Toll-like receptor 4 plays a crucial role in the immune–adrenal response to systemic inflammatory response syndrome

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Sepsis and septic shock are leading killers in the noncoronary intensive care unit, and they remain worldwide health concerns. The initial host defense against bacterial infections involves Toll-like receptors (TLRs), which detect and respond to microbial ligands. In addition, a coordinated response of the adrenal and immune systems is crucial for survival during severe inflammation. Previously, we demonstrated a link between the innate immune system and the endocrine stress response involving TLR-2. Like TLR-2, TLR-4 is also expressed in human and mouse adrenals. In the present work, by using a low dose of LPS to mimic systemic inflammatory response syndrome, we have revealed marked cellular alterations in adrenocortical tissue and an impaired adrenal corticosterone response in TLR-4$^{-/-}$ mice. Our findings demonstrate that TLR-4 is a key mediator in the crosstalks between the innate immune system and the endocrine stress response. Furthermore, TLR polymorphisms could contribute to the underlying mechanisms of impaired adrenal stress response in patients with bacterial sepsis.

lipopolysaccharide | stress axis | sepsis | corticoids | mice

Sepsis and septic shock, with a mortality of 20–80% (1), are leading killers in the noncoronary intensive care unit. In the United States, the incidence of sepsis has risen from 164,072 cases in 1979 to 659,935 in 2000, an increase of 13.7% per year (2). In the 1980s, Gram-negative bacteria were the predominant cause of sepsis; however, by the year 2000, Gram-positive bacteria accounted for >50% of all cases of sepsis in the United States. Despite the increasing incidence of sepsis and sepsis-related conditions, the overall mortality rate is declining, indicating the emergence of new, improved strategies.

The initial host defense against bacterial infections is executed essentially by pattern-recognition receptors, such as Toll-like receptors (TLRs), which detect and respond to microbial ligands. To date, 10 TLRs have been identified in humans. TLR-4 has been implicated in LPS signaling, innate immunity, and inflammation, whereas TLR-2 is involved in the recognition of Gram-positive bacteria (3–6). Clinical studies have demonstrated the existence of TLR mutations in humans (7). TLR-2 and TLR-4 polymorphisms are the most commonly identified, so far (8, 9). Moreover, TLRs have been implicated in the dysregulation of innate immunity during the pathological conditions of sepsis and cardiovascular disease (10–12).

The endocrine system is essentially involved in an intact adrenal response to stress, and it is crucial for the host defense against infection (13, 14). This involvement is supported further by the fact that adrenal insufficiency is associated with sepsis in a substantial number of cases (15, 16). Hypothalamic hormones, including corticotropin-releasing hormone and vasopressin, as well as inflammatory cytokines, such as IL-1, IL-6, and TNF-α, have been identified as important modulators of hypothalamic–pituitary–adrenal (HPA) axis function (13, 14). During inflammation, these cytokines mediate a high glucocorticoid output, indicating a change in regulation from the neuroendocrine to the immune–endocrine system (17). As a result, high levels of adrenal glucocorticoids are vital in preventing an uncontrolled inflammatory response to cytokines, which could have detrimental effects on the cardiovascular system. Therefore, during severe inflammation, a competent response of the adrenal and immune systems is important for survival (18–20).

Recently, we described the expression of TLR-2 in human and mouse adrenals (21). TLR-2 plays an important role in the adrenal stress response of mice because the absence of this receptor is associated with an enlarged adrenal gland and reduced corticosterone levels (22). Furthermore, plasma levels of adrenocorticotropic hormone (ACTH) are elevated in TLR-2$^{-/-}$ mice, indicating a possible impairment of the HPA axis at the adrenal level. Like TLR-2, TLR-4 is also expressed basally in human adrenals (21), suggesting that both receptors may be involved in HPA axis function. During the development of inflammatory conditions, a role for TLR-4 in the endocrine stress response has not yet been described. In the present work, we investigated the structure and function of the adrenal gland in TLR-4-deficient mice during experimental conditions of stress, e.g., systemic inflammatory response syndrome. In the past, commercial LPS preparations contaminated with TLR-2 ligands were not considered (23, 24); therefore, we also compared the effects of a pure LPS preparation with a crude LPS preparation on the endocrine stress response. Our results demonstrate that TLR-4 is a major mediator in the crosstalks between the innate immune system and the endocrine stress response.

Results

Structure and Function of the Adrenal Gland in TLR-4$^{-/-}$ Mice. Under control conditions, TLR-4 protein was expressed in the adrenal cells of WT mice (Fig. 1A). In TLR-4$^{-/-}$ mice, the adrenal gland was
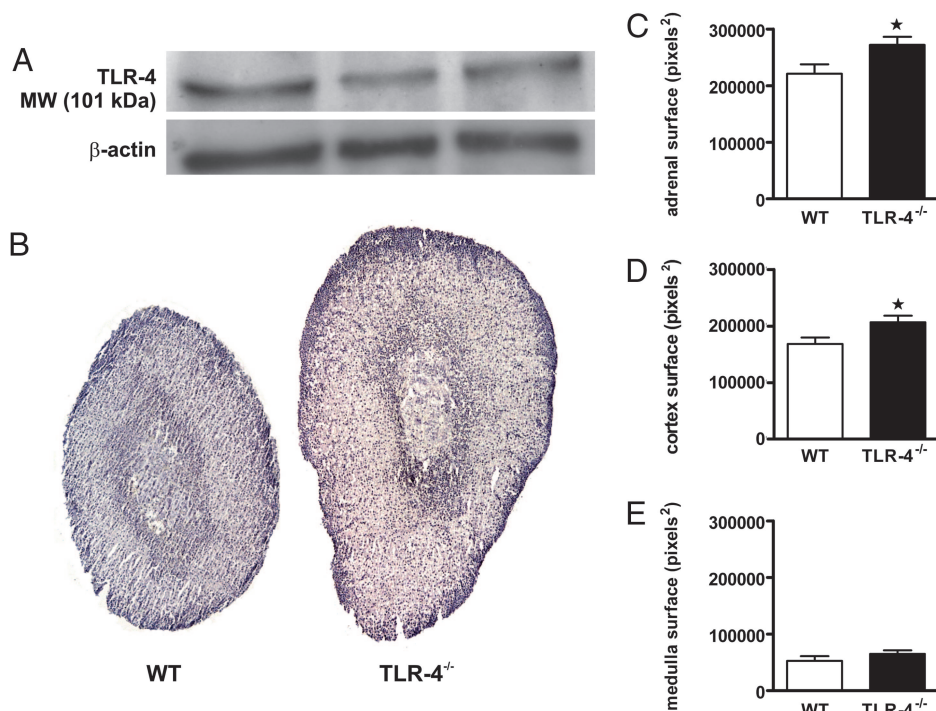
Fig. 1. Adrenal size in TLR-4$^{-/-}$ mice. (*A*) TLR-4 expression in adrenal lysates from three WT mice was analyzed by Western blotting. (*B*) Histological adrenal specimens of WT and TLR-4$^{-/-}$ mice are shown. (*C–E*) Determination of adrenal surface (*C*), cortex surface (*D*), and medulla surface (*E*) was performed in WT and TLR-4$^{-/-}$ mice (*n* = 8 per group). Data were analyzed with Student's *t* test; *, $P < 0.05$.

significantly larger ($272,000 \pm 14,000$ pixels^2) than in WT animals ($221,000 \pm 17,000$ pixels^2), as shown in Fig. 1 *B* and *C* ($P < 0.05$). This increase was caused by an enlarged adrenal cortex (Fig. 1*D*). No differences were observed in the size of the adrenal medulla between WT and TLR-4$^{-/-}$ mice (Fig. 1*E*).

During control conditions, adrenal corticosterone production in TLR4$^{-/-}$ mice (466 ± 26 ng/ml) was 4.5-fold higher ($P < 0.001$) than in WT mice (111 ± 19 ng/ml; Fig. 2*A*). In contrast, plasma

ACTH levels were similar in the two animal groups (Fig. 2*B*; $P > 0.05$).

The differences in corticosterone production were accompanied by marked morphological alterations in the adrenal cortex. In
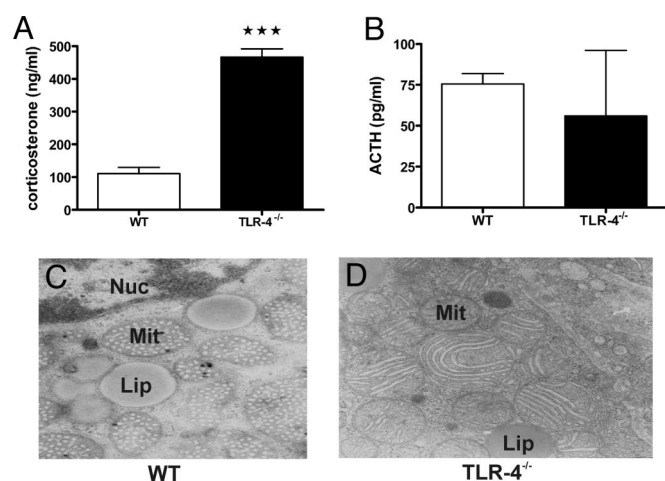

Fig. 2. Adrenal function and structure. (*A* and *B*) Corticosterone (*A*) and ACTH (*B*) levels in plasma were obtained from WT and TLR-4$^{-/-}$ mice (*n* = 8 per group). Data analysis was performed by using Student's *t* test; ***, $P < 0.001$. (*C*) An electron micrograph of adrenal cortical cells in the inner zona fasciculata in WT mice is shown. The cytoplasm is filled with characteristic round mitochondria with tubulovesicular cristae (MIT), ample smooth endoplasmic reticulum, and liposomes (LIP). (*D*) An electron micrograph of adrenocortical cells of TLR-4$^{-/-}$ mice in the zona fasciculata is shown. Mitochondria of the steroid-producing cells appear increased and more elongated with lamellar and even circular internal membranes bridging the mitochondrial matrix. Nuc, nucleus.
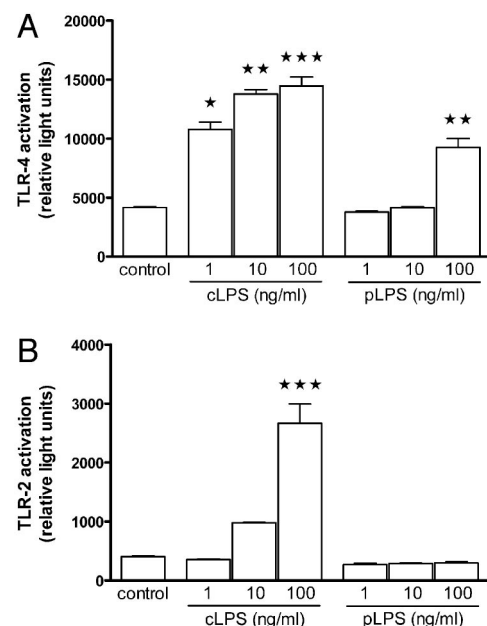
Fig. 3. Purity of LPS preparation. The purity of the LPS preparations was determined by using an NF-κB reporter gene assay in human embryonic kidney 293 cells transfected with either TLR-2 or TLR-4. (*A*) Effects of a pure (p) and a crude (c) LPS preparation (1–100 ng/ml) on NF-κB-driven luciferase activity in TLR-4-transfected cells (*n* = 3). (*B*) Effects of pLPS and cLPS (1–100 ng/ml) on luciferase activity in TLR-2-transfected cells (*n* = 3). Luciferase activity was normalized to the β-galactosidase control and is presented as relative light units. Data analysis was performed by using a one-way ANOVA followed by a Bonferroni posttest; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
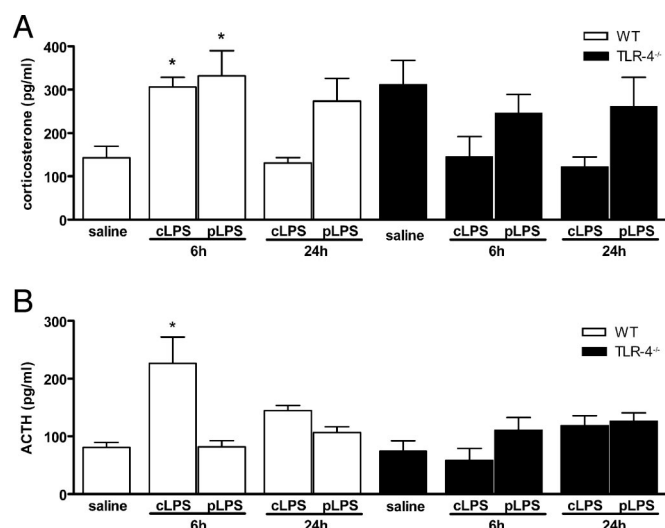
Fig. 4. Plasma corticosterone and ACTH response after LPS challenges. Plasma levels of corticosterone (*A*) and ACTH (*B*) were determined after 6 h or 24 h of i.p. treatment with 1 mg/kg cLPS or pLPS in WT animals (*n* = 8) and TLR-4$^{-/-}$ mice (*n* = 7). Data analysis was performed by using a one-way ANOVA followed by a Bonferroni posttest for each animal group; ∗, $P < 0.05$.

TLR-4$^{-/-}$ mice, endothelial cells and macrophages were found frequently in direct contact with adrenal cortical cells. In addition, contact zones between the cortex and medulla were widened, and particularly the zona reticularis appeared to be hypervascularized (data not shown). At the ultrastructural level, the most pronounced differences between WT and TLR-4$^{-/-}$ animals were found in the mitochondrial architecture (Fig. 2 *C* and *D*). The steroid-producing adrenocortical cells of WT animals revealed round mitochondria with characteristic tubovesicular cristae and some electron-opaque granules as described in ref. 25. In contrast, the 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. 2*D*). In addition, lipid-storing droplets constituting the substrates for steroidogenesis were abundant in adrenocortical cells of WT mice, but they were reduced conspicuously in TLR-4$^{-/-}$ mice.

Plasma Corticosterone and ACTH Response After LPS Challenges. Having observed an increase in corticosterone production of TLR-4$^{-/-}$ mice, we then investigated corticosterone and ACTH levels in response to LPS challenges. A comparison was carried out by using a cLPS and pLPS preparation because in the past, several observed effects of LPS have been attributed to contamination with TLR-2 ligands. We investigated carefully the purity of our LPS preparations by using a TLR-specific reporter gene assay. In TLR-4-transfected human embryonic kidney 293 cells, the commercial cLPS preparation as well as the pLPS caused a significant

increase in NF-κB-driven luciferase activity (Fig. 3*A*). In contrast, only cLPS but not pLPS induced TLR-dependent NF-κB activity in TLR-2-transfected cells (Fig. 3*B*). pLPS failed to increase luciferase activity even at a concentration of 100 ng/ml, which reflects approximately the *in vivo* dose we have used in this work. These data therefore suggest that the pLPS preparation was free of TLR-2 ligands, whereas the cLPS preparation activated both TLR-2 and TLR-4.

When cLPS and pLPS were injected into WT mice, both preparations induced a 2- to 3-fold increase in the release of adrenal corticosterone after 6 h compared with saline controls ($P < 0.05$). After 24 h, the stimulatory effect of LPS on adrenal corticosterone production decreased (Fig. 4*A*). In contrast, no elevation in the plasma levels of corticosterone was observed in TLR-4$^{-/-}$ mice, despite cLPS and pLPS challenges for 6 and 24 h (Fig. 4*A*). Similarly, plasma levels of ACTH were increased (2.5-fold) after cLPS but not pLPS treatment of WT mice (Fig. 4*B*). Like corticosterone, plasma levels of ACTH returned to baseline levels at 24 h. In TLR-4$^{-/-}$ mice, neither pLPS nor cLPS had any effect on the plasma levels of ACTH (Fig. 4*B*).

Profiles of Various Cytokines After cLPS and pLPS Challenges (6 and 24 h). We next determined the profile of various plasma cytokines in untreated and LPS-challenged WT and TLR-4$^{-/-}$ mice by employing a sensitive multiplex immunoassay. Under normal physiological conditions, control levels of the cytokines TNF-α and IL-12 were elevated significantly in TLR-4$^{-/-}$ mice (Fig. 5 *A* and *B*). IL-1β was also increased, although plasma levels were not of statistical significance (Fig. 5*C*).

When WT mice were challenged with cLPS, plasma levels of the inflammatory cytokines were increased dramatically; however, this effect was not observed with pLPS. After 6 h of treatment with cLPS, IL-1β was increased approximately by 3-fold, IL-6 by 200-fold, IL-12 by 80-fold, and TNF-α by 10-fold compared with saline treatment (Fig. 6 *A*–*D*, respectively). A similar trend was observed with the antiinflammatory cytokine IL-10. cLPS (6 h) increased the plasma levels of this cytokine (50-fold) significantly compared with the saline group (Fig. 6*E*). After 24 h of cLPS treatment, the plasma levels of all cytokines returned to levels that were similar to the pLPS and saline groups. In TLR-4$^{-/-}$ mice, neither cLPS nor pLPS had any effect on the plasma cytokines profiled. For IL-1β, cLPS appears to reduce its plasma level in TLR-4$^{-/-}$ mice, although the values were not statistically significant (Fig. 6*A*). All other cytokine levels were comparable among groups for 6 and 24 h (Fig. 6 *B*–*E*).

Adrenal Activation of NF-κB After cLPS and pLPS Challenges. NF-κB is an important regulator of proinflammatory cytokines; therefore, we compared activation of this transcription factor in adrenal extracts of WT and TLR-4$^{-/-}$ mice. In adrenal cells of WT mice, cLPS and pLPS treatment led to strong induction of NF-κB DNA binding activity, as evidenced by the appearance of a DNA–protein complex (Fig. 7). This complex was specific for NF-κB because incubation of the cell extracts with either recombinant IκB-α or a 20-fold excess of the unlabeled oligonucleotide abolished DNA
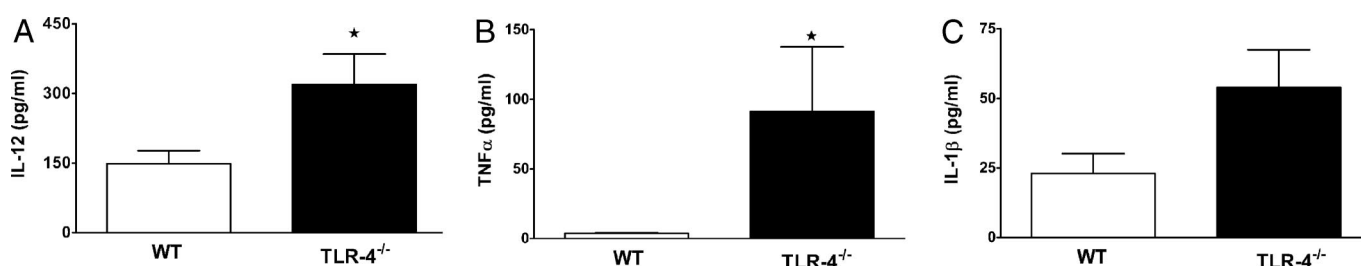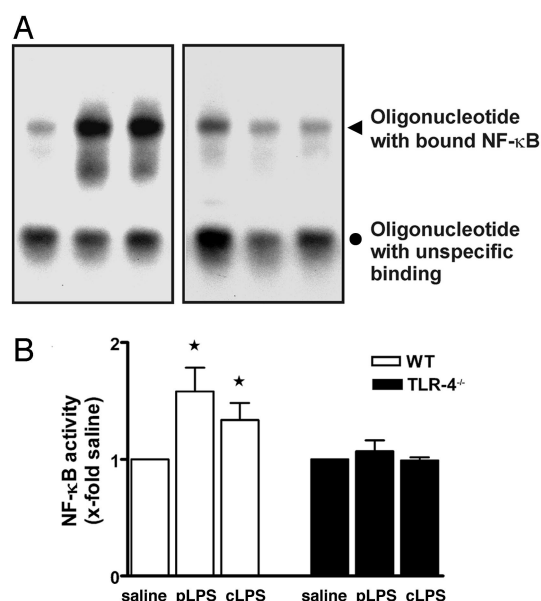

Fig. 5. Basal levels of plasma cytokines in WT and TLR-4$^{-/-}$ mice. Plasma cytokines IL-12 (*A*), TNF-α (*B*), and IL-1β (*C*) in untreated WT mice and TLR-4$^{-/-}$ mice (*n* = 5–8 per group) were determined by a multiplex microsphere-based immunoassay. Data analysis was performed by using Student's *t* test; ∗, $P < 0.05$.

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Fig. 6. Profile of plasma cytokines after LPS challenges in WT and TLR-4$^{-/-}$ mice. Plasma cytokines IL-1β (*A*), IL-6 (*B*), IL-12 (*C*), TNF-α (*D*), and IL-10 (*E*) were determined in WT and TLR-4$^{-/-}$ mice after an i.p. treatment with 1 mg/kg cLPS or pLPS or saline after 6 h or 24 h (*n* = 5–8 per group). Data analysis was performed by using a one-way ANOVA followed by a Bonferroni posttest for each animal group; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Fig. 7. Adrenal NF-κB activation in WT and TLR-4$^{-/-}$ mice. (*A*) EMSA autoradiographs of NF-κB activation in adrenal protein extracts from WT (*Left*) and TLR-4$^{-/-}$ (*Right*) mice (*n* = 5–8 per group) treated with 1 ml/kg i.p. saline or 1 mg/kg i.p. cLPS or pLPS for 1 h. DNA binding was analyzed with a ^{32}P-labeled NF-κB-specific oligonucleotide. The NF-κB–DNA complex is indicated by an arrowhead; a nonspecific DNA complex is marked by a circle. (*B*) Quantification of NF-κB activation was assessed by PhosphorImager analysis. Data were normalized to the intensity of the nonspecific DNA complex and are expressed as -fold induction of WT saline controls. Data analysis was performed by using a one-way ANOVA followed by a Bonferroni posttest; *, *P* < 0.05.

binding (data not shown). In contrast, activation of NF-κB was not detected in TLR-4$^{-/-}$ mice.

Discussion

The present work demonstrates a key role for TLR-4 in the adrenal stress response. Under control physiological conditions the adrenal gland is enlarged in TLR-4$^{-/-}$ mice, and this phenomenon presumably represents a compensatory mechanism for maintaining basal corticosterone release despite impaired adrenocortical function. It could also be argued that the altered structure of the adrenals reflects the enhanced synthesis and release of corticosterone in TLR-4$^{-/-}$ mice. Furthermore, it is possible that increased cytokine levels in TLR-4$^{-/-}$ mice stimulate the adrenal cortex directly to release corticoids. Intact steroidogenesis requires a defined spatial and conformational arrangement of mitochondria and their cristae, enabling optimal electron transfer and cytochrome P450 activity. Therefore, the mitochondrial cristae of steroid-producing cells are organized in a tubulovesicular pattern. In TLR-4-deficient mice, however, adrenocortical cells exhibit mitochondria with lamellar membranes or central dilatations. Furthermore, adrenocortical cells of TLR-4$^{-/-}$ mice show a marked reduction of liposomes, which may result in a rapid exhaustion of the adrenal lipid reserves on a massive stress stimulus such as endotoxemia.

LPS elicits its effects specifically through the activation of TLR-4 (26). The majority of previous studies using commercial LPS preparations did not take into consideration contamination with TLR-2 ligands, such as lipopeptides (23, 24). This fact raises doubts as to whether observations reported in earlier studies using LPS can be attributed solely to the TLR-4 pathway, and instead the observations may reflect the activation of TLR-2. To exclude these ambiguities, we compared the adrenal response in TLR-4$^{-/-}$ by employing two different LPS preparations. As confirmed by TLR-specific reporter assays, the pLPS preparation solely triggered

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TLR-4, but was devoid of TLR-2 agonistic activity, whereas the commercial cLPS preparation stimulated both receptors.

LPS stimulates various levels of the HPA axis, indicated by an increase in plasma ACTH and corticosterone levels (27, 28). Principally, it was thought that LPS exerts its main effects on hypothalamic corticotropin-releasing hormone stimulation by cytokine release. In turn, corticotropin-releasing hormone stimulates ACTH release from the pituitary gland (29). However, other studies have demonstrated endotoxin-stimulated effects on ACTH and corticosterone levels by corticotropin-releasing hormone-independent pathways (30, 31). Furthermore, corticosterone secretion by endotoxin is also mediated by both pituitary (ACTH stimulation) and extrapituitary mechanisms (e.g., histamine) (32, 33). There is also growing evidence to suggest that LPS elicits direct effects on adrenal cells. Human adrenal cells release cortisol by direct stimulation with LPS, an effect that is mediated by cyclooxygenase-dependent mechanisms (34, 35).

Under normal physiological conditions, 5 times higher basal corticosterone levels were observed in TLR-4$^{-/-}$ mice. The elevated corticoids levels must mean that these adrenals were being driven by some stimulus. ACTH was normal; therefore, increased ACTH could have initiated elevations. Negative feedback of the elevated levels of corticosterone returned the ACTH levels to near normal. However, in TLR-2$^{-/-}$ mice, which also have an enlarged adrenal cortex, corticosterone levels are suppressed (22). On the other hand, increased basal corticosterone levels in TLR-4$^{-/-}$ mice may also be caused by increased basal levels of IL-12 and TNF-α. In WT animals, both preparations of LPS elicited a profound effect on plasma corticosterone levels within the first 6 h; however, no effect was observed in TLR-4-deficient mice. In these mice, an inadequate response of corticosterone to pure LPS resulted from the absence of TLR-4 signaling. After treatment with cLPS, the lack of response to adrenal corticosterone could also have been caused by the abolished activation of IL-1β, TNF-α, and IL-6. These cytokines are released by peripheral immune cells in response to an endotoxin challenge, and through the activation of the HPA axis, they regulate corticosterone secretion (36, 37).

In contrast to corticosterone, the basal plasma levels of 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. The cLPS preparation used in this study contained contaminants with TLR-2 agonists. This possibility is in line with ref. 23, which demonstrated that commercial preparations of LPS often contain low amounts of impurities that can activate TLR-2 pathways. Two lipoproteins were found to be responsible for TLR-2-mediated cell activation by *Escherichia coli* LCD25 LPS (24).

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 (14). We profiled the plasma release of several cytokines during basal and LPS stimulation. Our study shows that both TNF-α and IL-12 are basally elevated in TLR-4$^{-/-}$ mice, which may provide another possible cause for elevated corticosterone levels, either by action in the hypothalamic–pituitary unit or directly on the adrenal cortex. However, increased output of these two cytokines did not alter the basal release of ACTH from the pituitary, perhaps because of negative feedback of the high basal corticoid levels in TLR-4$^{-/-}$ mice. Furthermore, another interesting observation is a marked reduction of IL-1β levels after 6 and 24 h after cLPS but not after pLPS treatment (Fig. 6). The underlying mechanisms are currently unknown, but a role for TLR-2 cannot be excluded.

Several studies have demonstrated the expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in tissue of the HPA axis (38, 39). Moreover, these cytokines regulate the hormonal

release and glucocorticoid output of the HPA axis (40). In rats, IL-1β, IL-6, TNF-α, and IL-12 are expressed in the pituitary and adrenal glands after cLPS stimulation (41). Therefore, it is not surprising that after 6 h of cLPS stimulation, all plasma cytokines were elevated in WT mice, indicating a competent inflammatory response. In addition, IL-10, which, aside from its recognized role in immunity also acts as an endogenous regulator of the HPA axis (42), was increased after a cLPS stimulus in WT animals. In contrast, pLPS had no effect in WT mice, indicating that TLR-2 signaling contributes to the observed activation of cytokines. This finding is supported by our previous work, which demonstrated that plasma levels of IL-1 and TNF-α are attenuated in TLR-2$^{-/-}$ mice but not abolished after cLPS treatment (22). In our work, the increased plasma cytokines may also have contributed to the elevated levels of ACTH.

Together with our previous work, this study reveals that during experimental conditions of systemic inflammatory response syndrome, TLR-4 is an essential mediator of the adrenal stress response. The absence of TLR-4 impairs the HPA axis primarily at the adrenal level, providing further evidence that LPS mediates its effects directly on adrenal cells; however, this impairment does not appear to compromise the phenotype of TLR-4$^{-/-}$ mice. Basal alterations in adrenal structure and corticosterone and cytokine activity suggest a functional role for TLR-4 in the HPA axis. Taking these results together with our previous findings, TLR-2 and -4 are shown to be key players in the immune and endocrine stress systems during inflammation. Mutations in the innate immune system are not rare events, and TLR polymorphisms may contribute to the underlying mechanism for impairment of the adrenal stress response in patients with sepsis.

Materials and Methods

Animals and Treatments. TLR-4$^{-/-}$ mice were generated by homologous recombination (43). WT (C57BL/6) and TLR-4$^{-/-}$ mice were housed under standard conditions (55% relative humidity, 12-h day–night rhythm, standard chow, and water ad libitum). All procedures were carried out in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International guidelines and *Guide for the Care and Use of Laboratory Animals* (44) and were approved by German government ethical and research boards. Animals (12–16 weeks old) were randomized ($n = 8$ per group) and treated with an i.p. injection for 6 or 24 h with 1 mg/kg saline, 1 mg/kg cLPS (*E. coli*; serotype O111:B4; Sigma–Aldrich), or a highly purified preparation of 1 mg/kg pLPS (*E. coli*; F515 LPS SB III,66). This dose of LPS was used to mimic the conditions of systemic inflammatory response syndrome. After each treatment, 0.5-ml blood samples were taken by aortic puncture under sodium pentobarbital anesthesia, and the adrenal glands were removed for analysis. Animals were killed by a terminal dose of sodium pentobarbital. Saline 6- and 24-h groups demonstrated similar values throughout the experiments; therefore, a saline control represents both groups.

Purity of LPS Preparation. To determine the purity of our LPS preparations we used a TLR-specific NF-κB reporter gene assay (45). Human embryonic kidney 293 cells stably expressing CD14 were cultured in DMEM supplemented with 10% heat-inactivated FCS/2 mM glutamine/50 μg/ml each penicillin and streptomycin. Cells were plated in triplicate onto 12-well plates with 1×10^5 cells per well. Transfection was performed by using FuGENE 6 reagent (Roche Diagnostics) with an NF-κB-controlled luciferase construct (120 ng), Rous sarcoma virus β-galactosidase (40 ng), and expression plasmids for either human TLR-2 or TLR-4 plus MD-2 (40 ng each). Twenty-four hours after transfection, cells were stimulated with a pLPS or cLPS preparation (0, 1, 10, or 100 ng/ml). After a further 20-h incubation, the activities of luciferase and β-galactosidase (which was used as a marker for transfection efficiency) were determined in cell extracts by using a chemiluminescence-based

assay (Roche Diagnostics). Luciferase activities were calculated and normalized to the β-galactosidase control.

Western Blotting. Tissues were lysed in ice-cold protein extraction buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.4/1 mM EDTA/5 μg/ml leupeptin/5 μg/ml aprotinin A/1 mM PMSF/0.1% SDS/1% sodium deoxycholate/1% Triton X-100). After a brief centrifugation, the supernatant was removed. Total protein was determined (Bradford assay), separated by SDS/PAGE, and blotted onto nitrocellulose membrane. The blots were probed with anti-TLR-4 antibody (1/1,000, L-14; Santa Cruz Biotechnology) and with horseradish peroxidase-conjugated anti-goat secondary antibody (1/3,000; Santa Cruz Biotechnology). After extensive washing, protein bands were visualized by enhanced chemiluminescence staining. Blots were also probed with anti-β-actin (Sigma–Aldrich; 1/3,000) to confirm equal loading of protein.

Morphometric Analysis. To determine the size of adrenal sections, morphometric analysis was performed by using a computer-supported imaging system connected to a light microscope (Eclipse TE300, LUCIA G Software, Nikon, and Jerome Industry Phototype P99135 camera, Digital Video Camera, Austin, TX). The area of several sections was measured in triplicate. The four largest sections were evaluated for an approximation of the longest diameter in each gland.

Electron Microscopy. Adrenal glands were fixed in 0.1 M phosphate buffer at pH 7.3 with 2% (vol/vol) formaldehyde and glutaraldehyde. Tissue slices were postfixed for 90 min (2% OsO_4 in 0.1 M cacodylate buffer, pH 7.3), dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at 80 kV in a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Plasma Corticosterone and ACTH. Plasma levels of corticosterone and ACTH were determined quantitatively with an RIA (Diagnostic Systems Laboratories, Webster, TX), as reported in ref. 22. The inter- and intraassay coefficient of variation for corticosterone was 4.9% and 4.1%, and for ACTH, it was 4.0% and 5.9%, respectively.

Plasma Cytokines. Plasma levels of IL-1β, -6, -10, and -12 and TNF-α (Mouse Cytokine multiPlex for Luminex laser; BioSource Europe, Nivelles, Belgium) were determined by using the microsphere array technique (Luminex 100 system; Luminex, Austin, TX). Assays were performed according to the manufacturer's protocols with an interassay coefficient of variation ranging from 5.2% to 10.4% for all of the different cytokines.

EMSA. Adrenal extracts were prepared in a high-salt buffer [20 mM Hepes, pH 7.9/350 mM NaCl/20% (vol/vol) glycerol/1% Nonidet P-40/1 mM $MgCl_2$/0.5 mM EDTA/0.1 mM EGTA/0.5 mM DTT/1 mM PMSF/2 μg/ml aprotinin/2 μg/ml leupeptin]. The extracts were cleared by centrifugation ($17,500 \times g$, 20 min, 4°C) and assayed for protein by the Bradford method. Equal amounts of protein (5 μg) were incubated at room temperature for 20 min with the ^{32}P-end-labeled NF-κB-specific oligonucleotide (Promega) in a 20-μl volume containing 4 μl of extract, 4 μl of 5× binding buffer [50 mM Hepes, pH 7.5/50 mM KCl/1 mM DTT/2.5 mM $MgCl_2$/50% (vol/vol) glycerol], 1 μg of poly(dI-dC), and 2 μg of BSA. The samples were separated on nondenaturing 4% polyacrylamide gels and quantified by PhosphorImager analysis.

Statistical Analysis. Data were analyzed by using Student's *t* test or one-way ANOVA in PRISM (GraphPad, San Diego). Results are presented as the mean ± SEM. Statistical significance was defined as follows: ∗, $P < 0.05$; ∗∗, $P < 0.01$; or ∗∗∗, $P < 0.001$.

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