The Role of Toll-like Receptors in the Immune–Adrenal Crosstalk

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ABSTRACT: Sepsis and septic shock remain major health concerns worldwide, and rapid activation of adrenal steroid release is a key event in the organism's first line of defense during this form of severe illness. Toll-like receptors (TLRs) are critical in the early immune response upon bacterial infection, and recent data from our lab demonstrate a novel link between the innate immune system and the adrenal stress response mediated by TLRs. Glucocorticoids and TLRs regulate each other in a bidirectional way. Bacterial toxins acting through TLRs directly activate adrenocortical steroid release. TLR-2 and TLR-4 are expressed in human and mice adrenals and TLR-2 deficiency is associated with an impaired glucocorticoid response. Furthermore, TLR-2 deficiency in mice is associated with marked cellular alterations in adrenocortical tissue. TLR-2-deficient mice have an impaired adrenal corticosterone release following inflammatory stress induced by bacterial cell wall compounds. This defect appears to be associated with a decrease in systemic and intraadrenal cytokine expression. 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 requiring an intact adrenal stress response.

KEYWORDS: toll-like receptors; knockout; bidirectional regulation; LPA

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THE BIOLOGICAL IMPORTANCE OF TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) play a crucial role in the innate immunity in mammals. Mammalian TLRs were originally found as homologues of the *Drosophila* Toll,[1] where it plays a key role in the establishment of dorsoventral polarity as well as in antifungal and antibacterial host defence. TLRs are type I transmembrane receptors that possess extracellular leucine-rich repeat domains flanked by cytoplasmatic domains. These extracellular domains can be considered as family pattern recognition receptors for the detection and response to microbial ligands. The initial host defense against bacterial infection by the innate immune system is essentially executed by these receptors. Particularly, TLR-2 and TLR-4 polymorphisms are frequent in humans. There is good evidence that impaired innate immunity mediated by TLRs is involved in sepsis and cardiovascular disease.[2–4] Several studies investigated the existence of TLR mutations in humans. TLR-2 Arg753Gln polymorphism, positively correlating with the incidence of sepsis in a white population, and a TLR-2 Arg677Trp polymorphism, correlating with the incidence of lepromatous leprosy in an Asian population, have been demonstrated.[4,5] A recent study reported a rate of nearly 10% heterozygosity for the TLR-2 Arg753Gln polymorphism,[6] suggesting clinical relevance of the TLR-2 gene during inflammation. Although the existence of a high rate of TLR-2 and TLR-4 polymorphisms in humans is shown, the role of TLR-2 and TLR-4 in the endocrine stress response during development and progression of inflammatory complications has only inadequately been investigated so far.

WHAT ARE THE FUNCTIONS OF THE DIFFERENT MEMBERS OF THE TLR FAMILY?

To date, at least 10 members of the TLR family have been identified in humans, and several ligands recognized by TLRs have been reported. TLR-2 is expressed mainly by peripheral blood monocytes and is involved in the recognition of gram-positive bacteria and other components of different pathogens.[7] It has been shown that TLR-1 coexpressed with TLR-6 enhances the TLR-2 response.[8] TLR-4 has been implicated in lipopolysaccharide (LPS) signaling, innate immunity and inflammation. TLR-3 and 5–9 are mainly involved in the recognition of a double-stranded RNA of viruses and certain bacterial components. The role of TLR-10 remains obscure, although an expression in the spleen and lung could be documented so far. For further reading on TLR, refer to Nishimura and Naito.[9]

During the last 10 years, our lab has focused on the interaction of immune cells and their mediators with the hypothalamic-pituitary-adrenal (HPA) axis employing *in situ* hybridization, microarray analysis, immunohistochemistry,

laser capture microdissection, organ perfusion, cell culture/co-culture systems, and animal models.[10–15] We have characterized the role of lymphocytes, cytokines, adipo-cytokines, and chemokines in the adrenal gland.[16–22] The nature of this immune–endocrine crosstalk is implicated in adrenal dysfunction and disease.[23–25] During inflammatory and autoimmune disorders, including sepsis, inflammatory bowel disease, and rheumatoid arthritis, immune–adrenal crosstalk becomes more critical in maintaining adequate adrenal stress response.[23,25–27]

GLUCOCORTICOIDS AND TOLL-LIKE RECEPTOR SHOW A BIDIRECTIONAL REGULATION

In addition to hypothalamic hormones, including corticotrophin-releasing hormone (CRH) and vasopressin, inflammatory cytokines such as IL-1, IL-6, and TNF-α have been identified as important modulators of the HPA axis in physiological as well as pathological situations. During inflammation, these cytokines are capable of maintaining high glucocorticoid output, suggesting a shift from neuroendocrine to immune–endocrine regulation of the adrenal during septicemia.[28] In turn, enhanced adrenal glucocorticoid release is required to prevent an uncontrolled response of inflammatory cytokines, which could result in severe damage to the cardiovascular system. Therefore, a coordinated response of the adrenal and immune system is crucial for survival during severe inflammation and sepsis.[29–31]

New data from other groups demonstrate that glucocorticoids have an influence on TLR expression. This suggests a role for glucocorticoids in the innate immune system. Although TNF-α and glucocorticoids are widely recognized as mutually antagonistic regulators of adaptive immunity and inflammation, they cooperatively regulate the components of the innate immunity like the TLR-2 expression via signal transducers and activators of transcription (STAT) and NF-κB signaling.[32] Furthermore, glucocorticoids synergistically enhance the IL-1β-induced TLR-2 expression, via upregulation of MAPK phosphatase-1 (MKP-1) expression, which, in turn, leads to the inactivation of both p38 and Jun kinase (JNK) signaling pathways, the negative regulators for TLR-2 induction.[32]

In one of our studies bidirectional action of glucocorticoids with both immunostimulatory, as well as immunosuppressive function during inflammation could be documented by gene expression profiling and reverse transcriptase polymerase chain reaction.[17] In this study, numerous newly discovered genes, playing critical roles in innate and adaptive immune responses, were found to be regulated by glucocorticoids in peripheral blood monocytes. In a global gene expression analysis, nearly 10,000 human-expressed genes were screened and 9% were considered to be down- and 12% to be upregulated by glucocorticoids. They could thus act as anti-inflammatory agents by

downregulation of proinflammatory cytokines as IL-1, lymphotoxin-β, IL-1-a, IL-8, IFN-α, and IFN-β, and upregulation of others, as, for example, the transforming growth factor (TGF)-β3, IL-10, and IL10-R. Additionally, many proinflammatory ligands were downregulated, whereas anti-inflammatory soluble mediators were mostly upregulated by glucocorticoids, demonstrating their immunosuppressive and protective role against inflammation expression. Of the six major clusters analyzed, immune response–related genes and unknown genes (ESTs) were mostly regulated. The immune cluster was divided into subclusters and subcategories. Considering the great importance for glucocorticoid therapy, these observations might help to design more specific and efficient treatment strategies in the future.

DIFFERENTIAL REGULATION OF HUMAN ADRENAL STEROID RELEASE BY BACTERIAL TOXINS

Lipopolysaccharide (LPS) stimulates various levels of the HPA axis, indicative of an increase in plasma ACTH and corticosterone levels.[33] Furthermore, LPS elicits direct effects on adrenal cells. Human adrenal cells release cortisol by direct stimulation with LPS, an effect mediated via cyclooxygenase-dependent mechanisms. Thus, LPS caused a dose-dependent stimulation of basal cortisol secretion by the human adrenocortical cell line, NCI-H295R, without affecting aldosterone. Additionally, both TLRs (2/4) and their specific ligands (Pam3Cys/purified LPS and lipid A) were found to be involved in this cortisol release. LPS was also found to stimulate prostaglandin E release by these cells via Cox-2 activation.[34]

Furthermore, it is worth knowing that LPS acts specifically through the activation of TLR-4.[35] In this respect, most of the former studies using commercial LPS preparations did not notice a possible contamination with TLR-2 ligands such as lipopeptides. Thus, in our studies we confirmed by TLR-specific reporter assays that a pure LPS preparation (pLPS) solely triggered TLR-4, but was devoid of TLR-2 agonistic activity. In contrast the commercial LPS preparation (cLPS) stimulated both receptors (K.Zacharowski, unpublished data).

THE ROLE OF TLRs IN THE HPA AXIS

TLR-2/4 Could be Localized in the Human Adrenal Gland

An intact adrenal stress response is critical for a host's defense to infection.[36,37] The family of TLRs are very important in the early immune response upon bacterial infection. In addition to an immune–adrenal crosstalk mediated through secretory products of immune cells acting on adrenal cells the endocrine system may have innate immune properties itself. Unlike other nonimmune cells, adrenal cells express not only cytokines and cytokine receptors,

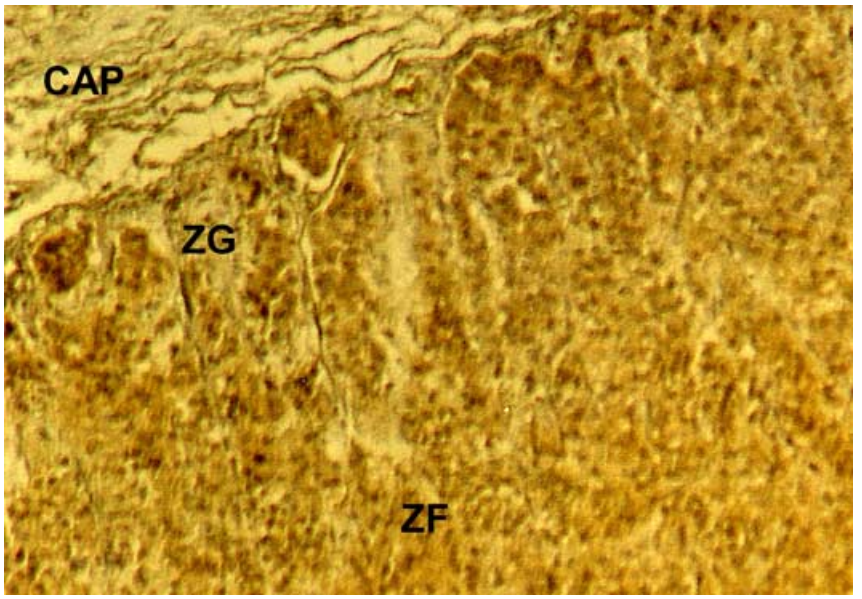

FIGURE 1. Human adrenal gland was stained with an antibody against TLR-2 (goat polyclonal S-16, Santa Cruz Biotechnology, Heidelberg, Germany). The brown staining shows DBC staining. C = capsule, ZG = zona glomerulosa, ZF = zona fasciculata.

but also major histocompatibility complex (MHC) class II molecules. Therefore, TLR-2 and TLR-4 receptors found on adrenal cells may be connected to a functional signaling system similar to the one characterized on immune cells and/or one that interacts directly with the activation of the steroid biosynthetic pathway in these cells. We could show that TLR-2/4 are expressed in the human adrenocortical cell line NCI-H295 and in human adrenal glands in the cortex, but not in the medulla[14] (FIG. 1).

Lessons Learned from TLR-2 Knockout Animals

Animal studies have shown that TLR-2-deficient mice are more susceptible to septicemia due to *Staphylococcus aureus* and *Listeria monocytogenes,* meningitis due to *Streptococcus pneumoniae,* and infection with *Mycobacterium tuberculosis,* suggesting that functional *TLR-2* polymorphisms may impair host response to a certain spectrum of microbial pathogens.

Detailed analyses show that the absence of TLR-2 in mice is associated with an enlargement of the adrenal gland and a reduction in corticosterone levels. Furthermore, plasma ACTH levels are elevated in TLR-2-deficient mice, indicating a possible impairment of the HPA axis on the level of the adrenal gland—even under basal conditions. Ultrastructural analysis from our

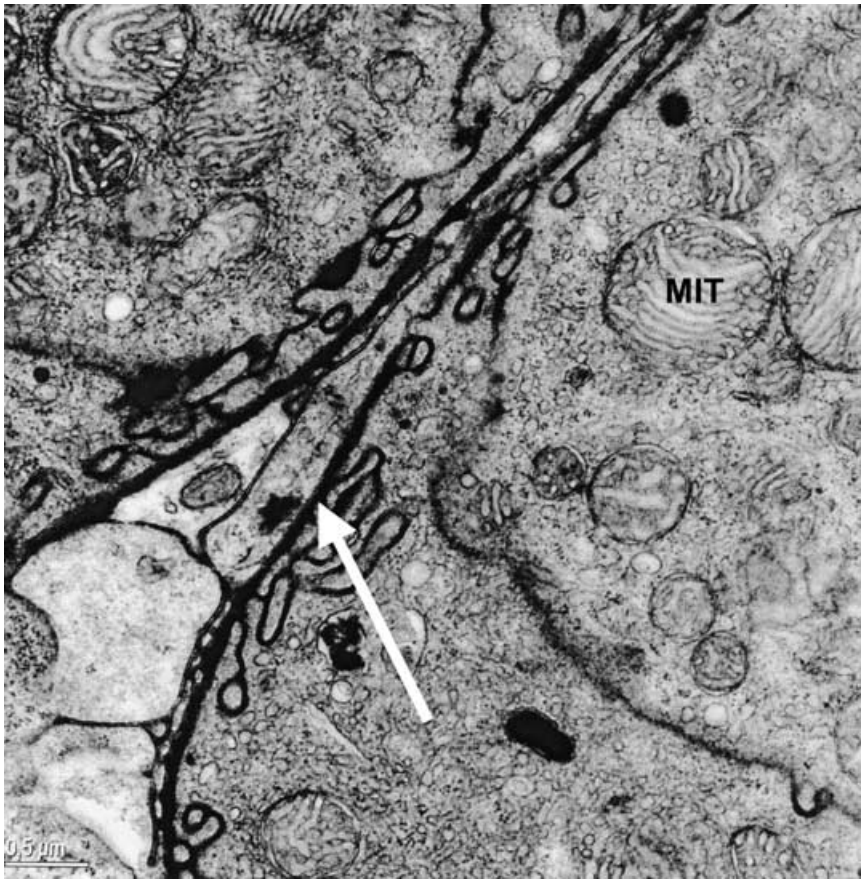

FIGURE 2. Ultrastructural image of adrenocortical cell in TLR-2-deficient animals. Cells demonstrate conspicuous alterations in membrane and mitochondrial structures in conjunction with impaired steroidogenic capacity (original magnification × 20,000). MIT = mitochondrium; *arrow* shows plasma membrane.

lab demonstrates that adrenocortical cells from TLR-2-deficient mice show marked changes of the plasma membranes with unusual interdigitations and infoldings of cell membranes (FIG. 2). This might suggest a primary defect in the adrenal function in TLR-2-deficient animals and could explain the reduction of corticosterone production.[15] Because cytokines are important in the regulation of the HPA axis, the TLR system might play a key role in the signal transduction of an adrenal stress response to inflammatory stimuli. This is in the context of the observation that mice with a targeted disruption of the TLR-2 gene are more susceptible to meningitis-induced intracranial complications. In addition, TLR-2-deficient mice have a more pronounced

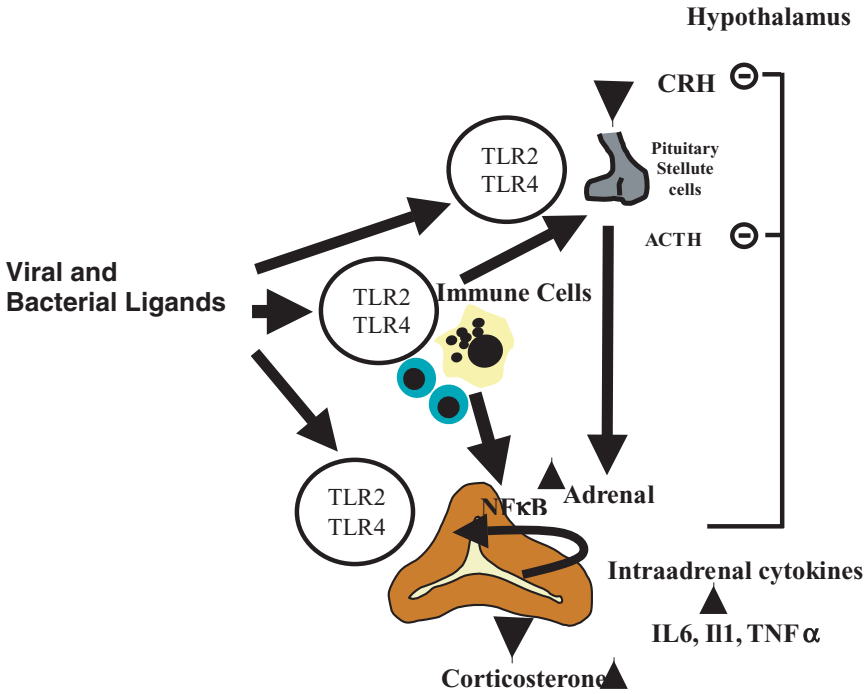

FIGURE 3. Artistic rendition of potential immune–endocrine interaction mediated by TLR-2 and TLR-4 during inflammation and sepsis. (Modified from Bornstein *et al.*[15])

reduction in body weight and a deterioration of motor impairment following experimental pneumococcal infection.[38] Similarly, TLR-2-deficient mice succumb to *M. tuberculosis* infection and are highly susceptible to *S. aureus* infection[39] compared to wild-type animals. Gram-positive bacteria such as *S. aureus* can trigger multiple organ failure and septic shock without causing endotoxemia.[40] In humans, two polymorphisms in the exon part of *TLR-2,* which attenuate receptor signaling, enhance the risk of acute severe infections, tuberculosis, and leprosy.[41]

In summary, our data demonstrate that TLR-2 and TLR-4 (R. Zacharowski, unpublished data) play important roles in the HPA axis. Thus, TLR-2 and TLR-4 constitute an important link between the immune and endocrine stress systems at both the central and peripheral levels, particularly during inflammation and sepsis (FIG. 3).

Clinical Implications

The crucial role of TLRs in the immune–adrenal crosstalk may have important clinical implications. Polymorphisms occur frequently in the TLR

system in humans and it is conceivable that these individuals will have more difficulties in mounting and/or maintaining an adequate activation of the HPA axis during the severe stress of inflammation. Patients with sepsis may develop adrenocortical insufficiency and benefit from glucocorticoid therapy.[42–45] The mechanisms of adrenal impairment during sepsis and other inflammatory states still remain enigmatic and it will be of great interest to test the role of TLRs in this setting. Currently cytokine antagonists are widely used in the treatment of rheumatoid arthritis and TNF-α antagonists have been shown to downregulate TLRs in synovial tissues of these patients.[46,47] Some patients, however, fail to respond favorably to these compounds. Therefore, there may be an impaired immune–adrenal communication in conjunction with the downregulation of the TLRs in some of these patients that should be considered in future studies. On the other hand, TLR antagonists may constitute a useful tool for treating chronic inflammation states with concomitant activation of the HPA axis.

Low-grade inflammatory states with an activated HPA axis form the basis of most modern health problems, including obesity, diabetes, allergies, depression, and cardiovascular disease. Therefore, TLR antagonists may become a powerful medication for a great variety of indications in the future.

CONCLUSION

The capacity to respond to external and internal stimuli with a rapid and efficient endocrine stress response has been a critical step for survival and evolution of higher organisms. The adrenal has an astonishing capacity to adapt to physiological stressors or disease with extensive hypervascularization, zonal transformation, cellular hyperplasia, and rapid hormone release. Similar to the steroid system the toll-like family of receptors predates the animal kingdom and is remarkably preserved through the evolutionary process. TLRs and in particular TLR-2 play a fundamental role in coordinating the organisms' first line of defense. The innate immune response to endogenous and or exogenous molecules or pathways indicates tissue injury, infection, and remodeling. Therefore, a coordinated and efficient response of both systems mediated through TLR-2 and TLR-4 may have been programmed early in evolution. In this course the interactions between the innate immune system and HPA axis may be characterized by a circuit that includes (i) activation of the HPA axis and initiation of the stress response, which, in turn, has immunomodulating properties; (ii) a feedback mechanism derived from the immune system that regulates the HPA axis.

Over the past few years, it has become evident that the adrenal gland itself, as the main effector organ of the HPA axis, is a major site for both the synthesis and action of numerous cytokines. In light of the future use of TLR agonists or antagonists for a variety of inflammatory and autoimmune

disorders, analyzing the adrenal function in TLR-2- and TLR-4-deficient mice[48] and human adrenal glands under normal conditions and following activation by bacterial lipids (i.e., LTA and LPS) is of great interest and has high clinical relevance.

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