Impact of Ambient Temperature on Inflammation-induced Encephalopathy in Endotoxemic Mice—Role of Phosphoinositide 3-Kinase Gamma

CURRENT STATUS: UNDER REVIEW

Guang-Ping Lang
Zunyi Medical University

Bernadin Ndongson-Dongmo
Universitetet i Oslo

Trim Lajqi
Heidelberg University

Michael Brodhun
HELIOS Klinikum Erfurt

Yingying Han
Goethe-Universitat Frankfurt am Main

Reinhard Wetzker
Universitätsklinikum Jena

Martin G. Frasch
University of Washington

Reinhard Bauer
Universitätsklinikum Jena

reinhard.bauer@med.uni-jena.de
CORRESPONDING AUTHOR
ORCID: https://orcid.org/0000-0002-4294-3758

DOI:
10.21203/rs.3.rs-21417/v1

SUBJECT AREAS
Neurobiology of Disease

KEYWORDS
neuroinflammation, ambient temperature, microglia, phosphoinositide 3-kinase γ, blood–brain barrier, matrix metalloproteinases, phagocytosis, migration
Abstract

**Background:** Sepsis-associated encephalopathy (SAE) is an early and frequent event of infection-induced systemic inflammatory response syndrome. Phosphoinositide 3-kinase γ (PI3Kγ) is linked to neuroinflammation and inflammation-related microglial activity. In homeotherms, variations in ambient temperature ($T_a$) outside the thermoneutral zone lead to thermoregulatory responses, mainly driven by a gradually increasing sympathetic activity, and may affect disease severity. We hypothesized that thermoregulatory response to hypothermia (reduced $T_a$) aggravates SAE in PI3Kγ-dependent manner.

**Methods:** Experiments were performed in wild-type, PI3Kγ knockout, and PI3Kγ kinase-dead mice, which were kept at neutral (30±0.5°C) or moderately lowered (26±0.5°C) $T_a$. Mice were exposed to lipopolysaccharide (LPS, 10 μg/g, from Escherichia coli serotype 055:B5, single intraperitoneal injection) - evoked systemic inflammatory response (SIR) and monitored 24 hours for thermoregulatory response and blood-brain barrier integrity. Primary microglial cells and brain tissue derived from treated mice were analyzed for inflammatory responses and related cell functions. Comparisons between groups were made with one-way or two-way analysis of variance, as appropriate. Post hoc comparisons were made with the Holm-Sidak test or t-tests with Bonferroni’s correction for adjustments of multiple comparisons. Data not following normal distribution was tested with Kruskal-Wallis test followed by Dunn’s multiple comparisons test.

**Results:** We show that a moderate reduction of ambient temperature triggers enhanced hypothermia of mice undergoing LPS-induced systemic inflammation by aggravated SAE. PI3Kγ deficiency enhances blood-brain barrier injury and upregulation of matrix metalloproteinases (MMPs) as well as an impaired microglial phagocytic activity.

**Conclusions:** Thermoregulatory adaptation in response to ambient temperatures below the thermoneutral range exacerbates LPS-induced blood-brain barrier injury and neuroinflammation. PI3Kγ serves a protective role in suppressing release of MMPs, maintaining microglial motility and reinforcing phagocytosis leading to improved brain tissue integrity. Thus, preclinical research targeting severe brain inflammation responses is seriously biased when basic physiological
prerequisites of mammal species such as preferred ambient temperature are ignored.

**Background**

Sepsis-associated encephalopathy (SAE) is the most common form of encephalopathy occurring in critical care settings and refers to acute neurological dysfunction that arises in the context of extracranial sepsis. SAE is an early feature of infection in the body, occurs quite often with a prevalence of up to 30% in septicemic patients at admission and SAE severity is associated with increased mortality of septic patients [1]. Although the symptoms of SAE are well recognized - it can take the form of delirium, coma, seizures, or late cognitive decline - its pathophysiology is incompletely understood [2].

Several mechanisms of SAE have been proposed. The hallmarks are thought to comprise diffuse neuroinflammation likely driven by an initial blood-brain barrier (BBB) leakage leading to microglial activation and altered neurotransmission [3]. Activation of cerebral microvascular endothelial cells as the primary constituent of the BBB is regarded as an early event, induced by interaction with a pathogen product such as lipopolysaccharide (LPS) via pattern recognition receptors and proinflammatory factors. The latter include activated complement components and cytokines and lead to an increased endothelial production of reactive oxygen and nitrogen species, accelerated transendothelial cytokine trafficking and enhanced endothelial permeability [4]. Microglial proinflammatory response further reinforces BBB breakdown and modifies it via PI3Kγ-dependent cAMP control [5].

Thermoregulation is a fundamental homeostatic function of all mammals; it includes afferent thermal sensing, central regulation, and an efferent response resulting in a tightly controlled body temperature within a narrow species-specific range [6]. Variations of core body temperature ($T_C$) outside this range trigger autonomic thermoregulatory responses, mainly via a gradually increased sympathetic activity to minimize radiant heat loss by skin vasoconstriction and maximize heat production by brown adipose tissue thermogenesis [7]. Clinical data clearly indicate that poor outcome of sepsis is associated with spontaneous $T_C$ lowering (hypothermia indicating energy depletion) [8-10]. Subgroups of patients with increased risk to develop sepsis such as trauma or burns
fail thermal regulation leading frequently to accidental hypothermia [11, 12]. However, in pathogenesis of SAE, the role of challenged thermoregulation upon exposure to a reduced ambient temperature \( (T_a) \) and the resulting accidental hypothermia have not been studied. Moreover, in mice, a widely used species for modeling SAE, the temperature range of standard practice in preclinical biomedical research [13] and legal recommendations [14, 15] can be outside the thermoneutral zone for this species. Consequently, the aim of this study was to examine whether exposure of mice to \( T_a \) outside their thermoneutral zone (but well within the practiced experimental guidelines) will affect BBB breakdown and brain inflammation triggered by LPS-induced systemic inflammatory response in mice. We hypothesized that a reduced \( T_a \) exacerbates the inflammation-triggered BBB dysfunction at the system’s, organ and molecular levels. To examine different traits of PI3K\( \gamma \) signaling on microglial activation, migration and phagocytic activity, PI3K\( \gamma \)-deficient mice [16] and mice carrying a targeted mutation in the PI3K\( \gamma \) gene causing loss of lipid kinase activity [17] were habituated to neutral or reduced \( T_a \). Subsequently, these animals were exposed to LPS to induce a systemic inflammatory response syndrome (SIRS). Our results demonstrate that challenging thermoregulation by exposure to reduced \( T_a \) during SIRS causes enhanced early BBB breakdown. This BBB dysfunction is mediated by PI3K\( \gamma \)-dependent microglial immune responses during acute systemic inflammation. Thus, preclinical research targeting severe brain inflammation responses is seriously biased when basic physiological prerequisites of mammal species such as preferred ambient temperature are ignored.

Methods

Animals and experimental procedures

PI3K\( \gamma \) knockout mice (PI3K\( \gamma \)-/-) [16] and mice carrying a targeted mutation in the PI3K\( \gamma \) gene causing loss of lipid kinase activity (PI3K\( \gamma \)\(^{KD/KD}\)) [17] were on the C57BL/6J background for >10 generations. Age-matched C57BL/6 mice were used as controls. The animals were maintained at 12 h light and dark cycles with free access to food and water. The animal procedures were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the committee of the Thuringian State
Government on Animal Research.

In order to ensure appropriate acclimatization, animal were introduced at least one week before starting the interventions [18]. Animals were divided into a cohort kept at neutral ambient temperature (30±0.5°C) [19] or another cohort kept at lowered ambient temperature (26±0.5°C) during the whole experimental period for appropriate acclimatization. Then, mice received LPS (10 mg/kg, intraperitoneal, from *Escherichia coli* serotype 055:B5, Sigma-Aldrich, St. Louis, USA, Lot #032M4082V) as a single intraperitoneal injection. Additionally, 500 µl saline was injected subcutaneously immediately after LPS administration as well as after 24h. Clinical status was assessed at baseline and 24 h after LPS administration according to [20].

As an in vitro correlate of hypothermia and neuroinflammation, primary microglia obtained from wild type, PI3Kγ⁻/⁻ and PI3KγKD/KD were exposed to an incubation temperature (T_{Inc}) of 33°C and LPS (100 ng/ml). (For overview, please refer to Tab.1.)

*Telemetric assessment of body core temperature (T_C) and heart rate*

T_C and heart rate were assessed by telemetric monitoring of electrocardiography (ECG) and abdominal temperature.

*Surgical procedure:* Mice were anesthetized with 2.5 % isoflurane in oxygen. A midline incision was made on the abdomen and the intraperitoneal cavity was gently opened. An implantable 1.6-g wireless radiofrequency transmitter (ETA-F10, Data Sciences International, St. Paul, MN) was inserted; the leads were transferred though the abdominal wall and the incision was closed by a surgical suture. The cathodal lead was looped forward subcutaneously to an area overlying the scapula and anchored in place with a permanent suture. The anodal lead was brought subcutaneously to rest near the heart apex. Thereafter, skin incision was sutured. A warming light was used to maintain body temperature between 36 and 37°C. Meloxicam was given for pain on the day of surgery and the following day. Experiments were initiated 10 days after recovery from surgical instrumentation. Animals were monitored continuously by telemetry by ECG as well as body temperature and motor activity recording.
Data acquisition and processing: For simultaneous ECG and body temperature, analog signals were digitalized by the telemetric receiver (model RPC-1, Data Sciences International, St. Paul, MN) and transferred via DSI Data Exchange Matrix at a sampling rate of 2 kHz with 12-bit precision (acquisition software: Ponemah Software 5.20) without a signal filter, and stored on PC for off-line data analysis. Instantaneous heart rate (HR) was derived from the reciprocal RR interval time series. Therefore, the individual R-waves, with the R-wave peak as the trigger point, were sequentially recognized (ATISApromo®, GJB Datentechnik GmbH, Langewiesen, Germany). Accurate R-wave peak detection was verified by visual inspection. Temperature was continuously measured by the implanted transmitter and stored in parallel to the ECG signal.

Measurement of blood-brain barrier permeability

BBB disruption was analyzed by measurement of Evans blue (EB) extravasation into brain tissue as described previously [5, 21]. In brief, EB (4 ml/kg of a 2% solution in PBS) was injected through the tail vein 1 h prior to killing. Deeply anaesthetized animals were transcardially perfused with ice-cold PBS (40 ml) 24 h after LPS administration. The brains were removed after blood removal, snap-frozen in liquid nitrogen and stored at -80°C. One hemisphere was homogenized in trichloroacetic acid (50%) and centrifuged (10,000 rpm, 20 min, 4°C). Supernatant was diluted in three volumes of ethanol. EB was quantified by fluorescence measurement (Tecan Infinite F200, excitation 620 nm, emission 670 nm) and compared to a standard curve. EB concentrations are presented as µg of EB per µl of brain tissue supernatant.

Blood and brain tissue cytokine assessment

The cytokines levels (TNF-α, IL-6, MCP-1, IL-10) in blood and brain tissues were determined using BD™ CBA Mouse Inflammation Kit (Dickinson and Company, San Jose, USA). Blood was obtained via direct heart puncture, collected in a heparinized syringe and immediately centrifuged at 1500×g for 10 min at 4°C. The plasma supernatant was taken immediately and kept at –80°C until measurement. The brain tissue was harvested after rinsing with cold PBS, immediately put in liquid nitrogen and kept at -80°C until processing. The brain tissue was then powdered, ice-cold diluted in PBS, homogenated and centrifuged at 1000 g for 10 min at 4°C. Supernatant was immediately kept at -80°C until
measurement.

**Primary microglial cells**

The microglial cells were isolated from neonatal mouse cerebral cortex (6-10 brains per measure) as described [22]. The cells were co-cultivated with astrocytes for 14 days at 37°C and 5% CO\textsubscript{2} in DMEM high glucose containing 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin and 2.5 mg/ml amphotericin B. After 14 days, adherent microglia were separated from astrocytes by adding PBS/EDTA and careful shaking. Purity of isolated microglia was more than 95%. Purity of microglia was in the range between 95 and 98%, as confirmed by Iba1 staining (data not shown). After harvesting, microglial cells were seeded in well plates.

**RNA Extraction and cDNA Synthesis**

For quantification of mRNA, cells were seeded into 6-well plates and incubated at 37°C (5% CO\textsubscript{2}) overnight. Afterwards, cells were disintegrated in Trizol reagent (QIAzol Lysis Reagent (#79306), Qiagen, Hilden, Germany), Germany). Total RNA was extracted from Trizol as recommended by the manufacturer. To prevent contamination of mRNA preparation with chromosomal DNA, mRNA samples were treated with DNase. RNA amount and purity were determined by Nano- DropTM 1000 (Peqlab, Erlangen, Germany). For first strand cDNA synthesis, 1µg total RNA was employed using the RevertAid First Strand cDNA Synthesis kit (#K1612) from Thermo Fisher Scientific (Waltham, MA, USA). Synthesis followed the protocol recommended by the manufacturer.

**Quantitative PCR**

Quantitative PCR (qPCR) was performed with Maxima SYBR Green/ ROX qPCR Master Mix Kit (Fermentas; St. Leon Rot, Germany) containing Maxima Hot Start Taq DNA polymerase and appropriate primer pairs. The following primer pairs were used: MMP-2 forward: TGGCAGTGCAATACCTGAAC and MMP-2 reverse: CCGTACTTGCCATCCTTCTC, MMP-3 forward: GTACCAACCTATTCCTGGTTGC and MMP-3 reverse: CCAGAGAGTTAGATTTGGTGGG, MMP-9 forward: ACCACTAAAGGTGCTCGGATGGTT, MMP-9 reverse: AGTACTGCTTGCCAGGAAGACGAA, MMP-13 forward: GGGCTCTGAATGGTTATGACATTC, MMP-13 reverse: AGCGCTCAGTCTTCTTCACCTCTT, as well as
GAPDH forward: CATGGCCTTCCGTTTCCCTA and GAPDH reverse: CCTGCTTCACCACCTTCTTGAT.

Relative mRNA expression was calculated in relation to mRNA levels of the housekeeping gene, GAPDH, according to 2-ΔΔCT method [23].

**In Vitro Chemotaxis Assay**

To investigate the influence of lipid kinase dependent and -independent functions of PI3Kγ on microglial migration, transwell assays were performed. Cells were seeded in 6-well plates. After attachment, cells were starved and incubated with intended substances. Following stimulation, 1 \( \times 10^5 \) cells were transferred in 300 µl serum-free medium into the upper chamber of a 12-well chemotaxis insert (ThinCert™, 8µm pores; Greiner-Bio-One GmbH, Frickenhausen, Germany). The chamber was placed in 700 µl serum-free medium containing chemoattractant (C5a; 10 ng/ml) and incubated at 37°C (normal T

inc

or at 33°C (reduced T

inc

) with 5% CO

2

for 2 h. Afterwards, cells on the lower side of the insert membrane were fixed with 100% ice-cold methanol and stained with 0.5% crystal violet solution (in 25% methanol) for 10 min. Average count of migrated cells was estimated through consideration of five independent visual fields.

**In Vivo Microglial Migration Assay**

Experiments were performed on adult (10–14 weeks) wild type, PI3Kγ

−/−

, and PI3Kγ

KD/KD

mice (7 mice per group) kept during the whole experimental period at neutral T

a

or reduced T

a

, respectively. To investigate the effect of targeted PI3Kγ mutation on microglial migration at neutral or reduced T

a

, an in vivo wound healing experiment was performed. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (16 mg/kg), and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Mice were then placed on a homeothermic heat blanket to maintain normal body temperature during surgery. The skull was exposed by a skin incision, and small burr holes were drilled through the skull. Using a micromanipulator focal stab, an injury was performed by gentle insertion of stainless steel pin (diameter 0.25 mm) into the parietal cortex at 3 mm below the dura mater [24, 25]. The pin was kept in place for two minutes and then removed. The burr holes were covered with bone wax, and the animals were returned to their cages. 12 h later, mice were
deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in phosphate buffer by cardiac puncture via the left ventricle. Brains were removed immediately after fixation and post-fixed for 5 h in 4% PFA at 4°C. After cryoprotection in phosphate-buffered saline (PBS) containing 30% sucrose, brains were frozen in methylbutane at -30°C and stored at -80°C. Whole brains were cut by horizontal sections at 40 μm on a freezing microtome (Microm International GmbH, ThermoScientific, Germany). The slices were immunostained with anti-Iba1 antibody to visualize microglia. Sections were photographed with a digital fluorescence camera (Nikon DSQii2) and mounted on the Nikon inverted research microscope Eclipse Ti (Nikon Instruments Europe B.V., Amstelveen, The Netherlands).

Quantitative measurements (ImageJ software, National Institutes of Health, Bethesda, MD) blinded to the treatment groups were used to count cell numbers per voxel and expressed in mm$^3$. At the injured region, three voxels were predefined as follows: Voxel 1, a cylinder with a diameter of 400 μm, center lying in the middle of injury, and an altitude of 40 μm; Voxel 2, hollow cylinder, subsequently on Voxel 1, with an inner diameter of 400 μm, an outer diameter of 800 μm, and an altitude of 40 μm; Voxel 3, hollow cylinder, subsequently on Voxel 2, with an inner diameter of 800 μm, an outer diameter of 1200 μm, and an altitude of 40 μm. Number of Iba1-positive cells was counted in all three voxels. Migratory index was estimated as the ratio of cell number in Voxel 1 divided by the sum of cell number in Voxels 1, 2 and 3 as described previously [24, 26].

**In Vitro Phagocytosis Assay**

Efficiency of phagocytosis at reduced versus neutral T$_a$ was investigated as previously described [24, 26]. Briefly, primary microglia cells obtained from wild type, PI3Kγ$^{-/-}$, and PI3Kγ$^{KD/KD}$ mice were seeded into 12-well plates and incubated at 37°C (5% CO$_2$) for 24 h. After attachment, cells were starved for 24 h in DMEM without FCS. Cell were subsequently stimulated with LPS (100 ng/ml) or left unstimulated. Phagocytosis assay was performed by using fluorescein isothiocyanate (FITC)-labeled Zymosan A (S. cerevisiae) BioParticles (9800U/ml) (#Z2841, Thermo Fisher Scientific, Waltham, USA). 7 μl of the suspended particles was added to the microglial cells and incubated 1 h at either 37°C or 33°C. After incubation the cells were fixed with 4% PFA, washed three times and stained with DAPI–
solution for 5 min (1:1000 in 1 İ PBS). Phagocytosed particles and cells of five independent visual fields were counted under a fluorescence microscope (Nikon Eclipse Ti, Nikon Instruments - Japan). The result of the phagocytosis of primary microglia was calculated by determining the phagocytic index (the uptake rate of FITC-Zymosan particles per cell).

**In Vivo Phagocytosis Assay**

Experiments were performed on adult (10–14 weeks) wild type, PI3Kγ⁻⁻, and PI3Kγ_KD/KD mice (7 mice per group) kept during the whole experimental period at neutral Tₐ or reduced Tₐ, respectively. To investigate the effect targeted PI3Kγ mutation on microglial phagocytosis FITC-labeled Zymosan particles (9800 U/ml) were administered into the brain as described previously [24]. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/ kg) and xylazine (16 mg/kg), and positioned in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The skull was exposed by a skin incision, and small burr holes were drilled through the skull. Using a micromanipulator a cannula (diameter 0.24 mm) attached on a Hamilton microsyringe (10 µl) was stereotaxically placed into the parietal cortex on both sides (stereotaxic coordinates were AP, - 2.0 mm; L, ± 0.5 mm; and V, - 2.5 mm, respectively [27]). Subsequently, 4 µl of FITC-labeled Zymosan particles suspended in artificial cerebrospinal fluid were infused within 120 seconds. The cannula remained in place for 5 minutes before removal. Twenty-four hours later mice were deeply anesthetized and perfused with 4% PFA in phosphate buffer by cardiac puncture via the left ventricle. Brains were removed immediately after fixation and post-fixed for 5 h in 4% PFA at 4°C. After cryoprotection in PBS containing 30% sucrose, brains were frozen in methylbutane at -30°C and stored at -80°C. Whole brains were cut by coronal sections at 40 µm on a freezing microtome (Microm International GmbH, Thermo Scientific, Germany). The slices were immunostained with anti-Iba1 antibody to visualize microglia. A voxel with an edge length of 400 µm, and an altitude of 40 µm were predefined as region of interest. Z-stack imaging was performed with a 20× objective using a digital fluorescence camera (Nikon DS-Qi2), mounted on the Nikon inverted research microscope Eclipse Ti (Nikon Instruments Europe B.V., Amstelveen, The Netherlands). Quantitative measurements (ImageJ software, National Institutes of Health, Bethesda,
MD) blinded to the treatment groups were used to count the percentage number of Iba-1 positive cells per mm$^3$ containing Zymosan particles.

*Histopathology and immunohistochemistry*

For determination of microglial activation, PMN homing, MMP-9 expression, and TUNEL positivity, brains were fixated in situ by transcardial perfusion with 4 % PFA after rinsing with PBS. Afterwards, they were immediately removed after fixation, post-fixated in 4 % PFA at 4 °C for 1 day, embedded in paraffin and cut into 6-μm-thick sections. After deparaffinization, the sections were heated with citrate buffer (0.01M, pH6.0) in the microwave (630W, 11min) for antigen removal and the nonspecific binding sites were blocked with blocking solution (5% NDS, 1% BSA-c, PBST). Then, the slide-mounted tissue sections were incubated with the desired primary antibody in antibody incubation solution (5% NDS, 1% BSA-c, PBST) at 4 °C overnight, followed by an incubation with the associated secondary antibody at 4 °C for 1 h. Negative control sections were incubated with goat serum in absence of the primary antibody. The following primary antibodies were used: goat polyclonal anti-Iba-1 (1:250) antibody (Abcam, Cambridge, UK) for Iba1 staining, rabbit polyclonal anti-MMP-9 (1:150) antibody (Cell Signaling Technology, Danvers, USA) for MMP-9 and rabbit anti-mouse PMN (ACCURATE CHEMICAL & SCIENTIFIC CO, USA) for neutrophils staining. For visualization, the secondary fluorescent goat anti-mouse isotype-specific antibody Alexa Fluor® 488 (Molecular Probes, Inc., Eugene, USA) and donkey anti-goat IgG antibody Alexa Fluor®568 (Thermo Fisher Scientific, Waltham, USA) were used. Method for TUNEL staining was described elsewhere [28]. Briefly, sections were deparaffinized and prepared for TUNEL-staining. Fragmented DNA was detected in situ by the TUNEL method using a commercially available kit according to the manufacturer’s protocol (In Situ Cell Death Detection Kit, POD; Roche, Germany). Deparaffinized sections were pretreated with 20 mg/ml proteinase K, and washed in PBS prior to TUNEL staining. TUNEL staining was performed by incubation with fluorescein-conjugated digoxigenin-UTP and terminal deoxynucleotidyltransferase at 37°C for 1 h. DNA fragmentation was visualized using converter-alkaline phosphatase, NBT/BCIP and counterstaining with Kernechtrot.

Cell counting for assessment of microglial cell activation: Cells were classified as ramified, amoeboid,
unipolar and bipolar. Ramified (normal) microglial cells are defined by thin, slender, radially projecting processes with well-developed ramifications. Amoeboid microglial cells are defined as having large soma, and short, thick and radially projecting processes. Unipolar and bipolar microglial cells were defined as having one or two thick process with well-developed ramifications [29, 30]. Estimation of cell counting migration and phagocytic index were performed by a co-author (G.-P. L.) blinded for genotype and treatment. In each case, evaluation was performed on three different slices obtained from frontal cortex, thalamus and hippocampus, each. Five separate fields of vision were counted with at least 100 cells.

Statistics

The statistical analysis was performed using SigmaPlot Software (Sigma-Plot Software, San Jose, USA). All data are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from the 10th to the 90th percentiles (extreme values are marked outside). Numbers of animals are given in figure legends for each group and time point. Comparisons between groups were made with one-way or two-way analysis of variance, if appropriate. In case of repeated measurements, one-way and two-way analysis of variance with repeated measures was used, if appropriate. Post hoc comparisons were made with the Holm-Sidak test or t-tests with Bonferroni's correction for adjustments of multiple comparisons. Data not following normal distribution was tested with Kruskal-Wallis test followed by Dunn’s multiple comparisons test.

Results

Impact of ambient temperature on degree of SIRS and SIRS-induced BBB disturbance

Intraperitoneal LPS administration induced a robust SIRS in mice kept at neutral as well as reduced $T_a$ as revealed by cytokine release in blood plasma and brain tissue (Fig. 1). IL-10 anti-inflammatory response was barely developed. However, reduced $T_a$ induced a worsened sickness state of SIRS in PI3Ky-deficient mice as measured by the clinical severity score (Tabl.1 Suppl.). Telemetric $T_C$ monitoring revealed that at baseline conditions all mice, regardless $T_a$, showed no differences in body temperature. However, reduced $T_a$ was accompanied by an enhanced sympathetic
tone to the heart already under baseline conditions, indicated by an increased HR regardless of the genotype (Fig. 2). Importantly, whereas the early period of LPS-induced SIRS was characterized by a rather similar HR elevation, HR remained elevated in the animals kept at neutral $T_a$, but not in the animals kept at reduced $T_a$, throughout the observation period. Furthermore, all mice kept at neutral $T_a$ exhibited a short-term period of mild hypothermia whereas the mice kept at reduced $T_a$ developed a markedly more pronounced and longer (24h versus 12h) lasting hypothermic period (Fig. 2).

At neutral $T_a$, LPS-induced SIRS provoked an increase of BBB leakage in wild type mice, whereas PI3K$\gamma$-deficient mice exhibited a significantly enhanced BBB disturbance compared with wild type mice (Figure 3). At baseline, this was also true for PI3K$\gamma$-kinase-dead mice.

In contrast, at baseline, housing at reduced $T_a$ induced BBB leakage in wild type mice to a similar degree as in the mutant mice (Fig 3B). Moreover, already at baseline, in the wild type mice the BBB leakage was more pronounced at reduced $T_a$ than at neutral $T_a$. LPS-induced SIRS provoked a substantially enhanced BBB leakage, which was most pronounced in PI3K$\gamma$-deficient mice. Of note, lipid kinase-dead mutant mice display similar degree of BBB breakdown as the wild type mice indicating a causal impact of lipid kinase-independent PI3K$\gamma$ signaling on the development of BBB disturbance during SIRS.

**Impact of $T_a$ and SIRS on degree of microglial activation, MMP expression, apoptosis, and PMN invasion**

To verify consequences of $T_a$ and LPS-induced SIRS, extent of neuroinflammation was assessed by different approaches. First we quantified the number of activated microglial cells assessed by shape characteristics [30]. As shown in Fig 4, a marked increase in microglial cell number with altered, mainly polarized shape occurred. Analysis of regional distribution revealed similarity in the extent of regional microglial cell activation in brain cortex, hippocampus and thalamus (Tabl. 2 Suppl.) suggesting a diffuse microglial activation due to LPS-induced SIRS. While we did not observe a significant genotype-related effect, the wild type mice showed an exacerbated response with regard to activated microglia counts at reduced $T_a$. 
In contrast, MMP expression in brain tissue obtained 24 hours after LPS injection displayed a significant $T_a$ as well as PI3Kγ dependency. There was an enhanced RNA expression in brains obtained from PI3Kγ -deficient mice kept at reduced $T_a$ in all MMPs under consideration compared to mice kept at neutral $T_a$ (Fig. 5). Furthermore, there was an increased mRNA expression in brains derived from PI3Kγ-deficient mice kept at reduced $T_a$ compared with wild type mice kept under same housing conditions. In contrast, PI3Kγ$^{KD/KD}$ mice showed a similar response as wild type mice, again suggesting a lipid kinase-independent mode of action.

Increased cerebral MMP expression appeared as a result of enhanced microglial activation in PI3Kγ$^{-/-}$ mice kept at reduced $T_a$ as is evidenced by an increased number of MMP-9 positive cells co-expressed in Iba-1 positive cells in these brains (Fig. 6A). Analysis of regional distribution revealed similarity in the extent of microglial MMP-9 expression in brain cortex, hippocampus and thalamus (Tabl. 3 Suppl.), again suggesting a uniform increase in activity.

To examine a possible $T_a$ -dependent impact on structural integrity of brain tissue due to LPS-induced SIRS, we quantified the extent of apoptosis in brain slices derived from wild type, PI3Kγ$^{-/-}$, and PI3Kγ$^{KD/KD}$ mice kept at neutral and reduced $T_a$. Albeit of small magnitude, already under baseline conditions, the rate of apoptotic cells was significantly increased in brains of PI3Kγ$^{-/-}$ mice kept at reduced $T_a$ compared with those kept at neutral $T_a$. LPS-induced SIRS exhibited consistently an increased number of apoptotic cells, which was most pronounced in PI3Kγ$^{-/-}$ mice kept at reduced $T_a$ (Fig. 6B, Fig 1 Suppl.). Regional comparison revealed that numbers of TUNEL-positive cells in PI3Kγ$^{-/-}$ mice kept at reduced $T_a$ were markedly higher in the hippocampus compared to cortex and thalamus (Tabl. 3 Suppl.).

To assess a contribution of blood-born immune cells to pathogenesis of SIRS-induced SAE we quantified the extent of invading PMN. Whereas under baseline conditions merely scattered PMN were encountered and neither $T_a$ nor genotype-related effects have been observed, LPS-induced SIRS was
accompanied by a distinct increase of invading PMN into the brain tissue. We found a significant $T_a$-dependent effect in PI3Kγ-deficient mice observing an enhanced PMN homing into brain tissue in mice kept at reduced $T_a$ (Fig. 6C, Fig 1 Suppl.). However, consistent with findings for Iba1-positive microglia cell numbers and MMP expression, we observed no brain regional differences in PMN invasion (Tabl. 3 Suppl).

**Impact of $T_a$ incubation temperature on microglial migration and phagocytosis**

The ability to migrate toward different chemotactic stimuli including those released by brain injuries is an important property of microglial cells, which is essential for biological functions. Our previous studies revealed a dependency of lipid kinase-related PI3Kγ signaling on directed motility of microglial cells owing to inflammatory stimulation [24]. Herein, we addressed the question if PI3Kγ-dependent migration of microglia is a result of different ambient temperature and its in vitro surrogate, i.e., the varied temperatures of incubation ($T_{\text{Inc}}$).

First, the *in vitro* cell motility was quantified toward migration to C5a added to bottom well of the transwell assay together with LPS stimulation. C5a acts as inflammatory peptide resulting in stimulation of microglial migration toward this chemo attractant. As demonstrated in Figure 7A, PI3Kγ deficiency as well as targeted knockout of the lipid kinase activity of PI3Kγ caused a markedly reduced migratory capacity by about 50% compared with cells derived from wild type mice. A moderately reduced $T_{\text{Inc}}$ provoked a further reduction in directed motility of primary microglial cells, whereas the PI3Kγ-related migratory alteration remained preserved.

To assess the effect of PI3Kγ-deficiency on directed cell motility in vivo, a wound healing assay using focal stab-injury [24] was carried out. Whereas the baseline number of microglial cells was similar in wild type, PI3Kγ deficient and -kinase dead brains (Table 4 Suppl.), migration of microglia in direction of the focal stab injury was clearly reduced in brains from PI3Kγ mutants as indicated by reduced microglial cell numbers in the inner region of interest (see Fig. 7A, Suppl. Fig. 2) of the injury site, which was markedly reduced in mice kept at reduced $T_a$. Taken together, the present data indicate an inhibitory role of reduced $T_a$ for the directional migration/chemotaxis of microglial cells from PI3Kγ
mutants.

Next, the role of modified $T_a$ ambient temperature for phagocytosis, another essential function of microglia, was analyzed. First, efficiency of phagocytosis was quantified by in vitro incubation of microglia with FITC-labeled Zymosan particles and subsequent counting of incorporated particles inside the microglial cells. PI3Kγ deficiency caused a distinct decrease of phagocytosis of microglial cells at reduced $T_{\text{Inc}}$ (Fig. 7B, Suppl. Fig. 3). At reduced $T_{\text{Inc}}$, quite similar effects have been ascertained. *In vivo* analysis of phagocytosis was performed by intracerebral administration of Zymosan particles. At neutral $T_a$, counting the number of cells with phagocytosed particles revealed a reduction of microglial phagocytic activity in brains derived from PI3Kγ$^{-/-}$ mice. Reduced $T_a$ caused an additional distinct inhibition of phagocytic activity which was even more pronounced in PI3γ-deficient mice.

**Discussion**

Our study identifies ambient temperature as a major impact factor for extent and clinical course of LPS-induced SIRS and concomitant blood-brain barrier breakdown as a key event in the development of SAE. We studied LPS-induced SIRS at a $T_a$ of 30°C (within thermoneutral zone for mice [31]) and at the upper edge of the recommended standard housing temperatures for laboratory mice, e.g. at 26°C [15]. Importantly, recent guidelines for preclinical studies in sepsis research [32, 33] and cerebrovascular research [34-36], with the emphasis to improve reproducibility and translational impact, neglected the impact of ambient temperature on pathogenesis of inflammatory diseases or did not consider that their recommended lower baseline limit for housing temperatures of small rodents causes chronic ‘cold’ stress [37, 38]. Furthermore, ignoring the role of ambient temperature on basal physiological responsiveness in small animals frequently used in preclinical sepsis models leads to far-reaching repercussions of profound misinterpretation: Time course of body temperature lowering and development of hypothermia is used for prognosis prediction while mice are under chronic cold stress for mice [39-43]. Clearly, statements of these pertinent reports are restricted merely on the subgroup of septic patients endangered for cold challenge leading to accidental
hypothermia [44]. Overall recommendations for endpoint decision on development of hypothermia in preclinical inflammatory and infection research while neglecting $T_a$ [32, 40-43] are clearly misleading.

The recent findings in mice showed that a reduced ambient temperature exacerbates SIRS-induced cardiac autonomic dysregulation and myocardial dysfunction [45]. This study in the heart supports the present results and conclusions on the brain response to cold stress.

We show for the first time that a reduction of $T_a$ of only 3-4 degrees below the lower critical $T_a$ for mice [46] increases the severity of BBB injury as a consequence of LPS-induced SIRS. This was clearly associated with a temporary disturbance of thermoregulation as a fundamental homeostatic function of all mammals, because body core temperature was markedly reduced early after LPS-induced SIRS and hypothermia persisted throughout the observed period (Fig. 2). Occurrence of hypothermia as a result of sepsis and overwhelming systemic inflammation characterizes a specific state of disturbed thermoregulation. Indeed, experimental and clinical studies clearly showed that severity of inflammation determines the magnitude of displacement from normothermia, e.g., mild to moderate SIRS induced by low and medium dosages of LPS provokes fever, whereas severe/life-threatening SIRS induces hypothermia [47-49]. The mechanisms regulating hypothermia are not fully understood, but cytokines such as TNF-a, interleukins (ILs) and interferon-gamma have been shown to induce or modulate hypothermia [50]. The herein presented data suggest that TNF-a may contribute to the hypothermic response because of a similar temporal profile.

However, other factors play apparently a more dominant role in the extent of hypothermia early after LPS-induced SIRS. Clearly, $T_a$ determines depth of $T_c$ reduction (Fig. 2). Therefore, hypothermic response appears to be a consequence of maladaptive thermoregulation leading to hypometabolism in order to avoid hypoxia [51].

PI3Kγ-dependent differences in extent of sickness suggest that disturbance in behavioral thermoregulation may contribute to the manifestation of hypothermia. Small rodents such as mice need an increased metabolic rate and periodic motor activity for appropriate heat production to maintain homeothermy because of its unfavorable surface area versus mass ratio [46, 52]. Mice
develop hypothermia when locomotor activity is diminished due to consequences of sickness induced by infection [53]. Our findings show that mice kept at reduced $T_a$ developed an exacerbated and prolonged hypothermia although they exhibited a markedly enhanced sympathetic tone. This might be related to a stronger impairment of thermoregulation with torpor-like traits elicited by LPS-induced SIRS [54], in addition to LPS-induced inhibition of brown adipose tissue thermogenesis [55].

In a previous study, we have shown that the kinase-independent control of cAMP phosphodiesterase activity by PI3Kγ acts as a crucial mediator of microglial cell activation, MMP expression and subsequent BBB deterioration [5]. The data obtained in the current study suggest that an aggravated BBB breakdown observed in mice kept at reduced $T_a$ during LPS-induced SIRS results from an intensified LPS-induced proinflammatory microglial response. This response is accompanied by the pronounced upregulation of brain MMP expression and perivascular MMP-9 release leading to increased PMN invasion with altered microglial migration and phagocytosis. These processes appear to be widespread because we made similar findings in quite different brain regions. Enhanced plasma protein extravasation in brains obtained from PI3Kγ-deficient mice kept at reduced $T_a$ suggests that the genotype-related differences in BBB breakdown appear to be related to microglial activation in response to systemic inflammation and concomitant brain tissue MMP upregulation. Compelling evidence suggests that early after SIRS manifestation, constitutive proteases are activated and begin the process of disassembling the extracellular matrix and opening the BBB [56, 57]. Immunohistological evaluation revealed that there is an increased number of Iba-1 positive cells which co-express MMP-9 in brains obtained from PI3Kγ-deficient mice kept at reduced $T_a$. These activated microglia may play a crucial role in exacerbating BBB deterioration “from the inside” by releasing MMP-3 and attacking the basal lamina and tight junction proteins [57, 58]. Our previous results revealed that the enhanced MMP-9 activity is of microglial origin and provoked by a deficient suppression of cAMP-dependent proinflammatory signaling in PI3Kγ-deficient mice [5]. MMP-9 is known to act as an executing protease for degrading matrix substrates and interrupting cell–cell or cell–matrix homeostatic interactions, which may directly trigger anoikis-like neuronal cell death by
interrupting cell-matrix survival signaling [59]. The current findings of aggravated SIRS-induced BBB impairment associated with reduced Tₐ are clearly PI3Kγ-dependent and induce an enhanced invasion of blood-born immune cells and an increased rate of apoptosis, especially in hippocampus, when the suppressive effect of PI3Kγ on cAMP as a critical mediator of immune cell functions is absent [5, 17, 60]. A previous report showed that deleting PI3Kγ suppressed BBB breakdown and MMP9 activity after experimental stroke with reduced infarct volumes and neurological scores at 24 hours after ischemia/reperfusion [61, 62]. In a subsequent study we documented an enhanced enlargement of infarct size in PI3Kγ KO mice 48 h after reperfusion [63]. Reasons of discrepancy between both studies have been discussed elsewhere [63]. Briefly, there are several differences in the experimental design which may responsible for the opposite findings. (i) In the previous report [61, 62], the documented enhanced BBB disturbance and associated brain damage in wild-type mice resulted from a longer-lasting period of focal ischemia (1 h vs. 45 min in our study) and occurred already earlier (24 h vs. 48 h in our study) after reperfusion. In contrast, our data showed that the enhanced enlargement of infarct size in KO mice was detectable clearly only at a later stage (48 h) of infarct maturation suggesting different underlying mechanisms. (ii) The authors of the previous study claimed a markedly enhanced PMN infiltration into the ischemic area of wild-type mice as the main source for increased MMP-9 secretion and ROS production and as the relevant executors for enlarged brain tissue damage [61]. In wild-type mice, our data clearly show that after a shorter period of brain ischemia (45 min) with probably reduced BBB disturbance an anticipated markedly increased PMN accumulation occurred within the ischemic core but was associated with smaller infarct size. KO mice with enlarged infarct size exhibited a reduced PMN accumulation. These findings suggest that PMN invasion is unlikely to play a decisive role for PI3Kγ-dependent differences in infarct development during later stages of reperfusion after brain ischemia. Another report showed neuroprotective effects against acute cerebral ischemia by inhibition of PI3Kγ-mediated superoxide generation in microglia [64]. While we did not study this issue in brain, we did perform a comparable study investigating the role of PI3Kγ in LPS-induced cardiomyopathy. We found that an activation of cardiac phosphodiesterases via PI3Kγ suppressed myocardial inflammatory responses including iNOS and
nitro-tyrosine levels [65]. Therefore, we conclude that the specific role of lipid kinase-dependent and -independent functions of PI3Kγ in inflammation-induced responses appears to be cell- and context dependent and may lead to opposite effects. Causal relations responsible for associated exacerbated brain injury cannot be drawn conclusively. Nevertheless, a reduced ability of directed motility and diminished phagocytic activity in brains obtained from PI3Kγ-deficient mice kept at reduced T_a suggest that these altered cell functions contribute to the phenotype of enhanced structural and functional cerebral disturbance leading to aggravated SAE symptoms. We identified the lipid kinase activity of PI3Kγ as an essential mediator of microglial migration [66]. Furthermore, diminished microglial phagocytic activity appears to contribute to the enhanced proinflammatory brain response to LPS-induced SIRS in PI3Kγ-deficient mice kept at reduced T_a because microglial phagocytosis represents a key factor for limiting excessive proinflammatory activation by clearance of dying cells and debris in injured brain tissue [29, 63, 67].

Conclusions
Our findings underline the importance of ambient temperature as a frequently neglected yet crucial environmental condition in translational inflammatory/infectious diseases research. The major significance of our findings is that a modest variation of an easily controllable parameter, i.e., the ambient temperature, led to a serious impact on the course of SAE. Furthermore, our data reveal the signaling protein PI3Kγ as a critical mediator of key microglial cell functions involved in LPS-induced BBB injury and accompanying neuroinflammation. PI3Kγ serves a protective role in that it suppresses MMP release, maintains microglial motility and reinforces phagocytosis leading to improved brain tissue integrity.

Thus, this study substantiates the importance of controlling T_a tightly to prevent serious bias in results from preclinical animal research on inflammation and infection. Accounting for T_a will improve the predictive power and value of the neuroinflammatory research and help overcome the ‘replication crisis’ [68].

Declarations
Ethics approval and consent to participate
This study involves animal experiment and includes a statement on ethics approval.

Consent for publication
Not applicable

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

Funding
The study was supported by the Deutsche Forschungsgemeinschaft [R.B.] [DFG, Grant RTG 1715].
B.N.-D. received funding from the European Union Seventh Framework Programme (FP7-PEOPLE-2013-COFUND) under grant agreement no 609020 - Scientia Fellows, G.-P.L. received a Scholarship by the China Scholarship Council.

Authors' contributions
RB and RW conceived and designed the study. G-PL performed in-vitro experiments and analyzed most of the experiments. RB and BN-D supervised the experimental program and performed animal experiments. RB prepared the manuscript. TL, MB, and YH performed some of the immunohistochemistry and data analysis. MGF and RW edited the manuscript. All authors approved the final version of the manuscript.

Acknowledgements
The authors acknowledge Mrs. R.-M. Zimmer for skillful technical assistance.

Abbreviations
References

1. Tauber SC, Eiffert H, Bruck W, Nau R: Septic encephalopathy and septic encephalitis. Expert Rev Anti Infect Ther 2017, 15:121-132.

2. Gofton TE, Young GB: Sepsis-associated encephalopathy. Nat Rev Neurol 2012, 8:557-566.

3. Annane D, Sharshar T: Cognitive decline after sepsis. Lancet Respir Med 2015, 3:61-69.

4. Kuperberg SJ, Wadgaonkar R: Sepsis-Associated Encephalopathy: The Blood-Brain Barrier and the Sphingolipid Rheostat. Front Immunol 2017, 8:597.

5. Frister A, Schmidt C, Schneble N, Brodhun M, Gonnert FA, Bauer M, Hirsch E, Muller JP, Wetzker R, Bauer R: Phosphoinositide 3-kinase gamma affects LPS-induced disturbance of blood-brain barrier via lipid kinase-independent control of
cAMP in microglial cells. *Neuromolecular Med* 2014, 16:704-713.

6. Nakamura K: *Central circuitries for body temperature regulation and fever.* *Am J Physiol Regul Integr Comp Physiol* 2011, 301:R1207-1228.

7. Nakamura Y, Nakamura K: *Central regulation of brown adipose tissue thermogenesis and energy homeostasis dependent on food availability.* *Pflugers Arch* 2018, 470:823-837.

8. Clemmer TP, Fisher CJ, Jr., Bone RC, Slotman GJ, Metz CA, Thomas FO: *Hypothermia in the sepsis syndrome and clinical outcome. The Methylprednisolone Severe Sepsis Study Group.* *Crit Care Med* 1992, 20:1395-1401.

9. Young PJ, Saxena M, Beasley R, Bellomo R, Bailey M, Pilcher D, Finfer S, Harrison D, Myburgh J, Rowan K: *Early peak temperature and mortality in critically ill patients with or without infection.* *Intensive Care Med* 2012, 38:437-444.

10. Schortgen F: *Fever in sepsis.* *Minerva Anestesiol* 2012, 78:1254-1264.

11. Cumming J, Purdue GF, Hunt JL, O'Keefe GE: *Objective estimates of the incidence and consequences of multiple organ dysfunction and sepsis after burn trauma.* *J Trauma* 2001, 50:510-515.

12. Soreide K: *Clinical and translational aspects of hypothermia in major trauma patients: from pathophysiology to prevention, prognosis and potential preservation.* *Injury* 2014, 45:647-654.

13. Overton JM: *Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters.* *Int J Obes (Lond)* 2010, 34 Suppl 2:S53-58.

14. CotEU EP: *Directive 2010/63/EU of the European Parliament and of the council on the protection of animals used for scientific purposes.* *Off J Euro Union L* 2010, 276:33-79.

15. NRC: *Guide for the Care and Use of Laboratory Animals.* In *Council of the
16. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP: **Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation.** *Science* 2000, **287**:1049-1053.

17. Patrucco E, Notte A, Barberis L, Selvetella G, Maffei A, Brancaccio M, Marengo S, Russo G, Azzolino O, Rybalkin SD, et al: **PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects.** *Cell* 2004, **118**:375-387.

18. Obernier JA, Baldwin RL: **Establishing an appropriate period of acclimatization following transportation of laboratory animals.** *ILAR J* 2006, **47**:364-369.

19. Gordon CJ, Becker P, Ali JS: **Behavioral thermoregulatory responses of single- and group-housed mice.** *Physiol Behav* 1998, **65**:255-262.

20. Gonnert FA, Recknagel P, Seidel M, Jbeily N, Dahlke K, Bockmeyer CL, Winning J, Losche W, Claus RA, Bauer M: **Characteristics of clinical sepsis reflected in a reliable and reproducible rodent sepsis model.** *J Surg Res* 2011, **170**:e123-134.

21. Comim CM, Vilela MC, Constantino LS, Petronilho F, Vuolo F, Lacerda-Queiroz N, Rodrigues DH, da Rocha JL, Teixeira AL, Quevedo J, Dal-Pizzol F: **Traffic of leukocytes and cytokine up-regulation in the central nervous system in sepsis.** *Intensive Care Med* 2011, **37**:711-718.

22. Giulian D, Baker TJ: **Characterization of ameboid microglia isolated from developing mammalian brain.** *J Neurosci* 1986, **6**:2163-2178.

23. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.** *Methods* 2001,
25:402-408.

24. Schneble N, Muller J, Kliche S, Bauer R, Wetzker R, Bohmer FD, Wang ZQ, Muller JP: The protein-tyrosine phosphatase DEP-1 promotes migration and phagocytic activity of microglial cells in part through negative regulation of fyn tyrosine kinase. *Glia* 2017, 65:416-428.

25. Seo JW, Kim JH, Kim JH, Seo M, Han HS, Park J, Suk K: Time-dependent effects of hypothermia on microglial activation and migration. *J Neuroinflammation* 2012, 9:164.

26. Sun SF, Pan QZ, Hui X, Zhang BL, Wu HM, Li H, Xu W, Zhang Q, Li JY, Deng XM, et al: Stronger in vitro phagocytosis by monocytes-macrophages is indicative of greater pathogen clearance and antibody levels in vivo. *Poult Sci* 2008, 87:1725-1733.

27. Paxinos G, Franklin KBJ: *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 2001.

28. Brodhun M, Fritz H, Walter B, Antonow-Schlorke I, Reinhart K, Zwiener U, Bauer R, Patt S: Immunomorphological sequelae of severe brain injury induced by fluid-percussion in juvenile pigs--effects of mild hypothermia. *Acta Neuropathol* 2001, 101:424-434.

29. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A: *Physiology of microglia*. *Physiol Rev* 2011, 91:461-553.

30. Zhang F, Vadakkan KI, Kim SS, Wu LJ, Shang Y, Zhuo M: Selective activation of microglia in spinal cord but not higher cortical regions following nerve injury in adult mouse. *Mol Pain* 2008, 4:15.

31. Gordon CJ: Toxic-induced hypothermia and hypometabolism: do they increase uncertainty in the extrapolation of toxicological data from experimental
animals to humans? Neurosci Biobehav Rev 1991, 15:95-98.

32. Zingarelli B, Coopersmith CM, Drechsler S, Efron P, Marshall JC, Moldawer L, Wiersinga WJ, Xiao X, Osuchowski MF, Thiemermann C: Part I: Minimum Quality Threshold in Preclinical Sepsis Studies (MQTiPSS) for Study Design and Humane Modeling Endpoints. Shock 2019, 51:10-22.

33. Osuchowski MF, Ayala A, Bahrami S, Bauer M, Boros M, Cavaillon JM, Chaudry IH, Coopersmith CM, Deutschman CS, Drechsler S, et al: Minimum Quality Threshold in Pre-Clinical Sepsis Studies (MQTiPSS): An International Expert Consensus Initiative for Improvement of Animal Modeling in Sepsis. Shock 2018, 50:377-380.

34. Percie du Sert N, Alfieri A, Allan SM, Carswell HV, Deuchar GA, Farr TD, Flecknell P, Gallagher L, Gibson CL, Haley MJ, et al: The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements Of in Vivo Experiments). J Cereb Blood Flow Metab 2017, 37:3488-3517.

35. Howells DW, Sena ES, Macleod MR: Bringing rigour to translational medicine. Nat Rev Neurol 2014, 10:37-43.

36. Vahidy F, Schabitz WR, Fisher M, Aronowski J: Reporting Standards for Preclinical Studies of Stroke Therapy. Stroke 2016, 47:2435-2438.

37. Hankenson FC, Marx JO, Gordon CJ, David JM: Effects of Rodent Thermoregulation on Animal Models in the Research Environment. Comp Med 2018, 68:425-438.

38. Hylander BL, Gordon CJ, Repasky EA: Manipulation of Ambient Housing Temperature To Study the Impact of Chronic Stress on Immunity and Cancer in Mice. J Immunol 2019, 202:631-636.

39. Rothe P, Sommerfeld O, Queissner CH, Otto GP, Sossdorf M, Richter A, Claus RA: Continuous non-invasive infrared monitoring in animal research during
infectious diseases. *Infection* 2015, **43**:S67–S68.

40. Trammell RA, Toth LA: **Markers for predicting death as an outcome for mice used in infectious disease research.** *Comp Med* 2011, **61**:492-498.

41. Laitano O, Van Steenbergen D, Mattingly AJ, Garcia CK, Robinson GP, Murray KO, Clanton TL, Nunamaker EA: **Xiphoid Surface Temperature Predicts Mortality in a Murine Model of Septic Shock.** *Shock* 2018, **50**:226-232.

42. Miao P, Kong Y, Ma Y, Zeng H, Yu Z: **Hypothermia predicts the prognosis in colon ascendens stent peritonitis mice.** *J Surg Res* 2013, **181**:129-135.

43. Drechsler S, Weixelbaumer KM, Weidinger A, Raeven P, Khadem A, Redl H, van Griensven M, Bahrami S, Remick D, Kozlov A, Osuchowski MF: **Why do they die? Comparison of selected aspects of organ injury and dysfunction in mice surviving and dying in acute abdominal sepsis.** *Intensive Care Med Exp* 2015, **3**:48.

44. Mallet ML: **Pathophysiology of accidental hypothermia.** *QJM* 2002, **95**:775-785.

45. Ndongson-Dongmo B, Lang GP, Mece O, Hechaichi N, Lajqi T, Hoyer D, Brodhun M, Heller R, Wetzker R, Franz M, et al: **Reduced ambient temperature exacerbates SIRS-induced cardiac autonomic dysregulation and myocardial dysfunction in mice.** *Basic Res Cardiol* 2019, **114**:26.

46. Gordon CJ: **The mouse thermoregulatory system: Its impact on translating biomedical data to humans.** *Physiol Behav* 2017, **179**:55-66.

47. Romanovsky AA, Shido O, Sakurada S, Sugimoto N, Nagasaka T: **Endotoxin shock: thermoregulatory mechanisms.** *Am J Physiol* 1996, **270**:R693-703.

48. Rudaya AY, Steiner AA, Robbins JR, Dragic AS, Romanovsky AA: **Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature.** *Am J Physiol Regul Integr Comp Physiol* 2005, **289**:R1244-
49. Kushimoto S, Yamanouchi S, Endo T, Sato T, Nomura R, Fujita M, Kudo D, Omura T, Miyagawa N, Sato T: **Body temperature abnormalities in non-neurological critically ill patients: a review of the literature.** *J Intensive Care* 2014, 2:14.

50. Leon LR: **Hypothermia in systemic inflammation: role of cytokines.** *Front Biosci* 2004, 9:1877-1888.

51. Corrigan JJ, Fonseca MT, Flatow EA, Lewis K, Steiner AA: **Hypometabolism and hypothermia in the rat model of endotoxic shock: independence of circulatory hypoxia.** *J Physiol* 2014, 592:3901-3916.

52. Cannon B, Nedergaard J: **Nonshivering thermogenesis and its adequate measurement in metabolic studies.** *J Exp Biol* 2011, 214:242-253.

53. Jhaveri KA, Trammell RA, Toth LA: **Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of C57BL/6J mice.** *Brain Behav Immun* 2007, 21:975-987.

54. Szentirmai E, Krueger JM: **Sickness behaviour after lipopolysaccharide treatment in ghrelin deficient mice.** *Brain Behav Immun* 2014, 36:200-206.

55. Okla M, Wang W, Kang I, Pashaj A, Carr T, Chung S: **Activation of Toll-like receptor 4 (TLR4) attenuates adaptive thermogenesis via endoplasmic reticulum stress.** *J Biol Chem* 2015, 290:26476-26490.

56. Rosenberg GA: **Neurological diseases in relation to the blood-brain barrier.** *J Cereb Blood Flow Metab* 2012, 32:1139-1151.

57. Candelario-Jalil E, Yang Y, Rosenberg GA: **Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia.** *Neuroscience* 2009, 158:983-994.

58. Gurney KJ, Estrada EY, Rosenberg GA: **Blood-brain barrier disruption by**
stromelysin-1 facilitates neutrophil infiltration in neuroinflammation.

*Neurobiol Dis* 2006, **23**:87-96.

59. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA:

*S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death.* Science 2002, **297**:1186-1190.

60. Perino A, Beretta M, Kilic A, Ghigo A, Carnevale D, Repetto IE, Braccini L, Longo D, Liebig-Gonglach M, Zaglia T, et al: **Combined inhibition of PI3Kbeta and PI3Kgamma reduces fat mass by enhancing alpha-MSH-dependent sympathetic drive.** Sci Signal 2014, **7**:ra110.

61. Jin R, Song Z, Yu S, Piazza A, Nanda A, Penninger JM, Granger DN, Li G:

*Phosphatidylinositol-3-kinase gamma plays a central role in blood-brain barrier dysfunction in acute experimental stroke.* Stroke 2011, **42**:2033-2044.

62. Jin R, Yu S, Song Z, Quillin JW, Deasis DP, Penninger JM, Nanda A, Granger DN, Li G:

*Phosphoinositide 3-kinase-gamma expression is upregulated in brain microglia and contributes to ischemia-induced microglial activation in acute experimental stroke.* Biochem Biophys Res Commun 2010, **399**:458-464.

63. Schmidt C, Frahm C, Schneble N, Muller JP, Brodhun M, Franco I, Witte OW, Hirsch E, Wetzker R, Bauer R: **Phosphoinositide 3-Kinase gamma Restrains Neurotoxic Effects of Microglia After Focal Brain Ischemia.** Mol Neurobiol 2016, **53**:5468-5479.

64. Wang L, Wang X, Li T, Zhang Y, Ji H: **8e Protects against Acute Cerebral Ischemia by Inhibition of PI3Kgamma-Mediated Superoxide Generation in Microglia.** Molecules 2018, **23**.

65. Ndongson-Dongmo B, Heller R, Hoyer D, Brodhun M, Bauer M, Winning J, Hirsch E, Wetzker R, Schlattmann P, Bauer R: **Phosphoinositide 3-kinase gamma controls**
inflammation-induced myocardial depression via sequential cAMP and iNOS signalling dagger. *Cardiovasc Res* 2015, **108**:243-253.

66. Schneble N, Schmidt C, Bauer R, Muller JP, Monajembashi S, Wetzker R: **Phosphoinositide 3-kinase gamma ties chemoattractant- and adrenergic control of microglial motility.** *Mol Cell Neurosci* 2017, **78**:1-8.

67. Neumann H, Kotter MR, Franklin RJ: **Debris clearance by microglia: an essential link between degeneration and regeneration.** *Brain* 2009, **132**:288-295.

68. Smart N, Pries AR, Dirnagl U: **Dr Smart talks to Prof Pries and Prof Dirnagl on animal experimentation in biomedical research.** *Cardiovasc Res* 2017, **113**:e12-e15.

Figures
Figure 1

Cytokine levels in blood plasma (A) and brain tissue (B). Values are given as medians and whiskers (minimum and maximum), n=3, for each group and experimental state. * † § p < 0.05, * significant differences versus baseline conditions within the same genotype, † significant differences versus neutral Ta within the same genotype, § significant differences versus wild type (Wt) mice within the same Ta condition).
Altered heart rate and augmented hypothermia in mice kept at reduced ambient temperature (Ta) after LPS-induced SIRS response compared with mice kept at neutral Ta irrespective of the genotype (wild-type mice, open boxplots, PI3Kγ-deficient mice (PI3Kγ−/−) filled boxplots, PI3Kγ-kinase-dead mice (PI3KγKD/KD) hatched boxplots). Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile, whiskers ranging from the 10th to the 90th percentiles (neutral Ta groups: wild type mice n=9, PI3Kγ−/− n=10, PI3KγKD/KD, n=8; reduced Ta groups: wild type mice n=12, PI3Kγ−/− n=10, PI3KγKD/KD n=9). * † p < 0.05, * significant difference between baseline and
LPS stimulation within each Ta state, † significant differences versus mice kept at neutral Ta (two-way repeated measures ANOVA, followed by Holm-Sidak test for post hoc multiple comparisons were performed).
Enhanced BBB leakage in PI3Kγ-deficient mice at reduced Ta 24h after LPS-induced SIRS.

(A) Mice kept at neutral Ta show mild disturbance of BBB integrity in PI3Kγ mutant. LPS-induced SIRS elicits small increase of BBB leakage in wild type mice (Wt, open boxplots). (B) Reduced Ta was accompanied by enhanced Evans blue (EB) extravasation into brain tissue indicating degree of BBB leakage in PI3Kγ-deficient (PI3Kγ-/−) mice (filled columns) in comparison with Wt and kinase-dead (PI3KγKD/KD, hatched boxplots) mice. (C) Representative pictures of native brain slices immediately after transcardial rinsing with physiological saline, brain removal and cutting into coronal slices using a brain matrix.

Values are presented as boxplots illustrating medians within boxes from first quartile (25th percentile) to the third quartile (75th percentile) and whiskers ranging from minimum to maximum, n=10 per group and time point. * † § p < 0.05, * significant differences versus baseline conditions within the same genotype, † significant differences versus neutral Ta within the same genotype, § significant differences versus Wt mice within the same Ta condition (Two-way ANOVA and one-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons was performed for comparison between respected groups, t-test was used for comparisons between states within same groups with Bonferroni’s correction for adjustments of multiple comparisons).
Figure 4

Marked increase of microglial cell activation due to LPS-induced SIRS in brains of mice kept at neutral Ta (A) and reduced Ta (B) (left panel; right panel: representative photomicrographs). The effect is exacerbated by reduced Ta in the wild type mice, but not in the genetically modified animals. Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (A,B: n=4-6, at each group and experimental state. * § † p < 0.05, * significant differences versus baseline within each group, § significant differences versus wild type mice kept at same Ta, † significant differences versus mice kept at neutral Ta at the same experimental state, two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, each).
Increased LPS-induced mRNA expression of MMP-2, MMP-3, MMP-9, and MMP-13 appeared 24 h post LPS mainly in brains obtained from PI3Kγ-deficient mice kept at reduced Ta. Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (A-D n=5 per each group and experimental state. § † p < 0.05, § significant differences versus wild type mice kept under same Ta, † significant differences versus mice kept at neutral Ta of the same genotype, two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, each).
Among the Iba1-positive cells, reduced Ta (upper plot) 24h after LPS administration resulted in an increased number of MMP-9 positive cells (A), number of TUNEL positive cells (B) and number of invading polymorphonuclear cells (PMN, C) appearing mainly in brains obtained from PI3Kγ-deficient mice. Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (lower panel: neutral Ta, upper panel: reduced Ta, n=4 per each group and
experimental state. * § † p < 0.05, * significant differences versus baseline of the same genotype and experimental state, § significant differences versus wild type mice kept under same Ta, † significant differences versus mice kept at neutral Ta at the same experimental state, two-way ANOVA, followed by Holm–Sidak test for post hoc multiple comparisons, each).

Figure 7
PI3Ky-dependent suppression of microglial motility and phagocytic activity: A. Lipid kinase-dependent reduction of migratory activity induced by brain injury (upper panel) and chemoattractant-stimulated migration of primary microglial cells (lower panel). Reduced ambient temperature as well as reduced temperature of incubation (TInc = 33°C) led to a decline of microglial motility. B. Lipid kinase-independent reduction of phagocytosis was not influenced by ambient/incubation temperatures: Reduced number of Iba-1 positive cell with phagocytized zymosan particles in brains obtained from PI3Ky/- mice (upper panel) as well as reduced uptake in number of zymosan particles by primary microglial cells obtained
from PI3Ky-/- mice (upper panel). Values are presented as boxplots illustrating medians within boxes from first quartile to the third quartile and whiskers ranging from the 10th to the 90th percentiles (A & B: n=5 for each group and experimental state. § † p < 0.05, § significant differences versus cells derived from wild type mice kept under same Ta or TInc, † significant differences versus cells derived from mice kept at reduced Ta or TInc at the same experimental state and genotype, two-way ANO-VA, followed by Holm-Sidak test for post hoc multiple comparisons, each).

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
End Table 1 new.pdf
End ##mgf Supplement_Guangping_Rev1 26032020 - RB.pdf