Chapter 2

Long-lasting pro-inflammatory suppression of microglia by LPS-preconditioning is mediated by RelB-dependent epigenetic silencing.

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Abstract

Microglia, the innate immune cells of the central nervous system (CNS), react to endotoxins like bacterial lipopolysaccharides (LPS) with a pronounced inflammatory response. To avoid excess damage to the CNS, the microglia inflammatory response needs to be tightly regulated. Here we report that a single LPS challenge results in a prolonged blunted pro-inflammatory response to a subsequent LPS stimulation, both in primary microglia cultures (100 ng/ml) and in vivo after intraperitoneal (0.25 and 1 mg/kg) or intracerebroventricular (5 μg) LPS administration. Chromatin immunoprecipitation (ChIP) experiments with primary microglia and microglia acutely isolated from mice showed that LPS preconditioning was accompanied by a reduction in active histone modifications AcH3 and H3K4me3 in the promoters of the IL-1β and TNF-α genes. Furthermore, LPS preconditioning resulted in an increase in the amount of repressive histone modification H3K9me2 in the IL-1β promoter. ChIP and knock-down experiments showed that NF-κB subunit RelB was bound to the IL-1β promoter in preconditioned microglia and that RelB is required for the attenuated LPS response. In addition to a suppressed pro-inflammatory response, preconditioned primary microglia displayed enhanced phagocytic activity, increased outward potassium currents and nitric oxide production in response to a second LPS challenge. In vivo, a single i.p. LPS injection resulted in reduced performance in a spatial learning task four weeks later, indicating that a single inflammatory episode affected memory formation in these mice. Summarizing, we show that LPS-preconditioned microglia acquire an epigenetically regulated, immune-suppressed phenotype, possibly to prevent excessive damage to the central nervous system in case of recurrent (peripheral) inflammation.
Introduction

Microglia are the principal innate immune effector cells of the CNS and constantly monitor their environment for homeostatic disturbances (Nimmerjahn et al., 2005). When exposed to infection or tissue damage, microglia become activated and in this executive state, they release increased amounts of cytokines, chemokines and neurotrophic factors along with changes in migration capacity and increased phagocytosis (Kettenmann et al., 2011). For CNS surveillance, microglia are equipped with a broad range of pattern recognition receptors, including the Toll-like receptors (TLRs), complement receptors, Fc receptors and scavenger receptors (Kettenmann et al., 2011; Lu et al., 2008; Lund et al., 2006; Nahid et al., 2011). To prevent an exacerbated inflammatory reaction, the response of (innate) immune cells needs to be tightly regulated (Foster et al., 2007). Exposure of innate immune cells to a microbial agent, such as LPS of Gram-negative bacteria, can lead to a significantly altered inflammatory response upon re-exposure to the agent, a phenomenon called endotoxin tolerance (ET). ET is observed in a variety of human diseases including hepatic ischemia and cystic fibrosis and has been associated with peripheral innate immune suppression and increased mortality (Biswas and Lopez-Collazo, 2009). ET has also affects the central nervous system; in 30% of septic intensive care unit patients suffering from septic encephalopathy, persistent cognitive dysfunction in verbal learning and memory as well as left hippocampal atrophy was observed (Semmler et al., 2013).

In monocytes/macrophages, it has been shown that TLR4 is critically involved in ET. TLR4 signals through MyD88- and TRIF-dependent pathways and in ET, MyD88/NF-κB signaling is inhibited leading to reduced pro-inflammatory cytokine expression. In macrophages, LPS/TLR4-induced ET is mediated by RelB, an NF-κB subunit known to suppress inflammatory gene expression through recruitment of a transcription-repressive complex (Chen et al., 2009). ET in human pro-monocytes is accompanied by RelB-mediated recruitment of the histone H3 lysine 9 methyl-transferase G9a (KMT1c), which leads to H3K9 dimethylation (H3K9me2) and recruitment of the DNA methyl-transferase DNMT3a/b, ultimately inducing heterochromatin formation and stable TNF-α and IL-1β gene silencing (El Gazzar et al., 2009, 2008; Kondilis-Mangum and Wade, 2012). In addition to epigenetic silencing, miRNAs have been implicated in ET in monocytes/macrophages. LPS-induced miRNA-146a has been shown to target IRAK1 and TRAF6, forming a negative feedback loop in NF-κB-dependent pro-inflammatory gene transcription (reviewed in Nahid et al., 2011). Furthermore, miRNA-146a regulates a pathway leading to RelB-dependent heterochromatin formation and silencing of the TNF-α promotor (El Gazzar et al., 2011). Other miRNAs reported to be involved in ET are miRNA-155, miRNA-221, miRNA-125b, miRNA-132 and miRNA-579 (Biswas and Lopez-Collazo, 2009; El Gazzar and McCall, 2010; Nahid et al., 2011; Quinn et al., 2012). In vitro pre-exposure of hippocampal slices to LPS has been reported to result in a blunted pro-inflammatory microglia response and to induce a more anti-inflammatory phenotype (Ajmone-
Cat et al., 2013). *In vivo*, LPS-preconditioning is associated with neuroprotection in animal models for ischemia (retinal ischemia and middle cerebral artery occlusion) (Halder et al., 2013; Rosenzweig et al., 2004). In addition, experimentally-induced sepsis and perinatal exposure to LPS have been related to detrimental effects on development and on learning and memory (Graciarena et al., 2010; Lin et al., 2012; Semmler et al., 2007). These reports show that exposure to LPS has long lasting effects on brain physiology, reducing hippocampal volume, causing neuronal loss within the hippocampus and prefrontal cortex and reduced cholinergic innervation in the parietal cortex, which can be linked to deficits in learning and memory (Graciarena et al., 2010; Lin et al., 2012; Semmler et al., 2007). The aim of our study was to unravel the molecular basis of reduced LPS sensitivity of pre-exposed microglia and if epigenetic alterations underlie this altered sensitivity.
Materials and methods

Animals
Wild-type, male C57Bl/6JolaHsd mice (7-9 weeks) were purchased from Harlan (Harlan, Horst, the Netherlands). All experiments were performed according to the experimental animal guidelines of the University of Groningen. Animals were housed under normal conditions in a 12/12 hr light/dark cycle and fed ad libitum in the central experimental animal facility of the University of Groningen. Animals were housed individually and randomly assigned to experimental conditions.

Primary microglia culture
Primary neonatal microglia were isolated from brains of postnatal day 1-3 C57Bl/6 mice (both female and male pups were used). After removal of the meninges and brain stem, the brains were minced and washed in dissection medium (Hanks bovine salt serum, PAA, Cat.nr. H15-010; D- (+)-Glucose solution, Sigma, Cat.nr. G8769; HEPES, PAA, 311-001) and incubated in dissection medium supplemented with 2.5% trypsin for 20 min. The trypsin treatment was stopped by addition of trypsin inhibition medium followed by washing with dissection medium supplemented with 10% FCS and 0.5 µg/ml DNase1. Cells were triturated using a glass pipette in 25 ml complete medium (Dulbecco’s Modified Eagle Medium (DMEM); Gibco, Cat.nr. 41965-039, supplemented with 10% FCS, 1mM sodium pyruvate and 1% pen/strep) and centrifuged for 12 min, 165 g at 12°C. After centrifugation, the cell pellet was resuspended in standard complete medium and plated as 1.5 brains per T75 culture flask. Medium was refreshed after 2 days and thereafter every 4 days. After 6-7 days of culture, medium was supplemented with 33% L929 fibroblast-conditioned medium to stimulate microglia proliferation. To obtain M-CSF containing L929 fibroblast-conditioned medium, 30 ml of normal medium was added to 80% confluent L929 cells and collected and filter sterilized after 2 days. After 8-10 days of culture, cultures reached 100% confluence and microglia were harvested 12 days after seeding by mitotic shake off for 1 hr at 150 rpm in an orbital shaker. After seeding, microglia were cultured in DMEM supplemented with 10% FCS and 1% pen/strep and medium collected from mixed glial cultures in a ratio of 50:50 at 37°C in a humidified atmosphere at 5% CO2.

Adult microglial cultures were obtained from confluent mixed glial cultures following an established protocol (Scheffel et al., 2012) based on a routine procedure for the preparation of neonatal microglia (Regen et al., 2011). Microglia were isolated from forebrains of 8-12 week old C57BL/6 mice, involving transient plating on microglia-free P0 astrocytes. To avoid variation caused by differences in LPS batches, a single batch of LPS (E. coli 0111:B4, Sigma-Aldrich, Cat#L4391) was used in all in vitro and in vivo experiments.
Microglia viability

The viability of primary microglia was determined using 1) a MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described previously (Kannan et al., 2013), 2) a LDH assay, and 3) cell enumeration. For MTT assays, microglia were seeded (75,000 cells/well of a 12-well plate) and treated according to the stimulation scheme depicted in Fig. 1A. After MTT was added, cells were incubated for 2 hr at 37°C in a 5% CO₂ incubator. Next, the cells were lysed in dimethyl sulfoxide; absorbance was measured at 540 nM and expressed per µg of cell lysate. Protein concentrations of the cell lysates were determined using a Bradford assay.

For LDH activity, microglia were seeded (75,000 cells/well of a 12-well plate) in conditioned medium, and the medium was collected from wells without cells (control) to determine the LDH activity in the conditioned medium used, and from the wells containing unstimulated (control) or LPS treated (LPS) microglia. LDH activity was determined after the first LPS stimulus (day 1), after a medium change at day 5 and at the second LPS stimulus (day 8). LDH activity was determined using the manufacturer’s protocol (Pierce™ LDH Cytotoxicity Assay Kit). Average LDH activity with the standard deviation is depicted.

For cell counting, microglia were seeded (75,000 cells/well of a 12-well plate) in conditioned medium, pictures were taken on days 2, 5, and 8 after seeding (20x magnification) and the cell number in six images per condition was counted (conditions were blind to the experimenter). The average cell number per microscopic field is depicted with the standard deviation.

BV-2 cell culture

BV-2 Cells were cultured in DMEM supplemented with 10% FCS and 1% pen/strep at 37°C in a humidified atmosphere at 5% CO₂. Puromycin (10μg/ml, Invitrogen) was used to select transduced BV-2 cells. BV-2 cells were stimulated for 24 hr with LPS, one day after seeding (3x10⁴ cells/10 cm²). After a 4 days interval, cells were rechallenged with LPS, 6 hr for RNA samples and 24 hr for protein samples. To avoid batch variation, single batches of LPS (E. coli 0111:B4, Sigma-Aldrich, Cat#L4391), Pam3CSK4 (InvivoGen, Cat#tlrl-pms) and polyinosinic:polycytidylic acid (poly I:C; InvivoGen, Cat#tlrl-pic) were used in all experiments performed in vitro and in vivo.

Lentiviral transduction

Plasmids encoding shRNA constructs targeting RelB or a scrambled vector control (piLentisilRNA-GFP, ABM©Good, Cat.nr. i038320) were packaged in VSV-G lentiviral (LV) coated particles in HEK293T cells, using a second generation LV system as described previously (Stewart et al., 2003). Briefly, BV-2 cells were seeded at a density of 5x10⁵ cells/well in a 6-well plate and treated with 0.45 µm filter sterilized non-purified LV supernatant for 12 hr in the presence of 8 µg/ml polybrene; transduced cells were selected using 10 µg/ml puromycin.

Acute live microglia isolation from adult murine brain
All steps were performed in dissection medium (Hanks bovine salt serum, PAA, Cat.nr. H15-010; D-(+)
Glucose solution, Sigma, Cat.nr. G8769; HEPES, PAA, 311-001). Mice were perfused with saline when anesthetized, brains were isolated and mechanically dissociated using a glass tissue homogenizer followed by passing through a 70 µm cell strainer (BD Falcon, Cat.nr. 352350). The cell suspension was centrifuged at 220 RCF at 4°C, acceleration 9 and brake 9. The supernatant was discarded and myelin was separated from the cell suspension using a 22% Percoll (GE Healthcare, Cat.nr. 17-0891-01) gradient where PBS was layered on top and centrifuged for 20 min. 950 RCF, acceleration 4, brake 0. Thereafter, the cell pellet was resuspended in 200 µl dissection medium. From this step onwards, all steps were performed using dissection medium without phenol red (Hanks bovine salt serum, PAA, Cat.nr.H15-009).

Microglia were stained with antibodies against CD11b (PE, eBioscience 12-0112) and CD45 (FITC, eBioscience, 11-0451) for 20 min. After washing, cells were passed through a 5 ml polystyrene round-bottom tube with a cell strainer cap (12x75mm style, Cat.nr.352235) and sorted using a Mo-flo XDP FACS (Beckman Coulter).

**Intracerebroventricular cannulation**

For intracerebroventricular (i.c.v.) LPS injections, C57Bl/6JOlaHsd mice were cannulated in the right lateral ventricle with 26 gauge stainless steel guide cannulas. For stereotactic surgery, mice were deeply anesthetized by intraperitoneal injection of ketamine/medetomidine (ketanest 100 mg/ml, Domitor 0.5 mg/ml) and bipuvacaine was used for local anesthesia. Implantation of the guide cannula was performed using stereotactic coordinates from bregma: anterior-posterior -0.3 mm; medio-lateral -1.0 mm; dorsal ventral -2.0 mm (Mouse Brain atlas). Directly adjacent to the guide cannula, an anchoring cranial screw was inserted and both guide cannula and cranial screw were fixed to the skull with dental cement. Anesthesia was antagonized by subcutaneous injection with antisedan (5 mg/ml). For pain prevention, mice were subcutaneously injected with Rimady (50 mg/ml) directly after surgery and the two following days. After recovery from anesthesia, mice were housed overnight in a recovery incubator and thereafter allowed to recover for at least a week prior to starting experiments. Mice were infused with saline or with 5 µg LPS (in saline) through a cannula (1 µl/min).

**RNA isolation and quantitative RT-PCR**

For quantitative RT-PCR analysis, total RNA was isolated using a Qiagen RNeasy Micro Kit according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using Random Hexamers (Fermentas), M-MuLV Reverse Transcriptase and Ribolock RNAse inhibitor (Fermentas). Quantitative PCR reactions were carried out using SYBR supermix (BIORAD) and a real time thermal cycler (ABI 7900HT, Applied Biosystems) with different sets of PCR primers (Table 1). For each gene, measurements were performed in triplicate in three independent experiments. PCR primers were designed in PRIMER EXPRESS 2.0. HMBS was used as an internal standard to calculate relative gene expression levels with the $2^{-\Delta\Delta CT}$
method (Schmittgen and Livak, 2008) which is depicted as fold-induction of control samples. A one-way ANOVA followed by a Fisher's Least Significant Difference (LSD) post hoc test was performed to determine statistical significance.

**Western blot analysis**
Microglia cell lysates were produced by addition of non-denaturing cell lysis buffer (Cell Signaling Technology, Cat# 9803) and scraping. Cell lysates were subsequently sonicated (1 x 5 sec, 80 W), centrifuged (10 min, 18,000 g, 4°C) and the supernatant was transferred to a fresh 1.5 ml eppendorf vial. Protein content was determined using a Bradford assay and 20 μg protein samples were mixed with Laemmli buffer and boiled for 5 min. Proteins were separated by SDS-PAGE gel electrophoresis and were transferred to polyvinylidene difluoride (PVDF) membranes equilibrated with transfer buffer (25 mM Tris, 150 mM glycine, 10% v/v methanol) by semi-dry electroblotting (Bio-Rad) at 3 mA/cm² for 1 hr. PVDF membranes were blocked with Odyssey blocking buffer (Odyssey Infrared Imaging system, blocking buffer, part#927-40000, Li-Cor Biosciences) diluted 1:1 with PBS for 1 hr at room temperature. After blocking, PVDF membranes were incubated overnight at 4°C with primary antibodies (NF-κB p65, Abcam, Cat# Ab13594; P-NF-κB P65 (S536), Cell Signaling Technology, Cat# 3033; Akt, Cell Signaling Technology, Cat# 2920s; P-Akt (s473), Cell signaling technology, Cat# 4060s; RelB, Cell Signaling Technology, Cat# 4954; β-actin, Abcam, Cat# Ab6276). Following primary antibody incubation, the membranes were washed 4 times with PBS 0.2% Tween-20 and incubated for 1 hr with secondary Infrared IRDye-labeled secondary antibodies (1:10000, LI-COR). After incubation with secondary antibodies, the membranes were washed 4 times with PBS 0.2% Tween-20 and scanned in a LI-COR Odyssey infrared imaging system. Blots were quantified using ImageJ software.

**ELISA**
Microglia supernatants were collected and used for ELISA analysis. For IL-1β ELISA of primary microglia, ATP (1 mM) was added during the last 15 min of the last LPS treatment to induce secretion. As a positive control for induction of inflammation, serum samples were collected from PBS and LPS injected mice used in *in vivo* experiments. In short, blood samples were collected in minicollect serum/plasma gel tubes (Greiner bio-one, Cat# 450472), left at room temperature for 30 min, subsequently centrifuged (10 min, 2000 rcf, 4°C) and serum was collected. ELISAs were performed according to the manufacturer’s instructions (Mouse TNF-α ELISA MAX™ Deluxe, Biolegend, Cat# 430906; Mouse IL-1β ELISA MAX™ Deluxe, Biolegend, Cat# 432606). Statistical analysis was performed using a one-way ANOVA followed by a LSD post hoc test.
**Phosphorylation studies**

Microglia (10^6 cells) were stimulated with LPS (100 ng/ml) for 24 hr and after a 5 days interval restimulated with LPS (100 ng/ml) for 15 min or 6 hr. Cells were lysed and assayed by ELISA kits for phosphorylation of p38, p42/44 (ERK1/2) and NF-κB according to the manufacturer’s instructions (Cell Signaling Technology). Absorbance was measured in an iMark plate reader (Biorad). Statistical differences were analyzed using one-way ANOVA followed by a LSD post hoc test.

**Chromatin immunoprecipitation**

ChIP was performed as described previously (Kooistra et al., 2010; Zanette et al., 2013) with minor modifications. Formaldehyde (1%, 10 min) was added to 10^6 microglia and crosslinking was stopped by addition of Glycine (125 mM) followed by two PBS washes. After washing, cells were lysed with cell lysis buffer followed by nuclear lysis buffer. Chromatin was sonicated using a Bioruptor (Diagenode), precleared using preblocked protein A agarose beads (25%; Protein A Agarose/Salmon Sperm DNA, Millipore, Cat# 16-157) and incubated overnight at 4°C with antibodies directed to specific histone modifications (AcH3, Millipore, Cat# 06-599; H3K4me3, Millipore, Cat# 07-473; H3K9me2, Millipore, Cat#17-648; H3K27me3, Millipore, Cat# 17-622; H3K9me3, Abcam, Cat#Ab8898; 5 μg/sample) and RelB (Cell Signaling Technology, Cat# 4954). Cross linked chromatin of 10^5 cells was immunoprecipitated and no antibody served as background control. The following day, immune complexes were precipitated with 80 μl pre-blocked protein A agarose beads for 2 hours at 4°C, washed and chromatin was eluted from the beads. The precipitated chromatin was de-crosslinked overnight and proteins digested. Quantitative PCR of immunoprecipitated DNA was performed using specific ChIP primers targeted to promoter regions of genes of interest (Table 1) and results are presented as % of input. Statistical differences were assessed by one-way ANOVA followed by a LSD post hoc.

**Phagocytosis assay**

Primary microglia were seeded in 2 well Lab-Tek™ II Chambered Coverglass (Nunc® Lab-Tek® II - CC2™ Chamber Slide™ system, Sigma, Cat# 6565) at a density of 5x10^5 cells per well. After treatment, culture medium was refreshed with medium containing 10 μg/ml of pHrodo™ E. coli BioParticles® conjugate (Life technologies, Molecular Probes, Cat.# P35361). At several time points, multiple bright field and red channel images (pHrodo emission) were acquired at 10x and 20x magnification. Mean cell fluorescence intensity was determined using ImageJ and data were plotted as mean fluorescence, relative units. Statistical differences were assessed by one-way ANOVA followed by a LSD post hoc test.
**Electrophysiology**

Microglia (5x10^4 cells) were seeded on glass coverslips and cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were treated with 100 ng/ml LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, Cat#L4391) once or twice for 24 hr. For patch-clamp recordings, coverslips were placed into a chamber with HEPES buffer (NaCl 150.0 mM, KCl 5.4 mM, MgCl\(_2\) 1.0 mM, CaCl\(_2\) 2.0 mM, HEPES 10.0 mM, Glucose 5 mM, adjusted with NaOH to pH 7.4) on the stage of a microscope (Axiovert FS, Zeiss). Cells were approached with microelectrodes, pulled from borosilicate capillaries with a resistance of 7-11 MΩ filled with intracellular solution (KCl 130 mM, MgCl\(_2\) 2 mM, CaCl\(_2\) 0.5 mM, Na-ATP 2 mM, EGTA 5.0 mM, and HEPES 10 mM, adjusted with NaOH to pH 7.3, 285 mosm/l). Cells were clamped at -70 mV and current traces were recorded upon application of series of depolarizing and hyperpolarizing steps (50 to -170 mV) with 10 mV increments. Uncompensated currents were measured at room temperature with an EPC 10 amplifier (HEKA electronics, Lambrecht/Pfalz, Germany). Statistical analysis was performed with the GraphPad Prism software and two-sided significance levels were determined based on the non-parametric Mann-Whitney U test.

**NO measurements**

Nitric oxide release was quantified by determining the levels of the stable metabolite nitrite with use of the Griess reagent system. Microglia were cultured in 96-well plates in a density of 1x10^5 cells/well and treated according to the scheme depicted in Fig. 1A. 24 hr after the last stimulation, culture medium was collected and mixed with an equal amount of Griess reagent (1% sulphanylamide, 5% phosphoric acid and 0.1% naphthylethlenediamine dihydrochloride) and left at room temperature for 5-10 min. Absorbance was measured at a wavelength of 550 nm using a labsystems, multiskan RC reader, and calculated using a nitrite standard curve. Statistical differences were assessed by one-way ANOVA followed by a LSD post hoc test.

**Immunohistochemistry**

Animals were transcardially perfused with saline 0.9% under isoflurane anesthesia. After perfusion, brains were fixed for 24h in 4% paraformaldehyde (PFA), whereafter they were stored in 1% PFA, until further use. Brains were cryoprotected by overnight incubation in 20% sucrose solution and cryosections of 50 μm thick were made. Free floating sections were blocked for 1 hour with 5% normal goat serum and thereafter incubated with primary antibody against Iba1 (Rb-anti-Iba1, Wako, Cat.#019-19741) for 72h at 4°C. Detection of primary antibody was done by incubation with Alexa fluor 488 donkey anti rabbit (Invitrogen, Cat.#A21206) secondary antibody. After secondary antibody incubation, sections were washes, incubated in Hoechst solution for 5 minutes, washed again and mounted on glass slides.
**Sholl analysis**

Confocal microglia Z-stack images (50 μm) were acquired on a Leica SP2 confocal laser scanning microscope with 1 μm intervals of microglia stained with Iba1 (3 mice per group). Maximal intensity projections were created using ImageJ and binary pictures were generated. Sholl analysis was performed using the Sholl analysis plugin of ImageJ. Intersection per radius increasing with steps of 1 pixel were used as a quantifiable units.

**T-maze learning**

Young adult (8 weeks) male C57Bl/6JOlaHsd mice were intraperitoneal injected with PBS or LPS (1 mg/kg; E. coli 0111:B4, Sigma-Aldrich, Cat#L4391)) and T-maze learning was performed 4 weeks later. First, animals were food restricted for 1 week until a weight reduction was of 10-15% was reached. The T-maze learning experiments were performed as described previously (Hagewoud et al., 2010). In short, a food reward was placed in one of the test arms of a cross maze consisting of four 90° angle tubular, transparent Plexiglas arms. A T-maze was created by blocking one of the starting arms. In both testing arms, food pellets were placed under perforations to prevent mice making the correct choice based on olfactory cues. Visual discrimination of baited vs non-baited arms was prevented by placing the food reward behind a small ridge. At the starting day, mice were subjected to two habituation trials where in the first trial one testing arm was baited and open while the other testing arm was closed and the second trial vice versa. Following habituation, which was only performed on the starting day, mice were subjected daily to a training session consisting of 6 trials where always the same arm was baited, randomly assigned to experimental animals. Training was performed until the mice reached a of ≥ 90% correct choices. For statistical analysis, a repeated-measures ANOVA was used.
Fig. 1) Reduced LPS-induced pro-inflammatory gene expression in preconditioned microglia. (A) Primary microglia were stimulated according to the depicted schedule. For RNA collection, samples were collected after a 6 hr LPS (100 ng/ml) stimulation, for protein collection, samples were collected after a 24 hr LPS (100 ng/ml) stimulation. (B) Pro-inflammatory IL-1β, TNF-α and IL-6 mRNA expression levels were determined in neonatal microglia stimulated with LPS using quantitative RT-PCR (−/− n=19, −/+ and +/+ n=20, +/− n=9). Expression levels were normalized to HMBS, average expression with standard errors is depicted. (C) LPS (100 ng/ml) induced IL-1β (n=4) and TNF-α (n=5) secretion by neonatal microglia were determined using ELISA. To induce cleavage of pro-IL-1β to IL-1β, ATP (1 mM) was added during the last 15 min of the LPS stimulus on day 8. Average secretion with standard errors is depicted. (D) Pro-inflammatory IL-1β and TNF-α mRNA expression levels were determined in adult microglia stimulated with LPS using quantitative RT-PCR (n=3). Expression levels were normalized to hypoxanthine-guanine phosphoribosyltransferase, average expression with standard errors is depicted; * p≤0.05, ** p≤0.01, ***; p≤0.001, **** p≤0.0001.

Results

LPS-preconditioning of primary microglia results in reduced responsiveness to a subsequent LPS stimulation.

LPS has been reported to induce ET in macrophages (Biswas and Lopez-Collazo, 2009). Here we first determined the effect of LPS-preconditioning on the inflammatory response of primary neonatal microglia. A LPS dose-response curve, ranging from 5 pg to 100 ng/ml, was generated based on IL-1β mRNA expression (Suppl. Fig. 1A). To determine the effect of preconditioning with LPS, microglia were first stimulated with LPS for 24 hr (5 pg/ml - 100 ng/ml) and after a 6 days interval re-exposed to LPS for 6 hr (100 ng/ml; Suppl. Fig. 1B). Preconditioning with LPS resulted in a concentration-dependent reduction in IL-1β mRNA expression in response to the second LPS stimulus and in all subsequent experiments with primary microglia, 100 ng/ml LPS was used as stimulus. To rule out that long-term culture affected the response of primary microglia to LPS, cells were stimulated with LPS either 1 or 6 days after seeding and IL-1β gene induction was determined by quantitative RT-PCR; no difference in the fold induction was observed (data not shown). To rule out that the observed reduction in LPS responsiveness in preconditioned microglia was due to reduced viability, MTT assays, LDH assays, and cell counting was performed. LPS preconditioned microglia displayed an almost 1.5-fold increase in MTT converting activity per μg of protein. These data indicate that LPS preconditioned microglia are still viable after 8 days in culture and that they are metabolically altered as they display increased mitochondrial activity (Suppl. Fig. 2A). To determine if LPS preconditioning resulted in increased cytotoxicity, lactate dehydrogenase (LDH) activity was determined in the medium of control (PBS) and preconditioned (LPS) microglia cultures. As these cells are grown in serum-containing, preconditioned medium, the LDH activity in medium alone was also determined as a control. No significant difference in the LDH activity was observed between medium alone and control and preconditioned microglia medium at 1, 5, and 8 days after seeding (Suppl. Fig. 2B). Finally, microscopic images of primary microglia 2, 5, and 8 days after seeding and stimulated with PBS or LPS were taken and the number of cells per
microscopic field were counted. A 15% reduction in the number of LPS preconditioned microglia was observed after 8 days of culture, irrespective of a second LPS stimulation (Suppl. Fig. 2C). Summarizing, LPS preconditioning did not induce microglia proliferation, cytotoxicity, or extensive cell loss.

Next, the effect of LPS-preconditioning on pro-inflammatory gene expression in primary neonatal microglia was determined. Microglia were treated with LPS (100 ng/ml) according to
the scheme depicted in Fig. 1A. A single LPS stimulation of primary microglia resulted in a significant induction of IL-1β, TNF-α and IL-6 mRNA expression compared to untreated control microglia (Fig. 1B). Compared to control microglia, preconditioned microglia showed a significantly reduced pro-inflammatory response to LPS; IL-1β, TNF-α and IL-6 expression were all significantly reduced (Fig. 1B). This reduced LPS responsiveness was observed both at 3 (data not shown) and 6 day intervals between the two LPS stimuli. Microglia that were preconditioned, but did not receive a second LPS stimulus, had transcription levels comparable to untreated control microglia. To determine whether LPS-preconditioning also resulted in reduced cytokine secretion, IL-1β and TNF-α protein levels were determined in supernatants of stimulated microglia. LPS preconditioning of primary microglia caused a significant reduction in the secretion of TNF-α compared to control microglia in response to LPS (Fig. 1C). Control and preconditioned (data not shown) microglia supernatants contained comparable TNF-α levels. When microglia were exposed to three subsequent LPS challenges, secreted TNF-α levels were significantly reduced with each additional LPS challenge (Suppl. Fig. 2A). It has previously been shown that LPS alone is not sufficient to induce the secretion of IL-1β by murine microglia and macrophages (Brough et al., 2002; Brough and Rothwell, 2007). To induce P2X7-dependent inflammasome formation and subsequent caspase-1 activation, ATP (1 mM) was added during the last 15 min of the LPS stimulus leading to cleavage of pro-IL-1β to IL-1β. The combined LPS/ATP stimulus resulted in significant IL-1β secretion and LPS-preconditioned microglia secreted significantly less IL-1β (Fig. 1C). To determine if adult microglia also display reduced responsiveness to LPS after a preconditioning stimulus, primary microglia obtained from 8-12 week old mice were stimulated according to the scheme depicted in Fig. 1A. Stimulation with LPS resulted in a significant induction of IL-1β and TNF-α expression where this induction was significantly less in preconditioned microglia (Fig. 1D). Control and preconditioned microglia express similar, low levels of IL-1β and TNF-α. Summarizing, these results show that preconditioning of both neonatal and adult primary microglia with LPS resulted in a prolonged attenuation of pro-inflammatory gene expression in response to a second LPS stimulus.

Fig. 2) In LPS preconditioned microglia, TLR4 signaling is intact, Pam3CSK4/TLR2 and LPS/TLR4 target gene expression is reduced and poly I:C/TLR3 target gene expression is not affected. (A). LPS-induced (100 ng/ml) phosphorylation of p65, p38 and p42/44 were determined using phospho-ELISAs (15 min group, n=6; 6 hr group, n=3). The average relative absorbance with standard errors are depicted; * p≤0.05, ** p≤0.01. (B). LPS (100 ng/ml) stimulated microglia protein lysates were western analyzed using the indicated antibodies (n=3); Actin was used as a loading control. (C) Pro-inflammatory IL-1β mRNA expression levels were determined in microglia stimulated with LPS (100 ng/ml)/Pam3CSK4 (100 ng/ml) using quantitative RT-PCR (n=5). Expression levels were normalized to HMBS, average expression with standard errors are depicted; ** p≤0.01. (D) Proinflammatory IL-1β and IFN-β mRNA expression levels were determined in microglia stimulated with LPS (100 ng/ml)/Poly I:C (50 μg/ml) using quantitative RT-PCR (n=3). Expression levels were normalized to HMBS, average expression with standard errors are depicted; * p≤0.05, ** p≤0.01, *** p≤0.001.
**LPS-induced microglial TLR4/NF-κB signaling is not attenuated after preconditioning**

To determine if the observed attenuated LPS response was caused by alterations in the LPS/TLR4 signaling cascade, LPS-induced activation of downstream signaling molecules was determined. Microglia were treated with LPS according to the scheme depicted in Fig. 1A. Cell lysates were generated 15 min and 6 hr after the second LPS stimulus. Phosphorylation levels of the p65 subunit of NF-κB and mitogen-associated protein kinases p38 and p42/44 (Erk1/2)
were determined using phosphoprotein-specific ELISAs. Control and preconditioned microglia both showed comparable and low levels of basal p65, p38 and p42/44 phosphorylation (Fig. 2A). In response to LPS, a significant increase in p65, p38 and p42/44 phosphorylation levels was observed in control and preconditioned microglia, both 15 min and 6 hr after the second LPS stimulation (Fig. 2A). Overall, LPS-induced p65, p38 and p42/44 phosphorylation levels were lower at 6 hr after LPS stimulation than after 15 min, reflecting the timed activation and inactivation of the TLR4 signaling cascade. In addition, LPS-induced phosphorylation of Akt and p65 was determined using immunoblotting. A low basal level of Akt and p65 phosphorylation was observed in control microglia. LPS stimulation resulted in comparable levels of Akt and p65 phosphorylation in both control and preconditioned microglia (Fig. 2B). Summarizing, these data indicate that the reduced pro-inflammatory gene expression observed in preconditioned microglia in response to a second LPS challenge was not caused by altered TLR4 signaling per se.

**LPS pre-conditioning results in reduced sensitivity to other TLR ligands**
To determine if the observed blunted pro-inflammatory response of microglia after a prior exposure to LPS is specific for TLR4-mediated signaling, LPS pre-conditioned microglia were stimulated with other TLR receptor ligands. Microglia were treated with LPS (24 hr; 100 ng/ml) and after a 6 day interval challenged with the TLR2 ligand Pam3CSK4 (100 ng/ml) or the TLR3 ligand polyinosinic:polycytidylic acid (poly-I:C; 50 μg/ml). Inflammatory cytokine expression levels were determined using quantitative RT-PCR. Stimulation with Pam3CSK4 alone resulted in a significant, approximately 3000-fold, induction of the IL-1β gene. In contrast, in LPS-preconditioned microglia, Pam3CSK4 induced expression of the IL-1β gene was significantly reduced to ~500 fold (Fig. 2C). In the reverse experiment, a similar effect was obtained. Pretreatment with Pam3CSK4 resulted in a significantly attenuated response to LPS (~3000- versus ~250-fold induction of IL-1β; Fig. 2C). Similar results were observed for pro-inflammatory genes TNF-α and IL-6 (data not shown).

As TLR4 (LPS) and TLR2 (Pam3CSK4) both signal through the MyD88/IRAK/NF-κB pathway, it was investigated whether LPS pre-conditioning also resulted in altered responsiveness to poly-I:C, a TLR3 ligand. The TLR3 receptor signals through a separate signaling cascade involving IRF3 (Kawai and Akira, 2006), which is independent of the TLR2/4 (non-)canonical NF-κB pathway. Microglia were first stimulated with LPS (24 hr; 100ng/ml) and after 6 days challenged with poly-I:C (50 μg/ml). Stimulation of microglia with poly-I:C resulted in significant induction of IL-1β and IFN-β expression (Fig. 2D). Remarkably, LPS pre-conditioning potentiated the induction of both IL-1β and IFN-β transcription by poly-I:C (Fig. 2D). Summarizing, these data show that LPS/TLR4 and Pam3CSK4/TLR2 pre-conditioned microglia display a reduced pro-inflammatory response to TLR2/4 ligands that signal through the same NF-κB signaling cascade, whereas the response
Fig. 3) RelB is expressed and enriched on the IL-1β promoter in preconditioned microglia and required for reduced LPS responsiveness of microglia. (A) RelB expression was determined using western blotting of LPS-stimulated (100 ng/ml) microglia cell lysates with Actin as a loading control (n=3). Densitometric analysis of the shown blots is depicted. (B) Microglia chromatin was immunoprecipitated with an antibody against RelB followed by quantitative PCR for the IL-1β promoter (n=3). RelB enrichment levels are depicted as % of input DNA; ** p≤0.01. (C) Quantification of western blots (n=4) for RelB expression in lysates of wildtype BV2 cells or BV-2 cells expressing scrambled shRNA or shRelB, with Actin as loading control, one of the westerns used for the quantification is depicted; ** p≤0.01. (D) Pro-inflammatory IL-1β mRNA expression levels were determined in wildtype BV2, scrambled-shRNA BV-2 and shRelB BV-2 microglia stimulated according to the scheme depicted in Fig. 1A with a 4 day interval using quantitative RT-PCR (n=3). Expression levels were normalized to HMBS, average expression with standard errors is depicted, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

induced by poly-I:C/TLR3 was not affected. Together with the findings obtained using phosphoprotein ELISAs and immunoblotting (Fig. 2A and B), these data indicate that the
reduction in pro-inflammatory gene expression after prior TLR2 and TLR4 activation most likely localizes downstream of NF-κB.

**Enrichment of RelB and repressive histone modifications at the IL-1β promoter chromatin in LPS-preconditioned microglia**

In macrophages, RelB has been reported to silence inflammatory gene expression by recruiting the H3K9 methyltransferase G9a (KMT1c) and the DNA methyl-transferase DNMT3a/b, resulting in stable TNF-α and IL-1β gene silencing (El Gazzar et al., 2009, 2008). In primary microglia, a single exposure to LPS already resulted in increased expression of RelB which was still detected after 8 days, irrespective of a second LPS stimulus (Fig. 3A). To determine if RelB binding was enhanced at the IL-1β promoter, chromatin immunoprecipitation (ChIP) assays were performed. In pre-conditioned microglia, RelB was enriched at the promoter region of IL-1β, regardless of a second LPS stimulation. In control and acutely stimulated microglia, no RelB enrichment at the IL-1β promoter was observed (Fig. 3B).

To determine if RelB was required for LPS preconditioning of microglia, RelB expression was downregulated using shRNAs. Western blot analysis indicated a ~50% reduction in RelB levels in preconditioned BV2 cells stably expressing RelB shRNAs (Fig. 3C). LPS stimulation of BV-2 cells resulted in increased IL-1β expression but LPS-preconditioning completely abrogated responsiveness to a subsequent LPS challenge. Similar results were obtained in BV-2 cells stably expressing scrambled shRNAs, although preconditioning did not completely block the induction of IL-1β after a second LPS challenge. Partial knock-down of RelB by shRNAs largely restored LPS-induced expression of IL-1β in preconditioned BV2 cells (Fig. 3D) indicating that RelB is required for LPS/TRL4-mediated ET in microglia.

Since RelB has been associated with epigenetic gene repression in macrophages, it was determined whether RelB enrichment was accompanied by altered histone modification levels. As expected, stimulation with LPS resulted in a significant enrichment of both H3K4me3 and AcH3 at the promoter region of IL-1β (Fig. 4A), both modifications generally being associated with permissive and transcriptionally active chromatin (Barski et al., 2007; Kouzarides, 2007). Interestingly, this enrichment was not or much less observed in response to LPS in preconditioned microglia (Fig. 4A). H3K4me3 and AcH3 enrichment levels in control and preconditioned microglia were comparable. Next, the enrichment of histone marks associated with transcriptionally repressed chromatin, H3K9me2, H3K9me3 and H3K27me3 was determined. Enrichment levels of H3K27me3 at the promoter of the IL-1β gene were reduced in control microglia in response to LPS, whereas enrichment levels between control, preconditioned and LPS-stimulated preconditioned microglia were not significantly different. H3K9me3 levels at the IL-1β promoter were comparable in control, LPS-stimulated microglia and LPS-stimulated preconditioned microglia. H3K9me3 levels were significantly reduced in preconditioned microglia, and hence unlikely to be responsible for the reduced LPS-induced
IL-1β expression in these cells (Fig. 4B). These data show that enrichment levels of “repressive” H3K27me3 and H3K9me3 modifications do not underlie the observed reduction in IL-1β mRNA expression in preconditioned microglia. Interestingly, H3K9me2 levels were significantly enriched in LPS-preconditioned microglia. In these pre-conditioned microglia, H3K9me2 levels were still enriched after a second LPS stimulus, albeit at slightly lower levels compared to pre-conditioned microglia. LPS stimulation of control microglia did not result in
H3K9me2 enrichment (Fig. 4B). Summarizing, these data show that LPS pre-conditioning resulted in epigenetic suppression of IL-1β gene expression, altering the LPS responsiveness of primary microglia.

**Increased phagocytosis in LPS-preconditioned microglia**

Microglia are regarded as the phagocytes of the brain and involved in removal of dead and damaged neurons as well as invading microorganisms (Kettenmann et al., 2011). We next determined if LPS-preconditioning, besides altering inflammatory cytokine gene expression levels upon a LPS rechallenge, also affected the phagocytic activity of microglia. Efficient microglia phagocytosis is required for pathogen removal and clearance of cell debris or apoptotic cells and eventually the initiation of the adaptive immune response by antigen presentation. Phagocytosis is reported to have a role in brain remodeling during the normal lifespan (Sierra et al., 2013, 2010). To assess phagocytic capacity of preconditioned microglia, in vitro stimulated microglia were tested in a phagocytosis assay using pHrodo *E. coli* conjugates, according to the scheme depicted in Fig. 1A. LPS treatment resulted in a significant increase in phagocytic activity in control microglia. Interestingly, phagocytic activity was enhanced in LPS-preconditioned microglia, independent of a second LPS stimulus (Fig. 5A), indicating that although suppressed in their pro-inflammatory gene expression, preconditioned microglia exhibit increased phagocytic activity.

**Increased iNOS release and outward currents in LPS-preconditioned microglia**

The membrane conductance of cultured primary microglia is dominated by inward rectifying K+ currents (Kettenmann et al., 2011; Pannasch et al., 2006). LPS treatment has been reported to trigger a change in membrane currents in microglia and to induce the expression of an outward current (Kettenmann et al., 2011). To determine if LPS preconditioning altered the electrophysiological properties of cultured microglia, membrane currents of control and LPS-stimulated microglia were determined using patch-clamp recording. The membrane was clamped at -70 mV and currents were recorded during de- and hyperpolarizing voltage steps ranging from 50 to -170 mV. As described previously, untreated cultured microglia elicit large inward rectifying currents and small outward currents. To determine the effect of LPS preconditioning, membrane currents of untreated cells and microglia treated either once or twice with LPS (with a 6 days interval) were recorded. Upon LPS treatment for 24 hr, compared to control cells, significantly decreased inward currents and increased outward currents were measured (Fig. 5B), an observation in agreement with earlier findings (Boucsein et al., 2000; Prinz et al., 1999). Challenging preconditioned microglia with LPS resulted in significantly increased outward currents compared to untreated microglia and cells treated with a single LPS exposure. Inward currents were not different between microglia exposed to LPS once or twice (Fig. 5B). It has been previously reported that the activation of potassium channels Kv1.3 and Kv1.5 is involved in increased outward K+ conductance. In agreement with the observed
outward currents, LPS stimulation of control and preconditioned cells resulted in increased expression of Kv1.5, compared to unstimulated microglia (Fig. 5C).

Activated microglia have been reported to produce increased amounts of nitric oxide (NO), mainly resulting from increased inducible nitric oxide synthase (iNOS). NO release by microglia is dependent on potassium channel Kv1.5 as Kv1.5−/− microglia display significantly
decreased iNOS transcription and NO production (Pannasch et al., 2006). To determine if the observed increase in outward currents was accompanied by increased iNOS expression and NO secretion, quantitative RT-PCR and nitrite measurements were performed. iNOS expression was increased by LPS stimulation of control microglia and this increase was even more pronounced in LPS stimulated preconditioned microglia. The increased iNOS expression was paralleled by NO production which was increased after LPS stimulation in control microglia and much more elevated by LPS in preconditioned microglia (Fig. 5D). Summarizing, these data show that although LPS preconditioning of primary microglia resulted in epigenetic suppression of pro-inflammatory cytokine genes, preconditioned microglia produce increased amounts of NO after a rechallenge with LPS.

**LPS-preconditioning (i.c.v.) results in reduced microglia responsiveness in vivo**

Primary neonatal microglia cultures are very amenable to *in vitro* manipulations, but next we determined if LPS preconditioning also altered microglia responsiveness *in vivo*. First, LPS was directly introduced into the mouse brain by i.c.v. injections. The right lateral ventricle was cannulated and after a two weeks recovery period, the mice received two i.c.v. injections with either PBS or 5 μg LPS, with a one week interval between both injections. I.c.v. injection of LPS induced a peripheral inflammation response as indicated by significantly elevated serum levels of TNF-α. This peripheral response was strongly reduced in preconditioned mice (Suppl. Fig. 2B). Three hr after the second i.c.v. LPS injection, microglia were isolated using FACS and inflammatory cytokine gene expression levels were determined using quantitative RT-PCR. A significant increase in IL-1β, TNF-α and IL-6 gene expression was observed in response to LPS (Fig. 6A). In preconditioned mice, the response to a subsequent i.c.v. LPS injection was significantly reduced (100-fold versus 400-fold induction for IL-1β; 50-fold versus 300-fold induction for TNF-α, 125-fold versus 480-fold for IL-6 mRNA levels). These data indicate that, in analogy to data obtained in primary microglia, i.c.v. preconditioning with LPS resulted in reduced pro-inflammatory gene expression in response to a subsequent LPS challenge.

**Intraperitoneal LPS injections induce microglia tolerance in vivo.**

There is ample evidence that peripheral inflammation induced by LPS results in inflammatory responses in the CNS (Cunningham, 2013). To determine if microglia also display reduced responsiveness to LPS after peripheral preconditioning, mice received two i.p. injections with LPS (0.25 mg/kg) or PBS, with 1, 4 or 32 week intervals. A single LPS injection in control mice resulted in a significant increase in serum TNF-α levels confirming peripheral inflammation. LPS injection of preconditioned mice showed an attenuated peripheral inflammatory response that was progressively restored with longer intervals between the LPS injections (Suppl. Fig. 2C). To determine if microglia displayed reduced LPS responsiveness
after preconditioning by i.p. LPS injection, microglia were isolated 3 hr after the LPS second injection using FACS, RNA was extracted and expression of inflammatory cytokines was determined using quantitative RT-PCR. As expected, injection with LPS resulted in a significant increase in IL-1β and TNF-α mRNA levels (Fig. 6B). Preconditioning by i.p. LPS injection resulted in significantly reduced expression levels of IL-1β and TNF-α after a 1, 4
and even 32 weeks interval with the second LPS challenge (Fig. 6B). Further supporting these observations, a mouse cytokine and chemokine array showed that the production of multiple pro-inflammatory cytokines and chemokines, including IL-1β, TNF-α and IL-6, was reduced in mice that were preconditioned before receiving a second LPS injection after 4 weeks (Table 2).

**Control and preconditioned microglia are morphologically very similar**

In terms of pro-inflammatory gene expression, preconditioned microglia respond very different from control microglia to LPS *in vivo*. To determine if preconditioned microglia adopted a different morphology, microglia were morphometrically analyzed. In the healthy resting brain, microglia have a small cell soma and highly ramified processes (Nimmerjahn et al., 2005). Upon activation, microglia retract their processes and become more hypertrophic with a larger cell soma and thicker processes (Kettenmann et al., 2011). To determine if microglia morphology was altered in preconditioned mice, differences in morphology were quantified using Sholl analysis (Morrison and Filosa, 2013). Four weeks after the first i.p. LPS injection, mice were rechallenged and after 24 hr brain sections were stained with Iba1 and cortical microglia were analyzed. Microglia in mice i.p. injected with LPS 24 hr prior to sacrifice, displayed an activated morphology with larger cell soma, reduced process lengths as well as a decrease in the sum of intersections reflecting reduced ramification in comparison to control microglia (Fig. 7A and B). Microglia in control and preconditioned mice displayed a similar morphology in terms of process length and sum of intersections. The morphology of microglia in preconditioned and control mice after i.p. LPS injection is very different; microglia in preconditioned mice have longer processes (more intersections at greater distances from the soma) and are much more ramified (higher sum of intersections; Fig. 7B). These data show that although preconditioned microglia are very different in their response to LPS, morphologically they are quite similar to control microglia.
Fig. 7) Preconditioned and control microglia have very similar morphologies and impaired T-maze performance in preconditioned mice. (A) Iba1 positive microglia Z-stack images (50 μm) were acquired with 1 μm intervals. (B) The degree of microglia ramification was quantified using Sholl analysis (-/- n=16 cells, -/+ n=18 cells, +/+ n=11 cells, +/- n=11 cells). The number of intersections with increasing distance from the cell soma and the total sum of intersections are depicted with the standard error; *** p≤0.001. (C) Learning of control PBS injected mice (N=18) and LPS injected mice (N=20) was assessed using a T-maze learning paradigm. Mice were subjected to 6 learning trials per day. Results are depicted as percentage of correct choices with SEM; * p≤0.05.
Fig. 8) Epigenetic silencing of the IL-1β promoter in LPS-preconditioned mice and a model for epigenetic suppression of inflammatory genes in preconditioned microglia. Microglia were FACS isolated after i.p. PBS or LPS (1mg/kg) injections as indicated with a 4 week interval. Chromatin was isolated and immunoprecipitated with antibodies against (A) active (n=3) and (B) repressive (n=3) histone modifications and followed by quantitative PCR for the IL-1β promoter. Enrichment levels are depicted as % of input DNA; * p≤0.05, ** p≤0.01, *** p≤0.001. (C) Proposed model: LPS-TLR4 signaling results in nuclear translocation of NF-κB (p50/p65), enrichment for “active” chromatin modifications and transcription of NF-κB target genes. RelB expression is also induced by p50/p65 and RelB binds to NF-κB target genes, where it recruits G9a (or related enzymes) that mediates H3K9 dimethylation, leading to “repressed” chromatin. In these preconditioned microglia, a subsequent LPS challenge results in a blunted transcriptional response. G9a recruitment is hypothesized, reflected by the dashed line.
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NF-κB target gene

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LPS

NF-κB activation

![Diagram of NF-κB activation](image2)

Active histone modifications (H3K4me3, AcH3)

Repressive histone modifications (H3K9me2)

Nucleosome
**Impaired T-maze learning by LPS preconditioned mice**

To address the effect of a single LPS challenge on learning, adult C57bl/6JOLA/Hsd mice were i.p. injected with PBS or LPS (1 mg/kg). Four weeks after PBS/LPS injection, the performance in T-maze learning of control PBS injected mice (n=18) and LPS injected mice (n=20) was determined. Both the PBS and LPS group started the first training days at chance levels, 48.2 ± 6.5% and 49.2 ± 3.9%, respectively. At the fifth training day, the PBS mice reached a level of correct choices of 97.2 ± 1.4%, which was significantly different from the LPS mice that scored 84.2 ± 5.7% (p<0.05) at day 5. The LPS group needed an additional day to reach a level of correct choices of 87 ± 10.5% (Fig. 7C).

**Stable epigenetic alterations in microglia of i.p. LPS-preconditioned mice**

To determine if the observed in vivo attenuation of pro-inflammatory LPS responsiveness was also accompanied by epigenetic alterations in the promoters of inflammatory genes similar to what was observed in primary microglia cultures, ChIP experiments were performed using microglia from mice i.p. injected with LPS (1mg/kg) or PBS. Microglia were FACS-isolated and the enrichment of histone modifications H3K4me3, AcH3, H3K27me3 and H3K9me2 at the IL-1β promoter was determined. In line with increased mRNA expression levels, a significant enrichment of the “active” marks H3K4me3 and AcH3 was observed at the IL-1β promoter after an i.p. injection of control mice with LPS (Fig. 8A). In preconditioned mice that received an i.p. LPS injection 4 weeks earlier, this enrichment was not observed in response to a second LPS injection and was similar to PBS injected control and preconditioned mice (Fig. 8A). This lack of enrichment of “on”-marks in preconditioned mice supports our observation that in these mice IL-1β gene expression is significantly attenuated in response to a second LPS challenge. To determine if preconditioning resulted in enrichment of “repressive” chromatin marks, the enrichment of H3K27me3 and H3K9me2 at the IL-1β promoter region was assessed. Microglia isolated from control and LPS-preconditioned mice showed no significant changes in H3K27me3 enrichment levels after PBS or LPS injections (Fig. 8B), an observation in agreement with data obtained in primary microglia. H3K9me2 levels were very low in control and in mice injected with LPS 3 hr earlier. Interestingly, enrichment of H3K9me2 was significantly increased at the IL-1β promoter region in microglia isolated from preconditioned mice, and enrichment levels were comparable in preconditioned mice that received PBS or LPS as a second injection (Fig. 8B). Summarizing, a single i.p. injection with LPS resulted in an epigenetically repressed IL-1β promoter and attenuated mRNA expression levels and cytokine secretion. These data show that a single LPS challenge in mice induced a repressive epigenetic imprint of the key pro-inflammatory cytokine gene IL-1β, in vivo.
Discussion

LPS-preconditioning has been reported to have both beneficial as detrimental effects in the CNS. *In vivo*, LPS-preconditioning of microglia has been associated with neuroprotection in animal models of ischemia by prevention of an exacerbated inflammatory response during retinal (Halder et al., 2013) or cerebellar ischemia (Rosenzweig et al., 2004). Several other reports showed a long-term negative impact on learning and memory, and neuroanatomy of prenatal LPS exposure or preconditioning by i.p. LPS injection in adult mice (Graciarena et al., 2010; Lin et al., 2012; Semmler et al., 2007). These observations indicate that the biological effects of LPS-preconditioning depend on the time of preconditioning and type of tissue challenged. Long term CNS tolerance to LPS or other inflammatory stimuli might serve as a means to prevent excess damage to the tissue caused by subsequent inflammatory insults. Preconditioned microglia still display an inflammatory response to a second LPS challenge, but much less pronounced, pointing to a means to limit excess damage that would be caused by repeated strong immune activation of microglia. The potentially detrimental effect of peripheral inflammation is illustrated by our observation that a single i.p. LPS injection affected the learning capacity of mice in a T-maze.

Acute or chronic LPS prestimulation of primary microglia cultures and organotypic hippocampal slices induced a downregulation of pro-inflammatory genes like TNF-α and IL6 when being re-stimulated with LPS after 7 days (Ajmone-Cat et al., 2013, 2003). The molecular basis for this altered LPS sensitivity is however largely unknown. In macrophages, Deng et al. (2013) showed that prestimulation with super low doses of LPS (5-50 pg/ml) or high doses of LPS (-100 ng/ml) have opposing effects in that low doses primes macrophages and high doses tolerizes them. They postulate that low dose of LPS increases the activation of IRAK-1, tollip and thereby induce the degradation of RelB. And it is thought that removal of RelB primes these cells. In contrast they show that high dose of LPS induces RelB production by activation of negative regulators such as PI3K and IRAK-M, which negatively regulate IRAK-1 (Deng et al., 2013). Although we did not check these upstream regulators by western blot analysis, the effect of low versus high LPS preconditioning on a second high LPS stimulus was assessed by quantitative RT-PCR. In primary microglia, we observed a concentration dependent tolerance induction. However, priming by low dose LPS (5-50 pg/ml) was not observed (Suppl. fig. 1). Using ligands for different TLR signaling pathways, we determined that LPS and Pam3CSK4 preconditioning affected a common target downstream of TLR2 and TLR4. Western analysis and phosphoprotein-specific ELISAs showed that LPS-induced phosphorylation of the p65 subunit of NF-κB was similar in control and preconditioned microglia (Fig. 3). Together these data suggest that the long-term reduction in endotoxin responsiveness induced by LPS or Pam3CSK4 occurs downstream of NF-κB in the TLR2/4 signaling pathways. Although these data strongly suggest that LPS-TLR4/Pam3CSK4-TLR2 signaling are mediating the observed preconditioning effects, non-TLR signaling could be implicated as well. Recently, an intracellular caspase 11 activation by LPS has been reported (Hagar et al., 2013). However,
TLR4− microglia are essentially not activated by LPS, indicating that TLR4 is the main LPS sensor of microglia. Taking together, for our paradigm, TLR4 would be the most relevant microglial ‘sensor’ of LPS. On the other hand, and most importantly, even if the phenomenon of LPS pre-conditioning would involve mechanisms additional to TLRs, the observed effect of LPS preconditioning would still be a finding of scientific and clinical relevance.

TLR2 is activated by peptidoglycans of gram-positive bacteria and Zymosan of yeast and TLR4 is activated by lipopolysaccharides of gram-negative bacterial cell walls, both yeast and bacteria-derived pathogen-associated molecular patterns (PAMPs). Interestingly, LPS preconditioning potentiated poly-I:C-TLR3 signaling, and poly-I:C mimics a viral PAMP, suggesting that LPS and Pam3CSK4 preconditioning might lead to an enhanced inflammatory response in case of a viral infection.

The observation that a reduced LPS response was still observed 32 weeks after the first LPS challenge in mice led us to determine if this was possibly caused by a repressive epigenetic imprint. The epigenome is of major importance in (stable) regulation of gene expression like memory imprinting of the adaptive immune system. The major epigenetic events influencing gene expression are covalent histone tail modifications and DNA methylation (Kondilis-Mangum and Wade, 2012). We assessed several histone tail modifications associated with transcriptionally active (H3K4me3 and AcH3) or repressed (H3K27me3, H3K9me2 and H3K9me3) chromatin to determine a possible epigenetic basis for the long-term effects of LPS stimulation on microglia responsiveness both in vitro and in vivo. Here, we show that enrichment of H3K4me3 and AcH3 at the promoter region of IL-1β in response to LPS was significantly lower in LPS preconditioned cells and mice when compared to control microglia. This observation is in agreement with the reduced IL-1β transcript levels in response to LPS in preconditioned microglia and mice. We did not observe an enrichment of H3K27me3 in chromatin of the IL-1β promoter in LPS-preconditioned primary microglia or mice, which makes it unlikely that epigenetic silencing is mediated by the Polycomb repressive complex 2.

Interestingly, a significant enrichment of “repressive” histone modification H3K9me2 was observed both in preconditioned primary microglia and in microglia acutely isolated from preconditioned mice. Mono- and di-methylation of H3K9 are mainly mediated by G9A (KMT1c), G9A-like protein 1 and SET domain bifurcated 1 and both these modifications have been associated with reversible gene silencing. H3K9me3 is regarded as a more constitutive mark of gene repression (Hirabayashi and Gotoh, 2010). In primary microglia, LPS-preconditioning resulted in enriched H3K9me2 levels but not in H3K9me3 levels, suggesting that the observed reduction in LPS responsiveness might be reversible.

Our data indicate that RelB is a key mediator of the reduced responsiveness to LPS in preconditioned microglia. In primary microglia, LPS induced a sustained expression of RelB, which was enriched at the promoter of IL-1β that also was enriched for H3K9me2, pointing to a similar mechanism of LPS-induced “tolerance” in microglia. Furthermore, expression of RelB
shRNAs in the microglia BV2 cell line restored LPS sensitivity showing that RelB is required for tolerance induction. How RelB leads to gene repression in microglia is unclear, but in macrophages, a direct interaction between RelB and G9A, the enzyme that mono- and dimethylates H3K9 has been reported (Chen et al., 2009; El Gazzar et al., 2009).

To determine if LPS preconditioning, in addition to altered inflammatory cytokine gene expression, affected other known microglia properties, phagocytic activity and electrophysiological characteristics of preconditioned primary microglia were determined. The expression of $K^+$ channels which mediate the in- and outward currents that can be recorded with whole cell patch clamping has been shown to change under pathophysiological conditions such as injury and epilepsy models (Kettenmann et al., 2011). One day after a pathologic event or after stimulation by LPS, microglial cells express the potassium channels Kv1.3 and Kv1.5. The expression of these channels is required for microglia iNOS transcription and NO production (Pannasch et al., 2006). In agreement with these results, in vitro electrophysiological analysis of primary microglia showed that preconditioned microglia display enhanced outward currents and increased iNOS expression and NO secretion. Showing that although suppressed in pro-inflammatory cytokines, these LPS preconditioned cells are not completely blunted in inflammatory responses. Furthermore, phagocytosis of pHrodo E. coli conjugates was significantly increased in preconditioned primary microglia. Summarizing, preconditioned primary microglia are suppressed in their pro-inflammatory gene expression but they exhibit increased phagocytic activity and iNOS and NO secretion, properties associated with inflammation-resolving microglia (Brown and Neher, 2010).

Changes in microglia morphology are associated with altered activity. As expected, a decrease in microglia ramification was observed in LPS-injected control mice. Microglia morphology in control mice and preconditioned mice was similar, although preconditioned microglia display a nearly significant increased degree of ramification. Most interestingly, LPS had no effect on microglia morphology in preconditioned mice; the degree of ramification and sum of intersections was not altered in preconditioned mice after a second LPS challenge. These data indicate that although preconditioned and control microglia are morphologically similar, in terms of endotoxin sensitivity they are very different.

I.p. injection of LPS can cause neuroinflammation through the neural route, circumventricular organs, cytokine transport across the blood brain barrier and secretion of substances by epithelial cells of the blood brain barrier (Quan and Banks, 2007). To directly administer LPS to the CNS, mice were i.c.v. cannulated. I.c.v. injection of LPS resulted in increased inflammatory cytokine expression that was significantly reduced in mice receiving two LPS injections with a 1-week interval. These data indicate that preconditioning of microglia in vivo results in reduced responsiveness to a second stimulus, irrespective of an i.p. or i.c.v. administration of the endotoxin. This is of particular interest in relation to observation made in humans where sepsis or generalized peripheral infections have been shown to be associated with cognitive decline (Semmler et al., 2013). If and how microglia are involved in the human...
situation is at present unclear, but our data demonstrate that peripheral inflammation has a long-lasting blunting effect on microglia responsiveness in mice.

Summarizing, our data show that stimulation of microglia with LPS resulted in an enrichment of “active” chromatin marks and increased expression these genes. After the initial pro-inflammatory response, (some of) these genes are occupied by RelB, become enriched for “repressive” chromatin modification H3K9me2 and are attenuated in their response to a subsequent challenge with LPS (Fig. 8C). Where the peripheral immune response to LPS was progressively restored after an LPS injection, this reduced LPS responsiveness was still observed in mice 32 weeks after the first LPS challenge. This indicates that peripheral ET is transient in nature but that a single LPS stimulation induced a stable repressive epigenetic imprint in microglia in vivo. In the peripheral immune system, ET is viewed as a mechanism to limit the inflammatory response to subsequent stimuli to prevent excessive tissue damage (Foster et al., 2007). On the other hand, long-term endotoxin tolerance in the periphery might be a potentially detrimental condition as it could hamper the ability to elicit a required immune response. However, many peripheral innate immune cells, like monocytes, are relatively short lived and replenished from the bone marrow, possibly explaining the progressively restored inflammatory response observed in mice i.p. injected with LPS (Suppl. fig. 3D). In contrast, microglia are cell population from a different embryonic origin (Ginhoux et al., 2010; Gomez Perdiguerro et al., 2015), with a low turnover and no or very limited contribution from peripheral myeloid cells to the CNS microglia population during adult life (Ajami et al., 2007). The observation that microglia display a sustained, altered LPS responsiveness might be a consequence of their low replacement rate, allowing for a long-term epigenetic imprint altering LPS responsiveness. Alternatively, repeated and extensive inflammatory activation of microglia is very detrimental for the CNS and should be avoided to prevent excess damage. So long-term tolerance might be a mechanism to prevent excess damage to the CNS in case of recurrent inflammatory stimulation. Importantly, our data show that LPS preconditioning results in a reduced, not a blocked, LPS response. We observed a long-term change in LPS preconditioned microglia from a pro-inflammatory to a more inflammation-resolving (increased outward currents, increased NO secretion and phagocytic activity) phenotype, possibly to limit potential excess CNS tissue damage as a consequence of repeated peripheral inflammation.

We show for the first time that a single inflammatory episode in mice leads to stable and long-lasting changes in the microglia epigenome, affecting endotoxin sensitivity and altering the response to a subsequent challenge. These observations might have significant implications for a better understanding of the long term cognitive effects observed in sepsis patients (Semmler et al., 2013) or the consequences of perinatal infections during pregnancy that are linked to reduced cognitive performance in the offspring (Williamson et al., 2011). Here, we show that a single i.p. LPS injection (1 mg/kg) of adult mice impaired T-maze learning 4 weeks post
injection. This observation illustrates the effect of a single inflammatory event on learning and the necessity for a better understanding of the mechanisms that underlie this effect.

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The authors declare that they have no conflict of interest.
References


Suppl. Fig. 1) Microglia preconditioning is LPS dose-dependent. (A) An LPS dose-response curve was generated in primary microglia. Primary microglia were stimulated with the indicated LPS dose for 6 hr, RNA was isolated and IL-1β mRNA levels were determined using quantitative RT-PCR. Expression levels were normalized to HMBS, average expression with standard errors is depicted. (B) Microglia preconditioning is LPS dose-dependent. Microglia were preconditioned with the indicated LPS dose for 24 hr and after 6 days again stimulated with LPS for 6 hr. IL-1β mRNA levels were determined using quantitative RT-PCR, expression levels were normalized to HMBS and average expression with standard errors is depicted.
Suppl. Fig. 2) Viability of primary microglia cultures. (A) Viability of long-term microglia cultures was determined using a MTT assay. Microglia were stimulated according to the scheme depicted in Fig. 1A. At day 9, cells were subjected to a MTT assay to determine mitochondrial activity, a measure for cellular viability and in parallel protein content was determined in a Bradford assay to correct for potential differences in protein content. The MTT converting activity is depicted per µg of protein. (B) Microglia culture viability was determined using an LDH assay. Microglia were seeded in conditioned medium and stimulated with LPS at day 1. Medium was collected from wells without microglia, wells with control microglia and wells with LPS treated microglia at 1, 5, and 8 days after seeding. LDH activity is depicted as Absorbance at 490 nm. Medium is conditioned medium without cells, control is unstimulated microglia, LPS is preconditioned microglia that received an LPS stimulus. (C) To determine if the number of microglia is very different between control and preconditioned conditions, microscopic images were taken of microglia cultures, stimulated according to the scheme depicted in Fig. 1A. Control: unstimulated microglia; LPS, day 8: microglia that received an LPS stimulus on day 8; LPS, day 1: microglia that received an LPS stimulus on day 1; LPS, day1 & day 8: microglia that received LPS stimulations on day 1 and day 8. The average number of microglia is depicted with the standard deviation. Representative images of primary microglia at day 8 of culture, stimulated as indicated, are shown.
A) TNF-α

- + +
- + +
- + +

ng/ml

+ day 1, LPS 24h
+ day 3.5, LPS 24h
+ day 6, LPS 24h

B) LPS injection after 1 week habituation
LPS injection after 1, 4 or 32 weeks
(3 hr RNA, 24 hr protein)

C) 1 wk

- - +
- - +
- - +

TNF-α pg/ml

- - +
- - +
- - +

week 1, LPS
week 2, LPS

D) 1 wk

- - +
- - +
- - +

1200
1200
1200

TNF-α pg/ml

- - +
- - +
- - +

week 1, LPS
week 2/5/33, LPS
Suppl. Fig. 3) ET after multiple LPS stimuli, and peripheral inflammation in mice i.c.v. and i.p. injected with LPS. (A) The effect of multiple LPS stimulation on TNFα secretion by primary microglia was determined. Cells were seeded and exposed to LPS for 24 hr at days 1, 3.5, and 6. At the end of each LPS stimulation, the supernatant was collected and TNFα levels were determined by ELISA. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. (B) Mice were injected with PBS or LPS (i.c.v. 5 μg, 1 μl/min or i.p. 0.25 mg/kg) with different intervals (1, 4, and 32 weeks) as depicted. RNA was isolated 3 hr after the second LPS injection, protein samples were generated 24 hr after the second LPS injection. (C) In mice i.c.v. injected with LPS, TNF-α serum levels were determined using an ELISA. Average serum levels with standard errors are depicted. A significant reduction in TNF-α serum levels was determined in response to LPS in preconditioned mice; * p ≤ 0.05 (D) In mice i.p injected with LPS, TNF-α serum levels were determined using an ELISA. A significant reduction in TNF-α serum levels was determined in response to LPS in preconditioned mice 1 and 4 weeks after the first LPS challenge. After a 32 weeks interval, TNF-α serum levels in response to LPS are comparable in control and preconditioned mice. Average serum levels with standard deviations are depicted; ** p ≤ 0.001, *** p ≤ 0.0001.
Table 1. Primers used for quantitative RT-PCR and chromatin immunoprecipitations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Reverse</th>
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<tr>
<td>HMBS</td>
<td>CCGAGCCAAGCACCAGGATA</td>
<td>CTCCCTCCAGGTGCCTCAGA</td>
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<tr>
<td>HPRT1</td>
<td>GACTTGCTCGAGATGTCA</td>
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<td>IFN-β</td>
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<td>IL-6</td>
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<td>Kv1.5</td>
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<td>IL-1β (ChIP)</td>
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<td>CXCL13/BLC/BCA-1</td>
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<td>58838 ± 8037</td>
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<td>G-CSF</td>
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<td>IFN-γ</td>
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<td>5108 ± 1296</td>
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<td>15995 ± 794</td>
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<td>8933 ± 235</td>
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<td>IL-13</td>
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<tr>
<td>IL-16</td>
<td>8469 ± 190</td>
<td>4157 ± 1054</td>
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</table>
Table 2. Mouse cytokine Antibody array, Panel A. Mice were twice i.p. injected with PBS or LPS with a 4 week interval between injections. Microglia were FACS isolated 6 hr after the second i.p. injection. Per condition, microglia from 3 mice were pooled and analyzed using the cytokine array. Results are shown as mean pixel density with standard deviations.

<table>
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<tr>
<th>Protein</th>
<th>PBS-PBS</th>
<th>LPS-PBS</th>
<th>PBS-LPS</th>
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<td>IL-17</td>
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<td>IL-23</td>
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<td>9237 ± 1158</td>
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<td>CXCL11/I-TAC</td>
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<td>M-CSF</td>
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<td>CXCL9/MIG</td>
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<td>CCL3/MIP-1α</td>
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<td>CCL4/MIP-1β</td>
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<td>CCL5/RANTES</td>
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Table 2. Mouse cytokine Antibody array, Panel A. Mice were twice i.p. injected with PBS or LPS with a 4 week interval between injections. Microglia were FACS isolated 6 hr after the second i.p. injection. Per condition, microglia from 3 mice were pooled and analyzed using the cytokine array. Results are shown as mean pixel density with standard deviations.