Characterization of immune cell profiles in meninges and brain parenchyma following injury in the developing mouse brain

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"Natura in minima maxima"

"Nature is the greatest in the smallest"

To my dear family

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ABSTRACT

Preterm newborns are particularly susceptible to complications such as hypoxia-ischemia (HI), which can result in brain injury and subsequent cognitive and/or motor function disabilities, including cerebral palsy. Immune cells have been shown to be involved in the development of perinatal brain damage, commonly with detrimental effects. There is recent evidence that the membranes around the brain parenchyma, the meninges, might also have important roles in the immune response after injury in the adult brain, for example, by being a site of peripheral immune cell infiltration into the brain parenchyma. However, the role of the meninges in preterm brain injury is not known. Thus, the aim of this doctoral thesis was to identify the roles of immune cells in the meninges and brain parenchyma after preterm brain injury using a mouse model of HI-induced preterm brain injury.

In **Paper I** we found that T and B cells accumulate in post-mortem brains and meninges in preterm infants with brain injury. Similarly, in mouse experiments we found that T and B cells respond to the HI injury and infiltrate into the parenchyma. Additionally, genetic deletion of T and B cells resulted in reduced white matter tissue loss 7 days after HI. **Paper II** shows that innate lymphoid cells subtype 2 (ILC2s) also accumulate in the meninges 7 days after HI, but ILC2-impaired mice show no differences in inflammatory response, tissue loss, or glial immunoreactivity compared to wild type mice after HI, demonstrating a non-essential role for this immune cell subtype after preterm brain injury. Using single cell RNA sequencing,

Paper III presents the cellular composition and the unique transcriptional identities of meningeal immune cells in neonatal mice such as border-associated macrophages, monocytes, and microglia. We also identify the possible involvement of neutrophils in the injury process 6 hours after HI.
To conclude, the findings of this thesis reveal the participation of immune cells in the brain parenchyma and in the meninges to the development of HI injury. We provide insights into the unique single cell profile in the meninges in the immature mouse brain and thus contribute to the understanding of immune cell involvement in the injury process and the inflammatory reactions after preterm brain injury.

Keywords: preterm brain injury, hypoxia-ischemia, immune response, neonatal meninges

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SAMMANFATTNING PÅ SVENSKA

Immunceller i hjärnan och hjärnhinnorna efter tidig hjärnskada

Förtidigt födda barn är särskilt känsliga för komplikationer som kan leda till hjärnskada och efterföljande kognitiva och/eller motoriska funktionsnedsättningar. Tack vare framsteg inom främst neonatal intensivvård så överlever för tidigt födda barn i högre utsträckning idag även efter extrem förtidsbörd (< 28 graviditetsveckor); myntets baksida är att fler barn löper risk att utveckla neurologiska och neuropsykistriska problem. Neuroprotektiv behandling har utvecklats för fullgångna barn med svår syrebrist (kylbehandling), men sådan terapi kan inte ges till för tidigt födda och mer basal kunskap om skadeutvecklingen i hjärnan är nödvändig. Immunceller har visat sig ha både skadliga och skyddande effekter och är involverade i utvecklingen av hjärnskador. Man har nyligen upptäckt att membranen runt hjärnan, hjärnhinnorna, är av betydelse i inflammatoriska processer hos vuxna med multipel skleros, vid hjärnans åldrande och för demensutveckling. Syftet med denna doktorsavhandling är att identifiera immuncellernas roll i hjärnan och hjärnhinnorna i en experimentell modell för hjärnskadeutveckling hos prematura barn. I denna *in vivo*-modell på mus inducerar vi syrebrist som orsakar inflammation och hjärnskada vilket även sker hos för tidigt födda barn. Dessa skeenden startar redan några timmar efter insulten men såväl inflammationen som skadeprocessen pågår under veckor-månader.

I **delarbete I** studerade vi hur adaptiva immunceller (B- och T-celler), som ansvarar för produktion av antikroppar respektive immunologiska minnesfuntioner, påverkas av hjärnskada efter förtidig födsel. Vi analyserade både hjärnan hos för tidigt födda barn som avlidit med och utan hjärnskada och hjärnan hos nyfödda möss som exponerats för syrebrist. Vi fann att antalet T- och B-celler ökade efter skadan i både hjärnvävnaden och i hjärnhinnorna. Med hjälp av andra *in vivo*-experiment visade vi att avlägsnande av T- och B-celler minskade hjärnskadan. Resultaten tyder på det inflammatoriska svaret efter syrebrist involverar T- och B-celler och dessa celltyper bidrar i skadeutvecklingen i den mycket omogna hjärnan.

I **delarbete II** fokuserade vi istället på hur en undertyp av immunceller som kallas medfödda (innata) lymfoida celler reagerar på skadan. Innata immunceller är bland de första som svarar i den inflammatoriska processen och immunförsvaret hos nyfödda barn är starkt beroende av denna typ av

celler vid inflammation och/eller infektion. Vi observerade att innata lymfoida celler ökar i den mycket omogna mushjärnan efter svår syrebrist. Cellerna var huvudsakligen lokaliserade i hjärnhinnorna och inte i hjärnvävnaden. Genom att tillämpa modern genetisk metodik kunde vi framställa möss med dysfunktionella innata lymfoida celler som kunde jämföras med möss som hade normala lymfoida innata celler. Det inflammatoriska svaret och hjärnskadan efter syrebrist skiljde sig dock inte nämnvärt mellan djur med dysfunktionella och normala innata lymfoida celler. Sammanfattningsvis har vi visat att innata lymfoid celler i hjärnhinnorna reagerar på svår syrebrist men är inte av betydelse i det inflammatoriska svaret eller i hjärnskadeprocessen.

I **delarbete III** hade vi två målsättningar: det första var att karakterisera immuncellsprofilen i hjärnhinnorna hos nyfödda under fysiologiska förhållanden. Det andra var att undersöka förändringar i hjärnhinnans immunceller efter syrebrist inducerad i den mycket omogna hjärnan genom att tillämpa samma musmodell som i delarbete I och II. Vi studerade leukocytfamiljen som innehåller både adaptiva och innata immunceller, inklusive de som vi studerat i tidigare delarbeten. Vi utförde RNA-sekvensering i singelceller vilket är en ny teknik som gör det möjligt att utforska olika populationer samtidigt och se vilka celltyper och signaleringsvägar som påverkas efter svår syrebrist som orsakar skada i den mycket omogna hjärnan. Vi fann att hjärnhinnans immunpopulation är heterogen och komplex, och att några av celltyperna endast förekommer i hjärnhinnorna och inte i hjärnan. Några av dessa celler, som mikroglia och gränsassocierade makrofager, verkar vara involverade i hjärnans utveckling. Dessa upptäckter kan vara viktiga för att bättre förstå mekanismerna bakom hjärnans utveckling, vilket är en mycket komplex och delvis okänd process. I detta projekt fann vi också en potentiellt ny mekanism i neutrofila celler som kan vara av betydelse i det innata immunsvaret vid inflammation och infektion.

Sammanfattningsvis visar våra studier att immunceller i såväl hjärnhinnorna som hjärnan deltar i den inflammatoriska processen efter svår syrebrist hos för tidigt födda och en del av dessa celler är av betydelse för hjärnskadeutvecklingen. För första gången identifierar vi den unika profilen för immunceller och deras genuttryck i hjärnhinnorna som omger den omogna hjärnan. Dessa immunceller kan vara av stor betydelse såväl för hjärnans utveckling som i skadeprocesser i hjärnan hos för tidigt födda barn.

SOMMARIO IN ITALIANO

Cellule immunitarie nel cervello e meningi a seguito di danno cerebrale nei neonati prematuri

I neonati prematuri sono particolarmente suscettibili a complicazioni, le quali possono risultare in danno cerebrale e seguenti disabilità cognitive e motorie. Grazie al miglioramento delle cure mediche, il numero di neonati che sopravvivono è in aumento; di conseguenza però, molti più neonati sono a rischio di complicazioni, anche gravi. Esistono delle terapie per neonati nati a termine, ma queste non sono sempre di successo, mentre per i nati prematuri non esiste una terapia approvata a livello clinico. La risposta infiammatoria al danno cerebrale è sostenuta da cellule immunitarie, le quali possono contribuire al danno o avviare alla guarigione dell'infiammazione. Recentemente è stato dimostato che le membrane che proteggono il cervello, chiamate meningi, sono coinvolte nell'infiammazione dovuta per esempio alla sclerosi multipla, all'invecchiamento e al declinio cognitivo in età avanzata.

Lo scopo di questa tesi di dottorato è quello di identificare il ruolo delle cellule immunitarie nel cervello e nelle meningi durante il danno al cervello prematuro, utilizzando un modello sperimentale. Con questo modello *in vivo*, si può simulare il quadro clinico osservato nei neonati, caratterizzato da morte cellulare, la quale può portare a perdita di massa tissutale, ed infiammazione, la quale inizia qualche ora dopo l'insulto e può continuare per mesi, diventando cronica.

Il **primo articolo** studia come le cellule facenti parte dell'immunità acquisita, chiamate cellule T e B, rispondono al danno al cervello prematuro. Queste cellule sono responsabili per la produzione di anticorpi e della risposta immunitaria a lungo termine. Gli esperimenti dimostrano che le cellule T e B aumentano dopo il danno non solo nel cervello ma anche nelle meningi. Ulteriori esperimenti mostrano che rimuovere le cellule T e B porta ad una riduzione della perdita tissutale, e di conseguenza ad una riduzione della lesione cerebrale. Si può quindi assumere che queste cellule hanno un effetto nocivo e vadano a peggiorare il danno cerebrale.

Il **secondo articolo** analizza il ruolo di una sottofamiglia di cellule dell'immunità innata, chiamate cellule linfoidi innate, durante il danno cerebrale nell'organismo prematuro. Queste cellule sono tra le prime a rispondere ad un'infiammazione e/o infezione, e i neonati dipendono

molto da loro per la risposta immunitaria. Innanzitutto, si osserva un aumento di cellule linfoidi innate nel cervello dei roditori. Inoltre, questo aumento è localizzato prevalentemente nelle meningi. Tuttavia, quando le funzioni di queste cellule vengono compromesse, non c'è nessuna differenza in danno tissutale o infiammazione nel cervello. In conclusione, sembra che le cellule linfoidi innate reagiscano al danno cerebrale ma non partecipino nella risposta infiammatoria dopo il danno ipossico-ischemico.

Il **terzo articolo** ha due obbiettivi: il primo è di caratterizzare le cellule immunitarie presenti nelle meningi neonatali a livello fisiologico, mentre il secondo è di studiare il ruolo delle cellule immunitarie nelle meningi dopo il danno al cervello prematuro, basandosi sui risultati precendemente ottenuti nel primo e secondo articolo. Usando lo stesso modello sperimentale impiegato negli articoli precendenti, viene studiata la famiglia dei leucociti, la quale racchiude le cellule immunitarie sia innate che acquisite, incluse le cellule studiate nei primi due articoli. I risultati dimostrano che la popolazione di cellule immunitarie nelle meningi è alquanto eterogenea, e alcuni sottotipi di queste cellule sembrano essere specifici per le meningi. Inoltre, alcune sottofamiglie di cellule, come la microglia e i macrofagi presenti al confine tra cervello e sistema circolatorio, sembrano essere coinvolte nello sviluppo fisiologico cerebrale. Questi risultati sono importanti al fine di capire i meccanismi molecolari alla base dello sviluppo cerebrale, il quale è un processo complesso e in parte ancora da delucidare. In questo progetto viene proposto un nuovo possibile meccanismo di risposta infiammatoria da parte dei neutrofili, il quale potrebbe essere importante nella reazione del nostro corpo a infiammazioni e/o infezioni.

In conclusione, i risultati di questa tesi dimostrano la partecipazione delle cellule immunitarie nel cervello e meningi del neonato prematuro dopo il danno cerebrale. Per la prima volta, vengono identificate le cellule immunitarie presenti nelle meningi neonatali e la loro potentiale participazione come risorsa di cellule immunitarie, le quali danno inizio alla risposta immuno-infiammatoria dopo il danno cerebrale nel neonato prematuro.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. "Lymphocytes contribute to the pathophysiology of neonatal brain injury"

Nazmi A., Albertsson AM., Rocha-Ferreira E., Zhang X., Vontell R., **Zelco A.**, Rutherford M., Zhu C., Nilsson G., Mallard C., Hagberg H., Lai J.C.Y., Leavenworth J.W., Wang X. *Front. Neurol., doi: 10.3389/fneur.2018.00159*

II. "Type 2 Innate Lymphoid Cells Accumulate in the Brain After Hypoxia-Ischemia but Do Not Contribute to the Development of Preterm Brain Injury"

Zelco A., Rocha-Ferreira E., Nazmi A., Ardalan M., Chumak T., Nilsson G., Hagberg H., Mallard C., Wang X. *Front. Cellular Neuroscience, 14. doi: 10.3389/fncel.2020.00249*

III. "Single-cell atlas reveals meningeal leukocyte heterogeneity in the developing mouse brain"

Zelco A., Börjesson V., de Kanter J., Lebrero-Fernández C., Lauschke V.M., Rocha-Ferreira E., Nilsson G., Nair S., Svedin P., Bemark M., Hagberg H., Mallard C., Holstege F.C.P., Wang X. *Submitted*

CONTENT

ABBREVIATIONS

BAM	Border-associated macrophage
BCR	B cell receptor
CD	Cluster of differentiation
CHETAH	CHaracterization of cEll Types Aided by Hierarchical classification
CNS	Central nervous system
CP	Cerebral palsy
CSF	Cerebral spinal fluid
EAE	Experimental autoimmune encephalitis
EoP	Encephalopathy of prematurity
FACS	Fluorescence-activated cell sorting
GO	Gene ontology
GW	Gestational week
HI	Hypoxic-ischemic
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate lymphoid cell
IPA	Ingenuity Pathway Analysis
LTi	Lymphoid tissue inducer

MAP-2	Microtubule-associated protein 2
MBP	Myelin basic protein
mRNA	messenger RNA
PND	Postnatal day
PVL	Periventricular leukomalacia
Rag1	Recombination activing gene 1
RNA	Ribonucleic acid
scRNA-seq	Single cell RNA-sequencing
TCR	T-cell receptor
Th	T helper cell
WT	Wild type

1 INTRODUCTION

1.1 Preterm brain injury

This section of the introduction describes the aspects relevant to the pathology studied in this thesis, namely the global trend of preterm birth and its relationship with cerebral palsy and the mechanisms behind the development of hypoxic-ischemic (HI) brain injury.

1.1.1 Preterm birth and preterm brain injury

The World Health Organization (WHO) defines preterm birth as birth before gestational week (GW) 37, and it can be further subdivided based on the GW at birth as extremely preterm (<28 weeks), very preterm (28–32 weeks), and moderately to late preterm (32–37 weeks)[1,2]. A report based on WHO data stated that in 2014 the global estimated rate of preterm births was 10.6% of all live births; however, the countries with the greatest numbers of preterm births are low-income countries, and these countries have estimated preterm birth rates that can reach up to 20% and account for around 80% of preterm births worldwide[3]. In the developed countries, the rate is estimated to be around 8.7%, although there is a global trend for increasing rates of preterm births. Moreover, preterm birth is responsible directly and indirectly for half of all neonatal deaths worldwide (Fig. 1)[1,4].

As a result of improvements in obstetric management and particularly neonatal intensive care in developed countries, most premature infants currently survive the neonatal period. However, preterm newborns are more susceptible to complications such as brain injury or developmental deficits compared to term neonates[5], and the high risk of brain injury and neurological and neuropsychiatric problems in preterm newborns is thus an important public health concern.

Preterm brain injury is a multifactorial process that can lead to motor and cognitive impairments[6,7]. One of the most common causes of preterm brain injury is periventricular leukomalacia (PVL)[7-12]. PVL is distinctly characterized by white matter damage surrounding the brain ventricles; however, oligodendrocyte, axonal, and neuronal deficits in multiple brain regions often accompany white matter injury, and therefore the collective term for these injuries has been defined as encephalopathy of prematurity (EoP)[13]. EoP has a complex etiology and it can derive from a multitude of

causes, such as infection, intrauterine complications, hyperoxia, hypoxia-ischemia, and genetic factors[14]. It is also notable that male newborns tend to have a higher incidence of brain injury than females, and they also tend to suffer more severe long-term consequences after brain injury[15-17]. Such differences might be due to a delayed cerebral maturation in male compared to female neonates[18].

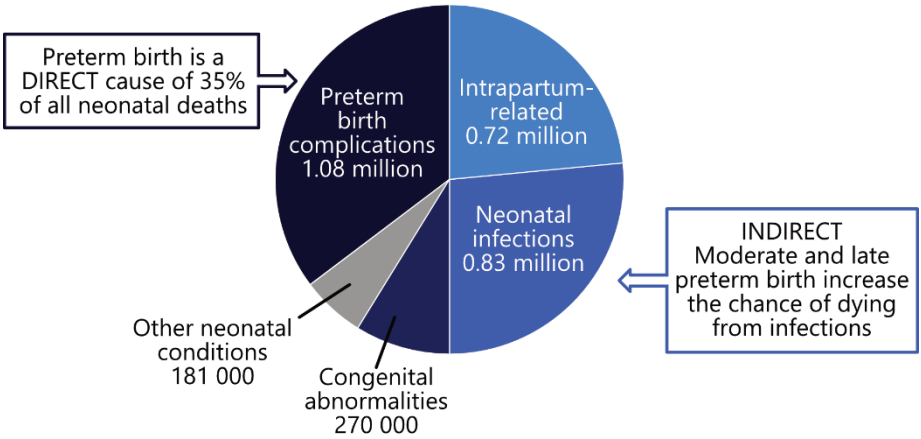

Figure 1. Estimated proportion of neonatal deaths in 2010. Permission from Blencowe et al.[1].

The outcomes for newborns who suffer from EoP include neurocognitive impairments and motor dysfunctions such as cerebral palsy (CP)[7,19]. In western Sweden, a series of studies have reported the CP prevalence since 1954. In the latest report (births in 2007-2010)[20] there were 1.96 CP cases for every 1000 births, and 38% of these cases were born preterm. The CP prevalence was the highest in the extremely preterm group (59.0 cases/1000 births) followed by the very preterm (45.7 cases/1000 births) and moderately preterm (6.0 cases/1000 births) groups. The absolute number of extremely preterm cases born with CP in the most recent report increased compared to the previous three years. Neuroimaging analysis revealed that diffuse white matter damage was more common in CP cases than in the past. In neonates born before 34 weeks, the majority had periventricular lesions[20].

1.1.2 Hypoxia-ischemia–induced neonatal brain injury

HI injury represents one of several risk factors for EoP[14]. The HI insult is a combination of ischemia (reduction or lack of blood flow) and hypoxia (reduced concentration of oxygen in the blood), which in preterm and term newborn brains can lead to chronic sequelae such as CP. The major components of this cascade of events are cell death, inflammation, mitochondrial dysfunction, pre-oligodendrocytes arrested maturation, excitotoxicity, and reduced neural connectivity[9,11,21,22].

HI injury can be characterized by three temporally distinct phases involving several processes[6] (Fig. 2). The first phase – latent or primary energy failure – takes place from a few minutes to a few hours after reperfusion following HI injury. During the primary energy failure, decreased blood flow and oxygen levels lead to glucose deprivation, which in turn triggers anaerobic metabolism, loss of mitochondrial adenosine triphosphate (ATP) production, and accumulation of lactic acid in the cell. Thus, there is a general state of excitotoxicity (toxicity due to the accumulation of excitatory amino acids such as glutamate), mitochondrial stress, and the production of reactive oxygen species[6,23], which begin to attract immune cells to the injury site[24]. During these first hours, these factors accumulate but do not yet lead to massive cell death, and the damage is still reversible. For this reason, this timepoint is the clinical intervention window in which therapeutic hypothermia can be applied to term newborns[25-27].

The second phase – referred to as secondary energy failure – lasts from a few hours to a few days after HI insult[28]. Excitotoxicity and oxidative stress due to mitochondrial failure is ongoing and as the cells can no longer compensate for this toxicity, they undergo cell death through several pathways such as apoptosis, necrosis, necroptosis, and autophagic cell death[29,30]. Therefore, the inflammatory response becomes even more prominent, including a surge in cytokine and chemokine production, resulting in infiltration of several types of immune cells into the brain and/or immune cells approaching the injury area[9,31-33].

The tertiary phase, which lasts from weeks to months after the HI insult, is mainly characterized by late cell death, remodeling and repair, and chronic inflammation[28,34] due to activated microglia[35], astrogliosis[36], and activated T cells[37].

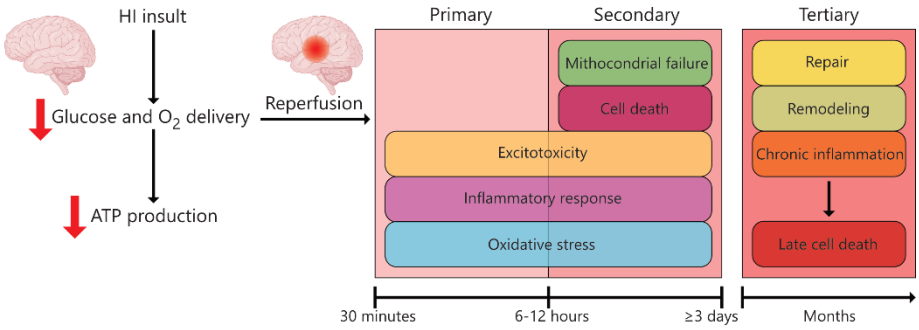

Figure 2. The timeline of HI injury divided into phases, adapted from Douglas-Escobar et al. 2015[28]. **Legend:** ATP: adenosine triphosphate; O_2: oxygen; ↓: decrease.

1.2 The central nervous system: the brain and meninges

The central nervous system (CNS) is constituted by the brain and spinal cord, and it is responsible for controlling autonomous functions, including heartbeat, breathing, and reflexes, and voluntary activities, including body movement and speech.

To be able to perform all of these function, the brain has evolved into the most complex and dynamic organ in our body. In this section, the general anatomy and development of the brain is described, followed by meningeal anatomy and development and the current knowledge of the meninges' role in brain pathologies.

1.2.1 Brain anatomy and development

The brain can be divided into gray and white matter, based on functions and myelination. The gray matter controls motor and cognitive functions, and disruption of these circuits can lead to a decline in these functions with age[38]. The gray matter constitutes the majority of the cerebral brain mass, and consists mainly of neurons[39]. However, the brain also contains other cell types, (e.g. microglia, oligodendrocytes astrocytes, and endothelial cells) that constitute up to 50% of the brain cells overall. These non-neuronal cells are mostly found in the white matter, and only a small portion are located in the gray matter[40,41]. Indeed, the vast majority of the white matter is constituted by oligodendrocytes, followed by astrocytes and microglia[41]. The white matter is characterized by the myelination of neuronal axons. The myelin wrapping, produced by oligodendrocytes[42],

permits faster signal transfer along the axons, therefore efficiently connecting several regions of the gray matter[43,44]. However, it has recently been suggested that the white matter also participates in cognition-related processes[45].

Brain development in human fetuses and mouse embryos occurs through similar phases, although at later stages some of the processes that progress postnatally in mice take place in the third trimester in humans[46,47]. Brain development starts at around GW3 in humans and around embryonic day (E)8–9 in mice when the first scaffold for CNS development is formed[48,49], followed by a period of neuronal growth and when connections are formed, surpassing the connectivity in the adult brain[50]. This phase is followed by a period in which non-pathological neuronal apoptotic death and connectivity refinement, called pruning, occurs. In this phase, approximately 50% of neurons and synaptic connections are removed[48,51,52]. In the later stages of the pregnancy, other cells come to seed the brain, such as immune cells, starting at around GW16 in humans and E8 in mice[53,54]. Processes like myelination, which is fundamental for white matter development, start in humans around GW20 and continue into postnatal life[48,55]. While the gray matter reaches maturation around 20 years of age, white matter development continues until mid-life[56]. In mice, evidence of myelination is found from around postnatal day (PND) 8[52].

Overall, the development of the brain is a complex and dynamic process involving the migration, differentiation, and maturation of several cell types, which have to be orchestrated with perfect timing in order to assure healthy development. Because brain development occurs over a long period during pregnancy and after birth, any disruption in physiological processes might impact greatly on the general development of the newborn. Additionally, females have more rapid cerebral maturation than male newborns, and thus the latter are more susceptible and for a longer time to any kind of brain injury occurring during the period of brain development[18].

1.2.2 Meningeal anatomy and development

The brain and spinal cord are protected by membranes called the meninges, but the precise function of the meninges in physiology and disease is still largely unknown.

The meningeal membranes are divided based on their histological appearances into three layers – the dura mater, the arachnoid mater, and the pia mater. The dura mater, also known as the pachymeninx, is the outer layer that is in contact with the skull[57,58]. The middle layer, the arachnoid mater, derives its name from its spider web-like appearance. The space in between the arachnoid mater and the pial layer is called the subarachnoid space, and it contains the cerebral spinal fluid (CSF). The innermost layer is the pia mater, which is in close contact with the brain parenchyma through the pial basement membrane, which allows the CSF to permeate the brain[59]. Collectively, the arachnoid mater, subarachnoid space, and pia mater are referred to as the leptomeninges (Fig. 3).

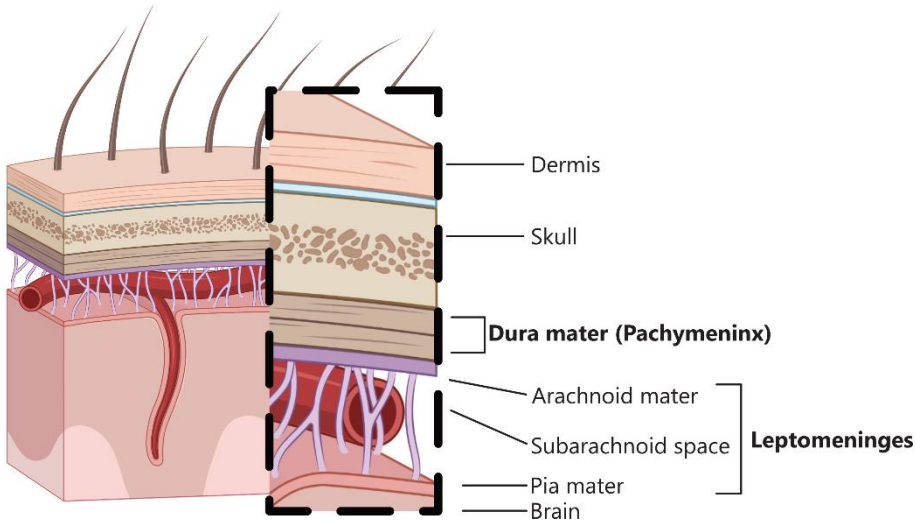

Figure 3. Representative illustration of anatomic layers of the meninges.

Rodent studies showed that in the later stages of development tissue differentiation progresses in a basal to apical direction in respect to the brain surface[60]. The leptomeninges differentiate into the arachnoid mater and subarachnoid space through cavitation, the dural layer becomes enriched in collagen[61], and the lymphatic vessels develop from the day of birth onwards for about 3–4 weeks until they stabilize into a final organization that remains through adulthood[62]. Regarding the inflammatory response, immune cells, specifically macrophages, are found in the mouse meninges from E9.5[54,63]. In humans, most of the meningeal structure is present at GW12[64], but to our knowledge no study has shown

whether immune cells appear in the meninges in the fetal or neonatal period.

1.2.3 The role of the meninges in development and disease

The meninges block potential threats from the periphery from entering the brain parenchyma, due to the different permeabilities based on the cellular organization of each meningeal layer[65]. However, recent studies have highlighted a variety of previously unknown meningeal functions such as acting as a niche for stem cells with neural differentiation potential[66-68] and playing a role in skull development[65]. Other studies have shown that the meninges can regulate the differentiation, migration, and positioning of neurons and seeding microglia through the activities of multiple factors[69-72].

Additionally, the meninges have been shown to be involved in the migration of oligodendrocyte precursors to the cerebral cortex through the activities of TGFβ1, BMP-4, and BMP-7[73] and to be involved in corpus callosum formation[74]. Lastly, mice with mutations in *Foxc1* (a major regulator of meningeal development[75-77]) show diminished blood vessel density in the brain at E14.5, indicating a potential role of the meninges in brain blood vessel development[75]. Although the meninges are intimately involved in many developmental processes, the specific players remain unknown.

Because they participate in several developmental processes, it is only natural that any defects in the meninges can result in neurodevelopmental pathologies. Mutations in the pial extracellular membrane genes[78] or deficiencies in zinc finger transcription factors[79] are the likely cause of cobblestone lissencephaly (lack of physiological gyrencephaly), which can result, for example, in Walker-Warburg syndrome, a congenital muscular dystrophy associated with cognitive disabilities[80]. In addition, Dandy-Walker syndrome results from chromosomal mutations that lead to the accumulation of CSF (hydrocephaly) and brain underdevelopment (hypoplasia)[81], with *Foxc1* as a major player in this pathology.

Despite having such a fundamental role in development and congenital pathologies, the role of the meninges in most brain pathologies is still not known, including their role in the inflammatory response to neonatal brain injury.

1.3 Immune cells, inflammation, and immune response in neonates

This section describes innate and adaptive immune cells studied in this thesis, the neonatal immune response and the immune cells that are found in the CNS, namely the brain and the meninges.

1.3.1 The first to respond: innate immune cells

Traditionally, immune cells are divided into two major categories, namely innate and adaptive subtypes. Innate immune cells are the first responders, and they react in a quick but unspecific manner to threats such as infection and/or sterile inflammation. Adaptive immune cells instead react more slowly than their innate counterparts, but they develop a targeted response through antigen presentation against the harmful stimuli[82]. The following sections focus on the main subtypes of cells studied in this thesis (Fig. 4).

Innate immune cells consist of several families of cells, including neutrophils, monocytes, macrophages, and innate lymphoid cells (ILCs). As mentioned above, these cells do not need antigen presentation; instead, they react in a similar manner to different direct stimuli, responding quickly but not specifically. These cells are usually the first to respond, and they recruit the adaptive immune cells that will later mount a specific immune response[83].

NEUTROPHILS

Neutrophils are in general considered to be the first responders in a variety of diseases throughout the body[84-86], and the lack of neutrophils (neutropenia) can lead to life-threating conditions[87,88]. Neutrophils are a highly heterogeneous population based on factors such as cell markers, maturity, and localization[89-94].

Such high heterogeneity can be an obstacle to researchers who study neutrophil development. The first hematopoietic progenitors appear around GW7-8, and they seed the fetal liver, spleen, and thymus where hematopoiesis will continue until GW28[95-97]. Later in life, the hematopoiesis will only continue in the bone marrow[95-97].

Once the neutrophils reach the site of inflammation, they engage in a swift and forceful response to any inflammatory event through degranulation

and the production of reactive oxygen species and neutrophil extracellular traps, effectively killing the bacteria or cancer cells and/or clearing the damaged tissue[93,98-100].

MONOCYTES AND MACROPHAGES

Monocytes are similar to macrophages and are the circulating counterpart of tissue-resident macrophages[101]. Monocytes are released from the bone marrow in response to acute or chronic inflammation[102], much like neutrophils.

Monocytes can be divided in classical and non-classical monocytes based on the expression of selected markers. In mice, the classical inflammatory monocytes are defined as $Ly6C^{high}CCR2^{+}$, and the non-classical patrolling monocytes are defined as $Ly6C^{low}CCR2^{low}$[102,103]. In adults, monopoiesis gives rise to the classical monocytes, which will then exit the bone marrow and enter the circulation[104,105].

Upon inflammation and/or infection, classical monocytes reach the inflamed site and start releasing factors such as tumor necrosis factor-α and inducible nitric oxide synthase, which are toxic to pathogens. They later become phagocytic cells and remove cell debris[106]. During infection, monocytes can also migrate to lymph nodes where they have an antigen-presenting function to activate T cells[107], and thus these cells may represent a bridge between innate and adaptive immunity.

Macrophages are tissue-resident cells that can have multiple ontogenies, and they show tissue-specific characteristics and have a variety of functions in development, homeostasis, and response to insults[108]. Regarding macrophage response, a gene expression study as part of the Immunological Genome Consortium highlighted that the macrophages from four different tissues (gut, lung, brain, and spleen) are extremely diverse, even when only comparing resting macrophages. Moreover, these subpopulations show unique transcription profiles for chemokines, Toll-like receptors, and downstream pathways[109].

INNATE LYMPHOID CELLS

The ILC subfamily has the common features of the innate branch of cells, but they also possess some characteristics that render them unique. They

are divided in three subtypes – ILC subtype 1, including natural killer (NK) cells, ILC2s, and ILC3s, which also include lymphoid tissue-inducer (LTi) cells[110].

ILCs can be classified according to their characteristic transcription factors, which are necessary regulators for their development and maturation. ILC development starts in the fetal liver and then continues in the bone marrow throughout adult life[111]. ILCs have a common innate lymphoid progenitor, which later specifies the progenitors of the ILC subtypes. Depending on the expression of specific transcription factors, these will in turn mature into NK cells (T-bet and EOMES), ILC1s (T-bet), ILC2s (Gata3), ILC3s (RORγT), or LTi cells (RORγT, TOX)[112].

The same classification is reflected in cytokine production, similar to what is observed in $CD4^+$ T helper (Th) cells: ILC1s and NK cells correspond to Th1 cells, ILC2s correspond to Th2 cells, and ILC3s together with LTi cells correspond to Th17/Th22[110,113] cells. Therefore, NK cells, ILC1s, and ILC3s are usually considered to be pro-inflammatory cells, whereas ILC2s have anti-inflammatory effects[114], and LTi cells are mainly involved in the secondary lymphoid organ formation and not to a great extent in immune responses[112].

Moreover, ILC2s have been shown to be able to influence and be influenced by other immune cells, thus creating a complex network of immune responses[115,116]. Most importantly, ILCs are among the first immune cells that appear in early life[117], and they have been shown to play an important role in the immune response against viruses, allergens, and intestinal inflammation[118], and they may be involved in spontaneous preterm labor[119], thus highlighting the possibility of ILCs' involvement in neonatal development and immune response after brain injury in preterm newborns.

1.3.2 The specific adaptive immune response

The adaptive immune response branch is mainly constituted by T and B cells, and these cells are responsible for cell-mediated and antibody-mediated responses, respectively. They are also responsible for long-term immune memory, and this feature allows the body to react much faster if the antigen is encountered again, thus permitting a quicker resolution of the inflammation and/or infection[120-122].

T CELLS

The T-cell receptor (TCR) is a molecule found on the surface of T cells. Because the TCR presents different isoforms that characterize different subtypes, T cells are subdivided into alpha-beta (αβ) and gamma-delta (γδ) T cells. Although similar, they have distinct developmental and functional traits[123]. T cell progenitors are detected in the fetal thymus at GW9 in humans, and in the following weeks mature T cells are found first in the thymus (GW12-13) and later in the secondary lymphoid organs, lymph nodes, and spleen (GW24)[124].

αβT cells can be divided in two major subfamilies – Th cells and T cytotoxic cells – which are characterized by the expression of the surface marker cluster of differentiation 4 (CD4) or 8 (CD8), respectively[125]. While $CD4^+$ Th cells mount a response without cell death, $CD8^+$ T cytotoxic cells kill the cells or the pathogen responsible for the inflammation and/or infection. $CD4^+$ Th cells are further divided into subtypes according to their cytokine production. Among them, the most studied are Th1, Th2, and Th17/22 cells. However, it has recently been shown that Th cells can also be plastic and change their pattern of cytokine production after differentiation in specific situations[126].

T cells react to a pathogen or to ongoing inflammation through the following three steps. First, naive T cells proliferate in the lymph nodes after they come in contact with antigen-presenting cells, which are specialized cells that train naive T cells to recognize parts of the pathogens (antigens)[127]. This phase is commonly called clonal expansion, where the T cells specific for a certain antigen (clones) exit the quiescent state and start dividing[120,128]. After clonal expansion, T cells infiltrate the damaged tissue where they perform their function depending on which T cell subtype they belong to. Afterwards, T cells will enter the contraction phase, where most of the cells become apoptotic once the antigen has been effectively neutralized. Lastly, a fraction of the clones will become long-term memory cells, allowing for a faster response in case the same antigen is encountered again[120,121,129].

γδT cells are also called innate-like cells because although they present a TCR and are able to mount a Th response, similar to the αβT cells, and can be classified according to the V(D)J receptor rearrangement[130], they have different development and activation processes. They are present in the fetal thymus before αβT cells start to appear, and they play important roles

in immune responses during early life[131-133]. γδT cells do not require MHC-presenting cells, and they can be activated either through ligands, with an antibody-like strategy, or through MHC-like proteins[134]. Because of their peculiarities, researchers have been studying these cells extensively in recent years[116,135-137].

B CELLS

B cells are the other arm of the adaptive immunity, and just like T cells they can mount a specific response and also play a major role in long-term immune memory through the production of highly specific recognition molecules, called antibodies or immunoglobulins.

Like other immune cells, B cell development starts in the bone marrow where progenitors and precursors go through a series of alternating states of activation and quiescence depending on the B cell receptor (BCR) and other receptor signaling[138-140]. However, there is a transient period where B cell pluripotent progenitors migrate to the fetal liver (around E12.5 in mice) and develop into specific B cell subtypes, such as B1a, to colonize specific organs and tissues[141,142].

B cells leave the bone marrow and move to the lymph nodes, spleen, and other secondary lymphoid organs[143,144]. Specifically, B cells in these organs form the germinal center, where B cells are presented to antigens through $CD4^+$ Th cells in order to start producing antibodies to fight off the pathogen. The specificity of the immunoglobulins is determined by the recombination of the BCR[145]. After antigen presentation and clonal proliferation, B cells producing immunoglobulins with high affinity for the antigen are selected. A portion of these cells will exit the lymph nodes to reach the site of inflammation and/or infection[146,147]. Another fraction will remain in the germinal center and become long-term memory B cells, ready to start proliferating if the same antigen is presented again; however, other germinal center- and Th-independent pathways of memory development have been reported[148,149]. The long-term memory B cells, together with the respective memory T cells, ultimately provide an essential tool for a highly targeted and fast response to a variety of antigens.

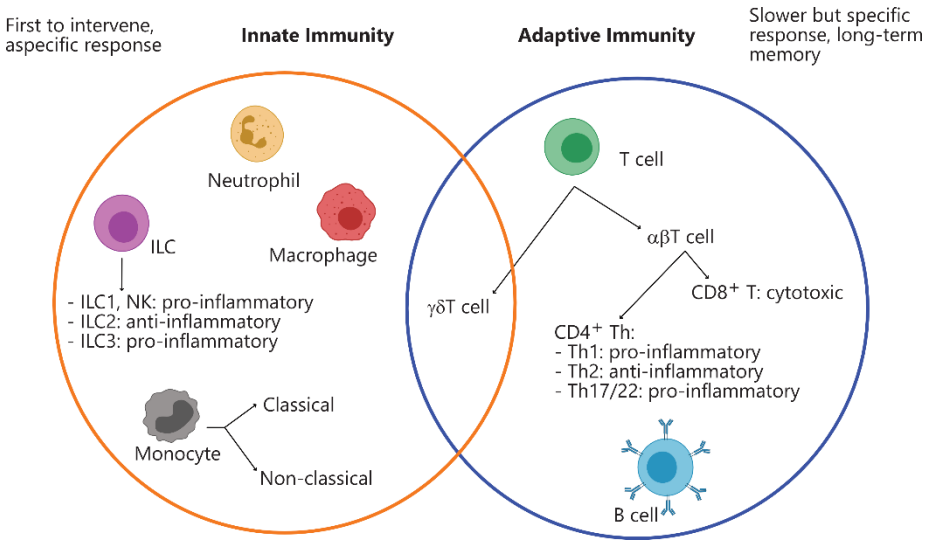

Figure 4. Innate and adaptive immune cells and their major subtypes.

1.3.3 Special features of neonatal immunity

Adaptive immunity is not functionally mature at the time of birth because it requires antigen presentation in order to be fully operational; therefore, neonates rely mainly on the innate immune response (Fig. 5)[150]. Furthermore, the neonatal immune response is usually skewed towards a Th2 response[151] because this allows the development of a tolerance to self-antigens without causing an extreme immune response for example[152].

INNATE IMMUNE CELLS

Neutrophils are already present in the amniotic fluid[153], and their number increases in the perinatal period, although only transiently[82,117]. Neutrophils, although present in high numbers in the neonatal period, show limited functions compared to adult neutrophils such as adhesion and rolling, chemotaxis, formation of neutrophil extracellular traps, and phagocytosis[154-157]. Although immature, neonatal neutrophils can still protect the newborn against infections[158,159].

Fetal and neonatal monocytes have impaired antigen presentation and decreased adhesion and thus show reduced infiltration to the inflammatory sites compared to adult monocytes[53,160,161]. However, neonatal monocytes

can have higher cytokine secretion and more sustained inflammatory response, which can lead to an exacerbation of inflammation[162,163].

Neonatal macrophages present similar phagocytic properties as adults[164], although pro-inflammatory cytokine expression is increased after LPS stimulation in neonatal macrophages compared to adult macrophages; however, their ability to activate T cell proliferation is still developing[165]. Overall, neonatal macrophages show a skewed response towards Th2-polarizing cytokines compared to Th1-polarizing cytokines, which could lead to a skewed Th2 response and thus higher neonatal susceptibility to infections[166], and this is even more likely to be the case in preterm newborns[167].

The γδT cells, which are innate-like cells, have typical T cell and innate immune cell features. Among the subtypes of γδT cells, the $V\delta1^+$ subtype is most common in neonatal human blood, while the most common adult subtype found in human blood is $V\gamma9V\delta2^+$, which is also present in utero in the second trimester of pregnancy, but then decreases around term delivery, which might indicate a predominant role in fetal immune response[134,168]. Overall, neonatal γδT cells are able to mount an immune response, although γδT cells from preterm newborns are immature compared to those in term newborns[169].

ILCs have important roles already during the fetal stage (GW9), and their numbers increase throughout the pregnancy and infancy. It has been shown that they are key players in a number of biological processes, including lymphoid tissue formation and microbiome colonization[117,170]. Furthermore, ILC2s and ILC3s are increased in the placenta during spontaneous preterm labor[119] and are found in high numbers in cord blood[171], and recently ILCs were found in human breast milk (mainly ILC1s)[172].

ADAPTIVE IMMUNE CELLS

Studies have shown that adaptive immune cells are present in fetal and neonatal compartments[150], showing that even if they are not the major immune component, these cells need to be taken into account when studying neonatal immunity[134].

T cell responses in neonates are usually skewed towards a Th2 response instead of Th1, e.g. in stimulated cord blood $CD4^+$ T cells[151] or after

vaccination, although many vaccines contain a Th2 adjuvant that can skew the system further towards a Th2 response[173]. Congenital cytomegalovirus infection triggers in utero T cell ($CD4^+$, $CD8^+$, and γδT) differentiation and mature-like responses to the infection[174-176]. B regulatory cells, which have immunosuppressive properties, have been shown to be involved in neonatal sepsis[177].

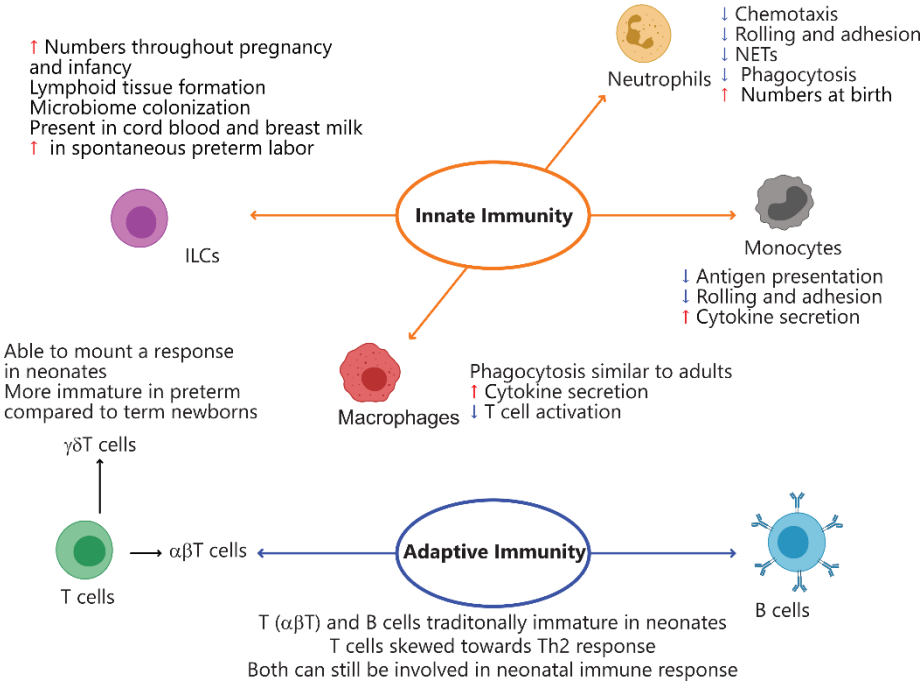

Figure 5. Neonatal immune cell functions compared to adults. Adapted from Tsafaras et al.[178]. **Legend:** ↑: increase; ↓: decrease.

1.3.4 Immune cells in the brain parenchyma and meninges

The brain immune response has been investigated in many different brain pathologies; however, until recently most studies have focused solely on the inflammatory response in the parenchyma, and the CNS border zones, such as the meninges, have generally been overlooked.

BRAIN IMMUNE CELLS

When it comes to immune cells in the brain, the brain-specialized macrophages, called microglia, dominate the inflammatory response. The

microglia seed the brain early on in life (GW4.5-5.5[72]) and are the main immune patrolling cells in the brain over the lifespan[53,54,179]. The first colonization routes used by microglia to infiltrate the brain parenchyma are the meninges, the choroid plexus, and the ventricles[72]; however, no study has analyzed in detail whether some of the microglial cells are retained in these border zones and what functions they might have.

Microglia are tissue-specific macrophages, and therefore they have similar functions when involved in the inflammatory response after an insult in the brain. However, microglia carry out a number of functions beyond the immune response, and studies have shown that several subtypes of microglia exist in different numbers throughout the lifespan and are involved in age-related white matter and cortical development and inflammatory responses[179-182].

These brain-specific macrophages have been studied in a wide range of brain pathologies such as Alzheimer´s disease, aging, multiple sclerosis, stroke, and neonatal brain injury[183-188]. In some of these pathologies microglia have been found to have a dual role. On one hand, they can sustain the inflammatory response via the production of inflammatory cytokines, while on the other hand they can promote inflammation resolution and recovery[183,184]. This dual response might be due to the high microglial heterogeneity that has recently been reported[179,180,185].

MENINGEAL IMMUNE CELLS

Only recently has the immune response in brain physiology and pathology been explored in the meninges, and this tissue has now been shown to be an active participant of many processes in brain development, physiology, and pathology. The meningeal immune landscape is quite diverse in the physiological state. Mast cells have been mainly reported in the dura mater, while dendritic cells and meningeal border-associated macrophages (BAMs) can be found in both the dura mater and leptomeninges. Additionally, some $CD4^+$ T cells have been reported in the leptomeninges at steady state, and microglial cells have been observed just under the pia mater[189]. ILCs have also been reported to be localized mainly in the meninges[190].

Prior to recent single cell studies[185,191], the mouse meningeal immune cells were studied only in a few pathologies, such as experimental autoimmune

encephalitis (EAE, a mouse model of multiple sclerosis), meningeal inflammations from various causes, and cancer[115,192-195]. Interestingly, neutrophils have been shown to infiltrate different layers depending on the pathology being studied; for example, in a stroke model they have been observed in the leptomeninges, whereas in the EAE model they were present in the dura[189,195]. Recently, studies have analyzed the role of BAMs in adult stroke models, and these cells seem to accumulate after stroke insult and trigger granulocyte accumulation and vascular leakage[196,197]. However, the role of most meningeal immune cells is still largely unknown in the majority of brain insults such as neonatal HI-induced brain injury.

2 AIMS

2.1 General aim

The overall aim of this doctoral thesis was to elucidate the inflammatory response of immune cells in a mouse model of preterm brain injury, both in the brain parenchyma and in the meninges.

2.2 Specific aims

The specific objectives were:

1) To study the adaptive immune response in human and mouse brains after preterm brain injury (**Paper I**)
2) To investigate the response from T and B cells in in preterm brain injury using both wild-type mice and mice deficient for T and B cells (**Paper I**)
3) To explore if ILCs react to HI-induced preterm brain injury (**Paper II**)
4) To study the immune response of ILC2s after HI insult using ILC2-impaired mice (**Paper II**)
5) To characterize the transcriptome of resident immune cells in neonatal mouse meninges at the single-cell level (**Paper III**)
6) To identify the early meningeal inflammatory responders at 6 hours after preterm HI-induced preterm brain injury (**Paper III**)

3 MATERIALS AND METHODS

This chapter describes the techniques and methods used in this thesis, focusing on how the technique or technology works and its advantages and disadvantages. The details of the methods can be found in each paper.

3.1 Patient samples

In **Paper I**, we validated our results using post-mortem human brain samples. The samples were collected under ethical permission (07/H0707/139) from the National Research Ethics Service, Hammersmith and Queen Charlotte's and Chelsea Research Ethics Services, London, UK. Six preterm post-mortem brains (<GW28) were used. A pathologist determined the primary cause of death. The details of the samples are reported in Paper I.

3.2 Animal experiments

3.2.1 Mouse strains

Papers I-III are based mainly on animal experiments approved under ethical permits from the animal ethics committee of the University of Gothenburg (5/2013, 58-2016, and 2042/18). Mouse models help us investigate physiological and pathological processes in great detail. Mice also have a short gestational period, which, combined with the large number of pups per litter, makes these models affordable. Because they have similarities in genetics to humans, it is also possible to create and employ a broad range of transgenic mice that allow researchers to study physiological and pathological mechanisms in detail, which is not possible to the same extent with other organisms. We used transgenic mouse strains in order to highlight the roles of different subtypes of immune cells, and the mouse strains used in this thesis are found in Table 1.

It is important to note that mouse models have their limitations, and there are some fundamental differences between mice and humans that must be taken into account. While the human brain is gyrenchephalic, the mouse brain is lissenchephalic, resulting in higher volumes of gray matter in mice compared to humans[198]. Additionally, processes such as human development of the immune system, blood-brain barrier formation, and oligodendrocyte maturation occur from the second half of the second

trimester up to term gestation in humans, while some of these processes take place in the first week of life of neonatal mice[46,47]. Due to the differences in brain size[40], some experiments can present technical difficulties, e.g. requiring pooling of multiple samples to reach the threshold for analysis. Lastly, while there are few differences in the immune system[199], both species present with immature immunity in the neonatal period, mainly relying on the innate immunity for an inflammatory response in the early stages after birth[82].

Mouse Strains	Referred as	Functional phenotype	Papers
C57Bl/6J	C57Bl/6J	Wild type mice	I, II, III
B6.129S7-Rag1^{tm1Mom}/J	*Rag1$^{-/-}$*	Mice deficient for mature T and B cells[200]	I
Ror$\alpha^{fl/fl}$;IL7R$^{+/Cre}$	ILC2-impaired	ILC2-impaired mice[201]	II

Table 1. Summary of mouse strains used in the studies. **Legend:** -/-: knock-out; fl: floxed; Cre: Cre recombinase.

3.2.2 Hypoxic-ischemic brain injury model

Preterm brain injury consists mainly of white matter injury, with occasional focal gray matter lesions[22]. Lately, diffuse white matter injury has become more frequently recognized in the clinical setting[20]. The Rice-Vannucci model is the most commonly used rodent model to study neonatal brain injury[202]. Although it was first developed in rats, it has been adapted to mice[203], and the HI-induced preterm brain injury model used in this thesis mimics the brain injuries seen in human preterm newborns, as previously published [204]. The model allows for variability: while the regional distribution of brain injury is rather consistent, there are some inter-individual differences, and this reflects the clinical situation with several degrees of severity, from mild to extensive brain damage[205-208].

The model starts with surgery to permanently occlude the left carotid artery, which by itself only reduces cerebral blood flow to a minor extent. The mice are then returned to their dam for one hour to recover from the surgery. Afterwards, the pups are placed in a hypoxic chamber, and the subsequent hypoxemia evokes a vasoconstrictor response in the left

hemisphere (ipsilateral to the occluded carotid artery) that, in combination with tissue hypoxia, causes brain injury[209].

There are several limitations of the HI-induced brain injury model, the most obvious being the differences in physiology between humans and murine species. Additionally, the HI brain injury model induces only unilateral injury, while preterm newborns can also present with more global and bilateral injury. However, this unilaterality of the injury in the model gives the advantage of using the other hemisphere as an internal non-injured control.

3.3 Immunoassays

3.3.1 Flow Cytometry and FACS

Flow cytometry allows for the identification and counting of cells and for the measurement of the protein abundance of certain antigens in a variety of populations based on emissions from fluorescent antibodies, usually conjugated. In **Papers I** and **II**, we used flow cytometry to determine the frequency of immune cells such as T and B cells (Paper I) and ILCs (Paper II) based on the expression of several markers (Table 2).

Fluorescence-activated cell sorting (FACS) is based on the same physics principles as flow cytometry, but this variant of the technique also allows for separation (sorting) of the cells of interest after analysis. We performed FACS in **Paper III** to sort the leukocyte populations from the meninges.

Flow cytometry and FACS have the advantage of simultaneously measuring the expression of multiple proteins, which can then be used to correctly identify the cell populations, their activation state, and much more. The disadvantages mainly come from technical limitations of the cytometer, e.g. the numbers of different emission spectra (colors) that can be detected. Another limitation regards the panel design: one has always to take into account how much the antigen is expressed *vs* the intensity of the fluorochrome. Ideally, the antigens which are lowly expressed should be targeted by an antibody with a strong fluorescent signal, but this can be limited by the commercial availability of antibodies.

In **Paper I** and **Paper II**, brains of neonatal mice were enzymatically and mechanically dissociated to obtain single cell suspensions of mononuclear cells, and these cells were stained with antibodies tagging typical markers

for the various cell types being studied. In **Paper III**, after mechanical dissociation, single cell suspensions of meninges were stained for CD45, a common marker for all leukocytes (Table 2)[210,211].

The analysis of the data was based on gating strategies. First, a selection was made based on the cell size and granularity, which was obtained from how the light, emitted by lasers, scattered forward or to the sides. Thereafter, the cells were selected for further analysis based on singularity (cells that were attached to each other were removed) and viability, typically using a DNA-binding dye. Lastly, the fluorescent emissions detected by the cytometer were used to identify and analyze the cells further based on their expression of markers. The final results were expressed as frequencies of the parent gate (the preceding gate) or of a specific population.

Cells	Markers	Papers
T cells	$CD3^+$	I
αβT cells	$CD3^+TCR\beta^+$	I
B cells	$CD19^+$	I
ILCs	$CD45^+Lin^-Thy1.2^+$	II
ILC2s	$CD45^+Lin^-Thy1.2^+NKp46^-SCA\text{-}1^+KLRG1^+$	II
Leukocytes	$CD45^{int+hi}$	III

Table 2. Summary of the flow cytometry markers used to identify the different immune cells in the papers.

3.3.2 Immunostaining

Immunohistochemistry (IHC) and immunofluorescence IHC (IF/IHC) staining are other antibody-based techniques used in all three papers. In contrast to flow cytometry, immunostaining is not a single-cell technique, but it rather gives us an overview of the expression of the target in the whole tissue section. Through IHC we can assess one protein at a time, while in IF/IHC we can target more proteins simultaneously in order to analyze details such as co-expression and co-localization of certain markers. Choosing one or the other depends on which kind of assessments we are trying to achieve, how specifically that protein is expressed, and in general on how much information we want to analyze from one

experiment. Here we employ primary antibodies, which bind to the target, and secondary antibodies, which amplify the signal either in coordination with other reagents (IHC) or through fluorescent-conjugation (IF/IHC). These two kinds of immunostaining require different types of microscopes – IHC is visualized in bright field, while IF/IHC requires fluorescence microscopes, commonly confocal microscopes, in order to be able to detect the fluorescence of separate signals and to obtain higher resolution.

To maintain structural integrity, the tissue needs to be fixed after collection, commonly with formaldehyde or similar compounds that bind to the protein and maintain the structure. In this thesis, the tissues were then sectioned using either a microtome for paraffin-embedded tissues (**Papers I-II**) or a cryostat (**Paper II**). The meninges were placed directly on the slide after removal from the parenchyma (**Papers II-III**).

Immunostaining is a versatile technique that can be used for a range of purposes and with many different combinations of antibodies, especially IF. Also, we can retain spatial information about the tissue we are analyzing, which can give us further insights into, for example, regional processes at the site of injury. However, immunostaining results can be affected by the levels of expression of protein, and therefore only the strongest signals will be detected. Furthermore, the risk of antibodies cross-reacting (binding to the wrong target) is always a possibility, especially if IHC/IF is used; however, this risk can be mitigated with the proper controls such as single antibody staining and by omitting the primary antibodies.

The choice of antibodies to use in immunostaining experiments will depend on the cells to be assessed, the availability of antibodies, and the targets' expression levels.

Microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP) are commonly used markers to stain for neurons in gray matter and white matter tissue, respectively. Loss of MAP-2 staining area indicates gray matter injury, tissue loss, and infarct because it is expressed in all neurons but is lost upon neuronal death[212]. MBP is expressed in the neural myelin sheath and is therefore used to measure brain white matter area[213].

Ionized calcium-binding adapter molecule-1 (Iba-1) and glial fibrillary acidic protein (GFAP) are well-known markers to identify microglia and astrocytes, respectively[214,215]. Iba-1 is expressed in both resting and activated microglia because it is expressed in the soma, and it allows not only the detection of microglial cells, but also the study of their

morphological changes based on Iba-1 immunoreactivity[214,216]. GFAP is present in the cytoskeleton, and astrocytes in activated states increase their expression of GFAP, and this can be used to identify activated astrocytes in response to injury[217].

ILC2s are more difficult to identify compared to other cells due to the fact that they share many features with Th2 cells[112]. Therefore, it is a common strategy to employ a marker to exclude T cells, typically CD3[190,218], and then to use a second marker such as ST2, which identifies ILC2s among the ILC subtypes[219,220].

Quantitative analysis of stained tissue sections highly depends on the scope of the experiment. We compared the injured hemisphere to the non-injured hemisphere in **Paper I** and **Paper II** as well as to naive animals in **Paper II** (IF/IHC). All analyses were performed using ImageJ software (Rasband, W.S., US NIH, United States).

For gray and white matter tissue loss (**Papers I-II**), the stained areas for MAP-2 and MBP were manually measured in multiple sections per animal, and then the volume loss was calculated.

Microglial staining by Iba-1 was assessed by measuring the ratio between the stained area and total area of the images around the site of injury (**Paper II**). Astrocytes, identified as GFAP-positive cells, were manually counted and were expressed as cell density (cells/mm^2, **Paper II**).

ILC2s, identified by using the ST2 and CD3 markers, were manually counted and expressed as numerical density (cells/mm^3, **Paper II**).

Meningeal immune cells were identified through IF/IHC with a combination of markers for BAMs ($CD206^+Lyve1^+Iba\text{-}1^+$) and microglia ($CD206^-Lyve1^+Iba\text{-}1^+$) as previously reported[185].

3.3.3 Cytokine and chemokine assays

In **Paper II**, the expression of cytokines and chemokines in the brain after HI was investigated using the third antibody-based technique in this thesis, the multiplex immunoassay. We used the Bio-Plex Pro™ Mouse Cytokine 23plex Assay (Bio-Rad). This method, like flow cytometry, analyzes several cytokines and chemokines simultaneously and can provide important information about the general inflammatory response in a certain tissue or cell type. This is achieved by using magnetic beads bound to antibodies specific to the proteins of interest; the beads are also fluorescent, emitting

specific wavelengths of light for each cytokine/chemokine analyzed. The beads are then combined in wells, giving the possibility to analyze all proteins simultaneously. Biotinylated antibodies are used to quantify the expression of the proteins by binding with streptavidin-phycoerythrin, which is also fluorescent. The quantitative data are obtained in a cytometer that measures the fluorescence from both the bead, indicating which protein, and the biotinylated streptavidin, measuring the quantity of protein present in the sample.

The technique is an advancement compared to the enzyme-linked immunosorbent assay (ELISA), which analyzes single proteins, and it provides the possibility for measuring several proteins at the same time and with higher sensitivity. This makes the multiplex method more time-efficient and makes it better for lower sample volumes, such as blood and CSF, and for proteins with low expression.

3.4 Gene expression analysis

3.4.1 Single-cell RNA-sequencing

In 2009, total RNA-sequencing (or bulk RNA-seq) was defined as a "revolutionary tool for transcriptomics"[221], and it has allowed researchers to explore in depth the cell's transcriptome in various species, tissues, and conditions. In the same year, the foundations of a new method – single cell RNA-sequencing (scRNA-seq) – were developed that would advance much further the information we can obtain from messenger RNA (mRNA)[222]. ScRNA-seq follows the same principles as bulk RNA-seq, and mRNA is extracted from the cells or tissue and then sequenced. The difference lies in how and when the RNA is extracted: bulk RNA-seq requires first the processing of cells or tissue followed by the RNA extraction, while in scRNA-seq this step is performed only after the cells have been isolated and separated (Fig. 6).

ScRNA-seq can be achieved in a number of ways, but the two most common are by creating a single-cell suspension (microdroplets) or by sorting each cell into a separate well. The two methods reflect different aims – if the cells are separated in wells, the sequencing depth is much greater and therefore more details of the mRNA can be analyzed, for example, mutations. However, due to the limited number of wells per plate, these studies usually analyze fewer numbers of cells. The microdroplets

methods are capable of processing thousands of cells and multiple samples at the same time, thus reducing the sample preparation time and increasing the number of cells sequenced. However, these advantages are counteracted by a reduced sequencing depth, and therefore only a limited number of analyses can be performed[223]. Overall, this new possibility to analyze cells separately has opened up new avenues for researchers to deepen our knowledge in fields such as neuroscience, immunology, and cancer research.

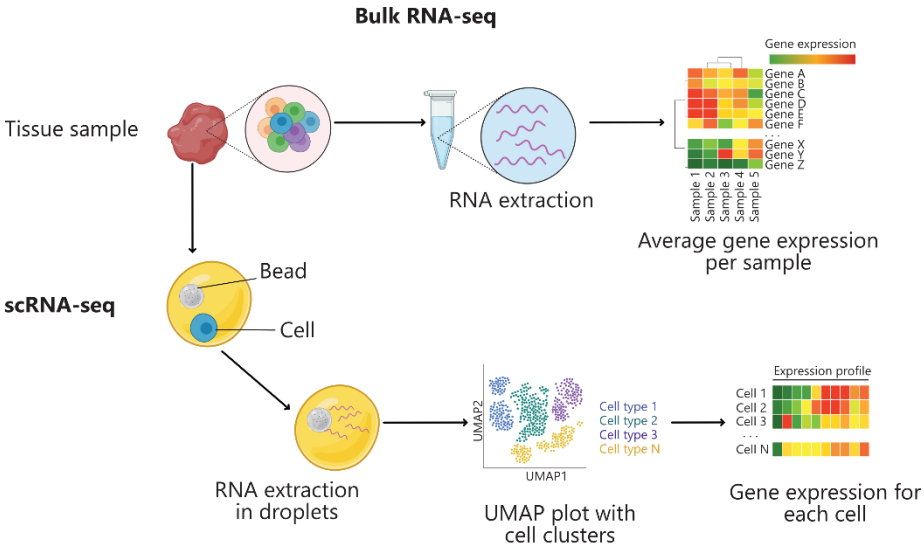

Figure 6. Differences between bulk RNA-seq and scRNA-seq (here represented as the droplet method). **Legend**: UMAP: Uniform Manifold Approximation and Projection.

There are several advantages to such an approach. First and foremost, bulk RNA-seq tissue samples are usually a pool of the different cell types present in that specific tissue; therefore, the gene expression we obtain from such experiments is an average expression of the tissue, with no possibility of breaking down the heterogeneity commonly present in all tissues. ScRNA-seq instead can disentangle such complexity because each cell is analyzed separately, and therefore we can understand much better the gene expression of a specific cell type. ScRNA-seq also allows the study of rare populations, where the expression is usually masked when averaging the gene expression in bulk RNA-seq. Also, the higher sensitivity of this method

requires fewer cells and therefore reduces the need for sample pooling. Furthermore, analyzing each cell separately unlocks a whole new range of analyses that were difficult to perform before, such as lineage tracing and developmental relationships (e.g. trajectory analyses). However, scRNA-seq reagents are costly and the analysis requires expert knowledge in bioinformatics, factors that make this technique less approachable than bulk RNA-seq. Furthermore, as for bulk RNA-seq, information regarding the regional organization of the tissue is lost.

In **Paper III**, we wanted to investigate the whole immune population in the meninges of neonatal mice. Because our aim was to characterize a quite broad cell population, we needed a method that allowed us to analyze the gene expression of thousands of cells. Therefore, we decided to use the microdroplets method. Using 10x Genomics instrument and reagents (CA, USA), sorted leukocytes were separated into single-cell suspensions called gel beads in emulsion (GEMs) in the Chromium Controller. The emulsion gel-in-oil is what separated the sample in individual droplets, each one containing one cell and one bead. The barcoded beads allow the identification of each cell at every step, from when GEMs are formed all the way to the bioinformatics analysis. The next steps were reverse transcription, cDNA amplification, and library preparation, all performed following the manufacturers' instructions and with quality controls in the recommended steps. The length of the sequencing read was paired-end 150 base pairs, meaning that 75 base pairs were read both from the 3'- and the 5'-end, and thus the mapping of the genome is more precise[224]. Phred quality score Q30, the probability that 1 in 1,000 base calls is incorrect[225,226], was 92.3%, indicating that our sequencing had a low probability of being incorrect and thus indicating the good overall quality of the sequencing.

3.4.2 Seurat analysis

Bioinformatics has evolved hand-in-hand with genomics techniques in order to analyze bigger and bigger datasets. This type of analysis is usually run in programming languages built to be able to run heavy statistical analysis, such as R and Python[227,228]. Indeed, because for each sample we have thousands of cells, and each cell has data regarding the expression of thousands of genes, these types of datasets have many variables, typically called dimensions. For scRNA-seq data, one of the most common workflows for data analysis is Seurat[229] (v3.027 used in **Paper III**).

Seurat, an R package, can be used for all steps from data filtering to visualization. A typical visualization of scRNA-seq data is the Uniform Manifold Approximation and Projection (UMAP), a type of principal component analysis plot. These plots are made by reducing the dimensions of the datasets according to specific parameters, which are chosen during the data processing in Seurat. The result of this dimensionality reduction is a plot where the cells are grouped by similarity in gene expression, and these groups are defined as clusters. Therefore, cells in the same cluster will have similar gene expression. Based on the dataset, the cluster numbers can vary depending on the heterogeneity of the examined cell population. In Seurat we can identify, for example, the gene expression pattern that distinguishes one cluster from the others, how the expression of the genes varies in the datasets, and the cell cycle status of the cells. It is also possible to further subdivide the clusters into subclusters, either because of a condition (e.g. treated *vs* controls) or other characteristics. Through Seurat, we can calculate the differentially expressed genes (DEGs) for any comparison, for which we get an average fold change (ratio of the gene expression levels between two groups), the p-value, and the adjusted p-value based on Bonferroni corrections.

3.4.3 Cluster annotation

Once the cells are filtered and clustered, we need to identify which cell populations we have in the dataset, especially in an experimental setting like ours where we want to analyze a broad range of multiple cell populations.

We first took a manual approach, and signature genes for several immune and brain populations were identified in the on-line database CellMarker[230], and the clusters were defined based on their expression patterns. However, interpretation of the expression levels can be a source of mistakes and biases. For example, if a small or rare cell population is not included in the manually annotated gene lists, it could be missed and important information could be lost.

CHaracterization of cEll Types Aided by Hierarchical classification (CHETAH) is an algorithm that unbiasedly and accurately annotates cells from scRNA-seq clusters[231]. Reference cells are used to create cell profiles, called reference profiles (RPs). The RPs are put together in a classification tree, constituted by nodes and branches. Cell populations are branches, and each RP in a branch represents a cell subtype, and two RPs are connected

through nodes. When there is no further subdivision, the cell types are called leaves (Fig. 7A).

Each cell (e.g. input cell *j*) enters a top-to-bottom stepwise process in the classification tree and proceeds until a classification is reached (Fig. 7B). The most distinctive genes for each RP are selected, defined as discriminatory genes, and used later on to annotate the input cells (Fig. 7C). Profile scores for each input cell are calculated based on the correlation between the input cell and all the potential RPs in the nodes (Fig. 7D).

The node with the highest sum of profile scores is selected to continue the annotation, but only if the confidence score is high enough. The confidence score is equal to the RP in the branch about to be chosen minus the average of the RPs in the other branches. The selected RP will be chosen if the confidence score is higher than the threshold (default 0.1; Fig. 7E). Then the cycle will be repeated again until a leaf is reached (e.g. cell type *x*). If the confidence score is lower than the threshold, then the input cell is defined as unassigned or intermediate, depending on the position of the last node in the classification tree[231].

The advantages of this method are a reliable, automated, and unsupervised annotation that takes into consideration the cell gene expression, and if not confident enough it will at least give an indication as to which cell type the input cell *j* might belong to.

3.4.4 Trajectory analysis

Trajectory analysis is useful for revealing relationships between cell populations. Based on gene expression, this analysis can help establish the order from progenitors to mature cells, for example, even without having actual data in a time series, and it is therefore also called "pseudotime" analysis. Therefore, it can be used, for example, to establish the sequence from progenitors to precursors to mature cells or, as in **Paper III**, to determine the relationships among similar immune cells and thus organizing the cells in a trajectory. We used such a method to understand the relationships among BAMs, microglia, and monocytes.

We used SCORPIUS, a R-package, to perform the pseudotime analysis[232]. An advantage of SCORPIUS compared to other similar packages is that it can be run in R in combination with Seurat and therefore allows the selection of exactly the same cells and clusters identified in the Seurat pipeline.

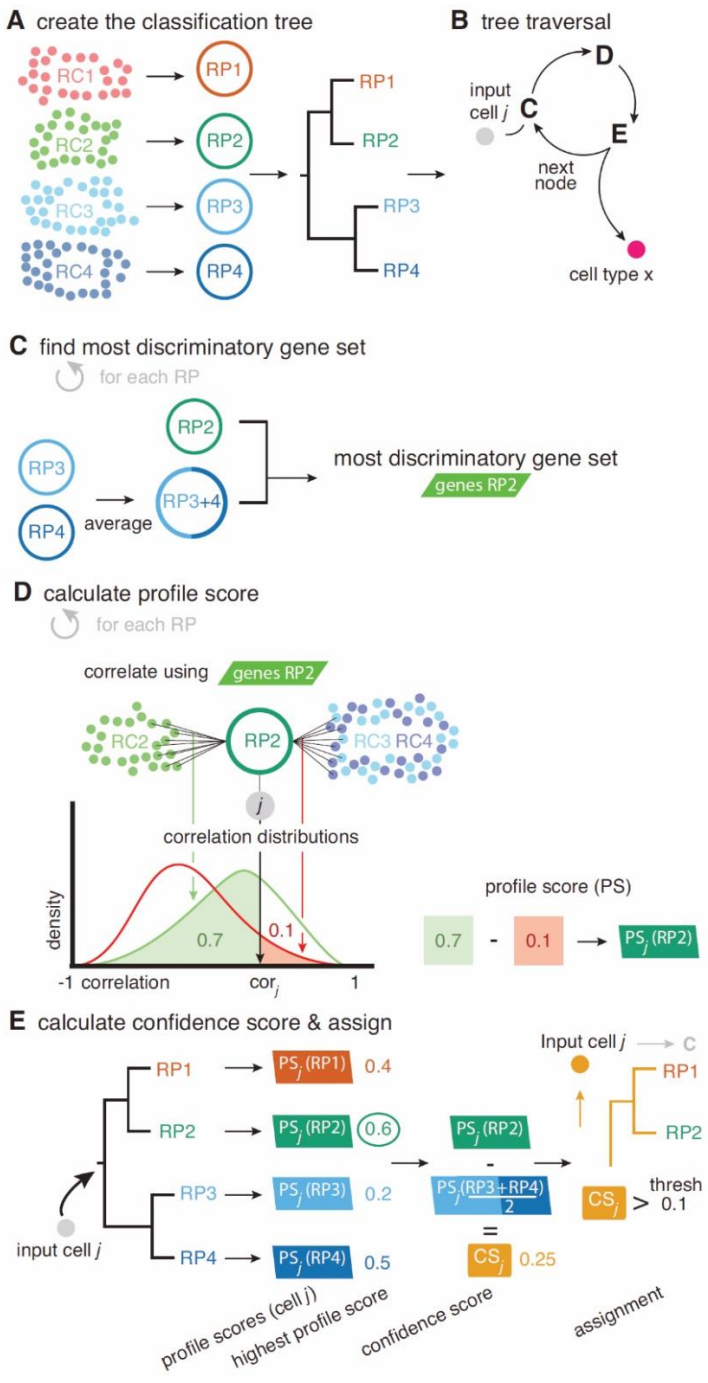

Figure 7. CHETAH algorithm, with permission from de Kanter 2019[231]. **Legend:** RC: reference cell; RP: reference profile; PS: profile score; CS: confidence score.

3.4.5 Transcriptome analysis

After we obtained the DEGs from Seurat, we needed to analyze these changes further. In **Paper III**, we used two types of analysis to understand the unique features of each cell population, and the differences between HI and controls, namely gene ontology (GO) and Ingenuity Pathway Analysis (IPA).

GO is a database containing information about genes and their respective translated products, if there are any, from several species. This information is categorized into which cellular component it is expressed, which molecular function the related protein has, and which biological process it is part of, and all genes are assigned a GO term for each category. We used gProfiler[233,234] to determine which GO terms were enriched, that is, which GO terms were more common based on the DEGs we analyzed. However, most GO term search tools only give us the enriched GO terms and the relative calculated p-value. To understand the directionality of the relevant GO term enrichment in our datasets, we used GOPlot R package[235], which calculates a directionality score (z-score) based on the DEGs and the GO term search results. If z-score > 0, the term is increased, and if z-score < 0 it is decreased.

However, GO terms are usually quite vague and do not provide information about specific pathways or molecules that could fully explain the differences between two groups. To answer this, we used IPA[236], which is a knowledge-based software for genomics data. IPA uses the same DEGs analyzed for the GO term search and provides predictions for changes between the two groups regarding, for example, canonical pathways, regulators, and upstream molecules. For each of these analyses, IPA gives p-values and other parameters to help understand on a much deeper level the changes seen between different cell populations and conditions in our paper.

3.5 Statistics and data visualization

Statistical analysis is usually the last step of data analysis, and the correct interpretation of the results heavily relies on appropriate statistical tests. We used GraphPad Prism software v6.02 (San Diego, CA; **Paper I**) and IBM SPSS Statistics 25 (IBM Corp, Armonk, NY, United States; **Paper II**). In **Paper**

III, most of the statistical analysis was run in R Seurat v3.027, and the remaining tests were run in gProfiler, GOPlot and IPA.

For normally distributed two-group comparisons, we used Student's t-test, and this was independent or paired depending on the comparison (**Paper I, II**). Non-normally distributed 2-group comparisons were tested with the Mann–Whitney U-test (**Paper II**).

For multiple group comparisons, we used ANOVA tests, and if the comparison contained both paired and independent data, we used mixed-model ANOVA, while independent groups were tested with 2-way ANOVA. Both used Games–Howell post-hoc corrections (**Paper II**).

In **Paper III**, gene expression comparisons between two groups were analyzed with the Wilcoxon Rank Sum test in Seurat. gProfiler, GOPlot and IPA p-values were reported as calculated by the original source[233-236].

The data in **Paper I** were presented as the mean ± SEM, while in **Paper II**, because we had non-parametric comparisons, we used 5^{th}–95^{th} percentile boxplots. In **Paper III**, the results from Seurat, CHETAH, and SCORPIUS were shown as UMAP plots. Volcano plots were used to visualize DEGs. A heatmap was used to represent the top 10 DEGs in each cluster compared to all others. GOPlot was used to represent the 10 most relevant GO terms from gProfiler. All IPA results were exported from the original software, except for canonical pathway graphs that were generated in GraphPad Prism based on the IPA predictions. Stacked bar charts were used to represent the frequencies of the cell populations between HI and naive mice, and histograms were used to show differences between gene expression of specific features in microglial populations between HI and naive animals.

The figures of this thesis, unless stated otherwise, were created with Biorender.com, including the summary figures of the results and conclusions.

4 RESULTS AND DISCUSSION

Numerous studies have contributed to our current knowledge on the role of immune cells after neonatal brain injury[24,31-33,37,204,237-242], but only a few studies have focused on preterm brain injury in animal models[31,204,238,243,244]. These latter studies have shown in rodent and sheep models that microglia[31,244], $CD4^+$ T cells with a Th response[204], γδT cells[238,243], and neutrophils[31,245] infiltrate the brain and show activation within 24 hours of the initial insult. However, we know from studies in other models that other immune cells, such as monocytes[33] and macrophages[196,197], also respond to brain injury. Furthermore, other immune cells which are vital for neonatal development, such as ILCs[117,170], have not previously been investigated following HI injury.

Using an established mouse model of HI-induced preterm brain injury, we investigated the role of adaptive and innate immune cells in the brain parenchyma and meninges after preterm HI injury.

4.1 T and B cells infiltrate the brain after HI

HI in neonatal PND9-10 mice and PND7 rats triggers the adaptive immune response with the infiltration of immune cells and the production of cytokines[24,37,242]. Based on the previous findings that $CD4^+$ T cells were increased in HI animals compared to naive animals at 24 hours and 7 days after preterm brain injury[204], we decided to further investigate the contribution of T and B cells to preterm brain injury. In **Paper I**, we investigated the presence of T and B cells in post-mortem PVL and control human brain samples with CD3 and CD20 antibodies, which are markers for T cells and B cells, respectively. We observed increased numbers of both T and B cells in PVL samples compared to controls, with accumulation in both the parenchyma and meninges. $CD3^+$ T cells were observed mainly in the white matter and in the meningeal blood vessels. Similarly, another study showed the accumulation of T cells in the white matter of PND7 rats after LPS/HI[246]. $CD20^+$ B cells were present in small numbers, and only in the meninges around the blood vessels (Fig. 9). Similar to our findings, B cells were found in human post-mortem brain samples after stroke injury in the ischemic and surrounding areas[247].

A previous publication from our group demonstrated that γδT cells are increased after preterm brain injury[243], indicating that lymphocytes react to

this type of brain injury and infiltrate into the brain in newborns. Additionally, the perivascular spaces, choroid plexus, and meninges have been proposed as infiltration routes for leukocytes into the brain[248-251], which might explain the accumulation of T and B cells in the meninges that we detected in PVL cases but not in controls.

The increase of T and B cells in the preterm brain was further confirmed by flow cytometry of neonatal mouse brains after HI injury. There was increased frequency of $CD3^+$ T cells had increased frequency in the ipsilateral compared to the contralateral brain hemisphere at 3 and 7 days after HI, and $CD3^+$ αβT cells were increased in the ipsilateral hemisphere at 7 days after HI. Similarly, $CD19^+$ B cells (corresponding to $CD20^+$ B cells in humans) also increased significantly within 7 days after HI (Fig. 8).

These findings are in line with a previous study showing that T cells were increased in the mouse brain after HI brain injury at PND10, and those authors showed the infiltration of $CD4^+$ and $CD8^+$ T cells for months after HI, with $CD4^+$ infiltration peaking at 17 and 100 days after HI and $CD8^+$ T cells peaking at 24 days after HI[37]. Another study of HI-induced injury in PND7 rats also showed the increased infiltration of $CD4^+$ T cells over time, persisting until 42 days after the insult. Furthermore, both T cells[252,253] and B cells[247] were present in the brain in adult stroke rodent models. T cells, including αβT cells, were found in the rat brain 24 hours after the stroke insult[253]. B cells infiltrate the mouse brain for weeks after the stroke insult, and immunoglobulins can be found in the border zone of the injury[247].

Altogether, we concluded that T cells, and to a lesser extent B cells, infiltrate the brain after neonatal brain injury both in human preterm newborns and in mice.

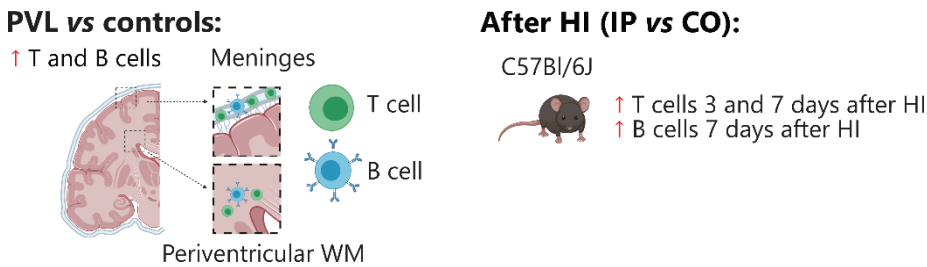

Figure 8. Accumulation of T and B cells after preterm brain injury in PVL post-mortem human brains and mice after HI. **Legend:** PVL: periventricular leukomalacia; IP: ipsilateral hemisphere; CO: contralateral hemisphere; WM: white matter; ↑: increase.

4.2 T and B cells may contribute to HI injury

In **Paper I** we examined the contribution of T and B cells to preterm brain injury in terms of gray and white matter tissue loss (using IHC for MAP-2 and MBP, respectively) at 7 days after HI in *Rag1*$^{-/-}$ mice, which do not possess mature T and B cells[200] (Fig. 9). We found that mice lacking T and B cells had partial white matter protection compared to wild type controls following injury.

T and B cells have previously been shown to worsen the injury in neonatal LPS/HI injury and adult mouse stroke models[246,254,255], similar to what we observed in this study. In all of these studies, the mice were lacking both T and B cells[246,254,255] or specific T cell subsets ($CD4^+$ or $CD8^+$ T cells)[254]. Furthermore, B cell deficiency has been shown to prevent cognitive decline in mice after stroke insult[247]. However, it has been shown that lacking or blocking the trafficking of only one subtype, either $CD4^+$ T or IL-10-producing B cells, can also worsen the severity of brain damage in neonatal or adult mice[32,256,257], indicating a complex balance between these immune cells. A recent study highlighted that depletion of peripheral T cells was actually detrimental for gray and white matter injury, and the damage was worsened by the absence of $CD4^+$ cells because the lack of T cells was compensated for by innate cells such as neutrophils and inflammatory macrophages[32].

Overall, our study showed that T and B cells are involved in preterm brain injury in post-mortem humans PVL samples and mice and that deleting these cells in mice provided moderate protection against white matter injury, although the exact mechanisms of these effects are still unknown.

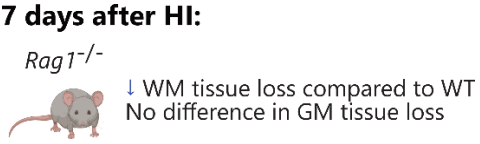

Figure 9. Combined deficiency of T and B cells (*Rag1*$^{-/-}$) leads to moderate neuroprotection in the white matter. **Legend:** GM: gray matter; WM: white matter; WT: wild type; ↓: decrease.

4.3 ILC2s increase in the brain after HI

In **Paper II**, we investigated a recently discovered family of innate immune cells called ILCs. We focused on the role of ILC subtype 2 (ILC2s) due to recent observations of their potential involvement in spontaneous preterm labor[119] and in adult CNS pathologies[115,190,258,259].

First, using flow cytometry analysis we investigated the presence of ILCs ($CD45^+Lin^-Thy1.2^+$) and ILC2s ($CD45^+Lin^-Thy1.2^+SCA\text{-}1^+NKp46^-KLRG1^+$) in the brain at different timepoints after HI-induced brain injury (Fig. 11). We found that both ILCs and ILC2s increased in the ipsilateral compared to contralateral brain hemisphere in a time-dependent manner, with the highest frequencies observed 7 days after HI. This finding is in agreement with previous studies in adult rodents, all of which observed an increase in ILC2s in the brain providing an anti-inflammatory response to brain pathologies such as spinal cord injury, cerebral malaria, EAE, and cognitive decline[115,190,258,259].

We also confirmed with IF/IHC that ILC2s (defined as $CD3^-ST2^+$) mainly resided in the meninges and not in the parenchyma of neonatal mice (Fig. 10), similarly to what was previously observed in adult mouse meninges[190,258]. These findings showed that the meninges could also be a site involved in the immune response after injury, as already observed in **Paper I**.

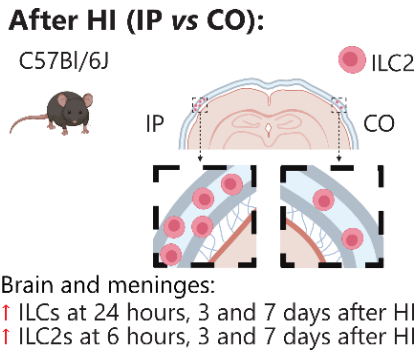

Figure 10. ILCs and ILC2s accumulate prevalently in the meninges after HI. **Legend:** IP: ipsilateral hemisphere; CO: contralateral hemisphere; ↑: increase.

4.4 ILC2s are non-essential to HI-induced brain injury

Based on these results, we investigated whether ILC2 deficiency could affect the inflammatory response and/or brain injury after HI by using ILC2-impaired mice (*Rorαfl/fl;IL7R*$^{+/Cre}$ mice[201]). We first wanted to investigate if ILC2s from neonatal pups of this mouse strain could respond to stimuli compared to wild type mice. IL-33 is a known trigger for ILC2s expansion[201], and therefore we used it as a stimulant to test this hypothesis. Flow cytometry experiments showed that neonatal ILC2s can respond to stimuli; we indeed observed an increase in cell frequencies after stimulation with IL-33 in wild type, but not in ILC2-impaired mice (Fig. 11). Thus, neonatal ILC2s are mature and are capable of responding to stimuli, which to our knowledge has not previously been shown in neonatal mice.

The inflammatory response was assessed by analyzing cytokine and chemokine levels in the brain and meninges of wild type and ILC2-impaired mice at 6 hours, 48 hours, and 7 days after HI. Overall, ILC2-impaired mice showed no overall difference in the inflammatory response except for a reduction in IL-13 expression in ILC2-impaired mice at 6 hours after HI compared to wild type controls (Fig. 13). IL-13 is one of the characteristic cytokines produced by ILC2s, and overall IL-13 production has been previously reported to be mainly sustained by ILC2s in several tissues[260-262], including the brain[190]. Higher levels of IL-13 have previously been associated with a worse outcome and increased severity of brain damage in newborns[263,264]. Thus, we hypothesized that this decrease in IL-13 in ILC2-impaired mice might lead to an amelioration of brain injury after HI.

However, we detected no difference between wild type and ILC2-impaired mice when we analyzed gray and white matter tissue loss, nor when microglial and astrocyte immunoreactivity was assessed 7 days after HI (Fig. 12). Thus, although neonatal ILC2s increase in the brain and meninges after HI, they do not seem to influence the extent of brain injury or the inflammatory response.

A similar finding has been reported where ILC3s increase after EAE insult, but ILC3-deficient mice showed no difference in EAE development compared to wild type mice[265]. Such results might be partially explained by considering the following. First, ILCs and ILC2s are generally low in numbers compared to other immune cells and are concentrated in brain border

regions such as the meninges and choroid plexus[115,190,258,266], as shown also in this study. Second, we cannot exclude the possibility of compensatory effects from other immune cells, such as what is seen in a lung infection model where the frequency of γδT cells and neutrophils increased in ILC2-deficient mice[116] or in an EAE model where mast cells and ILC2s together coordinated the response to injury[115]. However, we did not investigate if this kind of phenomenon occurs after HI insult.

ILC2s have recently been shown to have a different response based on the sex of the animal in an adult EAE model[115]. However, we did not detect any sex-dependent differences in any of our analyses.

ILC2-impaired *vs* WT mice:

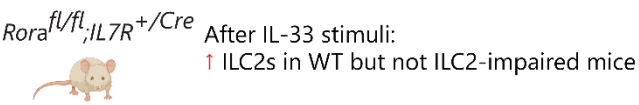

Figure 11. ILC2-impairment resulted in almost no changes compared to WT mice following HI. **Legend:** GM: gray matter; WM: white matter; WT: wild type; ↑: increase; ↓: decrease.

Overall, we concluded that ILC2s respond to HI by accumulating in the ipsilateral meninges, but they do not play an essential role in the development of brain injury in mice after neonatal HI.

4.5 Innate immunity is predominant in neonatal mouse meninges

Paper I and **Paper II** showed that immune cells respond to preterm brain injury and that they accumulate both in the parenchyma and meninges regardless of whether or not they contribute to the evolution of brain damage. However, both studies had limitations. By studying one immune cell subtype at a time, we could not rule out the possibility of compensatory mechanisms by other immune cell types, and we might have overlooked the role of other lesser known immune cells that might instead have a previously unknown role after preterm brain injury.

In **Paper III**, we therefore used a more unsupervised approach – scRNA-seq – to investigate the transcriptomes of total resident meningeal leukocyte populations in neonatal mice in **Paper III** (Fig. 12). We found that the majority of CD45$^+$ leukocytes in the meninges were BAMs, followed by microglia and monocytes, while neutrophils, ILCs, and T and B cells were present in much smaller populations. The neonatal mouse meninges thus appear to be similar in leukocyte composition to adult mouse meninges, where BAMs are also the main cell population and where other immune cell subtypes are detected in much smaller cell populations[191].

Adult BAMs have previously been divided into dural BAMs (D-BAMs) and sub-dural BAMs (SD-BAMs), based on their anatomical location[191]. Using the same signature genes, we further analyzed neonatal BAMs to see if we could observe a similar distinction among neonatal BAMs. Contrary to adult mice, we were not able to clearly separate the two subpopulations, in agreement with previous studies showing that BAM heterogeneity is present mainly in adults, and it increases with aging[185,191]. Thus, this differentiation might be possible only in adults but not in neonatal mice; although the majority of neonatal meningeal BAMs shared features with adult SD-BAMs.

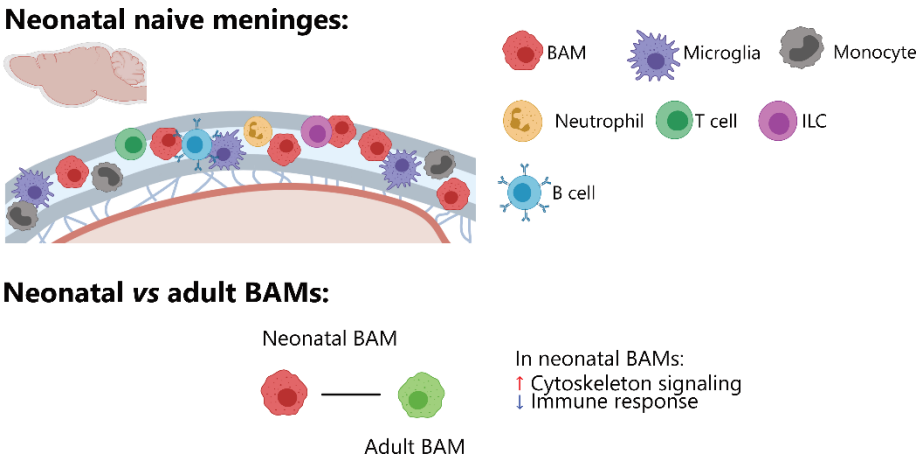

Figure 12. Naive neonatal meningeal leukocyte characterization and comparison between neonatal and adult BAMs. **Legend:** ↑: increase; ↓: decrease.

Based on differentially expressed genes, neonatal BAMs showed increased cytoskeleton signaling and reduced immune response compared to adult

mice (Fig. 12). Among other, *JUN* and *RhoB* were predicted to be regulators of the upregulation of cytoskeleton signaling in neonatal BAMs and both genes are known to regulate cytoskeleton organization through Rho and Rac signaling[267-269]. Regarding the downregulation of the immune response, macrophages steadily increase their immune response abilities over the course of embryonic development[54], and neonatal macrophages are capable of phagocytosis after LPS stimulation[164]. Overall, these findings suggest that BAMs can mount a response to insults early in life, but the immune response is still developing compared to BAMs from adult mice.

Furthermore, we found that BAMs, microglia, and monocytes were distinct from each other, although having some shared gene expression (Fig. 13). Recent studies have shown that these three immune cell subtypes, even if similar and with overlapping functions, have different ontogenies. Traditionally, it was thought that monocytes replenished the tissue-resident macrophage pools, but recently it has been shown that most macrophages have an embryonic origin[270,271], with only a few tissues as exceptions[272-277]. In mice, the first source of macrophages is the yolk sac, where these cells have been found in early gestation (E6.5-8.5)[278]. In later embryogenesis, the fetal liver becomes a site of hematopoiesis (E10.5), and after birth tissue-resident macrophages will self-renew without relying on bone marrow hematopoiesis[279]. Monocyte development is still debated, and it is currently thought that monocytes can arise from both granulocyte-macrophage and dendritic cell-macrophage progenitors in the bone marrow[280,281]. In the fetal mouse liver, monocyte progenitors appear around E9.5[282] and reach the circulation 4 days later (around E13.5) to start the colonization of tissues and organs, with the brain as an exception[160,282]. These fetal monocytes are similar to those of adults, although the pathogen recognition and antigen presentation genes are poorly expressed compared to adults[53,160]. Our trajectory analysis results showed that, based on gene expression, indeed these meningeal immune populations can be distinguished from one another already in the neonatal period.

The transcriptome of meningeal microglia was also further studied, and we found a possible intermediate population between BAMs and microglia as well as a white matter-specific subpopulation that was similar to the microglia found in the brain parenchyma in mice of similar age[179,180] (Fig. 13). The "BAM-like" microglial subtype expressed not only signature genes typical of BAMs, but also features (such as *Sall1* and *Tgfbr1*) that are uniquely expressed by microglia[54,191]. A BAM population has previously

been reported that expresses the typical microglial marker Sall1 in the choroid plexus[191], and it is possible that a similar intermediate population also exists in the neonatal meninges.

We also found a microglial subtype expressing features, such as *Spp1*, that are typical of parenchymal microglia from PND4-7 mice[179,180] (Fig. 13). This microglial population was found mainly in the parenchymal white matter only at this developmental age[179,180]. It has previously been reported that the meninges can influence brain development, oligodendrocyte migration, and corpus callosum formation[73,74]; however, the origin of this influence within the meninges remains poorly defined. It is possible that there is a microglial subtype in the meninges that shares similar gene expression to white matter-specific microglia and may contribute to white matter development. Further research is needed to explore the role of meningeal microglia in brain development.

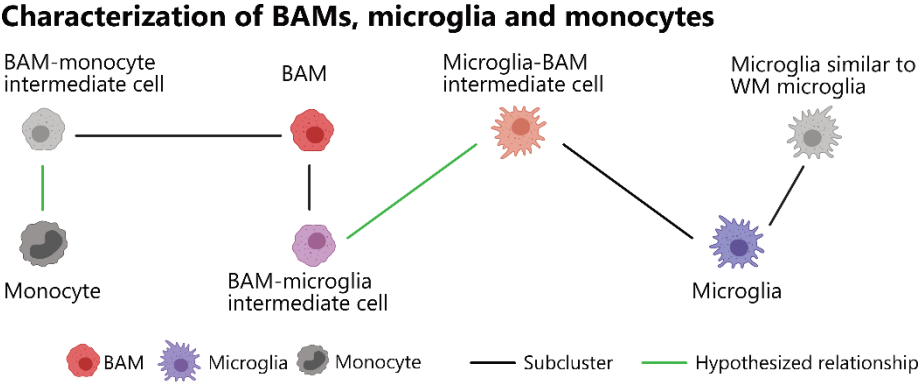

Figure 13. Proposed relationships among BAMs, microglia, and monocytes. **Legend:** WM: white matter.

4.6 Neutrophils are the major responders in the meninges 6 hours after HI

In **Paper III**, we also sought to understand how the previously identified leukocyte populations react to preterm brain injury early after HI insult.

As the major immune cells in the brain, microglial involvement has been studied extensively in neonatal brain injury[9,187,188,244,283], and preterm brain injury leads to the activation of microglia, which has been suggested to

contribute to cell death via mechanisms such as excitotoxicity and excessive production of pro-inflammatory cytokines[82,187,284]. However, microglia depletion increases the severity of brain injury in animal models of HI-induced brain injury in P10 mice[188] and neonatal stroke[186,285], thus highlighting the controversial role of microglial response after neonatal brain injury. In **Paper III** we described the heterogeneity of meningeal microglia, with their possible involvement in brain development. Although meningeal microglia were not significantly affected at 6 hours after HI, we cannot exclude that this meningeal cell population participates later in the inflammatory response after HI injury.

BAMs and monocytes showed moderate gene expression response after HI, with no changes in cell frequencies. IPA of DEGs showed an increased immune response for both cell populations. A recent study showed that monocyte infiltration into the brain peaked at 24 hours and at 7 days after term HI, but it decreased over the following weeks[33]. BAMs have been shown to increase after adult stroke 3 days after the insult and to be involved in the subsequent granulocyte recruitment[196,197]. However, their role in preterm brain injury is unknown. Based on these previous studies, it is likely that these two meningeal leukocyte subpopulations intervene at a later time point after HI injury. However, the mechanism of their inflammatory response needs to be further elucidated. Interestingly, BAMs also showed a downregulation of growth processes after HI, especially in terms of neuronal growth, indicating a possible role in brain development.

Although γδT cells have previously been studied in an adult mouse model of stroke[136], only recently have we confirmed their important role after preterm brain injury[238,243]. We previously found that depletion of γδT cells ameliorated the preterm brain injury, which is independent from the IL-17/IL-22 signaling pathway, suggesting mechanisms different from what was seen in the adult stroke brain injury model[243]. Additionally, γδT cells were found at the border zone of the injury 6 hours after HI, including the meninges, hippocampus, subcortical white matter, and ventricle area. However, the neuroprotective effect of γδT cell deficiency was only measured at 7 days after HI and not at earlier time points[243]. In **Paper III**, the meningeal γδT cell population was rather small at PND4, and only subtle changes were observed 6 hours after HI, which might be due to the relatively small number of γδT cells among the total meningeal leukocytes. Altogether, this indicates that γδT cells might first accumulate at 6 hours and start to mount an inflammatory response, but γδT cells might affect

the development of brain damage at a later stage in a noticeable manner. Similarly, ILCs also showed no difference in frequencies at 6 hours after HI in the meninges (**Paper III**). These results are in concordance with **Paper II,** where we demonstrated that ILCs and ILC2s tend to accumulate later in the meninges after HI, reaching a peak 7 days after the insult.

Overall, we identified neutrophils as the major responders 6 hours after HI in the meninges, based both on a three-fold increase in cell frequency and on differential gene expression. Neutrophils have previously been shown to be among the first responders in the brain in other brain injury animal models[32,85,251,286-288], although they were found to be increased in the parenchyma only 24 hours after HI-induced brain injury using PND9 mice[33].

In neutrophils, IPA predicted activation of the Stat3 signaling pathway and increased upstream genes such as *Csf2* and *Cebpβ*. Furthermore, we also observed an increase in G1 cell cycle-related genes in neutrophils from HI animals compared to naive neutrophils (Fig. 14), suggesting that HI-induced injury triggered proliferation of this immune subpopulation at 6 hours after insult, and not infiltration from the circulation.

Neutrophils are the most abundant circulating immune cells in the periphery, and they are characterized by a short life span (6–8 h). Therefore, to sustain their numbers in the circulation, the basal renewal rate by release from the bone marrow is high (5–10 x 10^{10} neutrophils per day)[289]. In the bone marrow, G-CSF is the main molecule responsible for physiological levels of granulopoiesis (neutrophil production). G-CSF is necessary for the progenitor cell commitment to the myeloid lineage and for precursor proliferation and release from the bone marrow[290,291], and it exerts these functions through the Jak/STAT pathway and the transcription factor *CEBPα*[292].

However, in order to mount a response to harmful stimuli, the bone marrow has an additional reservoir of immature neutrophils consisting of an estimated 6 x 10^{11} cells[293,294]. This massive pool can be released through emergency granulopoiesis, a process that generates neutrophils de novo in response to systemic inflammation and/or infection[292]. This pathway is triggered by *CEBPβ* and specifically STAT3, which under physiological conditions has an inhibitory effect on basal granulopoiesis[295-297].

Neutrophils have been found to be increased in the brain after preterm and term brain injury within 24 hours after the insult[31,33,245], but other studies have shown that neutrophils are increased as early as 6 hours after the

insult in neonatal and adult models of brain injury, with neutrophil activation and indications of neutrophil extracellular traps formation[85,298]. In **Paper III** we described a possible mechanism of early neutrophil response to HI insult involving emergency granulopoiesis in the meninges as part of the inflammatory response after HI-induced preterm brain injury.

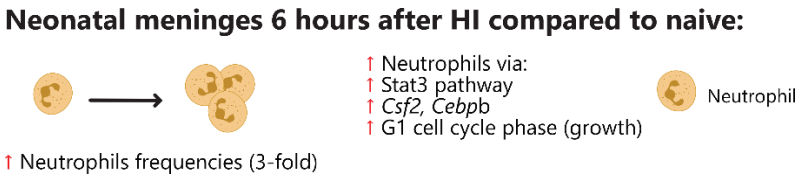

Figure 14. Meningeal neutrophil response after preterm HI in neonatal mice.
Legend: ↑: increase.

In summary, in this study we characterized the neonatal mouse meningeal leukocyte population, and we described for the first time the age-specific and region-specific features of BAMs and microglia. We also highlighted the possible involvement of these two cell populations in brain development. Lastly, we showed that neutrophils are the major responders early after HI insult in the neonatal mouse meninges and suggested a potential mechanism for their inflammatory response.

5 SUMMARY AND CONCLUSIONS

Preterm birth survival rates are increasing worldwide[4], with the consequence of a larger population with increased risks for neurological sequelae such as CP[20]. Although studies have investigated the role of immune response in neonatal brain injury[9,82,299], only few groups have studied the immune response in preterm brain injury, especially after HI[24,204,242-244], or how the meninges respond to HI[243].

We contribute with the following findings discussed in this thesis, as summarized in Fig. 15:

1. T and B cells respond to preterm brain injury and are increased in the parenchyma and meninges in post-mortem human PVL cases compared to controls.
2. αβT and B cells respond to the HI-induced preterm brain injury by infiltrating the mouse brain.
3. T and B cell deficiency is protective for the white matter tissue loss after HI insult, indicating that these cells might aggravate brain injury after HI.
4. ILCs and particularly ILC2s increase in the mouse brain after HI.
5. ILC2 impairment shows almost no changes in the inflammatory response in the CNS or brain damage compared to wild type mice.
6. Almost all known immune cell subtypes can be found in the neonatal mouse meninges, with a prevalence of innate immune cells (BAMs, microglia, and monocytes).
7. Neonatal meningeal BAMs, microglia, and monocytes are distinct cell populations but share similar gene expression patterns.
8. Neonatal meningeal BAMs show higher cytoskeleton signaling and immature immune responses compared to adult BAMs.
9. Microglial subpopulations show unique features compared to those in the brain parenchyma and might be involved in white matter development.

10. The early meningeal neutrophil response after HI is likely sustained by emergency granulopoiesis.

Overall, we show that both adaptive and innate immune cells respond to HI insult. In the brain parenchyma, we observed an increase of both adaptive (αβT and B) and innate (ILC2) immune cells. Additionally, T and B cell deficiency reduced the white matter tissue loss. Apart from the brain parenchyma, the meninges are an important site for immune response, with specific subtypes for this CNS compartment and overall complex immune cell composition. Furthermore, we show that the meninges are part of the early immune response in the brain after preterm brain injury.

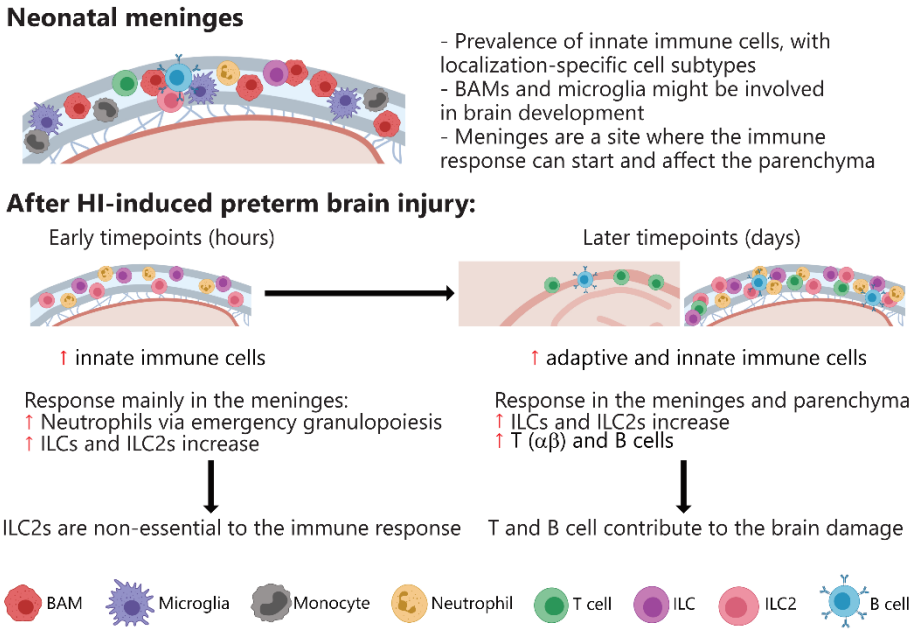

Figure 15. Graphical summary of the main results of this doctoral thesis. **Legend:** ↑: increase.

6 FUTURE PERSPECTIVES

From the results of this thesis, it is clear that the immune response both in the parenchyma and the meninges is of great significance after preterm brain injury and is therefore important to investigate further.

In **Paper I** we showed that T and B cells respond to preterm HI by infiltrating the brain parenchyma and the meninges and that removing both cell types can reduce the resulting white matter damage. Although this study gives us important insights into the adaptive immune response after brain damage, additional experiments are needed to better understand the mechanisms behind the T and B response to injury, for example, by using commercially available knock-out mice deficient in specific subsets of T or B cells. Being able to understand which T and/or B cell subset is particularly detrimental would be more relevant than just blocking both T and B cells at the same time. If we can unravel the key players necessary for the T and B cell response (e.g. secreted cytokines or antibodies), we could then design targeted drugs to prevent the detrimental chain reaction that leads to brain damage progression without disrupting the already fragile homeostasis of preterm newborns.

ILCs are highly involved in fetal development and pregnancy-related changes[117,119,170,171,300]. The results in **Paper II** show that neonatal ILCs respond to brain injury, especially ILC2s, but impairment of this cellular subtype did not affect tissue loss, inflammation, or cytokine/chemokine production after HI. However, because ILCs have been studied only for the past decade[112] and because of their shared profile with Th cells, it is extremely difficult to obtain a specific deficient or impaired mouse strain specific for certain ILC subsets without affecting other cell types. Because ILC2s were the predominant subtype in the ILC response to HI, we focused on this subtype, whereas NK cells, ILC1s, and ILC3s were not investigated further in this study. Further research using transgenic mouse strains could be performed to evaluate the role of these other subtypes, which were also increased in our study and have been shown to be involved in other brain pathologies[266,301] and in pregnancy and infancy-related biological processes[119,172,300]. Regarding ILC1s and ILC3s, according to the literature there are alternative approaches to impair or delete these subsets[302], but most of them are still not selective for ILC1s or ILC3s because the deletion in most cases affects also T, B, and NK cells[266,303]. Another alternative strategy may be antibody depletion; however, we still cannot exclude an

effect on T cells as well[304]. Therefore, the possibility of continuing these studies highly depends on whether specific mouse strains or depletion strategies are developed in the future.

In **Paper III** we investigated the resident immune population in the neonatal mouse meninges, and how meningeal leukocytes react to the HI insult. However, one of the main research questions that remains unanswered is what the human meningeal immune cell population composition is under physiological conditions and after preterm brain injury. Mouse models allow us to study changes in physiology and disease in many ways and with several techniques, but knowing how similar the mouse and human meningeal immune response are after preterm brain injury could provide necessary insights for further research. Spatial scRNA-seq experiments could answer this question: this technique allows the retention of the localization of immune cells and combines it with single cell sequencing by analyzing tissue sections. Thus, human post-mortem brain samples already stored in biobanks could be used to investigate the immune response after preterm brain injury, and this would greatly facilitate these experiments, since both frozen and paraffin-fixed samples can be analyzed using this methods. With such experiments, it would be possible to answer where (spatial information) and how (gene expression) immune cells are affected after preterm brain injury both in the brain parenchyma and the meninges.

Further research is needed to elucidate the function of the meningeal resident leukocyte population, especially the role of BAMs and meningeal microglia in CNS development. We show in **Paper III** that BAMs are the main subtype among neonatal leukocytes, similar to what has been observed in adult mice[191]. It is known that the meninges are involved in brain development[65,68,71], thus it would be relevant to study whether BAMs are involved in brain development, especially after we showed that HI might influence neuronal growth via BAMs. Ideally, this could be achieved by using transgenic mice deficient in BAMs; however, to our knowledge such a strain has not yet been developed. However, local depletion of BAMs might be possible, although technically difficult; for example, T cells have recently been locally depleted using nanodrugs for cancer treatment[305].

If this or a similar kind of targeted depletion becomes applicable to BAMs in the future, this could make it possible to study how the impairment or

depletion of BAMs affects brain development both during embryonic and postnatal stages.

The possible role of meningeal microglia in white matter formation needs to be elucidated as well. In **Paper III** we confirmed that *Tgfbr1* is predominantly expressed by microglial cells, and we hypothesized that one of the subclusters is involved in white matter development. Previous studies have shown that the meninges can affect oligodendrocyte migration through factors such as TGFβ1[73,74], but the upstream factors and/or cells affecting these biological processes remain unknown. Additional experiments might help to understand whether this specific subtype of microglia is involved in white matter development. Reporter mice for Sall1 could be used to selectively sort meningeal microglia, thus reducing the potential contamination from meningeal BAMs[306], although these mouse strains still show some problems with non-specific cell expression. Additionally, the same strain could be used in sequencing experiments to investigate the role of this cell type after preterm brain injury, and more specifically to investigate whether the meningeal microglia increase in number or undergo changes in gene expression at later time points after HI.

A recently published approach uses a binary Cre mouse strain, and by using two Cre promoters (*Sall1* and *Cx3cr1*) makes it possible to study microglia and macrophages simultaneously[307]. This mouse strain could allow the investigation not only of the role of microglia and BAMs in brain development, but also their immune response after preterm brain injury.

Lastly, scRNA-seq analysis of leukocytes from brain parenchyma and meninges at multiple timepoints after HI-induced preterm brain injury could tell us how the innate and adaptive immune response coordinate after the insult and could potentially provide information regarding the possibility of later therapeutic windows during the secondary phase (hours to days) or tertiary phase (days to months) of HI injury after the insult in order to reduce the damage caused by chronic inflammation.

In my opinion, the most relevant experiments are the ones involving the sequencing of human and mouse brain and meninges after preterm brain injury. This would give us a snapshot of how the immune system reacts to preterm brain injury in human newborns, combined with the insights on the inflammatory response timeline from the mouse experiments. Thus, we would be able to obtain a comprehensive picture of how the innate and

adaptive immune responses react to preterm brain injury, potentially giving us the most relevant immune cell/pathway and timepoint to target in order to counteract the brain damage.

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REFERENCES

1 Hannah Blencowe *et al.* Born Too Soon: The global epidemiology of 15 million preterm births. *Reprod Health*, doi:10.1186/1742-4755-10-S1-S2 (2013).

2 WHO: recommended definitions, terminology and format for statistical tables related to the perinatal period and use of a new certificate for cause of perinatal deaths. Modifications recommended by FIGO as amended October 14, 1976. *Acta Obstet Gynecol Scand* (1977).

3 Chawanpaiboon, S. *et al.* Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis. *The Lancet Global Health* **7**, e37-e46, doi:10.1016/s2214-109x(18)30451-0 (2019).

4 WHO. 15 Million Babies Born Too Soon. *Press Release* (2012).

5 Constable, R. T. *et al.* Prematurely born children demonstrate white matter microstructural differences at 12 years of age, relative to term control subjects: an investigation of group and gender effects. *Pediatrics* **121**, 306-316, doi:10.1542/peds.2007-0414 (2008).

6 Gopagondanahalli, K. R. *et al.* Preterm Hypoxic-Ischemic Encephalopathy. *Front Pediatr* **4**, 114, doi:10.3389/fped.2016.00114 (2016).

7 Volpe, J. J. Cerebral White Matter Injury of the Premature Infant—More Common Than You Think. *Pediatrics* **112** (2003).

8 Volpe, J. J. Brain Injury in the Premature Infant: Overview of Clinical Aspects, Neuropathology, and Pathogenesis. *Seminars in Pediatric Neurology* **5**, 21 (1998).

9 Hagberg, H. *et al.* The role of inflammation in perinatal brain injury. *Nat Rev Neurol* **11**, 192-208, doi:10.1038/nrneurol.2015.13 (2015).

10 Galinsky, R. *et al.* Complex interactions between hypoxia-ischemia and inflammation in preterm brain injury. *Dev Med Child Neurol* **60**, 126-133, doi:10.1111/dmcn.13629 (2018).

11 Haynes, R. L., Sleeper, L. A., Volpe, J. J. & Kinney, H. C. Neuropathologic studies of the encephalopathy of prematurity in the late preterm infant. *Clin Perinatol* **40**, 707-722, doi:10.1016/j.clp.2013.07.003 (2013).

12 Kinney, H. C. The near-term (late preterm) human brain and risk for periventricular leukomalacia: a review. *Semin Perinatol* **30**, 81-88, doi:10.1053/j.semperi.2006.02.006 (2006).

13 Volpe, J. J. Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. *The Lancet Neurology* **8**, 110-124, doi:10.1016/s1474-4422(08)70294-1 (2009).

14 Parikh, P. & Juul, S. E. Neuroprotection Strategies in Preterm Encephalopathy. *Semin Pediatr Neurol* **32**, 100772, doi:10.1016/j.spen.2019.08.008 (2019).

15 Marlow, N., Rose, A. S., Rands, C. E. & Draper, E. S. Neuropsychological and educational problems at school age associated with neonatal encephalopathy. *Arch Dis Child Fetal Neonatal Ed* **90**, F380-387, doi:10.1136/adc.2004.067520 (2005).
16 Lauterbach, M. D., Raz S Fau - Sander, C. J. & Sander, C. J. Neonatal hypoxic risk in preterm birth infants: the influence of sex and severity of respiratory distress on cognitive recovery. *Neuropsychology*, doi:D - KIE: 103717 OTO - KIE (2001).
17 Raz, S., Debastos, A. K., Newman, J. B. & Batton, D. Extreme prematurity and neuropsychological outcome in the preschool years. *J Int Neuropsychol Soc* **16**, 169-179, doi:10.1017/S1355617709991147 (2010).
18 Taylor, D. C. Differential rates of cerebral maturation between sexes and between hemispheres. Evidence from epilepsy. *Lancet*, doi:10.1016/s0140-6736(69)92445-3 (1969).
19 Rosenbaum P *et al.* A report: the definition and classification of cerebral palsy April 2006. *Dev Med Child Neurol* (2007).
20 Himmelmann, K. & Uvebrant, P. The panorama of cerebral palsy in Sweden part XII shows that patterns changed in the birth years 2007-2010. *Acta Paediatr* **107**, 462-468, doi:10.1111/apa.14147 (2018).
21 Johnston, M. V. *et al.* Plasticity and injury in the developing brain. *Brain Dev* **31**, 1-10, doi:10.1016/j.braindev.2008.03.014 (2009).
22 Back, S. A. White matter injury in the preterm infant: pathology and mechanisms. *Acta Neuropathol* **134**, 331-349, doi:10.1007/s00401-017-1718-6 (2017).
23 Gunn, A. J. & Bennet, L. Fetal hypoxia insults and patterns of brain injury: insights from animal models. *Clin Perinatol* **36**, 579-593, doi:10.1016/j.clp.2009.06.007 (2009).
24 Elsa Bona *et al.* Chemokine and Inflammatory Cell Response to Hypoxia-Ischemia in Immature Rats. *Pediatr Res* **45** (1999).
25 Shankaran, S. Therapeutic hypothermia for neonatal encephalopathy. *Curr Opin Pediatr* **27**, 152-157, doi:10.1097/MOP.0000000000000199 (2015).
26 Shankaran, S. *et al.* Whole-Body Hypothermia for Neonates with Hypoxic–Ischemic Encephalopathy. *The New England Journal of Medicine*, 1574-1584 (2005).
27 Edwards, A. D. *et al.* Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. *BMJ*, doi:10.1136/bmj.c363 (2010).
28 Douglas-Escobar, M. & Weiss, M. D. Hypoxic-ischemic encephalopathy: a review for the clinician. *JAMA Pediatr* **169**, 397-403, doi:10.1001/jamapediatrics.2014.3269 (2015).

29 Thornton, C. *et al.* Cell Death in the Developing Brain after Hypoxia-Ischemia. *Front Cell Neurosci* **11**, 248, doi:10.3389/fncel.2017.00248 (2017).
30 Hagberg, H., David Edwards, A. & Groenendaal, F. Perinatal brain damage: The term infant. *Neurobiol Dis* **92**, 102-112, doi:10.1016/j.nbd.2015.09.011 (2016).
31 Jellema, R. K. *et al.* Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. *Journal of Neuroinflammation* **10**, 19 (2013).
32 Herz, J. *et al.* Peripheral T Cell Depletion by FTY720 Exacerbates Hypoxic-Ischemic Brain Injury in Neonatal Mice. *Front Immunol* **9**, 1696, doi:10.3389/fimmu.2018.01696 (2018).
33 Smith, P. L. P. *et al.* Peripheral myeloid cells contribute to brain injury in male neonatal mice. *J Neuroinflammation* **15**, 301, doi:10.1186/s12974-018-1344-9 (2018).
34 Hassell, K. J., Ezzati, M., Alonso-Alconada, D., Hausenloy, D. J. & Robertson, N. J. New horizons for newborn brain protection: enhancing endogenous neuroprotection. *Arch Dis Child Fetal Neonatal Ed* **100**, F541-552, doi:10.1136/archdischild-2014-306284 (2015).
35 Bennet, L. *et al.* Chronic inflammation and impaired development of the preterm brain. *J Reprod Immunol* **125**, 45-55, doi:10.1016/j.jri.2017.11.003 (2017).
36 Bennet, L. *et al.* Cell therapy for neonatal hypoxia-ischemia and cerebral palsy. *Ann Neurol*, doi:10.1002/ana.22670 (2012).
37 Winerdal, M. *et al.* Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. *PLoS One* **7**, e36422, doi:10.1371/journal.pone.0036422 (2012).
38 Koini, M. *et al.* Grey-matter network disintegration as predictor of cognitive and motor function with aging. *Brain Struct Funct* **223**, 2475-2487, doi:10.1007/s00429-018-1642-0 (2018).
39 Herculano-Houzel, S. The human brain in numbers: a linearly scaled-up primate brain. *Front Hum Neurosci* **3**, 31, doi:10.3389/neuro.09.031.2009 (2009).
40 Azevedo, F. A. *et al.* Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J Comp Neurol*, doi:10.1002/cne.21974 (2009).
41 von Bartheld, C. S., Bahney, J. & Herculano-Houzel, S. The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *J Comp Neurol* **524**, 3865-3895, doi:10.1002/cne.24040 (2016).
42 van Tilborg, E. *et al.* Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia* **66**, 221-238, doi:10.1002/glia.23256 (2018).

43 Bean, B. P. The action potential in mammalian central neurons. *Nat Rev Neurosci* **8**, 451-465, doi:10.1038/nrn2148 (2007).
44 Wandell, B. A. Clarifying Human White Matter. *Annu Rev Neurosci* **39**, 103-128, doi:10.1146/annurev-neuro-070815-013815 (2016).
45 Filley, C. M. & Fields, R. D. White matter and cognition: making the connection. *J Neurophysiol* **116**, 2093-2104, doi:10.1152/jn.00221.2016 (2016).
46 Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M. & Noble-Haeusslein, L. J. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* **106-107**, 1-16, doi:10.1016/j.pneurobio.2013.04.001 (2013).
47 Mallard, C. & Vexler, Z. S. Modeling Ischemia in the Immature Brain: How Translational Are Animal Models? *Stroke* **46**, 3006-3011, doi:10.1161/STROKEAHA.115.007776 (2015).
48 Stiles, J. & Jernigan, T. L. The basics of brain development. *Neuropsychol Rev* **20**, 327-348, doi:10.1007/s11065-010-9148-4 (2010).
49 Sakai, Y. Neurulation in the mouse: Manner and timing of neural tube closure. *Anat Rec*, doi:10.1002/ar.1092230212 (1989).
50 Innocenti, G. M. & Price, D. J. Exuberance in the development of cortical networks. *Nat Rev Neurosci* **6**, 955-965, doi:10.1038/nrn1790 (2005).
51 Rakic, S. & Zecevic, N. Programmed cell death in the developing human telencephalon. *European Journal of Neuroscience* **12**, doi:10.1046/j.1460-9568.2000.00153.x (2000).
52 Chen, V. S. *et al.* Histology Atlas of the Developing Prenatal and Postnatal Mouse Central Nervous System, with Emphasis on Prenatal Days E7.5 to E18.5. *Toxicol Pathol* **45**, 705-744, doi:10.1177/0192623317728134 (2017).
53 Ginhoux, F. *et al.* Fate Mapping Analysis Reveals That Adult Microglia Derive from Primitive Macrophages. *Science* **330**, 841-845, doi:10.1126/science.1194637 (2010).
54 Utz, S. G. *et al.* Early Fate Defines Microglia and Non-parenchymal Brain Macrophage Development. *Cell*, doi:10.1016/j.cell.2020.03.021 (2020).
55 Lebel, C. & Deoni, S. The development of brain white matter microstructure. *Neuroimage* **182**, 207-218, doi:10.1016/j.neuroimage.2017.12.097 (2018).
56 Sowell, E. R. *et al.* Mapping cortical change across the human life span. *Nat Neurosci* **6**, 309-315, doi:10.1038/nn1008 (2003).
57 Protasoni, M. *et al.* The collagenic architecture of human dura mater. *J Neurosurg* **114**, 1723-1730, doi:10.3171/2010.12.JNS101732 (2011).

58 Absinta, M. *et al.* Human and nonhuman primate meninges harbor lymphatic vessels that can be visualized noninvasively by MRI. *Elife* **6**, doi:10.7554/eLife.29738 (2017).
59 Adeeb, N. *et al.* The pia mater: a comprehensive review of literature. *Childs Nerv Syst* **29**, 1803-1810, doi:10.1007/s00381-013-2044-5 (2013).
60 Vivatbutsiri, P. *et al.* Impaired meningeal development in association with apical expansion of calvarial bone osteogenesis in the Foxc1 mutant. *J Anat* **212**, 603-611, doi:10.1111/j.1469-7580.2008.00893.x (2008).
61 Angelov, D. N. & Vasilev, V. A. Morphogenesis of rat cranial meninges: A light- and electron-microscopic study. *Cell Tissue Res* **257** (1989).
62 Antila, S. *et al.* Development and plasticity of meningeal lymphatic vessels. *J Exp Med* **214**, 3645-3667, doi:10.1084/jem.20170391 (2017).
63 Goldmann, T. *et al.* Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nat Immunol* **17**, 797-805, doi:10.1038/ni.3423 (2016).
64 Weller, R. O., Sharp, M. M., Christodoulides, M., Carare, R. O. & Mollgard, K. The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS. *Acta Neuropathol* **135**, 363-385, doi:10.1007/s00401-018-1809-z (2018).
65 Dasgupta, K. & Jeong, J. Developmental biology of the meninges. *Genesis* **57**, e23288, doi:10.1002/dvg.23288 (2019).
66 Adeeb, N., Mortazavi, M. M., Tubbs, R. S. & Cohen-Gadol, A. A. The cranial dura mater: a review of its history, embryology, and anatomy. *Childs Nerv Syst* **28**, 827-837, doi:10.1007/s00381-012-1744-6 (2012).
67 Decimo, I., Fumagalli, G., Berton, V., Krampera, M. & Bifari, F. Meninges: from protective membrane to stem cell niche. *Am J Stem Cell* (2012).
68 Siegenthaler, J. A. & Pleasure, S. J. We have got you 'covered': how the meninges control brain development. *Curr Opin Genet Dev* **21**, 249-255, doi:10.1016/j.gde.2010.12.005 (2011).
69 Borrell, V. & Marin, O. Meninges control tangential migration of hem-derived Cajal-Retzius cells via CXCL12/CXCR4 signaling. *Nat Neurosci* **9**, 1284-1293, doi:10.1038/nn1764 (2006).
70 Chou, F. S., Li, R. & Wang, P. S. Molecular components and polarity of radial glial cells during cerebral cortex development. *Cell Mol Life Sci* **75**, 1027-1041, doi:10.1007/s00018-017-2680-0 (2018).
71 Siegenthaler, J. A. *et al.* Retinoic acid from the meninges regulates cortical neuron generation. *Cell* **139**, 597-609, doi:10.1016/j.cell.2009.10.004 (2009).
72 Verney, C., Monier, A., Fallet-Bianco, C. & Gressens, P. Early microglial colonization of the human forebrain and possible involvement in

periventricular white-matter injury of preterm infants. *J Anat* **217**, 436-448, doi:10.1111/j.1469-7580.2010.01245.x (2010).
73 Choe, Y., Huynh, T. & Pleasure, S. J. Migration of oligodendrocyte progenitor cells is controlled by transforming growth factor beta family proteins during corticogenesis. *J Neurosci* **34**, 14973-14983, doi:10.1523/JNEUROSCI.1156-14.2014 (2014).
74 Choe, Y., Siegenthaler, J. A. & Pleasure, S. J. A cascade of morphogenic signaling initiated by the meninges controls corpus callosum formation. *Neuron* **73**, 698-712, doi:10.1016/j.neuron.2011.11.036 (2012).
75 Mishra, S., Choe, Y., Pleasure, S. J. & Siegenthaler, J. A. Cerebrovascular defects in Foxc1 mutants correlate with aberrant WNT and VEGF-A pathways downstream of retinoic acid from the meninges. *Dev Biol* **420**, 148-165, doi:10.1016/j.ydbio.2016.09.019 (2016).
76 Kume, T. *et al.* The Forkhead/Winged Helix Gene Mf1 Is Disrupted in the Pleiotropic Mouse Mutation congenital hydrocephalus. *Cell* **93** (1998).
77 Zarbalis, K. *et al.* Cortical dysplasia and skull defects in mice with a Foxc1 allele reveal the role of meningeal differentiation in regulating cortical development. *PNAS* **104** (2007).
78 Devisme, L. *et al.* Cobblestone lissencephaly: neuropathological subtypes and correlations with genes of dystroglycanopathies. *Brain* **135**, 469-482, doi:10.1093/brain/awr357 (2012).
79 Inoue, T., Ogawa, M., Mikoshiba, K. & Aruga, J. Zic deficiency in the cortical marginal zone and meninges results in cortical lamination defects resembling those in type II lissencephaly. *J Neurosci* **28**, 4712-4725, doi:10.1523/JNEUROSCI.5735-07.2008 (2008).
80 Vajsar, J. & Schachter, H. Walker-Warburg syndrome. *Orphanet J Rare Dis* **1**, 29, doi:10.1186/1750-1172-1-29 (2006).
81 Imataka, G., Yamanouchi, H. & Arisaka, O. Dandy-Walker syndrome and chromosomal abnormalities. *Congenit Anom (Kyoto)* **47**, 113-118, doi:10.1111/j.1741-4520.2007.00158.x (2007).
82 Lai, J. C. Y. *et al.* Immune responses in perinatal brain injury. *Brain Behav Immun* **63**, 210-223, doi:10.1016/j.bbi.2016.10.022 (2017).
83 Jain, A. & Pasare, C. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. *J Immunol* **198**, 3791-3800, doi:10.4049/jimmunol.1602000 (2017).
84 Fuchs, T. *et al.* Immediate Neutrophil-Variable-T Cell Receptor Host Response in Bacterial Meningitis. *Front Neurol* **10**, 307, doi:10.3389/fneur.2019.00307 (2019).
85 Yao, H. W. & Kuan, C. Y. Early neutrophil infiltration is critical for inflammation-sensitized hypoxic-ischemic brain injury in newborns. *J Cereb Blood Flow Metab*, 271678X19891839, doi:10.1177/0271678X19891839 (2019).

86 Mulay, S. R. & Anders, H. J. Neutrophils and Neutrophil Extracellular Traps Regulate Immune Responses in Health and Disease. *Cells* **9**, doi:10.3390/cells9092130 (2020).
87 Crawford, J., Dale, D. C. & Lyman, G. H. Chemotherapy-induced neutropenia: risks, consequences, and new directions for its management. *Cancer* **100**, 228-237, doi:10.1002/cncr.11882 (2004).
88 Spoor, J., Farajifard, H. & Rezaei, N. Congenital neutropenia and primary immunodeficiency diseases. *Crit Rev Oncol Hematol* **133**, 149-162, doi:10.1016/j.critrevonc.2018.10.003 (2019).
89 Ng, L. G., Ostuni, R. & Hidalgo, A. Heterogeneity of neutrophils. *Nat Rev Immunol* **19**, 255-265, doi:10.1038/s41577-019-0141-8 (2019).
90 Chatfield, S. M., Thieblemont, N. & Witko-Sarsat, V. Expanding Neutrophil Horizons: New Concepts in Inflammation. *J Innate Immun* **10**, 422-431, doi:10.1159/000493101 (2018).
91 Christoffersson, G. & Phillipson, M. The neutrophil: one cell on many missions or many cells with different agendas? *Cell and Tissue Research* **371**, 415-423, doi:10.1007/s00441-017-2780-z (2018).
92 Garley, M. & Jablonska, E. Heterogeneity Among Neutrophils. *Arch Immunol Ther Exp (Warsz)* **66**, 21-30, doi:10.1007/s00005-017-0476-4 (2018).
93 Hellebrekers, P., Vrisekoop, N. & Koenderman, L. Neutrophil phenotypes in health and disease. *Eur J Clin Invest* **48 Suppl 2**, e12943, doi:10.1111/eci.12943 (2018).
94 Rosales, C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Front Physiol* **9**, 113, doi:10.3389/fphys.2018.00113 (2018).
95 Ciau-Uitz, A., Monteiro, R., Kirmizitas, A. & Patient, R. Developmental hematopoiesis: ontogeny, genetic programming and conservation. *Exp Hematol*, doi:10.1016/j.exphem.2014.06.001 (2014).
96 Haneline, L. S., Marshall, K. P. & Clapp, D. W. The Highest Concentration of Primitive Hematopoietic Progenitor Cells in Cord Blood Is Found in Extremely Premature Infants. *Pediatric Research* **39**, 820-825, doi:10.1203/00006450-199605000-00013 (1996).
97 Notta, F. *et al.* Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* **351** (2016).
98 Bajaj, M. S., Kew, R. R., Webster, R. O. & Hyers, T. M. Priming of human neutrophil functions by tumor necrosis factor: enhancement of superoxide anion generation, degranulation, and chemotaxis to chemoattractants C5a and F-Met-Leu-Phe. *Inflammation* (1992).
99 Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science*, doi:10.1126/science.1092385 (2004).
100 Segal, A. W. How neutrophils kill microbes. *Annu Rev Immunol* **23**, 197-223, doi:10.1146/annurev.immunol.23.021704.115653 (2005).
101 Jiyeon Yang, Lixiao Zhang, Caijia Yu, Yang, X.-F. & Wang, H. Monocyte and macrophage differentiation: circulation inflammatory

monocyte as biomarker for inflammatory diseases. *Biomarker Research*, doi:10.1186/2050-7771-2-1 (2014).
102 Guilliams, M., Mildner, A. & Yona, S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* **49**, 595-613, doi:10.1016/j.immuni.2018.10.005 (2018).
103 Geissmann, F., Jung, S. & Littman, D. R. Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties. *Immunity* **19**, 71-82, doi:10.1016/s1074-7613(03)00174-2 (2003).
104 Yona, S. *et al.* Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**, 79-91, doi:10.1016/j.immuni.2012.12.001 (2013).
105 Auffray, C. *et al.* Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*, doi:10.1126/science.1142883 (2007).
106 Shi, C. & Pamer, E. G. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* **11**, 762-774, doi:10.1038/nri3070 (2011).
107 Cheong, C. *et al.* Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* **143**, 416-429, doi:10.1016/j.cell.2010.09.039 (2010).
108 Epelman, S., Lavine, K. J. & Randolph, G. J. Origin and functions of tissue macrophages. *Immunity* **41**, 21-35, doi:10.1016/j.immuni.2014.06.013 (2014).
109 Gautier, E. L. *et al.* Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* **13**, 1118-1128, doi:10.1038/ni.2419 (2012).
110 Klose, C. S. & Artis, D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* **17**, 765-774, doi:10.1038/ni.3489 (2016).
111 Constantinides, M. G., McDonald, B. D., Verhoef, P. A. & Bendelac, A. A committed precursor to innate lymphoid cells. *Nature* **508**, 397-401, doi:10.1038/nature13047 (2014).
112 Vivier, E. *et al.* Innate Lymphoid Cells: 10 Years On. *Cell* **174**, 1054-1066, doi:10.1016/j.cell.2018.07.017 (2018).
113 Spits, H., Bernink, J. H. & Lanier, L. NK cells and type 1 innate lymphoid cells: partners in host defense. *Nat Immunol* **17**, 758-764, doi:10.1038/ni.3482 (2016).
114 Almeida, F. F. & Belz, G. T. Innate lymphoid cells: models of plasticity for immune homeostasis and rapid responsiveness in protection. *Mucosal Immunol* **9**, 1103-1112, doi:10.1038/mi.2016.64 (2016).
115 Russi, A. E., Ebel, M. E., Yang, Y. & Brown, M. A. Male-specific IL-33 expression regulates sex-dimorphic EAE susceptibility. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1710401115 (2018).

116 Van Dyken, S. J. *et al.* Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gammadelta T cells. *Immunity* **40**, 414-424, doi:10.1016/j.immuni.2014.02.003 (2014).
117 Yu, J. C. *et al.* Innate Immunity of Neonates and Infants. *Front Immunol* **9**, 1759, doi:10.3389/fimmu.2018.01759 (2018).
118 Bjorkstrom, N. K., Kekalainen, E. & Mjosberg, J. Tissue-specific effector functions of innate lymphoid cells. *Immunology* **139**, 416-427, doi:10.1111/imm.12098 (2013).
119 Xu, Y. *et al.* Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. *Am J Reprod Immunol*, doi:10.1111/aji.12820 (2018).
120 Kumar, B. V., Connors, T. J. & Farber, D. L. Human T Cell Development, Localization, and Function throughout Life. *Immunity* **48**, 202-213, doi:10.1016/j.immuni.2018.01.007 (2018).
121 Gourley, T. S., Wherry, E. J., Masopust, D. & Ahmed, R. Generation and maintenance of immunological memory. *Semin Immunol* **16**, 323-333, doi:10.1016/j.smim.2004.08.013 (2004).
122 Eibel, H., Kraus, H., Sic, H., Kienzler, A. K. & Rizzi, M. B cell biology: an overview. *Curr Allergy Asthma Rep* **14**, 434, doi:10.1007/s11882-014-0434-8 (2014).
123 Mombaerts, P., Arnoldi, J., Russ, F., Tonegawa, S. & Kaufmann, S. H. E. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen *Nature* **365** (1993).
124 Haynes, B. E., Martin, M. E., Kay, H. H. & Kurtzberg, J. Early events in human T cell onotgeny.Phenotypic Characterization and Immunohistologic Localization of T Cell Precursors in Early Human Fetal Tissues. *J Exp Med*, doi:10.1084/jem.168.3.1061 (1988).
125 Golubovskaya, V. & Wu, L. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers (Basel)* **8**, doi:10.3390/cancers8030036 (2016).
126 Cosmi, L., Maggi, L., Santarlasci, V., Liotta, F. & Annunziato, F. T helper cells plasticity in inflammation. *Cytometry A* **85**, 36-42, doi:10.1002/cyto.a.22348 (2014).
127 Harris, N. L., Watt, V., Ronchese, F. & Le Gros, G. Differential T Cell Function and Fate in Lymph Node and Nonlymphoid Tissues. *J Exp Med* (2002).
128 Chapman, N. M., Boothby, M. R. & Chi, H. Metabolic coordination of T cell quiescence and activation. *Nat Rev Immunol* **20**, 55-70, doi:10.1038/s41577-019-0203-y (2020).
129 Kaech, S. M. & Wherry, E. J. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27**, 393-405, doi:10.1016/j.immuni.2007.08.007 (2007).

130 Pang, D. J., Neves, J. F., Sumaria, N. & Pennington, D. J. Understanding the complexity of gammadelta T-cell subsets in mouse and human. *Immunology* **136**, 283-290, doi:10.1111/j.1365-2567.2012.03582.x (2012).
131 Chien, Y.-h., Meyer, C. & Bonneville, M. γδT Cells: First Line of Defense and Beyond. *Annual Review of Immunology* **32**, 121-155, doi:10.1146/annurev-immunol-032713-120216 (2014).
132 Dimova, T. *et al.* Effector Vgamma9Vdelta2 T cells dominate the human fetal gammadelta T-cell repertoire. *Proc Natl Acad Sci U S A* **112**, E556-565, doi:10.1073/pnas.1412058112 (2015).
133 Vantourout, P. & Hayday, A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* **13**, 88-100, doi:10.1038/nri3384 (2013).
134 Vermijlen, D. & Prinz, I. Ontogeny of Innate T Lymphocytes - Some Innate Lymphocytes are More Innate than Others. *Front Immunol* **5**, 486, doi:10.3389/fimmu.2014.00486 (2014).
135 Xu, W. *et al.* Mapping of gamma/delta T cells reveals Vdelta2+ T cells resistance to senescence. *EBioMedicine*, doi:10.1016/j.ebiom.2018.11.053 (2018).
136 Shichita, T. *et al.* Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat Med* **15**, 946-950, doi:10.1038/nm.1999 (2009).
137 Howard, J. *et al.* Human Vgamma9Vdelta2 T Lymphocytes in the Immune Response to P. falciparum Infection. *Front Immunol* **9**, 2760, doi:10.3389/fimmu.2018.02760 (2018).
138 Herzog, S., Reth, M. & Jumaa, H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat Rev Immunol* **9**, 195-205, doi:10.1038/nri2491 (2009).
139 Stein, M. *et al.* A defined metabolic state in pre B cells governs B-cell development and is counterbalanced by Swiprosin-2/EFhd1. *Cell Death Differ* **24**, 1239-1252, doi:10.1038/cdd.2017.52 (2017).
140 Heizmann, B., Kastner, P. & Chan, S. Ikaros is absolutely required for pre-B cell differentiation by attenuating IL-7 signals. *J Exp Med* **210**, 2823-2832, doi:10.1084/jem.20131735 (2013).
141 Kajikhina, K., Tsuneto, M. & Melchers, F. B-Lymphopoiesis in Fetal Liver, Guided by Chemokines. *Adv Immunol*, doi:10.1016/bs.ai.2016.07.002 (2016).
142 Boiers, C. *et al.* Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell* **13**, 535-548, doi:10.1016/j.stem.2013.08.012 (2013).
143 Shlomchik, M. J. Sites and stages of autoreactive B cell activation and regulation. *Immunity* **28**, 18-28, doi:10.1016/j.immuni.2007.12.004 (2008).
144 Wardemann, H. & Nussenzweig, M. C. B-cell self-tolerance in humans. *Adv Immunol*, doi:10.1016/S0065-2776(07)95003-8 (2007).

145 Melchers, F. Checkpoints that control B cell development. *J Clin Invest* **125**, 2203-2210, doi:10.1172/JCI78083 (2015).
146 Oropallo, M. A. & Cerutti, A. Germinal center reaction: antigen affinity and presentation explain it all. *Trends Immunol* **35**, 287-289, doi:10.1016/j.it.2014.06.001 (2014).
147 Victora, G. D. SnapShot: the germinal center reaction. *Cell* **159**, 700-700 e701, doi:10.1016/j.cell.2014.10.012 (2014).
148 Ochsenbein, A. F. *et al.* Protective long-term antibody memory by antigendriven and T help-dependent differentiation of long-lived memory B cells to short-lived plasma cells independent of secondary lymphoid organs. *PNAS*, doi:https://doi.org/10.1073/pnas.230417497 (2000).
149 Takemori, T., Kaji, T., Takahashi, Y., Shimoda, M. & Rajewsky, K. Generation of memory B cells inside and outside germinal centers. *Eur J Immunol*, doi:10.1002/eji.201343716 (2014).
150 Basha, S., Surendran, N. & Pichichero, M. Immune responses in neonates. *Expert Rev Clin Immunol* **10**, 1171-1184, doi:10.1586/1744666X.2014.942288 (2014).
151 Hebel, K. *et al.* CD4+ T cells from human neonates and infants are poised spontaneously to run a nonclassical IL-4 program. *J Immunol* **192**, 5160-5170, doi:10.4049/jimmunol.1302539 (2014).
152 Debock, I. & Flamand, V. Unbalanced Neonatal CD4(+) T-Cell Immunity. *Front Immunol* **5**, 393, doi:10.3389/fimmu.2014.00393 (2014).
153 Gomez-Lopez, N. *et al.* Are amniotic fluid neutrophils in women with intraamniotic infection and/or inflammation of fetal or maternal origin? *Am J Obstet Gynecol* **217**, 693 e691-693 e616, doi:10.1016/j.ajog.2017.09.013 (2017).
154 Anderson, D. C. *et al.* Diminished lectin-, epidermal growth factor-, complement binding domain-cell adhesion molecule-1 on neonatal neutrophils underlies their impaired CD18-independent adhesion to endothelial cells in vitro. *J Immunol* (1991).
155 Nussbaum, C. *et al.* Neutrophil and endothelial adhesive function during human fetal ontogeny. *J Leukoc Biol*, doi:10.1189/jlb.0912468 (2013).
156 Zhu, J. *et al.* Quantitative proteomics reveals differential biological processes in healthy neonatal cord neutrophils and adult neutrophils. *Proteomics*, doi:10.1002/pmic.201400009 (2014).
157 Abughali, N., Berger, M. & Tosi, M. F. Deficient total cell content of CR3 (CD11b) in neonatal neutrophils. *Blood* (1994).
158 Deshmukh, H. S. *et al.* The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat Med* **20**, 524-530, doi:10.1038/nm.3542 (2014).
159 Andrade, E. B. *et al.* TLR2-induced IL-10 production impairs neutrophil recruitment to infected tissues during neonatal bacterial

sepsis. *J Immunol* **191**, 4759-4768, doi:10.4049/jimmunol.1301752 (2013).
160 Hoeffel, G. *et al.* Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* **209**, 1167-1181, doi:10.1084/jem.20120340 (2012).
161 Török, C., Lundahl, J., Hed, J. & Lagercrantz, H. Diversity in regulation of adhesion molecules (Mac-1 and L-selectin) in monocytes and neutrophils from neonates and adults. *Archives of Disease in Childhood*, doi:10.1136/adc.68.5_spec_no.561 (1993).
162 Leiber, A. *et al.* Neonatal monocytes express antiapoptotic pattern of Bcl-2 proteins and show diminished apoptosis upon infection with Escherichia coli. *Pediatr Res* **76**, 142-149, doi:10.1038/pr.2014.74 (2014).
163 Roger, T. *et al.* High expression levels of macrophage migration inhibitory factor sustain the innate immune responses of neonates. *Proc Natl Acad Sci U S A* **113**, E997-1005, doi:10.1073/pnas.1514018113 (2016).
164 Speer, C. P., Gahr, M., Wieland, M. & Eber, S. Phagocytosis-Associated Functions in Neonatal Monocyte-Derived Macrophages. *Pediatr Res* **24**, doi:10.1203/00006450-198808000-00015 (1988).
165 Winterberg, T. *et al.* Distinct phenotypic features of neonatal murine macrophages. *Eur J Immunol* **45**, 214-224, doi:10.1002/eji.201444468 (2015).
166 Philbin, V. J. & Levy, O. Developmental biology of the innate immune response: implications for neonatal and infant vaccine development. *Pediatr Res* **65**, 98R-105R, doi:10.1203/PDR.0b013e31819f195d (2009).
167 de Jong, E., Strunk, T., Burgner, D., Lavoie, P. M. & Currie, A. The phenotype and function of preterm infant monocytes: implications for susceptibility to infection. *J Leukoc Biol* **102**, 645-656, doi:10.1189/jlb.4RU0317-111R (2017).
168 De Rosa, S. C. *et al.* Ontogeny of gamma delta T cells in humans. *J Immunol* **172**, 1637-1645, doi:10.4049/jimmunol.172.3.1637 (2004).
169 Gibbons, D. L. *et al.* Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. *Eur J Immunol* **39**, 1794-1806, doi:10.1002/eji.200939222 (2009).
170 Bank, U. *et al.* Cutting Edge: Innate Lymphoid Cells Suppress Homeostatic T Cell Expansion in Neonatal Mice. *J Immunol* **196**, 3532-3536, doi:10.4049/jimmunol.1501643 (2016).
171 Forsberg, A. *et al.* GATA binding protein 3(+) group 2 innate lymphoid cells are present in cord blood and in higher proportions in male than in female neonates. *J Allergy Clin Immunol* **134**, 228-230, doi:10.1016/j.jaci.2014.01.027 (2014).

172 Baban, B., Malik, A., Bhatia, J. & Yu, J. C. Presence and Profile of Innate Lymphoid Cells in Human Breast Milk. *JAMA Pediatr*, doi:10.1001/jamapediatrics.2018.0148 (2018).
173 Siegrist, C.-A. Neonatal and early life vaccinology. *Vaccine* **19**, doi:10.1016/s0264-410x(01)00028-7 (2001).
174 Marchant, A. *et al.* Mature CD8+ T lymphocyte response to viral infection during fetal life. *Journal of Clinical Investigation* **111**, 1747-1755, doi:10.1172/jci200317470 (2003).
175 Miles, D. J. *et al.* CD4(+) T cell responses to cytomegalovirus in early life: a prospective birth cohort study. *J Infect Dis* **197**, 658-662, doi:10.1086/527418 (2008).
176 Vermijlen, D. *et al.* Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J Exp Med* **207**, 807-821, doi:10.1084/jem.20090348 (2010).
177 Pan, X., Ji, Z. & Xue, J. Percentage of Peripheral CD19+CD24hiCD38hi Regulatory B Cells in Neonatal Sepsis Patients and Its Functional Implication. *Med Sci Monit* **22**, 2374-2378, doi:10.12659/msm.895421 (2016).
178 Tsafaras, G. P., Ntontsi, P. & Xanthou, G. Advantages and Limitations of the Neonatal Immune System. *Front Pediatr* **8**, 5, doi:10.3389/fped.2020.00005 (2020).
179 Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**, 253-271 e256, doi:10.1016/j.immuni.2018.11.004 (2019).
180 Li, Q. *et al.* Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. *Neuron* **101**, 207-223 e210, doi:10.1016/j.neuron.2018.12.006 (2019).
181 Staszewski, O. & Hagemeyer, N. Unique microglia expression profile in developing white matter. *BMC Res Notes* **12**, 367, doi:10.1186/s13104-019-4410-1 (2019).
182 Ueno, M. *et al.* Layer V cortical neurons require microglial support for survival during postnatal development. *Nat Neurosci* **16**, 543-551, doi:10.1038/nn.3358 (2013).
183 Cai, Z., Hussain, M. D. & Yan, L.-J. Microglia, neuroinflammation, and beta-amyloid protein in Alzheimer's disease. *International Journal of Neuroscience* **124**, 307-321, doi:10.3109/00207454.2013.833510 (2014).
184 Ma, Y., Wang, J., Wang, Y. & Yang, G. Y. The biphasic function of microglia in ischemic stroke. *Prog Neurobiol* **157**, 247-272, doi:10.1016/j.pneurobio.2016.01.005 (2017).
185 Mrdjen, D. *et al.* High-Dimensional Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in Health, Aging, and Disease. *Immunity* **48**, 380-395 e386, doi:10.1016/j.immuni.2018.01.011 (2018).

186 Faustino, J. V. *et al.* Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. *J Neurosci* **31**, 12992-13001, doi:10.1523/JNEUROSCI.2102-11.2011 (2011).
187 Tahraoui, S. L. *et al.* Central role of microglia in neonatal excitotoxic lesions of the murine periventricular white matter. *Brain Pathol*, doi:10.1111/j.1750-3639.2001.tb00381.x (2001).
188 Tsuji, S. *et al.* Aggravated brain injury after neonatal hypoxic ischemia in microglia-depleted mice. *Journal of Neuroinflammation* **17**, doi:10.1186/s12974-020-01792-7 (2020).
189 Rua, R. & McGavern, D. B. Advances in Meningeal Immunity. *Trends Mol Med* **24**, 542-559, doi:10.1016/j.molmed.2018.04.003 (2018).
190 Gadani, S. P., Smirnov, I., Smith, A. T., Overall, C. C. & Kipnis, J. Characterization of meningeal type 2 innate lymphocytes and their response to CNS injury. *J Exp Med* **214**, 285-296, doi:10.1084/jem.20161982 (2017).
191 Van Hove, H. *et al.* A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. *Nat Neurosci* **22**, 1021-1035, doi:10.1038/s41593-019-0393-4 (2019).
192 Russi, A. E., Walker-Caulfield, M. E. & Brown, M. A. Mast cell inflammasome activity in the meninges regulates EAE disease severity. *Clin Immunol* **189**, 14-22, doi:10.1016/j.clim.2016.04.009 (2018).
193 Russi, A. E., Walker-Caulfield, M. E., Ebel, M. E. & Brown, M. A. Cutting edge: c-Kit signaling differentially regulates type 2 innate lymphoid cell accumulation and susceptibility to central nervous system demyelination in male and female SJL mice. *J Immunol* **194**, 5609-5613, doi:10.4049/jimmunol.1500068 (2015).
194 Grisold, W. & Grisold, A. Cancer around the brain. *Neuro-Oncology Practice* **1**, 13-21, doi:10.1093/nop/npt002 (2014).
195 Coles, J. A., Myburgh, E., Brewer, J. M. & McMenamin, P. G. Where are we? The anatomy of the murine cortical meninges revisited for intravital imaging, immunology, and clearance of waste from the brain. *Prog Neurobiol* **156**, 107-148, doi:10.1016/j.pneurobio.2017.05.002 (2017).
196 Pedragosa, J. *et al.* CNS-border associated macrophages respond to acute ischemic stroke attracting granulocytes and promoting vascular leakage. *Acta Neuropathol Commun* **6**, 76, doi:10.1186/s40478-018-0581-6 (2018).
197 Rajan, W. D. *et al.* Defining molecular identity and fates of CNS-border associated macrophages after ischemic stroke in rodents and humans. *Neurobiol Dis* **137**, 104722, doi:10.1016/j.nbd.2019.104722 (2020).

198 White, T., Su, S., Schmidt, M., Kao, C. Y. & Sapiro, G. The development of gyrification in childhood and adolescence. *Brain Cogn* **72**, 36-45, doi:10.1016/j.bandc.2009.10.009 (2010).
199 Beura, L. K. *et al.* Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* **532**, 512-516, doi:10.1038/nature17655 (2016).
200 Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, doi:10.1016/0092-8674(92)90030-g (1992).
201 Oliphant, C. J. *et al.* MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* **41**, 283-295, doi:10.1016/j.immuni.2014.06.016 (2014).
202 Rice, J. E., Vannucci, R. C. & Brierley, J. B. The Influence of Immaturity on Hypoxic-Ischemic Brain Damage in the Rat. *Annal Neurol* **9** (1981).
203 Ditelberg, J. S., Sheldon, R. A., Epstein, C. J. & Ferriero, D. M. Brain injury after perinatal hypoxia-ischemia is exacerbated in copper/zinc superoxide dismutase transgenic mice. *Pediatr Res* (1996).
204 Albertsson, A. *et al.* The immune response after hypoxia-ischemia in a mouse model of preterm brain injury. *Journal of Neuroinflammation* **11** (2014).
205 Fawke, J. Neurological outcomes following preterm birth. *Semin Fetal Neonatal Med* **12**, 374-382, doi:10.1016/j.siny.2007.06.002 (2007).
206 Limperopoulos, C. Advanced neuroimaging techniques: their role in the development of future fetal and neonatal neuroprotection. *Semin Perinatol*, doi:10.1053/j.semperi.2009.12.001 (2010).
207 Kalpakidou, A. K. *et al.* Neonatal brain injury and neuroanatomy of memory processing following very preterm birth in adulthood: an fMRI study. *PLoS One* **7**, e34858, doi:10.1371/journal.pone.0034858 (2012).
208 Steinman, K. J. *et al.* Neonatal watershed brain injury on magnetic resonance imaging correlates with verbal IQ at 4 years. *Pediatrics* **123**, 1025-1030, doi:10.1542/peds.2008-1203 (2009).
209 Faye S. Silverstein, Lynn Torke, John Barks & Johnston, M. V. Hypoxia-ischemia produces focal disruption of glutamate receptors in developing brain. *Developmental Brain Research*, doi:doi: 10.1016/0165-3806(87)90192-1 (1987).
210 Altin, J. G. & Sloan, E. K. The role of CD45 and CD45-associated molecules in T cell activation. *Immunology and Cell Biology* **75** (1997).
211 Hathcock, K. S., Hirano, H., Murakami, S. & Hodes, R. J. CD45 expression by B cells. Expression of different CD45 isoforms by subpopulations of activated B cells. . *Journal of Immunology* **149** (1992).
212 Gilland, E., Bona, E. & Hagberg, H. Temporal Changes of Regional Glucose Use, Blood Flow, and Microtubule-Associated Protein 2 Immunostaining After Hypoxia-Ischemia in the Immature Rat Brain.

Journal of Cerebral Blood Flow and Metabolism, doi:10.1097/00004647-199802000-00014 (1998).
213 Back, S. A., Luo, N. L., Borenstein, N. S., Volpe, J. J. & Kinney, H. C. Arrested Oligodendrocyte Lineage Progression During Human Cerebral White Matter Development: Dissociation Between the Timing of Progenitor Differentiation and Myelinogenesis. *Journal of Neuropathology and Experimental Neurology* **61**, doi:10.1093/jnen/61.2.197 (2002).
214 Ahmed, Z. *et al.* Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. *J Histochem Cytochem* **55**, 687-700, doi:10.1369/jhc.6A7156.2007 (2007).
215 Baba, H. *et al.* GFAP Gene Expression during Development of Astrocyte. *Developmental Neuroscience* **19**, 49-57, doi:10.1159/000111185 (1997).
216 Norden, D. M., Trojanowski, P. J., Villanueva, E., Navarro, E. & Godbout, J. P. Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge. *Glia* **64**, 300-316, doi:10.1002/glia.22930 (2016).
217 Middeldorp, J. & Hol, E. M. GFAP in health and disease. *Prog Neurobiol* **93**, 421-443, doi:10.1016/j.pneurobio.2011.01.005 (2011).
218 Cardoso, V. *et al.* Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature* **549**, 277-281, doi:10.1038/nature23469 (2017).
219 Liu, J. *et al.* Local Group 2 Innate Lymphoid Cells Promote Corneal Regeneration after Epithelial Abrasion. *Am J Pathol* **187**, 1313-1326, doi:10.1016/j.ajpath.2017.02.010 (2017).
220 Bartemes, K., Chen, C. C., Iijima, K., Drake, L. & Kita, H. IL-33-Responsive Group 2 Innate Lymphoid Cells Are Regulated by Female Sex Hormones in the Uterus. *J Immunol*, doi:10.4049/jimmunol.1602085 (2017).
221 Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57-63, doi:10.1038/nrg2484 (2009).
222 Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* **6**, 377-382, doi:10.1038/nmeth.1315 (2009).
223 Kashima, Y. *et al.* Single-cell sequencing techniques from individual to multiomics analyses. *Exp Mol Med* **52**, 1419-1427, doi:10.1038/s12276-020-00499-2 (2020).
224 Katz, Y., Wang, E. T., Airoldi, E. M. & Burge, C. B. Analysis and design of rnA sequencing experiments for identifying isoform regulation. *Nature Methods* **7**, doi:10.1038/nmeth.1528 (2010).
225 Ewing, B. & Green, P. Base-Calling of Automated Sequencer Traces Using Phred. II. Error Probabilities. *Genome Res*, doi:10.1101/gr.8.3.186 (1998).

226 Ewing, B., Hillier, L., Wendl, M. C. & Green, P. Base-Calling of Automated Sequencer Traces Using Phred. I. Accuracy Assessment. *Genome Res*, doi:10.1101/gr.8.3.175. (1998).
227 Rossum, G. v. An Introduction to Python for UNIX/C Programmers. *Proc. of the NLUUG najaarsconferentie* (1993).
228 Ihaka, R. & Gentleman, R. R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics* **5** (1996).
229 Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411-420, doi:10.1038/nbt.4096 (2018).
230 Zhang, X. *et al.* CellMarker: a manually curated resource of cell markers in human and mouse. *Nucleic Acids Res* **47**, D721-D728, doi:10.1093/nar/gky900 (2019).
231 de Kanter, J. K., Lijnzaad, P., Candelli, T., Margaritis, T. & Holstege, F. CHETAH: a selective, hierarchical cell type identification method for single-cell RNA sequencing. *Nucleic Acids Research* **47**, doi:10.1093/nar/gkz543 (2019).
232 Cannoodt, R. *et al.* SCORPIUS improves trajectory inference and identifies novel modules in dendritic cell development. *bioRxiv*, doi:10.1101/079509 (2016).
233 Reimand, J. *et al.* g:Profiler-a web server for functional interpretation of gene lists (2016 update). *Nucleic Acids Res* **44**, W83-89, doi:10.1093/nar/gkw199 (2016).
234 Reimand, J., Kull, M., Peterson, H., Hansen, J. & Vilo, J. g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* **35**, W193-200, doi:10.1093/nar/gkm226 (2007).
235 Walter, W., Sanchez-Cabo, F. & Ricote, M. GOplot: an R package for visually combining expression data with functional analysis. *Bioinformatics* **31**, 2912-2914, doi:10.1093/bioinformatics/btv300 (2015).
236 Kramer, A., Green, J., Pollard, J., Jr. & Tugendreich, S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523-530, doi:10.1093/bioinformatics/btt703 (2014).
237 Albertsson, A. M. *et al.* The effect of osteopontin and osteopontin-derived peptides on preterm brain injury. *Journal of Neuroinflammation*, doi:10.1186/s12974-014-0197-0 (2014).
238 Zhang, X. *et al.* gammadeltaT cells but not alphabetaT cells contribute to sepsis-induced white matter injury and motor abnormalities in mice. *J Neuroinflammation* **14**, 255, doi:10.1186/s12974-017-1029-9 (2017).
239 Mirza, M. A., Ritzel, R., Xu, Y., McCullough, L. D. & Liu, F. Sexually dimorphic outcomes and inflammatory responses in hypoxic-

ischemic encephalopathy. *J Neuroinflammation* **12**, 32, doi:10.1186/s12974-015-0251-6 (2015).
240 Stridh, L., Smith, P. L., Naylor, A. S., Wang, X. & Mallard, C. Regulation of Toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. *Journal of Neuroinflammation* **8** (2011).
241 Winerdal, M. *et al.* Adenosine A1 receptors contribute to immune regulation after neonatal hypoxic ischemic brain injury. *Purinergic Signal* **12**, 89-101, doi:10.1007/s11302-015-9482-3 (2016).
242 Hedtjarn, M., Mallard, C., Arvidsson, P. & Hagberg, H. White matter injury in the immature brain: role of interleukin-18. *Neurosci Lett* **373**, 16-20, doi:10.1016/j.neulet.2004.09.062 (2005).
243 Albertsson, A. *et al.* Gammadelta T Cells Contribute to Injury in the Developing Brain. *Am J Pathol*, doi:10.1016/j.ajpath.2017.11.012 (2018).
244 Mallard, C., Welin, A., Peebles, D., Hagberg, H. & Kjellmer, I. White matter injury following systemic endotoxemia or asphyxia in the fetal sheep. *Neurochem Res* (2003).
245 Jinnai, M. *et al.* A Model of Germinal Matrix Hemorrhage in Preterm Rat Pups. *Front Cell Neurosci* **14**, 535320, doi:10.3389/fncel.2020.535320 (2020).
246 Yang, D. *et al.* Blocking lymphocyte trafficking with FTY720 prevents inflammation-sensitized hypoxic-ischemic brain injury in newborns. *J Neurosci* **34**, 16467-16481, doi:10.1523/JNEUROSCI.2582-14.2014 (2014).
247 Doyle, K. P. *et al.* B-lymphocyte-mediated delayed cognitive impairment following stroke. *J Neurosci* **35**, 2133-2145, doi:10.1523/JNEUROSCI.4098-14.2015 (2015).
248 Prinz, M. & Priller, J. The role of peripheral immune cells in the CNS in steady state and disease. *Nat Neurosci* **20**, 136-144, doi:10.1038/nn.4475 (2017).
249 Benakis, C., Llovera, G. & Liesz, A. The meningeal and choroidal infiltration routes for leukocytes in stroke. *Ther Adv Neurol Disord* **11**, 1756286418783708, doi:10.1177/1756286418783708 (2018).
250 Wilson, E. H., Weninger, W. & Hunter, C. A. Trafficking of immune cells in the central nervous system. *J Clin Invest* **120**, 1368-1379, doi:10.1172/JCI41911 (2010).
251 Mottahedin, A., Joakim Ek, C., Truve, K., Hagberg, H. & Mallard, C. Choroid plexus transcriptome and ultrastructure analysis reveals a TLR2-specific chemotaxis signature and cytoskeleton remodeling in leukocyte trafficking. *Brain Behav Immun* **79**, 216-227, doi:10.1016/j.bbi.2019.02.004 (2019).
252 Arumugam, T. V., Granger, D. N. & Mattson, M. P. Stroke and T-Cells. *NeuroMolecular Medicine* **7**, 229-242, doi:10.1385/nmm:7:3:229 (2005).

253 Marilena Campanella, Clara Sciorati, Glauco Tarozzo & Beltramo, M. Flow Cytometric Analysis of Inflammatory Cells in Ischemic Rat Brain. *Stroke*, doi:10.1161/hs0202.103399 (2002).
254 Yilmaz, G., Arumugam, T. V., Stokes, K. Y. & Granger, D. N. Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation* **113**, 2105-2112, doi:10.1161/CIRCULATIONAHA.105.593046 (2006).
255 Hurn, P. D. *et al.* T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J Cereb Blood Flow Metab* **27**, 1798-1805, doi:10.1038/sj.jcbfm.9600482 (2007).
256 Ren, X. *et al.* Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. *J Neurosci* **31**, 8556-8563, doi:10.1523/JNEUROSCI.1623-11.2011 (2011).
257 Bodhankar, S., Chen, Y., Vandenbark, A. A., Murphy, S. J. & Offner, H. IL-10-producing B-cells limit CNS inflammation and infarct volume in experimental stroke. *Metab Brain Dis* **28**, 375-386, doi:10.1007/s11011-013-9413-3 (2013).
258 Fung, I. T. H. *et al.* Activation of group 2 innate lymphoid cells alleviates aging-associated cognitive decline. *J Exp Med* **217**, doi:10.1084/jem.20190915 (2020).
259 Besnard, A. G. *et al.* IL-33-mediated protection against experimental cerebral malaria is linked to induction of type 2 innate lymphoid cells, M2 macrophages and regulatory T cells. *PLoS Pathog* **11**, e1004607, doi:10.1371/journal.ppat.1004607 (2015).
260 Fallon, P. G. *et al.* Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* **203**, 1105-1116, doi:10.1084/jem.20051615 (2006).
261 Kabata, H., Moro, K. & Koyasu, S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev* **286**, 37-52, doi:10.1111/imr.12706 (2018).
262 Klein Wolterink, R. G. *et al.* Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol* **42**, 1106-1116, doi:10.1002/eji.201142018 (2012).
263 Massaro, A. N. *et al.* Plasma Biomarkers of Brain Injury in Neonatal Hypoxic-Ischemic Encephalopathy. *J Pediatr* **194**, 67-75 e61, doi:10.1016/j.jpeds.2017.10.060 (2018).
264 Orrock, J. E. *et al.* Association of brain injury and neonatal cytokine response during therapeutic hypothermia in newborns with hypoxic-ischemic encephalopathy. *Pediatr Res* **79**, 742-747, doi:10.1038/pr.2015.280 (2016).
265 Mair, F. & Becher, B. Thy1+ Sca1+ innate lymphoid cells infiltrate the CNS during autoimmune inflammation, but do not contribute to disease development. *Eur J Immunol* **44**, 37-45, doi:10.1002/eji.201343653 (2014).

266 Hatfield, J. K. & Brown, M. A. Group 3 innate lymphoid cells accumulate and exhibit disease-induced activation in the meninges in EAE. *Cell Immunol* **297**, 69-79, doi:10.1016/j.cellimm.2015.06.006 (2015).
267 Tapon, N. & Hall, A. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Current Opinion in Cell Biology* **9**, 86-92, doi:10.1016/s0955-0674(97)80156-1 (1997).
268 Wheeler, A. P. & Ridley, A. J. RhoB affects macrophage adhesion, integrin expression and migration. *Exp Cell Res* **313**, 3505-3516, doi:10.1016/j.yexcr.2007.07.014 (2007).
269 Wheeler, A. P. & Ridley, A. J. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res* **301**, 43-49, doi:10.1016/j.yexcr.2004.08.012 (2004).
270 Ginhoux, F. & Guilliams, M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* **44**, 439-449, doi:10.1016/j.immuni.2016.02.024 (2016).
271 Hoeffel, G. & Ginhoux, F. Fetal monocytes and the origins of tissue-resident macrophages. *Cell Immunol* **330**, 5-15, doi:10.1016/j.cellimm.2018.01.001 (2018).
272 Molawi, K. *et al.* Progressive replacement of embryo-derived cardiac macrophages with age. *J Exp Med* **211**, 2151-2158, doi:10.1084/jem.20140639 (2014).
273 Bain, C. C. *et al.* Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Commun* **7**, ncomms11852, doi:10.1038/ncomms11852 (2016).
274 Scott, C. L. *et al.* Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat Commun* **7**, 10321, doi:10.1038/ncomms10321 (2016).
275 Tamoutounour, S. *et al.* Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* **39**, 925-938, doi:10.1016/j.immuni.2013.10.004 (2013).
276 Bain, C. C. *et al.* Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol* **15**, 929-937, doi:10.1038/ni.2967 (2014).
277 Haldar, M. *et al.* Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. *Cell* **156**, 1223-1234, doi:10.1016/j.cell.2014.01.069 (2014).
278 Samokhvalov, I. M. Deconvoluting the ontogeny of hematopoietic stem cells. *Cell Mol Life Sci* **71**, 957-978, doi:10.1007/s00018-013-1364-7 (2014).
279 Sawai, C. M. *et al.* Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals. *Immunity* **45**, 597-609, doi:10.1016/j.immuni.2016.08.007 (2016).

280 Yáñez, A. *et al.* Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity* **47**, 890-902.e894, doi:10.1016/j.immuni.2017.10.021 (2017).
281 Weinreb, C., Rodriguez-Fraticelli, A., Camargo, F. D. & Klein, A. M. Lineage tracing on transcriptional landscapes links state to fate during differentiation. *Science* **367**, doi:10.1126/science.aaw3381 (2020).
282 Hoeffel, G. *et al.* C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* **42**, 665-678, doi:10.1016/j.immuni.2015.03.011 (2015).
283 McRae, A., Gilland, E., Bona, E. & Hagberg, H. Microglial activation after neonatal hypoxia-ischemia. *Developmental Brain Research* **84** (1995).
284 Maj Hedtjärn *et al.* Interleukin-18 Involvement in Hypoxic–Ischemic Brain Injury. *The Journal of Neuroscience*, doi:10.1523/JNEUROSCI.22-14-05910.2002 (2002).
285 Fernandez-Lopez, D. *et al.* Microglial Cells Prevent Hemorrhage in Neonatal Focal Arterial Stroke. *Journal of Neuroscience* **36**, 2881-2893, doi:10.1523/jneurosci.0140-15.2016 (2016).
286 Christy, A. L., Walker, M. E., Hessner, M. J. & Brown, M. A. Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE. *J Autoimmun* **42**, 50-61, doi:10.1016/j.jaut.2012.11.003 (2013).
287 Palmer, C., Roberts, R. L. & Young, P. I. Timing of neutrophil depletion influences long-term neuroprotection in neonatal rat hypoxic-ischemic brain injury. *Pediatr Res* **55**, 549-556, doi:10.1203/01.PDR.0000113546.03897.FC (2004).
288 Povroznik, J. M., Engler-Chiurazzi, E. B., Nanavati, T. & Pergami, P. Absolute lymphocyte and neutrophil counts in neonatal ischemic brain injury. *SAGE Open Med* **6**, 2050312117752613, doi:10.1177/2050312117752613 (2018).
289 Summers, C. *et al.* Neutrophil kinetics in health and disease. *Trends Immunol* **31**, 318-324, doi:10.1016/j.it.2010.05.006 (2010).
290 Richards, M. K., Liu, F., Iwasaki, H., Akashi, K. & Link, D. C. Pivotal role of granulocyte colony-stimulating factor in the development of progenitors in the common myeloid pathway. *Blood* **102**, 3562-3568, doi:10.1182/blood-2003-02-0593 (2003).
291 Lord, B. I. *et al.* Haemopoietic progenitor and myeloid cell kinetics in humans treated with interleukin-3 and granulocyte/macrophage colony-stimulating factor in combination. *Int J Cancer*, doi:10.1002/ijc.2910590409 (1994).
292 Manz, M. G. & Boettcher, S. Emergency granulopoiesis. *Nat Rev Immunol* **14**, 302-314, doi:10.1038/nri3660 (2014).

293 Donohue, D. M., Reiff, R. H., Hanson, M. L., Betson, Y. & Finch, C. A. Quantitative Measurement of the Erythrocytic and Granulocytic Cells of the Marrow and Blood. *J Clin Invest*, doi:10.1172/JCI103750 (1958).
294 Athens, J. W. *et al.* Leukokinetic Studies. IV. The Total Blood, Circulating and Marginal Granulocyte Pools and the Granulocyte Turnover Rate in Normal Subjects. *J Clin Invest*, doi:10.1172/JCI104338 (1961).
295 Hirai, H. *et al.* C/EBPbeta is required for 'emergency' granulopoiesis. *Nat Immunol* **7**, 732-739, doi:10.1038/ni1354 (2006).
296 Panopoulos, A. D. *et al.* STAT3 governs distinct pathways in emergency granulopoiesis and mature neutrophils. *Blood* **108**, 3682-3690, doi:10.1182/blood-2006-02-003012 (2006).
297 Lee, C.-k. *et al.* STAT3 Is a Negative Regulator of Granulopoiesis but Is Not Required for G-CSF-Dependent Differentiation. *Immunity* **17**, doi:10.1016/s1074-7613(02)00336-9. (2002).
298 Perez-de-Puig, I. *et al.* Neutrophil recruitment to the brain in mouse and human ischemic stroke. *Acta Neuropathol* **129**, 239-257, doi:10.1007/s00401-014-1381-0 (2015).
299 Hagberg, H., Gressens, P. & Mallard, C. Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. *Ann Neurol* **71**, 444-457, doi:10.1002/ana.22620 (2012).
300 Miller, D., Motomura, K., Garcia-Flores, V., Romero, R. & Gomez-Lopez, N. Innate Lymphoid Cells in the Maternal and Fetal Compartments. *Front Immunol* **9**, 2396, doi:10.3389/fimmu.2018.02396 (2018).
301 Romero-Suarez, S. *et al.* The Central Nervous System Contains ILC1s That Differ From NK Cells in the Response to Inflammation. *Front Immunol* **10**, 2337, doi:10.3389/fimmu.2019.02337 (2019).
302 Song, C. *et al.* Unique and redundant functions of NKp46+ ILC3s in models of intestinal inflammation. *J Exp Med* **212**, 1869-1882, doi:10.1084/jem.20151403 (2015).
303 Cuff, A. O. & Male, V. Conventional NK cells and ILC1 are partially ablated in the livers of Ncr1 (iCre)Tbx21 (fl/fl) mice. *Wellcome Open Res* **2**, 39, doi:10.12688/wellcomeopenres.11741.2 (2017).
304 Bando, J. K. & Colonna, M. Innate lymphoid cell function in the context of adaptive immunity. *Nat Immunol* **17**, 783-789, doi:10.1038/ni.3484 (2016).
305 Gao, F. *et al.* Local T regulatory cells depletion by an integrated nanodrug system for efficient chem-immunotherapy of tumor. *Science China Chemistry* **62**, 1230-1244, doi:10.1007/s11426-019-9507-x (2019).
306 Buttgereit, A. *et al.* Sall1 is a transcriptional regulator defining microglia identity and function. *Nat Immunol* **17**, 1397-1406, doi:10.1038/ni.3585 (2016).

307 Kim, J. S. *et al.* A Binary Cre Transgenic Approach Dissects Microglia and CNS Border-Associated Macrophages. *Immunity*, doi:10.1016/j.immuni.2020.11.007 (2020).