management of severe sepsis and septic shock of the *Surviving Sepsis Campaign* are published in Critical Care Medicine (Dellinger et al. 2008), which provide an update to the guidelines of 2004. These guidelines represented phase II of the *Surviving Sepsis Campaign*, increasing awareness and improving the outcome of severe sepsis (Dellinger et al. 2004b;Dellinger et al. 2004a). The revised guidelines are based on the Grades of Recommendation, Assessment, Development and Evaluation (GRADE), which is a structured system for rating quality of evidence and the strength of grading recommendations in clinical practice (Guyatt et al.

2006;Schunemann et al. 2006). The GRADE system classifies the quality of evidence from high (A) to very low (D) and determines recommendations as strong (grade 1) or weak (grade 2). A strong recommendation implies that the desirable effect of an intervention (e.g. beneficial health outcomes, fewer burdens on staff and patients, and cost savings) clearly outweighs it undesirable effects (e.g. risk, harm, more burden and greater costs). A weak recommendation reflects that the trade-off between desirable and undesirable effects is less clear. The following tables 3-5 summarize issues of initial resuscitation and infection, haemodynamic support and adjunctive therapy, as well as other supportive therapies of severe sepsis.

	Strong recommendation or "we recommend"	**Weak recommendation or "we suggest"**
Initial resuscitation (first 6 h)	Begin resuscitation immediately in patients with hypotension or elevated serum lactate <4 mmol/l; do not delay pending ICU admission (1C) Resuscitation goals (1C): • CVP 8-12 mm Hg[a] • Mean arterial pressure ≥ 65 mm Hg • Urine output ≥ 0.5 ml/kg/h • Central venous (superior vena cava) oxygen saturation ≥ 70% or mixed venous ≥ 65%	If venous oxygen saturation target is not achieved (2C): • Consider further fluid • Transfuse packed red blood cells if required to haematocrit of ≥ 30% and/or • Start dobutamine infusion, maximum 20 µg/kg/min
Diagnosis	Obtain appropriate cultures before starting antibiotics provided this does not significantly delay antimicrobial administration (1C): • Obtain two or more BCs • One or more BCs should be percutaneous • One BC from each vascular access device in place >48 h • Culture other sites as clinically indicated Perform imaging studies promptly to confirm and sample any source of infection, if safe to do so (1C)	
Antibiotic therapy	Begin intravenous antibiotics as early as possible and always within the first hour of recognizing severe sepsis (1D) and septic shock (1B)	Combination therapy in *Pseudosomas* infections (2D) Combination empiric therapy in neutropenic patients (2D)

	Broad-spectrum: one or more agents active against likely bacterial/fungal pathogens and with good penetration into presumed source (1B) Reassess antimicrobial regimen daily to optimize efficacy, prevent resistance, avoid toxicity and minimize costs (1C) Duration of therapy typically limited to 7-10 days; longer if response is slow or there are undrainable foci of infection or immuno-logic deficiencies (1D) Stop antimicrobial therapy if cause is found to be non-infectious (1D)	Combination therapy ≤3-5 days and de-escalation following susceptibilities (2D)
Source identification and control	A specific anatomic site of infection should be established as rapidly as possible (1C) and within first 6 h of presentation (1D) Formally evaluate patient for a focus of infection amenable to source control measures (e.g. abscess drainage, tissue debridement) (1C) Implement source control measures as soon as possible following successful initial resuscitation (1C) (exception: infected pan-creatic necrosis, where surgical intervention is best delayed) (2B) Choose source control measure with maximum efficacy and minimal physiologic upset (1D) Remove intravascular access devices if potentially infected (1C)	

Table 3: Initial resuscitation and infection issues. Strength of recommendation and quality of evidence have been assessed using the GRADE criteria, presented in parentheses after each guideline. [a]A higher target CVP of 12-15 mm Hg is recommended in the presence of mechanical ventilation or pre-existing decreased ventricular compliance. BC, blood culture; CVP, central venous pressure; GRADE, Grades of Recommendation, Assessment, Development and Evaluation; ICU, intensive care unit. Modified from Dellinger et al. 2008.

	Strong recommendation or “we recommend”	Weak recommendation or “we suggest”
Fluid therapy	Fluid-resuscitate using crystalloids or colloids (1B) Target a CVP ≥8 mm Hg (≥12 mm Hg if mechanically ventilated) (1C) Fluid challenge technique while associated with a haemodynamic improvement (1D) Fluid challenge of 1000 ml of crystalloids or 300-500 ml colloids over 30 min. More rapid and larger volumes required in sepsis-induced tissue hypoperfusion (1D) Reduce rate of fluid administration if cardiac filling pressures increase without concurrent haemodymanic improvement (1D)	
Vasopressors	Maintain MAP ≥65 mm Hg (1C) Norepinephrine and dopamine centrally administered as the initial vasopressors of choice (1C) Do not use low-dose dopamine for renal protection (1A) In patients requiring vasopressors, insert an arterial catheter as soon as practical (1D)	No epinephrine, phenylephrine or vaso-pressin as the initial vasopressor in septic shock (2C). Vasopressin 0.03 units/min may be subsequently added to norepinephrine with anticipation of an effect equivalent to norepinephrine alone Use epinephrine as the first alternative agent in septic shock when blood pressure is poorly responsive to norepinephrine or dopamine (2B)
Inotropic therapy	Use dobutamine in patients with myocardial dysfunction as supported by elevated cardiac filling pressures and low cardiac output (1C) Do not increase cardiac index to predetermined supranormal levels (1B)	
Steroids	Hydrocortisone dose should be ≤300 mg/day (1A) No corticosteroids to treat sepsis in the absence of shock unless the patient’s endocrine or corticosteroid history warrants it (1D)	Intravenous hydrocortisone for adult septic shock when hypotension responds poorly to adequate fluid resuscitation and vasopressors (2C) No ACTH stimulation test recommended to identify subset of adults with septic shock who should receive hydrocortisone (2B) Hydrocortisone is preferred to dexa-methasone (2B)

		Fludrocortisone (50 µg/day; orally) if an alternative to hydrocortisone is being used that lacks significant mineralcorticoid activity. Fludrocortisone if optional if hydrocortisone is used (2C) Wean steroid therapy once vasopressors are no longer required (2D)
Recombinant human activated protein C	No rhAPC for adult patients with severe sepsis and low risk of death (typically APACHE II <20 or one organ failure) (1A)	rhAPC in adult patients with sepsis-induced organ dysfunction with clinical assessment of high risk of death (typically APACHE II ≥25 or multiple organ failure) if no contra-indications (2B, 2C for postoperative patients)

Table 4: Haemodynamic support and adjunctive therapy. Strength of recommendation and quality of evidence have been assessed using the GRADE criteria, presented in parentheses after each guideline. ACTH, adrenocorticotropic hormone; APACHE, Acute Physiology and Chronic Health Evaluation; CVP, central venous pressure; GRADE, Grades of Recommendation, Assessment, Development and Evaluation; MAP, mean arterial pressure; rhAPC, recombinant human activated protein C. Modified from Dellinger et al. 2008.

	Strong recommendation or "we recommend"	**Weak recommendation or "we suggest"**
Blood product administration	Give red blood cells when haemoglobin decreases to <7.0 g/dl (<70g/l) to target a haemoglobin of 7.0-9.0 g/dl in adults (1B). A higher haemoglobin level may be required in special circumstances (e.g. myocardial ischeamia, severe hypoxemia, acute haemorrhage, cyanotic heart disease or lactic acidosis) No antithrombin therapy (1B)	No erythropoietin to treat sepsis-related anemia (1B) No fresh frozen plasma to correct laboratory clotting abnormalities unless there is bleeding or planned invasive procedures (2D) Administer platelets when (2D): • counts are <5000/mm^3 (5 x 10^9/l) regardless of bleeding • counts are 5000-30,000/mm^3 (5-30 x 10^9/l) and there is significant bleeding risk • higher platelet counts (≥50,000/mm^3 [50 x 10^9/l]) are required for surgery or invasive procedures
Mechanical ventilation of sepsis-induced ALI/ARDS	Target a tidal volume of 6 ml/g (predicted) body weight in patients with ALI/ARDS (1B) Target an initial upper limit plateau pressure ≤30 cm H_2O. Consider chest wall compliance when assessing plateau pressure (1C) Allow $PaCO_2$ to increase above normal if needed to minimize plateau pressures and tidal volumes (1C)	Consider using the prone position for ARDS patients requiring potentially injurious levels of FIO_2 or plateau pressure, provided they are not put at risk from positional changes (2C) Consider non-invasive ventilation in the minority of ALI/ARDS patients with mild to moderate hypoxemic respiratory failure.

<table>
<tr><td></td><td>Set PEEP to avoid extensive lung collapse at end-expiration (1C)
Maintain mechanically ventilated patients in a semirecumbent position (head of the bed raised to 45°) unless contra-indicated (1B), between 30° and 45° (2C)
Use a weaning protocol and an SBT regularly to evaluate the potential for discontinuing mechanical ventilation (1A):
SBT options include a low level of pressure support with continuous positive airway pressure 5 cm H_2O or a T piece
Before the SBT, patients should:
• be arousable
• be haemodynamically stable without vasopressors
• have no new potentially serious conditions
• have low ventilatory and end-expiratory pressure requirement
• require FIO_2 levels that can e safely delivered with a face mask or nasal cannula

No pulmonary artery catheter for the routine monitoring of patients with ALI/ARDS (1A)

Use a conservative fluid strategy for patients with established ALI who do not have evidence of tissue hypoperfusion (1C)</td><td>Patients need to be haemodynamically stable, comfortable, easily arousable, able to protect/clear their airway and expected to recover rapidly (2B)</td></tr>
<tr><td>Sedation, anlagesia and neuro-muscular blockade in sepsis</td><td>Use sedation protocols with a sedation goal for critically ill mechanically ventilated patients (1B)

Use either intermittent bolus sedation or continuous infusion sedation to predetermined end points (sedation scales), with daily interruption/lightening to produce awakening. Re-titrate if necessary (1B)

Avoid neuromuscular blockers where possible. Monitor depth of block with train-of-four when using continuous infusions (1B)</td><td></td></tr>
<tr><td>Glucose control</td><td>Use intravenous insulin to control hyperglycemia in patients with severe sepsis following stabilization in the ICU (1B)</td><td></td></tr>
</table>

	Keep blood glucose <150 mg/dl (8.3 mmol/l) using a validated protocol for insulin dose adjustment (2C) Provide a glucose calorie source and monitor blood glucose values every 1-2 h (4 h when stable) in patients receiving intravenous insulin (1C) Interpret with caution low glucose levels obtained with point of care testing, as these techniques may overestimate arterial blood or plasma glucose values (1B)	
Renal replacement		Intermittent haemodialysis and CVVH are considered equivalent (2B) CVVH offers easier management in haemodynamically unstable patients (2D)
Bicarbonate thearapy	No bicarbonate therapy for the purpose of improving haemodynamics or reducing vasopressor requirements when treating hypoperfusion-induced lactic acidemia with pH≥7.15 (1B)	
Deep vein thrombosis prophylaxis	Use either low-dose UFH or LMWH, unless contra-indicated (1A) Use a mechanical prophylactic device such as compression stockings or an intermittent compression device, when heparin is contra-indicated (1A)	Use a combination of pharmacologic and mechanical therapy for patients who are at very high risk for deep vein thrombosis (2C) Use LMWH rather than UFH in patients at very high risk (2C)
Stress ulcer prophylaxis	Provide stress ulcer prophylaxis using H2 blocker (1A) or proton pump inhibitor (1B). Benefits of prevention of upper gastrointestinal bleed must be weighed against the potential for development of ventilator-acquired pneumonia	
Consideration for limitation of support	Discuss advance care planning with patients and families. Describe likely outcomes and set realistic expectations (1D)	

Table 5: Other supportive therapy of severe sepsis. Strenght of recommendation and quality of evidence have been assessed using the GRADE criteria, presented in parentheses after each guideline. ALI, acute lung injury; ARDS, acute respiratory distress syndrome; CVVH, continuous veno-venous haemofiltration; GRADE, Grades of Recommendation, Assessment, Development and Evaluation; ICU, intensive care unit; LMWH, low-molecular weight heparin; PEEP, positive end-expiratory pressure; SBT, spontaneous breathing trial; UFH, unfractionated heparin. Modified from Dellinger et al. 2008.

2.5.3 Diagnosis and Therapy in Infants

Sepsis in children is a major cause of mortality, estimated at about 10% in the United States, although the overall mortality from severe sepsis in infants is much lower than that in adults (Watson et al. 2003). It is important to note that the definitions for severe sepsis and septic shock in children are not identical to those in adults (Goldstein et al. 2005). Apart from age-appropriate differences in vital signs, the definition of SIRS obligates the presence of either temperature or leukocyte abnormalities. Severe sepsis in children is defined as sepsis plus cardiovascular dysfunction or acute respiratory distress syndrome or two or more other organ dysfunctions (Goldstein et al. 2005). Table 6 shows a summary of therapies of pediatric severe sepsis.

	Strong recommendation or "we recommend"	**Weak recommendation or "we suggest"**
Antibiotics	Administer antibiotics within 1 h of the identification of severe sepsis, after appropriate cultures have been obtained (1D)	
Mechanical ventilation	No graded recommendations	
Fluid therapy		Initial resuscitation begin with infusion of crystalloids 20 ml/kg over 5-10 min, titrated to clinical monitors of cardiac output, capillary refill, and level of consciousness (2C)
Vasopressors/Inotropic therapy		Dopamine as the first choice of support for pediatric patient with hypotension refractory to fluid resuscitation (2C) Use dobutamine in patients with low cardiac output and elevated systemic vascular resistance stated (cool extremities, prolonged capillary refill, decreased urine output but normal blood pressure following fluid resuscitation) (2C)
Therapeutic end points		Normalization of the heart rate, capillary refill of <2 s, normal pulses with no differential between peripheral and central pulses, warm extremities, urine output <1 ml/kg/h, and normal mental status (2C)
Steroids		Use hydrocortisone therapy in children with catecholamine resistance and suspected or proven adrenal insufficiency (50 mg/m^2/24 h (2C)
Recombinant human activated protein C	No rhAPC in children (1B)	

DVT prophylaxis		Use DVT prophylaxis in postpubertal children with severe sepsis (2C)
Stress ulcer prophylaxis	No graded recommendations	
Renal replacement therapy	No graded recommendations	
Glycemic control	No graded recommendations	
Sedation/Analgesia	Sedation protocols with a sedation goal when sedation of critically ill mechanically ventilated patients with sepsis is required (1D)	
Blood products	No graded recommendations	
Intravenous immunoglobulin		Consider immunoglobulin in children with severe sepsis (2C)
Extracorporeal membrane oxygenation (ECMO)		Limit use of ECMO to refractory pediatric septic shock and/or respiratory failure that cannot be supported by conventional therapies (2C)

Table 6: Therapies of pediatric severe sepsis. Strength of recommendation and quality of evidence have been assessed using the GRADE criteria, presented in parentheses after each guideline. DVT, deep vein thrombosis; ECMO, extracorporeal membrane oxygenation; GRADE, Grades of Recommendation, Assessment, Development and Evaluation; rhAPC, recombinant human activated protein C. Sources from Dellinger et al. 2008.

2.5.4 The Role of Cytokines in Inflammatory Reaction

The inflammatory reaction is a defensive response, which is directed against injury and infection. The innate immune system recognizes PAMPs and reacts with the release of mediators including anti-inflammatory cytokines, which protect against injury and infection (Gerard 2003). However, the extensive release of inflammatory mediators can cause excessive tissue injury and can have adverse effects toward the host itself. Ongoing activation of inflammation can result in MODS and frequently death (Munford and Pugin 2001). Additional syndromes such as compensatory anti-inflammatory response syndrome (CARS) and mixed antagonists response syndrome (MARS) have been described (Bone et al. 1997).
In the end, anti-inflammatory cytokines play a significant role in the outcome of patients with sepsis by counteracting ongoing inflammation. In Table 7, pro- and anti-inflammatory mediators are listed.

Pro-inflammatory	Anti-inflammatory
• TNF-α • IL-1β, 2, 6, 8, 12, 15, 18 • IFN-γ • Nitric oxide • Inducible nitric oxide synthase • Neutrophil elastase • Thromboxane • Platelet activating factor • Granulocyte macrophage-colony stimulating factor • Vasoactive neuropeptides • Phospholipase A_2 • Plasminogen activator inhibitor-1 • Prostaglandins • Prostacyclin	• IL-1 receptor antagonist • IL-4, 10, 13 • Type II IL-1 receptor • Transforming growth factor-β • Adrenaline • Soluble TNF-α receptors • Leukotriene B_4-receptor antagonist • LPS-binding protein

Table 7: Pro-inflammatory and anti-inflammatory mediators. IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

While anti-inflammatory cytokines are a prerequisite for controlling the inflammatory response, they also can lead to a depression of the immune system in patients. The condition in which these anti-inflammatory cytokines are produced in excess and become dominant systemically is called CARS or 'immunoparalysis' (Bone et al. 1997). Hartemink et al. (2003) described a 'biphasic immunological pattern' during sepsis, where an 'early hyper-inflammatory phase (SIRS)' followed by an anti-inflammatory response leads to a 'hypo-inflammatory state (CARS)'. The spectrum of the responses to sepsis is illustrated in Fig. 5 (Bone et al. 1997). This diagram shows the five stages of sepsis leading to MODS: Stage 1 is the initial insult producing local inflammation. Stage 2 is when severe insults lead to a systemic spillover of inflammatory mediators with few clinical signs or symptoms. When macrophages were primed for intense cytokine release, this amplified response is most likely the result of a second insult. In stage 3, the systemic reaction becomes apparent, leading to the clinical signs of SIRS and early organ dysfunction. Stage 4 is characterized by inappropriate immunosuppression or CARS overreaction with increased susceptibility to new infection. The last stage is characterized by an inappropriate, imbalance response and either excessive SIRS or CARS along with severe MODS.

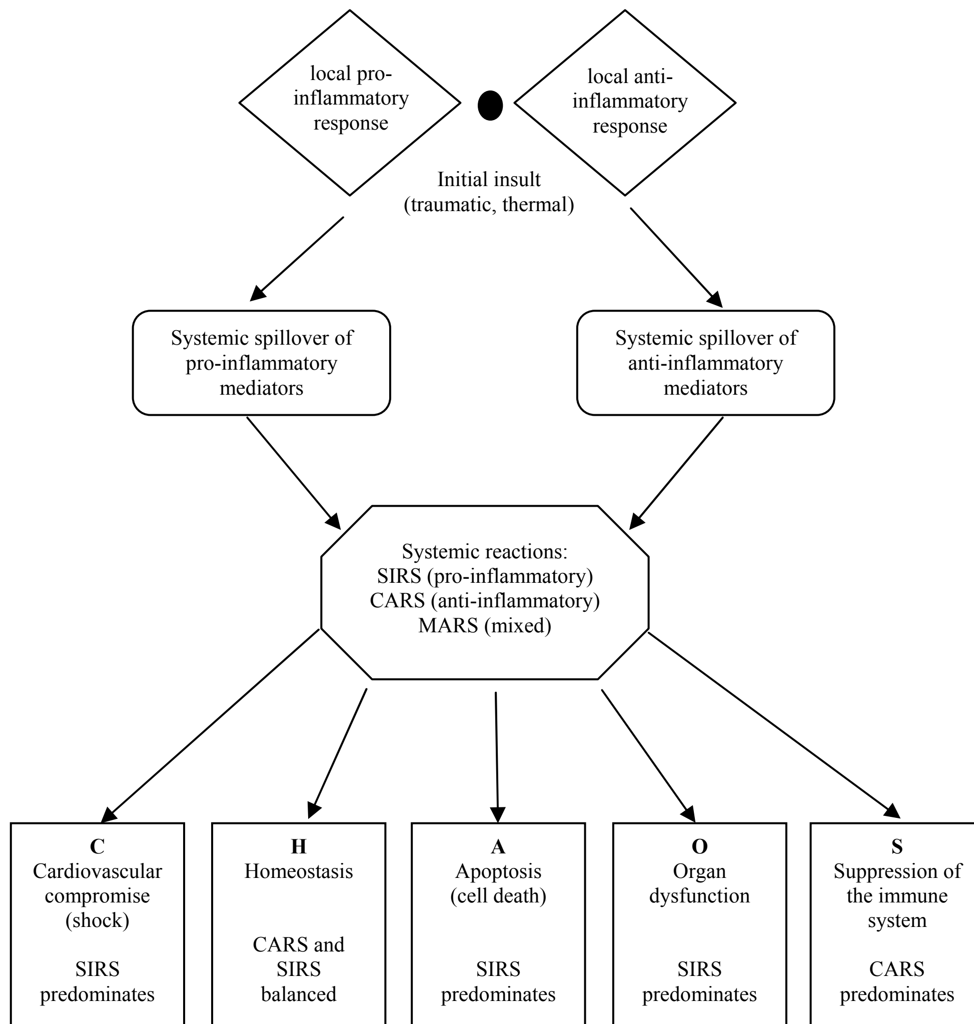


Figure 5: Progression of sepsis from the initial insult with local pro- and anti-inflammatory responses through the systemic spillover of mediators. The systemic reaction may have the features of an excess of either pro-inflammatory and anti-inflammatory mediators or a mixed pattern. The clinical sequelae of sepsis may vary depending on the balance of mediators. Excessive inflammatory response (SIRS) may produce shock and organ dysfunction. Excessive anti-inflammatory response (CARS) suppresses the immune system. A balanced response of inflammatory mediators produces homeostasis. CARS, compensatory anti-inflammatory response syndrome; MARS, mixed antagonists response syndrome; SIRS, systemic inflammatory response syndrome. From Bone et al. 1997.

2.5.5 The Role of Adrenal Gland during Sepsis

Adrenal insufficiency in patients with sepsis has gained more attention over the last few years. Absolute and relative adrenal insufficiency can be distinguished by using laboratory investigations. In most cases, testing the basal level of cortisol is able to identify the problem. In patients with critical illnesses, however, it is difficult to diagnose adrenal insufficiency. Although absolute adrenal insufficiency occurs only in a minority (1-3%) of critically ill

patients (Matot and Sprung 1998), relative adrenal insufficiency was found in up to 80% among those patients (Annane et al. 2000). During sepsis, subnormal adrenal corticosteroid production can occur without any obvious structural defects in the HPA-axis (Burchard 2001). Functional adrenal insufficiency can develop during the illness and is usually transient (Cooper and Stewart 2003). The major cause of relative adrenal insufficiency is an insufficient synthesis of cortisol due to cellular dysfunction. In contrast to absolute adrenal insufficiency, the morphological changes in relative adrenal insufficiency may be minor and sometimes are characterized by cellular hyperplasia within the adrenal cortex. This is often associated with a peripheral glucocorticoid resistance of the target cells caused by inflammatory conditions, although the absolute levels of cortisol might be normal (Meduri and Chrousos 1998). In septic shock, relative adrenal insufficiency may be due to impaired pituitary corticotropin release, attenuated adrenal response to corticotropin and reduced cortisol synthesis (Cooper and Stewart 2003). In patients with severe acute respiratory distress syndrome, it has been demonstrated that methylprednisolone treatment improved the decreased glucocorticoid response by reduction of NF-κB-mediated DNA binding and transcription of pro-inflammatory cytokines (Meduri et al. 2002). Therefore, if adrenal insufficiency can be identified, treatment with corticosteroids may be of benefit (Cooper and Stewart 2003). In critically ill patients, primary causes of adrenal insufficiency are often not detectable, if no specific hypothesis exists. In patients with sepsis and septic shock, the individual clinical course is different. Furthermore, dynamic testing is not always possible in ICU, which makes it difficult for the physician to consider glucocorticoids therapy due to the fact that decisions have to be made immediately in severe forms of sepsis in order to improve prognosis. Recently, Annane and co-workers tried to research more accurate diagnostic criteria for adrenal insufficiency in patients with severe sepsis or septic shock (Annane et al. 2006). They found that patients were very likely to have adrenal insufficiency when a baseline total cortisol was less than 10 µg/dl or a change in cortisol of less than 9 µg/dl (after cosyntropin) was observed. Conversely, adrenal insufficiency can be ruled out when patients after cosyntropin stimulation exhibited levels of total cortisol of 44 µg/dl or greater or a change in cortisol of 16.8 µg/dl or greater. When baseline cortisol level is between 10-44 µg/dl and the cortisol increment after cosyntropin stimulation is between 9-16.8 µg/dl, assessment of adrenal function requires metyrapone testing.

2.5.6 Corticosteroid Treatment

Administration of high doses of corticosteroids (e.g. 30 mg/kg of methylprednisolone) did not improve survival of patients with sepsis and worsens their outcomes by increasing the risk of secondary infections (Cronin et al. 1995). Despite the negative effects associated with the use of high-dose corticosteroids, a study by Annane and colleagues indicated that extremely ill patients with sepsis, who have persistent shock requiring vasopressors and prolonged ventilation may benefit from 'physiological' doses of corticosteroids (Annane 2001). It is postulated, that such patients may have 'relative' adrenal insufficiency, despite having elevated levels of circulating cortisol (Marik and Zaloga 2003;Shenker and Skatrud 2001). Annane and co-workers found that in the majority of critically ill patients, the body fails to mount an adequate stress response with respect to cortisol even with normal pre-existing adrenal function. The anti-inflammatory effects of corticosteroids include inhibition of pro-inflammatory cytokine synthesis, free radicals, prostaglandins, nitric oxide synthase and up-regulation of the vascular responsiveness to catecholamines (Annane et al. 2000;Annane and Cavaillon 2003). A study also by Annane and colleagues demonstrated that hydrocortisone (50 mg intravenous bolus, 4 times per day) and fludrocortisone (50 µg per day) administered for seven days to patients with sepsis shock improves survival. Combination therapy was beneficial even in patients, where baseline plasma cortisol did not increase by < 9 µg/dl when stimulated with ACTH (Annane et al. 2002). Meta-analysis of the trials conducted after 1997 as compared to those before 1989 (Annane et al. 2004;Minneci et al. 2004) showed a consistent improvement in overall mortality and shock reversal, whether they were 'responders' or 'non-responders'. The premise, on which earlier studies had administered high dose steroids, was that immunosuppression could alter the course of septic shock. However, this could have led to the harmful effects by for e.g. increasing the incidence of secondary infections (Minneci et al. 2004). In contrast, later studies were based on correcting the relative insufficiency of cortisol and to attenuate rather than suppress inflammation. This modified approach led to a lower dose of hydrocortisone (200-300 mg/daily) and over a longer duration of 5 days with tapering over 5-7 days (Keh and Sprung 2004;Minneci et al. 2004). A previous study demonstrated that corticosteroids in low doses only attenuate but do not suppress inflammatory responses (Keh et al. 2003). A large European multicentre trial (CORTICUS) tested the hypothesis that low dose steroids improves 28-day mortality in patients with septic shock, whose cortisol levels did not increase by more than 9 µg/dl in response to ACTH stimulation (Annane et al. 2003). Unfortunately, this trial failed to prove a

benefit in mortality using hydrocortisone therapy for septic shock. CORTICUS did demonstrate a faster resolution of septic shock in patients, who received steroids, but the use of the ACTH test (responders and non-responders) did not predict the faster resolution of shock (Sprung et al. 2008). Although corticosteroids seem to promote shock reversal, a lack of clear improvement in mortality, generally tempered the enthusiasm for their broad use, which is also due to the coupled side effects of corticosteroids, such as increased risk of infection and myopathy, immunosuppression, hypertension and metabolic disturbances (Rhen and Cidlowski 2005).

2.6 The Adrenal Gland

The adrenal gland is an endocrine organ that is essential for life. It secretes several glucocorticoids and mineralocorticoids that regulate both metabolism and electrolytes. The adrenal gland is located near the upper portion of the kidney (Fig. 6). Each gland is composed of two embryonic distinct regions, the adrenal cortex and the adrenal medulla.

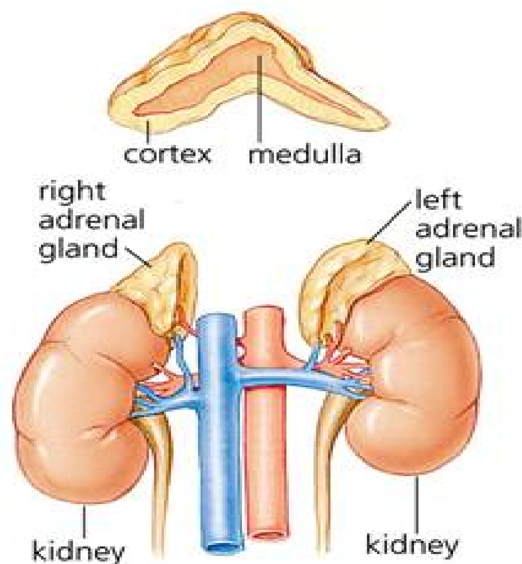


Figure 6: Location of the adrenal glands.
Obtained from http://jcsurgery.com/lap%20adrenal.htm.

The adrenal medulla, which arises from neuroectodermal cells, is located within the centre of the gland and ultimately gives rise to both the noradrenaline/adrenaline-secreting chromaffin cells and ganglia (James 1992;Parmer and Zinder 2002). The adrenal cortex, which will be the focus hereafter, is mesoderm-derived and comprises of three zones of anatomical and physiological distinct cells: the zona glomerulosa, zona fasciculata and zona reticularis (Fig. 7). The division of the mammalian adrenal cortex into these three distinct concentric zones was first described by Harley in 1858. The terms zona glomerulosa, zona fasciculata and zona reticularis were introduced by Arnold in 1866 (Neville and O'Hare 1982).

Zona glomerulosa cells reside in the outermost zone of the adrenal cortex. In response to increased potassium levels or decreased renal blood flow, cells of the zona glomerulosa secrete mineralocorticoids (primarily aldosterone) into the blood and therefore play a part in the renin-angiotensin system. Aldosterone regulates the body's concentration of electrolytes, primarily sodium and potassium. Immediately beneath the zona glomerulosa is the zona fasciculata. The cells of this zone produce glucocorticoids, such as corticosterone or cortisol. The primary glucocorticoid of the adrenal gland is cortisol. Upon binding to its target, cortisol enhances metabolism in several ways: stimulating the release of amino acids from the body, stimulating lipolysis (the breakdown of fat), stimulating gluconeogenesis (the production of glucose from newly released amino acids and lipids) and conserving glucose by inhibiting uptake into muscle and fat cells (Ehrenreich 1999;Ehrhart-Bornstein et al. 1998;Holmes et al. 2001). The third and innermost zone of the adrenal cortex is the zona reticularis. Cells of this zone provide a secondary source of androgens, such as testosterone, dihydrotestosterone, androstenedione and dehydroepiandrosterone. These androgens enhance muscle mass, stimulate cell growth and aid in the development of the secondary sexual characteristics (Rainey 1999;Rainey et al. 2002).

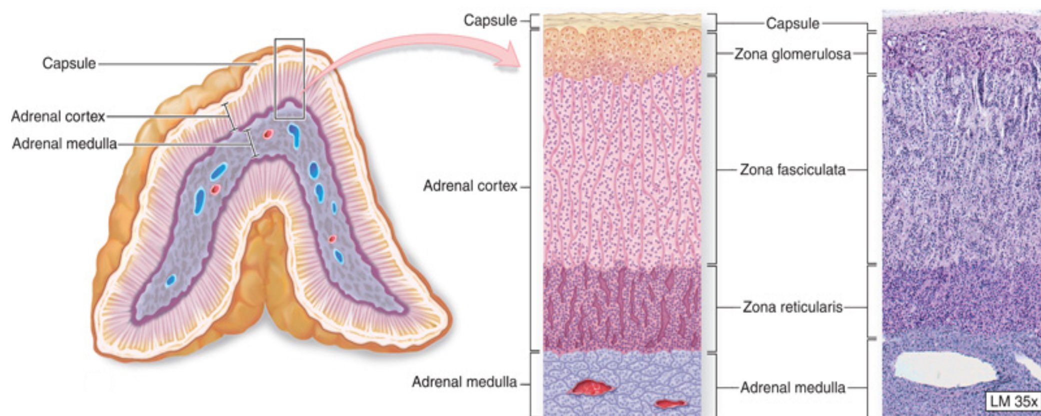


Figure 7: Histology of the adrenal gland. Illustrated are the diversity of the cells of the adrenal cortex and part of the adrenal medulla. Beneath the adrenal capsule are the three adrenal cortical zones: the zona glomerulosa, zona fasciculata, zona reticularis and their distinct morphology is apparent. The outermost zone is the zona glomerulosa. Cells within this zone tend to be columnar in shape and are arranged in irregular cords. The zona fasiculata is the middle and largest of the three zones in the cortex. Cells in the fasiculata are polyhedral and usually have a foamy appearance due to abundant lipid droplets. They also are arranged in distinctively straight cords that radiate toward the medulla. The innermost zone of the cortex is the zona reticularis. Cells within this zone are arranged in cords that project in many different directions and anastomose with one another. Obtained from http://academic.kellogg.cc.mi.us/herbrandsonc/bio201_McKinley/Endocrine%20System.htm.

2.7 The HPA-Axis

The HPA-axis is a major part of the neuroendocrine system that is activated in response to severe illness and stress situations (pain, fever, tissue damage, surgery, hypoxaemia, hypotension or hypoglycaemia). Under normal healthy conditions, the HPA-axis contributes to the maintenance of cellular and organ homeostasis. The hypothalamus releases CRH and vasopressin from an area along the median eminence. The hormones are then transported to the anterior lobe of the pituitary through the portal blood vessel system of the hypophyseal stalk, which descends from the hypothalamus. In the anterior pituitary gland, these hormones stimulate release of stored ACTH (also known as corticotrophin) which is transported by the blood to the cortex of the adrenal glands, where it rapidly stimulates the synthesis and secretion of glucocorticoids (Jurney et al. 1987;Reincke et al. 1993). Liberated glucocorticoids in turn will act on the HPA-axis, establishing a negative feedback mechanism with a resultant reduction in the release of CRH and ACTH (Fig. 8). Through the mechanism described, the body can control the secretion of glucocorticoids within relatively narrow limits and can respond with increased secretion of glucocorticoids to a variety of stresses and other signals (cytokines, endogenous peptides and other hormones).

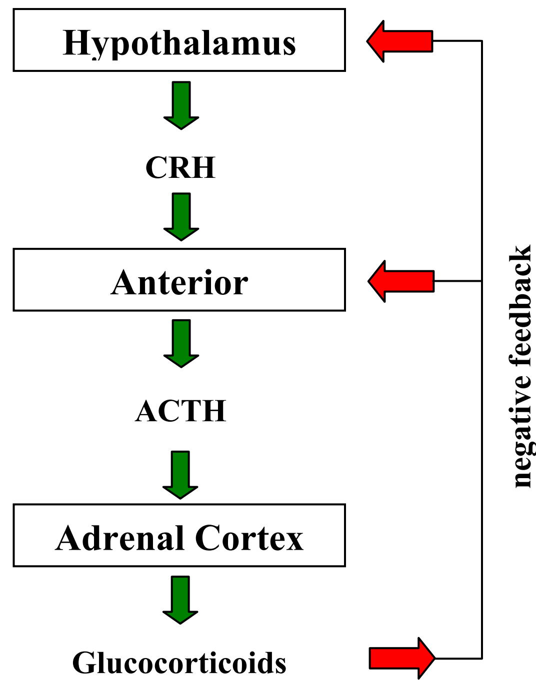


Figure 8: The HPA-axis. The hypothalamus releases CRH from an area along the median eminence. Release of this hormone is influenced by stress, by blood levels of cortisol and by the sleep/wake cycle. In the anterior pituitary gland, CRH stimulates release of ACTH, which is then transported to the cortex of the adrenal glands. Subsequent, cortisol is synthesized and secreted rapidly. Increased production of this glucocorticoid mediates alarm reactions to stress. Furthermore, cortisol exerts a negative feedback influence on the pituitary and the hypothalamus by inhibiting the synthesis and release of CRH and ACTH. ACTH adrenocorticotropic hormone; CRH, corticotropin-releasing hormone. Green arrow: stimulation; red arrow: inhibition.

3 Materials and Methods

3.1 Equipment and Materials

3.1.1 Chemicals

30% Acrylamide/Bis Solution	Bio-Rad, Munich, Germany
Albumin fraction V (Bovine; BSA)	Merck-Schuchardt, Hohenbrunn, Germany
Ammonium chloride (NH_4Cl; MW:53.49 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Ammonium persulfate (APS) ($(NH_4)_2S_2O_8$; MW: 228.2 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Aprotinin (Trypsin inhibitor) ($C_{284}H_{432}N_{84}O_{79}S_7$; MW: 6511.4 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Ascorbic acid (Vitamin C) (cell culture tested)	Sigma-Aldrich, Taufkirchen, Germany
Boric acid (H_3BO_3; MW: 61.8 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Bradford reagent (Protein dye reagent)	Sigma-Aldrich, Taufkirchen, Germany
Bromophenol blue ($C_{19}H_9Br_4NaO_5S$; MW: 691.9 g/mol)	Merck, Darmstadt, Germany
CpG-ODN (Thioat 1668)	TibMolBiol, Berlin, Germany
Deoxycholic acid ($C_{24}H_{40}O_4$; MW: 392.5 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
D-galactosamine (D-GalN)	Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO) (C_2H_6OS; MW: 78.1 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$; MW: 178.0 g/mol)	Merck, Darmstadt, Germany
DL-Dithiothreitol (DTT) ($C_4H_{10}O_2S_2$; MW: 154.3 g/mol)	Sigma-Aldrich, Taufkirchen, Germany

Entellan® (mounting medium)	Merck, Darmstadt, Germany
β-Estradiol (cell culture tested)	Sigma-Aldrich, Taufkirchen, Germany
Ethanol (absolute) (C_2H_5OH; MW: 46.1 g/mol)	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$; MW: 292.2 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Ethylene Glycol-bis (β-Aminoethylether)-N,N,N',N'-Tetraacetic acid (EGTA) ($C_{14}H_{24}N_2O_{10}$; MW: 380.4 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Ficoll 400	Sigma-Aldrich, Taufkirchen, Germany
Foetal bovine serum (FBS)	Gibco, Invitrogen GmbH, Karlsruhe, Germany
Foetal calf serum	PAA Laboratories, Pasching, Austria
Forskolin	Sigma-Aldrich, Taufkirchen, Germany
Glycerol ($C_3H_8O_3$; MW: 92.1 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Glycine ($C_2H_5NO_2$; MW: 75.1 g/mol)	Fluka, Buchs, Switzerland
Haematoxylin (modified acc. to Gill II)	Merck, Darmstadt, Germany
HEPES (N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid)	Gibco Life Technologies, Eggenstein, Germany
Leupeptin Hemisulfate Salt ($C_{20}H_{38}N_6O_4 \cdot \frac{1}{2} H_2SO_4$; MW: 475.6 g/mol)	Sigma-Aldrich, Steinheim, Germany
Lipopolysaccharides (LPS) (*Escherichia coli* 0111:B4)	Sigma-Aldrich, Taufkirchen, Germany
Lipopolysaccharides (LPS) (*Escherichia coli* F515 LPS SB III,66)	Prof. Zähringer, Borstel, Germany
Magnesium chloride ($MgCl_2$; MW: 95.2 g/mol)	Merck, Darmstadt, Germany
2-Mercaptoethanol ($HSCH_2CH_2OH$; MW: 78.1 g/mol)	Merck, Darmstadt, Germany

Methanol (CH_3OH; MW: 32.0 g/mol)	Merck, Darmstadt, Germany
Nonidet P40 Substitute (Nonylphenylpolyethylene glycol)	Fluka, Buchs, Switzerland
Paraffin (Paraplast tissue embedding media)	Fisher Scientific, Pittsburgh, PA
Paraformaldehyde ($(CH_2O)_n$; MW: 30.0 g/mol)	Merck, Darmstadt, Germany
Phenylmethylsulfonyl fluoride (PMSF) ($C_7H_7O_2SF$; MW: 174.2 g/mol)	Sigma-Aldrich, Steinheim, Germany;
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich, Taufkirchen, Germany
Poly dI-dC (poly desoxyinosin-desoxycytidin)	Sigma Chemical, St. Louis, USA.
Potassium chloride (KCl; MW: 74.6 g/mol)	Fluka, Buchs, Switzerland
Roti®-Histol	Carl Roth GmbH, Karlsruhe, Germany
Skimmed milk powder	Fluka, Buchs, Switzerland
Sodium chloride ($NaCl$; MWT: 58.4 g/mol)	Merck, Darmstadt, Germany
Sodium deoxycholate ($C_{24}H_{39}NaO_4$; MW: 414.6 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$; MW: 138.0 g/mol)	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}NaO_4S$; MW: 288.4 g/mol)	Merck, Darmstadt, Germany
Sodium orthovanadate (Na_3VO_4; MWT: 183.9 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
Sodium pentobarbital	University Pharmacy of Dusseldorf, Germany
Sodium selenite (cell culture tested)	Sigma-Aldrich, Taufkirchen, Germany
N,N,N',N'-Tetramethyl ethylenediamine (TEMED) ($C_6H_{16}N_2$; MW: 116.2 g/mol)	Merck, Darmstadt, Germany

Tris(hydroxymethyl)-aminomethane (Sigma 7-9®) ($C_4H_{11}NO_3$; MW: 121.1 g/mol)	Roth, Karlsruhe, Germany
Triton®X-100 ($C_3OH_{32}N_4O_8$; MW: 624.0 g/mol)	Sigma Chemical, St. Louis, USA
Trizma®hydrochloride ($C_4H_{11}NO_3.HCl$; MW: 157.6 g/mol)	Sigma-Aldrich, Taufkirchen, Germany
0.05% Trypsin/0.02% EDTA	Biochrom AG, Berlin, Germany
Tween®20 (polysorbate)	Merck-Schuchardt, Hohenbrunn, Germany
Vectashield (mounting medium)	Vector Laboratories, Peterborough, UK
Xylene ($C_6H_4(CH_3)_2$; MW: 106.2 g/mol)	Merck, Darmstadt, Germany

3.1.2 Antibodies, Enzymes, Protein Standard, DNA-oligonucleotides, Kits

Primary antibodies: see sections 3.3.8 and 3.3.15; Tables 12 and 14

Secondary antibodies: see section 3.3.8 and 3.3.15; Tables 13 and 15

DNA Oligonucleotides: specific for NF-κB	Promega, Mannheim, Germany

NF IgH-1comp	5'-AGT TGA GGG GAC TTT CCC AGG -3'
NF IgH-2comp	5'-TCA ACT CCC CTG AAA GGG TCC -3'

Enzymes:

Streptavidin-HRP	Dako Cytomation, Glostrup, Denmark
T4 Polynucleotide Kinase (T4 PNK)	Fermentas, St. Leon-Rot, Germany

Radioactive nucleotide:

dATP labelled with $\gamma[^{32}P]$ (3000 Ci/mmol)	Hartmann Analytic,Braunschweig, Germany

Kits:

Radioimmunoassay kit	Diagnostic Systems Laboratories, Webster, Texas, USA
Mouse Cytokine Luminex^{TM} multiplex kit (IL-1β, IL-6, IL-10, IL-12, TNF-α)	Biosource Europe, S.A. Nivelles, Belgium
Western blotting luminol reagent kit	Santa-Cruz Biotechnology, CA, USA
RNeasy Mini kit	QIAGEN, Hilden, Germany
RNase-free DNase set	QIAGEN, Hilden, Germany
OneStep RT-PCR kit	QIAGEN, Hilden, Germany

Water was generally of Millipore-purified or distilled quality, purchased from University Pharmacy Dusseldorf, Frankfurt (Germany) and Bristol (UK).

3.1.3 Equipments

Weighing balances (analytical and precision)	Sartorius, Göttingen, Germany
β-counter LS 1801	Beckman, Frankfurt, Germany
Centrifuge 5417R and 5810R	Eppendorf, Hamburg, Germany
Digital pH meter 646	Carl Zeiss, Oberkochen, Germany
Drying oven	Fisher Scientific, Pittsburgh, PA
Electronic ballast for mercury vapour compressed-arc lamp	Leistungselektronik Jena GmbH, Jena, Germany
Electrophoresis power supply- EPS 1001	Amersham Pharmacia Biotech, Piscataway, NJ, USA
Electrophoresis equipment	Bio-Rad, Munich, Germany
Film processor AGFA/CURIX 60	Agfa, Mortsel, Belgium
Galaxy mini centrifuge	VWR International, Darmstadt, Germany

Gel dryer 583	Bio-Rad, Munich, Germany
Heater	neoLab, Heidelberg, Germany
Jerome industry phototype P99135 camera	Digital Video Camera, Austin, TX, USA
Leica DM LB microscope	Leica Microsystems, Wetzlar, Germany
Leica DC300F digital camera	Leica Microsystems, Wetzlar, Germany
Luminex 100 system	Luminex Corporation, Austin, TX, USA
Mini-PROTEAN 3 system	Bio-Rad, Munich, Germany
Microtome HM330	Microm GmbH, Heidelberg, Germany
Nikon eclipse TE300 inverted microscope	Nikon, Tokyo, Japan
µQuant microplate Spectrophotometer	Bio-Tek, Winooski, Vermont, USA
Spindrive orbital shaker platform	Scienceware, Pequannock, New Jersey, USA
Variomag magnetic stirrers	H + P Labortechnik, Oberschleißheim, Germany
Vortex mixer	neoLab®, Heidelberg, Germany
Water bath	Memmert GmbH & Co. KG, Schwabach, Germany

3.1.4 Materials

Centrifuge tubes (15, 50 ml)	Falcon, Heidelberg, Germany
Cover slips (various sizes)	Fisher Scientific, Pittsburgh, PA
Cryo vials	Nunc, Wiesbaden, Germany
Gel blotting papers	Schleicher & Schuell, Dassel, Germany

Hybond ECL nitrocellulose membrane	Amersham Biosciences, Buckinghamshire, UK
Hyperfilm™ ECL high performance chemiluminescence film	Amersham Biosciences, Buckinghamshire, UK
Hyperfilm MP (X-ray film)	Amersham Biosciences, Buckinghamshire, UK
Intensifying screens	Eastman Kodak, Rochester, NJ, USA
KODAK READYMATIC dental chemicals (developer and fixer)	Eastman Kodak, Rochester, NJ, USA
Microscope slides	Engelbrecht, Edermünde, Germany
Microtest tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg, Germany
Multi-well cell culture plates	Nunc, Wiesbaden, Germany
Pipettes	Sarstedt, Nümbrecht, Germany
Sterile micropore filters	Millipore, Eschborn, Germany
Transparency films	Ahrend, Dreiech bei Frankfurt am Main, Germany
Whatman paper filters	Schleicher & Schuell, Dassel, Germany

3.2 Principle of Methods Applied

This chapter outlines the principle methods underlying the experiments performed in this study.

3.2.1 PCR

The purpose of a polymerase chain reaction (PCR) is to make a huge number of copies of a gene. PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two 'primers', short oligonucleotides that hybridize to the opposite strands of the target sequence and then prime synthesis of the complementary DNA sequence by DNA polymerase. The chain reaction is a three-step process, denaturation, annealing and extension, which is repeated in several cycles. At each stage of the process, the number of copies is doubled from two to four, to eight, and so on. The reactions are controlled by changing the temperature using a special heat-stable Taq polymerase.

1. Denaturation (at 94 °C):
During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (e.g. the extension from a previous cycle).

2. Annealing (at 54 °C):
The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly), and on that little piece of double stranded DNA (template and primer) the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond between the template and the primer is so strong that it does not break anymore.

3. Extension (at 72 °C):
72 °C is the ideal working temperature for the polymerase. The primers where there are a few bases built in already have a stronger ionic attraction to the template, more than the forces breaking these attractions. Primers that are in positions with no exact match become free again due to the higher temperature and so do not form a part of the extended fragment. The

bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds deoxyribonucleotide triphosphates from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

3.2.2 Bradford Assay (Quantification of Proteins)

The Bradford assay is a rapid and accurate method used to determine the total protein concentration of a sample. This assay uses the dye Coomassie brilliant blue G-250, and is based on the immediate absorbance shift from 465-595 nm when Coomassie brilliant blue G-250 binds to proteins in an acidic solution. The dye exists in a red and blue form. The red form is the predominant dye in solution and it converts to blue when its negative charge binds to the positive charges on protein. The binding of protein to the anionic form of the Coomassie dye results in an absorbance shift to 595 nm. The protein stabilizes the anionic form of the dye by hydrophobic and ionic interactions with arginine residues, and to a lesser extent histidine, lysine, tyrosine, tryptophan and phenylalanine residues. The absorption is proportional to the amount of protein present (Bradford 1976;Stoscheck 1990).

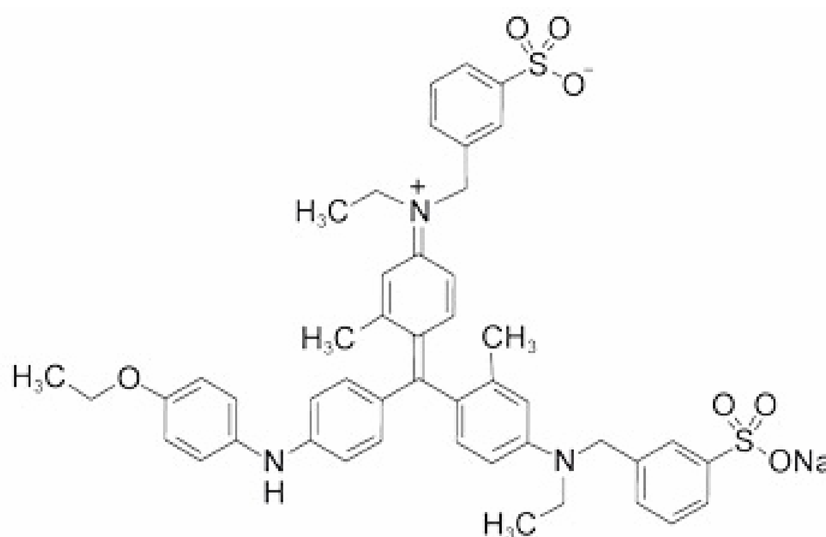


Figure 9: Chemical structure of Coomassie® Brilliant blue G-250.

Because the colour response is non-linear over a wide range of protein concentration, a standard curve must be run with each assay (Bradford 1976). Standard curves are commonly prepared using bovine serum albumin. A standard curve provides a reference for measuring the amount of protein in a solution of unknown concentration. It is constructed by measuring the absorption of several known concentrations of protein. Then a sample will be tested for its absorbance in the assay and its concentration will be determined from the standard curve.

3.2.3 Western Blotting (Immunoblotting)

Western blotting is a common technique for protein detection and the word blotting means the transferring of biological samples from a gel to a membrane and the detection of them on the surface of the membrane. The specificity of the antibody/antigen-interaction used to identify a specific protein in a complex protein mixture provides qualitative data about that protein. During a western blotting procedure, electrophoresis of a gel results in macromolecules being separated. Then, the separated molecules are transferred on to a second matrix, e.g. nitrocellulose membrane. The next step involves the blocking of the membrane to stop non-specific binding of antibodies to the surface of the membrane. The transferred protein is complexed with an enzyme-labelled antibody; an appropriate substrate is then added to the enzyme. This complex makes a detectable product on the membrane. One of the common substrate is chemiluminescence, which when combined with the enzyme complex produces light. The light can be captured using film that is designed for chemiluminescent detection. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane (Krebs et al. 2006).

3.2.4 EMSA

Electrophoretic mobility shift assay (EMSA) is used to investigate DNA/protein-interactions such as binding of transcription factors to their DNA binding sites. In principle, radioactivity-labelled DNA fragments containing binding sites specific for the transcription factor in question are incubated with a test sample, in which the presence or amount of DNA-binding

activity is determined. This mixture of components is then subjected to gel electrophoresis. The unbound DNA fragments will migrate relatively far away from the electrophoresis start site, whereas DNA/protein-complexes that may have formed during the incubation period will remain closest to the start site due to their larger size. Subsequently, autoradiography visualizes those complexes that contain radioactivity-labelled constituents. In order to confirm that the bands observed are specific and result from binding to a characterised DNA-site, competition assays may be performed. Unlabelled oligo-nucleotides with the specific DNA site are added to the sample, prior to the addition of the radioactivity-labelled oligonucleotides. The unlabelled specific oligonucleotides are added in molar excess over the labelled specific oligonucleotides and compete for binding. The observed band is weaker or not detectable at all, depending on the factor of molar ratio. Therefore concluding that the bands observed are indeed cause by a molecule that possesses a DNA-binding activity specific for the characterized oligonucleotide base sequence, but does not bind to other DNA.

3.2.5 RIA

The radioimmunoassay (RIA) is based on the competition between an unlabelled antigen and a fixed concentration of labelled antigen, which is called a tracer and usually labelled with I^{125}-isotope for a limited and constant number of binding sites on the specific antibody. The amount of tracer bound to the antibody will decrease as the concentration of unlabelled antigen increases. This can be measured after separating bound antibody from the free tracer and counting the bound fraction, the free fraction or both. A standard curve is set up with increasing amounts of known antigen and from this curve the amount of antigen in the unknown samples can be calculated.

RIA is not limited to mediators of the immune systems, but can also be extended to other systems in which there is a specific reactor or binding substance. This could be for example a specific binding protein, enzyme or receptor. This assay is also suitable for measurement of hormones, vitamins, drugs and other substances present in biological fluids like serum or plasma.

3.2.6 Haematoxylin Staining

Haematoxylin is a natural colour that is obtained from the logwood tree *Haemotoxylon*. There are several variations of the haematoxylin solution. The final colour of haematoxylin is blue. Haematoxylin can stain tissue only in acidic pH conditions. This staining makes it possible to visualize morphologic differences within tissues. Eosin is the most common colour which is used with haematoxylin. The intensity of eosin depending on its formula as well as the fixative varies very widely. Eosin stains cytoplasm pink to red; and also red blood cells to red. The contrast between these two colours makes it easy to see the difference between various parts of tissue.

3.2.7 Immunohistochemistry

Immunohistochemistry is a technique for the identification of specific tissue components by means of a specific antigen/antibody-reaction tagged with a visible label. Immunohistochemistry makes it possible to visualize the distribution and localization of specific cellular components (antigens) within a cell or tissue. The term immunohistochemistry is often used interchangeably with immunocytochemistry and immunostaining.

Fixation of tissue and cells

For immunohistochemical analysis, it is essential that the morphology of the tissues or cells is retained and that the antigenic sites (epitopes) are accessible. Tissue sections are typically immersed into a fixative solution. Fixation is necessary to prevent artifactual diffusion of soluble tissue components, to arrest enzymatic activity, to avoid decomposition of the structure and to protect the tissue against the deleterious effects involved in the immunohistochemical process.

Fixatives help to preserve tissue by preventing autolysis caused by lysosomal enzymes and bacterial or fungal growth. Fixatives can often cross-link the tissue, making it difficult for the immunoassay reagents to penetrate it. Each fixative procedure must to be optimized to minimize this problem, without altering the antigen or disturbing the endogenous location and the cellular detail of the tissue. There are a lot of different fixatives, for e.g. formaldehyde,

paraformaldehyde, glutaraldehyde, mercuric chloride, acetic acid-zinc chloride or precipitating fixatives like ethanol, methanol and acetone. The suitability of a fixative depends on the tissue or antigen studied.

Inhibition of endogenous tissue components

Tissue can contain materials that react with the various reagents used to develop an immunohistochemical stain. Cellular components, such as enzymes and the vitamin biotin can result in unwanted signals, falsely indicating the presence of an antigen. The immunoperoxidase method of signal detection is commonly used for the immunostaining of tissue. Final analysis of this staining method is complicated by the presence of endogenous peroxidase and 'pseudoperoxidase' activity in tissue. Peroxidase can react with H_2O_2 to reduce substrates, resulting in non-specific staining of tissue or false positives. The most common methods to inhibit or destroy endogenous peroxidase activity after tissue fixation are treatment with H_2O_2 (0.3%) alone or with methanol.

Blocking of non-specific sites (background staining)

Blocking the reactive sites in tissue is essential for the development of an immuno-histochemical reaction. Normal serum is the most popular blocking agent for immunohistochemical staining. The principle is that non-immune serum from the host species of the secondary antibody is applied to the tissue at the beginning of the procedure and will adhere to protein-binding sites either by non-specific adsorption or by binding of specific, but unwanted serum antibodies to antigens.

Labelled Streptavidin-Biotin

There are numerous immunohistochemical techniques that may be used to localize antigens. The selection of a suitable method should be based on parameters, such as the type of specimen under investigation and the degree of sensitivity required. In this study, the labelled streptavidin-biotin method was processed. Streptavidin derived from *Streptomyces avidinii* is a recent substitution to avidin. Unlike avidin, streptavidin molecule is uncharged, and therefore electrostatic binding to tissue is eliminated. In addition, streptavidin does not

contain carbohydrate groups which might bind to tissue lectins, resulting in a degree of background staining.
Labelled streptavidin-biotin is technically similar to the standard avidin-biotin complex-method. The first layer is unlabelled primary antibody, which reacts with tissue antigen. The second layer is biotinylated secondary antibody, which reacts with the primary antibody. This secondary antibody must be directed against the IgG of the animal species in which the primary antibody has been raised. The third layer is enzyme-streptavidin conjugates horseradish peroxidase (HRP)-streptavidin or alkaline phosphatase-streptavidin to replace the complex of avidin-biotin peroxidase. The enzyme is then visualized by application of the substrate chromogen which produces a colorimetric end product. Labelled streptavidin-biotin method is about 5-10 times more sensitive than the standard avidin-biotin-complex method.

Immunohistochemical substrates

Immunoenzymatic staining of tissue results in the reaction of a soluble substrate with an enzyme to produce an insoluble, coloured product. When a substrate is added, the intensity of the colour produced should correlate to the concentration of the primary antibody and the respective tissue antigen. Many enzymes are used for this application, but the most common selection is HRP (isolated from the root of the horseradish plant) and calf intestinal alkaline phosphatase. HRP has an iron-containing haeme group (haematin) as its active site and in solution is coloured brown. The haematin of HRP first forms a complex with H_2O_2 and then causes it to decompose resulting in water and atomic oxygen. HRP activity in the presence of an electron donor first results in the formation of an enzyme-substrate complex then in the oxidation of the electron donor. The electron donor provides the force in continuing catalysis of H_2O_2, while its absence effectively stops the reaction.
There are several electron donors that when oxidized become coloured products and are therefore called chromogens. 3,3'-diaminobenzidine is the most common electron donor in immunohistochemistry. Diaminobenzidine produces a brown end product, which is highly insoluble in alcohol and other organic solvents.

Controls

Special controls must be carried out in order to test the protocols and for the specificity of the antibody being used.

A positive control is to test for a protocol or procedure used. It is ideal to use a sample of tissue where the target antigen is present, and therefore can be used as a positive control. If the tissue shows negative staining, the protocol needs to be checked until a good positive staining is obtained.

A negative control is to test for the specificity of the antibody involved. Therefore, in the absence of the primary antibody or replacement of this by normal serum (derived from the same species as the primary antibody) no staining should occur. This control is easy to achieve and can be used routinely in immunohistochemical staining.

3.2.8 Luciferase Assay

The use of the firefly beetle *Photinus pyralis* luciferase gene in molecular biology has offered a method for utilizing light production in research. Luciferase interacts with the substrate luciferin and produces a light emission, peaking at 562 nm. The main flash reaction is finished within approximately 20 s. Therefore, luciferase determination needs rapid mixing and measurement of light emission. Light emission is measured most sensitively with luminometers in microtiter plates using scintillation counters or photographic films. This form of luminescence can provide a sensitive non-radioactive assay. Luciferase can be produced from different vectors and in various organisms as a reporter of gene regulation. Luciferase assays are one of the best non-toxic, rapid and sensitive methods to measure gene expression. The assay is based on the detection of luciferase activity which correlates with transcription due to DNA regulatory elements in genes, mutations within those elements as well as responses to extracellular and intracellular signals.

3.2.9 ELISA

Enzyme-linked immunosorbent assay (ELISA) employs the quantitative sandwich enzyme immuno technique. The antigen is detected by selective monoclonal antibodies, which interact with two different binding sites (epitopes). A specific antibody is coated on the surface of a

microtiter plate as the capture (immobilized) antibody. A secondary HRP-conjugated antibody serves as the detection antibody in the assay.

The antibodies in concert with the appropriate antigen form an antibody-antigen-antibody complex (sandwich-complex). The peroxidase then catalyzes the conversion of the chromogen substrate (e.g. tetramethylbenzidine) into a coloured product, which is detected by means of spectrophotometry. The intensity of the colour generated is directly proportional to the concentration of the presents in a sample. Sample values are then determined from the standard curve.

3.2.10 Luminex xMAP^{TM} Technology

The Luminex xMAP^{TM} technology employs microspheres. Each bead can be covered with a reagent specific to a particular bioassay, allowing the detection of specific analytes from a sample. Within the Luminex analyzer, lasers provoke the internal dyes that detect each microsphere particle. Many readings are made on each bead set, further validating the results. In this way, Luminex xMAP^{TM} technology allows multiplexing of up to 100 unique assays within a single sample, rapidly and precisely. This technique has ideal speed and sensitivity for cytokine measurements. Its sensitivity is comparable with traditional ELISA-based systems, but with additional advantages including an expanded dynamic range and smaller sample size.

3.3 Experimental Part

This chapter contains detailed descriptions of the experiments performed in this study as well as the contents of the reagents used.

3.3.1 Animals

TLR-2, TLR-4 and TLR-9 deficient (TLR-2$^{-/-}$, TLR-4$^{-/-}$ and TLR-9$^{-/-}$) mice were generated by homologous recombination. TLR-2$^{-/-}$ mice were provided by Tularik Inc. (South San Francisco, CA); TLR-4$^{-/-}$ and TLR-9$^{-/-}$ mice were kindly provided by Akira (Osaka University, Osaka, Japan) (Hoshino et al. 1999;Hemmi et al. 2000). TLR-2$^{-/-}$, TLR-4$^{-/-}$ and TLR-9$^{-/-}$ mice, plus their respective controls (wild-type = WT; C57BL/6 mice) were kept at 24 °C, 55% humidity, 12 h day-night rhythm and received a standard diet and water ad libitum. Male or female mice that were 12-16 weeks old (20-30 g) were used for the experiments.All procedures were carried out in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International guidelines and Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, National Institutes of Health, Publication no. 86-23) and were approved by the German government ethical and research boards.

3.3.2 Animal Interventions

TLR-2

WT and TLR-2$^{-/-}$ mice were randomized in 24 different treatment groups (n = 4-8/group) and treated intraperitoneally (i.p.) with saline (sal; 1 ml/kg), LPS (1 mg/kg; *Escherichia coli*, serotype 0.111:B4) or LTA (1 mg/kg; *Staphylococcus aureus*). After a period ranging from 30 min to 24 h, mice were killed by terminal anaesthesia using pentobarbital, provided by the University Pharmacy of Dusseldorf, Germany. Plasma samples and the adrenal glands were taken and frozen at -20 °C or snap frozen and stored at -80 °C respectively until assayed.

Treatment (i.p.)	30, 60, 90, 120 min	6 h	24 h
saline (1 ml/kg)	WT only	WT or TLR-$2^{-/-}$	WT or TLR-$2^{-/-}$
LPS (1 mg/kg)	WT only	WT or TLR-$2^{-/-}$	WT or TLR-$2^{-/-}$
LTA (1 mg/kg)	WT only	WT or TLR-$2^{-/-}$	WT or TLR-$2^{-/-}$

Table 8: Summary of treatment groups in the TLR-2 study.

TLR-4

WT and TLR-$4^{-/-}$ mice were treated i.p. with sal (1 ml/kg), crude 'commercialized' (c)LPS (1 mg/kg; *Escherichia coli*, serotype 0.111:B4) or pure (p)LPS (1 mg/kg; *Escherichia coli*, F515 LPS SB III, 66), a highly purified preparation kindly provided by Prof. U. Zähringer (Research Centre Borstel, Borstel, Germany). After 1, 6 and 24 h, mice were sacrificed by terminal pentobarbital anaesthesia. Plasma and the adrenal glands were taken and frozen at -20 °C or snap frozen and stored at -80 °C respectively until assayed.

Treatment (i.p.)	1 h	6 h	24 h
saline (1 ml/kg)	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$
cLPS (1 mg/kg)	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$
pLPS (1 mg/kg)	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$	WT or TLR-$4^{-/-}$

Table 9: Summary of treatment groups in the TLR-4 study.

TLR-9

WT and TLR-$9^{-/-}$ mice were i.p. sensitized with a hepatocyte-specific inhibitor of RNA synthesis D-galactosamine (D-GalN; 1 mg/kg). D-GalN sensitization was used to reduce the amount of bacterial DNA or CpG-ODN (Hemmi et al. 2000;Wang et al. 2006). Thirty minutes later, animals received i.p. either 1 ml/kg of sal or 1 nmol/g CpG-ODN (containing a "CG-motif": 5'-TCC ATG ACG TTC CTG ATG CT) for a period of 15 min to 6 h. After each time period, animals were killed by an overdose of pentobarbital. Adrenal glands, spleen and plasma samples were taken and frozen at -20 °C or snap frozen and stored at -80 °C respectively until assayed.

Treatment (i.p.)	15, 30, 45,60 min	2 h	6 h
saline (1 ml/kg)	WT only	WT or TLR-$9^{-/-}$	WT or TLR-$9^{-/-}$
CpG-ODN (1 nmol/g)	WT only	WT or TLR-$9^{-/-}$	WT or TLR-$9^{-/-}$

Table 10: Summary of treatment groups in the TLR-9 study.

3.3.3 Human Adrenal Tissue

Foetal (12-week) and adult human adrenal tissue were obtained from the adrenal tissue bank approved by the ethical committee of the University of Dresden, Germany.

3.3.4 Isolation of RNA

Total RNA from tissue or cells was isolated using RNeasy Mini kit including a DNase digestion step (RNase-free DNase set; Qiagen) according to the manufacturer's instructions. For reverse transcription reaction (RT), 1 µg of RNA was incubated at 37 °C for 1 h in buffer containing 200 U of M-MLV reverse polymerase, oligo$(dT)_{15}$ primers mix and dNTPs. RNA concentration was determined by absorbance at 260 nm. Until further processing, RNA was dissolved in 30 µl of RNase-free water and stored at -80 °C.

3.3.5 RT-PCR

The first-strand cDNA was synthesized using an Omniscript kit (Qiagen). Primers were designed by using the Primer3 software synthesised by MWG Biotechnology (Ebersberg, Germany) (see Tab. 11). All RT-PCR tests were run under the following conditions: initial denaturation at 94 °C for 60 s followed by 40 cycles of amplification with the denaturation step at 94 °C for 45 s, the annealing step for 60 s at 54 °C and the elongation step at 72 °C for 90 s or 30 cycles of 30 s at 94 °C, 30 s at 56 °C and 2 min extension at 72 °C. β-actin as a housekeeping gene was used to ensure the purity of the cDNA. The RT-PCR products were

separated in 2% (wt/vol) agarose gels and visualized with ethidium bromide. Quantitative analysis of TLR mRNA levels was performed by determination of the optical density using Scion Image software (Scion Corporation, Maryland, USA) and normalized to β-actin mRNA expression levels.

Gene	Forward	Reverse	Product size (bp)
mTLR-2	5'-AGCTCTTTGGCTCTTCTG-3'	5'-AGAACTGGGGGATATGC-3'	950
mTLR-4	5'-GCATGGCTTACACCACCTCT-3'	5'-GTGCTGAAAATCCAGGTGCT-3'	970
mTLR-9	5'-TGC AGG AGC TGA ACA TGA AC-3'	5'-TAG AAG CAG GGG TGC TCA GT-3'	297
mβ-actin	5'-GTGGGGCGCCCCAGGCACCA-3'	5'-CTCCTTAATGTCACGCACGAT-3'	540
mβ-actin	5'-TGT TAC CAA CTG GGA CGA CA-3'	5'-TCT CAG CTG TGG TGG TGA AG-3'	392

Table 11: Summary of primers.

3.3.6 Protein Preparation of Tissue for Western Blotting

Protein extraction buffer (Lysis buffer):

NaCl	150 mM
Tris-HCl (pH 7.4)	50 mM
Ethylenediaminetetraacetic acid (EDTA)	1 mM
Phenylmethylsulphonyl fluoride (PMSF)	1 mM
Triton X-100	1%
Sodium deoxycholate	1%
Sodium dodecyl sulfate (SDS)	0.1%
Leupeptin	5 µg/ml
Aprotinin A	5 µg/ml

The protease inhibitors leupeptin, aprotinin and PMSF were freshly added just before use. The lysis procedure was performed on ice.

Tissue (adrenal glands) was homogenized in 4 volumes of ice-cold protein extraction buffer, after being disrupted with a clean scalpel. The crude homogenate was then transferred to a microcentrifuge tube. After 20 min incubation on ice, the homogenate was centrifuged at 20000 x g for 20 min at 4 °C. The supernatant was removed and frozen at -80 °C until assayed. The total protein present in each sample was determined by the Bradford colorimetric assay.

3.3.7 Protein Quantification (Bradford Assay)

Protein concentration of samples for Western blotting or EMSA was measured using the method described by Bradford in a colorimetric assay. The unknown samples and the blank (consists of buffer with no protein) were diluted 1:10 or 1:25 in distilled water. For protein standards, a serial dilution of bovine serum albumin (BSA) was prepared. The BSA standard solutions ranged from 0.1-1.0 mg/ml. This assay was performed in 96-well plates and in duplicate for each sample and standard solution. 10 µl of the protein standards, the unknown samples and the blank were added to separate wells in the 96-well plate and mixed with 200 µl of the protein dye reagent (Bradford reagent). After incubation at room temperature (RT) for 5 min, the absorbance was measured at λ = 595 nm in a microplate reader. Protein concentrations in the samples were determined with KC4™ data analysis software using a standard curve.

3.3.8 Western Blotting

Stock solutions

Tris (hydroxymethyl)-aminomethane (Sigma 7-9®; 1.875 M):

Sigma 7-9®	45.4 g
distilled water	150.0 ml

was adjusted to pH 8.8 with 6 N HCl and to a total volume of 200 ml with distilled water.

Sigma 7-9® (0.625 M):

Sigma 7-9®	15.1 g
distilled water	150.0 ml

was adjusted to pH 6.8 with 6 N HCl and to a total volume of 200 ml with distilled water.

Ammonium persulfate (APS; 10%):

100 mg APS were dissolved in 1.0 ml of distilled water.

Tris-HCl (0.5 M):

Tris-HCl	15.76 g
distilled water	150.0 ml

was adjusted to pH 6.8 with 5 N NaOH and to a total volume of 200 ml.

Buffers

Separating gel buffer (200 ml):

1 g (0.5%) SDS was dissolved in 1.875 M Sigma 7-9® (pH 8.8) stock solution and stored at RT.

Stacking gel buffer (200 ml):

1 g (0.5%) SDS was dissolved in 0.625 M Sigma 7-9® (pH 6.8) stock solution and stored at RT.

Sample buffer (10 ml):

0.5 mM Tris-HCl (pH 6.8)	1.0 ml (final concentration: 50 mM)
Glycerol	2.0 ml (20%)
SDS	0.1 g (1%)
Bromphenol blue	5 mg (0.05%)
distilled water	6.0 ml
β-Mercaptoethanol	1.0 ml (10%)

β-Mercaptoethanol was added to sample buffer prior to use.

Running buffer (1000 ml):

Sigma 7-9®	6.1 g (50 mM)
Glycine	28.8 g (0.384 M)
SDS	1.0 g (0.1%)

All reagents were dissolved in distilled water.

Transfer buffer (1000 ml):

Sigma 7-9®	6.0 g (25 mM)
Glycine	14.4 g (0.192 M)
Methanol	200.0 ml (20%)
distilled water	800.0 ml

Solutions

Phosphate buffered saline (PBS) solution (1000 ml):

5 PBS tablets were dissolved in distilled water.

TPBS solution (400 ml):

PBS	399.0 ml
Tween® 20	1.0 ml

Blocking solution (200 ml):

PBS	200.0 ml
Skim milk	10.0 g (5%)
Tween® 20	0.5 ml

Antibodies

The primary and secondary antibodies used in Western blotting are listed in Tables 12 and 13, respectively.

Antibody	Isotype (Ig)	Dilution	Source
Anti-β-actin (monoclonal, mouse)	IgG1	1:3000	Sigma-Aldrich, St. Louis, USA
Anti-mouse TLR2 (polyclonal, goat)	IgG	1:1000	Santa Cruz, Heidelberg, Germany
Anti-mouse TLR4 (polyclonal, goat)	IgG	1:1000	Santa Cruz, Heidelberg, Germany
Anti-human TLR2 (polyclonal, goat)	IgG	1:500	Santa Cruz, Heidelberg, Germany
Anti-human TLR4 (polyclonal, rabbit)	IgG	1:500	Santa Cruz, Heidelberg, Germany
Anti-mouse TLR9/CD289 (polyclonal, rabbit)	IgG	1:1000	Imgenex Corp., San Diego, CA,USA

Table 12: Primary antibodies used in Western blotting analysis.

Antibody-HRP conjugated	Dilution	Source
Goat anti-mouse IgG-HRP	1:3000	Santa Cruz, Heidelberg, Germany
Rabbit anti-goat IgG-HRP	1:3000	Santa Cruz, Heidelberg, Germany
Donkey anti-rabbit IgG-HRP	1:3000	Santa Cruz, Heidelberg, Germany
Goat anti-rabbit IgG-HRP	1:5000	Imgenex Corp., San Diego, CA, USA

Table 13: Secondary antibodies used in Western blotting analysis. HRP, horseradish peroxidase.

Preparation of Gels

<u>Separating gel (7.5%; 20 ml):</u>

Acrylamide/Bis (30%)	5.0 ml
Separating gel buffer	4.0 ml
distilled water	10.9 ml
APS (10%)	100 µl
Tetramethylethylenediamine (TEMED)	20 µl

Stacking gel (4%; 10 ml)):

Acrylamide/Bis (30%)	1.3 ml
Stacking gel buffer	2.0 ml
distilled water	6.6 ml
APS (10%)	50 µl
TEMED	10 µl

The separating and stacking gels were prepared by mixing the respective reagents. Both monomer solutions were degassed under vacuum (in a 50 ml falcon tube) for at least 5 min. Then 10% APS and TEMED were gently added to the solutions immediately before pouring each gel.

First the gel cassette sandwich (Mini-PROTEAN cell 3) was assembled as recommended by the manufacturer. A comb was placed into the assembled gel cassette, and the glass plate was marked 1 cm below the comb to indicate the level to which the separating gel was to be poured. The comb was removed after marking. The separating gel solution filled (with a pipette) between the glass plate sandwich, until the mark was reached. Immediately, the solution was overlaid with water to remove air bubbles and to obtain an even interface between gels. Then the separating gel was allowed to polymerize for 45 min. The water on top of the separating gel was removed and the area above the gel was dried carefully with filter paper. The stacking gel was prepared just before use and then poured on top of the separating gel. The desired comb was inserted between the glass plate sandwiches, being careful not to trap bubbles under the teeth. The stacking gel was allowed to polymerize for 30-45 min.

SDS-PAGE and Membrane Transfer

Aliquots of cell lysates or tissue homogenates, equivalent to 40 µg proteins, were diluted in equal amounts of sample buffer and heated for 5 min at 95 °C. The samples were then loaded into each lane and separated by SDS-PAGE 7.5% polyacrylamide gels (1.5 mm). In the first lane, 7 µl of rainbow marker (Kaleidoscope Prestained Standards) were loaded. Gels were run at 100 V (constant voltage) for 1 h. After electrophoresis proteins were transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose membrane) by semi-dry-electroblotting. Transfer conditions were 1 h at a voltage of 100 V. When finished, the

nitrocellulose membranes were immersed in blocking solution and blocked overnight at 4 °C or for 2 h at RT on a shaker platform.

Antibodies and Detection

The membranes were washed 3 x 5 min with tween 20/PBS (TPBS). This and all other incubation steps were performed at RT on a shaker platform. The membranes were then incubated with the appropriate specific primary antibody in blocking solution for 1 h (see Tab. 12). After 3 x 5 min washes in TPBS, the nitrocellulose membranes were incubated with HRP-conjugated secondary antibody in blocking solution for 1 h as listed in Table 13. Finally, the membranes were washed with TPBS (3 x 5 min) and once in PBS for 5 min. Immunoreactive proteins were detected by enhanced chemiluminescence using the ECL detection system (Western blotting luminol reagent kit) and subsequent autoradiography. All steps of immunodetection were performed in the dark. Equal volumes of ECL solution A and B were mixed (1:1) and added to the membranes for 1 min. Membranes were placed in a film developer cassette and covered with transparent cover sheets. Film (Hyperfilm™ ECL) was placed on top and the cassette was closed. The film was developed for 5-20 min, respectively.

3.3.9 Nuclear Extraction for EMSA

EMSA totex high salt buffer:

4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.9)	20 mM
NaCl	350 mM
$MgCl_2$	1 mM
EDTA	0.5 mM
Ethylene glycol tetraacetic acid (EGTA)	0.1 mM
Dithiothreitol (DTT)	0.5 mM
PMSF	1 mM
Glycerol	20%
Nonidet P-40	1%
Aprotinin	2 µg/ml
Leupeptin	2 µg/ml

Leupeptin, aprotinin, DTT and PMSF as protease inhibitors were freshly added just before use. All procedures were performed on ice.

Nuclear extracts of tissues (adrenal glands) were prepared in a high salt buffer (50-100 µl). After homogenisation by slicing tissue into very small pieces or vigorous vortexing of the cells, the samples were incubated on ice for 15 min and then centrifuged at 18000 x g for 20 min at 4 °C. The supernatants were frozen and the protein content of the supernatants was determined by Bradford assay.

3.3.10 EMSA

Radiolabelling of Oligonucleotides

10x Klenow buffer (annealing buffer):

Tris-HCl (pH 7.5)	500 mM
NaCl	100 mM
$MgCl_2$	100 mM

Polynucleotide kinase reaction buffer (PNK buffer; 10x):

(as purchased from the supplier)

Tris-HCl (pH 7.6)	500 mM
$MgCl_2$	100 mM
DTT	50 mM
Spermidine	1 mM
EDTA	1 mM

Sodium/Tris/EDTA buffer (STE buffer):

NaCl	100 mM
Tris-HCl (pH 8)	10 mM
EDTA (pH 8)	1 mM

The DNA-oligonucleotides containing an NF-κB binding motif were synthesised by MWTG Biotech (Ebersberg, Germany). They were purchased single-stranded with the following sequence

5'- AGC TTC AGA GGG GAC TTT CCG AGA GGT CGA -3' or

5'- TCG ACC TCT CGG AAA GTC CCC TCT GAA GCT -3'.

These single-strand oligonucleotides were annealed with a complementary strand by heating 50 µg of either strand in 900 µl Millipore water and 100 µl Klenow buffer (10x) at 95 °C for 5 min and allowed to cool down at RT. They were then stored at -20 °C.

The oligonucleotides were radiolabelled with ^{32}P, using [γ ^{32}P]-adenosine triphosphate ([γ ^{32}P]-ATP) and T4 polynucleotide kinase. This enzyme is prepared from a cloned pseT gene of bacteriophage T4, which is carried by *Escherichia coli* cells. T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5'-hydroxylated terminus of DNAs (or RNAs). Thus, when the γ-phosphate group of the ATP was added in the reaction, containing a β-emitting ^{32}P-isotype, the DNA oligonucleotide was labelled radioactively. 1.5 µl of nucleotide (equivalent to 25-50 ng), 5 µl of [γ ^{32}P]-ATP (50 µCi), 1.5 µl (10-15 Units) T4 PNK and 10 µl millipore water were incubated in polynucleotide kinase reaction buffer (10x) at 37 °C for 30 min. The reaction was stopped by the addition of sodium/tris/EDTA buffer (40 µl). The vial was kept on ice immediately. The radiolabelled oligonucleotides were separated from unincorporated [γ ^{32}P]-ATP with QIAquick nucleotide removal kit. 10 volumes of binding buffer were added to 1 volume of the reaction sample and mixed. A QIAquick spin column was placed in a provided 2-ml collection tube. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min at 3800 x g. Then, the column was transferred into a clean tube, and the tube containing the radioactive flow-through was discarded appropriately. Thereafter, the column was washed by adding 500 µl of binding buffer and centrifuged for 1 min at 3800 x g. The wash procedure was repeated. The flow-through was discarded, and the QIAquick column was placed back in the same tube, which should been empty. It was centrifuged for an additional 1 min at ≥ 10000 x g and the column was placed in a clean 1.5-ml microcentrifuge tube. To elute DNA, 75 µl of distilled water was added to the centre of the QIAquick membrane subsequently. The column was then allowed to stand for 1 min before centrifugation. The labelled oligonucleotides were collected in a microcentrifuge tube and stored at -20 °C. 2 µl of elute were mixed with 1 ml of water in a flat-bottomed plastic vial. The radioactivity in the samples was determined in a β-counter.

Electrophoresis and Autoradiography

Gel shift marker (10 ml):

Xylincyanol	40 mg (final concentration: 0.4%)
Bromphenol blue	40 mg (0.4%)
Ficoll 400	2.5 g (25%)
distilled water	ad 10.0 ml

Gel shift buffer (20 ml):

HEPES (1 M)	1.0 ml (final concentration: 0.05 M)
EDTA (0.5 M)	0.2 ml (5 mM)
$MgCl_2$ (1 M)	0.5 ml (25 mM)
Glycerol (87%)	11.5 ml (50%)
Bromphenol blue (1%, pH 7.8)	1.0 ml
distilled water	5.8 ml

Binding reaction pre-mix:

Sample volume	9.5 µl
Gelshift buffer	4.0 µl
DTT (1 M)	0.1 µl
PMSF (200 mM)	0.2 µl
Poly deoxyinosinic-deoxycytidylic (dI-dC; 1 mg/ml)	1.0 µl
Bovine serum albumin (BSA; 1 mg/ml)	2.0 µl
distilled water	2.2 µl

The pre-mix was prepared as a master-mix for all samples and an additional one. Thereafter, the labelled probe ([^{32}P]-NF-κB oligonucleotide) was added to this binding reaction pre-mix, followed by incubation (15 min at RT). The amount of radiolabelled oligonucleotides used was determined by their radioactivity. An amount yielding 10000 cpm was used in the assays.

Tris-Borate-EDTA buffer (10x TBE buffer):

Tris	108.0 g
Boric acid	55.0 g
EDTA	7.4 g
distilled water	ad 1000 ml

Electrophoresis buffer (0.25 TBE buffer):

prepared by a 1:40 dilution of 10x TBE stock solution in distilled water.

10x TBE	25.0 ml
distilled water	975.0 ml

4.5% Polyacrylamide gels (4.5%; 200 ml):

distilled water	94.5 ml
Acrylamide 4K-Solution (40%, 19:1)	13.5 ml
10x TBE	3.0 ml
Glycerol (87%)	9.0 ml
APS (20%)	400 µl
TEMED	80 µl

The non-denaturing polyacrylamide gels were cast the day before the experiment and kept in a cooling room.

Binding reactions were prepared by combining the following in 1.5 ml micro-reaction tubes:

Binding reaction mixture (19 µl):

Nuclear extract (diluted with distilled water to 9.5 µl)	9.5 µl
Binding reaction pre-mix	9.5 µl

After 15 min of incubation at RT, all samples were loaded into the lanes of the pre-run polyacrylamide gel (220 V for 20 min) and 7 µl of the gel shift marker were loaded into the first lane to track the position of unbound probe. The gels were run with 0.25x TBE buffer for 3 h at 220 V until the bromphenol blue marker reached the mark of 12 cm from loading site. At

the end of electrophoresis, gels were carefully removed from the glass plates and transferred onto filter papers (Whatman papers). The gels were covered with aluminium foil and dried under vacuum (gel dryer) for 2 h at 80 °C. Then a radiographic film (Hyperfilm MP) was exposed to the dried gels, while kept at -70 °C for 4-6 h or overnight, and developed in a film processor.

3.3.11 Purity of LPS Preparation (Luciferase Assay)

To determine the purity of our LPS preparations, we used a TLR-specific NF-kB reporter gene assay (Hemmi et al. 2000;Opitz et al. 2001). Human embryonic kidney 293 (HEK293) cells stably expressing CD14 were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine and 50 µg/ml of each penicillin and streptomycin. Cells were plated into 12-well triplicate plates with 1 x 10^5 cells per well. Transfection was performed using FuGENE 6 reagent with an NF-kB-controlled luciferase construct (120 ng), *Rous sarcoma virus* β-galactosidase (40 ng) and expression plasmids for human TLR-2 or TLR-4 plus MD2 (40 ng each). 24 h after transfection, cells were stimulated with purified pLPS or crude cLPS preparation (0, 1, 10, 100 ng/ml). After a further 20 h of incubation, the activities of luciferase and β-galactosidase (which was used as a marker for transfection efficiency) were determined in cell extracts using a chemiluminescence based assay (Roche Diagnostics GmbH, Mannheim, Germany). Luciferase activities were calculated and normalized to the β-galactosidase control.

3.3.12 Tissue Preparation & Morphometric Analysis of Adrenal Glands

Tissue Embedding

<u>PBS:</u>

Solution A: 35.61 g of Na_2HPO_4 x 1 H_2O was dissolved in 1000 ml distilled water.

Solution B: 27.60 g of NaH_2PO_4 x 1 H_2O was dissolved in 1000 ml distilled water.

PBS (0.01 M):

Solution A	40.0 ml
Solution B	10.0 ml
NaCl	9.0 g

was adjusted to pH 7.3-7.4 and to a total volume of 1000 ml with distilled water.

Paraformaldehyde (4%):

Paraformaldehyde	80.0 g
distilled water	800.0 ml

Adrenal glands were fixed in 4% paraformaldehyde for 24 h at 4 °C. To remove any water before embedding in paraffin, dehydration was performed by soaking the tissue in a series of increasing alcohol concentrations. In this step, the tissues were incubated three times in 70%, 80%, 90% and 100% alcohol for 30 min at RT. Alcohol was removed from the tissue by incubating in xylol overnight. Thereafter, the tissues were incubated next in paraffin for 1 h and then in new paraffin overnight at 60 °C. Before embedding, the paraffin was changed four times. Finally, the adrenal gland was put in an embedding mould and melted paraffin was poured into the mould to form a block. The mould was left on a cold plate, until the tissue-paraffin block was set and removed from the mould. Blocks were stored at 4 °C.

Tissue Sectioning

The blocks were fixed firmly in a microtome and then sliced at a thickness of 5-8 µm. The sections were put on a surface of water (warmed at 45 °C) and allowed to spread out before placed on to slides. The slides were dried overnight at 45 °C and stored at RT until ready for staining.

Haematoxylin staining

Cross-sections of mouse adrenal gland were deparaffinized by incubating slides in xylol for 2 x 5 min and rehydrated by incubating in descending ethanol concentrations (100%, 90%, 70%, 50% ethanol and distilled H_2O). After rehydration, the sections were incubated in haematoxylin for 1 min, which resulted in a blue colour. The colour reaction was stopped by

incubating the slides in distilled H_2O for 1 min. All following incubation steps were done in a humid environment at RT to prevent the tissue sections from drying out during the staining process. Finally, the sections were dehydrated by incubating then in increasing concentrations of ethanol (50%, 70%, 90% and 100%). Sections were then incubated twice in Roti®-Histol (xylol substitute) for 1 min and mounted immediately with Entellan, a permanent mounting medium.

3.3.13 Morphometric Analysis

To determine the size of adrenal sections a computer-supported imaging system connected to a light microscope (Eclipse TE300 microscope, Nikon, LUCIA G Software, Nikon, Jerome Industry Phototype P99135 camera, (Austin, TX) was used. The area of tissue sections was measured in triplicates. The four largest sections were evaluated to give an approximation of the largest diameter in each gland.

3.3.14 Electron Microscopy for Functional Analysis of Adrenal Glands

Electron Microscopy was done in collaboration with Prof. S.R. Borstein, Department of Medicine, University of Dresden, Carl Gustav Carus, Dresden, Germany. Adrenal glands were fixed in phosphate buffer (0.1 M; pH 7.3) with 2% formaldehyde and glutaraldehyde. Adrenal slices were post-fixed for 90 min (2% OsO_4 in 0.1 M cacodylate buffer, pH 7.3), dehydrated in ethanol, and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined at 80 kV in a Phillips CM 10 electron microscope (Eindhoven, The Netherlands).

3.3.15 Immunostainings

Antibodies:

The primary and secondary antibodies used in immunostainings are listed in Tables 14 and 15, respectively.

Antibody	Isotype (Ig)	Dilution	Source
Anti-mouse TLR9/CD289 (polyclonal, rabbit)	IgG	1:100 (in IHC) 1:200 (in ICC)	Imgenex Corp., San Diego, CA, USA
Anti-mouse IL-1R (polyclonal, goat)	IgG	1:100	Santa Cruz, Heidelberg, Germany
Anti-mouse IL-6 (polyclonal, rabbit)	IgG	1:100	Chemicon, Temecula, USA
Anti-mouse TNF-α (polyclonal, goat)	IgG	1:100	R&D Systems Europe, Abingdon, UK

Table 14: Primary antibodies used in immunostainings.

Secondary antibody	Dilution	Source
Goat anti-rabbit IgG, biotinylated	1:300	Dako Cytomation, Glostrup, Denmark
Rabbit anti-goat IgG, biotinylated	1:300	Dako Cytomation, Glostrup, Denmark
Goat anti-rabbit IgG (H+L), Cy3-conjugated	1:250 (in IHC) 1:1000 (in ICC)	Jackson ImmunoResearch, Cambridgeshire, UK

Table 15: Secondary antibodies used in immunostainings.

Solutions :

Triton X-100 (0.3%) in PBS:

Triton X-100 0.3 ml

PBS (0.01 M) 99.7 ml

H_2O_2 (0.3%):

H_2O_2 (30%) 50 µl

0.3% Triton X-100/PBS 4950 µl

Normal serum (3%):

Normal serum (goat or rabbit)	150 µl
0.3% Triton X-100/PBS	4850 µl

DAB (stock; 4 mg/ml):

20 mg DAB were dissolved in 5 ml distilled water, aliquoted and stored at -20 °C.

DAB (0.2 mg/ml; 5 ml)

DAB (4 mg/ml)	500 µl
PBS (0.01 M)	4500 µl

For activating DAB, 5 µl of 1% H_2O_2 (33.3 µl of 30% H_2O_2 in 966.7 µl dH_2O) was added to each ml DAB solution before use.

Mouse adrenal gland cross-sections were deparaffinized by incubating slides twice in xylol for 5 min and rehydrated by incubating in descending ethanol concentrations (100%, 90%, 70%, 50% ethanol and dH_2O). After rehydration, the sections were washed in PBS for 5 min. Then the endogenous peroxidase activity was quenched by incubating the specimens for 30 min with 0.3% H_2O_2 in a humidity chamber. All following incubation steps were done in a humid environment at RT to prevent the tissue sections from drying out during the staining process. Sections were washed in PBS for 5 min, and then incubated with a blocking solution containing 3% normal serum (goat or rabbit serum; as they are the same species in which the secondary antibodies were raised) for 30 min. Thereafter, the specimens were incubated with the appropriate characterized primary antibody (Tab. 14) overnight at 4 °C. The primary antibody was diluted with 0.3% Triton X-100/PBS. The tissue sections were rinsed for 5 min in PBS and subsequently incubated with the respective biotinylated secondary antibody (Tab. 15) for 1 h. The sections were washed in PBS once again, followed by 30 min incubation with HRP-labelled streptavidin (1-2 drops solution per section). After washing in PBS, staining was completed by incubating with the substrate-chromogen, DAB (0.2 mg/ml) for 10 min. This resulted in a brown-coloured precipitate at the antigen site. Colour reaction was stopped by incubating the slides in PBS for 3 min. Sections were counterstained for 1 min in haematoxylin and washed 3 times in distilled water. Finally, the sections were dehydrated by incubating with increasing concentrations of ethanol (50%, 70%, 90% and 100%). Sections were then incubated (2x) in Roti®-Histol (xylol substitute) for 2 min and mounted

immediately with Entellan, a permanent mounting medium. Immunostaining of the sections was examined using a light or fluorescence microscope.

3.3.16 Determination of Plasma Corticosterone and ACTH by RIA

Plasma levels of corticosterone and ACTH were determined by RIA in collaboration with the Department of Endocrinology, Diabetes and Rheumatology, Heinrich-Heine University, Düsseldorf, Germany and Department of Medicine, University of Dresden, Carl Gustav Carus, Dresden, Germany, and according to the instructions of the manufacturer (Diagnostic Systems Laboratories, Webster, Texas, USA).

A standard curve was determined for each assay; 200 µl of standards, controls and samples were added to appropriate tubes and assayed in duplicates. Standards and controls were supplied by the manufacturer and present in each kit. To non-specific binding tubes, 300 µl of non-specific standard (buffer) was added. Immediately, 100 µl of the specific anti-serum (antibody) was added to all tubes except non-specific binding and 'Total Count' tubes. After incubation at 2-8 °C for 4 h, 100 µl of [^{125}I]-labelled ACTH reagent or [^{125}I]-labelled corticosterone reagent was added to all tubes. The tubes were vortexed and incubated for 16-24 h at 2-8 °C. Then, 1 ml of precipitating reagent (mixed before use) was added to all tubes, except 'Total Count' tubes. Tubes were vortexed and incubated for 15-20 min at RT. Then, all the tubes were centrifuged for 15 min at 1500 x g at 4 °C. The tubes were then poured off by simultaneous inversion with a sponge rack, which drained into the radioactive waste. The tubes were drained on absorbent material for 30 s and then gently blotted to remove any drops before returning to their upright position, as failure to blot tubes adequately may result in poor replication and inaccurate values. Tubes were then counted for 1 min in a γ-counter.

3.3.17 The Levels of IL-1β, IL-6 and TNF-α

The plasma levels of IL-1β, IL-6 and TNF-α were determined by using ELISA kits according to the manufacturer's instructions.

Sample Collection and Storage

Blood samples were allowed to clot for 2 h at RT or overnight at 2-8 °C before centrifuging for 20 min at approximately 2000 x g. Serum was then removed and assayed immediately or aliquoted and stored at -20 °C. Plasma was collected by using heparin as an anticoagulant and centrifuged for 20 min at 2000 x g within 30 min of collection. It was assayed immediately or aliquoted and stored at -20 °C or below.

Reagent Preparation

All reagents were brought to RT before use.

Kit control:

The kit's control was reconstituted with 1 ml distilled water.

Wash buffer:

To prepare wash buffer for one plate, 25 ml of concentrated wash buffer was diluted with 600 ml of distilled water.

Substrate solution:

Colour A and B was mixed together in equal volumes within 15 min of use. The solution has to be protected from light. 100 µl of the resultant mixture was required per well.

Standards:

The standard was reconstituted with 5 ml of buffered protein solution, with preservatives (Calibrator Diluent RD5T) to produce a stock solution of 500 pg/ml. The standard was allowed to sit for 5 min with gentle mixing prior to making dilutions to ensure complete reconstitution. The serial 1:2 dilutions of the standard were made (250, 125, 62.5, 31.2, 15.6, 7.8, 0 pg/ml).

Assay Procedure

All reagents and samples were brought to RT before use. Samples, controls and standards were assayed in duplicates. Assay diluent (RD1-14; 50 µl) was added to each well. In the next step, 50 µl of standards, samples or controls was added to the appropriate well of a 96-well plate. The wells were mixed by gently tapping the plate for 1 min. The plate was then covered

with the adhesive strip provided before incubating for 2 h at RT. Solution from the wells was then thoroughly aspirated or decanted. Each plate was washed 4 times with wash buffer (400 µl). After the last wash, any remaining wash buffer was removed by aspiration or decanting. The plate was inverted and blotted with a clean paper towel. After removing any unbound substances, 100 µl of a polyclonal antibody (specific for mouse IL-1β, IL-6 or TNF-α) conjugated to HRP was pipetted into each well. After 2 h of incubation at RT, the plate was aspirated and washed as previously described. Next, 100 µl of substrate solution was added to each well and the plate was incubated for 30 min at RT in the dark. Finally, 100 µl of stop solution was pipetted to each well and the plate was gently tapped to ensure thorough mixing. The solution changed from blue to yellow. The optical density was determined within 30 min by using a microplate reader set at a wavelength of 450 nm.

3.3.18 Determination of Plasma Cytokines by Luminex™ Microbead Assay

The plasma levels of cytokines (granulocyte macrophage-colony stimulation factor (GM-CSF), IL-1β, 2, 4, 5, 6, 10, 12, IFN-γ and TNF-α) were determined by using the Luminex™ microbead assay according to the manufacturer's instruction (BioSource Europe S.A. Belgium).

3.4 Analysis

All illustrations using EMSA, Western blots and immunohistochemical staining were done by using Adobe Photoshop Version CS2 (Adobe Systems Inc., San Jose, USA).

Quantitative analysis of NF-κB activation as well as TLR-2 and TLR-9 expression were performed by determination of optical density using Scion Image Version Alpha 4.0.3.2 (Scion Corporation, Federick, Maryland, USA).

The cytokine multiplex assays were analyzed with StarStation software Version 1.1 (Applied Cytometry Systems, Sheffield, UK).

3.5 Statistics

Results are expressed as mean ± standard error mean (SEM). Statistical significance was calculated by a Student's t-test, one sample t-test or an ANOVA followed by Bonferroni's or Dunnett's posttests where appropriate by using GraphPad Prism® Version 5.2 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at $P<0.05$.

4 Results

4.1 TLR-2 Study

4.1.1 TLR-2 Expression in Adrenal Glands

To determine whether TLR-2 exists in WT and TLR-$2^{-/-}$ mice, adrenal glands of both mouse groups were analyzed by Western blotting. Adrenal glands of WT mice expressed TLR-2 as demonstrated, however this protein was absent in TLR-$2^{-/-}$animals (Fig. 10).

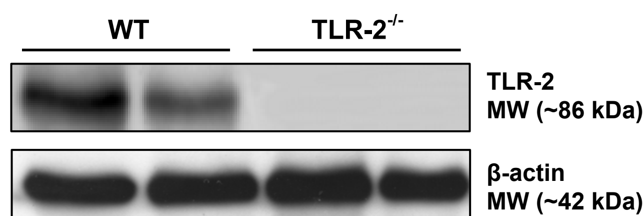


Figure 10: Expression of TLR-2 in adrenal glands by Western blotting analysis. A representative blot showing TLR-2 expression in adrenal glands of WT but not TLR-$2^{-/-}$ mice (n = 3/group). β-actin is used as house keeping protein.

4.1.2 Structure and Function of Adrenal Glands in TLR-$2^{-/-}$ Mice

4.1.2.1 Determination of Adrenal Gland Size

The adrenal size was determined to see if there were any differences between TLR-$2^{-/-}$ mice and their counterpart control animals. Morphometric analysis revealed that the adrenal gland was significant larger in TLR-$2^{-/-}$ mice (21.3 ± 1.9 mm^2) than in WT animals (11.5 ± 3 mm^2; Fig. 11B). As demonstrated in Fig. 11A, this increase was primarily due to the enlargement of the adrenal cortex and not the adrenal medulla.

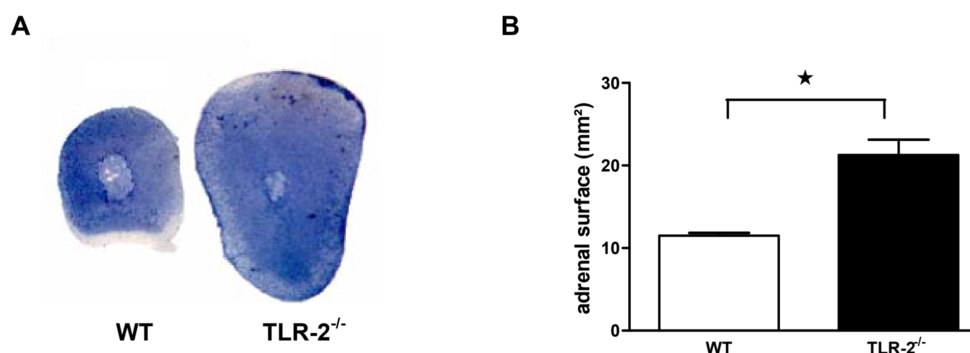


Figure 11: Enlargement of the adrenal gland in TLR-$2^{-/-}$ mice. (A) Measurement of adrenal size in WT (n = 5) and TLR-$2^{-/-}$ mice (n = 3) by morphometric analysis. (B) A graph of the adrenal surface of WT and TLR-$2^{-/-}$ mice. Data are presented as mean ± SEM. Statistical significance was determined by a Student's t-test. ★; $P< 0.05$.

4.1.2.2 Alterations in Adrenal Structure of TLR-2$^{-/-}$ Mice

Due to the facts that the adrenal glands were markedly enlarged in TLR-2$^{-/-}$ mice, it is of interest to investigate potential ultrastructural modifications of the afrenal glands in these animals. Electron micrographs of adrenal gland sections showed at the ultrastructural level that the adrenocortical cells of TLR-2$^{-/-}$ mice exhibited marked morphological alterations (Fig. 12A-D).

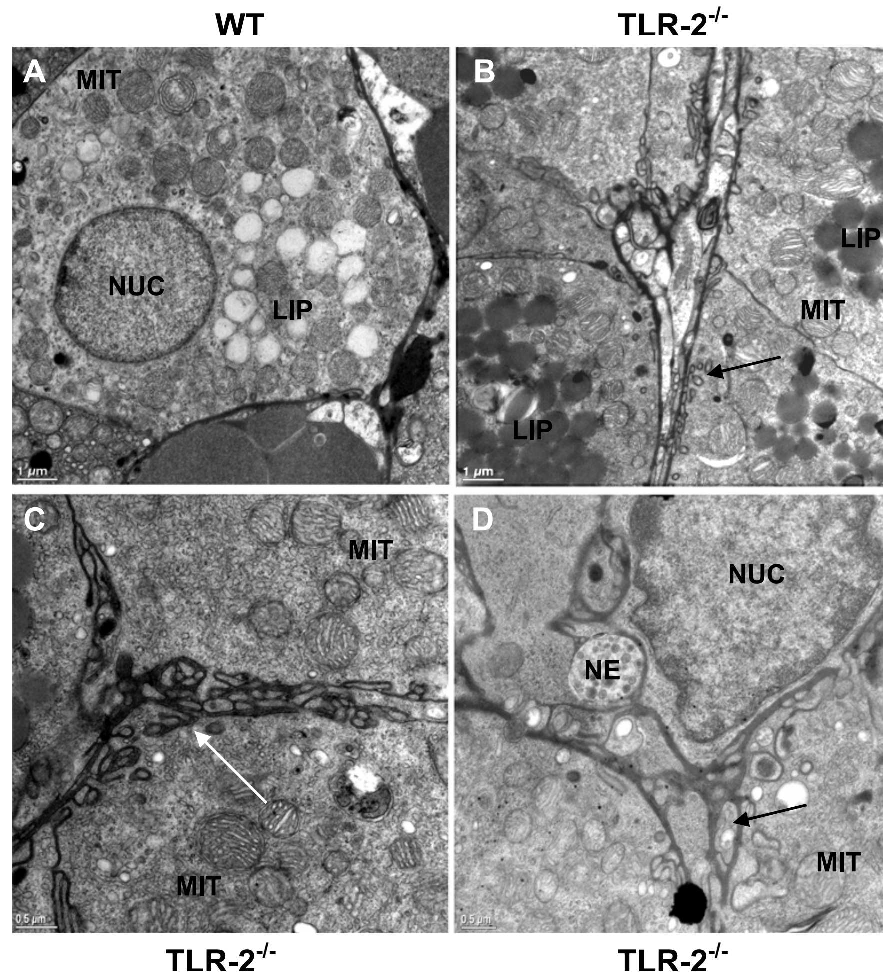


Figure 12: Adrenal structure in WT and TLR-2$^{-/-}$ mice. (A) Electronmicrographs of adrenocortical cells in the zona fasciculata in WT mice. Cytoplasm is filled with characteristic round mitochondria with tubulo-vesicular internal membranes (MIT), ample smooth endoplasmatic reticulums and liposomes (LIP). (B-D) Electronmicrographs of adrenocortical cells in the zona fasciculata in TLR-2$^{-/-}$ mice. Increase in plasma membrane folding with intracytoplasmic invaginations (arrows; bar = 1 µm; g). The electron-dense membranes form stratifications or girlande-like structures (bar = 0.5 µm). (D) Plasma membranes enveloping nerve endings (NE) (A-B). There are ample liposomes (LIP) storing cholesterol in both TLR-2$^{-/-}$ and WT mice (bar = 1 µm). The characteristic tubulovesicular mitochondria (MIT) of the steroid-producing cells in WT animals appear more elongated with tubular internal membranes bridging the mitochondrial matrix (MIT) in TLR-2$^{-/-}$ mice.

In control animals (WT), the most prominent organelles in steroid-producing adrenocortical cells were round mitochondria with characteristic tubulo-vesicular internal membranes and numerous lipid-storing droplets constituting the substrates for steroidogenesis (Fig. 12A). In

TLR-2$^{-/-}$ mice, there was a decrease in the number of mitochondria with a transformation of vesicular internal membranes to more tubular structures bridging the inner matrix (Fig. 12B). The most conspicuous changes, however, occurred at the cell membranes of adrenocortical cells in TLR-2$^{-/-}$ mice. While normal adrenocortical cells have the capacity to form filopodia under stimulation, changes in the plasma membrane of adrenocortical cells of TLR-2$^{-/-}$ mice were distinct and occurred under basal conditions (Fig. 12B-D). There were extensive interdigitations and in-folds engulfing cytoplasm, intracytoplasmic organelles or extracellular structures including nerve endings (Fig. 12D). In part, the abundant folding of the membrane appeared to form stratifications or vesicularization of membranes providing a complex labyrinth formed by interdigitating processes (Fig. 12D).

4.1.2.3 Alterations of Adrenal Function in TLR-2$^{-/-}$ Mice

Alterations of adrenal morphology in TLR-2$^{-/-}$ mice (Fig. 12) indicate that the absence of the TLR-2 may lead to alterations in adrenal function. To test this hypothesis, plasma corticosterone and ACTH levels in WT and TLR-2$^{-/-}$ mice were measured by RIA. Our results showed that the adrenal corticosterone release in TLR-2$^{-/-}$ versus WT mice was significantly decreased (Fig. 13A). In contrast, plasma levels of ACTH were elevated in TLR-2$^{-/-}$ animals (Fig. 13B) suggesting a potential primary impairment of the HPA-axis at the level of the adrenal gland.

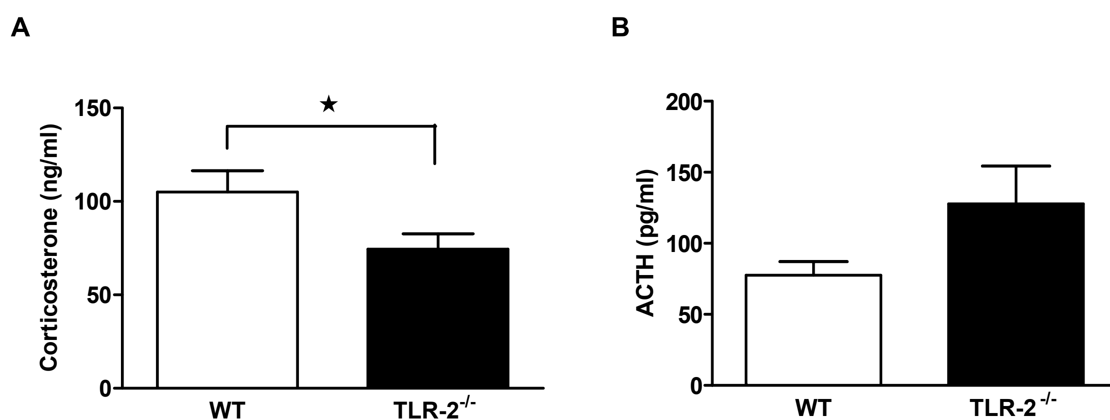


Figure 13: Plasma corticosterone and ACTH in WT and TLR-2$^{-/-}$ mice. Plasma from WT and TLR-2$^{-/-}$ mice (n = 6-8/group) was taken and (A) corticosterone and (B) ACTH concentrations were measured by RIA. Data are presented as mean ± SEM of 6-8 experiments assayed in duplicates. Statistical significance was determined by Student's t-test.★; $P<0.05$.

4.1.3 Effects of LPS or LTA on TLR-2 Expression in the Adrenal Gland

As demonstrated in previous experiments (Fig. 10), there was a basal expression of TLR-2 protein in the adrenal gland. The next step was to investigate the expression pattern of TLR-2 by determining its responsiveness to LPS or LTA at different time points. The time course of TLR-2 expression in adrenal glands of WT mice was analyzed by Western blot analysis. Animals were pretreated i.p. with saline (sal; 1 ml/kg), LPS (1 mg/kg) or LTA (1 mg/kg) for 0.5, 1, 1.5, 2, 6, or 24 h. As shown in Fig. 14, exposure to both LPS and LTA led to a time-dependent increase in TLR-2 protein expression, with maximum induction at 6 h and remaining significantly upregulated at 24 h.

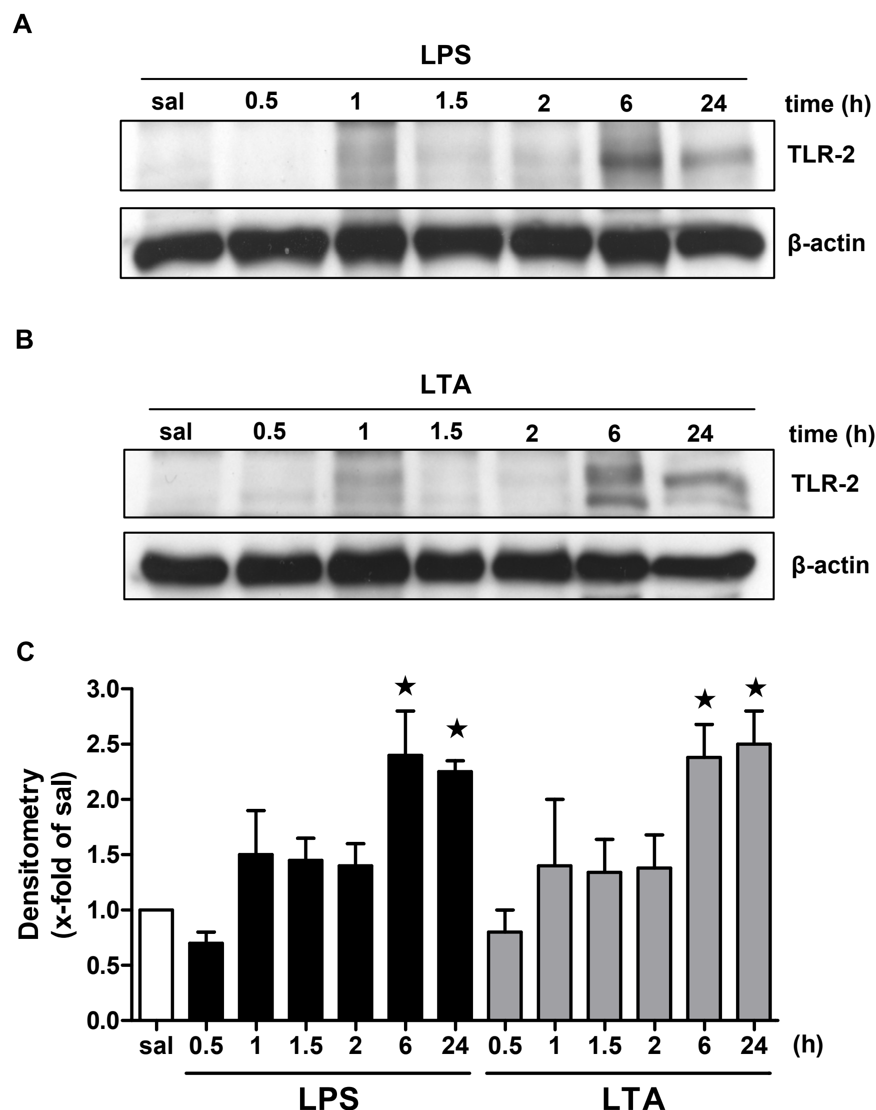


Figure 14: Effects of LPS or LTA treatment on TLR-2 expression in the adrenal gland. WT mice were challenged with (A) LPS (1 mg/kg; i.p.) or (B) LTA (1 mg/kg; i.p.) and saline (sal; 1 ml/kg; i.p.). After 0.5, 1, 1.5, 2, 6 or 24 h, protein extracts (40 µg) were subjected to immunoblotting. β-actin is shown as evidence of equal loading. (C) Densitometric analysis of TLR-2 protein expression in WT mice. Data are presented as mean ± SEM (n = 4/group).Statistical significance was determined by one-way ANOVA and Dunnet's post-test (sal versus each time point).★; $P<0.05$.

4.1.4 Plasma Levels of Corticosterone and ACTH after LPS or LTA Treatment

To investigate the role of TLR-2 in the adrenal stress response to bacterial cell wall fragments such as LPS or LTA, WT and TLR-$2^{-/-}$ mice were pretreated with saline (sal; 1 ml/kg), LPS (1 mg/kg; *Escherichia coli,* serotype 0.111:B4) or LTA (1 mg/kg; *Staphylococcus aureus*) by i.p. injection for 6 or 24 h. Thereafter, plasma concentrations of corticosterone and ACTH were determined by RIA.

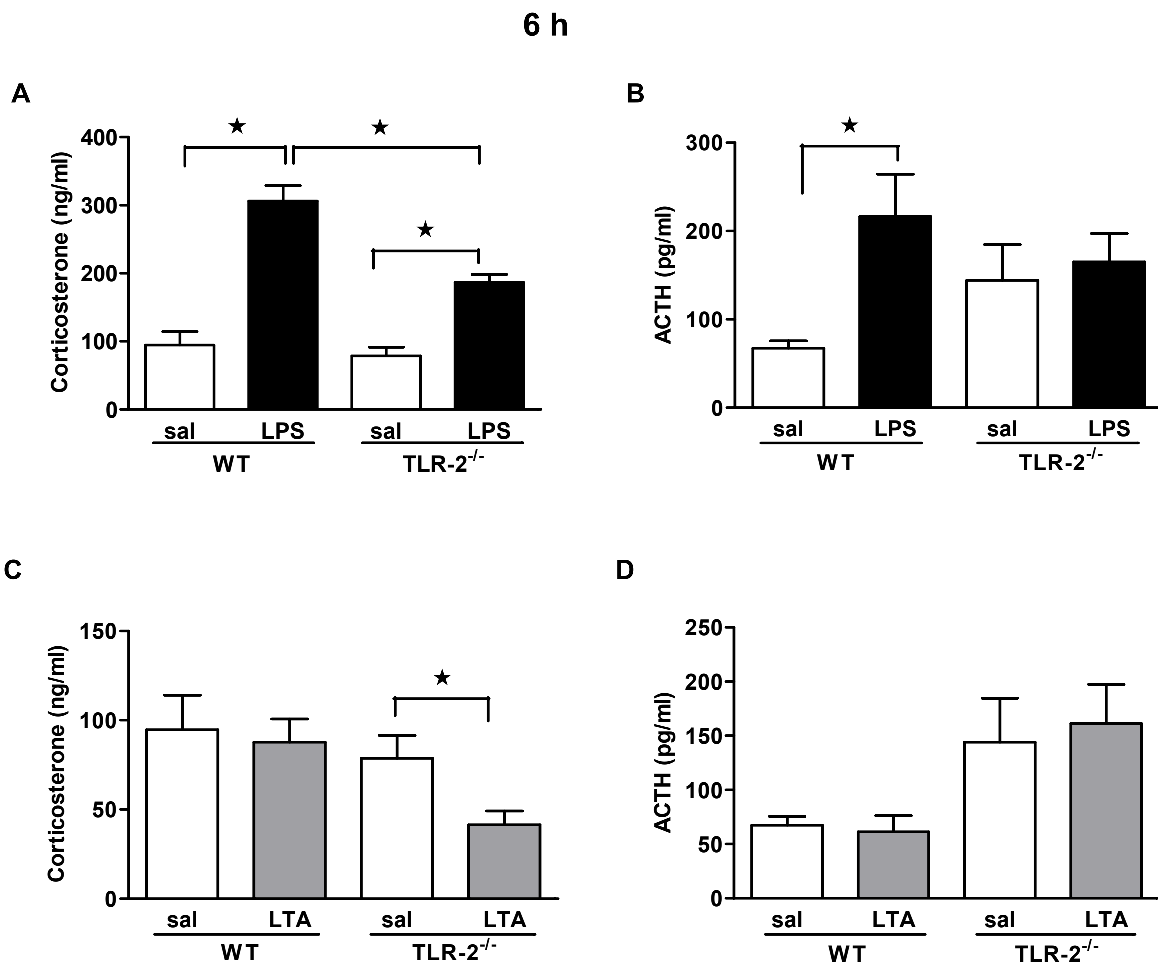


Figure 15: Plasma corticosterone and ACTH response after 6 h LPS or LTA challenges. WT and TLR-$2^{-/-}$ mice were challenged with saline (sal; 1 ml/kg; i.p.) or LPS (1 mg/kg; i.p.) and plasma corticosterone (A) and ACTH (B) levels were measured after 6 h. WT and TLR-$2^{-/-}$ mice were challenged with saline (sal; 1 ml/kg; i.p.) or LTA (1 mg/kg; i.p.) and plasma corticosterone (C) and ACTH (D) levels were measured after 6 h. Data are presented as mean ± SEM of 6-8 experiments assayed in duplicates (n = 6-8/group). Statistical significance was determined by Student's t-test.★; P<0.05.

We demonstrated that in WT mice following 6 h of LPS exposure, adrenal corticosterone release increased by three-fold as compared to saline treatment. This notable increase of plasma corticosterone after LPS was significantly higher in WT animals than in TLR-$2^{-/-}$

mice, where a two-fold increase in corticosterone after LPS was observed as compared to saline treatment only (Fig. 15A). Similarly, a significant increase in plasma levels of ACTH was observed in WT animals following LPS treatment. In contrast, ACTH levels in TLR-2$^{-/-}$ mice did not change after LPS treatment (Fig. 15B). Pretreatment with LTA for 6 h did not affect plasma corticosterone levels in WT mice (Fig. 15C). Conversely, TLR-2$^{-/-}$ animals exhibited a marked reduction in plasma corticosterone levels 6 h after LTA treatment (Fig. 15C). Furthermore, LTA had no significant effect on ACTH levels, neither in WT nor in TLR-2$^{-/-}$ mice (Fig. 15D).

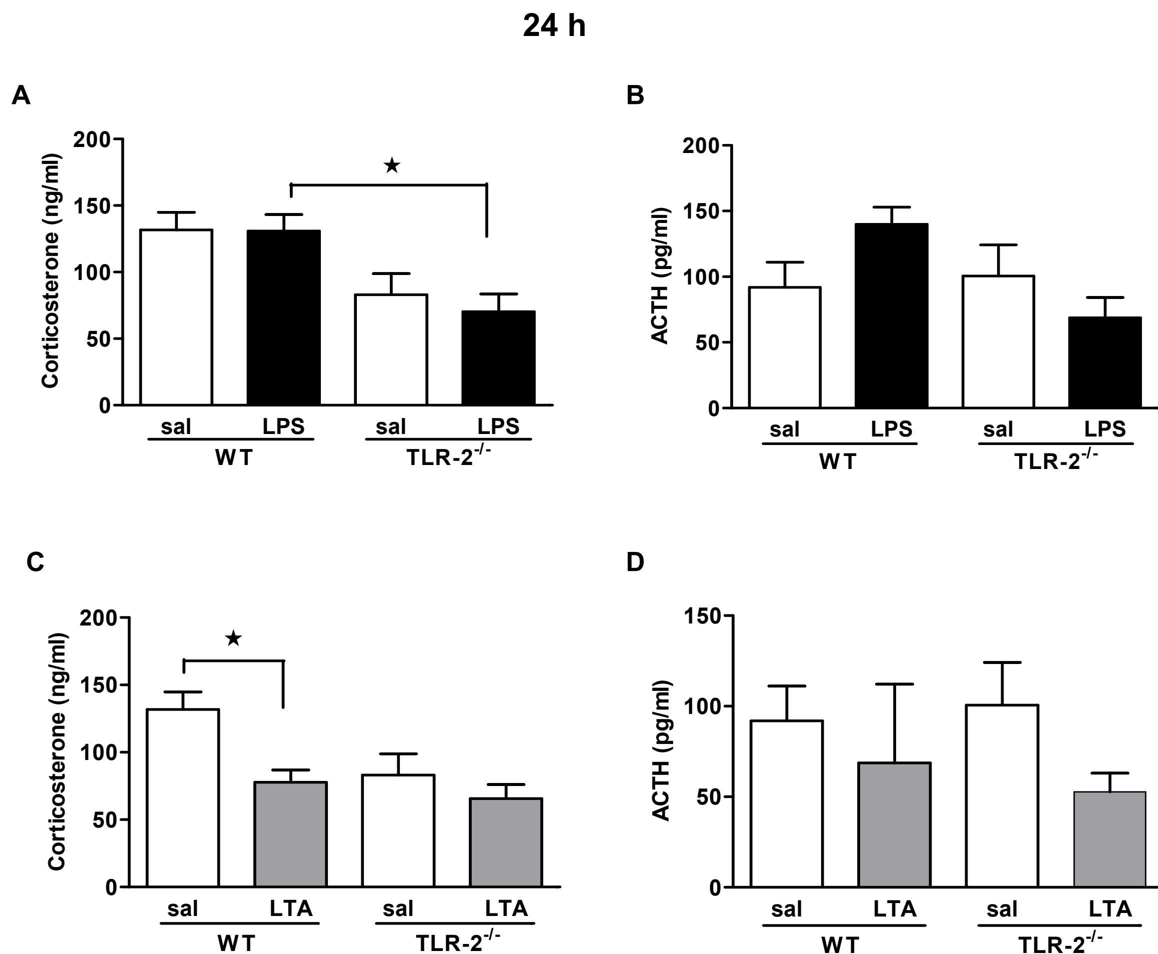


Figure 16: Plasma corticosterone and ACTH response after 24 h LPS or LTA challenges. WT and TLR-2$^{-/-}$ mice were challenged with saline (sal; 1 ml/kg; i.p.) or LPS (1 mg/kg; i.p.) and plasma corticosterone (A) and ACTH (B) levels were measured after 24 h. WT and TLR-2$^{-/-}$ mice were challenged with saline (sal; 1 ml/kg; i.p.) or LTA (1 mg/kg; i.p.) and plasma corticosterone (C) and ACTH (D) levels were measured after 24 h. Data are presented as mean ± SEM of 6-8 experiments assayed in duplicates (n = 6-8/group). Statistical significance was determined by Student's t-test.★; $P<0.05$.

In WT animals, plasma levels of corticosterone after 24 h of LPS treatment were comparable to saline treatment. In TLR-2$^{-/-}$ mice, a similar trend was observed; plasma corticosterone after

LPS was comparable to saline controls. However, it is interesting to note that in TLR-$2^{-/-}$ mice, LPS treatment significant attenuated corticosterone levels when compared to LPS treatment in WT mice (Fig. 16A). Plasma ACTH levels did not differ between groups after 24 h treatment with LPS (Fig. 16B). Surprisingly, LTA pretreatment caused a significant reduction of plasma corticosterone levels in WT mice by ~40%, whereas in TLR-$2^{-/-}$ mice, levels in saline and LTA treated animals were comparable (Fig. 16C). The adrenal ACTH response to LTA was not significantly different in WT or TLR-$2^{-/-}$ following 24 h of LTA treatment (Fig. 16D).

4.1.5 Adrenal Cellular Stress Response to LPS or LTA Challenges

Steroidogenesis takes place in mitochondria and the smooth endoplasmic reticulum in the steroid-producing cells. On the ultrastructural level, there was a marked increase in the number of mitochondria as well as in vesicularization in the adrenocortical cells of WT animals 24 h following LPS challenge (Fig. 17A). This increase in mitochondria, smooth endoplasmic reticulum and vesicularization of mitochondrial membranes is consistent with the increase in steroidogenesis. At the same time, the number of liposomes declined in accordance with the utilization of stored cholesterol pools as a substrate for glucocorticoid synthesis (Fig 17A). This process of increased SER, mitochondrial membranes and reduced liposomes was markedly less pronounced in TLR-$2^{-/-}$ mice, commensurate with their impaired steroid release (Fig 17B).

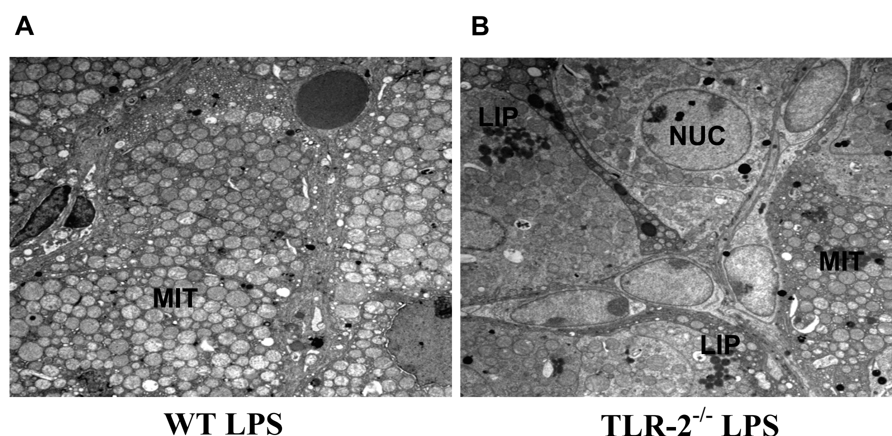


Figure 17: Adrenal structure in WT and TLR-$2^{-/-}$ mice following LPS stimulation. (A) Electronmicrographs of adrenocortical cells in the zona fasciculata in WT mice. Marked increase in mitochondria (MIT) filling the entire cytoplasm 24 h after LPS injection with a decline in liposomes (LIP). There is an increase in intercellular endothelial cells in direct contact with adrenocortical cells and an intracytoplasmic accumulation of lysosomes. (B) Electronmicrographs of adrenocortical cells of TLR-$2^{-/-}$ mice in the zona fasciculata. Increase in mitochondria is less pronounced following LPS treatment. NUC, nucleus

4.1.6 Adrenal NF-κB Activation by LPS or LTA Challenges

Activation of TLRs often results in the induction of the transcription factor NF-κB (Takeda 2005). Once induced, it translocates into the nucleus, where it binds to DNA and activates the induction of different genes. To confirm the important role of TLR-2 in adrenal response to LPS- or LTA-induced stress, NF-κB activation was determined by electrophoretic mobility shift assay (EMSA). This technique was performed with adrenal nuclear extracts from WT and TLR-$2^{-/-}$ mice. Animals were exposed to saline (sal, 1 ml/kg), LPS (1 mg/kg) or LTA (1 mg/kg) for 6 h. DNA-binding activity of NF-κB was analyzed by using a ^{32}P-labelled NF-κB specific oligonucleotide. The intensity of the bands (indicated by an arrow) reflects the amount of DNA-binding activity of NF-κB to its binding site in the nuclear extracts (Fig. 18A).

As shown in Fig. 18A, there was a low-level of background DNA-binding activity of NF-κB in saline-treated animals (WT mice). However, exposure to LPS as well as LTA led to a strong induction of NF-κB-DNA-binding activity in adrenal nuclear extracts, which was significantly higher than in saline controls (Fig. 18B). In contrast, DNA-binding activity of NF-κB was considerably impaired in TLR-$2^{-/-}$ mice. In addition, the response of adrenal cells to a LTA challenge was blunted, while LPS-mediated activation of adrenal NF-κB was preserved in TLR-$2^{-/-}$ mice (Fig. 18A, B).

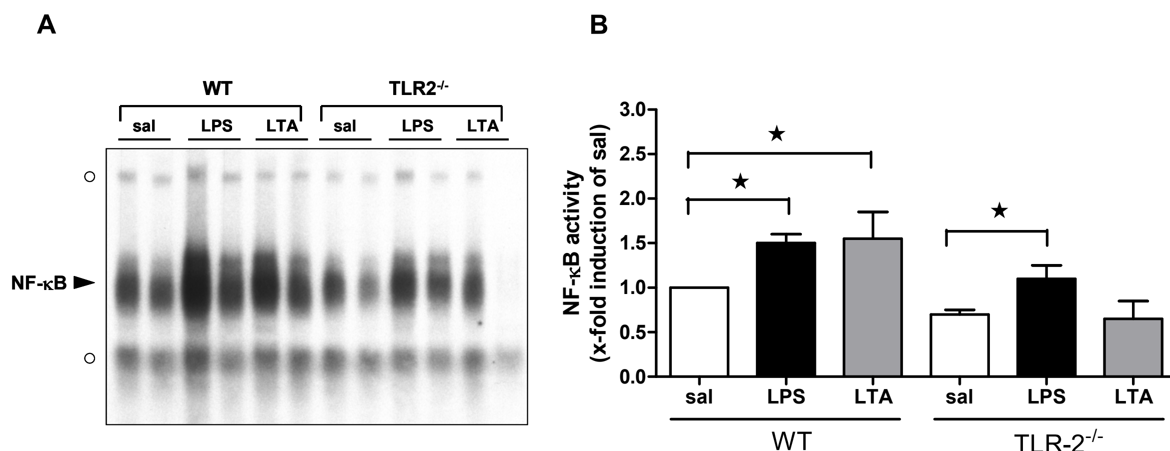


Figure 18: Activation of NF-κB in adrenal glands after LPS or LTA treatment. NF-κB activation was determined by EMSA in adrenal nuclear extracts from WT and TLR-$2^{-/-}$ animals (n = 5-8/group) after 6 h of treatment with saline (sal; 1 ml/kg; i.p.), LPS (1 mg/kg; i.p.) or LTA (1 mg/kg; i.p.). (A) Representative autoradiograph, NF-κB/DNA complex is indicated by the arrow, a non-specific DNA complex is marked by a circle. (B) Densitometric analysis, data were normalized to the intensity of the non-specific DNA complex and expressed as x-fold induction of WT saline-treated animals. Data are presented as mean ± SEM. Statistical significance was determined by Student's t-test. ★; $P<0.05$.

4.1.7 Plasma Levels of Various Cytokines in WT and TLR-2$^{-/-}$

DNA-binding of activated NF-κB regulates gene transcription and expression of several proteins. The target genes include cytokines (e.g. IL-1, 2, 6, 8, 12, 18 and TNF-α), immunoreceptors and acute phase proteins. Therefore, the next step of this study was to analyze the production of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6 in response to TLR-ligands, i.e. LPS and LTA. Mice (WT and TLR-2$^{-/-}$) were pretreated as indicated with saline (sal, 1 ml/kg), LPS (1 mg/kg) or LTA (1 mg/kg) for 6 or 24 h and plasma cytokine levels were determined by ELISA.

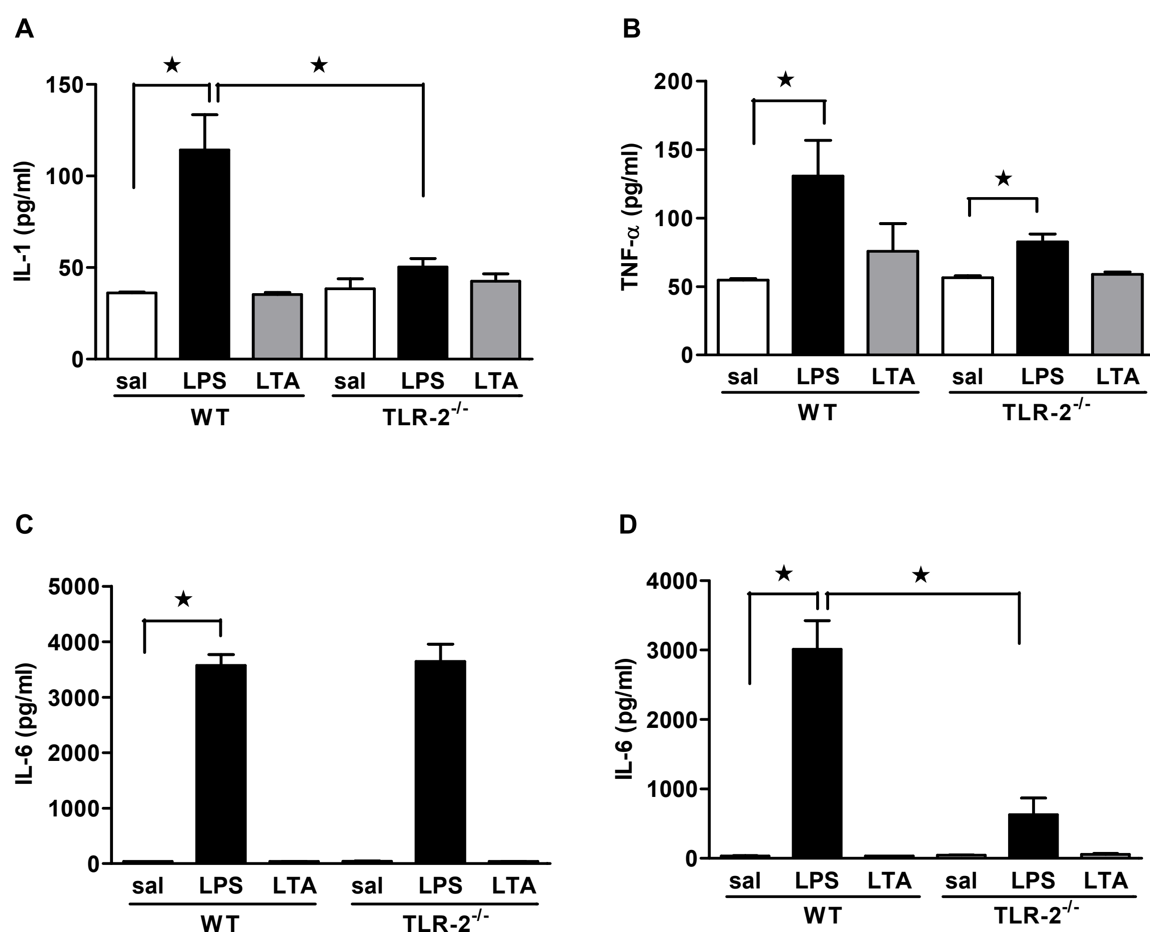


Figure 19: Plasma levels of cytokines in response to LPS or LTA treatment. Plasma levels of (A) IL-1, (B) TNF-α and (C) IL-6 after 6 h or (D) IL-6 after 24 h treatment with saline (sal; 1 ml/kg; i.p.), LPS (1 mg/kg; i.p.) or LTA (1 mg/kg; i.p.) in WT and TLR-2$^{-/-}$ mice (n = 5-8/group). Data are presented as mean ± SEM of 5-8 experiments assayed in duplicates. Statistical significance was determined by Student's t-test. ★; $P<0.05$.

WT mice showed a significant increase in the plasma levels of IL-1 (3-fold), TNF-α (2-fold) and IL-6 (1,000-fold) after 6 h exposure to LPS (Fig. 19A-C). Plasma levels of IL-1 and TNF-α returned to baseline concentrations after 24 h LPS treatment (data not shown), whereas plasma levels of IL-6 remained elevated in WT mice (Fig. 19D). The cytokine response (IL-1 and TNF-α) to LPS treatment (6 h) was significantly impaired in TLR-$2^{-/-}$ mice (Fig. 19A-C) However, LPS challenge caused an increase in plasma levels of IL-6 in TLR-$2^{-/-}$ mice, which was similar to the increase observed in WT animals (Fig. 19C). However, at 24 h after LPS administration, IL-6 levels were significantly reduced in TLR-$2^{-/-}$ mice when compared to WT mice (Fig. 19D). LTA challenge for 6 h or 24 h did not affect the plasma concentrations of IL-1, IL-6 or TNF-α neither in WT nor in TLR-$2^{-/-}$ mice (Fig. 19).

To support the association between TLR-2-deficiency and the impaired response of the inflammatory cytokines, we determined the local expression of the cytokines TNF-α, IL-1 and IL-6 on the adrenal gland. After saline or LPS treatment (24 h), immunohistochemical staining of adrenal cortices of WT and TLR-$2^{-/-}$ mice was performed (Fig. 20).

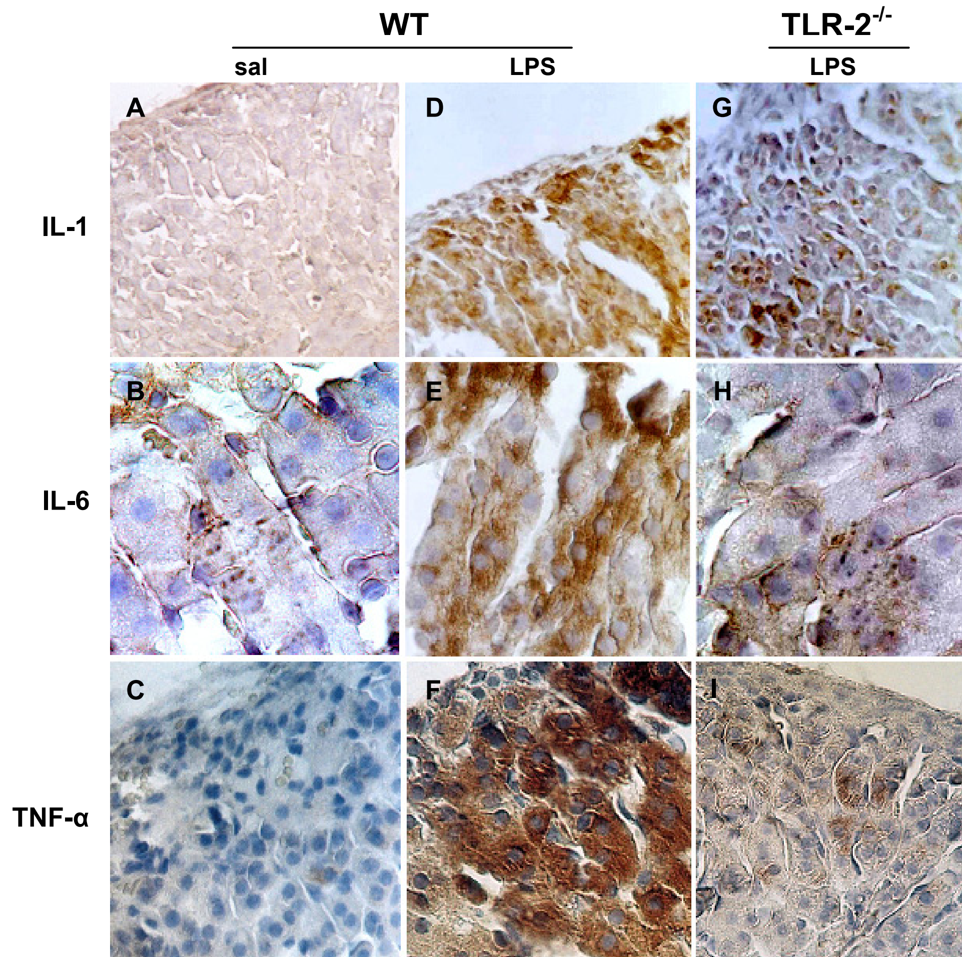


Figure 20: Detection of IL-1, IL-6 and TNF-α expression in adrenal cortices by immuno-histochemical staining. WT or TLR-$2^{-/-}$ were treated with saline (sal, 1 ml/kg; i.p.) or LPS (1 mg/kg; i.p.) for 24 h. Adrenal cortices were analyzed for immunoreactivity of IL-1 protein (A, D, G; 40x magnification), IL-6 protein (B, E, H; 100x magnification) and TNF-α protein (C, F, H; 100x magnification).

As shown in Fig. 20, there was only weak staining for IL-1, IL-6 and TNF-α on zona fasciculata cells of the adrenal cortex of WT mice following saline treatment (Fig. 20A-C). After LPS treatment (24 h), there was a marked increase in the expression of all three cytokine proteins, the most prominent staining was for TNF-α (Fig. 20D-F). In contrast, the expression of IL-1, IL-6 and TNF-α protein in TLR-$2^{-/-}$ animals following LPS-treatment was observably reduced (Fig. 20G-I).

4.2 TLR-4 Study

4.2.1 TLR-4 Expression in Adrenal Glands

We investigated whether TLR-4 is expressed in the adrenal glands of WT mice. Western blot analysis revealed that TLR-4 is expressed in these glands as demonstrated in Fig. 21.

Figure 21: Expression of TLR-4 in adrenal glands by Western blotting analysis. A representative blot showing TLR-4 expression in adrenal glands of WT mice (n = 3). β-actin is used as house keeping protein.

4.2.2 Structure and Function of Adrenal Glands in TLR-4$^{-/-}$ Mice

4.2.2.1 Determination of Adrenal Gland Size

Like in TLR-2 study, the adrenal size was determined by morphometric analysis to see if there were any differences between TLR-4$^{-/-}$ mice and their counterpart control animals. As demonstrated in Fig. 22A, the adrenal gland was significant larger in TLR-4$^{-/-}$ mice (272,000 ± 14,000 pixels^2) than in WT animals (221,000 ± 17,000 pixels^2).

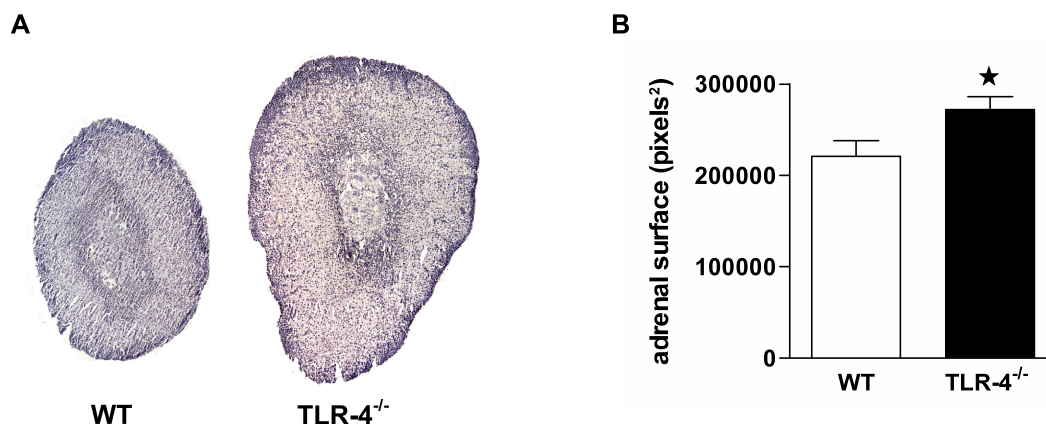


Figure 22: Enlargement of the adrenal gland in TLR-4$^{-/-}$ mice. (A) Measurement of adrenal size in WT and TLR-4$^{-/-}$ mice (n = 8/group) by morphometric analysis. (B) A graph of the adrenal surface of WT and TLR-4$^{-/-}$ mice. Data are presented as mean ± SEM. Statistical significance was determined by a Student's t-test. ★; $P< 0.05$.

4.2.2.2 Alterations in Adrenal Structure of TLR-4$^{-/-}$Mice

Seeing that the adrenal glands were markedly enlarged in TLR-4$^{-/-}$ mice, it is of interest to investigate potential ultrastructural modifications of the afrenal glands in these animals. At the ultrastructural level, the most pronounced differences of WT and TLR-4$^{-/-}$ animals were seen in the mitochondrial structure (Fig. 23A-B). The steroid-producing adrenocortical cells of WT mice revealed round mitochondria with characteristic tubovesicular cristae and electron-opaque granules (Fig. 23A). In contrast, mitochondria of TLR-4$^{-/-}$ adrenocortical cells showed a reorganization of the cristae to lamellar or even circular structures, bridging the inner matrix of the mitochondria (Fig. 23B). In addition, lipid-storing droplets constituting the substrates for steroidogenesis were abundant in adrenocortical cells of WT mice, but they were conspicuously reduced in TLR-4$^{-/-}$ mice.

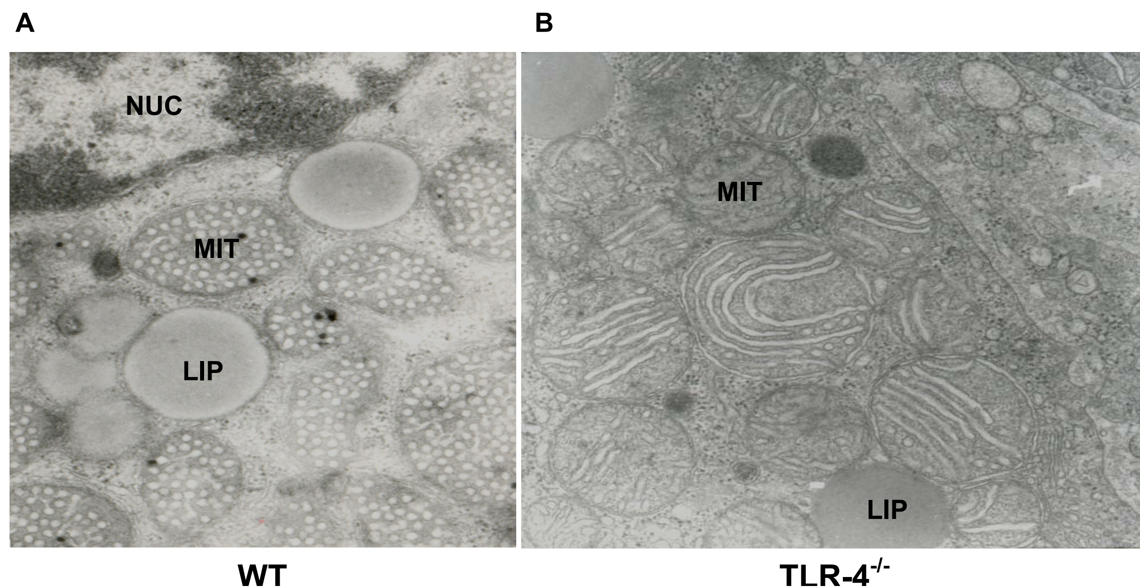


Figure 23: Adrenal structure in WT and TLR-4$^{-/-}$ mice. (A) Electronmicrographs of adrenocortical cells in the inner zona fasciculata in WT mice. The cytoplasm is filled with characteristic round mitochondria (MIT) with tubulovesicular cristae, ample smooth endoplasmic reticulum, and liposomes (LIP). (B) Electron micrographs of adrenocortical cells of TLR-4$^{-/-}$ mice in the zona fasciculata. Mitochondria of the steroid-producing cells appear increased and more elongated with lamellar and even circular internal membranes bridging the mitochondrial matrix. NUC, nucleus.

4.2.2.3 Alterations of Adrenal Function in TLR-4$^{-/-}$Mice

To examine if morphological modifications of adrenal gland in TLR-4$^{-/-}$ mice could lead to functional modifications, plasma levels of corticosterone and ACTH were determined by RIA in WT and TLR-4$^{-/-}$ mice. The results showed that adrenal corticosterone release in TLR-4$^{-/-}$

mice was significantly higher than in WT mice (Fig. 24A). In contrast, plasma levels of ACTH were similar in both groups (Fig. 24B).

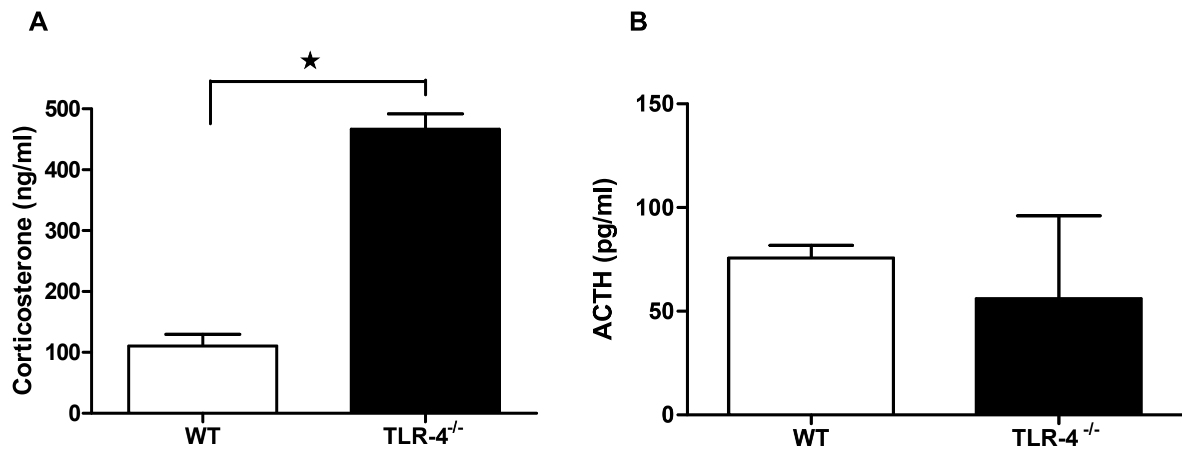
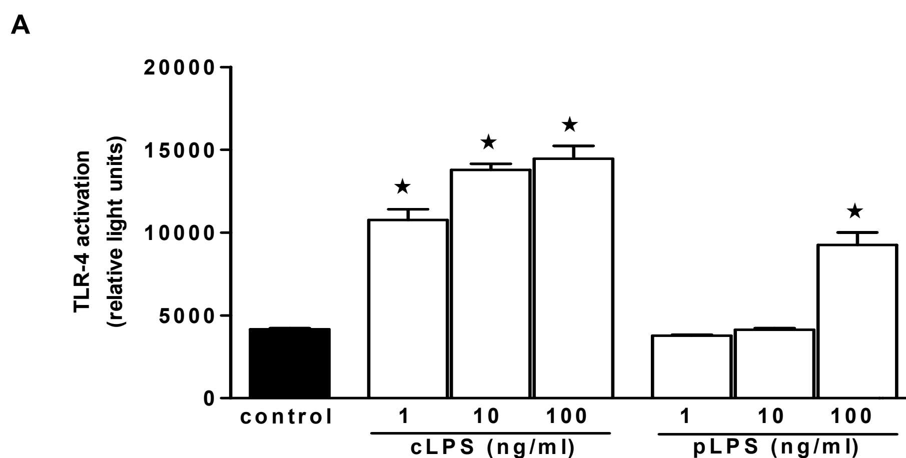


Figure 24: Plasma corticosterone and ACTH in WT and TLR-4$^{-/-}$ mice. Plasma from WT and TLR-4$^{-/-}$ mice (n = 8/group) was taken and (A) corticosterone and (B) ACTH concentrations were measured by RIA. Data are presented as mean ± SEM of 8 experiments assayed in duplicates. Statistical significance was determined by Student's t-test. ★; $P<0.05$.

4.2.3 Purity of LPS Preparations

To investigate whether TLR-4 plays a role in the adrenal stress response during inflammation, we used a low-dose of LPS that reflects the clinical condition of SIRS. Sometimes, commercial preparations of LPS contain TLR-2 agonists and effects could have been due to the activation of TLR-2 as well as TLR-4. To exclude this possibility, we first compared the agonist activity of TLR-2 and TLR-4 in the crude and pure LPS preparations (cLPS and pLPS) we obtained for this study.



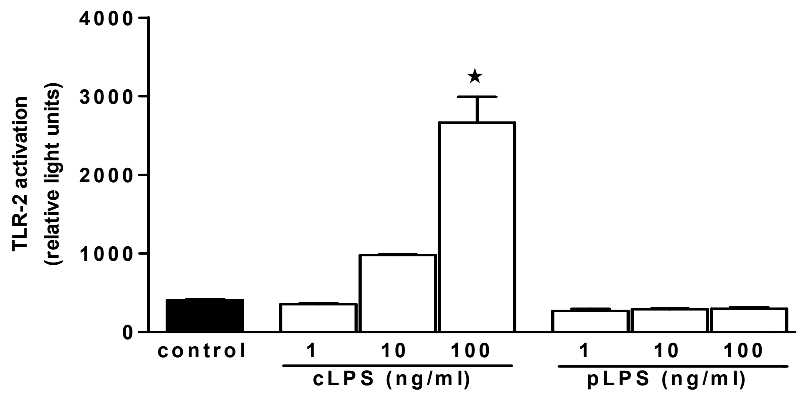


Figure 25: Purity of LPS preparations by luciferase assay. Human embryonic kidney 293 cells were transfected with either TLR-2 or TLR-4. (A) Effects of crude or pure LPS (cLPS or pLPS; 1-100 ng/ml) on NF-κB-driven luciferase activity in comparison to untreated control in TLR-4 transfected cells (n = 3). (B) Effects of cLPS and pLPS (1-100 ng/ml) on luciferase activity in comparison to untreated control in TLR-2 transfected cells (n = 3). Luciferase activity was normalized to the β-galactosidase control and is presented as relative light units. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni`s post-test (versus control). ★; P<0.05.

In TLR-4 transfected HEK293 cells, both cLPS (at all three concentrations) and pLPS (only at the highest concentration) increase significantly TLR-4 activation (NF-κB driven luciferase activity) (Fig. 25A), whereas only cLPS but not pLPS increased NF-κB activity in TLR-2 transfected cells. pLPS had no effect on luciferase activity even at the highest concentration (100 ng/ml) in TLR-2 transfected cells (Fig. 25B). These results demonstrate that the pLPS preparation was free of TLR-2 ligands. In contrast, the cLPS preparation activated both TLR-2 and TLR-4, demonstrating that this preparation was contaminated with TLR-2 agonist.

4.2.4 Effects of cLPS and pLPS on Plasma Corticosterone and ACTH

To determine the effects of LPS on the adrenal stress response, plasma concentrations of corticosterone and ACTH were measured by RIA, taken from WT and TLR-4$^{-/-}$ mice pre-treated with saline (1 ml/kg), cLPS (1 mg/kg) or pLPS (1 mg/kg) for 6 or 24 h.

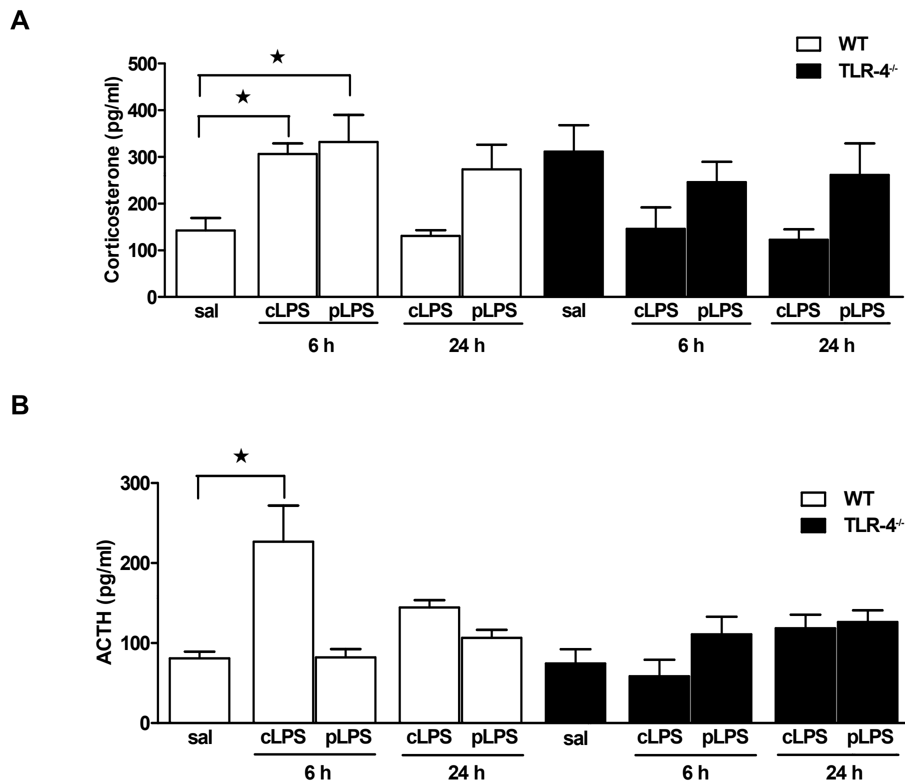


Figure 26: Plasma corticosterone and ACTH response to cLPS and pLPS challenges. Plasma levels of (A) corticosterone and (B) ACTH were determined after 6 or 24 h of treatment with saline (sal; 1ml/kg; i.p.), cLPS (1 mg/kg; i.p.) or pLPS (1 mg/kg; i.p.) in WT or TLR-4$^{-/-}$ mice (n = 7-8/group). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni`s post-test (versus saline). ★; $P<0.05$.

As shown in Fig. 26A, in WT mice corticosterone release increased significantly after both 6 h cLPS and pLPS treatment. After 24 h, plasma levels were similar to saline-treated controls in cLPS treated animals. In mice treated with pLPS, corticosterone plasma levels appeared to be raised, but were not more significant. In TLR-4$^{-/-}$ mice, cLPS and pLPS had no effect on corticosterone release at any time point. This could have been due to the fact that plasma levels of corticosterone were already elevated in saline-treated TLR-4$^{-/-}$ mice (3-fold higher versus WT animals). As demonstrated in Fig. 26B for plasma ACTH, at 6 h there was an increase with cLPS, but not pLPS in WT mice ($P<0.05$). However, at 24 h, plasma levels of ACTH were similar to saline-treated animals. In TLR-4$^{-/-}$ animals, both cLPS and pLPS had no effect.

4.2.5 Adrenal NF-κB Activation by cLPS or pLPS Challenges

To confirm NF-κB is involved in TLR-4 signalling during the adrenal stress response to LPS, we determined its activation by EMSA in adrenals. The intensity of the bands reflects the amount of DNA-binding activity of NF-κB to its binding site in the nuclear extracts (Fig. 27A). In the adrenal glands of WT mice, cLPS and pLPS treatment led to a strong induction of NF-κB-DNA binding protein complex after 1 h, but this effect was markedly impaired in TLR-4$^{-/-}$ animals (Fig. 27B).

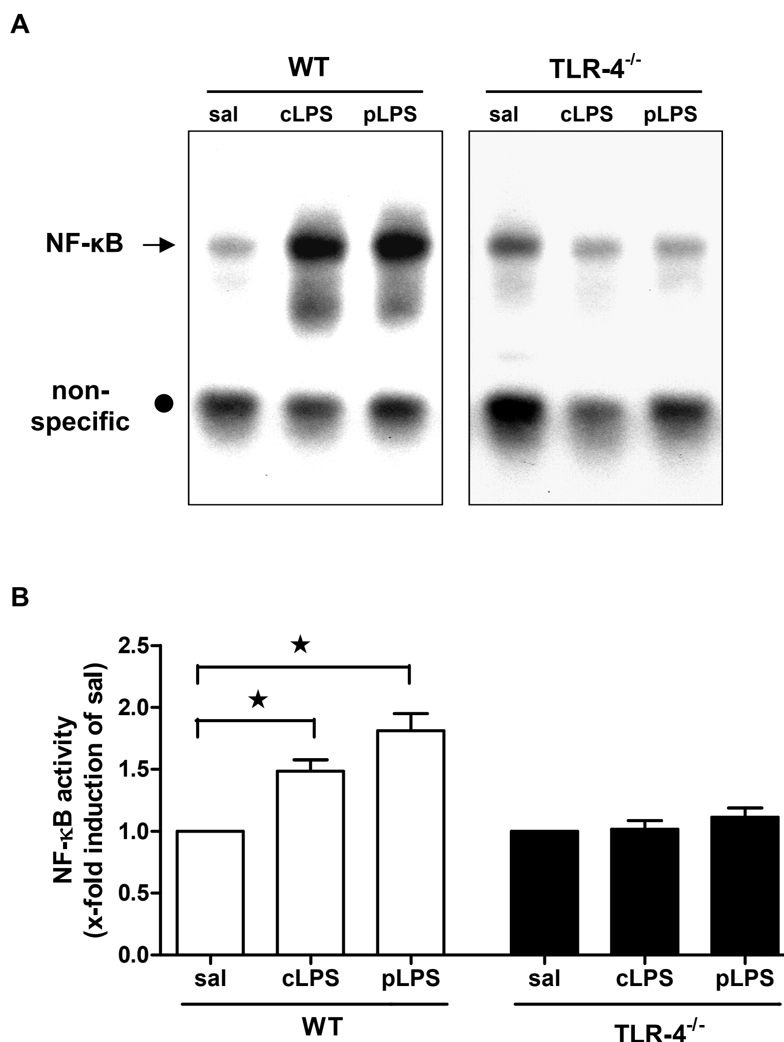
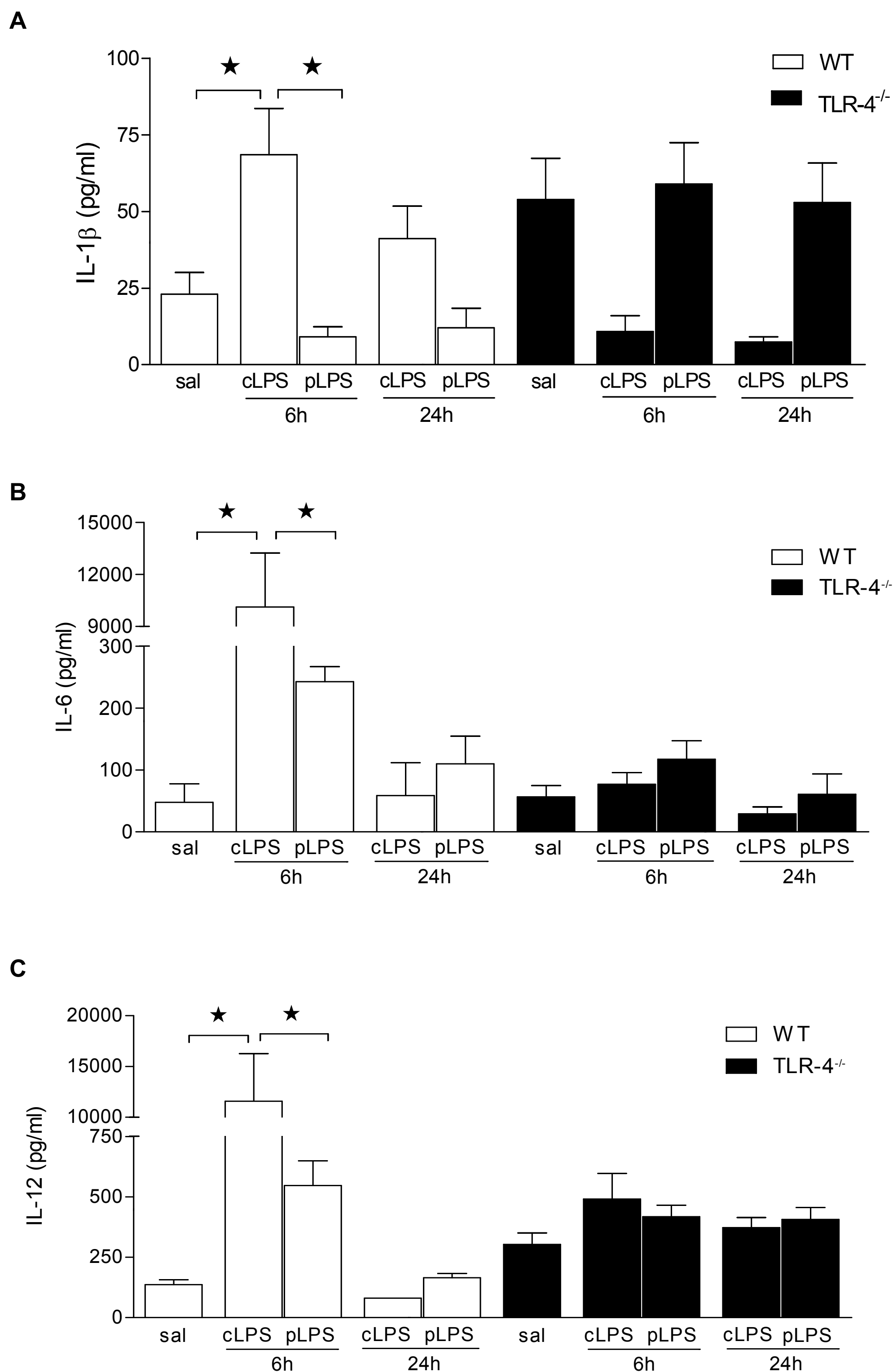


Figure 27: Activation of NF-κB in adrenal glands after cLPS or pLPS treatment. NF-κB activation was determined by EMSA in adrenal nuclear extracts from WT and TLR-4$^{-/-}$ animals (n = 5-8/group) after treatment with saline (sal; 1 ml/kg; i.p.), cLPS (1 mg/kg; i.p.) or pLPS (1mg/kg; i.p.). (A) Representative autoradiograph, NF-κB/DNA complex is indicated by the arrow, a non-specific DNA complex is marked by a circle. (B) Densitometric analysis of EMSA. Data were normalized to the intensity of the non-specific DNA complex and expressed as x-fold induction of WT saline-treated animals. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni's post-test for each animal group (versus saline). ★; $P<0.05$.

4.2.6 Plasma Levels of Various Cytokines in WT and TLR-4$^{-/-}$ Mice

Cytokines regulate the hormonal release and glucocorticoid output of the HPA-axis. Thus, the next step of this study was to analyze the systemic response of cytokines IL-1β, 6, 10, 12 and TNF-α after cLPS and pLPS treatment in WT and TLR-4$^{-/-}$ mice.



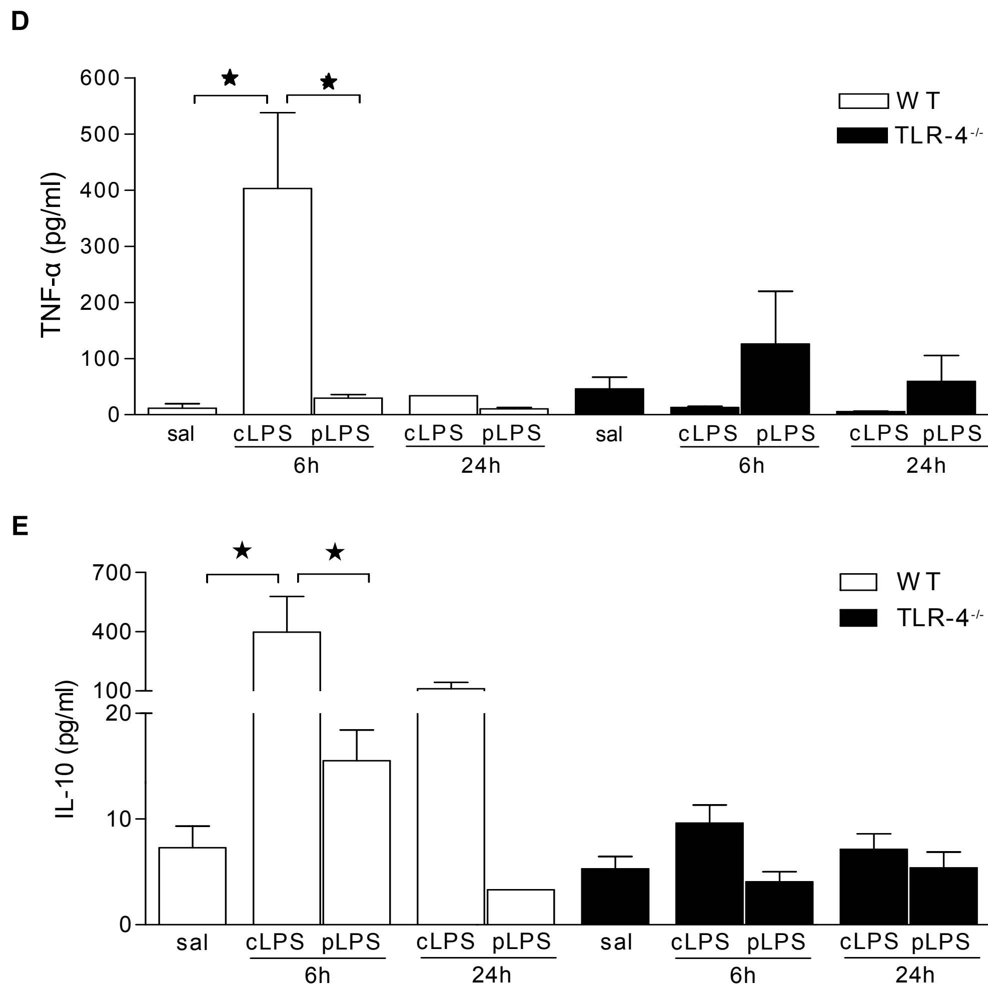


Figure 28: Plasma levels of cytokines in response to cLPS or pLPS treatment. Determination of plasma cytokines (A) IL-1β, (B) IL-6, (C) IL-12, (D) TNF-α, and (E) IL-10 in WT and TLR-4$^{-/-}$ mice (n = 5-8/group). Animals were treated with saline (sal; 1ml/kg; i.p.), cLPS (1 mg/kg; i.p.) or pLPS (1 mg/kg;i.p.) for 6 or 24 h. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni's post-test for each animal group. ★; P<0.05.

All plasma cytokines were increased in WT mice after a 6 h cLPS challenge. These results indicate an inflammatory response with cLPS, but the same could not be seen in pLPS-treated animals. After 6 h of cLPS treatment, IL-1β was increased approximately by 3-fold, IL-6 by 200-fold, IL-12 by 80-fold and TNF-α by 10-fold in comparison with saline- treated animals (Fig. 28A-D). The same result was shown with IL-10 (an anti-inflammatory cytokine): cLPS increased the plasma levels of this cytokine significantly (50-fold) after 6 h, in comparison to WT controls (Fig. 28E). After 24 h of pLPS treatment, plasma levels of all cytokines returned to the baseline and after cLPS treatment, plasma levels of all cytokine except IL-10 returned to the baseline after 24h. Plasma IL-10 was already high, but was not significant. In TLR-4$^{-/-}$ mice, neither cLPS nor pLPS had any effects on the plasma cytokines studied. Plasma levels of IL-1β were increased after pLPS treatment, but values were not of statistical significance.

4.3 TLR-9 Study

4.3.1 TLR-9 Expression in Adrenal Glands

In this study, we showed for the first time that murine adrenal glands expressed TLR-9 both at mRNA and protein level, as demonstrated by RT-PCR (Fig. 29A) and Western blot analysis (Fig. 29B). However, TLR-9 protein was absent in TLR-9$^{-/-}$ mice. Furthermore, both immunohistochemical and immunofluorescence staining for TLR-9 in the adrenal gland of untreated WT mice revealed that TLR-9 was expressed in the adrenal cortex, but not in the adrenal medulla. No staining was observed in negative controls where no specific TLR-9 antibody was used (Fig. 29C). As illustrated in Fig. 29D-E, the cortical zona fasciculata exhibited prominent staining for TLR-9.

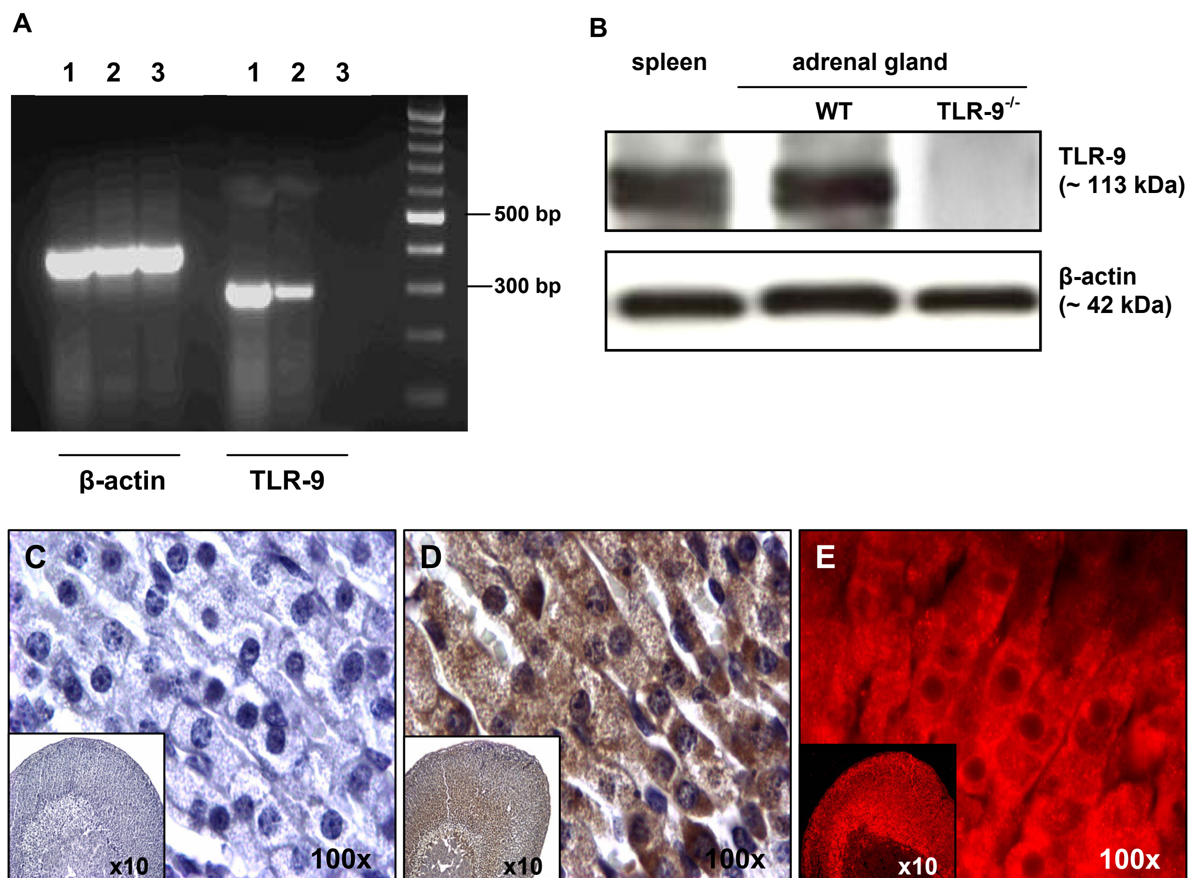


Figure 29: Expression of TLR-9 in the adrenal glands by (A) RT-PCR, (B) Western blotting analysis, (C-D) immunohistochemical and (E) immunofluorescence staining. Representative PCR gel (A) and western blot (B) respectively show TLR-9 expression in adrenal glands of WT but not TLR-9$^{-/-}$ mice. β-actin is used as house keeping protein. Depicted are the following groups: (1) spleen and (2) adrenal glands of WT mice and (3) adrenal glands of TLR-9$^{-/-}$ mice (n = 4/group). Spleen of WT mice served as positive control for the expression of TLR-9. The TLR-9 expression in the adrenal gland was visualized by (C-D) immuno-histochemical and (E) immuno-fluorescence staining. (C) Adrenal gland sections without the specific TLR-9 staining as negative control. (D-E) Sections stained by anti-mouse TLR-9 antibody revealed the expression of TLR-9 in adrenal cortex, particularly in zona fasciculata, but not in adrenal medulla. Overview, 10x magnification; sectionings of zona fasciculata, 100x magnification. Reproductive results were obtained in 3 independent experiments.

4.3.2 Structure and Function of Adrenal Glands in TLR-9$^{-/-}$ Mice

4.3.2.1 Determination of Adrenal Gland Size

Like in TLR-2 and TLR-4 studies, the adrenal size was determined by morphometric analysis to see if there were any differences between TLR-9$^{-/-}$ mice and their counterpart control animals. Morphometric analysis of the adrenal gland displayed no difference in adrenal size between WT and TLR-9$^{-/-}$ mice as shown in Fig. 30A-B.

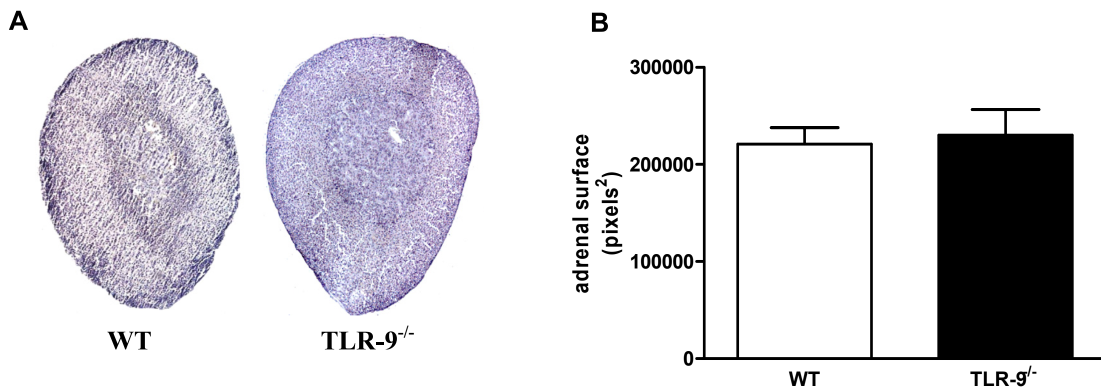


Figure 30: Size of the adrenal gland in WT and TLR-9$^{-/-}$ mice. (A) Measurement of adrenal size in WT and TLR-9$^{-/-}$ mice (n = 4-8/group) by morphometric analysis. (B) A graph of the adrenal surface of WT and TLR-9$^{-/-}$ mice. Data are presented as mean ± SEM.

4.3.2.2 Alterations of Adrenal Function in TLR-9$^{-/-}$Mice

Plasma corticosterone and ACTH levels in untreated WT and TLR-9$^{-/-}$ mice were measured by RIA. The results indicate comparably corticosterone and ACTH plasma levels in both animal groups (Fig. 31A-B).

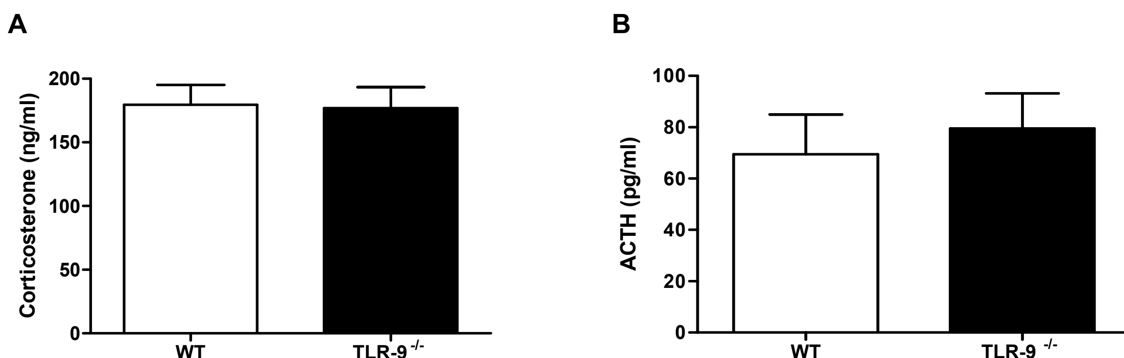
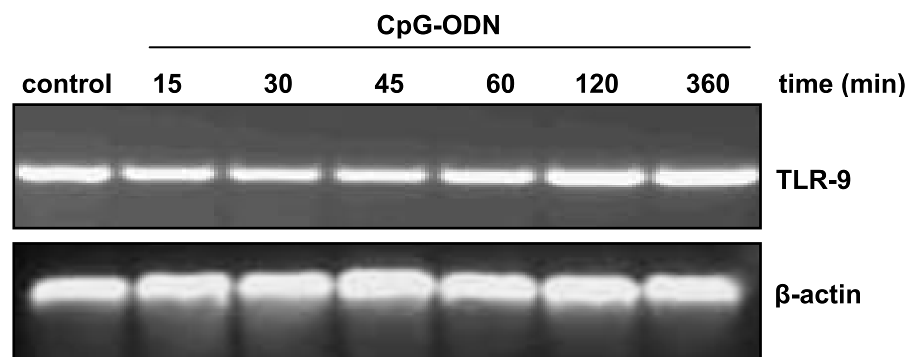


Figure 31: Plasma corticosterone and ACTH in WT and TLR-9$^{-/-}$ mice. Plasma from WT and TLR-9$^{-/-}$ mice (n = 4-8/group) was taken and (A) corticosterone and (B) ACTH concentrations were measured by RIA. Data are presented as mean ± SEM of 4-8 experiments assayed in duplicates.

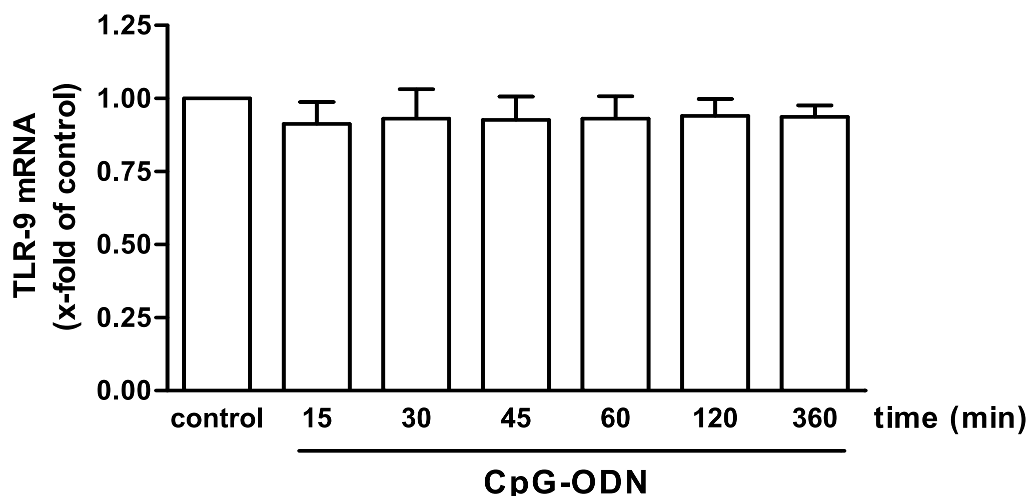
4.3.3 Effects of CpG-ODN on TLR-9 Expression in the Adrenal Gland

As showed in previous experiments (Fig. 29), there was a basal expression of TLR-9 in the adrenal gland. The next step was to investigate the expression pattern of this protein by determining their responsiveness to its appropriate ligand CpG-ODN at different time points. WT mice were challenged with CpG-ODN (30 min after sensitization with D-GalN) and sacrificed at several time points (15-360 min). In control experiments, D-GalN alone did not induce an inflammatory response (data not shown). Using RT-PCR and Western blot analysis, TLR-9 expression was studied. Fig. 32A depicts a typical autoradiograph where adrenal glands were analyzed using RT-PCR. TLR-9 band intensities were normalized to β-actin levels in the same sample. When compared to control, CpG-ODN challenge did not influence levels of TLR-9 mRNA over time (Fig. 32B). Similar results were obtained in Western blot analysis (Fig. 32C-D), demonstrating no significant change in TLR-9 protein expression pattern following CpG-ODN challenge.

A



B



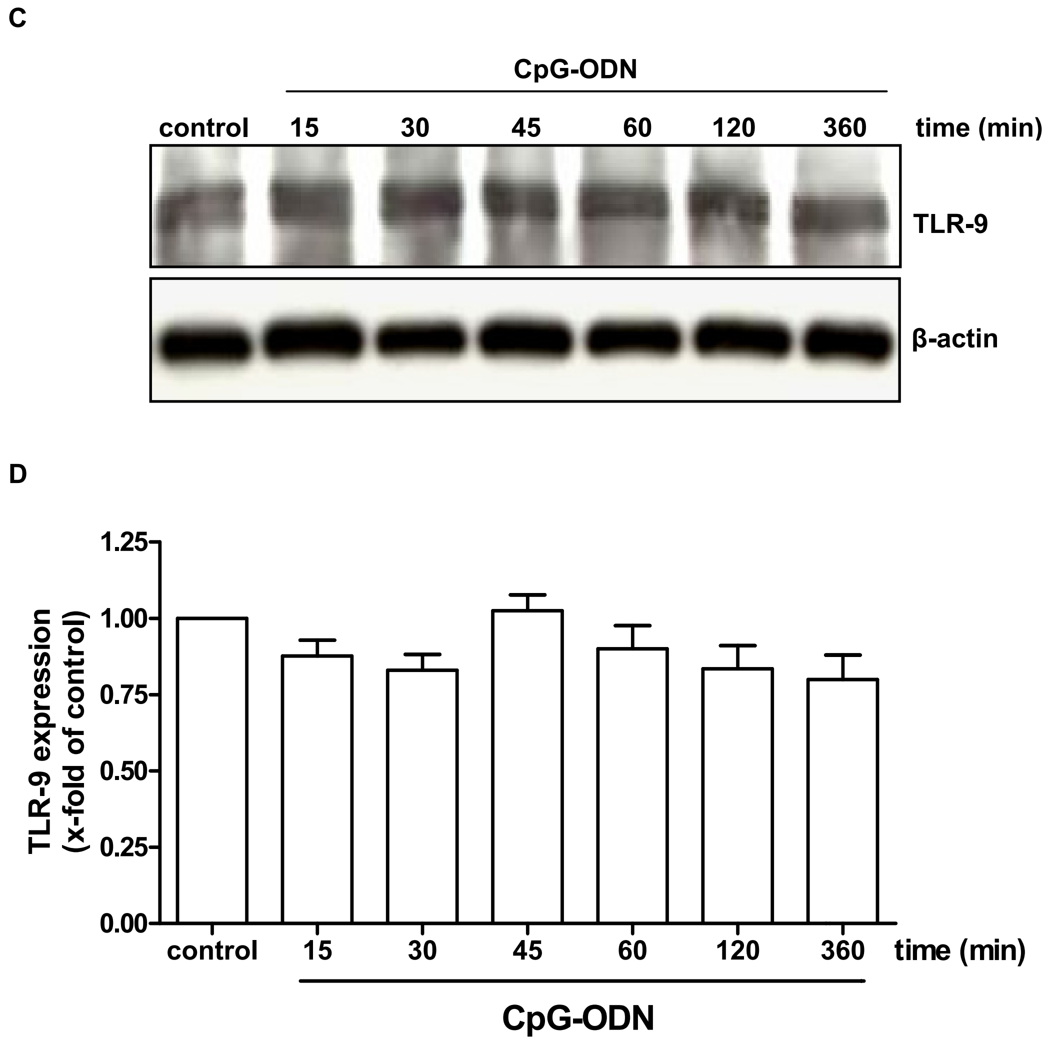


Figure 32: Effects of CpG-ODN on TLR-9 expression in the adrenal gland. D-GalN-sensitized (1 mg/kg; i.p.) WT mice were challenged with CpG-ODN (1 nmol/g; i.p.) for various time points as indicated (n = 8-10/group). (A) Using RT-PCR, adrenal glands were analyzed for TLR-9 expression. β-actin was used as a reference gene. (B) Quantification of autoradiographs. Band intensities were normalized to β-actin in the same samples. (C) Typical example of a Western blot showing TLR-9 protein expression. β-actin was used as a loading control. (D) Band intensities were normalized to β-actin expression in the same samples. Data are presented as mean ± SEM.

4.3.4 Plasma Levels of Corticosterone and ACTH after CpG-ODN Treatment

To investigate further the role of TLR-9 in the adrenal stress response to CpG-ODN, plasma levels of corticosterone and ACTH were determined by RIA. Under physiological conditions (control), the plasma levels of corticosterone (Fig. 33A) and ACTH (Fig. 33B) were similar in WT and TLR-9$^{-/-}$ mice. Following a CpG-ODN challenge, WT mice exhibited a three-fold increase in plasma corticosterone after 2 h and returned back to baseline values after 6 h. In

contrast, CpG-ODN-mediated effects were abolished in TLR-$9^{-/-}$ mice (Fig. 33A). CpG-ODN had little or no effect on ACTH plasma levels in WT animals. Similarly, no changes in plasma ACTH were observed after CpG-ODN treatment in TLR-$9^{-/-}$ mice (Fig. 33B).

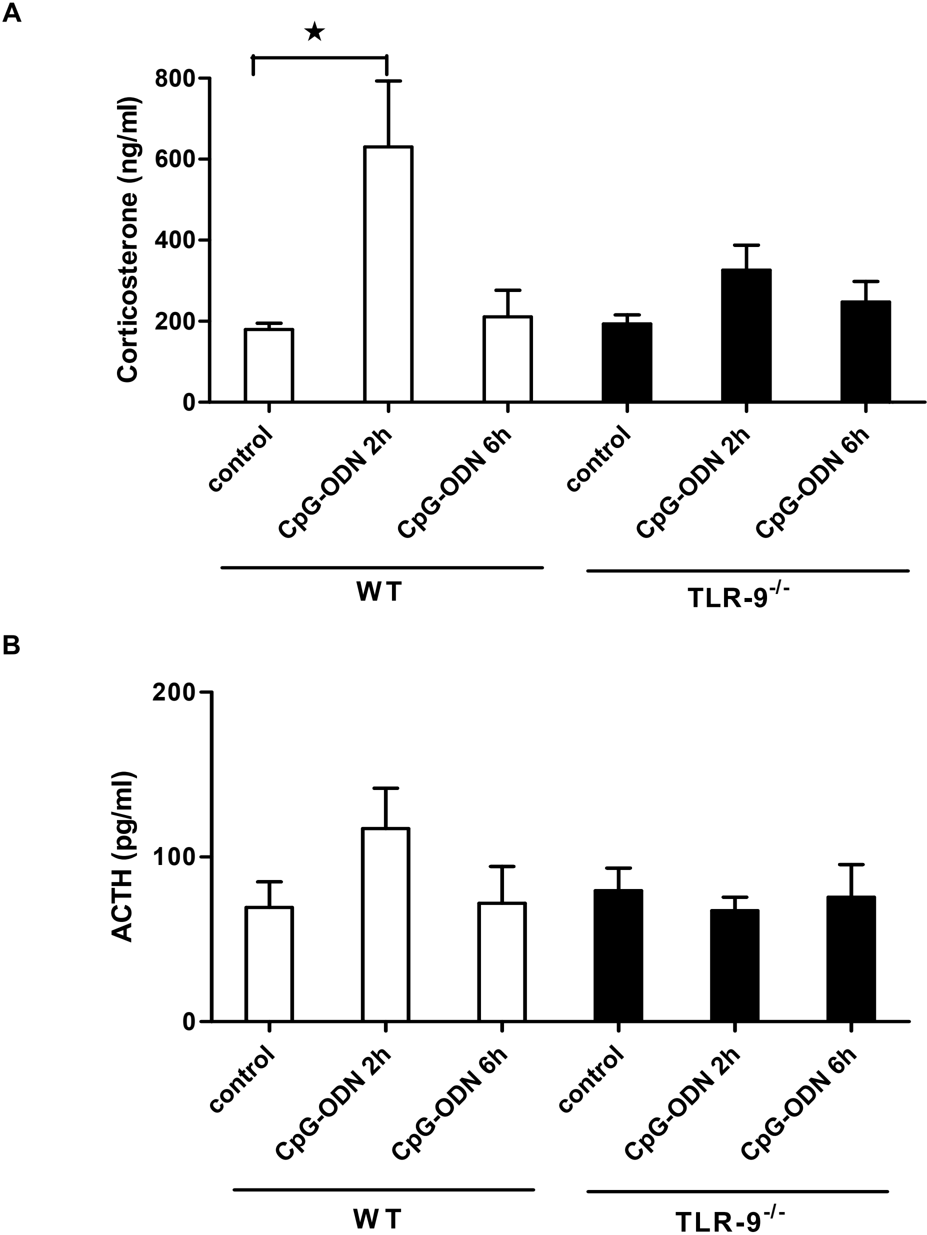


Figure 33: Plasma corticosterone and ACTH response to CpG-ODN challenge. Plasma levels of (A) corticosterone and (B) ACTH in D-GalN-sensitized (1 mg/kg; i.p.) WT and TLR-$9^{-/-}$ mice (n = 6-9/group) under control physiological conditions or after CpG-ODN challenge for 2 or 6 h (1 nmol/g; i.p.). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA and Bonferroni's post-test for each group (versus control). ★; $P<0.05$.

4.3.5 Adrenal NF-κB Activation after CpG-ODN Treatment

To investigate the role of NF-κB in TLR-9 signalling during the adrenal stress response to CpG-ODN, we determined its activation by EMSA in adrenals. We determined NF-κB activity in adrenal glands following a CpG-ODN challenge. As depicted in Fig. 34A (autoradiograph) and B (densitometry), CpG-ODN after 2 or 6 h had no detectable effect on NF-κB activity in WT or TLR-9$^{-/-}$ mice.

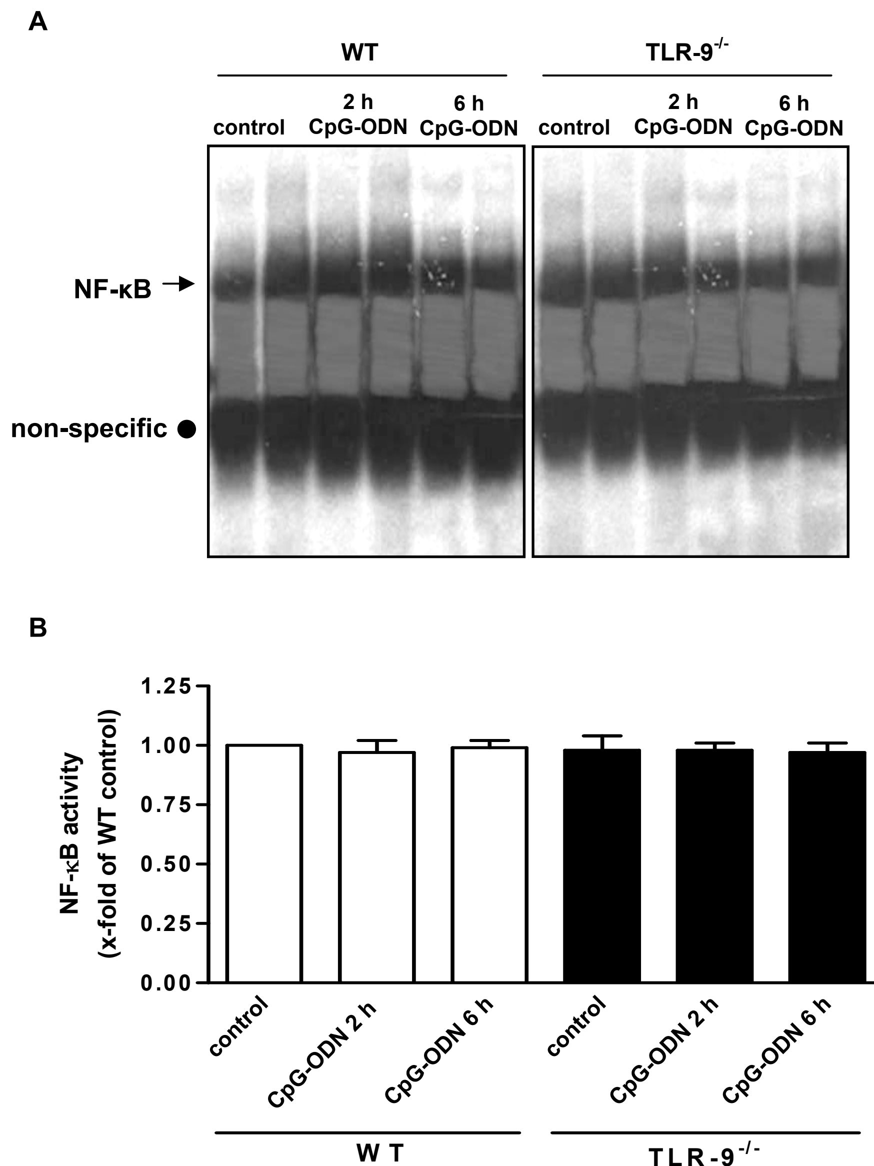


Figure 34: Activation of NF-κB in the adrenal glands in WT and TLR-9$^{-/-}$ mice after CpG-ODN treatment. NF-κB activation was determined by EMSA in adrenal nuclear extracts from D-GalN-sensitized (1 mg/kg; i.p.) WT and TLR-9$^{-/-}$ mice (n = 7/group) following 2 or 6 h challenge of CpG-ODN (1 nmol/g; i.p.). (A) Representative autoradiograph, NF-κB/DNA complex is indicated by the arrow, a non-specific DNA complex is marked by a circle. (B) Densitometric analysis of EMSA. Data were normalized to the intensity of the non-specific DNA complex and expressed as x-fold induction of WT control. Data are presented as mean ± SEM.

In another set of experiments, WT mice were challenged with CpG-ODN for various time points (15-360 min). When compared to control, no significant alterations of NF-κB activity were detected; Fig. 35A (autoradiograph) and B (densitometry). However, there was a 30% increase of NF-κB-DNA binding activity after 30 min which was of no statistical significance (P<0.0538).

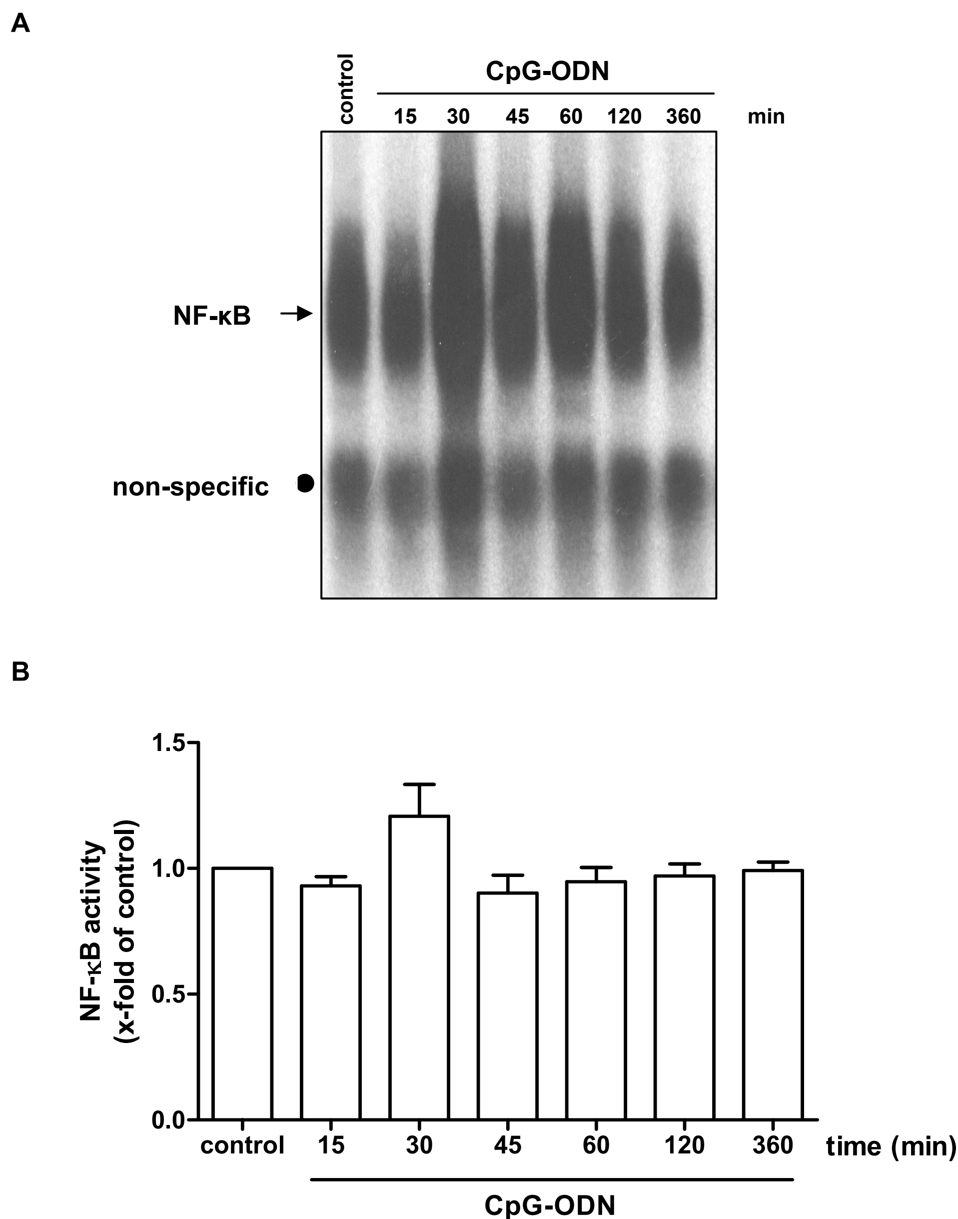


Figure 35: Activation of NF-κB in the adrenal glands in WT mice after CpG-ODN treatment. NF-κB activation was determined by EMSA in adrenal nuclear extracts from D-GalN-sensitized (1 mg/kg; i.p.) WT mice after CpG-ODN challenge (1 nmol/g; i.p.) for various time points as indicated (n = 4-7/group). (C) Representative autoradiograph, NF-κB/DNA complex is indicated by the arrow, a non-specific DNA complex is marked by a circle. (D) Densitometric analysis of EMSA. Data were normalized to the intensity of the non-specific DNA complex and expressed as x-fold induction of WT control. Data is presented as mean ± SEM.

4.3.6 Determination of Various Cytokines in WT and TLR-$9^{-/-}$

We next focused on the plasma activity of various cytokines in WT and TLR-$9^{-/-}$ mice. The animals were either challenged with saline or CpG-ODN for 15-360 min. As shown in Tab. 16, under control physiological conditions control WT and control TLR-$9^{-/-}$ mice exhibited similar levels of cytokines, such as GM-CSF, IL-5, 6, 12, IFN-γ and TNF-α. In contrast, a CpG-ODN challenge (120 min) in WT mice caused a marked and significant increase in all cytokines (except IL-1β and IL-2) ranging from 5- to 213-fold. Plasma levels of IL-6 (213-fold), IL-10 (37-fold), IL-12 (29-fold) IFN-γ (100-fold) and TNF-α (30-fold) were elevated in particular as compared with controls. After 360 min, levels of GM-CSF, IL-5, 6, 10, 12 and IFN-γ remained significantly increased. In contrast, a CpG-ODN mediated effects in WT mice were abolished in TLR-$9^{-/-}$ mice. Only IL-6 levels rose within 120 min, but 31-fold less than in WT animals. In addition, IL-6 levels were back to baseline after 360 min in TLR-$9^{-/-}$ mice and still up-regulated in WT mice.

We further investigated the effects of CpG-ODN challenge on plasma cytokine expression in WT mice at different time points (15-360 min). As summarized in Tab. 16, all cytokines were significantly up-regulated between 45 and 60 min (IL-1β approximately by 61-fold, IL-6 by 232-fold, IL-10 by 99-fold, IL-12 by 39-fold, GM-CSF by 6-fold and TNF-α by 288-fold), and plasma levels of IL-2, 5, 10, 12 and GM-CSF remained significantly high at 360 min. In contrast, IL-1β, 4, 6 and TNF-α levels declined to baseline already after 120 min.

Cytokine (pg/ml)	WT							TLR-9$^{-/-}$		
	control	CpG-ODN 15 min	CpG-ODN 30 min	CpG-ODN 45 min	CpG-ODN 60 min	CpG-ODN 120 min	CpG-ODN 360 min	control	CpG-ODN 120 min	CpG-ODN 360 min
GM-CSF	10±2	3±1	6±1	37±7*	63±3*	46±12*	46±30*	23±12	29±6	19±10
IL-1β	6±2	7±3	26±9	311±68*	366±18*	7±2	0.7±0.7	n.d.	0.5±0.5^{+}	n.d.
IL-2	45±12	n.d.	62±42*	n.d.	134± 6*	94±31*	79±18*	13±5^{+}	22±4	38±13
IL-4	1.0±0.3	1.0± 0.7	3.0±0.8	5.0±0.9	14±1*	18±11*	n.d.	n.d.	n.d.	n.d.
IL-5	9±2	11±3	8±3	16±2	50±4*	90±24*	105±38*	11± 3	61±19	62±42
IL-6	11±2	16±4	58±23	901±211*	2552±250*	2349±125*	168±76*	5±2	75±19*$^{,+}$	24±8
IL-10	5±1	11±3	25±8	419±134*	495±56*	186±65*	186±63*	n.d.	n.d.	n.d.
IL-12	180±15	201±26	191±21	1068±328*	6982±500*	5302±1687*	4411±1431*	174±60	190±71^{+}	173±21^{+}
IFN-γ	0.1±0.1	n.d.	n.d.	0.1±0.1	2.0±0.2	10±3*	14±5*	0.1±0.1	0.3±0.2^{+}	0.2±0.2^{+}
TNF-α	8±2	80±33	205±47	2103±522*	2301±174*	243±78*	44±13	2±1	4±2^{+}	13±8

Table 16: Plasma levels of cytokines in WT and TLR-9$^{-/-}$ mice in response to CpG-ODN treatment. D-GalN-sensitized (1 mg/kg; i.p.) WT and TLR-9$^{-/-}$ mice (n = 4-10/group) were challenged with CpG-ODN (1 nmol/g; i.p.) for various time points as indicated. Experiments were performed in duplicate. Data are presented as mean±SEM and were analyzed by one-way ANOVA for each time point vs. the control of WT or TLR-9$^{-/-}$ mice (* $P < 0.05$); Student's t-test for each time point of WT vs. TLR-9$^{-/-}$ mice ($^{+}$ $P < 0.05$).
GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; n.d., not detectable.

4.3.7 Expression of TLR-9 in Human Adrenal Glands

After characterizing the expression of TLR-9 in murine adrenal glands, it was of great interest to determine the expression of this receptor in the human adrenal gland. Here, we demonstrate for the first time by immunostaining the existence of TLR-9 in human adrenal glands. Normal human foetal and adult adrenal glands revealed TLR-9 expression in the adrenal cortex, but not in the adrenal medulla. As depicted in Fig. 36A, there was no detectable staining for TLR-9 detectable when the adrenal sections were stained without the specific TLR-9 antibody (negative control). Like in mouse adrenal glands, there was a staining of TLR-9 in zona fasciculata of both foetal (Fig. 36B) and adult (Fig. 36C) adrenal glands indicating constitutive expression of TLR-9.

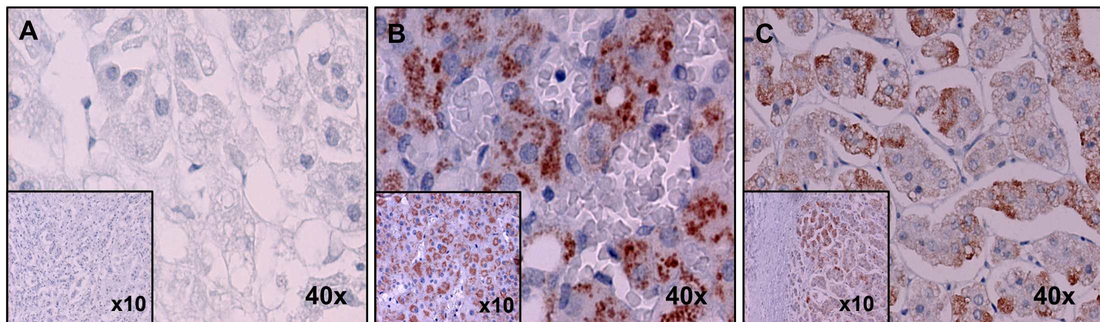


Figure 36: Expression of TLR-9 in human adrenal glands. (A) Negative control, (B) TLR-9 positive staining in foetal and (C) adult adrenal glands in the adrenal cortex (particularly in zona fasciculata, but not in adrenal medulla). Overview, 10x magnification; sectionings of zona fasciculata, 40x magnification. Results were obtained in 3 independent experiments.

5 Discussion

5.1 TLR-2 Study

In this study, we have demonstrated that TLR-2 is expressed in murine adrenal glands under basal conditions. Treatment of animals with a non-lethal dose of LPS or LTA (over a 24 h period) leads to time-dependent increases of TLR-2 expression. Several studies have reported that TLR-2 mediates LPS-responsiveness (Kirschning et al. 1998;Matsuguchi et al. 2000;Yang et al. 1998). However, the transcriptional regulation of TLR-2 in response to gram-negative bacteria has not been fully elucidated. Increased TLR-2 expression in response to LPS has been observed in brain, heart, lung, liver and skeletal muscle (Lang et al. 2003;Matsumura et al. 2000). LPS induces transcription of NF-κB, which leads to expression of inflammatory gene products, including cytokines (Mackman et al. 1991;Vincenti et al. 1992). Matsuguchi and colleagues demonstrated that an elevation in LPS-mediated TLR-2 mRNA was significantly impaired when NF-κB activation was inhibited by curcumin (Matsuguchi et al. 2000). These observations suggest that LPS-induced up-regulation of TLR-2 expression is mediated by NF-κB activation. Furthermore, a study from Oshikawa and co-workers hypothesized that TLR-2 up-regulation in alveolar macrophages following bacterial respiratory infections (e.g. LPS) may render the lung responsive to TLR-2 ligands. In addition, its up-regulation may contribute to the accelerated macrophage response seen at subsequent stages of infection, both of which may enhance the innate immunity against pathogens (Oshikawa and Sugiyama 2003). It is possible that after its own activation, TLR-4 promotes the activation of other TLRs and thus improves the host's defence against pathogens. In fact, it has been shown in adiopocytes activation of TLR-4 with LPS also results in induction of TLR-2 expression (Lin et al. 2000). Therefore in our study, LPS could have altered the expression of TLR-2 through a downstream mechanism, warranting further study. One further possible explanation could be that our LPS preparation could have been contaminated with TLR-2 ligands (Hirschfeld et al. 2000;Lee et al. 2002).

Furthermore, we show that TLR-2 was present in human adrenal NCI-H295 cells. This work was completed by the finding that TLR-2 is also expressed in human glands (Bornstein et al. 2004b). The presence of TLR-2 in adrenal glands may suggest a role for this receptor in the HPA-axis. Absence of TLR-2 in mice results in an enlargement of the adrenal gland, which is correlated to the elevation of plasma ACTH in TLR-2$^{-/-}$ mice. Only the adrenal cortex of TLR-

$2^{-/-}$ animals showed increased adrenal gland size, which may due to the prolonged release of ACTH from the pituitary, presumably to cope with a greater need for glucocorticoid production in adrenal cortex (Simpson and Waterman 1988). At the same time, the corticosterone levels in TLR-$2^{-/-}$ mice were reduced, which suggest a possible impairment of the HPA-axis at the level of the adrenal gland and even during basal conditions. Our ultrastructural analysis of adrenocortical cells indicates that TLR-$2^{-/-}$ mice also have marked changes at the level of the plasma membrane with unusual interdigitations and infoldings of cell membranes.

Adrenal glands have an astonishing ability to adapt to physiological stressors or diseases, with extensive hypervascularization, zonal transformations, cellular hyperplasia and rapid hormone release (Bornstein et al. 1992). Glucocorticoids (e.g. cortisol, corticosterone) are powerful anti-inflammatory compounds that have the ability to inhibit all stages of the inflammatory response. Thus, they play an important role in the modulation and suppression of the acute stress response (Munck et al. 1984). Glucocorticoids produce their effect by binding to glucocorticoid receptors inside the cell and forming a complex, which then moves to the nucleus, where it can directly or indirectly regulate the transcription of anti-inflammatory genes (Karin 1998). But it seems highly unlikely that the widespread anti-inflammatory actions of glucocorticoids can be explained by increased transcription of a small number of anti-inflammatory genes, such as annexin-1, IL-10 and IκBα (inhibitor of NF-κB). However, therapeutic doses of inhaled glucocorticoids do not increase annexin-1 concentrations in bronchoalveolar lavage fluid (Hall et al. 1999), and an increase in IκBα does not occur in most cell types (Heck et al. 1997).

Under basal conditions, impairment of adrenal structure and function in TLR-$2^{-/-}$ mice was only minimal and did not compromise the viability of the animals. However, an appropriate adrenal glucocorticoid response to inflammatory stimuli is critical for the organism to cope with disease. Therefore, we evaluated the adrenal stress response following activation of the HPA-axis by bacterial ligands, including LPS and LTA. Despite the fact that glucocorticoids induce gene transcription, the major anti-inflammatory effects of glucocorticoids are through suppression of inflammatory and immune genes, including cytokines such as TNF-α, IL-1β and IL-6 (Besedovsky and Del 1996;Wilckens and De 1997). The inhibitory effect of glucocorticoids appears to be due to the interaction between the activated glucocorticoid receptors and the transcription factors like NF-κB and activator protein-1, which mediate the expression of these inflammatory genes (Karin 1998;Konig et al. 1992;Ma et al.

2004;Scheinman et al. 1995;Yang-Yen et al. 1990). It has been known that there are cross-talks between the immune and endocrine system during acute infection, which is vital for homeostasis. Recent evidence indicates that after an inflammatory stimulus, the release of inflammatory cytokines (IL-1β, IL-6 and TNF-α) from immune cells or fibroblasts is impaired in TLR-$2^{-/-}$ mice (Darville et al. 2003;Kurt-Jones et al. 2004). It is well established that LPS is a potent activator of the HPA-axis (Beishuizen and Thijs 2003;Takemura et al. 1997). Therefore, we investigated the effect of LPS on the adrenal response. Interestingly, in the absence of TLR-2, the systemic response to LPS resulted in an impaired release of IL-1. Plasma levels of IL-6 and TNF-α were elevated after LPS treatment, although somewhat attenuated in TLR-$2^{-/-}$ animals. This finding was further supported by the fact that local adrenal expression of all three cytokines was significantly less after LPS stimulation when compared to WT animals.

Impaired IL-1 response in TLR-$2^{-/-}$ animals after exposure to LPS indicates an impairment of the HPA-axis, as it is a principal pro-inflammatory cytokine required for innate immunity (Suzuki et al. 2002). IL-1 can activate the HPA-axis at the central as well as the peripheral level (Chrousos 1995;Gonzalez-Hernandez et al. 1995). It has been reported that IL-1β has the ability to induce corticosterone in two ways: First, the cytokine can enter into the central nervous system and stimulate the HPA-axis, resulting in the release of ACTH and corticosterone (Parsadaniantz et al. 2000). Second, IL-1β can trigger adrenal corticosterone, either directly via IL-1 receptor (Mazzocchi et al. 1998;Nagano et al. 2000) or indirectly with the local release of catecholamines (Gwosdow 1995). These pathways may function independently of CRH (Sapolsky et al. 1987) or ACTH (Parsadaniantz et al. 2000) and thus be independent of the HPA-axis. Since LPS induces the synthesis and secretion of IL-1 (Laye et al. 2000), it has been proposed that IL-1 is the primary endogenous cytokine in the response to LPS. However, no significant alterations in plasma ACTH or corticosterone have been observed with the use of IL-1-receptor antagonist following LPS exposure (Dunn 2000). Hence, a blunted IL-1 response after LPS treatment in TLR-$2^{-/-}$ mice may not be the primary mechanism responsible for the impaired adrenal release of glucocorticoids.

Like IL-1, TNF-α is also an immune mediator which stimulates the HPA-axis during stress, increasing glucocorticoids and thus causing a decrease in the immune response. However, cytokines also suppress the HPA-axis. For instance TNF-α impairs ACTH release following CRH stimulation (Bateman et al. 1989;Gaillard et al. 1990). A number of clinical studies have reported low ACTH levels in patients with severe sepsis and SIRS (Bateman et al.

1989;Richards et al. 1999;Soni et al. 1995). Furthermore, TNF-α has been demonstrated to inhibit adrenal gland function (Jaattela et al. 1991;Zhu and Solomon 1992). It reduces adrenal cortisol synthesis by inhibiting the stimulatory action of ACTH on adrenal cells (Jaattela et al. 1990;Jaattela et al. 1991). Similarly, TNF-α has been shown to have a direct inhibitory effect on adrenal cells. Therefore, an impaired TNF-α response would then be expected to lead to an increase in corticosterone levels in TLR-$2^{-/-}$ mice rather than a blunted response that was observed in this study.

IL-6 is a potent stimulator of the HPA-axis during immune activation. After systemic immune induction by LPS, IL-6 enhances corticosterone release in mice in a CRH-independent way (Bethin et al. 2000). In the present study, we demonstrated that IL-6 is expressed in the adrenal cortex. Furthermore, Path and colleagues have shown that IL-6 receptor is present on the human adrenal gland and in adrenal cell cultures (Path et al. 1997). As the IL-6 receptor is expressed on adrenocortical cells, IL-6 may act directly on glucocorticoid synthesis via its receptor. There is increasing evidence that regulation of adrenal steroidogenesis may happen independently of the CRH/ACTH-axis (Chrousos 1995). Several studies have pointed out that diurnal variations in adrenal steroidogenesis do not seem to be directly related to plasma ACTH (Fehm et al. 1984a;Fehm et al. 1984b). After long-term administration of IL-6, plasma ACTH levels decrease, whereas cortisol levels remain high (Mastorakos et al. 1993;Spath-Schwalbe et al. 1994). Glucocorticoids are able to inhibit cytokine-induced ACTH release by acting at the hypothalamic level (Cambronero et al. 1989). Therefore, impairment of ACTH after an enduring challenge of IL-6 may be the result of inhibiting cortisol feedback mechanisms. The data in this study may also suggest a direct effect of IL-6 on the adrenal cortex.

In conclusion, there is an impairment of both systemic and local cytokine secretion, which would explain the significant attenuation of the HPA-axis in TLR-$2^{-/-}$ mice upon exposure to bacterial ligands. Nevertheless, we cannot exclude the fact that some of the effects observed in our study after LPS challenges are due to a contamination with lipopeptides. Recently, several studies reported contaminations of commercial LPS preparations with lipopeptides or lipoproteins (Hirschfeld et al. 2000;Lee et al. 2002;Tapping et al. 2000). It was demonstrated that modified phenol extraction of LPS eliminated TLR-2 mediated activity, and thus they concluded that components other than LPS were responsible for TLR-2 signalling (Hirschfeld et al. 2000;Tapping et al. 2000). Furthermore, Lee and colleagues investigated two lipoproteins isolated from *Escherichia coli*, lip12 and lip19, which were responsible for TLR-

2 mediated cell activation despite being major components in a LPS preparation (Lee et al. 2002).

The same investigations were performed by treating animals with LTA, a classic ligand for TLR-2 and a cell wall component of gram-positive bacteria. A significant impairment in adrenal glucocorticoid release after LTA stimulation was observed. Interestingly, this ligand significantly suppressed plasma corticosterone in TLR-2$^{-/-}$ mice 6 h after administration, in contrast to 24 h in WT animals. For future experiments, a higher dose of LTA should be used and therefore may elicit more prominent effects. In the current experiments, because there was no significant impairment at the pituitary level, the impaired glucocorticoid response suggests a direct action at the level of the adrenal gland.

5.2 TLR-4 Study

In the present study, we show for the first time that TLR-4 is expressed in adrenal glands of WT mice. Under normal physiological conditions, the adrenal gland is bigger in TLR-4$^{-/-}$ mice than in WT animals, which is due to the enlargement of the adrenal cortex. The enlargement of the adrenal glands in TLR-4$^{-/-}$ mice could represent a compensatory mechanism to maintain basal corticosterone release despite impaired adrenocortical function. One could also debate that the altered structure of the adrenal glands reflects the enhanced synthesis and release of corticosterone in TLR-4$^{-/-}$ mice. Nevertheless, it is possible that increased cytokine levels in TLR-4$^{-/-}$ mice stimulate directly the adrenal cortex to liberate corticosterone.

At the ultra-structural level of the adrenal gland, intact steroidogenesis requires a defined spatial and conformational arrangement of mitochondria and their cristae, enabling optimal electron transfer and cytochrome P450 activity. The mitochondrial cristae of steroid producing cells are organized in a tubulovesicular pattern. However, electron microscopy revealed that in TLR-4$^{-/-}$ mice, adrenocortical cells exhibit mitochondria with lamellar membranes or central dilatations. Adrenocortical cells of TLR-4$^{-/-}$ mice show a marked reduction of liposomes, which may result in a rapid exhaustion of the adrenal lipid reserves upon a massive stress stimulus like endotoxemia. Thus, these ultra-structural changes may provide an explanation for the innate defect of steroidogenesis in TLR-4$^{-/-}$ mice and their inability to respond adequately to external stimuli.

Furthermore, under physiological conditions the basal plasma corticosterone was significantly higher in TLR-4$^{-/-}$ mice, whereas ACTH levels were similar in both animal groups, WT and TLR-4$^{-/-}$. The elevated corticosterone levels indicate that these adrenals were being driven by some stimuli. Negative feedback of the elevated levels of corticosterone caused ACTH levels returned to nearly normal. However in TLR-2$^{-/-}$ mice, where an enlarged adrenal cortex was observed, corticosterone levels were suppressed (see chapter TLR-2 study). On the other hand, increased basal corticosterone levels in TLR-4$^{-/-}$ mice may also be due to increased basal levels of IL-12 and TNF-α.

The functional role of TLR-4 in murine adrenal glands was studied during inflammation. To mimic the clinical conditions of SIRS, a low-dose of LPS (1 mg/kg) was used. Crude LPS preparations are often contaminated with TLR-2 ligands such as lipopeptides (Hirschfeld et al. 2000;Lee et al. 2002). Therefore, it is possible that earlier observations reported using

crude LPS preparations may have been due to the activation of TLR-2 and TLR-4 signalling. To exclude these doubts, we used two different LPS preparations and compared their effects on the adrenal response in WT and TLR-4$^{-/-}$ mice. By using a chemiluminescence assay, we demonstrated that the purified pLPS preparation only activated TLR-4, whereas the crude LPS preparation stimulated both receptors, TLR-4 and TLR-2. These results indicated that cLPS was indeed contaminated with TLR-2 ligands and therefore not specific for TLR-4.

In WT animals, both preparations of LPS elicited a significant effect on plasma corticosterone levels within the first 6 h, however, no effect was observed in TLR-4$^{-/-}$ mice. In these mice, an inadequate response of corticosterone to pLPS might due to the absence of TLR-4 signalling. After treatment with cLPS, the lack of response to adrenal corticosterone could also have been due to the abolished activation of IL-1β, IL-6 and TNF-α. These cytokines are released by peripheral immune cells in response to an endotoxin exposure, and through the activation of the HPA-axis, they regulate the corticosterone liberation (van der Meer et al. 1995;van der Meer et al. 1996). Another possible explanation for the unresponsiveness to LPS could ascribe to the fact that plasma corticosterone is already elevated in saline-treated TLR-4$^{-/-}$ animals, so that corticosterone release is already exhausted even before stimulation with LPS. In contrast to corticosterone, basal ACTH remained unaltered in TLR-4$^{-/-}$ mice. Furthermore, unlike cLPS, pLPS did not affect pituitary ACTH release in WT animals, suggesting that a low dose of pLPS elicits its effects directly on the adrenal gland through the activation of TLR-4. At the pituitary level, cLPS-induced effects on ACTH release could have been mediated by TLR-4 and TLR-2, and thus confirm the contamination of the cLPS preparation with TLR-2 agonists.

Over the past few years, it has become evident that the adrenal gland is the main effector organ of the HPA-axis, and a major site for both the synthesis and action of numerous cytokines (Bornstein and Chrousos 1999). Various studies suggest that IL-6, either alone or in combination with other cytokines, is involved in many responses observed after LPS administration, including the activation of the HPA-axis (Lenczowski et al. 1997). Peripheral LPS may alter pituitary function indirectly by stimulating the secretion of hypothalamic hormones and neurotransmitters, which in turn modulate the secretion of pituitary hormones. Moreover, LPS may directly induce cytokines in the pituitary where they could act as autocrine or paracrine signals (Whiteside et al. 1999). It has been shown that immune activation of the adrenal gland by LPS is thought to occur by cytokine stimulation of CRH

production in the hypothalamus, which in turn stimulates the secretion of ACTH from the pituitary (Beishuizen and Thijs 2003;Gadek-Michalska and Bugajski 2004;Watanobe and Yoneda 2003). Binding of LPS directly to adrenal cells has been also reported (Enriquez de et al. 2000). Furthermore, another study has shown that LPS can act directly on human adrenal cells, and this direct effect leads to the stimulation of cortisol secretion (Vakharia et al. 2002).
Cytokines secreted by bacterial endotoxin-activated immune cells stimulate the HPA-axis, especially IL-1, IL-6 and TNF-α (Perlstein et al. 1993;Rivier et al. 1989). Immune cells including macrophages, monocytes, dendritic cells, mast cells and lymphocytes are located within the adrenal cortex. Macrophages are mostly located in the inner adrenocortical zone and express TNF-α (Gonzalez-Hernandez et al. 1996), IL-1β (Gonzalez-Hernandez et al. 1995) and IL-6 (Path et al. 1997). These cytokines influence adrenal function by exerting stimulatory or inhibitory effects (Judd 1998;Marx et al. 1998). Similarly, LPS-stimulated macrophages stimulate or inhibit glucocorticoid biosynthesis by human adrenocortical cells and may participate in a local immune-adrenal regulation. Adrenocortical cells themselves synthesize several cytokines. The distribution of the expression of these cytokines varies in a species-specific manner. In rats, high amounts of cytokines have been detected in the zona glomerulosa (Judd 1998), whereas in humans the main site of cytokine production is the inner zona reticularis (Gonzalez-Hernandez et al. 1994;Gonzalez-Hernandez et al. 1995;Gonzalez-Hernandez et al. 1996). In mice, studies indicate that potent activators of hormone synthesis like ACTH in the adrenal cortex induce IL-6 secretion, whereas TNF-α release is inhibited by ACTH (Judd 1998). The discovery of inhibitory factor expression in the rat adrenal may provide an explanation for the ability of adrenal lymphocytes to secrete these inflammatory cytokines in the presence of local glucocorticoids (Bacher et al. 1997). Most of the cytokines shown to be produced in the adrenal cortex are able to exert direct effects on adrenocortical cells (Judd 1998;Marx et al. 1998). These include effects on growth and differentiation of the adrenocortical cells. In particular the inflammatory cytokines TNF-α, IL-1β and IL-6, all seem to play a role in local immune-adrenal regulation. *In* vivo, IL-1β induced corticosteroid biosynthesis independently of ACTH and caused glucocorticoid secretion in hypophysectomized rats (Andreis et al. 1991), perfused rat adrenals (Roh et al. 1987) and dispersed human adrenal cells (Whitcomb et al. 1988). Recombinant human IL-6 also increased the secretion of cortisol and adrenal androgens in humans both via ACTH and directly (Path et al. 1997). TNF-α inhibited the secretion of aldosterone from rat adrenal cells

(Natarajan et al. 1989), whereas in human foetal adrenal cells, it decreased both basal and ACTH-stimulated cortisol production.

These findings suggest a key role for the adrenal gland and cytokines in the regulation of the HPA-axis. Therefore, the next step was to determine plasma levels of different cytokines under normal and pathological conditions. As mentioned, basal plasma levels of IL-1β, IL-12 and TNF-α are elevated in TLR-4$^{-/-}$ mice. However, elevation of these cytokines did not change the basal release of ACTH from the pituitary. In rats, IL-1β, IL-12 and TNF-α are expressed in the pituitary and adrenal gland after stimulation with a crude LPS preparation (Chen et al. 2005). Therefore, it is not surprising that after 6 h of cLPS stimulation, all plasma cytokines were raised in WT mice indicating an inflammatory response. Additionally, IL-10, which is recognized as a regulator of the HPA-axis (Smith et al. 1999), was elevated after a cLPS stimulus in WT animals. In contrast, pLPS had no effect in WT mice indicating that TLR-2 signalling contribute to the activation of these cytokines. This is supported by the previous study with TLR-2, demonstrating that plasma levels of IL-1β and TNF-α are decreased in TLR-2$^{-/-}$ mice, but not completely suppressed after cLPS stimulation (see chapter TLR-2 study).

LPS is known as an activator of NF-κB, which leads to expression of different inflammatory genes such as cytokines (Vincenti et al. 1992). Bauer et al. (2005) demonstrated that NF-κB activity was markedly decreased in TLR-4$^{-/-}$ mice in comparison with WT mice in response to an antioxidant (butylated hydroxytoluene). Furthermore, another study on TLR-4 in cardiac myocytes has shown that NF-κB was upregulated after LPS administration in WT control mice in comparison with non-functional TLR-4 mice (Baumgarten et al. 2006). Therefore, to confirm the role of TLR-4 signalling in adrenal stress response to LPS, NF-κB activation was also determined in this study. Our results show that pLPS and cLPS caused an induction of NF-κB activity in WT mice after 1 h, which was markedly impaired in TLR-4$^{-/-}$ mice. These results are consistent with previous findings, revealing an impairment of NF-κB induction after LPS treatment in TLR-4$^{-/-}$ mice.

5.3 TLR-9 Study

Here, we demonstrate for the first time that TLR-9 mRNA and protein are present in the murine adrenal gland. In order to study TLR-9 expression *in vivo*, tissue samples from healthy, non-treated animals (normal leukocyte blood count) were taken. Since histology of the adrenal gland revealed only a small number of leukocytes present in this organ, it can be excluded that adrenal TLR-9 mRNA and protein determined by PCR and Western Blot analysis was leukocyte-derived. TLR-9 was also detected in the human adrenal cortex. These findings are in line with a previous report that TLR-9 mRNA is expressed in human adrenal glands (Nishimura and Naito 2005). Additionally, it has been shown that TLR-9 is present in different organs, e.g. spleen, kidney, liver, lung and brain (Martin-Armas et al. 2006;Ono et al. 2004) and in various human tissue (Droemann et al. 2005;Nishimura and Naito 2005). However, the role and function of TLR-9 in the adrenal gland has not been studied.
Furthermore, we looked into adrenal morphology and function in WT and TLR-$9^{-/-}$ mice under control physiological conditions. Here, neither adrenal size nor plasma corticosterone or ACTH differed between animals. This is in contrast to our previous findings, where TLR-2 or TLR-4 deficient mice demonstrated marked differences in the above parameters when compared to WT animals (see chapters TLR-2 and TLR-4 studies). Although baseline function was abnormal in TLR-2 and TLR-4 mice, this did not influence the phenotypes of each strain. Therefore, we were interested in studying the role of TLR-9 in mice under conditions of inflammation, where appropriate adrenal function is very important for combating disease.

In a SIRS model, we investigated the effects of systemic inflammation using the TLR-9 agonist CpG-ODN on the HPA-axis in WT and TLR-$9^{-/-}$ mice. CpG-ODN caused a three-fold increase in levels of corticosterone in WT mice, comparable to the effects of LPS in a similar model (see chapter TLR-4 study). CpG-ODN-induced effect was absent in TLR-$9^{-/-}$ mice indicating that TLR-9 contributed either directly or indirectly to this hormonal response.
CpG-ODN had no effect on plasma levels of ACTH, either in WT or in TLR-$9^{-/-}$ mice. Although this fits with the lack of expression of TLR-9 in the murine pituitary gland (Ono et al. 2004) we cannot exclude a small indirect (IL-6 driven) stimulatory effect in WT mice. The HPA-axis can be influenced by both pituitary (ACTH) and extra-pituitary mechanisms (Gloddek et al. 2001b;Suzuki et al. 1986a;Gloddek et al. 2001a;Suzuki et al. 1986b). For example, LPS increases ACTH and corticosterone plasma levels (Watanobe and Yoneda

2003) as well as the release of various cytokines. The latter fact mediates the production of CRH in the hypothalamus. In turn, CRH stimulates the release of ACTH from the pituitary gland, which then increases glucocorticoid synthesis in the adrenal gland (Reincke et al. 1993). Based on our *in vivo* results, one could argue that corticosterone production was mediated by an extra-pituitary mechanism.

CpG-ODN challenge did not affect the expression of TLR-9 mRNA and protein in adrenal glands of WT mice. One of the reasons could be the dose of CpG-ODN administered (1 nmol/g). Several studies on the distribution of phosphorothioate oligodeoxynucleotides in rodents have shown that the majority of intravenously injected phosphorothioate oligodeoxynucleotides (96%) are cleared from the circulation as a result of rapid redistribution over the body fluid (Rifai et al. 1996). It has been demonstrated that 4 min after injection, 50% of ^{125}I-CpGs were eliminated from the blood stream with a very high uptake in liver and kidney (Butler et al. 1997;Martin-Armas et al. 2006;Peng et al. 2001;Rifai et al. 1996). These data suggest that the amount of CpG-ODN reaching the mouse adrenal gland is probably too low to affect the expression of TLR-9.

CpG-ODN treatment was associated with high plasma levels of corticosterone and various cytokines in mice. We profiled the cytokine expression (following a CpG-ODN challenge) in the mouse every 15 min for the first hour and than at 2 and 6 h, thereafter. Interestingly, most cytokines were already significantly up-regulated after 45-60 min (see time-course in Table 16). Inflammatory cytokines, such as IL-1, IL-6 and TNF-α have been identified as important modulators of HPA-axis function (Bornstein and Chrousos 1999;Chrousos 1995). Plasma levels of these three cytokines were increased by more than 10-fold compared to baseline supporting the concept of cytokine-mediated high glucocorticoid output and a change from neuron-endocrine to immune-endocrine function (Bornstein et al. 2004a). There is good evidence that CpG-ODN leads *in vitro* as well as *in vivo* to stimulation of immune competent cells resulting in the release of various cytokines (Anitescu et al. 1997;Hong et al. 2004;Kadowaki et al. 2001;Sparwasser et al. 1997;Stacey et al. 1996). In a model of CpG-DNA induced liver injury (D-GalN-sensitized mice), the pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α were generated within 1 h (Yi et al. 2006). However after 4 h, levels of these cytokines returned back to baseline. These findings correspond to our observation in WT mice (see Table 16). Cytokine levels were significantly increased between 45-60 min. After 2 h, levels of IL-1β, IL-6 and TNF-α declined to baseline, and only levels of IL-12

remained high. We cannot exclude whether the cytokine profile measured in this study was at least in part due to CpG-ODN-induced liver injury.

TLR-9 is somehow involved in the adrenal stress response following a CpG-ODN challenge. Absence of TLR-9 abolishes the corticosterone and cytokine response supporting the concept that CpG-ODN mediates its effects on adrenal cells via TLR-9. However, this does not appear to compromise the phenotype of TLR-9$^{-/-}$ mice. Whether this effect is mediated directly (via adrenal TLR-9), indirectly (via blood-derived TLR-9 and consequently cytokine release) or by a combination of both pathways, remains open for further study. However, we have at least strong evidence for a direct effect (30% increases in adrenal NF-κB activity after 30 min, significant plasma cytokine response after 45 min and significant corticosterone levels were detectable at 120 min). Taken together with our previous findings, TLR-9 activation contributes to an endocrine stress system response.

6 Perspective

According to the present findings, TLR-2, TLR-4 and TLR-9 are expressed in murine as well as in human adrenal glands. The presence of these TLRs in this endocrine organ suggests a potential role in HPA-axis function. Thus, they may constitute an important link between the innate immune and endocrine systems at two different levels, central as well as peripheral, particularly during inflammatory conditions. Our study with TLR-deficient mice shows that the absence of TLR-2 and TLR-4, but not TLR-9 leads to altered adrenal morphology. These alterations do not appear to compromise the phenotype of TLR-$2^{-/-}$ and TLR-$4^{-/-}$ mice. Moreover, TLR-2, 4 and 9 deficient animals were not able to respond adequately to inflammatory stress induced by their potential ligands LPS, LTA or CpG-ODN, respectively. These impaired adrenal stress responses appear to be associated with decreases in systemic and intra-adrenal cytokine expressions. In conclusion, TLR-2, TLR-4 and TLR-9 are shown to be key players in the immuno-endocrine interactions during inflammatory conditions.

Mutations in the innate immune system are not a rare event and may be the fundamental mechanism for an impaired adrenal stress response in patients with sepsis. Several studies which are relevant to TLR signalling showed that there is an important link between TLRs and human diseases. These studies demonstrate that manipulation of TLR pathways could have therapeutic potential for conditions such as hepatitis B and C, anthrax, influenza, HIV, allergic rhinitis, asthma, sepsis and various forms of cancer (reviewed by Kanzler et al. 2007). Currently, several TLR-based therapeutic agents are developed, e.g. TLR antagonists which are structural analogs of TLR-agonists but unable to induce signalling. Other possible approaches are antibodies directed against TLRs or small molecule antagonists selected from compound libraries. TLR antagonists appear quite promising for a number of inflammatory and autoimmune diseases. Two lipid A analogs which act as potent antagonists of TLR-4 have advanced into clinical trials for sepsis.

Based on the present study, it is of particular interest to clarify whether patients with TLR polymorphisms, in particular TLR-2, TLR-4 and TLR-9 polymorphism, have an impaired HPA-axis during stress. This could change the current therapy of sepsis, when patient’s genotypic profile is known. In summary, understanding of the relationships between TLR signalling pathways, related polymorphisms and severe diseases is of particular interest for development of new encouraging therapies.

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„The Role of Toll-like Receptors in the Adrenal Gland“

in der Klinik für Anästhesiologie des Universitätsklinikums der Heinrich-Heine-Universität Düsseldorf sowie der Klinik für Anästhesiologie, Intensivmedizin und Schmerztherapie des Klinikums der Johann Wolfgang Goethe-Universität Frankfurt am Main unter der Leitung von Herrn Univ.-Prof. Dr. Dr. Kai Zacharowski, FRCA ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in-oder ausländischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Vorliegende Arbeit wurde in folgenden Publikationsorganen veröffentlicht:

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