



Title	Altered inflammasome activation in neonatal encephalopathy persists in childhood
Authors	Kelly, Lynne A.;O'Dea, Mary I.;Zareen, Zunera;Melo, A. M.;McKenna, Ellen;Strickland, Tammy;McEneaney, V.;Donoghue, Veronica;Boylan, Geraldine B.;Sweetman, Deirdre;Butler, J.;Vavasseur, Claudine;Miletin, Jan;El-Khuffash, Afif;O'Neill, Luke;O'Leary, John;Molloy, Eleanor J.
Publication date	2021-07
Original Citation	Kelly, L. A., O'Dea, M. I., Zareen, Z., Melo, A. M., McKenna, E., Strickland, T., McEneaney, V., Donoghue, V., Boylan, G. B., Sweetman, D., Butler, J., Vavasseur, C., Miletin, J., El-Khuffash, A. F., O'Neill, L. A. J., O'Leary, J. J. and Molloy, E. J. (2021) 'Altered inflammasome activation in neonatal encephalopathy persists in childhood', <i>Clinical and Experimental Immunology</i> , 205 (1), pp. 89-97. doi: 10.1111/cei.13598
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1111/cei.13598
Rights	© 2021 The Authors. <i>Clinical & Experimental Immunology</i> published by John Wiley & Sons Ltd on behalf of British Society for Immunology., <i>Clinical and Experimental Immunology</i> , 205: 89–97. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made. - https://creativecommons.org/licenses/by/4.0/
Download date	2024-07-22 20:29:39
Item downloaded from	https://hdl.handle.net/10468/13117



UCC

University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Altered inflammasome activation in neonatal encephalopathy persists in childhood

L. A. Kelly ^{*,†,1} M. I. O'Dea,^{*,†,1}
Z. Zareen,^{*,†,1} A. M. Melo,^{*,†,1}
E. McKenna ^{*,†} T. Strickland,^{*,†}
V. McEneaney,^{*,†} V. Donoghue,[§]
G. Boylan,^{§,**} D. Sweetman,^{††}
J. Butler,^{‡‡} C. Vavasseur,^{††} J.
Miletin,^{§§} A. F. El-Khuffash,^{¶¶}
L. A. J. O'Neill,^{***} J. J. O'Leary^{†††} and
E. J. Molloy^{*,†,§,§†††}

^{*}Discipline of Paediatrics, Trinity College, University of Dublin, [†]Trinity Translational Medicine Institute (TTMI), Trinity College Dublin and Trinity Research in Childhood Centre (TRiCC), [‡]Children's Hospital Ireland (CHI) at Tallaght, [§]Radiology, National Maternity Hospital, Dublin, [¶]Department of Pediatrics and Child Health, University College Cork, ^{**}Infant Research Centre, Cork University Hospital, Cork, ^{††}National Maternity Hospital, Dublin, ^{‡‡}Meso-Scale Diagnostics, Manchester, UK, ^{§§}Neonatology, Coombe Women and Infants University Hospital, ^{¶¶}The Rotunda Hospital, ^{***}School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, ^{†††}Department of Histopathology, Trinity College Dublin, and ^{††††}CHI at Crumlin, Dublin, Ireland

Accepted for publication 16 March 2021
Correspondence: E. Molloy, Consultant Neonatologist and Paediatrician, Department of Paediatrics, Trinity Centre for Health Sciences, Tallaght University Hospital, Tallaght, Dublin 24, Ireland.
E-mail: elsean@hotmail.com

¹These authors contributed equally to this study.

Introduction

Neonatal encephalopathy (NE) is a clinically defined syndrome of altered neurological function in term infants manifested

Summary

Neonatal encephalopathy (NE) is characterized by altered neurological function in term infants and inflammation plays an important pathophysiological role. Inflammatory cytokines interleukin (IL)-1 β , IL-1ra and IL-18 are activated by the nucleotide-binding and oligomerization domain (NOD)-, leucine-rich repeat domain (LRR)- and NOD-like receptor protein 3 (NLRP3) inflammasome; furthermore, we aimed to examine the role of the inflammasome multiprotein complex involved in proinflammatory responses from the newborn period to childhood in NE. Cytokine concentrations were measured by multiplex enzyme-linked immunosorbent assay (ELISA) in neonates and children with NE in the absence or presence of lipopolysaccharide (LPS) endotoxin. We then investigated expression of the NLRP3 inflammasome genes, *NLRP3*, *IL-1 β* and *ASC* by polymerase chain reaction (PCR). Serum samples from 40 NE patients at days 1 and 3 of the first week of life and in 37 patients at age 4–7 years were analysed. An increase in serum IL-1ra and IL-18 in neonates with NE on days 1 and 3 was observed compared to neonatal controls. IL-1ra in NE was decreased to normal levels at school age, whereas serum IL-18 in NE was even higher at school age compared to school age controls and NE in the first week of life. Percentage of LPS response was higher in newborns compared to school-age NE. *NLRP3* and *IL-1 β* gene expression were up-regulated in the presence of LPS in NE neonates and *NLRP3* gene expression remained up-regulated at school age in NE patients compared to controls. Increased inflammasome activation in the first day of life in NE persists in childhood, and may increase the window for therapeutic intervention.

Keywords: hypoxic–ischaemic encephalopathy, inflammasome, inflammation, neonatal encephalopathy

by a subnormal level of consciousness or seizures, often associated with respiratory difficulties and slowed reflexes [1], and is associated with cerebral palsy and developmental delay.

Although therapeutic hypothermia (TH) has improved patient outcomes for infants with NE, morbidity remains high [2,3].

Inflammation plays an important pathophysiological role in perinatal brain injury and NE. During inflammation, expression of inflammatory mediators and proinflammatory cytokines activate the microglia which induce brain damage [4,5]. The tertiary stage of brain injury is presented weeks to years after the injury and is caused by persistent inflammation, resulting in impaired neurogenesis, cellular growth and maturation [6]. We have previously described systemic inflammation and alteration of cytokine production in neonates with NE which persists in childhood [7–10].

The nucleotide-binding and oligomerization domain (NOD)-like receptor family, NOD-like receptor protein 3 (NLRP3) is the most widely studied inflammasome and is expressed in the cytosol of monocytes, neutrophils, lymphocytes and dendritic cells. NLRP3 inflammasome mediates innate immune defence against pathogens including viruses, bacteria, reactive oxygen species (ROS) and damaged mitochondria [11–14]. The NLRP3 inflammasome activation leads to the cleavage of pro-caspase-1 into the active form of caspase-1. Caspase-1 then activates the proinflammatory cytokines interleukin (IL)-18 and IL-1 β [13]. The NLRP3 inflammasome has been implicated in the pathogenesis of a wide variety of diseases, including genetically inherited autoimmune and chronic diseases, such as inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis [15]. The inflammasome plays an important role in inflammation and brain injury, and has been shown to be up-regulated in neonatal hypoxic–ischaemic brain injury in mice [16]. IL-1 β has a key role in traumatic brain injury, cerebral ischaemia, subarachnoid, intracerebral haemorrhage and NE [9,17,18]. IL-1 β induces brain injury by the production of nitric oxide, activation of the necroptotic and apoptotic pathways and modulation of the mitogen-activated protein kinase (MAPK) pathway [19,20]. Manipulation of the inflammasome has showed promising results in traumatic brain injury [21]. Anakinra, canakinumab and rilonacept are safe and licensed immunomodulator drugs that interfere with the binding of IL-1 to its receptor in immune-mediated diseases, so understanding the role of the inflammasome in NE could lead to the identification of a therapeutic target [22,23]. We hypothesized that changes in the inflammasome responses are present in NE and may persist in later childhood. In this study, we describe the altered inflammasome response in neonates and school-age children with NE.

Materials and methods

Ethical approval

This study was approved by the ethics committees of Rotunda Hospital, Coombe Women and Infants University Hospital

and the National Maternity Hospital in Dublin, which are all tertiary neonatal intensive care units (NICUs) and national referral centres for therapeutic hypothermia, and Tallaght University Hospital. Families received verbal and written information on the study and written consent was obtained.

Patient groups

This multi-centre study recruited participants consecutively in two separate cohorts. The neonatal group was enrolled from the Neonatal Inflammation and Multiorgan dysfunction and Brain Injury Research (NIMBUS) study. The neonatal control group ($n = 19$) included samples which were obtained from healthy neonates in the first week of life. Healthy controls were born following a full-term normal delivery with normal Apgar scores, neurological examination and postnatal course and no underlying co-morbidities. The neonatal NE group ($n = 40$) included serial samples obtained during the first week of life from neonates with NE. The school-age post-NE cohort was recruited at follow-up from a neonatal group in the Multi-Organ Dysfunction and EEG (MODE) study: school-age control group ($n = 40$) samples were obtained from healthy children between 4 and 7 years of age. The school-age NE group ($n = 37$) samples were obtained from children (aged 4–7 years) after NE [9,24].

All NE groups were divided by severity of encephalopathy according to Sarnat and Sarnat [25]. Children in the NE II/III group received therapeutic hypothermia (TH) in the neonatal period in accordance with the TOBY (total body hypothermia for neonatal encephalopathy) criteria for 72 h duration [26]. Infants with congenital abnormalities, confirmed sepsis or evidence of maternal substance abuse were not enrolled.

Cytokine analysis

Peripheral blood was collected in a sodium citrate anticoagulated blood tube and analysed within 2 h of phlebotomy. Whole blood was incubated in the presence or absence of 10 ng/ml of lipopolysaccharide (LPS) (Sigma Life Science, Dublin, Ireland) at 37°C for 1 h. The plasma was then separated by centrifugation and stored at –80°C for later batch processing. IL-1 β , IL-18 and IL-1ra production was measured using a multiplex cytokine array customized for this study by Meso Scale Discovery (Manchester, UK) and analysed on the Sector imager and validated (Meso Scale Discovery) [7,9].

Quantitative real-time polymerase chain reaction (qRT-PCR) and analysis

Whole blood was incubated at 37°C for 1 h in the presence or absence of 10 ng/ml LPS. RNA was extracted using a Ribopure blood kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. RNA purity and concentration were determined using the

NanoDrop ND-100 spectrophotometer and analysed using ND-1000 version 3.1.2 software. qPCR was performed using TaqMan primer probes (*NLRP3*, *ASC* and *IL-1 β*); the evaluation of gene expression was performed by TaqMan[®] RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for data normalization. The 7900HT fast RT-PCR system (Thermo Fisher Scientific) was used and relative quantification (RQ) values were calculated using the $2^{-\Delta\Delta C_t}$ method [27].

Statistical analysis

Statistical analysis was carried out using the PASW statistical package version 24. Continuous normally distributed data were displayed as means and standard deviations (s.d.) and comparisons between groups (NE *versus* control) were made using the Mann-Whitney *U*-test; when comparing LPS responses we used Wilcoxon's signed-rank test and when comparing between cohorts (neonate *versus* child) we used the analysis of variance (ANOVA) Kruskal-Wallis test with Dunn's correction. Significance was assumed for values $P < 0.05$.

Results

Clinical characteristics

This study enrolled 136 newborns ($n = 59$) and school-age children ($n = 77$). Serum samples were taken on days 1 (D1) and 2 (D2) of life from newborns enrolled from the NIMBUS cohort (19 newborn controls and 40 NE). The grades of encephalopathy were as follows: moderate NE (grade II, $n = 38$) and severe NE (grade III, $n = 2$),

and all infants with NE received TH. Twenty-one neonate infants had seizures and 12 had abnormal magnetic resonance imaging (MRI) (Table 1). From the 19 neonatal controls five were male, with a mean (s.d.) gestation of 39.2 (1.6) weeks, birth weight 3.3 (0.5) kg.

School-age post-NE 37 children were also enrolled (age range = 4–7 years) from the MODE cohort, including 40 age-matched controls. The grades of encephalopathy in childhood post-NE were as follows: grade 0 ($n = 2$); mild NE (grade I, $n = 11$); moderate NE (grade II, $n = 22$); and severe NE (grade III, $n = 2$). Fifteen school-age children completed TH in the neonatal period, as some eligible infants died or had severe persistent pulmonary hypertension. Seventeen infants developed clinical seizures in the neonatal period and 13 had an abnormal MRI brain. There were no significant differences between the NE groups 0/I and II/III regarding gestational age, birth weight and gender. Infants with NE were significantly more likely to be delivered by lower-section caesarean section or instrumental delivery and had significantly lower Apgar scores at 1 and 5 min compared to controls [9] (Table 1). From the 40 school-age controls 28 were male, with a mean (s.d.) gestation of 40 (0.9) weeks, birth weight 3.6 (0.5) kg.

Alteration of immune system in patients with NE and remains altered in childhood

IL-18 and IL-1 β regulate cells of the immune system, guiding the subsequent immune responses [28]. IL-18, IL-1 β and IL-1ra concentrations were measured in serum from neonates. No difference in serum IL-1 β was observed between neonate controls and NE neonates at days 1 (D1) and 3 (D3) at baseline (Fig. 1a). However, IL-1 β was lower

Table 1. Demographics: newborns and school-age children with neonatal encephalopathy (NE)

Variables	Neonates		School age		P-value
	NE II/III ($n = 40$)		NE 0/I ($n = 13$)	NE II/III ($n = 24$)	
GA (weeks) ^a	37.15 (1.94)		39.7 (1.43)	40.5 (1.21)	0.06
BW (kgs) ^a	3.32 (1.50)		3.48 (1.6)	3.61 (1.6)	0.31
Gender, male, n (%) ^b	29 (72)		9 (76)	14 (58)	0.70
Delivery n (%)	LSCS	3 (7.5)	7 (53)	11 (45)	0.70
	SVD	37 (92)	3 (23)	7 (29)	0.73
	Inst	6 (15)	3 (23)	6 (25)	0.73
Apgar at 1 min Median (IQR) ^c	2 (1–7)		5 (3–6)	2 (1–5)	0.003
Apgar at 5 min Median (IQR) ^c	4 (2–9)		7 (5–8)	4 (2–7)	0.01
Apgar at 10 min Median (IQR) ^c	6 (3–10)		7.5 (6–9)	5 (3–7)	0.01
TH, n (%) ^b	40 (100)		0 (0)	15 (62)	< 0.001
Seizures, n (%) ^b	21 (52.5)		0 (0)	17 (70)	< 0.001
MRI: abnormal ^b	12 (30)		0/0	13 (50)	< 0.001

GA = gestational age; BW = birth weight; LSCS = lower section caesarean section; SVD = spontaneous vaginal delivery; Inst = instrumental delivery.

^aFor normally distributed data, mean \pm standard deviation is expressed and the independent Student's *t*-test was used for comparison, where $P < 0.05$ is significant with 95% confidence intervals.

^bFor binary variables, the χ^2 test was used for comparison.

^cFor skewed data, medians and interquartile ranges (IQRs) are expressed and the Mann-Whitney *U*-test was used for comparison.

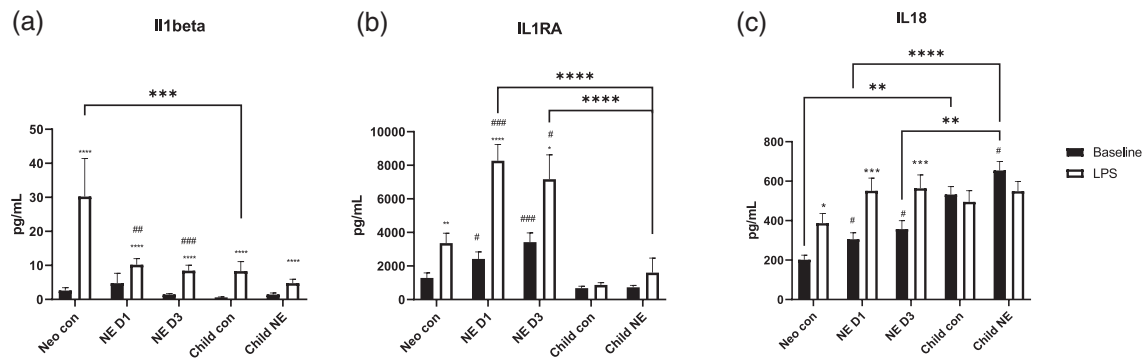


Fig. 1. Interleukin (IL)-18 was altered in neonatal encephalopathy (NE) patients compared to controls. Serum samples were obtained from patients with NE and controls on days 1 and 3 of life and at school age (4–7 years old). Cytokine concentrations were measured by multiplex enzyme-linked immunosorbent assay (ELISA). Graphs show median concentration of inflammasome related cytokines IL-1 β (a), IL-1ra (b) and IL-18 (c) in neonatal controls (Neo con, $n = 19$) and neonatal encephalopathy on days 1 (D1, $n = 40$) and 3 (D3, $n = 26$) as well as controls (Child con, $n = 40$) and NE patients at school age (Child NE, $n = 37$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using the Mann–Whitney compared test) and following lipopolysaccharide (LPS) stimulation.

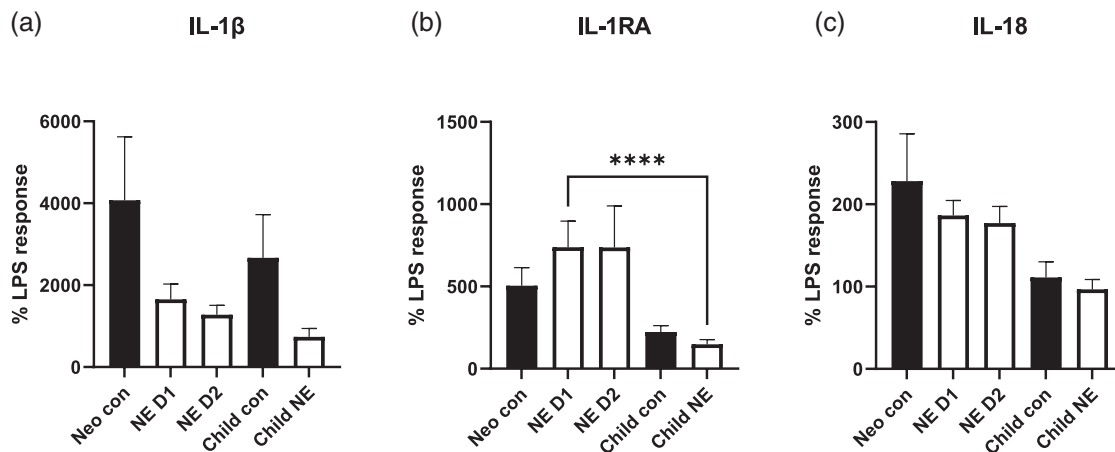


Fig. 2. Lipopolysaccharide (LPS) response was increased with age. Serum samples from neonatal encephalopathy (NE) patients and controls were stimulated with 10 ng/ml of LPS for 1 h, cytokine levels were measured by multiplex enzyme-linked immunosorbent assay (ELISA) and the percentage of LPS response was calculated. Graphs show the percentage of LPS response for IL-1 β (a), IL-1ra (b) and IL-18 (c) in neonatal controls (Neo con, $n = 19$) and neonatal encephalopathy on days 1 (D1, $n = 40$) and 3 (D3, $n = 26$) as well as controls (Child con, $n = 25$) and NE patients at school age (Child NE, $n = 34$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using the Mann–Whitney compared test).

in neonates with NE compared to controls in response to LPS at days 1 and 3 (D1, $P = 0.0034$ and D3, $P = 0.0004$, respectively; Fig. 2a).

IL-1ra concentration was increased threefold in NE on D1 ($P = 0.0242$) and D3 ($P = 0.0006$) compared to neonatal controls in the absence of stimulation (Fig. 1b). There were similar differences upon LPS stimulation at D1 ($P = 0.0004$) and D3 ($P =$ no difference was observed in IL-1ra concentration in response to LPS in neonates with NE and controls (Fig. 2b).

IL-18 was higher in NE neonates at D1 and D3 compared to controls at baseline (D1, $P = 0.0217$ and D3, $P = 0.0240$; Fig. 1c). When stimulated with LPS IL-18 response was similar in neonates with NE and neonatal controls (Fig. 2c).

We then measured IL-18, IL-1 β and IL-1ra concentrations in serum of children at school age post-NE. No difference was observed in serum IL-1 β at baseline between school-age controls and post-NE (Fig. 1a). A significant increase was shown in both control children and children at school age post-NE upon LPS stimulation compared to baseline ($P < 0.0001$, $P < 0.0005$, respectively). IL-1ra was similar in children with NE and age-matched controls in the absence or presence of LPS (Figs. 1b and 2b). IL-18 was higher in children with NE at school age than school-age controls at baseline ($P = 0.015$; Fig. 1c). The IL-18 LPS response was similar in controls and NE at school age (Fig. 2c).

When we compared IL-18, IL-1 β and IL-1ra concentration between neonates and school age children, we found that

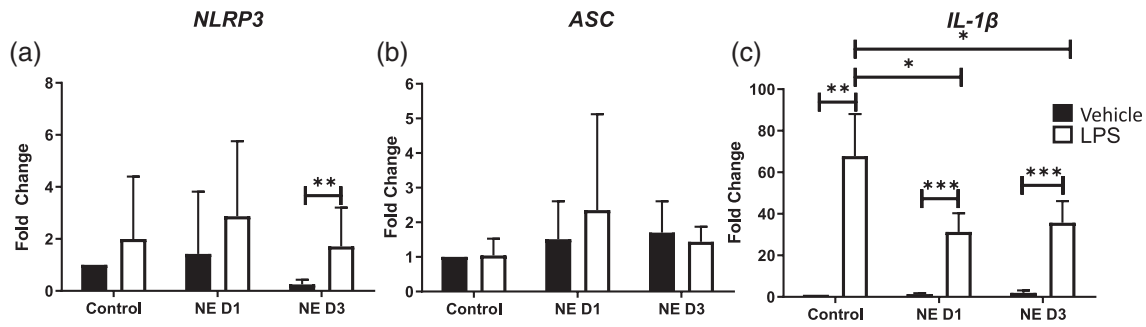


Fig. 3. *NLRP3* and *IL-1β* gene expression was increased in patients with neonatal encephalopathy (NE) in response to lipopolysaccharide (LPS). Serum samples from NE neonates and controls were stimulated with 10 ng/ml of LPS for 1 h. Gene expression of *NLRP3*, *ASC* and *IL-1β* was measured by polymerase chain reaction (PCR) neonatal controls ($n = 5$) and in NE on days 1 (D1, $n = 10$) and 3 (D3, $n = 10$) of life. Graphs show fold change expression of *NLRP3* (a), *ASC* (b) and *IL-1β* (c) when the control vehicle is normalized to 1 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using the Mann-Whitney compared test).

IL-1β was similar in school-age controls compared to neonatal controls at baseline; however, an increase of *IL-1β* was observed in neonatal controls in the presence of LPS ($P < 0.0005$; Figs. 1a and 2a). *IL-1ra* showed a decreasing trend in expression at school age compared to neonates at baseline. This decrease was significant between the NE groups at both D1 and D3 ($P < 0.0001$ and $P \leq 0.0001$, respectively) upon LPS stimulation. The percentage response to LPS from baseline was significantly lower in control children compared to neonatal controls ($P < 0.0001$); *IL-18* concentration in serum increased with age and was 2.5-fold higher in school age controls than control neonates ($P < 0.0013$). In contrast, *IL-18* was higher in NE than controls at D1 and D3 ($P < 0.0001$, $P = 0.0031$, respectively; Fig. 1c).

Inflammasome gene expression in neonates with NE

IL-18 and *IL-1β* are *NLRP3* inflammasome-related cytokines. No difference was observed in *NLRP3* expression in peripheral blood from NE neonates and healthy controls at baseline. However, in neonates with NE *NLRP3* expression was up-regulated on D3 in the presence of LPS in comparison to baseline ($P = 0.0028$; Fig. 3a). The expression of *ASC* was similar in controls and NE and was not altered after LPS stimulation compared to non-stimulation in newborns (Fig. 3b). *IL-1β* gene expression was up-regulated with LPS stimulation in neonatal controls ($P = 0.008$) and NE ($P < 0.0001$ at D1 and D3; Fig. 3c). However, *IL-1β* expression after LPS stimulation was significantly lower in NE on D1 ($P = 0.03$) and D3 ($P = 0.04$) compared to neonatal controls (Fig. 3c).

Persistent alteration in inflammatory responses in childhood following NE

Persistent alteration of *IL-18* was observed in school-age children after NE was compared to age-matched controls. To study if alteration of the inflammasome responses

following NE were persistent at school age, inflammasome gene expression in whole blood before and after stimulation with LPS was measured. *NLRP3* (Fig. 4a) and *ASC* (Fig. 4b) gene expression was not altered after LPS stimulation in school-age children at baseline. However, *NLRP3* was up-regulated in children with NE at school age after LPS stimulation compared to LPS-stimulated controls ($P = 0.008$; Fig. 4a). *NLRP3* gene expression was up-regulated in children with NE after LPS stimulation compared to unstimulated samples from children with NE ($P = 0.03$; Fig. 4a). No difference was observed in *ASC* gene expression between children with NE after LPS stimulation compared to baseline. However, LPS induced an increase in *ASC* expression in NE children at school age compared to controls (Fig. 4b). No difference was observed in *IL-1β* gene expression between children with NE or controls in the presence or absence of LPS (Fig. 4c). These results indicate that *NLRP3* gene expression is increased at D3 of life and remains elevated in childhood, inducing an altered and hyperresponsive immune response.

Discussion

The inflammasome-related cytokines *IL-18* and *IL-1RA* were up-regulated in newborns with NE compared to controls in the absence of stimuli. However, *IL-1ra* was reduced to normal levels at school age, whereas *IL-18* increased with age, and was higher in children post-NE at school age compared to school-age controls and NE at D1 and D3 and in neonatal *versus* child controls. In the presence of stimulus, *IL-1β* and *IL-1RA* decreased with age, and *IL-1ra* was also increased at D1 and D3 in neonatal NE *versus* NE at school age. When *IL-18* was compared between the groups, we found a persistent increase with age in both the control and the NE group.

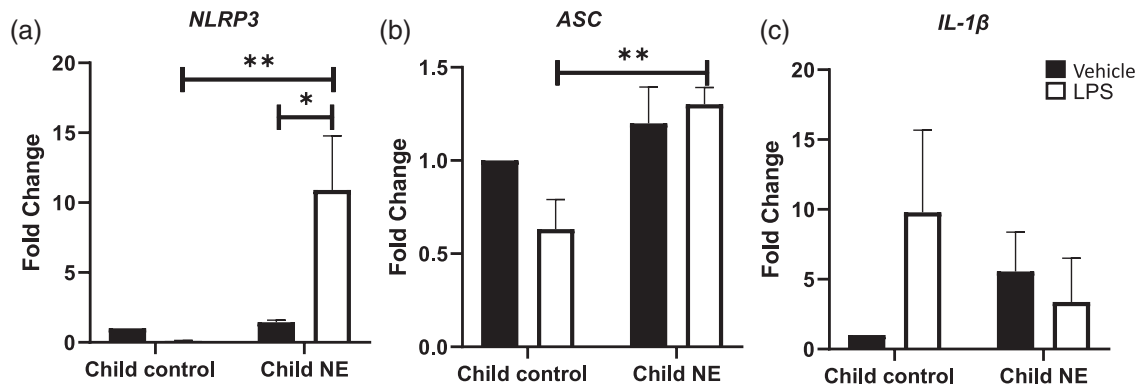


Fig. 4. Nucleotide-binding and oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome gene expression is higher in children with neonatal encephalopathy (NE). Serum samples from NE children and controls were stimulated with 10 ng/ml of lipopolysaccharide (LPS) for 1 h. Gene expression of *NLRP3*, *ASC* and *IL-1β* was measured by polymerase chain reaction (PCR) in control children (Child con, $n = 5$) and in NE children (Child NE, $n = 5$) after stimulation. Graphs show fold change expression of *NLRP3* (a), *ASC* (b) and *IL-1β* (c) when the control vehicle is normalized to 1 ($*P < 0.05$, $**P < 0.01$ using the Mann-Whitney compared test).

IL-1 is dramatically up-regulated in the brain during neuroinflammation [29]. IL-1RA antagonizes IL-1 signaling to maintain homeostasis and the IL-1/IL-1RA balance in healthy brains [29]. IL-1RA has been shown to protect rodents against ischaemia, traumatic brain injury and seizures [30]. In this study, we observed an increase of IL-1RA in patients with NE in the first week of life; however, this was reduced later in life, suggesting an acute neuroprotective role of IL-1RA in NE.

IL-18 was increased with age. Different studies have reported various cytokines such as IL-18, IL-2, IL-6, IL-8, IFN- γ and TNF- α to increase in older individuals [31,32]. Serum IL-18 was higher in children with NE than in age-matched controls. Elevated IL-18 has been associated in humans with multiple sclerosis and Alzheimer's disease [33,34].

Cytokines are known to activate leucocytes peripherally, which cross the blood-brain barrier (BBB) and produce more cytokines [35].

The BBB plays an important role in injury sustained in NE. An altered BBB allows leucocyte recruitment into the brain. Reperfusion post-ischaemia causes leucocyte activation and ROS release that further exacerbates the injury. Dammann *et al.* hypothesized that the leucocytes that contribute to brain injury are derived from outside the brain, and that by modulating leucocyte migration brain injury may be reduced [36].

We have previously reported that infants that require resuscitation are hyporesponsive to LPS stimulation [37]. Neonates that require resuscitation have increased neutrophil and monocytes CD11b and Toll-like receptor (TLR)-4 in response to LPS [8]. Persistent monocyte inflammation has been observed in school-age children with periventricular leucomalacia-induced cerebral palsy [38]. We have previously reported granulocyte-macrophage

colony-stimulating factor (GM-CSF), TNF- β , IL-2, IL-6 and IL-8 to be elevated in school-age children after NE compared to age-matched controls [9].

The use of LPS challenges the innate immune system and examines its response *ex vivo* following an endotoxin encounter. Prior exposure of innate immune cells to endotoxin causes them to become refractory to subsequent endotoxin challenge, and is termed 'endotoxin tolerance'. This previous endotoxin exposure inducing LPS tolerance is known as a form of innate immune memory. This may result in reduced response to inflammatory stimulus, reducing the inflammatory cytokine output causing a relative immunosuppression. The reduced inflammatory response may be beneficial in preventing excessive inflammation, which may cause tissue damage and be detrimental [9,39].

The inflammasome is a key regulator of proinflammatory responses and a potential immunomodulatory target without compromising anti-bacterial immunity completely. The cytokines secreted following inflammasome activation include IL-1 and IL-18 and regulate cells of both the innate and adaptive immune systems, guiding subsequent immune responses. IL-1 β and IL-18 activation depend upon the NLRP3 inflammasome [40]. The inflammasomes are complexes of proteins that induce inflammation in response to microbes and sterile stressors. Inflammasomes are formed by activation of nucleotide-binding oligomerization domain-like receptor family proteins (NLRP). In the presence of LPS or a fungal organism, the NLRP3 inflammasome induces the secretion of the proinflammatory cytokines IL-1 β and IL-18 [41]. The inflammasome genes *IL-1β* and *NLRP3* were altered in infants with NE during the first week of life. *IL-1β* and *NLRP3* were up-regulated after LPS stimulation in NE. Our study also showed significantly higher expression of the inflammasome genes *NLRP3* and *ASC*

in children with NE in comparison to age-matched controls, suggesting an altered inflammatory response in children with NE after LPS stimulation that persists. The inflammasome plays an important role in the innate immune response in the central nervous system [21] and has been explored in animal models of NE, but not human models [42–44]. Exploration of the inflammasome in NE will further explain the infant's inflammatory phenotype and potential future immunomodulation.

ASC deficiency is neuroprotective in neonatal hypoxic–ischaemic brain damage in mice, while NLRP3 deficiency increases brain damage, indicating a future potential immunomodulatory role of manipulating inflammasome components as therapy in NE [45]. In an animal model of traumatic brain injury, NLRP3 inflammasome complex assembly increased [46]. NLRP3-deficient mice were examined in comparison to controls, with no difference in the cerebral infarct size at 24 h [44]. However, when examined at a later time-point of 7 days, NLRP3-deficient mice had larger infarct volumes in comparison to control and ASC-deficient mice had smaller infarct sizes [45]. This suggests that NLRP3 deficiency is detrimental in brain injury, whereas ASC deficiency is neuroprotective, suggesting that future manipulation of ASC could be postulated as a target immunomodulatory therapy in NE.

The inflammasome plays an important role in the innate immune response in the central nervous system, with promising results demonstrating improved outcomes following its manipulation in traumatic brain injury [21]. There are safe and licenced immunomodulators manipulating the inflammasome in immune-mediated diseases, so understanding its role in NE could lead to a therapeutic target. At present, three IL-1 blockers have been Food and Drug Administration (FDA)-approved that effectively reduce IL-1 activation with limited side-effect profiles [47]. Abnormal inflammasome gene expression may suggest a future target for immunomodulation, with therapies already having an acceptable safety profile such as anakinra, an IL-1ra antagonist.

Anakinra has been shown to mitigate neuroinflammation and brain damage [48]. Che *et al.* showed that anti-IL-1 β attenuated tissue damage after perinatal hypoxic–ischaemic reperfusion by the reduction of caspase-3 activity in a sheep model of hypoxic–ischaemic encephalopathy [49]. Anakinra has a long-term effect even after treatment has been suspended [50].

This work provides important new findings in both describing the role of the innate immune system and provides new insights into the mechanisms that could provide new approaches to immunomodulation.

In conclusion, we have described dysregulated inflammation seen in newborns with NE which persists into childhood. Hypoxia–ischaemia induces IL-1 β and IL-18 production. For

the first time, to our knowledge, our study has described the alteration in the inflammasome response in NE and its persistence at school age, suggesting that targeting the inflammasome pathway or IL-1 may be a therapeutic option to manage persistent inflammation in children with NE.

Acknowledgements

This project was funded by the National Children's Research Centre, Crumlin, Dublin, CHI at Tallaght and the Health Research Board, Ireland. The authors thank all our patients, parents and nursing staff for their effort and time in this study. Open access funding provided by IReL.

Disclosures

The authors declare no conflicts of interest. The sponsor of the study has no role in the study design, collection, analysis and interpretation of data, writing the paper or in the decision to submit the manuscript for publication.

Author contributions

M. I. O'D., Z. Z., L. A. K., T. S., V. McE. and D. S. performed the experiments and analysed the data. L. A. K., A. M. M. and E. McK. analysed the results and prepared the manuscript, V. D. scored the MRIs, E. J. M. supervised the design and execution of the study, performed the final data analyses and writing of the manuscript.

Data availability Statement

Data are available on request due to privacy/ethical restrictions.

References

- 1 Nelson KB, Leviton A. How much of neonatal encephalopathy is due to birth asphyxia? *Am J Dis Child* 1991; **145**:1325–31.
- 2 Edwards AD, Brocklehurst P, Gunn AJ *et al.* Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ* 2010; **340**:c363.
- 3 Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. Cooling for newborns with hypoxic ischaemic encephalopathy. *Cochrane Database Syst Rev* 2013; **1**:CD003311.
- 4 Leviton A, Dammann O, Durum SK. The adaptive immune response in neonatal cerebral white matter damage. *Ann Neurol* 2005; **58**:821–8.

- 5 Berger I, Peleg O, Ofek-Shlomai N. Inflammation and early brain injury in term and preterm infants. *Isr Med Assoc J* 2012; **14**:318–23.
- 6 Davidson JO, Dean JM, Fraser M *et al.* Perinatal brain injury: mechanisms and therapeutic approaches. *Front Biosci* 2018; **23**:2204–26.
- 7 O'Hare FM, Watson RWG, O'Neill A *et al.* Serial cytokine alterations and abnormal neuroimaging in newborn infants with encephalopathy. *Acta Paediatr* 2017; **106**:561–7.
- 8 O'Hare FM, Watson RW, O'Neill A, Blanco A, Donoghue V, Molloy EJ. Persistent systemic monocyte and neutrophil activation in neonatal encephalopathy. *J Matern Fetal Neonatal Med* 2016; **29**:309–16.
- 9 Zareen Z, Strickland T, Eneaney VM *et al.* Cytokine dysregulation persists in childhood post neonatal encephalopathy. *BMC Neurol* 2020; **20**:115.
- 10 Sweetman DU, Onwuneme C, Watson WR, O'Neill A, Murphy JF, Molloy EJ. Renal function and novel urinary biomarkers in infants with neonatal encephalopathy. *Acta Paediatr* 2016; **105**:e513–e519.
- 11 Stutz A, Golenbock DT, Latz E. Inflammasomes: too big to miss. *J Clin Investig* 2009; **119**:3502–11.
- 12 Guarda G, Zenger M, Yazdi AS *et al.* Differential expression of NLRP3 among hematopoietic cells. *J Immunol* 2011; **186**:2529–34.
- 13 Piras V, Selvarajoo K. Beyond MyD88 and TRIF pathways in Toll-like receptor signaling. *Front Immunol* 2014; **5**:70.
- 14 Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013; **13**:397–411.
- 15 Ozaki E, Campbell M, Doyle SL. Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J Inflamm Res* 2015; **8**:15–27.
- 16 Serdar M, Kempe K, Rizazad M *et al.* Early pro-inflammatory microglia activation after inflammation-sensitized hypoxic–ischemic brain injury in neonatal rats. *Front Cel Neurosci* 2019; **13**:237.
- 17 Murray KN, Parry-Jones AR, Allan SM. Interleukin-1 and acute brain injury. *Front Cel Neurosci* 2015; **9**:18.
- 18 Jenkins DD, Rollins LG, Perkel JK *et al.* Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. *J Cerebrl Blood Flow Metab* 2012; **32**:1888–96.
- 19 Simoes AP, Duarte JA, Agasse F *et al.* Blockade of adenosine A2A receptors prevents interleukin-1beta-induced exacerbation of neuronal toxicity through a p38 mitogen-activated protein kinase pathway. *J Neuroinflammation* 2012; **9**:204.
- 20 Savard A, Brochu ME, Chevin M, Guiraut C, Grbic D, Sebire G. Neuronal self-injury mediated by IL-1beta and MMP-9 in a cerebral palsy model of severe neonatal encephalopathy induced by immune activation plus hypoxia–ischemia. *J Neuroinflammation* 2015; **12**:111.
- 21 Kerr N, Lee SW, Perez-Barcena J *et al.* Inflammasome proteins as biomarkers of traumatic brain injury. *PLOS ONE* 2018; **13**:e0210128.
- 22 Iannitti RG, Napolioni V, Oikonomou V *et al.* IL-1 receptor antagonist ameliorates inflammasome-dependent inflammation in murine and human cystic fibrosis. *Nat Commun* 2016; **7**:10791.
- 23 Dinarello CA, van der Meer JW. Treating inflammation by blocking interleukin-1 in humans. *Semin Immunol* 2013; **25**:469–84.
- 24 Sweetman DU, Onwuneme C, Watson WR, Murphy JF, Molloy EJ. Perinatal asphyxia and erythropoietin and VEGF: serial serum and cerebrospinal fluid responses. *Neonatology* 2017; **111**:253–9.
- 25 Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch Neurol* 1976; **33**:696–705.
- 26 Azzopardi D, Brocklehurst P, Edwards D *et al.*, the TOBY Study. Whole body hypothermia for the treatment of perinatal asphyxial encephalopathy: a randomised controlled trial. *BMC Pediatr* 2008; **8**:17.
- 27 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**:402–8.
- 28 Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev* 2018; **281**:8–27.
- 29 Spulber S, Bartfai T, Schultzberg M. IL-1/IL-1ra balance in the brain revisited – evidence from transgenic mouse models. *Brain Behav Immun* 2009; **23**:573–9.
- 30 Clark SR, McMahan CJ, Gueorguieva I *et al.* Interleukin-1 receptor antagonist penetrates human brain at experimentally therapeutic concentrations. *J Cerebr Blood Flow Metab* 2008; **28**:387–94.
- 31 Frayling TM, Rafiq S, Murray A *et al.* An interleukin-18 polymorphism is associated with reduced serum concentrations and better physical functioning in older people. *J Gerontol A Biol Sci Med Sci* 2007; **62**:73–8.
- 32 Koelman L, Pivovarova-Ramich O, Pfeiffer AFH, Grune T, Aleksandrova K. Cytokines for evaluation of chronic inflammatory status in ageing research: reliability and phenotypic characterisation. *Immun Ageing* 2019; **16**:11.
- 33 Alboni S, Cervia D, Sugama S, Conti B. Interleukin 18 in the CNS. *J Neuroinflammation* 2010; **7**:9.
- 34 Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and age-related diseases: role of inflammation triggers and cytokines. *Front Immunol* 2018; **9**:586.
- 35 de Boer AG, Breimer DD. Cytokines and blood–brain barrier permeability. *Prog Brain Res* 1998; **115**:425–51.
- 36 Dammann O, Durum S, Leviton A. Do white cells matter in white matter damage? *Trends Neurosci* 2001; **24**:320–4.
- 37 Molloy E, O'Neill A, Grantham-Sloan J *et al.* Neonatal encephalopathy is associated with altered perinatal systemic neutrophil apoptosis. *Am J Perinatol* 2007; **24**:525–30.
- 38 Lin CY, Chang YC, Wang ST, Lee TY, Lin CF, Huang CC. Altered inflammatory responses in preterm children with cerebral palsy. *Ann Neurol* 2010; **68**:204–12.

- 39 Zareen Z, Strickland T, Fallah L *et al.* Cytokine dysregulation in children with cerebral palsy. *Dev Med Child Neurol* 2021; **63**:407–12.
- 40 Ketelut-Carneiro N, Silva GK, Rocha FA *et al.* IL-18 triggered by the Nlrp3 inflammasome induces host innate resistance in a pulmonary model of fungal infection. *J Immunol* 2015; **194**:4507–17.
- 41 Song N, Liu Z-S, Xue W *et al.* NLRP3 phosphorylation is an essential priming event for inflammasome activation. *Mol Cell* 2017; **68**:185–97.e6.
- 42 Chen A, Xu Y, Yuan J. Ginkgolide B ameliorates NLRP3 inflammasome activation after hypoxic-ischemic brain injury in the neonatal male rat. *Int J Dev Neurosci* 2018; **69**:106–11.
- 43 Chen DI, Dixon BJ, Doycheva DM *et al.* IRE1 α inhibition decreased TXNIP/NLRP3 inflammasome activation through miR-17-5p after neonatal hypoxic-ischemic brain injury in rats. *J Neuroinflammation* 2018; **15**:32.
- 44 Ystgaard MB, Sejersted Y, Løberg EM, Lien E, Yndestad A, Saugstad OD. Early upregulation of NLRP3 in the brain of neonatal mice exposed to hypoxia-ischemia: no early neuroprotective effects of NLRP3 deficiency. *Neonatology* 2015; **108**:211–9.
- 45 Ystgaard M, Scheffler K, Suganthan R *et al.* Neuromodulatory effect of NLRP3 and ASC in neonatal hypoxic ischemic encephalopathy. *Neonatology* 2019; **115**:355–62.
- 46 Liu F, McCullough LD. Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacol Sin* 2013; **34**:1121–30.
- 47 Goldbach-Mansky R, Dailey NJ, Canna SW *et al.* Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med* 2006; **355**:581–92.
- 48 Sun M, Brady RD, Wright DK *et al.* Treatment with an interleukin-1 receptor antagonist mitigates neuroinflammation and brain damage after polytrauma. *Brain Behav Immun* 2017; **66**:359–71.
- 49 Chen X, Hovanesian V, Naqvi S *et al.* Systemic infusions of anti-interleukin-1 β neutralizing antibodies reduce short-term brain injury after cerebral ischemia in the ovine fetus. *Brain Behav Immun* 2018; **67**:24–35.
- 50 Neven B, Marvillet I, Terrada C *et al.* Long-term efficacy of the interleukin-1 receptor antagonist anakinra in ten patients with neonatal-onset multisystem inflammatory disease/chronic infantile neurologic, cutaneous, articular syndrome. *Arthritis Rheum* 2010; **62**:258–67.