Exploring the VISTA of glial cells

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General discussion and future perspectives

Contents

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Preface

Multiple sclerosis (MS) is characterized by complex pathological processes that depend on the interplay between different central nervous system (CNS)-resident cell types and peripheral immune cell subsets. The exact cause of MS remains unknown and treatment does not fully arrest the disease. The **immune system** undergoes changes from development, through adolescence, to adulthood, ageing, and disease. Early **perturbations during development** may affect the immune system later in life, and changes in immunity during adulthood could contribute to diseases such as MS. Immune responses in the CNS depend on **crosstalk between CNS-resident cell types and peripheral immune** cells, which requires soluble mediators as well as receptors. Immune checkpoints are essential immune-modulating receptors such as the negative checkpoint regulator (NCR) **V-type immunoglobulin domain-containing suppressor of T-cell activation (VISTA)** and understanding their role in cell-cell contact and MS will support the development of novel therapeutic interventions.

In this chapter, the **development of human microglia** with regard to their immune function and how it might correlate to immune perturbations during development and potentially during MS is discussed. The role of **astrocytes at different stages of experimental autoimmune encephalomyelitis (EAE) and MS** and their crosstalk with microglia is evaluated. Finally, **VISTA expression and function in microglia** and during MS and other CNS disease is examined and the potential of VISTA as a novel therapeutic target is discussed. To this end, this chapter is divided into four main parts. Experimental **chapters 2, 3,** and **4-6** are individually summarized, discussed, and future prospects are provided.

Microglia in neurodevelopment

In **chapter 2**, microglia during human fetal development from gestational week (GW) 9 to 18 were characterized using a combination of single cell gene expression profiling (single cell mRNA sequencing; scRNAseq) and chromatin accessibility assays (assay for transposase-accessible chromatin using sequencing; ATACseq). During early stages of development (GW<13), microglia exhibited a gene expression profile reminiscent of an immune-activated and phagocytic phenotype that was previously associated with neurodegeneration (Holtman et al., 2015; Keren-Shaul et al., 2017; Krasemann et al., 2017). As microglia matured during fetal development (GW>13), this activated, disease-associated profile declined, and a more homeostatic phenotype emerged. This emerging homeostatic subtype was similar to adult microglia and expresses sensome genes, a set of genes that encode for receptors involved in environmental sensing (Hickman et al., 2013). These receptors are essential for microglia immune surveillance, rendering microglia during early fetal development (GW>13) capable of sensing their environment. Microglia chromatin accessibility increased with increasing age and the DNA fragments underlying accessible regions were enriched for binding motifs of transcriptions factors (TF) associated with microglia from adult individuals. Thus, gene expression and chromatin accessibility profiles both demonstrate that early during fetal development (GW>13) microglia obtain an adult-like, immune surveillance signature, which may render microglia vulnerable towards environmental perturbations at these early stages of pregnancy.

Emergence of immune-surveilling microglia early in human fetal development

The main function of microglia in the healthy adult CNS is to scan their environment, sense potential perturbations, and restore homeostasis. To this end, microglia express a plethora of receptors that are collectively called the sensome, which includes genes associated with homeostatic adult microglia including *P2RY12*, *CSF1R*, *TMEM119*, *CX3CR1,* and *VISTA* (Hickman et al., 2013). These receptors respond to cellular components (e.g. ATP), secreted factors (e.g. cytokines and growth factors), or interact directly with receptors on other cells. Hence, the sensome is responsible for the immune-surveilling properties of microglia. Although the sensome was initially described for adult mouse microglia, similar genes are enriched in adult human microglia (Galatro et al., 2017; Gosselin et al., 2017). During ageing and disease, microglia downregulated sensome genes, which is associated with an immune-activated or phagocytic phenotype (Hickman et al., 2013; Keren-Shaul et al., 2017; Krasemann et al., 2017). Microglia are dysfunctional during neuroinflammation, since they cannot properly respond to environmental challenges and are unable to restore homeostasis. In early fetal human development (GW<13), microglia exhibit an activated/phagocytic transcriptional profile similar to the one observed in neurodegenerative diseases (NDD) and neuroinflammatory diseases, and these microglia do not abundantly express sensome genes (**Chapter 2**). These findings suggest that early fetal microglia (GW<13) do not yet fully exhibit the functionalities of microglia in the healthy adult CNS but are more involved in processes similar to those in neurodegeneration and inflammation. These processes may include phagocytosis, which is essential for neurogenesis and oligogenesis to clear apoptotic cells (Marín-Teva et al., 2004; Wakselman et al., 2008; Sierra et al., 2010; Cunningham et al., 2013). Therefore, microglia at this stage of development are not yet capable and likely not required to surveil their environment and respond to intruders or perturbations. From GW13 onwards in human fetal development, microglia start expressing sensome genes and exhibit transcriptional and regulatory profiles similar to microglia from juvenile and adult individuals (**Chapter 2**). It is important to note that this transition occurs quite early during fetal development at GW>13 (**Chapter 2**). The emergence of immune-sensing microglia implies that microglia obtain the capacity to respond to environmental changes already early during pregnancy which in turn will affect the entire CNS. These environmental perturbations could be infections, disrupted developmental processes, or fever, and microglia responses may lead to perturbed CNS development or dysfunctional microglia development which can affect the adult CNS (Tay et al., 2018).

Role of microglia in normal and perturbed CNS development

CNS development is an intricate process and perturbations due to environmental challenges can have severe consequences leading to neurodevelopmental disorders. Fevers and maternal infections during pregnancy are associated with increased risk of developing autism spectrum disorders (ASD), intellectual and learning disability, hyperactivity disorder, preeclampsia, and schizophrenia (Prinz and Priller, 2014; Prins et al., 2018; Tay et al., 2018). The fetus is most vulnerable in the second trimester of pregnancy, since fever and maternal infections at GW>12 particularly increase the risk of developing ASD (Hornig et al., 2018; Croen et al., 2019). This developmental period overlaps with the emergence of immune-sensing microglia (**Chapter 2**), indicating that perturbances of CNS development are associated with microglia responses towards fever and infections. During normal CNS development, microglia are

involved in neurogenesis, synaptogenesis, oligogenesis, and synaptic pruning (Tay et al., 2018; Cheadle et al., 2020; Diaz-Aparicio et al., 2020). Microglia reduction or depletion leads to dysfunctional synaptic transmission due to impaired synaptic pruning (Paolicelli et al., 2011; Squarzoni et al., 2014; Zhan et al., 2014). The emergence of immune-sensing microglia that can respond to fever and infections during pregnancy could cause issues in microglia- and CNS development. Perturbation of microglia during development could be long-lasting and affect microglia and the CNS later in life. An immune response of fetal microglia towards infections may also lead to inflammation, potentially perturbing CNS development. Furthermore, an inflammatory challenge during pregnancy can disrupt developmental microglia functions such as neurogenesis and synaptic pruning, which may result in perturbed CNS development and neurological issues later in life. Immune-surveilling properties of GW>13 microglia during human fetal development (**Chapter 2**) enable microglia responses towards infection and fever and may cause perturbed CNS development and have long-lasting consequences on CNS integrity.

Similar to fever and infections during pregnancy in humans, the offspring in mice after maternal immune activation (MIA) using poly I:C (toll-like receptor 3; TLR3) is prone to developing repetitive behavior such as self-grooming, schizophrenic-like and autism-like behavior, and impaired social interaction and memory (Choi et al., 2016; Mattei et al., 2017; Ikezu et al., 2020). Concomitantly, microglia from embryos and adult offspring after MIA exhibit altered transcriptional profiles and a pro-inflammatory phenotype with decreased phagocytic ability, suggesting long-lasting effects of perturbed CNS development (Mattei et al., 2017; Antonson et al., 2019; Ikezu et al., 2020). Depleting and repopulating microglia after MIA using CSF1R inhibitors abrogates behavioral abnormalities and restores microglia expression profiles (Ikezu et al., 2020), suggesting that microglia responses and changes during perturbed CNS development are involved in neurodevelopmental disorders. Interestingly, autism-like behavior in MIA offspring is also dependent on maternal IL17a (Choi et al., 2016), indicating a role for T helper (Th) 17 cells. Thus, multiple immune cell types of the CNS and the periphery and their potential crosstalk may be involved in neurodevelopmental disorders after prenatal infections and fever. Since human fetal microglia upregulate immune-surveillance genes including receptors involved in T-cell activation such as VISTA, a communication between microglia and T-cells at this gestational age (GW>12) is possible and may be involved in the vulnerability of the developing CNS towards perturbations.

Microglia development and implications for MS

Multiple studies have linked perturbed CNS development to a variety of neurodevelopmental disorders (Prinz and Priller, 2014; Prins et al., 2018; Tay et al., 2018) as described above, and the emergence of immune-surveilling microglia in human coincides with the critical phase during pregnancy in which the fetus is most vulnerable (GW>12) (**Chapter 2**) (Hornig et al., 2018; Croen et al., 2019). It is still unclear whether disturbances during fetal CNS development can affect the risk of developing other CNS diseases including MS and how microglia might be involved.

In mice, EAE disease onset is earlier and disease progression is more severe in adult offspring after MIA using lipopolysaccharide (LPS) (TLR4) or poly I:C (TLR3) (Solati et al., 2012; Mandal et al., 2013; Zager et al., 2015). Concomitantly, macrophage and T-cell infiltration is increased in EAE mice after MIA (Zager et al., 2015), especially of Th17 cells (Mandal et al., 2013). In

humans, caesarean delivery and prenatal exposure to pesticides may be associated with an increased risk of developing pediatric MS (Graves et al., 2017). The risk for MS may also be increased in case of maternal obesity, diabetes, and use of diethylstilbesterol (Gardener et al., 2009), a synthetic estrogen formerly used to prevent miscarriages. These epidemiological data, however, should be treated with much caution, since only two studies are available (Gardener et al., 2009; Graves et al., 2017), both featuring limited statistical power likely owing to a low number of cases (<1,000). A Danish study including more than 1.5 million individuals found that children from mothers with pregestational diabetes mellitus had a 2.3-fold increased risk for developing MS (Nielsen et al., 2020).

Not only environmental factors but also genetic predisposition, which may affect microglia development, can link the risk for MS with perturbed microglia and CNS development. A rare genetic disorder called hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS) links disrupted microglia development to demyelinating disease. HDLS is a rare genetic disease with adult onset characterized by demyelination, cerebral white matter (WM) degeneration, and axonal spheroids (Nicholson et al., 2013; Saitoh et al., 2013), an accumulation of axonal cytoplasm due to interrupted axonal transport. HDSL shares pathological features of MS including WM and grey matter (GM) demyelination and HDSL can be misdiagnosed as primary progressive MS (Saitoh et al., 2019). In most cases of HDLS, the disease is caused by a mutation in the *CSF1R* gene (Nicholson et al., 2013; Saitoh et al., 2013). CSF1 signaling is essential for neurodevelopment, microglia maturation, and survival (Erblich et al., 2011), and mutations in *CSF1R* may cause disrupted myelination during development and myelin maintenance in adulthood (Saitoh et al., 2013). Hence, perturbed microglia development may cause improper myelination during neurodevelopment and could increase the risk for developing demyelinating diseases such as MS later in life.

Genetic risk factors for MS have been analyzed extensively using GWAS and the latest study reported 233 genetic associations, single nucleotide polymorphisms (SNP), with MS susceptibility (Patsopoulos et al., 2019). These 233 SNP were associated with 551 putative MS susceptibility genes using cis-expression quantitative trait loci (cis-eQTL) analysis employing published eQTL datasets (Patsopoulos et al., 2019). Of note, these genes do not necessarily represent real MS susceptibility genes, as an eQTL is not always influenced by a single SNP, but potentially by multiple causal variants including environmental factors. Hence, functional studies are required to validate these putative MS susceptibility genes. The majority of putative MS susceptibility genes are associated with immune functions including adaptive immunity and cytokine production (Patsopoulos et al., 2019). Thirty-two SNP are associated with *major histocompatibility complex* (*MHC*) genes (Patsopoulos et al., 2019). Analyzing the expression of the genes associated with these 233 SNP in CNS cell types revealed that expression is enriched in microglia, but not neurons or astrocytes, pointing to an important role for microglia in MS susceptibility (Patsopoulos et al., 2019). During fetal microglia development, expression of a subset of these putative MS susceptibility increases with increasing age. These genes include immune-related genes such as genes involved in T-cell activation (e.g. *MHC*), genes involved in phagocytosis (e.g. *MERTK*), and genes related to IFN and TNF signaling (**Chapter 2**). Perturbed microglia development may alter expression of certain putative MS susceptibility genes long-term, potentially resulting in a microglia phenotype that contributes to MS susceptibility.

In summary, perturbed CNS development may affect microglia long-term, thereby enhancing the risk for developing MS during adulthood. These findings are observational and speculative

at this point, but highlight an unexplored potential relationship between disrupted microglia and CNS development and the risk for MS. Additional detailed analysis and future studies are warranted to further substantiate this hypothesis.

Microglia in neurodevelopment: future perspectives

In **chapter 2**, the first study reporting on human fetal microglia development is presented which yielded unexpected results of potential high relevance for fetal CNS development.

We isolated microglia from fetuses of GW9 to 18, which roughly covers the first half of human development. It would be highly valuable to study microglia development from conception to birth including embryonic stages (conception to GW9), late fetal stages (GW18 to birth), and early infancy. Characterizing microglia during the full developmental period will help to understand normal and perturbed CNS development, and potentially the development of neurodevelopmental disorders. Our current knowledge on human microglia development is based on scRNAseq but uses bulk microglia populations to assess chromatin accessibility (**Chapter 2**). Gaining more insights into heterogeneity in transcriptional regulation using single cell ATACseq and protein expression using, for example, CyToF, would greatly enhance our understanding of microglia development and developmental heterogeneity. Access to fresh tissue of very early and late GW fetuses is difficult and samples are very limited due to technical and ethical constraints. For ATACseq and RNAseq, an alternative approach could be the use of nuclei isolated from frozen tissue, which is still scarce but more readily available than fresh tissue.

We defined multiple developmental microglia subtypes in **chapter 2**, but functional annotation is largely based on transcriptional profiles, chromatin accessibility, and in situ protein expression. Due to lack of availability and small tissue sizes, in situ analyses are limited. Resolving the anatomical locations of microglia subtypes in the developing CNS, e.g. using spatial transcriptomics or in situ sequencing, will provide more insight into the developmental mechanisms that these subtypes could be involved in (e.g. phagocytosis of apoptotic cells in neurogenic areas). To further define these subtypes, they can be isolated to determine their function in vitro using assays to test their phagocytic ability and their ability to respond to pathogens and other foreign compounds. Alternatively, induced pluripotent stem cells (iPSC) offer another technology to test the function of these subtypes. Human iPSC-derived microglia can be generated efficiently in vitro and resemble human microglia transcriptionally and functionally (Abud et al., 2017). Furthermore, iPSC-derived microglia or iPSC-derived hematopoietic progenitors can be transplanted into transgenic mice, where they display a phenotype similar to human microglia (Abud et al., 2017; Hasselmann et al., 2019; Xu et al., 2020). These technologies offer a powerful tool to study human microglia genes in vivo. Genes or transcriptional regulators that are highly expressed in distinct developmental microglia subtypes could be depleted in iPSC followed by functional assays in vitro or in vivo. Assessing the function of these developmental microglia subtypes will provide insights into the dynamics of CNS development.

Chapter 2 focuses on microglia during normal CNS development and our findings may have implications for perturbed CNS development and resulting neurodevelopmental disorders and neurological deficits later in life. To further dissect the role of microglia in perturbed CNS development, it will be essential to characterize microglia of fetuses with impaired development. For example, miscarriages may occur due to perturbed CNS development after

infections, fever, or endogenous impaired development. Studying microglia during impaired development may provide novel strategies to counteract neurodevelopmental disorders.

In addition to neurodevelopmental disorders, it is interesting to assess whether perturbed CNS or microglia development increases the risk for developing CNS diseases such as MS later in life. As discussed above, there is limited epidemiological evidence and thus a link between MS and disrupted CNS development is not yet conclusive. Our novel analyses of genetic MS risk factors in conjunction with microglia development indicates that CNS and microglia development could play a role in MS risk later in life; however, epidemiological studies with large cohorts are required to provide more solid evidence that perturbed microglia or CNS development is associated with MS susceptibility. In addition to epidemiological studies, MIA offers an animal model that allows testing of this hypothesis. Analyzing the effect of MIA on the susceptibility and progression of MS by using EAE, cuprizone, or other MS models could elucidate whether perturbed CNS development increases the risk for MS. Furthermore, to assess the role of microglia in the long-term effect of perturbed CNS development on MS susceptibility, microglia could be isolated after MIA and EAE/cuprizone in offspring and assessed using transcriptional profiling, chromatin accessibility, proteomics, and functional assays such as myelin phagocytosis and inflammatory responses.

Astrocytes and their interaction with microglia in MS

Chapter 3 addressed astrocyte heterogeneity based on anatomical regions and two surface receptors ACSA-2/ATP1B2 and GLAST/SLC1A3, and differential transcriptional responses of astrocyte subtypes during EAE were characterized. Astrocytes exhibited considerable heterogeneity, both interregional (forebrain, hindbrain, spinal cord) and intraregional ($GLAST^{pos}$, $GLAST^{neg}$). Expression of GLAST distinguished transcriptionally distinct astrocyte subtypes, which were particularly different within the hindbrain. $GLAST^{pos}$ hindbrain astrocytes shared transcriptional features and anatomical location of Bergmann glia, which are specialized astrocytes of the cerebellar cortex. During EAE, hindbrain and spinal cord astrocytes acquired non-overlapping gene expression changes, demonstrating distinct transcriptional responses during neuroinflammation. Particularly spinal cord astrocytes were affected during EAE, which is likely due to the fact that most pathological changes occur in this anatomical region. During initial and acute phases of EAE, spinal cord astrocytes obtained a reactive phenotype similar to LPS-stimulated, neurotoxic (also called A1) astrocytes previously associated with infection and neurodegenerative diseases (Liddelow et al., 2017). These reactive astrocytes downregulated genes involved in blood-brain barrier (BBB) maintenance and neuronal support and upregulated MHC-II, suggesting a loss of homeostatic astrocyte function and an involvement in (re)activation of infiltrating T cells during EAE. In the chronic EAE phase, this reactive transcriptional profile was reduced and spinal cord astrocytes upregulated proliferation markers. Astrocyte proliferation in these stages promotes glial scar formation in an effort to promote regeneration and to shield healthy tissue from spreading damage such as cell debris, damage-associated molecular patterns, and inflammatory cells. Glial scars, however, can also inhibit regeneration and should therefore not be viewed binary as only beneficial or detrimental (Bradbury and Burnside, 2019).

Different roles of astrocytes in distinct stages of EAE and MS

Astrocytes were initially thought to only play a role at late stages of lesion development during MS by forming scar tissue, however, it is now appreciated that they are also involved in early lesion development (Brosnan and Raine, 2013). The exact role of astrocytes at different stages of lesion development and how they contribute to lesion formation is just beginning to emerge. Research on neuromyelitis optica (NMO) underlines the importance of astrocytes in demyelination and lesion-formation. NMO is an autoimmune disease caused by autoantibodies which leads to demyelination of the optic nerve and nerve fibers in the spinal cord. A common autoantibody found in NMO targets AQP4, a water channel expressed by astrocytes, and the autoimmune response against astrocytes can cause demyelination and the formation of lesions similar to those observed in MS (De Parratt and Prineas, 2010). It is unclear whether astrocytes are also implicated in demyelination in MS. In our EAE study presented in **chapter 3**, astrocytes acquire two different phenotypes: a highly reactive profile at early and acute stages, and a less reactive and more proliferative profile in late stages.

The reactive profile observed in early and acute stages of EAE is characterized by a transcriptional profile similar to LPS-stimulated (A1) reactive astrocytes, which were previously associated with a neurotoxic phenotype (Zamanian et al., 2012; Liddelow and Barres, 2017; Liddelow et al., 2017; Clarke et al., 2018). These reactive astrocytes in early and acute EAE lose their homeostatic signature including a reduction in expression of genes involved in cholesterol synthesis, BBB, and neuronal-support (**Chapter 3**). Facilitated entry of immune cells from the blood to the CNS via the BBB is a hallmark of MS (Compston and Coles, 2008; Dendrou et al., 2015; Thompson et al., 2018). Astrocytes are directly involved in forming the BBB as their end feet cover the endothelial layer, which is called the glia limitans. A downregulation of BBB genes in reactive astrocytes (**Chapter 3**) may facilitate transmigration of immune cells from the blood to the CNS. During MS (and NMO), many astrocytic end feet are lost or retracted and thus do not cover the entire endothelial layer (De Parratt and Prineas, 2010; Brosnan and Raine, 2013), likely rendering the BBB more accessible for cellular transmigration. A loss of cholesterol synthesis and lactate metabolism genes observed in reactive astrocytes during early and acute EAE (**Chapter 3**) may amplify oligodendrocyte and neuronal damage. Trophic support is a major task of astrocytes and is required for proper neuron and oligodendrocyte function. Especially oligodendrocyte death occurs early in MS lesion development and is caused by inflammation and resulting demyelination. A lack of trophic support by astrocytes likely further exacerbates oligodendrocyte damage, as restoring cholesterol synthesis ameliorates EAE (Itoh et al., 2017).

In addition to downregulation of homeostatic astrocyte genes, during early and acute stages of EAE reactive astrocytes upregulate genes involved in the complement cascade, inflammatory cytokine production, and antigen-presentation (**Chapter 3**). This reactive astrocyte phenotype is also observed in active MS lesions, reflected by co-expression of C3 and GFAP, and to a lesser extent also in chronic active and inactive lesions (Liddelow et al., 2017), suggesting this phenotype is mostly present during earlier phases of lesion pathology. Induction of this reactive astrocyte phenotype observed in EAE occurs through expression of the transcription factor MAFG (Wheeler et al., 2020). MHC-II expression is induced in these reactive astrocytes (**Chapter 3**), suggesting they are able to present antigens to infiltrating $CD4^{pos}$ Th cells in MS lesions. Astrocytes in active MS lesions contain myelin debris, which they take up through receptor-mediated endocytosis potentially using LRP1 leading to NFkB

activation (Ponath et al., 2017). Thus, astrocytes may present myelin antigens to infiltrating lymphocytes to stimulate (re)activation early during lesion formation. Mouse astrocytes can induce antigen-specific $CD4^{pos}$ and $CD8^{pos}$ T-cell proliferation in vitro after IFNg treatment which upregulates MHC-II and B7 co-stimulatory molecules (Cornet et al., 2000). Purified human astrocytes also upregulated MHC-II in response to IFNg and TNFa in vitro, but are unable to induce T-cell proliferation, likely due to a lack in co-stimulatory factor induction such as cytokines and co-stimulatory receptors (Weber et al., 1994). However, stimulation of human astrocytes in vitro using phosphorylated alpha synuclein, a protein that accumulates in Parkinson's disease (PD), induces surface expression of co-stimulatory and co-inhibitory molecules PDL1, CD80/CD86, and CD40, rendering them capable of inducing T-cell activation (Rostami et al., 2020). Further experiments are required to resolve if and to what extent astrocytes are involved in myelin antigen-presentation during lesion formation and progression in MS.

In chronic stages of EAE, astrocytes downregulate reactive genes compared to early and acute stages, albeit expression does not return to normal levels of naïve astrocytes (**Chapter 3**). Furthermore, astrocytes in chronic EAE upregulate genes involved in cell cycle and proliferation and are actively dividing (**Chapter 3**). Proliferation of astrocytes is important for maintaining inflammation and promoting repair, as a lack of astrocytes (GFAP knockout) (Liedtke et al., 1998) or ablating proliferating astrocytes (Voskuhl et al., 2009) during EAE disrupts glial scar formation and facilitates inflammation, BBB damage, neuronal loss, and demyelination. Glial scar formation, which occurs after demyelination predominantly in chronic MS lesions, requires astrocytic proliferation and seems to be beneficial for tissue repair. These changes suggest that astrocytes in late stages of MS lesion progression may slowly progress towards a homeostatic, proliferative phenotype, which might be involved in tissue regeneration. In line with this argument, astrocytes in remyelinating MS lesions show partly regenerated end feet, although structural abnormalities such as free-floating astrocytic processes remain (Brosnan and Raine, 2013).

In summary, during EAE astrocytes acquire different reactive phenotypes ranging from more detrimental in early and acute stages to more proliferative and tissue-supportive later during disease. These astrocyte phenotypes appear to correspond with astrocyte functions in different stages of lesion pathology in MS. **Chapter 3** is predominantly based on transcriptional changes in astrocytes during EAE and dissecting differences using functional assays will help to understand the role of astrocytes in EAE and during MS.

Potential role of VISTA in astrocyte interaction with microglia in MS

Neuroinflammation during MS depends on intricate interactions between multiple cell types including CNS-resident cells and infiltrating immune cells. Astrocyte and microglia interactions are important for orchestrating development, homeostasis, neuroinflammation, and regeneration processes through direct interactions.

Neurotoxic (LPS-stimulated; A1) reactive astrocytes, which show similarities to the reactive astrocytes we observed during early and acute EAE stages (**Chapter 3**), are induced by activated microglia (Liddelow et al., 2017). Following intraperitoneal LPS injections, microglia and potentially other CNS cell types secrete IL1a, TNFa, and C1q, which together induce a neurotoxic reactive astrocyte profile (Liddelow et al., 2017). Furthermore, microglia-derived TGFa and VEGFB regulate the pathogenic activity of astrocytes during EAE and potentially in

MS lesions (Rothhammer et al., 2018). TGFa induces a neuroprotective astrocyte phenotype, whereas VEGFB promotes a phenotype that exacerbates CNS inflammation (Rothhammer et al., 2018). Vice versa, astrocytes also secrete factors that modulate microglia behavior. IL33 secreted by astrocytes during postnatal development in mice is required for microglia to perform synaptic pruning in order to eliminate excess synapses (Vainchtein et al., 2018). In pathological environments, IL33 acts as a damage signal and can be beneficial in regeneration of spinal cord injury (Gadani et al., 2015). Other factors secreted by microglia and astrocytes that are used for communication were identified, which was reviewed in detail previously (Jha et al., 2019; Liu et al., 2020; Vainchtein and Molofsky, 2020).

In MS lesions, reactive astrocytes are often located in close proximity to CD68^{pos} microglia/macrophages (Liddelow et al., 2017), which indicates cell-cell interactions through direct contact in addition to crosstalk through soluble mediators. However, membrane-bound receptor interactions of microglia and astrocytes have not been studied in detail yet. As discussed above (see "Different roles of astrocytes in distinct stages of EAE and MS"), reactive astrocytes express MHC-II and co-stimulatory receptors and are capable of direct interaction with T cells in order to present antigens. It is conceivable that astrocytes also express receptors and ligands that can interact with microglia directly. Human and mouse astrocytes express *VSIG3* (Fig. 1), which is a potential counterreceptor of VISTA that inhibits T-cell activation. Therefore, astrocytes may be able to signal to microglia via VSIG3-VISTA interaction, which could affect microglia function. Of note, expression of *VSIG3* was unaltered in astrocytes during the course of EAE (data not shown). It remains unknown if VISTA has one or multiple receptors and ligands, and whether astrocytes and microglia interact via VISTA. Future directions to assess astrocyte and microglia interaction through receptors is given below (see "Astrocytes and their interaction with microglia in MS: future perspectives").

Astrocytes and their interaction with microglia in MS: future perspectives

Chapter 3 demonstrates that astrocytes acquire distinct transcriptional profiles at different stages of EAE. ScRNAseq of astrocytes during different stages of EAE (priming, peak, remission) yields similar results, as distinct astrocyte subtypes expand at different stages (Wheeler et al., 2020). How these astrocyte subtypes contribute to different stages of EAE

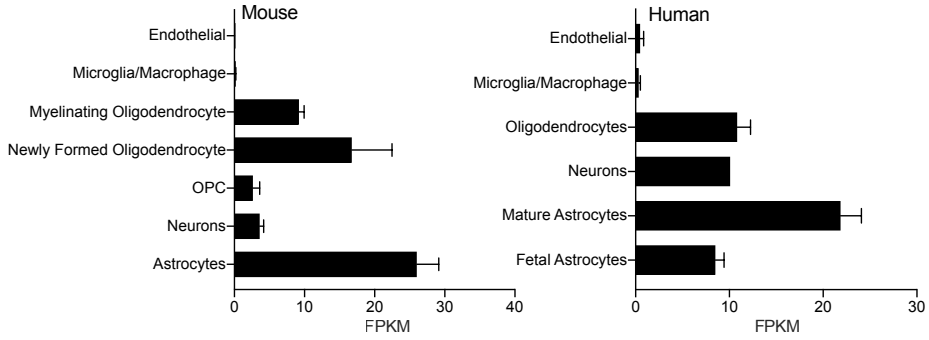

Figure 1. Expression of *VSIG3* in mouse and human CNS cell types. *VSIG3* mRNA levels (fragments per kilobase million; FPKM) in different CNS cell types in mouse and human, derived from published RNA-seq data (Zhang et al., 2014, 2016).

and MS is not completely understood. Pathogenic astrocytes, predominantly found during the peak of EAE, are driven by the transcription factor MAFG and disrupting this pathway alleviates EAE (Wheeler et al., 2020). Ablating proliferating astrocytes interferes in glial scar formation and exacerbates inflammation in EAE (Voskuhl et al., 2009). There is limited evidence that these astrocyte subtypes also exist in MS (Wheeler et al., 2020) and functional assessment of astrocyte subtypes in MS is lacking. To understand how astrocytes contribute to MS and specifically to pathology in different lesion stages, it will be necessary to determine whether astrocyte subtypes, analogous to the subtypes observed in EAE, are also present in MS. Single cell approaches (RNAseq, ATACseq, and protein-based assays) will help resolve astrocyte heterogeneity, followed by assessing functional differences of these subtypes. Characterizing astrocyte subtypes and their function at different lesions stages will provide great insights into astrocyte role in MS and may unravel novel treatment strategies.

Many studies have extensively characterized individual cell types in EAE and MS, and this knowledge can be harnessed to explore cell-cell interactions. Likely, the most studied direct cell-cell interaction is the activation of T cells through MHC-II and T-cell receptor (TCR) signaling in addition to co-stimulatory receptors and cytokines. **Chapter 3** demonstrates that astrocytes during EAE express MHC-II and other studies suggest that at least in vitro astrocytes are able to stimulate T-cell proliferation. It is yet unknown whether ablating MHC-II expression in astrocytes affects T-cell infiltration and (re)activation in EAE. This could be assessed using transgenic mice in future experiments. Besides potential interactions with infiltrating T cells, astrocytes are found in close proximity to microglia in MS lesions (Liddelow et al., 2017), suggesting cell-cell interactions. Crosstalk of microglia and astrocytes through cytokines is well-studied, but cell-cell interactions via receptors are not well described (see above). These cell-cell interactions may facilitate activation or inhibition of distinct astrocytes and microglia phenotypes during disease. Astrocytes express receptors that could directly bind to receptors expressed on microglia, such as VSIG3 and VISTA. Whether VISTA and VSIG3 actually interact and if this interaction has functional consequences for microglia or astrocytes should be assessed in the future using cell type-specific knockout (KO) experiments. Astrocyte and microglia transcriptional profiles during homeostasis, EAE, and MS are available, and these data could be harnessed to identify potential receptor-ligand pairs that are used for communication. Cell type-specific KO in co-cultures and transgenic mice could be used to test whether these receptors indeed affect microglia and astrocyte phenotypes, the expansion of subtypes, or modulate EAE progression. Integrating our knowledge on individual cell types to gain insights into cell-cell interactions in homeostasis and EAE and MS will enhance our understanding of cell crosstalk during MS and potentially how to interfere in pathogenic interactions.

VISTA role in microglia and the CNS and its therapeutic potential for MS

In **chapter 4**, VISTA expression in the healthy CNS and during neuroinflammation was characterized using a combination of in situ, in vitro, and in vivo analyses of human, mouse, and macaque tissue. VISTA was predominantly expressed by microglia and to lesser extent endothelial cells in healthy CNS. After TLR ligation in vitro, macaque and mouse microglia downregulated VISTA expression. In mice, freshly isolated microglia exhibited significantly

decreased *VISTA* expression after LPS exposure, during EAE, and in Ercc1-deficient mice. ATACseq revealed that microglia VISTA is potentially regulated by PU.1 and MAFB, two transcription factors important for microglia development and function. Finally, VISTA expression was decreased in a chronic active lesion in post-mortem tissue of an MS patient. To expand on these observations, in **chapter 5**, expression of VISTA in multiple CNS inflammatory and neurodegenerative in humans and respective animal models was assessed using public domain mRNAseq data sets. *VISTA* expression was higher in microglia compared to peripheral myeloid cells. During MS, stroke, neurodegeneration, and other CNS diseases, microglia *VISTA* was decreased. To understand how decreased VISTA expression may affect MS and microglia function, **chapter 6** assessed VISTA expression in distinct MS lesion stages, and VISTA function in microglia was characterized using a novel microglia VISTA KO mouse model. VISTA expression was increased in immunological active lesions, but decreased in inactive lesions. Expression of VISTA more strongly correlated with expression of microglia markers IBA1 and TMEM119 than with expression of inflammatory markers CD68 and HLA-DR. Microglia VISTA KO did not affect the microglia transcriptional response to LPS and during EAE. Furthermore, microglia VISTA KO did not influence the development or progression of EAE. In naïve mice, however, VISTA KO resulted in decreased microglia ramification, increased expression of cell cycle genes, and elevated levels of genes associated with microglia immune-activation. In vitro, VISTA KO in microglia reduced their ability to phagocytose myelin, but did not affect phagocytosis of early apoptotic Jurkat cells or *E. coli* particles. These findings demonstrate that microglia VISTA expression is downregulated during CNS disease in mice, and differentially regulated in distinct MS lesion stages. Since VISTA regulates microglia myelin phagocytosis and induces a more regulatory, homeostatic microglia phenotype, VISTA might be involved in CNS homeostasis, neuroinflammation, and MS lesion pathology.

Functions of VISTA in microglia versus peripheral myeloid cells

Microglia are myeloid 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). As opposed to other tissue-macrophage subsets, microglia also exhibit a range of CNS-specific functions including synaptic pruning, and the release of neurotrophic as well as neurotoxic factors (Colonna and Butovsky, 2017). During homeostasis, microglia are constantly scanning their environment and are highly sensitive and responsive towards any perturbations (Colonna and Butovsky, 2017). Hence, regarding resting microglia as inactive tissue macrophages has become obsolete. Classically, microglia have been classified as M1-M2, in analogy to nomenclature in macrophages, but with emerging insights in microglia heterogeneity and functions, this classification system has become too limited or even obsolete (Ransohoff, 2016). There is an urgent need for a better classification system, in analogy to what was recently reported for astrocytes (Escartin et al., 2021).

One function of VISTA expressed by myeloid cells as a ligand is the inhibition of T-cell activation, thus it acts as an NCR (Wang et al., 2011) (Fig. 2). Microglia are capable of presenting antigens and expressing other NCR. It is thus conceivable that VISTA as a ligand also acts as an NCR in microglia, where it binds to a counterreceptor on T cells leading to inhibition of T-cell activation (Fig. 2). VISTA functioning as an NCR in microglia might be of particular relevance for CNS-peripheral immunity interactions (discussed below), which predominantly occur during immune cell infiltration in CNS diseases such as MS. Whether

VISTA on microglia acts as an NCR needs to be assessed in future studies. In CNS development, brain-resident CD4 T cell interaction with microglia is required for microglia development (Pasciuto et al., 2020). In view of VISTA as an NCR in the adult CNS, it is intriguing that microglia express VISTA at such high levels during steady state, since peripheral immune cells including T cells are sparse in healthy brain parenchyma. Therefore, it is not surprising that in addition to potentially inhibiting T-cell activation, VISTA in microglia has a function as a receptor.

In peripheral myeloid cells, VISTA has multiple additional functions including phagocytosis of apoptotic cells (efferocytosis) (Yoon et al., 2015; Cohen et al., 2016), cytokine production (Bharaj et al., 2014, 2018), tolerance induction (ElTanbouly, Schaafsma, et al., 2020), and chemotaxis (Sergent et al., 2018; Broughton et al., 2019) (Fig. 2). **Chapter 6** demonstrates that VISTA in microglia regulates myelin phagocytosis, but not efferocytosis of early apoptotic Jurkat cells. It is yet unknown whether VISTA also regulates amyloid or synapse uptake (Fig. 2), which would be of particular importance for CNS development and Alzheimer's disease (AD). Microglia are responsible for clearing cellular and molecular debris in the CNS, especially during development and disease (Colonna and Butovsky, 2017). Microglia also regulate the size of the neural progenitor pool using phagocytosis in the developing cortex (Cunningham et al., 2013). Furthermore, microglia are involved in synaptic pruning (synaptophagy) (Colonna and Butovsky, 2017), a unique form of phagocytosis to eliminate viable synapses, which is required for learning and memory. In view of its role in microglia myelin phagocytosis, VISTA as a receptor might also be involved in other phagocytic processes such as uptake of neural progenitors or synaptic pruning.

Microglia are highly capable of producing pro and anti-inflammatory cytokines and chemokines upon receiving a wide variety of stimuli (Colonna and Butovsky, 2017). The intracellular pathways leading to the production of these signaling molecules is mostly conserved between microglia and other tissue macrophages. Since overexpression of VISTA in vitro leads to spontaneous cytokine secretion (TNFa, IL1b) in human monocytes (Bharaj et al., 2014), and KO of VISTA is associated with an altered cytokine and chemokine profile (CCL2, CCR2, IL23) (Ceeraz, Eszterhas, et al., 2017; Ceeraz, Sergent, et al., 2017; Li et al., 2017; Liu et al., 2018), VISTA may also be involved in microglia cytokine and chemokine production. Indeed, VISTA KO microglia exhibit a more amoeboid morphology and increased expression of genes associated with immune-activated microglia during neuroinflammation and neurodegeneration (**Chapter 6**) (Fig. 2). Together these findings suggest that VISTA is required to maintain a more regulatory, homeostatic microglia profile. However, deletion of VISTA does not affect EAE development or microglia responses to LPS and during EAE, both of which are severe peripheral inflammatory challenges. It is possible that immune responses of microglia are more tightly regulated than those of peripheral macrophages in order to prevent excessive CNS tissue damage.

VISTA as a receptor is not only involved in the production, but also in the response to chemokines. Blocking VISTA in mice leads to enhanced migratory capacity of monocytes in response to CCL2 in vitro (Sergent et al., 2018). CCL2 in the CNS is produced by astrocytes, microglia, endothelial cells (Semple et al., 2010), and can be produced by neurons during stress such as impairment of oxidative metabolism (Yang et al., 2011). Microglia express CCR2 and respond to CCL2 by migrating and producing cytokines (Semple et al., 2010). This response can be both beneficial and detrimental as it leads to clearance of debris, but also contributes to neuroinflammation by production of pro-inflammatory cytokines (Semple et

al., 2010; Yang et al., 2011). VISTA could be involved in the microglia response and migration to CCL2, which would have consequences particularly during CNS diseases where CCL2 production is increased, such as MS, traumatic brain injury, and stroke (Semple et al., 2010). The transcriptional profiles of VISTA KO microglia did not reveal a change in chemokine production or expression of chemokine receptors (**chapter 6**). However, functional assays are required to determine whether VISTA in microglia is also involved in chemotaxis in future studies.

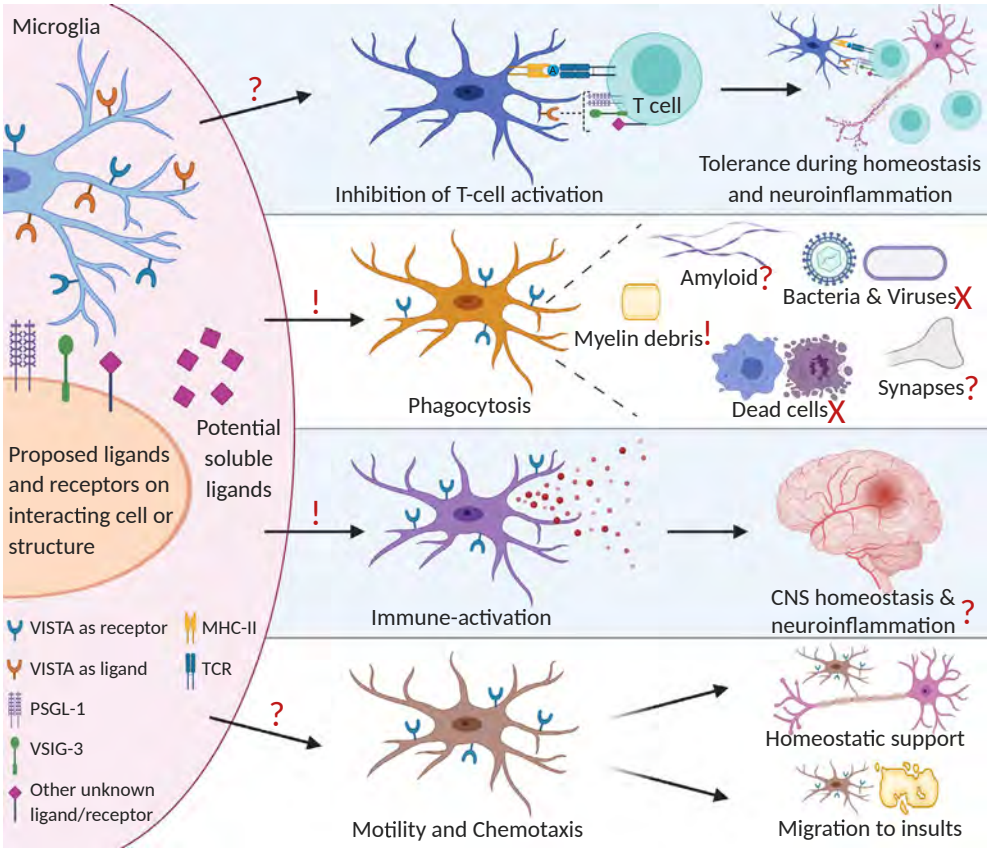

Figure 2. Known and potential functions of VISTA in microglia. VISTA expressed on microglia may act as a receptor and a ligand, binding to proposed and unknown ligands/receptors. Similar to VISTA function in peripheral myeloid cells, VISTA regulates phagocytosis and induces a more regulatory, homeostatic microglia phenotype. VISTA KO decreases uptake of myelin, but not of apoptotic cells or *E. coli* particles. It is unknown whether VISTA also regulates uptake of amyloid or synapses. VISTA has more functions in peripheral myeloid cells, which have not been studied in microglia. These functions include antigen-presentation, and motility and chemotaxis. All known and potential functions of VISTA in microglia are important for maintaining CNS homeostasis including synaptic pruning, removal of metabolic waste and cell debris and immune tolerance. Furthermore, these VISTA functions in microglia could be essential during CNS disease, in which microglia are responsible for antigen presentation, defense against pathogens, protective versus destructive neuroinflammation and for tissue regeneration.

Roles of VISTA and other NCR in CNS-peripheral immunity interactions

NCR are pivotal signaling molecules that aid in balancing immune responses to limit autoimmunity while maintaining an effective immune response. Therefore, it is important to discuss the role of NCR and VISTA with regard to their direct signaling capacity through cell-cell interactions; in this case the interaction between CNS and peripheral immunity. There are two main types of CNS-peripheral immunity interactions: indirect (e.g. through cytokines and other secreting signaling molecules) and direct (cell-cell contact through receptors) (Greenhalgh et al., 2020). We will focus on direct interactions of glia and endothelial cells with the peripheral immunity via NCR and VISTA. Box 1 briefly summarizes recent views and debate on CNS immune privilege, as extensive discussion is beyond the scope of this chapter.

The initial contact of peripheral immune cells with the CNS is via endothelial cells, which directly interact with immune cells. Endothelial cells can regulate the transmigration of peripheral immune cells into the CNS, which is of particular importance during CNS diseases such as MS (Dong and Yong, 2019). Blocking the adhesion of immune cells to the endothelium by natalizumab blocking VLA4 emerged as an effective therapy to limit neuroinflammation (Steinman, 2005; Duan et al., 2013). Endothelial cells are capable of presenting antigens through MHC-II and can facilitate the transmigration of T cells into the brain parenchyma (Lopes Pinheiro et al., 2016). As capable antigen-presenting cells (APC), endothelial cells also express a range of NCR such as PDL1 and PDL2 which suppress T-cell responses in vitro (Rodig et al., 2003) and may inhibit T-cell transmigration. VISTA as a ligand expressed by endothelial cells may provide inhibitory signals to passing T cells as well, thereby fine-tuning T-cell reactivity in the CNS, which is of particular importance in CNS diseases with immune cell infiltration (e.g. MS). The function of VISTA in endothelial cells has not been studied to date but should be investigated particularly with regard to peripheral immune cell infiltration into the CNS and antigen presenting capability of the endothelium.

Other CNS cell types that can actively communicate with peripheral immune cells via direct contact are astrocytes and microglia. Both cell types can express MHC-II (induced/upregulated during inflammation notably by interferons) and are capable of presenting antigens to T cells (Antel et al., 2020), and both cell types express NCR, as previously mentioned. Microglia PDL1 expression regulates T-cell (re)activation in the CNS during EAE (Schreiner et al., 2008; Schachtele et al., 2014). In the transgenic APP/PS1 (APPswe/PS1dE9) mouse model for AD, depletion of microglia using a CSF1R inhibitor (PLX5622) results in an increase in parenchymal T cell numbers and a reduction of anti-inflammatory cytokines (Unger et al., 2018). It is thus conceivable that microglia provide inhibitory signals to T cells, which is essential to limit T-cell (re)activation in the CNS. Functional evidence on whether VISTA expressed by microglia has co-inhibitory effects on T-cell activation is lacking; however, based on extensive characterization of VISTA NCR functions in other myeloid cells, it is highly likely that blocking or depleting VISTA on microglia will enhance T-cell (re)activation in the brain.

Box 1. Immune-privilege features of the CNS

The healthy CNS has long been regarded as an immune-privileged organ. This assumption originated from classic observations that tissue allografts placed in the brain are not subject to immune rejection (Medawar, 1948; Billingham and Boswell, 1953), hence peripheral immune cells were thought to be excluded from the healthy CNS. Over the past decades however, this dogma has been challenged and modified. Although the CNS is immune privileged to some extent to limit tissue damage by carefully balancing protective versus excessive immunity, the CNS is also actively monitored by immune cells and does not exclude access and effector functions of peripheral immune cell subsets. Misconceptions on CNS immune-privilege and the role of the BBB were previously reviewed in detail (Bechmann et al., 2007; Galea, Bechmann, et al., 2007).

Resident CNS immune cell types

The healthy CNS has multiple lines of immunological defenses that provide protection from intruders, aid in clearing waste, and promote regeneration. CNS resident immunity is mainly composed of microglia, astrocytes, and brain border macrophages. These cells can phagocytose, secrete cytokines, recruit immune cells from the periphery, and present antigens. Peripheral immune cells patrol the perivascular space in the CNS (Loeffler et al., 2011; Smolders et al., 2013), ready to receive signals from CNS-resident immune cells to migrate into the parenchyma. $CD8^{pos}$ T cells that migrate into the parenchyma can acquire a memory-like profile ($CD103^{pos}$, $CD127^{pos}$) consistent with long-term tissue resident T cells (Wakim et al., 2010, 2012; Steinbach et al., 2016; Smolders et al., 2018). These T cells are likely involved in the defense against viruses (Wakim et al., 2010, 2012; Steinbach et al., 2016; Smolders et al., 2018).

The blood-brain barrier

The concept of the BBB is based on experiments performed by Paul Ehrlich using hydrophilic dyes that did not enter the CNS parenchyma (Ehrlich, 1885). Misconceptions on the BBB include that this tissue barrier inhibits both entry of cells and molecules from the periphery. While the BBB indeed shields the CNS from certain soluble molecules using tight junctions especially at capillary levels, it is possible for peripheral immune cells to enter the CNS preferentially at postcapillary venules (Bechmann et al., 2007). Endothelial cells are part of the BBB and are actively involved in antigen presentation (Galea, Bernardes-Silva, et al., 2007), which is of particular relevance during CNS disease that involve peripheral immune cell infiltration such as MS. Furthermore, the BBB can have reduced integrity during CNS inflammation, thereby facilitating infiltration of peripheral cells (Bechmann et al., 2007).

Lymphatic drainage of the CNS

The discovery of lymphatic drainage of the CNS is another case for reconsidering the immune-privileged status of the CNS. Solutes, tracers, and proteins drain from the brain to cervical lymph nodes resulting in immune responses (Cserr et al., 1992; de Vos et al., 2002; Laman and Weller, 2013; Louveau et al., 2015; Engelhardt et al., 2016; Dolgin, 2020), which suggests a functional lymphatic drainage of the CNS. The brain parenchyma itself lacks lymphatic vessels, however, lymphatic vessels are present in the meninges, which carry fluid, macromolecules, and immune cells from the cerebrospinal fluid (CSF) to the draining cervical lymph nodes (Louveau et al., 2015, 2018; Da Mesquita et al., 2018). Ablating these lymphatic vessels reduces T-cell infiltration during EAE and ameliorates symptoms (Louveau et al., 2018) and promotes amyloid-beta deposition in AD mouse models (Da Mesquita et al., 2018).

In summary, although the CNS features a certain degree of immune-privilege, likely to prevent excessive tissue damage, these immune-privileges are not absolute, since the CNS is actively monitored and capable of mounting immune responses tailored to infectious or sterile insults.

VISTA during microglia and CNS development

VISTA expression increases during mouse and human microglia development with highest expression during adulthood (**Chapters 2** and **4**). Homeostatic microglia markers and sensome genes follow similar expression dynamics during development suggesting that VISTA may play a role in healthy CNS development and in microglia homeostasis and immune-surveilling capacity. VISTA is a receptor and a ligand involved in several immune functions of myeloid cells including T-cell activation, cytokine response, phagocytosis, and chemotaxis (Flies et al., 2011; Wang et al., 2011; Bharaj et al., 2014; Yoon et al., 2015; Sergent et al., 2018). As such, increasing expression of VISTA matches the enhanced immune-surveilling capability of microglia later during human fetal development (**Chapter 2**). Since VISTA regulates microglia phagocytosis of myelin (**Chapter 6**), VISTA may play a role in neurodevelopmental processes. These processes may include clearance of cellular or molecular debris, which is crucial for neuro-, oligo-, and astrogenesis (Marín-Teva et al., 2004; Wakselman et al., 2008; Sierra et al., 2010; Cunningham et al., 2013), and synaptic pruning, which is essential for learning and memory. Most likely, VISTA is not a key regulator in these developmental processes, since generic VISTA KO mice are able to generate seemingly healthy offspring (Wang et al., 2014; Yoon et al., 2015); however, studies have not assessed cognitive abilities such as learning, attention, and memory formation in VISTA KO mice. Furthermore, the CNS of VISTA KO mice has not been studied in detail regarding structural changes during neurodevelopment and in adulthood.

The CNS is surveilled by peripheral immune cells and T cells with resident-like characteristics can be found in the brain parenchyma (Box 1) (Wakim et al., 2010, 2012; Steinbach et al., 2016; Smolders et al., 2018), albeit the presence of T cells in the parenchyma is still controversial. During mouse development, T cells receive activation signals in the periphery and migrate to the CNS where they acquire resident-like features (Pasciuto et al., 2020). Ablating T cells from periphery and CNS using MHC-II KO mice alters microglia transcriptional profiles and inhibits microglia fetal-to-adult transition and synaptic pruning capacities (Pasciuto et al., 2020). This T cell-mediated regulation of microglia development is likely due to secreted factors (Pasciuto et al., 2020). Hence, T cells and their interaction with microglia are crucial for mouse microglia development and important neurodevelopmental processes such as synaptic pruning. It is unknown whether microglia and T cells interact through VISTA in the adult and developing CNS. Yet, due to its function in myeloid cells, it is likely that microglia VISTA is involved in interaction with T cells and T-cell activation. Thus, VISTA may be involved in T-cell mediated regulation of microglia development.

Targeting VISTA in autoimmunity and cancer

In mouse studies, VISTA has successfully been used as a target for immunotherapy in cancer and autoimmunity. The two main approaches that are used to block or enhance VISTA signaling are employing immunoenhancing anti-VISTA antibodies (antagonists) or immunosuppressive anti-VISTA antibodies (agonists), respectively. In addition to antibodies, small molecules or constructs can also be designed to target VISTA, leading to enhanced or suppressed immunity.

In multiple mouse models of cancer, an immunoenhancing anti-VISTA antibody (clone 13F3) leads to a reduction in tumor size and increased overall survival (Le Mercier et al., 2014). Blocking VISTA using immunoenhancing antibodies leads to increased infiltration of

tumor-specific T cells, a decrease in myeloid-derived suppressor cell (MDSC) numbers and suppressive capacity, and a decrease in tumor-specific Tregs (Le Mercier et al., 2014). Using these immunoenhancing anti-VISTA antibodies in mouse models of autoimmunity including EAE (Wang et al., 2011) and murine lupus nephritis (Sergent et al., 2018) exacerbates disease. A small molecule CA-170 inhibits VISTA, PDL1, and PDL2, and showed antitumor effects in clinical studies (NCT02812875, clinicaltrials.gov) (Musielak et al., 2019).

Immunosuppressive anti-VISTA antibodies reduce the severity of inflammatory disease in mice including autoimmunity. Graft-versus-host disease (GvHD) is prevented when targeting VISTA on donor T cells using an immunosuppressive anti-VISTA antibody (Flies et al., 2011). Using this immunosuppressive antibody (clone MH5A or 8G8), disease severity of experimental asthma (Liu et al., 2018), lupus, hepatitis, psoriasis, and arthritis (ElTanbouly, Zhao, et al., 2020) are reduced, and autoimmunity in systemic and discoid lupus erythematosus is alleviated (Han et al., 2019).

In summary, VISTA can be used as a therapeutic target for both enhancing the immune response in case of cancer and inhibiting the immune response during inflammation and autoimmunity.

Immunotherapy in the CNS

Immunotherapy using immune checkpoint inhibitors is currently established as an effective treatment against several cancer types, and targeting immune checkpoints is more recently being explored as new treatment options for autoimmune diseases such as rheumatoid arthritis and MS. Studies mainly focus on the effects of immunotherapy on peripheral immunity; however, evidence strongly suggests that immune checkpoint inhibitors affect the CNS as well.

Currently, there is no U.S. food and drug administration (FDA)-approved immunotherapy for glioblastoma (GBM), but initial preclinical studies have yielded some encouraging results (Ratnam et al., 2019). Since GBM tumor cells and infiltrating T cells express a range of NCR, targeting these checkpoints may boost the anti-tumor immunity. In CNS metastatic diseases, immune checkpoint inhibitors targeting PD1 (pembrolizumab, nivolumab) and CTLA4 (ipilimumab) have been shown to slow down progression or reduce tumor size (Kamath and Kumthekar, 2018).

Immunotherapy may not only be beneficial in CNS-associated tumors, but also in NDD and MS. In AD, neuroinflammation is associated with increased hyperphosphorylated tau burden and microglia-mediated recruitment of peripheral immune cells can help in clearing amyloid-beta plaques (Dionisio-Santos et al., 2019). Anti-PD1 antibody therapy facilitates clearance of amyloid-beta and improves cognitive performance in AD mouse models (Baruch et al., 2016). However, conflicting data exist that suggest there is no effect of anti-PD1 therapy in AD (Latta-Mahieu et al., 2018). 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, deplete subsets of immune cells, or modulate immune signaling pathways; however, immune checkpoints are not used as a target for MS immunotherapy yet. Agonistic antibodies targeting NCR such as VISTA may enhance immune inhibition signals and therefore present an effective treatment for MS.

Interestingly, immune checkpoint inhibitors used in oncology have adverse effects on the CNS. Nivolumab treatment of melanoma induced spontaneous, reversible CNS demyelination in a patient (Pillonel et al., 2019). Immune checkpoint inhibitor-treated patients are also more susceptible to developing other CNS diseases including paraneoplastic neurological symptoms, encephalitis, MS, and hypophysitis, an inflammation of the pituitary gland (Yshii et al., 2017). In melanoma patients treated with immune checkpoint inhibitors, these neurological adverse events occur in 1% (anti-CLTA4), 3% (anti-PD1), or 14% (anti-CTLA4 and anti-PD1) of the population (Yshii et al., 2017). These complications are likely caused by augmented immune responses leading to neurotoxicity. However, it is incompletely understood whether the immune checkpoint inhibitor-associated CNS adverse effects and beneficial effects of immune checkpoint inhibitors on CNS-associated tumors are mediated indirectly via infiltrating immune cells, directly by therapeutic antibodies gaining access into the CNS parenchyma at meaningful concentrations, or by both. Since the BBB is compromised during MS and many CNS-associated tumors, it is highly likely that immune checkpoint inhibitors can act on CNS-resident cells directly. Therefore, studying NCR expression and function in CNS-resident cells is crucial to developing immune checkpoint inhibitor therapies for CNS diseases and to predict and mechanistically understand CNS adverse events.

Approaches to modulate VISTA in MS and other CNS diseases

Based on effectiveness of targeting VISTA in cancer and autoimmunity in mice and the use of immune checkpoint inhibitors in CNS-associated tumors and NDD, it is conceivable that VISTA may offer a novel therapeutic target for treating CNS disease.

When targeting VISTA as a therapeutic strategy to treat NDD, CNS-associated tumors, or MS, it is important to consider the complex expression dynamics and functions of VISTA. Using monoclonal antibodies against VISTA will not only target various peripheral immune cells (myeloid cells, neutrophils, T cells), but also microglia and CNS endothelial cells. Research has been focused on the function of VISTA in peripheral immune cell subsets, whereas there is no knowledge on the effects in the CNS of targeting VISTA using monoclonal antibodies.

Regarding the potential role of VISTA in microglia and the CNS (**Chapter 6**), multiple functional outcomes of VISTA modulation are plausible. Targeting VISTA on endothelial cells may be a viable option to inhibit or enhance T-cell activation during MS or NDD and cancer, respectively. In mice, VISTA KO enhances anti-glioma responses in mice (Flies et al., 2014). During MS, peripheral immune cell infiltration may be reduced upon enhancing VISTA signaling in endothelial cells. Microglia are APC and responsible for (re)activation of T cells in the CNS. Modulating VISTA on microglia may have similar effects as on endothelial cells. However, in microglia VISTA is involved in phagocytosis, cell cycle and immune-activation. These functions make it difficult to predict the outcome of modulating VISTA during peripheral and CNS disease. Using anti-VISTA antibodies systemically may affect microglia function unpredictably. It is therefore important to further dissect VISTA function in microglia in order to understand potential CNS responses to VISTA modulation. On the other hand, the large variety of functions that VISTA has in myeloid cells and potentially microglia may also open up treatment possibilities. For example, antibodies targeting different VISTA epitopes may have distinct functional consequences.

VISTA in microglia and as a therapeutic target for MS: future perspectives

VISTA represents an NCR with unique characteristics which in the CNS is predominantly expressed by microglia. Expression of VISTA is differentially regulated in ageing, neuroinflammation, and multiple CNS diseases including neurodegeneration, stroke, and cancer. In microglia, VISTA is involved in myelin uptake and the maintenance of a more regulatory, homeostatic microglia phenotype. Effective targeting of VISTA in cancer and autoimmunity opens wide possibilities to modulate VISTA as a therapeutic strategy in CNS disease. However, more knowledge on the functions of VISTA in the CNS and the effects of systemic VISTA modulation on the CNS is necessary to evaluate the therapeutic potential of targeting VISTA in CNS diseases. VISTA's roles in microglia and the CNS are currently only beginning to be explored; hence, we have formulated remaining open questions in Box 2. Answering these questions will provide insights into the function of VISTA in microglia and in CNS disease, which will potentially yield novel therapeutic strategies and mechanistic insights into CNS homeostasis and disease.

Box 2. Remaining open questions on VISTA in the CNS (continued on next page)

Does microglia VISTA regulate chemotaxis and T-cell activation?
VISTA regulates microglia phagocytosis of myelin and is involved in maintaining a more regulatory microglia phenotype. In macrophages, VISTA inhibits T-cell activation and is required for macrophage chemotaxis and migration. Microglia VISTA KO did not affect EAE progression or a dysregulated chemokine production. However, functional experiments such as chemotaxis- and T-cell activation assays are needed to determine whether VISTA in microglia is involved in chemotaxis and T-cell activation.

What is the role of VISTA in microglia and CNS development?
VISTA expression decreases in microglia during development and regulates myelin phagocytosis and regulatory, homeostatic microglia functions. Myelin formation, synapse pruning, neurogenesis and other developmental processes rely on microglia functions and activities. VISTA might contribute to a healthy CNS development. Depleting VISTA in microglia during development followed by extensive evaluation of CNS developmental processes such as neurogenesis and myelination including compaction will help to determine the role of VISTA in CNS development.

What is the function of VISTA in endothelial cells?

Brain endothelial cells express low levels of VISTA. Since endothelial cells are involved in antigen presentation and cell migration into the CNS, VISTA may play a role in the communication between endothelial cells and peripheral immune cells, particularly during diseases such as MS. Endothelial cell-specific VISTA KO in vivo and in vitro models could be developed to assess whether VISTA is involved in endothelial function including activation and transmigration of leukocytes.

Can VISTA expression be induced in other CNS-resident cells?

In non-diseased CNS tissue, VISTA is predominantly expressed by microglia and to a lesser extent by endothelial cells. Other NCR are known to be upregulated or induced during inflammation in other CNS cell types. Although VISTA expression is decreased in microglia during disease, bulk tissue VISTA expression is increased, suggesting induction or upregulation of VISTA on other CNS cell types. Extensive in situ analyses or single cell proteomics could be used to determine whether other CNS cell types upregulate VISTA under disease conditions.

Box 2 (continued). Remaining open questions on VISTA in the CNS

Which cell types express VISTA in CNS diseases and specifically in distinct MS lesion types?

Microglia and endothelial cells express VISTA, but other CNS-resident cells potentially induce VISTA expression in disease, which is currently unknown. During CNS disease and particularly MS, where peripheral immune cells infiltrate the CNS, VISTA expression by different cell types and subsets should be dissected to understand the role of VISTA in neuroinflammation. Single cell transcriptomic or proteomic as well as in situ analyses could help resolve which cell types express VISTA in MS lesions.

What are functional binding partners of VISTA?

Multiple VISTA binding partners have been proposed, however, many of these counterreceptors could not be replicated in other studies. To elucidate VISTA biology and develop VISTA-targeted treatment strategies, it will be essential to confidently identify potential binding partner(s).

Does modulating VISTA using immunotherapy affect the CNS?

Preclinical studies are investigating the therapeutic potential of targeting VISTA in cancer and autoimmunity. Neurological adverse events after blocking other NCR (PD1, CTLA4) in patients have been reported. Currently, it is unknown whether targeting VISTA can affect the healthy CNS or modulate CNS disease progression. CNS responses upon therapeutic targeting of VISTA should be studied to resolve whether neurological adverse events can be expected with an anti-VISTA therapy.

How can VISTA be we modulated during CNS diseases as a therapeutic strategy?

Several therapeutic strategies to target VISTA in CNS disease are conceivable. Using agonist and antagonist monoclonal antibodies or small molecules, VISTA signaling could be enhanced or suppressed to treat CNS-associated tumors, NDD, or MS. Research on possibilities to target VISTA in CNS disease is lacking.