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Neonatal Systemic Inflammation Induces Inflammatory Reactions and Brain Apoptosis in a Pathogen Specific Manner

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Key words: Inflammation, neonatal sepsis, neurological outcome, neuroprotection, lipopolysaccharide, term new-born, temperature, rat model.

Abstract

Background: After neonatal asphyxia, therapeutic hypothermia (HT) is the only proven treatment option. Although established as a neuroprotective therapy, benefit from HT has been questioned when infection is a comorbidity to hypoxia-ischaemic (HI) brain injury. Gram-negative and gram-positive species activate the immune system through different pathogen recognition receptors and subsequent immunological systems. In rodent models, gram-negative (lipopolysaccharide, LPS) and gram-positive (PAM_3CSK_4 (PAM)) inflammation similarly increase neuronal vulnerability to HI. Interestingly, while LPS pre-sensitisation negates HT neuroprotective effect, HT is highly beneficial after PAM-sensitised HI brain injury.

Objective: We aimed to examine whether systemic gram-positive or gram-negative inflammatory sensitisation, affects juvenile rat pups per se, without an HI insult.

Methods: Neonatal P7 rats (n=209) received intraperitoneal injections of vehicle (0.9%NaCl), LPS (0.1mg/kg) or PAM (1 mg/kg). Core temperature and weight gain was monitored. Brain cytokine expression (IL-6, IL-1β, TNF-α, IL-10) (PCR), apoptosis (cCas3 3) (western blots), and microglial activation (Iba-1) (immunohistochemistry) was examined.

Results: LPS induced an immediate drop in core temperature followed by poor weight gain, not seen after PAM. Furthermore, LPS induced brain apoptosis, while PAM did not. The magnitude and temporal profile of brain cytokine expression was differed between LPS- and PAM-injected animals.

Conclusion: These findings reveal sepsis-like conditions and neuro-inflammation specific to the inflammatory stimulus (gram-positive versus gram-negative), in the neonatal rat. They emphasize the importance of pre-clinical models being carefully tailored to their clinical scenario.

Background

In industrialised countries, early onset sepsis (EOS) have an incidence of 0.5-1.2 per 1000 live-borns [1]. Systemic inflammation increases the vulnerability of the neonatal brain to hypoxic-ischaemic (HI) insults, and is considered a risk factor for neurodevelopmental sequelae [2]. Although therapeutic hypothermia (HT) is an effective neuroprotective strategy after HI injury, 40-50% of patients still have poor developmental outcome including death [3]. As clinical trials of HT in parts of the world where infection rates are higher failed to show benefit, clinicians and researchers are questioning whether comorbidities such as perinatal infection could negate the neuroprotective effect of HT [2,4]. Exposure of the 7-day-old (P7) rat to LPS prior to a mild HI insult significantly increased brain injury and abolished the neuroprotective effects of HT [5], supporting that hypothesis. However, LPS only represent gram-negative type bacterial infections. Gram-negative and gram-positive species activate the immune system through different pathogen recognition receptors and subsequent immunological pathways [6]. While LPS binds primarily to toll-like receptor (TLR)-4, gram-positive bacterial cell wall molecules adheres to TLR-2 on the host immune cells, to activate the inflammatory cascade, and have been shown to be TLR-4 independent (Fig.1) [6]. Pre-sensitising with the synthetic TLR-2 agonist, PAM_3CSK_4 (PAM), in the same neonatal rat model of HI brain injury, simulated gram-positive type infection and induced brain injury of the same severity, but the neuroprotective effect of HT was preserved [7].

Although in most cases of EOS the causative agent remains unidentified, a recent population-based study showed that 91% of culture-positive sepsis cases among term-born babies were caused by gram-positive bacterial species [8].

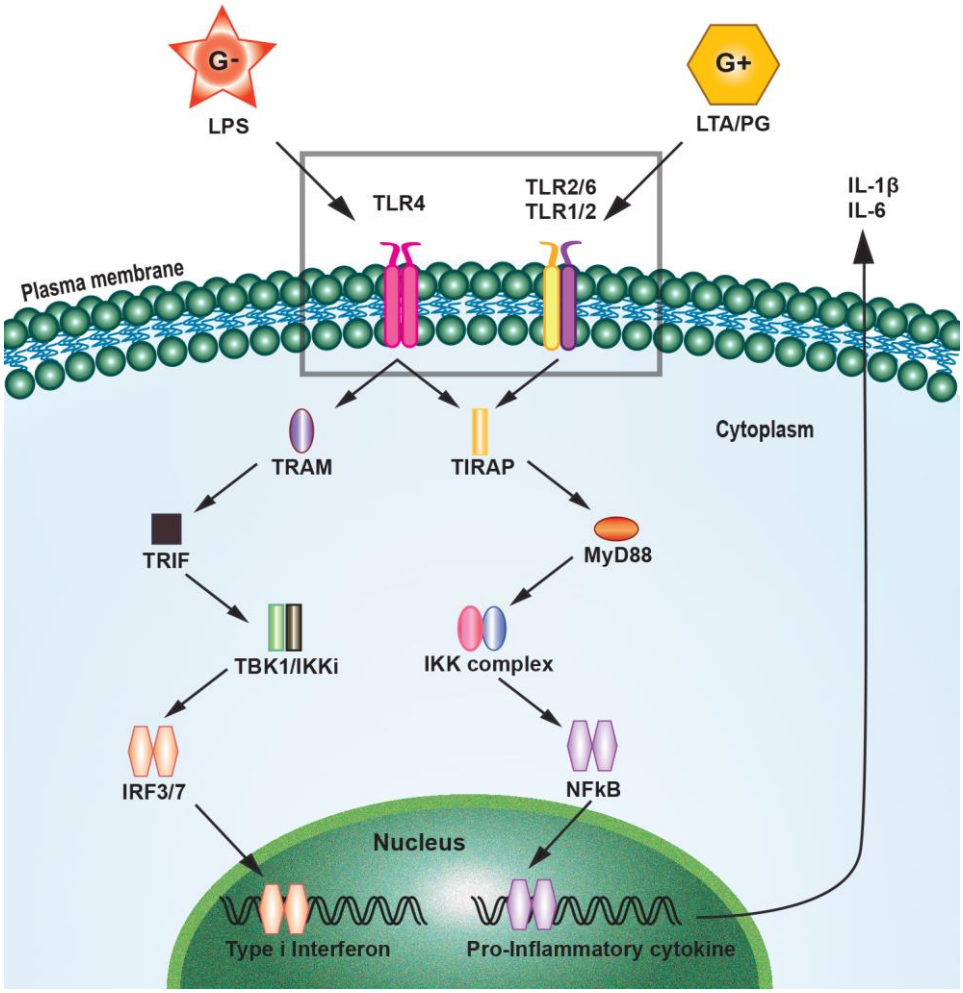

Figure 1. Inflammatory activation by gram-positive and gram-negative bacteria

Recognition of gram-negative (LPS) and gram-positive (LTA, PG, PAM_3CSK_4) bacterial pathogen associated molecular patterns (PAMPs) by plasma membrane-localized TLR-4 and TLR-2 (TLR-2 forms a heterodimer with TLR-1 or TLR-6 to form a functional receptor complex). TLR-2 and TLR-4 both act through the MyD88-dependent signalling pathway, where the active IκB kinase (IKK) complex activates nuclear factor kappa B (NF-κB) subunits to initiate the transcription of inflammatory cytokines. TLR-4 also activates MyD88-independent signalling by recruiting TIR-domain-containing adaptor-inducing interferon-β (TRIF). Here activation of the TANK-binding kinase 1 (TBK1)/IKK inhibitor (IKKi) complex results in the production of inflammatory cytokines and type I interferons (modified from Kumar *et al.* [33]).

Using neonatal rat pups without inducing HI brain injury, we investigated differences in inflammatory response to triggers of TLR-2 and TLR-4 respectively, with focus on temporal core temperature changes, development of intracerebral apoptotic cell death and neuro-inflammatory markers, and weight gain representing well-being in the neonate.

Material and Methods

Animals and injections

All experiments were approved by the University of Oslo's Animal Ethics Research Committee. Experiments were performed on P7 Wistar rats (Charles River Laboratories, Sulzfeld, Germany) of both genders. All pups were kept in an animal facility with a 12:12-h dark:light cycle at 21°C environmental temperature with food and water ad libitum. Animals were always randomised across litter, sex and weight before the experiments commenced.

We used LPS from Escherichia coli 055:B5 (Sigma) (0.1mg/kg), and the synthetically manufactured TLR-2/1 agonist PAM_3CSK_4 (*Vaccigrade, Sigma-Aldrich*) (1mg/kg). Vehicle (Veh) for dilutions was sterile 0.9% NaCl. The LPS dose is one that previously sensitised the neonatal brain to HI brain injury [5]. We based the PAM dose on previous publications [9], as well as our own dose-response experiments from developing the model of PAM-sensitised HI brain injury [7]. The PAM model was developed to explore neuroprotective effect of hypothermia after PAM-sensitised HI brain injury. We therefore aimed for a dose which induced the same level of infection-sensitised injury as in our LPS-sensitised model, where hypothermic neuroprotection was negated [5]. Control groups received a single dose of Veh. All injections were given intraperitoneally (i.p.) in a volume of 10µl/g body weight, at room temperature (21°C).

Core temperature recordings

P7 rats (n=29) received a Veh (n=9), LPS (n=10), or PAM (n=10) injection. Core temperature was monitored using a rectal probe (IT-21, Physitemp Instruments, Clifton, NJ, USA) at 9 selected time points after injection (0, 1, 2, 4, 6, 8, 10, 12, and 24h). All groups were handled similarly throughout the experiment, performed in a temperature-controlled room (21±0.5°C). To record the individual nesting temperature at a given time, one pup was removed from the dam at a time for temperature recording before returnal to the dam.

Weight gain analysis

In a separate study, P7 pups (n=36) received injections as described, and returned to their dams. At P14 all pups were weighed separately. Weight gain was calculated as percentage gain from P7 - P14.

Brain apoptosis

The apoptotic protein marker, cleaved caspase 3 (cCas3), was examined in brain tissue at 24 and 48h survival post injections using western blot (WB) technique as previously described [10]. Three groups were examined at 24h (n=36); Veh, LPS and PAM. For the 48h follow-up only LPS and PAM data were available (n=6 per group). Image Lab (Image Lab Software, version 5.2.1; BioRad, Calif., USA) was used for optical density measurements of protein signals on scans in ChemiDoc™ Touch Imaging Systems (BioRad).

Brain Cytokine expression

Using qRT-PCR, we studied the time course of pro- (IL-6, IL-1ß, TNF-α) and anti-inflammatory (IL-10) cytokines expressed in brain tissue after systemic LPS-injection (n=50), over a 48h period. Subsequently, the same cytokines were examined in brain tissue after systemic injections of PAM (n=50), or Veh (n=50). Nine post-injection time points were selected for analysis in LPS- and PAM-injected animals (0, 2, 4, 6, 12, 18, 24, 36 and 48h). Four time points (0, 4, 8 and 24h) were selected in the Veh group (Fig. 4). Brains were harvested at the selected time points, and snap frozen in liquid nitrogen before storage at -80°C.

Using RNeasy mini kit (Qiagen), total RNA was extracted, and concentration measured with NanoDrop spectrophotometer. cDNA was synthesised from 1µg RNA using the qScript™ cDNA Synthesis Kit (Quanta Biosciences). qRT-PCR was performed with the ABI7900 sequence detection system (PE applied biosystems, Foster City, CA, USA) in a 10µl total volume, using commercial TaqMan® Gene Expression Assays (Applied Biosystems) and the Universal TaqMan Master Mix (PE Applied Biosystems, CAS # 67-68-5). PCR cycling conditions were: 2min at 50°C and 10min at 95°C, before 40 x (15 seconds at 95°C and 1min at 60°C). Using relative quantification method, all values were normalized to the housekeeping gene, GAPDH, in the same sample. The inflammatory response in terms of expression of these cytokines was plotted against time, and expressed relative to their level at time point zero.

Microglial activation

Ionized calcium binding adaptor molecule 1 (Iba1) was examined by WB technique at 48h post injections as described previously (n=18) [10].

Iba1 immunoreactivity was analysed in animals with 7 days' survival (n=30), as described previously [10]. Virtual slides were exported as high-resolution tiff images for further analysis with ImageJ software (ImageJ, version 1.46r, National Institutes of Health, Bethesda, MD), detecting

Iba1 immunoreactivity. The summed intensity detected was analysed by two individual observers blinded to the treatment groups. Inter-rater reliability was crosschecked using Pearson correlation coefficient analysis**.** An average of the two was taken for comparison across treatment groups. Microglial activation was expressed as Iba1 detected relative to hemispheric area in the same brain.

Statistical Data analyses

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, Ca, USA). Temperature measurements are presented as mean ±SEM. For weight gain as well as cytokine and WB analysis, descriptive data are presented as median with 95% confidence intervals (CI) as these data were not normally distributed. Multi-group comparisons were done using Kruskal-Wallis test, and Mann-Whitney-Wilcoxon rank sum tests for comparing two groups to get exact two-tailed p-values. Due to the variable spread in cytokine expression data, Kolmogorov-Smirnov test was used for group-to-group comparisons. A p-value <0.05 (two-sided) was considered statistically significant.

Results

Core temperature changes

Already 2h after injection of LPS, mean core temperatures had dropped by 4.3°C (2.7-6.4), a significantly greater temperature reduction than in Veh- and PAM-injected animals, which dropped by 2.5°C (0.2-3.0)($p< 0.01$) and 2.1°C (1.1-4.8)($p<0.01$), respectively. It took 8h before core temperatures in the LPS-injected group increased to the same value as PAM and Veh (Fig. 2).

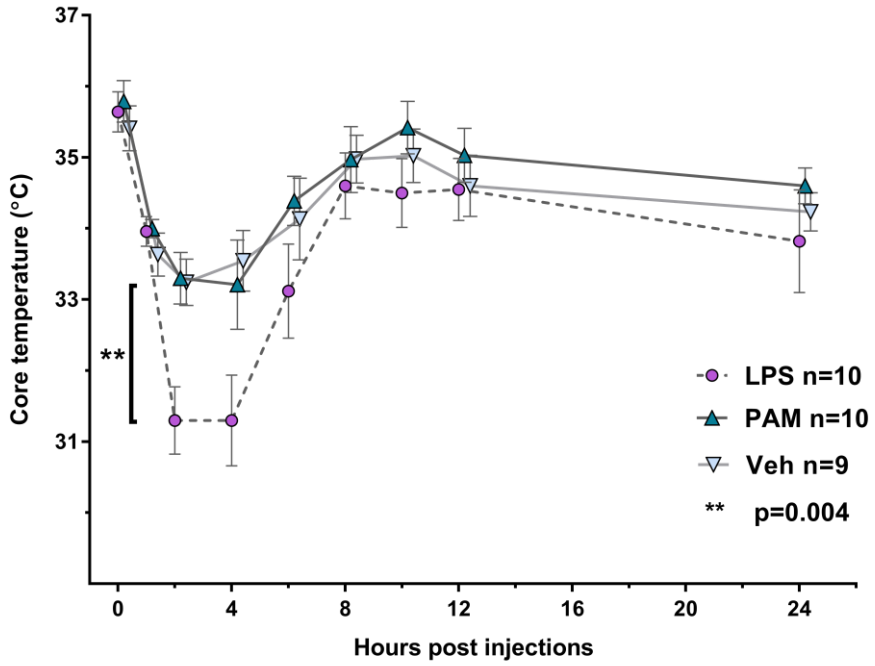

Figure 2. Core temperature developments after systemic injections

Sequential core temperature measurements (°C) of P7 rat pups over 24 h following i.p. injections of Veh (n=9), LPS (n=10), or PAM (n=10) expressed as mean ± SEM. ** $p < 0.01$.

Differences in weight gain

The Veh- and PAM-groups had similar median weight gain one week after injections, 138% (130.5-145.5) and 145.6% (134.1-137.1) respectively. The LPS-injected pups, however, had significantly poorer weight gain at median 115.2% (91.5-138.9) compared to the Veh-group ($p=0.02$) and the PAM-group ($p<0.01$).

Intracerebral apoptosis

cCas3 was significantly increased in the brains of LPS-injected animals after 24h, compared to the Veh ($p<0.0001$) and the PAM groups ($p<0.0001$). cCas3 continued to increase the next 24h in LPS animals. After PAM there was no elevation of cCas3 at 24 h post-injection, similar to after injection of Veh, nor did it elevate over the next day (Fig.3B).

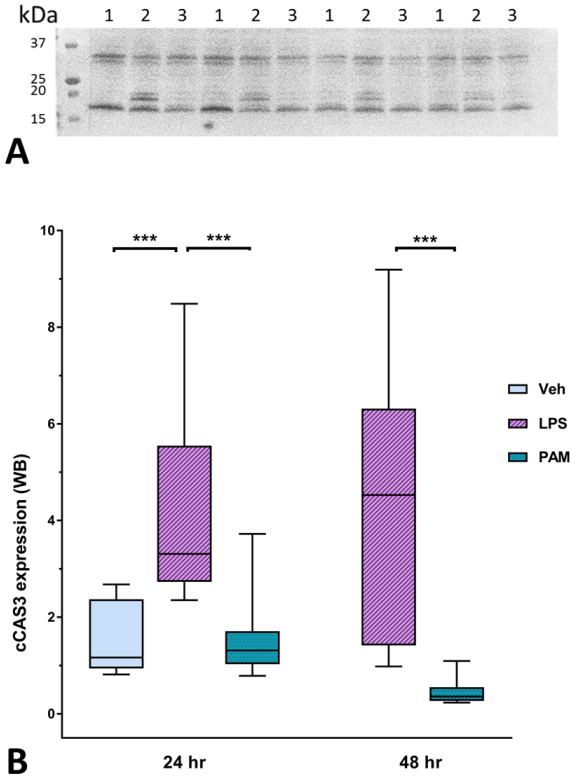

Figure 3. Apoptotic activation in brain after systemic injections (WB)

The Western blot with ladder on top (**A**), loaded with Veh (1), LPS (2), PAM (3) in repeated sequences. The first band is the un-cleaved caspase 3 protein at 36 kDa. Below are the cleaved subunits after activation with bands at 19 and 17 kDa. **B**: Box-&-Whiskers plot of cCas3 expression in brain tissue at 24 and 48 h after injections. *** $p < 0.001$.

Cytokine expression in brain tissue

The temporal changes in cerebral cytokine expression (IL-6, IL-1β, TNF-α, IL-10) after peripheral injections of PAM and LPS are shown in table 1. After injection of Veh, none of the four cytokines were significantly elevated at any time (Fig. 4A).

Up-regulation of cerebral cytokines was found to be specific to the stimulus (Fig. 4B). After LPS, IL-6 expression increased rapidly within 2h, and after a second peak at 12h returned to baseline levels. After PAM-injection there was a later (6h) significant change in the IL-6 level. Expression of TNF-α was also significantly increased already 2h after LPS injection. The TNF-α peak induced by PAM-injection was seen later, at 6-12h. IL-1β expression was strongly up-regulated in both groups, but while also this pro-inflammatory cytokine immediately rose in the LPS-group, the response was somewhat delayed in the PAM-group.

The pattern was different for IL-10. A small but significant change was seen 6h after LPS-injection, while in PAM-animals the IL-10 response was immediate and sustained.

	IL-6	IL-1β	TNF-α	IL-10
LPS	0.001* (KW)	0.049* (KW)	0.002* (KW)	0. 135 (KW)
2 h	0.008*	0.008*	0.008*	0.05
4 h	0.338	0.015*	0.03*	0.242
6 h	0.084	0.015*	0.03*	0.026*
12 h	0.026*	0.015*	0.015*	0.061
18 h	0.264	0.286	0.079	0.286
24 h	0.873	0.048	0.079	0.05
36 h	0.079	0.008*	0.714	0.167
48 h	0.286	0.008*	0.079	0.079
PAM	<0.001* (KW)	0.001* (KW)	0.002* (KW)	0.05 (KW)
2 h	0.286	0.061	0.357	0.008*
4 h	0.896	0.026*	0.069	0.069
6 h	0.026*	0.008*	0.004*	0.004*
12 h	0.08	0.004*	0.008*	0.008*
18 h	0.286	0.016*	0.1	0.048*
24 h	0.351	0.286	0.108	0.108
36 h	0.81	0.143	0.008*	0.357
48 h	0.873	0.357	0.079	0.047

Table 1. Changes in cerebral cytokine expression (p-values) after systemic injections of PAM or LPS (hours, h).

Kruskal-Wallis test (KW) for multi-group comparisons. Changes in expression of each specific cytokine was compared against the same cytokine at 0 h (n=5), using Kolmogorov-Smirnov test. * significant, $p<0.05$.

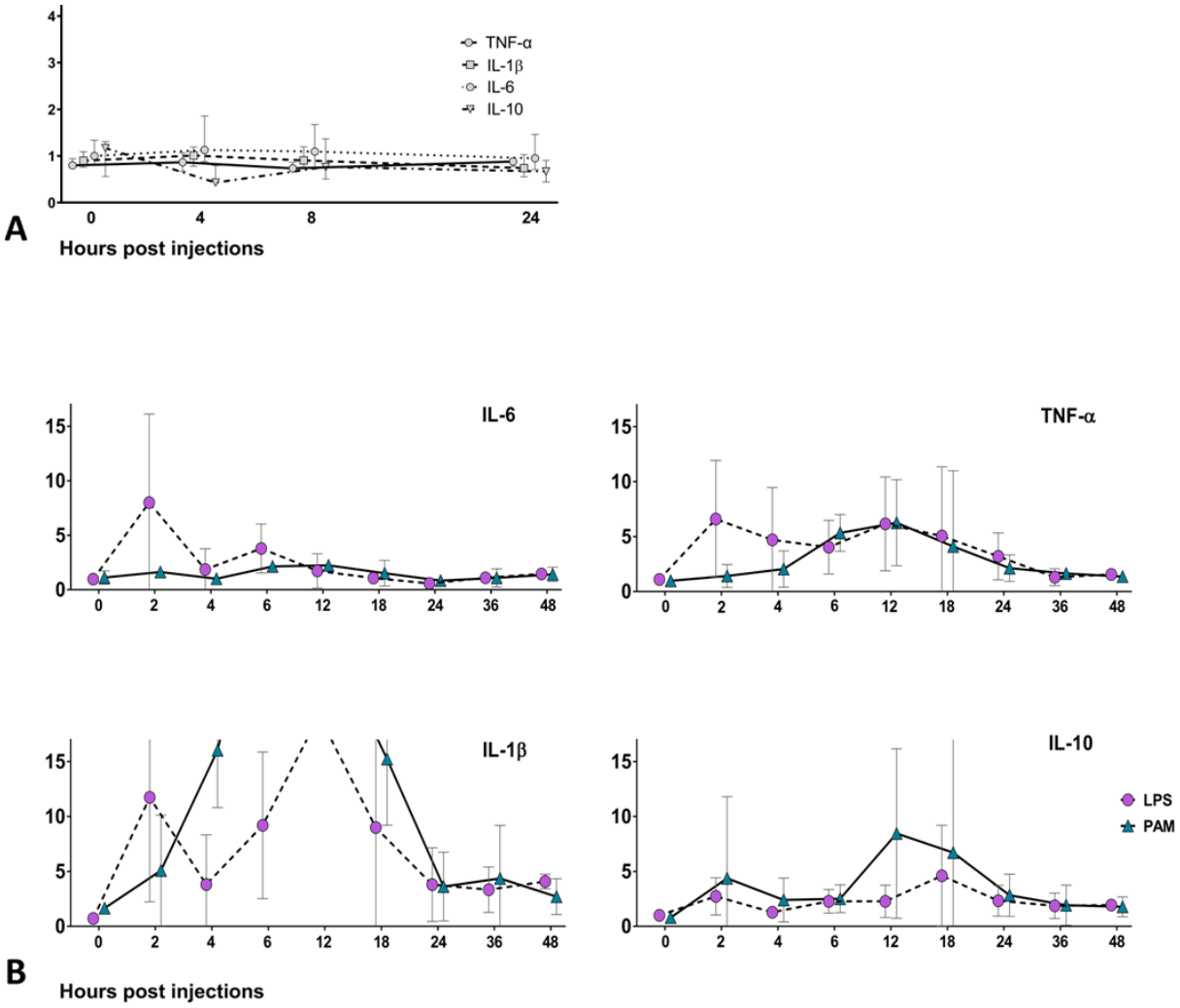

Figure 4. Cytokine expressions in brain tissue (PCR)

Y-axis values are cytokine expression relative to expression of a house keeping protein (GAPDH) in the same tissue sample (arbitrary units). The lines are drawn through the median for each time point, with error bars showing 95% CI. **A**: Temporal expression of IL-6, TNF-α, IL-10 and IL-1β after i.p. injection of Veh (n=7-14 per time point). **B**: Graphs show temporal profiles of specific cytokines (IL-6, TNF-α, IL-10 or IL-1β) after a single i.p. PAM- (triangles, complete line) or LPS- (circles, dotted line) injection (n=5-6).

Microglial activation in response to systemic injections

Western blots from snap frozen brain tissue collected 48h post injections showed no differences between LPS- (1.5; 1.2-1.9) and PAM animals (1.4; 1.3-1.6). Iba1 was however significantly higher in animals which had received LPS or PAM compared to Veh (1.2; 1.0-1.4) ($p=0.04$ for both comparisons)(Fig. 5A).

Immunofluorescence-labelled Iba1-specific antibodies revealed microglia throughout the brains of pups from all groups at P14. Again there was no difference between LPS- (87.8; 40.4-136) and PAM-injected (71.5; 56.4-108.3) animals. Median Iba1-labelling detected was higher in pups which had received LPS or PAM, than in Veh animals (33.1; 24.6-138.6), although not statistically significant ($p=0.21$ and $p=0.28$). By appearance there was no obvious difference in number of microglia across the three groups, however microglia in the activated state were found in LPS- and PAM-injected animals, as opposed to in the Veh group (Fig. 5B-D).

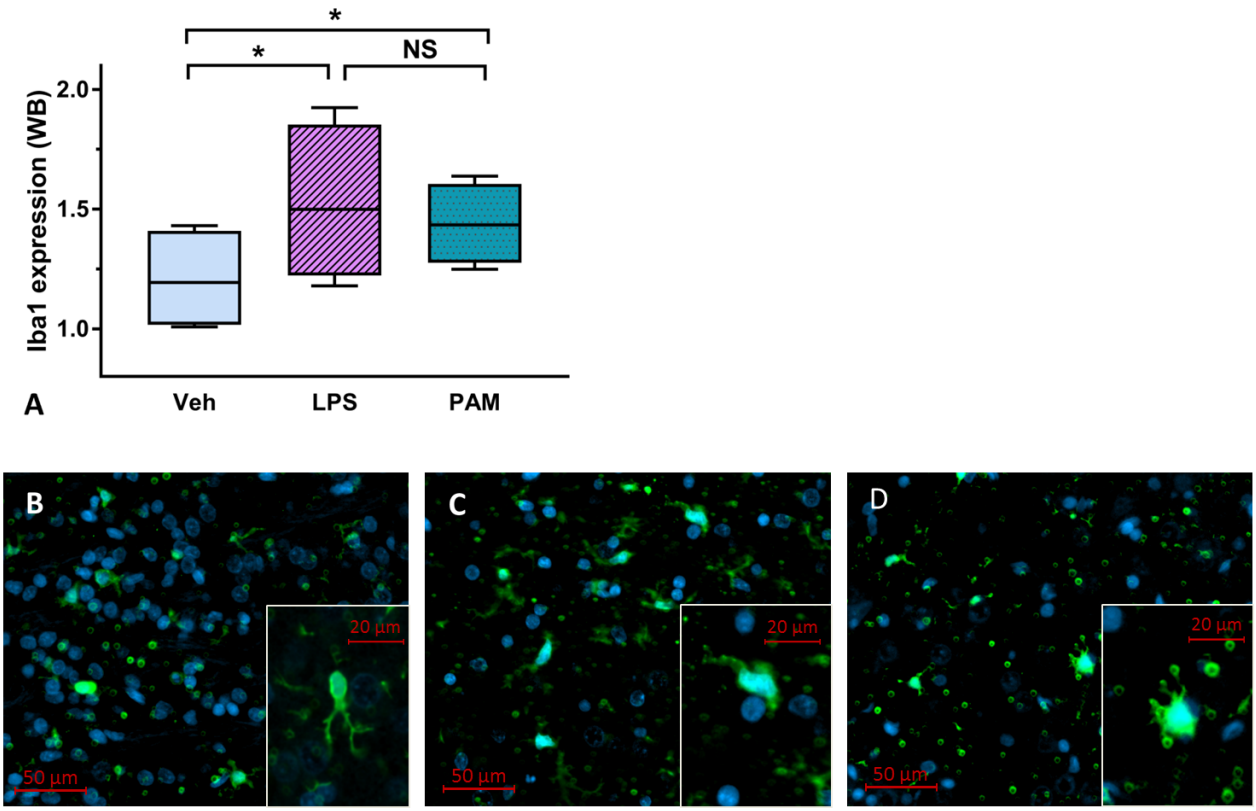

Figure 5. Iba1 expressions after systemic injections (IHC)

A: Box-&-Whiskers plot of Iba1 expression in brain tissue 48h after injections (WB). *p <.05. Representative IHC images from the Veh-group (**B**), the LPS-group (**C**) and the PAM-group (**D**). Iba1 expression is seen as green. DAPI (blue) stains nuclei. Magnified in picture A is a typical ramified resting microglia. Picture B and C show microglia in the activated state, with larger rounded somata and withdrawn dendritic processes.

Discussion

In this study of juvenile rats with brain maturation equal to near-term humans, we found pathogen dependent inflammatory responses after either LPS (a gram-negative type stimulus) or PAM (a gram-positive stimulus) administration.

When term new-born infants need HT after perinatal asphyxia, cooling starts within a few hours. It is a major question whether infection negates the neuroprotective effect of HT. To a similar degree, pre-sensitisation with LPS and PAM increased injury at normothermic recovery [7,11]. With experimental HI followed by HT, LPS negated neuroprotection [5], unlike PAM, where HT had significant effect [7]. With current diagnostic methods, the causative pathogen in case of a concomitant infection cannot be revealed in time to impact the decision of whether to cool or not. However, if most infections in term born neonates in the industrialised part of the world are caused by gram-positive pathogens [8,12], the decision to cool should not be delayed by these diagnostic challenges.

To further explore differences between two clinically relevant immune response pathways, the current study addresses the effect of LPS and PAM on physiology and neuropathology in juvenile animals without an HI injury.

Within 2h after LPS administration core temperature dropped significantly in these P7 rat pups, unlike in the Veh- or PAM-injected animals. With the exception of a brief temperature reduction following injection of a room-tempered solution (21°C), the temperature development of Veh- or PAM animals remained steady. Rodents have previously been shown to develop hypothermia in response to a significant systemic infection [13]. However, in most studies on rodent sepsis the stimulants have been gram-negative bacteria or LPS injection. In human sepsis, loss of core

temperature (“cold sepsis”) is thought to indicate a more severe generalised disease state with higher mortality [14]. When spontaneous drop in core temperature is a result of HI brain injury, it has been shown to be a strong predictor of poor outcome [15]. It is reasonable to interpret the temperature changes seen after LPS here as a sign of a more severe generalised disease state, than what is seen in littermates who received PAM.

Microglial activation was seen both at 48h and at 7 days after PAM and LPS injections, and to a similar degree. This supports the idea that inflammatory activation in blood leads to activation of the monocyte line in the CNS [16]. Some, or even a majority, of the Iba1 positive cells seen in the brain after systemic inflammation are peripheral monocytes [17]. TNF-α was shown to play a major role in recruitment of these cells from blood to brain [18]. IL-6 is a key factor stimulating microglial activation and proliferation [19]. Both LPS and PAM induced significant elevations of TNF-α and IL-6 well within the time point where we analysed microglial activation, and can therefore explain the similarity of Iba1 density.

The activation of monocytes/microglia and their release of pro-inflammatory molecules induce cellular death [20]. Kim *et al*. attributed LPS-induced neurotoxicity and apoptosis to microglial density [20]. Interestingly however, apoptosis was induced in LPS-injected animals, but not in the PAM-injected ones (Fig.3). This suggests that the mechanism of inflammatory induced apoptosis is not restricted simply to microglial/monocytal activation, but might be modified by microglial phenotype or other immunological events, especially in gram-positive type inflammation.

The LPS-induced apoptosis demonstrated above is in line with previous studies [21]. The authors concluded that the LPS-induced changes could be interpreted as downstream effects of sepsis. The

profound differences between these two main pathways of inflammatory activation has clinical importance in the context of injurious impact of systemic infection on the immature brain; in sensitisation of the term neonatal brain to HI injury, as well as in white matter injury induced by systemic inflammation in the premature [22]. Our findings suggest that the mechanisms behind these phenomena are complex and not only the inflammation *per se*. The differing temporal patterns of various pro- and anti-inflammatory cytokines might play an important role.

IL-6 and TNF-α play important roles in thermal response to inflammation [23], and increased sickness behaviour [24]. Our findings of intracerebral IL-6 and TNF-α surges already 2h after LPS-injection, which coincide with a drop in core temperature, supports the thermoregulatory role of these cytokines, and explains a reduction in food intake. The increased IL-6 and TNF-α level in the brains of PAM-injected pups only reach statistical significance after a 6-12 h delay. Here, however, they peak without a concomitant change in core temperature, and with satisfactory weight gain. As opposed to in LPS animals, the increased IL-6 and TNF-α in PAM animals was accompanied by an elevated IL-10 level.

IL-1β expression was significantly increased after both LPS and PAM injections. IL-10 was briefly elevated after LPS, while significantly increased at 2h and maintained elevated until 18h, after PAM. Several studies suggest a protective role of IL-10 through modulation of on-going inflammation. IL-10 reduced excitotoxic brain injury triggered by IL-1β in neonatal mice [25]. A genetic polymorphism that results in increased production of IL-10 has been associated with decreased white matter injury and reduced risk of CP in studies on very premature infants [26], also supporting the neuroprotective role of IL-10.

Due to the limitation of crushed tissue, we have not studied the intracerebral responses regionally. Specifically, LPS induced apoptosis in cultured neurons and microglia, but not in astrocytes [27], and apoptosis have been shown to be dependent on cell type density for various brain regions [20]. Exploring regions known to be particularly vulnerable to HI like the hippocampus and cortex could also help elucidate inflammatory sensitisation and its relation to temperature changes. Another significant limitation to this study is the challenge of interpretation. Current knowledge on specific cytokines and their action in pathologic situations are uncertain. Additionally, studies on translation of immune responses from rodents to humans are scarce [28].

Researchers have approached a sepsis-like scenario by using LPS in various animal models spanning a wide range of clinical fields [29,30]. LPS is relatively inexpensive, and thoroughly investigated as a potent inflammatory trigger. However, the limitation that LPS exclusively represents gram-negative infections has not often been addressed. Our findings raise the question of how other inflammatory triggers, both acute and chronic, including viral and parasitic infections, may affect outcome after HI. Both hypoxia and LPS prior to the HI insult have displayed pre-conditioning activities, and the timing is determinant for the outcome [31,32]. The physiological and neuroinflammatory responses in various settings of inflammation are under constant investigation. How they as co-morbidities to HIE might modify hypothermic neuroprotection is still unknown.

We can conclude that the temporal upregulation of these mediators of cellular death and inflammation are different for analogues of a gram-positive and gram-negative systemic infection, with different downstream thermoregulatory effects, in the neonatal rat. Therefore, it is important to acknowledge that using LPS in pre-clinical models of inflammation may not always reflect the clinical scenario appropriately.

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Disclosure Statement

The authors declare no competing financial interests.

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