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Diverse action of lipoteichoic acid and lipopolysaccharide on neuroinflammation, blood-brain barrier disruption, and anxiety in mice

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ABSTRACT

Microbial metabolites are known to affect immune system, brain, and behavior via activation of pattern recognition receptors such as Toll-like receptor 4 (TLR4). Unlike the effect of the TLR4 agonist lipopolysaccharide (LPS), the role of other TLR agonists in immune-brain communication is insufficiently understood. We therefore hypothesized that the TLR2 agonist lipoteichoic acid (LTA) causes immune activation in the periphery and brain, stimulates the hypothalamic-pituitary-adrenal (HPA) axis and has an adverse effect on blood-brain barrier (BBB) and emotional behavior. Since LTA preparations may be contaminated by LPS, an extract of LTA ($LTA_{extract}$), purified LTA (LTA_{pure}), and pure LPS ($LPS_{ultrapure}$) were compared with each other in their effects on molecular and behavioral parameters 3 h after intraperitoneal (i.p.) injection to male C57BL/6N mice.

The $LTA_{extract}$ (20 mg/kg) induced anxiety-related behavior in the open field test, enhanced the circulating levels of particular cytokines and the cerebral expression of cytokine mRNA, and blunted the cerebral expression of tight junction protein mRNA. A dose of $LPS_{ultrapure}$ matching the amount of endotoxin/LPS contaminating the $LTA_{extract}$ reproduced several of the molecular and behavioral effects of $LTA_{extract}$. LTA_{pure} (20 mg/kg) increased plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 and interferon-γ, and enhanced the transcription of TNF-α, interleukin-1β and other cytokines in the amygdala and prefrontal cortex. These neuroinflammatory effects of LTA_{pure} were associated with transcriptional down-regulation of tight junction-associated proteins (claudin 5, occludin) in the brain. LTA_{pure} also enhanced circulating corticosterone, but failed to alter locomotor and anxiety-related behavior in the open field test.

These data disclose that TLR2 agonism by LTA causes peripheral immune activation and initiates neuroinflammatory processes in the brain that are associated with down-regulation of BBB components and activation of the HPA axis, although emotional behavior (anxiety) is not affected. The results obtained with an LTA preparation contaminated with LPS hint at a facilitatory interaction between TLR2 and TLR4, the adverse impact of which on long-term neuroinflammation, disruption of the BBB and mental health warrants further analysis.

1. Introduction

There is abundant evidence that bacterial infection of peripheral tissues causes innate immune cells to produce pro-inflammatory cytokines which act on the brain to cause sickness as well as molecular and behavioral perturbations (Dantzer et al., 2008). The immune system senses bacterial intrusion via pattern recognition receptors (PRRs) which recognize evolutionarily highly conserved structures on pathogens, so-called pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) represent the best characterized family of PRRs which are expressed on the

Abbreviations: ACTB, beta actin; BBB, blood-brain barrier; CA, central area; CCL2, chemokine (CC motif) ligand 2; CLDN5, claudin 5; DMSO, dimethyl sulfoxide; EU, endotoxin unit; GAPDH, glycerinaldehyde-3-phosphate-Dehydrogenase; HEK, human embryonic kidney; HPA, hypothalamic-pituitary-adrenal; hTLR, human Toll-like receptor; IFN, interferon; IL, interleukin; i.p., intraperitoneal; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NOD, nucleotide-binding oligomerization domain; OCLN, occludin; PAMP, pathogen associated molecular patterns; PFCT, prefrontal cortex; PPIL3, peptidyl-prolyl cistrans isomerase-like 3; PRR, pattern recognition receptor; TJP1, tight junction protein 1; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

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cell surface and thus can initiate a first-line immune response against invading pathogens (Medzhitov et al., 1997). Toll-like receptor 4 (TLR4), for instance, is responsible for the recognition of lipopolysaccharide (LPS) on the cell wall of gram-negative bacteria (Poltorak et al., 1998). Upon binding of LPS to TLR4, which requires the presence of myeloid differentiation 2, the signaling cascade targets myeloid differentiation primary response protein 88 and results in the release of pro-inflammatory cytokines (Kawai et al., 1999). If LPS is endocytosed, the TRIF-related adaptor molecule/TIR-domain-containing adapter-inducing interferon-β pathway is activated and causes release of type 1 interferons (Kawai and Akira, 2010). Through these immune mediators, LPS is known to cause sickness and evoke signs of anxiety- and depression-like behavior in rodents (Bluthé et al., 1994; O'Connor et al., 2009; Painsipp et al., 2010; Sulakhiya et al., 2016). In humans, symptoms of depression and anxiety are correlated with LPS exposure and subsequent cytokine release (Vogelzangs et al., 2016), and increased IL-6 and INF-α levels correlate with severity of depression and anxiety (Capuron et al., 2009; Raison et al., 2006).

The family of TLRs comprises 12 members (10 in humans) (Pandey et al., 2015) which are targeted by different PAMPs (Kawai and Akira, 2010). Under conditions of bacterial invasion it is likely that different PRRs are activated in parallel and that the ensuing immune and brain responses are the result of the positive and/or negative interactions between the PRR-mediated reactions. For instance, the sickness response to LPS is enhanced by synergism between TLR4 and the nuclear-binding domain (NOD)-like receptors NOD1 and NOD2, which recognize peptidoglycan elements (Farzi et al., 2015b). In contrast, lipoteichoic acid (LTA) is a major cell wall component of gram-positive bacteria and a PAMP that is primarily recognized by Toll-like receptor 2 (TLR2) (Hermann et al., 2002). LTA is a surface-associated adhesion amphiphile composed of a soluble polymer, consisting of polyhydroxy alkane units, such as ribitol and glycerol, attached to the cell membrane with a diacylglycerol. The sequence of glycerol and ribitol repeat units varies between species (Schneewind and Missiakas, 2014). Bacteriolysis leads to the release of LTA into the bloodstream, which occurs in response to β-lactam antibiotic treatment (Van Langevelde et al., 1998). LTA induces the secretion of cytokines such as IL-1β and TNF-α, which can contribute to the disruption of the blood-brain barrier (BBB) (Boveri et al., 2006). In addition, LTA is required for anchoring microorganisms to brain microvascular endothelial cells that disrupt the BBB (Sheen et al., 2010).

Despite the deleterious impact mediated by LTA on BBB function, the effects of this PAMP on molecular changes in the immune-brain axis and on behavior have not yet been explored. As concerns regarding the contamination of commercial LTA preparations by LPS have been raised (Gao et al., 2001; Morath, 2001), the present study was conducted with an extract ($LTA_{extract}$) and a purified preparation of LTA (LTA_{pure}). The effects of these LTA preparations on the immune-brain axis were compared with the effects of ultrapure LPS ($LPS_{ultrapure}$). The first specific aim was to examine whether behavior in the open field, indicative of sickness and/or anxiety, is affected by intraperitoneally (i.p.) injected LTA from *Bacillus subtilis*. The second aim was to examine the effect on immune activation in the periphery and brain as reflected by the expression of cytokines in the plasma, amygdala and prefrontal cortex. Given that the HPA axis is activated by extrinsic and intrinsic stressors (Borrow et al., 2016) including immune activation (Farzi et al., 2015b; Lehmann et al., 2013), the third aim was to assess the effect of LTA on circulating corticosterone. The fourth aim was to evaluate the potentially deleterious effect of LTA on BBB composition by studying the transcriptional regulation of tight junction-associated proteins in the amygdala and prefrontal cortex.

2. Methods and materials

2.1. Experimental animals

The experiments were performed with 10-week-old male C57BL/6N mice (n = 188; 22–27 g body weight) obtained from Charles River (Sulzfeld, Germany). The animals were housed in pairs in a vivarium under controlled conditions: temperature set point at 22 °C, air humidity set point at 50% and a 12 h light/dark cycle. Tap water and standard laboratory chow were provided *ad libitum* throughout the experiment.

2.2. Ethics statement

The experimental procedure and number of animals used were approved by the ethical committee at the Federal Ministry of Science, Research, and Economy of the Republic of Austria (BMWF-66.010/0026-WF/II/3b/2014) and conducted according to the Directive of the European Parliament and of the Council of September 22, 2010 (2010/63/EU). The experiments were designed in such a way that both the number of animals used and their suffering was minimized.

2.3. Reagents

LTA from *Bacillus subtilis* was obtained from two different vendors (Sigma-Aldrich, Vienna, Austria, catalog number L3265, from here on referred to as $LTA_{extract,}$ and Invivogen, Toulouse, France, catalog number tlrl-lta, from here on referred to as LTA_{pure}). LPS from *Escherichia coli* O111:B4 extracted by successive enzymatic hydrolysis steps and purified by the phenol-TEA-DOC extraction protocol ($LPS_{ultrapure}$) was obtained from Invivogen (catalog number tlrl-3pelps). For TLR4 antagonism experiments, the TLR4 antagonist TAK-242 was used (Calbiochem/Merck Millipore, Darmstadt, Germany; catalog number US1614316, resatorvid, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate) (Ii et al., 2006; Kawamoto et al., 2008).

2.4. Activation of TLR2 and TLR4 in the HEK-Blue® reporter cell assay

HEK-Blue® (Invivogen, Toulouse, France) hTLR2 and hTLR4 cells were grown in Dulbecco's Modified Eagle Medium (Gibco, ThermoFisher, Waltham, MA, USA) containing 4.5 g/l glucose, 2 nM L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin and 1 x HEK-Blue® Selection at 37 °C and 5% CO_2. After confluency was reached, cells were seeded into 24-well plates, 2.5×10^5 cells/well. Cells were then treated with 10^2 pg/ml, 10^4 pg/ml, or 10^6 pg/ml of LTA_{pure}, $LTA_{extract}$, or $LPS_{ultrapure}$ and incubated for 24 h. Sterile, distilled H_2O was used as control.

To assess the TLR4 specificity of the agonists, cells were incubated overnight (12 h) with 3 μM TAK-242 dissolved in DMSO. DMSO was used as control at a concentration that did not exceed 0.2%. Following the overnight treatment with TAK-242 or DMSO, $LTA_{extract}$ (10^6 pg/ml) or $LPS_{ultrapure}$ (10^4 pg/ml, or 10^6 pg/ml) was added, after which the cells were incubated for 24 h.

For quantitation of TLR2 and TLR4 activation, 180 μl of HEK-Blue® Detection medium was added to a 96-well plate, and 20 μl of supernatant from the treated cells was added. Alkaline phosphatase activity was subsequently measured with a Victor plate reader (PerkinElmer, Rodgau, Germany) at 655 nm.

2.5. Quantitation of endotoxin in $LTA_{extract}$ with the EndoLISA® endotoxin detection assay

To determine the amount of endotoxin present in $LTA_{extract}$, the EndoLISA® (Hyglos, Bernried am Starnberger See, Germany)