Exploring the VISTA of glial cells

Borggrewe, Malte

DOI:
10.33612/diss.168886037

1

General introduction and outline of the thesis

Contents

Partly adapted from review *"Exploring the VISTA of microglia"*
published in Journal of Molecule Medicine, 2020

Preface

Multiple sclerosis (MS) is a chronic, demyelinating, autoimmune disease of the central nervous system (CNS), which manifests at a young age (usually <40 years). The cause of MS is unknown and in combination with a highly individual disease course, this heterogeneous disease is difficult to study and to treat. **MS treatment** has improved dramatically over the past 25 years but does not fully arrest the disease. Current treatments focus on limiting inflammatory responses, which can alleviate symptoms and aid in resolving CNS inflammation. However, as MS course is different in each patient, these treatments are not always beneficial and can have severe side effects, since physiological immune responses can become impaired. **Immune checkpoints** are receptor/co-receptor pairs that provide a balance between maintaining a properly functioning immune system, while inhibiting aberrant inflammation or autoimmunity. These immune checkpoints can be targeted therapeutically to modulate the immune response, e.g. to induce immunity against tumors, or by reducing immunity during inflammation or autoimmune diseases. **V-type immunoglobulin domain-containing suppressor of T-cell activation (VISTA)** is a negative checkpoint regulator (NCR) that provides inhibitory signals to T cells in order to reduce immunity. Modulating VISTA in MS may offer a novel strategy to limit autoimmunity and alleviate symptoms. This thesis will shed light on the function and expression of VISTA in the CNS and how it is deregulated in MS.

The three types of **CNS glial cells** are oligodendrocyte, microglia and astrocytes, which are involved in maintaining CNS homeostasis and are directly implicated in MS and other CNS disease. **Microglia and astrocytes** contribute to the neuroinflammatory cascade in MS but can also be beneficial as they aid in resolving inflammation and promoting tissue repair. In MS, there is an intricate interaction of different cell types including glial cells and infiltrating immune cells such as T cells and monocytes. Receptors such as NCR and VISTA are essential molecules for cell-to-cell communication and regulating immune responses during MS; however, these mechanisms are not well understood. **In this thesis**, transcriptional profiles and heterogeneity of astrocytes during homeostasis and experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, are characterized. Furthermore, the function and expression of VISTA in the CNS and microglia during health and neuroinflammation (especially MS) are delineated. Together, these data will help to understand the function of glial cells and their communication with distinct infiltrating immune cell types during MS and extend the rationale for novel immunotherapy targeting VISTA.

From development in utero to disease in adulthood, microglia are essential for resolving perturbations in order to maintain CNS homeostasis. Microglia are already present in the developing CNS before neurogenesis, astrogenesis, and myelination occur, but their role in CNS development in humans remains largely unexplored. **CNS development** is a dynamic and intricate process, and microglia dysfunction has directly been coupled to neurodevelopmental disorders and neurological complications later in life. This thesis will provide insights into human microglia development and how they might be involved in neurodevelopmental disorders.

Glial cells: more than neurons little helper

Neurons are the CNS cell type that conducts electrical impulses, resulting in sensation, muscle contraction, memory formation, learning, and more. Neurons signal via a dense network using specialized connections of axons and synapses. However, neurons are dependent on glial cells for their survival and proper functioning.

The role of glial cells in the CNS

Glial cells (also called neuroglia), named after initial theories that these cells merely act as "nerve glue", were discovered by Rudolf Virchow (Virchow, 1858), and further characterized by Santiago Ramon y Cajal, Pio del Rio-Hortega, and others in the 19th and early 20th century (Del Rio-Hortega, 1919; Cajal, 1995). It is now appreciated that glial cells are essential for healthy brain homeostasis, propagation of neuronal signaling, and directly involved in ageing and virtually all CNS diseases including neurodegenerative diseases (NDD; e.g. Alzheimer's disease (AD), Parkinson's disease (PD), fronto-temporal dementia (FTD)), MS, CNS-associated tumors, and psychiatric diseases (e.g. depression, schizophrenia).

The three main types of glial cells in the CNS are astrocytes, microglia, and oligodendrocytes.

Astrocytes are star-shaped cells with hundreds of thousands of processes that are involved in synaptic transmission, blood-brain barrier (BBB) function, and have many more functions (see "Focus on astrocytes").

Microglia are the tissue macrophages of the CNS parenchyma and have immune functions such as antigen presentation, cytokine production, and phagocytosis. In addition to functions similar to other tissue macrophages, microglia have CNS-specific abilities that are essential for synapse maintenance and neuronal functions. Microglia origin, development, and function during homeostasis and disease are reviewed below (see "Microglia in the spotlight").

Oligodendrocytes are the myelinating cells of the CNS. When oligodendrocyte progenitor cells (OPC) differentiate into oligodendrocytes, they will form sheaths of myelin that wrap around axons in order to provide insulation, which is essential for saltatory action potential conduction (Bradl and Lassmann, 2010). Furthermore, oligodendrocytes provide trophic support to neurons by producing neurotrophic factors and metabolites. In the autoimmune disease MS, the immune system attacks myelin sheaths and oligodendrocytes, which results in death of these cells and damaged axons (Bradl and Lassmann, 2010) (see "A brief guide to multiple sclerosis"). Myelination mainly occurs during development, but OPC also exist in adult brains and can differentiate into oligodendrocytes to form new myelin sheath. Therefore, regeneration of myelin e.g. during MS is possible, albeit less effective compared to the original myelination (Franklin and Ffrench-Constant, 2017).

Focus on astrocytes

Astrocytes are involved in multiple functions of the CNS including synaptic transmission and plasticity, myelination, BBB, structural-, and trophic support. The star-shaped cells have a complex morphology with millions of thin processes that extend through the CNS parenchyma and are in contact with other CNS-resident cells. In the human brain, an individual astrocyte is in contact with up to two million neuronal synapses (Oberheim et al., 2009), which is essential for proper signal transduction.

Astrocytes are part of the tripartite synapse where they regulate synaptic transmission by releasing and sequestering ions and metabolites. Activity of synapses triggers a Ca^{2+}-dependent response in astrocytes leading to the secretion of neuroactive compounds called glia transmitters (Allen and Eroglu, 2017; Xin and Bonci, 2018). Astrocytes express a range of receptors for neurotransmitter such as glutamate, ATP, and GABA rendering them capable of directly responding to synaptic signaling (Allen and Eroglu, 2017). Synaptic transmission requires a fine balance of ions and neurotransmitters and astrocytes are involved in keeping this balance by sequestering potassium and glutamate released by neurons (Kuffler, 1967; Allen and Eroglu, 2017). An accumulation of potassium due to, for example, improper uptake by astrocytes can lead to epileptic neuronal activity (Tanaka et al., 1997; Allen and Eroglu, 2017).

Neurons also depend on astrocytes for their energy supply since astrocytes provide lactate that neurons are unable to produce on their own. Astrocytes metabolize glycogen to generate lactate which is secreted and can be taken up by neurons when they need additional energy (Tsacopoulos and Magistretti, 1996; Xin and Bonci, 2018). This mechanism is important for learning and synaptic plasticity. Astrocytes are also the main cholesterol producer which is required by neurons and oligodendrocytes. Oligodendrocytes need cholesterol and other lipids provided by astrocytes for proper myelination, especially during development (Camargo et al., 2017).

Astrocytes are part of the BBB providing structural support and regulating ion, protein, and water homeostasis (C. Y. Liu et al., 2018). The BBB is a tissue barrier that controls the influx of solutes from blood and consists of endothelial cells and astrocytes (Bechmann et al., 2007). Astrocytes cover the endothelial layer with projections called astrocytic end feet and form the glia limitans. Therefore, astrocytes are essential in bridging the communication between the periphery and the CNS.

During CNS disease and ageing, astrocytes can lose their aforementioned homeostatic functions and acquire a reactive phenotype. A reactive astrocyte phenotype can contribute to CNS dysfunction (Pekny et al., 2016), but may also be beneficial by containing local damage and supporting tissue regeneration (Alilain et al., 2011; Liddelow and Barres, 2017). Astrocytes can acquire at least two different reactive phenotypes, detrimental and beneficial, sometimes also referred to as "A1/A2" reactive astrocytes, respectively (Zamanian et al., 2012). A beneficial reactive astrocyte phenotype was observed in transient middle cerebral artery occlusion (tMCAO) in mice (Zamanian et al., 2012), suggesting astrocytes may promote regeneration after stroke. A detrimental reactive astrocyte phenotype was characterized after lipopolysaccharide (LPS) administration in mice, mimicking and inflammatory insult (Zamanian et al., 2012). This reactive phenotype is induced by microglia through secretion of IL1a, TNF, and C1q (Liddelow et al., 2017). Markers of this detrimental phenotype are also expressed by astrocytes during ageing, in NDD (AD, PD), and MS, suggesting that an astrocyte phenotype consistent with LPS-stimulated neurotoxic astrocytes can occur in human disease and that reactive astrogliosis may contribute to CNS dysfunction in ageing and during disease.

Reactive astrocytes upregulate a variety of proteins and genes involved in immune response, neurotoxic factors, and cytokines. For example, reactive astrocytes induce expression of MHC-II (Zamanian et al., 2012), suggesting that they are able to stimulate T-cell activation. Mouse astrocytes are capable of inducing antigen-specific $CD4^{pos}$ and $CD8^{pos}$ T-cell proliferation

in vitro after induction of MHC-II and B7 co-stimulatory molecules by IFNg (Cornet et al., 2000). Human astrocytes also upregulated MHC-II after IFNg and TNF stimulation in vitro; however, they do not induce T-cell proliferation, likely due to a lack of expression of co-stimulatory receptors (Weber et al., 1994). In contrast, in vitro stimulation of human astrocytes with phosphorylated alpha synuclein, a protein that accumulates in PD, induces co-stimulatory and co-inhibitory receptor expression (PDL1, CD80/CD86, CD40) (Rostami et al., 2020). Hence, whether astrocytes can act as antigen-presenting cells (APC) to induce T-cell activation remains controversial.

Glial cell heterogeneity

The division of glial cells into the major cell types (oligodendrocytes, microglia, astrocytes) is based on functionality and morphological observations. Since multiple functions are attributed to each of these glial cell types, it is conceivable that different subtypes within these compartments exist. Rapid technological advances in recent years enabled further dissection and refinement of cellular subpopulations. Cellular heterogeneity can be examined using single cell approaches such as single cell mRNA sequencing (scRNAseq) or mass cytometry time-of-flight (CyTOF). These technologies are now widely accessible, and their increasingly high throughput allows detection of low-frequency cellular subtypes. Single cell approaches are extensively used in glial cell research and it is now appreciated that within each glial cell type population there is considerable and dynamic cellular heterogeneity (Zeisel et al., 2015), which depends on anatomical region, contact to other cell types, and disease condition. Crudely dividing glial cell types into major classes (oligodendrocytes, astrocytes, microglia) and regarding these cell types as homogeneous populations is obsolete. It is clear that glial cells are highly heterogeneous and are able to rapidly adjust to their environment.

Astrocyte heterogeneity was already observed several decades ago, where astrocytes based on GFAP immunohistochemical staining were divided into fibrous and protoplasmic types, associated with white matter (WM) and grey matter (GM), respectively (Miller and Raff, 1984). Interregional heterogeneity of astrocytes is well-described and changes in transcriptomic profiles follow the dorsoventral axis from cortex to thalamus (Morel et al., 2017). Furthermore, ion channel expression is different in astrocytes across different anatomical regions (Zhang and Barres, 2010). Commonly used astrocyte markers such as GFAP and GLT1/SLC1A2, which were initially regarded as pan-astrocyte markers, only label subsets of astrocytes and can distinguish astrocyte subtypes (Zhang and Barres, 2010; Morel et al., 2019). More extensive flow cytometry analysis of multiple surface markers revealed that astrocytes can be further divided into five subtypes with varying frequency based on anatomical region (John Lin et al., 2017). It is likely that using different or larger panels of surface markers, many more astrocyte subtypes can be detected. ScRNAseq offers a more unbiased approach to define cellular subtypes as it does not depend on specific surface markers. However, to enrich for a specific cell population such as astrocytes, widely expressed markers (e.g. ALD1L1 or ACSA-2 /ATP1B2 for astrocytes) are still frequently used. Examining 1800 individual ACSA-2^{pos} astrocytes in mouse cortex and hippocampus identified five subtypes that differ in expression of genes involved in synapse function, neurotransmission, ion transport, immune functions, and more (Batiuk et al., 2020). Including more cells in scRNAseq analyses will allow detection of even smaller subsets of glial cells that may have unique functions in the CNS. As described above, reactive astrocytes subtypes also exist and can be divided into at least two subtypes with distinct functions (Zamanian et al., 2012; Liddelow and Barres, 2017; Liddelow et al., 2017), suggesting that distinct astrocyte subtypes differentially contribute during CNS disease.

A similar cellular heterogeneity is attributed to microglia, since they are highly dynamic and dependent on local environmental cues provided, for example, by different anatomical regions or contact to different cell types. Microglia from different regions exhibit distinct transcriptional profiles and varying phagocytic capacity (Grabert et al., 2016; Ayata et al., 2018). Recent scRNAseq studies described a more homogeneous population of microglia in healthy adult mouse and human brain (Krasemann et al., 2017; Hammond et al., 2019; Li et al., 2019; Masuda et al., 2019), leaving the issue of regional diversity of microglia contentious. During CNS development and disease in mice, however, microglia heterogeneity is apparent and subtypes likely differentially contribute to various biological processes (Matcovitch-Natan et al., 2016; Keren-Shaul et al., 2017; Krasemann et al., 2017; Hammond et al., 2019; Li et al., 2019) (see "Microglia development follows pathway distinct from macrophages" and "Microglia during CNS disease").

Single nuclear mRNA sequencing (snRNAseq) emerged as an alternative approach to scRNAseq to capture CNS cell heterogeneity during health and disease using nuclei instead of whole cells (Lake et al., 2016; Hu et al., 2017; Mathys et al., 2019). Advantages of snRNAseq are that it can be done on frozen tissue without the use of cell surface markers and cell sorting. Although expression profiles of microglia nuclei and cells are very similar (Gerrits et al., 2020), expression of specific sets of genes might not be captured properly in microglia nuclei (Thrupp et al., 2020). Additionally, efficiency of nuclei isolation may be different for nuclei from distinct cell subsets, potentially resulting in biased data.

Future technological advances allowing more detailed tracking of even larger numbers of cells/nuclei over time will likely reveal that glial cells cannot be separated into discrete subtypes but rather are highly dynamic and plastic. Thus, compartmentalization of cell populations should be done with great caution, especially when studying highly complex and dynamic environments, which are present, for example, in CNS development and disease.

Microglia in the spotlight

Microglia are the resident immune cells of the CNS and possess similar functions as tissue macrophages such as antigen presentation, phagocytosis, respiratory burst, and release of cytokines and chemokines (Colonna and Butovsky, 2017). In contrast to other tissue-macrophages, microglia exhibit a range of CNS-specific functions including synaptic pruning (the elimination of unused synapses), and the release of neurotrophic as well as neurotoxic factors (Colonna and Butovsky, 2017). Microglia are evenly dispersed throughout the CNS parenchyma and once every few hours scan the entire parenchyma for damage or intruders (Colonna and Butovsky, 2017). Scanning of the parenchyma is facilitated by dynamically moving processes which give microglia a typical homeostasis-associated ramified morphology (Glenn et al., 1992). Since microglia are constantly scanning their environment under homeostasis, and are highly sensitive and responsive towards any perturbations, the notion of viewing resting microglia as inactive tissue macrophages has become obsolete.

Ontogeny and maintenance of microglia

Microglia are CNS-resident tissue macrophages with unique functions and origin. Whereas most tissue macrophages are constantly renewed by monocytes derived from bone marrow

hematopoiesis, microglia colonize the CNS early during embryonic development and are a largely self-maintaining population under homeostatic conditions.

During embryonic development, there are three waves of hematopoiesis: primitive hematopoiesis occurring in the extra-embryonic yolk sac, a transient wave where erythromyeloid progenitor cells migrate from yolk sac to fetal liver, and definitive hematopoiesis resulting in hematopoietic stem cells and occurring throughout human life (Bennett and Bennett, 2020). Microglia originate from primitive hematopoiesis in the yolk sac and colonize the CNS at embryonic day 9.5 (E9.5) in mice (Ginhoux et al., 2013; Ginhoux and Prinz, 2015; Hoeffel et al., 2015). However, a subset of microglia may also derive from erythromyeloid progenitor cells in the fetal liver during the transient wave and seed the CNS at E12.5 (De et al., 2018; Bennett and Bennett, 2020).

Insights into the origin of microglia is mainly based on genetic fate mapping experiments conducted in mice and it is difficult to assess whether a similar ontogeny can be attributed to microglia in humans. During human development, amoeboid microglia can be detected at the edge of the CNS in the leptomeninges, the ventricular edge, and the choroid plexus as early as gestational week 4.5 (GW4.5) (Monier et al., 2007; Menassa and Gomez-Nicola, 2018), which overlaps with yolk sac-derived hematopoiesis in humans (Tavian et al., 1999). Microglia enter the CNS from these sites and appear in the parenchyma at GW9-12, depending on the anatomical region (Monier et al., 2006, 2007; Menassa and Gomez-Nicola, 2018).

After local proliferation during development, microglia are a self-maintaining population that does not depend on hematopoiesis-derived turnover like most other tissue macrophages. Microglia are long-lived cells with a lifespan of several months up to years in mice (Füger et al., 2017; Tay et al., 2017). In humans, it has been predicted that some microglia can live up to 20 years (Réu et al., 2017), however, much higher turn-over rates of less than a year were also proposed (Askew et al., 2017). The lifespan of human microglia is therefore not completely resolved and requires further research. Ablating microglia in mice using a CSF1R inhibitor revealed that microglia renewal is dependent on resident cells rather than on infiltrating monocytes (Ajami et al., 2007). After CSF1R inhibition, a small proportion (approximately 1%) of microglia survives which proliferates and replenishes the microglia population following withdrawal of the inhibitor (Huang et al., 2018; Najafi et al., 2018).

Microglia development follows pathway distinct from macrophages

The appearance of microglia in the CNS at E9.5 in mice and GW4.5 in humans precedes neurogenesis, astrogliogenesis, oligodendrogenesis, and myelination (Fig. 1) (Menassa and Gomez-Nicola, 2018); hence, microglia may be involved in these developmental processes.

In mice, microglia development is driven by transcriptional and epigenetic changes. Early during development (E10.5-12.5), transcriptional programs responsible for microglia proliferation are active (Matcovitch-Natan et al., 2016; Hammond et al., 2019; Li et al., 2019), whereas programs for support of neuronal development (e.g. synaptic pruning) are active in later embryonic stages (E14.5-16.5) (Matcovitch-Natan et al., 2016). At early postnatal stages, mouse microglia acquire a homeostatic and immune-surveillance phenotype (Matcovitch-Natan et al., 2016; Hammond et al., 2019; Li et al., 2019) (see "Microglia roles in development and homeostasis") and deletion of the transcriptional regulator MAFB disrupts this homeostasis (Matcovitch-Natan et al., 2016). Heterogeneity of microglia is much higher

during mouse development compared to adult CNS (Hammond et al., 2019; Li et al., 2019). One microglia subtype that features a proliferative and phagocytic phenotype is associated with WM tracts (Hammond et al., 2019; Li et al., 2019) and might be involved in phagocytosis of newly formed oligodendrocytes. This phagocytic developmental microglia subtype shares similarities with an immune-activated/phagocytic microglia phenotype observed in NDD (Keren-Shaul et al., 2017) (see "Microglia during CNS disease"). During neurogenesis, uptake of newly formed and apoptotic cells is essential to allow proper neuronal development (Marín-Teva et al., 2004; Wakselman et al., 2008; Sierra et al., 2010; Cunningham et al., 2013). Especially in postnatal development, synaptic pruning is essential for learning and formation of memory and is performed by microglia (Paolicelli et al., 2011; Zhan et al., 2014) (see "Microglia roles in development and homeostasis").

Microglia development in humans is not characterized in detail to date. Most observations of human microglia development are based on immunohistochemical studies (Monier et al., 2006, 2007; Menassa and Gomez-Nicola, 2018), likely due to unavailability of fresh post-mortem fetal and embryonic CNS tissue. One study investigated gene expression profiles of microglia in bulk from GW14-23 fetuses which suggested most overlap with mouse E16.5 microglia (Thion et al., 2018). These GW14-23 microglia already express markers associated with an immune-surveillance microglia phenotype (Thion et al., 2018). However, investigating a bulk microglia population cannot capture cellular heterogeneity and shifts in microglia subtypes. Since mouse microglia are highly heterogeneous during development and specific subtypes are associated with developmental functions, a single cell approach will be necessary to dissect human microglia development in detail.

Microglia roles in development and homeostasis

Like other tissue macrophages, microglia exhibit classical innate immune functions such as phagocytosis, antigen presentation, cytokine production, and chemotaxis. They will respond to infiltrating viruses and bacteria by eliciting an immune response and promoting tissue regeneration after the infection is cleared. Tissue regeneration is achieved by phagocytosing apoptotic cells (efferocytosis), clearing waste, and releasing anti-inflammatory factors. It is important to note that microglia are distinct from other tissue macrophages not only due to their unique origin and self-maintenance capacity (see "Ontogeny and maintenance of microglia"), but also due to their specialized CNS-associated functions. These functions include synapse maintenance through pruning and modulation, and release of neurotrophic factors, which is essential for proper CNS functioning and homeostasis (Li and Barres, 2017).

Microglia are crucial for neurogenesis and synapse modulation, which is particularly relevant for CNS development (Cheadle et al., 2020; Diaz-Aparicio et al., 2020). The P2Y12 receptor (P2RY12) expressed on microglia senses neuronal-derived ATP which acts as a chemoattractant (Dissing-Olesen et al., 2014; Li and Barres, 2017). Through the classical complement cascade (CR3 and C3) (Stevens et al., 2007; Schafer et al., 2012), microglia eliminate unused synapses, a process called synaptic pruning. Synaptic pruning is essential for developing and maintaining a properly functioning neuronal network (Paolicelli et al., 2011). As such, synaptic pruning is particularly important during development and early childhood, but also occurs later in life, where it is involved in disease-associated synapse loss (Stephan et al., 2012). Synaptic pruning depends on neuronal-microglia interaction via CX3CR1 (Reshef et al., 2017). Depleting or reducing microglia numbers impairs synaptic

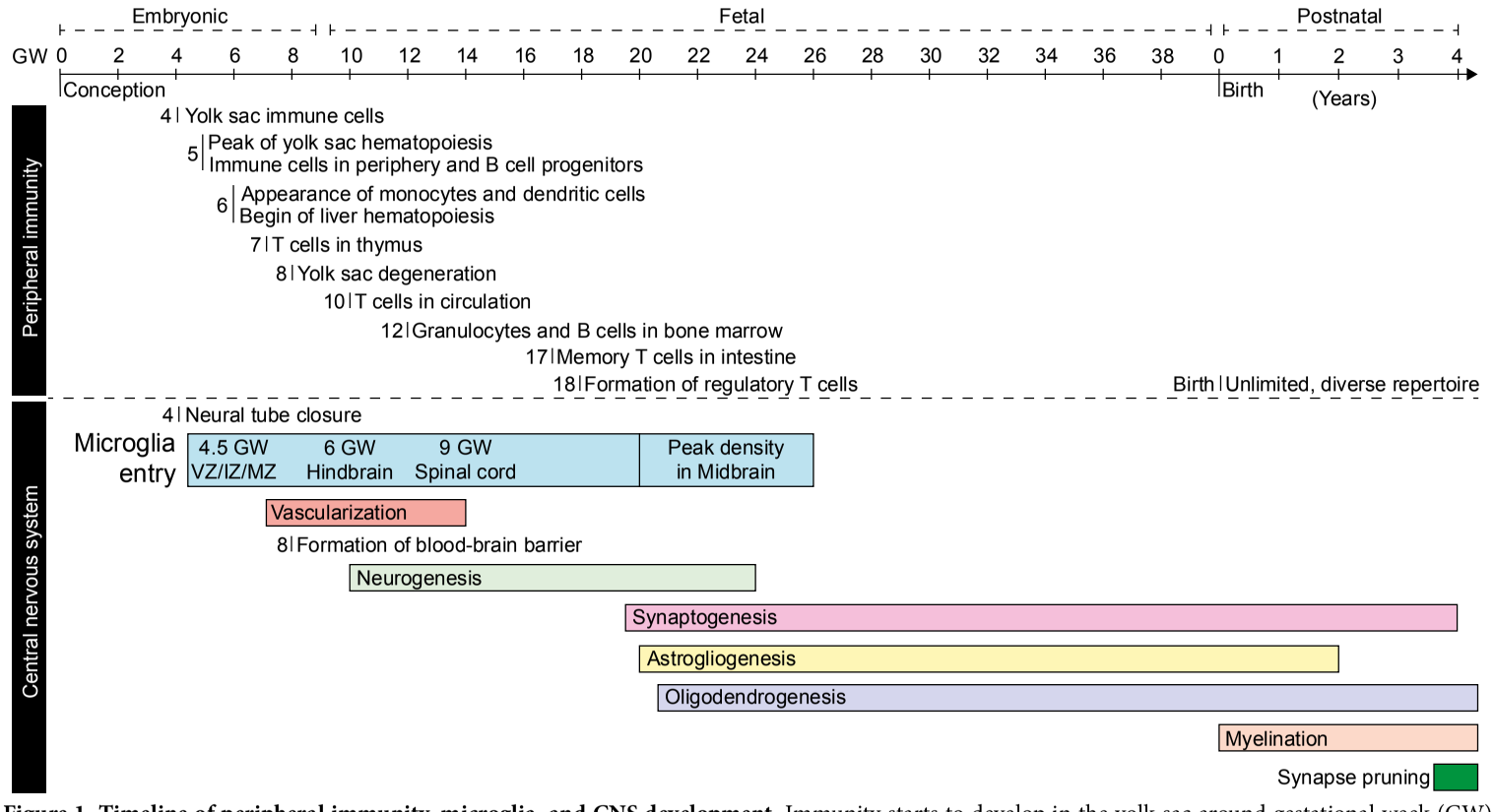

Figure 1. Timeline of peripheral immunity, microglia, and CNS development. Immunity starts to develop in the yolk sac around gestational week (GW) 4, following appearance of B cell progenitors, monocytes, and dendritic cells around GW 5-6. Hematopoiesis transitions to the liver at GW 6 and the yolk sac degenerates shortly thereafter at GW 8. First immune cells appear in the bone marrow at GW 12 and around GW 17-18, memory and regulatory T cells are formed. Microglia enter the CNS at 4.5 weeks of gestation before neurogenesis, astrogliogenesis, oligodendrogenesis and other development processes. By 9 weeks of gestation, microglia have infiltrated other regions of the CNS including the hindbrain and spinal cord. This scheme is based on illustrations from published reviews (Menassa and Gomez-Nicola, 2018; Park et al., 2020).

pruning, leading to weak synaptic transmission and impaired circuitry, which is associated with autism-like behavior in mice (Paolicelli et al., 2011; Squarzoni et al., 2014; Zhan et al., 2014). Microglia also support neurons by releasing BDNF (Coull JA et al., 2005) and taking up apoptotic cells, especially in neurogenic zones (Marín-Teva et al., 2004; Wakselman et al., 2008; Sierra et al., 2010; Cunningham et al., 2013). Phagocytosis of apoptotic cells supports tissue homeostasis and is facilitated by microglia TAM receptors TYRO3, AXL, and MER (Fourgeaud et al., 2016).

During homeostasis, human and mouse microglia exhibit a characteristic gene expression profile, with low expression of immune-related genes and high expression of homeostatic genes such as CX3CR1, P2RY12/13, and TMEM119 (Hickman et al., 2013; Galatro et al., 2017; Gosselin et al., 2017; Dubbelaar et al., 2018). Approximately 100 of these homeostatic genes encode for receptors that are involved in environmental sensing, called the sensome, which are downregulated during ageing and disease (Hickman et al., 2013, 2018; Keren-Shaul et al., 2017) (see "Microglia during CNS disease"). Interestingly, a sexual dimorphism was observed in microglia transcriptomes. Female microglia exhibit higher expression of immune-related genes than males at steady-state, and sex differences influence the morphology and response of microglia towards environmental perturbations such as infection (Hanamsagar et al., 2017; Thion et al., 2018).

In conclusion, microglia exhibit multiple CNS-specific functions that are important for neurogenesis, maintenance of neuronal circuitries, and homeostasis. Disruption of proper microglia function is therefore associated with neurodevelopmental disorders such as autism, neurodegeneration, and neuroinflammation in adulthood.

Microglia during CNS disease

Microglia are highly dynamic cells that respond quickly to environmental perturbations in an effort to restore homeostasis. Genome-wide association studies (GWAS) have placed microglia in focus of CNS-associated disease research, since many disease risk genes, for example for AD and MS, are predominantly expressed by microglia in the CNS (Marioni et al., 2018; McQuade and Blurton-Jones, 2019; Nott et al., 2019). As knowledge on microglia in neurological disease expands, it is now appreciated that microglia are involved in ageing and most CNS diseases including neurodevelopmental disorders (autism, intellectual disabilities), psychiatric disorders (schizophrenia, depression), CNS inflammation (infections, MS), and NDD (AD, PD, FTD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS)) (Li and Barres, 2017; Mondelli et al., 2017; Dubbelaar et al., 2018; Voet et al., 2019; Masuda et al., 2020).

The microglia disease phenotype deviates from the homeostatic phenotype and is highly dependent on the local microenvironment such as disease condition, anatomical region, and contact with other cell types. Activated microglia during disease can thus acquire a range of phenotypes with different functions including antigen presentation, phagocytosis, and release of pro- and anti-inflammatory cytokines. According to macrophage nomenclature, this activated microglia phenotype was initially defined in the M1-M2 continuum; however, the wide range of their activation phenotypes rendered this definition unproductive and contentious for microglia biology (Ransohoff, 2016).

During ageing and CNS disease, microglia can acquire different morphologies, pointing towards a deviation from the homeostatic phenotype. The homeostatic ramified microglia morphology can change towards a more amoeboid morphology, which is often associated with neuroinflammation and an activated/phagocytic microglia phenotype (Nimmerjahn et al., 2005; Tremblay et al., 2011; Dubbelaar et al., 2018). During ageing, microglia can acquire dystrophic morphology, characterized by de-ramification, spheroid formation, and fragmentation of processes (Streit et al., 2004). Furthermore, a hyper-ramification of microglia is present during accelerated ageing and priming (Raj et al., 2014), where microglia are impaired in homeostatic and tolerogenic functions such as clearance of debris and neuronal support.

During NDD, microglia acquire an activated phenotype which is also called disease-associated microglia (DAM) or microglia neurodegenerative phenotype (MGnD) (Holtman et al., 2015; Keren-Shaul et al., 2017; Krasemann et al., 2017). In mouse models for AD (and ALS), a subtype of microglia expands which exhibits this phagocytic (Keren-Shaul et al., 2017) and potentially synaptotoxic phenotype (Frigerio et al., 2019). DAM/MGnD are induced after phagocytosis of apoptotic neurons, which sets off a TREM2-APOE dependent pathway (Keren-Shaul et al., 2017; Krasemann et al., 2017). Blocking APOE inhibits the development of this phenotype (Krasemann et al., 2017; Frigerio et al., 2019). Hallmarks of DAM/MGnD are a downregulation of homeostatic microglia genes (e.g. *Cx3cr1*, *P2ry12*, *Tmem119*) and an upregulation of immune-activation/phagocytosis genes (e.g. *Apoe*, *Axl*, *Trem2*, *Lpl*) (Chiu et al., 2013; Holtman et al., 2015; Srinivasan et al., 2016; Keren-Shaul et al., 2017; Krasemann et al., 2017; Mathys et al., 2017). In human AD, a subset of microglia exhibits gene expression profiles similar to DAM/MGnD in mice (Grubman et al., 2019; Mathys et al., 2019).

Research on microglia in NDD greatly enhances the understanding of microglia in other CNS diseases, since neuroinflammatory processes are often (partly) overlapping (Holtman et al., 2015). For example, in MS and mouse models for MS, subsets of microglia exist which share transcriptional features with DAM/MGnD (Hammond et al., 2019; Masuda et al., 2019). More detail on microglia and astrocytes in MS is provided below (see "Microglia and astrocytes in MS").

A brief guide to multiple sclerosis

MS is a chronic, inflammatory, demyelinating disease of the CNS characterized by the formation of demyelinated areas called lesions that contain peripheral immune cell infiltrates dominated by macrophages (Compston and Coles, 2008; Dendrou et al., 2015; Thompson et al., 2018). MS is most likely of autoimmune origin, although no CNS antigens driving disease have been established unequivocally. The heterogeneous nature of MS manifests in highly individual disease courses, a diversity of clinical symptoms, and the emergence of different types of lesions. These lesions can be staged based on variations of neuropathological assessments, such as the degree of demyelination and inflammation as used in this thesis (Fig. 2) (Van Der Valk and De Groot, 2000; Van Der Valk and Amor, 2009; Kuhlmann et al., 2017).

The complex disease MS

MS is characterized by chronic inflammation of the CNS and the development of lesions which are demyelinated areas of WM and GM. In addition to oligodendrocyte death and resulting demyelination, neurodegeneration is another characteristic of MS.

MS is a chronic disease and usually presents in a relapsing-remitting pattern (RRMS), in which periods of symptoms are alternating with symptom-free periods (Compston and Coles, 2008; Thompson et al., 2018). However, during RRMS there is also a worsening of disability that is independent of relapses (Kappos et al., 2020). The relapsing phase is usually followed by a phase of progressive increase in disability called secondary-progressive MS (SPMS). A low number of patients (10-20%) presents without a relapsing phase but experiences an uninterrupted progression of disease called primary-progressive MS (PPMS) (Compston and Coles, 2008; Thompson et al., 2018). Neurological symptoms include movement disability and paralysis, vision impairment, fatigue, numbness, and abnormal sensation.

The cause of MS is unknown, but many risk factors have been identified. The prevalence of MS in families and monozygotic twins suggests that the a priori risk of developing MS is partly (up to 25%) caused by genetic factors (Willer et al., 2003; Dendrou et al., 2015). GWAS identified 233 single-nucleotide polymorphisms (SNP) associated with MS, 32 of which were associated with MHC genes (Patsopoulos et al., 2019). This high number of MHC-associated SNP already suggests a strong involvement of antigen-presentation and T-cell activation in MS. The majority of the non-MHC SNP are also associated with immune functions including adaptive immunity ($CD4^{pos}$ and $CD8^{pos}$ T-cells, B cells, APC), microglia functions, and TNF and IFN pathways (Patsopoulos et al., 2019). Environmental factors associated with MS include smoking, obesity in early life, vitamin D deficiency, and infections (Dendrou et al., 2015; Thompson et al., 2018). Epstein-Barr virus (EBV) infection with occurrence of mononucleosis is strongly associated with MS, possibly contributing via molecular mimicry on the B-cell level (Thompson et al., 2018).

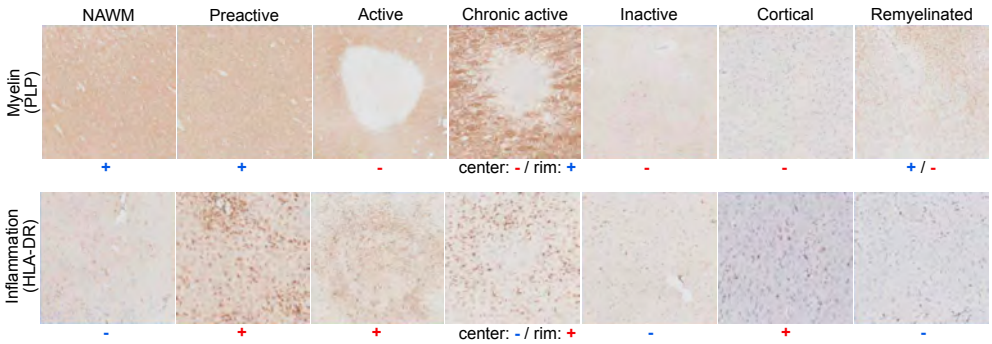

Figure 2. Different types of MS lesions. MS lesion staging according to degree of demyelination and inflammation (Van Der Valk and De Groot, 2000; Van Der Valk and Amor, 2009; Kuhlmann et al., 2017). Presence or absence of demyelination (PLP) and inflammation (HLA-DR) are indicated with colored plus and minus signs. NAWM = Normal-appearing white matter, PLP = Proteolipid protein, HLA-DR = Human leukocyte antigen DR isotype (MHC-II)

Heterogeneous pathology of MS

Pathological hallmarks of MS are inflammation, demyelination, axonal and neuronal damage/loss, and atrophy; however, the sequence of these processes is still unknown. Demyelination and resulting lesions in MS are caused by infiltrating immune cells that set off a cascade of acute inflammation, which is further exacerbated by CNS resident glial cell types astrocytes and microglia (see "Microglia and astrocytes in MS"). Infiltrating immune cell subsets include T cells, B cells, monocytes (Dendrou et al., 2015), and more recently neutrophils were also associated with MS lesion pathology (Pierson et al., 2018). Early during lesion formation, microglia activation and infiltration of $CD8^{pos}$ T cells is observed. During this stage, T cells become (re)activated in the parenchyma and an autoimmune reaction towards myelin is initiated (Goverman, 2009; Lassmann et al., 2012; Dendrou et al., 2015; Thompson et al., 2018). Subsequently, more immune cells (B cells, T cells, neutrophils, and predominantly macrophages) are recruited which results in continuation and amplification of myelin destruction (Lassmann et al., 2012; Thompson et al., 2018). This destruction of myelin is facilitated by autoreactive cytotoxic T cells and cytotoxic cytokines, excitotoxins, and reactive oxygen species (ROS) and nitric oxide (NO) released by microglia and macrophages (Lassmann et al., 2012; Dendrou et al., 2015). Enhanced permeability or leakiness of the BBB further facilitates immune cell infiltration to the CNS parenchyma. Lesions with active inflammation are present in all types of MS and at all stages of the disease, including in patients deceased after living with MS for up to three decades (Luchetti et al., 2018).

Over the course of the disease, axonal loss and neurodegeneration become more extensive, possibly exacerbated by inflammatory processes (Lassmann et al., 2012; Dendrou et al., 2015). Demyelination and loss of oligodendrocytes promotes axonal damage including transection, resulting in neuronal death. Axonal damage as well as oxidative stress caused by microglia and macrophages can cause mitochondrial dysfunction in neurons, eventually leading to neurodegeneration (Lassmann et al., 2012; Dendrou et al., 2015). Furthermore, oligodendrocytes release iron upon death, which amplifies oxidative stress (Lassmann et al., 2012).

MS lesions can be divided into different types based on the occurrence of demyelination (Luxol Fast Blue staining or PLP) and inflammation (MHC-II) (Fig. 2) (Van Der Valk and De Groot, 2000; Van Der Valk and Amor, 2009). Preactive lesions are defined by a lack of immune cell infiltrates and demyelination and the presence of immune-activated microglia. Active lesions feature immune cell infiltrates, immune-activated microglia, and demyelination, whereas inactive lesions are demyelinated but lack aberrant inflammation. Mixed active/inactive lesions, also called chronic lesions, contain an inactive core and an active rim. After demyelination occurred and inflammation is resolved, remyelination occurs, albeit with lower efficiency than the original myelin ensheathment. These remyelinated lesions are also called shadow-plaques.

Mouse models mimicking individual aspects of MS biology

The exact cause of MS is unknown, and MS naturally only occurs in humans, hence the development of valid animal models has proven difficult. Animal models of MS are commonly used to mimic certain aspects or pathological hallmarks such as CNS autoimmunity, acute CNS inflammation, and de-/remyelination, but no single model captures the entire pathological spectrum of MS. A detailed comparison of the broad variety of these models is beyond the

scope of this general introduction, and is available in comprehensive reviews (Steinman and Zamvil, 2006; Mix et al., 2010; Ransohoff, 2012; Lassmann and Bradl, 2017). In this thesis, MOG_{35-55} induced EAE in C57Bl/6 mice was employed.

EAE is one of the most commonly used animal models for MS and can be elicited in many species including rodents and non-human primates. Initially, EAE was induced in animals by injecting brain tissue emulsions, which caused MS-like symptoms in a fraction of animals (Ransohoff, 2012; Lassmann and Bradl, 2017). EAE induction was further refined by using complete Freund's adjuvant (CFA) as an immune booster to break self-tolerance. Furthermore, the antigens that cause EAE were identified, purified, and used for immunization to further increase reproducibility. Nowadays, EAE in mice is induced by myelin-autoreactive $CD4^{pos}$ T cells either by using transgenic T-cell receptors (TCR), passive transfer of autoreactive T cells, or by active immunization with myelin-components such as PLP or MOG (Lassmann and Bradl, 2017). Active sensitization EAE is mostly induced by immunization with MOG peptides in addition to CFA and pertussis toxin (PTX) to increase permeability of the BBB and promote development of T helper 1 (Th1) and Th17 cells (Agarwal et al., 2012). Immunization leads to activation and expansion of autoreactive $CD4^{pos}$ T cells which infiltrate the spinal cord and initiate an acute myelin-directed inflammation (Lassmann and Bradl, 2017). Hallmarks of active sensitization EAE are monocyte infiltration, microglia activation, and active demyelination leading to paralysis within 10 days of immunization. Although EAE covers multiple aspects of MS, many pathological components are not present, depending on the species. In mice, EAE only results in WM lesions of the spinal cord, but does not cause iron accumulation and demyelination of brain and GM (Lassmann and Bradl, 2017), which is observed in MS. The rapid and acute development of EAE does not match the chronic and progressive disease pathology of MS either. Nevertheless, well-selected state of the art EAE models can mimic several of the autoimmune and acute inflammatory and demyelinating aspects of MS and have been indispensable in elucidating MS pathogenesis and development of novel treatments, such as natalizumab (anti-VLA4).

Microglia and astrocytes in MS

Both microglia and astrocytes are involved in inflammatory processes in MS by antigen presentation, secretion of bioactive compounds such as cytokines, ROS, NO, and other inflammatory mediators, and glial scar formation.

Based on studies using a wide variety of EAE models, microglia are thought to be beneficial during initial disease responses in order to resolve inflammation and promote tissue regeneration, which was reviewed previously (Voet et al., 2019). In later stages of disease, microglia may contribute to chronic neuroinflammation and neurodegeneration. In active MS lesions, microglia lose some of their homeostatic markers such as P2RY12 and upregulate pro-inflammatory and phagocytic genes, whereas a shift towards a more homeostatic and anti-inflammatory microglia phenotype is observed in inactive lesions (Zrzavy et al., 2017; Guerrero and Sicotte, 2020). In several mouse models recapitulating aspects of MS pathology (EAE, cuprizone, and lysolecithin), microglia subsets are present that exhibit an immune-activated/phagocytic phenotype characterized by downregulation of homeostatic markers and upregulation of immune-related and phagocytosis genes (Hammond et al., 2019; Jordão et al., 2019; Masuda et al., 2019). Of note, these activated phenotypes differ between models, likely due to different disease mechanisms (Masuda et al., 2020). Microglia subsets similar

to the immune-activated/phagocytic phenotype observed in mouse MS models were also identified in MS patients (Masuda et al., 2019). Uptake of MS myelin might be the driver for a phagocytic microglia phenotype (Hendrickx et al., 2014) and promotes more regenerative, anti-inflammatory functions (Boven et al., 2006; Guerrero and Sicotte, 2020). Furthermore, microglia from MS WM upregulate genes involved in lipid handling and iron homeostasis (van der Poel et al., 2019), potentially reflecting their response to myelin damage and iron deposition.

Astrocytes are important players in MS since they are involved in many mechanisms that are impaired in MS such as BBB maintenance, oligodendrocyte function, and myelination. Reactive astrocytes undergo astrogliosis, an abnormal increase in astrocytes, thereby forming a glial scar during later stages of inflammation in MS lesions. A glial scar is beneficial as it shields healthy CNS tissue from spreading inflammation and supports tissue regeneration (Ponath et al., 2018). However, glial scars can also inhibit regeneration and hence should not be viewed as only beneficial or detrimental (Bradbury and Burnside, 2019).

Early in lesion formation, astrocytes acquire a hypertrophic morphology and express MHC-II components, suggesting they are able to (re)activate infiltrating T cells by presenting myelin components (Ponath et al., 2018). In EAE and MS, subtypes of astrocytes upregulate genes involved in neuroinflammation, mitochondrial dysfunction, and oxidative stress, which is induced by the transcription factor MAFG (Wheeler et al., 2020). These findings are in line with previous observations that a neurotoxic (LPS-stimulated) reactive astrocyte subtype is present in MS lesions (Liddelow et al., 2017; Schirmer et al., 2019). In chronic stages of EAE, astrocytes downregulate cholesterol synthesis genes (Itoh et al., 2017), which is important for oligodendrocyte function. A decrease in cholesterol availability may further impair myelination, since restoring cholesterol synthesis in astrocytes alleviates EAE symptoms (Itoh et al., 2017).

The multifaceted molecule VISTA

Immune checkpoints are important receptors that provide stimulatory or inhibitory signals to keep the immune system in balance between protection and autoimmunity (Fig. 3). NCR are inhibitory receptors that can be modulated to regulate immunity. VISTA is a unique NCR as it has many other functions in addition to inhibiting T-cell activation. In myeloid cells, VISTA acts not only as a ligand but also as a receptor and is involved in phagocytosis, cytokine response, and chemotaxis. Multiple NCR are expressed in the CNS; however, expression and function of VISTA remains unknown.

Immune checkpoints: essential regulators of immunity

Immune checkpoints are critical in maintaining the balance between protective immune responses of appropriate magnitude versus excessive inflammation with undue tissue damage and autoimmune disease. Co-stimulatory and co-inhibitory receptors provide T cells with activating or suppressing signals, respectively, and a disruption of this balance can lead to autoimmunity or prevent specific immune responses (Fig. 3). NCR are receptors that provide co-inhibitory signals to T cells, which leads to inhibition of T-cell activation. Immune checkpoints and particularly NCR are intensely pursued as therapeutic targets for cancer

and autoimmunity. Blocking NCR enhances anti-tumor immunity, whereas enhancing NCR signaling offers a strategy to alleviate autoimmunity (Fig. 3). Studies mainly focus on NCR biology in cancer and peripheral immunity; however, multiple NCR are also expressed by CNS-resident cell types including neurons, oligodendrocytes, astrocytes, and microglia (Yshii et al., 2017). Expression of most NCR in the CNS is upregulated or induced during inflammation (Yshii et al., 2017). A subset of cancer patients develops neurological adverse effects after NCR treatment including encephalitis and MS (Cuzzubbo et al., 2017; Yshii et al., 2017), demonstrating that NCR modulation can affect the CNS. Inhibition of NCR has proven to mount an anti-tumor response in certain types of CNS-associated human tumors (Kamath and Kumthekar, 2018; Ratnam et al., 2019). Furthermore, modulating NCR activity affects CNS autoimmunity such as EAE (Latchman et al., 2004; Carter et al., 2007; Joller et al., 2012; Aarts et al., 2017) (Fig. 3). Detailed clinical studies assessing the effectiveness of modulating NCR in CNS inflammation, ageing, and neurodegeneration are lacking.

Immune checkpoints in the CNS

Expression and function of NCR in peripheral immunity especially during cancer and autoimmunity are extensively studied and are beginning to be understood. In contrast, the physiological and pathological functions of NCR in the CNS are in its infancy, although NCR are involved in a variety of CNS functions including communication with peripheral immune cells.

Multiple inhibitory immune checkpoints are expressed by mouse and human CNS-resident cells at least on mRNA level with varying abundancies including *A2AR*, *B7H3*, *BTLA*, *CTLA4*, *LAG3*, *NOX2*, *PD1*, *PDL1*, *PDL2*, and *TIM3* (Fig. 4). Every major CNS cell type (neurons, oligodendrocytes, microglia, astrocytes, endothelial cells) expresses inhibitory immune checkpoints, but microglia express the largest diversity (Fig. 4). Expression of many of these inhibitory immune checkpoints is induced or upregulated during inflammatory conditions including PD1, PDL1, PDL2, and TIM3 (Yshii et al., 2017).

Function and in-depth expression dynamics of the majority of immune checkpoints has not been studied in detail in the CNS. The best studied NCR in the CNS is PDL1 (also known as CD274 and B7H1), which is predominantly expressed by microglia and neurons (Fig. 4). During inflammation, PDL1 expression is induced in astrocytes, oligodendrocytes (Phares et al., 2009) and endothelial cells (Pittet et al., 2011), and upregulated in microglia and neurons. Upregulation or induction of PDL1 in microglia and astrocytes during inflammation limits CNS inflammation and pathology by inhibition of T-cell activation (Schachtele et al., 2014; Chauhan and Lokensgard, 2019). In EAE, for example, responses of infiltrating PD1-expressing T cells are suppressed by microglia PDL1 expression (Schreiner et al., 2008). Conversely, deletion of PDL1 or PDL2 in mice reduces the infarct volume after tMCAO, due to reduction of immune-activated microglia and infiltrating peripheral immune cells (Bodhankar et al., 2013). Hence, it is possible that expression of PDL1 has different functional consequences for different cell types (T cells versus myeloid cells). In PDL1 deficient mice, PD1 and PDL2 expression is increased (Bodhankar et al., 2013), suggesting a compensatory mechanism. Therefore, genetic depletion of one NCR can likely be balanced by upregulation of functionally similar NCR.

TIM3 is a co-inhibitory receptor that suppresses T-cell activation. In microglia, TIM3 regulates inflammatory responses such as inducible nitric oxide synthase (iNOS) production

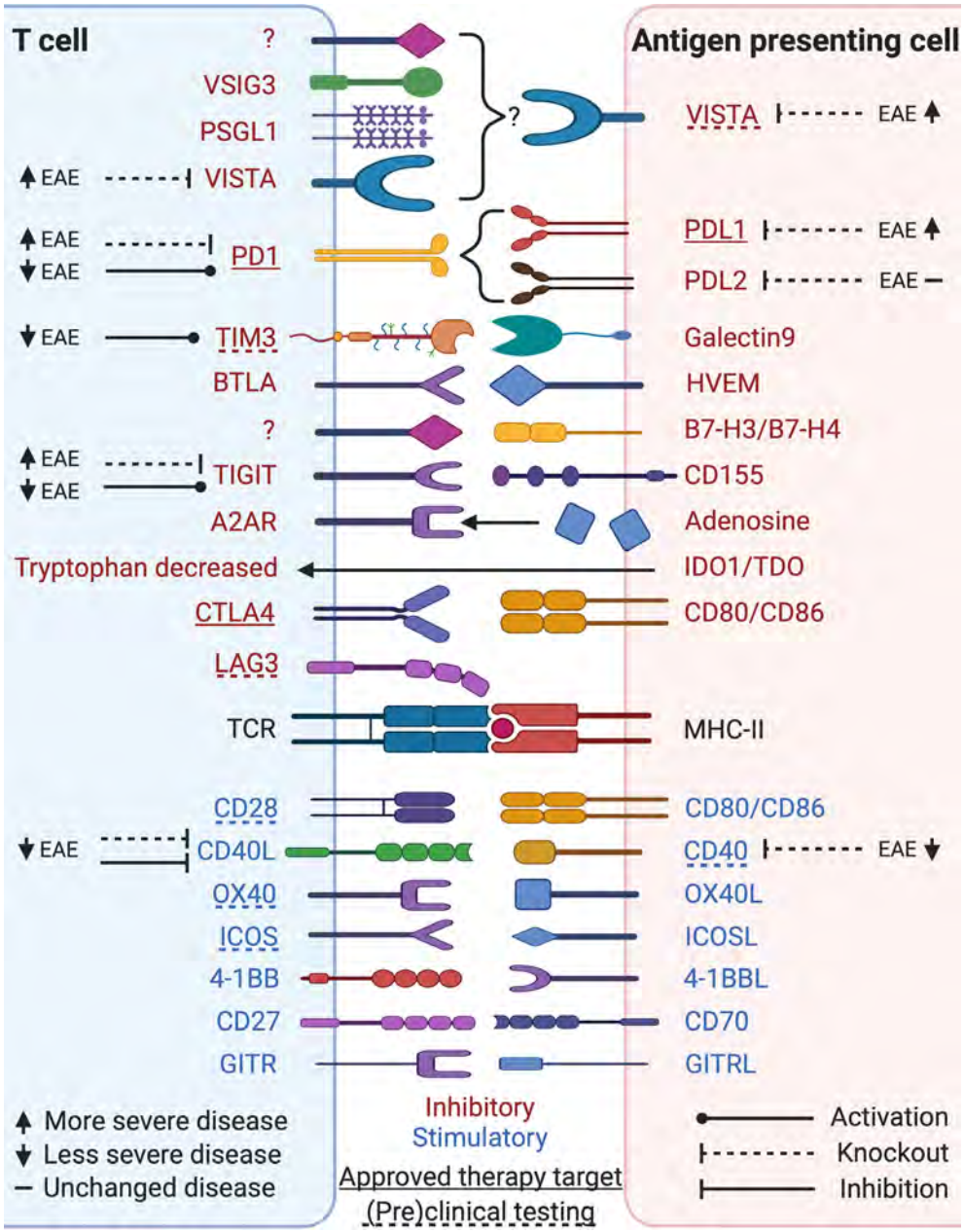

Figure 3. Immune checkpoints regulate T-cell responses and are involved in EAE development. Immune checkpoints and their counterreceptors are depicted, which provide stimulatory and inhibitory signals to T cells in order to regulate their responses. Knockout of immune checkpoints and activation or inhibition using antibodies affect EAE disease progression (Latchman et al., 2004; Carter et al., 2007; Joller et al., 2012; Aarts et al., 2017). Inhibiting the activity of negative checkpoint regulators PD1, PDL1, and CTLA4 using monoclonal antibodies is used in clinics to treat cancer (Murciano-Goroff et al., 2020). Additional immune checkpoints are currently under investigation in (pre)clinical trials for their therapeutic potential in cancer and autoimmunity (Paluch et al., 2018; Murciano-Goroff et al., 2020).

after exposure to glioma-conditioned medium (Kim et al., 2020), suggesting that NCR have intrinsic functions in microglia biology. In melanoma brain metastases, microglia are the principal IDO-expressing cell type compared to infiltrating immune cells (Herrera-Rios et al., 2020). IDO is an immunomodulatory enzyme that facilitates conversion of tryptophan to kynurenine, resulting in antimicrobial and immunosuppressive environments (Frumento et al., 2002). This high expression of IDO indicates that microglia are potent immunomodulatory cells especially during CNS diseases that include immune cell infiltration, such as MS.

VISTA structure and binding partners

VISTA (also known as PD1H (Flies et al., 2011), DD1a (Yoon et al., 2015), DIES1 (Aloia et al., 2010), GI24 (Sakr et al., 2010), C10orf54, VSIR, B7H5, and 4632428N05Rik) is an NCR that is expressed in multiple tissues at varying levels. Multiple counterreceptors have been proposed, but not proven beyond doubt.

VISTA is a transmembrane protein that contains an immunoglobulin variable (IgV)-like fold and shares similarities with B7 family members PD1, PDL1, CD28, and CTLA4 (Mehta et al., 2019). The extracellular domain of VISTA contains four conserved cysteines that are not present in other B7 family members (Mehta et al., 2019). Across species, VISTA is highly conserved with 96% identical protein sequence comparing human to other primates (rhesus macaque, cynomolgus monkey, common marmoset) and 77% between human and mouse (unpublished). The VISTA gene is located on chromosome 10 within the intronic region of Cadherin23 (CDH23).

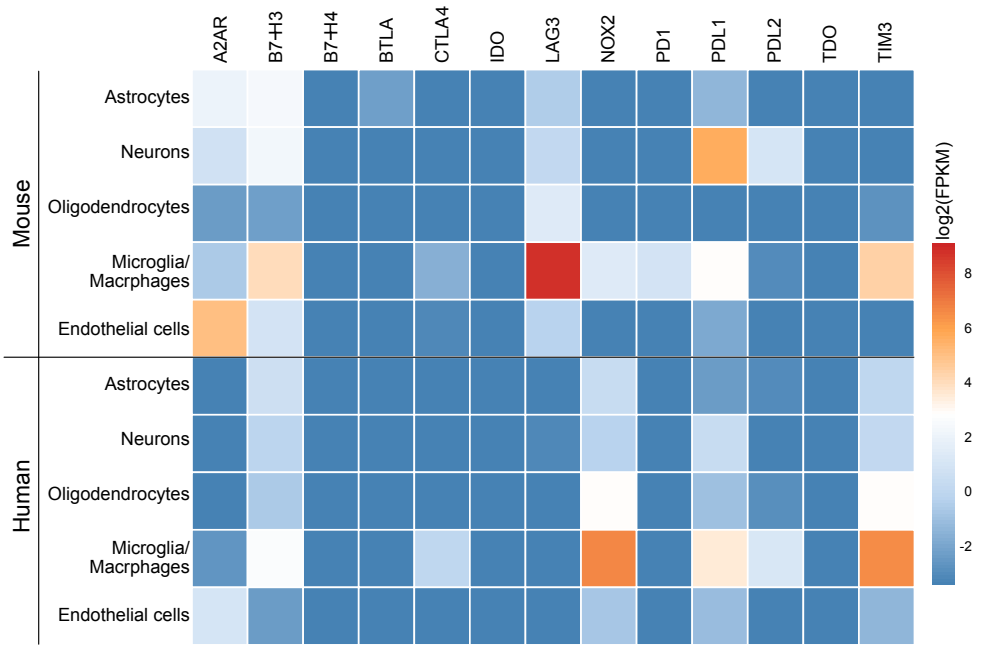

Figure 4. Expression of inhibitory immune checkpoints in mouse and human CNS during homeostasis. Heatmap illustrates mRNA levels as log2(FPKM) in different types of CNS cells, derived from published mRNA sequencing data (Zhang et al., 2014, 2016).

Although the counterreceptor of VISTA remains elusive, multiple candidate binding partners have been proposed: VSIG3/IGSF11 (Mehta et al., 2019; Wang et al., 2019), VISTA itself through homophilic interaction (Yoon et al., 2015), and PSGL1 (Johnston et al., 2019). VSIG3 binds to VISTA in ELISA assays (Mehta et al., 2019; Wang et al., 2019), and plate-bound VSIG3 inhibits anti-CD3-induced cytokine secretion by T cells (Wang et al., 2019). However, evidence for functional cellular interactions through VISTA and VSIG3 in vitro and particularly in vivo is lacking. A homophilic VISTA interaction between apoptotic cells and macrophages has been suggested to be necessary for facilitating uptake of apoptotic cells (Yoon et al., 2015). However, this homotypic binding could not be replicated in another study (Johnston et al., 2019). In this study, PSGL1 was proposed as a binding partner via histidine residues within the extracellular domain of VISTA (Johnston et al., 2019). Binding of PSGL1 and VISTA leads to inhibition of T-cell activation and only occurs at acidic pH in vitro and in vivo (pH 6.0) (Johnston et al., 2019). Hence, binding of VISTA to PSGL1 selectively occurs in acidic environments, e.g. theoretically provided by tumors and inflammation (Johnston et al., 2019).

It is possible that VISTA has multiple binding partners, but additional evidence and replication studies will be necessary to unequivocally demonstrate functional binding of VISTA to one or more of these potential counterreceptors.

VISTA expression across cell types and tissues

VISTA mRNA is expressed in multiple organs and tissues including thymus, spleen, heart, kidney, lung, bone marrow, and the brain (Wang et al., 2011). Predominantly the hematopoietic compartment expresses VISTA with highest levels in myeloid cells (monocytes, macrophages, dendritic cells), neutrophils, followed by naïve $CD4^{pos}$ and $CD8^{pos}$ T cells, as well as regulatory $Foxp3^{pos}$ T cells (Flies et al., 2011; Wang et al., 2011; ElTanbouly et al., 2019). Whereas expression of other NCR is increased upon T-cell activation, VISTA is constitutively expressed on resting T cells. VISTA expression in other hematopoietic cell types is detectable but low, including natural killer (NK) cells, thymocytes, and plasma cells, whereas no VISTA expression is observed in B cells (Flies et al., 2011; Wang et al., 2011; ElTanbouly et al., 2019).

Of note, VISTA expression is not restricted to the cell surface, but is also observed in high levels intracellularly in myeloid cells (ElTanbouly et al., 2019). Here, it colocalizes with markers for early endosomes (EEA1) and recycling endosomes (RAB11) (ElTanbouly et al., 2019), suggesting that VISTA is actively recycled and/or has other functions in the cytoplasm.

Several studies demonstrated expression of VISTA in various types of cancer including gastric carcinoma (Böger et al., 2017), colorectal carcinoma (Xie et al., 2018; Deng et al., 2019), hepatocellular carcinoma (Zhang et al., 2018), ovarian and endometrial cancer (Mulati et al., 2019), prostate cancer (Gao et al., 2017), pancreatic cancer, and melanoma (Blando et al., 2019). In some types of cancer, VISTA is expressed by cancer cells themselves, including gastric, ovarian, and endometrial tumors (Böger et al., 2017; Mulati et al., 2019). However, VISTA expression is predominantly found on myeloid-derived suppressor cells (MDSC) in the tumor microenvironment (Green et al., 2015; Wu et al., 2017; Latta-Mahieu et al., 2018; Xu et al., 2019). In MDSC, VISTA expression is induced by hypoxic tumor environments via HIF1a (Deng et al., 2019). Moreover, VISTA expression is induced in apoptotic cells as a downstream target of p53 and is required for engulfment by phagocytes (Yoon et al., 2015). VISTA is also involved in differentiation as reducing VISTA expression using siRNA

or miRNA-125b inhibits the differentiation of mouse embryonic stem cells (Aloia et al., 2010; Battista et al., 2013) and preadipocytes (Ren et al., 2013).

VISTA as a negative checkpoint regulator

Multiple studies have demonstrated that VISTA inhibits T-cell activation and therefore functions as an NCR. VISTA-Ig fusion protein or VISTA-overexpressing A20 cells both reduce proliferation and cytokine production (IL2 and IFNg) in ovalbumin (OVA) or anti-CD3-stimulated T cells in vitro (Wang et al., 2011). Furthermore, blocking VISTA in mice using an antagonistic anti-VISTA antibody (clone 13F3) increases T-cell proliferation in response to OVA and exacerbates the development of EAE (Wang et al., 2011). Concomitantly, targeting VISTA on T cells in mice using an agonistic anti-VISTA antibodies (clone MH5A or 8G8) protects mice from graft-versus-host disease (GvHD) (Flies et al., 2011), hepatitis (Flies et al., 2014), lupus (Han et al., 2019; ElTanbouly, Zhao, et al., 2020), psoriasis (ElTanbouly, Zhao, et al., 2020), and arthritis (ElTanbouly, Zhao, et al., 2020). This protection from GvHD is independent of host cells (Flies et al., 2011), but is due to engagement of VISTA on donor T cells, inhibiting their activation (Flies et al., 2015; ElTanbouly, Zhao, et al., 2020).

In addition to inhibition of T-cell activation, VISTA is also involved in T-cell differentiation and expansion. In GvHD, for example, activation of VISTA on donor T cells expands regulatory T cells (Tregs) (Flies et al., 2011). Concordantly, T cells in generic VISTA knockout (KO) mice exhibit a reduced ability to form iTregs (Wang et al., 2017). The generation of natural Tregs, however, is not impaired (Wang et al., 2017). The iTregs of generic VISTA KO mice are more prone to conversion into Th17 and Th1 cells during inflammation compared to wildtype iTregs (Wang et al., 2017). This overall reduction in iTreg formation and induction of Th1 and Th17 cells supports the notion of a more reactive T-cell compartment in VISTA KO mice. It is likely that this reactivity is caused by both intrinsic effects of VISTA deficiency in T cells, and indirect effects of an altered cytokine profile and depletion of VISTA in other cell types (e.g. dendritic cells, DC). Consistent with this argument, DC in VISTA KO mice produce more IL23, leading to augmented IL17a production by Th17 and γδ T cells, resulting in the exacerbation of psoriasiform plaques in mice induced by imiquimod (Li et al., 2017).

In contrast to other NCR, which are expressed upon T-cell activation, VISTA is constitutively expressed on resting T cells, suggesting distinct functionalities. Underscoring this non-redundant role of VISTA, double KO of VISTA and PD1 significantly increases T-cell responses to foreign antigens and exacerbates EAE compared to VISTA or PD1 single KO mice (Liu et al., 2015). Detailed analysis of the T-cell compartment in VISTA KO mice using single cell transcriptomic and epigenetic approaches demonstrate that VISTA is crucial for maintaining naïve T cell quiescence (ElTanbouly, Zhao, et al., 2020). Therefore, VISTA regulates T-cell tolerance before activation occurs, whereas other NCR such as CTLA4 and PD1 only act after T-cell activation to inhibit priming and effector functions. VISTA is the first known NCR that acts at such an early stage in the T-cell activation cascade and hence offers a novel, non-redundant target for therapeutic interventions (ElTanbouly, Zhao, et al., 2020).

Function of VISTA in myeloid cell biology

VISTA was initially discovered as an NCR, but since then a role for VISTA in a variety of other processes in myeloid cells has been proposed, including cytokine response, chemotaxis, and efferocytosis (Fig. 5).

In multiple mouse models of inflammation, VISTA KO is associated with an increase in pro-inflammatory cytokines (Ceeraz, Eszterhas, et al., 2017; Ceeraz, Sergent, et al., 2017; Li et al., 2017; H. Liu et al., 2018). These cytokines derive from T cells and myeloid cells. In a psoriasis mouse model, for example, VISTA KO enhances the production of IL23 by DC (Li et al., 2017). Surprisingly, overexpression of VISTA in human monocytes in vitro leads to spontaneous cytokine production (TNF, IL1b) on mRNA (Bharaj et al., 2018) and protein level (Bharaj et al., 2014). It is unclear whether these opposing findings are due to differences between species (human versus mouse), or because of distinct approaches of studying VISTA (KO versus overexpression). Nonetheless, these studies demonstrate that VISTA is involved in the cytokine response of myeloid cells (Fig. 5).

Emerging evidence suggests that VISTA is involved in chemotaxis and migration through direct and indirect signaling in myeloid cells (Fig. 5). In VISTA KO mice, elevated levels of inflammatory cytokines and chemokines such as CCL2 (MCP1) are observed in the lung, which is associated with the development of experimental asthma (H. Liu et al., 2018). CCL2 is a chemoattractant for monocytes and thus VISTA may indirectly regulate the recruitment of monocytes. VISTA also directly regulates monocyte chemotaxis, since blocking VISTA on monocytes using an antagonistic antibody (clone 13F3) enhances their migration ability (Sergent et al., 2018). Concordantly, expression of the CCL2 receptor CCR2 was increased in 13F3-treated mice (Sergent et al., 2018).

In macrophages, expression of VISTA is required for the engulfment and uptake of apoptotic cells (Yoon et al., 2015; Cohen et al., 2016). VISTA is upregulated in a p53-dependent manner in apoptotic cells and a homophilic interaction with VISTA on macrophages facilitates efferocytosis (Yoon et al., 2015) (Fig. 5). A lack of VISTA on either phagocytes or apoptotic cells impairs dead cell clearance (Yoon et al., 2015). However, as mentioned, a homophilic interaction of VISTA could not be replicated to date (Johnston et al., 2019). Concordantly, blocking VISTA on macrophages using a neutralizing antibody also reduces the uptake of neutrophils in vitro (Cohen et al., 2016).

Activating VISTA enhances endotoxin tolerance and decreases the septic shock lethality in mice (ElTanbouly, Schaafsma, et al., 2020). Endotoxin tolerance describes a mechanism of innate immune memory, in which innate immune cells are less sensitive towards an immune stimulus such as LPS, if they have been stimulated with LPS previously. Using an agonistic anti-VISTA antibody (clone 8G8) leads to epigenetic reprogramming of macrophages, which results in an anti-inflammatory profile and increases endotoxin tolerance (ElTanbouly, Schaafsma, et al., 2020).

Many of the presented experiments are based on a generic VISTA KO mouse model or systemically administered VISTA-modulating antibodies. Therefore, it cannot be excluded that some of the observed changes in myeloid cells are due to a lack of VISTA on other cell types as opposed to a cell-intrinsic role of VISTA. However, most studies additionally used cell-specific in vitro assays to verify their results, suggesting a cell-intrinsic function. Using conditional depletion of VISTA in a cell-type specific manner will be important to further dissect the function of VISTA in myeloid cells in vivo.

Summarized, VISTA functions beyond being an NCR and is involved in multiple aspects of the innate immune response of myeloid cells.

Dual role of VISTA as receptor and ligand

VISTA has a large spectrum of expression and functions across multiple tissues and cell types. This diverse function and expression may in part be attributed to the dual role of VISTA as a receptor and a ligand (Fig. 5).

Regarding the function of VISTA as an NCR, both ligand and receptor activities on APC and T cells can lead to T-cell inhibition. VISTA-Ig fusion proteins and VISTA-overexpressing A20 cells both reduce proliferation of anti-CD3-stimulated T cells (Wang et al., 2011; Lines et al., 2014). Therefore, VISTA expressed on APC can act as a ligand and upon binding to a counterreceptor on T cells this leads to T-cell inhibition. Conversely, engaging VISTA expressed on naïve T cells can also inhibit T-cell activation (Fig. 5), which has been shown in the context of hepatitis (Flies et al., 2014) and GvHD in mice (Flies et al., 2011; ElTanbouly, Zhao, et al., 2020). As mentioned, treatment of mice with agonistic anti-VISTA antibody (clone MH5A) activating VISTA signaling protects mice against GvHD (Flies et al., 2011). Passive

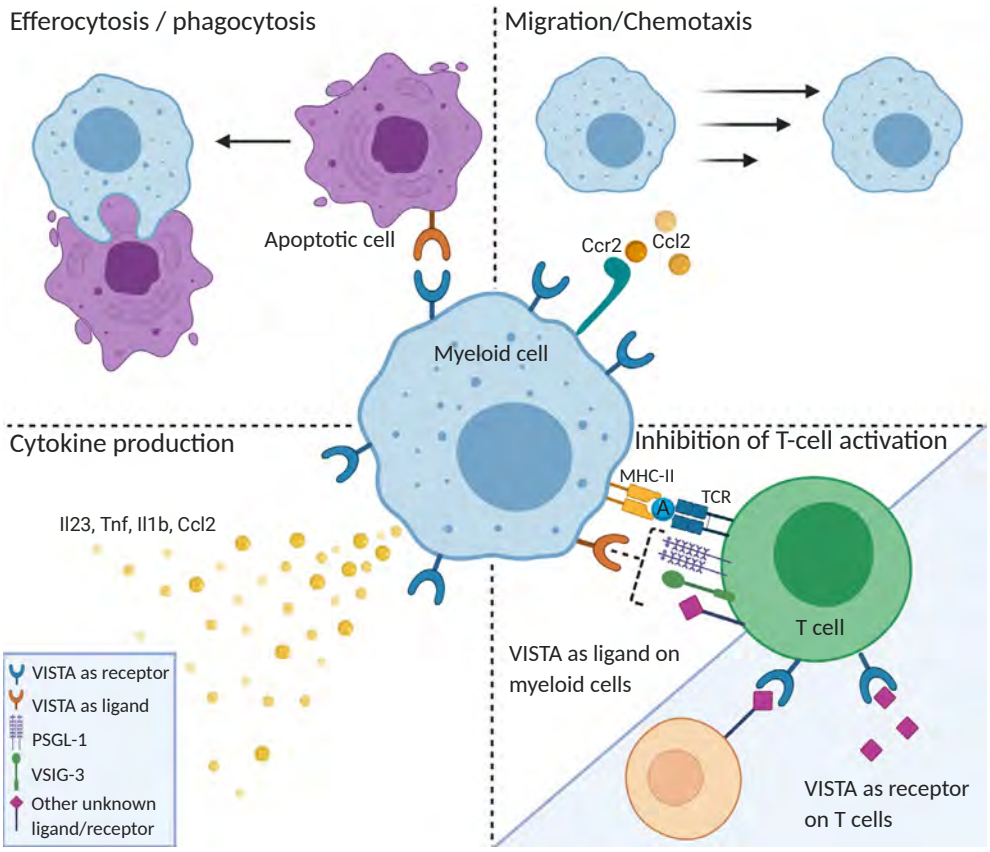

Figure 5. Ligand and receptor functions of VISTA in myeloid cells. VISTA is involved in multiple myeloid cell functions where it acts as a receptor and ligand. As a ligand on myeloid cells and as a receptor on T cells, VISTA inhibits T-cell activation. As a receptor on myeloid cells, VISTA is involved in cytokine production, migration/chemotaxis, and phagocytosis/efferocytosis. PSGL1, VISTA and VISG3 were proposed as VISTA receptor/ligands and it remains unknown if VISTA has additional receptors/ligands.

transfer of wildtype T cells into VISTA KO mice and subsequent anti-VISTA treatment also reduced GvHD, demonstrating that host cells are not involved in this protective effect (Flies et al., 2011). Thus, VISTA as a receptor on T cells and as a ligand on T cells as well as APC inhibits T-cell activation and thereby exerts its role as an NCR.

In myeloid cells, VISTA also acts as a receptor thereby exerting functions beyond inhibition of T-cell activation (Fig. 5). Overexpression of VISTA in human monocytes/macrophages leads to spontaneous inflammatory cytokine secretion, which is abrogated after deleting the cytoplasmic domain (Bharaj et al., 2014). Although the cytoplasmic domain of VISTA does not contain any immunoreceptor tyrosine-based signaling motifs, multiple casein kinase 2 and phosphokinase C phosphorylation sites are present (Mehta et al., 2019). These data demonstrate that engagement of VISTA on myeloid cells results in downstream cellular signaling through the cytoplasmic tail, which has functional ramifications for the cell such as cytokine production (Bharaj et al., 2014).

This dual role of VISTA as a receptor and ligand has important consequences for studying its function and the therapeutic potential of anti-VISTA antibodies. The effect of VISTA KO and VISTA-targeted treatment must be studied for individual cell types and with regard to VISTAs broad functions.

A VISTA on MS treatments

There is currently no cure for MS, only limited treatment options for RRMS, and no treatment for progressive MS. Thus, there is a high need in developing novel treatment strategies.

Currently, there are more than 10 FDA-approved immunomodulatory therapies for MS (Baecher-Allan et al., 2018). These drugs interfere with peripheral immune cell trafficking to the CNS (e.g. Natalizumab; anti-VLA4), deplete subsets of immune cells (e.g. ocrelizumab; anti-CD20), or modulate immune signaling pathways (e.g. IFNb, cladribine, dimethyl fumarate); however, immune checkpoints are not used as a target for MS immunotherapy yet (Baecher-Allan et al., 2018; Thompson et al., 2018).

Immunotherapy using immune checkpoint inhibitors is already established as an effective treatment against several cancer types, and more recently against autoimmune diseases such as rheumatoid arthritis. Studies mainly focus on the effects of immunotherapy on peripheral immunity, but evidence strongly suggests that immune checkpoint inhibitors affect the CNS as well, since neurological side effects are observed after immunotherapy treatment of cancer (Cuzzubbo et al., 2017; Yshii et al., 2017).

Targeting NCR using monoclonal antibodies may offer novel therapeutic strategies to limit autoimmunity while retaining a beneficial immune response. PDL1 KO exacerbates passive transfer EAE due to an increase in T cell infiltration (Latchman et al., 2004). Similarly, VISTA KO also exacerbates passive transfer EAE (Wang et al., 2014). Agonistic antibodies that activate NCR signaling may enhance immune inhibition signals and therefore present an effective treatment strategy for MS. The functions of VISTA in the CNS during health and disease are currently unknown. As VISTA can be exploited as a therapeutic target for cancer and autoimmune diseases, it is conceivable that VISTA may offer a novel therapeutic target for MS.

Outline of the thesis

MS is a chronic, demyelinating autoimmune disease of the CNS and treatment options are limited. Targeting NCR may offer novel strategies to modulate inflammatory responses during MS, while maintaining a properly functioning immune system. Treatment of cancer using currently approved immune checkpoint inhibitors can affect the CNS and lead to various neurological symptoms, suggesting that monoclonal antibodies have direct effects on CNS-resident cell types and hence on CNS function. VISTA is a more recently discovered NCR and is involved in controlling T-cell quiescence and the development of autoimmune disease. Modulating VISTA in MS may offer a novel tool of modulating the autoimmune response and alleviate symptoms. However, the role of VISTA in the CNS is unknown. Furthermore, in MS there is an intricate interplay of multiple cell types including CNS-resident cells such as astrocytes and microglia, and the infiltrating immune cell subsets. The exact functions of microglia and astrocytes in MS and how they communicate with other CNS-resident cells and infiltrating immune cells are not well understood.

The main research questions of this thesis are: (i) how do human microglia develop with regard to transcriptional- and chromatin accessibility profiles, immune competence including antigen recognition and NCR such as VISTA, and how do they contribute to CNS development, (ii) what is the transcriptional response of different astrocyte subtypes in EAE, and (iii) how does VISTA contribute to neuroinflammation (especially in MS/EAE), what is the function of VISTA in the CNS and microglia, and what are the potential implications for immunotherapy.

Chapter 1 provides a general introduction and outline to astrocytes and microglia in the CNS during health and disease, MS, and the function and role of VISTA is provided.

Chapter 2 characterizes microglia during human fetal development using a combination of single cell gene expression profiles and chromatin accessibility assays. Microglia acquire an immune-sensing, homeostatic phenotype early during fetal development, which may partly explain the vulnerability of the developing CNS towards fever and infections.

Chapter 3 delineates astrocyte heterogeneity based on surface molecules ACSA-2/ATP1B2 and GLAST/SLC1A3 during homeostasis and characterizes the phenotype of astrocytes during EAE. To dissect the role of astrocytes in MS, gene expression profiles of astrocyte subtypes at different stages of EAE are characterized. GLAST surface expression distinguishes transcriptional distinct astrocyte subtypes and spinal cord astrocytes are highly reactive during acute stages of EAE, whereas they switch to a more proliferative phenotype in chronic stages.

Chapter 4 describes VISTA expression in the CNS during health and disease with focus on microglia VISTA expression and changes of expression during MS. Microglia are the principal VISTA-expressing CNS-resident cell type and their VISTA expression decreases during innate immune receptor ligation in vitro, in mouse models of microglia activation and neuroinflammation, and in chronic MS lesions.

Chapter 5 assesses VISTA expression in multiple human CNS inflammatory and neurodegenerative diseases and respective animal models using published mRNA sequencing datasets. In almost all investigated CNS diseases, microglia VISTA expression is decreased.

Chapter 6 characterizes VISTA function in microglia and investigates expression of VISTA in different MS lesion stages. VISTA is differentially expressed in distinct MS lesions and correlates mainly with other microglia markers and not inflammatory markers. The role of VISTA in microglia during health and disease is delineated using microglia-specific VISTA KO mice and different models of acute peripheral inflammation (LPS) affecting the CNS versus autoimmune disease (EAE). VISTA KO does not affect microglia transcriptional responses after LPS or during EAE and does not alter EAE progression. However, VISTA regulates myelin phagocytosis and induces a more regulatory transcriptional microglia profile in naïve mice.

Chapter 7 summarizes all experimental findings, discussing them in the context of recent literature and future perspectives on VISTA-directed therapy and differential contributions of glia subsets to MS pathology and progression.