Altered distributions and functions of natural killer T cells and γδ T cells in neonates with neonatal encephalopathy, in school-age children at follow-up, and in children with cerebral palsy

Nawal A.B. Taher a,b,c, Lynne A. Kelly b,c, Alhanouf I. Al-Harbi a,c,1, Mary I. O’Dea b,c,d,e,f,g, Zunera Zareen b,c,d, Emer Ryan b,c,d,e, Eleanor J. Molloy b,c,d,e,f,g, Derek G. Doherty a,c,*

a Discipline of Immunology, School of Medicine, Trinity College Dublin, Ireland
b Discipline of Paediatrics, School of Medicine, Trinity College Dublin, Ireland
c Trinity Translational Medicine Institute, Trinity College Dublin, Ireland
d Trinity Research in Childhood Centre, Trinity College Dublin, Ireland
e Paediatrics, Children’s Health Ireland at Tallaght & Crumlin, Dublin, Ireland
f Paediatrics, Coombe Women and Infants University Hospital, Dublin, Ireland
g National Children’s Research Centre, Crumlin, Dublin, Ireland

1 T cells were depleted from children with cerebral palsy. Upon stimulation ex vivo, T cells, natural killer cells and Vδ2 T cells from neonates with NE more readily produced inflammatory cytokines than their counterparts from healthy neonates, suggesting that they were previously primed or activated. Thus, innate and conventional lymphocytes are numerically and functionally altered in neonates with NE and these changes may persist into school-age.

1. Introduction

Neonatal encephalopathy (NE) is a neurological syndrome in term babies characterised by altered levels of consciousness, seizures, abnormal tone and reflexes, and/or failure to initiate or maintain respiration (Pfister et al., 2012; D’Alton et al., 2014). The incidence of NE ranges from 2 to 5 per 1000 live term births in advanced countries and up to 26 per 1000 live births in less developed countries (Kurinczuk et al., 2010). NE carries an overall mortality during postnatal period of 28% and approximately a third of surviving infants exhibit permanent neurodevelopmental delay in the form of cerebral palsy (CP), cognitive disabilities, or epilepsy (Kurinczuk et al., 2010; Korzeniowski et al., 2018; O’Dea et al., 2020). Affected new-borns may exhibit multiorgan dysfunction (Shah et al., 2004; O’Dea et al., 2020).

NE has multiple aetiologies including hypoxia-ischemia, infection during the perinatal period, placental abnormalities, metabolic disorders, coagulopathies and neonatal vascular stroke, however, the cause is unidentified in more than half of cases and is likely to be a combination of factors (MacDonald et al., 1980; Shankaran et al., 1991). The only standard of care available is therapeutic hypothermia, the maintenance of the infant at 33.5–34.5 °C for 72 h, which regardless of aetiology gives a maximal effect if started within 6 h from delivery (Tagin et al., 2012; Jacobs et al., 2013). However, therapeutic hypothermia is effective only in a proportion of patients, therefore, there is an urgent need to discover new adjunctive therapies for NE.

Inflammation is a complex outcome of immune defence that can occur in any tissue in response to damage, infection or ischemia (Nathan, 2002). It is initiated by the detection of products of pathogens or damaged tissues by cells of the innate immune system and results in the recruitment and activation of multiple immune cell types and the release of soluble factors. If it is not followed by an anti-inflammatory...
phase, with immune cell inactivation and death and tissue repair, inflammation can lead to persistent tissue damage. The inflammatory reaction in NE and CP can be provoked by hypoxia-ischaemia or infection in the brain and is mediated by numerous effectors, such as phagocytes, cytotoxic cells, chemokines and cytokines (Chew et al., 2006; Morkos et al., 2007; Hagberg et al., 2015; O’Hare et al., 2016; Bajnik et al., 2017; Li et al., 2017; O’Hare et al., 2017; Perrone et al., 2018; Zareen et al., 2020a; Zareen et al., 2020b). An understanding of the causes and mechanisms of inflammation in infants with NE and CP may facilitate the discovery of adjunctive therapies and preventative strategies. Blocking of persistent inflammation may boost neuroprotection by treating the tertiary mechanisms of brain damage which prevent endogenous repair and regeneration and predispose patients to further cognitive dysfunction and sensitisation to further injury (Fleiss and Gressens, 2012; Chevin et al., 2016).

Central to the maintenance of the inflammatory response are lymphocytes, which include B cells and conventional T cells of the adaptive immune system and natural killer (NK) cells, natural killer T (NKT), mucosal-associated invariant T (MAIT) and gamma/delta (γδ) T cells of the innate immune system. These lymphocyte subsets play essential roles in the inflammatory process by activating other immune cells through contact-dependent or cytokine-mediated interactions and they are currently being tested for the treatment of several inflammatory and autoimmune disorders (Salio et al., 2014; Godfrey et al., 2015; Tyler et al., 2015). However, little is known about the relative roles of lymphocyte subsets in inflammation associated with NE.

2. Materials and methods

2.1. Ethical approval

Ethical committee approval for this study was granted from four tertiary referral, university-affiliated children’s and maternity hospitals in Dublin, Ireland – the Coombe Women & Infants University Hospital; National Maternity Hospital; Children’s Health Ireland at Tallaght and the Rotunda Hospital. Written informed consent was obtained from all parents of children who took part in the study.

2.2. Study participants

Blood samples were obtained within the first 3 days of life from 30 neonates with NE and 17 healthy neonates. New-born babies with congenital anomalies or evidence of maternal substance abuse were excluded. Blood samples were also obtained from 10 school-age children post NE, 10 children with CP and 23 healthy school-age children (Zareen et al., 2020a, 2020b; Dietrick et al., 2020).

2.3. Blood sampling

Blood samples were collected in sodium citrate anticoagulated blood tubes and processed within 2 h of sample acquisition. Serum samples were centrifuged at 450 g for 5 min and the supernatants were stored at –80 °C until batch cytokine analysis was carried out.

2.4. Lymphocyte subset enumeration and phenotyping

50–100 μL of whole blood was stained for 15 min in the dark at room temperature with a live/dead cell stain (Fixable Viability Dye eFlour 506, Invitrogen, California, USA) diluted 1/1000 with phosphate buffered saline (PBS). Cells were then stained for 15 min at room temperature with monoclonal antibodies (mAb) specific for CD3 (clones UCHT1 or BW264/56), CD4 (OKT4), CD8 (SK1), CD19 (HB19), CD56 (HCD56), CD69 (FN50), CD161 (HP-3G10), and the Vo7.2 (clone REA179), Vo24Ja18 (6B11), V61 (REA173) and V62 (B6) T cell receptors (TCR) found on MAIT cells, invariant NKT (iNKT) cells and the two most common subsets of human γδ cells, respectively. mAbs were purchased from BioLegend (San Diego, USA and Miltenyi Biotec, Bergische Gladbach, Germany) and were diluted to pre-determined concentrations in PBA buffer (PBS containing 2% fetal calf serum and 0.02% sodium azide). After staining, cells were washed twice in PBA buffer and red cells were lysed in 1 mL FACS lysis buffer (BD Biosciences, Oxford, UK). Finally, the cells were washed with PBA buffer, fixed with 1% paraformaldehyde and analysed on a Becton Dickinson FACSCanto II flow cytometer. Gate limits were determined using unstained and fluorescence-minus-one controls and analysed with FlowJo software (Tree Star, Ashland, USA). T cells were defined as CD3+ cells. NK cells were defined as CD3–CD56+ cells, B cells were defined as CD3–CD19+ cells. MAIT cells were defined as CD3+Vo7.2+CD161+ cells and iNKT cells were defined as CD3+Vo24Ja18+ cells. V61 and V62 T cells were defined as cells expressing CD3 and the V61 or V62 TCRs, respectively. Circulating cell frequencies (%) were determined by flow cytometry and absolute numbers (per litre of blood) were calculated from viable cell counts as determined by fluorescence microscopy. The gating strategy for identification of T cells and the V62 T cell subset by flow cytometry is shown in Fig. 1 and representative flow cytometry dot plots showing each lymphocyte subset are shown in Figs. 2–5 and 7–9. The minimum information about the flow cytometry experiments, as required by Lee et al. (2008) are shown in the supplementary information.

2.5. Measurement of serum cytokine levels

Serum cytokine levels were analysed using the U Plex biomarker group 1 multiplex assay for human interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-5, IL-6, IL-8, IL-9, IL-10, IL-15, IL-17A, IL-21, IL-22 and IL-23, purchased from Mesoscale Discovery (Rockville, USA), according to the manufacturers’ instructions. This method employs a 96-well sandwich immunoassay which can quantify up to 10 analytes in 25 μL samples. Non-specific binding between assays was typically <0.1%. U-plex sample recovery was within the acceptable range (70–130%) with samples diluting linearly from 2 to 16-fold. The limits of detection for the individual assays were within expected ranges.

2.6. Analysis of intracellular cytokine production

Whole blood (50 μL) was plated in wells of a 96-well flat bottom microtiter plates and stimulated for 18 h at 37 °C, 5% CO2 with either medium alone, 50 ng/mL phorbol myristate acetate with 1 μg/mL ionomycin (PMA/I), recombinant human IL-12 (50 ng/mL, R&D Systems) and IL-18 (50 ng/mL, R&D Systems), IL-12 (30 ng/mL) and IL-15 (100 ng/mL), or the ligand recognised by the V62 TCR (E)-4-hydroxy-3-
methyl-but-2-enyl pyrophosphate (HMB-PP; 10 nM) in the presence of brefeldin A (eBioscience) to prevent cytokine secretion from the cells. Cells were then stained with mAbs specific for cell-surface markers, as described above, fixed for 20 min in 4% paraformaldehyde and permeabilised in 0.2% saponin in PBA buffer. Cells were then stained with antibodies specific for IFN-\(\gamma\), TNF-\(\alpha\), IL-17A and granzyme B in the dark at room temperature for 30 min. After washing with PBA buffer, the cells were analysed by flow cytometry.

2.7. Statistical methods

P values between groups were obtained using the unpaired Mann-Whitney U test, and paired t-test where appropriate. P values of \(<0.05\) were considered statistically significant.

3. Results

3.1. Patient demographics

Thirty neonates with NE and 17 healthy neonates were recruited. The mean (±SD) gestational age of the neonates with NE was 39.4 ± 1.5 weeks and the mean birth weight was 3.5 ± 0.5 kg. The mean gestational age of the healthy neonates was 38.5 ± 1.0 weeks and the mean birth weight was 3.5 ± 0.0 kg. Ten school-age children with a history of NE, all of whom were were clinically stable, 10 children with CP, and 23 age-matched healthy children were also recruited. Patient demographics, including sex, Apgar scores at 1, 5 and 10 min after birth, and history of seizures, are shown in Table 1. Apgar scores of the CP patients and school-aged healthy children were not available. All control subjects had no significant medical history and there was no recent history of fever or infection at the time of blood sampling.

3.2. Distribution of circulating lymphocyte populations in neonates and school-age children with NE

Whole blood from the patients and control subjects was stained with mAbs specific for CD3, CD4, CD8, CD19 and CD56 and analysed by flow cytometry. After exclusion of dead cells and doublets, T cells, B cells and NK cells were identified as CD3\(^+\), CD19\(^+\) and CD3\(^-\)CD56\(^+\) cells, respectively (Fig. 2A and B). Fig. 2C shows that the percentage frequencies and absolute numbers of T cells were significantly higher in children with CP compared to healthy children. The frequencies of B cells were significantly higher in neonates with NE compared to healthy neonates and significantly lower in school-age children with CP compared to healthy school-age children, however, these differences were not significant when absolute cell numbers were compared (Fig. 2D). Both the frequencies and numbers of B cells were higher in school-age children with NE compared to age-matched control children (Fig. 2D). The frequencies, but not absolute numbers, of NK cells were significantly lower in neonates with NE compared to healthy neonates (Fig. 2D).

Flow cytometric analysis of CD4 and CD8 expression by T cells from the different subject groups (Fig. 3A) indicated that the frequencies of CD4\(^+\) and CD8\(^-\) T cells, as percentages of total T cells, were similar in all
Fig. 2. Frequencies and absolute numbers of circulating T cells, B cells and NK cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A and B, Flow cytometry dot plots showing the enumeration of T cells and B cells (A) and NK cells (B) within peripheral blood mononuclear cells. C-E, Scatter plots showing the frequencies (left) and absolute numbers (right) of T cells (C), B cells (D) and NK cells (E) in the five subject groups.
subject groups (Fig. 3C). However, the frequencies of double-negative CD4⁺CD8⁻ and double-positive CD4⁺CD8⁺ T cells were higher in school-age children post NE compared to healthy children. Additionally, CD4⁻CD8⁻ T cell frequencies were raised in children with CP (Fig. 3A, D and E).

3.3. Innate T cells in neonates and school-age children with NE

Whole blood from the neonates with NE, healthy neonates, school-age children with NE and CP and healthy school-age children was stained with mAbs specific for CD3 and the two most common TCR δ-chains and analysed by flow cytometry (Fig. 4 A and B). The frequencies and absolute numbers of Vδ1 T cells were lower in children with CP compared to age matched healthy children (Fig. 4C). Strikingly, the frequencies and absolute numbers of Vδ2 T cells were significantly higher in neonates with NE, in school-age children post NE and in children with CP compared to age-matched controls (Fig. 4D).

Whole blood from the subject groups was also stained with mAbs specific for the Va7.2 TCR, which together with CD161 identifies MAIT cells (Fig. 5A), and the Vα24Jα18 TCR which identifies iNKT cells (Fig. 5B) using flow cytometry. Fig. 5C shows that MAIT cells were found in very low numbers in neonates and that the frequencies and numbers
of MAIT cells were similar in healthy school-age children and in school-age children who were previously diagnosed with NE. However, the frequencies, but not absolute numbers of MAIT cells were lower in children with CP. The frequencies and absolute numbers of iNKT cells were significantly higher in neonates with NE, in school-age children post NE and in children with CP compared to age-matched controls (Fig. 5 D).

3.4. Serum cytokine levels in neonates and school-age children with NE

The levels of IFN-γ, TNF-α, IL-2, IL-5, IL-6, IL-8, IL-9, IL-10, IL-15, IL-17A, IL-21, IL-22 and IL-23 in serum samples from all subjects were measured by multiplex immunoassay. Fig. 6 shows that the serum levels of IFN-γ, TNF-α, IL-2, IL-5, IL-6, IL-10, IL-15, IL-17A, IL-21, IL-22 and IL-23 were similar in all subject groups. The levels of IL-8 were found to be significantly higher in both neonates and school-age children with NE compared to age-matched control subjects. This increase was not found

Fig. 4. Frequencies and absolute numbers of circulating Vδ1+ and Vδ2+ γδ T cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A and B, Flow cytometry dot plots showing the expression of CD3 and the Vδ1 (A) and Vδ2 (B) T cell receptors by peripheral blood mononuclear cells. C and D, Scatter plots showing the frequencies (left) and absolute numbers (right) of Vδ1 (C) and Vδ2 T cells (D) in the five subject groups.
in children with CP. The levels of IL-9 were also higher in the patients with NE, but this difference was only significant in the school-age children.

3.5. Ex vivo cytokine and granzyme B production by T cells, NK cells and Vδ2 T cells from healthy neonates and neonates with NE

Since Vδ2 T cells were found to be significantly expanded in neonates with NE compared to healthy neonates, we next investigated cytokine and cytotoxic mediator production by these cells taken from 7 healthy neonates and 10 neonates with NE following activation ex vivo. We also measured these functional readouts in total T cells and NK cells. Whole blood was stimulated with medium alone, PMA/I, IL-12 + IL-18, IL-12 + IL-15, or HMB-PP in the presence of brefeldin A. Cells were then stained with mAbs specific for cell-surface CD3, CD56 and the Vδ2 TCR and intracellular IFN-γ, TNF-α, IL-17A and granzyme B and analysed by flow cytometry. Fig. 7 (A and B) shows that significantly higher numbers of total T cells from neonates with NE produced IFN-γ in response to stimulation with PMA/I or IL-12 and IL-18, compared to T cells from healthy neonates. The same was true for TNF-α and IL-17A production.
when cells were stimulated with PMA/I. Stimulation with PMA/I or IL-12 with IL-18 also resulted in significantly higher frequencies of granzyme B production by total T cells from neonates with NE (Fig. 7B). Thus, although the numbers of T cells are similar in neonates with NE and healthy neonates, the neonates with NE patients had significantly higher numbers of effector T cells capable of producing inflammatory cytokines and cytotoxic mediators.

A similar finding was obtained with NK cells. Upon stimulation with IL-12 with IL-15, and to a lesser degree with PMA/I, NK cells expressed the activation marker CD69 and produced IFN-γ and TNF-α and this occurred more frequently in NK cells from neonates with NE compared to healthy neonates (Fig. 8A and B). NK cells from neonates with NE also stained more intensely for granzyme B in response to either stimulus compared to NK cells from healthy neonates (Fig. 8C).

We next investigated cytokine production by the Vδ2 subset of T cells, which typically accounts for less than 3% of circulating T cells (Tyler et al., 2015). After stimulation with PMA/I or IL-12 and IL-18, significantly higher frequencies of Vδ2 T cells from neonates with NE produced IFN-γ compared to Vδ2 T cells from healthy neonates (Fig. 9A and B). The frequencies of Vδ2 T cells that produced TNF-α in response to all 3 stimuli were also significantly higher in the neonates with NE compared to healthy neonates (Fig. 9B). Vδ2 T cells from neonates with NE also more frequently produced IL-17 in response to stimulation with PMA/I, whereas stimulation with IL-12 and IL-18 or HMB-PP failed to induce significant IL-17 production by these cells from patients or controls. Interestingly, activation of Vδ2 T cells with IL-12 with IL-18 or HMB-PP, but not with PMA/I, resulted in granzyme B production and this was significantly higher among Vδ2 T cells from neonates with NE compared to control neonates. Collectively, these results show that Vδ2 T cells are both expanded and activated in neonates with NE.

4. Discussion

Inflammation plays a major role in brain injury associated with NE. It is characterised by astrocyte and microglial cell activation and the release of inflammatory cytokines which result in the recruitment of neutrophils and monocytes to the brain (Chew et al., 2006; Hagberg et al., 2015; Li et al., 2017). Circulating immune cell activation is also associated with poor outcome in brain injury. Neonates with NE have increased numbers of monocytes and neutrophils with dysregulated functions (Morkos et al., 2007; O’Hare et al., 2016) and elevated serum levels of inflammatory cytokines, which are associated with poor developmental outcomes and mortality (Bajnok et al., 2017; O’Hare et al., 2017; Perrone et al., 2018). The importance of neutrophils in brain injury following hypoxia ischemia is clear from animal studies, where neutrophil depletion can reduce brain injury (Doycheva et al., 2014; Yao and Kuan, 2020). The serum cytokine levels in the subjects in the present study are consistent with a role for neutrophils in NE. We found that IL-8, which promotes neutrophil recruitment and activation, was present at higher levels in neonates and children with NE compared to healthy age-matched controls. Cytokine dysregulation by neutrophils in neonates with NE is long-lasting and persists into childhood (Li et al., 2017; Zareen et al., 2020a; Zareen et al., 2020b).

While effector cells of the innate immune system clearly contribute to the pathogenesis of NE, little is known about the role of lymphocytes, the central controllers of innate and adaptive immune responses. In view of the persistent nature of inflammation in NE and CP (Fleiss and Gressens, 2012; Li et al., 2017; Zareen et al., 2020a, 2020b; Winerdal et al., 2012), it is likely that conventional T cells and B cells of the adaptive immune system control the actions of neutrophils and monocytes through cytokine and antibody production. Activated CD4+ and CD8+ T cells infiltrate the brain in the tertiary phase of injury following hypoxia-ischemia in mice (Winerdal et al., 2012; Nazmi et al., 2018). Depletion studies have demonstrated pathological (Yang et al., 2014) and protective (Herz et al., 2018) roles for T cells, suggesting distinct roles for different T cell subsets (Liesz et al., 2009). B cells also infiltrate the brains of mice following hypoxia ischemia (Nazmi et al., 2018) and regulatory B cells may contribute to protection against brain injury via the release of IL-10 (Bodhankar et al., 2013).

In the present study, we measured the absolute numbers and percentage frequencies of circulating B cells and T cells in neonates with NE, healthy neonates, school-age children with NE, school-age children with CP, and healthy school-age children. We found that B cell frequencies and numbers were significantly higher in neonates and school-age children with NE compared to healthy age-matched children. The numbers and frequencies of total T cells were higher in children with CP but similar in the other subject groups. Analysis of CD4 and CD8 expression by these T cells revealed that CD4+ and CD8+ T cell frequencies were similar in the five subject groups, but CD4+ CD8− and CD4+ CD8− T cells were present in greater numbers in school-age who had NE compared to healthy school-age children and CD4+ CD8− T cells were also expanded in children with CP. These data suggest a role for unconventional T cells, such as iNKT cells and γδ T cells, which frequently are negative for CD4 and CD8, in the pathogenesis of NE.

Functional studies showed that, upon stimulation ex vivo, T cells from neonates with NE more frequently produced IFN-γ, TNF-α, IL-17 and granzyme B than T cells from healthy neonates, suggesting that they have previously been primed or activated. IFN-γ and TNF-α are T helper type 1 (Th1) cytokines which promote monocyte, macrophage and CD8+ T cell activation and cytotoxicity, whereas IL-17 is the signature Th17 cytokine, which promotes the recruitment and activation of neutrophils. The enhanced ability of T cells from patients with NE to produce Th1 and Th17 cytokines and the cytotoxic mediator granzyme B is consistent with a role for T cells in the maintenance of the inflammatory responses seen in NE. However, we did not observe increases in the levels of Th1 or Th17 cytokines in the serum of NE patients, suggesting that T cell activation may be restricted to the brain. Future studies are required to determine if the numerical changes reflect movement of B cells and T cells to the brain or whether particular subsets of these cells, such as regulatory B cells (Bodhankar et al., 2013) or Th1, Th2, Th17 or regulatory T cells (Su et al., 2018), are numerically altered. Future studies are also required to determine if the increased numbers of B cells in patients with NE are associated with higher serum immunoglobulin levels. However, the changes in B and T cell numbers and their altered state of activation suggest roles for the adaptive immune system in the pathogenesis of NE.

Our data also show that innate lymphocytes, such as NK cells, γδ T cells, MAIT cells and iNKT cells, may also contribute to neuro-inflammation in NE and CP. Innate T cells initiate, regulate and maintain innate and adaptive immune responses (Salio et al., 2014; Godfrey et al., 2015; Tyler et al., 2015). They recognise and respond to changes in the levels of metabolites, including lipids, pyrophosphates, riboflavin precursors and proteins, that are synthesised in response to cell stress, such as hypoxia, infection or tumour transformation. They respond by rapid cytotoxicity, potent cytokine release, and contact-dependent interactions with neutrophils, monocytes, macrophages, dendritic cells
and B cells. The innate lymphocytes, NK cells (Fathali et al., 2013), γδ T cells (Nazmi et al., 2018; Albertsson et al., 2018) and iNKT cells (Wang et al., 2016) infiltrate the brains of mice following experimental hypoxia ischemia and contribute to brain injury.

We found that the frequencies of circulating NK cells were significantly lower in neonates with NE. Furthermore, NK cells from neonates with NE more readily expressed the activation marker CD69 and produced IFN-γ, TNF-α and granzyme B in response to stimulation ex vivo than NK cells from healthy neonates.

As previously reported (Ben Youssef et al., 2018), we found that MAIT cells were found in very low numbers in neonates. These cells expanded in childhood and their frequencies and numbers were similar in healthy children and in children with NE but were less frequent in children with CP. The frequencies and absolute numbers of Vδ1 T cells were also lower in children with CP compared to age-matched healthy children. Future studies are required to determine if the depletions of these cells from blood are due to their trafficking to the brain.

A striking observation in the present study was significant increases in the frequencies and absolute numbers of iNKT cells and Vδ2 T cells in neonates and school-age children with NE and CP, compared to healthy children. These increases may account for the observed increases in CD4+ CD8− T cells in NE patients and may promote neuroinflammation.
via their well-documented abilities to produce early bursts of inflammatory cytokines, which lead to downstream activation of other cells of the immune system (Salio et al., 2014; Godfrey et al., 2015; Tyler et al., 2015). iNKT cells and Vδ2 T cells typically expand in response to glycolipids and pyrophosphates, respectively, produced by bacteria or by host cells responding to bacterial or viral infection, however future studies are required to determine if infection underlies the inflammation found in NE.

Whereas iNKT cells are found in insufficient numbers for functional studies using blood samples, we found that after stimulation ex vivo, significantly higher frequencies of Vδ2 T cells from neonates with NE produced IFN-γ, TNF-α, IL-17 and granzyme B compared to Vδ2 T cells from healthy neonates. Neonates have reduced capacity for mounting conventional αβ T cell responses, but Vδ2 T cells are functionally competent during early development and are important in early-life immunity. Vδ2 T cell numbers and functions are altered in several neurological diseases, including infectious meningitis, ischemic stroke and multiple sclerosis (Dieli et al., 1999; Peterfalvi et al., 2009; Albertsson et al., 2018; Maimaitijiang et al., 2018; Wo et al., 2020). Vδ2 T cells are also capable of producing IL-9 (Peters et al., 2016), which was found at higher levels in the serum of NE patients, but future work is required to determine if Vδ2 T cells are the source of this inflammatory...
cytokine. Invariant NKT cells are thought to play essential roles in the pathogenesis of ischemic stroke, neurodegenerative disease and auto-immune diseases such as multiple sclerosis and myasthenia gravis (Wang et al., 2016; De Biasi et al., 2016; Cui and Wan, 2019). Multiple clinical trials involving in vivo activation or adoptive transfer of ex vivo activated Vδ2 T cells and iNKT cells are ongoing (Exley and Nakayama, 2011; Richter et al., 2013; Pauza et al., 2018; Kabelitz et al., 2020) and may in future be applied as a novel treatment of NE.

Limitations to this study include the small sample sizes and the restricted numbers of tests that could be carried out on the 50–100 μL blood samples obtained. Interpretation of the results is further confounded by the multiplicity of aetiologies and manifestations thought to underlie NE, which could involve different components of the immune system. A further limitation is the use of peripheral blood to attempt to assess the immunological basis of an inflammatory process that occurs in the brain. Furthermore, while innate T cells display semi-invariant TCRs, the present study has not investigated TCR clonality or antigen fine-specificity. Future longitudinal studies are also required to measure fluctuations in lymphocyte numbers and functions from birth into childhood and to correlate these with developmental changes.

5. Conclusions

Our data suggest that both innate and conventional lymphocytes are numerically and functionally altered in neonates with NE and that these changes may persist into school age. iNKT cells and Vδ2 T cell numbers

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**Fig. 8.** Cytokine and granzyme B production by NK cells from patients with neonatal encephalopathy (NE). A, Representative flow cytometry dot plots showing CD56 and IFN-γ expression by gated CD3-negative cells in whole blood after stimulation with medium or IL-12 + IL-18. B, Mean (±SEM) percentages of NK cells from healthy neonates (n = 7) and neonates with NE (n = 10) that produce cytokines upon stimulation ex vivo. C, Mean (±SEM) fluorescence intensity staining for granzyme B in NK cells from healthy neonates (n = 7) and neonates with NE (n = 10) that produce cytokines upon stimulation ex vivo.
and frequencies were higher in neonates and children with NE and CP compared to healthy children, while MAIT cells and Vδ1 T cells were depleted from children with CP. Upon stimulation ex vivo, T cells, NK cells and Vδ2 T cells from neonates with NE more readily produced inflammatory cytokines than their counterparts from healthy neonates, suggesting that they were previously primed or activated. In view of their limited diversity, multifunctionality and ease of activation, expansion and manipulation, innate T cells make attractive potential targets for therapeutic modulation and may ultimately prove amenable for the treatment of NE and the subsequent inflammatory processes.

**Conflict of interest statement**

All authors declare that there are no conflicts of interest.

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