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Examining the role of lymphocytes in health and in neonatal encephalopathy and the influence of mucosal associated T cells on B cell functions

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Nawal Abdullah B Taher

For My husbund Abduladheem

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List of abbreviations used

α-GalCer	α-galactosylceramide
ASC	Antibody-secreting cells
5-A-RU	5 5-amino-6-D-ribitylaminouracil
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
BDFACS	BD Biosciences Flexible Flow Cytometry System
CBA	Cytometric Bead Array
CP	Cerebral palsy
CNS	Central Nervous System
Con	Control
DAMPS	Damage-Associated Molecular Patterns
dNK	Decidual NK
EB/AO	Ethidium bromide / acridine orange
EGA	Estimated gestational age
ELISA	Enzyme Linked Immunoassay
FSc	Forward scatter
GC	Germinal centre
GFI-1	Growth factor independent-1
HIE	Hypoxic-ischaemic encephalopathy
HMB-PP	(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
I FNγ	Interferon-gamma
ICU	Intensive care unit
IGF-1	Insulin-like growth factor-1
IL- 1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-9	Interleukin-9
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-17A	Interleukin-17A
IL-18	Interleukin18
IL-21	Interleukin-21
IL-22	Interleukin-22
IL-23	Interleukin-23
iNKT	Innate natural killer T cells
iNOS	Inducible nitric oxide synthase

IRI	Ischaemia-reperfusion injury
KIR	killer immunoglobulin-like receptors
LLPC	Long-lived plasma cells
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MAC	Terminal membrane attack complex
MG	Microglia
MHC	Major histocompatibility complex
MS	Multiple scelerosis
MR1	MHC-related molecule 1
MRI	Magnetic resonance imaging
MSD	Meso Scale Discovery
MAIT	Mucosal associated invariant T
Nab	Natural antibodies
NE	Neonatal encephalopathy
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa beta
NICE	National Institute for Clinical Excellence
NK	Natural Killer
NO	Nitric Oxide
PAMPS	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBA	Phosphate buffer acetate
PBS	Phosphate buffer saline
DC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptors
PFA	Paraformaldehyde 4%
PMA/I	Phorbol myristate acetate with ionomycin
RORα	RAR-related orphan receptor alpha
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
SLO	Secondary lymphoid organs
SSC	Side scatter
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TSLP	Thymic stromal lymphopoietin
Th	T helper
TH	Therapeutic hypothermia
TNF-α	Tumour Necrosis Factor-α
T regs	Regulatory T cells
VEGF	Vascular endothelial growth factor

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General Introduction

1.1 Neonatal encephalopathy

1.1.1 Clinical features of NE

Neonatal encephalopathy (NE) is a complex syndrome defined clinically by a disturbance in neurological functions, in the earliest days of life in a term infant. An altered degree of consciousness, convulsions, difficulties in initiating, sustaining breathing and diminished tone and reflexes, are common symptoms of this condition [1]. NE can be a consequence of a wide variety of causes and is a clinical term that does not specify aetiology. The terms NE and hypoxic-ischaemic encephalopathy (HIE) are frequently used interchangeably in the literature [2-4]. NE has several aetiologies, but hypoxic ischemia is the most common factor. Others include perinatal infections, placental abnormalities, inborn errors of metabolism, coagulopathies and neonatal vascular stroke. However, in almost half of NE cases, the exact underlying reason is not identified and is usually a mixture of multiple factors [5]. The brain is not the only organ affected following the hypoxic-ischemic insult. Infants can also exhibit co-existing multi-organ dysfunction affecting the immune, respiratory, endocrine, renal, hepatic, and cardiac systems, which can result in severe mortality and morbidity [6, 7]. Neonatal neurological examinations rely on a standardized scoring method that has been associated with clinical outcomes in order to make a clinical diagnosis of NE. In 1976, Sarnat and Sarnat divided the phases of encephalopathy into mild, moderate, and severe to define the clinical signs of NE. Sarnat came to the conclusion that mild encephalopathy that persisted for more than 7 days was linked to poor neurologic prognosis or mortality (Table 1) [8].

Table 1-1 Sarnat scoring system [8]

Sarnat Stage	Stage 1	Stage 2	Stage 3
Level Of Consciousness	Hyperalert Lethargic	Obtunded Stupor	Coma
Activity	Normal	Decreased	Absent
Neuromuscular Control -Muscle Tone	Normal	Mild Hypotonia	Flaccid
Posture	Mild Distal Flexion	Strong Distal Flexion	Intermittent decerebration(extension)
Stretch Reflexes	Overactive	Overactive	Decreased or absent
Primitive Reflexes Suck	Weak	Weak or Absent	Absent
Moro	Moro Strong, low threshold	Weak/Incomplete, High Threshold	Absent
Atonic Neck Reflex	Slight	Strong	Absent
Autonomic Function-Pupils	Mydriasis	Miosis Variable	unequal, poor light reflex, fixed dilated
Heart Rate	Tachycardia	Bradycardia	Variable
Seizures	None	Common	Uncommon, excluding decerebration

1.1.2 Epidemiology of NE

In children under the age of five, it is estimated that NE is one of the top ten contributing factors for deaths, according to the WHO [9]. NE affects 1.15 million babies every year worldwide with 96% of NE cases born in the developing world. Its incidence varies from 1 to 3 per 1,000 live births in high-income countries and up to 20 per 1000 live births in low-resource settings [10-12].

1.1.3 Pathogenesis of NE

A reduction in cerebral perfusion is caused by intermittent anoxia or acute hypoxia leads to initial drop in cerebral blood flow causes a primary energy failure [13]. Reduced cerebral blood flow leads to low oxygen and glucose levels, resulting in significantly less energy (adenosine triphosphate (ATP)) and increased lactate production [14]. Low ATP levels lead to disruption of many of the cell's defence mechanisms, especially the sodium/potassium (Na/K) pumps and mechanisms that keep intracellular calcium low [15]. When the Na/K pumps fail, an excess of positively charged sodium ions floods the neurons, causing massive depolarization. This causes glutamate, an excitatory amino acid, to be released. The glutamate binds to glutamate receptors, allowing more calcium and sodium to enter the cell [15]. Elevated intracellular calcium has serious consequences, causing cerebral oedema, ischemia, and microvascular damage, as well as necrosis and/or apoptosis [16]. Primary energy failure causes cellular necrosis by disrupting the cytoskeleton and cell membrane [15, 17]. As a result, cells swell and rupture, resulting in cellular death. When a cell ruptures, the contents of the cell are released, leading to inflammation [18]. Inflammatory mediators can damage white matter and cause scar tissue [19]. The cells may recover or progress to apoptosis [15]. Apoptosis results in cell shrinkage and the preservation of cellular membranes in the absence of inflammation. Apoptosis will occur days after the initial infection [18].

After the primary energy failure phase, there is a short period of recovery once blood flow has been restored [13]. Normal cerebral metabolism characterizes this short recovery, known as the latent period. The length of the latent period is believed to be related to the severity of the hypoxic-ischemic insult, with more severe insults shortening the latent time [20]. The precise start and end times of the primary energy failure phase, latent period, and secondary energy failure phase are unknown at this time [21]. Thus, the typical time for therapeutic intervention is during the latent period [17] (Figure 1).

Between 6 and 48 hours after the initial damage, secondary energy failure stage occurs. The pathophysiology of secondary energy failure is unknown [17]. Generally, it seems to link to oxidative stress, excitotoxicity, and inflammation. Free radicals generated by oxidative stress damage neuronal cell membranes, resulting in necrosis or apoptosis. The neonatal brain is extremely vulnerable to oxidative stress due to low antioxidant concentrations [22] and high oxygen consumption during the transition from foetal to neonatal life [23]. Neonates have high concentrations of unsaturated fatty acids, which makes more oxygen free radicals. During a hypoxic-ischemic state, iron bound to proteins is released, allowing free iron (Fe^{2+}) to react with peroxides and form free radicals [13]. The ability of the neonatal brain to remove free radicals is reduced [22].

Excitotoxicity develops when excitatory receptors are overstimulated by high amounts of extracellular neurotransmitters, particularly glutamate. High influx of sodium and calcium to neuronal cells occurs as a result of overstimulation. Several neuronal pathways use glutamate, involving hearing, vision, somatosensory function, learning, and memory, suggesting why HIE has such a detrimental impact on neonates' future growth [24]. Inflammation has a role in the evolution of HIE-related brain injury morbidities, but its exact role is unidentified [23]. In animal models, accumulation of neutrophils into the brain parenchyma during the early stages of damage (4–8 hours) has been reported to

cause cerebral oedema [25]. The role of inflammation in the pathogenesis of NE is a major subject of this thesis and will be discussed in detail below.

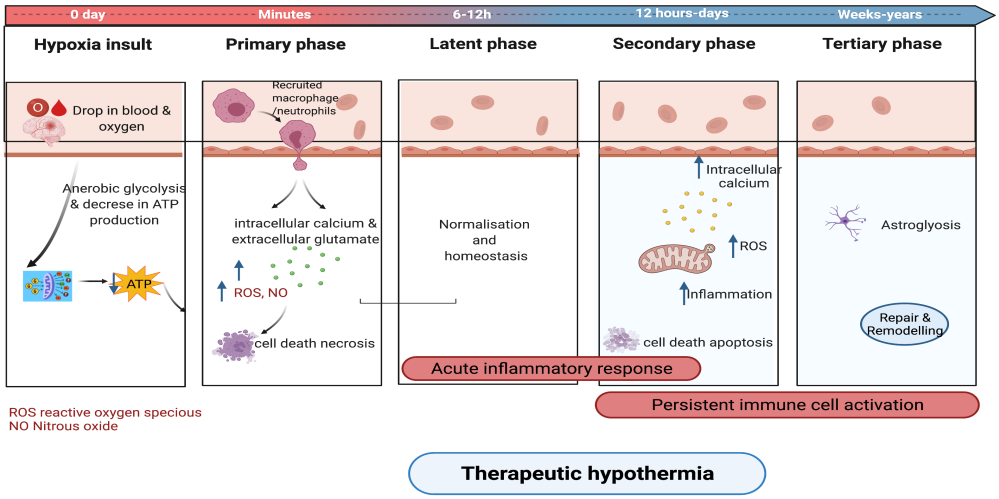

Figure 1.1Diagram depicting the various pathogenic stages of brain damage following cerebral HI. Acute hypoxic insult (HI) is the major phase, followed by latent, secondary energy failure, secondary brain damage, and tertiary brain injury phases. Figure created with BioRender.com

1.1.4 Treatment of NE

The only standard of care available for NE is therapeutic hypothermia (TH), the maintenance of the infant at 33.5-34.5^{o}C for 72 hours. TH lowers temperature of the brain's most critical structures, the basal ganglia. For neonatal HIE, there are two key methods of TH: whole-body cooling and selective head cooling with mild systemic hypothermia. Since the brain generates more than two-thirds of total body heat, selective head cooling is advantageous to whole-body cooling. To prevent the harmful effects of systemic cooling, cooling the brain rather than the rest of the body is highly recommended [26]. Theoretical modelling of cooling, on the other hand, examines the temperature distribution within the neonatal head. The only situation that resulted in a major reduction in deep brain temperature was when the body temperature was reduced to 34°C, suggesting that systemic temperature must be reduced to achieve deep brain cooling [27]. The core body and deep brain temperatures must be identical for whole-body cooling to work.

The neuroprotective effect of TH can be due to a number of mechanisms. Hypothermia can protect cells against apoptosis. After resuscitation, twelve hours of mild hypothermia in neonatal piglets considerably reduced the numbers of apoptotic cells but not the number of necrotic cells [11]. TH may also protect neurons by lowering the metabolic rate in the brain, lowering toxic nitric oxide (NO) and free radical production, mitigating the release of excitatory amino acids (glutamate, dopamine) and ameliorating the ischaemia-impaired uptake of glutamate [28].

The key benefits for neuroprotection are that hypothermia be initiated as early as possible in the latent phase, within the first six hours, before secondary deterioration, and that it is continued for a sufficient period, typically around 72 h [29, 30]. These studies show that, as currently applied, ~15% of infants will have better neurological outcome

after cooling compared to standard care. Despite impressive neuroprotective effects, questions remain on how to optimize TH for neonatal HIE. Although mortality and significant neurologic disability rates have decreased since the pre-TH era, they remain unacceptably high at 40 per cent to 55 per cent [31, 32].

In 2010, the National Institute for Clinical Excellence (NICE) recommended that TH is the treatment for NE. TH reduces the infants' body temperature to 33.5°C for 72 hours. It must commence within a six-hour window after delivery [33, 34]. TH reduces the co-morbidities in the long term, such as death and significant neuro-developmental impairment by 18 months [35], lowers the risk of developing CP [36] and prolongs the maintenance of an IQ of >85 [37]. Conversely, recent evidence has demonstrated that school-age children aged between six to eight years of age, who were cooled at birth for NE and did not develop CP, had lower motor and cognitive test outcomes than healthy subjects [38, 39]. In addition, they struggle with concentration, have slower response times, and have low visuospatial processing skills [40]. 18-month developmental scores do not predict these motor and cognitive delays [33, 39].

1.2 Inflammation in NE

1.2.1 Neuroinflammation

Local inflammation in the brain contributes to damage initiated by hypoxia-ischemia and contributes to the development of neurodegenerative diseases including schizophrenic behaviour and memory and attention deficits in animal models [41, 42]. In humans, perinatal inflammation is also linked to several neuropsychological disorders and it is suggested that inflammation has long term sequelae on the brain functions during childhood (Hagberg et al., 2012).

Neuroinflammation is characterized by the induction of an immediate and robust activation of brain-resident glial cells, microglia and astrocytes. In humans, microglia are central to the initiation and resolution of inflammation in the brain [43]. Proinflammatory mediators released by microglia and astrocytes such as interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) [44] lead to recruitment of neutrophils into the central nervous system (CNS) and the further impairment of the blood-brain barrier permeability (BBB) [45, 46]. The presence of TNF-α in the cerebral endothelium interrupts blood flow to the brain leading to the exaggeration of brain injury after ischemic-hypoxic attacks [47].

The hippocampus, responsible for memory and cognitive brain functions, responds specifically to inflammatory signalling through cytokine receptors. [48, 49]. Cerebral infection is linked to irreversible morphological changes in brain structures in the hippocampus. [50]. A growing group of evidence from experimental studies suggests that intrauterine infection and systemic inflammation are involved in the development of brain white matter injury and subsequent cerebral palsy [51].

1.2.2 The innate immune system

1.2.2.1 Introduction

The innate immune system provides an early first line of defence against invading microorganisms. It has the capacity to act rapidly in a nonspecific way every time the body is exposed to a pathogen. Furthermore, it lacks the ability to build and generate life-long immunological memory to help prevent reinfection, although recent studies are challenging this notion [52].

The innate immune system is activated by the binding of pathogen associated molecular patterns (PAMP) to a variety of pattern recognition receptors (PRR) found in

cell membranes, endosomes and the cytoplasm. In addition to PAMPs, PRRs can also recognise molecules produced by damaged or injured cells – these are termed damage-associated molecular pattern (DAMPs). Innate immunity is the first line of defence against invading microorganisms and cellular stress or damage [53, 54] .

The body has several barriers - physical, chemical and microbiological - in order to prevent pathogen entry. Physical barriers include the skin, epithelial layers of the digestive tract, respiratory system and nasopharynx, cilia, eyelashes, and other body hair. Secretions include mucus, bile, gastric acid, saliva, tears, and sweat. The complement cascade is composed of several soluble and membrane-bound proteins released mostly by the liver but also by leukocytes, adipocytes, and cells in the central nervous system (such as neurons, astrocytes, and microglia [55, 56]. Complement is activated by the classical, lectin, and alternative pathways, all of which are triggered by various stimuli. Complement activation contributes to pathogen removal in 3 ways. Pathogen-bound complement components, such as C3b and C4b, can opsonise pathogens by binding to cells that express complement receptors (such as complement receptor type 1, CR1) to facilitate their removal by phagocytosis. Anaphylatoxin proteins, such as C3a and C5a are proinflammatory peptides that communicate with and activate immune cells by binding to their receptors (C3a receptor, C3aR, and C5a receptor, C5aR). The terminal membrane attack complex (MAC) damages lipid bilayers in bacterial cell membranes leading to their lysis [56, 57].

1.2.2.2 Cells of innate immune system

The cells of the innate immune system include neutrophils, monocytes, macrophages, dendritic cells, eosinophils, mast cells as well as epithelial cells, endothelial cells and tissue parenchymal cells. In addition, natural killer (NK) cells and innate, unconventional T cells demonstrate primary effector functions upon pathogen exposure. Phagocytes such

as macrophages and neutrophils eliminate microbes and secrete inflammatory mediators. Neutrophils, monocytes, macrophages and dendritic cells develop and mature during foetal life, but at different times, and the activities of all components of innate immunity are weak in newborns compared to those in later life. There is a steep rise in the number of mature neutrophils from around 12 weeks of gestation until shortly before birth [58].

Neutrophils

The most frequent type of white blood cell is neutrophils. The neutrophil has a central function of clearance of extracellular pathogens, by phagocytosing pathogens and/or releasing antimicrobial factors present in cytoplasmic granules. By degranulation and the extrusion of nucleic acids, neutrophils release neutrophil extracellular traps (NETs), which capture and destroy extracellular pathogens [59]. Neutrophils can be present in the vascular endothelium surrounding the peri-injury area in the brain as soon as 2 hours after the injury and subsequently invade the brain parenchyma. Tissue-resident microglia interact with infiltrating neutrophils, which polarize microglia into classical (M1) versus alternatively activated (M2) pathway subtypes [60].

Dendritic cells

Dendritic cells (DCs) are unique subsets of immune cells that bridge innate and adaptive immunity through antigen presentation. DCs are responsible for capturing, processing, and presenting antigens to naïve T cells, as well as mediating their differentiation into effector cells [61]. Based on differences in expression of pattern recognition receptors (PRR), such as toll-like receptors, C-type lectins, and intracellular nucleic acid sensors, and function, there are two distinct types of human blood DCs: plasmacytoid (pDC) and myeloid (mDC) DCs, which originate from separate progenitors [62]. In response to bacterial and viral infections, mDCs promote and regulate pro-inflammatory responses by activating T-helper 1 and cytotoxic T lymphocyte responses

[63]. In response to viral infection pDCs produce type I interferons (IFNs) which stimulate the expression of proteins that block viral replication [64]. It is thought that the rise in the frequency of severe influenza infections in term babies may be explained in part by reduced pDCs numbers [65].

Macrophages

Macrophages are differentiated cells of the mononuclear phagocytic lineage that have a role in the detection, phagocytosis and destruction of harmful microorganisms. They also can also present antigens to T lymphocytes. Macrophages can be stimulated by PAMPs, such as bacterial lipopolysaccharide or cytokines, such as interferon-γ (IFN-γ) and TNF-α, and regulated by interleukin IL-4, IL-5, and IL-10. Macrophages can be divided into M1 and M2 macrophages [66, 67]. TNF-α, IL-1, IL-6, IL-12, IL-18, and IL-23 are cytokines secreted by M1 macrophages giving them proinflammatory and anticancer functions [68, 69]. M2 macrophages have anti-inflammatory properties that can be triggered by cytokines such as IL-4, IL-13, glucocorticoids, macrophage colony stimulating factor 1 (M-CSF/CSF1), IL-10, IL-33, IL-21, and TGF-β, [70, 71]. Meanwhile, tissue damage caused by intense M1 macrophage-mediated responses is the primary factor of atherosclerosis and other chronic inflammation [69, 72, 73].

Microglia

Microglia (MG) constitute about 10-15% of all cells in the brain and are known to reside permanently in the CNS [74] They are cells of the myeloid cell linage and arise from yolk sack (YS) primitive macrophages and possibly also progenitor cells derived from hematopoietic cells [75]. MG have two main functions: (1) innate immune defence and (2) development and homeostasis. The former role includes responses to invading pathogens and removal of debris from wounds, while the latter concerns maintaining

cortical lamination, oligodendrocyte maturation, and phagocytosis-mediated learning and memory acquisition [76, 77]. MG populations vary in different regions of the brain with regard to gene expression, frequency [78] and shape [79]. The cortex and hippocampus have two-fold higher frequencies of MGs than the thalamus and midbrain [79, 80].

The weak immune system in neonates

There is a steep rise in the number of mature neutrophils from around 12 weeks of gestation until shortly before birth [58]. Then their frequencies return to a stable level after a few days of the postnatal period. However, they have weak bactericidal functions. These weak functions are evident as inadequate responses to inflammatory stimuli, reduced adhesion to endothelial cells and diminished chemotaxis [81]. The inadequate innate immune response in preterm infants also reflects an immaturity of monocytes and macrophages. Macrophages in preterm infants have lower TLR4 expression lower than term babies. Preterm infants also have lower serum IgG and complement compared to term infants. Impaired innate immunity in neonates, contributes significantly to the high risk of life-threatening infections in premature and newborn infants [58, 82].

1.2.3 Innate immunity in NE

1.2.3.1 The role of neutrophils and monocytes in NE

Several studies have highlighted roles for neutrophils and monocytes in the pathogenesis of NE. O'Hare et al reported a link between poor outcomes in neonatal NE and circulating immune cell activation [83]. There is higher expression of CD11b and toll-like receptor (TLR)-4 on neutrophils and monocytes of infants who need supportive resuscitation in comparison with neonatal controls [83]. Antenatal infections, such as urinary tract infections, can lead to higher concentrations of IL-1, IL-6 and TNF-α released by astrocytes in brain and is related to preterm delivery [84]. Neonates with

NE have elevated serum cytokine levels, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, IL-1β, IL-6, IL-10, TNF-α and VEGF, which are associated with high mortality and morbidity and poor neurodevelopmental consequences such as cerebral palsy [83, 85]. Dysregulation of GM-CSF, IL-18, IL-2, IL-6, IL-8 and TNF-β production by neutrophils in response to LPS persists into childhood following NE [86].

Neutrophils are the first cells to migrate to the infarction area following brain ischemia, where they contribute to brain injury [87]. Antibody depletion of neutrophils leads to a reduction of brain injury following hypoxia ischemia (HI) in mice [88-90]. Morkos et al. reported a link between poor neurological consequences after NE and the higher neutrophil counts on the first day of life [91]. Hudome et al. found a reduction in HI brain swelling when the neutrophils were depleted. They recognised that neutrophils were recruited to the brain as early as 8 hours in brain injury [92, 93], suggesting that neutrophils have a crucial role in developing brain swelling after ischemic injury. Several findings have shown that neutrophils play a negative role in the human adult ischemic brain [94, 95]. The neutrophil-lymphocyte ratio in peripheral blood, for example, has been found to predict stroke outcomes, with high neutrophil-lymphocyte ratio values attributed to poor neurological recovery [96].

Brain injury can be due to neutrophil extracellular traps (NETs), which, once released by neutrophils, inhibit thrombolysis following ischemia [97]. Reactive oxygen species (ROS) production and interactions with endothelial and glial cells are among suggested effector mechanisms of brain injury. New studies on neutrophil heterogeneity in stroke, however, have challenged this long-held belief. The concept of N1/N2 neutrophils was first presented in adult stroke pathophysiology [98-101].

Previous reports showing that neonatal neutrophil extravasation into the brain parenchyma following HI is rare [93, 102] led to the possibility that neutrophils are clinically irrelevant. However, a more recent study by Smith et al., found that many neutrophils accumulate outside the vasculature [103] which show that the majority of neutrophils are present in the parenchyma 24 hours after HI. An early peripheral neutrophilia preceded the infiltration of these cells to the brain. These findings are also consistent with clinical observations of an increased number of peripheral neutrophils, which correlated with poor neurodevelopmental outcomes in hypoxic-ischemic encephalopathy in term infants [104]. This early increase in the blood followed by a rapid decline after 24 hours, when neutrophil numbers peaked in the brain, implies that blood neutrophils migrated to the brain. The cause of the increased number of circulating neutrophils is unknown, but may result from increased release from or production in the bone marrow, as seen in adult stroke models [105]. This is in line with a recent study by Mülling et al., 2021, who found that neonatal neutrophils are rapidly activated in the hypoxic-ischemic brain, which is linked to a significant increase in ROS production and a high frequency of over-activated neutrophils. Experimental depletion of neonatal neutrophils 12 hours after HI suppressed acute neurodegeneration, as well as micro-and astrogliosis [106].

1.3 The role of lymphocytes in NE

While monocytes and neutrophils clearly contribute to brain injury in NE, less is known about the roles of lymphocytes, which have a central role in tissue homeostasis and innate and adaptive immune responses in all tissues in the body. However, their influence on CNS functioning has been largely ignored, owing to T cells' sporadic presence in the brain parenchyma in healthy people and the concept that the CNS is an

"immune privileged" site. On the other hand, the CNS is no longer regarded as an organ with only sporadic interactions with the peripheral immune system.

1.3.1 Lymphocyte ontogeny

The foetal immune system is ontogenetically regulated to allow the development of the foetus and avoid foetal tissue rejection by the mother's immune system. It also needs to remain tolerant of maternal alloantigen. After birth, the sudden change in antigen exposure, many of them related to the gut microbiota, leads to the induction of the host immune system [58]. There are two arms of the body's defence; the innate and adaptive immune systems, which interact to defend against harmful pathogens.

Human foetal T cell maturation occurs between 8 and 12 weeks estimated gestational age (EGA). Indeed, double-positive $CD4^+CD8^+$ thymocytes expressing αβ or γδ T cell receptors (TCR) and single-positive $CD4^+$ and $CD8^+$ thymocytes can be detected at 12.5 weeks EGA [107]. Foetal T cells then begin to migrate to the peripheral tissues, with T cell zones appearing in the spleen from 18 weeks EGA [108]. NK cells develop by nine weeks EGA. Innate lymphoid cells (ILCs), γδ T cells and mucosa-associated invariant T cells (MAIT cells) develop at 18 weeks post gestation and B cells detected by 8 weeks gestation in fetal liver [109, 110].

The adaptive immune system relies on B and T lymphocytes, which express clonotypic antigen-specific receptors generated by genetic rearrangements and somatic recombination of germline gene segments, generating a massive diversity. These receptors enable B cells and T cells mount appropriate antigen-specific responses, which are enhanced upon subsequent detection of the same pathogen and they persist enabling immunological memory. This process is exclusive to T cells and B cells of the adaptive immune system and allows for the generation of a diverse repertoire of antigen-specific

receptors. B cells can further undergo somatic mutation to increase affinity for antigen binding. It estimated that as many as 10^{12} antigen specificities of B cells and T cells might be generated in this manner (Tonegawa, 1983).

1.3.2 Conventional T cells

Coordination of adaptive immunity is the function of conventional T lymphocytes. Conventional T cells express TCRs consisting of an α and a β polypeptide associated with a number of membrane-bound polypeptides, which are collectively termed CD3 and mediate signal transduction upon antigen binding to the TCR. The αβ TCR recognises peptide fragments of protein antigens bound to major histocompatibility complex (MHC) class I or class II molecules [111]. These molecules activate cluster of differentiation 8 ($CD8^+$) T lymphocytes and $CD4^+$ T lymphocytes respectively [112, 113]. The MHC class I and class II molecules are highly polymorphic and have the ability to bind and interact with a wide range of peptides derived from pathogens [114-116]. TCR diversity is achieved through variable (V), diversity (D), and joining (J) recombination in the gene segments encoding for the TCRα and TCRβ proteins which occurs in the thymus during T cell development in the thymus. Further diversity is achieved through random addition and removal of nucleotides from the junctions between these gene segments and random pairing of TCR α-chain and TCR β-chains. These processes are responsible for producing a large repertoire of antigen receptors encoding a wide array of specificities [115, 117]. After thymic selection, whereby potentially autoreactive and potentially useless T cells are removed, there are approximately $2x10^7$ TCRs present in a human [115]. This diversity and complexity is fundamental to the normal functioning of the immune system [117]. However, with this level of diversity, each antigen specificity is present on a very small number of cells, therefore, a period of several days is required to generate sufficient numbers of antigen-specific effector cells to mediate immunity against the antigen-

expressing pathogen. This means the adaptive immune system does not provide rapid and immediate protection to the host. Rather, once bound to the antigen, clonal expansion of T cells will occur to create a large population of effector T cells, which can take between 7-10 days [118]. Following activation, $CD8^+$ T cells have key roles in eradication of pathogens by killing pathogen-infected and tumour cells. $CD4^+$ T cells function as helper cells, producing cognate and cytokine signals to enhance the responses of $CD8^+$ T cells, B cells, phagocytes and other cytotoxic cells, such as eosinophils and mast cells. This is known as adaptive immunity and this time-lapse may leave the host vulnerable to infection in the early stages [112, 114-116]. Following activation, a proportion of the effector T cells persist as memory T cells and upon subsequent encounter with the same pathogen, can be rapidly re-activated allowing a much faster secondary adaptive immune response [119].

CD4 T helper cells orchestrate immune responses against a broad range of pathogens by activating other effector cells of the immune system. They also regulate/suppress immune responses to control both the amplitude and duration of immune responses to limit autoimmunity. CD4 T cells carry out their roles primarily in peripheral tissues and secondary lymphoid organs [120].

CD4 T helper (Th) cells are divided into five primary subclasses based on the expression of hallmark cytokines and lineage-specific master transcription factors in the cells. Th1 (IFN-γ and T-bet), Th2 (IL-4/IL-5/IL-13 and GATA3), Th17 (IL-17/IL-22 and RORγt), Tfh (IL-21 and Bcl6) and Treg (T regulatory) (IL-10/ TGF-β/IL-35 and Foxp3) [121]. Some groups have also described some other subsets, such as Th3 (TGF-β) [122], Tr1 (IL-10) [123], Th9 (IL-9) [124, 125] and Th22 (IL-22) [126].

1.3.2.1 Th1 cells

TCR activation in a cytokine milieu containing IL-12 and IFN-γ induces naive T cells differentiation into Th1 cells. NK cells or other T cells may be the initial providers of IFN-γ, while DCs and macrophages produce IL-12 during T cell priming [127, 128]. IFN-γ stimulates signalling through the STAT1 pathway, resulting in T-bet production [129-131], a master regulator that enhances IFN-γ -expression, and downregulates IL-4, a cytokine, which promotes Th2 differentiation. The STAT4 pathway is activated when IL-12 binds to the IL-12 receptor heterodimer, boosting Th1 responses and increasing IFN-γ production by responding Th1 cells [132, 133].

CXCR3 is an essential chemokine receptor expressed by Th1 cells and is commonly utilised to identify human Th1 cells as they migrate toward inflammatory areas following pathogen invasion [134].

Th1 cytokines include IFN-γ, TNF-α, lymphotoxin, and IL-2, all of which are involved in the host immune response to intracellular infections, including all viruses, intracellular bacteria such as *Salmonella typhimurium* and *Mycobacterium tuberculosis* [135] and intracellular protozoa, such as *Leishmania major*, *T. gondii* and many others [136, 137].

A feature of Th1 cells is their expression of the transcription factor T-bet [138]. Together with another transcription factor, H2.0 like homeobox (Hlx), T-bet promotes the synthesis of IFN-γ and expression of IL-12 receptor-β2 chain. T-bet additionally reduces the expression of other master transcription factors, including GATA3 and RORγt [121, 139] leading to to suppression of Th2 and Th17 cell development [140].

Th1 cells promote the activation of CD8 cytotoxic T cells and NK cells, making them crucial for the antiviral immunity [136].

A variety of human disorders have been linked to Th1 cell pathogenesis. Hashimoto's thyroiditis, multiple sclerosis, type-1 diabetes mellitus, rheumatoid arthritis, sarcoidosis,

atherosclerosis, *Helicobacter pylori*-induced and autoimmune chronic gastritis, transplant rejection, Crohn's disease, and contact dermatitis are all associated with increased numbers and activities of Th1 cells [141].

Through cell interaction and localized IFN-γ secretion, Th1 cells activate infected macrophages. This triggers a cascade of metabolic reactions that transform the macrophage into a powerful antibacterial effector cell. IFN-γ activates resting macrophages to produce NO, ROS, and lysosomal enzymes, which kill ingested microorganisms. This is the classical pathway of activation by Th1 cell. This synergism is also caused by the attachment of macrophage molecules CD80/CD86 and CD40 to T cell molecules CD28 and CD40L which resulted in different effector functions of macrophages [142].

1.3.2.2 Th2 cells

Another distinct subgroup of $CD4^+$ effector T cells is Th2 cells. Th2 cells secrete IL-4, IL-5, and IL-13 as well as amphiregulin and IL-17E/IL-25. These cells have a crucial role in humoral immunity and contribute significantly to the orchestration of the immune response to multicellular parasites and worms [143]. ST2/IL-1 R4, CXCR4, CCR3, CCR4, and CCR8 are some of the cell surface receptors displayed by Th2 cells and the production of cytokines, which enable these cells to be distinguished from other types of T cells [144].

Upon receiving signals from DCs, naive CD4 T cells undergo expansion into Th2 cells [143], under the effect of IL-4, which triggers the release of GATA-3 and growth factor independent-1 (GFI-1) in a STAT6-dependent manner [145]. IL-4 and IL-2, IL-7, or thymic stromal lymphopoietin (TSLP) play an essential role in Th2 expansion [146]. GATA-3 is a crucial gene that promotes Th2 cell differentiation and proliferation. Differentiation of other T cell subpopulations is inhibited by GATA-3, [147]. IL-13,

induces antibody class switching and IgE production by B cells as well as alternative macrophage activation throughout the body. Th2 cell-produced cytokines, also boost eosinophil activation and survival, whereas IL-5, and IL-9 play a vital role in activating mast cells [146].

Th2 cell responses are sometimes imbalanced, resulting in pathologies including [143, 148] persistent allergic inflammation, asthma, atopic dermatitis, rhinitis, food allergies [149], and eczema [150]. There are a variety of nonmicrobial stimuli that trigger Th2 cells, such as food allergens [151], venoms [152], house dust mite [153], pollen [154] and vaccination adjuvants can also cause pathological Th2 responses (Alum, MF59) [155].

1.3.2.3 Th17 cells

The Th17 cell lineage is a more recently-described subpopulation of $CD4^+$ effector lymphocytes. They are distinct from Th1 and Th2 lineages and characterised by the production of pro-inflammatory cytokines such as IL-17 (A and F), IL-21, and IL-22 [156, 157], expression of master transcription regulator RORγt [158, 159] and cell-surface expression of CD161, CCR4 and CCR6 [160, 161]. Th17 cells are widely regarded as essential for mounting immune responses against extracellular fungi and bacteria. Th17 cells are required for protective immunity against *Bacillus anthracis* [162] *Staphylococcus aureus* [163] and *Candida albicans* [164].

Differentiation and expansion of naive $CD4^+$ T cells into Th17 cells involves initial priming by TGF-β and IL-6, expansion by IL-21, and stabilisation by IL-23 [165]. Mucosal and epithelial tissues are enriched with Th17 cells. Increased expression of chemokine receptors such as CCR4 and CCR6 assists in the recruitment of Th17 cells to mucosal inflammatory areas such as the intestines, lungs, and skin.

In order to eliminate extracellular infections, Th17 cytokines recruit and activate neutrophils which internalise and destroy bacteria and fungi by phagocytosis. Th17 cells also stimulate immune and non-immune cells to release matrix metallopeptidases, nitric oxide, mediators, and proteins that contribute to immunity against these pathogens [165-168].

Th17 cells are also implicated in the pathogenesis of several chronic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis [169, 170], Graves' disease [171], ankylosing spondylitis [172], ANCA vasculitis [173], and Crohn's disease [174]. In addition to these disorders, several neuropsychiatric and neurodegenerative disorders, including schizophrenia [175] and multiple sclerosis [176] have been associated with Th17 pathogenesis. In numerous inflammatory and autoimmune conditions, targeting Th17 cells or their effector mediators promises to treat these conditions. Targeting IL-12p40 is inhibiting Th1 responses. Antibodies specific for IL-17A/F, anti-IL-17RA, and RORγt inhibitors are being employed to treat various inflammatory conditions [177].

1.3.2.4 T-Regulatory Cells

Regulatory T cells (Tregs) are unique subsets of effector CD4 characterized by their ability to control the immune system, and they are crucial in the maintenance of immunological homeostasis [178-180]. The thymic-derived Treg (tTreg) cells are generated from the thymus during negative selection [181] and inducible Treg cells originate from the peripheral circulation [182, 183]. tTreg cells maintain immunological tolerance by regulating self-reactive effector T cells that have eluded thymic selection. Differentiation and maintenance of Treg cells is controlled by the master transcription factor Foxp3 [184].

There are several non-specific surface markers for Treg cells such as CD25 (IL-2 α-chain receptor) and CD127 (IL-7 α-chain receptor). However, *FOXP3* is the "universal standard" for detecting a Treg cell [185]. According to studies in murine and human, lethal autoimmunity is mediated by genetic mutations in FOXP3 [186, 187].

In autoimmune diseases and graft-versus-host disease or graft rejection. Tregs hold a therapeutic promise because of their immune response regulation. Efficacy has been proven in several proof-of-principle pre-clinical investigations, which have been published [188-190]. The safety and tolerability of these drugs have been proven in various phase I/II studies [191] and a large number of clinical trials are now underway.

1.3.2.5 Follicular helper T (Tfh) cells

Tfh are a unique subtype of $CD4^+$ T cells found in the germinal centres of secondary lymphoid organs (SLOs), which include the tonsil, spleen, and lymph nodes, among other places. Germinal centres (GCs) are the structural and functional lymphoid units within B cell follicles in SLOs and are composed of T and B cell zones. Tfh cells are unique in that they are concentrated in the B cell zone and interact with B cells. In addition to SLOs, Tfh can also be detected in the bloodstream and circulation [192].

Tfh cells play a critical function in the development of germinal centres in the body. They promote the establishment of long-lasting serological memory where B cells go through affinity maturation and class-switch recombination to generate high-affinity antibodies [9, 193-195]. This is mediated in part by interaction of the co-stimulatory molecule CD40 with the CD40-ligand (CD40-L) on the B cell and by the production of IL-21 which leads to B cells proliferation.

The formation of Tfh cells occurs in the T cell zone of SLOs [9, 193, 194]. The interaction of naive $CD4^+$ T cells with antigen-presenting dendritic cells (DCs) within T

cell follicles is the very first step of the process leading to Tfh cell development [196]. This stage of Th cell formation, known as pre-Tfh, is characterised by upregulation of CXC chemokine receptor 5 (CXCR5) expression as well as B-cell lymphoma 6 protein (Bcl-6), Achaete-scute homolog 2 (Ascl2), the inducible T cell co-stimulator (ICOS), programmed cell death-1 (PD-1), and Batf, and downregulation of CCR7. These modifications direct the pre-Tfh cells to the T/B cell border, where signals received from B cells cause a further rise in the Tfh-associated gene expression (Bcl6, PD-1, ICOS, and CXCR5), commitment to the functional GC Tfh cell programme, and subsequent GC development. Tfh formation is dependent on TGF-β, IL-12, IL-23, and activin A signalling in humans, whereas in mice, IL-6, IL-21, and Bcl6 signalling are required for the formation of Tfh cells [197-201].

1.3.3 B cells

B cells play a critical function in humoral immunity by producing antibodies. They can directly identify antigens via their cell-surface antigen receptors. They express MHC class II molecules enabling them to activate $CD4^+$ Tfh cells which reciprocally promote B cell maturation into plasma cells, antibody isotype class switching and antibody release . B cells are generated from lymphoid progenitor cells in the bone marrow, where they undergo differentiation into mature lymphocytes. They subsequently move to the spleen and secondary lymphoid organs, where they develop and differentiate into distinct cell types [202]. Naive B cells, germinal centre (GC) B cells, memory B (BM) cells, and antibody-secreting cells (ASC) make up the human mature B compartment [203] All human B cell subsets seen in lymphoid organs, except GC cells, can also be found in the peripheral circulation.

Activation of naive B cells is dependent on antigen identification by the Ig receptor and the presence of additional signals, which can originate from a $CD4^+$ T cell (thymus-

dependent) or, in certain situations, directly from microbial components (thymus-independent). In order for B cells to respond, both B cells and T cells must recognize the same antigen. B cells detect and internalise protein antigens that enter the lymph nodes via the lymphatic system. They do this by presenting Tfh cells with antigenic peptides bound to MHC class II proteins. As mentioned above, Tfh cells are distinguished by the expression of the Bcl-6 transcription factor, CD40 ligand, ICOS, the chemokine receptors CXCR4 and CXCR5, the inhibitory receptor PD-1 and the signalling lymphocytic activation molecule-associated protein (SAP) [204, 205]. The Tfh cells then provide signals that reciprocally induce B cell differentiation into memory cells and antibody secreting plasma cells.

Antibodies provide protection against invading pathogens and are the foundation of successful immune responses [206]. One of the hallmarks of humoral immunity is the progressive increase in the affinity of antibodies for antigen over time, a process that occurs in germinal centres (GCs) within B cell follicles of secondary lymphoid organs [207, 208]. As a result of pathogenic infection, B cells mature in the GCs into memory B cells and long-lived plasma cells (LLPCs). LLPCs produce long-lived, high affinity antibodies. Memory B cells rapidly mature into plasmablasts, which produce protective antibodies, but do not undergo affinity maturation and are short-lived [9]. Within the GC, B cells undergo proliferation and somatic hypermutation of Ig genes, followed by a process in which the “fittest” B cells, those best able to capture antigen via surface Ig and present it on MHC class II molecules, are selected by Tfh cells The strength of interaction between Tfh cells and GC B cells, which is proportional to the amount of antigen presented by GC B cells, determines the cell cycle speed and number of divisions of GC B cell clones [209-211].

In both humans and mice, B cells are divided into three main categories. B-1 cells, B-2 cells, and regulatory B cells. Their classification is based on ontogeny and anatomical localisation. B1 B cells stem from B1 progenitors present in the foetal liver and continue as a self-renewing population even after the neonatal period. B-1 B cells are mainly localized in the intraperitoneal cavity, and some are present in the spleen. B-1 B cells, which include B-1a and B-1b cells, have a role in the innate immunity by secreting natural antibody, which can bind to self-antigens with low specificity, without the need for T cells help [212-214].

Natural antibodies (NAb) are low-affinity immunoglobulins that identify both external and self-antigens [215, 216]. Most NAbs are IgM and IgG antibodies, but IgA NAbs have also been described. [217]. NAb play a role in inducing apoptosis [218], activating complement [219, 220], opsonizing antigen [221], enhancing antigenicity [222], directing antigen to lymph nodes [223], and participating in FcR-mediated phagocytosis [220]. They also support dendritic cell growth and maturation by acting as general neutralizing mediators [218] and endogenous adjuvants for $CD8^+$ T-cell responses [224].

B-2 cells systemically circulate through the bloodstream, and some reside in follicles of the spleen. Follicular (FO) B cells and marginal zone (MZ) B cells of lymph node both fall under the B2 classification [214]. There is a major contribution of B-2 cells to adaptive immunity by secreting antigen-specific antibodies. Upon stimulation by T cells, they differentiate into plasma cells; in order to produce high specific antibodies. A subset of plasma cells differentiates into memory B cells to react with rapid immune responses. A large body of evidence, has documented that B-2 cells are implicated in the pathophysiology of autoimmune conditions by secreting autoantibodies [225].

Regulatory B cells have immunosuppressive activity by secreting IL-10, IL-35, and transforming growth factor (TGF)-β. While regulatory B cells numbers are low, they have

powerful immunosuppressive activity. Regulatory B cells are present in the spleen, lymph nodes, and blood [226]. Recent studies demonstrate that regulatory B cells are implicated in the pathophysiology of autoimmune diseases.

1.3.4 B cells and T cells in neuroinflammation

Lymphocytes have an essential role in the support of CNS homeostasis. A growing body of evidence has implicated lymphocytes in various neuro-physiological functions. T cells and B cells are found in the meningeal space and choroid plexus of mice and humans, [227, 228]. Recombination-activating gene (RAG)-deficient mice, which lack T and B lymphocytes, have poor social recognition and memory [229]. IFN-γ-producing T cells control neuronal communication as well as behavioural patterns [230]. Using IL-4-deficient mice and bone marrow transplantation, researchers have shown that cognitive tasks expand the number of T cells in the meningeal space and that IL-4-producing T cells control learning and memory [231]. Adult neurogenesis is impaired in nude mice, which lack T cells but have normal B cell numbers [232].

According to these studies, T cells in the meningeal space and choroid plexus are thought to be responsible for social and cognitive activities. However, it is unknown if the brain dysfunctions caused by T-cell deficiency are developmental in nature. T cells are implicated in many CNS pathologies due to their roles in controlling innate and adaptive immune responses [233]. The peripheral lymphocytic response plays a significant role in facilitating post-ischemic injury [234] and can aggravate brain damage [235, 236]. Neonates and infants with NE caused by acute ischaemic insults are 25 times more likely to have higher lymphocyte counts in blood in the first 12 hours of life, than aged-matched controls, regardless of the type of intrapartum asphyxia, [237, 238]. The fact that adult lymphocyte-deficient mice are preserved from ischaemic damage adds to

the evidence that T cells play a significant role in ischaemic brain injury [239]. Stroke studies in chimeric mice have shown that brain injury is attributable to T cells rather than B cells. $CD4^+$ IL-17^+ Th17 cells enter the damaged brain in inflammation-sensitized hypoxic-ischemic newborn brain damage and enhance neuroinflammation through microglia activation [240]. Interestingly, the blocker of the sphingosine-1-phosphate (S1P) receptor, fingolimod (FTY720), which sequesters lymphocytes in lymph nodes, preventing them from contributing to autoimmune reactions, suppressed Th17 cell infiltration and motor impairments in the brain. γδ T lymphocytes, according to another study, have a role in brain injury in preterm infants caused by hypoxia ischemia [241]. Hypoxic-ischemic brain injury does not cause white matter injury or neuronal death in TCR-deficient mice. In the white matter layer, γδ T cells were present in human preterm newborns with periventricular leuomalacia and neonatal mice with HIE [241].

Several lines of evidence indicate how crucial lymphocytes are in the progression of secondary brain damage [242, 243]. There was a significant neuroprotective effect of spleen removal in newborn rats before HI [244], suggesting that lymphocytes from the spleen can promote the progression of brain damage. Furthermore, previous research by the same group using splenic irradiation to kill splenic cells without splenectomy reported substantial advantages in minimizing the brain's immune cell infiltration after cerebral ischemia [245].

The roles of the various sub-groups of lymphocytes has not yet been clarified. Recent work by Nazmi and his co-workers using $RAG1^{-/-}$ mice, which are deficient in B cells and T cells, revealed a significant decrease in HI brain injury compared to control wild-type mice [246]. However, the relative contributions of T or B cells to the development of brain lesions was uncertain. Albertsson and co-workers, by targeting gamma delta (γδ) T lymphocytes demonstrated that the depletion of these innate T lymphocytes provided

neuroprotection in mice on the fourth day after birth [241]. Yilmaz and co-workers, confirmed the above findings and reported that RAG1$^{-/-}$ mice, had significantly less severe cerebral infarction and neurologic sequalae compared to WT mice when they were subjected to middle cerebral artery occlusion. After cerebral ischemia, Yilmaz et al. discovered that RAG1-deficient animals had a significantly smaller infarct size and a better neurological prognosis than wild type (WT) mice [236, 247].

Several studies support the hypothesis, that T lymphocytes play a pivotal role in maintaining the chronic immuno-inflammatory activation after perinatal HI. Some studies suggest, that an adequate adaptive immune response is initiated after 7 days in mouse models, while others show a very early T cell response, however, all studies were able to show the presence and the activation of T lymphocytes in damaged areas for at least a month after the initial injury [248, 249]. Effector T cells were able to exert neurotoxicity via several mechanisms such as the production of perforin and granzyme B, the release of free radicals, the triggering of apoptotic pathways within neurons, and most importantly, the production of pro- and anti-inflammatory cytokines [250, 251].

1.3.5 NK cells

NK cells are a distinct lineage of innate lymphoid cells (ILCs), lacking CD3, but expressing CD56 and NKp46 and mediate innate immunity against viruses and tumours. Although, both NK cells and type 1 ILCs are IFN-γ and TNF producers, NK cells are distinguished from ILCs by having a cytolytic role. NK cells lack antigen-specific receptors. Their activities are controlled by signals through a variety of stimulatory, costimulatory and inhibitory receptors. Once activated NK cells kill target cells by perforin and granzyme-mediated or death receptor-induced apoptosis [118]. Activating receptors include NKG2D, CD94-NKG2C heterodimers and natural cytotoxicity

receptors, whereas inhibitory receptors include killer immunoglobulin-like receptors (KIR) and CD94-NKG2A heterodimers. NK cells also express a number of co-stimulatory receptors [252, 253].

NK cells are rigorously controlled and hypo-responsive to target cells during pregnancy to protect the foetus during antenatal development [254-256]. NK cell numbers and cytolytic activities increase throughout gestation. At birth, neonatal NK cells are present in high numbers but display lower cytotoxic activity compared to those in the initial stages of pregnancy. Following the neonatal period there is a decrease in circulating NK cell frequencies up to 5 years of age [252, 255]. However, they possess a lower level of activation than term infants which make the infant vulnerable to viral infection.

Decidual NK (dNK) cells are a distinct subpopulation of NK cells, that are tissue-resident at the maternal-foetal placental side during early gestation. They constitute about 70–90% of the immune cells in the uterus and are the most frequent maternal leukocyte population during the first 3 months of gestation in humans [257, 258]. dNK cells are thought to have multiple roles in human pregnancy [258, 259]. They have a strong relationship with trophoblast cells and release cytokines that stimulate trophoblast growth while also mediating trophoblast differentiation, invasion, and spiral artery remodelling [259, 260]. dNK cells are also involved in maintaining angiogenic balance by secreting VEGF and placental growth factor (PlGF) and maintaining maternal-foetal tolerance throughout pregnancy [261-263].

1.3.6 γδ T cells

γδ T cells are T cells characterised by the expression of a heterodimeric TCR composed of γ and δ chains and are primarily activated in an MHC-independent manner [264, 265]. The γδ TCR can recognise and respond to various compounds, including non-peptide

metabolites of isoprenoid biosynthesis, lipids, stress molecules (MICA and MICB), heat-shock proteins, and others [266].

Based on the arrangement of TCR δ genes, human γδ T cells are classified into three predominant populations based on their γδ TCRs chain usage. Vδ1 and Vδ2 are the most abundant subsets, while lower numbers of Vδ3, Vδ4, Vδ5 and Vδ6 T cells have been described [267, 268]. Both Vδ1 and Vδ2 subsets have been studied extensively in humans, whereas research is still lacking on the other subsets, despite their presence in the peripheral circulation.

γδ T cells develop in the thymus during gestation. [269-272]. The Vγ9 and Vδ2 V gene segments can be detected in foetal liver between 5^{th} and 6^{th} weeks of gestation, and in the foetal thymus after eight weeks [273]. The $V\gamma9V\delta2^{+}$ lymphocytes are among the first T-cells to develop in the human foetus and are the predominant peripheral blood γδ T-cell population in most adults [274]. At term, around 40-week gestation, there is a significant expansion of Vδ1 T cells, which represent the majority of γδ cells in cord blood. At the same time, the $V\gamma9^{+}V\delta2^{+}$ and Vδ3 T cells comprise a minor proportion of total γδ T cells in cord blood [275].

γδ T cells, account for 1-10% of mature circulating $CD3^{+}$ cells in healthy human adults, and are often abundant as resident cells within solid organs and mucosal tissues, [276, 277]. γδ T cells are rich at mucosal sites such as the skin, gut, lung, and reproductive genital tract. The γδ TCRs in the human colonic mucosa make up approximately 20% of intraepithelial lymphocytes [278]. In contrast to αβ T cells, more than 70% of γδ T cells are double-negative for CD4 and CD8, with a third of them expressing CD8, but $CD4^{+}$ γδ T cells comprise less than 1% [279].

Vδ1 T cells make up approximately 10% of total γδ T cells in adults. They sustain epithelial tissue integrity by expressing rearranged receptors that are selected in the thymus. They function in part by recognizing the stress-inducible ligands MICA and MICB expressed by virus-infected and transformed cells, as well as self-glycolipids presented by CD1c/d molecules [280].

In humans, most Vδ2 T cells co-express the Vγ9 chain, forming Vγ9Vδ2 heterodimers [281]. This TCR chain combination can either be configured before birth or by antigen presentation [282] and accounts for 50-95% of γδ T cells in peripheral blood [283, 284]. The Vγ9Vδ2 TCR recognises non-peptide antigens associated with the MHC-like antigen presenting molecule butrophilin 3A1. These antigens include (*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the non-mevalonate pathway of isoprenoid biosynthesis, generated by some bacteria and protists [285], but not expressed in human cells. Vδ2 T cells expand to up to 50% of all peripheral T cells, in some infectious states [286]. Upon activation, they rapidly kill target cells and secrete cytokines, including IFN-γ, TNF-α, GM-CSF, IL-4, IL-10, and IL-17, thereby providing early signals that activate and polarise innate and adaptive immune responses of other cells. Furthermore, they have a crucial role in bridging innate and adaptive immunity by accelerating differentiation of several subgroups of antigen-presenting cells (APCs) [287].

Vδ3 T cells, the third most abundant T cell type, make up about ~0.6 per cent of peripheral blood γδ T lymphocytes and can also be identified in the liver and gut [288, 289]. Activation of Vδ3 T cells has been shown to occur through cognate engagements with MHC-class I as well as CD1d [264], an MHC-like protein effective at presenting lipid moieties, despite the fact that no specific antigens have been identified as ligands for the Vδ3 TCR. In renal and stem cell transplant recipients with CMV reactivation

[290], as well as those with B cell chronic lymphocytic leukaemia [291], the frequency of Vδ3 T cells is frequently increased in the peripheral circulation. Vδ3 T cells, like Vδ2 T cells, may act as a link between the innate and adaptive branches of immunity by modulating B cell and dendritic cell (DC) function [292, 293].

1.3.7 γδ T cells in NE

γδ T cells are the most common innate T cell which has been examined in NE. Albertsson and his co-workers found higher numbers of γδ T cells in the brains of animal models of ischemia and in the post-mortem preterm infants brain with periventricular leukomalacia [294]. γδ T cells are found in mouse brains as early as six hours and up to 1 week after HI [295]. Despite higher mRNA expression levels of IL-17 and IL-22 in a mouse model of preterm brain damage, the production of these cytokines by γδ T cells did not appear to contribute to brain injury. Furthermore, there were no neuroprotective effects of reducing IL-17 or IL-22 levels in these mice models, suggesting that these cells do not play a role in the brain damage that leads to premature brain injury [294].

IL-17 is implicated in the inflammatory process following ischemic brain injury. Shichita et al. reported that IL-17-producing γδ T cells play a critical role in the delayed phase of brain damage after ischemia. They are the primary producers of IL-17, rather than Th17 cells [296]. Higher serum concentrations of IL-17 are found in stroke patients than in healthy subjects. IL-23 is an essential regulator of IL-17 production by γδ T cells. Following stroke, macrophages or monocytes secrete IL-23 during the delayed phase of encephalopathic ischemia. IL-23-deficient mice showed a reduction in the infarction volume at 24 hours following HI, whereas, IL-17 depletion led to a decrease in infarction size by the 4th day [297]. Targeting γδ T cells might be a novel therapeutic potential in neonatal brain injury, because they have shown promising results in the immunotherapy of other inflammatory diseases and cancer.

1.3.8 MAIT cells

Mucosal-associated invariant T (MAIT) cells are a unique subset of innate-like unconventional T cell. They express the semi-invariant TCR α-chain Vα7.2, which recognises the MHC-related molecule 1 (MR1), presenting transitory intermediates of the riboflavin synthesis pathway [298, 299]. MR1 is a non-polymorphic MHC class 1-like protein which regulates the development and function of MAIT cells. In humans, MAIT cells comprise 1-10% of peripheral T cells [112, 114-116, 300]. MAIT cells primarily reside at mucosal tissues, and they make up to 40% of T cells in liver, and up to 10% of T cells in the lamina propria of the intestine and the respiratory and digestive systems [114, 299, 301-303].

MAIT cells respond faster than conventional T cells. They can be activated either via TCR recognition of MR-1 presenting antigen or in the absence of antigen recognition through a host of innate inflammatory and antiviral cytokines such as interleukin-12 (IL-12), IL-18 and IL-15 and interferon (IFN) α/β produced in response to infections [304, 305]. Thus, MAIT cells play crucial roles in the initiation of immune responses. Interestingly, MR1 is the most conserved antigen-presentation molecule, showing up to 80% amino acid sequence homology of the antigen-binding, α1 and α2, domains in mammals, implying MAIT cells play an essential physiological role in the immune response [114, 306, 307]. Their TCRα chain is invariant and composed of Vα7.2-Jα33 and Vα19-Jα33 in humans and mice respectively, which combine with the TCRβ chain [298, 305].

5-amino-6-D-ribitylaminouracil (5-A-RU) is a metabolite in the riboflavin synthesis pathway which binds non-enzymatically with endogenous compounds such as methylglyoxal to produce a profoundly unstable adduct known as 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) or 5-(2-oxoethylideneamino)-

6-d-ribitylaminouracil (5-OE-RU) [304, 308]. 5-OP-RU and 5-OE-RU can be captured by a lysine residue on MR1 resulting in formation of a second Schiff base, covalently attaching the molecule to the MR1 protein. These complexes act as antigens which potently activate MAIT cells through the TCR [304]. The riboflavin synthesis pathway is mainly present in bacteria and fungi and is absent in humans, making it an essential indicator of pathogen invasion to the host immune system [116, 299, 304, 308]. Following MR1-mediated activation, MAIT cells rapidly produce an array of cytokines conforming with a T helper 1 (Th1) type, Th17-type or mixed profile including IFN-γ, TNFs, IL-17 and IL-22 which sequentially promote cytolysis of the infectious agent [301, 304]. MAIT cells also produce granzymes such as granzyme A (GzmA), granzyme K (GzmK) and granzyme B (GzmB) and perforin enlisting them with the ability to lyse infected cells via granular exocytosis [299]. Microbes that make use of the riboflavin synthesis pathway are capable of activating MAIT cells. Some of these include *Escherichia coli, Mycobacterium tuberculosis and Staphylococcus aureus* [309, 310].

1.3.9 MAIT cells in NE

The role of MAIT cells in neonatal brain injury has not yet been elucidated. A study done by Ben Youssef *and* his co-workers found that in neonates, the frequencies of MAIT cells are 30 times lower than in adults, and this population expands thoughout childhood and peaks in adolescence. However, Chen *et al.* reported that MAIT cells from children are capable of producing more IFN-γ than MAIT cells from young adults (20-40 years old), whereas TNF-α and granzyme B production levels are similar [311, 312].

Despite the large numbers of MAIT cells in human circulation and tissues, there remains a paucity of evidence on their roles in the pathogenesis of CNS disease especially brain injury. In multiple sclerosis (MS) patients, $CD8^+$ MAIT cells are recruited to the CNS [313], leading to reduced $CD8^+$ MAIT-cell frequencies in the blood. Therefore,

$CD8^+$ MAIT cells seem to play a role in the innate arm of immunopathology in MS. However, MAIT cell functions were found to be impaired in patients with MS, with reduced IFN-γ and TNF-α production [314]. Mexhitaj and his group used MR1-tetramers to detect MAIT cells in blood of children with MS. They reported an abnormal increase in circulating MAIT frequencies, with a greater tendency of these MAIT cells to release IL-17 and IFN-γ compared to MAIT cells of both children with (monophasic) acquired demyelinating syndrome and healthy controls [315]. Circulating MAIT cells are reduced in patients with alcoholic or non-alcoholic fatty liver disease-related cirrhosis while they accumulate in liver fibrotic septa [316]. More studies are required to understand the role of MAIT cells, if any, in the brain.

1.3.10 NKT cells

NKT cells are a heterogeneous group of innate-like T cells whose TCRs recognise glycolipid antigens presented by CD1d [317, 318]. They are characterized by the expression of TCRs and NK cell receptors, such as CD161 in humans and NK1.1 in mice [319] and contribute to the activation and regulation of other immune cells and have roles in tumour immunity, autoimmunity and infectious diseases [320]. Based on TCR expression, NKT cells are categorized into two types. Invariant NKT (iNKT) cells, also known as type I NKT cells, express a semi-invariant TCRα-chain (Vα24Jα18 in humans and Vα14Jα18 in mice), while, type II NKT cells have more variable TCR-α and -β chain repertoires [321, 322]. Only 0.1% of human peripheral blood T cells are iNKT cells [323]. iNKT cells recognise a number of autologous and microbial glycolipids, but α-galactosylceramide (α-GalCer) is the most widely studied antigen recognised by iNKT cells. α-GalCer is not found in mammalian systems but is found in marine sponges [114]. Upon activation by α-GalCer, iNKT cells respond rapidly by killing tumour cells and producing Th1, Th2, Th17 and Treg cytokines. iNKT cells initiate the immune response

by regulating other immune cells such as DCs, T cells and B cells [320] by contact-dependent mechanisms and by the rapid secretion of a diverse array of Th1, Th2, Th17 and Treg cytokines [319].

Type II NKT cells thought to be more prevalent than iNKT cells in humans. They recognize lipid antigens presented by CD1d, but this subset of NKT cells does not recognize α-GalCer. Instead, they can recognize the mammalian glycolipids sulfatides and lysophosphatidylcholine [324]. Both types of NKT cells can secrete a range of cytokines that modulate NK, T and B cell responses [324]. Glycosphingolipids are abundant in brain. An understanding of the role of NKT cells in brain injury is required [325].

Evidence from previous research suggests that NKT cells have several roles in CNS disorders including neurodegenerative diseases: MS, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) and CNS viral infection, and in ischemic stroke [326]. In MS, NKT cell frequencies and cytokine profiles vary between the various stages of the disease. For example, in relapsing-remitting patients, IL-4 production was found to be higher than in the progressive stages of MS and control subjects. However, NKT cells express proinflammatory cytokines in secondary progressive MS patients [327, 328]. Finkelstein et al. and his coworkers reported that in an ALS mouse model, an analogue of α-GalCer prevented death of motor neurons and induced T cell recruitment to the spinal cord the life span of the animals [329].

Little about the role of NKT cells in NE. A study done in mice after HI insults found that there was a steady recruitment of iNKT cells to the ischemic brain parenchma suggesting that they play a role. iNKT cell recruitment appears as early as 48 h post-infarction but only after major injury to the blood brain barrier. TNF-α and IFN-γ

concentrations produced by iNKT cells gradually increased and peaked at 48 h after persistent cerebral ischemia. In addition, an elevation of TNF-α and IFN-γ protein expression in the infarction region were detected by immunohistochemical analyses [330].

In a study of renal injury after ischaemia-reperfusion injury (IRI), Yang and co-workers [331] found that sulfatide-induced activation of type II NKT cells protected the kidneys from IRI, by mechanisms that involved hypoxia-inducible factor (HIF)-1α and IL-10 [331]. These findings suggest that type 2 NKT cells may be targeted to treat ischaemic injury. Another study done by Wong et al. [332] found a strong link between stroke severity, peripheral iNKT cell activation, IL-10 production, and immunosuppression in stroke patients. They showed that stroke patients had increased CD69 expression by circulating iNKT cells compared to healthy and hospital controls. Indeed, in patients with stroke, iNKT cell activation was positively connected with IL-10 production, and peripheral IL-10 levels were closely correlated with poor outcome. These findings suggest that iNKT cells trigger an immunosuppressive response by releasing IL-10, making the patient more susceptible to infection [333]. This suggests that the manipulation of NKT cells could have treatment potential in NE, however, further investigation of these cells should be carried out in patients with NE.

As motioned above, innate T cells play important role in the activation and polarization of adaptive immune responses and are likely to influence the susceptibility and outcome of NE. In particular, innate T cells can control conventional T cells activation via effects on antigen presentation by DCs and others APCs. Furthermore, iNKT cells and some γδ T cells can promote antibody production by B cells. At the outset of this project, it was not known if MAIT cells could similarly influence antibody responses. A possible role of MAIT cells in B cell maturation, cytokine production, antigen presentation, antibody

isotype switching, and antibody release could have important influence on the development and outcomes of NE.

1.4 Hypothesis and aims

1.4.1 Hypothesis

Although most studies to date on the immunology of NE in humans have focussed on the possible role of neutrophils in the responses to cerebral ischemia, there is evidence that lymphocytes also accumulate in the postischemic brain within the first 24 hours after reperfusion. However, the contribution of specific lymphocyte subpopulations and their products to the pathogenesis of neonatal encephalopathy has not been systematically addressed. We hypothesise that lymphocytes control the inflammatory responses that contribute to NE and that immunological interventions which target these cells will serve as adjunct therapies to TH. We also hypothesize that inflammatory response in NE may involve crosstalk between adaptive and innate lymphocytes. Crosstalk between B cells and either iNKT cells or γδ T cells promotes B cell differentiation and antibody responses, but at the outset of this project, it was not known if MAIT cells can similarly contribute to B cell functions.

1.4.2 Aims

The overall aim of the present research was to perform an in-depth phenotypic and functional characterization of the circulating lymphoid cell compartment and serum cytokine profiles from healthy donors grouped according to age, from pregnancy to adulthood and to compare these to those in in neonates with NE, school-age children post-NE, and children with cerebral palsy. A second aim was to investigate if one innate T cell population, MAIT cells, can influence the function of B cells. The specific objectives are as follows:

1. To compare circulating innate and conventional lymphocyte numbers and phenotypes and cytokine levels in cord blood and in blood from healthy neonates, school-age children and young and older adults.

2. To enumerate and phenotypically characterize circulating innate and conventional lymphocyte numbers, phenotypes and functions and serum cytokine levels in neonates with NE, school-age children post NE, school-age children with CP and in age-matched healthy control subjects.

3. To investigate functional interactions between MAIT cells and B cells from healthy donors.

CHAPTER 2
Materials and Methods

2.1 Study Groups

The study focused on infants and children with neonatal encephalopathy and children with cerebral palsy. Additionally, we investigated the following control groups: healthy term infants, school age-children, adults and elderly subjects. Blood samples were also obtained from consenting adults and from used buffy coat packs obtained from the Irish Blood Transfusion Service. Standard operating techniques were practiced using healthy adult donations of blood to ensure optimisation of experiments prior to their use on neonatal blood.

To identify the innate and adaptive immune cells across the human life span, we recruited five human cohorts: Cord blood CB (n = 10, through the Coombe Women and Infants University Hospital, Dublin), neonates (n = 17, through the Coombe Women and Infants University Hospital, Dublin), school-age children (n = 23; aged 1–16; median age = 7; through Tallaght Children's Hospital; Dublin), young adults (n = 25; age range 23–45, median age 30 through The Trinity Translation Medicine Institute, Dublin), and older people (age range 71-92; median 78 they were recruited from St. James's hospital. All donors were healthy and had non-complicated term births.

2.1.1 Newborn infants with NE

Newborns with NE were recruited from the Coombe Women and Infants University Hospital, the National Maternity Hospital, Holles Street Hospital, the National Children's, Hospital Tallaght and the Rotunda Hospital. The inclusion criteria of Huang et al for the diagnosis of NE were applied [334]. These criteria require the presence of unusual neurological symptoms in the immediate postnatal period, such as hypotonia or seizures, or the presence of other organ problems (kidneys, liver, lung, heart, haematological) as well as at least two of the three requirements listed below: (i) evidence

or suspicion of hypoxic-ischaemic injury based on a history of foetal distress, i.e. abnormal basal heart rate within 10 minutes window as measured by cardiotocography (CTG), loss of beat-to-beat variability on the cardiotocograph, and/or abnormal scalp pH; (ii) need for resuscitation after birth, i.e. bag and mask ventilation; and (iii) base deficit >15 mmol/l or pH <7.2 in cord blood or in a peripheral arterial sample. Infants with serious congenital defects and mothers who use illicit drugs are among those who are excluded from participating. Table 4.1 in chapter 4 of this thesis, shows demographic data for neonatal NE & school-age children with NE, who were included in this study.

2.1.2 School-age children with NE and CP

Ten school-age children with a history of NE, all of whom were clinically stable, were studied. Additionally, 10 children with CP were recruited from Tallaght Hospital. 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. Table 4.2 in chapter 4 of this thesis, shows demographic data for school-age children with NE, who were included in this study.

2.2 Control subjects

2.2.1 Healthy term Neonates

Blood samples from 17 normal term infant healthy controls were taken following normal spontaneous vaginal delivery. All infants had an uncomplicated postnatal course after birth with normal neurological examination at birth and normal Apgar scores, used to summarize the health of newborn children against infant mortality.

2.2.2 Cord Blood Samples

Umbilical cord blood samples from 10 normal term infants were taken following normal spontaneous vaginal delivery, or after non-complicated Caesarean section. All infants had an uncomplicated postnatal course with Apgar scores of 9 at 5 minutes after birth.

2.2.3 Healthy school age children

The paediatric control group consisted of 23 children aged 15 years or less, admitted to the surgical day ward for minor elective surgical procedures. None of the children had significant medical conditions. Samples were taken in theatre during cannula insertion by the anaesthetic team. The age range of the school-age paediatric controls was 7-16 years (median = 10).

2.2.4 Healthy adults

25 healthy adult volunteers were recruited from consenting students and laboratory staff at Trinity Translational Medicine Institute. The age range of the donors was age range between 23–45, the median 30 [n = 25].

2.2.5 Healthy older adults

We studied 10 older adults subjects aged 7-92 years (median 83 years) who were recruited from St. James's Hospital. Their clinical states were stable at time of phlebotomy, with no signs of infection, and their white blood cell counts were within normal range. They were at the hospital because of fall and fracture, others for intracranial haemorrhage, gastritis, abdominal pain, depression and pneumonia, none of them on immunotherapy. Importantly, many of the older adults were not illness-free they have co-morbidities as it shown in table in chapter 3.

2.3 Ethical Approval

Approval to conduct this study was obtained from the Ethics Committees of 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 - and from St. James's Hospital. All participants were informed of the nature of the study and informed written consent was obtained from the parents of all infants and children prior to recruitment. Ethical approval for the use of adult and elderly controls was obtained from the Research Ethics Committee of Trinity College Dublin School of Medicine.

2.2 Materials

Equipment used in this investigation and their sources are listed in Table 2.1. General reagents are listed in Table 2.2. Consumable plasticware is listed in Table 2.3. Antibodies used for cell surface phenotypic analysis by flow cytometry are listed in Table 2.4. Antibodies used for intracellular phenotypic analysis by flow cytometry are listed in Table 2.4.

Table 0-1 Equipment used in this investigation

Equipment	Model	Company
Balance	Adventure Pro	Ohaus, NJ, USA
Blood packs	MSE 6500L	macopharma
Centrifuge	Eppendorf 5415 D	Eppendorf, Hamburg, Germany
Haemocytometer slides.	Neubaur improved	Germany
CO2 incubator	180i	Thermo Fisher scientific, MA, USA
Flow cytometer	FACS Canto II	Becton Dickinson, Oxford
Laminar air flow class II safety cabinet	Clean Air MSC BSS6-2	Thermo Fisher scientific, MA, USA
Laminar air flow class II safety cabinet	MSC 1.2	Thermo Electron LED Gmbh, Germany
Magnet	LS Magnet	Miltenyi Biotech, Bergisch Gladbach, Germany
Magnet stand	MACS Multi stand	Miltenyi Biotech, Bergisch Gladbach, Germany
Magnet	The big Easy EasySep	Stemcell technologies, France
Microscope	Inverted; NAO 30	Olympus Corporation, Japan
Multichannel pipette	Finn pipette F2	Thermo Fisher Scientific, MA, USA

PH meter	Benchtop PH20-01	Hanna instruments, RI, USA
Pipettors p20, p100, p200, p1000	Eppendorf Research plus	Thermo Fisher Scientific, MA, USA
Pipette filler	Motorised, Fisher brand	Thermo Fisher Scientific, MA, USA
UV microscope	E200 Eclipse	Nikon, USA
Vortex	Vortex Genie	Scientific industries, NY, USA
Water Bath	YCW-010E	Germany Industries corp; Taiwan

Table 0-2 General reagents used in this investigation

Reagents	Company	Location
1M HEPES	Gibco Invitrogen	Paisley, UK
100 mM sodium pyruvate	Gibco Invitrogen	Paisley, UK
100x MEM non-essential amino acids (NEAA)	Gibco Invitrogen	Paisley, UK
200 mM (100x) l-glutamine	Gibco Invitrogen	Paisley, UK
30% bovine serum albumin	Sigma-Adrich	Poole, UK
4% paraformaldehyde	Santa Cruz	Santa Cruz, CA, USA
50 mM ß-mercaptoethanol	Gibco Invitrogen	Paisley, UK
50x MEM amino acids	Gibco Invitrogen	Paisley, UK
Rapamycin	Gibco Invitrogen	Paisley, UK
Anti-CD14 microbeads	Miltenyi Biotech	Bergisch- Gladbach,Germany
Brefeldin A	Sigma-Adrich	Poole, UK
Dimethyl sulfoxide (DMSO)	Sigma-Adrich	Poole, UK
Ethidium bromide	Sigma-Adrich	Poole, UK

Ethylenediaminetetraaceteic acid (EDTA)	Sigma-Adrich	Poole, UK
Hyclone fetal bovine serum	Thermo-scientific	Logan, UT, USA
Ionomycin	Sigma-Aldrich	Poole, UK
LIVE/DEAD Fixable Dead Cell Stain	Gibco Invitrogen	Paisley, UK
Lymphoprep	Nycomed Pharma AS	Oslo, Norway
Monensin	Biolegend	San Diego, CA, USA
Penicillin-streptomycin	Gibco Invitrogen	Paisley, UK
Phorbol-12-myristate-13 acetate (PMA)	Sigma-Adrich	Poole, UK

Table 0-3 ***Disposable plasticware used in this investigation***

Item	Source	Company
10 ml plastic pipettes	Sarstedt, Nümbrecht, Germany	
5 ml plastic pipettes		
3.5 ml transfer piptettes		
25ml plastic pipettes		
6 -well flat bottom tissue culture plates	Corning Life sciences	MA, USA
Sterilin Sterile Graduated pipette (10 ml)	ThermoFisher Scientific	Waltham, MA.USA.
Costar Stripette Sterile Graduated pipette (25 ml and 5 ml)	Sigma- Aldrich	Poole, UK
96 -well flat bottom tissue culture plates	Corning life sciences	MA, USA
96 -well round bottom tissue culture plates	Corning life sciences	MA, USA
Cryovials	Thermo-scientific Nunc	Roskilde, Denmark
FACS tubes	BD Biosciences	Oxford, UK

Table 2-4 Antibodies used for cell surface phenotyping by flow cytometry

Specificity (mouse anti-human)	Conjugate	Clone	Company	location
CD3	APC-CY7	UCHT1	Biolegend	San Diego, CA, USA
CD3	Pacific blue	HIT3a	Biolegend	San Diego, CA, USA
CD4	PECY7/APC	OKT4	Biolegend	San Diego, CA, USA
CD8	PerCP-CY5.5	SKI	Biolegend	San Diego, CA, USA
CD8	APC Cy7	6B11	Biolegend	San Diego, CA, USA
Vα24/Jα18	APC	6B11	Biolegend	San Diego, CA, USA
CD19	APC/CY7	HIB19	Biolegend	San Diego, CA, USA
CD19	PE Cy7	HIB19	Miltenyi Biotec	Biotec GmbH, Bergisch Gladbach, Germany
CD19	Allophycocyanin (APC)	HIB19	Biolegend	San Diego, CA, USA

CD20	APC	2H7	Miltenyi	Biotec GmbH, Bergisch Gladbach, Germany.
CD69	FITC	FN50	Biolegend	San Diego, CA, USA
CD56	PECY7	HCD56	Biolegend	San Diego, CA, USA
CD161	PerCP-CY5.5	CHP3G10	Biolegend	San Diego, CA, USA
CD161	FITC/APC	HP-3G10	Miltenyi	Biotec GmbH,Bergisch Gladbach, Germany.
V*α*7.2	APC/PerCeP		Miltenyi	Biotec GmbH, Bergisch Gladbach, Germany.
V*α*7.2	PE	B6	Biolegend	San Diego, CA, USA
CD40	PE	HB-7	Biolegend	San Diego, CA, USA
Vδ1	FITC	REA173	Biolegend	San Diego, CA, USA
Vδ2	PE/FITC/BV510	B6	Biolegend	San Diego, CA, USA
CD38	PE-CY7	HB-7	Biolegend	San Diego, CA, USA
CD86	FITC/APC	-	Immunotools	

HLA-DR	PerCP-CY5.5	L243	Biolegend	San Diego, CA, USA
CD3	No fluorochrome	OKT3	BD Biosciences	Oxford, UK
CD28	No fluorochrome		BD Biosciences	Oxford, UK
anti-Vα7.2	Anti-PE	Miltenyi , Biotec, Germany		
anti-CD19				
anti-PE, anti-CD3				
anti-PE, anti-CD8				

Table 2-5 Antibodies used for intracellular phenotypic analysis by flow cytometry

Specificity	fluorochrome	Source
IFN-γ	APC	BD Bioscience
IFN-γ	PE	Biolegend, San Diego, CA, USA
IFN-γ	FITC	
IFN-γ	PercP/Cy5.5	
TNF- α	PE Cy7	
IL-2	PE	BD Bioscience
IL-4	PE	Biolegend, San Diego, CA, USA
IL-10	FITC	
IL-17	APC	
GzmB	Amcyan	BD Bioscience
IgD	PE	Biolegend, San Diego, CA, USA
DCS	eFluor506	Invitrogen, ThermoFisher Scientific
anti-human Foxp3	PE	Biolegend, San Diego, CA, USA
anti-human RORγt	PECy7	Biolegend, San Diego, CA, USA
anti-human T-bet	FITC	Biolegend, San Diego, CA, USA
anti-human GATA-3	APC	Biolegend, San Diego, CA, USA
CBA assays Kit		BD Bioscience
MSD ELISA kits		MSD Diagnostics, USA

2.2.3 Preparation of Solutions and Media

2.2.3.1 Foetal calf serum (FCS).

Small quantities of 20 different batches of FCS were obtained from Hyclone and tested before bulk buying of two batches that optimally supported the growth and differentiation of dendritic cells and innate T cells in the laboratory. These were stored at -80ºC for future use. Before use, the FCS was and heated at 56ºC for 30 minutes in water bath to inactivate complement and filtered through a 0.2 µm sterile nylon filter to remove large particles of proteins and fibrin aggregates.

2.2.3.2 Complete RPMI (cRPMI).

cRPMI consisted of RPMI 1640 medium containing 50 μM L-Glutamine, supplemented with 25 mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 µg/ml Fungizone and 10% FCS. cRPMI was stored at 4ºC and warmed to 37ºC before each use.

2.2.3.3 MAIT cell medium.

MAIT cell medium consisted of cRPMI, prepared as above, and supplemented with 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture. MAIT cell medium was stored at 4ºC and warmed to 37ºC before each use.

2.2.3.4 Complete DMEM (cDMEM).

cDMEM consisted of DMEM medium containing high glucose and Glutamine supplemented with 10% FCS, 25 mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 µg/ml Fungizone. cDMEM was stored at 4ºC and warmed to 37ºC warmed before use.

2.2.3.5 Miltenyi Buffer.

Miltenyi buffer (500 ml) was used for magnetic bead sorting of cells and was made up fresh for every use. Miltenyi buffer consisted of PBS containing 0.5% bovine serum

albumin and 2 mM EDTA. It was prepared by adding, 8.3 ml of 30% BSA and 2 ml of 500 mM EDTA to 500 ml PBS buffer. BSA was previously made by dissolving 30 g BSA powder in 100 ml PBS and filtering it through a 0.2 μm filter. Miltenyi buffer was stored at 4°C

2.2.3.6 Ethidium bromide / acridine orange (EB/AO) viability stain.

A stock solution of EB (3 mg/ml) and AO (5 mg/ml) in PBS was made. A working solution of EB/AO was made up by diluting each stock 1:100 in PBS. The working solution was stored at RT in the dark and used at 1:20 dilution for cell counting in haemocytometer.

2.2.3.7 PBA (PBS with BSA and azide) buffer.

PBA buffer was used for staining cells for flow cytometry. PBA buffer consists of PBS containing 1% bovine serum albumin and 0.02% sodium azide. 25.86 ml of 30% BSA and 1.035 ml of 10% sodium azide were added to 500 ml PBS and the solution was stored at 4°C.

2.2.3.8 Cell freezing Mixture.

Cells were cryopreserved in a mixture of 90% FCS and 10% dimethyl sulphoxide (DSMO).

2.2.3.9 Saponin solution.

0.1 g of saponin powder were dissolved in 50 ml PBA to give a concentration of 0.2% used to permeabilise cells.

2.3.1 Sterile Technique

Cell culture is the process by which cells are grown under controlled conditions and is an essential laboratory technique necessary for the maintenance of viable cells for later use in experiments. Sterile technique is a key factor in all cell culture procedures. All cell culture work was performed inside a biocontainment level 2 cell culture hood to prevent

contamination, and the hood was thoroughly decontaminated prior to use. All materials were sterile before use and any materials that were not sterile either were autoclaved or filtered. 70% ethanol in distilled water (dH_2O) was used to wipe down all surfaces and materials before and after cell culture procedures. All cell cultures were incubated in a 5% CO_2 incubator set at 37°C. Liquid waste was decontaminated using suitable dose of Virkon, then discarded down in the sink.

2.3.2 Preparation of Human Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy subjects and from buffy coat packs provided by the Irish Blood Transfusion Service (St. James's Hospital, Dublin 8). PBMCs were prepared by density gradient centrifugation over Lymphoprep. 15 ml of Lymphoprep was added to a 50 ml Falcon tube. 35 ml of blood diluted 1:1 with PBS containing 1% FCS was carefully layered onto the Lymphoprep and centrifuged for 30 minutes at 400xg with the brake off (acceleration and deceleration set to zero). The buffy coat layer was then carefully harvested and washed twice with PBS and centrifuged for 10 minutes at 450xg to remove any remaining Lymphoprep. The final PBMC pellets were resuspended in complete RPMI 1640.

2.3.3 Enumeration of Cells

Cells were stained with EB/AO and enumerated by fluorescence microscopy using a haemocytometer slide. When viewed under ultraviolet light, live cells fluoresce green while dead cells appear orange. 10 µl of the cell suspension was added to 190 µl of EBAO solution (working solution prepared as described in section 2.2.3.6 and 10 µl of this mixture placed into the well of a haemocytometer slide. Live cells were counted in two large squares (1 mm x 1 mm sections). Each of these squares accommodates 0.1 mm of liquid so the number of cells per 1 ml in the original suspension can be calculated.

2.3.4 Cryopreservation and Recovery of Cells

Cells were pelleted by centrifugation for 7 minutes at 450 xg with brake and acceleration at maximum. Fresh freezing mixture, consisting of 90% FCS and 10% dimethyl sulphoxide (DMSO) was made, cooled on ice, and the cell pellet was resuspended in 2 ml of freezing mixture. This cell mixture was rapidly transferred to labelled cryovials and placed in a Mr Frosty container in an -80^{o}C freezer. For long-term storage, cells were then transferred to liquid nitrogen after 24 hours. Cells were recovered from cryopreservation by rapid thawing under running water. The vial was removed from the hot water before the contents completely thawed and transferred to a universal tube. Complete RPMI was added dropwise, while shaking the tube to mix continuously. This was then topped up with RPMI to 20 ml. Tubes were centrifuged for 7 minutes at 450 xg with brake and acceleration at maximum. Then supernatants were discarded and cells were resuspended in an appropriate amount of medium.

2.4 Flow Cytometry

2.4.1 Principles of Flow Cytometry

Flow cytometry is a widely used technique which allows for the analysis of many different parameters of single particles via fluorescent tagging. The name flow cytometry is derived from the words ‘flow’, ‘cyto’ and ‘metry’ which correspond to the fluidics, cells and measurements respectively. This technique had a wide variety of applications in the present project, including intracellular and surface staining with fluorochrome-labelled antibodies and other ligands, and CBA immunoglobulin assays. All standard flow cytometry was carried out on a Becton Dickinson FACS Canto II flow cytometer. Flow cytometric cell sorting was carried out on a Becton Dickinson FACSMelody flow cytometer.

Flow cytometry exploits a laser based, analytic technique which makes it possible to examine a variety of cell characteristics within a heterogeneous population such as whole blood. The flow cytometer can assess and detect a wide range of cells and particles ranging from 0.5-100 μm. The fluidic mechanism involves hydrodynamic focusing of cells into the middle of a stream, in single file, and passing them by multiple lasers, thousands of cells per second. As the cells pass by the lasers, they absorb the light and fluorescence is emitted from each cell. The way in which the cells scatter light in different directions can be exploited to obtain information about the physical shape of the cells. Large objects will scatter more light in the forward direction which is detected by the forward scatter (FSC) detector. Light scattered at 90º to the laser will be detected by the side scatter (SSC) detector. The more irregular, rough and granular a cell is, the more light it will scatter to the side. This measurement can be used to determine the granularity or internal complexity of the cell. Flow cytometry is useful for obtaining other information such as phenotype, viability of a cell and cell signaling patterns. These characteristics are determined using fluorochromes, such as fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerythrin (PE) that are conjugated to antibodies specific for cell-surface or intracellular antigens. The fluorescence intensity is relative to the concentration of fluorochrome, which is determined by the relative abundance of the detected proteins. These data are read as the mean fluorescence intensity (MFI) value. The flow cytometer translates this optical information into graphical data for further interpretation and analysis.

The FACS Canto II works by operating three different systems. The fluidics system is responsible for sample acquisition, where the cells are pressurized into single file by way of hydrodynamic focusing through the injection tube, where laser light intercepts the cell. The optics system encompasses three lasers; blue, violet and red, as well as dichroic

mirrors which reflects scatter light into filters, which further directs the light into the detectors. Lastly, the electronics system is comprised of detectors responsible for converting light signals into electrical signals. The FACSDiva software can transform these electrical signals into graphical data.

2.4.2 CS&T Beads

On a weekly basis BD Cytometer Setup and Tracking (CS&T) Research Beads were run after fluidics was started up in order to monitor and enhance the FACS Cantos II's performance. One drop of CS&T beads was diluted in 350 ml PBA and 'run' in the CST performance test. The CS&T beads enhance the cytometers set up as well as automatically characterize, track and report measurements of BD digital flow cytometers. CS&T beads also define a baseline for the fluorescence detectors and optical configuration.

2.4.3 Parameters

Parameters were set to ensure that the flow cytometer could distinguish between cells positive and negative for each fluorochrome. Fluorochromes which were not used in the experiment were unselected. The voltages for FSC area and SSC area were set to 350 and 450, respectively. In order to remove debris from the analyses, the threshold was set to 40,000.

2.4.4 Compensation

Fluorescence cross over can occur between two fluorochromes, that are both expressed on a single cell. This is a product of an overlap of emission spectra of fluorochromes which are detected by the same detector, which may result in a false positive 'spill over'. Compensation controls were run, made up of OneComp beads stained with a single fluorochrome, to allow the computer to calculate any spectra overlap and generate compensation.

2.4.5 Cell Surface Staining of Cells for Phenotypic Analysis

Phenotypic analysis of lymphocytes within whole blood from patients with NE and control subjects was carried out by flow cytometry within 24 hours of blood sample collection. 50 µl of whole blood were pipetted into flow cytometry tubes. Cells were stained for 15 minutes in dark at 4°C at room temperature with a live/dead cell stain (Flexible viability dye diluted 1/1000 with PBS). Cells were then washed with PBA buffer, pelleted by centrifugation and incubated with fluorochrome-conjugated monoclonal antibodies (mAb) specific for, CD3, CD4, CD8, CD161, TCR-Vα7.2, CD56, TCR-Vδ1, TCR-Vδ2, TCR-Vδ3, CD19, CD69 and TCR-Vα24/Jα18 in PBA buffer for 15 minutes at room temperature. A fluorochrome-conjugated antibody specific for glycophorin A was included in all tubes where cord blood or neonatal blood was being analysed, to stain immature red blood cells. This amounted to 3 flow cytometry tubes per subject and an unstained sample and fluorescence-minus-one (FMO) controls where required. 1 ml of FACS lysis buffer was then added to lyse red blood cells, then the cells were washed with PBA buffer. Finally, the cells were fixed with 1% paraformaldehyde (PFA) in PBA buffer for 20 minutes and then washed with PBA buffer and acquired by flow cytometry on a Becton Dickinson FACSCanto II flow cytometer.

2.4.6 Intracellular Staining

Once surface staining was complete, cells requiring intracellular staining were washed and pelleted by adding approximately 2 ml of PBA, vortexing and centrifuging at 450xg for 7 minutes. The supernatant was discarded. Cells were fixed using ~0.5 ml/tube of 4% PFA and vortexed. Tubes were incubated in the dark for 10 minutes to allow fixation to occur. Cells were washed and pelleted again. Cells were permeabilized with 1 ml of 0.2% saponin in PBA buffer, freshly made as described in section 2.2.3.9 and incubated in the dark for a further 10 minutes. Following this incubation period, cells were washed and

pelleted. Tubes were then stained with a cocktail of intracellular staining mAbs in saponin to bring the total volume to be added to each tube to 50 µl. Tubes were vortexed and left in the dark for 30 minutes. Cells were then washed and pelleted and resuspended in ~0.5 ml PBA. The tubes were covered with aluminum foil and kept in the fridge until used for flow cytometry analysis.

2.2.7 Sample Acquisition & Gating Strategy

FACS tubes containing stained cell samples were vortexed, loaded, run through the machine and their data were recorded. FlowJo 10 software was used for analyses of these FCS files. Polygon gates were used to gate on the lymphocyte population, then the live cells and single cells (Figure 2.1).

Data were analysed with Flow Jo software. The gating strategy employed is shown in Figure 2.1. T cells were defined as $CD3^+$ cells. NK cells were defined as $CD3^-$ $CD56^+$ cells. B cells were defined as $CD3^-$ $CD19^+$ cells and Vδ1, Vδ2 and Vδ3 T cells were defined as $CD3^+$ $V\delta1^+$, $CD3^+$ $V\delta2^+$, and $CD3^+$ $V\delta3^+$ cells, respectively. MAIT cells were defined as $CD3^+$ $V\alpha7.2^+$ $CD161^+$ cells and iNKT cells were defined as $CD3^+$ $V\alpha24/J\alpha18^+$ cells.

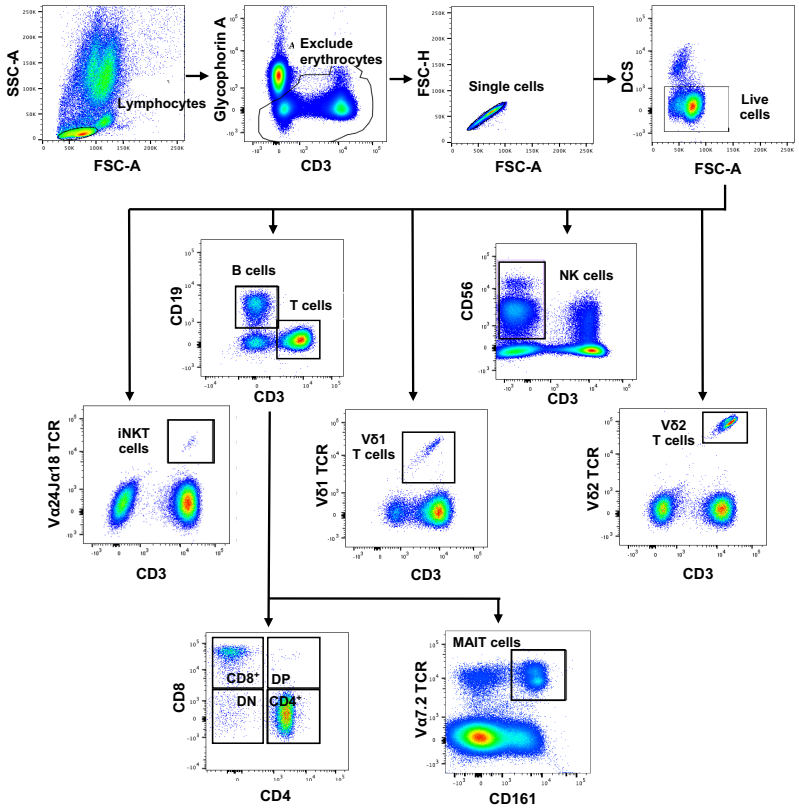

Figure 2.1 Gating strategy for the detection of T cells, B cells, NK cells, CD4+ T cells, CD8+ T cells, CD4-CD8- T cells, CD4+CD8+ T cells, iNKT cells, MAIT cells, Vδ1 T cells and Vδ2 T cells by flow cytometry. *Whole blood was stained with a dead cell stain (DCS) and monoclonal antibodies specific for glycophorin A, CD3, CD4, CD8, CD19, CD56, CD161 and the Vα7.2, Vα24Jα18, Vδ1 and Vδ2 T cell receptors and analysed by flow cytometry. Upper panels, left to right: flow cytometry dot plots showing forward scatter area (FSC-A) plotted against side scatter area (SSC-A) with an electronic gate drawn around the lymphocytes; Dot plot showing CD3 plotted against glycophorin A with a gate drawn around the glycophorin-negative cells to exclude erythrocytes; Dot plot showing FSC-A plotted against FSC-height (FSC-H) for gated lymphocytes with a gate drawn around the single cells; Dot plot showing FSC-A plotted against the dead cell stain for gated single cells with a gate drawn around the live cells. Centre panels: Five dot plots showing expression of CD3 and either CD19, CD56, Vα24Jα18, Vδ1 or Vδ2 by gated live singlet lymphocytes with gates drawn around the B cells, T cells, NK cells, iNKT cells, Vδ1 and Vδ2 T cells. Lower panels, left and right: Dot plot showing expression of CD4 and CD8 by gated T cells with gates drawn around the $CD4^+$ T cells, $CD8^+$ T cells, double negative (DN) $CD4^-CD8^-$ T cells and double positive (DP) $CD4^+CD8^+$ T cells; Dot plot showing expression of CD161 and Vα7.2 by gated T cells with a gate drawn around the MAIT cells.*

2.5 Cell Enrichment and Sorting

2.5.1 Magnetic isolation of B cells

B cells were isolated from PBMCs. The cell number was determined as outlined in section 2.3.3. The cell suspension was centrifuged at 400 xg for 10 minutes and the supernatant discarded. The cell pellet was resuspended in 320 µl of Miltenyi buffer per 10^7 total cells. 80 µl of CD19 Microbeads per 10^7 total cells was added. Cells were incubated for 15 minutes in the rotator in the fridge (4−8°C). After incubation the cells were washed with 1 ml Miltenyi buffer and pellet was resuspended in 500 µl Miltenyi buffer for up to 10^8 cells.

An LS column was set up in a magnet and washed 3 times with 500 µl of Miltenyi buffer, allowing the buffer to completely flow through the column each time. The magnetic bead-labelled cells were then applied to the column and the liquid was allowed to completely flow through the column. The B cells expressing CD19 were retained in the column due to the magnetic field. The column was then removed from the magnet. 1 ml of Miltenyi buffer was added and the $CD19^+$ B cells were eluted from the column using a syringe plunger. The cells were then washed and centrifuged at 400 xg for 10 minutes and the cell number was determined as described in section 2.3.3. The cells were either used immediately for co-culture or incubated in cRPMI in the 5% CO_2 incubator, or cryopreserved until required.

2.5.2 Magnetic Bead Isolation of MAIT Cells from PBMCs

2.5.2.1 Magnetic bead isolation of MAIT cells. MAIT cells were isolated from PBMC by magnetic bead sorting of the $V\alpha7.2^+$ cells followed by flow cytometric cell sorting of the $CD161^+$ cells. PBMCs were isolated and the cell number was determined as described in section 2.3.2 and 2.3.3 respectively. The cell suspension was centrifuged at 300 xg for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 100 µl

of Miltenyi buffer, per 10^7 total cells. The cells were stained with 40 µL of anti-Vα7.2 antibody PE and incubated for 15 minutes in a rotator at 4 °C, in the fridge. Cells were then washed by adding 1-2 ml of Miltenyi buffer and centrifuging at 400 xg for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in 240 µl of Miltenyi buffer for up to 10^8 cells. 60 µl of magnetic beads coated with anti-PE mAb per 10^7 total cells was then added to the cells. Once again cells were incubated for 15 minutes in the rotator in the fridge 4°C. Cells were then washed again as above.

The magnetically labelled Vα7.2$^+$ cells were separated from the other cells using the LS column. The LS column was placed in the magnetic field, with a collection tube placed underneath and a pre-separation filter was placed on top of the column. The LS column was washed three times with 3 ml of Miltenyi buffer prior to the addition of the cell suspension. Once completed, the cell suspension was added to the filter followed by a further 5 ml of Miltenyi buffer to ensure all the suspension had run through the column. The Vα7.2 labelled cells were then retained in the column as they bound to the anti-PE coated magnetic bead. The column was then removed from the field and the magnetically labelled Vα7.2$^+$ MAIT cells were eluted from the column in 5 ml Miltenyi buffer using a plunger. Cells were then washed and centrifuged at 400 xg for 10 minutes. In some cases, cells were further stained for CD161 in order to further purify MAIT cells via sorting using FACS Melody by gating on the Vα7.2 and CD161 double positive population. After sorting, cells were either used immediately or resuspended in 2 ml cRPMI and stored in the CO_2 incubator until needed. The Vα7.2 negative cells that were collected in the collection tube were washed and pelleted and used to obtain B cells via another round of magnetic separation using CD19 magnetic beads (see section 2.2.8.2).

2.5.2.2 Magnetic isolation of CD3$^+$ cells.

PBMCs were isolated and counted as outlined in section 2.3.2 and 2.3.3. The cells were pelleted by centrifugation and resuspended in 80 µl of Miltenyi buffer per 10^7 total cells. 20 µl of CD3 Microbeads was added per 10^7 total cells and incubated in the rotator in the fridge for 15 minutes (4−8°C). The cells were then washed and pelleted. The cells were then resuspended in 500 µl of Miltenyi buffer per 10^8 cells.

The LS column was set up and run as described above for B cells. All T cells expressing CD3 were retained in the column due to the magnetic field. $CD3^+$ T cells were eluted from the column by removing it from the magnet, adding 1 ml of Miltenyi buffer and pushing it out of the column using the plunger. The cells were then washed and centrifuged at 400 xg for 10 minutes and the cell number was determined as described in section 2.3.3. In some instances, the CD3 T cells were further sorted using the FACS Melody as described in section 2.5.2.3. The cells were either used immediately for co-culture or incubated in cRPMI in the 5% CO_2 incubator until required.

2.5.2.3 Magnetic isolation of $CD8^+$ T Cells.

PBMCs were isolated and counted as outlined in section 2.3.2 and 2.3.3 respectively. The cells were pelleted by centrifugation and resuspended in 40 µl of Miltenyi buffer per 10^7 total cells. 10 µl of biotin-labelled antibody specific for CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A) was added per 10^7 cells and incubated in the rotator in the fridge for 5 minutes (4−8°C). This allows magnetic bead labelling of all non-CD8 T cells. After incubation, 30 µl of Miltenyi buffer per 10^7 cells were added. This was followed by the addition of 20 µl of $CD8^+$ T Cell Microbead Cocktail, containing antibodies specific for biotin, was added and the cells were incubated in the rotator in the fridge for 10 minutes (4−8°C). The cells were then washed and pelleted. The cells were then resuspended in 500 µl of Miltenyi buffer per 10^8 cells.

The LS column was set up and run as described above. All non-CD8 T cells were retained in the column due to the magnetic field. $CD8^+$ T cells passed through the column. The cells were then washed and centrifuged at 400 xg for 10 minutes and the cell number was determined as described in section 2.3.3.

2.5.2.4 Flow cytometric cell sorting

Flow cytometric cell sorting on a MoFlo XDP cell sorter (Beckman Coulter) was used to sort highly purified MAIT from PBMC. In this method, the cell sample was labelled with mAbs specific for CD3, CD161 and Vα7.2 and acquired on the cell sorter. Cells were passed through a column of pressurised sheath fluid and as they are ejected from the nozzle on the cell sorter, they passed through the analysis point consisting of laser beams which provide scattered light and fluorescence signals, which are then compared to pre-set sort criteria for the cells of interest. There is time delay between the analysis point and where the droplets break off at the end of the stream and it is referred to as the drop delay. Once the drop delay is calculated, an electric charge is applied to the stream at the precise moment the first drop forms, allowing individual drops to be independently charged. Once the charged droplets reach the end of the stream, they are passed through high-voltage charged deflection plates which cause the charged droplets to be deflected towards the oppositely charged plate and into collection tubes, while uncharged droplets are aspirated to waste (Davies, 2007).

PBMC were enriched for MAIT cells by positive magnetic bead separation of Va7.2+ cells (Miltenyi Biotec) as described previously. The Va7.2-enriched cells were stained with mAb specific for CD3 and Va7.2 in PBS for 15 min. The cells were washed in PBS and the pellets were re-suspended in 0.5 ml PBS per 1 x 10^7 cells and sorted using a MoFlo XDP cell sorter (Beckman Coulter) which allowed retrieval of pure (>97%)

populations of Vα7.2^{+} CD161^{+} cells. The cells were either used immediately for co-culture or incubated in cRPMI in the 5% CO_2 incubator until required.

2.6 In Vitro Expansion of MAIT Cells

2.6.1 Expansion of MAIT cells from PBMCs using 5 ARU

Protocols developed by Dr Andrew Hogan and colleagues at Maynooth University were used to enrich MAIT cells from PBMCs of healthy donors. PBMCs were isolated as described in section 2.3.2. counted and resuspended in 1 ml of MAIT cell medium at a density of 1.5x10^{6} PBMCs/ml. The MAIT cell antigen 5-amino-6-(D-ribitylamino)uracil (5-A-RU) was added at a concentration of 4 µg/ml along with 100 µM of 9 µl methylglyoxyl was added to 5 ml sterile water. 100 µl of this was added to 890 µl of MAIT cell Then 10 µl of 5-ARU was added to this to make a total of 1,000 µl. 100 µl was added to each ml of cells. 2 ml of PBMC were transferred (containing 3 x 10^{6} cells) to the wells of a 6-well plate and incubated at room temperature and 5% CO_2. After 48 hours, 25 U/ml IL-2 was added to the 5-ARU-treated monocytes. After 5 days, 250 U/ml IL-2 was added. Cells were fed and split as needed (media turns yellow very quickly). On day 10, the MAIT cell purity was between 60 – 90% as assessed by flow cytometry (Figure 2.2).

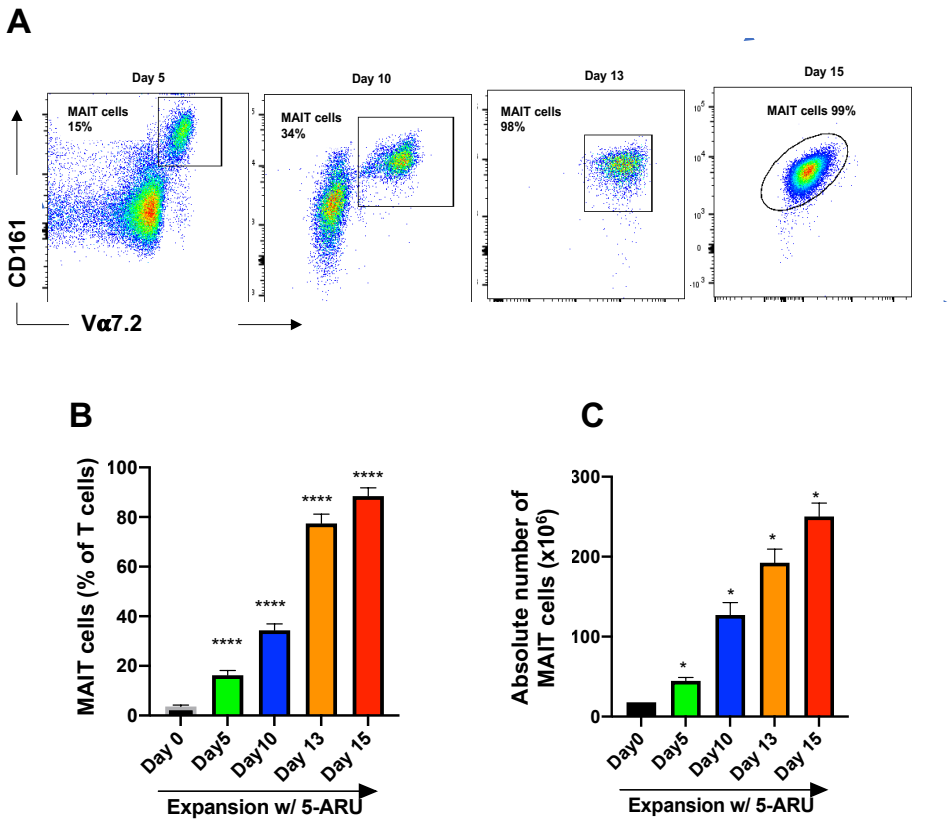

Figure 2.2 In vitro expansion of MAIT cells. *MAIT cell lines were generated by stimulating PBMC with 5-ARU and culturing them with IL-2 as described in section 2.6.1. A, Flow cytometry dot plots showing Vα7.2 and CD161 expression by gated CD3+ cells within PBMC after stimulation with 5-ARU for 5, 10, 13 and 16 days. B and C, Bar graphs showing percentage frequencies (A) and absolute numbers (B) of MAIT cells generated after 5, 10, 13 and 15 days post stimulation of PBMC. Data are expressed as means (±SEM) of 12 MAIT cell expansions. *p ≤ 0.05, ****p ≤0.0001 using the Wilcoxon signed rank test*

2.6.2 Validating the purity of MAIT cells

$0.5x10^6$ cells from MAIT cell expansions transferred to a flow cytometry tube. Cells were stained with mAbs specific for CD3 (labelled with Pacific Blue), CD161 (labelled with FITC), and Vα7.2 (labelled with PE) and analyzed by flow cytometry. MAIT cells are positive for CD3, CD161 and Vα7.2 figure 2.2.

2.6.3 Activation of MAIT cells using other modes of stimulation

Sorted MAIT cells or MAIT cells within PBMCs were stimulated with medium alone (unstimulated), IL-12 with IL-18 (IL-12/IL-18), anti-CD3 and anti-CD28 mAbs (anti-CD3 & CD28), 5-A-RU or phorbol myristate acetate with ionomycin (PMA/Ionomycin), as described below.

2.6.3.1 Stimulation of cells with IL-12/IL-18

PBMCs or MAIT cells were transferred to the wells of microtiter plate in cRPMI. 50 ng/ml each of IL-12 and IL-18 was added to each well. The cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.6.3.2 Stimulation of cells using anti-CD3 and anti-CD28 antibodies

Anti-CD3 and anti-CD28 mAbs were pre-bound to the wells of a microtiter plate 1 day prior to the experiment. PBS containing 3 µg/ml of anti-CD3 functional mAb and 3 µg/ml of anti-CD28 mAb was added to each well. The cells were then incubated for 24 hours at 37°C and 5% CO_2. The PBS containing the antibodies was then removed and the wells were washed with PBS before adding cells.

2.6.3.3 Stimulation of cells using 5-A-RU

PBMC or MAIT cells were transferred to the wells of a microtiter plate in cRPMI. 1 µl of methylglyoxal (100 µM) was added to 0.5 ml sterile water. 10 µl of this solution was

added to 89 µl of MAIT cell medium. 1 µl of 5-A-RU 4 µg/ml was added to this solution to bring the final volume to 100 µl. 10 µl of this was added to each well containing $1.5x10^6$ cells/ml then the cells were incubated for 24 hours at 37°C and 5% CO_2.

2.6.3.4 Stimulation of PBMCs with PMA/ionomycin.

Cells were plated in microtitre plates in cRPMI. 10 ng/ml of PMA and 1 µg/ml of ionomycin was added to each well. The cells were then incubated for 24 hours at 37 °C and 5% CO_2.

2.7 Activation of B cells

2.7.1 Stimulation of B cells with LPS.

PBMCs or sorted B cells were transferred to wells of a microtiter plate in cRPMI at a density of $0.5x10^6$ cells/ml. LPS was added to a final concentration of 20 ng/ml and the cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.2 Stimulation of B cells with BAFF.

PBMCs or sorted B cells were transferred to the wells of a microtiter plate in cRPMI. BAFF was added to a final concentration of 50 ng/ml and the cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.3 Stimulation of B cells with Pam3Cysk.

PBMCs or sorted B cells were transferred to the wells of a microtiter plate in cRPMI. Pam3Cysk4 was added to a final concentration of 1 µg/ml and the cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.4 Stimulation of B cells with Pam3Cysk/PolyIC.

PBMCs or sorted B cells were transferred to the wells of a microtiter plate in cRPMI. 1 μg/ml of Pam3Cysk4 and 25 μg/ml of PolyIC was added to each well. The cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.5 Stimulation of B cells with CD40/IL-4/IL-21.

PBMCs or sorted B cells were transferred to the wells of a microtiter plate in cRPMI. 10 μg/ml of anti-CD40 mAb, 100 ng/ml of IL-4 and 20 ng/ml of IL-21 were added to each well. The cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.6 Stimulation of B cells with resting MAIT cells.

Sorted B cells were transferred to the wells of a microtiter plate in cRPMI. Varying numbers of sorted MAIT cells were added to the wells. The cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.7.7 Stimulation of B cells with activated MAIT cells.

Sorted B cells were transferred to the wells of a microtiter plate in cRPMI. Sorted MAIT cells were added at various ratios to the wells. 50 ng/ml of both IL-12 and IL-18 were also added to each well. The cells were then incubated for 24 hours at 37°C and 5% CO_2.

2.8 Measurement of Cell Activation

2.8.1 Analysis of intracellular cytokine production. See section 2.4.6.

2.8.2 Analysis of cytotoxicity (CD107a assay)

CD107a is a lysosomal membrane protein that becomes expressed on the cell surface as a result of cytolytic degranulation by T cells and natural killer cells [335, 336]. The presence of CD107a on the cell surface is connected to cell lysis capacity and therefore, CD107a can be used as a marker for cells that have undergone cytolytic

degranulation and cell activation. Cells were stimulated as described in section 2.6.2 then 2.5 µl of PerCP Cy5.5-labeled anti-CD107a mAb was added to the stimulated and unstimulated wells in the co-cultures. The cells were then incubated for 1 hour at 37°C and 5% CO_2, before adding the 10 µg/ml brefeldin A. The cells were then incubated for a further 4 hours. CD107a expression was analyzed by flow cytometry.

2.8.3 Cytometric Bead Array (CBA) Immunoglobulin Assay (IgM, IgA, IgG)

The BD CBA Human Immunoglobulin Master Buffer Kit assay was used to measure antibody production and immunoglobulin class switching by B cells. This assay uses capture beads, with a known size and fluorescence, to capture soluble immunoglobulin which can then be detected and analysed by flow cytometry. Supernatants from B cell cultures were transferred to 96 well plates and stored at -80℃. These supernatants were later thawed and 25 µl was used for the assay. The vial of the cytokine standard was reconstituted by adding 1 ml of assay diluent swirled gently to mix and diluted to obtain the following dilutions: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 0, 1:32, 1:64, 1:128, 1:256, 0. The capture antibody was bound to the wells of 96-well plates for 1h followed by either the sample or the standard, and incubated for 1 hour at room temperature. After that, the plate was rinsed and covered with PE detection reagent before being incubated at room temperature for 2 hours, protected from light. The samples were fixed in 4% PFA and then re-suspended in wash buffer. The instrument setup beads, which contained unstained beads and single stained beads for each APC, APC-Cy7, and PE, were used to set up the flow cytometry.

2.8.4 Measurement of cytokine release by MSD technology

The principles of the multiplex ELISA assay involved examining cytokines via a sandwich immunoassay format, where capture antibodies were coated in a patterned array

on the bottom of the wells of a plate to which the serum sample was added. The multi-spot array allowed simultaneous measurement of multiple analytes in the same well, therefore conserving sample volume. The plate was then analysed on the SECTOR Imager, via electrochemiluminescence detection technology.

This multiplex ELISA technique allows for the simultaneous detection of several cytokines by coating several particular capture antibodies on matching spots on a microplate that has been electrified. A proprietary tag (MSD Gold™ Sulfo-tag) is attached to the detecting antibody, and when the tag is activated, it produces a signal [337] which is proportionate to the amount of analyte in the sample.

The following Serum cytokines levels were measured 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-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-15, IL-17A, IL-21, IL-22 and IL-23, Mesoscale created a unique MSD®MULTI-SPOT assay plate for this study kit (catalogue number K15067L-1), which was used for analysis (MSD Diagnostics, USA). Following the extraction of peripheral blood serum, it was transferred to a 96-well MSD plate and the cytokines were measured according to the manufacturer's instructions. After that, the plate was analysed and verified using the SECTOR Imager (Meso Scale Discovery, Rockville, MD, USA; www.meso-scale.com). All of the assays had limits of detection within the predicted ranges for their respective tests.

The U-plex plate was prepared through the use of the U-plex Coupled Antibody Solutions that were created. The linker self-assembles to unique spots on the multiplex plate.. This combination was vortexed and allowed to incubate at room temperature for 30 minutes before using. Following this incubation step, 200 µl of Stop Solution was added, vortexed, and allowed to incubate at room temperature for another 30 minutes. To produce the multi

plex coating solution, 600 µl of each U-Plex linked antibody solution was added to a single tube and vortexed. A total of up to ten antibodies were pooled and made up to 6ml with stop solution.. Each well received 50 µl of multiplex coating solution, the plate was sealed with an adhesive plate seal and left to incubate at room temperature for one hour with shaking. During this time the calibator solution was prepared. Using the product certification information, the calibrators were chosen which were specific to the antibodies of interest. These were reconstituted with diluent and combined to form a calibrator master mix to a final volume of 500µl. Using serial dilutions, a 7-point calibrator curve was generated for use on the plate. Following plate incubation with coating solution, a total of three washes were performed with 150 µl/well of 1X PBS/0.05% Tween-20. The plate was now ready to be used.

Diluent 43 was used to produce the test, and 25 µl of it was applied to each well. Gentle taps were given to the plate on both sides. It was necessary to fill each calibration well in duplicate with 25 µl of the previously produced Calibrator solution plus two extra wells of diluent as the blank reading, and to fill each serum well with a corresponding amount of serum samples (25µl/well). An adhesive seal was used to secure the plate, which was then incubated at room temperature with shaking for 1 hour. During this time, the detection antibody was made as follows; 60µl of each detection antibody to a final volume of 6 ml with sample diluent. The plate was washed three times with 1X PBS/0.05% Tween-20. Each well received 50µl of detection antibody solution, which was added to each well. An adhesive seal was used to secure the plate, which was then incubated at room temperature with shaking for 1 hour. After three more wash steps, 150 µl of Read Buffer was added to each well. The plate was subjected to an MSD instrument for examination. With the use of GraphPad prism software, the data were presented in picograms per millilitre of fluid and analysed (www.graphpad.com).

2.9 Analysis of Cell Death

Direct killing of B cells by MAIT cells was measured using a Total Cytotoxicity and Apoptosis Detection Kit from Immunochemistry Technologies (MN, USA) and flow cytometry. MAIT cells were activated with IL-12 with IL-18 for 30 minutes at 37°C. $2x10^6$ cells/ml target cells (B cells) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, signal in FL-1) as described in the kit instructions. The number of stained target cells per well was adjusted to $1.5x10^6$ cells per ml. 100 µl of MAIT cells were added to obtain four MAIT cell:B cell ratios 1:1, 0.5:1, 0.2:1 and 0.05:1). The cells were co-cultured for 5 hours. Apoptotic cells were identified using Annexin V, while dead cells were detected using 7-aminoactinomycin D (7-AAD). 7-AAD is a dye that attaches to DNA and intercalates between cytosine and guanine nucleotides, giving fluorescence that can be detected by flow cytometry [338]. Apoptotic cells externalise phosphatidylserine to the surface of the extracellular membrane. Annexin V binds to phosphatidylserine [339]. For 10 minutes in the dark, 3 µl of Annexin V was applied to $1x10^5$ cells. The cells were then washed with binding buffer and 2 µl of 7AA-D was added, and cells were immediately analysed by flow cytometry. An apoptosis-positive control was created by incubating cells with 4 g/ml camptothecin for 5 hours. Target cells were treated at 56°C for 6 minutes to generate necrotic positive controls. CFSE was used to mark target cells, and flow cytometry was used to quantify apoptosis and cell death, detected using 7-AAD and annexin V. The following formula was used to calculate the percent specific lysis [338].

$$\%\ of\ specific\ lysis = \frac{\%\ (dead) target\ cells - \%\ spontaneous\ (dead) target\ cells}{100 - \%\ spontaneous\ (dead) target\ cells} X\ 100\%$$

2.10 Toxicity of MAIT cells Against B cells

Annexin V and propidium iodide (PI) were used to measure apoptosis and necrosis respectively by flow cytometry. MAIT cells, co-cultured with B cells were plated at a density of 0.1 $x10^6$ cells per well and starved overnight with serum depleted medium. Cells were treated for 24 h, 48 h and 72 h with different concentrations of MAIT cells After treatment cells were stained for Annexin V and PI to measure apoptosis and necrosis respectively. Cells were centrifugated at 1500 rpm for 7 min. Cells were washed with 1 ml of 1X of binding buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM calcium chloride, pH 7.4. 3 µl of Annexin V was added to each sample and incubated for 15 min in the dark. Cell were then washed with 1X of binding buffer. The pellets were resuspended in 250 µl of binding buffer. Immediately before flow cytometry acquisition, 1 mg/ml of PI was diluted 1:4000 in binding buffer and 250 µl was added to the appropriate tubes. Cells were incubated at 56°C for 6 min to generate the necrosis positive controls.

2.11 power calculations

To compare cell numbers and phenotypes in NE chapter for subject groups, sample sizes were calculated from data obtained in our previous studies using R software. Using the results of an unpublished study comparing Vδ1 T cell frequencies in uninfected and HIV-infected children from Uganda, a power calculation (n=(((Zα+Zβ)^2)*(2*(σ^2)))/(δ^2)) with 0.95 power and $P<0.05$ (δ=4.1; SD=4.6) gave a sample size of 34 subjects in each group (2-tailed test). But because of a study limitation we only got 30 NE subjects in the neonatal period.

A pilot study of intracellular IL-17 expression by Vδ1 T cells within PBMC from patients with HIV infection and control subjects stimulated with phorbol myristate acetate (PMA) and ionomycin has given preliminary data allowing calculation of sample size requirements for comparing cytokine expression between NE patients and control

subjects. Using a conservative estimate of the magnitudes of the differences in IL-17^{+} Vδ1 T cells between patients and controls, we found that 8 subjects in each group will be adequate (α=0.05; β=0.1; power = 90%).

2.12 Statistical Analysis

Statistical analysis was done using GraphPad Prism Version 9.0 (GraphPad Prism, San Diego, California). All data is expressed as mean ± SEM. Statistical tests used are indicated in the relevant figure legend. Statistical significance was considered at $p<0.05$. Kruskal-Wallis testing (non-parametric 1 way ANOVA) with Dunn's multiple comparison testing analysis was used when multiple groups were compared based on the variance of the dataset for control chapter. The Mann Whitney U test was used to compare NE patients and controls.

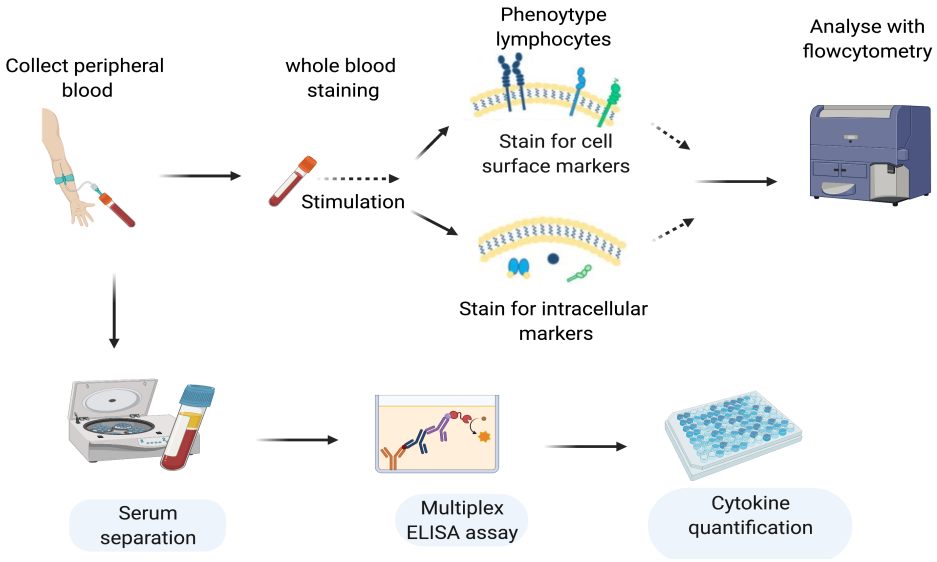

Figure 2.3 Diagram depicting the methodology which used for results chapter. *Peripheral blood was collected and stained for surface and intracellular markers expression, then analysed by flowcytometry, the rest of serum was tested for different cytokines assay by multiplex ELISA assay. Figure created with BioRender.com*

CHAPTER 3

Comparison of the lymphoid cell compartments in cord blood and in peripheral blood from healthy neonates, children, young adults and older adults

3.1 Introduction

Development of the immune system commences during pregnancy and continues into adulthood. The foetus lives in a semi-allogeneic, protected environment, whereas neonates are subjected to a sudden onslaught of colonisation by the microbiota and infection by pathogens. Each year, infections are linked to around 40% of the 3 million neonatal global deaths [38, 340].

The functioning of both innate and adaptive immunity in humans is influenced by a variety of elements. Environmental factors, infectious disease exposure, diet, pharmaceutical therapies, psychological stress, sleep quality, and chronological age are among the most researched topics [58, 341-345]. Several studies have found that the changes in immune responses are defined by three major stages of life: childhood, adulthood, and old age [58, 346-349]. The immune system is considered inexperienced at birth, with a predisposition to be more tolerogenic and driven by the innate immune response, while the adaptive immune system is still developing. When faced with several external stressors during development, the adaptive immune system develops to produce a more pathogen-specific response. A significant change occurs at later ages, characterized by an augmented pro-inflammatory milieu, T-cell senescence, and a decrease in T and B cell diversity [350-353].

Because exposure to foreign antigens is confined to maternal alloantigens, foetal and neonatal T cell activity has been primarily described as tolerogenic. Therefore, naive $CD4^+$ T cells tend to develop into $Foxp3^+$ $CD25^+$ regulatory T cells (Treg) [354, 355]. The numbers of circulating mature $CD4^+$ and $CD8^+$ T cells increases just before birth, most likely as a response to a change in exposure to environmental antigens [356, 357]. Other subpopulations of T cells such as γδ T cells and iNKT have been reported to have

a higher presence in neonates compared to adults [358-360]. Their capacity to rapidly induce production of interferons (IFNs) may serve as a compensatory mechanism for the poorly developed adaptive T cell response at that age.

B cell function in neonates has been described to be reduced due to low expression of antigen receptors on the cells, impaired somatic hyper-mutation and long-term plasmablast survival and differentiation, and predominance of low-affinity IgM producing cells [361-364].

As a child grows, the immune response tends to move toward T and B cell reactive repertoires as well as a more established immunological memory, which is primarily characterized by infection and vaccination over the course of the child's lifespan [365, 366]. The induction of effector responses and regulatory mechanisms, as well as epigenetic reprogramming of T and B cells, have been connected to exposure to various antigens and microbiota in the gut, respiratory tract, and skin [367, 368]. It has been documented that numbers of circulating Treg cells decrease, whereas numbers of naive T cells and memory Th1, Th17, and Th2 cells increase during childhood [9, 58, 369]. Antigen presentation, class switching, and somatic hypermutation in B cells, enhance the generation of antibodies, with improved capability for neutralizing invading pathogenic organisms. An individual's memory T and B cell repertoire will evolve throughout childhood and adulthood to recall previous infections and immunizations [367]. Migration of antibody-producing plasma cells to the bone marrow is required for memory cell maintenance throughout life and appears to be assisted by asymptomatic viral infections such as influenza, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and others [58, 370, 371]. This growth of the adaptive immune response will last throughout adulthood.

The immunological response deteriorates as people get older. Some of the age-related factors involved in the decline of the immune response appear to be related to poor regulation of damaged cells, increased gut permeability, changes in microbiota, cellular senescence, immune cell dysregulation, dysfunctional inflammasome activation or oxidative stress, chronic infections, and genetic susceptibility [350, 352]. Long-term immunological memory diminishes along with other adaptive immune mechanisms, limiting the response to infections. Inflammageing, now recognized as chronic morbidity, disability, and frailty, has been linked to a predominant proinflammatory environment due to persistent inflammation, [350, 352, 372]. As a result of a decreased production of naive T cells in a shrinking thymus and accumulation of highly differentiated senescent T cells, T cell diversity in the elderly is significantly reduced [353, 373]. In vitro studies have also revealed that the NF-κB signalling pathway of $CD4^+$ T cells from elderly people is altered, resulting in an increase in the production of pro-inflammatory cytokines as compared to younger people [374, 375]. In addition, IFN-γ production and cytotoxicity by NK and NKT cells has been reported to decline at this stage of life [376]. The peripheral B cell pool, like the T cell pool, accumulates antigen-experienced memory cells while drastically reducing the number of naive B cells, resulting in reduced clonal diversity and, as a result, higher frequencies of non-specific antibodies [351].

Although there are several reviews that compare adaptive immune cell types at various ages, few research studies specifically examine how they develop over time. We compared lymphocyte frequencies in cohorts of healthy donors from different age groups to see how the innate and adaptive immune systems behave in healthy conditions over the ages. Changes in B cells, NK cells, and distinct subpopulations of T cells, as well as pro and anti-inflammatory cytokines levels in serum, were measured in cord blood, neonates, school-aged children, young adults, and the older adults.

3.2 Aims and hypothesis

The overall aim of this chapter was to characterise the lymphoid cell compartments in cord blood and in peripheral blood from healthy term neonates, school age children, young adults and older adults.

The specific aims were:

1. To enumerate and phenotypically characterize circulating T cells, B cells, NK cells, γδ T cell subsets, MAIT cells, and iNKT cells in healthy subjects grouped according to age.
2. To measure serum cytokine levels in the five subject groups.

3.3 Specific methods

3.3.1 Patient specimens

Cord blood samples were obtained from 10 term neonates and peripheral blood and serum samples were obtained from 17 neonates, 23 school-age children, 25 young adults and 10 elderly subjects aged 7-92 years (median 83 years) who were recruited from St. James's Hospital. Blood from neonates was taken immediately after normal vaginal delivery. All neonates had normal neurological examination at birth and normal Apgar scores. Newborn babies with congenital anomalies or evidence of maternal substance abuse were excluded. Blood and serum samples were also obtained from 23 healthy school-age children (aged 1-16 years) coming for day case surgeries or undergoing phlebotomy, from 25 young adults (aged 23-45 years) working at St. James's Hospital, and from 10 older subjects (aged 71-92) attending St. James's Hospital. All subjects had no history of chronic infection or immune-mediated illnesses. The clinical state of the older adults was stable at time of phlebotomy, with no signs of infection, and their white blood counts were within normal range. They were attending hospital because of fall and fracture, for

intracranial haemorrhage, gastritis, abdominal pain, depression or pneumonia. None of these patients were on any form of immunotherapy. The demographics of the older adults studied are shown in Table 3.1. The demographics of the neonates studied are shown in Table 3.2.

Table 3-1 Demographic characteristics of older adults cohort

Category	Parameter
Age, years; median (IQR)	83 (71-92)
Sex	N=5 male, N=5 female
Co-morbidity count, n; median (IQ)	4 (2-7)
Co-medication count, n; median (IQ)	6.5 (3-10)
WBC count (average ±SD)	6.8 (1.36±)
Lymphocyte count (average ±SD)	1.45 (±0.37)
Cause of admission	4= fall 1= intracranial haemorrhage 1= heart failure 1= abdominal pain 1= worsening 1= confusion 1= depression 1= cognitive decline

Table 3-2 Demographics of neonatal Control

Variables		Control (n=17)
GA (wks) [a]		38.56 ± 1.03
BW (kg)		3.52 ± 313
Gender, male, n (%)[b]		56%
Mode of delivery (%)	LSCS	5 (29.4)
	SVD	11(64)
	inst	1 (6)
Apgar @1 min [a]		9
Apgar @5 min [a]		10
Apgar @10 min [a]		N/A
TH, n (%)[b]		N/A
Seizures, n (%)[b]		N/A
MRI-Abnormal, n (%)[b]		N/A
Cord Arterial PH [a]		N/A
Cord Arterial BE [a]		N/A
CPR, yes (%)[b]		N/A
Intubated, yes (%)[b]		N/A

GA = Gestational Age; BW = Birth Weight; LSCS = Lower Section Caesarean Section; SVD = Spontaneous Vaginal Delivery; Inst = Instrumental delivery

3.3.2 Antibodies and flow cytometry

Whole blood was stained with a dead cell stain followed by fluorochrome-conjugated monoclonal antibodies (mAb) specific for glycophorin A to exclude immature erythrocytes, human CD3, CD4 and CD8 to detect T cells, CD19 to detect B cells, CD3 and CD56 to detect NK cells, CD3, CD161 and Vα7.2 to detect MAIT cells, CD3 and Vα24Jα18 to detect iNKT cells, and CD3 with Vδ1 or Vδ2 to detect Vδ1 T cells and Vδ2 T cells, respectively (Table 2.4 in methods chapter). The gating strategy used for the enumeration of total, $CD4^+$, CD8+, $CD4^-CD8^-$ and $CD4^+CD8^+$ T cells, B cells, NK cells, MAIT cells, Vδ1 T cells, Vδ2 T cells and iNKT cells is shown in Figure 2.1.

3.3.3 Measurement of serum cytokine levels

Serum cytokine levels were measured using the U Plex biomarker group 1 multiplex assay for IFN-γ, TNF-α, IL-2), IL-4, 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. As described in section 2.8.4 in methods chapter.

3.3.4 Statistical analysis

GraphPad Prism Version 9 (GraphPad software, San Diego, CA) was used to perform statistical analyses. Kruskal-Wallis testing (non-parametric 1 way ANOVA) with Dunn's multiple comparison testing analysis was used when multiple groups were compared based on the variance of the dataset. Statistical significance is described as *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3.4 Results

3.4.1 Lymphocyte frequencies in healthy donors grouped according to age

The frequencies of T cells, B cells and NK cells, as percentages of lymphocytes, in cord blood and peripheral blood from different age groups were analysed by flow cytometry. The frequencies of T cells were lower in cord blood samples compared to blood samples taken from all subject groups and this decrease was significant when compared with T cell frequencies in all subjects (Figure. 3.1.A, $p < 0.05$). The frequencies B cells were significantly higher in CB compared to neonatal blood (Figure. 3.1 B; $p < 0.05$) but they increased significantly during childhood at ($p < 0.001$) and then decreased again during adulthood and in older adults (Figure. 3.1 B; $p < 0.01$). We also observed expansions of NK cells in blood, starting at the neonatal stage, when they comprise up to 15% of peripheral lymphocytes and peaking in young adults when they represented up to 30% of total lymphocytes (Figure. 3.1C; $p<0.001$). These results show that T, B and NK cell frequencies fluctuate with age, and are likely to lead to age-related changes in immunocompetence.

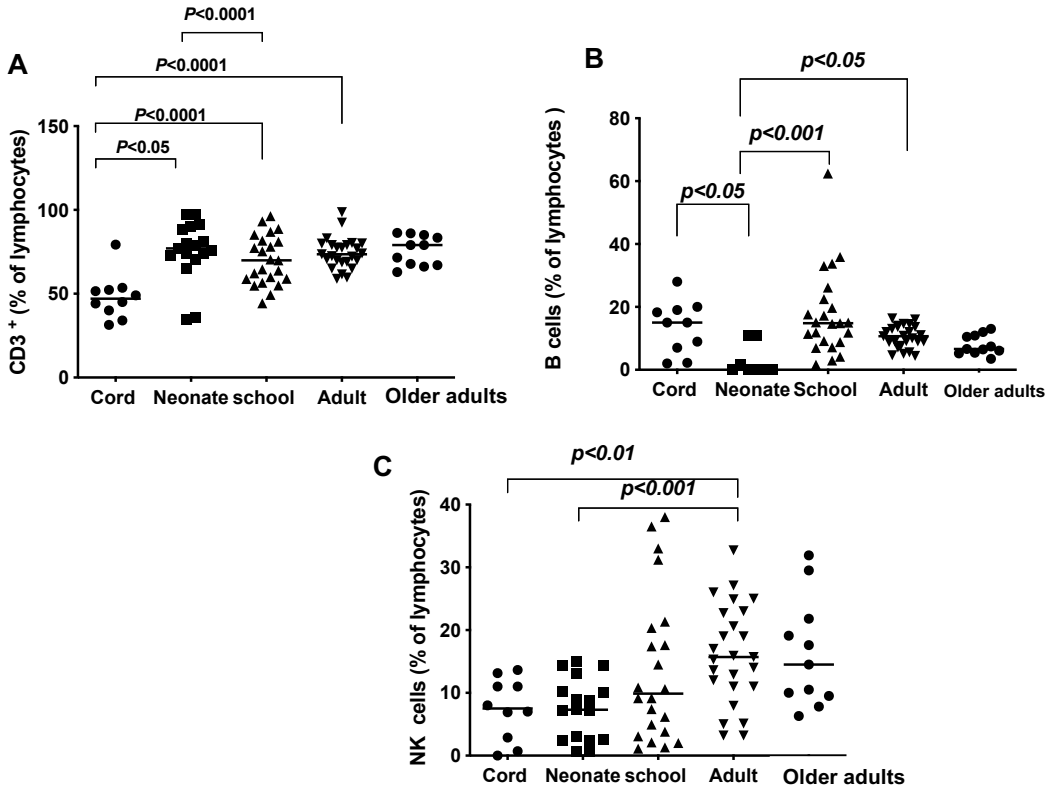

Figure 3.1 Lymphocyte frequencies in healthy donors grouped according to age. *T cell, B cell and NK cell frequencies in cord blood (n=10) and in peripheral blood of neonates (n=17), school-age children (n=23), young adults (n=25) and older adults (n=10). Whole blood was stained with mAbs specific for CD3, CD19 and CD56 and analysed by flow cytometry. Scatter plot show frequencies of $CD3^+$ T cells (A), B cells (B) and NK cells (C) as percentages of total lymphocytes. Dots represent individual patients, bars represent median; Data between groups are compared using the Kruskal-Wallis test with post hoc Dunn's test.*

3.4.2 Conventional T lymphocyte subset frequencies in healthy donors grouped according to age

Umbilical cord blood samples were collected from 10 term infants and venous blood was obtained from 17 healthy term babies, 23 school-age children, 25 adults and 10 older people. T cells were further examined on the basis of CD4 and CD8 positivity to assess whether there were variations in helper ($CD4^+$), cytotoxic ($CD8^+$) and unconventional ($CD4^+CD8^+$ and $CD4^-CD8^-$) between different age groups. Samples of whole blood were stained with mAbs specific for CD3, CD4 and CD8 and analysed by flow cytometry. After gating on singlet, live, $CD3^+$ lymphocytes, the frequencies of $CD4^+$ and $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$, as percentages of total $CD3^+$ cells, were determined (Figure 3.2). We observed that the frequencies of $CD8^+$ were similar at all stages of life (Figure. 3.2B). Figure 3.2 A shows that $CD4^+$ T cells, account for 60 - 80% of T cells in CB, but were lower in neonates, and significantly lower in school age children ($p< 0.001$). CD4+ T cells then remained at ~60% in adults and older adults. There was a significant increase in the frequencies of T cells with $CD4^-$ $CD8^-$ phenotypes from cord blood to adulthood and from neonatal life to school age children and adulthood, However, $CD4^+CD8^+$ T cell frequencies were higher among older adults when it compared with school-age children (Figure. 3.2C and 3.2D).

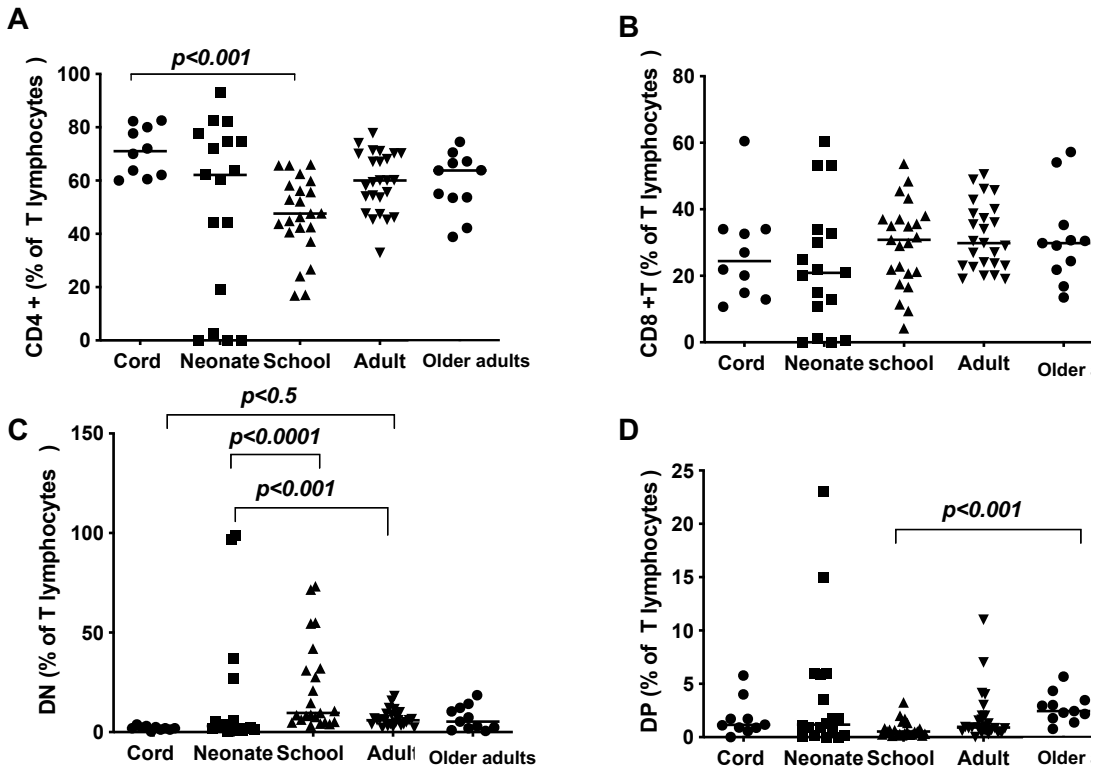

Figure 3.2 Conventional and unconventional T cell frequencies in healthy donors grouped according to age. *Frequencies of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (double positive or DP) and $CD4^-CD8^-$ (double negative or DN) T cells in cord blood (n=10) and in peripheral blood of neonates (n=17), school-age children (n=23), young adults (n=25) and older adults (n=10). Cells were stained with mAbs specific for CD3, CD4 and CD8 and analysed by flow cytometry. Scatter plot show frequencies of $CD4^+$ T cells (A), $CD8^+$ T cells (B), DN T cells (C) and (D) DP T cells as percentages of total lymphocytes. Dots represent individual patients, bars represent median; Data are compared using the Kruskal-Wallis test with post hoc Dunn's test.*

3.4.3 Innate T cells frequencies in healthy donors grouped according to age

Cord blood was obtained from 10 healthy term infants and venous blood was taken from 17 neonates, 23 children at a school-age, 25 adults, 10 older subjects for analysis of innate T cell frequencies. Whole blood was stained with a dead cell stain followed by mAb specific for CD3, CD161 and Vα7.2 to detect MAIT cells, CD3 and Vα24Jα18 to detect iNKT cells, and CD3 with Vδ1 or Vδ2 to detect Vδ1 T cells and Vδ2 T cells, respectively. Cells were analysed by flow cytometry. Figures 3.3A shows that Vδ1 T cells are present at very low frequencies (typically <0.1% of T cells) in cord blood and in peripheral blood from neonates, but their numbers increased throughout life significantly among all groups, accounting for up to 3% of T cells at school age and adulthood ($P<0.01$) and reaching highest levels in the older adults ($P<0.0001$). A similar pattern was seen for Vδ2 T cells, except that their frequencies were highest in young adults ($P<0.0001$) and then they declined slightly into old age (Figure 3.3B). iNKT cells also exhibited a similar dynamic, being undetectable or present at very low frequencies (<0.1% of T cells) in cord blood and peripheral blood from neonates but they expanded to a mean of 0.3% of T cells in young adults (Figure 3.3C). iNKT cell frequencies were not determined in the elderly subjects. Similar to iNKT cells, MAIT cells were undetectable or present at very low frequencies on cord blood and peripheral blood from neonates, but they expanded to up to 8% of total T cells in school-aged children and young adults ($P<0.01$; Figure 3.3D). MAIT cells were not studied in older adults. Thus, all of the unconventional T cells enumerated in the present investigation, are extremely rare in cord blood and in neonates, but they expand after birth, presumably in response to environmental antigens.

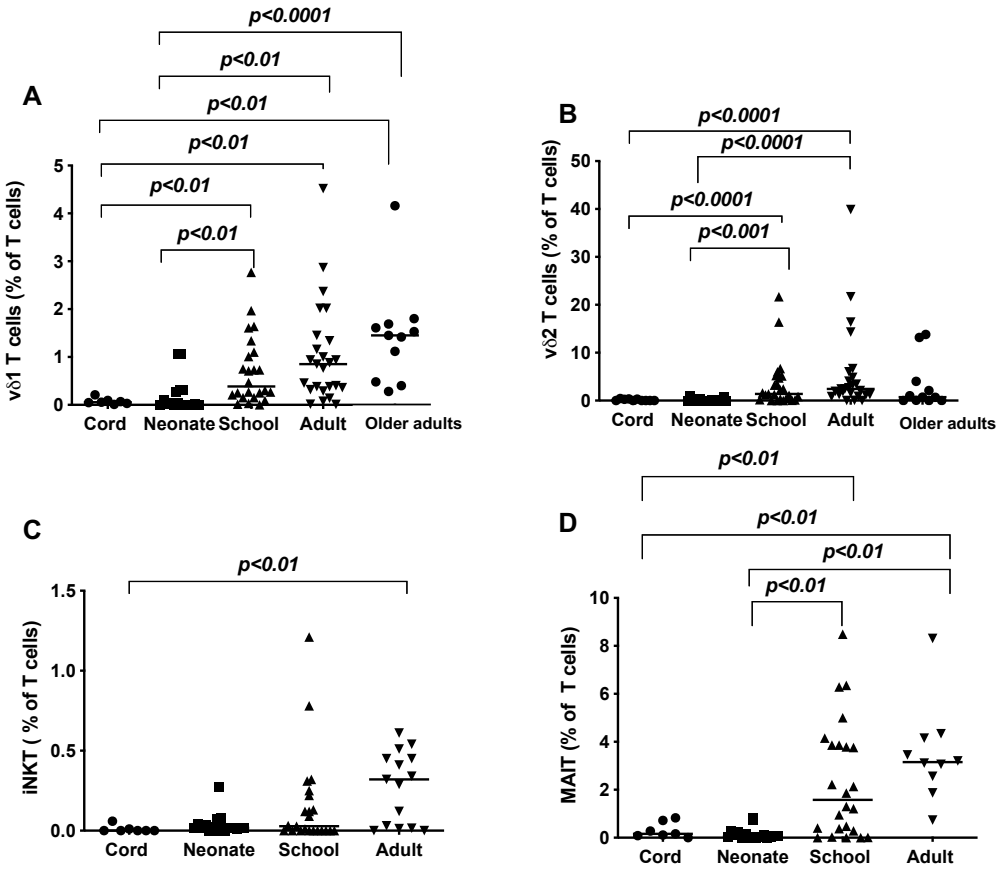

Figure 3.3 Innate lymphocyte frequencies in healthy donors grouped according to age. *Cord blood samples were obtained from 10 term babies and venous blood samples were obtained from 17 neonates, 23 school age children and young adults (n=25) and older adults (n=10). Cells were stained with mAb specific for CD3 and TCR Vδ1 to detect Vδ1 Tcells, CD3 and TCR Vδ2 for Vδ2 T cells, CD3, CD161 and Vα7.2^{+} for MAIT cells, and CD3 and Vα24Jα18 TCR for iNKT cells, then analysed by flow cytometry. Scatter plot show frequencies of Vδ1 T cells (A), Vδ2 T cells (B), MAIT cells (C), and iNKT cells (D). Dots represent individual patients, bars represent median; Data are compared using the Kruskal-Wallis test with post hoc Dunn's test.*

3.4.4 Phenotype of Circulating MAIT Cells in Healthy Subjects

As mentioned above, we defined human circulating MAIT cells as $CD3^+$ $TCRV\alpha7.2^+$ $CD161^{hi}$ cells by flow cytometry (Figure 3.3 D) as indicated in a previous report [377]. We also subdivided MAIT cells into $CD4^+$, $CD8^+$ and double negative (DN) subsets [31, 51, 240, 302, 359, 377-380]. We also investigated CD4 and CD8 expression by MAIT cells in the different subject groups. CD4+ MAIT cells were extremely rare (data not shown), whereas most MAIT cells were found to express CD8 or neither CD4 nor CD8. Interestingly, the frequencies of CD8+ MAIT cells as percentages of total MAIT cells, were higher in the circulation of neonates compared to cord blood ($P<0.001$) and they increased significantly into school age and adulthood ($P<0.001$; Figure 3.4A). A corresponding decrease in the frequencies of CD4- CD8- MAIT cells with age was observed ($P<0.001$; Figure 3.4B).

3.4.5 MAIT cells from young adults display higher expression of CD69

Analysis of activation status of MAIT cells, by measurement of CD69 expression, revealed that <8% of MAIT cells from cord or neonatal blood exhibited activated phenotypes, but these frequencies rose to means of 12% in school-age children and 18% of young adults ($P<0.001$; Figure 3.5). These data suggest that CD8+ MAIT cells are selected and activated in response to environmental antigens, starting at birth. Of note, there is a considerable variation for each subset of MAIT cells among individuals across all the age groups. Notably also, we did not detect $CD4^+$ MAIT cells.

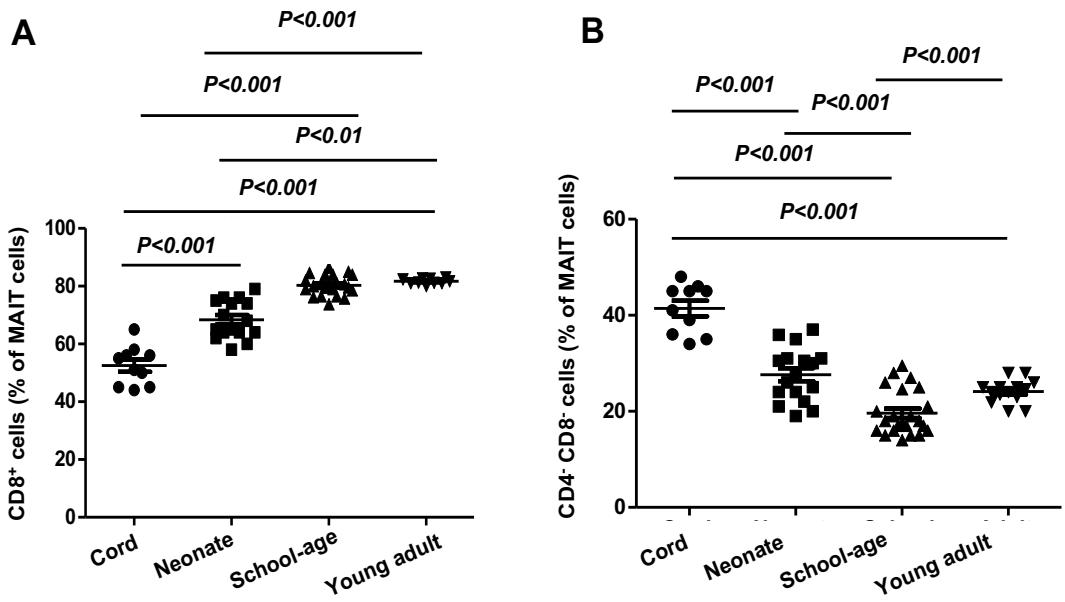

Figure 3.4 Frequencies of CD8$^+$ and double negative MAIT cells in cord blood (n=10) and in peripheral blood of neonates (n=17), school-age children (n=23), young adults (n=25). *Cells were stained with a dead cell stain and mAb specific for CD3, CD4, CD8, CD161, Vα7.2 and analysed by flow cytometry. Scatter plot show frequencies of CD8 MAIT cells (A), CD4$^-$CD8$^-$ MAIT cells (B), as percentages of MAIT cells. Dots represent individual patients, bars represent median; Data are compared using the Kruskal-Wallis test with post hoc Dunn's test.*

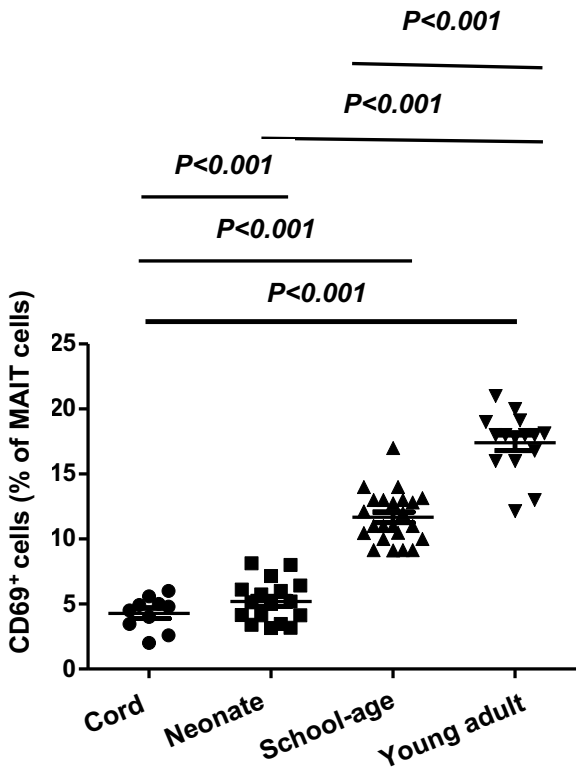

Figure 3.5 Activation status of MAIT cells in cord blood and peripheral blood of neonates, children and adults*. Cord blood (n=10) and peripheral blood of neonates (n=17), school-age children (n=23) and young adults (n=15) was stained with a dead cell stain and mAb specific for CD3, CD161, Vα72 and CD69 and analysed by flow cytometry. Scatter plot shows frequencies of CD69 MAIT cells as percentages of total MAIT cells. Dots represent individual patients, bars represent median; Data are compared using the Kruskal-Wallis test with post hoc Dunn's test.*

3.4.6 Serum cytokine levels in healthy subjects

The levels of IFN-γ, TNF-α, IL-12, IL-4, IL-6, IL-15, 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. Figure 3.6 shows that the serum levels of the Th1-associated cytokines IFN-γ and TNF-α were significantly higher in school-age children and adults compared to neonates and CB samples ($p < 0.0001$).

The levels of the Th2 cytokines IL-4 and IL-5 were similar in all subject groups, but the levels of IL-9 level fluctuated, being significantly lower in neonatal blood compared to CB ($p < 0.05$) and the levels dropped significantly in the school children ($p < 0.001$) and increased in adult life ($p < 0.05$; Figure 3.7).

No differences in the serum levels of the Th17-associated cytokines, IL-17a and IL-21 were found in the different age groups. Serum levels of IL-22 were significantly higher in neonates and children compared to cord blood, while IL-23 levels were significantly lower in all the cohorts compared to cord blood at ($p < 0.001$ and $p < 0.05$ respectively; Figure 3.8).

The levels of the proinflammatory cytokine IL-6 were considerably higher in adults compared to CB, neonates and children ($p < 0.0001$, $p < 0.01$ and $p < 0.01$, respectively). The same pattern was observed with IL-8 ($p < 0.0001$, $p < 0.0001$ and $p < 0.0001$). Similarly, IL-10 showed a raising trend with significantly higher levels in adults compared to CB samples and neonates respectively ($p < 0.0001$; Figure 3.9).

The levels of the growth factor for T and NK cells IL-2 were slightly elevated in neonates and adults compared to CB control subjects but this was not significant. Lastly IL-15 levels were significantly higher in neonates compared to the CB and higher in school-age children compared to adults ($p < 001$ and $p < 0.0001$, respectively; Figure 3.10).

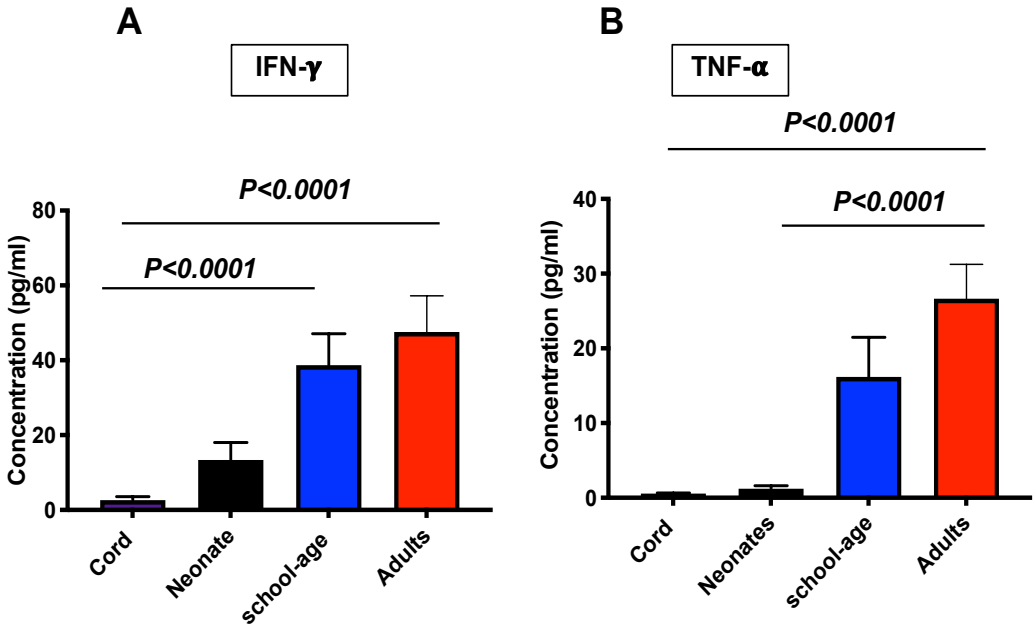

Figure 3.6 Serum Th1 cytokines levels in in healthy donors grouped according to age. *Concentrations of IFN-γ and TNF-α were measured in 10 cord blood samples and peripheral blood from 10 neonates, 10 school age children and 10 adults using multiplex ELISA. Graphs show mean ± SEM levels of IFN-γ (A) and TNF-α (B) in the subject groups. Bars represent median, and whiskers represent the range; Data were compared using the Kruskal-Wallis test with post hoc Dunn's test.*

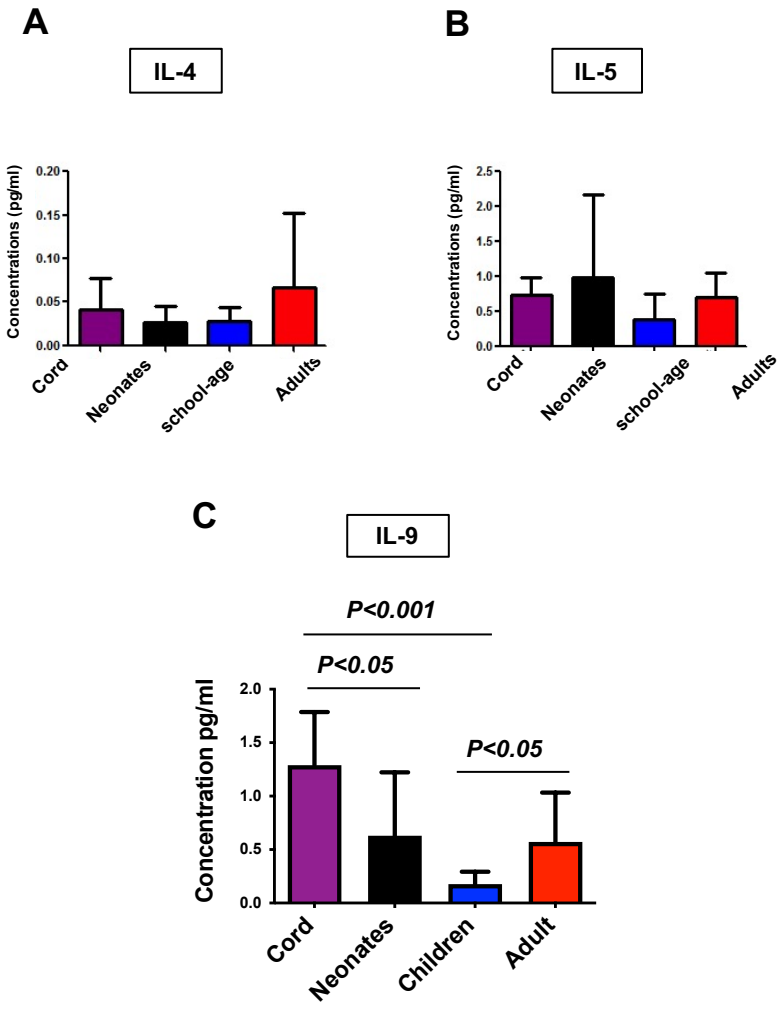

Figure 3.7 Serum Th2 cytokine levels in healthy donors grouped according to age. *Concentrations of IL-4, IL-5, and IL-9 were measured in 10 cord blood samples and peripheral blood from 10 neonates, 10 school age children and 10 adults using multiplex ELISA. Graphs show mean ± SEM levels of IL-4 (A), IL-5 (B), and IL-9 (C), in the subject groups. Bars represent median, and whiskers represent the range; Data were compared using the Kruskal-Wallis test with post hoc Dunn's test.*

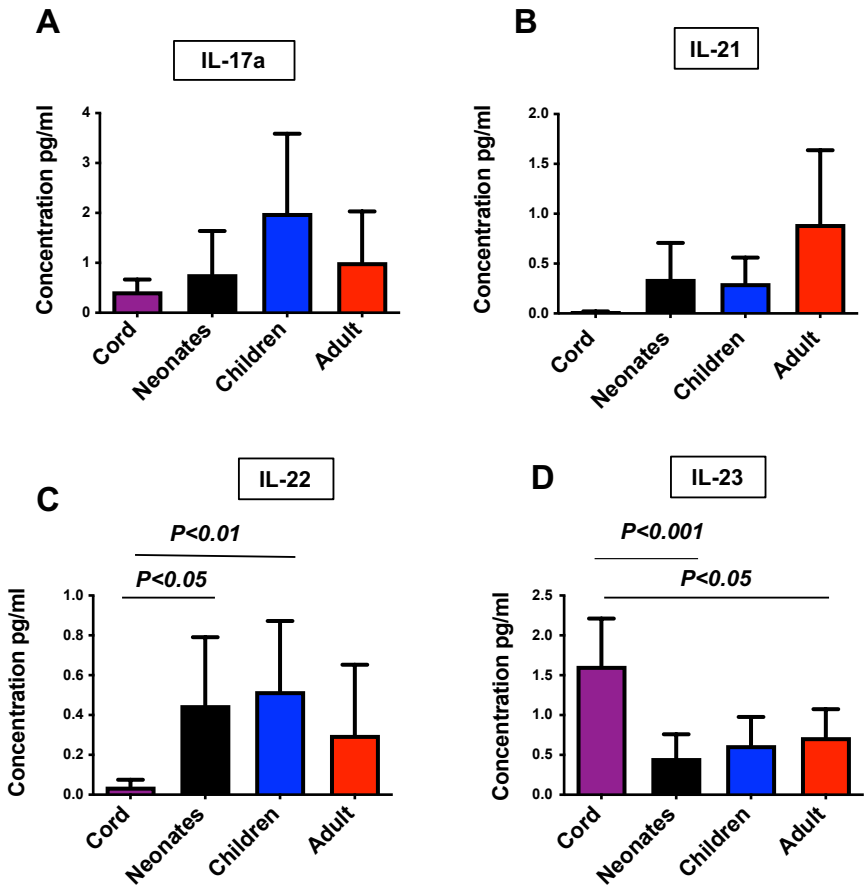

Figure 3.8 Serum Th17 cytokines levels in in healthy donors grouped according to age. *Concentrations of Interleukin-17a (IL-17a), Interleukin-21 (IL-21), Interleukin-22 (IL-22) , Interleukin-22 (IL-23) were measured in 10 cord blood samples and peripheral blood from 10 neonates, 10 school age children and 10 adults using multiplex ELISA. Graphs show mean ± SEM levels of IL-17a (A), IL-21 (B), IL-22 (C), and IL-23 (D), in the subject groups. Bars represent median, and whiskers represent the range; Data were compared using the Kruskal-Wallis test with post hoc Dunn's test.*

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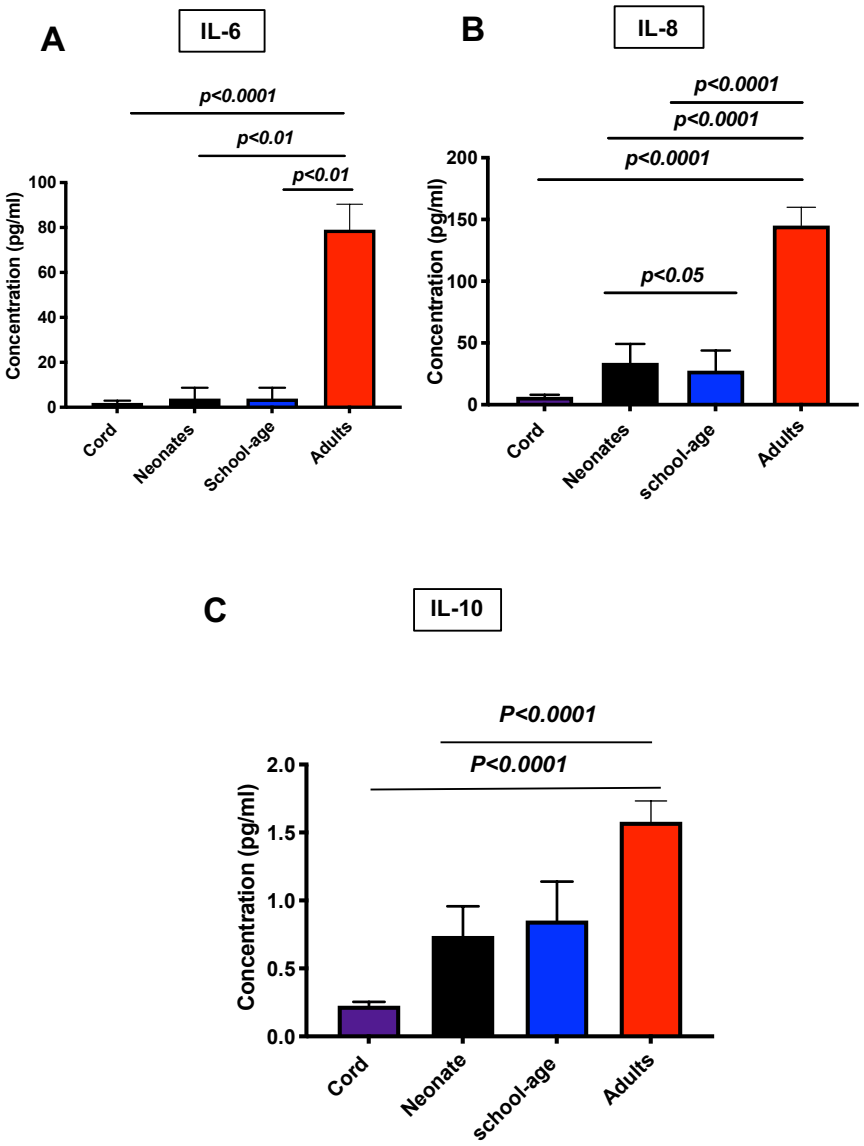

Figure 3.9 Serum Proinflammatory cytokines levels in healthy donors grouped according to age. *Concentrations of Interleukin-6 (IL-6), Interleukin-8 (IL-8), and Interleukin-10 (IL-10) were measured in 10 cord blood samples and peripheral blood from 10 neonates, 10 school age children and 10 adults using multiplex ELISA. Graphs show mean ± SEM levels of IL-6 (A), IL-8 (B), and IL-10 (C), in the subject groups. Bars represent median, and whiskers represent the range; Data were compared using the Kruskal-Wallis test with post hoc Dunn's test.*

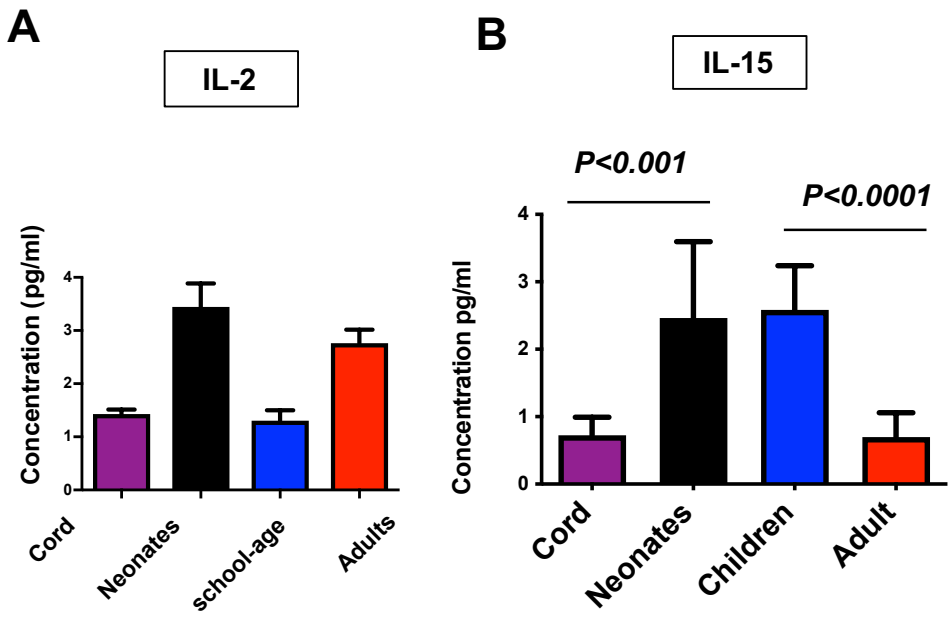

Figure 3.10 Serum T and NK cell growth factors levels in healthy donors grouped according to age. *Concentrations of interleukin-2 (IL-2), and interleukin-15 (IL-15) were measured in 10 cord blood samples and peripheral blood from 10 neonates, 10 school age children and 10 adults using multiplex ELISA. Graphs show mean ± SEM levels of IL-2 (A), and IL-15 (B) in the subject groups. Data were compared using the Kruskal-Wallis test with post hoc Dunn's test.*

3.5 Discussion

Formation of the adaptive immune system begins early in fetal life. B cells are present in the blood and spleen by 12 weeks of gestation and T cells start to depart from the thymus from about 14 weeks. In the fetus, responsiveness to infection is low and is frequently associated with spontaneous abortion [381-383]. Consequently, the immune system is inexperienced at birth, with a tendency to be tolerogenic, rather than immunogenic. During the neonatal period, the infection response is skewed towards a pro-inflammatory response and the immune system acquires adaptive features as a result of exposure to microbes [384]. As old age progresses, major components of the immune system are impaired and persistent inflammation can lead to chronic morbidity, disability and frailty [346, 352, 372-374].

We compared the potential functional effectiveness of the innate and adaptive immune systems in cohorts of healthy donors from different age groups. Flow cytometry was employed to profile the frequencies of conventional T cells, B cells and NK cells, and subpopulations of innate T cells in cord blood samples and peripheral blood samples from neonates, school-aged children, young adults and elderly people. The results are summarised in Table 3.3. We found that the frequencies of T cells were lower in cord blood compared to peripheral blood at all age groups. T cell frequencies remained relatively constant throughout life, and we did not observe any significant changes in their expression of CD4 and CD8 from the neonatal to older adult stages. Similar findings have been reported by Santagostino and co-workers (2003), but other workers have reported reductions in the numbers of $CD4^+$ and $CD8^+$ T cells with age [385, 386]. Similar to previous reports [387-390]. we found that B cell frequencies increased after birth, reaching highest levels at school age and then declining gradually throughout adulthood. On the other hand, NK cell numbers were low in cord blood and in neonatal

blood, but they gradually increased throughout adulthood. This increase in NK cells with age has also been reported by others [391-394]. Previous studies have demonstrated that the expansions in the frequencies of NK cells seen in older adults patients are mostly attributable to expansions of the $CD56^{dim}$ subset and reductions of the $CD56^{bright}$ subset [391, 395-397].

The four innate T cell populations investigated in the present study - Vδ1 T cells, Vδ2 T cells, iNKT cells and MAIT cells all showed similar dynamics across the age groups. All four T cell populations were undetectable or present at very low numbers in cord blood and in peripheral blood of neonates, but they expanded after birth reaching highest levels in adulthood. Using next-generation sequencing of γδ TCRs in cord blood and in infants, Davey and co-workers (2017) and Ravens et al (2020) demonstrated that Vδ1 T cells express naïve phenotypes at birth and differentiate into memory cells in response to antigenic exposure, typically becoming strongly focused on a few high-frequency clonotypes by adulthood. In contrast, Vδ2 T cells are the predominant population of γδ T cells in the fetus and in adults. Unlike CD4 T cells, they are poised for rapid Th1-like responses even before birth, which allows them to play a key role in the first line of defense against pathogens in early life [275]. The majority of Vδ2 T cells express Vγ9Vδ2 T cell receptors that recognise the phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, an intermediate in the 2-C-methyl-d-erythritol 4-phosphate pathway of isoprenoid synthesis found in some bacteria and protists and postnatal expansion of Vγ9Vδ2 T cells is thought to reflect exposure to phosphoantigen-containing bacteria [398, 399]. Although fetal γδ T cells are distinct from most adult γδ T cells in terms of TCR specificity and function [400-402], phosphoantigen-responsive Vγ9Vδ2 T cells are also present in the fetus before postnatal microbial exposure occurs [275]. Shortly after birth, Vδ2 T cells acquire more potent proliferative and cytotoxic activity, which is

associated with downregulation of programmed death-1 (PD-1) and upregulation of NKG2A [403].

Confirming a previous report by Prabhu and co-workers (2016), we found that iNKT numbers are very low in cord blood and in neonates but they expand after birth, reaching highest levels in adulthood. iNKT cells have important influences on adaptive immune responses via their ability to rapidly release Th1, Th2, Th17 and Treg cytokines and to mediate contact-dependent maturation and activation of dendritic cells, macrophages and B cells. For this reason, iNKT cells play multiple roles in the pathology of and protection against multiple diseases and are activated and expanded in neonates with preterm birth and neonatal encephalopathy [404, 405]. In germ-free mice, iNKT cells colonise the intestinal and pulmonary mucosa and contribute to inflammatory bowel disease and asthma, but colonisation of these organs with a conventional microbiota at birth, but not in adulthood, protected the mice against these inflammatory disorders [406].

Another innate T cell subset that expanded from very low numbers after birth is MAIT cells. Similar to the findings of Walker and co-workers (2012) and Chen et al (2019), we found that MAIT cell frequencies increase from birth to adulthood and other studies have demonstrated that their numbers decline in elderly subjects, in part due to an increased susceptibility to apoptosis [380, 407]. Interestingly, we found that the proportions of MAIT cells that expressed CD8 increased from the neonatal stage up to adulthood and $CD8^+$ MAIT cells constituted the majority of MAIT cells in adults. Conversely, the proportions of MAIT cells that expressed double negative $CD4^-$ $CD8^-$ phenotypes decreased with age. $CD4^+$ MAIT cells were found to be very rare at all stages of development. Consistent with previous reports [407], we found that the expression of the T cell activation marker CD69 on MAIT cells increased progressively from cord blood to elderly populations, suggesting that they are selected and activated by exposure to

microorganisms during development. Circulating MAIT cells can produce multiple cytokines that were differentially regulated with age, showing the lowest expression IFN-γ in the young, compared with older children [407]. Thus, the numbers, phenotypes and functions of MAIT cells appear to depend on age and vary in different clinical settings such as human immunodeficiency virus and *Helicobacter pylori* infection.

In addition to measuring lymphocyte subset frequencies, we measured the levels of serum cytokines, which may be produced by these and other cells. Using a multiplex ELISA assay, we found that levels of Th1 associated cytokines, including IFN-γ and TNF-α, were high in adults and school children but low in cord blood and in neonates. The low IFN-γ and TNF-α levels in neonates were quantitatively comparable to those reported previously [408-410] and may explain why neonates have greater susceptibility to intracellular infections [411, 412]. Similar levels of the Th2 cytokines IL-4 and IL-5 were observed at all ages, but the levels of IL-9 were high in cord blood but low in serum from neonates and decreased in school children before increasing in adults. A study conducted by Kleiner and colleagues (2013) in healthy donors ranging in age from one year to more than eighteen years old discovered that children aged 7 to 17 years had higher levels of the Th2 cytokine IL-4 than adults in the same study.

In the present study, levels of the Th17 associated cytokines, IL-17A and IL-21 did not change significantly from the neonatal stage to adulthood, but levels of IL-22 were found to be lower and levels of IL-23 were higher in cord blood compared to peripheral blood of neonates, school-age children and adults. Kleiner and co-workers (2013) reported that IL-17 levels rise with age, and we found a non-significant increase in IL-17 levels in adults compared to children.

Finally, the levels of the proinflammatory cytokines IL-6 and IL-8 were high in adults compared to other cohorts. These data are in line with other publications reporting an enhancement of Th1 and proinflammatory cytokines activity with age [413, 414].

Our data show that specific lymphocyte populations and cytokine levels vary according to age from the fetal stage to adulthood and into older stages of life. These changes are likely to affect immunocompetence, which will vary according to the type of infection or danger. A thorough understanding of these dynamics is critical, as this may be a major component of disease initiation and progression. There is a level of similarity in the immune system between aged people and newborns, with decreases in antimicrobial activities of neutrophils and macrophages, a decline in antigen presentation by dendritic cells, reduced NK cell killing, and impaired adaptive lymphocyte responses. Therefore, both young and old age people could be considered to have compromised immune systems which may make them susceptible to certain infections [58]. In older adults, a particularly striking feature of the immunosenescence process is the development of a low-grade pro-inflammatory state, characterised by an increase in serum inflammatory mediators such as IL-6, IL-1Ra, TNF, IL-1, and C-reactive protein [372, 415] As a result of these impairments, older people are more prone to cancers, autoimmune diseases and poor outcomes of infectious diseases, such as SARS-CoV-2 [372, 416-418]. Although we did not test inflammatory cytokine levels in our older adult cohort, we did note a tendency for inflammatory cytokine levels to increase over the age ranges of our cohorts.

3.6 Conclusion

Table 3.3 shows that, compared to young adults, neonates have lower levels of circulating innate T cells and lower levels of inflammatory and Th1 cytokines. Thus, the neonatal immune system appears to be impaired, leaving infants susceptible to infectious

disease . At later stages of life, there are expansions of innate lymphocytes and increased levels of inflammatory cytokines. These data suggest that innate T cells do not represent a significant component of the foetal immune system but that they emerge rapidly after birth, presumably in response to microbial colonisation. It is possible that innate T cells play pivotal roles in the changing immune system from birth to old age. Innate T cells respond rapidly to conserved antigens and can influence the activities of T cells, B cells, NK cells, neutrophils, macrophages and dendritic cells. Their paucity in infants may underlie the frequent failure of the innate and adaptive systems to respond to antigenic challenge, whereas the high numbers of these cells in older individuals may result in overstimulation of innate immune responses leading to inflammatory disease. Future studies are required to determine if iNKT cells, subsets of γδ T cells or MAIT cells can most be most appropriately targeted. Our data suggest that distinct cell types may optimally control homeostatic and disease-associated immunity at different stages of life and support the view that age is an important determinant of susceptibility to disease.

Table 3.3 Summary of circulating lymphocyte numbers fluctuate with age

	Cord blood	Neonates	Children	Adults (18-64)	Adults > 65 years
T cells	↓			60-80%	
B cells		↓		5-15%	
NK cells		↓		5-30%	
Innate T Cells	↓	↓		<10%	
Th 1 cytokines	↓	↓			Not tested
Th2 cytokines					Not tested
Th17 cytokines					Not tested
Proinflammatory cytokines	↓	↓	↓		Not tested

Arrows indicate significantly higher or lower frequencies of cells populations and cytokines levels compared with those in the young adults with % being included in the table for Adults.

CHAPTER 4
Characterisation of the lymphoid cell compartments in blood from NE and healthy subjects

4.1 Introduction

Neonatal encephalopathy (NE), is the primary cause of neonatal mortality and long-term neuro-impairment such as cerebral palsy [378, 419]. NE is a syndrome characterized clinically by a disruption in neurological function, including an alteration in the level of consciousness, abnormal neurological signs such as seizures, weak reflexes, inadequate feeding and difficulty in maintaining adequate respiration [420]. The incidence of NE varies according to geographical location and socioeconomic status. It occurs at approximately 1 to 3.5 per 1000 live term newborns in developed nations and up to 26 per 1000 live term newborns in less developed countries [23, 421, 422].

NE has multiple aetiologies but perinatal hypoxia-ischemia is responsible for the most cases leading to HIE. Other causes include infections, metabolic conditions, coagulopathies, and neonatal stroke [423]. In half of NE cases, the primary reason is unidentified and is likely to be a combination of many factors [424].

There is an association between multiorgan failure and the severity of asphyxia. About 50% of infants with moderate to severe NE develop prolonged-term morbidity and mortality [29]. Survivors of NE are at risk of several long-term neurodevelopmental impairments, including cerebral palsy, intellectual disability, impairment of language skills or working memory, and problematic behaviour [425]. The only gold standard evidence-based therapy available for these new-borns is therapeutic hypothermia [11]. Until now, no single biomarker has proved to be sufficiently sensitive and specific to help in the prediction of NE occurrence and severity of brain injury in infants both with and at risk of NE [85, 425]. Therefore, there is an urgent need to develop new biomarkers and therapeutics for NE.

Emerging evidence has suggested that the inflammatory responses that occur due to hypoxia-ischemia are a crucial contributor in the pathophysiology of NE in both term and preterm neonates. Hypoxia ischemia elicits immediate and robust activation of brain resident cells, followed by peripheral leukocyte recruitment. This results in secondary neuronal damage that can persist for several months or even years, and subsequently, the anti-inflammatory response is initiated in order to control the inflammation [426-429]. There is a positive correlation between the severity of brain injury in infants with NE and the magnitude of the inflammatory response in the first seven days of life [430]. There is ample evidence that inflammation in preterm new-borns may contribute to detrimental neurodevelopmental outcomes [83].

An understanding of the causes and mechanisms of inflammation in NE may accelerate the discovery of adjunctive therapies and preventative strategies. Elimination of inflammation may lead to boosted neuroprotection and has potential for NE treatment [431].

Neutrophils are the first immune cells to be recruited to the area of infarction following brain ischemia, contributing to brain injury [87]. Morkos and co-workers reported that elevated peripheral neutrophil counts on the first day of life in asphyxiated neonates are a risk factor for neurological disabilities [91]. Hudome and co-authors reported that neutrophils are recruited to the brains of neonatal rats subjected to hypoxic-ischemic brain injury as early as 8 hours and that depletion of neutrophils led to a reduction in brain swelling [92, 93]. This suggests that neutrophils play a crucial role in brain swelling after ischemic injury. A large body of evidence from clinical studies and preclinical models of hypoxia have documented that neutrophils aggregate in the brain's blood vessels after hypoxic ischemic

encephalopathy (HIE), causing impairment in the ability of erythrocytes to carry oxygenated blood [25, 102, 432].

In addition to neutrophils, monocytes and other immune cells are recruited to the brain during neonatal hypoxic-ischemic attacks and can cross the blood-brain barrier (BBB) through parenchymal blood vessels [432, 433]. During hypoxia-ischemia, the immature brain expresses several chemoattractant molecules, involving CCL2, CCL3 and CCL7 that recruit monocytes from the circulation to infiltrate and accumulating in the brain, where they mediate inflammation [434, 435].

Lymphocytes are also central to the maintenance of the inflammatory response. The repertoire of lymphocytes includes B cells and conventional T cells of the adaptive immune system and NK cells, NKT cells, MAIT cells and γδ T cells of the innate immune system. These lymphocyte subsets play crucial roles in the inflammatory process by activating other immune cells through contact-dependent or cytokine-mediated interactions. Cellular therapies with lymphocytes are currently being tested to treat several inflammatory and autoimmune disorders [112, 287, 436]. Nonetheless, there is paucity of information about the relative roles lymphocyte subsets in inflammation associated with NE.

Under normal physiological conditions, the BBB is not permeable to T lymphocytes, however, high levels of $CD4^+$ T cells in the brain are characteristic of the immune response after ischemia in mice [102]. In one week after HIE, $CD4^+$ T cells reached their peak amount and remained within the brain for up to 35 days. This led to the recruitment of more immune cells, particularly $CD8^+$ T cells, as observed in an HIE rat model. Notably, these cells stayed in the CNS for up to 3-months after the initial insults [248]. Albertsson and his workers supported this by showing that a biphasic influx of $CD4^+$

occurred on day 1 day and a week after hypoxic-ischemic insult in the experimental mouse model [437].

Cytokines play fundamental roles in regulating the immune system, by promoting the proliferation, differentiation, activation and regulation of most types of leukocytes [438]. Many cytokines, such as IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α, are involved in inflammatory responses in vivo [439]. These cytokines can activate inflammatory cells such as neutrophils and monocytes/macrophages. These cytokine-activated cells may release toxic substances, such as reactive oxygen species (ROS) and toxic granules including proteolytic enzymes and [440]. Thus, various cytokines are associated with the pathophysiology of ischemia-reperfusion-associated injury [441].

Several studies have demonstrated that cytokines and chemokines mediate neuroinflammation in NE [442-445]. Cytokines are released in animal models of brain injury due to infection, trauma, excitotoxicity, and hypoxia-ischemia [442]. Animal and human studies have also demonstrated specific cytokine trajectories after a hypoxic-ischemic insult. Typically, cytokine levels peak within 12–24 hours post-insult, but some cytokines have shown a biphasic pattern [443]. Given the implicated role of cytokines in the evolution of neonatal brain injury and the dynamic nature of cytokine release after a hypoxic-ischemic insult, investigation of serial cytokine levels offers a promising avenue for identifying biomarkers of ongoing brain injury in newborns with HIE.

Dammann and co-workers (2011) have observed that in humans and experimental animal models of NE that there is an increase in the expression of the pro-inflammatory cytokines IL-1β and TNF-α, within the brain, following perinatal brain damage by pathogens or hypoxic injury [4]. O'Hare and co-workers confirmed a significant alteration in serum cytokine profiles in neonates who need resuscitation during delivery. They also showed that these neonates had raised GM-CSF levels during the first 24 hours [83],

while Jenkins and co-workers demonstrated that serum levels of IL-8, IL-6 and IL-10 were higher in the first two days of life during treatment with TH in comparison to non-cooling NE babies. In these babies, TNF and vascular endothelial growth factor (VEGF) levels were lower than control group at 72-96 hour [443]. Cytokine and chemokine actions may play speific roles in each phase of injury and recovery.

Understanding the responses of pro-and anti-inflammatory cytokines in NE may facilitate strategies to modulate the inflammatory response and decrease brain injury. Further research is needed to develop diagnostic, predictive, and prognostic serum biomarkers of brain injury in high-risk groups of neonates that are sensitive, specific, reliable, accurate and reflect the degree of brain injury and the injury's timing. These biomarkers may be used to predict the response to treatment and prediction of long-term outcomes.

4.2 Hypothesis and aims

4.2.1 Hypothesis

Lymphocytes control the inflammatory responses that contribute to NE and immunological interventions which target these cells will serve as an adjunct therapy to hypothermia.

4.2.2 Aims

The overall aim is to perform an in-depth phenotypic and functional characterization of the lymphoid cell compartment in blood and to analyse serum cytokine profiles in neonates with NE, school-age children post-NE and children with cerebral palsy and to compare them with those in age matched control subjects. The specific objectives are as follows:

1. To enumerate and phenotypically characterize circulating T cells, B cells, NK cells, γδ T cell subsets, MAIT cells, and iNKT cells in healthy neonates, neonates with NE, healthy school-age children, school-age children with a history of NE but who are clinically stable, and school-age children with cerebral palsy.

2. To measure serum cytokine levels in the five subject groups.

3. To compare the abilities of selected lymphocyte populations, form neonatal group to produce cytokines and cytotoxic mediators in response to stimulation *in vitro*.

4. To compare the expression of transcription factors that polarise adaptive immune responses by selected populations of lymphocytes in the patient and control groups.

4.3 Specific methods

4.3.1 Ethical Approval

Ethical committee approval for this study was granted from four tertiary referral, university-affiliated maternity hospitals in Dublin, Ireland – the Coombe Women and Infants University Hospital, the National Maternity Hospital, Holles Street Hospital, the National Children's Hospital Tallaght and the Rotunda Hospital. All parents of children who participated in the current study gave were informed written consent.

4.3.2 Patient specimens

Venous blood samples were obtained from 30 term neonates with NE, 17 healthy term neonates, 10 school age-children with a history of NE but who were clinically stable, 10 children with cerebral palsy, and 23 healthy school age children. The neonates with NE included 2 infants at stage I, 25 at stage II and 3 for stage III. Since subject numbers in some stages were low, we combined the patients in stages I, II and III. Blood from neonates was taken after normal vaginal delivery. Blood from healthy school-age children was taken on the day of surgical procedures for example, tonsillectomy, adenoidectomy.

4.3.3 Blood sample processing

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

4.3.4 Lymphocyte subset enumeration and phenotyping

50-100 µl of whole blood was stained for 15 minutes in the dark at room temperature with a live/dead cell stain (Fixable Viability Dye eFlour 506) 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 (HIB19), CD56 (HCD56), CD69 (FN50), CD161 (HP-3G10), and the Vα7.2 (clone REA179), Vα24Jα18 (6B11), Vδ1 (B1), and Vδ2 (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 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 Finally, the cells were washed with PBA buffer, fixed with 1% paraformaldehyde and analysed on a BD FACS Canto II flow cytometer. Gate limits were determined using unstained and fluorescence-minus-one controls and analysed with FlowJo software. 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 $CD3^+$ $V\alpha7.2^+$ $CD161^+$ cells and iNKT cells were defined as $CD3^+$ $V\alpha24J\alpha18^+$ cells. Vδ1 and Vδ2 T cells were defined as cells expressing CD3 and the Vδ1 or Vδ2 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 flow cytometric gating strategy used to detect these cells is shown in Figure 2.1.

4.3.5 Measurement of serum cytokine levels

Serum cytokine levels were analysed using the U Plex biomarker group 1 multiplex assay for human IFN-γ,TNF-αIL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, 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. As described in section 2.8.4.

4.3.6 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 hours at 37°C, 5% CO_2 with either medium alone, 50 ng/ml phorbol myristate acetate with 1 µg/ml ionomycin (PMA/I), recombinant human IL-12 (50 ng/ml) with IL-18 (50 ng/ml), IL-12 (30 ng/ml) with IL-15 (100 ng/ml) or the ligand recognised by the Vδ2 TCR (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP; 10 nM). Brefeldin A was added for the last 4 hours to prevent cytokine secretion from the cells. Full details are described in section 2.4.6.

4.3.7 Statistical analysis

GraphPad Prism Version 9 was used to perform statistical analyses. Mann–Whitney and Student t tests were used where appropriate for comparison between two groups, as indicated. ANOVA or Kruskal–Wallis analysis was used when multiple groups were compared based on the variance of the dataset. Statistical significance was described as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

4.4 Results

4.4.1 Patient demographics

Thirty neonates with NE and 17 healthy neonates were recruited. The mean (±SD) gestational ages 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 weeks and the mean birth weight was 3.5 ±3.0 kg. Patient demographics, including sex, Apgar scores at 1, 5 and 10 minutes after birth, and history of seizures, are shown in Table 4.1 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 minutes after birth, and history of seizures, are shown in Table 4.2. 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.

Table 0-1 Demographics of neonatal NE patients

Variables		NE-I (n=2)	NE-II (n=23)	NE-III (n=3)
GA (wks) [a]		39.48 ± 1.47	39.44 ± 1.45	39.87± 1.54
BW (kg)		3.55 ± 519	3.55 ± 519	3.72 ± 46
Gender, male, n (%)[b]		1 (50)	56%	1 (33)
Mode of delivery (%)	LSCS	0 (0)	9 (39)	0 (0)
	SVD	2 (100)	10 (43)	3 (100)
	Inst	0 (0)	4 (18)	0 (0)
Apgar @1 min [a]		3.25 ± 2.29	3.4 ±2.42	3.28 ± 2.58
Apgar @5 min [a]		4.74 ± 2.53	4.89 ± 2.61	4.14 ± 2.74
Apgar @10 min [a]		5.74 ± -2.58	5.87 ± 2.60	5.21 ± 2.93
TH, n (%)[b]		2 (100)	23 (100)	3 (100)
Seizures, n (%)[b]		N/A	13 (57)	3 (100)
MRI-Abnormal, n (%)[b]		1 (50)	17 (74)	3 (100)
Cord Arterial PH [a]		6.962 ± 0.05	$7.14^{+/-}$ 0.22	$7.47^{+/-}$ 0.12
Cord Arterial BE [a]		-14.55 ± 2.5	$-10.94^{+/-}$ 10.86	$-11.34^{+/-}$ 9.4
CPR, yes (%)[b]		0 (0)	13 (57)	3 (100)
Intubated, yes (%)[b]		0 (0)	19 (83)	3 (100)

Mild NE = Grade NE-1, Moderate to severe NE = NEII/III, GA = Gestational Age; BW = Birth Weight; LSCS = Lower Section Caesarean Section; SVD = Spontaneous Vaginal Delivery; Inst = Instrumental delivery

.

Table 4-2Demographics of school age children with NE and their age control

Variables		Control (n=23)	NE I-II (n=4)	NE II -III (n=6)	CP (n= 10)
GA (wks) [a]		40 ± 0.6	39.7 ± 1.4	40.5± 1.21	39.7 ± 1.5
BW (kg)		3.5 ± 0.5	3.46 ± 0.58	3.62 ± 0.61	3.69 ± 0.09
Gender, male, n (%)[b]		18 (78)	4(100)	3(50)	7 (70)
Mode of delivery (%)	LSCS	20 (87)	2 (50)	4 (67)	6 (60)
	SVD	2 (100)	1 (25)	1 (25)	2 (20)
	Inst	1 (4)	1 (25)	1 (25)	1 (10)
Mean age at examination, year		10	N/A	4.5 ± 0.5	7 ± 3.4
Apgar @1 min [a]		9	5	2±3	N/A
Apgar @5 min [a]		9	7 ± 1.61	4 ± 2.74	N/A
Apgar @10 min [a]		N/A	7.5 ± 1.80	5 ± 2.4	N/A
TH, n (%)[b]		N/A	1 (25)	5 (83)	N/A
Seizures, n (%)[b]		N/A	0(0)	4 (67)	N/A
MRI-Abnormal, n (%)[b]		N/A	1 (25)	3 (50)	

Mild NE = Grade NE-1, Moderate to severe NE = NEII/III, GA = Gestational Age; BW = Birth Weight; LSCS = Lower Section Caesarean Section; SVD = Spontaneous Vaginal Delivery; Inst = Instrumental delivery

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

4.2.2.1 T cell numbers and frequencies are raised in children with CP, but normal in neonates and children with NE

Samples of whole blood obtained from 30 term infants with NE, 17 healthy neonates, 10 school-age children with NE, 10 patients with CP and 23 healthy school-age children were stained with mAb specific for CD3 and CD19 (Figure. 4.1A). After gating on lymphocytes and exclusion of doublets and dead cells, the percentage frequencies of lymphocytes that expressed CD3 in the absence of CD19 were determined by flow cytometry (Figure. 4.1B) and absolute numbers of T cells were calculated from viable cell counts as determined by fluorescence microscopy (Figure. 4.1C). There was no significant variation between percentages or numbers of T cells in NE patients compared with age-matched controls. However, both T cell frequencies and absolute counts were significantly higher in the CP group compared to age matched controls ($P<0.05$).

4.2.2.2 B cell frequencies are higher in NE samples from neonatal patients and school age-children patients but lower in the CP group

Samples of whole blood obtained from 30 term infants with NE, 17 healthy neonates, 10 school-age children with NE, 10 patients with CP and 23 healthy school-age children were stained with mAbs specific for CD3 and CD19. After gating on lymphocytes, doublets and exclusion of dead cells, B cell frequencies were determined from the percentages of lymphocytes that expressed CD19 in the absence of CD3 (Figure 4.2A and B) and absolute numbers were calculated from the viable cell counts (Figure. 4.2C).

Figure 4.2B shows that the frequencies of B cells were significantly higher both in neonatal and school-age children with NE compared to their healthy counterparts ($p < 0.05$). However, a statistically significant difference in the absolute cell numbers was

only observed in school-age children (Figure 4.2C). Interestingly, B cell frequencies were significantly lower in children with CP compared to age matched healthy children (Figure 4.2B),

4.2.2.3 NK cells frequencies are significantly lower in neonatal NE compared to age-matched healthy neonates

Samples of whole blood collected from 30 term neonates with NE, 17 healthy term babies, 10 school-age children with NE, 10 patients with CP and 23 healthy school-age children were stained with mAbs specific for CD3 and CD56. After gating on lymphocytes and exclusion of doublets and dead cells, NK cell frequencies were determined from the percentages of live lymphocytes that expressed CD56 in the absence of CD3 using flow cytometry (Figure 4.4A) The percentages but not absolute numbers of NK cells, were significantly lower in neonates with NE compared to healthy neonates (Figure 3 B and C). NK cell numbers and frequencies were similar in the other subject groups.

4.2.2.4 T cell expression of CD4 and CD8 in the subject groups

T cells were further examined on the basis of CD4 and CD8 positivity to assess whether there were any variations between NE and CP patients and age-matched controls. Samples of whole blood were stained with mAbs specific for CD3, CD4 and CD8 and analysed by flow cytometry. After gating on singlet, live, $CD3^+$ lymphocytes, the frequencies and absolute numbers of $CD4^+$ and $CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ were determined (Figure 4.4 - 4.7). The absolute numbers of $CD4^+$ T cells were similar in healthy neonates and neonates with NE but higher in school-aged children who had NE and children with CP compared to healthy age-matched children (Figure 4.4C). However, the frequencies of the $CD4^+$ T cells as percentages of total T cells were similar

in all subject groups (Figure 4.4B). The frequencies and absolute numbers of $CD8^+$ T cells were similar in all subject groups, except for the children with CP who had higher numbers of these cells compared to healthy school-age children (Figure. 4.5). The frequencies of double-negative $CD4^-$ $CD8^-$ and double-positive $CD4^+CD8^+$ T cells were elevated in school-age children post NE compared to healthy subjects (Figure 4.6 and 4.7). Double negative (DN) $CD4^-$ $CD8^-$ T cell frequencies were also elevated in the CP group; however, these increases were not significant when absolute numbers of DN cells were compared. In contrast, the absolute numbers of double positive $CD4^+CD8^+$ T cells were significantly higher in the school age children who had a history of NE and in those with CP.

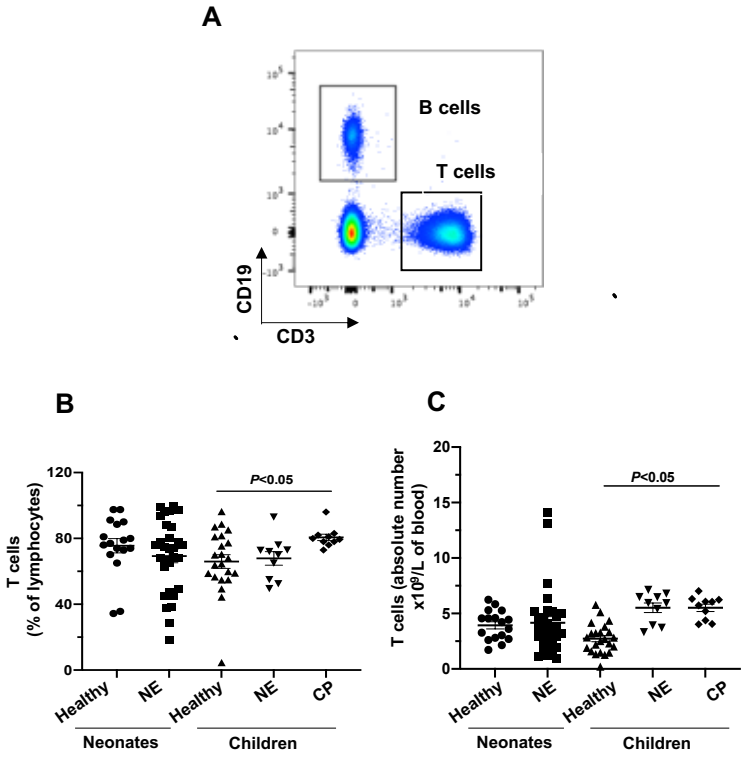

Figure 4.1 Enumeration of circulating T cells in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3 and CD19, and a dead cell stain and the frequency of T cells ($CD3^+CD19^-$) were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

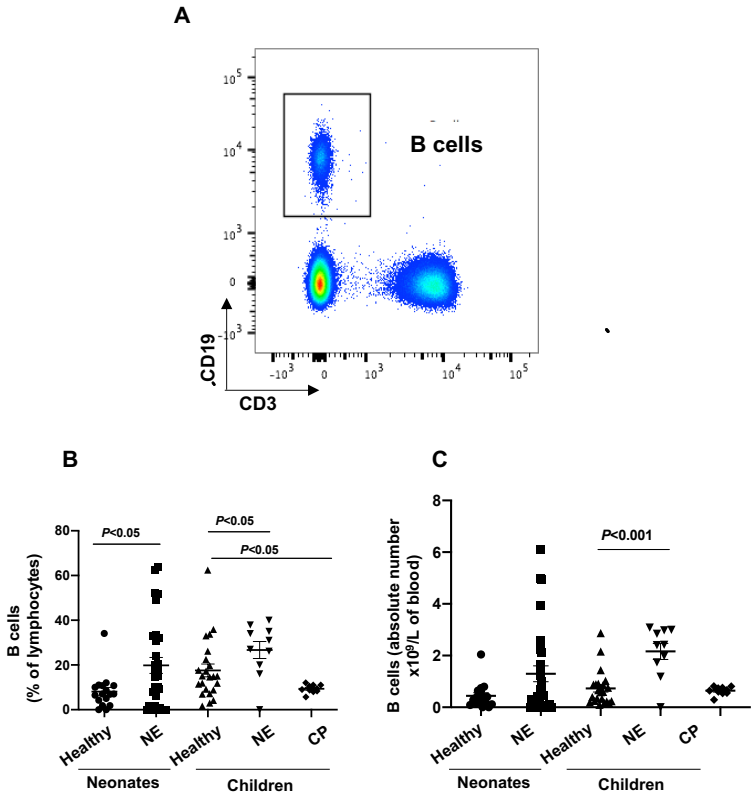

Figure 4.2 Enumeration of circulating B cells in neonatal and school-age children with NE, children with CP and healthy control subjects*. Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3 and CD19 and a dead cell stain (A). The frequency of B cells ($CD19^+CD3^-$) were analysed by flow cytometry. B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of B cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

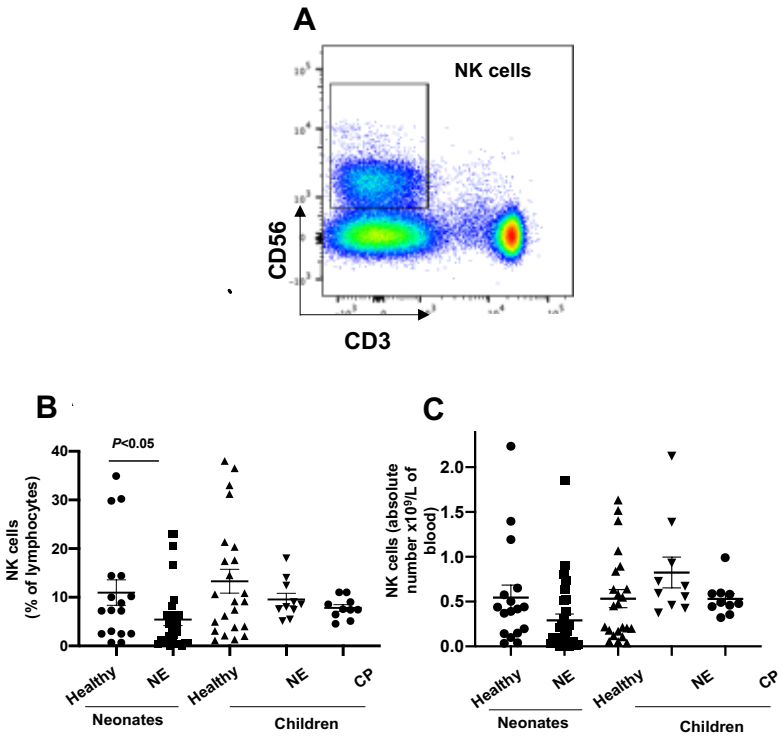

Figure 4.3 Enumeration of circulating NK cells in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD56 and a dead cell stain and analysed by flow cytometry. The frequencies of NK cells ($CD56^{+}CD3^{-}$) were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of NK cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

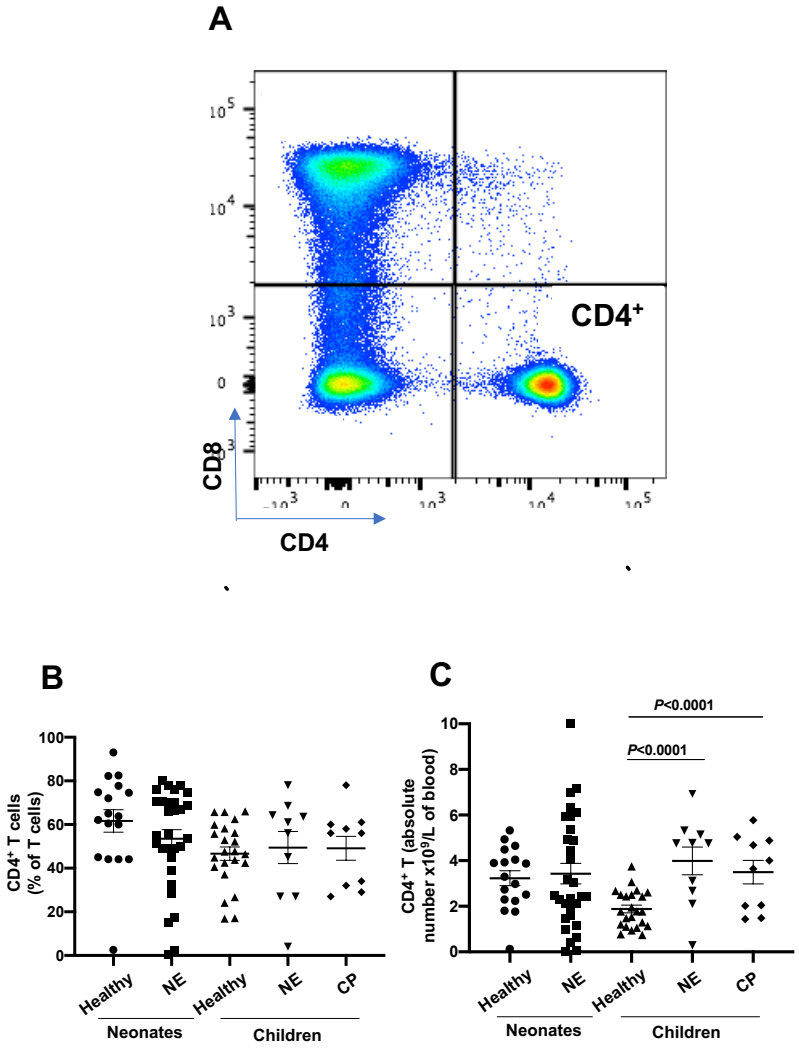

Figure 4.4 Enumeration of circulating $CD4^+CD8^-$ T cells in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD4, CD8 and a dead cell stain. The frequency of CD4 cells ($CD4^+CD8^-CD3^+$) were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of CD4 T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

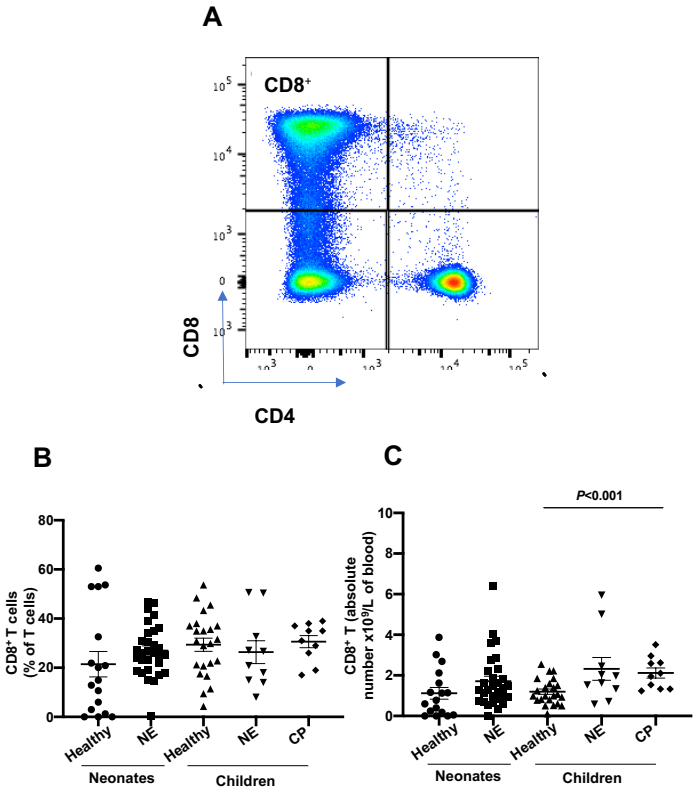

Figure 4.5 Enumeration of circulating $CD8^+CD4^-$ cells in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD4 and CD8 and a dead cell stain. The frequency of CD8 T cells ($CD8^+CD3+$) were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of CD8 cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

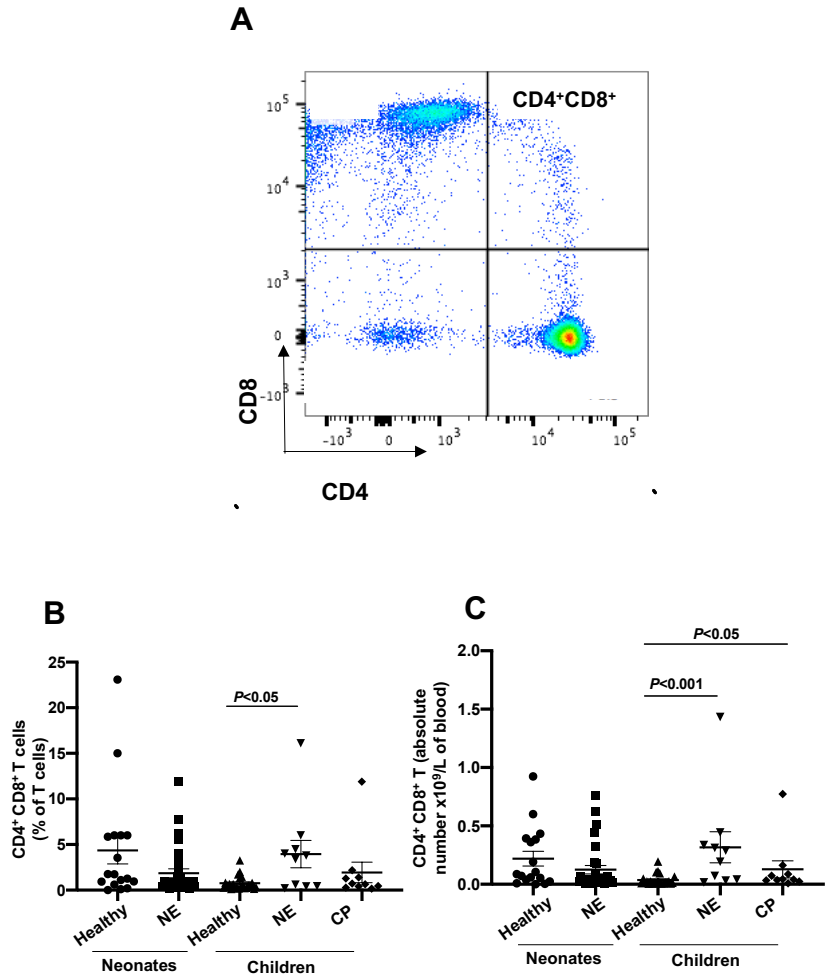

Figure 4.6 Enumeration of circulating double positive T cells ($CD4^+CD8^+$) subsets in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD4 and CD8 and a dead cell stain. The frequency of double positive T cells ($CD4^+CD8^+CD3^+$) were analysed by flowcytometry (A). A and B, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of double positive T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

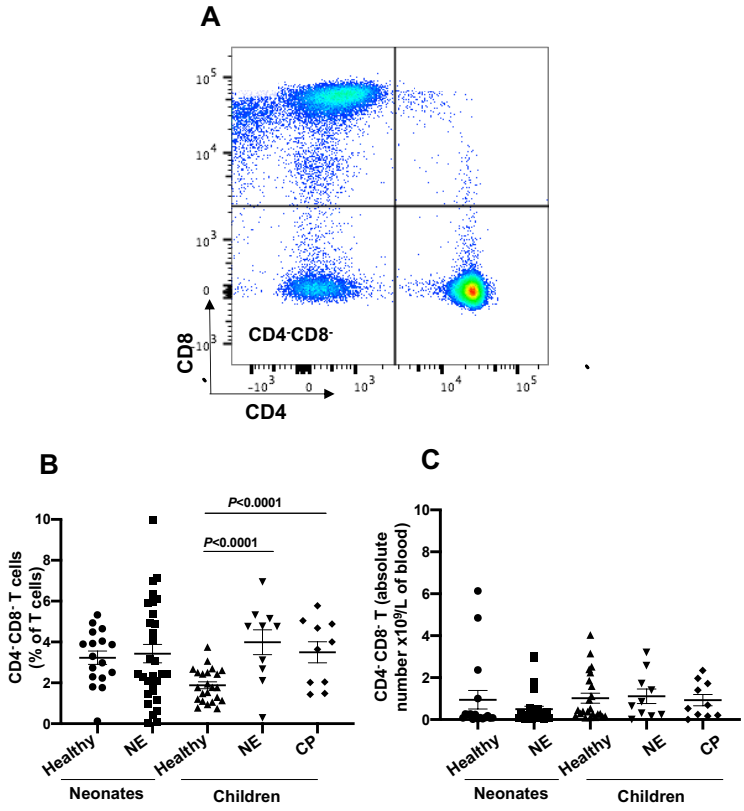

Figure 4.7 Enumeration of circulating double negative ($CD4^-CD8^-$) T cells in neonatal and school-age children with NE, children with CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD4 and CD8 and a dead cell stain. The frequencies of DN T cells ($CD4^-CD8^-CD3^+$) were analysed by flowcytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of DN T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

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

4.4.3.1 Vδ1 T cell frequencies are depleted in children with CP compared to age matched healthy children

Whole blood from the neonates with NE, healthy neonates, school-age children with NE and CP and healthy school-age children was phenotyped to assess any variations in circulating Vδ1 T cells in patients compared to healthy controls. Cells were stained with anti-Vδ1 and anti-CD3 mAbs and analysed by flow cytometry after gating on lymphocytes and exclusion of doublets and dead cells (Figure. 4.8A). Figure 4.8 B and C show that the frequencies and absolute numbers of circulating Vδ1 T cells were significantly decreased in the CP patients compared to healthy controls. The frequencies and numbers of Vδ1 T cells were similar in neonates and school-age children with NE compared to age-matched controls.

4.4.3.2 The frequencies and absolute numbers of Vδ2 T cells were significantly higher in neonates and school-age children with NE and CP compared to age-matched controls

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 TCR Vδ2-chain and analysed by flow cytometry after gating on lymphocytes and exclusion of doublets and dead cells. Vδ2 T cell frequencies were determined from the percentages of T cells that expressed Vδ2 and CD3 (Figure. 4.9A).

Strikingly, the Mann-Whitney U test revealed that the frequencies and absolute numbers of Vδ2 T cells were significantly higher in neonates and school-age children with NE compared to age-matched controls ($p<0.05$ in all cases) (Figure 4.9 B and C).

Vδ2 T cell frequencies and absolute numbers were also significantly higher in the school-age children with CP compared to healthy children, (Figure 4.9 B and C).

4.4.3.3 Circulating MAIT cell frequencies are similar in NE patients and healthy controls

Whole blood from the neonates with NE, healthy neonates, school-age children with NE and CP and healthy school-age children was phenotyped to assess any variations in circulating MAIT cells. Cells were stained with antibodies specific for CD161, CD3 and the Vα7.2 TCR, and analysed by flow cytometry. After gating on lymphocytes and exclusion of doublets and dead cells MAIT cells were defined as cells positive for CD3, CD161 and Vα7.2 (Figure. 4.10A and B). Figure 4.10 C shows that MAIT cells frequencies were very low numbers in healthy neonates and neonates with NE. These cells expanded to up to 9% of circulating T cells in healthy school-age children. MAIT cells were found at slightly lower frequencies and numbers in school-age children with NE. However, MAIT cell frequencies were significantly lower in children with CP compared to healthy age matched children ($p<0.05$). Absolute numbers of MAIT cells showed the same trend in the different groups, as shown in (Figure 4.10 D), but the differences did not reach statistical significance.

4.4.3.4 Circulating iNKT cell frequencies and numbers are higher in neonates and children with NE

Whole blood samples from patients with NE, CP and healthy donors were stained with anti- Vα24Jα18 and anti-CD3 mAb and analysed by flow cytometry. After gating on lymphocytes, dead cells and doublets were excluded and iNKT cells were defined as $CD3^+$ $Vα24Jα18^+$ cells (Figure 4.11A). Figure 4.11B shows that iNKT cell frequencies were significantly higher in neonates and school age children with NE and CP when

compared with age-matched healthy controls ($p<0.05$, 0.05 and 0.001, respectively). Figure 4.11C shows that the absolute numbers of circulating iNKT cells in NE and CP patients were also significantly higher than in age-matched control subjects. Collectively, these data suggest that Vδ2 T cells and iNKT cells may have roles in the inflammatory processes that lead to NE and CP.

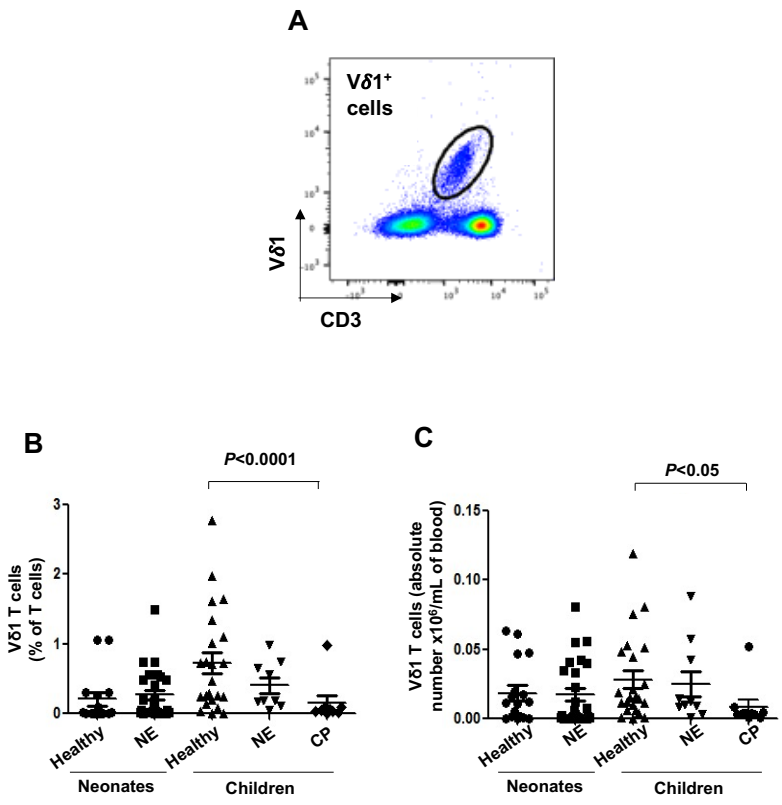

Figure 4.8 Enumeration of circulating Vδ1 T cells in neonatal and school-age children with NE and CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, TCR Vδ1 and a dead cell stain. The frequencies of Vδ1 T cells were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of Vδ1 T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test*

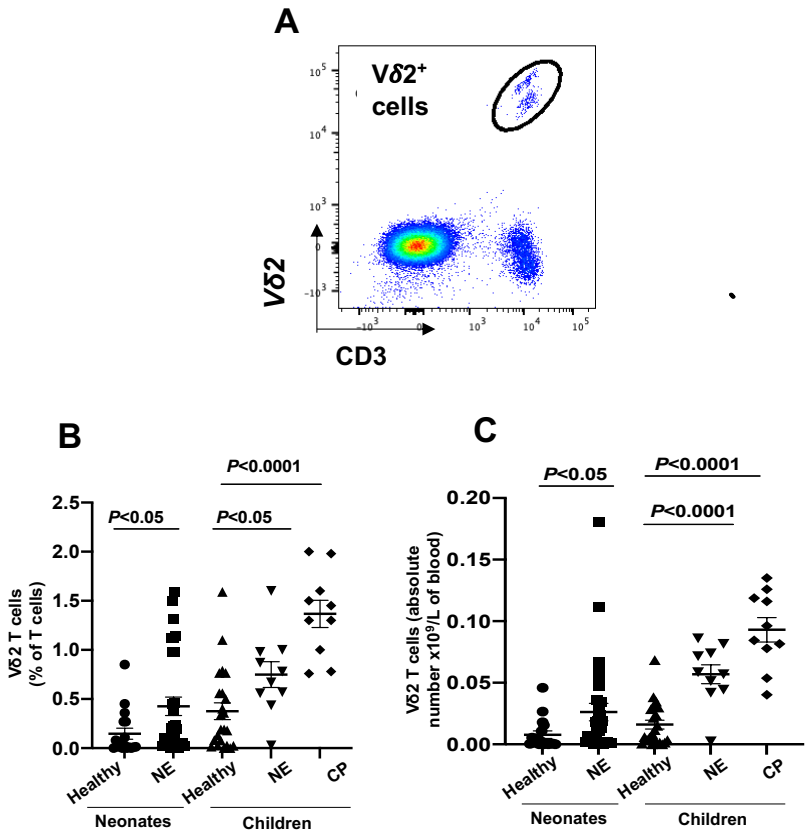

Figure 4.9 Enumeration of circulating Vδ2 T cells in neonatal and school-age children with NE and CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, Vδ2 and a dead cell stain. The frequencies of Vδ2 T cells were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of Vδ2 T cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

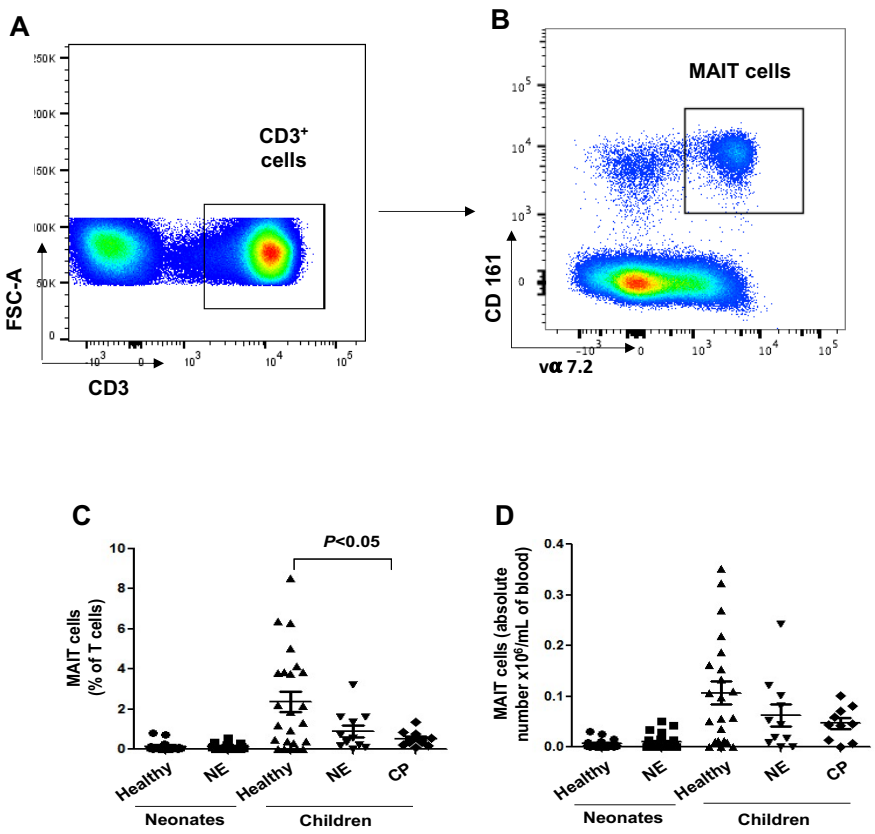

Figure 4.10 Enumeration of circulating MAIT cells in neonatal and school-age children with NE and CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD161, Vα7.2 and a dead cell stain. The frequencies of MAIT cells ($CD3^+$ $CD161^+$ $Vα7.2^+$) were analysed by flow cytometry (A and B). C and D, Scatter plots showing percentages frequencies (C) and absolute numbers (D) of MAIT cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

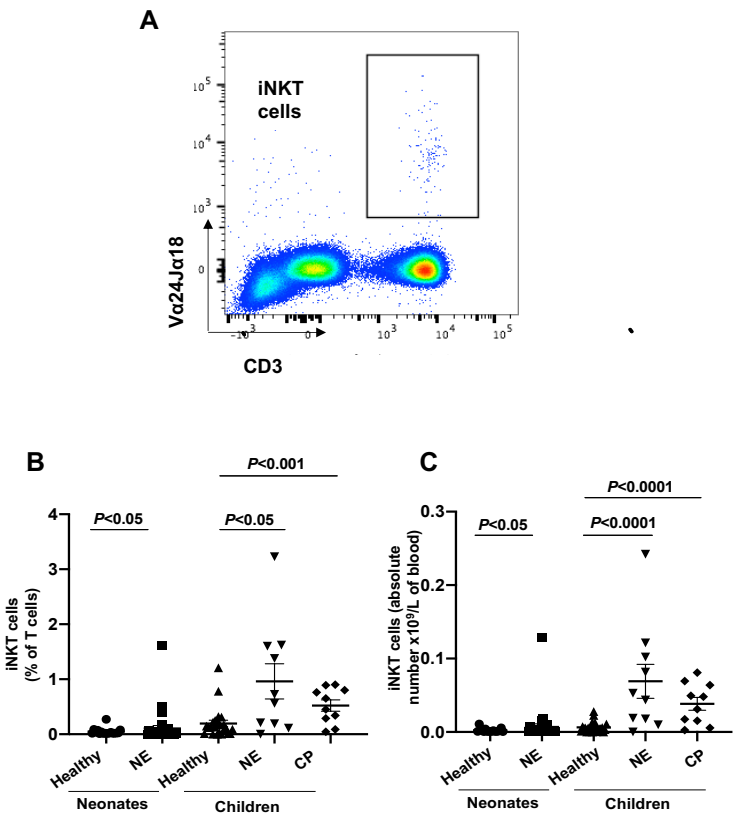

Figure 4.11 Enumeration of circulating iNKT cells in neonatal and school-age children with NE and CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, Vα24Jα18 TCR and a dead cell stain. The frequency of iNKT cells (CD3+ Vα24Jα18^{+}) were analysed by flow cytometry (A). B and C, Scatter plots showing percentages frequencies (B) and absolute numbers (C) of iNKT cells in each subject group. Error bars show means ± SEM. Data were compared using Mann-Whitney U test.*

4.4.4 Lymphocytes from NE patients display higher expression of CD69

To investigate if T cells, NK cells, MAIT cells, and Vδ2 T cells are activated, or primed for activation in vivo during NE, cell-surface expression of CD69 by these cells was measured in whole blood by flow cytometry (Fig. 4.12A). CD69 expression on freshly isolated T cells was found significantly more frequently in neonatal and school-aged NE patients compared to T cells from age-matched healthy controls ($p<0.0001$ in all cases, Figure. 4.12B). NK cells from NE patients with NE and healthy subjects upregulated CD69 expression significantly in all groups compared to their healthy counterparts ($p<0.05$-0.0001 in all cases; Figure 4.12C). Similarly, with MAIT and Vδ2 T cells, CD69 expression was significantly more frequent in all groups when compared to healthy controls ($p< 0.0001$), (Figure 4.12 D and E).

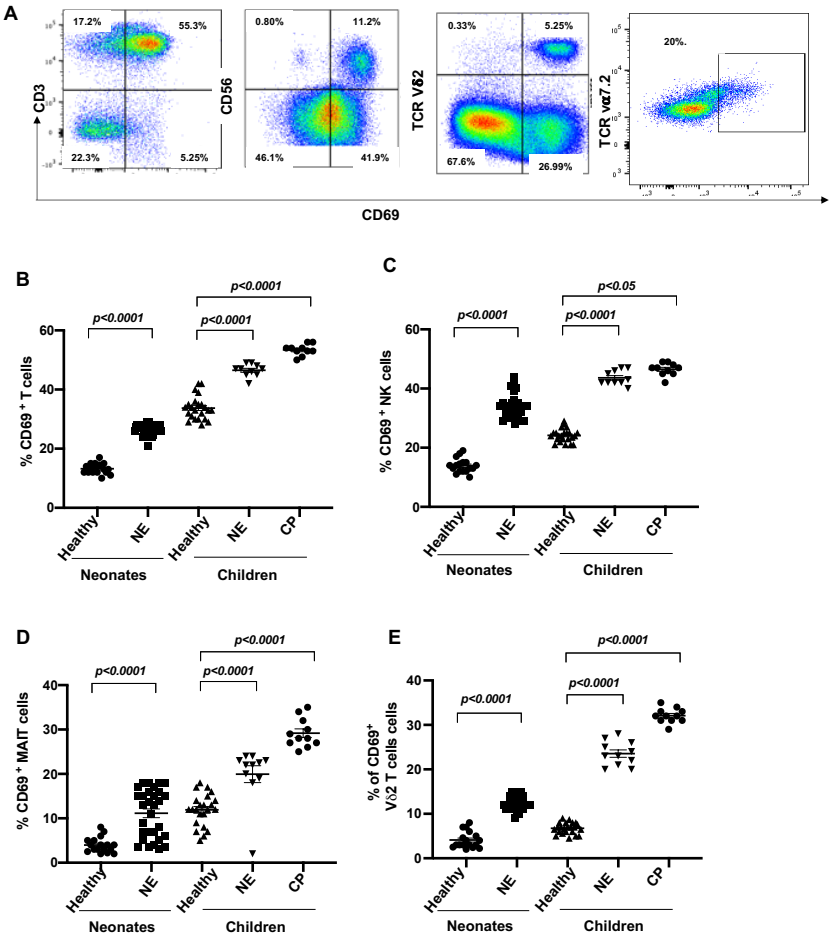

Figure 4.12 Expression of activation marker CD69 by T cells, NK cells, MAIT cells and Vδ2 T cells in neonatal and school-age children with NE and CP and healthy control subjects. *Blood samples were obtained from 30 neonatal NE patients, 10 school-age children with NE, 17 healthy term neonates, 10 children with CP and 23 healthy school-age children. Whole blood was stained with antibodies specific for CD3, CD56, CD161, Vα7.2, TCR Vδ2, CD69 and a dead cell stain. The frequencies of T cells, NK cells, Vδ2 and MAIT cells were analysed by flowcytometry (A). B-E, Scatter plots showing percentages of T cells (B), NK cells (C), MAIT cells (D), and Vδ2 T cells (E) in each subject group that expressed CD69. Error bars show means ± SEM. Data were compared using Mann-Whitney U test*

4.4.5 Lymphocytes from NE patients display higher expression of the transcription factors T-bet, Foxp3 and RORγt than lymphocytes from age-matched control subjects

Selective activation of transcription factors plays a fundamental role in the development of neurodevelopmental diseases, including epilepsy, intellectual impairment, autism spectrum disorders and CP [446]. We examined the expression of key transcription factors associated with CD4 T cell activation in whole blood taken from 7 healthy neonates and 10 neonates with NE following activation *ex vivo*. We examined the production of T-bet, Foxp3 and RORγt, which drive Th1,Tregs and Th17 cells respectively [10–12].

Whole blood from 7 healthy neonates and 10 neonates was stimulated with medium alone, PMA/I, or IL-12 + IL-18 in the presence of brefeldin A. Cells were then stained with mAbs specific for cell-surface CD3, CD4 and intracellular T-bet, GATA3, Foxp3 and RORγt and analysed by flow cytometry

Figure 4.13 shows that $CD4^+$ T cells from neonates with NE, frequently expressed T-bet in response to stimulation with PMA/I or IL-12 with IL-18 compared to CD4 T cells from their age matched healthy controls (Figure. 4.1B; $p<0.0001$). Thus, although the numbers of T cells are similar in babies with NE and healthy neonates, the babies with NE patients had significantly higher numbers of effector T cells capable of inducing Th1 responses. These data suggest that T-bet may also play a significant role in the development of inflammation of NE.

As seen in Figure 4.14, $CD4^+$ and $CD4^-$ T cells from neonates with NE more frequently expressed Foxp3 in response to stimulation with PMA/I, but not IL-12 and IL-18, when compared to CD4 T cells from their age matched healthy controls ($p<0.0001$).

RORγt was also expressed by higher proportions of $CD4^+$ and $CD4^-$ T cells from neonates with NE than from their counterparts from healthy neonates after stimulation with PMA/I. Stimulation with IL-12 with IL-18 also resulted in slightly higher frequencies of RORγt production by $CD4^+$ T– cells from babies with NE compared to unstimulated $CD4^+$ T cells (Figure 4.14).

Taken together, these results provide evidence for increased production of t-bet, RORγt, and Foxp3, key promoters of Th1, Th17 cell and Treg differentiation respectively, in newborn babies with NE.

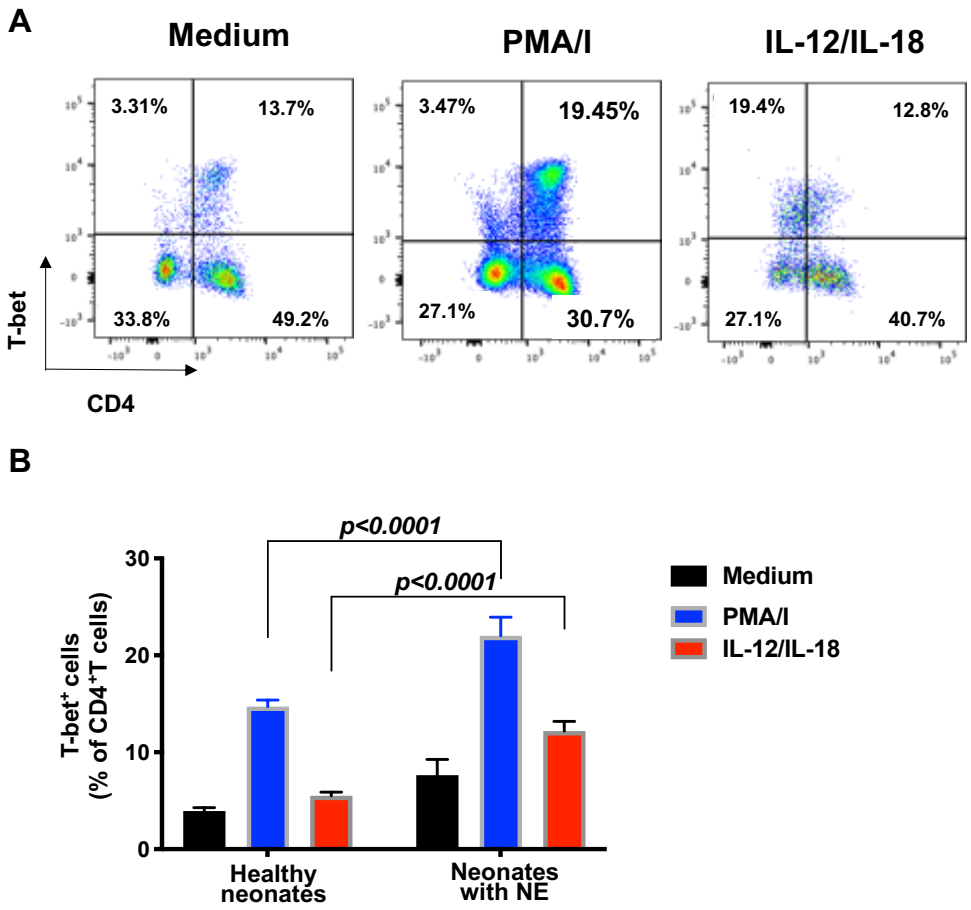

Figure 4.13 Flow cytometric analysis of T-bet transcription factor production by CD4⁺ T cells in whole blood. *A, Representative flow cytometry dot plots showing CD4 and T-bet⁺ expression by gated CD4 T cells in whole blood after stimulation with medium, PMA/I, or IL-12 + IL-18 for 18 h. B, Mean (± SEM) percentages of CD4 T cells from healthy neonates (n = 7) and neonates with NE (n = 10) that expressed T-bet upon stimulation ex vivo. Data were compared using Mann-Whitney U test.*

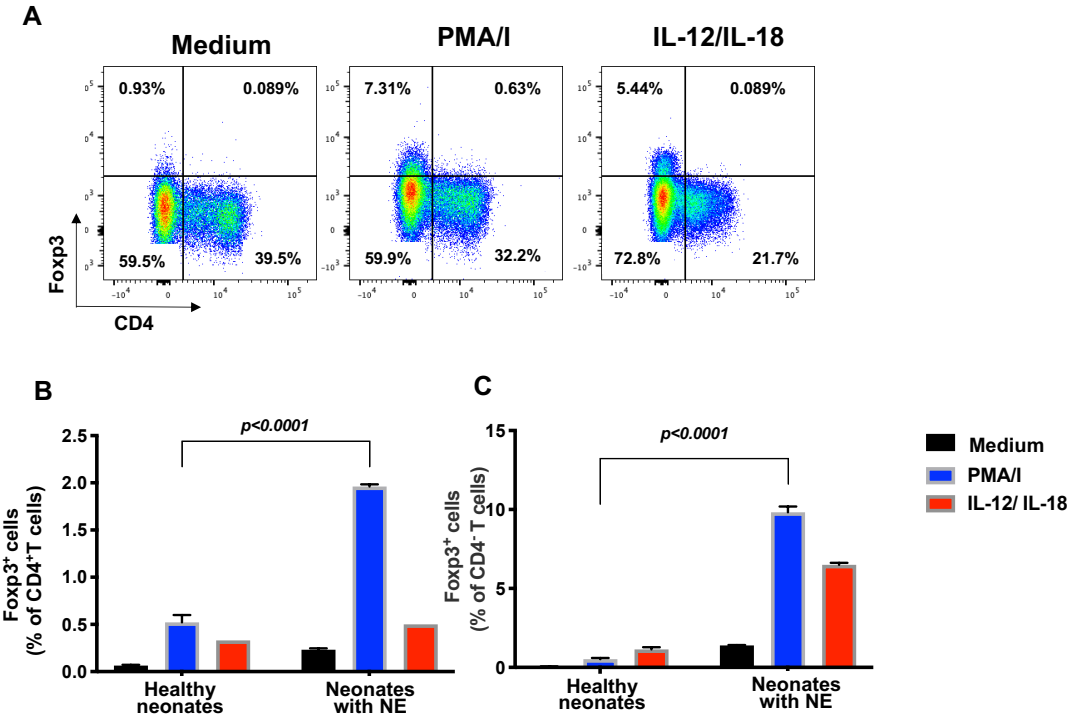

Figure 4.14 Flow cytometric analysis of Foxp3 transcription factor production by CD4⁺ T cells in whole blood. *A, Representative flow cytometry dot plots showing CD4 and Foxp3⁺ expression by gated CD3⁺ T cells in whole blood after stimulation with medium PMA/I, or IL-12 + IL-18 for 18 h. B and C, Mean (± SEM) percentages of CD4⁺ T cells (B) and CD4⁻ T cells (C) from healthy neonates (n = 7) and neonates with NE (n = 10) that express FoxP3 upon stimulation ex vivo. Data were compared using Mann-Whitney U test.*

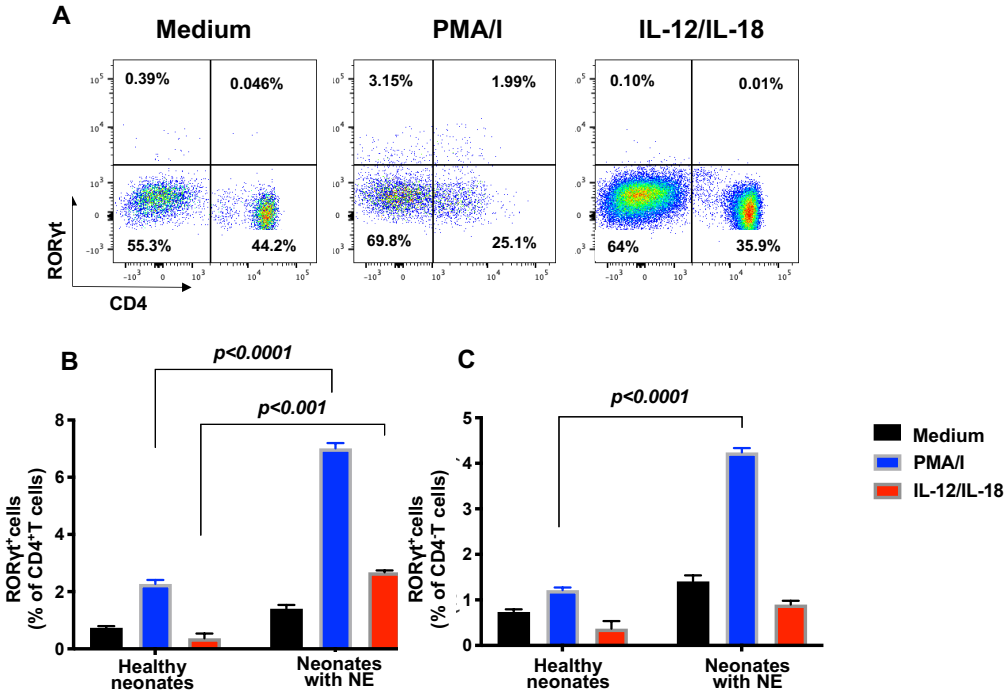

Figure 4.15 Flow cytometric analysis of RORγt transcription factor production by CD4⁺ T cells in whole blood. *A, Representative flow cytometry dot plots showing CD4 and RORγt⁺ expression by gated CD4 T cells in whole blood after stimulation with medium PMA/I, or IL-12 + IL-18 for 18 h. B and C, Mean (± SEM) percentages of CD4⁺ T cells (B) and CD4⁻ T cells (C) from healthy neonates (n = 7) and neonates with NE (n = 10) that express RORγt upon stimulation ex vivo. Data were compared using Mann-Whitney U test.*

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

The levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-15, IL-17A, IL-21, IL-22 and IL-23 in serum samples from healthy neonates, neonates with NE, healthy school-age children and school-age children with NE and CP (n=10 in each group) were measured by multiplex immunoassay. Figure 4.16 shows that the serum levels of the Th1-associated cytokines, IFN-γ, TNF-α and IL-12p70, were similar in all subject groups. However, the levels of IL-2 were significantly lower in neonates with NE compared to healthy neonates ($P<0.001$) and were elevated in school-age children with NE and CP compared to age-matched control subjects ($P<0.001$).

Analysis of serum levels of Th2 cytokines (Figure 4.17) indicated that the levels of IL-4, IL-5 and IL-10 were similar in all subject groups, but the levels of IL-9 were higher in the school-age children with NE compared to age-matched control subjects. A similar elevation in IL-9 levels was detected in the CP children but this difference did not reach statistical significance.

No differences in the serum levels of the Th17-associated cytokines, IL-17, IL-21, IL-22 and IL-23 were found in any of the subject groups (Figure 4.18). Analysis of the proinflammatory cytokines IL-6 and IL-8 (Figure 4.19) revealed that IL-6 levels were slightly higher in the neonates with NE compared to healthy term infants and IL-8 levels were significantly higher in neonates and school children compare to the age-matched control groups ($P<001$ and $P<0.01$ respectively). However, IL-8 levels were normal in the children with CP.

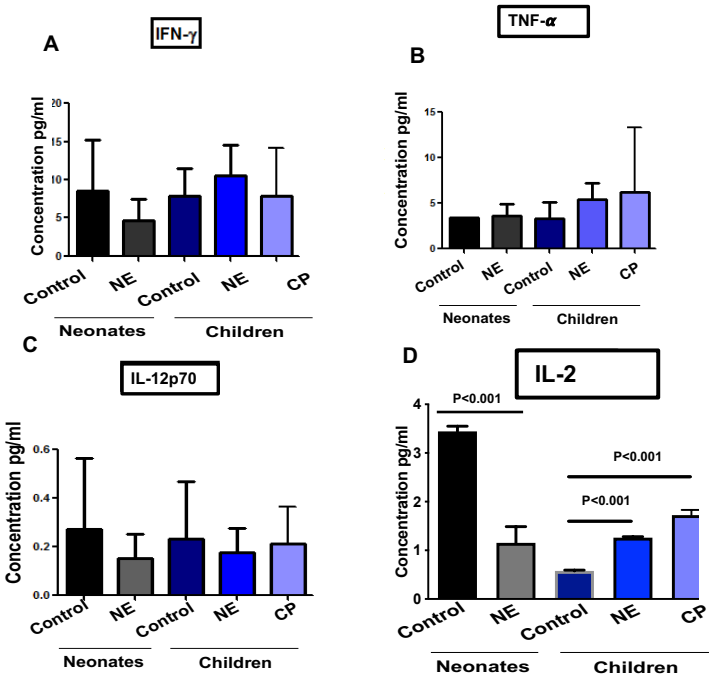

Figure 4.16 Serum Th1-associated cytokine levels in neonates and school-age children with NE and CP and in aged-matched healthy donors. *Serum from healthy neonates, neonates with NE, healthy school-age children and school-age children with NE and CP (n=10 in each group) were assayed for levels of IFN-γ, TNF-α, IL-12p70 and IL-2 using multiplex ELISA. Bars show the mean (±SEM) cytokine levels. Data were compared using Mann-Whitney U test.*

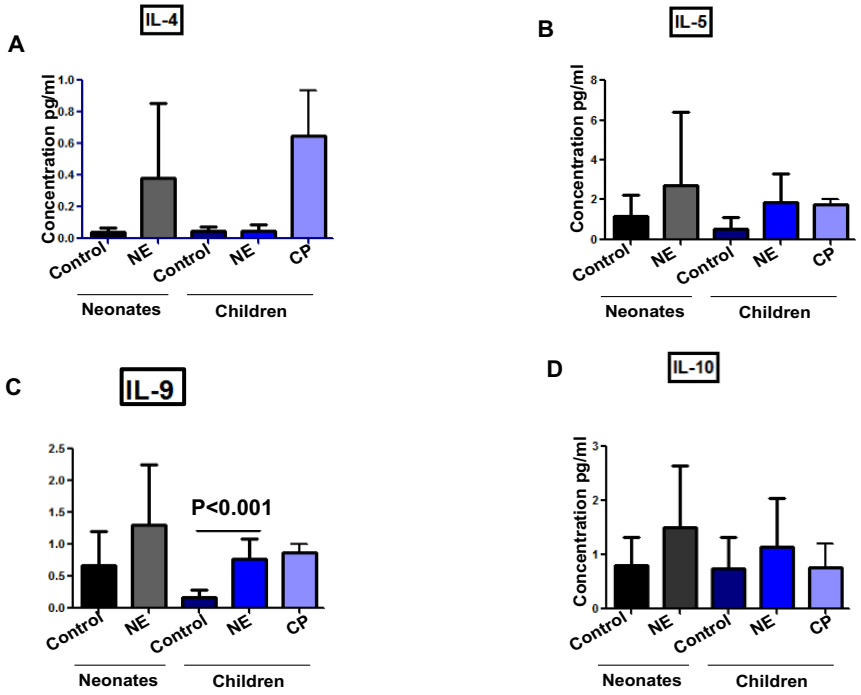

Figure 4.17 Serum Th2 cytokine levels in neonates and school-age children with NE and CP and in aged-matched healthy donors. *Serum from healthy neonates, neonates with NE, healthy school-age children and school-age children with NE and CP (n=10 in each group) were assayed for levels of IL-4, IL-5, IL-9 and IL-10 using multiplex ELISA. Bars show the mean (±SEM) cytokine levels. Data were compared using Mann-Whitney U test*

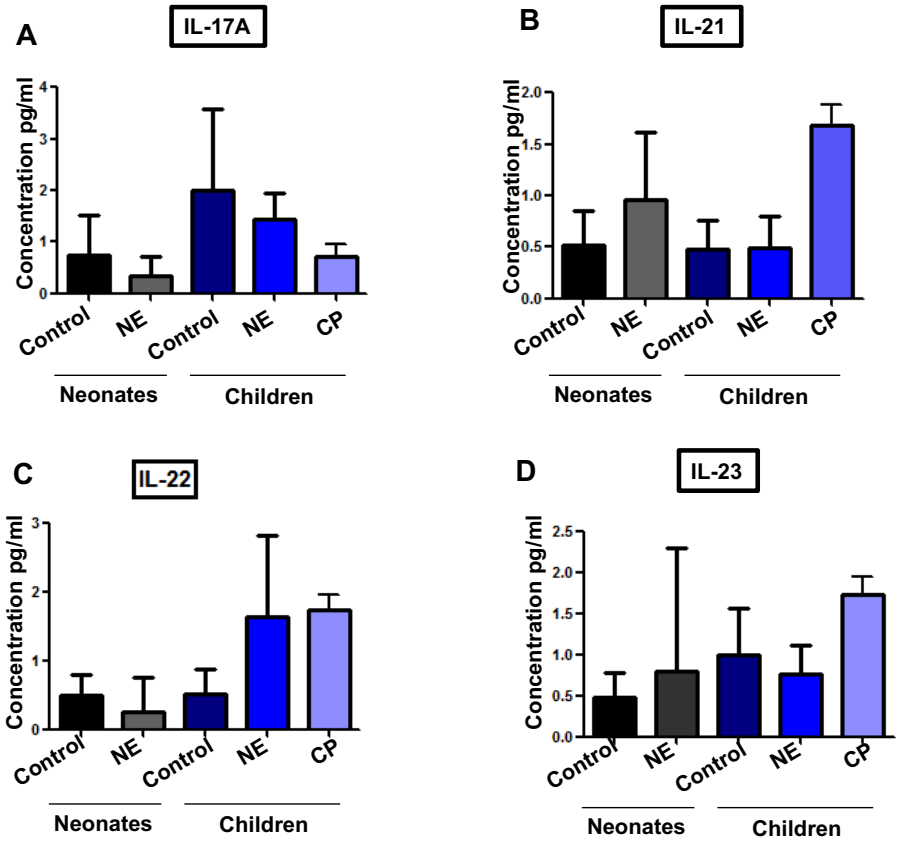

Figure 4.18 Serum Th17 cytokine levels in neonates and school-age children with NE and CP and in aged-matched healthy donors. *Serum from healthy neonates, neonates with NE, healthy school-age children and school-age children with NE and CP (n=10 in each group) were assayed for levels IL-17A, IL-21, IL-22, and IL-23 using multiplex ELISA. Bars show the mean (±SEM) cytokine levels. Data were compared using Mann-Whitney U test. No significant differences were observed between the cytokine's levels from patient-matched groups.*

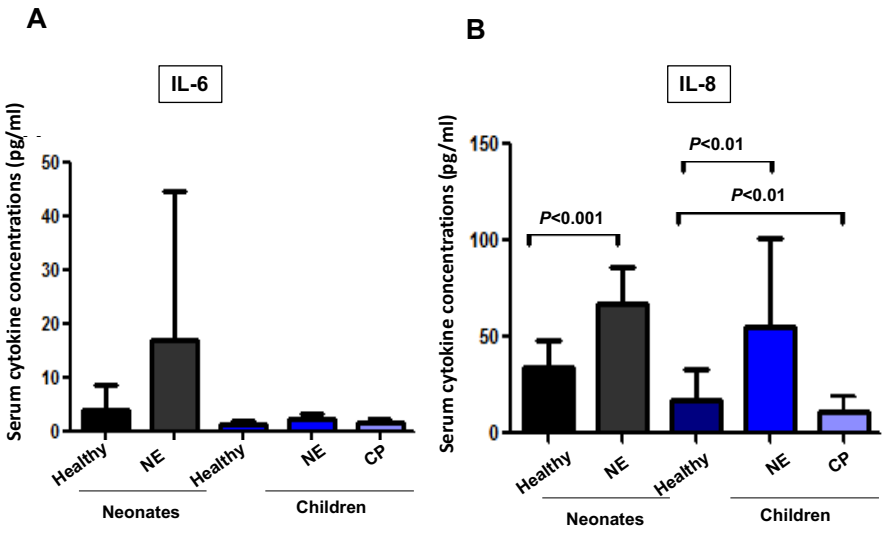

Figure 4.19 Proinflammatory cytokine levels in neonates and school-age children with NE and CP and in aged-matched healthy donors. *Serum from healthy neonates, neonates with NE, healthy school-age children and school-age children with NE and CP (n=10 in each group) were assayed for levels of IL-6 and IL-8 using multiplex ELISA. Bars show the mean (±SEM) cytokine levels. Data were compared using Mann-Whitney U test.*

4.4.7 Ex vivo cytokine and granzyme B production by T cells from healthy neonates and neonates with NE

Since Vδ2 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 (for T cells and Vδ2 T cells), IL-12 + IL-15 (for NK cells) or HMB-PP (for Vδ2 T cells) 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 (Figure. 4.20A). Figure 4.20B demonstrates that IFN-γ was produced by significantly higher numbers of total T cells from neonates with NE in response to stimulation with PMA/I or IL-12 and IL-18, compared to T cells from their age matched healthy controls. Similar results were observed for TNF-α and IL-17A (Figure. 4.20B), which were produced by higher frequencies of T cells from neonates with NE than from healthy neonates after stimulation 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 babies with NE (Figure 4.20C). Thus, although the numbers of T cells are similar in babies with NE and healthy neonates, the babies with NE patients had significantly higher numbers of effector T cells capable of producing inflammatory cytokines and cytotoxic mediators.

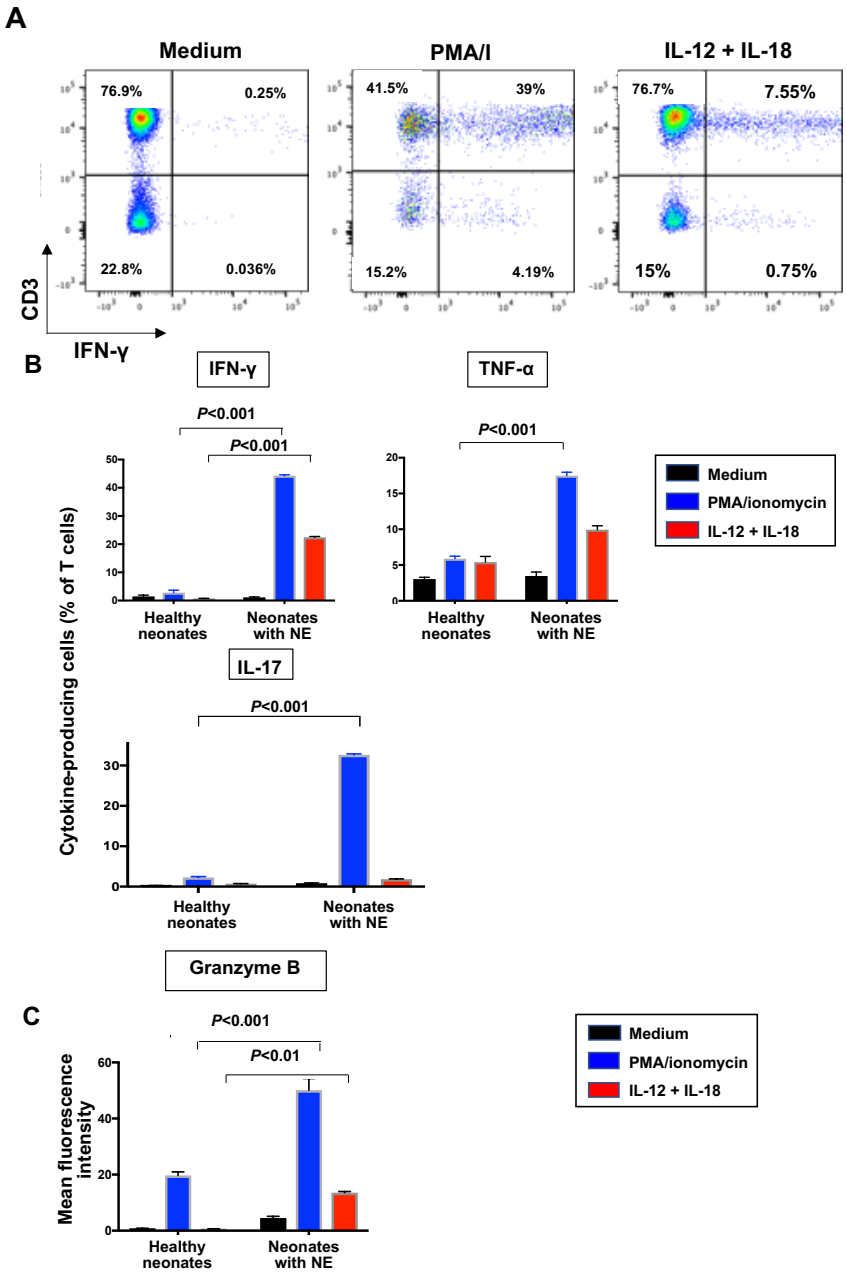

Figure 4.20 Cytokine and granzyme B production by T cells from patients with neonatal encephalopathy (NE). *A, Representative flow cytometry dot plots showing CD3 and IFN-γ expression by gated lymphocytes in whole blood after stimulation with medium PMA/I, or IL-12 + IL-18. B, Mean (± SEM) percentages of T 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 T cells from healthy neonates (n = 7) and neonates with NE (n = 10) upon stimulation ex vivo. Cell frequencies in subject groups were compared using the Mann Whitney U statistical test.*

4.4.8 Ex vivo cytokine and granzyme B production by NK cells from healthy neonates and neonates with NE

Since the percentages but not absolute numbers of NK cells, were significantly lower in neonates with NE compared to healthy neonates (Figure 4.3B) and the expression of CD69 expression on NK cells was significantly higher in NE patients compared to NK cells from controls in both groups (Figure 4.12C). we investigated the ability of NK cells to produce cytokines and granzyme B *ex vivo* by intracellular flow cytometry. Fresh blood samples were stimulated for 18 hours with medium alone PMA/I or IL-12 and IL-15 in the presence of brefeldin A. Cells were then stained with mAbs specific for cell-surface CD3, CD56 and intracellular IFN-γ, TNF-α, and granzyme B and analysed by flow cytometry (Figure 4.21A).

Upon stimulation with IL-12 + IL-15, but not with PMA/I, NK cells produced IFN-γ and TNF-α and this occurred more frequently in NK cells from neonates with NE compared to healthy neonates (Figure 4.21B). NK cells from neonates with NE also stained more powerfully for granzyme B in response to either stimulus compared to NK cells from healthy neonates (Figure. 4.21C).

These data suggest that, although the frequencies of NK cells are significantly lower in babies with NE and healthy neonates, the babies with NE patients had significantly higher numbers of effector T cells capable of producing inflammatory cytokines and cytotoxic mediators.

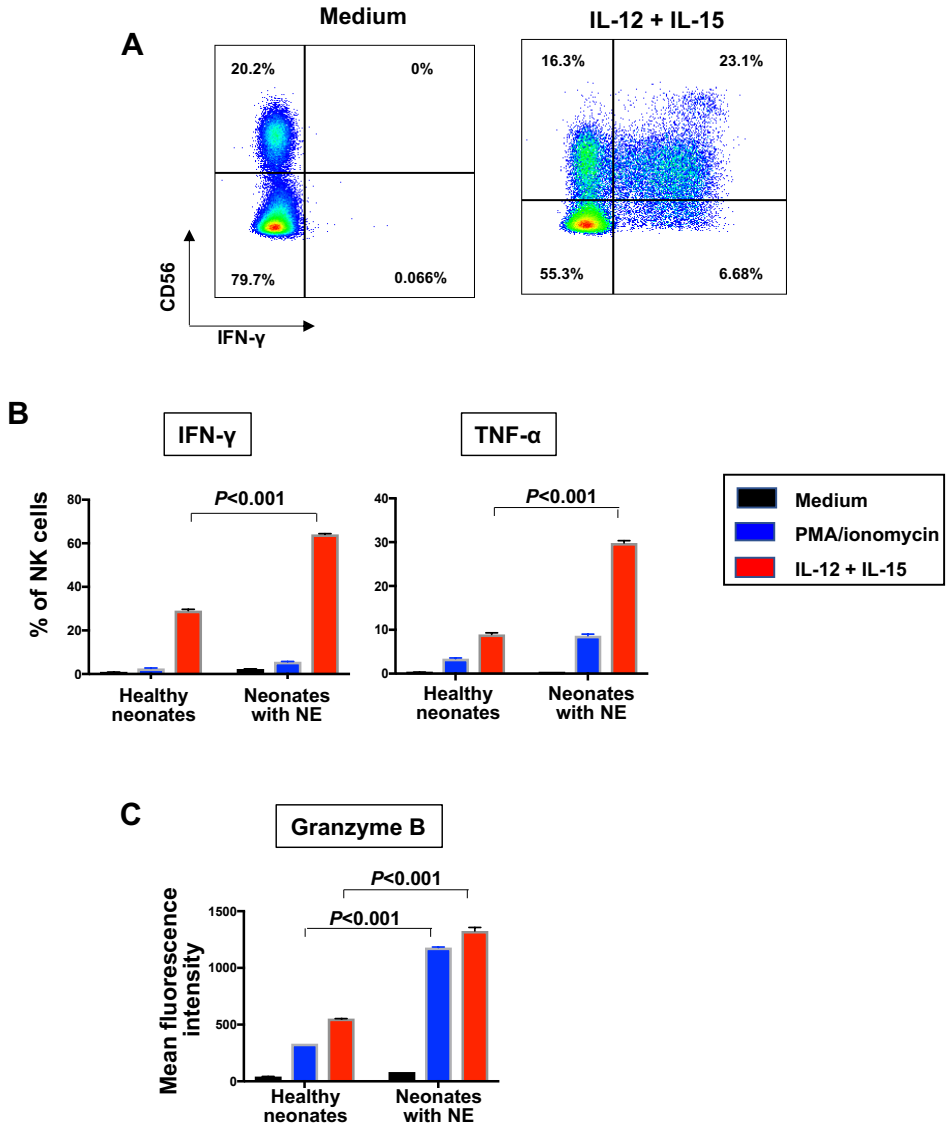

Figure 4.21 Cytokine and granzyme B production by NK cells from patients with neonatal encephalopathy (NE). *A, Representative flow cytometry dot plots showing CD56 and IFN-γ expression by gated CD3-negative cells in whole blood after stimulation with medium or IL-12 + IL-15. B, mean (±SEM) percentages 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 granzyme B upon stimulation ex vivo. Samples were compared using a non-parametric Mann-Whitney test analysis, P <0.001.*

4.4.9 Activation of Vδ2 T cells from patients with NE and healthy controls

In section 4.4.3.2, we showed that the frequencies and absolute numbers of circulating Vδ2 T cells are significantly higher in neonates and school-age children with NE and CP compared to age matched control subjects (Figure. 4.9). We next investigated activation, cytokine and cytotoxic mediator production by these cells taken from 7 healthy term babies and 10 neonates with NE following activation *ex vivo.*

Fresh whole blood samples were stimulated with PMA/I, IL-12 and IL-18 or HMB-PP for 18 hours and expression of the activation marker CD69 by gated Vδ2 T cells was analysed by flow cytometry (Figure. 4.22 A and B). CD69 was induced on some Vδ2 T cells following activation by PMA/I, the cytokines, or HMB-PP. Higher frequencies of Vδ2 T cells from neonates with NE expressed this activation marker than Vδ2 T cells from healthy neonates (Figure. 4.22C).

Significantly higher frequencies of Vδ2 T cells from babies with NE produced IFN-γ and TNF-α compared to Vδ2 T cells from healthy neonates (Figure 4.23). This was found after stimulation with PMA/ionomycin, IL-12 + IL-18, or the Vδ2 T cell antigen HMB-PP. Vδ2 T cells from babies with NE also more frequently produced IL-17 in response to stimulation with PMA/I. 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 babies with NE compared to control neonates. Collectively, these results show that Vδ2 T cells are both expanded and activated in babies with NE.

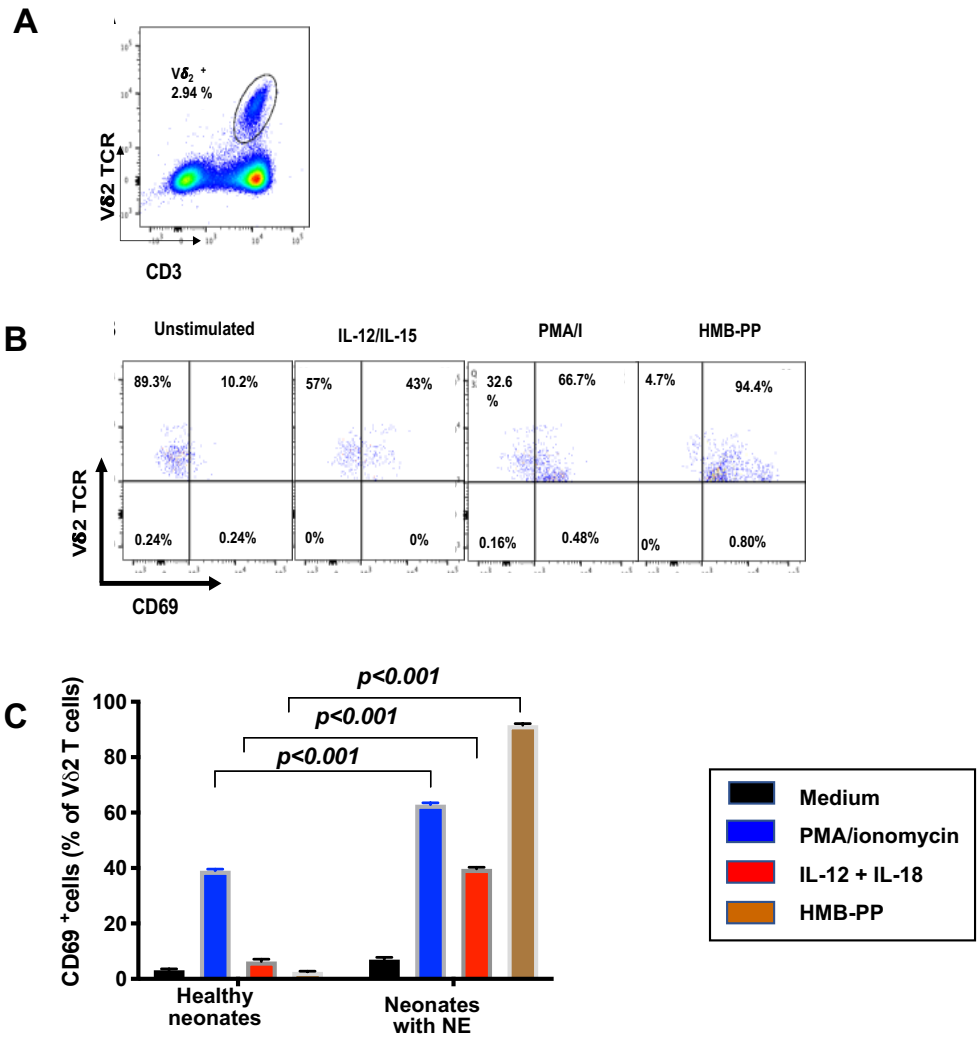

Figure 4.22 ***Mean (± SEM) percentages of Vδ2 T cells from healthy neonates (n=7) and neonates with NE (n=10) that express CD69 upon stimulation ex vivo.*** *A, Representative flow cytometry dot plots showing gated Vδ2^{+}CD3^{+} positive cells in whole blood. B, Representative flow cytometry dot plots showing CD69 expression by gated Vδ2^{+}CD3^{+} positive cells in whole blood after stimulation with medium or IL-12 with IL-18, PMA/I, or HMB-PP for 24 hours at 37°C. Brefeldin A was added for the last 4 hours. Cells were then stained for cell surface CD3, Vδ2 TCR and CD69 and analysed by flow cytometry*. C, Bar charts showing *mean (± SEM) percentages of Vδ2 T* cells that express CD69 upon stimulation. S*amples were compared using Mann-Whitney statistical analysis.*

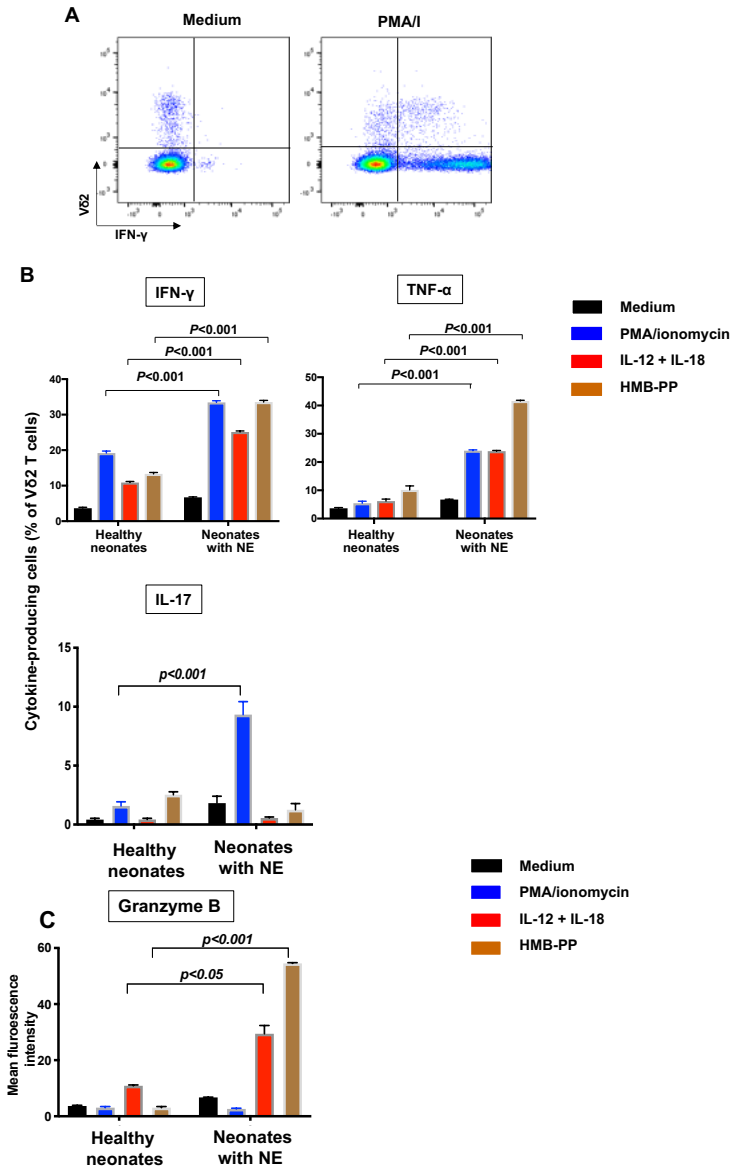

Figure 4.23 ***Cytokine and granzyme B production by Vδ2 T cells from patients with neonatal encephalopathy (NE).*** *A Representative flow cytometry dot plots showing Vδ2 TCR and IFN-γ expression by gated CD3^{+} positive cells in whole blood after stimulation with medium or PMA with ionomycin (PMA/I) B, mean (±SEM) percentages of Vδ2 T 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 Vδ2 cells from healthy neonates (n = 7) and neonates with NE (n = 10) upon stimulation ex vivo. Samples were compared using the Mann-Whitney test.*

4.5 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 [427, 447, 448]. 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 [92, 449] and elevated serum levels of inflammatory cytokines, which are associated with poor developmental outcomes and mortality [450, 451]. The importance of neutrophils in brain injury following hypoxia ischemia is clear from animal studies, where neutrophil depletion can reduce brain injury [452, 453]. 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 [86, 448, 454].

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 [86, 448, 454, 455], 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 [248, 295] Depletion studies have demonstrated pathological [378] and protective [456] roles for T cells, suggesting distinct roles for different T cell subsets [457]. B cells also infiltrate the brains of mice following

hypoxia ischemia [295] and regulatory B cells may contribute to protection against brain injury via the release of IL-10 [458].

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.

To further analyse functional subsets of T cells in neonates with NE and healthy neonates, we treated whole blood samples with T cell activators and compared the production of transcription factors, cytokines and cytotoxic mediators.

Functional studies showed that, upon stimulation *ex vivo*, T cells from neonates with NE also 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 [458] or Th1, Th2, Th17 or regulatory T cells [459] 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 neuroinflammation in NE and CP. Innate T cells initiate, regulate and maintain innate and adaptive immune responses [112, 287, 436]. 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 [244], γδ T cells [294, 295] and iNKT cells [460] 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 more readily produced IFN-γ, TNF-α and granzyme B in response to stimulation *ex vivo* than NK cells from healthy neonates.

As previously reported [461], 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 from the blood 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 [112, 287, 436]. 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 [462]. Vδ2 T cell numbers and functions are altered in several neurological diseases, including infectious meningitis, ischemic stroke and multiple sclerosis [463-466]. Vδ2 T cells are also capable

of producing IL-9 [467], 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 autoimmune diseases such as multiple sclerosis and myasthenia gravis [460, 468, 469]. Multiple clinical trials involving *in vivo* activation or adoptive transfer of *ex vivo* activated Vδ2 T cells and iNKT cells are ongoing [470-473] and may in future be applied as a novel treatment of NE.

4.6 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 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.

CHAPTER 5

An investigation into the reciprocal interactions between B cells and mucosal associated invariant T cells

5.1 Introduction

In chapter 3, we found that MAIT cells are rare in CB and in peripheral blood from neonates, but they expand significantly in early age (Figure. 3.3) until reaching the highest frequencies during adulthood. These findings are in accordance with previous studies [380, 474-476], which reported similar findings. We also found that the frequencies of B cells were low in neonatal blood but they increased during childhood and then decreased again during adulthood and in older adult people (Figure 3.1).

We report in chapter 4 that B cell frequencies are higher in NE samples at neonatal and school age compared to their age-matched healthy counterparts (Figure 4.2), but are lower in children with CP. While MAIT cells were rarely present in neonates, their numbers and frequencies were lower in school-age children with NE and CP compared to healthy age matched children.

As other innate T cells, such as iNKT and gd T cells, have been shown to have follicular helper activity for B cells [477, 478], we hypothesised that MAIT cells might also influence B cell numbers and function [479].

B cells are generated from lymphoid progenitor cells in the bone marrow, where they undergo differentiation into mature lymphocytes. They subsequently move to the spleen and secondary lymphoid organs, where they develop and differentiate into distinct cell types [202]. Activation of naive B cells is dependent on antigen identification by the Ig receptor and the presence of additional signals, which usually originate from a $CD4^+$ T cell. In order for B cells to respond, both B cells and T cells must recognize the same antigen. B cells detect surface antigens on microorganisms that enter the lymph nodes via the lymphatic system. This leads to internalisation and digestion of the microorganism and presentation of antigenic peptides bound to MHC class II molecules to Tfh cells. The

Tfh cells then provide signals that reciprocally induce B cell differentiation into memory cells and long-lived plasma cells (LLPC). LLPCs produce long-lived, high affinity antibodies. Memory B cells rapidly mature into plasmablasts, which produce protective antibodies, but do not undergo affinity maturation and are short-lived [207]. Within the GC, B cells undergo proliferation and somatic hypermutation of Ig genes, followed by a process in which the "fittest" B cells - those best able to capture antigen via surface Ig and present it on MHC class II molecules, are selected by Tfh cells [480]. The strength of interaction between Tfh cells and GC B cells, which is proportional to the amount of antigen presented by GC B cells, determines the cell cycle speed and number of divisions of GC B cell clones [209-211].

B cells are considered professional antigen-presenting cells (APCs) for $CD4^+$ T cells because they are capable of selectively recognizing low amounts of antigens with their cell-surface immunoglobulin and constitutively express MHC class II and co-stimulatory molecules. This allows B cells to prime T cells, which then in turn stimulate the maturation of B cells into memory cells and plasma cells that produce antibodies [481].

B cells can be categorised into subsets based on their differentiation stage and on the cytokines they secrete [482]. The functional B cell subsets are divided into effector B (Be) cells that activate immunity and regulatory B (B reg) cells that inhibit immunity. Be cells are further classified into Be-1 cells, which generate T helper (Th)1-type cytokines, and Be-2 cells, which produce T helper (Th)2-type cytokines.

B cells are also divided into B1 cells and B2 cells, which are distinguished by their developmental origin and location [483]. B1 cells have self-renewing ability and live longer. These cells are the primary source of natural antibodies that mediate innate-like immunity. B2 cells which represent the majority of B cells, recirculate and have a short life span. These cells are metabolically quiescent and can be triggered to produce

antibodies in secondary lymphoid organs with the assistance of T cells [483, 484]. B2 cells can be classified into two types depending on their distribution in lymphoid tissues: marginal zone B cells and follicular B cells. Immature B cells in the marginal zone play a vital role in innate immunity, whereas follicular B cells are essential for antigen-specific acquired immunity [485, 486]. Breg cells are believed to have an immunological suppressive effect, as evidenced by their production of suppressive cytokines such as interleukin-10, interleukin-35, and transforming growth factor-β [487-489].

Follicular helper T (Tfh) cells are a unique subtype of $CD4^+$ T cells, which are found in the GCs of secondary lymphoid organs (SLOs), including the tonsil, spleen, and lymph nodes. Tfh cells are unique in that they are concentrated in the B cell zone and interact with B cells. Tfh cells can also be detected in the circulation [192].

Tfh cells play a critical function in the development of germinal centres in the body. Naïve T cells interact with antigen-presenting DCs in the T cell zones of the SLOs, leading to Tfh cell development [9, 193, 194, 196, 490]. They then move to the B cell zones, where they promote B cell maturation and the establishment of long-lasting serological memory through affinity maturation and class-switch recombination to generate high-affinity antibodies [193-195]. This is mediated in part by interaction of the co-stimulatory molecule CD40 with the CD40-ligand (CD40-L) on the B cell and by the production of IL-21 which leads to B cells proliferation.

TCR signalling is required for activation, proliferation, differentiation, migration, survival, and effector functions in Tfh cells. As a result of a discrepancy between stimulatory and inhibitory costimulation signals, an increase in the number of Tfh cells is sometimes created, resulting in Tfh-driven autoimmunity [491, 492]. Understanding the development and function of Tfh cells is important for generating new vaccine strategies

against pathogens as well as targeted approaches to abrogate the inappropriate activity of these cells in patients with various autoimmune diseases.

MAIT cells are a subgroup of unconventional innate-like T cells that recognize highly conserved metabolites produced by bacteria and yeasts, giving them a broad range of microbe specificities [310, 493, 494]. Many bacteria, but not humans nor other mammals, generate riboflavin precursors, which are capable of activating MAIT cells [494]. These products are presented to the MAIT cell TCR by the major histocompatibility complex class-I related protein, MR1. MAIT cells represent 1-15% of peripheral blood T cells and reside mainly at mucosal sites [495]. MAIT cell TCRs consist of an invariant Vα7.2-Jα33 TCRα chain combined with a limited number of TCRβ chains [496]. In addition to TCR-mediated activation, MAIT cells can be activated by cytokines, such as IL-12, IL-18 and IL-23 [497]. Upon activation, MAIT cells respond rapidly and release a host of pro-inflammatory cytokines and cytolytic products, which aid in clearing bacterial infections [498]. MAIT cells can also kill tumour cells and stressed target cells, enhance inflammation and tissue repair, enhance wound healing, augment monocyte, neutrophil, and dendritic cell survival, and activate and prime $CD8^+$ T lymphocytes [499-503]

B cells are required for MAIT cell accumulation in the periphery [504]. In the human gut, MAIT cells were discovered in high numbers, and investigations in mice have revealed that their expansion in the periphery depends on B lymphocytes and a microbiota [505]. In support of this notion, one study investigated the role of *Enterobacteriaceae* bacteria to trigger MAIT cell activation and cytokine production, and confirmed that B cells acted as APCs [303, 306, 307, 481, 506-508]. MAIT cells are activated when exposed to bacterially infected B cells in an MR1-dependent manner. MAIT cell activation resulted in expression of CD69 and the production of IL-17A, IFN-γ, and TNF-α [505].

Although B cells have been shown to modify and influence MAIT cell activity, the first research indicating that MAIT cells may be B cell helpers were published in 2017 [9, 495]. Leung et al., (2014) had discovered that MAIT cell frequencies in humans with acute *Vibrio cholerae* infection and correlated with the presence of lipopolysaccharide (LPS)-specific IgA and IgG antibody responses [509]. Bennett and co-workers observed that MAIT cells activated with *Vibrio cholerae* upregulate CD40L, a crucial factor in the activation of B cells by T cells . This research team also showed that human MAIT cells can induce B cell antibody production *in vitro*. They did so by stimulating MAIT cells with bacteria and cytokines then applying the supernatants to autologous B cells and testing for B cell stimulatory cytokines. This gave the first direct evidence that MAIT cells can influence plasmablast differentiation and antibody production, implying that MAIT cells play a role in B cell maturation [510].

5.2 Hypothesis, aims & objectives

5.2.1 Hypothesis

MAIT cells are innate T cells with functional similarities to other innate T cells, such as iNKT cells and Vδ2 T cells, which are capable of promoting B cell maturation into antibody-producing plasma cells [477, 511]. Thus, iNKT cells and Vδ2 T cells have potential therapeutic value as vaccine adjuvants. We investigated if MAIT cells also have similar B cell stimulatory or costimulatory activities.

5.2.2 Aims

In order to determine whether MAIT cells have the capacity to activate B cells, human MAIT and B cell co-culture experiments were performed. Preliminary experiments using PBMCs were carried out to determine optimal conditions for activating B cells and MAIT cells. Subsequent studies utilised sorted lines of MAIT cells and B cells, investigating the effects of resting and activated MAIT cells on activation, cytokine and antibody

production by B cells, and killing of B cells. A cartoon figure depicting these objectives is shown in Figure 5.1.

5.2.3 The specific aims were:

1. To generate MAIT cell lines and purified B cells from human blood samples.

2. To optimise conditions to stimulate MAIT cells to produce IL-17 and IFN-γ.

3. To optimise conditions to activate B cells to express activation markers CD38 and CD69.

4. To compare co-stimulatory marker expression and cytokine production by B cells following culture with resting or activated MAIT cells or with control stimuli.

5. To examine antibody class switching and antibody release by B cells cultured with resting or activated MAIT cells.

6. To determine if MAIT cells can kill B cells *in vitro*.

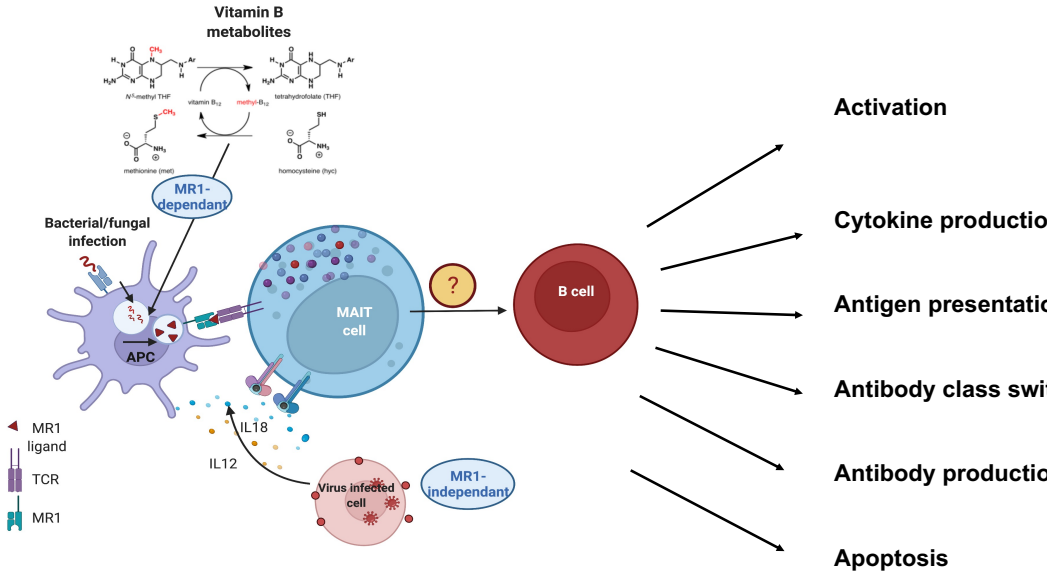

Figure 5.1. Schematic representation of MR1-dependent and MR1-independent MAIT cell activation and their possible influence on B cell activation. *The functions shown on the right are the objectives being addressed in the present chapter.*

5.3 Methods

The methods employed in the present investigation are summarised in Figure 5.2.

5.3.1 Analysis of MAIT cells by flow cytometry

PBMC or expanded MAIT cells were surface stained with the following mAbs: anti-CD3 PacBlue, anti-CD161 FITC and anti-Vα7.2 PE. Cells were analyzed by flow cytometry. MAIT cells were defined as $CD3^+$ $CD161^+$ $V\alpha7.2^+$ cells. Full methods are described in section 2.4.5.

5.3.2 Generation of MAIT cell lines

Protocols developed by Dr Andrew Hogan and colleagues at Maynooth University were used to expand MAIT cells from PBMCs from healthy donors. Full methods are described in section 2.6.1.

5.3.3 Generation of B cell cultures

PBMC were extracted from blood samples obtained from healthy donors or buffy coat packs (kindly donated by the Irish Blood Transfusion Service). B cells were purified using CD19 Microbeads as described in section 2.5.1 and cultured for up to 7 days in complete RPMI medium. Flow cytometric examination of CD20 expression revealed that the purity of B cells was >99%.

5.3.4 Activation of B cells

$0.5x10^6$ PBMCs or sorted B cells were transferred to wells of a microtiter plate in cRPMI and stimulated with BAFF, LPS, Pam3Csk4, Poly I:C + Pam3Csk4, IL-21, anti-CD40 mAb + IL-4 + IL-21, MAIT cells or IL-12 + IL-18-activated MAIT cells it is described in section 2.7.

5.3.5 Activation of MAIT cells

Sorted MAIT cells or MAIT cells within PBMCs were stimulated with medium alone (unstimulated), IL-12 with IL-18 (IL-12/IL-18), anti-CD3 and anti-CD28 mAbs (anti-CD3 & CD28), 5-A-RU or phorbol myristate acetate with ionomycin (PMA/Ionomycin), as described in section 2.6.3.

5.3.6 Analysis the expression of markers of antigen presentation by B cells

B cells were stimulated as described above or cultured with equal numbers of MAIT cells for 24 h, 72 h and 7 days in cRPMI. The cells were then stained with mAbs specific for CD3, CD20, CD161 and Vα7.2 and markers of antigen presentation CD40, CD86 and HLA-DR and analysed by flow cytometry. Mean fluorescence intensity (MFI) values of CD40, CD86 and HLA-DR expression by $CD3^{-}CD20^{+}$ cells (B cells) were measured. CD20 was used to detect B cells, rather than CD19 because this marker was frequently blocked by the anti-CD19 antibody used to purify B cells. The results were interpreted as a fold change in the co-culture compared to B cells alone.

5.3.7 Analysis of cytokine production

PBMC, MAIT cells, B cells or co-cultures of MAIT cells and B cells, were stimulated as described above for 24 or 72 h. The cells were then cultured with monensin overnight and then stained for cell surface expression of CD3, CD161 and Vα7.2, CD19. The cells were then fixed and permeabilised and stained for intracellular expression of IFN-γ, IL-4, IL-6, IL-10, IL-12p40, IL-13 and TNF-α for analysis by flow cytometry (see section 2.4.6).

5.3.8 Analysis of cytokine release By ELISA

MAIT cells were stimulated as described above for 24 h. The supernatants were then harvested and assayed for levels of IFN-γ, IL-4, IL-6, IL-10, IL-12p70, and TNF-α by enzyme-linked immunosorbent assay (ELISA) multiplex ELISA kits (MesoScaleDiagnostics, USA). Full details of the methods are described in section 2.8.4.

5.3.9 Analysis of MAIT cell degranulation

Anti-CD107a PerCP-Cy5.5 labeled mAb was added to cultures of MAIT cells or co-cultures of MAIT cells and B cells. The cells were then incubated for 1 hour at 37°C and 5% CO_2. After 1 hour, monensin (2 mM) was added to prevent the mAb conjugate from being proteolytically digested when CD107a was reinternalized. CD107a expression by $CD3^+$ $CD161^+$ $V\alpha7.2^+$ cells (MAIT cells) was analyzed by flow cytometry. Full details of the methods are described in section 2.8.2.

5.3.10 Measurement of antibody production by B cells

MAIT cells were cultured with B cells at various ratios in the presence or absence of B stimulators, as described above, for 7 days. The supernatants were harvested and analysed using immunoglobulin Cytometric Bead Array kits for IgA, IgM, IgE and total IgG levels (see section 2.8.3).

5.3.11 Examination of immunoglobulin class switching after treatment with various stimuli

Total B cells were cultured in medium alone or with equal numbers of expanded MAIT cells for 3 days. Cells were then stained for cell surface expression of CD19, CD27, and IgD. Flow cytometry was used to examine changes in the percentages of naive ($CD27^-$ IgD^+), unswitched memory ($CD27^+IgD^+$), switched memory ($CD27^+IgD^-$), and CD27 memory ($CD27^-IgD^-$) B cells.

5.3.12 Analysis of cell death

Direct killing of B cells by MAIT cells was measured using a Total Cytotoxicity and Apoptosis Detection Kit from Immunochemistry Technologies and flow cytometry as described in section 2.9.

Lytic ability of MAIT cells against B cells was also tested by staining the cells with annexin V and propidium iodide (PI) to measure apoptosis and necrosis respectively by flow cytometry. Full details of these methods are described in section 2.10.

5.3.13 Statistical analysis

Statistical analysis was done using GraphPad Prism Version 9.0 (GraphPad Prism, San Diego, California). Treatments of cells were compared using a paired t test or all statistical tests, a p value ≤ 0.05 was considered significant.

Summary methods

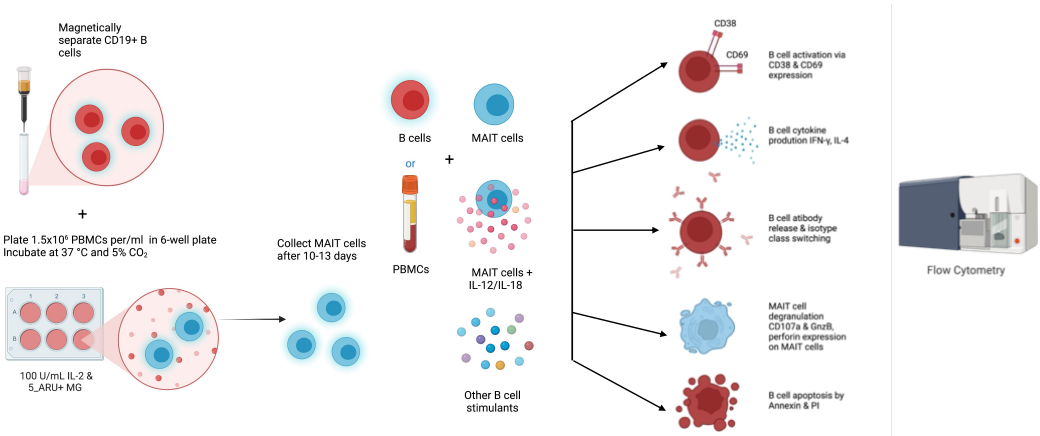

Figure 5.2. Summary of investigations carried out in the present chapter. *B cells were isolated from using CD19 microbeads. More PBMCs from the same donor were treated with 5-ARU as described in section 2.6.1 for 10-13 days, Purified CD19 B cells were stimulated under different conditions or co-cultured with MAIT cells or IL-12 + IL-18-activated MAIT cells. The readout from each experiment was shown in the figure. Figure created with BioRender.com*

5.4 Results

5.4.1 Activation of MAIT cells within PBMC

To optimise conditions for the analysis of cytokine production by MAIT cells, PBMCs from 6 healthy donors were stimulated for 24 hours with PMA/ionomycin, IL-12/IL-18, anti-CD3/anti-CD28 or 5-A-RU with methylglyoxal. We used flow cytometry to examine which of these MAIT cell stimulators could optimally induce the expression of the early activation marker CD69 by MAIT cells within PBMC (Figure 5.3).

The unstimulated control shows baseline expression of CD69 on the MAIT cells in PBMCs. IL-12/IL-18 stimulation resulted in a mean 30% increase in the number of MAIT cells expressing CD69 after 24h, ($p \leq 0.01$). Anti-CD3/anti-CD28 induced a ~60% increase in the number of MAIT cells expressing CD69 ($p \leq 0.0001$). 5-ARU also increased the percentage of activated MAIT cells to >60% ($p \leq 0.0001$). PMA/ionomycin was responsible for an 85% increase in CD69 expression by MAIT cells ($p \leq 0.0001$). All the stimulants successfully activated MAIT cells within the PBMC sample with IL-12/IL-18 being the least effective and PMA/ionomycin being the most effective for inducing CD69 expression.

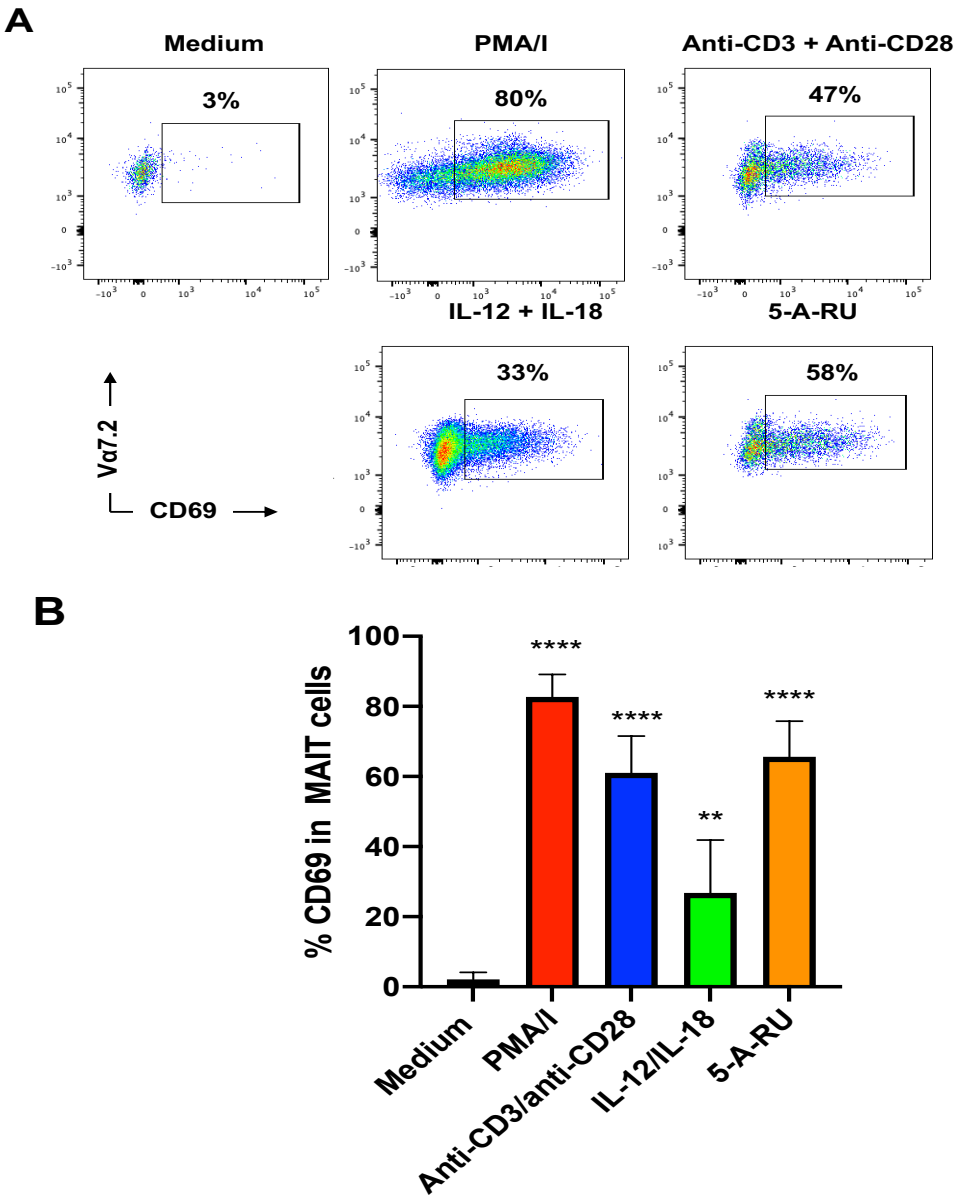

Figure 5.3 Activation of MAIT cells within PBMC. *PBMC were stimulated for 24 hours with medium alone, PMA and ionomycin (PMA/I), mAbs specific for CD3 and CD28, IL-12 with IL-18 or 5-A-RU. Cells were then stained with mAbs specific for CD3, CD161, Vα7.2 and CD69 and the percentages of MAIT cells that expressed CD69 were determined by flow cytometry. A, Flow cytometry dot plots showing CD69 expression by gated MAIT cells, defined by positivity for CD3, CD161 and Vα7.2, within PBMC after stimulation. B, Bar graph showing mean ± SEM percentages of MAIT cells from 6 donors that expressed CD69 after each treatment. **, P<0.01; ****, P<0.0001 using the paired t test.*

5.4.2 IFN-γ production by MAIT cells

We next sought to examine the production of IFN-γ by MAIT cells within PBMC in response to TCR-dependent and TCR-independent stimulation. PBMC were stimulated with medium alone, IL-12 and IL-18, PMA and ionomycin, anti-CD3 and anti-CD28 mAbs, or 5-A-RU with methylglyoxal for 24 h (Figure 5.4). The frequencies of MAIT cells that expressed IFN-**γ** were determined by flow cytometry (Figure 5.4A). Unstimulated T cells showed little IFN-**γ** production. When PBMCs were stimulated with PMA/ionomycin, a mean of 18% of MAIT cells produced IFN-**γ**. 5-ARU stimulation caused a significant increase in the frequencies of MAIT cells that produced IFN-**γ** (27%) compared to unstimulated cells. Anti-CD3/anti-CD28 mAbs and IL-12/IL-18 were less stimulatory, inducing IFN-γ production by means of 9% and 14% of MAIT cells, respectively (Figure 5.4B).

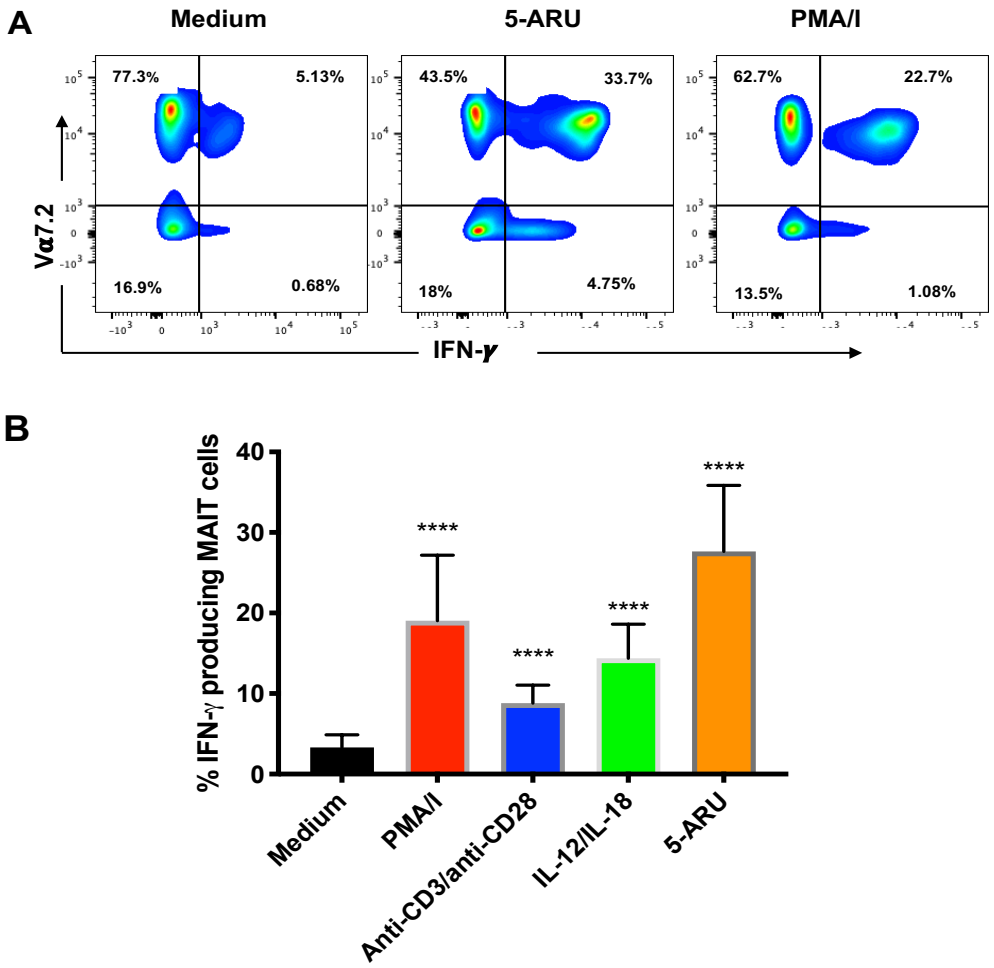

Figure 5.4 Induction of IFN-γ production by MAIT cells within PBMC*. PBMCs were treated for 48 hours with medium alone, PMA and ionomycin (PMA/I), mAbs specific for CD3 and CD28 (anti-CD3/anti-CD28), IL-12 with IL-18, or 5-ARU. Cells were then stained with mAbs specific for cell surface CD3, CD161 and Vα7.2 and intracellular IFN-γ and analysed by flow cytometry. A, Flow cytometry dot plots showing expression of IFN-γ by Vα7.2^{+} cells after gating on CD3^{+}CD161^{+} cells (MAIT cells). B, Bar graph showing frequencies of MAIT cells that expressed IFN-γ after each treatment. Data are expressed as mean (±SEM) of MAIT cells from 15 donors that expressed IFN-γ. ****p ≤0.0001 using* the paired t test.

5.4.3 IL-17 production by MAIT cells

Both IFN-γ and IL-17 are characteristically secreted by activated MAIT cells [343, 502, 512]. PBMCs were stimulated with PMA and ionomycin, anti-CD3 and anti-CD28 mAbs, IL-12 and IL-18, 5-ARU or medium alone as an unstimulated control for 24 h and the frequencies of MAIT cells that produced IL-17 were determined by flow cytometry (Fig. 5.5A). Unstimulated MAIT cells showed almost no IL-17 production. When MAIT were stimulated with PMA/ionomycin, ~7 % of MAIT cells produced IL-17. About 8% of MAIT cells produced IL-17 when stimulated with 5-ARU. Again, anti-CD3/anti-CD28 mAbs were the least stimulatory, inducing IL-17 production by a mean of 3% of MAIT cells.

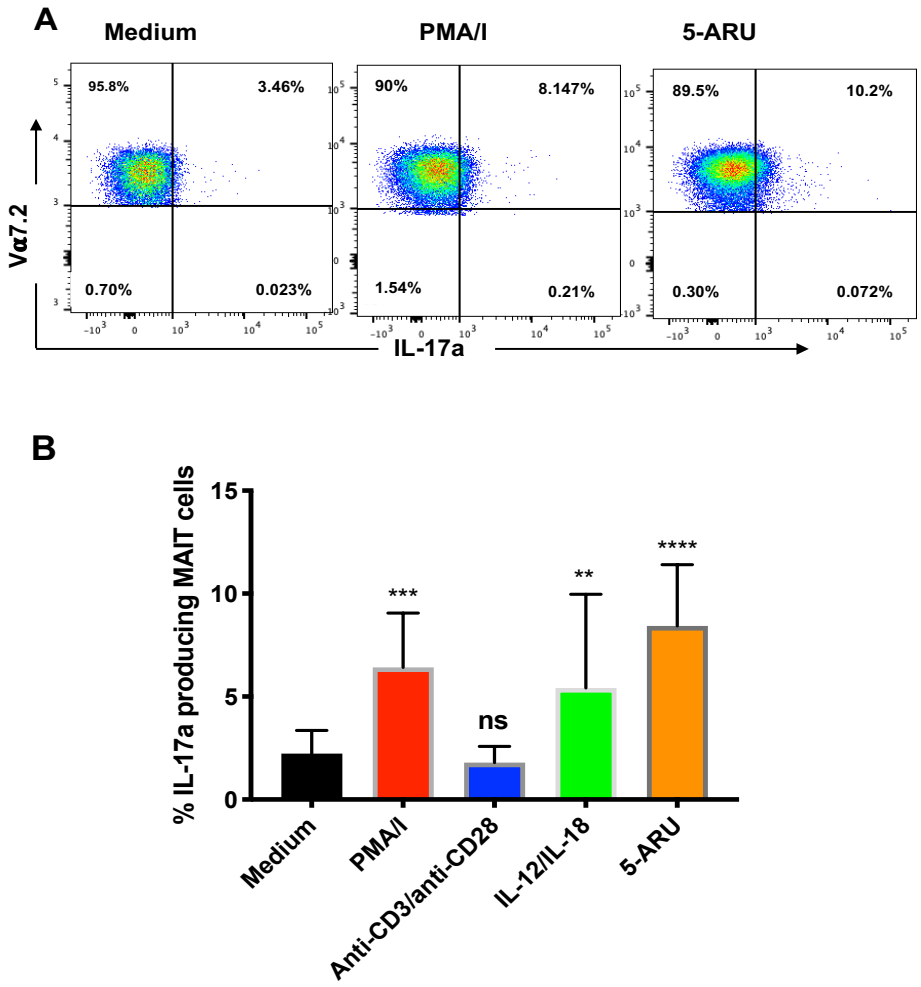

Figure 5.5 Induction of IL-17 production by MAIT cells within PBMC*. PBMCs were treated for 24 hours with medium alone, PMA and ionomycin (PMA/I), mAbs specific for CD3 and CD28 (anti-CD3/anti-CD28), IL-12 with IL-18, or 5-ARU. Cells were then stained with mAbs specific for cell surface CD3, CD161 and Vα7.2 and intracellular IL-17 and analyzed by flow cytometry. A, Flow cytometry dot plots showing expression of IL-17 by Vα7.2$^+$ cells after gating on CD3$^+$CD161$^+$ cells (MAIT cells). B, Bar graph showing frequencies of MAIT cells that expressed IL-17 after each treatment. Data are expressed as mean (±SEM) of MAIT cells from 15 donors that expressed IL-17. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤0.0001 using the paired t test.*

5.4.2 Expansion of MAIT cells from PBMCs using 5-A-RU

In order to optimise MAIT cell expansion from PBMCs *in vitro*, PBMCs were treated with 5-ARU as described in section 2.6.1. Figure 5.6A shows that MAIT cells expanded potently in response to 5-AR-U in the presence of methylglyoxal. This method of expansion resulted in a high purity of MAIT cells, with the starting purity of about 3-4% increasing to about 75% on day 13 and 90% on day 15 (Figure 5.6B). Over $200x10^6$ MAIT cells of high purity could routinely be generated in 2 weeks using this method (Figure 5.6C).

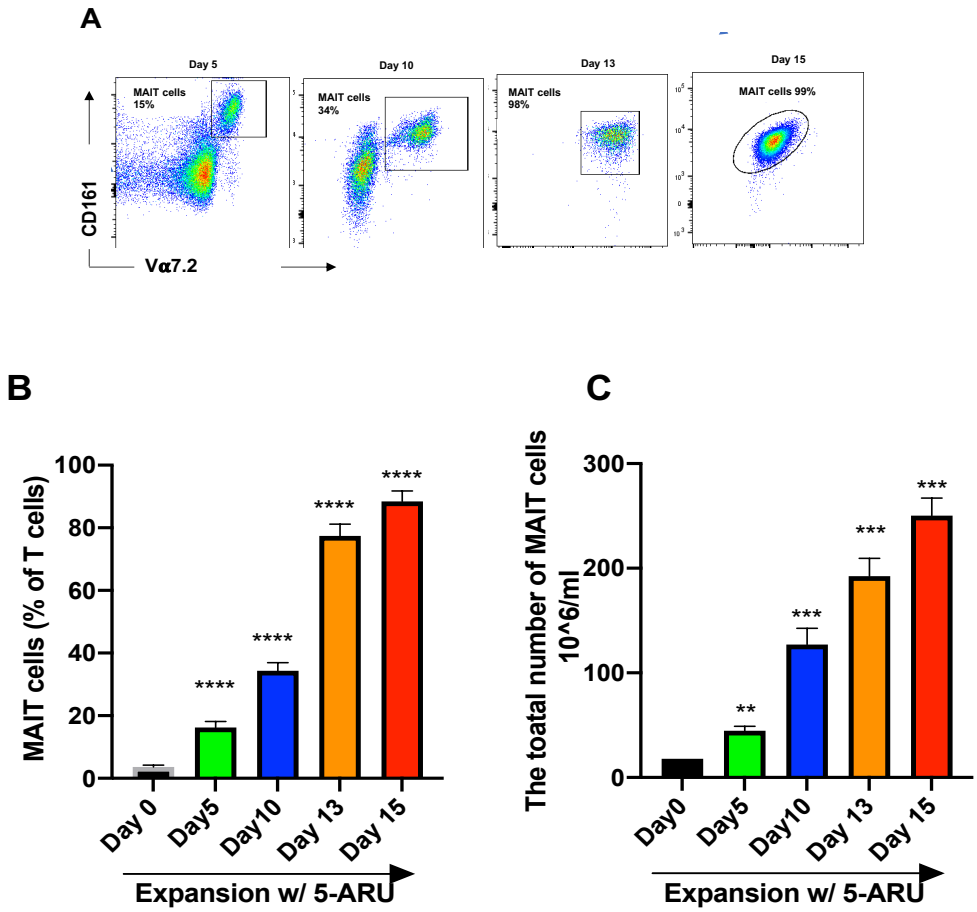

Figure 5.6. In vitro expansion of MAIT cells. MAIT cell lines were generated by stimulating PBMC with 5-ARU and culturing them with IL-2 as described in section 2.6.1. *A, Flow cytometry dot plots showing Vα7.2 and CD161 expression by gated CD3+ cells within PBMC after stimulation with 5-ARU for 5, 10, 13 and 15 days. B and C, Bar graphs showing percentage frequencies (B) and absolute numbers (C) of MAIT cells generated after 5, 10, 13 and 15 days post stimulation of PBMC. Data are expressed as means (±SEM) of 12 MAIT cell expansions . **p ≤0.01, ***p ≤0.001, ****p ≤0.0001 using the paired t test.*

5.4.2.1 Optimisation of IFN-γ production by MAIT cells lines

MAIT cells naturally secrete IFN-γ upon activation (Figure 5.4). To establish optimal conditions for inducing IFN-γ production by MAIT cell lines, MAIT cell lines from 12 healthy donors were generated as described in section 2.6.1. The cells were cultured for 24 h with either medium, 10 ng/ml IL-7 or 50 ng/ml each of IL-1β and IL-23 [244]. The cells were then stimulated with either PMA and ionomycin, anti-CD3/CD28 mAb, IL-12 with IL-18 or 5-ARU or left untreated for 4 h or 96 h. The cells were then stained using mAb specific for cell surface CD3, CD161 and Vα7.2 to detect MAIT cells and intracellular IFN-γ and analysed by flow cytometry. For each culture condition, the highest percentage of IFN-γ production by MAIT cells occurred when the cells were stimulated with PMA/ionomycin. Little IFN-γ was detected in MAIT cells stimulated with anti-CD3/CD28 mAb or IL-12+IL-18, however, after culture with IL-7, MAIT cells stimulated under these conditions produced IFN-γ in both after 4 h and 96 hrs. This study shows that PMA/I was the most potent stimulator of IFN-γ by MAIT cells. However, because of the non-physiological nature of PMA/I as a T cell stimulant, routine activation of MAIT cells was carried out IL-12/IL-18. Similarly, at 96 h, PMA/I was the most potent stimulator of IFN-γ production by MAIT cells, but interestingly, 5-ARU also induced IFN-γ secretion by similar frequencies of MAIT cells when IL-7 was added to the preculture. Conversely, IL-12/IL-18 was the most potent stimulator of IFN-γ by MAIT cells when we precultured them with IL-1β + IL-23 ($p<0.001$; figure 5.7). Again, because of the non-physiological nature of PMA/I as T cell stimulant, routine activation of MAIT cells was carried out using IL-12/IL-18.

5.4.2.2 IL-17 production by MAIT cells

We also investigated if MAIT cells can produce IL-17 in response to different modes of culture and stimulation. MAIT cell lines from healthy donors (n=12) were cultured for 24 h with either medium alone IL-7, or IL-1β + IL-23. The cells were then stimulated with either PMA and ionomycin, anti-CD3/anti-CD28, IL-12 with IL-18 or 5-ARU or left untreated for 4 h or 96 h. Then the cells were stained using mAb specific for cell surface CD3, CD161 and Vα72 to detect MAIT cells and intracellular IL-17 and analysed by flow cytometry. As for IFN-γ, we found that there was significant increase in the frequencies of IL-17 production by MAIT cells at 4 hours under stimulation with PMA/I and this trend also has been seen when we precultured the MAIT cells with IL-7 or IL-1β + IL-23 (Figure 5.8). Similarly, at 96 h PMA/I induced IL-17 production by <15% of MAIT cells but IL-12/IL-18 also induced IL-17 secretion precultured with IL-1β + IL-23 $p<0.001$ (Figure 5.8).

These data show that MAIT cells can be stimulated undervarious conditions to produce IFN-γ and IL-17.

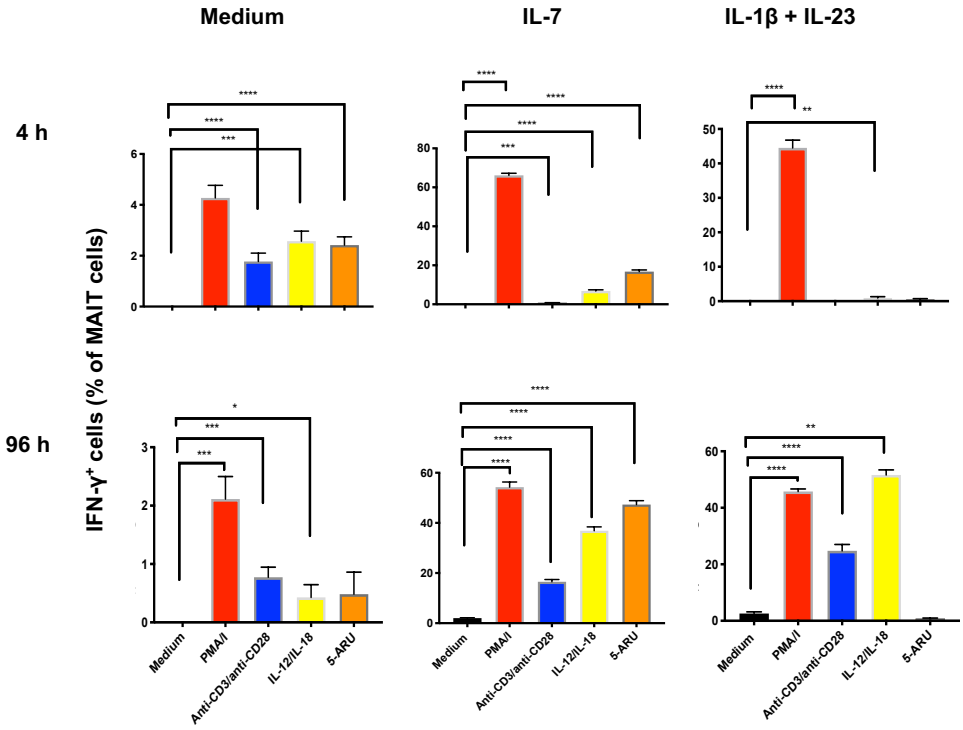

Figure 5.7 Induction of IFN-γ production by expanded MAIT cells. *Expanded MAIT cells from 12 healthy donors were cultured for 4 or 96 h with either medium, IL-7, or IL-1β + IL-23. The cells were then stimulated with medium alone, PMA and ionomycin (PMA/I), anti-CD3/CD28 mAb, IL-12 + IL-18, or 5-ARU for 4 h or 96 h. Cells were then stained with mAbs specific for CD3, CD161 and Vα.72 to detect MAIT cells and intracellular IFN-γ and analysed by flow cytometry. Bars represent means ±SEM percentages of MAIT cells that produced IFN-γ after each treatment. *P<0.05; **P<0.01; ***P<0.001;****P<0.0001 using the paired t test.*

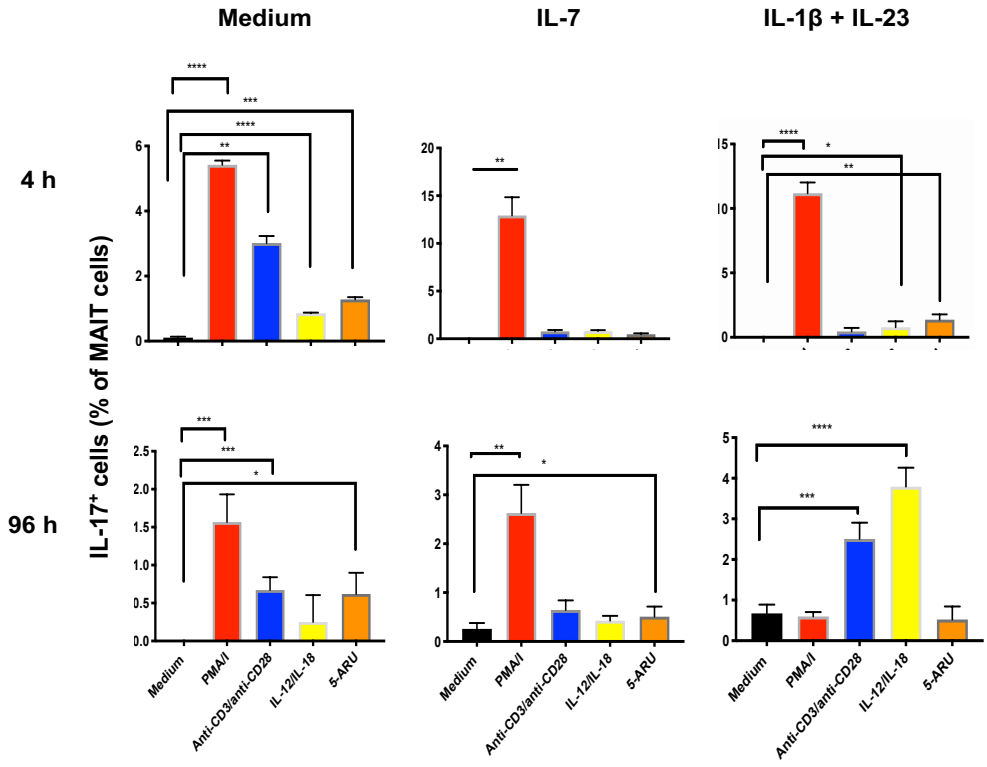

Figure 5.8 Induction of IL-17A production by expanded MAIT cells. *Expanded MAIT cells from 12 healthy donors were cultured for 4 or 96 h with either medium, IL-7, or IL-1β + IL-23. The cells were then stimulated with medium alone, PMA and ionomycin (PMA/I), anti-CD3/anti-CD28 mAb, IL-12 + IL-18, or 5-ARU for 4 h or 96 h. Cells were then stained with mAbs specific for CD3, CD161 and Vα72 to detect MAIT cells and intracellular IL-17 and analysed by flow cytometry. Bars represent means ±SEM percentages of MAIT cells that produced IFN-γ after each treatment. *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001 using the paired t test.*

5.4.3 MAIT cells secrete pro- and anti-inflammatory cytokines

While the flow cytometric cytokine assay revealed the percentage of cells expressing cytokines, we also quantified the levels of cytokine production in cell supernatants. Expanded MAIT cells from 10 donors were stimulated with PMA/I, anti-CD3/anti-CD28 and IL-12/IL-18 or left unstimulated for 24 h, and then supernatants were analysed for levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17a, IL-21, IL-22, IL-23, by multiplex ELISA. We found that MAIT cells stimulated with PMA/I produced significant amounts of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17a, IL21 and IL-22 (Figures 5.9 and 5.10) but not IL-23, after 42 h. In contrast, MAIT cells stimulated with anti-CD3/CD28 produced significant amounts of IFN-γ, IL-2, IL-4, IL-5 and IL-6 but did not augment TNF-α, IL-8, or IL-9, IL-13, IL-17, IL-22 or IL-23 (Figures 5.7 and 5.8) production compared with MAIT cells cultured with medium alone. IL-12/IL-18 induced significant secretion of IL-5, IL-6, IL-8, IL-9, IL-13, IL-21 and IL-22 but not IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-17a, nor IL-21 after 72h (Figures 5.9 and 5.10). These data indicate that many factors are released by activated MAIT cell that may influence B cell differentiation and antibody production.

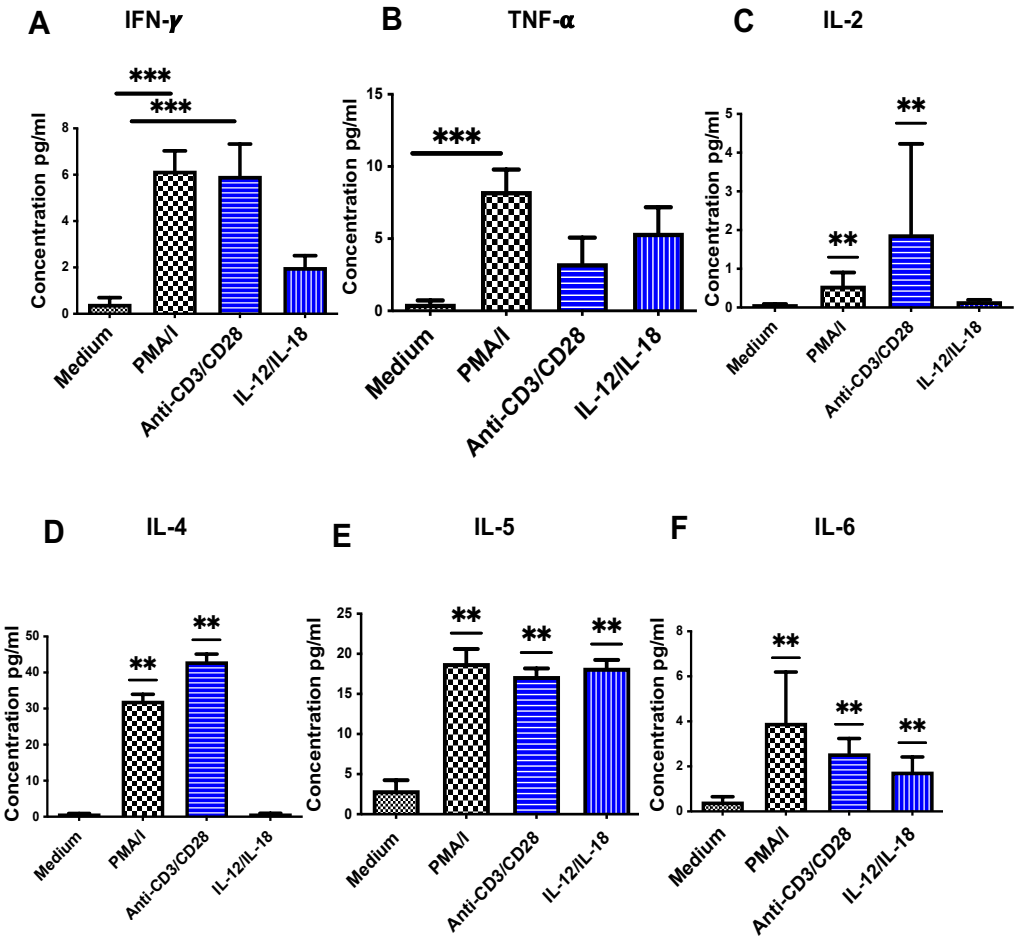

Figure 5.9 Cytokine production by expanded MAIT cell lines. *Expanded MAIT cells were stimulated with PMA/I, Anti-CD3/CD28 and IL-12/IL-18 or left unstimulated for 24 h, then cell supernatants assayed for cytokine levels by multiplex ELISA. Graphs show mean (±SEM) levels of IFN-γ (A) TNF-α, (B) IL-2, (C) IL-4, (D) IL-5, (E) and IL-6 (F). Data shown are mean (±SEM) cytokine levels measured in supernatants of 10 MAIT cell lines. Groups were compared using the paired t test. **p < 0.01, ***p < 0.001. Data from 10 separate experiments (n = 10).*

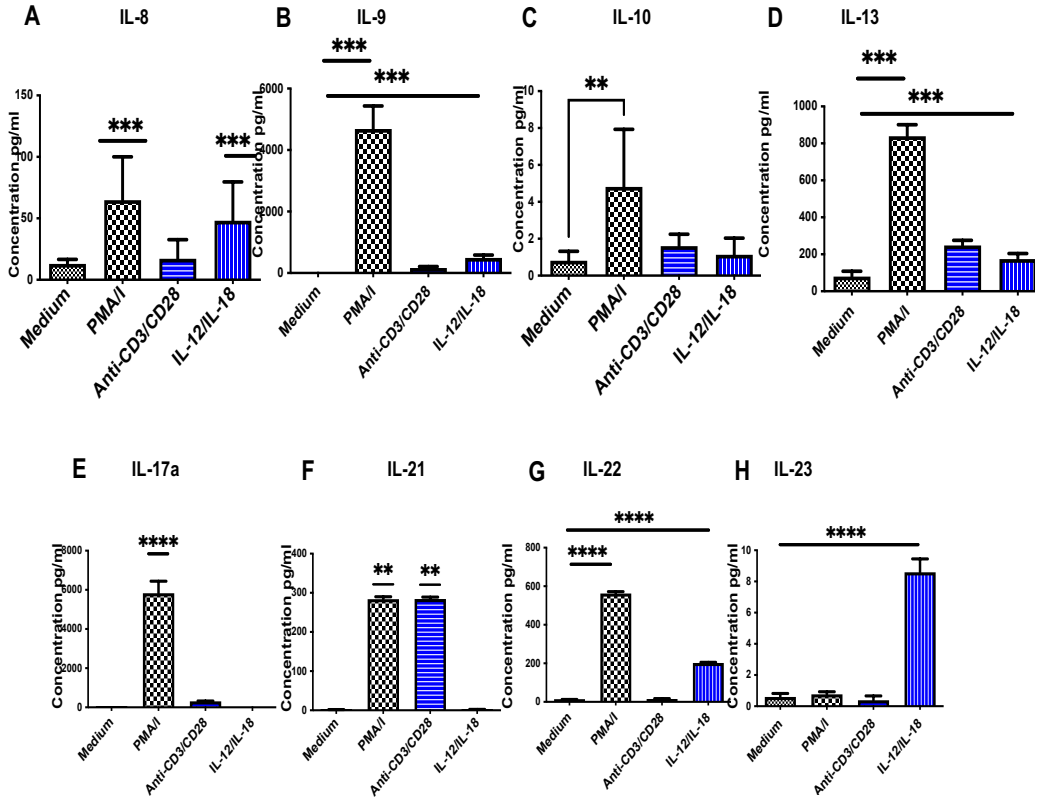

Figure 5.10 ***Cytokine production by expanded MAIT cell lines induce distinct cytokine.*** *Expanded MAIT cells were stimulated with PMA/I, Anti-CD3/CD28 and IL-12/IL-18 or left unstimulated for 24 h, then cell supernatants assayed for cytokine levels by multiplex ELISA. Graphs show mean (±SEM) levels of IL-8 (A) IL-9 (B) IL-10 (C) IL-13, (D) IL-17a, (E), IL-21 (F), IL-22 (G),and IL-23(H). Data shown are mean (±SEM) cytokine levels measured in supernatants of 10 MAIT cell lines. Groups were compared using the* paired t test. ***p < 0.01, ***p < 0.001, ****p < 0.0001. Data pulled from 10 separate experiments (n = 10).*

5.4.3 Co-culture experiments investigating MAIT and B cell synergistic actions

Recent studies have shown that some innate T cells, including iNKT cells, Vδ2 T cells and Vδ3 T cells can provide B cell help for antibody production [477, 478, 511]. We aimed to determine if MAIT cells also are capable of inducing B cell maturation, antibody isotype switching and antibody production. We also investigated if MAIT cells have the potential to promote maturation of B cells into APC and if MAIT cells can kill autologous B cells. These experiments were carried out by co-culturing lines of 5-ARU-expanded MAIT cells and sorted B cells. The effects of MAIT cells, either resting or activated with IL-12 and IL-18, on B cell functions were compared with those of a number of stimulants known to activate B cells, including medium alone (unstimulated), LPS, BAFF, Pam3Cysk4 the TLR3 agonist Poly:IC, PamCysk with poly:IC (PolyIC/PamCysk), and anti-CD40 mAb with IL-4 and IL-21 (CD40/IL-4/IL-21).

5.4.3.2 Activation of B cells by MAIT cells

B cells were sorted from PBMC by magnetic selection of $CD19^+$ cells (Figure. 5.11). Sorted B cells were cultured for 1, 3 or 7 days with resting or activated autologous MAIT cells and as controls, a variety of B cell stimulators. Cells were then stained with mAbs specific for CD20, CD69 and CD38 and the percentages of B cells that expressed CD69 were determined (Figure 5.12A).

Figure. 5.10B shows the mean (± SEM) percentages of B cells that expressed CD69 over time for all B cell stimulations. Unstimulated B cells showed baseline expression of CD69, which is approximately 10% and this increased gradually up to a maximum of 90% with BAFF, LPS, PolyI:C/Pam3Csk4, IL-21, and anti-CD40/IL-4/IL-2, by 7 days (p = 0.0001). Up to 30% of B cells expressed CD69 expression after stimulation for 1 day. Co-culture of B cells with MAIT cells, either resting or activated with IL-12 and IL-

18 also induced significant CD69 expression ($p \leq 0.0001$). Thus, MAIT cells are capable of activating B cells.

Figure 5.11A shows that B cell activation also led to the expression of CD38. Similar to CD69, unstimulated B cells showed baseline expression of CD38, which was approximately 10% of the cells, and this increased slowly from this baseline to a maximum of 80%, when stimulated with BAFF, PolyI:C/Pam3Csk4, anti-CD40/IL-4/IL-21 after 7 days. In contrast, LPS and IL-21 on its own did not significantly increase CD38 expression at any time point. Lastly, resting or IL-12/IL-18-activated MAIT cells induced a significant ~60% increase in CD38 expression ($p \leq 0.0001$). Thus, both resting and activated MAIT cells are capable of activating autologous B cells *in vitro*.

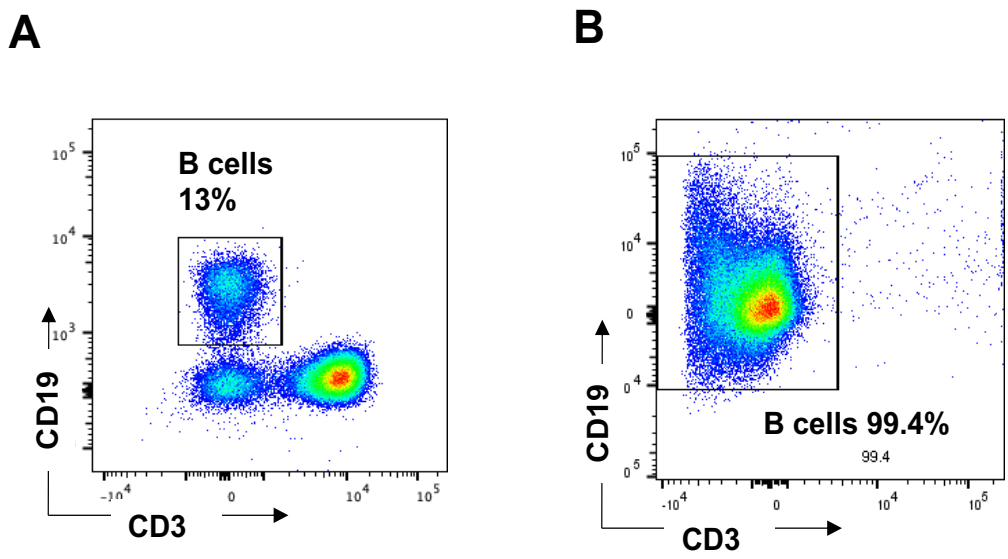

Figure 5.11 Isolation of B cells. *PBMC were labelled with mAbs specific for CD3 and CD19 and the $CD3^{-}CD19^{+}$ cells were isolated using a CD19 magnetic beads. Flow cytometry dot plots show the expression of CD3 and CD19 by unsorted PBMC (A) and sorted B cells (B).*

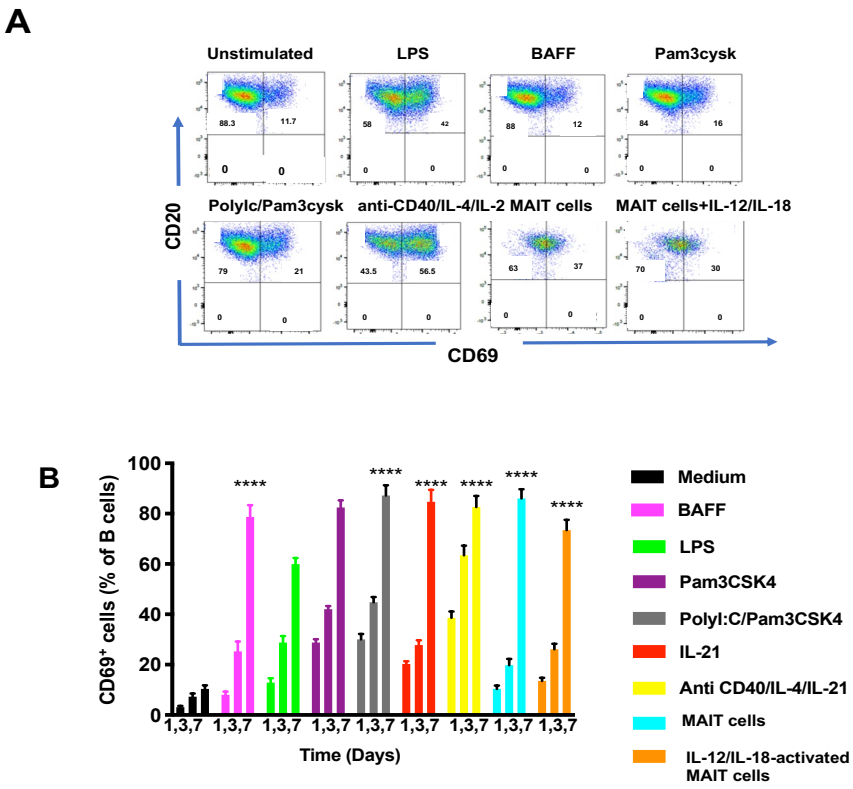

Figure 5.12 Activation of sorted B cells in vitro. *Sorted B cells were stimulated for 1, 3 or 7 days with medium alone, BAFF, LPS, Pam3Csk4, Poly I:C + Pam3Csk4, IL-21, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20 and CD69 and the percentages of B cells that expressed CD69 were determined by flow cytometry. A, Flow cytometry dot plots showing CD69 expression by gated B cells, defined by positivity for CD20. B, Bar graph showing mean ± SEM percentages of B cells from 15 donors that expressed CD69 after each treatment. ****P<0.0001 using a paired t test.*

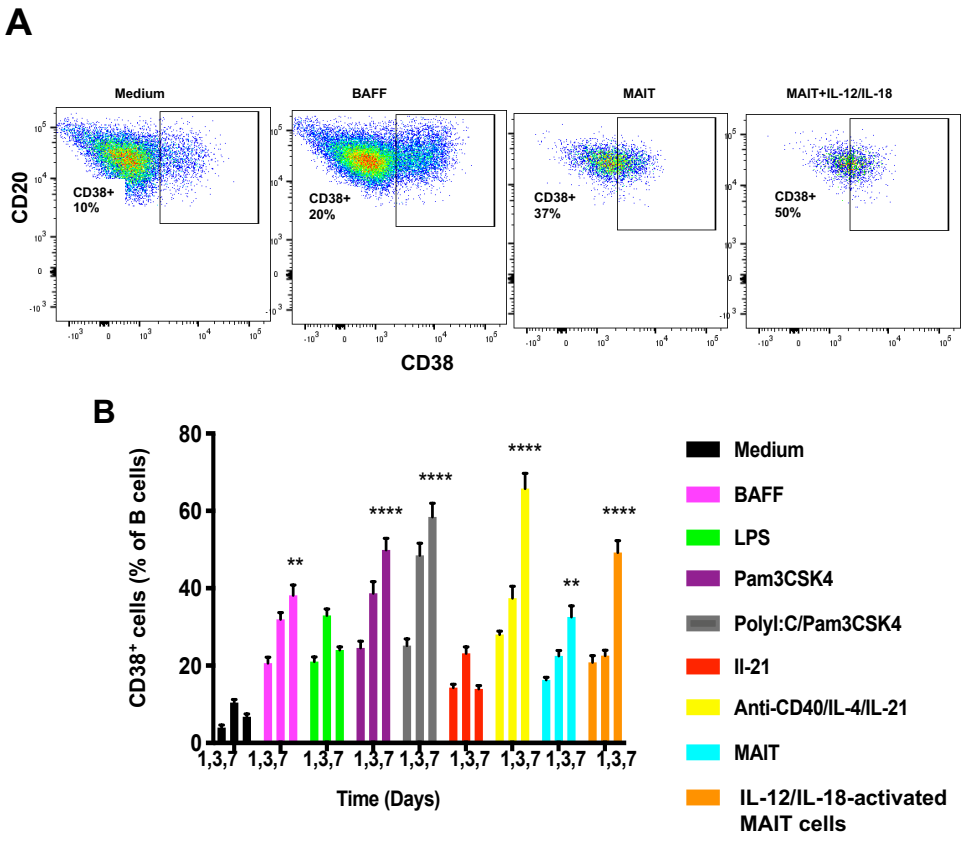

Figure 5.13 Activation of sorted B cells in vitro. *Sorted B cells were stimulated for 1, 3 or 7 days with medium alone, with medium alone, BAFF, LPS, Pam3Csk4, Poly I:C + Pam3Csk4, IL-21, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20 and CD38 and the percentages of B cells that expressed CD38 were determined by flow cytometry. A, Flow cytometry dot plots showing gating of B cells defined by positivity for CD20and CD38 expression by gated B cells after stimulation. B, Bar graph showing mean ± SEM percentages of B cells from 15 donors that expressed CD38 after each treatment. ** P<0.01; ****P<0.0001 using a paired t test.*

5.4.3.2 MAIT cells induce the expression of markers of antigen presentation by B cells

We next investigated if MAIT cells can induce differentiation of B cells into cells with phenotypes of APC. Purified CD19 B cells were stimulated for 1, 3 or 7 days with medium alone, LPS, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, or co-cultured with MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20, HLA-DR, CD40 and CD86 and the MFI of HLA-DR, CD40 and CD86 on B cells was determined by flow cytometry. Figure 5.14 shows that HLA-DR expression on B cells was significantly upregulated by each of these conditions in comparison to medium alone except for anti-CD40 mAb which showed no difference when compared to the control. HLA-DR expression on B cells peaked at 3 days post incubation for each condition apart from Poly I:C + Pam3Csk4 which saw a significant increase in expression by day 7. The most potent inducer of HLA-DR expression on B cells was unactivated MAIT cells. These data suggest that resting and IL-12/IL-18-activated MAIT cells are capable of inducing HLA-DR on B cells.

Figure 5.15 shows that CD86 expression on B cells was significantly upregulated by stimulation with LPS 3 or 7 days, compared with unstimulated B cells. No CD86 upregulation was induced by poly I:C + Pam3Csk4 treatment. The most potent inducer of CD86 expression on B cells was unactivated MAIT cells, which induced significant CD86 expression by day 3. IL-12/IL-18-activated MAIT cells also induced CD86 expression by B cells, but to a lesser degree than resting MAIT cells.

CD40 expression was not induced on B cells after stimulation with LPS or poly I:C + Pam3Csk4. However, co-culture of B cells with MAIT cells led to induction of CD40

expression on the B cells. CD40 expression peaked at 3 days post incubation resting MAIT cells and with MAIT cells activated with IL-12/IL-18 (Figure 5.16).

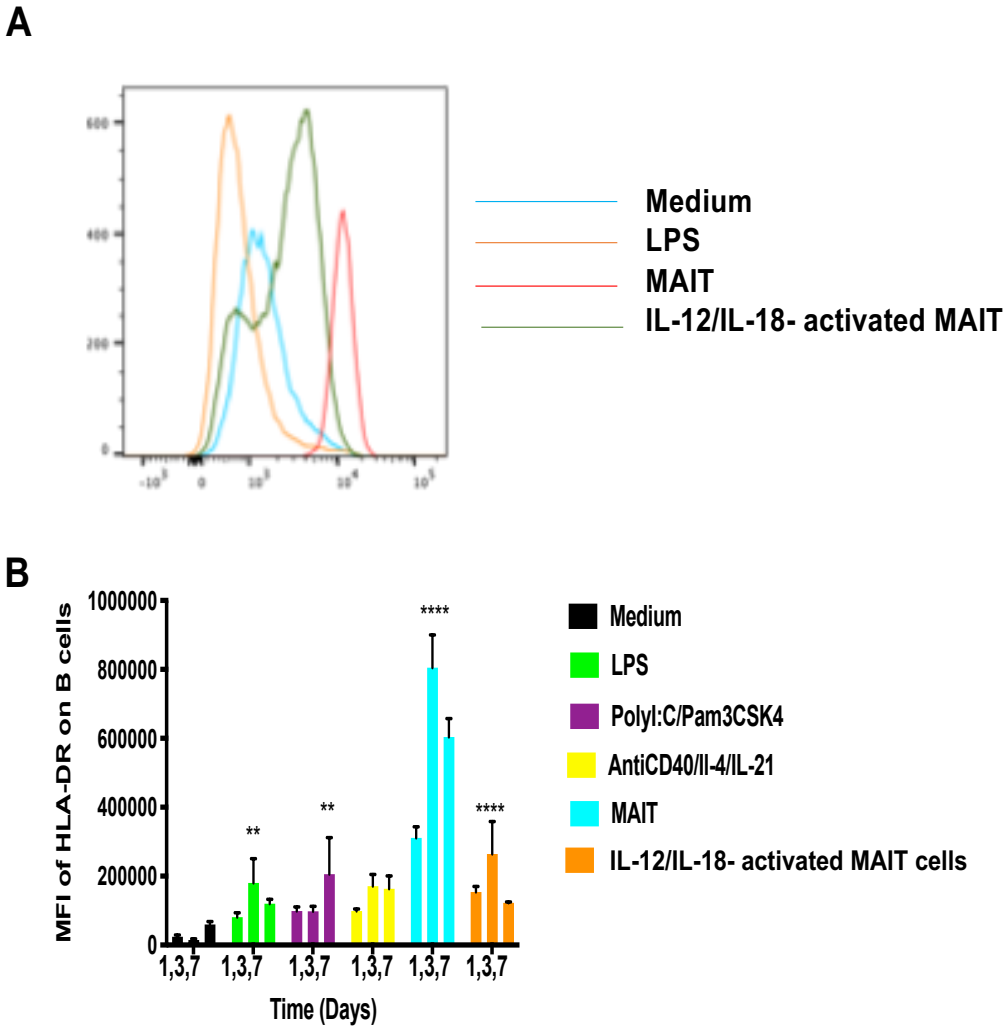

Figure 5.14 Induction of HLA-DR on B cells by MAIT cells. *Sorted B cells were stimulated for 1, 3 or 7 days with medium alone, LPS, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20 and HLA-DR and the MFI of HLA-DR expression on B were determined by flow cytometry. A, Flow cytometry histograms showing expression of HLA-DR by gated $CD20^+$ cells after stimulation. B, Bar graph showing mean ± SEM MFI of HLA-DR expression on B cells from 15 donors after each treatment. **P<0.01; ****P<0.0001 compared to unstimulated sample at the same time point, using a paired t test.*

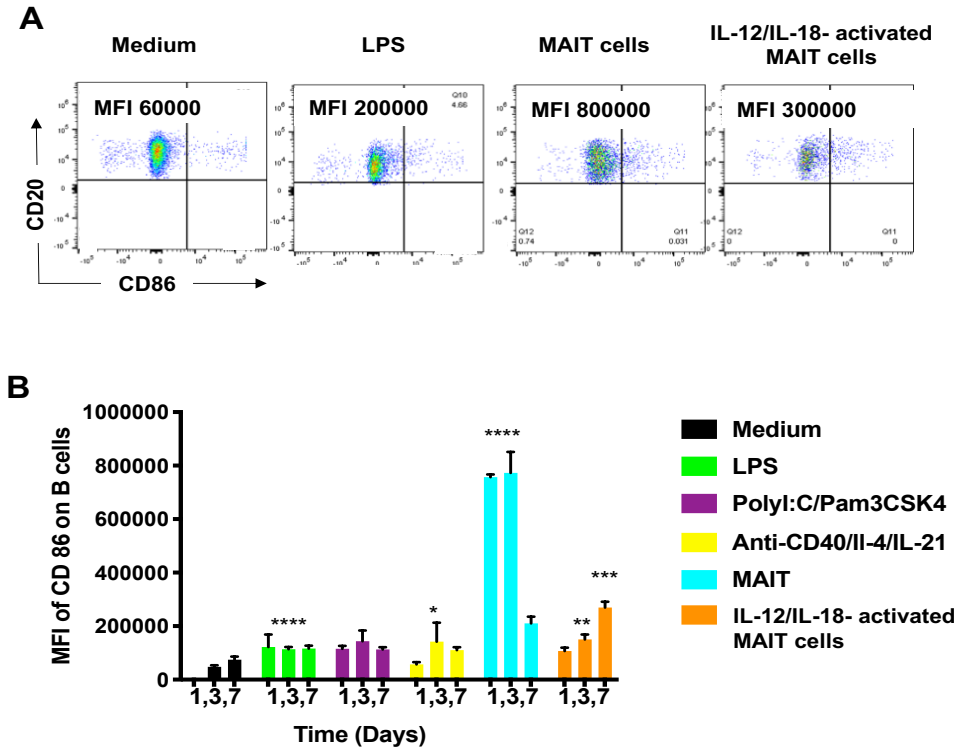

Figure 5.15 Induction of CD86 on B cells by MAIT cells. *Sorted B cells were stimulated for 1, 3 or 7 days with medium alone, LPS, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20 and CD86 and the MFI of CD86 expression was determined by flow cytometry. A, Flow cytometry dot plots showing expression of CD86 by $CD20^{+}$ cells after stimulation. B, Bar graph showing mean ± SEM MFI of CD86 expression by B cells from 15 donors after each treatment. *p<0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 compared to unstimulated sample at the same time point, using a paired t test*

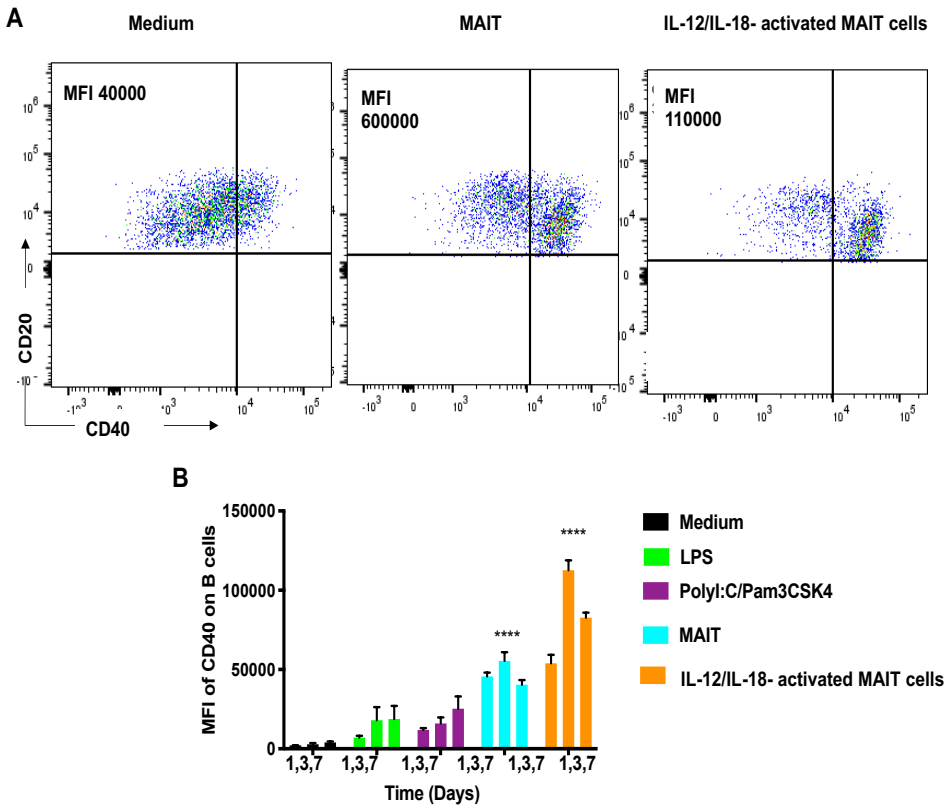

Figure 5.16 Induction of CD40 on B cells by MAIT cells. *Sorted B cells were stimulated for 1, 3 or 7 days with medium alone, BAFF, LPS, Poly I:C + Pam3Csk4, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for CD20 and CD40 and the MFI of CD40 expression was determined by flow cytometry. A, Flow cytometry dot plots showing expression of CD40 by gated $CD20^+$ cells after stimulation. B, Bar graph showing mean ± SEM MFI of CD40 expression on B cells from 15 donors after each treatment. ****P<0.0001 compared to unstimulated sample at the same time point, using a paired t test*

5.4.3.3 MAIT cells promote cytokine production by B cells

Magnetic bead sorted B cells were subjected to stimulation for 24 hours with medium alone, BAFF, LPS, polyI:C with Pam3Cysk4, anti-CD40 mAb with IL-4 and IL-21, or co-cultured with equal numbers of resting or IL-12 + IL-18-activated MAIT cells. The production of IFN-γ, IL-4, IL-10, and TNF-a by gated B cells was analysed by flow cytometry. Figure 5.17 shows that less than 10% of unstimulated B cells produced IFN-γ, but this number increased significantly when the B cells were treated with LPS or anti-CD40 mAb with IL-4 and IL-21, but not BAFF or polyI:C with Pam3Cysk4. Co-culture of B cells with resting MAIT cells did not lead to significant IFN-γ production, but co-culture with IL-12 + IL-18-activated MAIT cells resulted in IFN-γ production by about 40% of B cells.

In order to investigate whether MAIT cells can induce IL-4 production by B cells, B cells were treated with the above-mentioned stimuli over a 24-hour period and IL-4 production by B cells was examined by flow cytometry (Figure 5.18). Figure 5.18B shows that none of the stimuli, except for LPS were capable of inducing IL-4 and little change from the unstimulated control was observed. Baseline percentage of B cells expressing IL-4, was 1% and LPS stimulation resulted in an approximate 6-fold increase in IL-4 production. Stimulation with resting or IL-12/IL-18-activated MAIT cells induced a significant increase in IL-4 production with up to 7% of B cells producing this cytokine ($p \leq 0.001$; Figure 5.18).

Collectively, these data show that MAIT cells can induce cytokine production by B cells, but while activated MAIT cells are required to induce IFN-γ production, both resting and activated MAIT cells can induce IL-4 production.

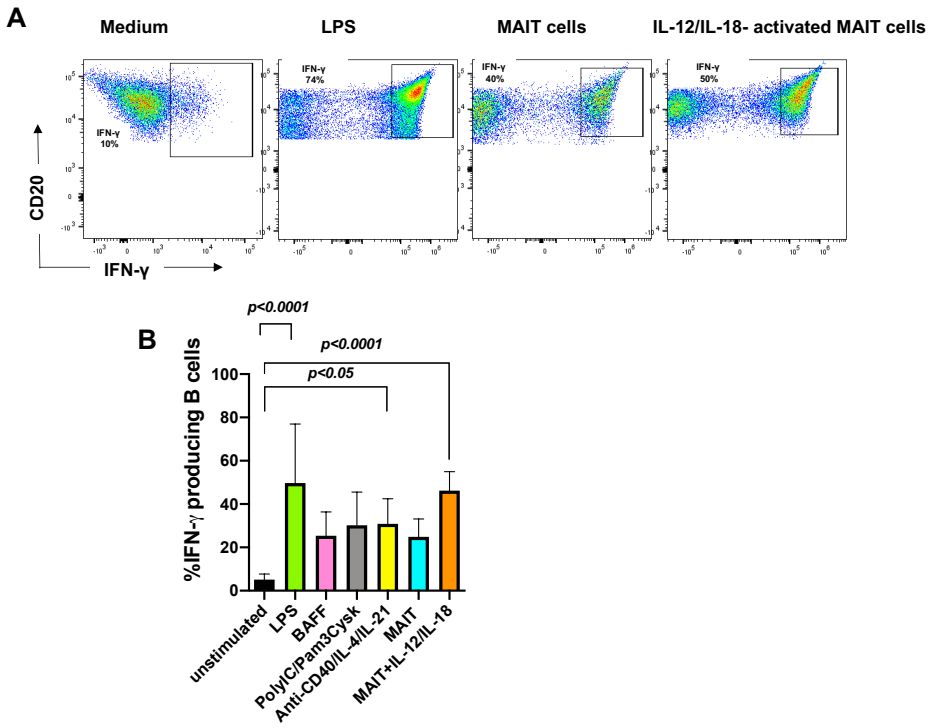

Figure 5.17 Induction of IFN-γ production by B cells by MAIT cells. *Sorted B cells were stimulated for 24 hours with medium alone, LPS, BAFF, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for cell-surface CD20 and intracellular IFN-γ and the percentages of B cells that expressed IFN-γ were determined by flow cytometry. A, Flow cytometry dot plots showing expression of IFN-γ by $CD20^+$ cells after stimulation. B, Bar graph showing mean ± SEM percentages of B cells from 6 donors that expressed IFN-γ after each treatment. Data were compared using a paired t test.*

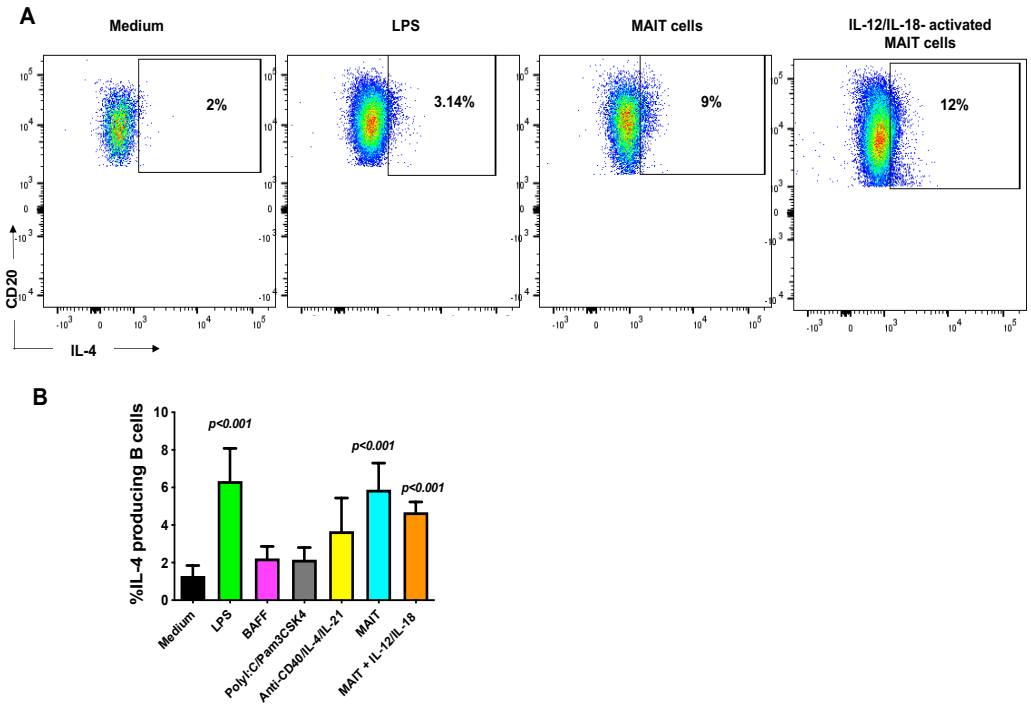

Figure 5.18 Induction of IL-4 production by B cells by MAIT cells. *Sorted B cells were stimulated for 24 hours with medium alone, LPS, BAFF, Poly I:C + Pam3Csk4, IL-21, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for cell-surface CD20 and intracellular IL-4 and the percentages of B cells that expressed IL-4 were determined by flow cytometry. A, Flow cytometry dot plots showing expression of IL-4 by $CD20^+$ cells after stimulation. B, Bar graph showing mean ± SEM percentages of B cells from 6 donors that expressed IL-4 after each treatment. Data were compared using a paired t test.*

5.4.4 MAIT cells promote immunoglobulin class switching by B cells

As section 5.5.2 and 5.5.3 suggest, resting MAIT cells and MAIT cells activated with IL-12 and IL-18 are capable of activating B cells. Therefore, further investigations were carried out to gain insight into if MAIT cells may induce immunoglobulin class switching in B cells and antibody secretion. Sorted B cells were stimulated for 7 days with medium alone, LPS, BAFF, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for cell-surface IgD and CD27 and the percentages of B cells that expressed $CD27^{-}IgD^{+}$ (naïve B cells), $CD27^{+}IgD^{+}$ (unswitched memory), $CD27^{+}IgD^{-}$ (switched memory) and $CD27^{-}IgD^{-}$ ('$CD27^{-}$ memory B cells') phenotypes were determined by flow cytometry. Figure 5.19 shows that about 70% of unstimulated B cells expressed the $CD27^{-}IgD^{+}$ phenotype (naïve B cells). Stimulation with LPS, BAFF, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21 resulted in a significant induction of CD27 expression, giving the B cells unswitched memory phenotypes. These changes were not associated with downregulation of IgD, indicating that the stimuli did not induce immunoglobulin class switching. Upon co-culture with resting MAIT cells, the majority of B cells acquired CD27+IgD- or CD27-IgD- phenotypes, indicating that MAIT cells induce immunoglobulin class switching. Interestingly, co-culture of B cells with IL-12/IL-18-activated MAIT cells resulted in immunoglobulin class switching but progression to the CD27-IgD- phenotype did not occur, as seen when the B cells were co-cultured with resting MAIT cells. Thus, MAIT cells can activate B cells to induce immunoglobulin class-switching.

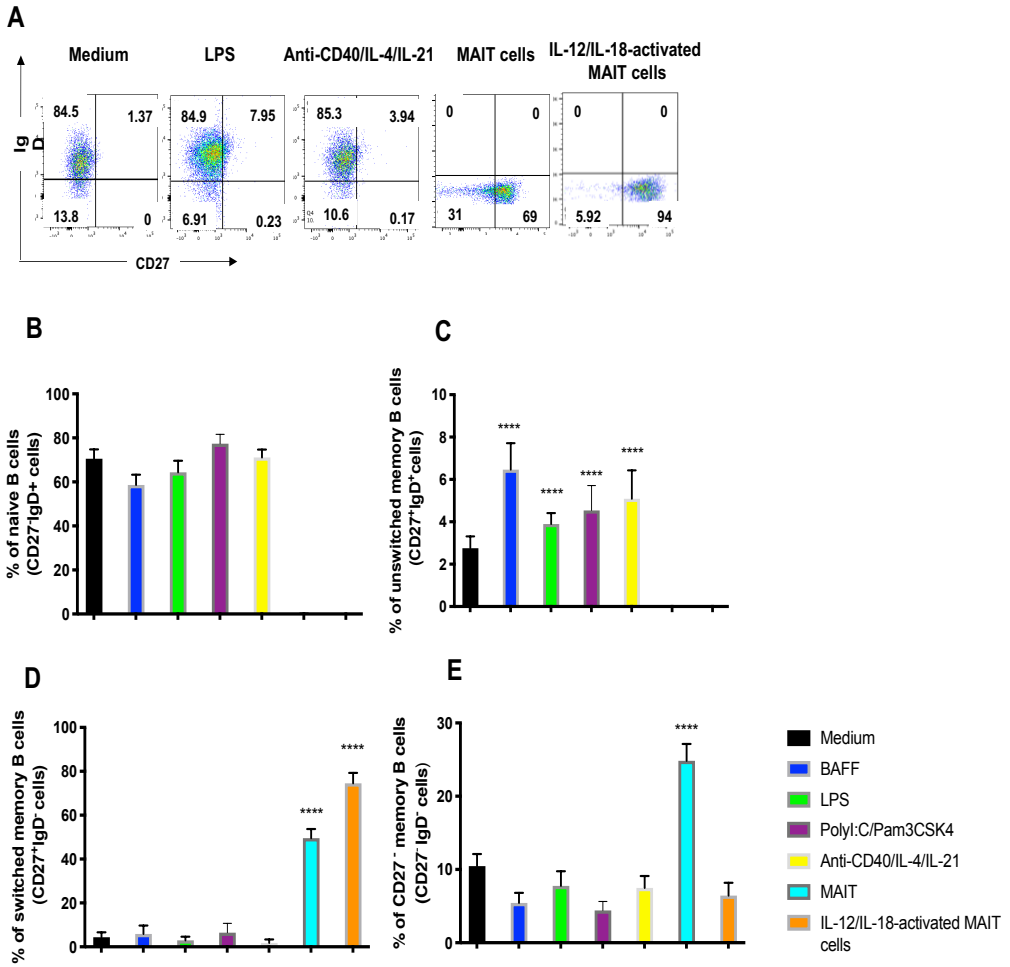

Figure 5.19 Induction of antibody isotype switching in B cells by MAIT cells. *Sorted B cells were stimulated for 7 days with medium alone, LPS, BAFF, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Cells were then stained with mAbs specific for cell-surface IgD and CD27 and the percentages of B cells that expressed $CD27^-IgD^+$ (naïve B cells), $CD27^+IgD^+$ (unswitched memory), $CD27^+IgD^-$ (switched memory) and $CD27^-IgD^-$ ('$CD27^-$ memory B cells') phenotypes were determined by flow cytometry. A, Flow cytometry dot plots showing expression of IgD and CD27 by gated $CD20^+$ cells after stimulation. B - E, Bar graph showing mean ± SEM percentages of B cells from 15 donors that expressed $CD27^-IgD^+$, $CD27^+IgD^+$, $CD27^+IgD^-$ and $CD27^-IgD^-$ phenotypes after each treatment. Data were compared using a paired t test (not significant). ****$P<0.0001$.*

5.4.4. MAIT cells induce antibody production by B cells

During infection, the first antibodies to be produced by B cells are IgD and IgM. In order to monitor the production of antibodies in response to various stimuli, a CBA immunoglobulin multiplex assay was used to quantify IgM, IgA, IgE and IgG in supernatants of B cells. No IgE was detected in any of the B cell supernatants (data not shown). Stimulation of B cells with LPS, polyI:C + Pam4Cysk3 and resting MAIT cells resulted in a significant increase in IgM. Stimulation with LPS and either resting or activated MAIT cells led to increased production of IgG, while stimulation with LPS, BAFF, Pam4Cysk3, and either resting or activated MAIT cells resulted in increased production of IgA. Interestingly, co-culture of B cells with IL-12/IL-18-activated MAIT cells resulted in the highest increases in IgG and IgA, which were increased 30-fold and 40-fold, respectively. These data suggest that while the soluble B cell stimulators can induce antibody secretion, they do not induce isotype switching by B cells. MAIT cells are capable of inducing both immunoglobulin class switching and antibody secretion (Figure 5.20).

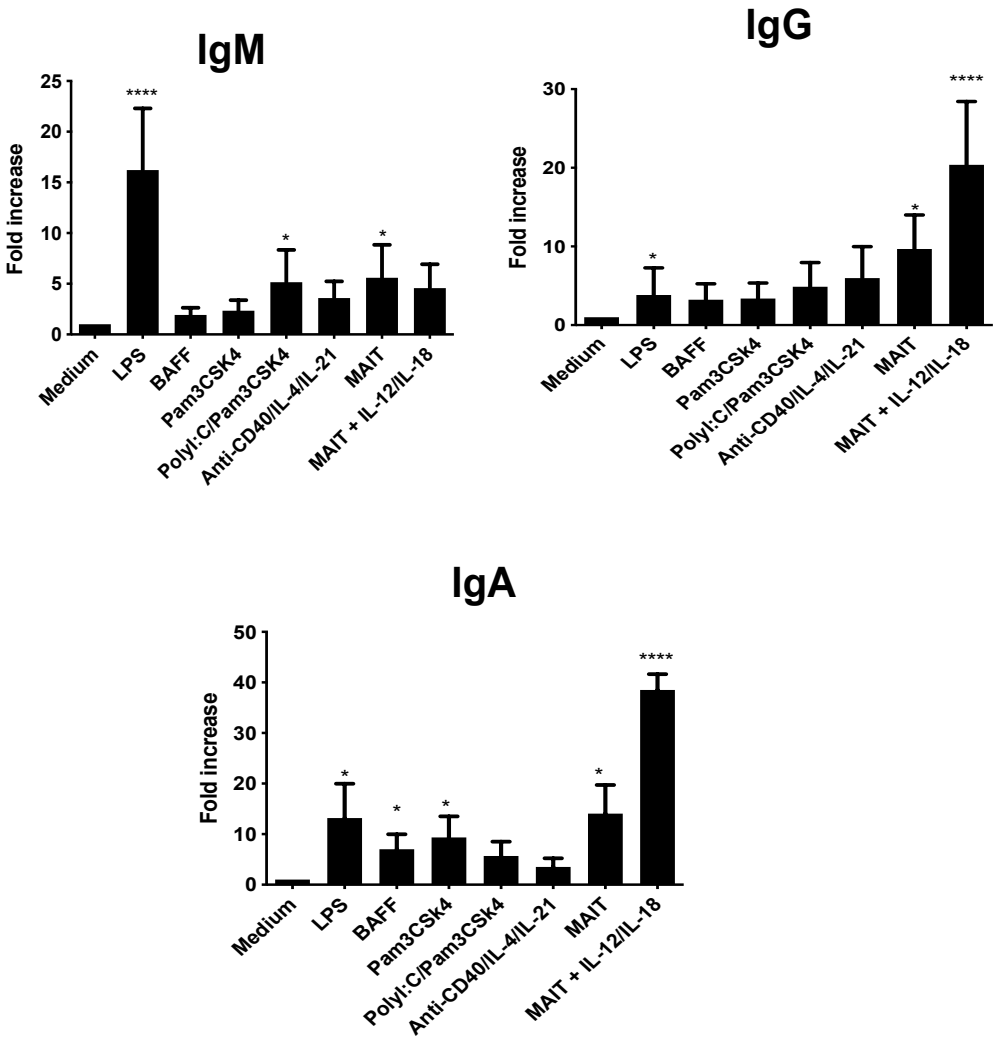

Figure 5.20 Induction of IgM, IgG and IgA release from B cells by MAIT cells*. Sorted B cells were stimulated for 7 days with medium alone, LPS, BAFF, Pam3Csk4, Poly I:C + Pam3Csk4, anti-CD40 mAb + IL-4 + IL-21, resting MAIT cells or IL-12 + IL-18-activated MAIT cells. Supernatants from B cell cultures were harvested, stored at -80°C, and later assayed for IgM, IgG and IgA using the CBA Immunoglobulin Assay. Results show mean ± SEM fold increases in antibody production. *P<0.05; ****P<0.0001 using a paired t test.*

5.4.5 MAIT cells degranulate in the presence of B cells

To evaluate if MAIT cells degranulate when co-cultured with B cells, we measured the expression of lysosomal protein marker CD107a, which is externalised during which the cells release perforin, granzyme B and various other cytolytic effector molecules. B cells were co-cultured with resting MAIT cells or IL-12 + IL-18 activated MAIT cells at various ratios (1:1, 0.5:1, 0.2:1 and 0.05:1) for 24, 48 and 72 hours. The percentage of MAIT cells expressing CD107a was examined by flow cytometry (Figure 5.21 A). After 24h, exposure to B cells resulted in an increase in CD107a expression on resting MAIT cells. As expected, the frequency of CD107a expression by MAIT cells did not increase with increasing MAIT/B cell ratio but increased over time up to 72 hours (Figure 5.21 B). Interestingly, IL-12 and IL-18 activated MAIT cells cultured alone did not upregulate CD107a, but when they were cultured with B cells, CD107a expression was significantly increased demonstrating that, within this cohort of donors, MAIT cells underwent degranulation in the presence of B cells but not when activated using cytokines (Figure 5.21 B).

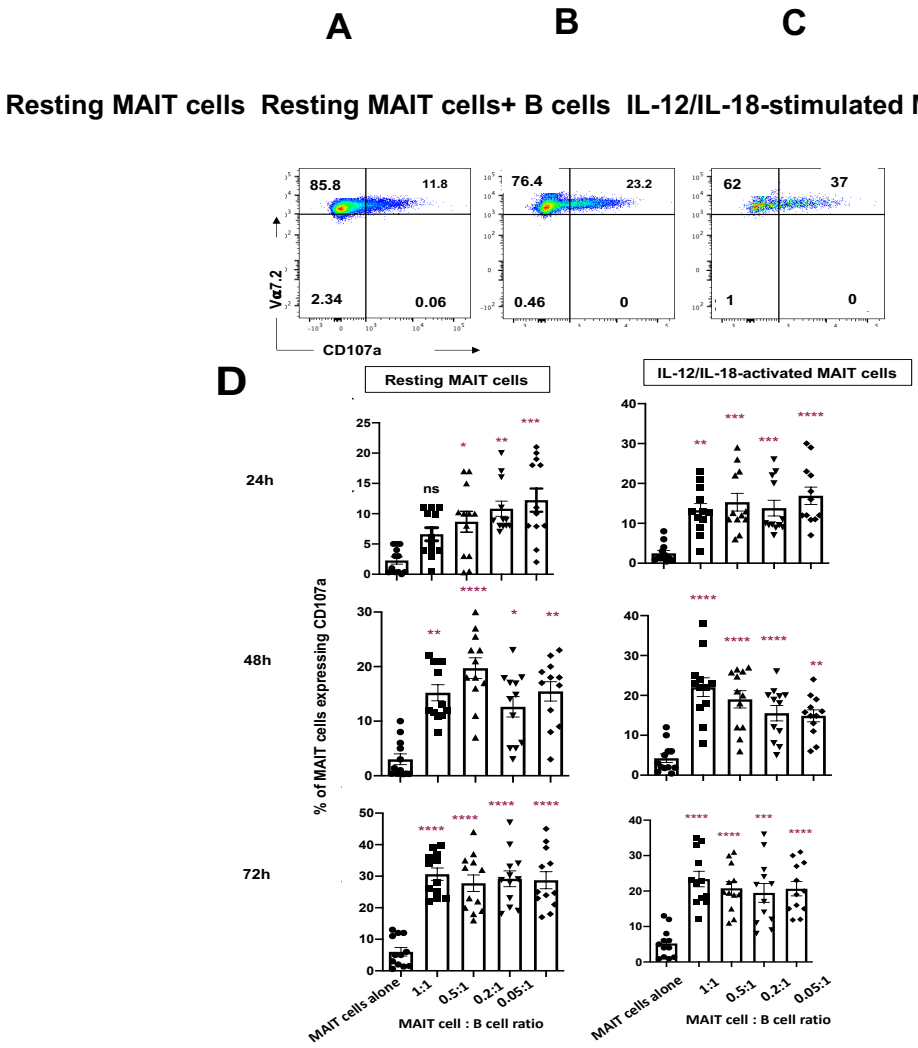

Figure 5.21 MAIT cells degranulate in the presence of autologous B cells. *Lines of expanded MAIT cells were incubated with medium alone or activated with IL-12 and IL-18 and cultured for 24 h, 48 h, and 72h with medium alone or with sorted autologous B cells at various MAIT:B cell ratios. A, Flow cytometry dot plot showing CD107a expression on MAIT cells incubated in the absence (*A*) and presence of B cells or B cells in the presence of IL-12 and IL-18 activated MAIT cells* (C). D*, Graphs shows mean (±SEM) percentage of MAIT cells from 12 donors that express CD107a under each condition. Each data point represents an individual blood donor. (*P<0.05; **p<0.01, ***p<0.001 using one-way ANOVA with Tukey's multiple comparisons test.*

5.4.6 MAIT cells upregulate granzyme B and perforin expression in the presence of B cells

In a continued attempt to understand these cytolytic interactions between MAIT cells and B cells another coculture experiment was performed. In this experiment B cells were co cultured with resting MAIT cells or IL-12 + IL-18 activated MAIT cells at various ratios (1:1, 0.5:1, 0.2:1 and 0.05:1) for 24, 48 and 72 hours. Gated MAIT cell populations were then examined for granzyme B expression by flow cytometry (Figure 5.22 B).

At 24 hours the percentage of resting MAIT cells expressing granzyme B was increased at all ratios, with a 0.05:1 ratio of resting MAIT cells:B cells demonstrating the largest increase (roughly 20%). The percentage of MAIT cells that expressed granzyme B was found to be similar at the 48 and 72 hr time points.

Activated MAIT cells demonstrated a similar pattern with higher levels of Granzyme B expression when co cultured with B cells at the same timepoints and ratios. At 24 hrs, the highest expression of granzyme B was seen at a ratio of 0.05:1. At 48 hrs, the highest increase in GzmB expression was seen at a ratio of 0.2:1 (roughly 35% increase) and at 72 hrs the highest increase was seen at a 1:1 ratio with an increase in 30%. These significant results combined with that of the CD107a assay (Fig. 5.22) suggest that MAIT cells have cytolytic tendencies towards B cells and achieve this via release of GzmB.

Analysis of perforin expression by MAIT cells exposed to B cells demonstrated a similar pattern to that of granzyme B expression with higher levels of perforin expression when cocultured with B cells at the same timepoints and ratios (Figure 5.23). When resting MAIT cells were co-cultured with B cells, at 24 hrs, high expression of perforin was seen at ratios of 1:1, 0.5, and 0.2:1, before it dropped down to the lowest expression at a ratio of 0.05:1. At 48 and 72 hrs increased expression of perforin was seen at all MAIT cell:B cell ratios, with up to 60% of MAIT cells expressing perforin. Similar

results were observed when IL-12 + IL-18-activated MAIT cells were co-cultured with the B cells (Figure 5.21B). The presence of IL-12 and IL-15 was not sufficient to induce perforin expression by MAIT cells – B cells were also required. These significant results combined with those of the CD107a assay (Fig. 5.20) and granzyme B (Fig. 5.22) suggest that MAIT cells have cytolytic capacities towards B cells and achieve this via release of lytic granules as granzyme B and perforin.

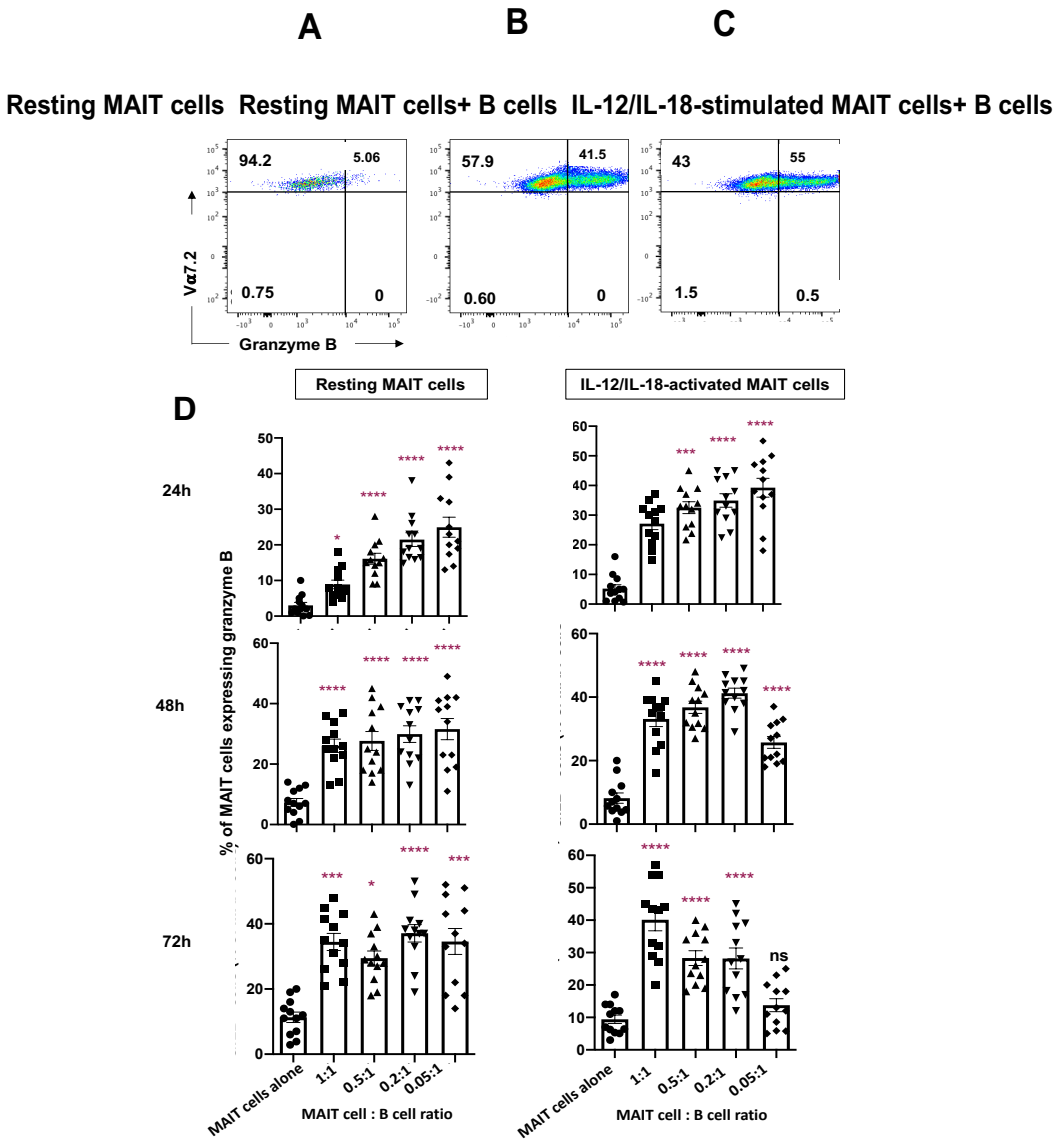

Figure 5.22 B cells induce granzyme B expression by MAIT cells. *Lines of expanded MAIT cells were incubated with medium alone or activated with IL-12 and IL-18 and cultured for 24 h, 48 h, and 72h with medium alone or with sorted autologous B cells at various MAIT:B cell ratios. Cells were then stained with mAbs specific for CD3, CD161, Vα7.2 and granzyme B and analysed by flow cytometry. A, Flow cytometry dot plot showing* granzyme B *expression on MAIT cells incubated in the absence (A) and presence of B cells (B, and B cells plus IL-12/IL-18 activated MAIT cells (C). D, Graphs shows mean (±SEM) percentage of MAIT cells from 12 donors that express granzyme B under each condition. Each data point represents an individual blood donor. (*P<0.05; **p<0.01, ***p<0.001 using one-way ANOVA with Tukey's multiple comparisons test.*

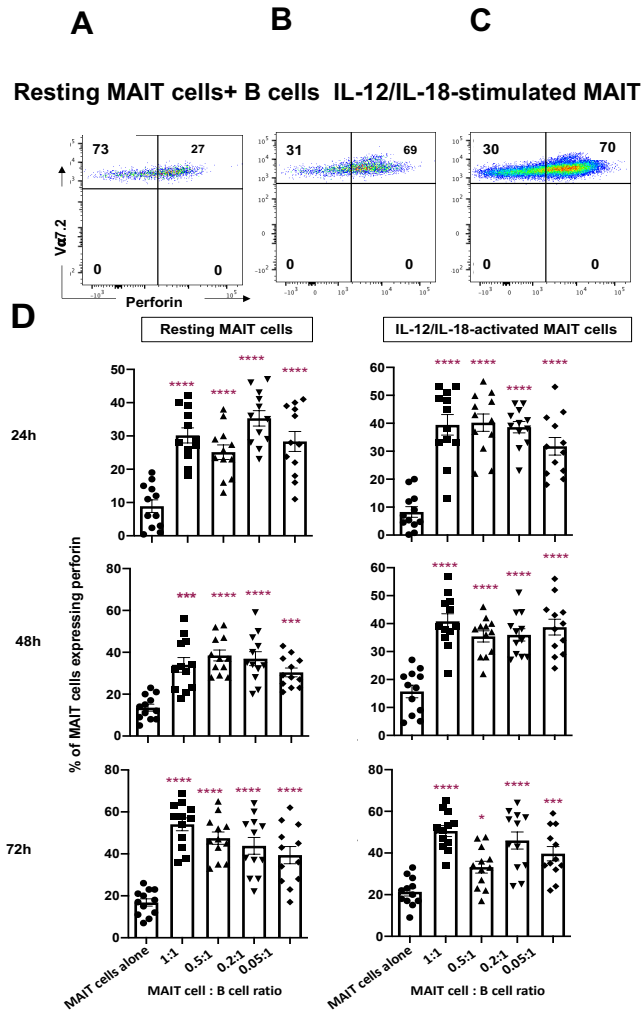

Figure 5.23 B cells induce perforin expression by MAIT cells. *Lines of expanded MAIT cells were incubated with medium alone or activated with IL-12 and IL-18 and cultured for 24 h, 48 h, and 72h with medium alone or with sorted autologous B cells at various MAIT:B cell ratios. Cells were then stained with mAbs specific for CD3, CD161, Vα7.2 and perforin and analysed by flow cytometry. A, Flow cytometry dot plot showing perforin expression on MAIT cells incubated in the absence (A) and presence of B cells(B, and B cells plus IL-12/IL-18 activated MAIT cells (C). D, Graphs shows mean (±SEM) percentage of MAIT cells from 12 donors that express granzyme B under each condition. Each data point represents an individual blood donor. *P<0.05; **p<0.01, ***p<0.001 using one-way ANOVA with Tukey's multiple comparisons test.*

5.4.7 MAIT kill autologous B cells

The above results indicate that MAIT cells upregulate expression of the degranulation marker CD107a and the cytotoxic effector molecules GzmB and perforin, when exposed to autologous B cells, suggesting that MAIT cells can kill B cells. To determine if MAIT cells can actually kill B cells, we carried out an apoptosis assay, by staining the cells with annexin V and propidium iodide (PI), which stain apoptotic and necrotic cells, respectively.

Sorted B cells from six donors were co-cultured for 24 hours, 48 hours, and 72 hours with medium alone or equivalent numbers of autologous resting or IL-12/IL-18-activated MAIT cells. Cells were then stained with mAbs specific to CD3 and CD20, propidium iodide (PI), and annexin V and analysed by flow cytometry. The frequencies of apoptotic or dead cells increased from less than 10% when B cells were cultured alone, to almost 40% when B cells were cultured for 48 h with equal numbers of resting MAIT cells. This number increased to 50% when IL-12/IL-18 activated MAIT cells were used. Cytotoxicity was not enhanced by co-culturing B cells with MAIT cells for 72 h (Figure 5.24). To confirm that this B cells death was MAIT cell-dependant, we also tested the viability of cells after coculture with total PBMCs. Figure 5.25 shows that addition of total PBMCs had little effect on B cell viability, with less than 6% of B cells expressing markers of apoptosis or death even after treatment IL-12/IL-18.

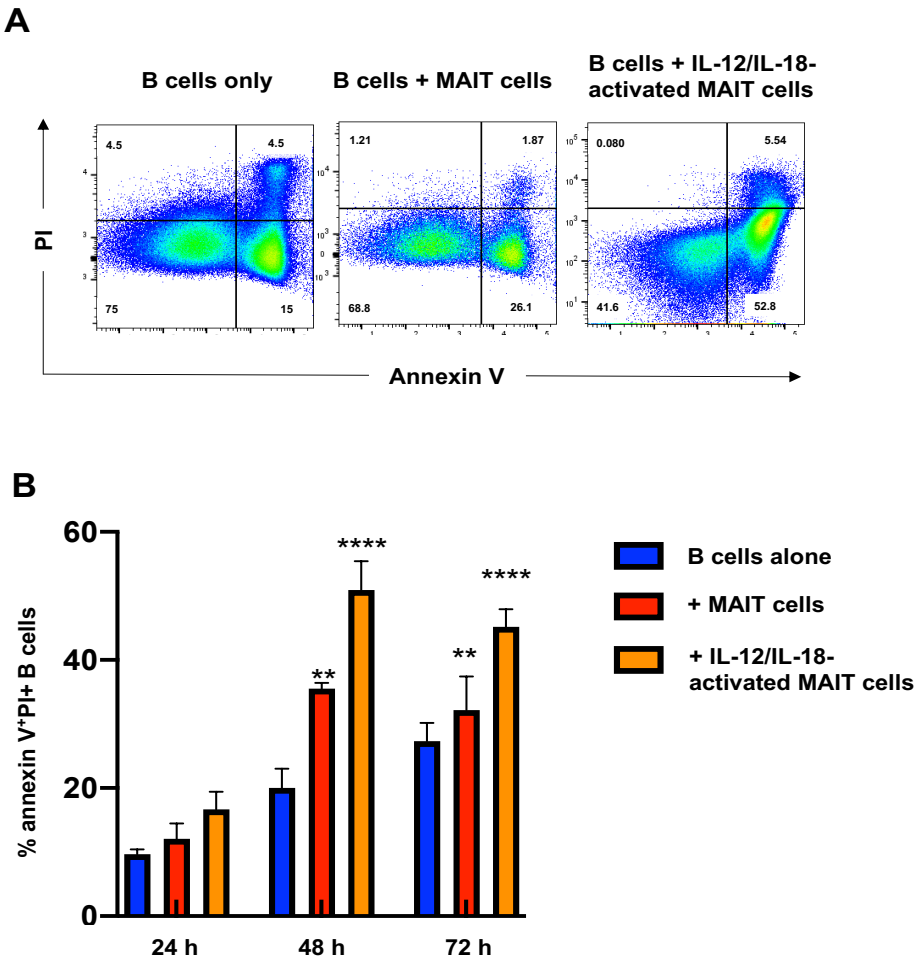

Figure 5.24 MAIT cells induce apoptosis in autologous B cells. *Sorted B cells from 6 donors were cultured for 24 h, 48 h, and 72h with medium alone or with equal numbers of autologous resting or IL-12/IL-18-activaterd MAIT cells. Cells were then stained with mAbs specific for CD3 and CD20 and propidium iodide (PI) and annexin V and analysed by flow cytometry. A, Flow cytometry dot plots showing annexin V and PI staining of gated B cells incubated in the absence (left) and presence of resting (centre) and activated (right) MAIT cells. B, Graphs shows mean (±SEM) percentage of MAIT cells from 6 donors that express annexin V under each condition. **p<0.01, ****p<0.0001 using using a paired t test.*

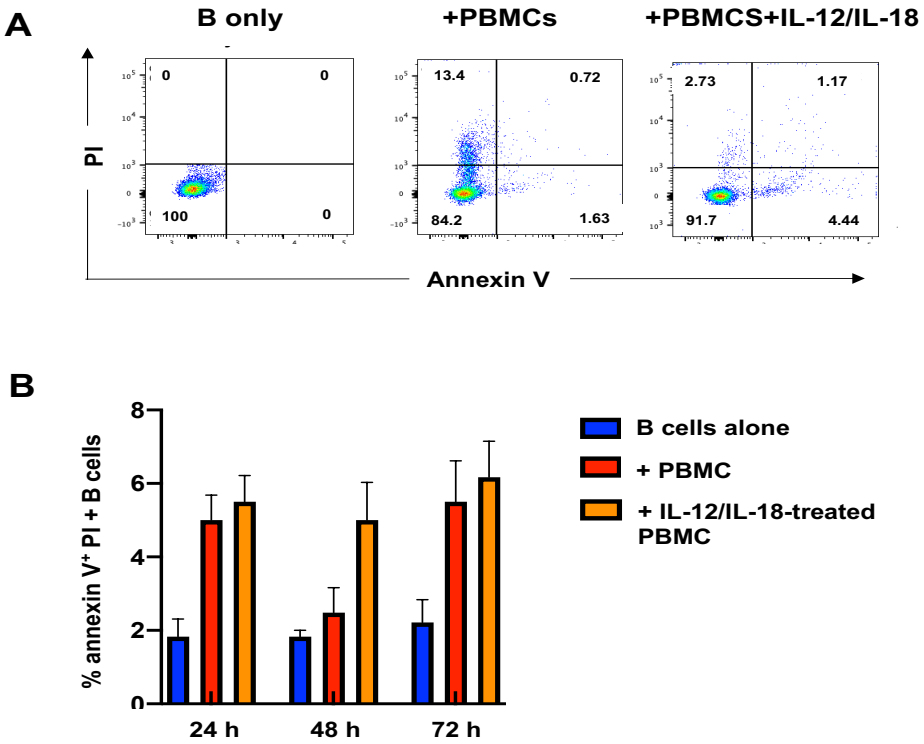

Figure 5.25 Total PBMC do not induce apoptosis in autologous B cells*. Sorted B cells from 6 donors were cultured for 24 h, 48 h, and 72h with medium alone or with equal numbers of autologous resting or IL-12/IL-18-treated PBMC. Cells were then stained with mAbs specific for CD3 and CD20 and propidium iodide (PI) and annexin V and analysed by flow cytometry. A, Flow cytometry dot plots showing annexin V and PI staining of gated B cells incubated in the absence (left) and presence of resting (centre) and IL-12/IL-18-treated (right) PBMC. B, Graphs shows mean (±SEM) percentage of MAIT cells from 12 donors that express annexin V under each condition. Differences were not significant using a paired t test.*

5.4.8 MAIT cells kill autologous B cells

Sorted B cells from 6 donors were cultured for 5h with medium alone or with autologous resting or IL-12/IL-18-treatred MAIT at various ratios. Cells were then tested for direct cytotoxicity by MAIT cells using the total cytotoxicity and apoptosis detection kit by flow cytometry (Figure 5.26A). Using this method, apoptotic cells stain positive for annexin-V and necrotic cells stain positive for 7-AAD.

An approximate 55% of B cells showed specific lysis when co-cultured with similar numbers of unstimulated MAIT cells (Figure 5.26B). This specific lysis was higher at MAIT:B cell ratios of 0.5 but lower at E/T ratios of 0.02:1. Unexpectedly, when MAIT cells were activated with IL-12 and IL-18, a the % specific lysis was not increased over the specific lysis observed with resting MAIT cells. This further suggests that MAIT cells have the ability to kill B cells.

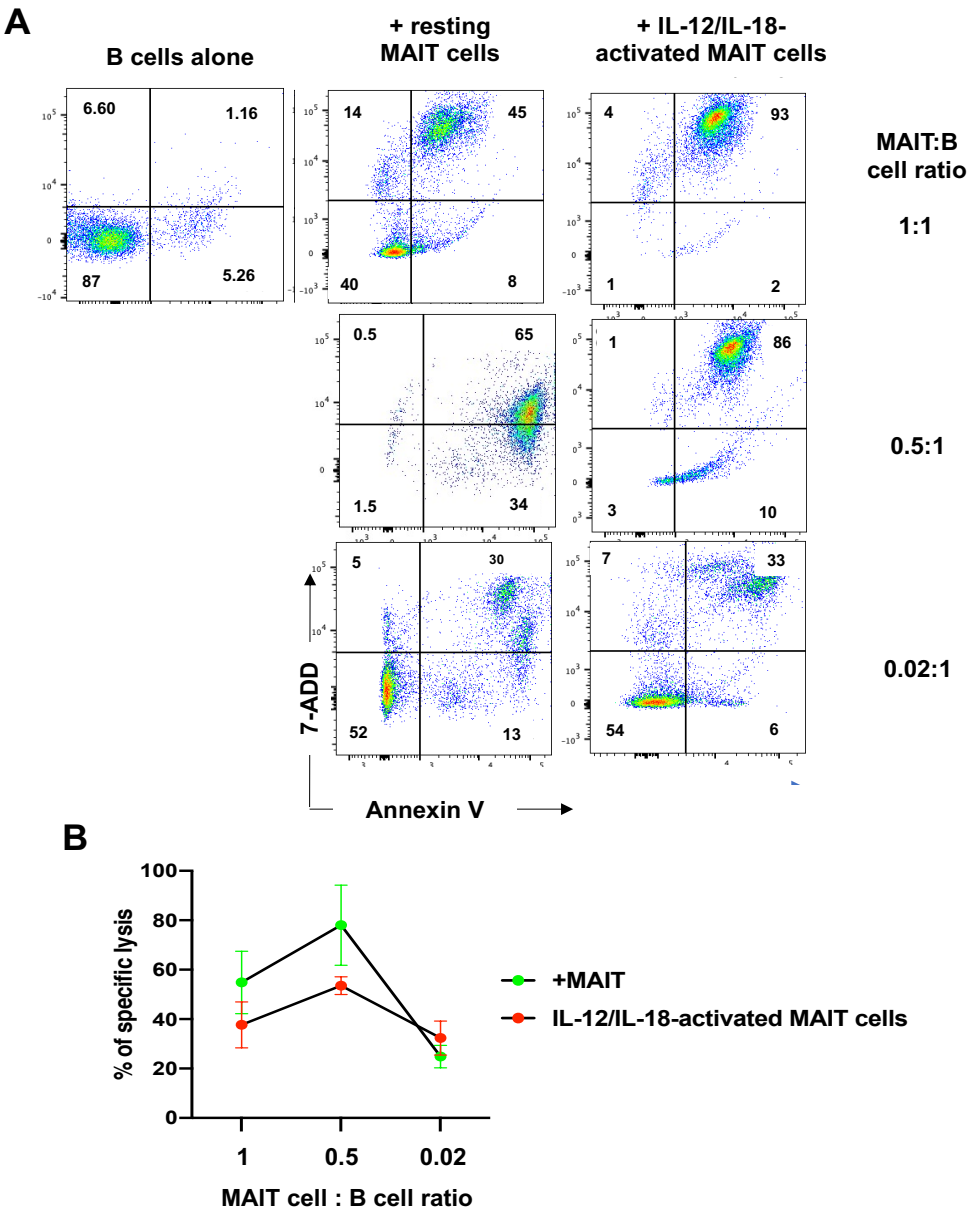

Figure 5.26 MAIT cells kill autologous B cells. *Sorted B cells from 6 donors were cultured for 5h with medium alone or with autologous resting or IL-12/IL-18-treatred MAIT cells at various ratios. Cells were then tested for direct cytotoxicity by MAIT cells using the total cytotoxicity and apoptosis detection kit analysed by flow cytometry. A, Flow cytometry dot plots showing annexin V and 7-AAD staining of gated B cells incubated in the absence (left) and presence of resting (centre) and IL-12/IL-18-treated (right) MAIT cells. B, Graph shows mean (±SEM) specific lysis of B cells from 6 donors under each condition.*

5.5 Discussion

MAIT cells are innate-like lymphoid cells that play roles in early stages of immune responses within the tissues. MAIT cells are abundant in humans and can be found throughout the body, including the bloodstream and mucosal areas [513]. MAIT cells can perform a variety of effector functions. They can kill bacterially and virally infected cells such as cells infected by Dengue virus, hepatitis C, and influenza A [305], and human immunodeficiency virus [514, 515].

MAIT cells can link innate and adaptive immune responses by promoting differentiation of different types of cells into APC that are capable of initiating antigen-specific T cell responses and long-term immunological memory [436]. These findings implicate MAIT cells as candidate targets for development of novel therapies and vaccines. Previous studies demonstrate that MAIT cells can aid B cells by boosting plasmablasts and promoting Ig production in an MR1-dependent manner, most likely through increasing memory B cell development [510], suggesting that they can promote humoral immune responses in vivo. Thus, we sought to further investigate the role of MAIT cells in providing B cell helper activity and the mechanisms involved in this.

According to more recent research, B cell maturation and immunoglobulin class switching can both be promoted by MAIT cells . In this chapter we examined the synergistic activities of MAIT cells and B cells to determine whether and how they may be manipulated and harnessed to contribute to the prevention and treatment of infectious diseases in a clinical setting. We postulated that MAIT cells would stimulate the maturation and activation of B lymphocytes. We therefore investigated if MAIT cells can induce B cell activation and maturation into cells capable of secreting cytokines, presenting antigen, undergoing antibody class-switching and releasing antibody.

MAIT cells can be triggered by either their TCR or cytokines. In this section, we have described their reactions to these two types of activation. TCR activation of MAIT cells resulted in a robust activation with the generation of several proinflammatory cytokines; in contrast, cytokine-mediated activation was slower and less polyfunctional. First, a study was conducted in order to determine the ideal parameters for MAIT cell activation. MAIT cells that have been activated typically release inflammatory cytokines such as IL-17 and IFN-γ [516, 517]. Therefore, measuring the percentage of MAIT cells producing IL-17 and IFN-γ in PBMCs, after stimulation with different agents revealed the best conditions for MAIT cell activation. Very few unstimulated MAIT cells secreted IL-17 and IFN-γ and the optimal stimulation occurred when MAIT cells were stimulated with the 5-A-RU for both IL-17 and IFN-γ production, as also reported [512, 518]. MAIT cells were also potently activated with PMA/I. PMA activates protein kinase C, while ionomycin is a calcium ionophore [188, 313, 497, 519-521], so stimulation with these compounds circumvents the cell membrane receptor complex and initiates intracellular signalling pathways [188, 313, 497, 519-521]. Anti-CD3 and anti-CD28 mAbs were the least effective stimulant for MAIT cell activation, in terms of cytokine production. Anti-CD3 and anti-CD28 stimulate lymphocytes by partially mimicking stimulation by APCs [523]. IL-12 and IL-18 also provided little stimulation for MAIT cells to produce IL-17 and IFN-γ, leaving 5-A-RU as the optimum stimulatory condition for activating cytokine production by MAIT cells.

We also examined MAIT cell activation by flow cytometric analysis of their expression of the activation markers CD69 and CD38 [516]. The optimal conditions for MAIT cell stimulation was PMA and ionomycin, followed by anti-CD3/anti-CD28 and 5-A-RU.

We next looked at the role of MAIT cells in secreting pro- and anti-inflammatory cytokines. From our results we can conclude that MAIT cells, when stimulated with PMA/I produced significant amounts of IFN-γ, TNF-α IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-17a, IL-21 and IL-22, but not IL-23. In contrast, MAIT cells when stimulated with anti-CD3/anti-CD28 produced IFN-γ, IL-2, IL-4, IL-5 and IL-6 but did not augment TNF-α, IL-8, or IL-9, IL-13, IL-17, IL-22 or IL-23 production compared with MAIT cells cultured alone. This is in line with a previous study which showed that activated MAIT cells from healthy people produce IL-21, which promotes B cell development into plasmablasts PB and antibody production in vitro [9]. Therefore, from our data we can show that many factors are released by activated MAIT cell that may influence B cell differentiation and antibody production.

We analysed the effects of several B cell and MAIT cell stimulators, tracked cytokine release, and investigated antibody production and immunoglobulin class switching in order to better understand how to optimise and characterise B cell activation.

All stimulants including MAIT cells and IL-12 + IL-18-activated MAIT cells resulted in significant B cell activation. These results demonstrated that the B cell population present in PBMCs can be successfully stimulated with the above B cell ligands. These results are in line with those of other studies [524, 525].

Previous studies have demonstrated that γδ T cells can induce maturation of B cells into antibody-secreting plasma cells [511, 526, 527], suggesting that they can promote humoral immune responses *in vivo*. Thus, we sought to further investigate if MAIT cells also have a Tfh-like function capable of inducing B cell maturation, antibody isotype switching and antibody production.

B cells were co-cultured with either resting or IL-12/IL-18-stimulated MAIT cells and the results showed that both treatments induced expression of HLA-DR, CD86 and CD40

on B cells, suggesting that MAIT cells can drive maturation of B cells to acquire markers of APC function and possibly boost adaptive immunity.

Analysis of cytokine production by ELISA revealed that stimulation of MAIT cells with IL-12/IL-18 drove production of IFN-γ, IL-2, IL-6, TNF-α, IL-8, IL-21 and IL-23 but not IL-4., IL-6 has been shown to result in significantly increased IgG levels, and IL-10 has been shown to promote B cell survival, proliferation, and antibody production [510]. IL-21 is well known for its capacity to stimulate B cell proliferation, plasma cell differentiation, and antibody production, among other functions [528]. We also found that MAIT cells can induce cytokine production by B cells. Interestingly, activated MAIT cells were required to induce IFN-γ production but resting and activated MAIT cells could induce IL-4 production.

Next, we investigated the effects of MAIT cells on antibody class switching and antibody release by B cells. Over seven days, B cells were stimulated with resting or IL-12 + IL-18-activated MAIT cells and as controls, LPS, BAFF, Poly I:C + Pam3Csk4 and anti-CD40 mAb + IL-4 + IL-21. Interestingly, when B cells were co-cultured with resting MAIT cells, the majority of B cells expressed class-switched switched phenotypes and acquired CD27, indicating that MAIT cells can induce immunoglobulin class switching. Co-culture with IL-12/IL-18-activated MAIT cells resulted in even more potent immunoglobulin class switching to memory type at about 90%. Thus, this is the first report that MAIT cells can activate B cells to acquire memory phenotypes and induce immunoglobulin class-switching.

We also investigated if MAIT cells could promote antibody release by B cells. We found that B cells co-cultured with MAIT cells did not produce IgE but they released copious amounts of IgG, IgA and IgM. One of the most significant outcomes observed was the 30-fold and 40-fold increase of IgG and IgA when B cells were co-cultured with

IL-12/IL-18-activated MAIT cells. Hence from our data, we can conclude that, whereas soluble B cell stimulators can induce antibody secretion but not isotype switching by B cells, MAIT cells are capable of inducing both immunoglobulin class switching and antibody secretion. These findings all support the hypothesis that MAIT cells provide help to B cells.

Much previous research has documented that infected epithelial cells [529] monocytes [530], and embryonic renal cell lines [531] are susceptible to cytotoxicity by MAIT cells. This activity was dependent on both MR1 and cytolytic mediators such as perforin and granzymes [530]. We therefore investigated if MAIT cells can kill B cells. We analysed if MAIT cells degranulate in the presence of B cells by examining the cell-surface expression of lysosomal protein marker CD107a after culturing MAIT cells with B cells. Interestingly, when B cells were co-cultured with IL-12 and IL-18 activated MAIT cells, CD107a was significantly increased on the MAIT cell population demonstrating that within this cohort of donors, MAIT cells underwent degranulation in the presence of B cells.

To understand the cytolytic activity in more detail, we evaluated if MAIT cells produce granzyme B and perforin in the presence of B cells. We found that when cultured with B cells, MAIT cells expressed granzyme B and perforin. These findings confirm previous data [530], that MAIT cells can rapidly transition to a more classical cytotoxic cell type, displaying high levels of GrzB and perforin.

Having shown that MAIT cells upregulate expression of the degranulation marker CD107a and the cytotoxic effector molecules GzmB and perforin, when exposed to autologous B cells, we next carried out an apoptosis assay to determine if MAIT cells can kill B cells. We observed that the frequency of dead cells increased to 50% when IL-

12/IL-18 activated MAIT cells were co-cultured with B cells. In contrast, total PBMC cocultured with B cells did not lead to B cell death.

Our data suggest that MAIT cells may have the ability to both activate and kill B cells. As a result of these findings, there may be therapeutic opportunities to use MAIT cells to augment or control B cell responses in patients with B cell immunodeficiencies and in vaccination. This study lends credence to the idea that MAIT cells can be used therapeutically to target B cells. B cells are essential for the development of protective immunity against infections, and the generation of antibodies by these cells is vital. The role of T cells, particularly Tfh cells, in regulating the processes of affinity maturation and isotyping switching has been demonstrated in a significant number of studies [532]. iNKT cells have also emerged as a source of both cognate and noncognate support for B cells in recent studies [533, 534]. This chapter demonstrates the potential of MAIT cells, a family of innate-like T cells, to differentially promote or regulate B cell and antibody responses through selective interactions with B cells. MAIT cells are relatively abundant in humans (5% of circulating T cells) compared to other immune cells such as iNKT or Tfh cells, which account for less than 1% of circulating T cells. Our data signify that MAIT cells may be a possible candidate for improving B cell responses in humans and may be beneficial for reactivation and differentiation of memory B cells.

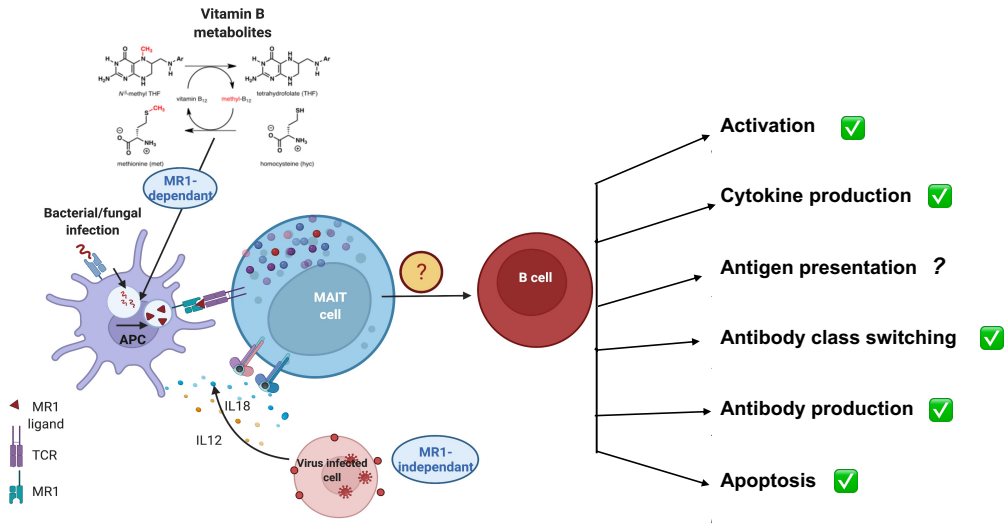

Figure 5.27 Diagram depicting the summary of this chapter. *MAIT cells can promote B cell activation, maturation, the expression of markers of antigen presentation, cytokine production, antibody isotype switching and antibody release. MAIT cells can also kill B cells. Figure created with BioRender.com*

Chapter 6
General summary and conclusions

NE affects 3 in 1000 live births leading to more than half a million infant deaths worldwide every year [490, 535]. Although there has been remarkable improvement in the management of NE, over 50% of affected infants will suffer moderate to severe disabilities [536]. Therapeutic hypothermia for moderate-to-severe NE will only save one out of seven infants from major neuro-developmental delay or death by age of 18 months old [35, 448, 451]. Therefore, there is a great need for new therapies and novel medical management approaches. Advances in these areas require a knowledge of the neonatal immune system.

Infectious diseases are the leading cause of death among children under five globally [537] . Each year, infections are linked to around 40% of the 3 million neonatal global deaths [538]. The vulnerability of neonates and infants to infectious diseases is believed to be due to the immaturity of the immune system [9]. In comparison to adults with primary infections, adaptive immune responses specific for HSV and CMV in newborns and infants are delayed and diminished [539-542]. Infection of young children with *Bordetella pertussis*, respiratory syncytial virus (RSV), enterovirus 71, or influenza is more likely to lead to hospitalization or death than infection of adults with the same pathogens [481, 543, 544].

In older adults, there is a decline in the ability of the innate and adaptive immune systems to combat new infections as well as persistent inflammation, a process known as inflammaging [545]. These modifications to the immune system result in higher incidence of infectious, malignant, and autoimmune disorders that have all been linked to age-related morbidity [546]. Additionally, it has been found that there are elevated plasma levels of IL-6, IL-1, and TNF in the older adults [547]. These factors lead to constant

activation of the innate immune response, leading to continued inflammation and impaired adaptive immune responses.

As lymphopoiesis diminishes, the number of naive T cells declines, causing an increase in the number of memory cell subsets in elderly subjects. The accumulation of CD4 and CD8 T cells that have lost the ability to express the costimulatory surface protein CD28, is thought to be a fundamental trait of senescent T cells [548]. Chronic low-grade inflammation is enhanced by IL-6, IL-1, TNF, acute phase proteins and antioxidants [549, 550]. However, inflammaging is also associated with down-regulated innate immune responses, driven by regulatory subtypes of T and B cells as well as regulatory subtypes of macrophages, dendritic cells, NK cells, and type II NKT cells [551]. The hyper-inflammatory response seen in COVID-19 patients is likely triggered by loss or impaired function of $CD4^+$ T cells, $CD8^+$ T cells, and B cells. However few studies have compared these immune features directly in cohorts from younger age ranges to older age ranges.

In chapter 3 of this thesis, we explored the functional effectiveness of the innate and adaptive immune systems in 5 healthy cohorts from different age groups. Flow cytometry was employed to profile the frequencies of conventional T cells, B cells and NK cells, and subpopulations of innate T cells in cord blood samples and peripheral blood samples from neonates, school-aged children, young adults and older adults. We found that the frequencies of T cells were lower in cord blood than in peripheral blood from subjects at all ages and that overall T cell frequencies remained relatively constant throughout life. We did not observe any significant changes in their expression of CD4 and CD8 from the neonatal to older age ranges. These findings are in agreement with Santagostino and co-workers (2003), but others have reported decreases in the numbers of $CD4^+$ and $CD8^+$ T cells with age [385, 386]. Similar to previous reports [387-390], there were significant

expansions of B cells from birth to childhood and reaching peak levels at school age and then decreasing gradually throughout adulthood. Thus, B cell numbers are reduced in neonates and the older adults and these changes may predispose these age groups to more severe infections [552].

We found that NK cell numbers were low in cord blood and in neonatal blood, but they steadily expanded throughout middle age. This increase in NK cells with age has also been reported by others [391, 393]. The expansions in the frequencies of NK cells seen in elderly patients are mostly attributable to expansions of the $CD56^{dim}$ subset and reductions of the $CD56^{bright}$ subset. These results show that T, B and NK cell frequencies fluctuate with age, and are likely to lead to age-related changes in immunocompetence which can contribute to inflammaging and development of diseases and cancer in elderly populations.

Analysis of four innate T cell populations – Vδ1 T cells, Vδ2 T cell, MAIT cells and iNKT cells – revealed that all of these cell types are found at very low frequencies in cord blood and in peripheral blood of neonates, but they all expand after birth and throughout childhood and adulthood. Innate T cells have important influences on adaptive immune responses to infection via their ability to rapidly release Th1, Th2, Th17 and Treg cytokines and to mediate contact-dependent maturation and activation of dendritic cells, macrophages and B cells. The paucity of innate T cells in infants may contribute to their increased susceptibility to infection, whereas their increased numbers in elderly subjects may contribute to inflammation and the development of illnesses and cancer. Phenotypic and functional changes to innate T cell populations may also underlie the altered immunocompetence seen in infants and elderly subjects. In this regard, we found that the proportions of MAIT cells that expressed CD8 expanded from the neonatal stage to

adulthood, and $CD8^+$ MAIT cells were the majority of MAIT cells in adults. On the other hand, the fraction of MAIT cells that displayed double negative $CD4^-CD8^-$ phenotypes reduced with age.

As expected, the levels of the proinflammatory cytokines IL-6 and IL-8 were found to be high in adults compared to yougner cohorts. These data are in line with other publications reporting an enhancement of Th1 and proinflammatory cytokines activity with age **[413, 414]**. A prevalent issue in the world of global public health is the co-occurrence of chronic illnesses among the seniors [553]. According to one study, more than half of all senior people in developed nations have more than three chronic diseases, which are illnesses with distinct pathologies and no interdependence on one another [554, 555]. Indeed, the older adults studied in the present investigation suffered from a number of comorbidities, as described in chapter 3. Another study has indicated that more than 70% of the senior population in China's major cities, such as Beijing and Shanghai, suffer two or more chronic conditions [556, 557]. Those with comorbidities had greater hospitalization and death rates and considerably worse clinical prognoses when compared to patients with a single condition. Comorbidities commonly entail several medications, and interactions between medications and disorders frequently result in lower ultimate effectiveness, poorer prognosis, more unpleasant responses, and higher health care costs [558].

Our data suggest that innate T cell function should be investigated in the context of inflamamging in future studies. Their numbers increase into old age and they express markers of maturation and activation. Since chronic inflammation and associated immune dysregulation is a major risk factor for diseases that commonly affect older people, such as atherosclerosis, cancer and type 2 diabetes, manipulation of innate T cell functions

may have therapeutic value. Clinical trials targeting iNKT cells and Vδ2 T cells are ongoing in cancer patients and these strategies may in future be used to treat inflammatory disease.

Inflammation plays a vital role in the evolution of brain injury associated with NE. Inflammatory cytokines are released by activated astrocytes and microglial cells, which leads to the recruitment of neutrophils and monocytes to the brain **[427, 447, 448]**. The activation of immune cells in the circulation is also associated with a poor outcome following brain injury **[450, 559]**. Although it is evident that innate effector cells, such as monocytes and neutrophils, play important roles in the pathophysiology of NE less is known about T cells, which are the master controllers of both innate and adaptive immune responses. In chapter 4 of this thesis, we explored the roles of lymphocyte in neonatal encephalopathy. In this study, children with NE, CP, and healthy age-matched neonates and children were examined for circulating conventional and innate lymphocytes counts and percentage frequencies. We found a significant increase in B cell frequencies and numbers in neonates and school-age children with NE compared to their healthy counterparts. However, we did not detect any changes in frequencies of total T cells in all groups except that they were higher in children with CP.

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. However, we found higher numbers of both $CD4^-CD8^-$ and $CD4^+CD8^+$ T cells in school-age who had NE compared to healthy school-age children, and higher numbers of $CD4^-CD8^-$ T cells in children with CP. In light of these findings, it is possible that unconventional T cells, such as iNKT cells, MAIT cells and γδ T cells, which are frequently negative for CD4 and CD8, play an important role in the pathophysiology of NE.

We also reported changes in the frequencies and numbers of innate T cells in neonates and children with NE. As previously reported [461], we found that MAIT cells were found in very low numbers in neonates but they 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. Analysis of Vδ2 T cells and iNKT cells revealed for the first time a significant increase in the frequencies and absolute numbers of both cell types in neonates and school-age children with NE and CP, compared to healthy age-matched neonates and children. These striking increases in Vδ2 and iNKT cell numbers in patients with NE and CP provide compelling evidence for a role for these cells in the neuroinflammatory events that lead to NE and CP. In support of this notion, γδ T infiltrate the brains of mice following experimental hypoxia ischemia and contribute to brain injury [246, 330].

In the present study, we also investigated functional readouts of key cell types from neonates with NE and healthy neonates. Whole blood samples taken from neonates with NE and healthy neonates were stimulated with pharmacological stimulators and T cell activators and the expression of transcription factors, cytokines, and cytotoxic mediators by total T cells, NK cells and Vδ2 T cells was examined in order to further characterize effector subsets in both groups. Insufficient numbers of iNKT cells were available for similar functional studies using blood samples. We found that stimulated NK cells from neonates with NE produced IFN-γ, TNF-α, and granzyme B at higher frequencies than stimulated NK cells from healthy age-matched donors. Although the frequencies of circulating NK cells were significantly lower in neonates with NE, they were primed and expressed higher levels of CD69 comparing to healthy infants. We also found a significant increase in the production of Th1 and Th17 cytokines by total T cells from

neonates with NE. These cells generated more IFN-γ, TNF-α, granzyme B and IL-17 when stimulated *ex vivo* than T cells from healthy neonates. Th1 cytokines promote monocyte, macrophage and $CD8^+$ T cell activation and cytotoxicity, whereas Th17 cytokines promotes the recruitment and activation of neutrophils. The serum levels of Th1 and Th17 cytokines were not increased in NE patients, suggesting that T cell activation may be limited to the brain. Future research will be necessary to understand whether the numerical changes represent the transfer of B cells and T cells to the brain or whether specific subsets of these cells, such as regulatory B cells, are responsible for the numerical changes [458].

Although neonates have a limited potential for establishing typical T cell responses, Vδ2 T cells are functionally competent during early development and have a role in early-life immunity [462]. Vδ2 T cell numbers and functions are altered in several other neurological diseases, including infectious meningitis, ischemic stroke and multiple sclerosis [294, 463, 465]. Although there are several distinct T cell subsets and types, the majority of studies have focused on Th1, Th2, Th17, and Treg cells, there is limited research on these cells in neonatal encephalopathy. We detected that Vδ2 T cells from neonates with NE generated significantly higher amounts of proinflammatory cytokines such as IFN-γ, TNF-α, IL-17 and GnzB upon stimulation ex vivo, when compared to Vδ2 T cells from their healthy counterparts. These T cells with varied phenotypes may also have an impact on neuroinflammation through a variety of mechanisms, including controlling the immune response, interacting with CNS resident immune cells, and influencing neurogenesis and angiogenesis at various stages after brain injury.

A further understanding of the functionality of these key immune cell subsets may offer the possibility that altering the ratio of proinflammatory to anti-inflammatory

cytokines produced by innate T cells with different phenotypes may develop into a possible therapeutic strategy in future studies that lowers mortality and enhances functional outcomes and prognosis in patients with neonatal encephalopathy.

We also found iNKT cell frequencies were significantly higher in neonates and school age children with NE and CP when compared with age-matched healthy controls and the absolute numbers of circulating iNKT cells in NE and CP patients were also significantly higher than in age-matched control subjects. Collectively, these data suggest that iNKT cells may have roles in the inflammatory processes that lead to NE and CP. Invariant NKT cells are thought to play essential roles in the pathogenesis of ischemic stroke, neurodegenerative disease and autoimmune diseases such as multiple sclerosis and myasthenia gravis [460, 468]. Multiple clinical trials involving *in vivo* activation or adoptive transfer of *ex vivo* activated Vδ2 T cells and iNKT cells are ongoing [472, 560] and may in future be applied as a novel treatment of NE. It has recently been shown that iNKT cells and Vδ2 T cells can enhance adaptive immunity *in vitro* and *in vivo*. They drive maturation of B cell into plasma cells capable of secreting antibodies. [561-563] and DC maturation into antigen presenting cells. Our group has demonstrated that Vδ2 T cells and iNKT cells can increase the production of IgG, IgM and IgA, but not IgE, by B cells in vitro. They also induced HLA-DR, CD40, and CD86 expression by DC and B cells, indicating that Vδ2 T cells and iNKT cells can influence the development of APCs [477].

In chapter 5 of this thesis, we examined the synergistic activities of MAIT cells and B cells We postulated that MAIT cells, like Vδ2 T cells and iNKT cells, would stimulate the maturation and activation of B lymphocytes. We investigated if MAIT cells can induce B cell activation and maturation into cells capable of secreting cytokines,

presenting antigen, undergoing antibody class-switching and releasing antibodies. We found that MAIT cells are capable of activating B cell maturation, cytokine production, immunoglobulin class switching and antibody secretion. We also found that MAIT cells can kill B cells. These results show that MAIT cells can control several aspects of B cell biology and that they may contribute to the B cell abnormalities found in neonates and children with NE. The results identify a potential place for MAIT cells, along with Vδ2 T cells and iNKT cells, as targets for immunotherapy for infectious and immune-mediated disease. As demonstrated in chapter 5, MAIT cells can be easily isolated from blood, being found in relatively high numbers compared to Vδ2 T cells and iNKT cells and can be cultured for up to 2 months while retaining their viability and functionality. However, future studies are required to determine how their functions may be exploited for the treatment or prevention of NE.

A major limitation of the present study is the use of peripheral blood to attempt to assess the immunological basis of an inflammatory process that occurs in the brain. Future studies are required to determine if the depletions of these cells from blood are due to their trafficking to the brain. To directly investigate the roles of lymphocyte subsets in neuroinflammation requires the use of animal models or postmortem brain tissue. Some excellent animal models of neuroinflammation and NE have been developed and these could be exploited for a more detailed analysis of the roles of innate T cells. However, caution must be exercised because humans and mice display major differences in their innate T cell repertoires. For example, mice do not have counterparts for human Vδ2 T cells. Therefore, humanised mice are required to study these cells *in vivo*. Murine studies are also required to determine if infection underlies the inflammation found in NE.

There are several limitations to the present study that need to be addressed in future studies. Further investigation of larger cohorts of patients and control subjects is required to confirm the findings of chapters 3 and 4. Interpretation of the results is confounded by the multiplicity of aetiologies and manifestations thought to underlie NE, which could involve different components of the immune system, therefore detailed stratification of patients according to clinical features is required. Because of the restricted numbers of tests that can be carried out on the 100-250 μl blood samples obtained from neonates, future studies could employ transcriptomic analysis of selected immune cell populations, in order to probe more deeply the changes in gene expression. In this regard, single cell mRNA sequence analysis of innate T cell populations from cohorts grouped according to age or from patients with NE, CP and age-matched controls might identify biological pathways associated with the immunological changes observed. Longitudinal studies utilising samples taken from subjects at different time points are also required to measure fluctuations in lymphocyte numbers and functions from birth into childhood and to correlate these with developmental changes.

Conclusion

These studies revealed novel roles for innate T cells in humans and in particular, in neonatal encephalopathy. The ultimate impact of this research would be to translate the observations of the present thesis into novel therapies that will benefit patients with NE and CP. Although much can be learned from the observational studies in the present thesis, therapeutic strategies that target individual lymphocyte subsets require the use of animal models in which expression of specific genes can be disrupted leading to 'knockout mice' lacking individual cell types, cytokines or other factors, which can then

be replaced to confirm the findings of our observations. Experimental immunotherapies could then be tested in humans in clinical trials.

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8. Appendix 1

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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

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ABSTRACT

We enumerated conventional and innate lymphocyte populations in neonates with neonatal encephalopathy (NE), school-age children post-NE, children with cerebral palsy and age-matched controls. Using flow cytometry, we demonstrate alterations in circulating T, B and natural killer cell numbers. Invariant natural killer T cell and Vδ2$^+$ γδ T cell numbers and frequencies were strikingly higher in neonates with NE, children post-NE and children with cerebral palsy compared to age-matched controls, whereas mucosal-associated invariant T cells and Vδ1 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; Korzeniewski 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

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Abbreviations

CP	cerebral palsy
γδ	gamma/delta
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IFN-γ	interferon-γ
IL	interleukin
iNKT	invariant NK T
mAb	monoclonal antibody
MAIT	mucosal-associated invariant T
NE	neonatal encephalopathy
NK	natural killer
NKT	natural killer T
PBA	PBS containing bovine serum albumin and sodium azide
PBS	phosphate buffered saline
PMA/I	phorbol myristate acetate with ionomycin
TCR	T cell receptor
TNF-α	tumour necrosis factor-α

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-ischemia 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; Bajnok 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), 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 control 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 centrifugated 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 (HIB19), CD56 (HCD56), CD69 (FN50), CD161 (HP-3G10), and the Vα7.2 (clone REA179), Vα24Jα18 (6B11), Vδ1 (REA173) and Vδ2 (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 $CD3^+$ $V\alpha7.2^+$ $CD161^+$ cells and iNKT cells were defined as $CD3^+$ $V\alpha24J\alpha18^+$ cells. Vδ1 and Vδ2 T cells were defined as cells expressing CD3 and the Vδ1 or Vδ2 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 Vδ2 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% CO_2 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 Vδ2 TCR (E)-4-hydroxy-3-

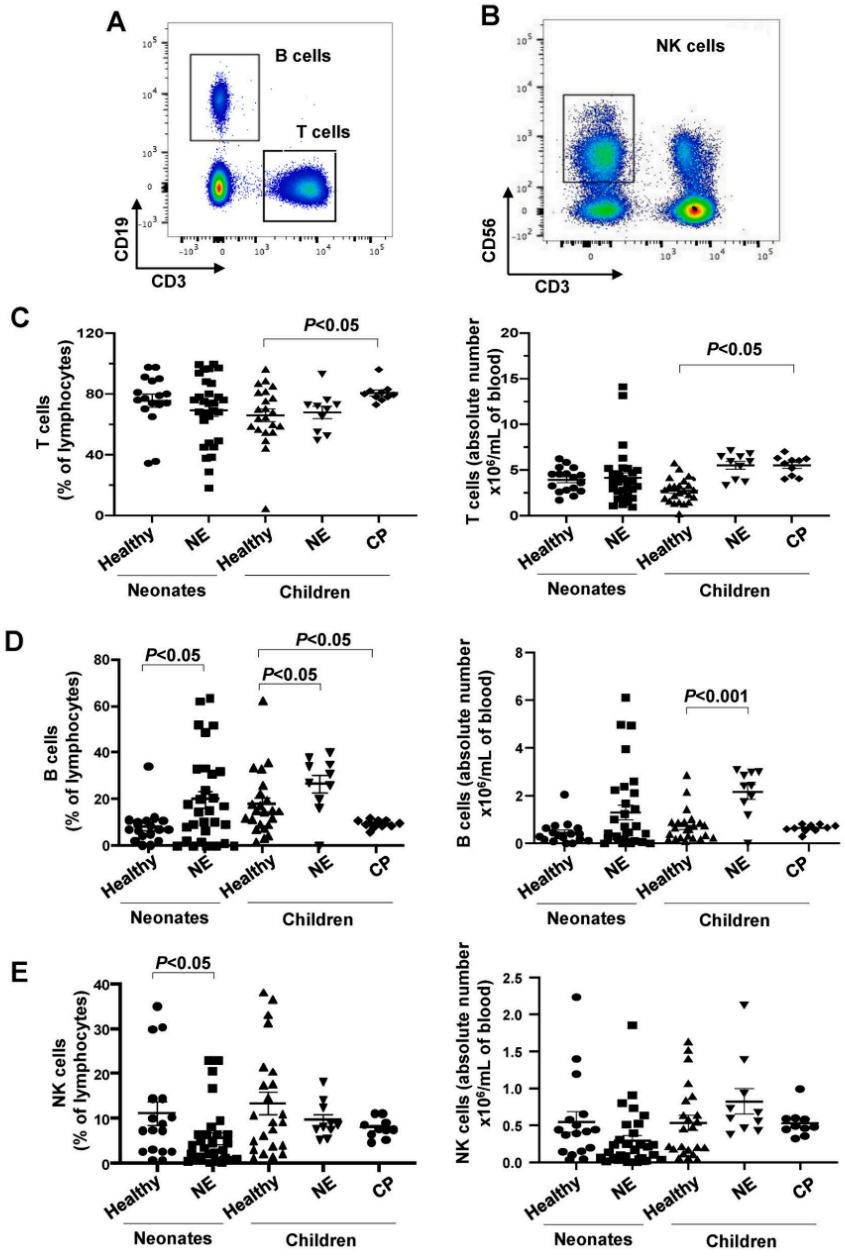

Fig. 2. Frequencies and absolute numbers of circulating T cells, B cells and NK cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A and B, Flow cytometry dot plots showing 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.

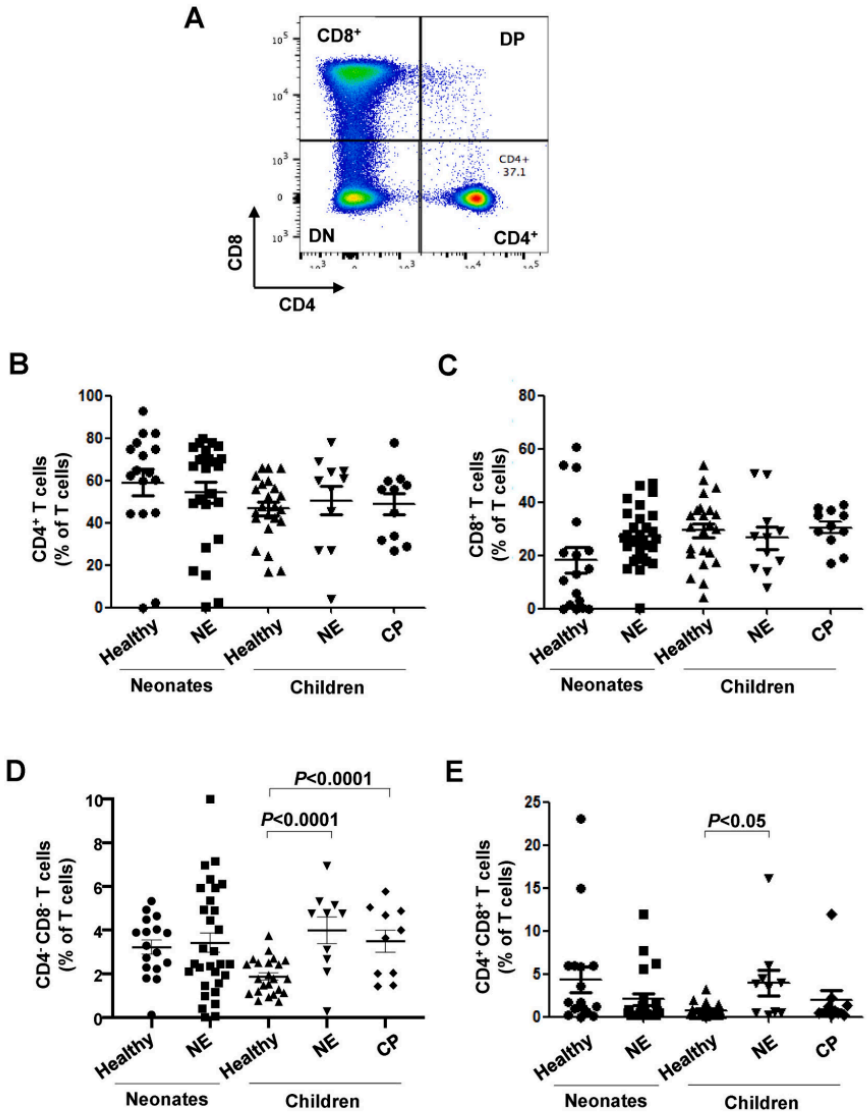

Fig. 3. Frequencies of circulating $CD4^+$, $CD8^+$, $CD4^-CD8^-$ double negative and $CD4^+CD8^+$ double positive T cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A, Flow cytometry dot plot showing CD4 and CD8 expression by gated T cells. B-E, Scatter plots showing the frequencies of T cells that expressed $CD4^+$ (B), $CD8^+$ (C), $CD4^-CD8^-$ (D) and $CD4^+CD8^+$ (E) phenotypes 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. 3 A, 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 Vα7.2 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

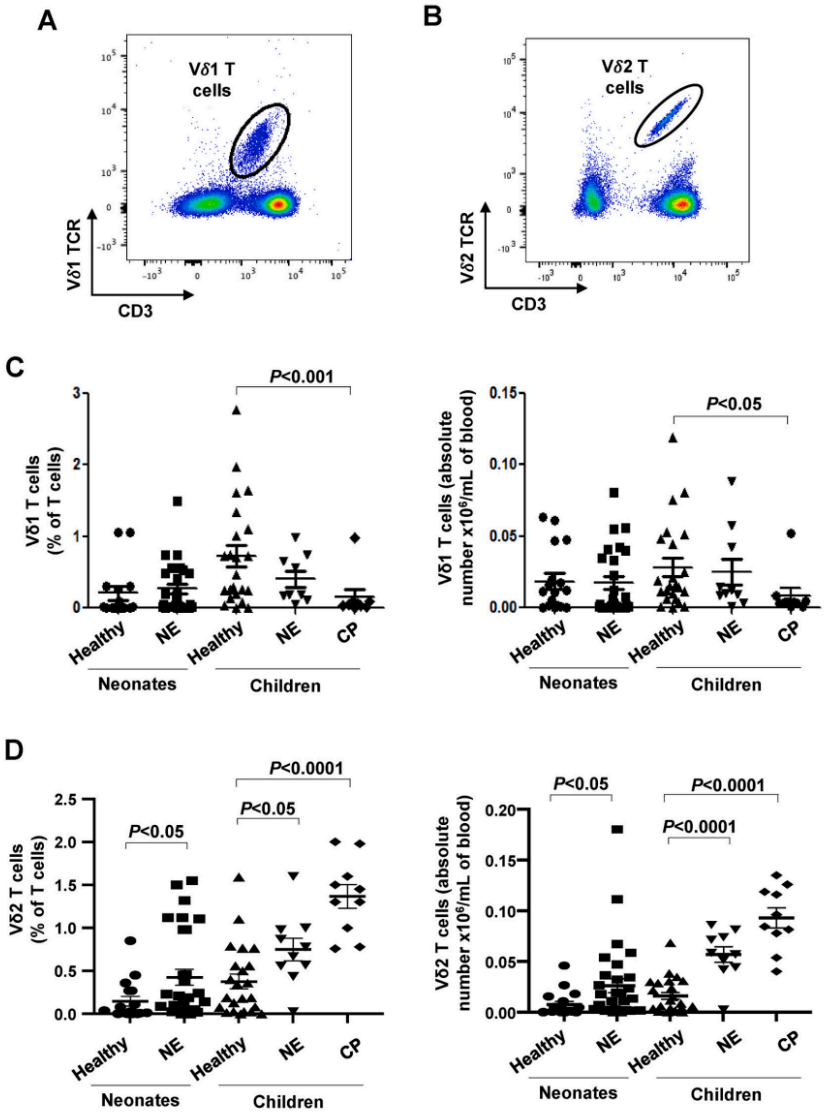

Fig. 4. Frequencies and absolute numbers of circulating Vδ1$^+$ and Vδ2$^+$ γδ T cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A and B, Flow cytometry dot plots showing 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.

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. 5D).

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

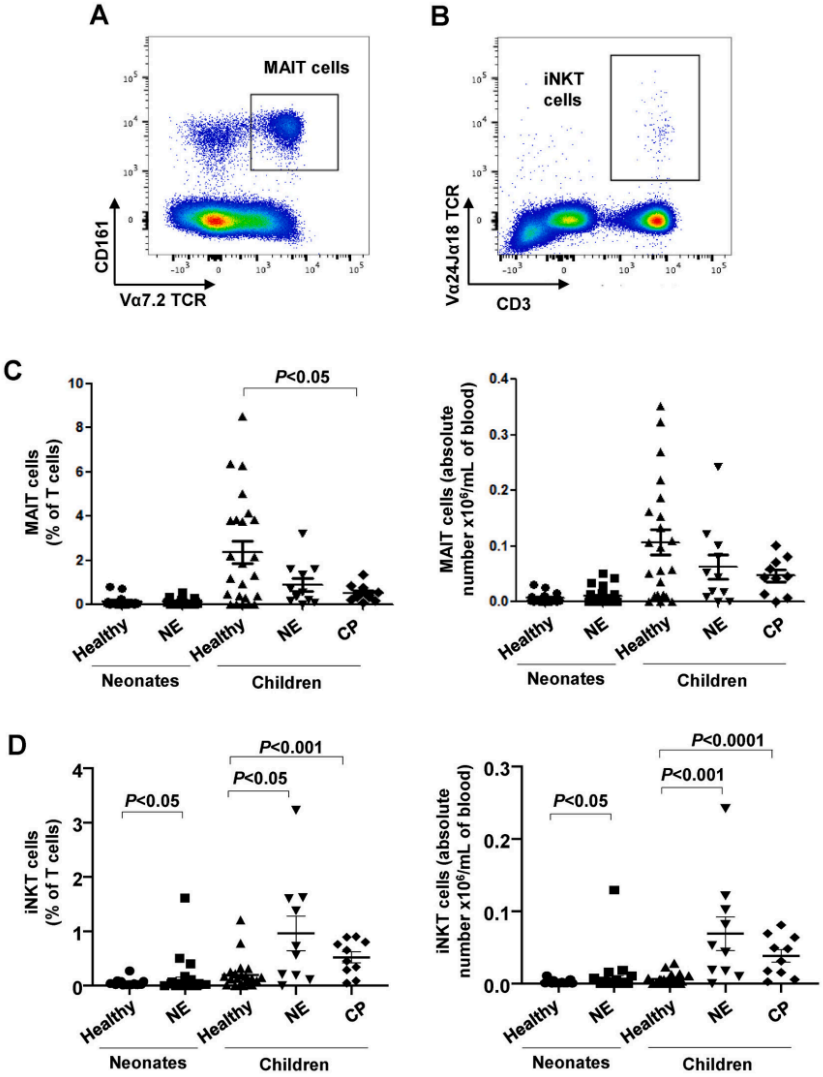

Fig. 5. Frequencies and absolute numbers of circulating MAIT cells and iNKT cells in 30 neonates with neonatal encephalopathy (NE), 17 healthy neonates, 10 school-age children post NE, 10 children with cerebral palsy (CP) and 23 healthy school-age children. A and B, Flow cytometry dot plots showing the detection of MAIT cells (A) and iNKT (B) within peripheral blood mononuclear cells. C and D, Scatter plots showing the frequencies (left) and absolute numbers (right) of MAIT cells (C) and iNKT 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*

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

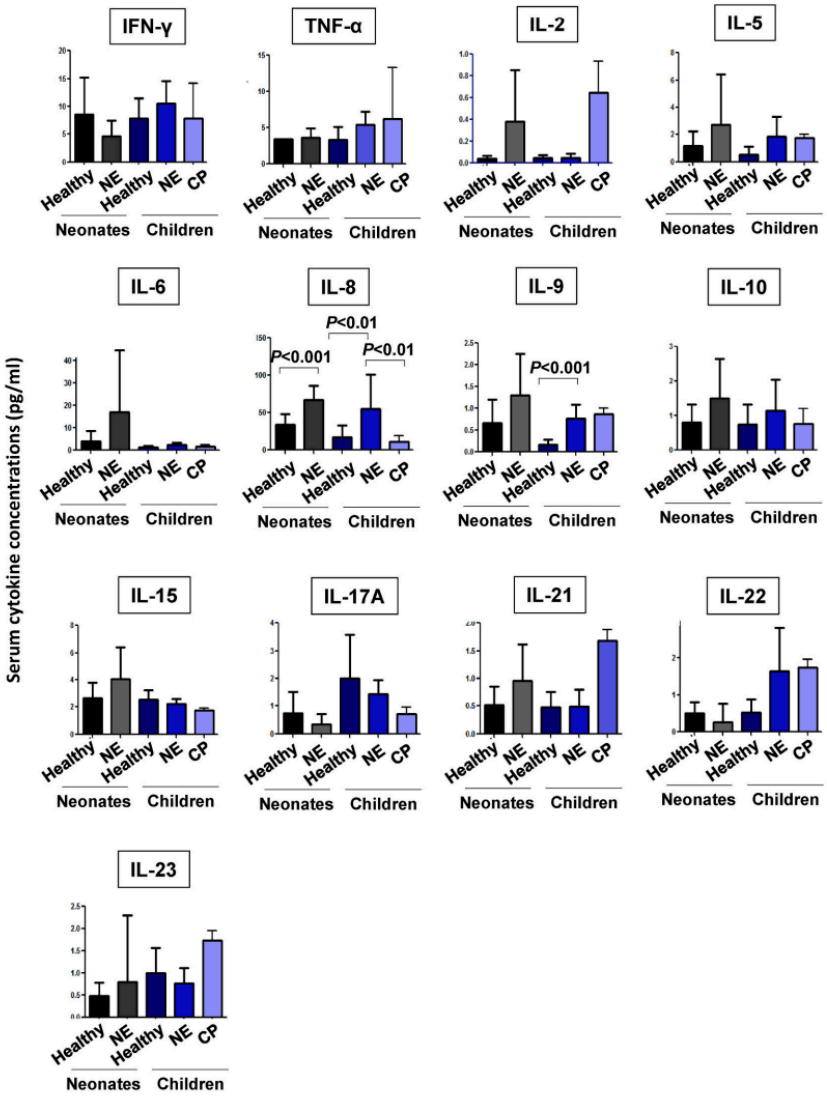

Fig. 6. Serum cytokine concentrations in neonates and school-age children with neonatal encephalopathy (NE) and cerebral palsy (CP) and in aged-matched healthy donors.

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

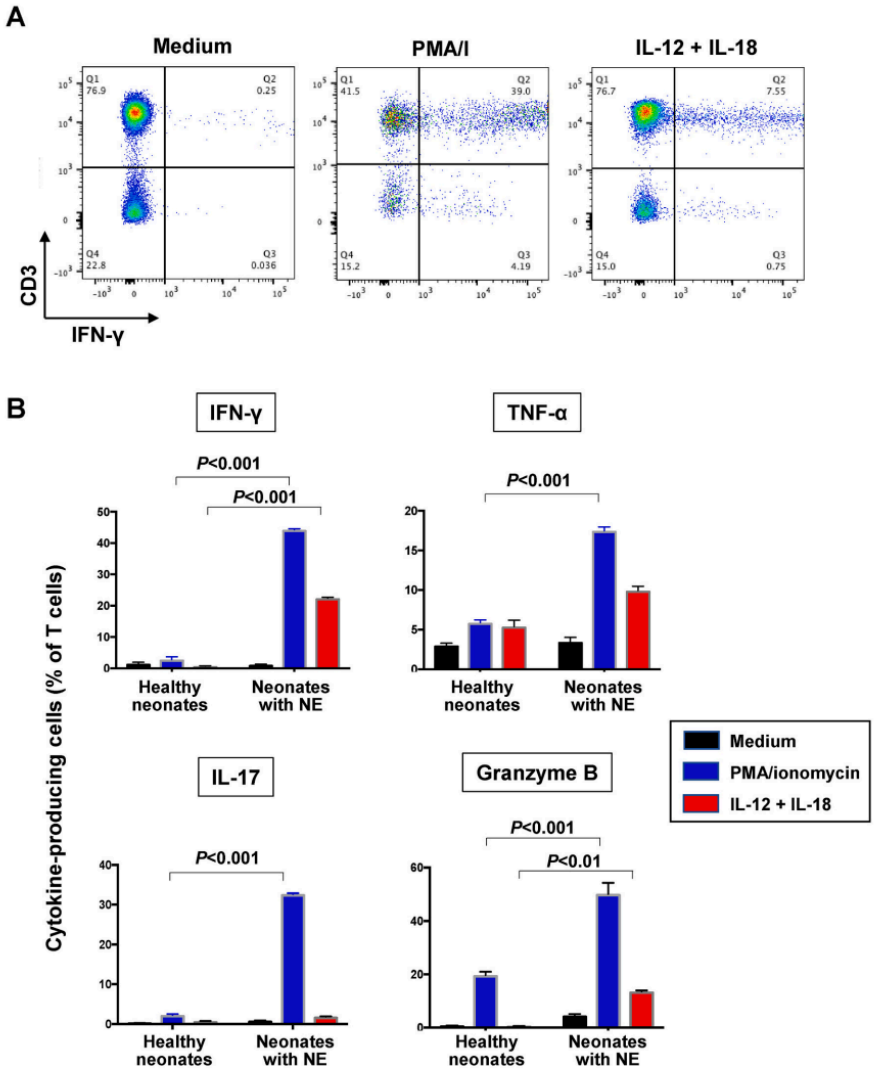

Fig. 7. Cytokine and granzyme B production by T cells from patients with neonatal encephalopathy (NE). A, Representative flow cytometry dot plots showing CD3 and IFN-γ expression by gated lymphocytes in whole blood after stimulation with medium, PMA/I or IL-12 + IL-18. B, Mean (±SEM) percentages of T cells from healthy neonates (n = 7) and neonates with NE (n = 10) that produce cytokines or granzyme B upon stimulation *ex vivo*.

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

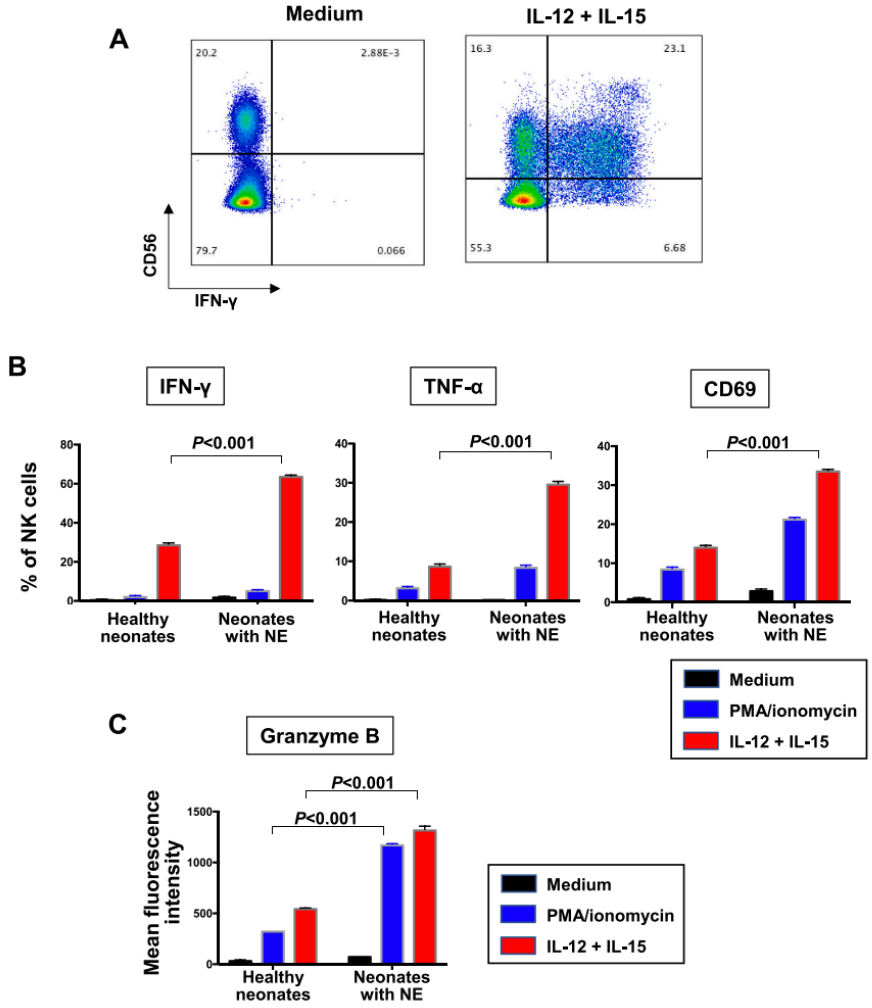

Fig. 8. Cytokine and granzyme B production by NK cells from patients with neonatal encephalopathy (NE). A, Representative flow cytometry dot plots showing CD56 and IFN-γ expression by gated CD3-negative cells in whole blood after stimulation with medium or IL-12 + IL-18. B, Mean (±SEM) percentages of 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*.

cytokine. Invariant NKT cells are thought to play essential roles in the pathogenesis of ischemic stroke, neurodegenerative disease and autoimmune 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

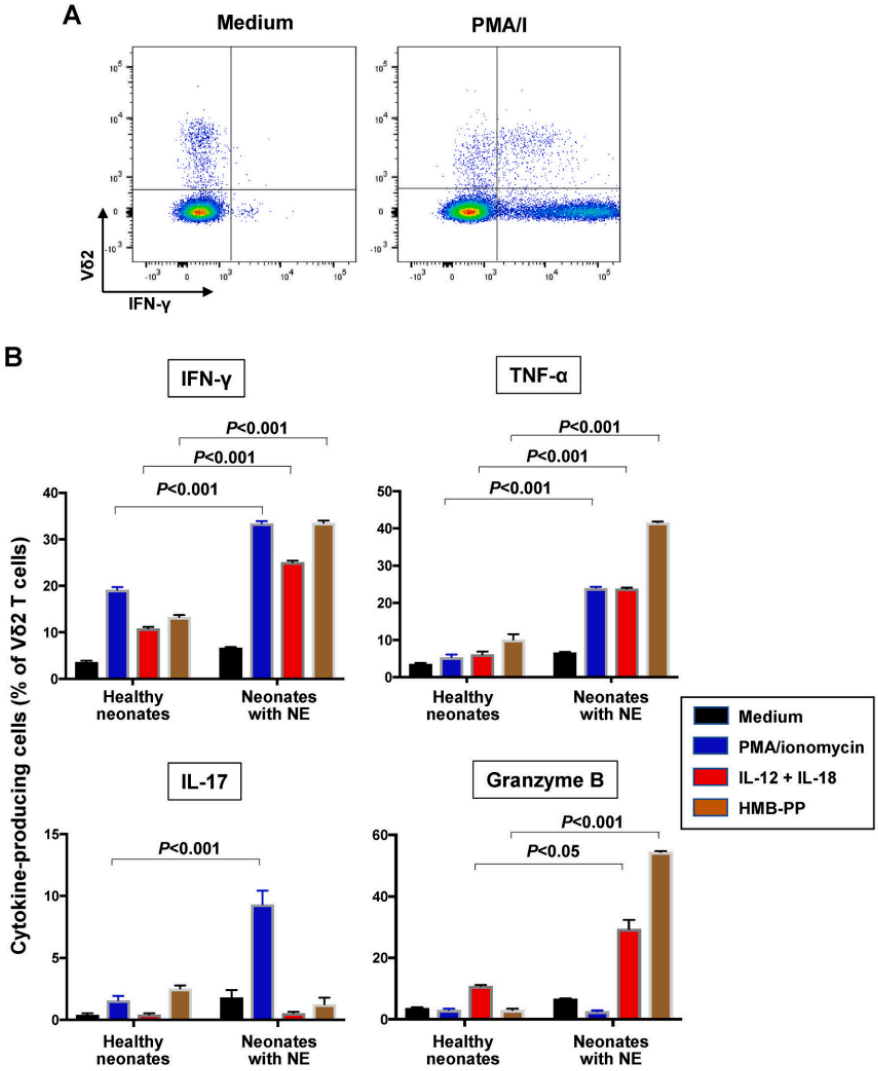

Fig. 9. Cytokine and granzyme B production by Vδ2 T cells from patients with neonatal encephalopathy (NE). A, Representative flow cytometry dot plots showing Vδ2 and IFN-γ expression by gated CD3-positive cells in whole blood after stimulation with medium or PMA/I. B, Mean (±SEM) percentages of Vδ2 T cells from healthy neonates (n = 7) and neonates with NE (n = 10) that produce cytokines or granzyme B 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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Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2021.577597.

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