Exploring the VISTA of glial cells

Borggrewe, Malte

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Appendices

Contents

Abbreviations

4OHT	4-hydroxytamoxifen
A2AR	Adenosine A2A receptor
ACSA2	Astrocyte cell surface antigen 2
AD	Alzheimer's disease
ALDH1L1	10-formyltetrahydrofolate dehydrogenase
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APOE	Apolipoprotein E
AQP4	Aquaporin 4
ARM	Activated response microglia
ATACseq	Assay for transposase-accessible chromatin using sequencing
ATP	Adenosine triphosphate
ATP1B2	ATPase Na+/K+ transporting subunit beta 2
AUC	Area under curve
AXL	Tyrosine-protein kinase receptor UFO
B2M	Beta-2-microglobulin
B7H3	B7 homolog 3
BBB	Blood-brain barrier
BC-TSO	Biotinylated barcoded template switching oligo
BDNF	Brain-derived neurotrophic factor
BM	Bone marrow
BTLA	B- and T-lymphocyte attenuator
C	Control
C10orf54	Chromosome 10 open reading frame 54
C3	Complement factor 3
Ca	Calcium
CCL2	Chemokine ligand 2
CCR2	Chemokine receptor 2
CD	Cluster of differentiation 4
CDH23	Cadherin 23
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CHEA	ChIP enrichment analysis
ChIPseq	Chromatin immunoprecipitation using sequencing
CHRDL1	Chordin-like 1
CLEC7A	C-type lectin domain family 7 member A
CNS	Central nervous system
CNX43	Connexin 43
CPM	Counts per million
CR3	Complement receptor 3
CRYM	Mu-crystallin homolog
CSF	Cerebrospinal fluid
CSF1R	Colony stimulating factor 1 receptor
CTLA4	Cytotoxic T-lymphocyte-associated protein
CX3CR1	Fractalkine receptor
CytoD	Cytochalasin D
CyTOF	Cytometry by time of flight
DAM	Disease-associated microglia
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cell
DE	Differential expression
DEG	Differentially expressed gene
DIES1	Differentiation of embryonic stem cells 1

DMEM	Dulbecco's modified eagle medium
DRAQ5	Deep red anthraquinone 5
DTT	Dithiothreitol
DUSP1	Dual specificity protein phosphataase 1
E1	EAE score 1
E4	EAE score 4
E9.5	Embryoic day 9.5
EAE	Experimental autoimmune encephalomyelitis
EAJ	Early apoptotic Jurkat cells
EBV	Epstein-Barr virus
Ech	Chronic EAE
EDTA	Ethylenediaminetetra-acetic acid
EEA1	Early endosome antigen 1
EMP	Erythro-myeloid progenitor
ENCODE	Encyclopedia of DNA elements
eQTL	Expression quantitative trait locus
ERCC1	Excision repair cross-complementation group 1
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FB	Forebrain
FCR	Fc receptor
FDA	U.S. food and drug administration
FGFR3	Fibroblast growth factor receptor 3
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FPKM	Fragments per kilobase million
FTD	Frontotemporal dementia
GABA	Gamma-aminobutyric acid
GBM	Glioblastoma
GDF10	Growth differentiation factor 10
GEO	Gene expression omnibus
GFAP	Glial fibrillary acidic protein
GI24	Platelet receptor GI24
GLAST	Glutamate aspartate transporter
GLT1	Glutamate transpoter 1
GM	Gray matter
gMFI	Geometric mean fluorescence intensity
GO	Gene ontology
GvHD	Graft-versus-host disease
GW	Gestational week
GWAS	Genome-wide association study
HB	Hindbrain
HBA/G	Hemoglobin A/G
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HDLS	Hereditary diffuse leukoencephalopathy with axonal spheroids
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1a	Hypoxia inducible factor 1 subunit alpha
HLA-DR	Human leukocyte antigen DR isotype
Homeo	Homeostatic
HOMER	Hypergeometric optimization of motif enrichment
HOX	Homeobox
IBA1	Ionized calcium-binding adapter molecule 1
IDO	Indoleamine 2,3-dioxygenase
IEG	Immediate early gene

IFN	Interferon
Ig	Immunoglobulin
IGSF11	Immunoglobulin superfamiliy member 11
IL	Interleukin
iNOS	Induced nitric oxide synthase
iPSC	induced pluripotent stem cells
ITGAX	Intergrin, alpha X
iTreg	Induced regulatory T cells
KI67	Marker of proliferation Ki-67
KO	Knockout
LAG3	Lymphocyte-activation gene 3
LCCM	L929 cell-conditioned medium
LCMV	Lymphocytic choriomeningitis virus
LCN2	Lipocalin 2
logFC	Log2 fold change
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP1	Lipoprotein receptor-related protein 1
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B
MAP1B	Microtubule-associated protein 1B
MBP	Myelin basic protein
MDSC	Myeloid-derived suppressor cell
ME	Module Eigengene
MER/MERTK	Proto-oncogene tyrosine-protein kinase MER
MFP2	Multifunctional protein 2
MG	Microglia
MGnD	Microglia neurodegenerative phenotype
MHC	Major histocompatibility complex
MIA	Maternal immune activation
miRNA	MicroRNA
MKI67	Marker of proliferation Ki-67
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MRPL23	39S ribosomal protein L23, mitochondrial
MS	Multiple sclerosis
MTX1	Metaxin 1
MYBPC1	Myosin-binding protein C, slow-type
NAGM	Normal-appearing gray matter
NAMPT	Nicotinamide phospho-ribosyltransferase
NAWM	Normal-appearing white matter
NCBI	National center for biotechnology information
NCR	Negative checkpoint regulator
NDD	Neurodegenerative diseases
NDRG1	N-myc downstream regulated 1
NFkB	Nuclear factor kappa B
NK cell	Natural killer cell
NMO	Neuromyelitis optica
NO	Nitric oxide
NOX2	NADPH oxidase 2
OLIG1/2	Oligodendrocyte transcription factor 1/2
OPC	Oligodendrocyte progenitor cell
OVA	Ovalbumin
P2RY12	Purinergic receptor P2Y, G-protein coupled, 12
PARP4	Poly ADP-ribose polymerase 4

PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Parkinson's disease
PD1H	Programmed death 1 homolog
PDGFRA	Platelet derived growth factor receptor alpha
PDL1	Programmed death ligand 1
PE	Phycoerythrin
PGE2	Prostaglandin-E 2
PI	Propidium iodide
PKM	Pyruvate kinase isozyme
PLP	Proteolipid protein
PPMS	Primary progressive MS
PSGL1	P-selectin glycoprotein ligand 1
PTX	Pertussis toxin
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S100B	S100 calcium-binding protein B
SC	Spinal cord
SCENIC	Single-cell regulatory network inference and clustering
scRNAseq	Single cell RNA sequencing
SERPINA3N	Alpha 1-antichymotrypsin
SLC1A2	Solute carrier family 1 member 2
SNP	Single nucleotide polymorphism
SOX4	Transcription factor SOX4
SPC24	Kinetochore protein Spc24
SPF	Specific-pathogen free
SPMS	Secondary progressive MS
SPP1	Secreted phosphoprotein 1
TAM	Tyrosine-protein kinase receptors
TCR	T-cell receptor
TF	Transcription factor
TGF	Transforming growth factor
Th1	T helper cell 1
TIM3	T-cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
tMCAO	Transient middle cerebral artery occlusion
TMEM119	Transmembrane protein 119
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells 2
TYRO3	Tyrosine-protein kinase receptor TYRO3
TYROBP	Protein tyrosine kinase-binding protein
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
VEGFB	Vascular endothelial growth factor B
VISTA	V-type immunoglobulin domain-containing suppressor of T-cell activation
VLA4	Very late antigen 4
VSIG3	V-set and immunoglobulin domain containing 3
VSIR	V-set immunoregulatory receptor
WGCNA	Weighted gene co-expression network analysis
WIF1	Wnt inhibitory factor 1
WM	White matter
WT	Wildtype
ZP3	Zona pellucida sperm-binding protein 3

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Nederlandse samenvatting

Multiple sclerose (MS) is een chronische en vaak progressieve ziekte van het centrale zenuwstelsel (CZS), die zich manifesteert op jonge leeftijd, meestal tussen de 20 en 40 jaar. Bij MS komen celtypes van het perifere immuunsysteem, waaronder macrofagen en lymfocyten, het CZS binnen en beschadigen de myeline-schede, die fungeert als isolatie voor axonen en cruciaal is voor snelle verspreiding van saltatoire actiepotentiaal, en myelineschade resulteert ook in verlies van axonen. MS-patiënten vertonen een reeks symptomen, waaronder verminderd gevoel, zichtproblemen en verlamming. Hoewel algemeen wordt aangenomen dat MS van auto-immuun oorsprong is, is de primaire oorzaak van MS onbekend. De meest voorkomende typen MS zijn relapsing-remitting MS en primaire / secundair progressieve MS. Relapsing-remitting MS wordt gekenmerkt door perioden van plotselinge toename van neurologische symptomen, gevolgd door perioden van remissie. Bij primair-progressieve MS verergert de neurologische handicap zonder recidieven en kan optreden na relapsing-remitting MS (= secundair progressief) of zonder eerdere relapsing-remitting MS (= primair progressief). Behandelingen die de afweer onderdrukken zijn de afgelopen 25 jaar sterk verbeterd en zijn het meest effectief bij relapsing-remitting MS. Deze behandelingen zijn echter minder effectief bij progressieve MS en stoppen de progressie van de ziekte niet.

Immune checkpoints zijn receptoren die een balans bieden tussen het versterken en beperken van de immuunrespons om enerzijds een goede beschermende functie mogelijk te maken, maar anderzijds zonder onnodige ontsteking of auto-immuunziekten. Het moduleren van de activiteit van immune checkpoints is een krachtig hulpmiddel gebleken om bijvoorbeeld de immuunrespons tegen kanker te versterken of om de immuunrespons bij auto-immuunziekten te verminderen. VISTA is een immune checkpoint dat remmende signalen afgeeft aan T-cellen, wat leidt tot verminderde immuniteit. Daarom biedt het verbeteren van VISTA-signalering bij MS een nieuwe mogelijke behandelingsstrategie om auto-immuniteit te beperken en symptomen te verminderen.

MS-pathologie is afhankelijk van een dynamisch en complex samenspel tussen verschillende celtypen in het CZS en subtypen van perifere immuuncellen. Naast neuronen bestaat het CZS uit andere belangrijke celtypen, gliacellen genaamd. De belangrijkste typen CZS-gliacellen zijn ependymale cellen, oligodendrocyten, astrocyten en microglia. Ependymale cellen vormen een laag die het centrale kanaal van het ruggenmerg en de ventrikels van de hersenen bekleedt en zijn betrokken bij de productie van cerebrospinale vloeistof. Oligodendrocyten vormen de myeline-schede rond neuronale axonen, die saltatoire impulsgeleiding mogelijk maakt, waardoor de propagatie van saltatorische actiepotentiaal wordt vergemakkelijkt. Astrocyten hebben honderdduizenden uitlopers en zijn betrokken bij een grote verscheidenheid aan CZS-processen, zoals synaptische transmissie, bloed-hersenbarrièrefunctie en ondersteuning van andere CNS-celtypen. Microglia zijn de belangrijkste immuuncellen van het CZS, die beschermen tegen indringers, weefselherstel ondersteunen en helpen bij de neuronale functie, bijvoorbeeld door het 'snoeien' van synaptische verbindingen. Het bestuderen van gliacellen en immune checkpoints zoals VISTA in het CZS zal helpen om de rol van VISTA tijdens CZS-aandoeningen zoals MS te begrijpen en kan mogelijk leiden tot de ontwikkeling van nieuwe behandelingsstrategieën. Daarom behandelt dit proefschrift de functie van VISTA, microglia en astrocyten in het zich gezond ontwikkelende en volwassen CZS en in MS met behulp van post-mortem menselijk weefsel, bioinformatica, celkweek systemen en diermodellen.

Hoofdstuk 1 geeft een overzicht van de rol van microglia en astrocyten in het zich ontwikkelende en het volwassen, gezonde CZS, evenals bij MS. Pathologische kenmerken en moleculaire mechanismen bij MS worden beschreven en de huidige literatuur over VISTA wordt besproken.

In **hoofdstuk 2** wordt de ontwikkeling van menselijke foetale microglia gekarakteriseerd met betrekking tot hun genexpressie en epigenetische profielen. Genexpressieprofielen van individuele cellen (single cell sequencing) geven een integrale momentopname van de functionele toestand van een cel, omdat transcriptie het mechanisme is waarmee de gencode vertaald wordt naar eiwitten, de functionele eenheden van een cel. Epigenetische analyses richten zich op de regulerende systemen die genexpressie en de structuur van het genoom reguleren zonder de genetische code te veranderen. Epigenetische profielen en veranderingen van deze profielen hebben een directe invloed op genexpressie. We analyseerden genexpressieprofielen van circa 15.000 individuele microglia-cellen en epigenetische profielen van microglia van 20 foetussen van 9 tot 18 weken van de zwangerschap (eerste en tweede trimester). Weefsel werd verkregen uit electieve abortussen en microglia werden geïsoleerd middels fluorescentie-geactiveerde celsortering (FACS), een techniek om individuele celtypen op te zuiveren. Gezamenlijke genexpressie en epigenetische profielen toonden aan dat een subset van foetale microglia al uitrijpt in deze vroege periode van foetale ontwikkeling. Daarmee verkrijgt deze subset waarschijnlijk het vermogen om immunologisch te reageren op (verstoringen in) hun omgeving, een kenmerk van microglia dat doorgaans werd geassocieerd met volgroeide menselijke hersenen. Maternale koorts en infecties tijdens deze ontwikkelingsperiode worden in verband gebracht met neurologische ontwikkelingsstoornissen (bijv. autisme en verstandelijke beperking) en mogelijk met de ontwikkeling van MS op latere leeftijd. We veronderstellen dus dat deze vroege immunologische rijping van microglia bijdraagt aan de gevoeligheid van de foetale hersenen voor omgevingsverstoringen tijdens de zwangerschap.

Hoofdstuk 3 behandelt de functies van astrocyten in verschillende stadia van experimentele auto-immuun encefalomyelitis (EAE), een muismodel voor auto-immuun- en inflammatoire kenmerken van MS. De ontwikkeling van EAE kan worden onderverdeeld in verschillende stadia, van vroege ziekte tot acute ziekte en chronische ziekte. FACS werd gebruikt om twee verschillende populaties astrocyten te isoleren op basis van ACSA- en GLAST-expressie. Deze twee astrocytpopulaties vertoonden differentiële genexpressieprofielen, wat verschillende functionaliteiten suggereert. Astrocyten vertoonden een sterk reactief profiel bij acute EAE geassocieerd met een neurotoxische functie die waarschijnlijk bijdraagt aan EAE-pathologie en mogelijk aan MS. In chronische stadia kregen astrocyten echter een meer proliferatieve signatuur in plaats van dit neurotoxische profiel. De proliferatie van astrocyten is een kenmerk van MS, waarbij astrocyten littekenweefsel vormen (astrogliose) om gezond weefsel tegen beschadiging te beschermen. De vorming van gliale littekens door astrocyten ondersteunt waarschijnlijk weefselregeneratie en bevordert dus herstelmechanismen, maar het kan ook axonale regeneratie en remyelinisatie voorkomen.

In **hoofdstuk 4** werd de expressie van VISTA in het CZS geëvalueerd. In de literatuur wordt beschreven dat VISTA sterk tot expressie wordt gebracht door immuuncellen (monocyten, macrofagen, T-cellen). Het is echter niet bekend welke celtypen in het CZS VISTA tot expressie brengen. Om VISTA-expressie in het CZS te analyseren, gebruikten we post-mortem menselijk weefsel en muisweefsel en ontdekten dat VISTA voornamelijk tot expressie wordt gebracht door de aanwezige immuuncellen van de hersenen, microglia. Tijdens MS en andere inflammatoire CZS-aandoeningen was de VISTA-expressie op microglia drastisch

verminderd. Aangezien VISTA een immune checkpoint is die de immuunrespons negatief reguleert, is het mogelijk dat het verlies van VISTA ontsteking bevordert in ziekten van het CZS.

Om VISTA-expressie in microglia en tijdens CZS-ziekten nadere te evalueren, biedt **hoofdstuk 5** aanvullende analyses en meer diepgaande informatie over VISTA-expressie in een breder scala aan CZS-ziekten bij mensen en muizen. De expressie van VISTA door microglia VISTA nam af bij alle onderzochte ziekten van het CZS of respectieve en corresponderende diermodellen, waaronder MS, neurodegeneratieve ziekten (de ziekte van Alzheimer, amyotrofische laterale sclerose), sepsis en beroerte. Deze breinziekten vertonen neuro-inflammatie, en een gebrek aan VISTA-expressie in aangetaste hersengebieden kan ontsteking, T-celactivering en weefselschade bevorderen.

VISTA is niet alleen een immune checkpoint, maar heeft ook tal van andere functies. VISTA is bijvoorbeeld betrokken bij de opname van dode cellen, wat een cruciale stap is in fysiologische neurologische ontwikkeling en weefselherstel. Bovendien reguleert VISTA de migratie van immuunceltypes naar signaalmoleculen, die de locatie van weefselschade markeren. Ten slotte remt VISTA de productie van cytokinen, signaalmoleculen van het immuunsysteem die ontsteking kunnen bevorderen en afremmen. Afhankelijk van de functie die VISTA heeft in microglia, kan de vermindering van VISTA-expressie bij breinziekten verschillende functionele uitkomsten hebben. Daarom werd de functie van VISTA in microglia onderzocht in **hoofdstuk 6**. Om de VISTA-functie in microglia te ontrafelen, werd een model ontwikkeld waarin VISTA-deficiëntie werd gericht op microglia. VISTA-deficiënte microglia vertoonden een veranderde morfologie, gekenmerkt door een lagere vertakking. Expressie van genen betrokken bij de celcyclus en immuunactivering van microglia nam toe door uitschakeling van VISTA enkel in microglia. Microglia VISTA-deficiëntie had geen invloed op de progressie van EAE en had geen invloed op de microglia-respons tijdens EAE of op stimulatie met de bacteriële component lipopolysaccharide (LPS). In celkweek vertoonden microglia die deficiënt zijn voor VISTA een verminderd vermogen om myeline op te nemen. In verschillende MS-laesiestadia werd VISTA differentieel tot expressie gebracht. Een ontregelde homeostase van microglia en verminderde opname van myeline zou kunnen bijdragen aan de progressie van MS en aan verminderde homeostase van het CZS.

Hoofdstuk 7 vat de mogelijke implicaties van de bevindingen in dit proefschrift samen en bespreekt deze in de context van de recente literatuur. Verder worden toekomstperspectieven en mogelijke vervolgexperimenten besproken. Het in kaart brengen van de ontwikkeling van menselijke microglia is een belangrijk element om de gevolgen van verstoringen tijdens de zwangerschap te doorgronden, waaronder neurologische ontwikkelingsstoornissen, maar ook mogelijk de ontwikkeling van auto-immuniteit en CZS-ziekte op latere leeftijd. Een verstoorde ontwikkeling van muis embryo's door ontstekingsinsulten verhoogt bijvoorbeeld de gevoeligheid voor het ontwikkelen van EAE. Het nieuwe feit dat humane microglia al in een vroeg stadium van de ontwikkeling immuunfuncties ontwikkelen, kan microglia en dus het zich ontwikkelende CZS kwetsbaar maken voor infecties en koorts, waardoor de vatbaarheid voor het ontwikkelen van aandoeningen zoals MS toeneemt. Niet alleen microglia, maar ook andere gliatypes, namelijk astrocyten, zijn betrokken bij ontsteking en weefselherstel in MS. Het bestuderen van de verschillende celtypen in het CZS en hun interactie is essentieel om nieuwe therapeutische interventiestrategieën te ontwikkelen. Astrocyten hebben mogelijk schadelijke functies in het begin van EAE ontwikkeling, maar kunnen nodig zijn voor weefselregeneratie in latere ziektestadia, en dit kan vergelijkbaar zijn bij MS. Microglia

en astrocyten zijn functioneel zeer heterogeen, vooral tijdens de ontwikkeling en onder pathologische omstandigheden. Het begrijpen van astrocyten- en microglia-subgroepen tijdens pathologie is belangrijk, aangezien subset-specifieke behandelingen een veelbelovende weg zijn voor onderzoek en mogelijke therapie.

Aangezien microglia en astrocyten elkaars functie beïnvloeden door uitgescheiden factoren en mogelijk directe receptorinteracties, is het denkbaar dat het blokkeren of induceren van specifieke interacties de ziekteprogressie bij MS kan stoppen. VISTA zou een dergelijk receptor kunnen zijn die cruciaal is voor microglia-interactie met astrocyten en infiltrerende immuuncellen tijdens MS. Verminderde VISTA-expressie in microglia tijdens ontsteking leidt tot verminderde myeline-fagocytose en verhoogde activering van immuuncellen, die gezamenlijk ontsteking bevorderen en herstelmechanismen remmen. Het manipuleren van VISTA-signalering in microglia tijdens MS verdient nadere bestudering in de toekomst, omdat dit ontstekingsprocessen kan verminderen. Aangezien VISTA echter alomtegenwoordig tot expressie wordt gebracht door immuuncellen in de periferie en verschillende functies heeft in verschillende celtypen, is het ook essentieel zijn om de functionele gevolgen van het moduleren van VISTA-functie tijdens MS zorgvuldig te evalueren.

English summary

Multiple sclerosis (MS) is a chronic and often progressive disease of the central nervous system (CNS), which manifests at a young age, usually between 20 and 40 years. In MS, immune cell types including macrophages and lymphocytes enter the CNS and damage the myelin sheath, which acts as an insulation for axons and is crucial for rapid saltatory action potential propagation, and myelin damage also results in axonal loss. MS patients display a range of symptoms including impaired sensation, vision problems, and paralysis. Although MS is generally thought to be of autoimmune origin, the primary cause of MS is unknown. Most common types of MS are relapsing-remitting MS and primary/secondary progressive MS. Relapsing-remitting MS is characterized by times of sudden increase of neurological symptoms, followed by remission periods. In progressive MS, neurological disability is worsening without relapses and can present after relapsing-remitting MS (= secondary progressive) or without previous relapsing-remitting MS (= primary progressive). Immune suppressive treatments have improved dramatically over the past 25 years and are most effective in relapsing-remitting MS. These treatments, however do not arrest disease progression, and are less effective in progressive MS.

Immune checkpoints are receptors that provide a balance between enhancing and limiting the immune response to allow proper protective function without aberrant inflammation or autoimmune disease. Modulating immune checkpoint activity is a powerful tool to, for example, increase the immune response against cancer, or to reduce the immune response in autoimmune diseases. VISTA is an immune checkpoint that provides inhibitory signals to T cells leading to reduced immunity. Therefore, enhancing VISTA signaling in MS offers a novel treatment strategy to limit autoimmunity and reduce symptoms.

MS pathology is dependent on a dynamic and complex interplay between different CNS-resident cell types and peripheral immune cell subsets. In addition to neurons, the CNS consists of other important cell types, called glial cells. The main types of CNS glial cells are ependymal cells, oligodendrocytes, astrocytes, and microglia. Ependymal cells form a layer that lines the central canal of the spinal cord and the ventricles of the brain and are involved in cerebrospinal fluid production. Oligodendrocytes form the myelin sheaths around neuronal axons, facilitating saltatory action potential propagation, which enables neuronal signaling. Astrocytes have hundreds of thousands of processes and are involved in a large variety of CNS processes such as synaptic transmission, blood-brain barrier function, and support of other CNS cell types. Microglia are the principal immune cells of the CNS, that protect the CNS against intruders, support tissue repair, and assist in neuronal function for instance by synaptic pruning. Studying glial cells and immune checkpoints such as VISTA in the CNS will help to understand the role of VISTA during CNS disease such as MS and can potentially lead to the development of novel treatment strategies. Hence, this thesis addresses the function of VISTA, microglia, and astrocytes in healthy developing and adult CNS, and in MS using post-mortem human tissue, bioinformatic approaches, cell culture assays, and animal models.

Chapter 1 provides an overview of the role of microglia and astrocytes in developing and adult healthy CNS, as well as in MS. Pathological hallmarks and molecular mechanisms in MS are outlined and the current literature on VISTA is reviewed.

In **chapter 2,** human fetal microglia development is characterized with respect to their gene expression and epigenetic profiles. Single cell gene expression profiles provide a snapshot

of the functional state of a cell, as transcribed genes are translated to proteins, which are the functional units of a cell. Epigenetic analyses focus on the regulatory systems that regulate gene expression and the structure of the genome without changing the genetic code. Epigenetic profiles and changes of these profiles directly affect gene expression. We assessed gene expression profiles of approximately 15,000 individual microglia cells and epigenetic profiles of microglia from 20 fetuses of gestational weeks 9 to 18 (first and second trimester). Tissue was obtained from elective abortions and microglia were captured using fluorescence-activated cell sorting (FACS), which is a technique to isolate individual cell types. Gene expression and epigenetic profiles jointly demonstrated that a subset of fetal microglia already mature in this early period of fetal developmental and likely obtain the ability to immunologically respond to (perturbations in) their environment, a hallmark of microglia typically associated with adult human brains. Maternal fever and infections during this developmental period are associated with neurodevelopmental disorders (e.g. autism and intellectual disability) and potentially with the development of MS later in life. Thus, we postulate this early maturation of microglia contributes to the sensitivity of the fetal brain towards environmental perturbations during pregnancy.

Chapter 3 addresses the function of astrocytes at different stages of experimental autoimmune encephalomyelitis (EAE), which is a mouse model for autoimmune and inflammatory features of MS. The development of EAE can be separated into distinct stages from early disease, to acute disease, and chronic disease. FACS was used to isolate two different populations of astrocytes based on ACSA and GLAST expression. These two astrocyte populations showed differential gene expression profiles, suggesting distinct functionalities. Astrocytes displayed a strongly reactive profile in acute EAE associated with a neurotoxic function likely contributing to EAE pathology, and potentially to MS. In chronic stages, however, instead of this neurotoxic profile, astrocytes acquired a more proliferative signature. Astrocyte proliferation is a hallmark of MS as astrocytes form scar tissue (astrogliosis) to protect healthy tissue from damage. The formation of glial scars by astrocytes likely supports tissue regeneration and hence promotes repair mechanisms, however, it can also prevent axonal regeneration and remyelination.

In **chapter 4**, the expression of VISTA in the CNS was evaluated. It is described in literature that VISTA is highly expressed by immune cells (monocytes, macrophages, T cells). However, it is unknown which CNS-resident cell types express VISTA. To analyze VISTA expression in the CNS, we used post-mortem human and mouse tissue and found that VISTA is predominantly expressed by the resident immune cells of the brain, microglia. During MS and other inflammatory CNS disease, VISTA expression on microglia was drastically decreased. Since VISTA is an immune checkpoint that negatively regulates the immune response, it is possible that loss of VISTA on microglia promotes inflammation during CNS diseases.

To further evaluate VISTA expression in microglia and during CNS diseases, **chapter 5** provides additional analyses and more in-depth information on VISTA expression in a wider range of CNS diseases in humans and mice. Microglia VISTA expression decreased in all assessed CNS diseases or respective animal models including MS, neurodegenerative diseases (Alzheimer's disease, amyotrophic lateral sclerosis), sepsis, and stroke. These CNS diseases feature neuroinflammation, and a lack of VISTA expression in affected brain regions might promote inflammation, T-cell activation and tissue damage.

VISTA is an immune checkpoint, but it also has a variety of other functions. For example, VISTA is involved in the uptake of dead cells (efferocytosis), which is a crucial step in

neurodevelopment and tissue repair. Furthermore, VISTA regulates migration of immune cell types towards signaling molecules, which is used to attract immune cells to sites of damage. Finally, VISTA inhibits the secretion of cytokines, which are signaling molecules of the immune system that can promote inflammation. Depending on the function that VISTA has in microglia, the reduction in VISTA expression during CNS disease could have different functional outcomes. Therefore, the function of VISTA in microglia was investigated in **chapter 6.** To unravel VISTA function in microglia, a model was developed where VISTA-deficiency was targeted to microglia. VISTA-deficient microglia exhibited an altered morphology, characterized by a lower ramification. Genes involved in cell cycle and immune-activation of microglia were increased after VISTA depletion. Microglia VISTA-deficiency did not affect the course of EAE and did not alter the microglia response during EAE or to the inflammatory bacterial component lipopolysaccharide (LPS). In cell culture, microglia deficient for VISTA exhibited reduced ability to phagocytose myelin. In distinct MS lesion stages, VISTA was differentially expressed. Dysregulated microglia homeostasis and impaired myelin uptake might contribute to MS progression and to disturbed CNS homeostasis.

Chapter 7 summarizes and discusses the potential implications of the findings presented in this thesis. Furthermore, future perspectives with potential follow-up experiments are discussed. Delineating human microglia development is an important quest to understand the consequences of perturbances during pregnancy, which include neurodevelopmental disorders, but also potentially the development of autoimmunity and CNS disease later in life. For example, perturbed mouse development increases the susceptibility to developing EAE. The fact that microglia develop immune-sensing properties early in human development may render microglia and the developing CNS vulnerable towards infections and fever, thereby enhancing the susceptibility to developing CNS disease such as MS. Not only microglia, but also other glial cell types, namely astrocytes, are involved in MS inflammation. Studying CNS-resident cell types and their interaction is essential to develop potential therapeutic intervention strategies. Astrocytes are potentially detrimental early during EAE but may be required for tissue regeneration in late disease stages, which might be similar in MS. Microglia and astrocytes are highly heterogeneous particularly during development and under pathological conditions. Understanding astrocyte and microglia subsets during pathology is important, since subset-specific treatments are a promising avenue of research and potential therapy.

Since microglia and astrocytes affect each other's phenotype using secreted factors and potentially direct receptor interactions, it is conceivable that blocking or inducing specific interactions may halt disease progression in MS. VISTA could be such a receptor that is crucial for microglia interaction with astrocytes and infiltrating immune cells during MS. Decreased VISTA expression during inflammation in microglia may lead to reduced myelin phagocytosis and increased immune cell activation, together promoting tissue inflammation and inhibiting repair mechanisms. Restoring VISTA signaling in microglia during MS should be studied in the future as it might reduce inflammation. However, as VISTA is ubiquitously expressed by immune cells in the periphery and has different functions in distinct cell types, it will be essential to carefully evaluate functional consequences of modulating VISTA expression during MS.

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