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# Lack of Toll-like receptor 2 results in higher mortality of bacterial meningitis by impaired host resistance

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ABSTRACT

Bacterial meningitis is - despite therapeutical progress during the last decades - still characterized by high mortality and severe permanent neurogical sequelae. The brain is protected from penetrating pathogens by both the blood–brain barrier and the innate immune system. Invading pathogens are recognized by so-called pattern recognition receptors including the Toll-like receptors (TLR) which are expressed by glial immune cells in the central nervous system. Among these, TLR2 is responsible for the detection of Gram-positive bacteria such as the meningitis-causing pathogen *Streptococcus pneumoniae*. Here, we used TLR2-deficient mice to investigate the effects on mortality, bacterial growth and inflammation in a mouse model of pneumococcal meningitis.

Our results revealed a significantly increased mortality rate and higher bacterial burden in TLR2-deficient mice with pneumococcal meningitis. Furthermore, infected TLR2-deficient mice suffered from a significantly increased pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and Chemokine (C-C motif) ligand 2 (CCL2) or CCL3 chemokine expression and decreased expression of anti-inflammatory cytokines and antimicrobial peptides. In contrast, glial cell activation assessed by glial cell marker expression was comparable to wildtype mice.

Taken together, the results suggest that TLR2 is essential for an efficient immune response against *Streptococcus pneumoniae* meningitis since lack of the receptor led to a worse outcome by higher mortality due to increased bacterial burden, weakened innate immune response and reduced expression of antimicrobial peptides.

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## 1. Introduction

Bacterial meningitis is a life-threatening infection which is characterized by strong inflammation and neuronal damage with high mortality and long-term sequelae affecting up to 50% of survivors (Mook-Kanamori et al., 2011). The entering of pathogens into the subarachnoid space after crossing the blood-brain-barrier (BBB) leads to release of pro-inflammatory compounds by autolysis and secretion of mediators which enhance inflammation further and attract immune cells. Next to the direct toxic effects of the bacteria, the immune response also contributes to CNS injury (Gerber and Nau, 2010). The main effector cells of the innate immune response within the CNS are the glial cells. Resident microglia and astrocytes exert multiple functions including protective and restorative effects in response to CNS infection or injury (Ransohoff and Brown, 2012). Among the liberation of pro- and anti-inflammatory mediators such as cytokines as well as antimicrobial peptides (AMP) as endogenous antibiotics and immunostimulatory mediators, glial cells control and orchestrate the course of the immune response in the CNS (Brandenburg et al., 2012, 2009, 2008; Farina et al., 2007; Mariani and Kielian, 2009). For the initiation of the innate immune response by glial cells, the detection of pathogens is a prerequisite. Immune cells are able to recognize an infinite antigenic repertoire of broadly defined molecular motifs from pathogens. These molecular determinants, so-called pathogen-associated molecular patterns (PAMP) are conserved patterns which are sensed by pattern recognition receptors (PRR) that comprise multiple receptor families located in both extracellular and intracellular milieus. In addition to detecting molecular patterns associated with different microorganisms, PRR have also been implicated in recognizing an array of endogenous molecules termed danger-associated molecular patterns (DAMP) (Hanke and Kielian, 2011). These include receptor families such as the Toll-like receptors. The TLR family members, 13 described in mice and 11 in humans, can be distinguished by their subcellular localization and motif spectrum (Konat et al., 2006). To date, TLR2 and TLR4 are the

*Abbreviations:* AMP, antimicrobial peptide; CRAMP, cathelicidin-related antimicrobial peptide; Defb4, b-defensin 4; FPR, formyl peptide receptor; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule 1; Itgam, integrin alpha M; KO, knock out; TLR, Toll-like receptor; WT, wildtype.

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best characterized receptors which recognize the microbial motif peptidoglycan/lipoprotein/dectin (PGN) and lipopolysaccharide (LPS), respectively. Other TLR like TLR9 recognize non-methylated CpG motifs of bacterial and viral DNA (Brandenburg et al., 2013; Hanke and Kielian, 2011; Kumagai et al., 2008). All glial cells (microglia, astrocytes as well as oligodendrocytes) have been shown to express TLR but they differ in the distribution and expression of the receptors (Bottcher et al., 2003; Konat et al., 2006). The importance of TLR for the immune response in bacterial meningitis was recognized by publications revealing that lack of TLR2 or polymorphisms in TLR 2, 4 or 9 genes can lead to an unfavorable course of bacterial meningitis in both rodents and humans (Echchannaoui et al., 2002; Klein et al., 2008; Koedel et al., 2003; Sanders et al., 2011, 2012; van Well et al., 2012).

Therefore, this study was designed to complete and expend the data of previous works to the effects of TLR2 deficiency in a model of pneumococcal meningitis. We investigated the course of the infectious disease by evaluating the mortality rate, bacterial growth, and meningeal neutrophil granulocyte infiltration of $TLR2^{-/-}$ and wild type (WT) mice and characterized the innate immune response by investigating changes in glial cell activity and expression of AMP as well as pro- or anti-inflammatory marker expression.

## 2. Materials and methods

### 2.1. Animals

$TLR2^{-/-}$ or WT mice were bred in the Central Animal Care Facility of the RWTH Aachen University. $TLR2^{-/-}$ mice have been originally presented by Werts et al. (2001) and provided by Reiling et al. (2002). The strain has a C57BL/6 background. WT mice were backcrossed on the C57B/6J background for at least five generations.

### 2.2. Mouse model of experimental pneumococcal meningitis (Gerber et al., 2001)

$TLR2^{-/-}$ or WT mice (weight 19–23 g, aged 2–3 months) were anaesthetised with ketamine (100 mg/kg) and xylazine (20 mg/kg) and infected by injecting $10^4$ colony-forming units (CFU) of a *Streptococcus pneumoniae* D39 (type 2) strain in the subarachnoid space through the right frontolateral skull. Uninfected control animals were injected with 10 μl of a sterile saline solution. All infected mice developed clinical signs of infection within 24 h. In the first set of experiments (mortality groups, $n = 14$ for $TLR2^{-/-}$ and $n = 17$ for WT), the clinical state and spontaneous survival time were closely monitored without antibiotic treatment. In a second set of experiments with the same infection regime (histological and gene expression group, $n = 5$ each group), mice were infected and sacrificed 12 or 30 h after induction of meningitis without antibiotic treatment and were either perfused with 4% formalin for immunohistochemical analysis or with 0.9% NaCl solution for RNA isolation. Bacterial titers were evaluated 30 h after infection in tissue homogenates of cerebellum and spleen and in blood samples by plating 10-fold dilutions on blood agar plates and incubation for 24 h at 37 °C and 5% $CO_2$ (detection limit $10^2$ CFU/ml in tissue homogenates and $10^3$ CFU/ml in blood samples (Merres et al., 2014, Schmidt et al., 2014)). All animal experiments were approved by the Animal Care Committee of the University Hospital of Aachen and by the District Government in Recklinghausen, North Rhine-Westphalia, Germany.

### 2.3. Meningeal inflammation score

Meningeal inflammation was estimated by the invasion of granulocytes into the frontal interhemispheric region, the whole hippocampal fissure (both sides), 3 superficial meningeal regions over the convexities, and the third ventricle (complete distribution) 30 h after infection. One high power field (20 × objective) was scored in each region: no granulocytes: 0; <10 granulocytes: 1; 10 to 50 granulocytes: 2; and >50 granulocytes: 3. The scores of the individual regions were added (range of the score: 0–21) (Oldekamp et al., 2014; Tauber et al., 2005).

### 2.4. Immunohistochemistry

Formalin-fixed and paraffin-embedded 5-μm whole coronary brain sections were examined. For immunofluorescence staining, sections were deparaffinized, for Iba-1 staining pre-treated for 10 min with microwaving in Tris/EDTA/Tween 20 buffer and after blocking with 5% normal goat serum in PBS incubated with either polyclonal rabbit anti-GFAP (1:9000; EnCor, Gainesville, FL, USA) or anti-Iba1 (1:10,000; Wako, Neuss, Germany) overnight at 4 °C. This was followed by incubation with the biotinylated secondary antibody (1:400; DAKO, Hamburg, Germany) and peroxidase-labelled streptavidin-biotin staining technique. For staining, Diaminobenzidine (DAB) was used. After counterstaining with hematoxylin, the slides were finally mounted with Aquatex (Boehringer, Mannheim, Germany). For neutrophil granulocyte staining, slices were stained with naphthol AS-D chloroacetate esterase (91-C Kit; Sigma-Aldrich) according to the manufacturer's protocol.

### 2.5. Quantification of immunoreactive cells

The sections were examined blinded using a 10 × objective up to a 40 × objective. Only immunoreactive cells within the hippocampal formation were counted. An Analysis Software Imaging System (microscope Keyence BZ-9000; Keyence, Neu-Isenburg, Germany) was used to measure the area of the hippocampal formation. The densities of immunolabelled cells were expressed as the number of marked cells per square millimeter of the area measured. The density of labelled cells was evaluated in 4 coronal sections from each mouse.

### 2.6. RNA isolation and realtime RT-PCR

Total RNA was isolated using the peqGold Trifast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA samples were reverse-transcribed by moloney murine leukemia virus (MMLV) reverse transcriptase (Fermentas, Burlington, Canada) and random hexamer primers (Invitrogen, Darmstadt, Germany). The cDNA products were used immediately for SYBR green (Applied Biosystems, Darmstadt, Germany) real-time RT-PCR. Gene expression was monitored using the StepOne Plus apparatus (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. Relative quantification was performed using the ΔCt method which results in ratios between target genes and a housekeeping reference gene index including (TATA box binding protein (TBP)). The primer for Glial fibrillary acid protein (GFAP), Integrin alpha M (Itgam), cathelin-related antimicrobial peptide CRAMP, Formyl peptide receptor 1 (FPR1) and 2 (FPR2) and Defensin b4 (Defb4) were manufactured by Qiagen (QT00101143, QT00156471, QT00156571, QT01165899, QT00171514 and QT00257656; QuantiTect Primer Assay; Qiagen, Hilden, Germany). The primer for TBP, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1RA), hemoxygenase 1 (HO-1), chemokine (C-C motif) ligand 2 (CCL2), CCL3 (Buschmann et al., 2012) were manufactured by Eurofins MWG Operon (Ebersberg, Germany; for primer sequences for TBP, TNF-α and IL-6 see Merres et al., 2014; for IL-1RA and HO-1 see Oldekamp et al., 2014). The specificity of the amplification reaction was determined by melting curve analysis. Amplification efficiency was calculated with the LinRegPCR software package (version 12.7).

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