Multifactorial development of microglia and its dysregulation

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Multifactorial development of microglia
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ABBREVIATIONS

A

ASD: Autism Spectrum Disorder

B

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

C

CENA: Co-expression network analysis

CNS: Central Nervous System

cKO: Conditional knock-out

CP: Cortical plate

CPM: Count per million

D

DC: Dendritic cell

DEG: Differentially Expressed Genes

E

E: Embryonic day

EAE: Experimental autoimmune encephalomyelitis

EMPs: Erythro-myeloid precursors

F

FC: Fold change

FUCCI: Fluorescent ubiquitination-based cell-cycle indicator

G

GF: Germ-free

GFP: Green fluorescence protein

GO: Gene ontology

I

IFN-I: type I interferon

IgA: Immunoglobulin A

L

LCMV: Lymphocytic choriomeningitis virus

LPS: Lipopolysaccharides

M

M: Meninges

MCA: Middle cerebral artery

MIA: Maternal immune activation

MMP: Matrix metalloproteinases

MS: Multiple sclerosis

N

NCX: Neocortex
NGFI-A: Nerve growth factor-inducible clone A

P

P: Postnatal day
PCA: Principal component analysis
POA: Preoptic area
PolyIC: Polyinosinic-polycytidylic acid

R

RTqPCR: Real-time quantitative reverse transcription PCR
Runx1: Runt-related transcription factor 1

S

SFB: Segmented filamentous bacteria
SPF: Specific-pathogen-free
SVZ: Subventricular zone

T

TAM: Tamoxifen
TF: Transcription factor

V

V: Ventricle
VZ: Ventricular zone

W

WGCNA: Weighted gene co-expression network analysis

Y

YS: Yolks Sac
YSM: Yolk Sac Macrophage

ABSTRACT

Microglia, the resident macrophages of the central nervous system (CNS), provide immune protection and contribute to brain development and homeostasis by constantly sensing and interacting with their environment. They are implicated in numerous neurological disorders and thus fully understanding how they are regulated could allow therapies that can circumvent these disorders. Many factors play a role in the proper development and function of microglia, however it is still unclear how these factors modulate microglia exactly. Furthermore, prenatal stress such as maternal inflammation activation (MIA) has been shown to affect the behaviors of offspring. In our study, we observe that microglia development involves distinct phases of differentiation, distinguishable by specific transcriptomic signatures. Comparing male and female mice confirmed the sexual dimorphism in microglia, which is more apparent in adults, where females were more immunologically responsive. Using germ-free (GF) mice, the absence of microbiota had a stage and sex-dependent effect on microglia: microglia were found to be significantly perturbed in male embryos or female adults. Comparing human fetal microglia with murine microglia revealed a common gene signature and their importance in neuronal regulation before birth. In all, our study showed that proper microglia development is dependent on multiple factors including both intrinsic or extrinsic factors. These findings have major implications for our understanding of microglia contribution to health and disease.

Keywords: microglia, development, sexual dimorphism, microbiota, ontogeny, dysregulation, neurological disorders

1. Introduction

Microglia are the resident macrophages of the Central Nervous System (CNS). These cells, which constitute 5-20% of the total glial (non-neuronal/supporting) population, were defined as the first line of defense against foreign pathogens (Perry, 1998) and thus play important roles in inflammation and homeostasis of the CNS, as we will discuss below. As a consequence, dysregulated microglial functions are associated with the pathogenesis of neuroinflammatory diseases and neurodegenerative disorders, advocating for the modulation of microglial response as an attractive therapeutic target. However, developing rational interventions through manipulation of microglia biology requires a comprehensive and accurate knowledge of their origin, development and functions of this unique and specialized resident macrophage population.

1.1. The identity of microglia

1.1.1. Historical perspective

Microglia were first described in the work of Franz Nissl, in which rod-like cells in the brain were depicted as reactive glial units that were migratory, proliferative and phagocytic in nature (Nissl, 1899). Santiago Ramon y Cajal later renamed these cells as the “third element of the nervous system” to discriminate them from neurons and neuroglia cells, adding that they were possibly of mesodermal origin (Cajal, 1913). Del Rio-Hortega, a student of Ramon y Cajal later discovered that two non-neuronal cell types were contained in such “third element of the nervous system” using modified silver staining techniques that labeled them differently (Rio-Hortega, 1932). The majority of these cell types were oligodendroglia that do not display phagocytic activity and the minority were described as resting ramified cells. The minor population was subsequently characterized as the “third element of the CNS” with mesodermal origin that is migratory and phagocytic (Rio-Hortega, 1932). Del Rio-Hortega later introduced the term “microglia” to characterize these cells as non-neuronal, non-astrocytic third element

different from neuroectodermal oligodendroglia or oligodendrocytes that make up macroglia (Rio-Hortega, 1939).

1.1.2. Microglia are resident tissue macrophages

For decades, many studies postulated that microglia shared the same origin as astrocytes and oligodendrocytes (Ginhoux et al., 2013). However, later studies using light/electron microscopy and immunohistochemistry distinguished the differences in morphology between microglia and macrophages at different stages of development (Murabe and Sano, 1982). Shortly after, another study showed that microglia can be recognized by antisera raised against monocytes or macrophages antigens (Hume et al., 1983). Microglia were further shown to have a similar phenotype to monocytes and macrophages as reported by immunohistochemical expression of macrophage markers, such as CD11b, in mice (Perry et al., 1985) and also in human microglia (Akiyama and McGeer, 1990). Importantly, a genetic study showed that by knocking out PU.1, an essential transcription factor for myeloid cell development resulted in the absence of microglia in addition to other macrophages (Beers et al., 2006; McKercher et al., 1996). This confirmed the myeloid and macrophage nature of microglia, thus suggesting that microglia were brain-associated macrophages.

1.1.3. Markers of microglia

Being the resident macrophage of the CNS, microglia express numerous classical macrophage markers such as the integrin CD11b, the CSF-1 receptor (CD115), the surface glucoproteins F4/80, CD200R and signal regulatory protein alpha (CD172 α) (Gautier et al., 2012) as well as the fractalkine receptor CX3CR1 (Jung et al., 2000) and the calcium-binding protein IBA1 (Imai et al., 1996). Interestingly, IBA1 is commonly used as a microglia-specific marker but is also expressed by other tissue-resident macrophages (Imai et al., 1996). The main difference between microglia and other tissue macrophages is the expression of CD45, a pan-hematopoietic marker. Microglia have an intermediate expression of CD45 as opposed to the

high expression in other macrophages (Dijkstra et al., 1985). Other differences include the higher expression of CX3CR1 in microglia compared to other macrophages and monocytes (Jung et al., 2000). Recently, more specific markers of microglia were reported such as scavenger receptor FCRLS, G-coupled receptor P2ry12 (Butovsky et al., 2014) and transmembrane protein TMEM119 (Bennett et al., 2016).

1.2. Ontogeny of microglia

1.2.1 Yolk sac ancestors

The emergence of microglia have been observed in the developing brain prior to hematopoiesis of the fetal liver and bone marrow (Alliot et al., 1991), but coinciding with the initiation of yolk sac (YS) primitive hematopoiesis (Takahashi and Naito, 1993). Primitive hematopoiesis takes place within the blood islands of the extra-embryonic YS at murine embryonic day 7 (E7.0) and produces erythro-myeloid precursors (EMPs) (Parkhurst et al., 2013) that give rise to mostly primitive nucleated erythrocytes and primitive macrophages (Hoeffel et al., 2015; Orkin and Zon, 2008). A seminal study from Ginhoux et al showed that microglia, in contrast to other tissue-resident macrophages, arise from YS primitive macrophages (Ginhoux et al., 2010). This study used a fate-mapping model based on the expression of runt-related transcription factor 1 (Runx1), a transcriptional regulator that is important for development of hematopoietic stem cells, was manipulated to trace YS progenitors including YS macrophages. The tagging of the YS progenitors was done during early embryogenesis as the expression of Runx1 is restricted to the extra-embryonic YS during this short window period (Samokhvalov et al., 2007). The study of this mouse model showed that majority of embryonic and adult microglia came from YS progenitors, with little or no contribution from either fetal or adult monocytes in the brain (Figure A). Later studies have confirmed the ontogeny of microglia using fate-mapping models that could trace yolks sac (YS) progenitors accurately (Schulz et al., 2012; Sheng et al., 2015). Importantly, most other tissue macrophages do not come from YS progenitors but rather arise from fetal monocytes (Hoeffel et al., 2015; Sheng et al., 2015). Fetal monocytes do not give rise to microglia

possibly due to the absence of intrinsic potential or denial of entry into the developing brain due to the formation of the blood-brain barrier that starts as early as E13.5 (Daneman et al., 2010) (Figure A), further discussed in a review (Hoeffel and Ginhoux, 2015).

Figure A. Brain and microglia development in parallel with the different stages of fetal hematopoiesis.

This figure depicts the development of brain from early embryogenesis from E9.5 to adulthood. In parallel, the appearance of different source of hematopoiesis is shown. At E9.5, YS macrophages start to colonize the brain. From about E13.5, pericytes and astrocytes start to form the blood-brain barrier and initiation of the hematopoiesis in the fetal liver begins. After birth, hematopoiesis occurs in the bone marrow. During inflammation, bone marrow derived progenitors are able to enter the inflamed brain to provide additional protection. (From Ginhoux et al., 2013)

In particular, a later study showed that microglia derived from CSF-1R-expressing YS precursors that were present between E8.5 and E9.5 (Schulz et al., 2012). As all previous studies focused on committed CX3CR1⁺ YS progenitors that were considered immature macrophages, a study was carried out to further elucidate the earlier progenitors prior to acquiring macrophage phenotype. It was discovered that YS CD45^c-Kit⁺ EMPs could give rise to microglia in the brain. These YS EMPs differentiate into CD45⁺c-Kit^{lo}CX3CR1⁻ precursors that mature into and colonize the brain as CD45⁺c-Kit⁺CX3CR1⁺

cells (Kierdorf et al., 2013). Interestingly, using the Runx1 lineage tracing model, and tagging YS progenitors at either E7.5 or E8.5, two waves of EMPs that arise sequentially in the YS were described (Hoeffel et al., 2015). The first wave at E7.5 differentiated locally into YS macrophages and was identified as the main source of microglia. The second wave at E8.5 could either differentiate locally into YS macrophages or enters the newly formed blood circulation to migrate and seed the fetal liver. In essence, microglia predominantly arise from YS macrophages that came from the first wave of EMPs as precisely described and discussed in a review (Ginhoux and Guillemin, 2016).

Other complementary experimental models such as newborn transplant (where host newborn CD45.2 mice were sublethally irradiated and subsequently reconstituted with hematopoietic cells from congenic CD45.1 mice) and parabiosis (mice are surgically attached together to allow a shared blood circulation) confirmed that postnatal radio-resistant microglia are independent of circulating hematopoietic cells and self-renew locally (Ajami et al., 2011; Ginhoux et al., 2010; Hoeffel et al., 2015)

1.2.2. Inflammation

Previous work on microglia focused on its origin and homeostasis during development and steady state. However, when situations such as inflammation occur, bone marrow-hematopoietic cells such as monocytes may infiltrate the brain and give rise to microglial-like cells. Studies using bone marrow transplantation to distinguish between radio-resistant microglia and radiosensitive blood-derived cells reported that bone-marrow-derived precursors colonized the CNS and differentiate into microglia, leading to the wrong assumption that microglia arise from blood monocytes (Ginhoux and Jung, 2014; Greter et al., 2015) (Figure A). This was observed when the blood-brain barrier is compromised due to inflammation of the CNS or lethal irradiation (ionization), as well as the concomitant transfer of numerous donor bone marrow cells in the circulation (Ajami et al., 2007; Mildner et al., 2009). These monocytes or even bone marrow derived precursor cells are then able

to differentiate into microglia-like cells with similar phenotypes and functions to resident microglia (Takahashi et al., 2015). However, it is yet unknown whether they are as effective as the initial embryonic derived resident cells in maintaining CNS homeostasis and in neuroinflammation. Of note, in a mouse model of experimental autoimmune encephalomyelitis (EAE), such microglia-like cells do not persist locally once the inflammation is over (Ajami et al., 2011). In addition, when observing Alzheimer's disease mice brains, monocyte-derived microglia-like cells aggregate around amyloid plaques to a significantly smaller extent when compared to resident microglia (Prokop et al., 2015; Varvel et al., 2015). Further work is required to define the precise experimental conditions in which peripheral cells are allowed to engraft in the brain and become "bona fide" microglial cells or just macrophages with shared overlapping phenotypes and functions with embryonic endogenous microglia. It is speculated that niche availability will determine such outcome as discussed further in a review (Guilliams and Scott, 2017).

1.3. Development of microglia

Deriving from the YS and colonizing the CNS parenchyma, microglia migrate to different regions of the brain in a temporal and spatial manner. These microglia then proliferate and mature into ramified cells that form a surveillance network around the CNS (Mosser et al., 2017).

1.3.1. Migration of embryonic microglia

Microglia form focal accumulations around white-matter regions as they first enter the CNS parenchyma and are highly proliferative (Verney et al., 2010). Here, the immature microglial cells exist in an amoeboid form that maintains minimal cellular processes to promote better cell migration (Monier et al., 2007). Thereafter, microglia colonize the brain in two distinct phases (as seen in human fetal brain): the tangential migration followed by the radial migration (Figure B). During the tangential migration, microglia travel along the CNS parenchyma surface and spread out evenly covering the whole CNS layer (Cuadros and Navascues, 2001). Next, during the radial migration, microglia

move perpendicularly to the surface and distribute throughout the CNS layers. Within the developing cortex, the cells migrate towards the sub-cortical plate and assemble at the junction between the pre-subplate and the cortical plate, transient fetal zones that becomes the cerebral cortex (Monier et al., 2007). Finally, microglia penetrate the cortical plate to various parts of the brain (Monier et al., 2007).

Numerous factors contribute to microglia migration during brain development. The expression of matrix metalloproteinases (MMP) 8 and 9, typically involved in remodeling of the extracellular matrix, facilitates the migration of microglia and blocking of MMPs prevents the microglia expansion (Kierdorf et al., 2013). A gradient of signals such as semaphorins/netrins (Spassky et al., 2002), MCP1/MIP-1 α (Rezaie et al., 2002) and ligands of CSF-1R and VEGFR1 (Lelli et al., 2013) also help to guide microglia and promote their migration. CX3CR1 has also been linked to the migration of microglia, as knocking out CX3CR1, led to the delay of microglia recruitment in the early murine postnatal brain (Hoshiko et al., 2012). Finally, there may be developmental dependency between blood vessels and microglia as microglia colonization coincides with CNS vascularization during embryonic development (Earle and Mitrofanis, 1998) and microglia release soluble factors that stimulate the growth of blood vessels (Rymo et al., 2011).

Figure B. Migration of microglia during brain development.

At the start of primitive hematopoiesis in the YS, yolk sac progenitors give rise to immature microglia that begin to colonize the brain. Upon arrival, they migrate in a tangential fashion along the surface of the CNS and subsequently travel radially towards the cortical plate. CP: Cortical plate; NCX: Neocortex; M: Meninges; V: Ventricle. (From Mosser et al., 2017)

1.3.2. Proliferation of embryonic microglia

In order to colonize the entire CNS, microglia proliferate profusely, promoted by numerous factors such as CSF-1, GM-CSF, IL4 and IL5 (Navascues et al., 2000). During embryonic development, microglia proliferation is observed in various brain regions including the cortex and hippocampus (Dalmau et al., 2003). The microglial proliferation continues during the early postnatal phase (Dalmau et al., 2003) at a diminishing rate until the second week of postnatal development where proliferation is low, similar to that of the adult rate (Ginhoux et al., 2010; Marin-Teva et al., 1999).

1.3.3. Maturation of Microglia

After spreading out evenly in the CNS, microglia mature from an amoeboid to a ramified form. An *in vitro* study showed that microglia cultured on top of a layer of astrocytes start to develop processes after a week, which subsequently elongate and branch out (Sievers et al., 1994). In fact, astrocyte conditioned media that contain purines and cytokines such as GM-CSF, CSF-1 and TGF- β could lead to the ramification of microglia (Schilling et al., 2001; Wollmer et al., 2001). In a more recent study, *ex vivo* experimentation confirmed that astrocyte-derived factors such as CSF-1/IL-34, TGF- β 2 and cholesterol lead to ramification and survival of microglia (Bohlen et al., 2017).

Upon ramification of microglia, their electrophysiological properties change. Immature amoeboid microglia express inward K⁺ current channels, while maturing postnatal microglia show reduced expression of inward K⁺ channels and transient expression of outward K⁺ channels, finally resulting in negligible voltage-gated channels expression during the adult phase (Arnoux et al.,

2013; Boucsein et al., 2000; Rigato et al., 2012). Based on transcriptomic analysis of microglia, TGF- β has been highlighted to play a role in modulating microglia maturation (Butovsky et al., 2014). The gene signature of mature microglia is expressed after 4 days post-partum and vastly differs from other myeloid cells including recruited macrophages during inflammation and also microglia cell lines and cultures. It may thus be inaccurate to obtain information from simplified models such as microglial cell cultures.

1.4. Microglia shapes the central nervous system through development

In addition to their immune-related functions during brain inflammation, microglia exhibit specific functions that support neuronal differentiation and function in steady state that are regulated alongside brain development. Here we describe their involvement with the CNS from early embryogenesis to the adult phase and their involvement in the various processes along development (Figure C).

Figure C. Developmental clock of neuronal activity and the interaction with microglia.

The illustration describes how microglia regulate various aspects of neuron biology in a chronological order from early development to adulthood. This includes neurogenesis of neural progenitors, promoting survival or death of neurons, synaptic pruning of maturing neurons and modulating the synaptic activity via ATP. (Hoeffel et al., 2014)

1.4.1. Microglia regulates neurogenesis in embryos

During neurogenesis in the cerebral cortex, an abundant number of progenitors are produced in the ventricular (VZ) and subventricular zones (SVZ). This production of progenitors is abated at the terminal phase of cortical development (Bayer and Altman, 1991). It has been reported that in rodents and primates, microglia are responsible for this reduction by phagocytosing the cortical neuronal precursors and thus regulating their numbers (Cunningham et al., 2013). Of note, during the late phase of prenatal

development in rodents (E17.5 onwards), microglia in the cortex specifically target neuron progenitors that express the transcription factor Pax6 or Tbr2. Reduction in neuron progenitors due to local apoptosis was ruled out as following depletion of microglia, the number of progenitors increased, while upon maternal immune activation (MIA) by lipopolysaccharide (LPS) which activates microglia, led to further reduction of progenitors.

The process of neurogenesis continues after birth and it has been shown that microglial regulation of neuronal precursor numbers continue throughout adulthood (Sierra et al., 2013; Sierra et al., 2010). Microglia are involved in the regulation of the density of immature neurons arising in the SVZ of the forebrain, which is a known site of postnatal neurogenesis.

1.4.2. Shaping of the embryo brain

A study showed that embryonic microglia are able to regulate the wiring of the forebrain in a region specific manner such as controlling the outgrowth of dopaminergic axons and the positioning of neocortical interneurons (Squarzoni et al., 2014). Using models of embryonic microglia depletion, it was observed that in the absence of microglia, there was an excessive outgrowth of TH-positive (dopaminergic) axons into the striatum, leaving those in the mid-brain unaffected. Both the absence of microglia and maternal immune activation, where microglia is activated, led to Lhx6-expressing interneurons penetrating the cortical plate prematurely and distributing themselves sparsely around layer V of the neocortex. In mice, it was observed that knocking out CX3CR1 (important for neuronal-microglial communication (Hoshiko et al., 2012)) also led to a minor extension of dopaminergic axons at birth. Furthermore, inactivation of CX3CR1 and DAP12 (an adaptor protein implicated in microglia function (Thrash et al., 2009)) led to the defective distribution of Lhx-6 expressing interneurons at E18.5.

1.4.3. Microglia involvement in postnatal brain

1.4.3.1. Microglia regulates both the death and survival of neurons

During the early postnatal phase, microglia were shown to phagocytose apoptotic Purkinje cells that are part of the cerebellar efferent pathway. Interestingly, there was increased survival of Purkinje cells (van Rooijen et al., 1997) in the absence of microglia, suggesting their role in inducing neuronal death. In another study, microglia were reported to produce reactive oxygen species that enhanced the apoptosis of neurons in the hippocampus through the CD11b/DAP12 signaling pathway (Wakselman et al., 2008).

On the other hand, microglia have also been shown to promote neuronal survival. Depleting or inactivating microglia by minocycline, resulted in an increase in death of layer V cortical neurons. Additionally, microglia activation did not cause any apoptosis in layer V, confirming that only inactivation, and not activation, of microglia led to cell death of layer V neurons (Ueno et al., 2013). Such microglia-mediated neuron survival results from the IGF1-IGF1Ra interaction that initiates the PI3K-AKT pathway involved in cell survival.

1.4.3.2. Modulation of synaptic pruning and neuronal activity

Besides being involved in controlling the density of neurons, microglia also contribute to remodeling the connections between neurons in the postnatal phase. Postnatal synaptic pruning is an important developmental process where excessive synapses are removed to ensure appropriate connections between neurons for proper CNS function. For instance, microglia are able to eliminate weaker pre-synaptic connections that were established during early development, thus allowing the division of visual inputs from both eyes which regulate the formation of the retinogeniculate system (Schafer et al., 2012). Notably, this shows that microglia have the ability to discriminate between weak and strong synaptic connections. Such synaptic modifications by microglia were also observed in the mice hippocampus (Paolicelli et al., 2011). Expression of post-synaptic protein PSD95 and pre-synaptic protein

SNAP95 using immunohistochemistry were observed in microglia of the postnatal phase, confirming phagocytosis. The microglia density drop in CX3CR1 knockout mice led to a delayed and impaired synaptic pruning process, resulting in the formation of excess dendritic spines and immature synapses that are characteristics of an immature brain circuit.

Microglia are also able to sense neuronal activities and elicit responses to modulate homeostasis in an experience-dependent manner. For example in the context of visual experience, microglia interactions with axons and synaptic connections are very dynamic (Tremblay et al., 2010). In the absence of light, microglia had decreased motility and migrated to regions of larger dendritic spines that were constantly shrinking. Upon re-exposure to light, these microglia behaviors are reversed. In zebrafish larvae, microglia are in constant contact with neuronal cell bodies of the optic tectum (Li et al., 2012). This is due to the release of ATP from neurons that are highly active, causing the microglial processes to enclose the neurons for a short period of time and thus stably decreasing their activity. Another example shown that microglia can indirectly modulate hippocampal neuronal activity in vitro (Pascual et al., 2012). Following treatment with LPS, activated microglia release ATP that leads to the attraction of astrocytes, which subsequently discharge glutamate. The release of glutamate causes a quick and transient surge in the spontaneous postsynaptic currents in neurons from the hippocampus.

Altogether, it is thus well documented that microglia at different phases in development are involved in various CNS activities and this could be due to a developmental change in microglia and also in part due to the environment surrounding microglia that likely control and regulate microglia development in a feed-forward loop.

1.5. Sexual dimorphism and their consequences

1.5.1. Sexual dimorphism in immune responses

A recent review highlighted the differences in immune responses of both the innate and adaptive immunity against self-antigens and foreign pathogens

(Klein and Flanagan, 2016) in a sex-dependent manner. Further, these differences appear to change with age (Figure D).

The number and response of innate cells are different between males and females: for example, there are a higher number of natural killer cells in males than females (Abdullah et al., 2012). In contrast, neutrophils and macrophages are more phagocytic in females than males (Spitzer, 1999). Also, antigen-presenting cells present peptides more efficiently in females compared to males (Weinstein et al., 1984). The generation of chemokines and cytokines by innate immune cells also varies between the different genders. Upon stimulation by LPS, human PBMCs from males generate more TNF as opposed to females (Moxley et al., 2002). On the other hand, LPS stimulated mice peritoneal macrophages showed higher expression levels of TLR4 and produce higher levels of CXCL10 in males than females (Marriott et al., 2006).

Figure D. Differential immune responses observed in men and women throughout their lifespan.

Difference in the innate and adaptive immune responses were observed since before birth and through growing up till old age. (From Klein et al., 2016)

Factors such as age and the reproductive maturity influence sexual dimorphism and the differential immune responses. For instance, female fetuses are able to cope with more intra-uterine stress than male fetuses. It was noted that the female premature fetuses had higher survival rates compared to male premature fetuses, as the placentas from male premature fetuses appear to be more chronically inflamed (Gluckman et al., 2005). Postnatally, there were a higher number of monocytes and basophils in human male infants till 13 months old upon exposure to pathogens (Bellamy et al., 2000). The number of NK cells is also higher in male children compared to females (Lee et al., 1996). Following stimulation to LPS, male infants have higher proinflammatory responses than female infants (Casimir et al., 2010). These results suggest that males establish a more potent innate immunity compared to females during the postnatal phase. During puberty, the effects of sex hormones on the immunity have a great impact on sex differences in inflammation. Infant males may produce greater inflammatory responses, however, pubescent females have greater inflammatory responses (Yang and Kozloski, 2011). Females go on to have more CD4⁺ T cells and greater CD4/CD8 T cell ratios than males through adulthood (Uppal et al., 2003), while males have higher frequencies of T_{reg} cells (Afshan et al., 2012). Finally, during ageing, the immune system enters a chronic low-grade pro-inflammatory state that appears to be more prevalent in females than males (Furman et al., 2014). In addition, the number of NK cells increase with age, this rise being greater in females compared to males (Hirokawa et al., 2013).

Figure E. Sex bias seen in cancers, autoimmune and infectious diseases.

Female bias is observed more in diseases related to autoimmunity while male bias were more prevalent in cancers. Males and females displayed differential susceptibility to different infectious diseases. (From Klein et al., 2016)

Under pathological conditions, males and females display distinct differences in their susceptibility to autoimmune diseases, infections and cancers (Figure E). Broadly, males are more susceptible to non-reproductive malignant cancers (Cook et al., 2009) while females are more susceptible to autoimmune diseases (Jacobson et al., 1997). To a lesser extent, sexual biases are also observed in vulnerability to different infectious diseases as described in the review (Klein and Flanagan, 2016).

1.5.2. Sexual dimorphism in neurological diseases.

In agreement with the observed sex bias in various disorders, several studies have shown that there are sex biases in CNS-related disorders such as neurodevelopment disorders, neurodegenerative diseases, multiple sclerosis and responsiveness to pain (Zagni et al., 2016).

Autism Spectrum Disorder (ASD), a type of neurodevelopment disorder, affects 4 males to each female with the ratio increasing with severity of this disorder (Kim et al., 2011). A cohort study, involving approximately 2500 autistic children, was designed to understand how different males and

females with autism behaved. They found that females had lower social communication skills, lower cognitive competence, dampened adaptive skills and more externalizing problems as compared to males (Frazier et al., 2014).

Neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease, also display sexual biases. The prevalence and incidence of Parkinson's disease are higher in men compared to women (Pavon et al., 2010). Studies have also shown that lethargy, uneasiness, sorrow, constipation and fidgeting legs and pain are more prominent in females. However, symptoms such as daytime fatigue, leaking saliva, interest in sex and sexual dysfunction were symptoms found more predominantly in men (Martinez-Martin et al., 2012; Solla et al., 2012). In Alzheimer's disease, on top of having a higher prevalence for the disease in comparison to age-matched men, women also displayed a faster decline with age. Post-mortem studies of humans revealed that men with Alzheimer's disease had more severe pathology in the right hemisphere whereas in women, a more global pathology was observed, affecting more areas of the brain (Phung et al., 2010).

Interestingly, while women appear to face an increased risk of developing multiple sclerosis, men typically encounter a worse disease progression with their white matter observed to be more extensively and severely affected as seen by severity of white matter diffusion abnormalities (Schoonheim et al., 2014).

Pain, which is a health problem faced in developed countries also exhibit sexual dimorphism. Research has shown that women have greater sensitivity and facilitation to pain and decreased inhibition of pain (Mogil, 2012). Microglia is involved in pain processing by releasing BDNF induced by P2X₄R (Coull et al., 2005). A study showed that microglia-mediated pain processing is critical only in male mice; while in female, microglia are not required for mechanical pain hypersensitivity (Sorge et al., 2015). Female mice were able to reach equivalent levels of pain hypersensitivity through adaptive immune cells such as T cells. T cells have been reported to release pro-algesic mediators that may have similar effects on neuronal activity (Grace et al., 2014).

1.5.3. Sexual dimorphism in microglia

Besides sexual difference observed in microglia's role in pain hypersensitivity, microglia have also been shown to differ between males and females in brain colonization (Schwarz et al., 2012) and brain masculinization (Lenz et al., 2013). Schwarz reported that in rat brains, there are more microglia in males during early postnatal phase while there are more amoeboid microglia in females in their later development (Schwarz et al., 2012). In a separate study of middle cerebral artery (MCA) occlusion, it was reported that both astrocytes and microglia displayed a distinct sex-dependent response to MCA occlusion. Immunohistochemistry analysis of mice after MCA occlusion showed a higher expression of CD11b in male microglia as opposed to female (Morrison and Filosa, 2016).

Sex-dependent differences in cell populations, such as microglia, thus clearly display how the intrinsic nature can affect their development and functions differently, which have a differential impact of the well-being and behavior of an individual.

1.6. Microbiome and the central nervous system

On the other hand, extrinsic factors such as the commensal microbiota also have an effect on immune cells and health. In recent years the gut flora, or microbiome, has emerged as a key regulator of brain circuitry, neurophysiology and behavior (Braniste et al., 2014; Diaz Heijtz et al., 2011; Yano et al., 2015). Accordingly, the lack of microbiome in germ-free (GF) mice severely impacts on microglial maturation and function (Erny et al., 2015; Matcovitch-Natan et al., 2016). In adults, microbiome was shown to influence microglia indirectly via the release of short chain fatty acids (Erny et al., 2015).

1.6.1. Microbiome affects brain development and behavior

The microbiome is able to regulate normal brain development and behavior (Diaz Heijtz et al., 2011). In the absence of microbiota, GF mice exhibit increased motor activity and lowered anxiety-like behavior. GF mice were shown to have higher monoamine turnover in the striatum, such as noradrenaline, dopamine and serotonin (5-HT). The change in transmission of these monoamines are linked to anxiety-like behavior. GF mice displayed lower expression of genes linked to synaptic plasticity such as nerve growth factor-inducible clone A (NGFI-A) and brain-derived neurotrophic factor (BDNF). They also showed changes in genes involved in four canonical pathways such as synaptic long-term potentiation and cAMP-mediated signaling. There was also a reduction in proteins linked to synaptogenesis such as synaptophysin and PSD-95 in the striatum of GF mice.

Microbiota has also been shown to alleviate behavioral and physiological aberration linked to neurodevelopmental disorders (Hsiao et al., 2013). When commensal bacteria, *Bacteriodes fragilis*, were administered to MIA offspring (which displayed signs of various core behavioral characteristics of ASD), ASD-like behaviors were ameliorated. The treated mice did not display anxiety-like behavior in the open field test. They also had improved sensorimotor gating and proper communicative behavior. Treatment of MIA offspring also improved intestinal permeability and re-established the composition of microbiota to that of control mice.

1.6.2. Communication of microbiota with the immune system

Besides protecting us from foreign pathogens, the immune system is also involved in regulating the composition and homeostasis of commensal microbiota in the host (Figure F). Various immune effector cells work together to stratify the luminal microbes and bring epithelial and bacterial interaction to the minimum by secreting mucus layers, immunoglobulin A (IgA) and anti-bacterial proteins that regulate the composition of microbiota (Cash et al., 2006; Johansson et al., 2008; Macpherson and Uhr, 2004). Commensal microbes that infiltrate the intestinal epithelial barrier are phagocytosed by macrophages and dendritic cells (DCs) (Kelsall, 2008). DCs carrying the commensal bacteria induce the secretion of protective IgAs which are spread along the mucosal layers through the re-circulated B and T cells (Macpherson and Uhr, 2004). This reflects the ability of the immune cells to permit immune responses against commensals to be distributed extensively yet restricting them to the mucosal layers.

On the other hand, commensal microbiota are able to emit signals to regulate the immune cells. Microbiota have an influence on T cell subsets, for example, colonization of mice with segmented filamentous bacteria (SFB) led to the accumulation of Th17 cells and to a smaller extent Th1 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Microbiota are also capable of influencing the systemic immunity and innate lymphoid cells (Lorenz et al., 2003; Mazmanian et al., 2005) (Figure G). All in all, these show the two-way communication of microbiota and immune cells in maintaining homeostasis in the body.

Figure F. Regulation of microbiota by immune cells

Immune cells regulate the commensal microbiota in 2 ways: 1) Stratification through the production of mucus or IgA or 2) compartmentalization by surveillance of immune cells like DCs and macrophages. (Hooper et al., 2012)

Figure G. Regulation of immune cells by microbiota

Microbiota release signals to immune cells influencing their increase or reduction such as in the increase of Th17 and Treg and the reduction in iNKT cells. (Hooper et al., 2012)

1.6.3. Microbiome affects microglia biology

Microglia, immune cells of the brain that is constantly sensing their environment, are influenced by the microbiome. Colonization of the heterogeneous microbiota regulates microglia maturation and activation during homeostasis (Erny et al., 2015). Constitutive or transient depletion of microbiota significantly affected microglia morphology and development, which resulted in a delayed response against microbial-associated molecular pattern and pathogens. Performing RNA sequencing of microglia from GF and control mice, it was observed that

198 genes were downregulated and 173 genes were upregulated in GF mice. Genes that were downregulated included genes that were related to cell activation such as Cd86, Ly86 and Mapk8. Genes that were upregulated consist of microglial transcription and survival genes such as Sfp1 and Csf-1r. In GF mice, the innate immunity and resistance against bacterial and viral infection are dampened, as seen when treated with bacterial LPS or lymphocytic choriomeningitis virus (LCMV) that resulted in a significant reduction in the expansion of microglia density. Also both the transient depletion and reduced complexity of microbiota displayed an immature phenotype such as changes in morphology of microglia.

Matcovitch-Natan et al. further tested the effect of the microbiome on microglia development (Matcovitch-Natan et al., 2016). Comparing microglia from newborns and adult of GF and control mice, they observed a larger number of genes with decreased expression in adult microglia when compared to newborns. This could be due to the change in microbiota composition during the weaning period. In the absence of microbiota, a decrease in expression of genes linked to inflammation and defense response in microglia was observed. It was noted that a large portion of developmental genes that were down-regulated belonged to the late adult microglia phase. These show the effect of microbiome on development of microglia from birth to adulthood.

1.7. Dysregulation of embryonic microglia

Microglia play an important role in maintaining the CNS such as the formation of functional brain circuits and thus when there is dysregulation in microglia functions, the consequences could be detrimental. Epidemiological studies have provided data linking maternal viral or bacterial infections with children's risk of developing disorders such as autism spectrum disorders or epilepsy (Knuesel et al., 2014). Microglia may be implicated in such maternal immune activations by disrupting neuronal synaptic connections and as a consequence, lead to

neurodegeneration.

1.7.1. Maternal stress during pregnancy affects the brain and behavior

Studies have reported that maternal inflammation during pregnancy can contribute to the overgrowth of the offspring's brain and lead to the acquisition of autism-related behaviors (Le Belle et al., 2014). When pregnant mice were treated with low doses of LPS, capable of inducing maternal immune activation, the offspring showed an average increase in brain size compared to non-treated controls. In particular, these offspring display an increase in cortical area and thickness. Additionally, microglia were observed to increase in numbers throughout the brain as well as elevated proliferation of Nestin+ progenitors in the SVZ. Importantly, MIA lead to the display of abnormal behavior in these offspring as seen in a deficit in ultrasonic vocalization when separated from their dams and increased anxiety as observed in the elevated plus maze test.

In a separate study, prenatal infection resulted in ASD-like behavior and modified synaptic pruning in the offspring (Fernandez de Cossio et al., 2017). LPS-treated offspring displayed early onset of altered social behavior (ultrasonic vocalization) and increased amount of repetitive movement, typical features of ASD (Pearson et al., 2011). There is high activity of synaptic pruning during the postnatal period (Paolicelli et al., 2011). Intriguingly, male offspring of LPS-treated mice displayed significantly greater number of spines in granule cells of the dentate gyrus at P15, which could explain the change in behavior.

1.7.2. The effects of maternal inflammation on microglia

Treatment of pregnant mice with viral mimic polyinosinic-polycytidylic acid (polyIC) also lead to an increased density of cholinergic neurons in the basal forebrain of the offspring and activation of fetal microglia (Pratt et al., 2013). qRT-PCR analysis of the microglia revealed significant

increase in expression of IL-6, a proinflammatory cytokine. A 32-plex Luminex assay to assess the production of cytokines and chemokines was performed and reported the increase in numerous cytokines such as IL-1 α , Gm-csf/M-csf, IL-4 and IL-9 and also chemokines including eotaxin, MIP1 β and RANTES. These data confirms the activation of microglia following MIA.

MIA also led to a change in microglial transcriptomic signature in the offspring (Mattei et al., 2017). mRNA sequencing of brains from PolyIC treated offspring and control offspring revealed differential expression of genes between the 2 groups. 1398 DEGs were identified and genes that were upregulated in PolyIC treated offspring were related to embryonic development and positive regulation of long-term neuronal plasticity, while down-regulated genes were linked to cell migration, phagocytosis and inflammatory response. Again, increased pro-inflammatory signals were observed in the hippocampus of MIA offspring where there was significant increase in IL-6 expression. Assessing the phagocytic activity of microglia in these mice using fluorescent latex beads, it was noted that MIA offspring displayed a lower phagocytic index compared to controls.

1.8. Microglia in neurological diseases

Microglia play crucial roles in shaping the proper development of the brain and any dysregulation would inevitably lead to possible complications. Microglia have been shown to be implicated in numerous neurological disorders such as Alzheimer's disease and Amyotrophic Lateral Sclerosis (ALS) (Li and Barres). In the CNS, microglia exclusively express certain genes such as CD33, CR1 and TREM2, which have been identified as risk genes for Alzheimer's disease (Hollingworth et al.; Lambert et al.; Sims et al.). Additionally, microglia are also involved in tauopathy, a manifestation of Alzheimer's disease, where microtubule-associated protein tau are seen to spread across various brain regions (Asai et al.).

Microglia have been linked ALS, a degenerative disease characterized by progressive loss of motor neurons (Prinz et al.). Genes such as C9orf72 and TAR DNA-binding protein (TARDBP) are often mutated in ALS (Al-Chalabi et al.). C9orf72 is involved in the regulation of microglial phagosome and lysosome pathways (O'Rourke et al., 2016), while TARDBP regulates microglial phagocytosis (Paolicelli et al.). Thus, it is important to understand how these various factors are implicated to allow better manipulation for the treatment of neurological diseases that involves microglia dysregulation.

1.9. Objective of this study

Microglia, the resident macrophage of the CNS, form a network surrounding the brain and spinal cord. They are present from early embryogenesis to adulthood and are involved in the protection of the brain as well as shaping the development and maintaining the homeostasis of the CNS. Their crucial roles make them implicated in numerous neurological disorders when they are dysregulated and thus are a crucial therapeutic target. However to accurately manipulate the response of microglia, we have to fully understand their development and the intrinsic and extrinsic factors that affect them.

It has been reported that intrinsic or extrinsic factors such as sexual identity or the microbiome respectively affects microglia development and function. However, how exactly do these factors modulate microglia and the possible interplay of these various factors needs to be addressed.

In this study, we thus designed experiments using a combination of various mouse models, transcriptomic profiling, flow cytometry and immunohistochemistry techniques to answer the following questions:

- 1) What are the changes in microglia along development? What genes and transcription factors are involved in their normal developmental process?
- 2) What are the sexual differences between male and female microglia along side development and what implications do they have in microglial functions?
- 3) The effect of microbiome on the development and maturation of microglia has not been fully investigated. How different are the effects in males and females?

2. Materials and methods

2.1. Experimental model and subject details

2.1.1. Animal models

For microarrays, specific-pathogen-free (SPF) C57BL/6 mice from A*STAR Biological Resource Centre (BRC) were used for the different time points. FUCCI mice were also obtained from in-house breeding in BRC. Microglia proliferation was investigated using the fluorescent ubiquitination-based cell-cycle indicator (FUCCI) transgenic mouse model in which the green-emitting fluorescent protein Azami Green is fused to Geminin, a ubiquitination oscillator whose expression is regulated by cell-cycle-dependent proteolysis, resulting in the expression of fluorescence in cells in S/G2/M phases (Sakaue-Sawano et al., 2008). Experiments done on these mice are approved under the IACUC protocol 151071. Animals were bred under proper regulations, following recommendations of the local ethics committee.

SPF and GF were maintained in sterile plastic isolators at the National Cancer Centre's germ-free facility (NCC) housed in the SingHealth Experimental Medicine Centre. Animals were maintained on autoclaved R36 Lactamin Chow (Lactamin, Sweden) and kept under 12-h light cycles condition. *Sal11^{gfp/+}* mice were from Dr. Melanie Greter of University of Zurich (Takasato et al.).

The day of vaginal plug was considered as embryonic day (E) 0.5 and day of birth as postnatal day (P)0. Sex determination of mouse embryos (E18.5) was assessed visually based on the presence of male or female reproductive organs and later confirmed by expression of X and Y specific genes.

2.1.2. Human samples

Mid-Trimester-Pregnancy-Termination (MTPT) fetuses were kindly donated by patients for the purpose of this study, which was explained

clearly to the patients to get their consent. The fetuses were of healthy conditions and aborted for social reasons. The samples taken are from KK Hospital and National University Hospital, Singapore and work done are approved with a CIRB (2013/837/D).

2.2. Methods details

2.2.1. Cell suspension preparation

Mice and human tissues were cut into small pieces, incubated in RMPI containing 10% fetal bovine serum and Collagenase type IV (0.2 mg/ml, working activity of 770U/mg; 1 hour for human tissues, adult mice and newborns and 30 minutes for embryonic mice tissues) and then passed through a 19G needle to obtain a homogeneous cell suspension as previously described. (Ginhoux et al., 2010) In addition, adult brain cell suspensions were resuspended in 40% isotonic Percoll and underlayered with 80% isotonic Percoll centrifuges at 2800rpm for 20 minutes at room temperature. Cells at the interphase were collected and washed for FACS.

2.2.2. Flow Cytometry

Cells of interest were sorted using the BD-FACS ARIA for transcriptomic analysis. Cell suspensions were analyzed using the BD-LSRII. Data were analyzed using FlowJo software (Treestar). Conjugated antibodies used to identify cell populations are listed as follows: anti-CD45 APC Cy7 (BD Pharmingen), anti-CD11b BV650 (Biolegend), anti-F4/80 Biotin (Biolegend), Anti-Ly6C (Ebioscience), anti-CD64 (BD Pharmingen).

2.2.3. Immunohistochemistry

For immunohistochemistry, dissected brains were fixed in 4% PFA at 4°C from 4h to overnight, depending on the developmental stage. Immunohistochemistry was performed on 40-80 µm free-floating vibratome sections as described before (Deck et al., 2013). Sections

were blocked 2 hours with PBS1X-10% fetal bovine serum-0.01% TritonX-100 and then incubated overnight with primary antibodies. Sections were rinsed twice in PBS1X-0.01% TritonX-100 followed by several PBS1X washes before overnight incubation with secondary antibodies (1/400 in PBS1X). Hoechst (1/1000) was used for fluorescent nuclear counterstaining. Primary antibodies used: anti-rabbit IBA1 (Wako), anti-rat CTIP2 (Abcam), anti-rabbit CUX1 (Santa Cruz), anti-rat L1 (Millipore). Secondary antibodies used: AF488/Cy3/Cy5-conjugated (Jackson ImmunoResearch)

2.2.4. Image acquisition

For microglia cell density quantifications, images of the somatosensory neocortex, preoptic area and striatum were acquired with a confocal microscope (Leica TCS SP5).

2.2.5. Mouse Microarrays

Cells from several embryos or animals (>3) were pooled for each replicate to derive triplicates for each stage. Total RNA was extracted using the Ambion *mirVana*™ miRNA Isolation Kit (Ambion® Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. All mouse RNAs were analyzed using an Agilent Bioanalyser (Agilent, Santa Clara, CA, USA) for quality assessment. Biotinylated cRNA was prepared according to the protocol by TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip® (**Epicentre® (an Illumina company)**) using 50ng of total RNA. 1500ng of cRNA was hybridized on Illumina Mouse WG-6 Version 2 chips (Illumina, San Diego, CA, USA) for 17 hours at 58°C. The arrays were then washed and stained according to Illumina Wash Protocol. The chips were scanned using BeadArray Scanner 500GX (Illumina, San Diego, CA, USA).

2.2.6. Human Microarrays

Ten MTPT human fetal samples were selected. Total RNA was isolated from Human fetal microglia cells following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific) followed by a Qiagen RNeasy Micro clean-up procedure according to the manufacturer's protocol. All RNAs were analyzed using an Agilent Bioanalyser (Agilent, Santa Clara, CA, USA) for quality assessment. Biotinylated cRNA was prepared according to the protocol by Epicentre TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 (**Epicentre® (an Illumina company)**) using 500 pg of total RNA. 750 ng of cRNA was hybridized on Illumina Human-HT12 Version 4 chips (Illumina, San Diego, CA, USA) for 17 hours at 58°C. The arrays were then washed and stained according to Illumina Wash Protocol. The chips were scanned using BeadArray Scanner 500GX (Illumina, San Diego, CA, USA).

2.2.7. RNA-sequencing

Cells from several embryos or animals (>3) of each sex were pooled at each stage for each replicate. Sex were identified by visual inspection and later confirmed by expression of X and Y specific genes. Total RNA was extracted using the Ambion *mirVana*™ miRNA Isolation Kit (Ambion® Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. All mouse RNAs were analyzed using an Agilent Bioanalyser (Agilent, Santa Clara, CA, USA) for quality assessment. Embryonic samples were processed using smartseq whereas adult samples were processed using truseq.

For smartseq, cDNA libraries were prepared from 2ng total RNA starting material and 1µl of a 1:50,000 dilution of ERCC RNA Spike in Controls (Ambion® Thermo Fisher Scientific) using the SMARTSeq v2 protocol (Picelli et al., 2014) with the following modifications: 1. Addition of 20 µM TSO; 2. Use of 250pg cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, San Diego, CA, USA). The length distribution of the cDNA

libraries was monitored using a DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip (Perkin Elmer, Waltham, MA, USA). All samples were subjected to an indexed paired-end sequencing run of 2x51 cycles on an Illumina HiSeq 2000 system (Illumina) (16 samples/lane).

For truseq, cDNA libraries were prepared using 50 ng of total RNA and 2ul of a 1:2000 dilution of ERCC RNA Spike in Controls (Ambion). The fragmented mRNA samples were subjected to cDNA synthesis using Illumina TruSeq® RNA sample preparation kit version 2 (Low-Throughput protocol) (Illumina, San Diego, CA, USA) according to manufacturer's protocol, except for the following modifications: 1. use of 13 PCR cycles, 2. two additional round of Agencourt Ampure XP SPRI beads (Beckman Courter) to remove >600bp double stranded cDNA. The length distribution of the cDNA libraries was monitored using DNA 1000 kits on the Agilent bioanalyzer. All samples were subjected to an indexed PE sequencing run of 2x51 cycles on an Illumina HiSeq 2000 (12 samples/ lane).

2.2.8. RT-qPCR

cDNA was synthesized with random hexamer from 15 to 100 ng total RNA using SuperScript II Reverse Transcriptase (Life Technologies). Real-time quantitative PCR (RT-qPCR) was performed using KAPA SYBR Fast qPCR kit (KAPABiosystems). Three primer sets were tested for each gene and showed the same results. Quantitative PCR was performed in a Roche LightCycler 480 with SYBR reagent in 20µl final volume per well. Each sample was measured in triplicate for all primers. Melt curve analysis and agarose gel electrophoresis were performed to verify the specificity of PCR amplicons. List of primers can be found in Table 4.

2.3. Quantification and statistical analysis

2.3.1. Mouse Microarray Data Analysis

Raw intensity values of gene expression were extracted from Microarray data using Illumina GenomeStudio software and quantile normalized using R package lumi. Normalized expression values were subsequently subject to log₂ transformation. Hierarchical clustering was performed using Pearson correlation and ward.D2 agglomeration method on log₂ transformed expression values. Principal Component Analysis (PCA) was performed on the log₂ transformed expression values using prcomp function in R with parameter scale=TRUE. For mouse microarray data, genes differentially expressed across different developmental stage were selected using ANOVA followed by post-hoc Tukey test and *Benjamini-Hochberg* adjusted p value less than 0.05. Differentially expressed genes were grouped to 7 distinct clusters by hierarchical clustering. Molecular functions significantly enriched by genes in each cluster were identified using Ingenuity Pathway Analysis (IPA). Genes differentially expressed between human early versus late microglia or human female vs male microglia were identified using Limma and adjusted p value less than 0.05. Hierarchical clustering, PCA and differentially expressed genes (DEG) analysis were all performed on log₂-transformed values.

2.3.2. RNA sequencing Data Analysis

For RNA-sequencing data analysis, raw reads were aligned to mouse reference genome MM10 using STAR aligner. Read count per gene was calculated using featureCount program and gencode gene annotation version M9. Count per million reads (CPM) values were calculated using edgeR package. Differentially expressed genes showing adjusted p value less than 0.05, fold change greater than 1.5 and a minimum of 25 average cpm were identified using edgeR package.

2.3.3. Co-expression network analysis (CENA)

For the generation of gene regulatory networks, we took the union of DEGs (814 genes) for all comparison and all present transcription factors (TFs) (473 genes). Expression values of these genes were used for co-expression network analysis (CENA) for all samples using BioLayout3D (www.biolayout.org) for DEG and TF network respectively. Applying a correlation cutoff of 0.85 for DEG network and 0.7 for TF network resulted in a coexpression network with 753 nodes (DEG) and 431 nodes (TF). The calculated gene-gene pairs along with their Pearson correlation coefficient were exported from BioLayout3D and imported into Cytoscape [doi:10.1101/gr.1239303] using force-directed layout for visualization. Group fold change (FC) values (based on the comparison of each condition with the mean of all conditions) were mapped for each condition individually onto the network. To identify commonalities and differences between microglia at different stages based on co-expression network analysis, we colored genes based on their group FCs > 1.5 or < -1.5 .

2.3.4. WGCNA Analysis

Weighted gene co-expression network analysis was performed on normalized expression data using the R package WGCNA v1.51 [DOI: 10.1186/1471-2105-9-559] following the standard workflow. For computational efficiency, genes were filtered according to the subsequent procedure: First, for each comparison the expression table was subsetting by all samples associated with the respective comparison and the 1000 most variable genes between those samples were determined. The resulting 11 sets of 1000 most variable genes were then united in a total amount of 2395 genes representing the relevant variance within the dataset. To obtain a signed network fulfilling the scale free topology the soft-thresholding power parameter was set to 20. Co-expression modules were defined using a minimum module size of 30 genes and by merging modules with a module eigengene dissimilarity below 0.1 resulting in 10 modules having sizes between 48

and 499 genes. The modules were characterized by GO enrichment with a focus on biological processes using the R package cluster-Profiler v3.0.5 [<https://doi.org/10.1089/omi.2011.0118>]. The co-expression network was generated based on the Topological Overlap Matrix requiring an edge weight of more than 0.3 to at least one other gene and excluding genes belonging to the grey module that contains those genes that do not fit in any other module. Network visualization was carried out in Cytoscape v3.4.0 applying the edge-weighted springembedded layout and hiding smaller clusters with less than 5 genes. In the base network nodes were colored according to module membership while in all other networks differentially-expressed genes (Fold-Change > 1.5 or < -1.5 and CPM > 25) were highlighted by red (positive FC) or blue (negative FC) fill color. Transcription factors were marked by a triangular node shape.

2.3.5. Images analysis

Iba1-positive cells were quantified using ImageJ software. Iba1-positive cells morphology were quantified using Imaris software.

2.3.6. Signaling pathway analysis

Pathway analysis and biological function enrichment analysis were performed using Ingenuity Pathway Analysis (IPA) or Database for Annotation, Visualisation and Integrated Discovery (DAVID). DAVID functional annotation was performed on the list of DEGs by Pathway analysis GO Term. Only the pathway having a FDR<0.05 were represented.

2.3.7. RT-qPCR analysis

Quantitative values were obtained from the cycle number (Ct value). Quantities were determined as $Q_{\text{target}} = 2^{\Delta C_{\text{t sample}}}$, where the ΔC_{t} value was determined by subtracting the average Ct value of target gene from the average Ct value of the housekeeping gene.

2.3.8. Human Microarray Data Analysis

Raw intensity values of gene expression were extracted from Microarray data using Illumina GenomeStudio software and quantile normalized using R package lumi. Normalized expression values were subsequently subject to log2 transformation. Hierarchical clustering of samples was performed using Pearson correlation and ward.D2 agglomeration method on log2 transformed expression values. Hierarchical clustering identified two major groups, one corresponds to early stage and the other corresponds to late stage. Female and male microglia samples were identified by the expression of DDX3Y and XIST genes. Genes differentially expressed between early versus late stages, female versus male were selected using Limma and adjusted p value less than 0.05. Human gene ids and mouse gene ids were converted to HomoloGene group ID (HID) based on NCBI HomoloGene. Converted human and mouse microarray gene expression data were subject to CIBERSORT analysis wherein mouse data were used as reference gene expression for identifying gene signatures of each developmental stage. The CIBERSORT analysis determined the proportion of each mouse gene signatures that were represented in the human microglia samples. Genes that were expressed across all human samples were identified and overlapped with core microglia signature genes derived from mouse microarray data (details given in the following section).

2.3.9. Core microglia signature gene identification

In order to identify core signature genes of mouse microglia, we first identified genes that were expressed across all developmental stages from York Sac to adult. From our microarray data of mouse microglia development, median gene expression values were identified for each sample. Genes with expression values higher than median gene expression were considered as being expressed. Genes that were expressed in all samples were selected as genes that were expressed

across all developmental stages. However, thus determined gene set includes genes that are also highly expressed in other cell types. Therefore, we identified significantly up-regulated genes in microglia as compared to other immune cell types from ImmGen data. The overlap between microglia up-regulated genes and genes expressed across microglia development was identified as candidate core signature of microglia. To further remove putative house-keeping genes, we identified genes that were expressed in all cell types from ImmGen data and exclude them from the candidate signature.

2.3.10. Statistical analyses

All data are presented as mean $\pm$ SEM. Two-tailed Kruskal-Wallis tests were used to compare groups of data and non-parametric two-tailed Mann-Whitney *U*-tests were used to compare two distributions. Variances were similar between compared distributions. All graphs and statistical analyses were obtained using GraphPad Prism software, unless otherwise stated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. Results and Discussion

3.1 Microbiome influences prenatal and adult microglia in a sex-specific manner

Here we sought to understand how microglia differentiation and functions are temporally regulated by intrinsic and extrinsic factors, such as sexual imprinting and microbiome respectively.

3.1.1. Microglia differentiation follows distinct developmental stages

First, we define the embryonic microglial differentiation program in normal (SPF conditions) development. To examine this, we FACS-sorted yolk sac macrophages (YSM) and microglia from seven developmental stages (E10.5, E12.5, E14.5, E16.5, E18.5, P0 and adult – P60) and ran microarray to probe their gene expression (Figure 1A).

Figure 1. Clustering of microglia from different developmental stages.

(A) Sorting strategy of microglia from mouse brain. Microglia and YS progenitors were gated on $CD45^{int}CD11b^{+}F4/80^{+}CD64^{+}Ly6C^{-}$ cells. (B) Unsupervised hierarchical clustering of microglia sorted from various stages of mouse development. Height of dendrogram represents the distance of differences between clusters. $n=3-4$ per stage. (C) PCA analysis of corresponding samples from (B) showing clustering of

microglia from different age. Each dot represents an independent biological sample composed of a pool of 3-4 embryos.

Unsupervised hierarchical clustering revealed that microglia samples clustered in a stage-dependent fashion, allowing us to classify the different developmental stages into four phases: progenitor (YSM – E10.5), embryonic 1 (E12.5 – E14.5), embryonic 2 (E16.5 – P0) and adult phase (Figure 1B). This is consistent with the observations from a previous study (Matcovitch-Natan et al, 2016). We performed a Principal component analysis (PCA) and in agreement with the hierarchical clustering, the samples grouped together by developmental stages along the PC1 axis (Figure 1C). In contrast, PC2 was rather able to reflect the difference in tissues, as the YSM appears to segregate further away from the rest of the microglia samples. This suggested that although E10.5 microglia are similar to YSM, they are already transcriptomically different, supporting the concept that the neural environment can influence very early, the colonizing YSM that react quickly to local cues (Gosselin et al, 2014,2017; Lavin et al, 2014).

To better understand the biological differences between the microglia from each phase, we performed a gene ontology (GO) analysis based on the differentially expressed genes (DEGs) among the various phases. The progenitor phase had an up-regulation of 4582 genes that are involved in processes such as cell cycle, proliferation and DNA replication (Figure 2A and Appendix 1). These genes are progressively down-regulated with time. Using FUCCI mice that tags proliferating cells with a green fluorescence, Azami Green (Sakaue-Sawano et al., 2008), we confirmed that the proliferative state of the microglia is highest at the progenitor phase and decrease with time to adulthood (Figure 2B and 2C). Embryonic phases 1 and 2 were both linked closely to genes that were involved in brain development and function. We observed an up-regulation of 1299 genes, including *PlexinA2* and *Igf1r* that are involved in nervous system development and function as well as cell-to-cell

signaling (Figure 2A and Appendix 1). In embryonic phase 2, 2116 genes that included *Cxcr4* and *Igf1* were up-regulated (Figure 2A and Appendix 1) and related to nervous system development and function and cellular development. Finally, the adult phase, with an up-regulation of 3508 genes, is mostly related to genes involved in immune functions such as *Jun*, *Ccr5* and *Mafb* (Figure 2A and Appendix 1). These results reveal the differentiation of microglia following developmental stages, having potentially different functions at each phase of development that likely reflects their functional adaptation to the evolving and maturing environment.

Figure 2. Microglia follow several developmental phases.

(A) Gene ontology (GO) analysis of microglia samples from different stages as seen in Figure 1. Heatmap shows the gene expression of the

various phases. Each group of genes is linked to different biological processes and is divided into 7 clusters. The line graph on the right represents the progression of expression of that cluster through development. n=3-4 per stage. (B) Gating strategy of microglia from FUCCI mice to observe their proliferative state. Cells positive for Azami Green (fluorescence) are proliferating. (C) Quantification of Azami green+ microglia from FUCCI mice. Data are represented as mean $\pm$ SEM; n=3-5 per stage; Two-tailed Kruskal-Wallis tests; ***p<0.001. Embryo. Ph = Embryonic phase.

3.1.2. Embryonic maturation and prenatal expression of sensome genes

In order to gain more insights into embryonic microglia differentiation, we performed co-expression network analyses (CENA) (Ulas et al., 2017) by mapping group fold changes for each time point onto the network. Using either all present transcription factors (TFs) or the union of DEGs, we were able to highlight the clusters of genes with strong expression in each phase and thus allowing us to follow the temporal evolution of these gene expressions (Figure 3).

The analysis reveal that the control of TF clusters across the different phases of development was dynamic. In a recent publication, the transcription factor *Sall1* was shown to control the activation and function of microglia in adult mice (Buttgereit et al., 2016). Here, we show that the expression of *Sall1* increases steadily during development and peaked at the adult stage (Figure 4A). Likewise, using *Sall1*^{gfp/+} mice, it is observed that there is negligible GFP expression in microglia at E11.5, progressing to 72.8% of microglia expressing GFP at E14.5 and attaining near 100% expression at adulthood (Figure 4B).

Figure 3. The developmental “clock” of microglia.

(A) Co-expression network analysis (CENA) based on the expression of 431 transcription factors and (B) CENA based on the expression of total DEGs among different developmental stages ($n=3-4$ per stage). Expression differences (fold change) relative to overall mean expression were indicated by the colour scale ($-0.15 < \text{fold-change} < 1.5$ and false discovery rate (FDR) corrected p -value < 0.05).

Figure 4. Microglia development and maturation.

(A) Gene expression level of microglia along development. (B) Using *Sall1^{gfp/+}* mice, the expression of *Sall1* in microglia was quantified from YSM to adult microglia. (C) Heat map of sensome gene expression of microglia along development. Each row represent a biological replicate. $n=3-4$ per stage. (D) List of selected sensome genes up-regulated in each phase. Cluster number correlates to the cluster in Figure 2.

To assess the sensing capabilities of microglia along their development, we analyzed the expression of the sensome genes. Microglia express a unique cluster of transcripts encoding proteins for sensing endogenous

ligands and microbes. These genes are defined by Hickman et al as the sensome genes (Hickman et al., 2013). It was noteworthy that several sensome genes were already expressed even before birth (Figure 4C and 4D). As early as the progenitor phase, 9 genes (C3ar1, Clec7a, Itgb2, Icam4, Cd52, Pilra, Ifitm6, Ptprc and Cd79b) were shown to be expressed and another 9 genes (A630033H20Rib, Cd101, Cd79b, Fcer1g, Fcgr4, Clec7a, Emr1, Lgals9 and Ptprc) in embryonic phases 1 and 2 (Figure 4C and 4D). Expectedly, most of the sensome genes had the highest expression levels in the adult stage (Figure 4C and 4D, Appendix: Table 1). These data suggest that microglia are able to express the sensome *in utero* and that these cells are able to sense and respond to local or systemic changes early during embryogenesis.

3.1.3. Adult microglia acquire a transcriptomic sex-specific signature

Sex-specific differences in microglia have been shown to be consequential to variation in mechanical pain and brain masculinization (Lenz and McCarthy, 2015; Mapplebeck et al., 2016). To probe if sexual dimorphism is implicated in microglia development, we sorted microglia from males and females of E18.5 (embryonic phase) and adult mice for RNA-sequencing analysis. Microglia of E18.5 mice had only 13 DEGs between males and females, most of which are sex-specific genes present on the X and Y chromosomes. Nonetheless, a few genes such as Rac1 and Lamp1 that are involved in cellular remodeling and phagocytosis respectively had a higher expression in the females (Figure 5A). In contrast, microglia from adult mice had 519 DEGs between males and females (Figure 5A and 5B), suggesting that sexual imprinting starts later during development. To understand the biological differences between the males and females in adult mice, we ran a pathway analysis and observed that female microglia showed an upregulation of genes that were link to apoptotic process, inflammatory response and response to lipopolysaccharide (LPS) (Figure 5C). Thus, microglia appears to be in a more immune active state in females

compared to males. This further supports a previous study that revealed females are more “inflammatory” than males (Klein and Flanagan, 2016). We validated the DEGs using real-time quantitative reverse transcription PCR (RTqPCR) (Figure 5D), confirming the sexual dimorphic transcriptomic profiles of microglia, which are prevalent after birth in normal SPF conditions.

Figure 5. Microglia display distinct sexual dimorphism later in development.

(A) Number of DEGs (at least 1.5 FC) observed between males and females of E18.5 and adult mice. Increase or decrease in expression is

based on females compared to males. $n=2-3$ per stage (B) Heat map of corresponding DEGs shown in (A). $n=2$ per condition. (C) DAVID pathway analysis of genes upregulated (at least 1.5 FC) in adult females compared to male. (D) Validation of gene expression between adult males and females by RTqPCR. $n=2-3$ per condition *** $P < 0.001$, nd = not determined.

We generated a heat map of the sensome between male and female adult mice and found that amongst the 100 sensome genes, there were 12 genes that were differentially expressed including *Ccr12*, *Gpr34* and *P2ry13*, suggesting that microglia from different gender could respond differently to environmental cues (Figure 6A).

Figure 6. Differences of male and female microglia in sensing and colonization

(A) Heatmap of expression of sensome genes in microglia of adult females and males. $n=2$ per condition. (B) Coronal sections of somatosensory cortex from E18.5 and adult mice stained for IBA-1 to observe colonization of microglia (Representative of 6 samples per condition). Scale bar (E18.5)=100 μ m (P20)=200 μ m. (C) Quantification of microglia density as observed in (B). Two-sided unpaired Mann-Whitney test was performed. $n=6$ per condition. * $P < 0.05$

Having seen sex-specific gene expression differences in microglia, we wanted to know if such sexual dimorphism affects microglia in terms of colonization pattern and morphology. We studied the density and distribution of microglia, using IBA1 immunostaining, from brains of

embryonic to adult mice of both genders. We examined 3 distinct regions of the brain that control key functions such as motor functions and thermoregulation (Borich et al., 2015; Boulant, 2000); the somatosensory neocortex, the striatum and preoptic area (POA), regions which have been reported to have differential densities between males and females (Lenz et al., 2013). Imaging the neocortex, we noted a minor transient increase in the density of microglia in the females of E18.5 compared to the males (Figure 6B and 6C). This increase was not seen in the adults, where the density of both sex in adults are similar. Moreover, the transient increase was not observed in striatum or POA either (data not shown). Thus, while microglia acquire a divergent sexual transcriptomic signature during development, only a minor transient difference is seen during early development.

3.1.4. Time and sex-specific impact on the microglia transcriptome of GF mice

It has been shown that adult microbiota has an impact on the density, morphology and function of adult microglia (Erny et al., 2015; Matcovitch-Natan et al., 2016). We thus sought to elucidate if there would be an effect of the absence of microbiota on microglia gene expression profile before birth and also if microbiome absence might impact the observed microglial sexual dimorphism. To address this, we sorted microglia from SPF and GF mice from 3 phases of development (E14.5 (embryonic 1), E18.5 (embryonic 2) and adults) for RNA-seq analysis. Microglia from males and females were sorted separately from E18.5.

Interestingly, we observed that the absence of maternal microbiota also has an impact on embryonic microglia. At E14.5, a subtle effect with only 19 DEGs found between SPF and GF microglia (Figure 7A). Most of these genes, which include *Aoah* and *Ly86*, are involved in LPS processing and response (Janelisins et al., 2014; Ortiz-Suarez and Bond, 2016), suggesting that the maternal gut microbiome may indirectly regulate the expression of genes to control its own detection in

the developing embryo. However, at E18.5 just before birth, we observe a major impact on microglia transcriptomic profile that is sex-dependent. There were 1216 DEGs between SPF and GF microglia of males, in contrast to 20 DEGs found in females (Figure 7A-C). Out of the 1216 DEGs in males, a majority of them (1169) are downregulated and only 47 upregulated and also around 20% of the sensome genes were affected. On the other hand, we observed an opposite effect in the adults with 433 genes downregulated in female GF microglia in comparison to female SPF and 26 genes downregulated in males (Figure 7A, D-E). The changes seen in expression levels of the selected genes were validated by RTqPCR (Figure 7F-G). Pathway analysis using the DEGs revealed that the embryonic male GF microglia were linked to translation and metabolism, while adult female GF microglia had a decrease in expression of genes related to inflammatory response in particular to LPS and also translation and enhanced expression of genes linked with regulation of transcription (Figure 7C and 7E). Of note, one gene, Ly86 was significantly differentially expressed at all stages between GF and SPF microglia independent of sex and age. Ly86 have been shown to required for LPS recognition and signaling (Nagai et al., 2002) and also associated with inflammation, insulin resistance and obesity (Su et al., 2014).

Figure 7. Differential gene expression among GF and SPF mice of different ages and sex.

(A) Number of DEGs (1.5 FC) observed in GF and SPF mice at E14.5, E18.5 (male and female) and adult stage (male and female). $n=2-3$ per condition and stage. (B) Heatmap of genes expressed by GF vs SPF and Male vs Female at E18.5. (C) DAVID pathway analysis of enriched genes in microglia of GF females at E18.5. (D) Heatmap of genes expressed by GF vs SPF and Male vs Female at adult stage. (E)

DAVID pathway analysis of enriched genes in microglia of GF female at adult stage. (F-G) Validation of DEGs by RTqPCR between SPF and GF mice (male and female, E18.5 and adult). * $P < 0.05$, *** $P < 0.001$, ns = not significant. FDR= False Discovery Rate.

To determine which networks are affected by development (age), sexual identity and the presence of microbiota, we conducted a weighted gene co-expression network analysis (WGCNA). This approach allowed us to establish genetic modules that are different across conditions i.e. embryonic phase to adult, between males and females and between SPF and GF mice (Appendix 2). Four modules were found to be specific to the embryonic stages, which were mostly associated with cell organization, metabolic processes, translation, protein organization and biogenesis. On the other hand, the adult stage was defined by five modules related to cell migration/chemotaxis, cell morphogenesis, regulation of transcription and immune system response (Appendix 2). This supports our previous finding that microglia follow several developmental phases (Figure 2). Subsequently, we mapped the DEGs of E18.5 SPF/GF and adult SPF/GF microglia. This illustration suggests that during embryonic stages, the absence of microbiota have a drastic effect on genes that are linked to cell/protein organization and translational metabolic processes (Figure 8A-B). Several genes involved in the adaptive immune response were also affected. However, in the E18.5 GF female, no major changes were observed in the network. At the adult stage, many genes that were related to adaptive immune response, regulation of transcription, cell morphogenesis, migration and chemotaxis were altered in GF female microglia (Figure 8A-B). A negligible difference was observed in the adult male microglia. Thus, absence of microbiota has a considerable impact on embryonic male microglia and adult female microglia. Collectively, our work reports that microglia senses the presence or absence of microbiota even before birth during the prenatal phase and subsequently respond in either males or females in a age-dependent manner.

Figure 8. Absence of microbiota has age and sex-dependent effect on microglial transcriptomic profiles.

(A) Illustration of gene co-expression network using Cytoscape. Nodes are coloured according to modules. Co-expression network is based on all genes in WGCNA with a topological overlap of more than 0.3 to at least 1 other gene. Clusters with < 5 nodes were excluded. (B) Differential expression between GF and SPF microglia from E14.5 to adult stage within the gene co-expression network. Blue <1.5 FC, Red >1.5 FC.

3.1.5. Time and sex-specific impact on microglial brain colonization in GF mice

To determine if changes in our gene expression data were linked to modifications in morphology and colonization as reported in adults (Erny et al., 2015), we examined by immunohistochemistry the brains of GF/SPF mice in both embryonic and postnatal stages. Firstly, using NKX2.1 and CTIP2 immunohistochemistry, which label subdivisions of the basal ganglia and neocortex respectively, we confirmed that GF

mice displayed a preserved global forebrain patterning (Figure 9A). Using Isolectin B4 (IB4) staining, we showed that the formation of blood vessels was not severely altered by the absence of the microbiome (Figure 9A). Next, using L1 and TAG1 co-immunostaining, we further confirmed that the axonal tracts of the main forebrain were not drastically altered (Figure 9A). Finally, we examined the cortical layering of the somatosensory cortex in E18.5 brains using deep and upper-layer molecular markers (CTIP2 and CUX1 respectively) (Figure 9B) and quantified the thickness of each of the 5 cortical layers (Figure 9C). While there was no major difference, the thickness level of layer V was increased in all GF mice in comparison to SPF mice (Figure 9C). These data showed that while some minor deficits were observed, GF mice show a relatively preserved brain morphogenesis.

Figure 9. Brain patterning in GF and SPF mice.

(A) Coronal sections of brains from E14.5 GF and SPF mice with NKX2.1, CTIP2, IB4, L1 and TAG1 immunostaining. Scale bar = 500 μ m. (B) Coronal sections of somatosensory cortex of brains from E18.5 GF and SPF (male and females) with staining of CUX1+ layer II, III, IV and CTIP2+ layer V. Scale bar = 100 μ m. (C) Thickness of cortical layer of brains from E18.5 SPF and GF mice. $n=4$, Two-sided unpaired Mann-Whitney test was performed. $*P < 0.05$.

We then proceeded to assess and quantify the microglia density, morphology and colonization in different brain regions during embryogenesis (E14.5, E16.5 and E18.5) and at the postnatal phase (P20) in SPF and GF conditions. In the absence of microbiota, the density of microglia in embryonic GF mice were increased in all 3 regions examined, i.e. the somatosensory cortex, the striatum and POA (Figure 10-12). Similar to a study that reported the role of microbiota on adult microglia morphology (Erny et al., 2015), GF embryonic microglia was described to display excessive ramifications. This phenotype was still observed later on in the somatosensory cortex and moreover appears to have a sexual bias, in line with our transcriptomic analyses. At E18.5, microglia density was significantly higher in males compared to females. However, in adults, we observed an opposite effect where the density was higher in females (Figure 10D-E). Such sex and age-dependent regulation of microglia density is also region-specific, as seen in the striatum and POA, both male and female had an increased in microglia density at E18.5 (Figure 11-12). Collectively, both our transcriptomic and colonization analyses converge to support the idea of sex and age dependent regulation of microglia in GF mice.

Figure 10. Immunohistochemistry analysis of the somatosensory cortex in SPF and GF mice.

(A) Coronal sections of the somatosensory cortex of SPF and GF mice at different ages. Scale bar = 100 μ m for E14.5 to E18.5, = 200 μ m for P20. (B-E) Quantification of IBA-1+ cells in the somatosensory cortex of SPF and GF mice at different ages and sex. $n=3-6$. Two-sided unpaired Mann-Whitney test was performed. * $P < 0.05$, ** $P < 0.01$.

Figure 11. Immunohistochemistry analysis of the striatum in SPF and GF mice.

(A) Coronal sections of the striatum of SPF and GF mice at different ages. Scale bar = 100 μ m for E14.5 to E18.5, = 200 μ m for P20. (B-D) Quantification of IBA-1+ cells in the striatum of SPF and GF mice at different ages and sex. $n=3-6$. Two-sided unpaired Mann-Whitney test was performed. * $P < 0.05$.

Figure 12. Immunohistochemistry analysis of the POA in SPF and GF mice.

(A) Coronal sections of the POA of SPF and GF mice at different ages. Scale bar = 100 μ m for E14.5 to E18.5, = 200 μ m for P20. (B-D) Quantification of IBA-1+ cells in the POA of SPF and GF mice at different ages and sex. $n=3-6$. Two-sided unpaired Mann-Whitney test was performed. * $P < 0.05$.

3.1.6. Microbiota influences the acquisition of sex-specific transcriptomic signatures

Our results prompted us to further probe if the microbiota had a sex-linked effect on the microglia transcriptome. To better understand this, we looked at microglia sexual dimorphism in GF mice at the embryonic age (E18.5) and the adult stage. At E18.5, 283 DEGs were identified between GF males and females, with most of the genes downregulated in GF male (Figure 13A). Analyzing the DEGs via pathway analysis, it was of interest that the microglia in GF males had a decrease in gene

expressions that were related to immune system response (Appendix: Table 2). This implies that the male microglia immune functions are more defective in absence of microbiota. At the adult stage, 322 DEGs that were related to regulation of transcription and immune system response were upregulated in microglia from female GF mice compared to males (Figure 13A). Interestingly, when comparing with SPF controls, the absence of microbiota in GF mice caused an increase in the number of DEGs between males and females of embryonic phase (13 to 283, 8 overlap), while decreasing the number of DEGs in adults (519 to 391, 226 overlap) (Figure 13B). When we perform hierarchical clustering and PCA analysis, we noted that microglia harvested from male or female mice formed distinct clusters, regardless of the presence or absence of microbiota (Figure 13 C-D). This suggests that in the adult stage, the impact of the microbiome is not as prevalent as the sexual identity.

Figure 13. Microbiota's impact on the sexual dimorphism of microglia.

(A) Number of DEGs with a 1.5 fold-change between GF females compared to males at E18.5 and adult. $n=2-3$. (B) Venn diagram of DEGs (with 1.5 FC) between female and male in SPF or GF mice (E18.5 and adult) $n=2-3$. (C) Dendrogram displaying the clustering of male and female of SPF and GF mice at the adult stage. (D) PCA analysis of corresponding samples from (C). PC= Principal component.

Using WGCNA, we performed a gene co-expression analysis that confirmed that the absence of microbiota had an impact on both the embryonic stage (E18.5) and the adult stage of microglia sexual dimorphism (Figure 14). To have a better interpretation of the difference in sexual dimorphism that are specifically affected by the absence of microbiota, we compared the DEGs between males and females in both

SPF and GF mice. Before birth at E18.5, the lack of microbiota had a gender-based effect on immune system. In the adult, this affected genes related to transcription regulation (Appendix: Table 2). However, we also noted that some differences in the transcriptome related to immune response and transcription were similarly affected in females compared to males in both SPF and GF conditions. In all, our data defies the current notion that sex-specificity are controlled intrinsically by demonstrating that microglia sexual dimorphism can be regulated by the gut microbiome, limiting it at the embryonic stage and promoting it at the adult stage.

Figure 14. Co-expression analysis of DEGs (female vs male) from E18.5 to adult in SPF and GF mice. Blue and red nodes represent a fold change of less than or more than 1.5 respectively.

3.1.7. A conserved transcriptomic signature in mid-trimester fetal human microglia

In order to correlate our study of murine microglia development with human microglia development, we also investigated the gene

expression signature of human fetal microglia. We sorted human fetal microglia from 14 weeks to 22 weeks for microarray purposes. Using hierarchical clustering, we observed two main clusters that correlated with early and late stage of gestation, regardless of the sex of the fetuses (Figure 15A). Transcriptomic analysis revealed only 63 DEGs between the early and late clusters that had no meaningful associated pathways (Figure 15B). Pearson correlation amongst the human fetal microglia samples was high (0.97), suggesting that the males and females at this stage were highly similar. There were only 9 DEGs and they were all present on the X and Y chromosomes (Figure 15C). This is in agreement that sexual dimorphism is not established in mid-gestational human microglia, as seen in mice.

We generated a murine core microglial signature of 568 genes, after excluding housekeeping and non-protein coding genes (Appendix: Table 3). Among these genes, 387 genes were found to be expressed in all human fetal microglial samples (Figure 16A). These common genes between murine and human microglia were linked to phagocytosis and immune response. These results thus showed that mouse and human microglia share a highly conserved transcriptomic profile through their core gene signature. We sought to understand the biology behind the 387 common genes. In order to do so, we first divided the 568 mouse core genes into five distinct clusters based on their change in expression across development. For example, cluster 1 consist of genes that had a progressive increase along development while cluster 2 genes had repressed expression before birth and is upregulated at the adult stage (Figure 16B). Running a comparative analysis of the 387 common genes based on these five clusters, revealed that there was a significant enrichment in cluster 1 ((Fisher's exact test; BH adjusted p-value < 0.05). 98 out of 126 genes present in cluster 1 were common to the human fetal microglial genes and are involved in neuron apoptosis and immune response (Figure 16C). Interestingly, cluster 1 genes peaked at E16.5 of murine development, suggesting mid-trimester human fetal microglia are closely related to murine E16.5 stage.

Figure 16. Comparing murine and human microglia.

(A) Venn diagram of mouse microglia core genes and human fetal microglia expressing genes. Gene ontology of 387 common genes analyzed by DAVID. (B) Expression levels of mouse-human microglia common genes based on 5 clusters of mouse core signatures. (C) Gene ontology analysis of cluster 1 from (B). (D) CIBERSORT analysis of human fetal microglia gene expression with mouse developmental signatures. FDR = False discovery rate. ns= not significant. Age shown is weeks + days

In order to investigate further the corresponding stage of mid-trimester human fetal microglia when compared to murine microglia, we employ the CIBERSORT algorithm, which allowed us to understand the contribution of the expression of genes in a dataset (Newman et al., 2015). Using our mouse microglia development microarray dataset (Figure 2) as a reference, the CIBERSORT analysis suggested that human mid-trimester microglia translate closely to the murine microglia of E16.5 stage (Figure 16D). Of note, these stages before birth in both

mouse and human appears to have no sexual dimorphism (Figure 5A and 15).

In our previous mouse data, we showed that microglia sensing capabilities started even before birth with the expression of sensome genes. To elucidate if this applies to human fetal microglia, we analyzed their expression of sensome genes (Appendix 3). The analysis revealed that some sensome genes were highly expressed (Csf-1r, Cx3cr1, Ifngr1, Cxcl16 and Ly86) and some lowly expressed (Tlr4, Tlr6, Tlr7, Cd86, Cd14 and Cd68). In general, the sensome expression along development in mouse and human seems to be similar. Altogether, our results suggest that the mid-trimester human fetal microglia share a core microglia signature common to mouse and are already able to sense and respond to environmental cues *in utero*, but do not exhibit sexual dimorphism as observed in mice.

3.1.8 DISCUSSION

Our results showed that microglia, the major regulator of the central nervous system in normal and pathological conditions, mature along development and subsequently acquire sexual dimorphism. Interestingly, such maturation is regulated by maternal gut microbiota and the lack of maternal gut microbiota affected the normal development of embryonic microglia in a time and sex-dependent fashion. Since many neurological disorders have been shown to have sexual biasness, our findings have major implications with regards to the roles of microglia in health and disease.

3.1.8.1. Embryonic maturation of microglia and expression of sensome genes

A recent study has now shown the maturation of microglia along development (Matcovitch-Natan et al., 2016). Likewise, we have shown that from the yolk sac progenitor stage, to colonization of the brain in the early embryonic stage, and finally to adulthood, we observed a phase-dependent maturation of microglia. During the progenitor phase, we observed an up-regulation of the cell cycle processes associated with early progenitors. Once the progenitors colonize the brain, between E14.5 to birth, the embryonic phase is linked to neuronal development and morphogenesis, suggesting a crucial role of microglia in shaping the brain circuit, as we seen in our previous study which showed the importance of microglia and their interaction with axons and their effect on the adequate positioning of neurons in cortical layers (Squarzoni et al., 2014). Due to the limitation of our human sample being restricted to mid-trimester fetal samples, we were unable to delve deeper into the various stages of human microglia development. However, it was still enriching to know that we were able to translate the mid-trimester human fetal microglia to the E16.5 stage of murine microglia and this common phase in both species are linked to neural development. As our group has recently established a way to derive human microglia-like cells *in vitro* from Induced pluripotent Stem Cells (IPSC), this would provide a platform for better analysis of human microglia development and function in health and disease. Also, in both mice and human microglia, we observed that several sensome genes are already expressed before birth, suggesting that embryonic microglia could have a central role in integrating the local and systemic signals in the developing brain. As described in a previous study, tissue environments typically encompass a unique combination of signaling factors that are able to drive differing programs of gene expression by differential activation of a common set of gene enhancers (Gosselin et al., 2014).

3.1.8.2. Progressive acquisition of sexual dimorphism

It is now known that microglia have varying phenotypes and properties in males and females. They have been shown to have different colonization pattern in males and females, contribute to brain masculinization and appear to be responsible for the differences in pain sensitivity between male and female mice (Lenz et al., 2013; Rebuli et al., 2016; Schwarz et al., 2012). Here, we observed that there was little sexual dimorphism between males and females at the embryonic phase, with only a few X and Y-linked DEGs, whereas during the adult stage, a distinct difference between males and females emerged. In mice, consistent with past studies that showed that females have a higher level of immune activity compared to males (Klein and Flanagan, 2016), our results also suggest that female microglia are more activated in comparison to male microglia, with increased immune responses. Noteworthy, genes related to type I interferon (IFN-I) pathways such as *Irfn1*, *Cxcl10* and *Ccl2*, had a higher expression in females. It was of interest that in a recent study, the authors detected a transcriptional response to IFN-I in astrocytes during experimental CNS autoimmunity and also in CNS lesions from patients with multiple sclerosis (MS) and its murine model, experimental autoimmune encephalomyelitis (EAE) (Rothhammer et al., 2016). In this study, the authors used female mice, commonly used in EAE studies, since only female develop symptoms of the disease (Dalal et al., 1997). By displaying a higher level of IFN-I basal activation in steady state, microglia could also contribute to this sexually dimorphic disease phenotype and this remains to be investigated. In steady state, our data suggest that the role of microglia in regulating proper brain circuit functions might be sexually biased. When looking at the sensome genes between males and females, it was noted that several genes were differentially expressed that thus could cause microglia from different gender to sense and respond differently to foreign and environmental cues during development. As sex differences in microglia remain inadequately described in literature, it is crucial to better understand the underlying mechanisms as well as the effects in additional conditions and species.

3.1.8.3. Sex and time-specific impact of the microbiome

Unexpectedly, adult microglia have been shown to respond to the lack of gut microbiota and thereby quickly changing their morphology, density and gene expression (Erny et al., 2015). In our data, we observed that the maternal microbial environment already has an impact on the fetal microglia. Remarkably, microglia displayed sex-dependent temporal windows of sensitivity to colonization of the microbiome: microglia in the male brain were more affected before birth by the *in utero* microbiota environment while the females were affected more by the microbiome after birth. Regardless of the stage, microglia from GF mice were affected differentially and displayed a dampened immune response that was more striking in the adult stage suggesting hypoactivation or immaturity due to the lack of microbiota. At the adult stage, we saw an increase of basal immune activation state in the female SPF microglia and this could be the reason why they were more affected by the absence of microbiome compared to males. In contrast, embryonic microglia show minor sexual dimorphism in physiological conditions, prompting us to call into question the origin of their sexually differential responses in GF embryos. Further experiments are required to address whether there are additional sexual dimorphic responses to other signals or if environmental cues are relayed to the CNS differentially. No matter the underlying mechanism, we have identified distinct temporal windows that affect microglia in response to environmental cues. Epidemiological studies have shown an increased risk of early onset of schizophrenia or ASD in males due to their susceptibility to early life challenges such as inflammation (Halladay et al., 2015; Ochoa et al., 2012). On the other hand, auto-immune diseases, multiple sclerosis or depression which arise more during the adolescents or adult stage, affects the females more (Ngo et al., 2014; Zagni et al., 2016). Our results, revealing that microglia are affected by environmental challenges in a time and sex-dependent manner, thus provide a

potential link or causative factor to the sex bias in neurological disorders.

3.1.8.4. Microbiome influences microglial sexual dimorphisms

Sexual dimorphism has generally been known to arise due to intrinsic factors such as genetic programming and hormonal influence (Freedman et al., 2004; Sinclair et al., 1990). Intriguingly, in our study, we found that the lack of microbiota has a drastic effect on the sexual dimorphism of microglia, affecting the males prenatally and the females after birth. We hypothesize that microglia may first initiate the expression of some genes to recognize the external signals including microbiome-derived metabolites, which conversely promote the establishment of sexual dimorphism (Figure 17). Some potential microbiota metabolites include short-chain fatty acids or metabolism of tryptophan that may travel to the brain through the circulation (Cryan and Dinan).

Our data thus demonstrated that in addition to the prevailing concept that sex biasness in gene expression is essentially due to intrinsic genetic programs, extrinsic factors are also able to influence the sexual dimorphism of microglia. This discloses a novel interaction between sex-specific properties and environmental factors that has major implications in health and disease. In light of studies that link microbiota to neurological diseases such as Alzheimer's disease (Harach et al.), we hypothesize that microbiota could be a factor leading to sexual biasness in such diseases. All in all, this study highlights the need to precisely identify and take into consideration the sex of subjects when diagnosing and treating neurological disorders.

Figure 17. Interplay between sensing microglia, microbiota and sexual dimorphism.

In the brain, immature microglia express the sensome genes and are able to sense and respond to environmental signals including metabolites from gut microbiota. The signals from the microbiota promote sexual dimorphism in microglia leading to differences seen in the transcriptome between male and female microglia.

4. Conclusion and future directions

The development of microglia is not as linear as we imagined. Numerous factors, intrinsic or extrinsic, influence their maturation and obstacles may perturb their development. We confirmed that microglia differentiation involves distinct developmental phases and start to acquire sexually dimorphic transcriptomic signatures in adulthood. This could explain the sexual bias seen in neurological disorders.

Even external factors such as the presence or absence of commensal microbiota have an impact on microglia. We know the gut-brain axis exist and that the microbiota have an effect on the brain through the enteric nervous system that is in direct contact with the microbiome (Carabotti et al., 2015). However, it is still unclear how exactly do these commensal microbes send signals to the microglia that are in constant contact with neurons of the CNS. Microbiota have been shown to be implicated in neurological disorders such as Parkinson's disease and other neuropsychiatric disorders (Mulak and Bonaz, 2015; Petra et al., 2015). Thus, the exact mechanism behind the interaction between microbiota, microglia and neurological disorders needs to be elucidated.

Furthermore, our study highlighted that microbiota affects sexual dimorphism, thus suggesting the possibility that sexual biasness seen in neurological disorders may be caused by the microbiome. Further research includes elucidating if specific commensal bacteria are involved in causing sexual dimorphism in microglia and the exact mechanism of microbiota–microglia communication. Using males and females of ageing models, such as Senescence Accelerated Mouse-Prone 8 (SAMP8) (Butterfield and Poon) that is susceptible to neurological disorders like Alzheimer's disease, and subjecting them to germ-free conditions could allow us to understand the interplay of factors in diseases.

Thus, when targeting microglia and their responses for treatment of diseases or disorders, there are many considerations. Treatments for

male or female patients of different ages will be different. This is in line with the trend of personalized medication, which will be more effective and efficient in treating patients.

5. Appendix

Appendix 1: The seven clusters characterizing the different developmental phases of microglia. The plots show the expression of the corresponding genes that are associated with these functions during development.

Appendix 2: A) Module trait correlation analysis. Each row represents a module eigengene (ME) and each column a trait. The corresponding correlation (top) and p-value (bottom) are indicated in each cell. B) GO terms associated with each module. GO terms were ranked by p-value and the top 5 processes were listed.

Appendix 3: Heatmap of expression of sensome genes in human microglia. n=10.

Table 1: Available online here

(<https://www.dropbox.com/s/44hgy4jrlz9i8d7/Table%201.pdf?dl=0>)

Table 2: Available online here

(<https://www.dropbox.com/s/fb9p8az92z882k6/Table%202.xlsx?dl=0>)

Table 3: Available online here

(<https://www.dropbox.com/s/6uhv5znc56vwojc/Table%203.pdf?dl=0>)

Table 4: Available online here

(<https://www.dropbox.com/s/srcxliq20m69v4l/Table%204.xlsx?dl=0>)

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Zagni, E., Simoni, L., and Colombo, D. (2016). Sex and Gender Differences in Central Nervous System-Related Disorders. *Neuroscience journal* 2016, 2827090.

7. List of publications

Below is a list of journal articles published during the course of PhD:

1. **Low D**, Ginhoux F. Recent advances in the understanding of microglial development and homeostasis. *Cellular Immunology*. 2018. [In proof]
2. Thion M*, **Low D***, Silvin A, Chen J, Grisel P, Ulas T, Squarzoni P, Scholz C, Sophie-David F, Lum J, Amoyo A, Larbi A, Poidinger M, Buttgereit A, Greter M, Chan J, Schultze J, Schlitzer A, Pettersson S, Ginhoux F, Garel S. Microbiome influences prenatal and adult microglia in a sex-specific manner. *Cell*. 2017 Dec 21. pii: S0092-8674(17)31432-0. doi: 10.1016/j.cell.2017.11.042. [Epub ahead of print] ***Equal contribution**
3. McGovern N, Shin A, Low G, **Low D**, Duan K, Yao LJ, Msallam R, Low I, Shadan NB, Sumatoh HR, Soon E, Lum J, Mok E, Hubert S, See P, Kunxiang EH, Lee YH, Janela B, Choolani M, Mattar CNZ, Fan Y, Lim TKH, Chan DKH, Tan KK, Tam JKC, Schuster C, Elbe-Bürger A, Wang XN, Bigley V, Collin M, Haniffa M, Schlitzer A, Poidinger M, Albani S, Larbi A, Newell EW, Chan JKY, Ginhoux F. Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. *Nature*. 2017 Jun 29;546(7660):662-666.
4. Lum FM, **Low DK**, Fan Y, Tan JJ, Lee B, Chan JK, Rénia L, Ginhoux F, Ng LF. Zika Virus Infects Human Fetal Brain Microglia and Induces Inflammation. *Clin Infect Dis*. 2017 Apr 1;64(7):914-920.
5. Xu X, Tay Y, Sim B, Yoon SI, Huang Y, Ooi J, Utami KH, Ziaei A, Ng B, Radulescu C, **Low D**, Ng AY, Loh M, Venkatesh B, Ginhoux F, Augustine GJ, Pouladi MA. Reversal of Phenotypic Abnormalities by CRISPR/Cas9-Mediated Gene Correction in Huntington Disease

- Patient-Derived Induced Pluripotent Stem Cells. *Stem Cell Reports*. 2017 Mar 14;8(3):619-633.
6. Andreasson KI, Bachstetter AD, Colonna M, Ginhoux F, Holmes C, Lamb B, Landreth G, Lee DC, **Low D**, Lynch MA, Monsonego A, O'Banion MK, Pekny M, Puschmann T, Russek-Blum N, Sandusky LA, Selenica ML, Takata K, Teeling J, Town T, Van Eldik LJ. Targeting innate immunity for neurodegenerative disorders of the central nervous system. *J Neurochem*. 2016 Sep;138(5):653-93.
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