Contribution of SIGLEC-11 and SIGLEC-16 receptors to neuro-inflammation and neurodegeneration

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List of Abbreviations

4-HNE	4-hydroxynonenal
AD	Alzheimer’s disease
Aif1	Allograft inflammatory factor-1
BSA	Bovine serum albumin
C1qa/b	Complement component 1 with q subcomponent, alpha/beta polypeptide
C1qc	Complement component 1 with q subcomponent, c chain
C3	Complement component 3
C4	Complement component 4
CA3	Cornu Ammonis 3
Cd68	Cluster of differentiation 68
CNS	Central nervous system
Cy3	Cyanine 3
Cyba	Cytochrome B-245 Alpha Chain
Cybb	Cytochrome B-245 Beta Chain
DAPI	4',6-diamidino-2 phenylindole
DAP-12	DNAX-activating protein of 12 kDa
ddH2O	Double-distilled water
DG	Dentate gyrus

FC	Fold change
Fcer1g	Fc Epsilon Receptor Ig
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GlcNAc	N-acetylglucosamine
HP	Hippocampus
Iba1	Ionized calcium binding adaptor molecule 1
IL1b	Interleukin 1 beta
ITAM	Immunoreceptor tyrosine based activation motif
Itgam	Integrin alpha M
itgb2	Integrin Subunit Beta 2
ITIM	Immunoreceptor tyrosine based inhibition motif
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein Kinase
MS	Multiple Sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide

Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NeuN	Neuronal nuclei
NF-κB	Nuclear factor-kappa B
NGS	Normal goat serum
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PFA	Paraformaldehyde
PSD-95	Post-synaptic density protein 95
ROS	Reactive oxygen species
SEM	Standard error of the mean
SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase-2
Siglec	Sialic-acid-binding immunoglobulin-like lectin
SN pc	Substantia nigra pars compacta
SN pr	Substantia nigra pars reticulate
sqRT-PCR	semi-quantitative real-time PCR
TBE	Tris/Borate/EDTA
TH	Tyrosine hydroxylase

Tmem119	Transmembrane protein 119
TNF-α	Tumor necrosis factor-α
TREM2	Triggering receptor expressed on myeloid cells 2
vGLUT-1	Vesicular glutamate transporter 1
WT	Wild type

1. Introduction

1.1 Microglia cells

1.1.1 The origin and function of microglia cells

Microglia are the resident immune cells within the central nervous system (CNS) (see fig.1). It is believed that in the brain parenchyma, they arise from the primitive macrophages in the fetal yolk sac. During the early stages of fetal development, they will make a distinct population of cells in the CNS which has different features from the bone-marrow derived macrophages (Ginhoux et al. 2010; Saijo and Glass 2011). The population of those immune cells with ionized calcium-binding adaptor molecule-1 (Iba1) on their cell surface, will proliferate as the Iba1-positive microglial precursors within the CNS (Chen et al. 2010). During the embryonic development, the Iba1-positive cells differentiate into adult microglia. They act as regulators of CNS development and neuronal survival by releasing cytokines, chemokines and growth factors as well as by carrying out silent phagocytosis of apoptotic material. The function of these cells is critical for maintaining a healthy condition in the brain, whereas they scan the brain and could recognize the plaques, damaged neurons and synapses as well as other brain injuries (Linnartz-Gerlach, Kopatz, and Neumann 2014; Wake, Moorhouse, and Nabekura 2011).

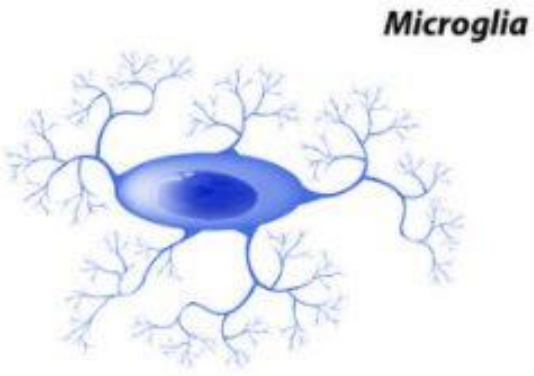


Fig. 1: Microglia, the resident immune cells in the CNS. Microglia play an essential role in the brain by maintaining the steady and stable condition in the brain. They keep the brain healthy via phagocytosis of damaged markers (Modified according to Stratoulias et al., EMBO Journal, 2019).

1.1.2 Different subtypes of microglia cells

Microglia are single-cell types that have a high level of plasticity, which allows them to acquire multiple phenotypes and thus, perform several functions in health and disease (Stratoulias et al. 2019). Furthermore, each member of the population of microglia cells serves a distinct function (Kierdorf et al. 2013; Lawson et al. 1990; Perdiguero et al. 2015; Schulz et al. 2012). Here are some examples of the different microglia phenotypes.

Ramified morphology is one of the features of microglia (Parakalan et al. 2012). They are also known as resting microglia. Microglia with ramified morphology are regularly distributed throughout the CNS under normal physiological conditions. They are characterized by long-distance branches and small cellular body. These branches are constantly moving and are extremely sensitive to even the smallest changes in the physiological conditions of their surrounding environment. Ramified microglia are a more stable state of microglia cells that secrete only low levels of immune molecules and do not perform phagocytosis. They might become acutely activated when detect a threat or damage to the CNS.

In pathological conditions, microglia transform from a ramified to an ameboid phenotype. It is proposed that microglia undergo a cascade of reactions and changes in gene transcription and expression to develop the hypertrophic and ameboid phenotypes of activated microglia (see fig.2). (Flanary et al. 2007; Graeber 2010). These activated microglia cells, known as ameboid microglia, are characterized by being proliferative, phagocytic and migratory (Parakalan et al. 2012). In contrary to ramified microglia, ameboid microglia have a larger cellular body and do not have branches. In addition, the presence of these cells during normal embryonic development and brain injury suggests that they could act as effective linkers between the immune system and CNS (Streit 2002; Tay, Mai, et al. 2017; Tay, Savage, et al. 2017).

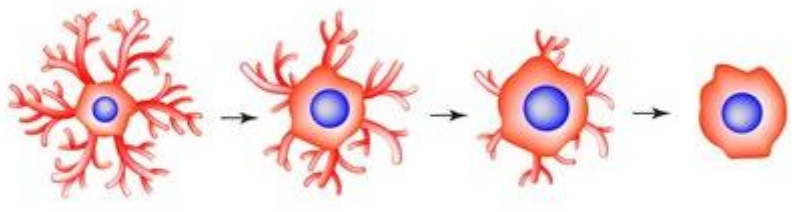


Fig. 2: Microglia activation. The process of microglia activation from its ramified morphology (long-distance branches and small cellular body, *left*) to the ameboid microglia morphology (*right*) with large cellular body is required for easier movement in different regions of the brain (Modified according to activation of microglia, neuron cells, nerve, infographics, shutterstock, 2021).

Throughout a lifetime, microglia can have varying morphologies based on their distribution in different regions in the CNS. Their phenotype is influenced by the physiological conditions surrounding them, such as LPS exposure, as well as the presence of diverse cellular structures in their vicinity, such as myelinated and unmyelinated axons, dendrites and neuronal cell bodies (Fuger et al. 2017; Graeber 2010; Lawson et al. 1990). Furthermore, it has been established that the morphology and function of microglia cells are regulated by specific genes encoding signaling molecules and transcription factors (Parakalan et al. 2012). In the ameboid microglia, studies have observed the expression of genes which are involved in nervous system development (such as *Dpysl3* and *Smarca*) (Barak et al. 2003; Wang and Strittmatter 1996), immune system development (such as *Hmgb3* and *Sla*) (Dragone et al. 2009; Nemeth et al. 2005; Nemeth et al. 2003; Sosinowski et al. 2000), cell migration during neurodevelopment (*Dcx*) (Bai et al. 2003) and the immune response. Contrarily, the ramified microglial cells express genes that are involved in myelination (such as *Mbp*) enabling oligodendrocyte precursor cells (OPCs) to differentiate into the mature oligodendrocytes (Cross et al. 1997; Ng, Jeppesen, and Bird 2000). Generally, Parakalan and colleagues observed in their study that genes which are involved in cell proliferation, death, and differentiation are highly expressed in ameboid microglia (Parakalan et al. 2012). The

ramified microglia, however, highly express a large number of genes involved in cytoskeletal organization and cell differentiation.

1.1.3 Microglia cells in the normal procedure of brain aging

Aging is a multifactorial process which involves the accumulation of molecular damages that leads to age-related disability and diseases. This process is associated with inflammation in the brain and activation of the microglia. As mentioned before, microglia play a significant role to eliminate inflammation and infection in the brain. It has been observed that aged brains are often characterized by the presence of activated microglia (Perry, Matyszak, and Fearn 1993; Rio-Hortega 1939). As compared to younger brains, these aged activated microglia may exhibit an altered cytokine profile (Sierra et al. 2007). A key point is that activated microglia in the aged brain may be responsible for exaggerated inflammatory responses through accumulating damage signals like oxidative damages and neurotoxins (Perry, Cunningham, and Holmes 2007). As a result, the inflammatory responses prolong the cycles of proliferation and production of pro-inflammatory cytokines rendering them as neurotoxic factors (Godbout et al. 2005). These factors may cause toxic inflammation condition inside the CNS which may result in more neuronal damages and greater amounts of debris (see fig.3). The outcome of this process could ultimately trigger excessive microglia activation and the repetition of above-mentioned procedure would lead to neurodegenerative diseases and eventually death (Monje, Toda, and Palmer 2003).

Furthermore, chronic microglial activation causes the impairment of adult neurogenesis in hippocampus (Monje, Toda, and Palmer 2003) and damages to the periventricular white matter (PWMD) in the early postnatal brain (Kaur and Ling 2009). As a result, activated microglia in both postnatal and adult stages can cause excessive inflammation in the CNS (Parakalan et al. 2012). Excessive microglia activation, prolonged inflammatory responses and a wide range of neurotoxins may be the reasons that why aging process is an important risk factor in several types of neurodegenerative diseases. Similar to the other types of immune cells, microglia have a variety of receptors on their cell surface and activation of

microglia is dependent on these receptors (Pocock and Kettenmann 2007). Hence, microglial cell-surface receptors can either activate or inhibit the immune response pathway via binding to their specific ligands (Bachiller et al. 2018; Hanisch and Kettenmann 2007).

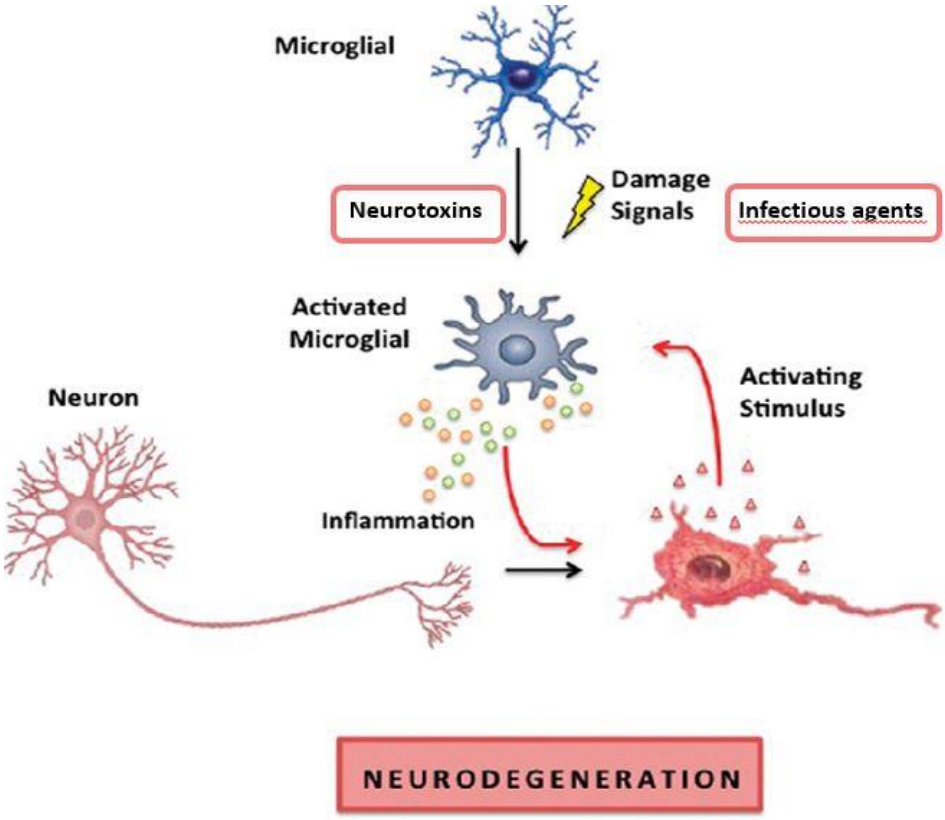


Fig. 3: Microglia cells are a specialized population of macrophages-like cells in the CNS. Microglia cells act as the resident immune cells in the CNS and could clear the brain from damages such as neurotoxins and infectious agents. Higher activation of microglia cells could cause irreversible damages inside the brain. Activation of microglia is a normal procedure occurring in the aged brain (Modified from Morales et al., 2018, Semantic scholar).

1.1.4 Microglial cell-surface receptors

As described in section 1.1.1, microglia are the intrinsic immune cells of the brain and act as the first line of defense in the CNS. Upon stimulation, these cells express receptors and release chemokines and cytokines which might affect the neurons and their function (Pocock and Kettenmann 2007). Microglia have various types of receptors that execute different functions when activated by their specific ligands (see fig. 4). These receptors will allow microglia to distinguish their surrounding environment, thus protecting brain against injuries. Listed below are a few microglial receptor examples.

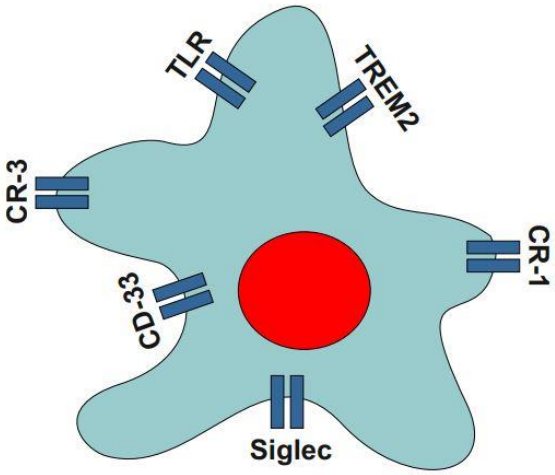


Fig. 4: Receptors on the surface of microglia cells. TLR (Toll like receptors), TREM2 (Triggering receptor expressed on myeloid cells 2), CR-1 (Complement 3b/4b Receptor 1), Siglec (Sialic acid-binding immunoglobulin-type lectins), CD-33 (Sialic acid binding immunoglobulin-like lectin 3) and CR-3 (Complement 3b/4b Receptor 3) are examples of different receptors on the surface of microglia cells. Each receptor has its particular function, which when activated via its ligand would lead to several functional changes of microglial cells.

Complement C3b/C4b receptor 1 (CR-1) is expressed at higher levels on the activated microglia and eventually leads to induction of neuronal death (Crehan, Hardy, and Pocock 2013). Increased levels of tumor necrosis factor-a (*TNF-α*) and Interleukin-1β (*IL1-β*) could

be responsible for activating microglia and CR-1 on their surface. Thereby, polymorphism in CR-1 locus may be correlated with the increased levels of amyloid-β (Aβ) (Brouwers et al. 2012; Crehan et al. 2012; Rogers et al. 2006).

CR3 is another receptor on the surface of microglia. Studies demonstrated that the inactivated complement receptor 3 (CR3) is related to the effective uptake of Aβ and pathogenesis of Alzheimer's disease (AD). In the brains of AD patients, CR3 tends to colocalize with Aβ plaques, which suggests the direct interaction between the CR3 and Aβ (Fu et al. 2012; Strohmeyer et al. 2002).

In the CNS, there are detrimental cells which would secrete exogenous and endogenous molecules. Toll like receptors (TLRs) on the surface of microglia are crucial to distinguish and fight against these deleterious cells (Lehnardt 2010). TLRs are a family of membrane proteins that can detect a variety of molecules known as danger- and pathogen-associated molecular patterns (Lemaitre et al. 1996; Morisato and Anderson 1994). Microglia expressed a wide range of TLRs, starting from TLR1 to TLR9. Several studies have shown that these receptors are involved in activating microglia that ultimately may cause neurotoxicity (Hanke and Kielian 2011; Olson and Miller 2004).

Triggering receptor expressed on myeloid cells 2 (TREM2) is a transmembrane protein expressed on microglia (Chen et al. 2020). TREM2 is involved in phagocytosis and could inhibit the production of inflammatory mediators by microglia cells (Hsieh et al. 2009; Sessa et al. 2004; Takahashi, Rochford, and Neumann 2005). Linnartz-Gerlach and her colleagues indicated a link between TREM2 and neuronal loss and other age-related changes which might describe TREM2's role in the process of aging (Linnartz-Gerlach et al. 2019).

Sialic acid binding immunoglobulin (Ig)-like lectin 3 (Siglec-3 or CD33) is a transmembrane protein, expressed on microglia cells (Estus et al. 2019). A study has described the role of CD33 in the uptake of Aβ, similar to CR1 and CR3, by surrounding the aggregated Aβ plaques (Griciuc et al. 2013). Furthermore, deletion of CD33 in human induced-pluripotent stem cell-derived microglia showed an increase in phagocytosis activity of these cells. This would further support the role of CD33 in the uptake of Aβ (Wissfeld et al. 2021).

There are different types of Siglec receptors expressed on the surface of human microglia cells. Investigations represented that the proper activation of human Siglec receptors is vital. Their proper activity triggers a cascade of reactions, leading to activation or inhibition of microglia, which allows our immune response to be refined against debris and neuronal damages as we age (Linnartz-Gerlach, Kopatz, and Neumann 2014; Siddiqui et al. 2019).

1.2 What are Siglecs?

Siglecs are type I transmembrane receptors expressed on the innate immune cells, including microglia. Siglecs could bind to sialic acid residues of glycoproteins and glycolipids. They belong to a large family of Ig-like lectins, which contain two or three unique domains (Bornhofft et al. 2018; Bull et al. 2021; Crocker, Paulson, and Varki 2007; Lubbers, Rodriguez, and van Kooyk 2018; Varki and Angata 2006; Angata et al. 2004). According to previous studies, Siglec receptors can be divided into two subsets based on their sequence similarity. The first group consists of sialoadhesin (Siglec-1) expressed on macrophages, CD-22 (Siglec-2) on the surface of B cells, myelin associated glycoprotein (MAG/Siglec-4) on myelin and Siglec-15 which is expressed on osteoclasts, macrophages and dendritic cells. This group shares a low sequence similarity (approximately 25-30%). Despite of their low sequence similarity, they are conserved among several mammals (Angata et al. 2004; Bornhofft et al. 2018; Crocker, Paulson, and Varki 2007).

On the other hand, the CD-33 related Siglecs (CD-33r Siglecs), is the main subset of Siglec receptors (Crocker, Paulson, and Varki 2007). They have approximately 50-99% identity, but it has been discovered that this group is evolving quickly. Angata and colleagues suggested that in mammals there are multiple variations of CD-33r Siglec group due to the evolving process. This process contains gene duplication, exon shuffling and gene conversion (Angata et al. 2004). CD-33r Siglecs are expressed on innate mature immune cells such as neutrophils, monocytes and macrophages. They can regulate the expansion, differentiation and activation of the cells (Cao and Crocker 2011; Cognasse et al. 2015; Zhang et al. 2007). Due to the signaling domain of CD-33r Siglecs, most of these receptors could inhibit the

immune responses. They contain two conserved motifs of Src homology region 2 domain containing phosphatase-1 (SHP-1) and Src homology region 2 domain containing phosphatase-2 (SHP-2) tyrosine phosphatases as well as the suppressor of cytokine signaling 3 (SOCS3) (Estus et al. 2019).

As discussed earlier, each Siglec receptor consists of two or three domains. The extracellular domain has an N-terminal Ig domain of “variable set” (V-set) region containing a sialic acid specific binding site and a different length of repeated Ig “constant set” (C-set) domains. Different basic amino acids are found in the transmembrane domain, which allows for the interaction of membrane modifications. Finally, the intracellular domain consists of an immunoreceptor tyrosine-based inhibitory motif (ITIM) or ITIM–like motif which could perform an inhibitory signaling response or immunoreceptor tyrosine-based activation motif (ITAM) that would cause activation of the immune system. Overall, these tyrosine-based motifs are involved in cell signaling (Bornhofft et al. 2018; Bull et al. 2021; Crocker, Paulson, and Varki 2007; Lubbers, Rodriguez, and van Kooyk 2018; Varki and Angata 2006).

As mentioned earlier, ITAM signaling receptors could activate the down-stream pathway which may result in induction of the immune responses. After the interaction of ligand with the ITAM receptors, the ITAM motifs in the cytoplasmic tail, together with association of an adaptor molecule such as DAP-12, could phosphorylate the tyrosine residues by Src family kinases (family of tyrosine kinases). Phosphorylated ITAM motifs could transmit the signal to the Mitogen-Activated Protein Kinase (MAPK) pathway via phosphatidyl-inositol-3 (PI3) activation. This would cause activating the down-stream cascade which would lead to initiating immune system responses (Crocker 2005; Crocker, Paulson, and Varki 2007; Ravetch and Lanier 2000).

On the other hand, Siglecs containing ITIM motifs get phosphorylated by the Src family kinases following the ligand specific linkage. This phosphorylation leads to intracellular signaling involving SHP-1 and SHP-2. After that, they could block the MAPK signaling pathway resulting in inhibitory effects to quench the pro-inflammatory pathways (Crocker 2005; Crocker, Paulson, and Varki 2007; Crocker and Varki 2001; Pillai et al. 2012; Schwarz

et al. 2015). It has been mentioned that ITIM and ITAM signaling pathways have a balance between their inhibitory and activatory signaling receptors in a healthy brain condition, while in neurodegenerative diseases this balance is disrupted (Rawal and Zhao 2021).

As shown in figure 5, Siglec-3, -9 and -11 are members of the ITIM signaling receptor family. On the other hand, Siglec-14 and -16 have ITAM motifs which together with an adaptor molecule, DAP-12/TYROBP, lead to activation of immune responses (see figure 5) (Daeron et al. 2008; Lubbers, Rodriguez, and van Kooyk 2018; Pillai et al. 2012).

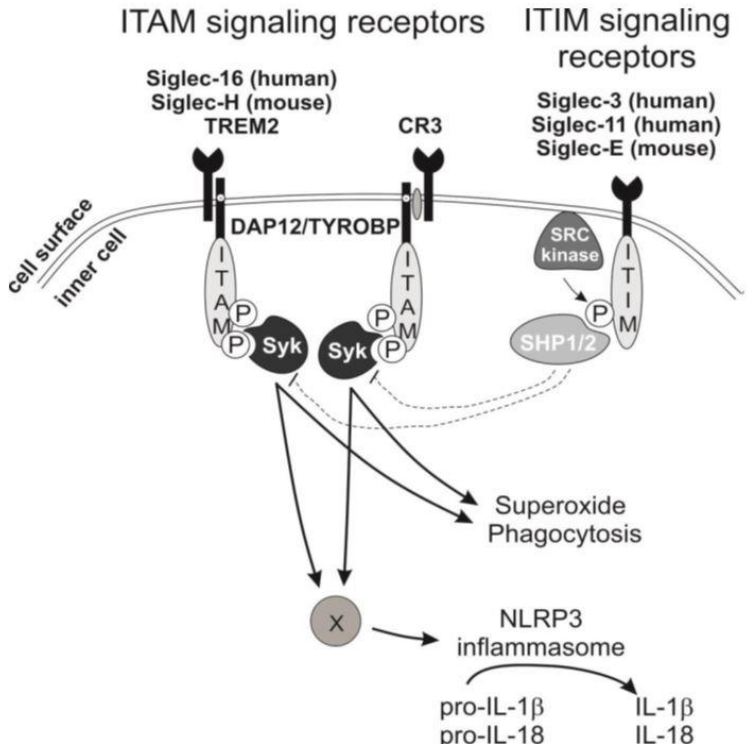


Fig. 5: ITIM (Immunoreceptor tyrosine-based inhibitory motif) and ITAM (Immunoreceptor tyrosine-based activation motif) receptors on microglia cells. ITAM signaling receptors associated with their adaptor molecule, DNAX-activating protein of 12 kDa (DAP-12/ TYROBP), to activate the oxidative stress, pro-inflammatory markers and phagocytosis. However, ITIM signaling receptors via phosphorylation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and Src homology region 2 domain-containing phosphatase-2 (SHP-2) inhibit the pro-inflammatory cascade (Modified from Linnartz-Gerlach et al, Glycobiology, 2014).

1.3 SIGLEC-11 and SIGLEC-16

SIGLEC-11 is one of the ITIM signaling receptors and it was first described by Angata and colleagues in 2004 (Angata et al. 2004). This single-pass transmembrane receptor has several disulfide bonds and glycosylation in its structure. As well as other CD-33r Siglecs, SIGLEC-11 gene, is located on chromosome 19 in humans. The extracellular domain of SIGELC-11 seems to resemble human SIGELC-10, while the cytoplasmic tail does not show this similarity (Angata et al. 2002; Whitney et al. 2001; Yousef et al. 2002).

As mentioned earlier, SIGLEC-16 was described by Cao and colleagues as a functional as well as non-functional pseudogene SIGLEC-16P variant in humans for the first time. They realized that the lineage of SIGLEC-11 gene after obtaining various gene duplication and conversion, could build the inhibitory (SIGLEC-11) and activatory (SIGLEC-16) role of receptor pair in both chimpanzee and human being SIGLEC-16(Cao et al. 2008). Thereby, SIGELC-11 and SIGLEC-16 were referred as paired receptors (Cao et al. 2008; Schwarz et al. 2017; Wang et al. 2012). As with SIGLEC-11, SIGLEC-16 is located on human chromosome 19. While SIGLEC-11 signals via its own ITIM- signaling motif, SIGLEC-16 is activated with association of the adaptor molecule DAP-12/TYROBP, which contains the ITAM motif (Cao et al. 2008).

Even though SIGELC-11 and SIGLEC-16 receptor sequences share a high degree of similarity, the sequence similarities are lost in the transmembrane domain. SIGLEC-11 displays a neutral charge in its transmembrane protein, as do most transmembrane receptors. However, SIGLEC-16 illustrated the positive charged lysine which binds to DAP-12 and could activate the SSIGLEC-16s' members. In contrast, this ITAM signaling receptor will encode the negatively charged glutamate at four positions from lysine (Blasius et al. 2006; Crocker and Redelinghuys 2008; Schwarz et al. 2017; Takamiya et al. 2013).

1.3.1 SIGLEC-11- an inhibitory Siglec receptor

SIGLEC-11 is widely expressed on tissue macrophages in humans and microglia in the human brain (Hayakawa et al. 2005). It binds preferentially to oligo- and polysialic acid. Sialic

acids are a subset of nine-carbon backbone sugars and form a diverse family of over 40 neuraminic acid derivates (Holst et al. 2016; Kooner, Yu, and Chen 2019; Zanetta et al. 2001). They are commonly part of glycoproteins, glycolipids or gangliosides (Karlstetter et al. 2017). Overall, sialic acids often bind to the oligosaccharides as terminal sugars by different linkages such as α2.3, α2.8 and α2.9 (Szymanski, Schnaar, and Aebi 2015; Varki and Gagneux 2015; Varki et al. 2015; Varki, Schnaar, and Schauer 2015). The functions of sialic acids depend on their interactions with various glycolipids and glycoproteins (Linnartz-Gerlach, Kopatz, and Neumann 2014; Varki and Gagneux 2015; Varki, Schnaar, and Schauer 2015) including the transportation of micro- and macro-molecules, cellular communication and most importantly being a biological recognizer (Cavalcante et al. 2021). There was a study showed that hyper sialylation in a proper mode is a brilliant way for tumor cells to escape from the immune system, which may account for tumor metastasis and its malignancy (Zhou, Yang, and Guan 2020). This would claim that healthy cells that have the specific pattern of sialic acids on their glycocalyx, are recognized by inhibitory Siglec receptors which may prevent the immune system from being activated for a long time and eventually result in the immune-protective effects.

SIGLEC-11 specifically binds to its ligand, □ -2, -8-linked oligosialic acid (Angata et al. 2002; Hayakawa et al. 2005). As in most of the CD-33r Siglecs, SIGLEC-11 contains ITIM motifs in its cytoplasmic domain. Upon ligand binding, the ITIM motifs interact with the SHP-1 and SHP-2 domains and subsequently inhibiting inflammatory responses and production of cytokines and oxidative stress markers, as well as preventing the loss of neurons and synapses (Angata et al. 2002). In terms of this inhibition, they may serve protective functions (Bisht, Sharma, and Tremblay 2018; Edler et al. 2018; Heshmati-Fakhr et al. 2018; Hopp et al. 2018; Katsumoto et al. 2018; Moore, Taylor, and Crack 2019).

There were various studies describing the significant role of SIGLEC-11 receptor in the neurodegenerative diseases. Earlier studies indicated a negative regulatory role of SIGLEC-11. They observed lower transcription level of the inflammatory mediator IL-1ß in murine microglia expressing human SIGLEC-11. Co-culturing of these microglia with neurons demonstrated higher neurite density and increased neuronal cell bodies comparing to the co-

culture of control microglia, which do not express human SIGLEC-11, with neurons indicating the neuroprotective effect of SIGLEC-11 (Wang and Neumann 2010).

SIGLEC-11 was also mentioned in another study as a central marker for preventing the progression of Alzheimer's disease. In this research, the authors described SIGLEC-11 to interact with sialic acid epitopes of neuronal gangliosides. This may help to clear or evade aggregating degenerative plaques such as Aß-plaques (O'Neill, van den Berg, and Mullen 2013; Raj et al. 2014; Salminen and Kaarniranta 2009). In a recent study, Liao and colleagues demonstrated the inhibitory role of SIGLEC-11 in their animal model of Parkinson's disease. Based on their findings, soluble polysialic acid with an average degree of polymerization 20 (polySia avDP20) could prevent inflammation and oxidative burst in human macrophages via SIGLEC-11 receptor and interferes with alternative complement activation. The authors checked the effects of polySia avDP20 on day 5 and 19 after the first injection of polySia avDP20. They found that both Cd68 and Iba1 levels were decreased in the substantia nigra *pars reticulata* (SNpr) in SIGLEC-11 transgenic mice at both time points. However, the wild type mice only exhibited a decreased level of Iba1+ cells and a lower expression level of Cd68 at day 19 (Liao et al. 2021). This may represent the confirmation of SIGLEC-11's inhibitory functions in neurodegenerative disorders.

1.3.2 SIGLEC-16- a paired receptor for SIGLEC-11

There is evidence that SIGLEC-16 is expressed on macrophages and microglia of the CNS, but it is a polymorphic gene either encoding a non-functional pseudogene or an intact gene in a percentage of humans with potential activatory properties. With over 99% sequence identity at the first two Ig-like domains to SIGLEC-11, it appears to be a "paired" receptor to SIGLEC-11 for the fine tuning of human microglial responses (Linnartz-Gerlach, Kopatz, and Neumann 2014). There is an evolutionary theory of SIGLEC-16 and SIGLEC-11, suggesting that these genes are originally emerged via a gene duplication event (Angata et al. 2002; Cao et al. 2008). In humans, this might have led to the functional and non-functional polymorphic alleles in the SIGLEC-16 locus (Wang et al. 2012). The non-functional

pseudogene SIGLEC-16P consists of a deletion of 4 base pair in exon 2 (Hayakawa et al. 2017). Studies by Hayakawa et al. and Wang et al. suggest that the functional SIGLEC-16 gene might have appeared later in evolution having a minor beneficial function, while the non-functional pseudogene still has a higher frequency compared with the functional ones in human populations (Hayakawa et al. 2017; Wang et al. 2012).

While Cao and colleagues describing that unlike most of CD-33r Siglecs, SIGLEC-16 associates with DAP-12 leading to the activation of the immune system (Cao et al. 2008). Therefore, DAP-12 may interfere with the ITIM pathway triggered by Siglec signaling.

1.4 Role of Siglec receptors in neurodegenerative diseases

Recent investigations have shown that the Siglec receptors on the surface of microglia cells play a critical role in neurodegenerative diseases and normal aging process (Fehervari 2015; Lubbers, Rodriguez, and van Kooyk 2018). Interestingly, expression levels of Siglec receptors varies throughout lifespan (Crocker, Paulson, and Varki 2007; Schwarz et al. 2015).

CD-33, also known as Siglec-3, is an inhibitory member of CD-33r Siglecs that demonstrated interactions with sialylated amyloid plaques. Thus, it could decrease microglia's ability to phagocytose these detrimental plaques (Estus et al. 2019). Wissfeld and colleagues described that deletion of CD-33 in human induced pluripotent stem cell-derived microglia could increase phagocytosis of Aβ aggregations. Ultimately, this will lead to the beneficial phagocytosis of amyloid-beta1-42 and the critical function of the CD-33, a microglial cell surface receptor, in the pathogenesis of AD (Wissfeld et al. 2021).

There are several risk factors that could contribute to neurodegenerative diseases including gender, nutrition, infection and inflammation, tumor, and trauma (Brown, Lockwood, and Sonawane 2005). The aging is one of these main risk factors (Datki et al. 2020; Guerreiro and Bras 2015). Even though there have been studies on the role of the Siglec receptors in neurodegenerative conditions, their efficacy during the normal aging process of the brain remains unclear. In order to better understand the changes of microglia cells over the time,

it would be essential to investigate the molecular mechanism of microglia and the accurate functioning of Siglec receptors in the process of aging.

1.5 Aging markers

As mentioned in many studies, aging of the brain might be one of the major risk factors of neurodegenerative diseases (Kamphuis et al. 2012; Machado et al. 2016). Therefore, studying the normal process of aging could be beneficial to understand the molecular changes of it. In addition, investigations related to the aging process may reveal new therapeutic approaches for treating neurodegenerative diseases. Despite the fact that many investigations carried out to understand the molecular mechanisms of these diseases, most of them pointed to the neuro-inflammatory responses mediated by microglia as the main reason for molecular changes during aging (Davies et al. 2017; Griffin et al. 2006; Streit et al. 2004; Wong et al. 2005). In most of these studies, researchers examined differences in inflammatory and oxidative markers between adult and aged mice.

In adult and aged C57BL/6 mice, Wong and colleagues found increased Cd68 levels in activated microglia cells (Wong et al. 2005). In another study, Griffin and colleagues reported that aged rats have a higher level of Cd68 protein compared to the adult ones (Griffin et al. 2006). They checked three time points of 3-, 9- and 15-months old rats and observed a higher inflammation rate in the 15-months old versus the other groups. In addition to higher protein levels of Cd68, they also showed that aged rats had higher levels of *TNF-α, IL-1ß, IL-6, and IL-10* (Griffin et al. 2006).

Lipofuscin (LF), known as age pigment (Moreno-Garcia et al. 2018), was described as a secondary aging marker (see fig.6) by O'Neil and colleagues' study (O'Neil et al. 2018). In this study, the authors evaluated the accumulation level of LF in both neurons and microglial cells of adult and aged mice. In comparison with the adult group, their results showed a higher accumulation of LF in both examined cells in their aged mice. Additionally, Sierra and colleagues described the increased expression level of the pro-inflammatory cytokines including TNF-α, IL1β and IL-6 in microglial cells isolated from the aged mice compared to

the younger mice. As they pointed out, the characterization of aged microglia is not limited to a higher level of pro-inflammatory cytokines, but they can also be identified by a greater accumulation of LF granules in the aged microglia compared to the younger microglia (O'Neil et al. 2018; Sierra et al. 2007).

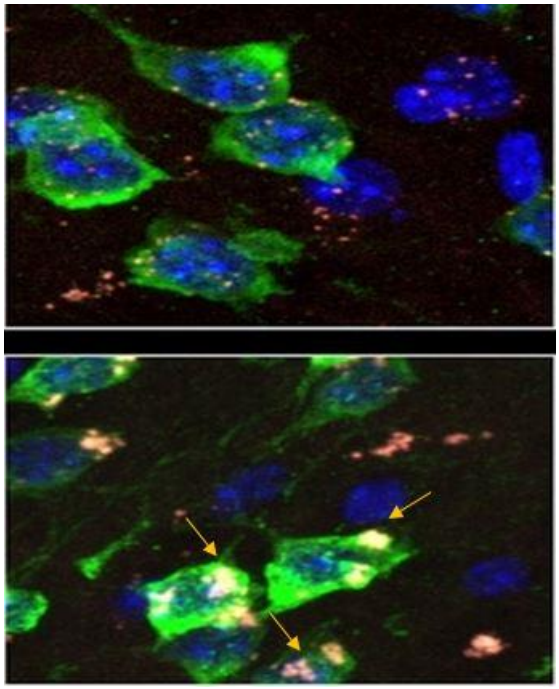


Fig. 6: Lipofuscin (LF) granules. Accumulation of LF granules in the microglial cells are visible with the pink color at the age of 18 months old mice (bottom) compared to the 9 months old (top). (Modified from Sudmant et al., Cell Reports, 2018.)

Free radicals are compounds that process an unpaired electron causing oxidative damages to all macromolecules including lipids, proteins and DNAs. Reactive oxygen species (ROS) are a major family of these free radicals (Sudmant et al. 2018). These oxidative markers are capable to attack poly unsaturated fatty acids resulting in the formation of lipid peroxides. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are the products of the lipid peroxidation. 4-HNE which is used as an aging marker, is also an important factor for the pathogenesis of Alzheimer's disease (McGrath et al. 2001). According to one Korean study

performed in 2018, the 14-months-old C57BL/6 mice had lower levels of oxidative damages in comparison with their older counterparts. They have examined the effects of radiofrequency electromagnetic fields (RF-EMFs) from mobile phones on both mouse groups over an extended period, and they have found that these RF-EMFs are not associated with oxidative damage to the brain. They described that the only factor associated with higher levels of 4-HNE in older mice was aging (Jeong et al. 2018).

1.6 Aims of this study

Microglia are the first line of defense in the CNS and play a key role during the normal physiological aging procedure (Ginhoux et al. 2013). These immune cells express SIGLEC receptors on their surface and could recognize damaged neurons within the brain and phagocytose them (Crocker, Paulson, and Varki 2007). To maintain brain homeostasis, microglia and the SIGLEC receptors must perform their accurate functions. Several studies have shown that during aging, microglia might become dysfunctional (Koellhoffer, McCullough, and Ritzel 2017; Mosher and Wyss-Coray 2014). Although these CNS resident immune cells and specifically their surface SIGLEC receptors are implicated in the aging process of the brain (Koellhoffer, McCullough, and Ritzel 2017; Krstic and Knuesel 2013; Mosher and Wyss-Coray 2014; Perry, Cunningham, and Holmes 2007), the particular role of Siglec receptors is still unclear. As human SIGLEC-11 and SIGLEC-16 were reported to be paired receptors, the purpose of this study was to determine their possible opposite roles during adulthood and aging. Thereby, I planned to use two different time points of 6- and 24-months old transgenic mice, expressing either SIGLEC-11 or SIGLEC-16 on microglia and macrophages, to investigate the effect of these specific SIGLEC receptors in the brain. The 6 months old group represents the normal adult mice in this study and the 24 months old group represents the aged mice. An initial goal was to investigate the possible inhibitory role of SIGLEC-11 and activatory role of SIGLEC-16 on microglia cells and their function in the transgenic mice at the adult and aged stages of their life. I therefore decided to analyze both transcription and expression level of microglia activation, pro-inflammatory cytokines and oxidative stress markers. The second aim was to determine the number of neurons in the experimented mice groups to investigate any age-related neuronal loss. Finally, this study

aimed to describe the function of these two Siglec receptors at both adult and aging populations in order to describe the role they play in determining the differences between age-related markers. These analyses might provide information to understand the mechanism of SIGLEC-11 and -16 receptors in the brain throughout the lifespan of mice.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Name	**Company**
4',6-diamidino-2-phenylindole (DAPI)	Sigma, Germany
Agarose (PeqGOLD Universal)	PeqLab, Germany
AquaPoly/Mount	Polysciences Europe, Germany
Bovine serum albumin (BSA)	Sigma, Germany
Double-distilled water (ddH2O)	autoclaved and filtered in the institute
Dulbecco's Phosphate Buffer Solution (PBS)	Gibco, Germany
Ethanol (C2H6O)	Roth, Germany
Ethidium Bromide (10g/l)	Roth, Germany
Hexanucleotide Mix (10x)	Roche, Germany
Isopropanol	Roth, Germany
Normal goat serum (NGS)	Sigma, Germany
Paraformaldehyde (PFA)	Sigma, Germany
QIAzol Lysis Reagent	Qiagen, Germany
Rompun® 2% (Xylazin)	Bayer, Germany
Sucrose 10%	Roth, Germany
SuperScript® III Reverse Transcriptase	Invitrogen, Germany
SYBR® Green ERTM qPCR Super Mix	Invitrogen by Life Technologies, USA
Trichloromethane/chloroform	Roth, Germany
Tris base	Roth, Germany
Tris buffer, 0.2 M	Roth, Germany
Triton X-100	Sigma, Germany
Tissue Tek®	Labtech, Germany

2.1.2 Consumables

Name	**Company**
1 ml syringe	Braun, Germany
10 µl, 100 µl, 1000 µl pipette tips	StarLab, Germany
15 ml plastic tubes	Greiner, Germany
50 ml plastic tubes	Sarstedt, Germany
Hand examination gloves	Meditrade, Germany
Glass cover slips (25 x 75 mm)	Thermo Scientific, USA
Injection needles	Braun, Germany
Polymerase chain reaction (PCR) tubes, 500 µl	Biozym Scientific GmbH, Germany
QPCR Seal optical clear film	PeqLab, Germany
Stainless steel beads (7 mm)	Qiagen, Germany
SuperFrost® Plus microscope slides	Thermo Scientific, USA

2.1.3 Technical equipment

Name	**Company**
AxioImager.Z1/ApoTome microscope	Zeiss, Germany
Biofuge Fresco/ pico (centrifuges)	Heraeus, Germany
Electrophoresis Power Supply EPS-301	Amersham Bioscience, Germany
Leica SP8 lightening Confocal microscope	Leica, Germany
Freezer -20 °C ProfiLine	Liebherr, Germany
Freezer -80 °C Herafreeze	Heraeus, Germany
GelDoc	BioRad, Germany
Mastercycler epgradient S	Eppendorf, Germany

Microm Cryo Star HM 560	Thermo Scientific, USA
NanoDrop 2000c spectrophotometer	Thermo Scientific, USA
NextSeq 500	Illumina, USA
Perfect BlueTM Horizontal Midi/Mini Gel Systems	PeqLab, Germany
Thermocycler T3 (PCR maschine)	Biometra, Germany
Vortex Genie2	Scientific Industries Inc., USA

2.1.4 Buffers and Solutions

Name	**Ingredients**
1 % Agarose gel	0.6 g agarose, 3 µl ethidium bromide, 60 ml Tris/Borate/EDTA buffer (1x)
10x BSA	10 g BSA in 100 ml 1x PBS
10x Tris/Borate/EDTA (TBE) buffer	1.78 M Tris base, 1.78 M boric acid, 0.04 M EDTA (pH 8.0), up to 2 litre ddH2O
30 % sucrose solution	30 g sucrose in 100 ml 1x PBS, 0.1 % sodium azide
4 % paraformaldehyde (PFA), pH 7.3	20 g PFA, 30 ml NaOH, 50 ml PBS (1x) up to 1 litre ddH2O
Anaesthesia solution (Ketamin/Xylazin)	1 ml Ketmin 10%, 0.5 ml Xylazin 2%, 8.5 ml ddH2O

2.1.5 Primers

Name	Sequence
ms-Aif1-Forward	GAAGCGAATGCTGGAGAAAC
ms-Aif1-Reverse	AAGATGGCAGATCTCTTGCC
ms-aldh1l1-Forward	GCGAATTCTGCCCAATGTCC
ms-aldh1l1-Reverse	TCCTTCACCTCCTCCACCAG
ms-C1qA-Forward	AGAGGGGAGCCAGGAGC
ms-C1qA-Reverse	CTTTCACGCCCTTCAGTCCT
ms-C1qB-Forward	GACTTCCGCTTTCTGAGGACA
ms-C1qB-Reverse	CAGGGGCTTCCTGTGTATGGA
ms-C1qC-Forward	GCCTGAAGTCCCTTACACCC
ms-C1qC-Reverse	GGGATTCCTGGCTCTCCCTT
ms-C3-Forward	TAGTGCTACTGCTGCTGTTGGC
ms-C3-Reverse	GCTGGAATCTTGATGGAGACGCTT
ms-C4-Forward	TGGAGGACAAGGACGGCTA
ms-C4-Reverse	GGCCCTAACCCTGAGCTGA
ms-CD68-Forward	CAGGGAGGTTGTGACGGTAC
ms-CD68-Reverse	GAAACATGGCCCGAAGTATC
ms-Cyba-Forward	CCTCCACTTCCTGTTGTCGG
ms-Cyba-Reverse	TCACTCGGCTTCTTTCGGAC
ms-Cybb-Forward	GGGAACTGGGCTGTGAATGA
ms-Cybb-Reverse	CAGTGCTGACCCAAGGAGTT
ms-DAP-12-Forward	ATGGGGGCTCTGGAGCCCT
ms-DAP-12-Reverse	TCATCTGTAATATTGCCTCTGTGT
ms-fcer1g-Forward	CTGTCTACACGGGCCTGAAC
ms-fcer1g-Reverse	AAAGAATGCAGCCAAGCACG
ms-GAPDH-Forward	ACAACTTTGGCATTGTGGAA
ms-GAPDH-Reverse	GATGCAGGGATGATGTTCTG

ms-GAPDH genotyping-Forward	ACAACTTTGGCATTGTGGAA
ms-GAPDH genotyping-Reverse	GATGCAGGGATGATGTTCTG
ms-Il1ß-Forward	CTTCCTTGTGCAAGTGTCTG
ms-Il1ß-Reverse	CAGGTCATTCTCATCACTGTC
ms-iNOS-Forward	AAGCCCCGCTACTACTCCAT
ms-iNOS-Reverse	GCTTCAGGTTCCTGATCCAA
ms-itgam-Forward	CATCAAGGGCAGCCAGATTG
ms-itgam-Reverse	GAGGCAAGGGACACACTGAC
ms-itgb2-Forward	GTCCCAGGAATGCACCAAGT
ms-itgb2-Reverse	CCGTTGGTCGAACTCAGGAT'
ms-S100B-Forward	GTCTTCCACCAGTACTCCGG
ms-S100B-Reverse	ACGAAGGCCATGAACTCCTG
ms-Sig11genotyping-Forward	GGAGATGTCAGGGATGGTTC
ms-Sig11genotyping-Reverse	AGCAGCGTATCCACATAGCGT
ms-Sig11-qRT-PCR-Forward	CACTGGAAGCTGGAGCATGG
ms- Sig11-qRT-PCR - Reverse	ATTCATGCTGGTGACCCTGG
ms-Sig16genotyping-Forward 3	CCGGTGACGGTCATCTGTG
ms-Sig16genotyping-Reverse 3	AGTTCCAGGGAGGCCACAC
ms-Sig16genotyping-Forward 5	GAAGATCCTGCTTCTCTGC
ms-Sig16genotyping-Reverse 5	TTAGTCCGTGACAGCGTC
ms-Sig16-qRT-PCR-Forward	CCTCGGGTTCAAATGGAGCA

ms- Sig16-qRT-PCR - Reverse	TCTTCATAGCCACCTCCCCA
ms-TMEM 119-Forward	GTGTCTAACAGGCCCCAGAA
ms-TMEM 119-Reverse	AGCCACGTGGTATCAAGGAG
ms-TNFa-Forward	GGTGCCTATGTCTCAGCCTC
ms-TNFa-Reverse	TGAGGGTCTGGGCCATAGAA

2.1.6 Antibodies

2.1.6.1 Primary antibodies

Antigen	**Host**	**Company**	**Dilution**
4-hydroxynonenal (4-HNE)	Mouse	Abcam, ab48506	1:15 from 10µg/ml
CD68	Rat	Bio-Rad, MCA1957	1:500
Ionized calcium binding adaptor molecule 1 (Iba1)	Rabbit	Wako, #019-19741	1:500
Neuronal nuclei (NeuN)	Mouse	Millipore #MAB377	1:500
Postsynaptic density protein 95 (PSD-95)	Mouse	Thermo Scientific, MA1046	1:200
Tyrosine hydroxylase (TH)	Rabbit	Sigma #T8700	1:500
Vesicular glutamate transporter 1 (vGLUT-1)	Rabbit	Synaptic Systems GmbH #135303	1:500

2.1.6.2 Secondary antibodies

Fluorophore	Reactivity	Host	Company	Dilution
Alexa Fluor® 488	rabbit	Goat	Invitrogen, A11008	1:500
Alexa Fluor® 488	mouse	Goat	Invitrogen, A11001	1:500
Cyanine 3 (Cy3)-conjugated F(ab')2 fragment	mouse	Goat	Dianova, #115-166-072	1:200 , 1:500
Cy3-conjugated F(ab')2 Fragment	Rat	Goat	Dianova, #112-166-072	1:200
Cy3-conjugated F(ab')2 fragment	rabbit	Goat	Dianova, #111-165-047	1:200

2.1.7 Kits and Markers

Name	Company
100 base pair DNA ladder	Invitrogen, Germany
KAPA mouse genotyping Hot Start Kit	PeqLab, Germany
RNase-Free DNase Set	Qiagen, Germany
RNeasy® Mini Kit	Qiagen, Germany
SuperScript First-Strand Synthesis System	Invitrogen, Germany

2.1.8 Software

Name	**Company**
AxioVisio V4.8.2.0	Zeiss, Germany
Corel Draw® X5 + X8	Corel GmbH, Germany
GraphPad QuickCalcs	GraphPad Software Inc., USA
IBM SPSS Statistics (v.23)	IBM Corporation, Germany
ImageJ 1.43m; Java 1.6.0_14	National Institute of Health, USA
Master cycler ep realplex	Eppendorf, Germany
Microsoft Office 2021	Microsoft, USA
NanoDrop 2000/2000c	Thermo Fisher Scientific, USA
Prism 9 (v9)	GraphPad Software Inc., USA

2.2 Methods

2.2.1 Mice

All animal experiments have been approved by the authors´ institutional review boards and by the local government and have been conducted according to the principles expressed in the Helsinki Declaration. We used 6 and 24 months old male Siglec11 transgenic mice (SIGLEC-11) [19], Siglec16 transgenic mice (SIGLEC-16) (see generation of SIGLEC-16 transgenic mice in Johannes Peter Ackermann thesis, University of Bonn, 2016) and C57/BL6 as control [wild-type(wt)] group. Animals were housed in the institute's animal facility in specific pathogen free environment with water and food adlibitum at 12 hours' dark/night cycle. Housing and breeding of the animals were performed in accordance with

the German guidelines of the animal care and use committee. All efforts were made to minimize the number of animals used and their suffering.

2.2.1.1 Genotyping of mice

Tissue of either ear or tail was taken for the isolation of genomic DNA using the KAPA mouse genotyping Hot Start Kit according to the manufacturer's protocol. Genomic DNA was eluted in ddH2O. Then, PCR with the genotype-specific primers (see Table 1) was performed using the KAPA2G Fast Hot Start Genotyping Mix from the same kit as described in Table 2.

Tab. 1: Genotyping primer mix

Mouse line	**Primer mix**	**Expected product length**
SIGLEC-11	ms-Sig11 genotyping-Forward ms-Sig11 genotyping-Reverse ms-GAPDH genotyping-Forward ms-GAPDH genotyping-Reverse	SIGLEC-11 (+): ~200 bp GAPDH: 133 bp
SIGLEC-16	ms-Sig16 genotyping-Forward3 ms-Sig16 genotyping-Reverse3 ms-Sig16 genotyping-Forward5 ms-Sig16 genotyping-Reverse5 ms-GAPDH genotyping-Forward ms-GAPDH genotyping-Reverse	SIGLEC-16 (+): 250bp and 111 bp GAPDH: 133 bp

Tab. 2: Genotyping PCR reaction mix

Master Mix for SIGLEC-11 and GAPDH	**1x**
PCR (DEPC) Water	8.2µl
DMSO	1.3µl
KAPA (green) master mix	12.5µl
DNA	1µl
Primer Forward	1µl
Primer Reverse	1µl

Master Mix for SIGLEC-16	**1x**
PCR (DEPC) Water	8.2µl
DMSO	1.3µl
KAPA (green) master mix	12.5µl
DNA	1µl
Primer Forward 3	0.5µl
Primer Reverse 3	0.5µl
Primer Forward 5	0.5µl
Primer Reverse 5	0.5µl

Tab. 3: Genotyping programs for thermocycler for SIGLEC-11

Step	Temperature	Time (minutes)	
Initial denature	94°C	5:00	
Denature	94°C	00:30	32x
Annealing	58°C	00:40	
Elongation	72°C	00:45	
Final elongation	72°C	8:00	

Tab. 4: Genotyping programs for thermocycler for SIGLEC-16

Step	Temperature	Time (minutes)	
Initial denature	94°C	5:00	
Denature	94°C	00:45	38x
Annealing	58°C	00:45	
Elongation	72°C	00:45	
Final elongation	72°C	10:00	

PCR products were loaded onto 1 % agarose gel containing ethidium bromide. A 100 base pair ladder was used as reference marker. The samples were run at 100-120 V and 110 mA for 45 minutes. The gels were visualized using GelDoc with QuantityOne software.

For SIGLEC-11 mouse genotyping, presence of around 200 bp bond was important. For SIGLEC-16 there should be two bonds of 111 and 250 bp to refer the sample as the positive SIGLEC-16. For both PCR reactions GAPDH should present 133 bp bond.

2.2.1.2 Tissue preparation

Unless stated otherwise male animals were weighed routinely, followed by an anesthesia with 200 µl per 10 g body weight anesthesia solution (Ketamin/Xylazin; for more details, see 2.1.4) and then perfused with 1x PBS. The brain was directly removed and cut into both hemispheres.

Left hemisphere was directly used for RNA isolation, while the right hemisphere was stored in 4 % PFA for 24 hours at 4 °C, followed by incubation in 30 % sucrose solution at 4 °C until the brain was fully soaked and sank to the bottom of the tube. The fixed brains were embedded in Tissue-Tek® and stored at -80 °C for immunohistochemistry.

2.2.2 Analysis of gene transcription

2.2.2.1 RNA isolation of brain samples

RNA from freshly taken hemispheres of male transgenic mice or wildtype littermates (see section 2.2.1) was extracted using the RNeasy® Mini kit from Qiagen. Shortly, tissue was directly homogenized twice with 1 ml Qiazol Lysis Reagent and a stainless-steel bead (mean diameter: 7 mm) in a Tissue Lyser LT for 6 minutes at 50 Hz. After 5 minutes' incubation at room temperature, 200 µl chloroform were added. The mixed suspension was again incubated for 3 minutes at room temperature before centrifuging at 12000 x g for 15 minutes at 4 °C. The upper, aqueous phase was transferred into a new tube and 500 µl 70 % ethanol were added and mixed. Then, 600 µl of the solution were transferred into a RNeasy column and centrifuged at 10000 x rpm for 20 seconds at room temperature. The flow-through was discarded and the remaining solution was transferred in the same way until the complete lysate was loaded onto the column. The RNA isolation procedure was then continued according to the manufacturer´s protocol. 50 µl eluted RNA was either stored at -80 °C or directly used for reverse transcription.

2.2.2.2 cDNA synthesis by reverse transcription of RNA

Reverse transcription of RNA was performed with SuperScript III reverse transcriptase and random hexanucleotide mix according to the Invitrogen protocol for SuperScript First-Strand Synthesis. The concentration of transcribed cDNA was measured using NanoDrop and was diluted to a working concentration of 100 ng/µl.

2.2.2.3 Semi-quantitative real-time polymerase chain reaction (sqRT-PCR)

Semi-qRT-PCR with specific oligonucleotides was performed using the SYBR® Green ERTm qPCR Super Mix. Gene transcripts of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were applied as internal RNA loading control. For quantitative real-time PCR the following mix was prepared in a 96-well-plate:

SYBR Green Mix	12.5 µl
Forward and reverse mixed primers (each 10 pmol/µl)	2 µl
cDNA	1 µl
ddH2O	9.5 µl

For the non-template control cDNA was replaced with ddH2O. The plate was covered with a plastic lid and analyzed in a Mastercycler epindorf S with the following program:

Cover T° = 105 °C

Initial denature	95 °C	10:00 minutes	
Denature	95 °C	00:15 minutes	40x
Annealing	60 °C	00:30 minutes	
Elongation	72 °C	00:30 minutes	

To ensure that a specific product was obtained, a dissociation curve analysis was performed using the following program:

95°C	1:00 minute
55 °C	00:15 minutes
95 °C	00:15 minutes
Ramp rate	20:00 minutes

Amplification specificity was confirmed by analysis of the melting curves. Results were analyzed with the Master cycler ep realplex software after establishing the reaction efficiency for each primer pair. Values were normalized with their respective GAPDH values. Delta cycle threshold (CT) values were normalized to 3 months wildtype (=100 %) and quantification using the ΔΔCt method was applied

2.2.3 Immunohistochemistry

Mouse half brain tissues of PBS-perfused mice were immersed in 4% paraformaldehyde (PFA) for 24 hours, followed by 30% sucrose supplemented with 0.1% sodium azide until processed into frozen sections. The hemisphere was embedded in O.C.T.™ Compound, Tissue Tek®, cut into 20 µm coronal sections and stored at −20°C before staining.

2.2.3.1 Immunohistochemistry of microglia cells

For microglia activation and phagocytosis analysis, sections were blocked and permeabilized using 10% BSA and 0.25% TritonX-100 in PBS followed by the primary antibodies rabbit-anti-ionized calcium-binding adapter molecule 1 (Iba1, 1:500; Wako) and rat-anti-cluster of differentiation 68 (CD68, 1:500; BioRad) in incubation solution (IS; 5% BSA and 0.05% TritonX-100 in PBS) overnight at 4°C. After three washing steps in IS, the sections were incubated in the corresponding Alexa488-coupled secondary antibodies (1:400; Invitrogen) and Cy3-conjugated F(ab')2 fragment (1:200; Dianova) in IS for 2 hours at room temperature. After two washing steps with 1x PBS, nuclei of cells were labeled with 4´ ,6-diamidino-2-phenylindole (DAPI, 1:10,000; Sigma) for 30 s followed by another washing step 2 with 1× PBS. Sections were embedded with Moviol (Sigma, Germany) and stored at 4°C. Images were taken with a confocal microscope (LEICA SP8). All number-coded images were analyzed by a blinded investigator using the ImageJ software. The levels were defined according to the mouse brain atlas of Paxinos & Franklin. For analysis of microglia activation and phagocytosis of microglia cells, z-stacks of the stained hippocampus and the SN *pars reticulate* (SN*pr*) were taken at the Bregma level −2.88 mm to −2.9 mm. The intensity of Iba1 and CD68 per area was measured and background intensity was subtracted. Additionally, the number of Iba1- and CD68-positive cells were counted. Five of these positive cells were chosen in each picture and soma intensities of both Iba1 and CD68 were measured in the selected cells. The corresponding background intensities were subtracted for each soma.

2.2.3.2 Immunohistochemistry of Iba1 cell clusters

For Iba1 cluster analysis, the same procedure of Iba1/CD68 staining was performed for the samples but this time only Iba1 was used as the primary antibody and the Alexa488-coupled as the secondary antibody. The microscope LEICA SP8 was used for checking the stained samples. 3 or more cells that are close to each other (50µm) were defined as cluster. These clusters were checked in both Substantia nigra *pars reticulate* (SN*pr*) and hippocampus.

2.2.3.3 Immunohistochemistry of oxidized lipids

For oxidized lipids, sections were blocked and permeabilized using 10% BSA, 0.20% TritonX-100 and 5% NGS in PBS (blocking solution, bs) followed by the primary antibody mouse-anti-4-hydroxynonenal (4-HNE, 1:15 from 10µg/ml; abcam) in bs for 2 hours at room temperature. After three washing steps in 1x PBS, the sections were incubated in the corresponding Cy3-conjugated F(ab')2 fragment (1:500; Dianova) in bs for 2 hours at room temperature. After two washing steps with 1x PBS, nuclei of cells were labeled with 4´ ,6-diamidino-2-phenylindole (DAPI, 1:10,000; Sigma) for 30 s followed by another washing step 2 with 1× PBS. Sections were embedded with Moviol (Sigma, Germany) and stored at 4°C. Images were taken with a confocal microscope (LEICA SP8). All number-coded images were analyzed by a blinded investigator using the ImageJ software. The levels were defined according to the mouse brain atlas of Paxinos & Franklin. For analysis of oxidized lipids, z-stacks of the stained hippocampus and the SN *pars reticulate* (SN*pr*) were taken at the Bregma level −2.94 mm to −2.98 mm. The intensity of 4-HNE per area was measured and background intensity was subtracted.

2.2.3.4 Immunohistochemistry of lipofuscin and Iba1

For lipofuscin analysis, sections were blocked and permeabilized using 10% BSA and 0.25% TritonX-100 in PBS followed by the primary antibody rabbit-anti-ionized calcium-binding adapter molecule 1 (Iba1, 1:500; Wako) in incubation solution (IS; 5% BSA and 0.05% TritonX-100 in PBS) overnight at 4°C. After three washing steps in IS for analysis of lipofuscin analysis, the sections were incubated in the corresponding Alexa488-coupled secondary antibodies (1:400; Invitrogen) in IS for 2 hours at room temperature. After two washing steps with 1x PBS, nuclei of cells were labeled with 4´ ,6-diamidino-2-phenylindole (DAPI, 1:10,000; Sigma) for 30 s followed by another washing step 2 with 1x PBS. Sections were embedded with Moviol (Sigma, Germany) and stored at 4°C. Images were taken with a confocal microscope (LEICA SP8). All number-coded images were analyzed by a blinded investigator using the ImageJ software. The levels were defined according to the mouse brain atlas of Paxinos & Franklin. For analysis of lipofuscin accumulation, z-stacks of the stained

hippocampus and the SN *pars reticulate* (SNpr) were taken at the Bregma level −2.7 mm to −2.8 mm. The intensity of lipofuscin per area was measured and background intensity was subtracted. Additionally, the number of Iba1/lipofuscin-double positive cells were counted. Five of the Iba1/lipofuscin double positive cells were chosen in each picture and soma intensities of lipofuscin were measured in these selected cells. The corresponding background intensities were subtracted for each soma.

2.2.3.5 Immunohistochemistry of neurons

For staining of neurons, sections were blocked and permeabilized using 10% BSA, 0.20% TritonX-100 and 5% NGS (blocking solution, bs) in PBS followed by the primary antibodies rabbit-anti-tyrosine hydroxylase (TH, 1:500; Sigma) and mouse-anti-neuronal nuclei (NeuN, 1:500, Millipore) in bs for 2 hours at room temperature. After three washing steps in PBS, the sections were incubated in the corresponding Alexa488-coupled secondary antibodies (1:500; Invitrogen) and Cy3 conjugated F(ab')2 fragment (1:200; Dianova) in bs for another 2 hours at room temperature. After two washing steps with 1x PBS, nuclei of cells were labeled with 4´ ,6-diamidino-2-phenylindole (DAPI, 1:10,000; Sigma) for 30 s followed by another washing step 2 with 1× PBS. Sections were embedded with Moviol (Sigma, Germany) and stored at 4°C. Images were taken with an Apotome microscope (Zeiss). All number-coded images were analyzed by a blinded investigator using the ImageJ software. The levels were defined according to the mouse brain atlas of Paxinos & Franklin. For analysis of general and cell-type specific neuronal loss, z-stacks of the stained hippocampus at the Bregma level −2.3 mm to −2.46 mm and the SN *pars compacta* (SN*pc*) at the Bregma level -2.92 were taken. The number of NeuN-positive cells at both hp and SN*pc* was measured and TH-positive cells were counted only in the SN*pc* region.

2.2.3.6 Immunohistochemistry of synapses

For synapse analysis, sections were blocked and permeabilized using 2% BSA and 0.20% TritonX-100 (blocking solution, bs) in PBS followed by the primary antibodies mouse-anti-

post synaptic density protein-95 (PSD-95, 1:200; Thermo Scientific) and rabbit-anti- Vesicular glutamate transporter 1 (vGLUT-1, 1:500; Synaptic Systems GmbH) in diluent solution (ds, 0.5% BSA and 0.05% TritonX-100 in PBS) for 3 days at 4°C. After three washing steps in 1x PBS, the sections were incubated in the corresponding Alexa488-coupled secondary antibodies (1:500; Invitrogen) and Cy3 conjugated F(ab')2 fragment (1:20; Dianova) in ds overnight at 4°C. After three washing steps with 1x PBS, nuclei of cells were labeled with 4´,6-diamidino-2-phenylindole (DAPI, 1:10,000; Sigma) for 30 s followed by another washing step 2 with 1x PBS. Sections were embedded with Moviol (Sigma, Germany) and stored at 4°C. Images were taken with an Apotome microscope (Zeiss). All number-coded images were analyzed by a blinded investigator using the ImageJ software. The levels were defined according to the mouse brain atlas of Paxinos & Franklin. For analysis of pre- and post-synapse and co-puncta synapse loss, z-stacks of the stained samples at the Bregma level −2.3 mm to −2.5 mm. The number of pre- and post- synapse and co-puncta synapses were measured in CA3 region.

2.2.4 Statistical analysis

Data of at least three independent experiments were normalized to 6 months old BL6 mice. Results were presented as mean ± SEM (= standard error of the mean) or mean + SEM of at least three independent experiments. Significant outliers were discovered by using the outlier calculator of GraphPad QuickCalcs and were excluded from further analysis. Graphs were designed using Prism 9. Data with more than one variable were analyzed by multiple linear regression including an interaction term followed by a pairwise comparison with LSD-post hoc correction using IBM SPSS Statistics (v.23) computer software (IBM Corporation, Germany). The Breusch–Pagan/Cook–Weisberg test for heteroscedasticity was performed to assess the equality of variances in the linear model. If variances were significantly different, a robust linear model including an interaction term was chosen for further analysis. Data were in general regarded as significant if p-values were below or equal 0.05 (*$p \leq 0.050$; **$p \leq 0.010$; ***$p \leq 0.001$).

3. Results

3.1 Characterization of the mouse lines

It is believed that microglia cells, as the resident immune cells in the CNS, could recognize different sialylated structures via their surface Siglec receptors (Linnartz-Gerlach, Kopatz, and Neumann 2014). It has also been suggested that ITAM and ITIM-signaling receptors may work together to maintain brain homeostasis (Siddiqui et al. 2019). Accordingly, SIGLEC-16 and SIGLEC-11 are thus regarded as paired receptors for activating ITAM-signaling and inhibiting ITIM-signaling (Schwarz et al. 2017). Prof. Neumann's laboratory produced transgenic mouse lines expressing either human SIGLEC-11 or SIGLEC-16 under an Iba1-minipromoter. At the first level, gene transcription and protein expression levels of the SIGLEC-11 and SIGLEC-16 receptors in the experimental groups SIGLEC-11(+/-) [SIGLEC-11 tg] and SIGLEC-16(+/-) [SIGLEC-16 tg] as compared to wildtype control C57BL/6 [wt] was analyzed.

3.1.1 RNA transcription results confirmed SIGLEC-11 gene transcription in SIGLEC-11 transgenic mice.

RNA samples from the brain of SIGLEC-11 and C57BL/6 mice were collected (for details see section 2.2.2.1) and the complementary DNA (cDNA) was synthesized. Then, qRT-PCR was performed to check the transcription level of SIGLEC-11 in both SIGLEC-11 transgenic mice and control mice brain samples. In SIGLEC-11 transgenic mice, SIGLEC-11 gene transcripts were detected (see fig.7).

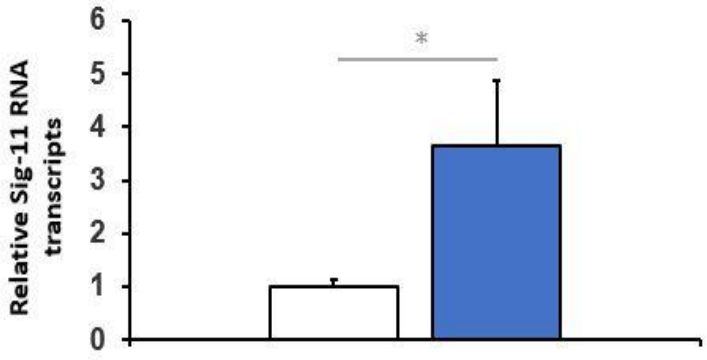
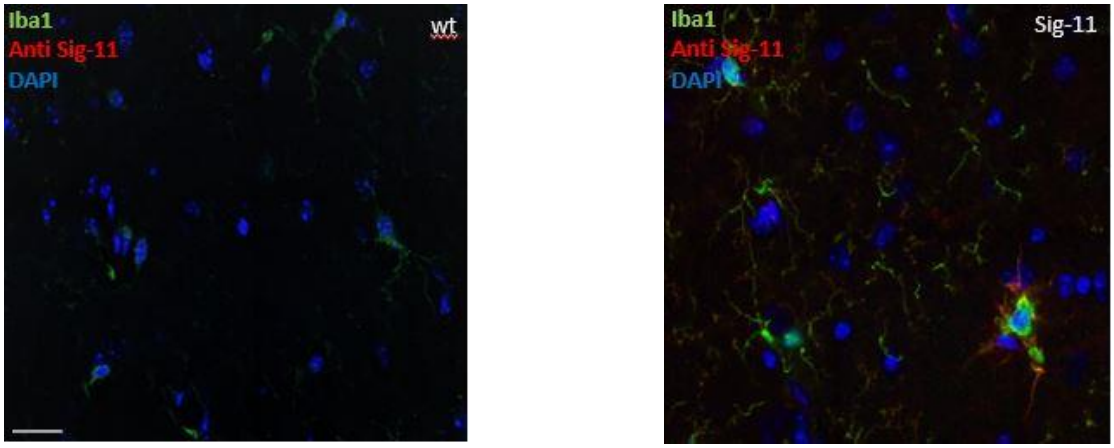


Fig. 7: Transcription level of SIGLEC-11 gene, White: BL6, Blue: SIGLEC-11, The samples were checked by the SIGLEC-11 specific primer. The SIGLEC-11 samples showed the transcription of SIGLEC-11 compared to the background signal of BL6 samples as the control group. Data shown as mean ± SEM; normalized to BL6 samples; n=6-8; *p ≤ 0.050.

3.1.2 Expression of SIGLEC-11 protein in the SIGLEC-11 transgenic mice.

Aside from RNA analysis, protein expression levels of SIGLEC-11 were examined in the experimented mice groups. An anti-SIGLEC-11/16 antibody was used to stain the fixed brain slices of SIGLEC-11 and BL6 mice. The immunostaining results showed similar results to the RNA level results. In the SIGLEC-11 mice group, the protein was expressed in cells with a microglial-like morphology, while in the control animals, this protein was not detected (see fig. 8 A and B). This double-labelling assay with anti-Iba1 antibodies confirmed that SIGLEC-11 is expressed in microglia (see fig. 8).

A



B

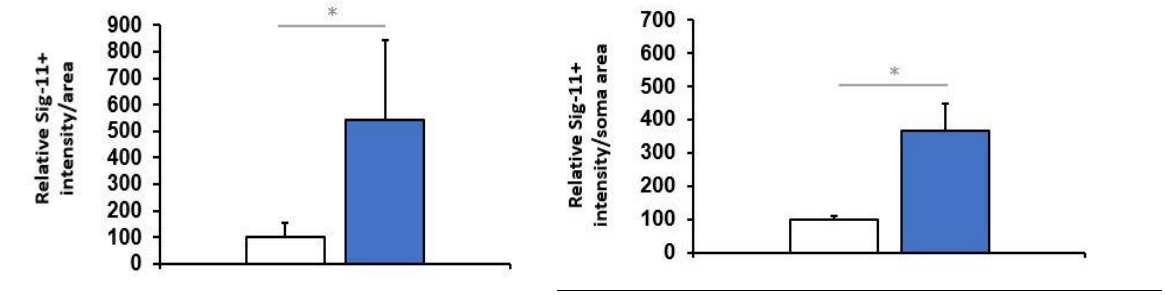


Fig. 8: Expression level of SIGLEC-11 protein. A Immunostaining with an anti- SIGLEC-11/16 antibody, Blue: DAPI, Green: ionized calcium binding adaptor molecule 1 (Iba1) and Red: anti SIGLEC-11/16. BL6 and SIGLEC-11 mice slices stained with Iba1 and anti-SIGLEC-11/16 primary antibodies. 4´ ,6-diamidino-2-phenylindole (DAPI) is used as the nucleus marker in the staining. Expression of SIGLEC-11 protein is visible in the SIGLEC-11-positive samples by the red fluorescence color of Cy3-conjugated F(ab')2 Fragment secondary antibody against the anti siglec-11 antibody in the microglia cells. Substantia nigra pars reticulate (SNpr), Bregma level: -2.5_-2.6. Scale bar: 10 µm. **B** Expression level of SIGLEC-11 protein, White: BL6, Blue: SIGLEC-11, Intensity of SIGLEC-11 per area (left) /soma area (right) showed increased intensity of this protein in SIGLEC-11 samples compared to the BL6 samples. Data shown as mean ± SEM; normalized to BL6 samples; n=6-9; *p ≤ 0.050.

3.1.3 RNA transcription level of SIGLEC-16 gene in the SIGLEC-16 transgenic mice.

As with the SIGLEC-11 samples, the transcription and expression of SIGLEC-16 were also analyzed. The transcription level of the SIGLEC-16 gene was assessed by qRT-PCR using specific primers developed for the SIGLEC-16 gene. Comparing the SIGLEC-16 samples with the BL6 group, we detected the presence of the SIGLEC-16 gene transcripts in the brains of SIGLEC-16 tg mice (see fig.9).

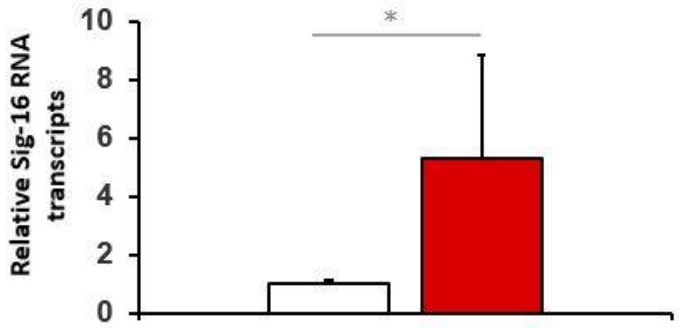
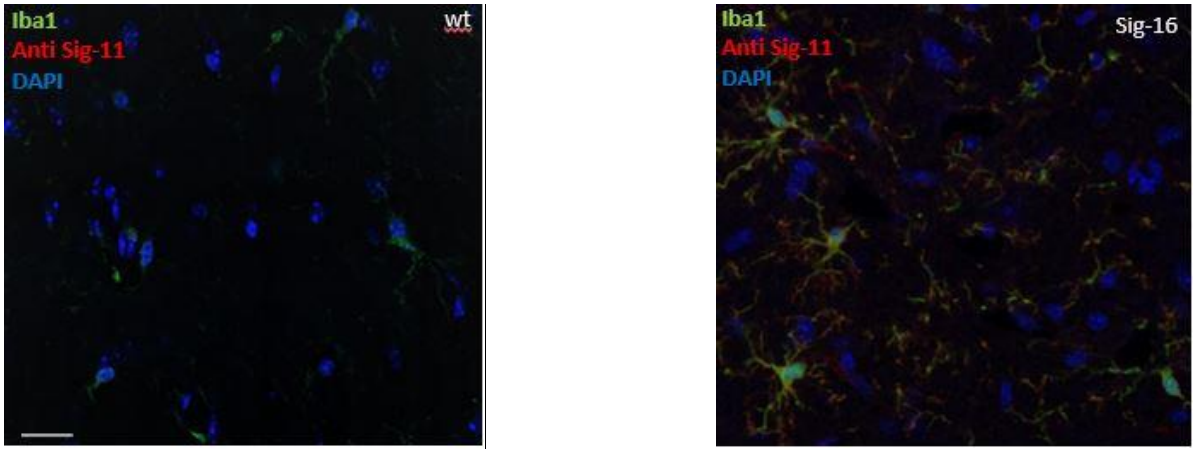


Fig. 9: Transcription level of SIGLEC-16 gene, White: BL6, Red: SIGLEC-16, The samples were checked by the SIGLEC-16 specific primer. The SIGLEC-16 samples showed transcription level of SIGLEC-16 compared to the background signal of BL6 samples as the control group. Data shown as mean ± SEM; normalized to BL6 samples; n=6-8; *p ≤ 0.050.

3.1.4 Expression of SIGLEC-16 protein in the SIGLEC-16 transgenic mice.

Previously it has been mentioned that SIGLEC-11 and SIGLEC-16 have a high degree of similarity in their sequences. In addition, because there are no antibodies specific to SIGLEC-16, we used the anti-SIGLEC-11/16 antibody to demonstrate the expression levels of SIGLEC-16. Figure 10 A shows the expression of SIGLEC-16 protein in SIGLEC-16 samples compared with the wildtype control brain samples.

A



B

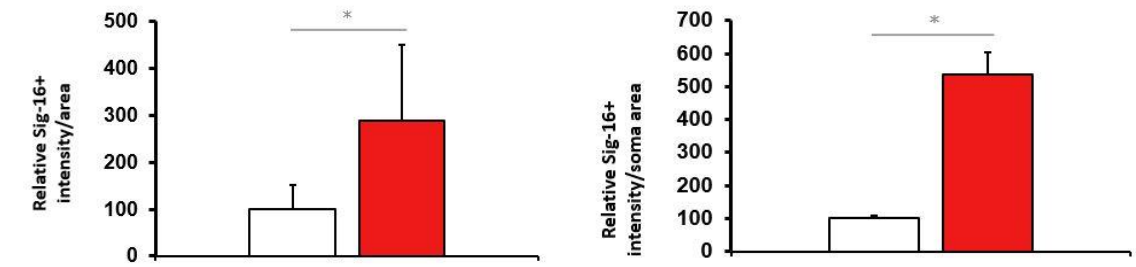
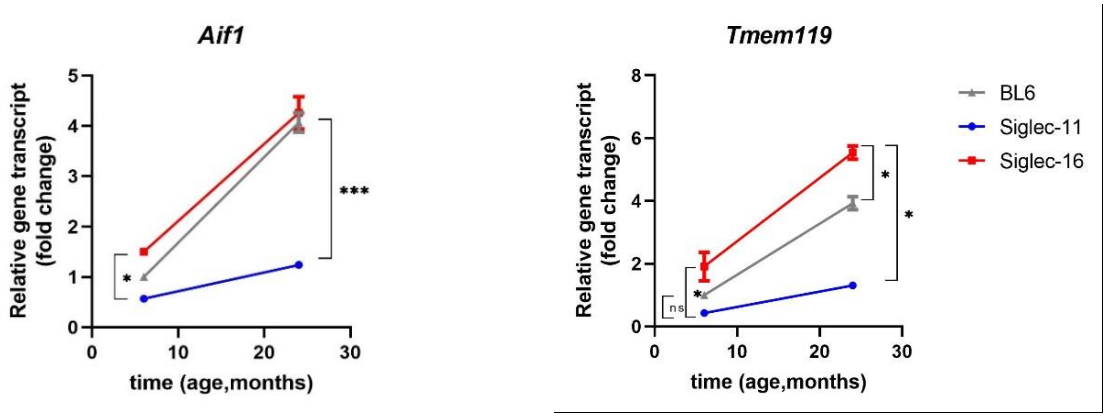


Fig. 10: Expression level of SIGLEC-16 protein. A Immunostaining of anti SIGLEC-11/16 antibody in the SIGLEC-16 samples, Blue: 4´ ,6-diamidino-2-phenylindole (DAPI), Green: ionized calcium binding adaptor molecule 1 (Iba1) and Red: anti SIGLEC-11/16. BL6 and SIGLEC-16 stained with Iba1 and anti SIGLEC-11/16 primary antibodies. The expression level of SIGLEC-16 protein is visible in the SIGLEC-16 samples by the red fluorescence color of Cy3-conjugated F(ab')2 Fragment secondary antibody against the anti siglec-11 antibody. Substantia nigra pars reticulate (SNpr), Bregma level: -2.5_-2.6. Scale bar: 10 µm. **B** Expression level of SIGLEC-16 protein, White: BL6, Red: SIGLEC-16, Intensity of SIGLEC-16 per area (left) /soma area (right) showed increased intensity of this protein in SIGLEC-16 samples compared to the BL6 samples. Data shown as mean ± SEM; normalized to BL6 samples; n=6-8; *p ≤ 0.050.

3.2 Influence of SIGLEC-11 and SIGLEC-16 on the microglia activation and pro-inflammation.

Throughout the aging process, microglial markers and pro-inflammatory cytokines are highly expressed (Lively and Schlichter 2018). SIGLEC-11 signaling receptor, as an ITIM signaling receptor, may inhibit the production and expression of these markers. Therefore, we analyzed *Aif1*/*Iba1* and *Tmem119* as macrophage/microglial markers and *Tnfα* and *Il1ß* as pro-inflammatory cytokines. Consequently, qRT-PCR results demonstrated lower transcription levels of all mentioned investigated markers in SIGLEC-11 versus the SIGLEC-16 group at 24 months old (*Aif1*: p = 0.00054, *Tmem119*: p = 0.0106, *TNFα:* p = 0.0071, *IL1ß*: p = 0.00090). Also, a lower transcription level of these markers was detected in the 6 months old animals than in the 24 months old animals (age-specific: for all of the markers: p < 0.0001; Fig. 11 **A** and **B**).

A



B

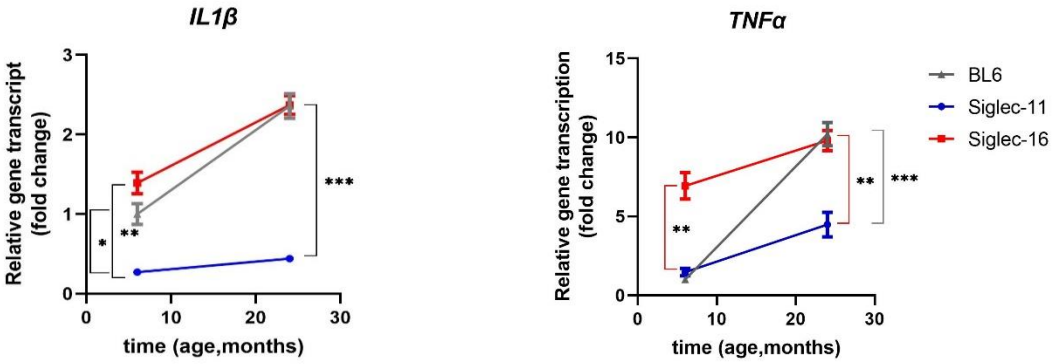
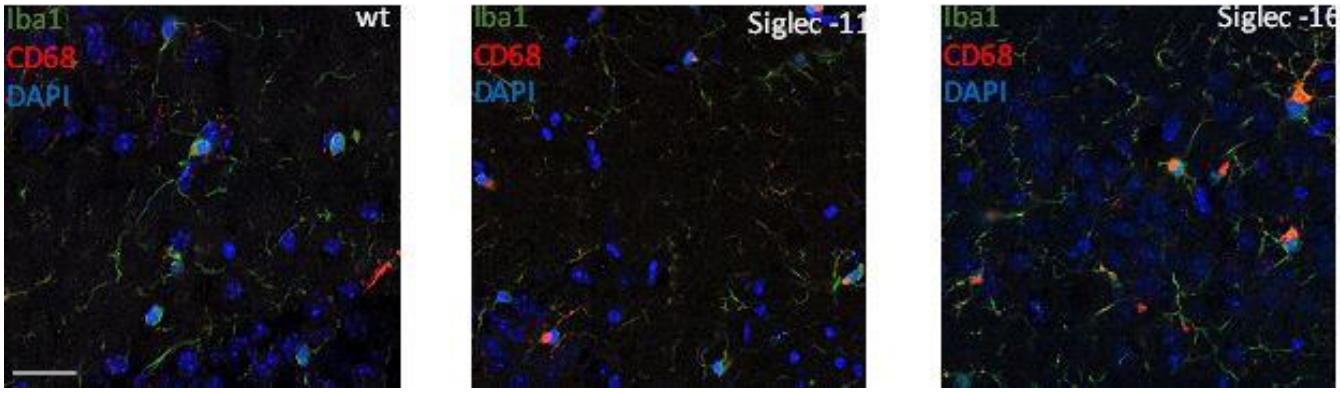


Fig. 11: Gene transcripts of microglial markers and pro-inflammatory cytokines. Transcription level of *Aif1* and *Tmem119* (**A**), *Il1ß* and *TNFa* (**B**). **A** SIGLEC-16 at the age of 24 months old is showing the higher transcription level of the microglial markers. However, SIGLEC-11 demonstrated the lower transcription level of these markers at the 24 months old compared to both BL6 and SIGLEC-16. Data shown as mean ± SEM; normalized to BL6 samples; n=6-13; n.s. not significant, *p ≤ 0.050, ***p ≤ 0.001. **B** SIGLEC-16 24 months old have the highest transcription level of pro-inflammatory markers versus the SIGLEC-11 and control group at both examined ages. Data shown as mean ± SEM; normalized to BL6 samples; n=6-14; *p ≤ 0.050, **p ≤ 0.010, ***p ≤ 0.001.

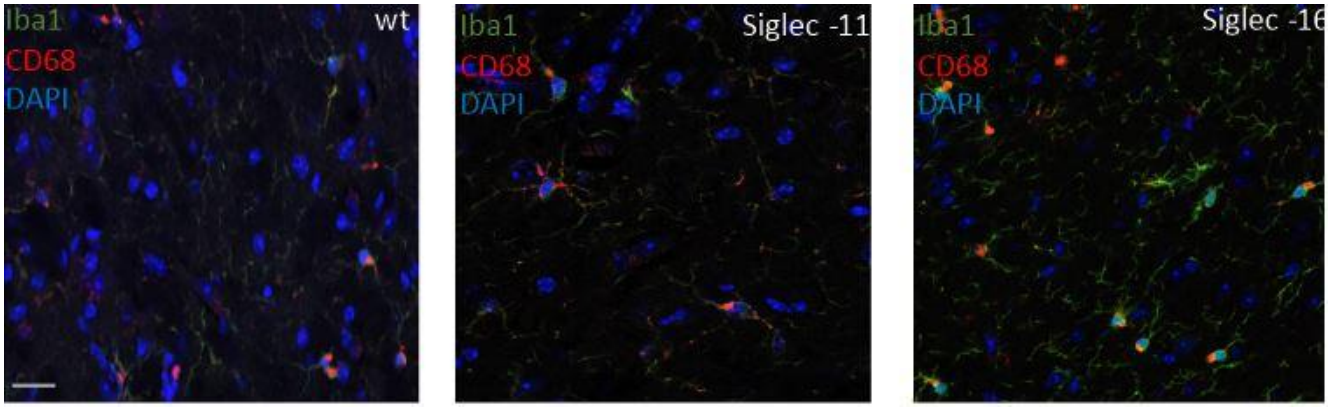
Iba1 is the specific calcium-binding protein of microglia and macrophages (Hovens, Nyakas, and Schoemaker 2014; Jurga, Paleczna, and Kuter 2020). Therefore, it is an appropriate marker for assessing the number of microglia cells in our experimental groups. Using the Iba1/CD68 staining method, the number of Iba1-positive cells was determined in the present study. SIGLEC-11 showed fewer Iba1-positive neurons in the hippocampus (Figure.12 A) as well as in the SNpr (Figure.12 B) when compared with SIGLEC-16 and the control group (genotype-specific: p [Hp] = 0.0062 and p [SNpr] = 0.0023). This decrease was significant at 24 months old (Hp: wt 204.736 ± 12.70%, SIGLEC-11 84.05 ± 5.91% and SIGLEC-16 219.20 ± 22.10%, p [Sig-11 vs. BL6] = 0.0048, p [Sig-11 vs. Sig16] = 0.0029 SN*pr*: wt 277.19 ± 37.07%, SIGLEC-11 157.16 ± 31.52% and SIGLEC-16 411.99 ± 60.78%, p [Sig-11 vs. BL6] = 0.0613, p [Sig-11 vs. Sig-166] = 0.0038). Additionally, the results (see fig. 12 C) of the SIGLEC-16 and BL6 animals showed higher number of Iba1-positive cells at both hippocampus and SNpr areas of the 24 month old animals in comparison to all other

genotypes examined (age-effect: Hp: p [Sig-11-6mo vs. Sig-11-24mo] = 0.805 p [Sig-16-6mo vs. Sig-16-24mo] = 0.029, p [BL6-6mo vs. BL6-24mo] = 0.0439 and SNpr: p [Sig-11-6mo vs. Sig-11-24mo] = 0.0512, p [Sig-16-6mo vs. Sig-16-24mo] = 0.0196, p [BL6-6mo vs. BL6-24mo] = 0.0277).

A



B



C

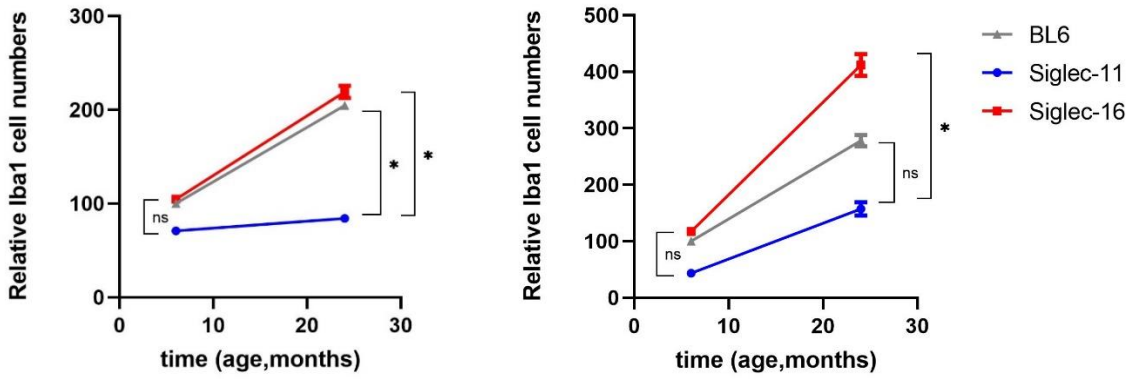
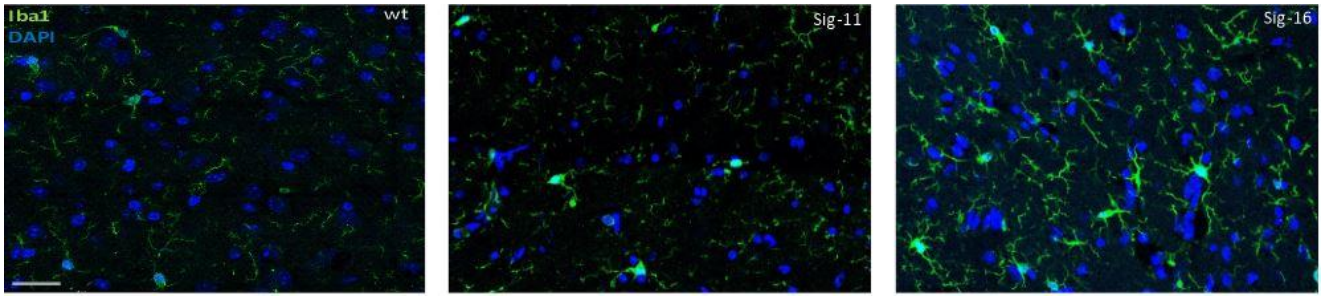


Fig. 12: Expression level of microglial markers. A and **B** Stained samples of Iba1/CD68 staining, Blue: 4´ ,6-diamidino-2-phenylindole (DAPI), Green: ionized calcium binding adaptor molecule 1 (Iba1) and Red: Cluster of differentiation 68 (CD68). SIGLEC-16 demonstrated higher number of Iba1+cells in both of the hippocampus (Hp) (A) and Substantia nigra pars reticulata (SN*pr*) (B) regions at the 24 months old compared to the other groups. Bregma level: -2,88 _ -2.9 mm. Scale bar: 20 µm. **C** Iba1+ cell numbers. Number of the Iba1-positive cells per area in the Hp (left) and SN*pr* (right) were increased in SIGLEC-16 at the 24 months old versus the control and SIGLEC-11 groups. Data shown as mean ± SEM; normalized to BL6 samples; n=6-12; n.s. not significant, *p ≤ 0.050.

Our experimental mice groups were also screened for other aging markers such as Iba1-positive cell clusters. SIGLEC-11-24 months old mice showed the lower amount of these clusters compared to SIGLEC-16 in both hippocampal (p = 0.0016; see fig.13 **A**) and SN*pr* regions (p = 0.0038; see fig.13 **B**). In other words, SIGLEC-16 at 24 months old compared to the SIGLEC-11 demonstrated increased Iba1-positive cell clusters at both hippocampus (p = 0.0026) and SN*pr* (p = 0.0013), although these differences were not statistically significant compared to the BL6 (Hp: p = 0.0588 and SNpr: p = 0.0701). Moreover, there is a noticeable increase in the number of these clusters specifically at 24 months old mice (Hp: wt 224.688 ± 42.56%, SIGLEC-11 96.73 ±17.97% and SIGLEC-16 295.91 ± 30.96%, p = 0.0376, SNpr: wt 256.84 ± 26.95%, SIGLEC-11 108.41 ± 14.60 and SIGLEC-16 302.27 ± 22.26%, p = 0.0152).

A



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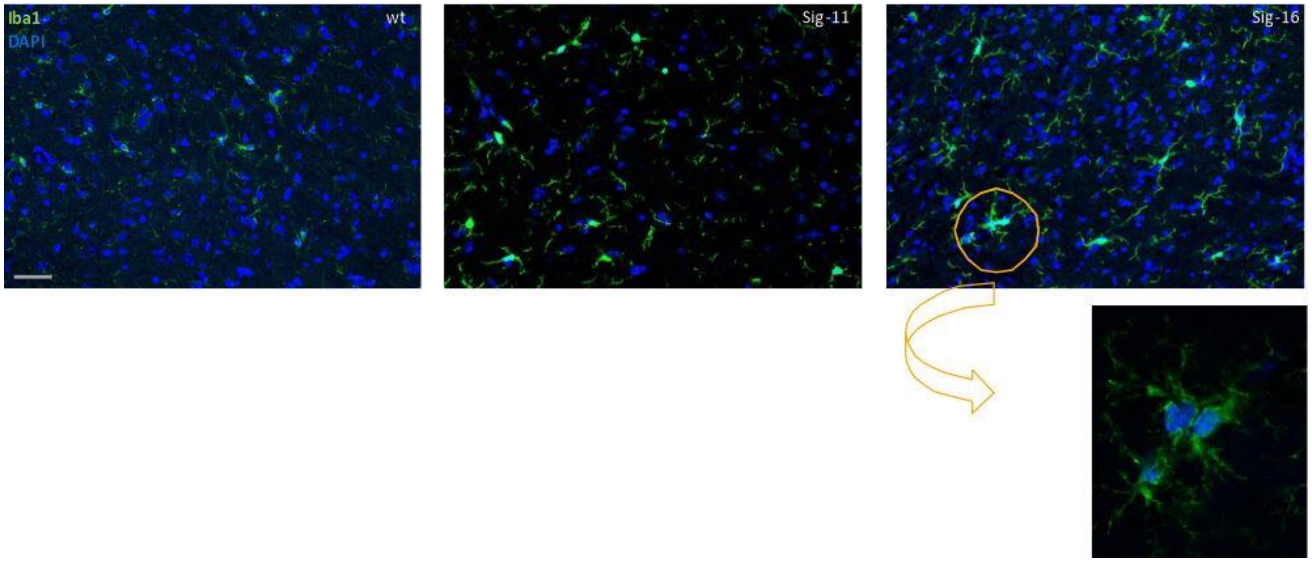

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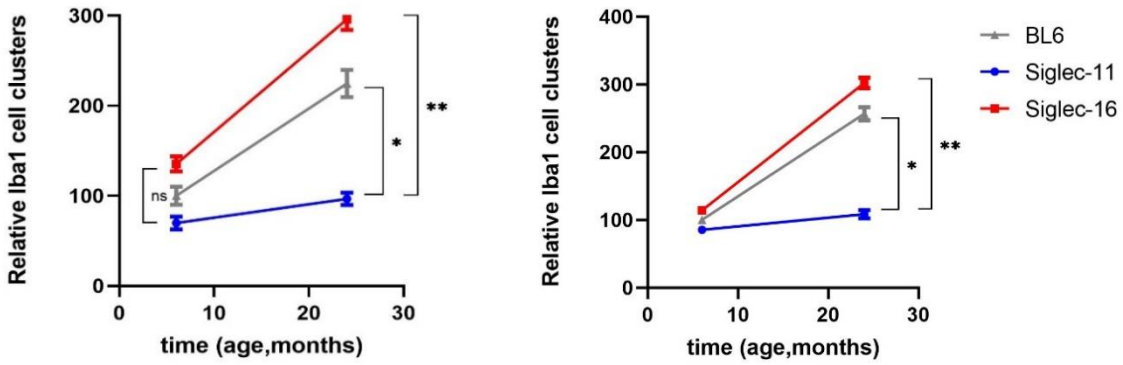
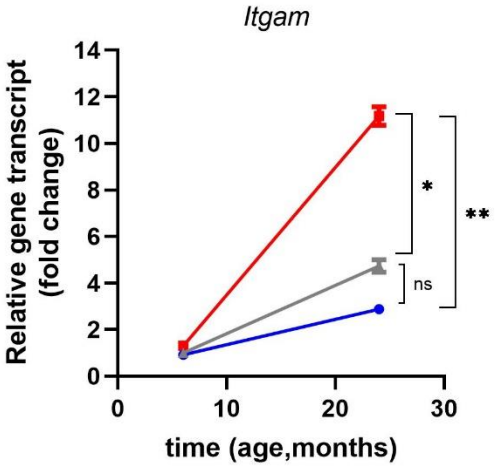


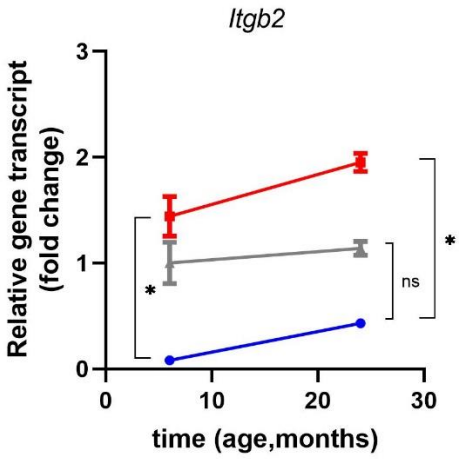
Fig. 13: Immunostaining of Iba1+ cell clusters, Blue: 4´ ,6-diamidino-2-phenylindole (DAPI), Green: ionized calcium binding adaptor molecule 1 (Iba1). **A** Iba1+ cell clusters in Hp region stained with Iba1 antibody in SIGLEC-11, SIGLEC-16 and BL6. Hippocampus (Hp), Scale bar: 10 µm. Bregma level: -2.78 _ -2.88 mm. **B** The Iba1+ cell clusters in Substantia nigra pars reticulata (SNpr) region shown in all three experimented groups. In SIGLEC-16 one of the clusters presented in larger size for better visibility of clusters. SNpr, Scale bar: 10 µm, higher magnification: 50 µm. Bregma level: -2.78 _ -2.88 mm. **C** The number of Iba1+ cell clusters per area in Hp (left) and SNpr (right) increased at the 24 months old age of SIGLEC-16 versus BL6. However, SIGLEC-11 showed lower number of these clusters comparing to the control group in both examined areas. Data shown as mean ± SEM; normalized to BL6 samples; n=6-9; n.s. not significant, *p ≤ 0.050, **p ≤ 0.010.

Based on protein level results, these results were in consistent with the data obtained from the transcription level of microglia markers, *Aif1* and *Tmem119*. These data showed increase in gene transcripts of *Aif1* and *Tmem119* in SIGLEC-16 while the transcription level of them were decreased in SIGLEC-11. Increased level of expression and transcription of microglial markers would lead to the higher microglia activation in SIGLEC-16, while reduced levels of RNA and protein of mentioned markers hint towards a lower activation of these immune cells in SIGLEC-11s' CNS transgenic mice.

3.3 Activation of complement cascade

As we grow older, the neurons within our brain may suffer from oxidative damages. It is essential for the immune system to purge tissues from the dead cells and their secreted factors, as well as bacteria. Importantly, the ability of the immune system to deplete tissue from these apoptotic cells will diminish due to aging (Tezel et al. 2010). However, a highly activated complement cascade could also be detrimental for the healthy cells in the brain. As resident immune cells in the CNS, microglia can activate the complement system. Thus, we analyzed the complement markers in order to investigate the relation between microglial Siglec receptors and immune system activity. By using qRT-PCR, we assessed the transcription level of *C1qa*, *C1qb*, *C1qc*, *C3*, *C4, Itgam* and *Itgb2*. All these markers are related to the activation of the complement system, either through the expression of the initial marker in the complement cascade or through the expression of the complement receptors. At both ages of six and 24 months old, SIGLEC-11 showed reduced transcriptional level of all these complement factors, indicating that this receptor plays an inhibitory role in complement activation. However, transcription level of these markers in our SIGLEC-16 at 24 months of age were higher than the BL6 mice at the same age (*C1qa*: $p = 0.003$, *C1qb*: $p = 0.0077$, *C1qc*: $p = 0.0036$, *C3*: $p = 0.0041$, *C4*: $p = 0.02$, *Itgam*: $p = 0.0061$ and *Itgb2*: $p = 0.110$; see fig.14). It would be worthwhile to mention that some of the markers showed significant differences between the SIGLEC-11 and SIGLEC-16 at the age of 6 months old (*Itgb2*: $p = 0.0365$, *C1qc*: $p = 0.0290$, *C3*: $p = 0.0482$ and *C4*: $p = 0.0119$). These data presented that compared with SIGLEC-16 mice, SIGLEC-11 mice possess less complement activation at either 6- or 24-months of age, resulting in lower immune damage to the brain. Because fewer immune damages are occurred in SIGLEC-11 transgenic mice, the brain cells are more protected from unnecessary immune responses compared to the SIGLEC-16 animals.


Itgam
Relative gene transcript (fold change)
time (age,months)
ns
*
**


Itgb2
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time (age,months)
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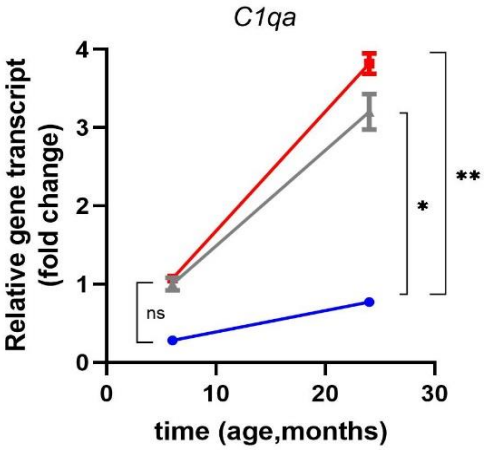
C1qa
Relative gene transcript (fold change)
time (age,months)
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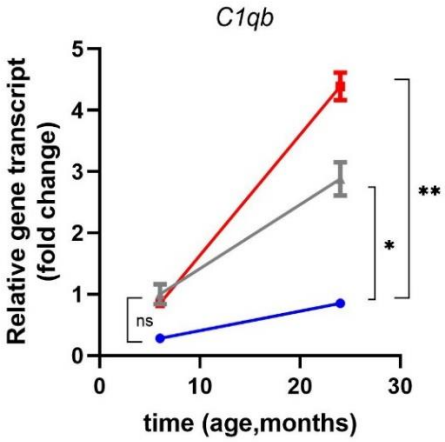
C1qb
Relative gene transcript (fold change)
time (age,months)
ns
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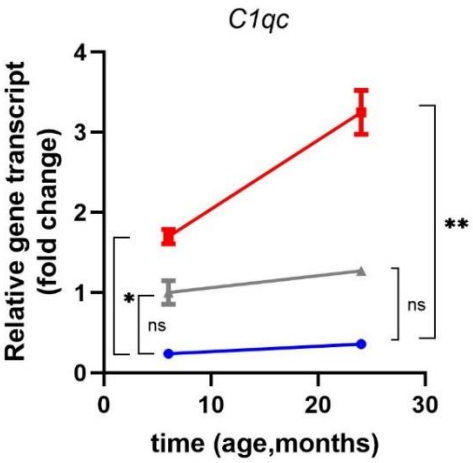
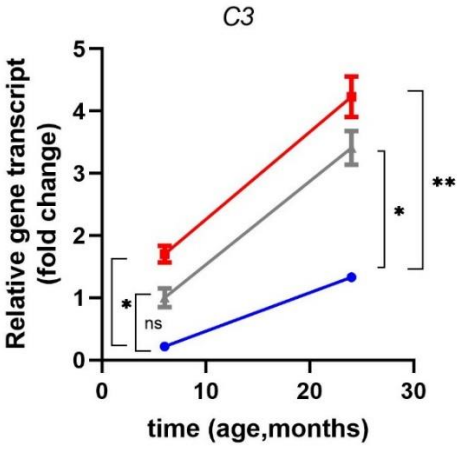

C1qc
Relative gene transcript (fold change)
time (age,months)
*
ns
ns
**


C3
Relative gene transcript (fold change)
time (age,months)
*
ns
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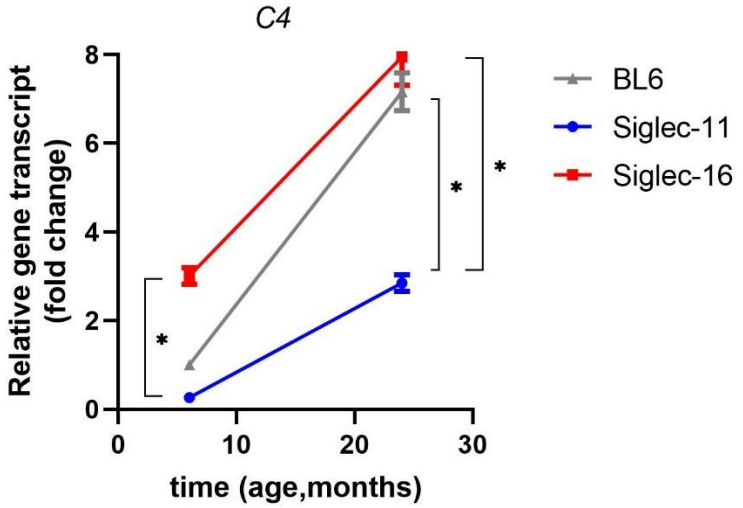
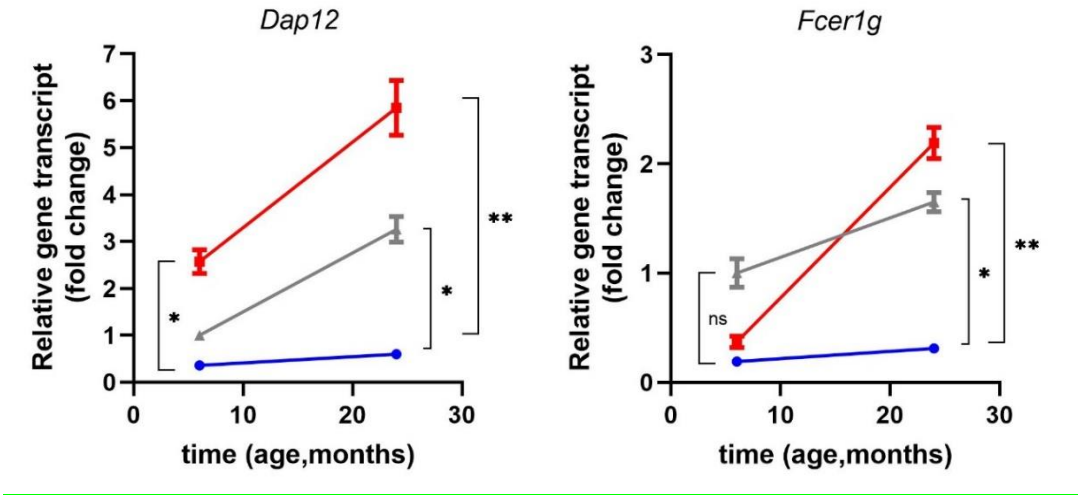


Fig. 14: qRT-PCR of complement components. Transcription level of the complement components [Complement component 1 with q subcomponent, alpha/beta polypeptide (C1qa/b), Complement component 1 with q subcomponent, c chain (C1qc), Complement component 3 (C3), Complement component 4 (C4), Integrin alpha M (Itgam) and Integrin Subunit Beta 2 (Itgb2)] were analyzed by qRT-PCR in our experimented mice groups. SIGLEC-11 showed lower transcription level of these markers at both 6 and 24 months old comparing to SIGLEC-16 and BL6. Data shown as mean ± SEM; normalized to BL6 samples; n=6-14; n.s. not significant, *p ≤ 0.050, **p ≤ 0.010.

3.4 Expression of phagocytosis-associated lysosomal/endosomal Cd68

Phagocytosis is an immune response in which its cells could recognize large particles such as bacteria. In addition, these phagocytic cells are capable of identifying and digesting the cellular debris and apoptotic cells (Castelli et al. 2019). Due to the fact that aging is associated with neuronal loss, phagocytosis is vital in engulfing and removing these damaged and apoptotic cells via its cells (Loera-Valencia et al. 2019; Schafer and Stevens 2013). We investigated the transcription level of *Dap12*, *Fcer1g* and *CD68*. The transcription level of these phagocytic markers were significantly decreased in SIGLEC-11 at the 24 months of age compared to both BL6 and SIGLEC-16 transgenic mice groups (*Dap12*: p = 0.0058, *Fcer1g*: p = 0.0018 and *Cd68*: p = 0.00209; see fig.15).



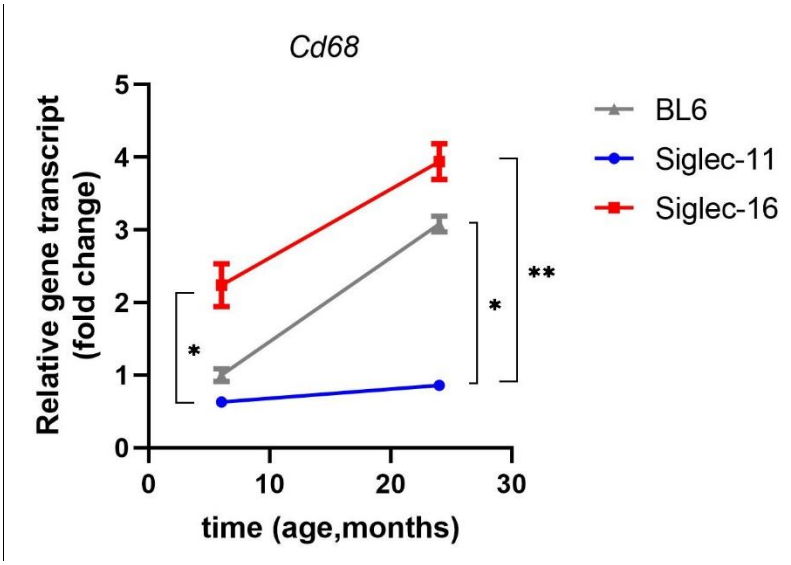


Fig. 15: qRT-PCR of phagocytotic markers. SIGLEC-16 at the age of 24 months old represented higher transcription level of DNAX-activating protein of 12 kDa (Dap12), Fc Epsilon Receptor Ig (Fcer1g) and Cluster of differentiation 68 (Cd68) versus the other groups. Data shown as mean ± SEM; normalized to BL6 samples; n=6-10; n.s. not significant, *p ≤ 0.050, **p ≤ 0.010.

Moreover, the double immunostaining was performed to investigate the expression level of CD68 in our experimented mice groups. In figure 12 A, the intensity of CD68 is shown per soma area in the hippocampus, while figure 12 B illustrates the CD68 intensity per soma area in the SN*pr*. The double staining of Iba1/CD68 revealed the reduced expression levels of Cd68 intensity /soma area in hippocampal (see fig.12 **A**) and SN*pr* (see fig.12 **B**) regions in SIGLEC-11 when compared to both control and SIGLEC-16 groups at 24 months old ($p = 0.0400$, $p = 0.0295$ and SN*pr*: $p = 0.162$, $p = 0.0104$, accordingly). It is visible from the staining in figure 12 **A** and **B** that CD68 is highly expressed in SIGLEC-16 samples compared to the SIGLEC-11 and BL6 samples (genotype-specific: p [Hp] = 0.0064 and p [SN*pr*] = 0.02100). Figure 16 illustrates the CD68 intensity level per soma area in both hippocampus (Hp) and substantia nigra pars reticulate (SN*pr*).

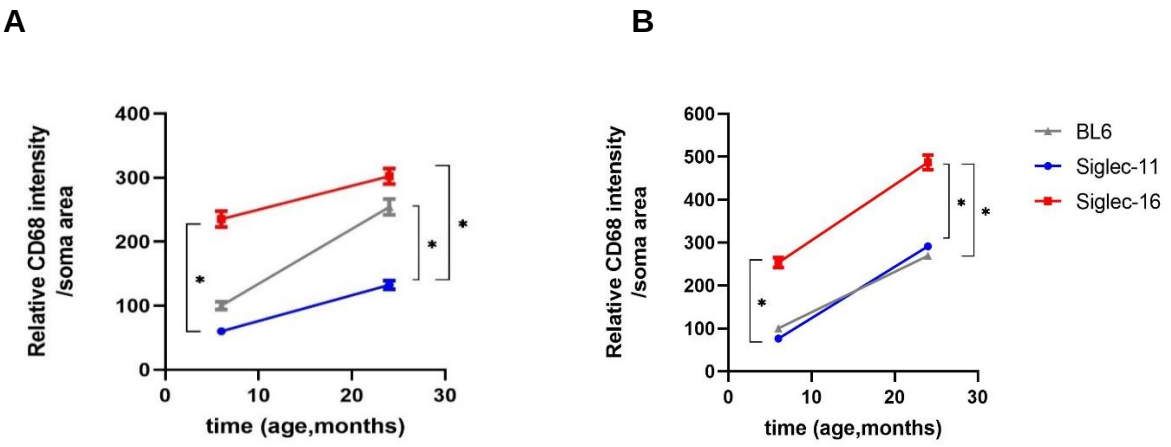
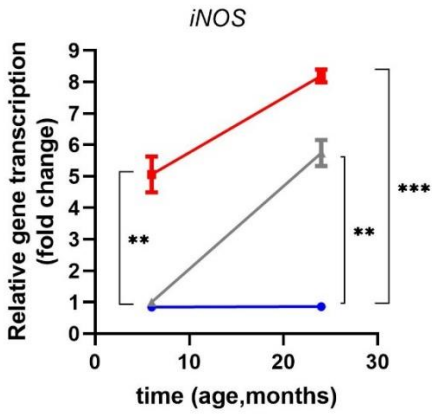


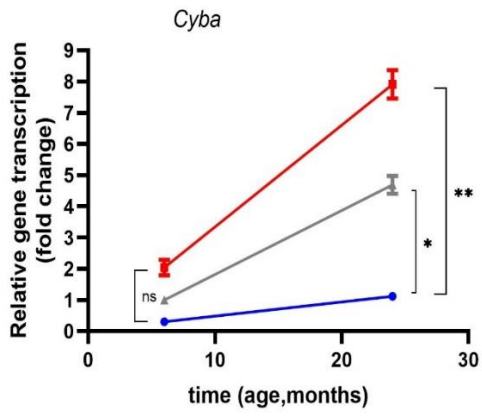
Fig. 16: Expression level of phagocytotic markers. Cluster of differentiation 68 (CD68) intensity per soma area. Analysis of double staining of Iba1/CD68 of our experimented transgenic mice demonstrated increased intensity of CD68 in the SIGLEC-16 at the age of 24 months old compared to the SIGLEC-11, control group at 24 months old and their 6 months old samples at both hippocampus (Hp) (**A**) and substantia nigra pars reticulate (SNpr) (**B**). Data shown as mean ± SEM; normalized to BL6 samples; n=6-11; *p ≤ 0.050.

Overall, transcription and translation levels of examined phagocytotic markers were decreased in SIGLEC-11, while they were at higher levels in SIGLEC-16 transgenic mice. Due to these outcomes, it is possible that SIGLEC-16 mice might display a higher rate of phagocytosis compared to the control and SIGLEC-11 groups.

3.5 Investigation of oxidative stress markers

As part of the oxidation process, reactive oxygen species (ROS) attack lipids, DNA, and proteins. This mechanism contains of free radicals stealing electrons from lipids and proteins which could lead to oxidative damages during aging (Niki 2009). To investigate the transcription level of oxidative stress markers; *iNOS*, *Cyba* and *Cybb* were quantified by qRT-PCR (see fig.17). In contrast to the SIGLEC-16 mice groups, the SIGLEC-11 mice at 6 months of age displayed significantly lower levels of transcription for all examined markers (*iNOS*: p = 0.00139, *Cyba*: p = 0.065, and *Cybb*: p = 0.0272). Similarly, transcription of the mentioned oxidative stress markers in SIGLEC-11 mice were decreased compared to SIGLEC-16 and the control group at the age of 24 months old (*iNOS*: p = 0.00105, *Cyba*: p = 0.0071 and *Cybb*: p = 0.0090). In other words, in SIGLEC-16 transgenic mice, transcription levels of these markers were tendentially increased in both adult and aged groups (genotype-specific: p = 0.0066).





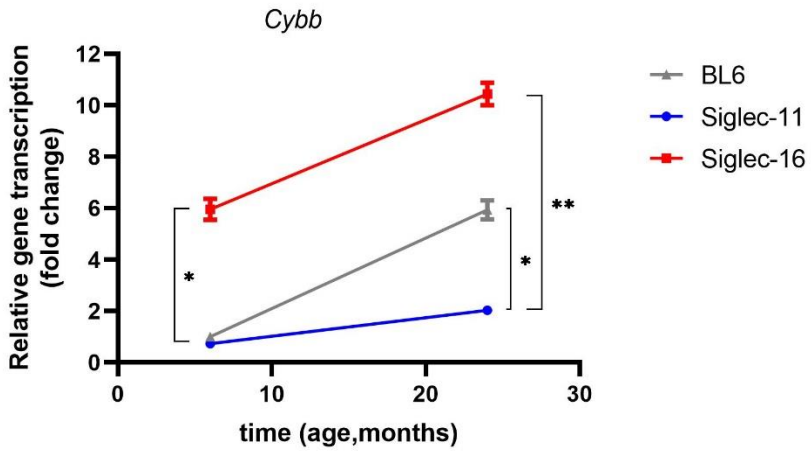
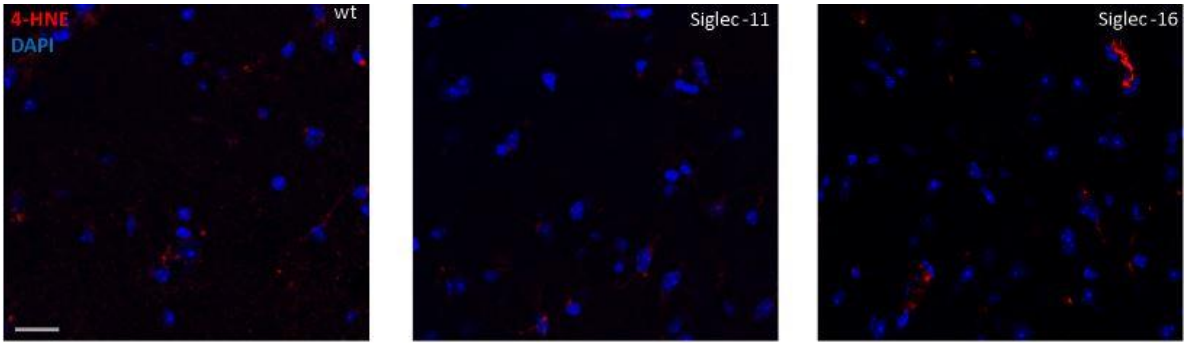


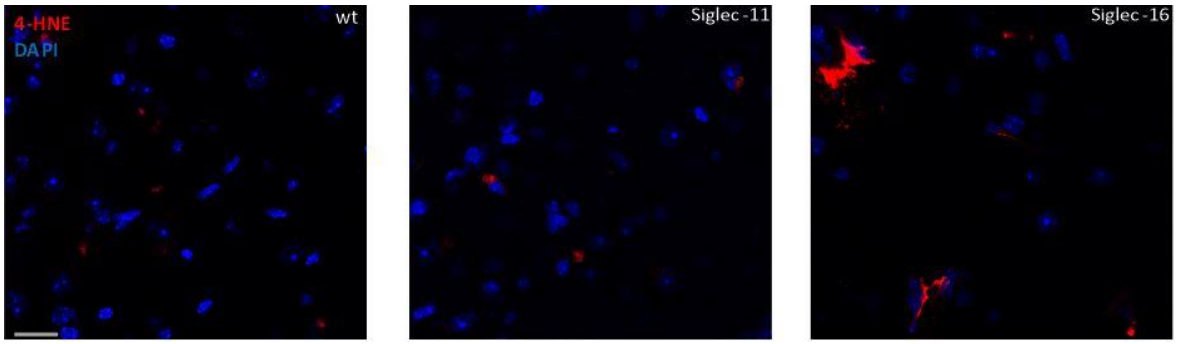
Fig. 17: qRT-PCR of oxidative stress markers. Inducible nitric oxide synthase (iNOS), Cytochrome B-245 Alpha Chain (Cyba) and Cytochrome B-245 Beta Chain (Cybb) were transcribed higher in SIGLEC-16 at 24 months old comparing to the control group, while SIGLEC-11 had the lower transcription level of the above-mentioned markers. Data shown as mean ± SEM; normalized to BL6 samples; n=6-13; n.s. not significant, *p ≤ 0.050, **p ≤ 0.010, ***p ≤ 0.001.

Besides the RNA levels, it would be essential to measure the expression level of oxidative stress markers in our transgenic mice groups. 4-hydroxynonenal (4-HNE) and malondildehyde (MDA) are produced together throughout the lipid peroxidation. Since lipid peroxidation is an age-related phenomenon, reduction of 4-HNE could be one way to verify the oxidative damages. (Zhang and Forman 2017). Hence, 4-HNE staining was carried out to investigate the role of our Siglec receptors in oxidative damage responses (see fig.18 **A**: Hp and **B**: SN*pr*). As shown in Fig.18 C, SIGLEC-16-24 months old samples showed higher intensity of 4-HNE than the SIGLEC-11 transgenic animals in both hippocampus and SN*pr* areas (Hp: p = 0.0186 and SN*pr*: p = 0.0369). However, in our younger mice groups, the 6 months of age, intensity of 4-HNE was not significantly different in SIGLEC-16 transgenic mice compared to BL6 and SIGLEC-11 at both experimented regions (see fig.18 **C**). Therefore, this would confirm the hypothesis that the oxidative stress process is occurred due to aging and during its later periods.

A


4-HNE
DAPI
wt
Siglec-11
Siglec-16

B


4-HNE
DAPI
wt
Siglec-11
Siglec-16

C

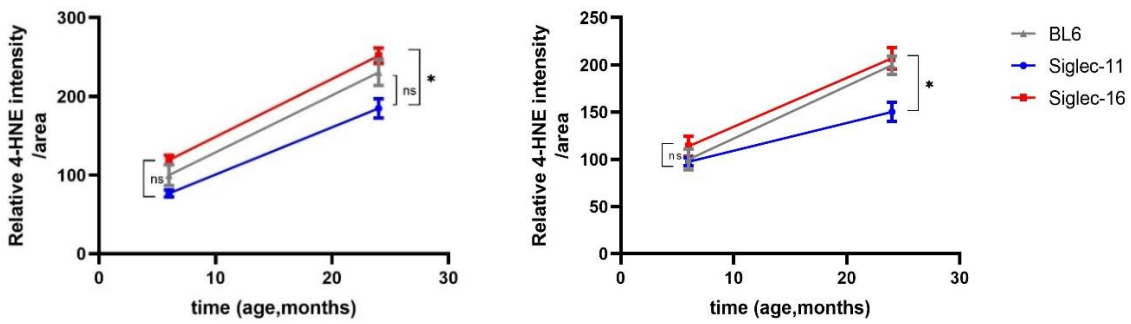
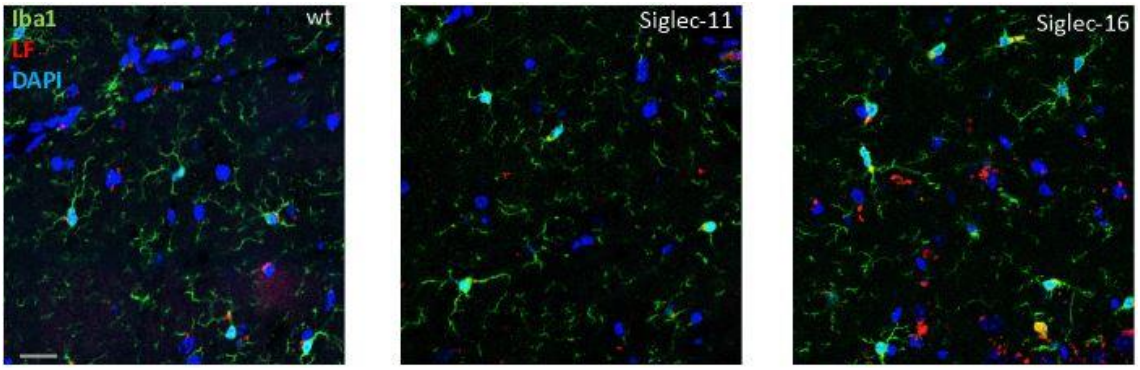

Relative 4-HNE intensity /area
time (age,months)
ns
*
BL6
Siglec-11
Siglec-16

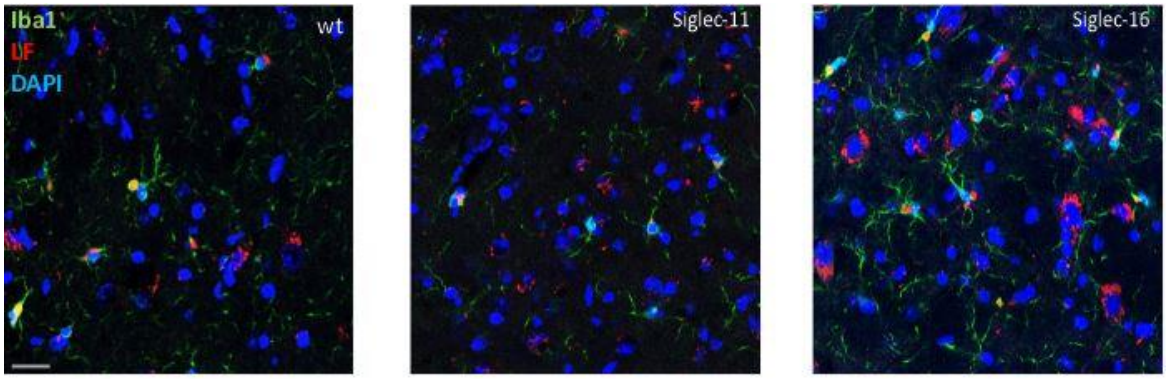
Fig. 18: Expression level of oxidative stress markers. 4-hydroxynonenal (4-HNE) staining of experimented mice groups, Blue: DAPI, Red: 4-HNE. **A** Immunostaining of 4-HNE in SIGLEC-11, SIGLEC-16 and BL6 in hippocampus (Hp) showed higher levels of 4-HNE intensity in SIGLEC-16 at 24 months old. Scale bar: 20 µm. Bregma level: -2.94 _ -2.98 mm. **B** Staining of 4-HNE in substantia nigra pars reticulata (SNpr) area described lower 4-HNE intensity in SIGLEC-11 compared to the other groups at 24 months old. Scale bar: 20 µm. Bregma level: -2.94 _ -2.98 mm. **C** SIGLEC-16 at the 24 months old showed higher 4-HNE intensity versus the other groups at 24 months old at both Hp (left) and SNpr (right). Our results clarified that the 6 months old mice were not showing significant differences among the transgenic animal groups. Data shown as mean ± SEM; normalized to BL6 samples; n=6-13; n.s. not significant, *p ≤ 0.050.

Accumulation of lipofuscin (LF) is one of the major brain's age-related markers. LF, enriched in brain, skin, and muscle cells, is composed of lipids, proteins and metals (Singh Kushwaha, Patro, and Kumar Patro 2018). Furthermore, studies described the auto-fluorescent feature of this age-related factor. Recent investigations demonstrated that LF accumulated predominantly within microglia (O'Neil et al. 2018; Singh Kushwaha, Patro, and Kumar Patro 2018). Accordingly, we characterized the auto-fluorescence of LF and its accumulation in the microglia cells of our study groups both in hippocampal and SN*pr* areas (see fig.19 **A** and **B**). Our results showed that in SNpr of SIGLEC-16 24 months old, LF intensity/soma area is higher compared to SIGLEC-11 and BL6 control (p [Sig-16 vs. Sig-11] = 0.0094 and p [Sig-16 vs. BL6] = 0.0316). Moreover, SIGLEC-16 transgenic mice demonstrated the increased number of LF-positive/Iba1-positive cells compared to the other groups at both hippocampus and SN*pr* regions (genotype-specific: p [Hp] = 0.0331 and p [SN*pr*] = 0.00376) specifically at 24 months old (Hp: wt 150.85 ± 8.15, SIGLEC-11 123.53 ± 9.84 and SIGLEC-16 192.38 ± 3.85, p = 0.0720, SN*pr*: wt 174.80 ± 11.00, SIGLEC-11 128.83 ± 8.37 and SIGLEC-16 197.52 ± 8.09, p = 0.049) (see fig.19 **D**). Figure 19 D shows that at the age of 6 months, SIGLEC-16 mice had more Iba1-positive cells expressing LF than SIGLEC-11 mice (Hp: p = 0.0340 and SN*pr*: p = 0.0255). Overall, these age-related lipid markers are more aggregated in the activated microglia cells (Iba1+ cells) in SIGLEC-16 than in the other groups.

A


Iba1
LF
DAPI
wt
Siglec-11
Siglec-16

B


Iba1
LF
DAPI
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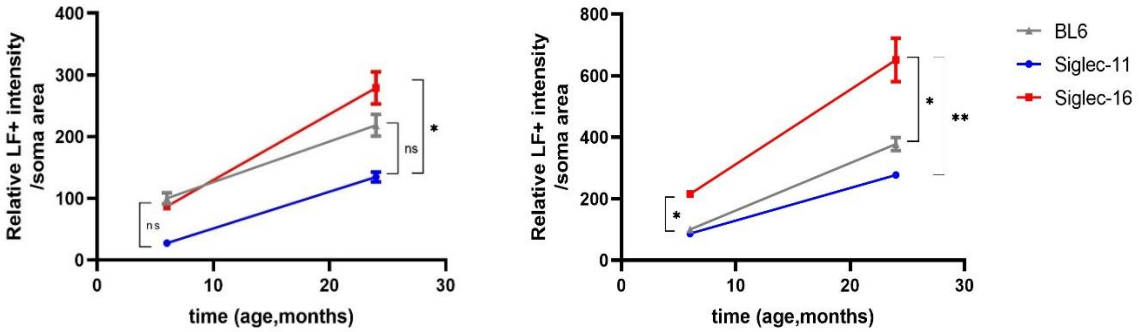
Siglec-11
Siglec-16

C


Relative LF+ intensity /soma area
400
300
200
100
0
0
10
20
30
time (age,months)
ns
ns
*
Relative LF+ intensity /soma area
800
600
400
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0
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time (age,months)
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BL6
Siglec-11
Siglec-16

D

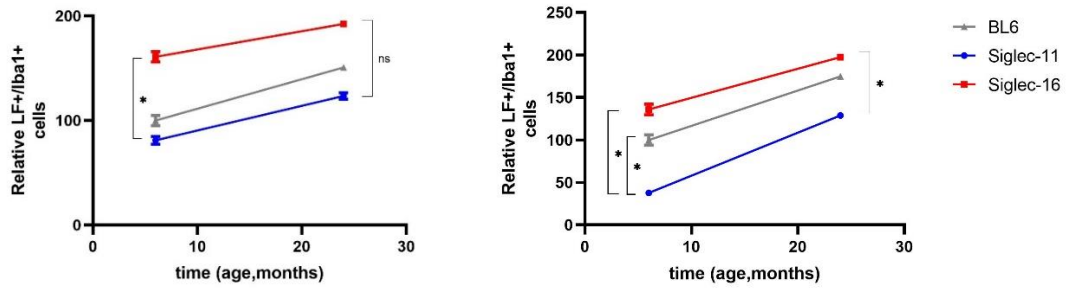


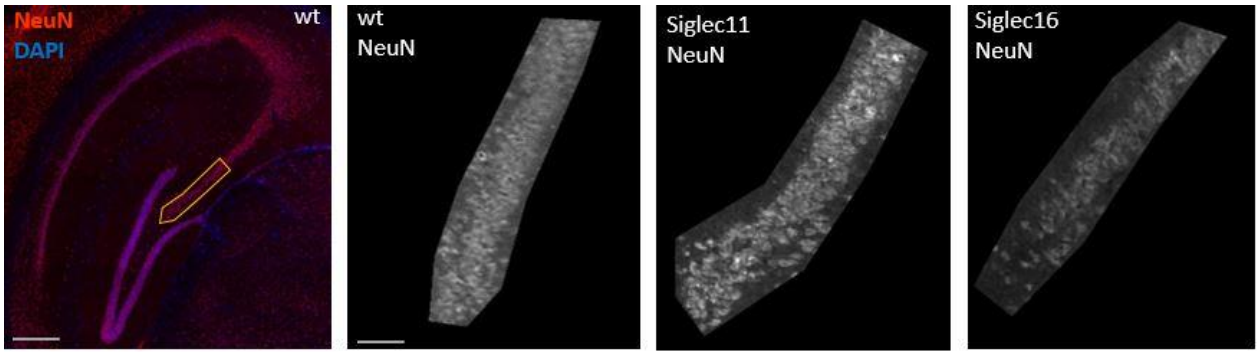
Fig. 19: Lipofuscin staining. Auto-fluorescence of lipofuscin (LF) in Ionized calcium binding adaptor molecule 1 (Iba1)-positive cells, Blue: DAPI, Green: Iba1 and Red: auto-fluorescence of LF. **A** Auto-fluorescence of LF in Iba1+ cells in hippocampus (Hp) area of the experimented mice at 24 months old. Scale bar: 20 µm. Bregma level: -2.7 _ -2.8 mm. **B** Auto-fluorescence of LF in Iba1+ cells in substantia nigra pars reticulate (SNpr) area of the experimented mice at 24 months old. Scale bar: 20 µm. Bregma level: -2.7 _ -2.8 mm. **C** Intensity level of LF in the Iba1+ cells was higher in the SIGLEC-16 24 months old at both Hp (left) and SNpr (right), while SIGLEC-11 demonstrated lower LF intensity in the microglia cells. **D** Increased number of LF+/Iba1+ cells represented in SIGLEC-16 at 24 months old versus SIGLEC-11 and BL6 at both 6 and 24 months old in HP (left) and SNpr (right). Data shown as mean ± SEM; normalized to BL6 samples; n=6-10; n.s. not significant, *p ≤ 0.050, **p ≤ 0.010.

3.6 Age-related neuronal loss

Loss of neurons, typically associated with normal aging and degenerative diseases, was examined within two different areas: the hippocampal and substantia nigra *pars compacta* (SN*pc*). There is evidence that age-related damage to glial cells in the white matter could contribute to damage of the hippocampus (Metzler-Baddeley et al. 2019). Furthermore, investigations observed that age-related damages occurred most rapidly in the hippocampus, while other brain regions showed no damages at early time points (Pandya and Patani 2021; Metzler-Baddeley et al. 2019). Wang and colleagues illustrated that neurons in the substantia nigra are susceptible to the oxidative stress which itself is an aging marker (Wang and Michaelis 2010). A possible explanation for this high sensitivity to oxidative stress markers stands in the metabolism of dopamine in the neurons of the

substantia nigra (Kastner et al. 1992). According to previously mentioned studies, specific regions such as hippocampus and SN areas are more susceptible to neurodegeneration and age-related damages compared to the other brain regions (Noda et al. 2020; Vaillancourt et al. 2012). Therefore, we analyzed the neurons in these areas for any evidence of modification. Neurons were stained with the neuronal nuclei marker (NeuN) in the hippocampus as shown in figure 20 **A** and in the SN*pc* in figure 20 **B**. Dopaminergic neurons were stained with tyrosine hydroxylase (TH) antibody in SN*pc* which are shown in figure 20 **C** in green. In Figure 20 **D** and E, we calculated the relative density of NeuN-positive cells in the hippocampus area as well as NeuN- or TH-positive cells in the SN*pc* region. In SIGLEC-16 mice, the number of NeuN-positive cells were generally decreased comparing to the other groups (genotype-specific: Hp: p = 0.0054 and SN*pc*: p = 0.0072) specifically at the 6 months old (Hp: wt 1.00 ± 0.1, SIGLEC-11 1.05 ± 0.1 and SIGLEC-16 0.65 ± 0.1, p [Sig-16 vs. Sig-11] = 0.0381 and SN*pc*: wt 1.00 ± 0.086, SIGLEC-11 1.40 ± 0.131 and SIGLEC-16 0.89 ± 0.115, p [Sig-16 vs. Sig-11] = 0.0348; Fig 20 **D**). Moreover, checking the density of TH-positive cells in SN*pc* area demonstrated a greater number of these cells in SIGLEC-11 at the 24 months old group (see fig.20 **E**) compared to SIGLEC-16 (p = 0.0068), while there was a tendency of more dopaminergic neurons in SN*pc* of SIGLEC-11- 6 months old mice compared to SIGLEC-16 and BL6 at 6 months old (p [Sig-11 vs. Sig-16] = 0.053 and p [Sig-11 vs. BL6] = 0.0800). In general, SIGLEC-16 transgenic mice have greater loss of neurons as compared to SIGLEC-11 and BL6 mice.

A



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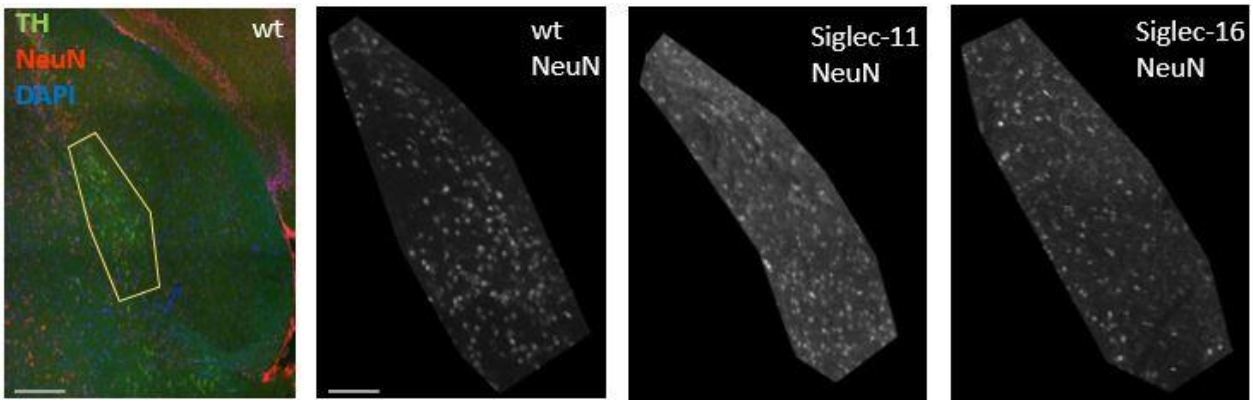
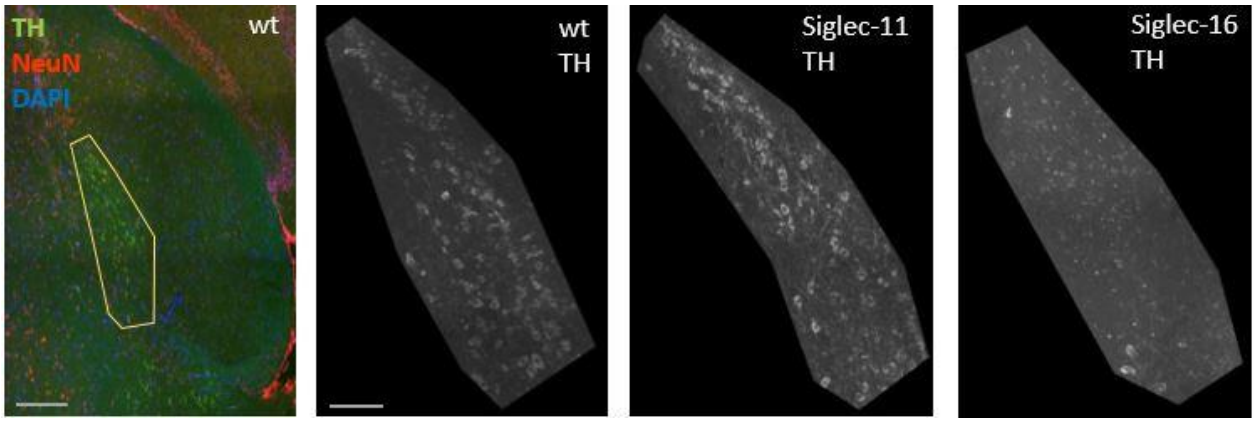

TH
NeuN
DAPI
wt
wt
NeuN
Siglec-11
NeuN
Siglec-16
NeuN

C


TH
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TH
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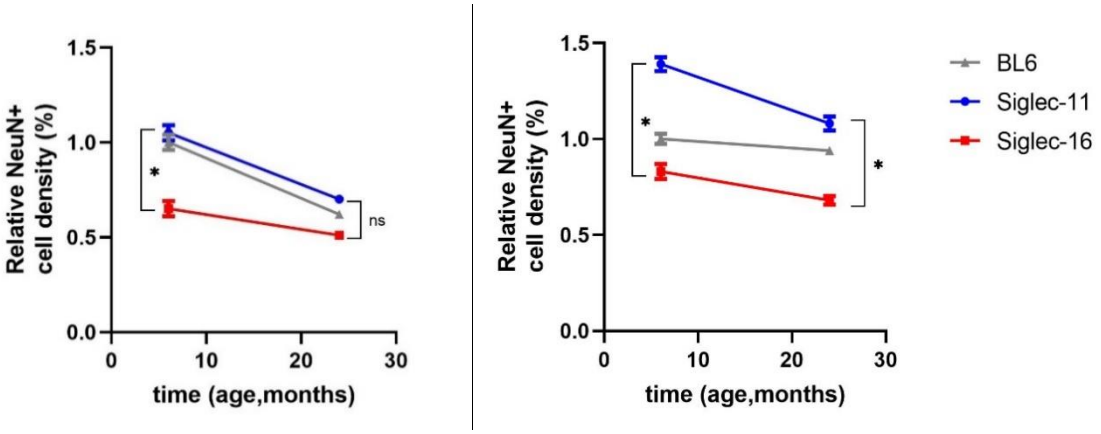
TH
Siglec-16
TH

D


Relative NeuN+
cell density (%)
1.5
1.0
0.5
0.0
0
10
20
30
time (age,months)
*
ns
Relative NeuN+
cell density (%)
time (age,months)
*
*
BL6
Siglec-11
Siglec-16

E

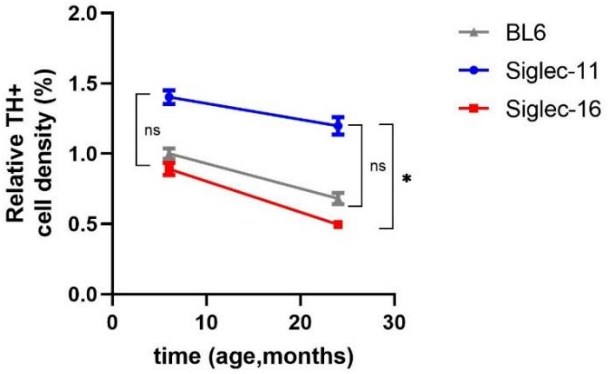
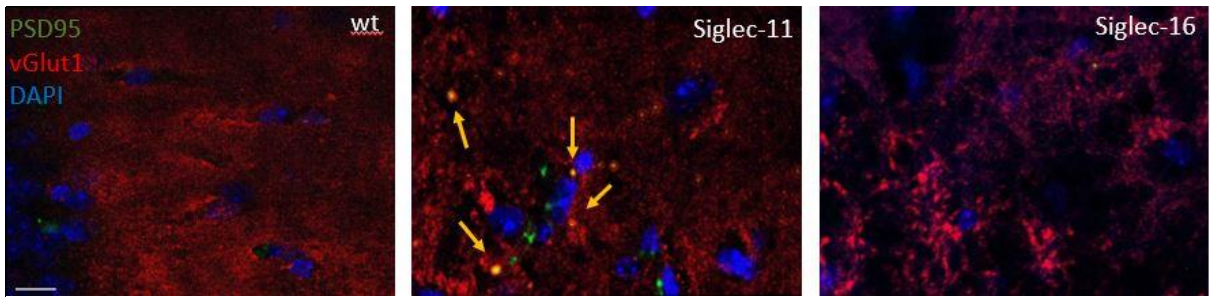


Fig. 20: Neuronal loss, Blue: 4',6-diamidino-2-phenylindole (DAPI), Green: Tyrosine hydroxylase (TH) and Red: Neuronal nuclei (NeuN). **A** Immunostaining of NeuN+ cells in hippocampus (Hp) showed decreased number of NeuN+ cells in SIGLEC-16 at 24 months old compared to the other two groups. Scale bar: 100 µm and higher magnification 200 µm, Bregma level: -2.3_ -2.46. **B** Immunostaining of NeuN+ cells in substantia nigra pars compacta (SN*pc*) demonstrated lower number of NeuN+ cells in SIGLEC-16 at 24 months old compared to the other two groups. Scale bar: 100 µm and higher magnification 200 µm, Bregma level: -2.92. **C** Immunostaining of TH+ cells in SN*pc* represented higher number of these type of neurons in SIGLEC-11 at 24 months old versus BL6 and SIGLEC-16 at the same age. Scale bar: 100 µm and higher magnification 200 µm, Bregma level: -2.92. **D**-left Number of NeuN+ cells (red) were at lower levels in the hippocampus of SIGLEC-16 mice at both 6 and 24 months old compared to the other groups. **D**-right In Sn*pc* the number of NeuN+ cells was higher in SIGLEC-11 at 6 compared to the SIGLEC-16 and BL6 and stayed at higher levels during the aging process (at 24 months old). **E** Number of the TH+ cells in SIGLEC-11 was higher than in the other examined groups at the age of 6 months old. Dopaminergic neurons were still higher at the 24 months old of SIGLEC-11 transgenic mice versus the other two groups in which the number of these cells was reduced over the procedure of aging. Data shown as mean ± SEM; normalized to BL6 samples; n=6-12; n.s. not significant, *p ≤ 0.050.

3.7 Age-related synapses loss

It is possible for the nervous system to be impaired when synapses are deleted or dysfunctional. Since an impaired nervous system is associated with aging, it is critical to understand the amount of these synapses (Morrison and Baxter 2012). Therefore, we checked the synapse loss in cornu ammonis 3 (CA3) region in SIGLEC-11, SIGLEC-16 and BL6 mice at 6- and 24-months old of ages by the antibodies against the glutamatergic pre- and post-synapses and after that the co-puncta density was quantified (see fig.21 **A**). Although SIGLEC-16 mice at 24 months of age have a greater loss of synapse compared to SIGLEC-11 24 months old group ($p = 0.0411$; see figure 21.B), C57BL/6 mice and SIGLEC-11 mice of the same age showed no significant differences in loss of synapse. In figure 21 **B** it is visible that the number of synapses decreased in BL6 at 6 months old versus the BL6 mice at 24 months old ($p = 0.0173$). Likewise, SIGLEC-16 animals at 24 months old indicated a significant synapse loss compared to their 6 months old age ($p = 0.0032$). Taken together, synapse loss was significantly higher in SIGLEC-16 compared to SIGLEC-11 or control group.

A



B

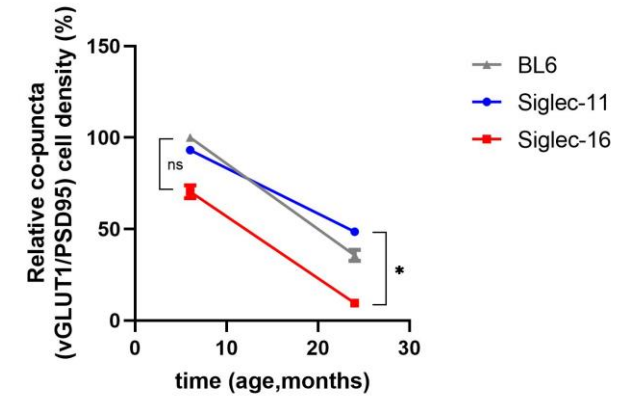


Fig. 21: Synapse loss. Immunostaining of pre- and post-synapses, Blue: 4',6-diamidino-2-phenylindole (DAPI), Red: Vesicular glutamate transporter 1 (vGLUT1) and Green: Post-synaptic density protein-95 (PSD-95). **A** Double immune staining of vGLUT1 as the pre-synaptic marker and PSD-95 (green) as the post-synaptic marker in the CA3 region of hippocampus (Hp) in all three experimented groups (SIGLEC-11, SIGLEC-16 transgenic mice and BL6 as wt group). The yellow dots are showing the location of co-puncta synapses. Scale bar: 10 µm, Bregma level: -2.3_ -2.5. **B** Increased synapse loss were described in SIGLEC-16 at 24 months old comparing to SIGLEC-11 and BL6 mice at 24 months old. However, at 6 months old, SIGLEC-11 showed similar levels of the number of co-puncta synapses versus BL6. Comparing SIGLEC-16 to both SIGLEC-11 and BL6 mice groups displayed that at the age of 6 months old there are fewer number of co-puncta synapses in SIGLEC-16 transgenic mice. Data shown as mean ± SEM; normalized to BL6 samples; n=6-11; n.s. not significant, *p ≤ 0.050.

4. Discussion

The increase in aging population has been documented by numerous studies, which may be attributable to improvements in medical care around the world. The aging process is, however, a complex and unknown process involving impaired or incomplete cellular and physiological functions, which leads to irreparable deficiencies (Bishop, Lu, and Yankner 2010). Microglia dysfunction is also considered to be one of the major causes of aging leading to neuronal and synaptic loss, and age-related damages including oxidative stress, impaired phagocytosis as well as dysregulation of complement components. Hence, it is necessary to study the precise function of microglia and their cell surface receptors as the driving force of above-mentioned events.

4.1 Microglia cells and their Siglec receptors

A population of resident immune cells in the CNS known as microglia expresses a set of recognition receptors that are responsible for the proper response to the various processes in the brain (Linnartz-Gerlach, Kopatz, and Neumann 2014). As mentioned in section 1.2, the ITAM-signaling receptors play the key role in activating microglia via phosphorylation of their ITAM motifs in the cytoplasmic tail. Thereby these motifs could cause the MAPK pathway to be activated and eventually it may lead to the phagocytosis of the pathogens, cellular debris and apoptotic materials. On the other hand, the ITIM receptors, express on the cell surface of microglia will inhibit the excess activation of these immune cells by inhibiting the downstream cascade which were triggered by ITAM signaling, via dephosphorylating the multiple downstream members (Billadeau and Leibson 2002). During a healthy brain condition, there is a great balance between the ITIM and ITAM signaling pathways, which is disrupted due to aging (Siddiqui et al. 2019). Therefore, it is critical to discover the exact mechanism of these ITIM/ITAM receptor signaling throughout the lifespan. Due to the fact that both SIGLEC-11 and SIGLEC-16 are identified as examples of ITIM and ITAM receptors, the effect of these two receptors is investigated on a number of age-related markers.

4.1.1 SIGLEC-11 and SIGLEC-16

In the cytoplasmic tail of SIGLEC-11 expressed on microglial cell lines, there is an ITIM motif. This ITIM domain may cause the inhibitory effects of SIGLEC-11. Regardingly, several studies demonstrated this inhibitory effect of SIGLEC-11 in neurodegenerative diseases (Wang and Neumann 2010). As an example we could point to O'Neil and colleagues' study which described the interaction of SIGLEC-11 with neuronal gangliosides and this will result in the uptake of Aß-plaques by microglia cells (Chiricozzi et al. 2020; O'Neill, van den Berg, and Mullen 2013; Rubovitch et al. 2017; Sipione et al. 2020). This would elucidate the neuroprotective effects of the SIGLEC-11 receptor.

On the other hand, there is SIGLEC-16 as an ITAM signaling receptor expressed on the cell surface of microglia. It is believed that a high percentage of amino acids in SIGLEC-16's protein are identical to those in SIGLEC-11's protein (Cao et al. 2008). Due to their high sequence similarity, these two receptors are referred to as paired receptors (Cao et al. 2008). It may describe the opposite function of Siglec16 and SIGLEC-11 signaling receptors. Thus, as opposed to SIGLEC-11, which exhibits the neuroprotective effects, SIGLEC-16, as an ITAM signaling receptor, possesses neurodegenerative attributes. Moreover, there is an evolutionary theory of SIGLEC-16 and SIGLEC-11 which illustrated the gene duplication event as the origin of these genes (Angata et al. 2002; Cao et al. 2008). It was reported that in humans, SIGLEC-16 has two polymorphic alleles, one functional and one non-functional (Wang et al. 2012). Due to the possible neuroprotective effects of the non-functional SIGLEC-16 allele (SIGLEC-16P), it is more commonly found in the human population (Hayakawa et al. 2017).

In spite of the neuroprotective effects of SIGLEC-11 (Hayakawa et al. 2017; Wang and Neumann 2010) and neurodegenerative consequences of SIGLEC-16 (Lin, Yeh, and Yang 2021; Linnartz-Gerlach, Kopatz, and Neumann 2014; Siddiqui et al. 2019; Wang and Neumann 2010), the exact function of SIGLEC-11 and SIGLEC-16 in the normal process of

aging is still unknown. In this study, I examined the inhibitory and activatory role of SIGLEC-11 and SIGLEC-16 in the normal process of aging.

Accordingly, our experiments were conducted on transgenic SIGLEC-11 and SIGLEC-16 mouse models and C57BL/6 as the control group at two different time points of 6- (referred as adult group) and 24- (referred as aged group) months old. In the first step it was important to identify the transcription and expression level of these two receptors on our mouse microglia cells. We analyzed the transcription of SIGLEC-11 and SIGLEC-16 genes by using their specific primers in transgenic mice. BL6 mice were taken as the control samples. By qRT-PCR, we determined that the increased level of gene transcripts of the SIGLEC-11 and SIGLEC-16 gene was observed in the SIGLEC-11 and SIGLEC-16 transgenic mice, respectively.

Moreover, immunostaining of SIGLEC-11 and SIGLEC-16 samples revealed the expression of these proteins in the transgenic mouse models. These results showed the stable expression level of SIGLEC-11 and SIGLEC-16 at both RNA and protein level. This is the first step of this project to investigate the role of these Siglec receptors on the aging procedure of the brain.

4.2 Microglia activation and inflammatory markers under the control of SIGLEC-11 and SIGLEC-16

As previously described in section 3.2, checking the microglia activation marker and pro-inflammatory cytokines at the RNA level demonstrated reduced transcripts of these markers in SIGLEC-11 compared to other genotypes. The reason behind this may be due to the inhibitory role of the SIGLEC-11 receptor. However, SIGLEC-16 receptors illustrated their inflammatory role via the higher transcription level of these examined markers. It has been also mentioned in one study the inhibitory effect of SIGLEC-11 on microglial markers by comparing the microglia cells expressing SIGLEC-11 receptor to those without it. In this study, microglia cells were co-cultured with neurons. Microglia in presence of SIGLEC-11 compared to the those lacking it, display lower transcription level of IL1-β (Wang and

Neumann 2010). The results of this study are in line with our data of inhibitory role of SIGLEC-11 in the experimented transgenic mice. In other words, all the previous and recent investigations are presenting the neuroprotective function of SIGLEC-11.

In addition, comparing the 6- and 24-month-old animals, inflammatory markers showed lower transcription levels in the 6 months-old transgenic mice as compared to their 24-month-old counterparts. It can be concluded from this little transcription level of these markers that inflammation occurs at a lower level in adulthood than it does at older ages. It is also likely that the higher transcription of microglia markers indicates higher microglia activation in the elderly compared to adults. These results are in consistent with previous investigations. As Zöller and her colleagues reported in their study, the aged mice (C57BL/6 24 months old) displayed an increased transcription level of Iba1 in comparison to the younger ones (C57BL/6 6 months old). They also mentioned that the certain immune function genes, such as Cd244 and Lilrb4 were transcribed at lower levels in the 6 months old mice comparing to the older ones (Zoller et al. 2018).

Furthermore, Linnartz-Gerlach and colleagues found that activating ITIM signaling receptors such as SIGLEC-11 could reduce phagocytosis, whereas activation of ITAM signaling receptors might induce phagocytic procedure in microglia cells (Linnartz-Gerlach, Kopatz, and Neumann 2014). An increase in the expression of Iba1 was also observed in another study. It was noted in this study that up-regulation of Iba1 as the microglial marker is due to the age-related changes including higher inflammation in the aged mice (Lee et al. 2017). Similarly, double immunostaining of Iba1/CD68 as the microglia and phagocytic markers represented elevated protein levels of these markers in 24 months old SIGLEC-16 mice compared to the other examined groups. Two main outcomes could be described by these results. To begin with, the increased expression level of microglia in SIGLEC-16 transgenic mice illustrated the inflammatory role of the SIGLEC-16 receptor. However, the lower number of activated microglia in SIGLEC-11 animals suggested that it inhibits microglia activation. These results are in consistent with those of Wang and colleagues (2010), who investigated the inhibitory role of SIGLEC-11 in microglia phagocytosis (Wang and Neumann 2010).

Additionally, these data could provide the explanation for higher expression level of Iba1 in the aged mice compared to the adult group. This outcome is in parallel with previous studies which have noted a greater activation of microglia in the aged group comparing to the adult ones (Norden and Godbout 2013). In Norden and her colleagues' study, the increased pro-inflammatory cytokines and accordingly the increased expression level of inflammatory receptors on microglia cells were elucidated. Thus, they observed higher activation of microglia during the normal aging process (Norden and Godbout 2013).

In summary, comparing the SIGLEC-11 animals with the SIGLEC-16 and BL6 groups, it might be concluded that SIGLEC-11 as an ITIM signaling receptor could reduce the microglia activation. Furthermore, in accordance with the above-mentioned studies, the aged microglia are more activated compared to the adults. From these data, we could conclude that older mice, in agreement with the recent investigations, may illustrate more microglial activation and inflammatory responses. In addition, SIGLEC-11 transgenic mice due to the presence of this ITIM signaling receptor and its inhibitory effect, would display lower inflammation and microglia activation in their aged group compared to other aged, examined mice. This outcome would suggest that the SIGLEC-11 receptor may mediate the inflammatory responses during the aging process.

4.2.1 Iba1+ cell accumulation as the aging marker

Haass and his colleagues examined Iba1-positive cell clusters in the aged mouse model of Alzheimer's disease (Brendel et al. 2017; Parhizkar et al. 2019). In the Trem2 knock out aged mouse, fewer microglia were clustered around the new apolipoprotein-E plaques than in the wild type (Brendel et al. 2017). Paassila and his colleagues also observed clusters of microglia in Alzheimer's disease concurrent with amyloid-beta (Aß) plaque formation and earlier than dystrophic neurites. They have shown that clustering of microglia plays a key role in the early stages of Alzheimer's disease (Paasila et al. 2020). This would suggest that accumulation of Iba1+ cells are the important key in both aging procedure and neurodegenerative diseases. These data were in parallel to the investigation of these

immune cell clusters in this project, which both were describing that presence of Iba1+ cell clusters is a hallmark of aging. In line with these investigations, our 6 months old mice showed no or extremely low number of these clusters versus the other aged groups. Our study identified Iba1-positive cell clusters, as a marker of aging, which were evidently more prevalent in our 24-months-old mice groups. This is also in agreement with the Fernández and colleagues' study which has shown the higher number of age-related markers including Iba1+ cell clusters in older APP/PS1 mice (10 months of age) compared to younger mice (2 months of age) (Fernández et al. 2021).

In accordance with these studies, our examined SIGLEC-11 mice group were showing the fewer number of these clusters at the 24 months old age comparing to the SIGLEC-16 and control group. Accordingly, in SIGLEC-16 transgenic mice, due to higher inflammatory responses, there are more activated microglia and, eventually, more microglia cell clusters in comparison to SIGLEC-11 transgenic mice. In other words, it explains the inhibitory effect of ITIM motifs in SIGLEC-11, while demonstrating the activatory role of SIGLEC-16 in association with its adaptor molecule, DAP-12. Possibly, this may once again illustrate the SIGLEC-11's neuroprotective effect from earlier time periods.

4.3 Role of microglia in activating the complement cascade

There is no doubt that as we age, the number of apoptotic and damaged cells are increased. These cells should be phagocytosed by the immune cells after their activation. Complement cascade is one mechanism for phagocytosis of these damaged and apoptotic cells with the help of its chief member, microglia (Fishelson, Attali, and Mevorach 2001; Kouser et al. 2015; Trouw, Blom, and Gasque 2008). It has been proved that there is a homeostatic balance between the neurons and microglia cells in the CNS. Though, every little factor which could disruptively activate the complement system may lead to disruption of this balance via activating microglia (Zabel and Kirsch 2013). Complement cascade may be activated in three different pathways of classical, alternative and/or lectin. Depending on the specific recognition markers such as pathogens or markers from injured tissues and cells, one of the

mentioned pathways may be activated. Along with recognition markers, each of the complement components could play a critical role in activating the proper complement pathway. The most important component of complement cascade is C3, which participates in all three complement pathways (Nishida, Walz, and Springer 2006). In addition, C1q plays a role as an initiator to activate the whole complement system (Zabel and Kirsch 2013). C1q could specifically recognize the markers from the apoptotic cells in order to induce their clearance (Kouser et al. 2015). Hence, it would be essential to analyze the transcription level of complement components as the markers which are expressed to activate microglia. The examined complement components were expressed at higher levels in the 24 month old mice compared to the 6 months of ages. This may be related to an increased activation of the complement cascade due to aging. Likewise, it has been shown in a study performed in 2009 that the mRNA levels of complement components such as C1qa, C3 and C4 are increased with aging in the C57BL/6 mice (Reichwald et al. 2009).

Meanwhile, aging may cause higher levels of inflammation in the brain due to an increase in apoptotic and damaged cells. These inflammatory reactions may promote the expression of complement cascade genes. The complement components could activate the immune system to deplete the damaged cells and accordingly reduce the inflammation within the brain (Dunkelberger and Song 2010; Schartz and Tenner 2020; Warwick et al. 2021). In comparison with SIGLEC-11 and the control group, transcription levels of complement components increased in SIGLEC-16. Regarding the increased complement components gene transcripts in SIGLEC-16, the complement cascade is more activated in SIGLEC-16 than SIGLEC-11 and wt mice. This may suggest that higher complement cascade activation may lead to more neuronal damages. In conclusion, SIGLEC-16 signaling receptor could cause the complement cascade to become increasingly active, which may be related to the increased number of damaged cells in these transgenic animals.

4.4 The effect of microglia cells in phagocytosis

Considering that microglia cells are the resident immune cells in the central nervous system, they are also essential for phagocytosing damaged neurons and granules caused by aging. Studies have indicated microglia dysfunction, as one of the hallmarks of aging, that is responsible for the imperfect phagocytosis of damaged factors (Waller et al. 2019; Yanguas-Casas et al. 2020). In the Yanguas-Casas study, the researchers assessed the phagocytic activity of microglia in adult (5 months old) and aged (18 months old) mice. They showed that microglia cells isolated from aged mice exhibit increased phagocytosis activity as compared to those isolated from the adult group (Yanguas-Casas et al. 2020). Similarly, phagocytosis activity of the experimented aged microglia cells was higher than the adult microglia. It would mean that aged mice have more activated microglia that can perform phagocytosis at greater level than adult mice.

In order to understand the other side of this story, it is necessary to compare the different transgenic mice groups of SIGLEC-11, SIGLEC-16, and BL6 according to their genotypes. Therefore, the transcription level of DAP-12 is higher in SIGLEC-16 compared to SIGLEC-11 and BL6. Since DAP-12 acts as an adapter molecule for SIGLEC-16 activation, it may result in increased activity of SIGLEC-16 on microglia and, in turn, higher inflammation and phagocytosis (Abtahian et al. 2006; Mócsai et al. 2006; Zou et al. 2007). Hence, it will be confirmed that activation of SIGLEC-16 is capable to cause higher phagocytic responses in the experimented animals in this study.

Moreover, as mentioned before in section 4.2, the immunostaining of Iba1/Cd68 demonstrated that the expression level of Cd68 is increased in SIGLEC-16 transgenic mice versus the SIGLEC-11 mice. As a result of this, the higher phagocytic activity in SIGLEC-16 mice is not limited to the RNA level. These results may prove that SIGLEC-16 exerts an activatory effect on phagocytosis, while representing the inhibitory functions of SIGLEC-11.

The increased expression level of Cd68 is another hallmark of aging (Safaiyan et al. 2016). In addition, activation of the ITAM signaling receptors has been described as the major risk factor in neurodegenerative disorders (Ivashkiv 2009). At the age of 24 months, the results

of SIGLEC-16 transgenic mice provide this evidence to support both facts that containing the SIGLEC-16 receptor as a signaling receptor for ITAM, as well as being aged are factors that contribute to the higher transcription levels of Cd68.

4.5 Role of microglia cells in removing the oxidative stress markers and oxidized lipids.

As described by Schwartz and colleagues, deletion of Siglec-E, as an ITIM signaling receptor, could cause accelerated aging in their mouse model (Siglec-E-/-), which is associated with an unbalanced ROS metabolism. This will result in damages to cellular DNA, proteins and lipids (Schwarz et al. 2015). Based on their results, ITAM signaling receptors can accelerate the aging process. Comparing the results of ITIM versus ITAM signaling receptors in our study, we found that the transcription level of oxidative stress markers were lower in SIGLEC-11 mice compared to SIGLEC-16 mice and the control group. These results would indicate the inhibitory effect of SIGLEC-11 as an ITIM signaling receptor in our examined SIGLEC-11 transgenic mice. Moreover, the results obtained from immunostaining of 4-HNE at the lipid level confirmed this hypothesis regarding the SIGLEC-11's inhibitory role. As Schwarz mentioned in his research, Siglec-E, as a member of CD-33r Siglecs in mice, could reduce the speed of aging in the mice. This function is due to the decreased level of oxidative stress markers compared to the Siglec-E knocked-out mice. In line with previous study, our data demonstrated that SIGLEC-11, as a member of CD-33r Siglecs in humans, may play a similar role to Siglec-E in mice, namely, reduction of oxidative stress markers at both RNA and lipid levels. In other words, SIGLEC-11 may reduce the speed of the aging in SIGLEC-11 transgenic mice as compared to control and SIGLEC-16. However, SIGLEC-16 mice, in our project, with higher transcription and expression level of oxidative stress markers might display the accelerated aging.

Furthermore, as mentioned in other studies, the increased level of oxidative stress markers and oxidized lipids are regarded as hallmarks of brain aging (Simpson and Oliver 2020; von Bernhardi, Eugenin-von Bernhardi, and Eugenin 2015; von Leden et al. 2017). In d'Avila and colleague's study the neuropathological features were investigated that are associated with

age-related cognitive decline in their mouse model. In comparison to their younger group, the 4-HNE level, as an oxidized lipid, was significantly higher in the aged mice. In addition, they found that the learning of aged mice was slower and at the lower levels versus the young mice (Ahmad et al. 2022; d'Avila et al. 2018). In the study at hand, the 4-HNE intensity level was significantly higher in the aged SIGLEC-16 mice when compared to the other aged groups. Similarly, the aged transgenic mice showed increased transcription level of oxidative stress markers comparing to the 6 months old mice. In the 6-month-old mice group, the 4-HNE concentration was remarkably similar in all groups, indicating that changes in oxidative stress markers at the expression level may reveal themselves at the later time points. It is possible that these markers are not visible in the adulthood which may represent aging as an important risk factor for cognitive impairment via higher oxidative damages.

4.6 Lipofuscin granules in the microglia cells.

Lipofuscin, the age pigment, accumulates over time specifically in post-mitotic cells including neurons or phagocytes and is regarded as a secondary marker of aging (Brunk and Terman 2002; Singh Kushwaha, Patro, and Kumar Patro 2018). In the study of Singh and colleagues, they observed different accumulation patterns of female rats in different ages (from 3 months old to 30 months old). They described accumulation of LF within neurons in hippocampus area specifically in CA1 and CA3 of the aged mice. O'Neil also observed accumulated LF inside the microglia cells. In their study, they checked the accumulation of LF in both neurons and microglia of adult and aged mice. In comparison to the adult cells, a higher proportion of LFs were accumulated in the aged NeuN+ and Iba1+ cells (Brunk and Terman 2002; O'Neil et al. 2018; Singh Kushwaha, Patro, and Kumar Patro 2018). Our results represented the accumulation of LF in the microglia cells at both hippocampus and SNpr regions of all three experimented transgenic mice. Our aged mice showed more accumulated LF in the Iba1-positive cells compared to the 6 months old mice as was observed in O`Neil’s study. As Singh described, LF is taken as waste materials which the lysosomal hydrolyses are not able to digest it. This is the reason of accumulation of these pigmented granules. In short-lived mammals such as rats and mice the rate of the accumulated LF is higher compared to the

long-lived mammals. It is believed that this is due to the higher mitochondrial activation in short-lived mammals which may lead to higher levels of superoxide and hydrogen peroxide radicals. This discovery is in agreement with the presence of LF in microglia cells of our 6 months old mice. Most importantly, due to the microglia dysfunction during aging, the accumulation of LF granules is much higher in aged microglia versus the adults. In addition, Singh and colleagues stated that LF granules could also accumulate in young mice, whereas as they age, these aggregates grow to larger sizes (3-5µm) (Singh Kushwaha, Patro, and Kumar Patro 2018). Thus, accumulated LFs are visible at all ages of mice, albeit in different range sizes. The reason that accumulated LFs are also present in our six-months-old mice could be explained in this way. Despite the fact that in the aged groups (24 months old) these accumulated granules were at higher levels compared to the adult samples (6 months old).

In addition, the auto-fluorescent intensity of LF decreased in SIGLEC-11 group versus the wt and SIGLEC-16 groups. As LF is the age pigment and is accumulated due to incomplete phagocytosis of microglia cells, the results of this study suggest that SIGLEC-11 receptors could inhibit the accumulation of LF granules, which may diminish the signs of aging. On the other hand, it may explain SIGLEC-16's inflammatory role leading to higher levels of aging markers in the SIGLEC-16 mouse population.

4.7 Age-related neuronal and synapse loss.

Accumulation of the aggregated LF and higher 4-HNE intensity levels as the oxidative stress markers (see fig.22) would result in lower antioxidant defense or higher reactive oxygen species (ROS) (Halliwell 2001). Moreover, it was visible that the accumulated LFs were present at greater levels in neurons of the aged time points compared to the adult mice (O'Neill, van den Berg, and Mullen 2013). These events lead to increased vulnerability of neurons (Castelli et al. 2019). Several studies have documented loss of neurons during aging (Pakkenberg and Gundersen 1997; West et al. 1994). The study of Edler and colleagues is an example of the age-related neuronal loss studies. It was observed in their research that the neuronal densities are decreased in the CA1 and CA3 regions as a consequence of aging

(Edler et al. 2020). Another example is the study performed in Brazil in 2012 by Mortera' and Herculano-Houzel. According to Mortera' and Herculano-Houzel investigation, rats of 24 months' ages experienced greater levels of neuronal loss than rats of 3 months, 9 months, and 12 months old. They also described that the increased transcription and expression level of oxidative stress markers could contribute to the age-related loss of neurons (Mortera and Herculano-Houzel 2012). According to another study, Mattson and colleagues observed that higher level of stress may lead to increased damages to macro molecules including DNA. This might confirm the link between higher ROS levels and more neuronal loss related to aging (Mattson and Magnus 2006).

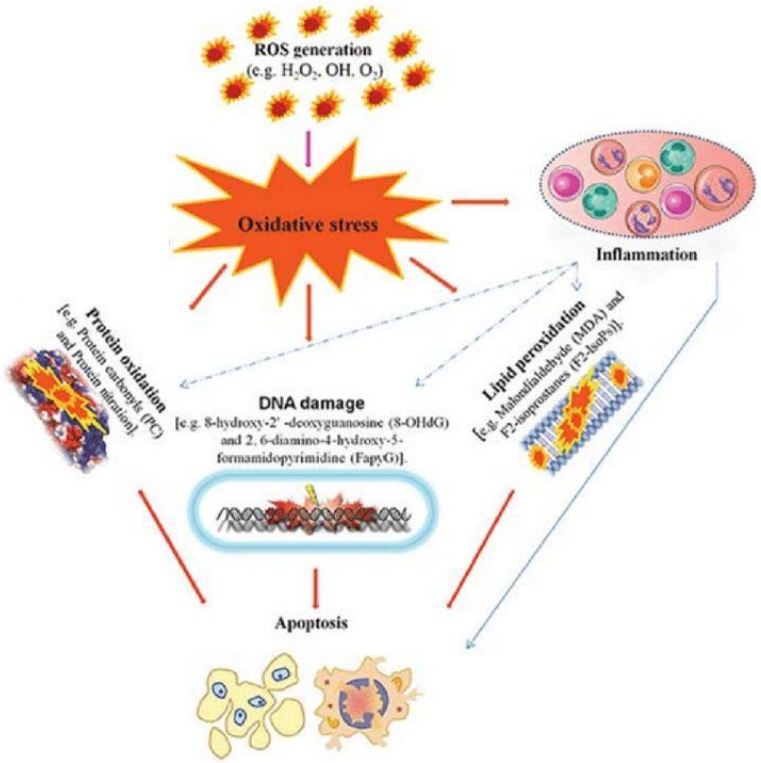


Fig. 22: Age-related reactive oxygen species and inflammation affects neuronal viability. It has been described that the higher levels of oxidative stress and inflammatory responses cause DNA damages, lipid and protein peroxidation that is leading to apoptosis of neurons. This figure shows a cascade of multiple events causing neuronal loss during aging. Modified from Madkour, Journal of targeted drug delivery, 2019.

In comparison with other genotypes and aged groups, we observed an increase in the number of neurons in SIGLEC-11 at 6 months of age. SIGLEC-16, on the other hand, showed the greatest loss of neurons at the age of 24 months. Likewise, the results were repeated for the dopaminergic neurons, the critical cells within the brain for the memory. In general, the increased number of neurons may be due to the inhibition of excessive microglia activation and more oxidative stress responses by SIGLEC-11. However, SIGLEC-16 because of its inflammatory effect might activate microglia leading to reduced number of NeuN+ and TH+ cells at both experimented regions. The process of neuronal loss is starting earlier than the time point that animals are referred as aged animals (Mortera and Herculano-Houzel 2012). It is therefore important to examine the differences in the number of neurons at earlier time points. In the experimented groups, the increased number of neurons in SIGLEC-11 at 6 months old could demonstrate the effect of SIGLEC-11 at the earlier time points than 6 months old. Therefore, future studies should be conducted to investigate the effect of Siglec receptors during the development of the brain.

Among the hallmarks of aging, we could point to synaptic injuries. In previous studies, synaptic loss has been observed prior to neuronal loss (Kashyap et al. 2019; Brady and Morfini 2010). These studies stated that the loss of synapses may restrict the ability of neurons to communicate with each other, which it is likely the reason of neuronal loss. Therefore, it is essential to investigate the number of synapses throughout the lifespan. Accordingly, checking the pre- and post-synaptic markers revealed that SIGLEC-16 group have fewer synapses than the other groups, especially at the 24 months old. Compared to SIGLEC-11, which is an inhibitory Siglec receptor, SIGLEC-16 as an ITAM signaling receptor has a lower number of synapses. These data would prove the neuroprotective properties of SIGLEC-11, while describing the neurodegenerative characteristics of SIGLEC-16. As well, these investigations were in line with the previous studies which demonstrated a decrease in the number of synapses and neurons in the aged brain compared to the younger brain (Bondareff and Geinisman 1976; Geinisman, de Toledo-Morrell, and Morrell 1986; Geinisman et al. 1992; McWilliams and Lynch 1984).

We can conclude from our outcomes that the higher number of synapses in SIGLEC-11 transgenic mice at 24 months of age might be due to their lower synapse loss. It would describe the inhibitory role of SIGLEC-11 signaling receptor, while its paired receptor, SIGLEC-16, could cause greater synapse loss due to its activating effect. Since the number of synapses in the experimented mice groups at the 6 months of age are not statistically significant, it would be essential to investigate the number of synapses at the earlier time points.

4.7.1 Role of microglial cells in neurogenesis.

A major event in the development of the nervous system is neurogenesis, which refers to the formation of new neurons from neural stem cells. The process of neurogenesis occurs during the prenatal and postnatal periods, although recent research suggests that the generation of new neurons can also occur in certain areas of the brain throughout the adult life (Gemma and Bachstetter 2013; Ming and Song 2011; Mori, Buffo, and Gotz 2005). However, the data of our project could target the embryonic neurogenesis which might oriented future studies.

As mentioned before, human brain microglia are regularly distributed throughout the CNS and originate from primitive macrophages in the yolk sac (Ginhoux et al. 2013). Following migration of primitive macrophages into the neural tube, specific modifications, including Iba1 expression, will occur to distinguish these cells from macrophages in other organs. Because of the main function of microglia in the production, maturation, and integration of new neurons, they may play an essential role in neurogenesis (Ginhoux et al. 2013) including synaptic pruning (Paolicelli et al. 2011), neuronal connectivity (Schafer and Stevens 2013) and behavioral development, as well (Tremblay, Lowery, and Majewska 2010).

Since microglia are involved in the apoptosis of neural progenitor stem cells (NPS), they could play a pivotal role in maintaining the healthy population of cells during the developmental stages. Hashimoto et al. study. observed that Macrophage-colony

stimulating factor (M-CSF) and granulocyte-macrophage (GM-CSF) are the origins of tissue macrophages such as microglia in brain. They also described that CSF1 is secreted via neurons and will bind to its receptor (CSF1R) on the cell surface of microglia (Hashimoto et al. 2013). Therefore, CSF1 signaling is crucial for microglia cells. Moreover, studies have shown that genetic disorders in CSF1R can adversely affect the number of microglia (Elmore et al. 2014; Ginhoux et al. 2010) and decreased in number of these immune cells could result in an imperfect neurogenesis. There was another study describing the role of microglia in healthy and proper neurogenesis. They demonstrated that depleting microglia by utilizing the CSF1R technique may lead to reduction of the neuronal cells. These studies were intelligibly described the role of microglia in neurogenesis (Ginhoux et al. 2013; Panchision et al. 2001).

Despite the fact that these studies mention the pivotal role of microglia in improving neurogenesis, there is also some evidence describing microglia's negative effect on neurogenesis (Miller et al. 2008; Tran and Miller 2003). Marín-Teva and colleagues realized the role of microglia cells in the programmed cell death at the developmental stages of the brain. They described that microglia phagocytosis would induce the death of neurons which are involved in synaptogenesis (Marin-Teva et al. 2004). Therefore, microglia are the important regulators for neurogenesis during embryonic developmental stages.

In general, microglia play a key role in modulating neurogenesis, which extends beyond the clearance and removal of the newly born cells (see figure 23). They are involved in every specific step of neurogenesis including neuroprotection and neurodegeneration. They are able to phagocytose damaged neural progenitor stem cells. Most importantly, studies have proved the role of microglia in synaptic connections. During the process of neurogenesis, microglia cells could remodel synaptic connections which is performed by phagocytosis the inefficient synapses (Schafer et al. 2012).

In this project, I demonstrated that SIGLEC-11 transgenic mice had a significantly higher number of neurons than the adult control group. Likewise, the number of dopaminergic neurons were increased in the SIGLEC-11 animals compare to BL6, at the 6 months old.

Whilst SIGLEC-16 illustrated lower number of these cells at 6 months old comparing to both SIGLEC-11 and control group. Moreover, the differences of transcription level of several examined markers including microglia markers, pro-inflammatory cytokines, complement components, oxidative stress and phagocytic markers at the 6 months old would describe the already earlier effect of the SIGLEC-11 and -16 receptors in the experimented mice groups. Taken together the above-mentioned studies and the results of the project, it could be possible that these Siglec receptors have potent to function at the premier stages of the experimented mice. Due to its inhibitory effects, SGLEC-11 could result in neuroprotective effects from an embryonic stage. However, its paired receptor, SIGLEC-16, may have neurodegenerative effects at the developmental stage of the embryos. Nevertheless, further studies are needed to fully determine the involvement of microglia and their receptors in healthy synaptic connections and healthy neurogenesis.

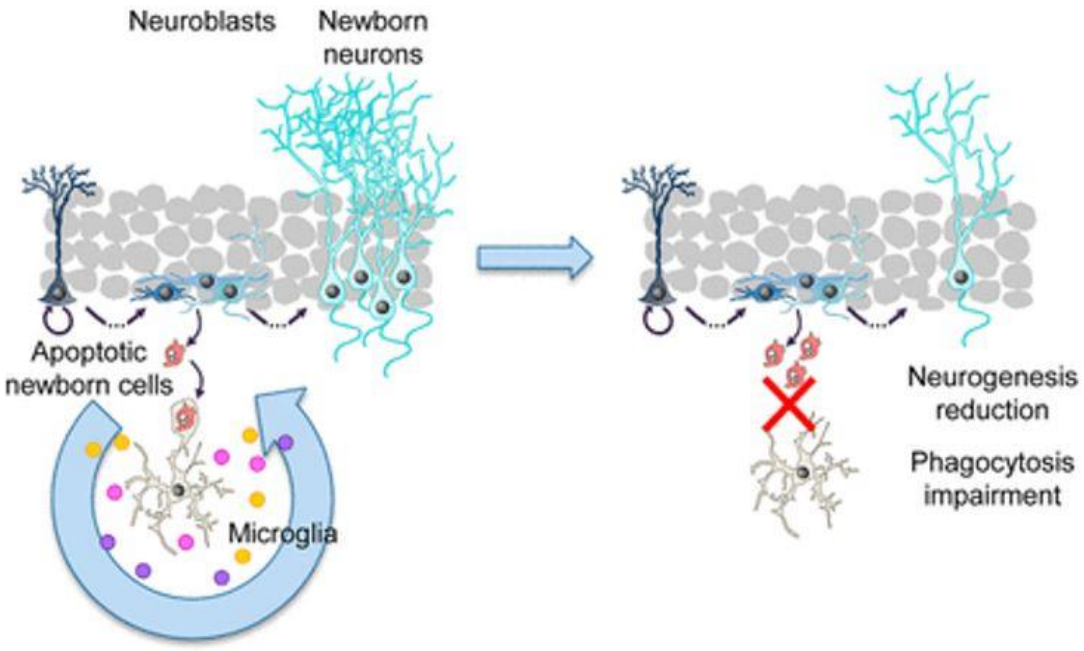


Fig. 23: Role of microglia cells in neurogenesis. The healthy microglia cells will perform the accurate apoptosis of newborn cells and lead to a complete flawless neurogenesis procedure. However, the impaired phagocytosis of microglia cells may cause an unfavorable apoptosis and decreasing the number of newborn neurons. Modified from Diaz-Aparicio et al, Journal of Neuroscience, 2019.

4.8 SIGLEC-11 and SIGLEC-16: The outlook.

The present study was an attempt to investigate the inhibitory role of SIGLEC-11 in the normal process of aging of the brain. As is demonstrated by the findings, the inhibitory effect is evident as early as six months of age. However, SIGLEC-16 plays a key role in the activation of microglia cells, which in turn results in neuronal damages in the brain. According to our results, the activatory effect of SIGLEC-16 signaling receptor might accelerate the aging procedure at the earlier time points in the SIGLEC-16 transgenic mice compared to the other mice groups. Furthermore, in line with the previous research, which has indicated SIGLEC-11 and SIGLEC-16 as paired receptors, the current study also confirmed the feasibility of this hypothesis by demonstrating the inhibitory effect of SIGLEC-11 and the activatory role of SIGLEC-16. We reported that the opposite effect of these two Siglec receptors is responsible to mediate the activation of microglia cells in the experimented mouse groups. This could describe the possibility that Siglec receptors are involved in activating or silencing microglial cells during the aging process.

As described in 4.7.1, studies have established the role of microglia in the neurogenesis process. In a recent study performed in 2020, Marsters and colleagues observed the increased Iba1 and Cd68 expression level in the ventricles. They have realized that the number of the ameboid microglia are higher in the ventricles at day E15.5, representing their role in oligodendrocyte differentiation. It is proved that in the absence of microglia, the number of the oligodendrocyte precursor cells are increased (Marsters et al. 2020). According to Marsters's investigation and other related mentioned studies, microglia play a major role in both neurogenesis and gliogenesis. Moreover, studies have reported the effect of microglia on the synaptic pruning and eventually the neuronal connections as well as neuronal circuits (Paolicelli et al. 2011; Schafer and Stevens 2013). However, further investigation is required

to discover the onset effect of the two Siglec receptors. Since 6 weeks of age is considered to be the onset of mice adulthood, it would be pivotal to examine the role of Siglec receptor at this age. Therefore, investigation at this time point may elucidate the fact whether Siglec receptors' effect may initiate from the adulthood on. The outcomes of studying 6-weeks old mice might be compared with the results of 6-month-old and 24-month-old ones. Based on these data, the recent comparison would indicate whether Siglec receptors are capable of impacting microglia activity throughout their lifespan or that their effect begins much earlier, during the brain's development stage.

5. Abstract

Brain aging is a multifactorial process including accumulation of aged lipids and proteins, neuroinflammation and loss of neurons. SIGLEC-11 and SIGLEC-16 are paired opposite inhibitory- versus activatory-signaling receptors on human microglia without any direct homology in mice. Contribution of these paired receptors to brain aging is still unclear.

In this study, the effect of these paired receptors during normal aging was investigated at two different time points (6 and 24 months of age) in humanized inhibitory-signaling SIGLEC-11 versus activatory-signaling SIGLEC-16 transgenic (tg) mice, that ectopically were expressing the respective human receptors in microglia and macrophages. Immunohistochemical analysis of hippocampus and substantia nigra showed that the number of Iba1-positive cells was increased at the age of 24 months in SIGLEC-16 compared to the SIGLEC-11 tg mice and wildtype group. In addition, protein levels of Cd68 were increased in SIGLEC-16 tg mice at 24 months versus the other both groups. In line with the protein level results, gene transcription of the microglial markers, Iba1 and Cd68, increased in SIGLEC-16 tg at 24 months old versus the wildtype control and SIGLEC-11 tg groups. Results also demonstrated that 6 months old SIGLEC-11 tg mice have lower gene transcription levels of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1β and the complement proteins including C3 and C4 compared to SIGLEC-16 tg mice. Furthermore, oxidative markers presented higher levels in SIGLEC-16 tg at an age of 24 months compared to the other groups at the similar age. Moreover, 24 months old SIGLEC-16 tg mice showed increased loss of neurons in the hippocampus compared to the wildtype control and SIGLEC-11 tg mice. In parallel, staining of pre- and post-synaptic markers demonstrated higher number of synapses in SIGLEC-11 tg mice at 6 months of age compared to the other experimented groups. Results suggest that activation of microglia as well as the complement cascade are better controlled in SIGLEC-11 tg mice and that increased inflammation might contribute to loss of neurons in SIGLEC-16 tg mice.

Taken together, our study described inhibitory-signaling SIGLEC-11 as neuro- and synapse-protective receptor through aging, while activatory-signaling SIGLEC-16 promotes inflammation and neuronal loss.

6. List of Figures

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8. References

Abtahian, F., N. Bezman, R. Clemens, E. Sebzda, L. Cheng, S. J. Shattil, M. L. Kahn, and G. A. Koretzky. 2006. 'Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells', *Mol Cell Biol*, 26: 6936-6949.

Ahmad, M. A., O. Kareem, M. Khushtar, M. Akbar, M. R. Haque, A. Iqubal, M. F. Haider, F. H. Pottoo, F. S. Abdulla, M. B. Al-Haidar, and N. Alhajri. 2022. 'Neuroinflammation: A Potential Risk for Dementia', *Int J Mol Sci*, 23.

Angata, T., S. C. Kerr, D. R. Greaves, N. M. Varki, P. R. Crocker, and A. Varki. 2002. 'Cloning and characterization of human Siglec-11. A recently evolved signaling molecule that can interact with SHP-1 and SHP-2 and is expressed by tissue macrophages, including brain microglia', *J Biol Chem*, 277: 24466-24474.

Angata, T., E. H. Margulies, E. D. Green, and A. Varki. 2004. 'Large-scale sequencing of the CD33-related Siglec gene cluster in five mammalian species reveals rapid evolution by multiple mechanisms', *Proc Natl Acad Sci U S A*, 101: 13251-13256.

Bachiller, S., I. Jimenez-Ferrer, A. Paulus, Y. Yang, M. Swanberg, T. Deierborg, and A. Boza-Serrano. 2018. 'Microglia in Neurological Diseases: A Road Map to Brain-Disease Dependent-Inflammatory Response', *Front Cell Neurosci*, 12: 488.

Bai, J., R. L. Ramos, J. B. Ackman, A. M. Thomas, R. V. Lee, and J. J. LoTurco. 2003. 'RNAi reveals doublecortin is required for radial migration in rat neocortex', *Nat Neurosci*, 6: 1277-12783.

Barak, O., M. A. Lazzaro, W. S. Lane, D. W. Speicher, D. J. Picketts, and R. Shiekhattar. 2003. 'Isolation of human NURF: a regulator of Engrailed gene expression', *EMBO J*, 22: 6089-60100.

Billadeau, D. D., and P. J. Leibson. 2002. 'ITAMs versus ITIMs: striking a balance during cell regulation', *J Clin Invest*, 109: 161-168.

Bishop, N. A., T. Lu, and B. A. Yankner. 2010. 'Neural mechanisms of ageing and cognitive decline', *Nature*, 464: 529-535.

Bisht, K., K. Sharma, and M. E. Tremblay. 2018. 'Chronic stress as a risk factor for Alzheimer's disease: Roles of microglia-mediated synaptic remodeling, inflammation, and oxidative stress', *Neurobiol Stress*, 9: 9-21.

Blasius, A. L., M. Cella, J. Maldonado, T. Takai, and M. Colonna. 2006. 'Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12', *Blood*, 107: 2474-2476.

Bondareff, W., and Y. Geinisman. 1976. 'Loss of synapses in the dentate gyrus of the senescent rat', *Am J Anat*, 145: 129-136.

Bornhofft, K. F., T. Goldammer, A. Rebl, and S. P. Galuska. 2018. 'Siglecs: A journey through the evolution of sialic acid-binding immunoglobulin-type lectins', *Dev Comp Immunol*, 86: 219-231.

Brady, S., and G. Morfini. 2010. 'A perspective on neuronal cell death signaling and neurodegeneration', *Mol Neurobiol*, 42: 25-31.

Brendel, M., G. Kleinberger, F. Probst, A. Jaworska, F. Overhoff, T. Blume, N. L. Albert, J. Carlsen, S. Lindner, F. J. Gildehaus, L. Ozmen, M. Suarez-Calvet, P. Bartenstein, K.

Baumann, M. Ewers, J. Herms, C. Haass, and A. Rominger. 2017. 'Increase of TREM2 during Aging of an Alzheimer's Disease Mouse Model Is Paralleled by Microglial Activation and Amyloidosis', *Front Aging Neurosci*, 9: 8.

Brouwers, N., C. Van Cauwenberghe, S. Engelborghs, J. C. Lambert, K. Bettens, N. Le Bastard, F. Pasquier, A. G. Montoya, K. Peeters, M. Mattheijssens, R. Vandenberghe, P. P. Deyn, M. Cruts, P. Amouyel, K. Sleegers, and C. Van Broeckhoven. 2012. 'Alzheimer risk associated with a copy number variation in the complement receptor 1 increasing C3b/C4b binding sites', *Mol Psychiatry*, 17: 223-233.

Brown, R. C., A. H. Lockwood, and B. R. Sonawane. 2005. 'Neurodegenerative diseases: an overview of environmental risk factors', *Environ Health Perspect*, 113: 1250-1256.

Brunk, U. T., and A. Terman. 2002. 'Lipofuscin: mechanisms of age-related accumulation and influence on cell function', *Free Radic Biol Med*, 33: 611-619.

Bull, C., R. Nason, L. Sun, J. Van Coillie, D. Madriz Sorensen, S. J. Moons, Z. Yang, S. Arbitman, S. M. Fernandes, S. Furukawa, R. McBride, C. M. Nycholat, G. J. Adema, J. C. Paulson, R. L. Schnaar, T. J. Boltje, H. Clausen, and Y. Narimatsu. 2021. 'Probing the binding specificities of human Siglecs by cell-based glycan arrays', *Proc Natl Acad Sci U S A*, 118.

Cao, H., and P. R. Crocker. 2011. 'Evolution of CD33-related siglecs: regulating host immune functions and escaping pathogen exploitation?', *Immunology*, 132: 18-26.

Cao, H., U. Lakner, B. de Bono, J. A. Traherne, J. Trowsdale, and A. D. Barrow. 2008. 'SIGLEC16 encodes a DAP12-associated receptor expressed in macrophages that evolved from its inhibitory counterpart SIGLEC11 and has functional and non-functional alleles in humans', *Eur J Immunol*, 38: 2303-2315.

Castelli, V., E. Benedetti, A. Antonosante, M. Catanesi, G. Pitari, R. Ippoliti, A. Cimini, and M. d'Angelo. 2019. 'Neuronal Cells Rearrangement During Aging and Neurodegenerative Disease: Metabolism, Oxidative Stress and Organelles Dynamic', *Front Mol Neurosci*, 12: 132.

Cavalcante, T., M. M. Medeiros, S. N. Mule, G. Palmisano, and B. S. Stolf. 2021. 'The Role of Sialic Acids in the Establishment of Infections by Pathogens, With Special Focus on Leishmania', *Front Cell Infect Microbiol*, 11: 671913.

Chen, S. K., P. Tvrdik, E. Peden, S. Cho, S. Wu, G. Spangrude, and M. R. Capecchi. 2010. 'Hematopoietic origin of pathological grooming in Hoxb8 mutant mice', *Cell*, 141: 775-785.

Chen, S., J. Peng, P. Sherchan, Y. Ma, S. Xiang, F. Yan, H. Zhao, Y. Jiang, N. Wang, J. H. Zhang, and H. Zhang. 2020. 'TREM2 activation attenuates neuroinflammation and neuronal apoptosis via PI3K/Akt pathway after intracerebral hemorrhage in mice', *J Neuroinflammation*, 17: 168.

Chiricozzi, E., G. Lunghi, E. Di Biase, M. Fazzari, S. Sonnino, and L. Mauri. 2020. 'GM1 Ganglioside Is A Key Factor in Maintaining the Mammalian Neuronal Functions Avoiding Neurodegeneration', *Int J Mol Sci*, 21.

Cognasse, Fabrice, Kim Anh Nguyen, Pauline Damien, Archibald McNicol, Bruno Pozzetto, Hind Hamzeh-Cognasse, and Olivier Garraud. 2015. 'The Inflammatory Role of Platelets via Their TLRs and Siglec Receptors', *Frontiers in Immunology*, 6.

Crehan, H., J. Hardy, and J. Pocock. 2013. 'Blockage of CR1 prevents activation of rodent microglia', *Neurobiol Dis*, 54: 139-149.

Crehan, H., P. Holton, S. Wray, J. Pocock, R. Guerreiro, and J. Hardy. 2012. 'Complement receptor 1 (CR1) and Alzheimer's disease', *Immunobiology*, 217: 244-250.

Crocker, P. R. 2005. 'Siglecs in innate immunity', *Curr Opin Pharmacol*, 5: 431-437.

Crocker, P. R., and P. Redelinghuys. 2008. 'Siglecs as positive and negative regulators of the immune system', *Biochem Soc Trans*, 36: 1467-1471.

Crocker, P. R., and A. Varki. 2001. 'Siglecs, sialic acids and innate immunity', *Trends Immunol*, 22: 337-342.

Crocker, Paul R., James C. Paulson, and Ajit Varki. 2007. 'Siglecs and their roles in the immune system', *Nature Reviews Immunology*, 7: 255-266.

Cross, Sally H., Richard R. Meehan, Xinsheng Nan, and Adrian Bird. 1997. 'A component of the transcriptional represser MeCP1 shares a motif with DNA methyltransferase and HRX proteins', *Nature Genetics*, 16: 256-259.

d'Avila, J. C., L. D. Siqueira, A. Mazeraud, E. P. Azevedo, D. Foguel, H. C. Castro-Faria-Neto, T. Sharshar, F. Chretien, and F. A. Bozza. 2018. 'Age-related cognitive impairment is associated with long-term neuroinflammation and oxidative stress in a mouse model of episodic systemic inflammation', *J Neuroinflammation*, 15: 28.

Daeron, M., S. Jaeger, L. Du Pasquier, and E. Vivier. 2008. 'Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future', *Immunol Rev*, 224: 11-43.

Datki, Z., Z. Galik-Olah, E. Janosi-Mozes, V. Szegedi, J. Kalman, A. G. Hunya, L. Fulop, H. Tamano, A. Takeda, P. A. Adlard, and A. I. Bush. 2020. 'Alzheimer risk factors age and female sex induce cortical Abeta aggregation by raising extracellular zinc', *Mol Psychiatry*, 25: 2728-2741.

Davies, D. S., J. Ma, T. Jegathees, and C. Goldsbury. 2017. 'Microglia show altered morphology and reduced arborization in human brain during aging and Alzheimer's disease', *Brain Pathol*, 27: 795-808.

Dragone, L. L., L. A. Shaw, M. D. Myers, and A. Weiss. 2009. 'SLAP, a regulator of immunoreceptor ubiquitination, signaling, and trafficking', *Immunol Rev*, 232: 218-228.

Dunkelberger, J. R., and W. C. Song. 2010. 'Complement and its role in innate and adaptive immune responses', *Cell Res*, 20: 34-50.

Edler, M. K., E. L. Munger, R. S. Meindl, W. D. Hopkins, J. J. Ely, J. M. Erwin, E. J. Mufson, P. R. Hof, C. C. Sherwood, and M. A. Raghanti. 2020. 'Neuron loss associated with age but not Alzheimer's disease pathology in the chimpanzee brain', *Philos Trans R Soc Lond B Biol Sci*, 375: 20190619.

Edler, M. K., C. C. Sherwood, R. S. Meindl, E. L. Munger, W. D. Hopkins, J. J. Ely, J. M. Erwin, D. P. Perl, E. J. Mufson, P. R. Hof, and M. A. Raghanti. 2018. 'Microglia changes associated to Alzheimer's disease pathology in aged chimpanzees', *J Comp Neurol*, 526: 2921-2936.

Elmore, M. R., A. R. Najafi, M. A. Koike, N. N. Dagher, E. E. Spangenberg, R. A. Rice, M. Kitazawa, B. Matusow, H. Nguyen, B. L. West, and K. N. Green. 2014. 'Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain', *Neuron*, 82: 380-97.

Estus, S., B. C. Shaw, N. Devanney, Y. Katsumata, E. E. Press, and D. W. Fardo. 2019. 'Evaluation of CD33 as a genetic risk factor for Alzheimer's disease', *Acta Neuropathol*, 138: 187-199.

Fehervari, Zoltan. 2015. 'A Siglec link to aging', *Nature Immunology*, 16: 598-98.

Fernández, Andrés, Elena Quintana, Patricia Velasco, Belén Moreno-Jimenez, Belén de Andrés, Maria Luisa Gaspar, Isabel Liste, Marçal Vilar, Helena Mira, and Eva Cano. 2021. 'Senescent accelerated prone 8 (SAMP8) mice as a model of age dependent neuroinflammation', *Journal of Neuroinflammation*, 18: 75.

Fishelson, Z., G. Attali, and D. Mevorach. 2001. 'Complement and apoptosis', *Mol Immunol*, 38: 207-219.

Flanary, B. E., N. W. Sammons, C. Nguyen, D. Walker, and W. J. Streit. 2007. 'Evidence that aging and amyloid promote microglial cell senescence', *Rejuvenation Res*, 10: 61-74.

Fu, H., B. Liu, J. L. Frost, S. Hong, M. Jin, B. Ostaszewski, G. M. Shankar, I. M. Costantino, M. C. Carroll, T. N. Mayadas, and C. A. Lemere. 2012. 'Complement component C3 and complement receptor type 3 contribute to the phagocytosis and clearance of fibrillar Abeta by microglia', *Glia*, 60: 993-1003.

Fuger, P., J. K. Hefendehl, K. Veeraraghavalu, A. C. Wendeln, C. Schlosser, U. Obermuller, B. M. Wegenast-Braun, J. J. Neher, P. Martus, S. Kohsaka, M. Thunemann, R. Feil, S. S. Sisodia, A. Skodras, and M. Jucker. 2017. 'Microglia turnover with aging and in an Alzheimer's model via long-term in vivo single-cell imaging', *Nat Neurosci*, 20: 1371-1376.

Geinisman, Y., L. de Toledo-Morrell, and F. Morrell. 1986. 'Loss of perforated synapses in the dentate gyrus: morphological substrate of memory deficit in aged rats', *Proc Natl Acad Sci U S A*, 83: 3027-3031.

Geinisman, Y., L. deToledo-Morrell, F. Morrell, I. S. Persina, and M. Rossi. 1992. 'Age-related loss of axospinous synapses formed by two afferent systems in the rat dentate gyrus as revealed by the unbiased stereological dissector technique', *Hippocampus*, 2: 437-444.

Gemma, C., and A. D. Bachstetter. 2013. 'The role of microglia in adult hippocampal neurogenesis', *Front Cell Neurosci*, 7: 229.

Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov, and M. Merad. 2010. 'Fate mapping analysis reveals that adult microglia derive from primitive macrophages', *Science*, 330: 841-845.

Ginhoux, F., S. Lim, G. Hoeffel, D. Low, and T. Huber. 2013. 'Origin and differentiation of microglia', *Front Cell Neurosci*, 7: 45.

Godbout, J. P., J. Chen, J. Abraham, A. F. Richwine, B. M. Berg, K. W. Kelley, and R. W. Johnson. 2005. 'Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system', *FASEB J*, 19: 1329-1331.

Graeber, M. B. 2010. 'Changing face of microglia', *Science*, 330: 783-8.

Griciuc, A., A. Serrano-Pozo, A. R. Parrado, A. N. Lesinski, C. N. Asselin, K. Mullin, B. Hooli, S. H. Choi, B. T. Hyman, and R. E. Tanzi. 2013. 'Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta', *Neuron*, 78: 631-643.

Griffin, R., R. Nally, Y. Nolan, Y. McCartney, J. Linden, and M. A. Lynch. 2006. 'The age-related attenuation in long-term potentiation is associated with microglial activation', *J Neurochem*, 99: 1263-1272.

Guerreiro, R., and J. Bras. 2015. 'The age factor in Alzheimer's disease', *Genome Med*, 7: 106.

Halliwell, B. 2001. 'Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment', *Drugs Aging*, 18: 685-716.

Hanisch, U. K., and H. Kettenmann. 2007. 'Microglia: active sensor and versatile effector cells in the normal and pathologic brain', *Nat Neurosci*, 10: 1387-1394.

Hanke, M. L., and T. Kielian. 2011. 'Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential', *Clin Sci (Lond)*, 121: 367-387.

Hashimoto, D., A. Chow, C. Noizat, P. Teo, M. B. Beasley, M. Leboeuf, C. D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S. W. Boyer, E. C. Forsberg, M. Tanaka, N. van Rooijen, A. Garcia-Sastre, E. R. Stanley, F. Ginhoux, P. S. Frenette, and M. Merad. 2013. 'Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes', *Immunity*, 38: 792-804.

Hayakawa, T., T. Angata, A. L. Lewis, T. S. Mikkelsen, N. M. Varki, and A. Varki. 2005. 'A human-specific gene in microglia', *Science*, 309: 1693.

Hayakawa, T., Z. Khedri, F. Schwarz, C. Landig, S. Y. Liang, H. Yu, X. Chen, N. T. Fujito, Y. Satta, A. Varki, and T. Angata. 2017. 'Coevolution of Siglec-11 and Siglec-16 via gene conversion in primates', *BMC Evol Biol*, 17: 228.

Heshmati-Fakhr, N., F. Sotoodehnejadnematalahi, N. Yousefi, M. Sayyah, S. M. Hosseini, and H. G. Pourbadie. 2018. 'Triggering microglia through toll-like receptor 2 pathway induced interferon beta expression in cell and animal model of Alzheimer's disease', *Neuroreport*, 29: 1456-1462.

Holst, S., A. J. Deuss, G. W. van Pelt, S. J. van Vliet, J. J. Garcia-Vallejo, C. A. Koeleman, A. M. Deelder, W. E. Mesker, R. A. Tollenaar, Y. Rombouts, and M. Wuhrer. 2016. 'N-glycosylation Profiling of Colorectal Cancer Cell Lines Reveals Association of Fucosylation with Differentiation and Caudal Type Homebox 1 (CDX1)/Villin mRNA Expression', *Mol Cell Proteomics*, 15: 124-140.

Hopp, S. C., Y. Lin, D. Oakley, A. D. Roe, S. L. DeVos, D. Hanlon, and B. T. Hyman. 2018. 'The role of microglia in processing and spreading of bioactive tau seeds in Alzheimer's disease', *J Neuroinflammation*, 15: 269.

Hovens, Iris Bertha, Csaba Nyakas, and Regien Geertruida Schoemaker. 2014. 'A novel method for evaluating microglial activation using ionized calcium-binding adaptor protein-1 staining: cell body to cell size ratio', *Neuroimmunology and Neuroinflammation*, 1: 82-88.

Hsieh, C. L., M. Koike, S. C. Spusta, E. C. Niemi, M. Yenari, M. C. Nakamura, and W. E. Seaman. 2009. 'A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia', *J Neurochem*, 109: 1144-56.

Ivashkiv, L. B. 2009. 'Cross-regulation of signaling by ITAM-associated receptors', *Nat Immunol*, 10: 340-347.

Jeong, Y. J., Y. Son, N. K. Han, H. D. Choi, J. K. Pack, N. Kim, Y. S. Lee, and H. J. Lee. 2018. 'Impact of Long-Term RF-EMF on Oxidative Stress and Neuroinflammation in Aging Brains of C57BL/6 Mice', *Int J Mol Sci*, 19.

Jurga, A. M., M. Paleczna, and K. Z. Kuter. 2020. 'Overview of General and Discriminating Markers of Differential Microglia Phenotypes', *Front Cell Neurosci*, 14: 198.

Kamphuis, W., M. Orre, L. Kooijman, M. Dahmen, and E. M. Hol. 2012. 'Differential cell proliferation in the cortex of the APPswePS1dE9 Alzheimer's disease mouse model', *Glia*, 60: 615-629.

Karlstetter, M., J. Kopatz, A. Aslanidis, A. Shahraz, A. Caramoy, B. Linnartz-Gerlach, Y. Lin, A. Luckoff, S. Fauser, K. Duker, J. Claude, Y. Wang, J. Ackermann, T. Schmidt, V. Hornung, C. Skerka, T. Langmann, and H. Neumann. 2017. 'Polysialic acid blocks mononuclear phagocyte reactivity, inhibits complement activation, and protects from vascular damage in the retina', *EMBO Mol Med*, 9: 154-166.

Kashyap, G., D. Bapat, D. Das, R. Gowaikar, R. E. Amritkar, G. Rangarajan, V. Ravindranath, and G. Ambika. 2019. 'Synapse loss and progress of Alzheimer's disease -A network model', *Sci Rep*, 9: 6555.

Kastner, A., E. C. Hirsch, O. Lejeune, F. Javoy-Agid, O. Rascol, and Y. Agid. 1992. 'Is the vulnerability of neurons in the substantia nigra of patients with Parkinson's disease related to their neuromelanin content?', *J Neurochem*, 59: 1080-1089.

Katsumoto, A., H. Takeuchi, K. Takahashi, and F. Tanaka. 2018. 'Microglia in Alzheimer's Disease: Risk Factors and Inflammation', *Front Neurol*, 9: 978.

Kaur, C., and E. A. Ling. 2009. 'Periventricular white matter damage in the hypoxic neonatal brain: role of microglial cells', *Prog Neurobiol*, 87: 264-280.

Kierdorf, K., D. Erny, T. Goldmann, V. Sander, C. Schulz, E. G. Perdiguero, P. Wieghofer, A. Heinrich, P. Riemke, C. Holscher, D. N. Muller, B. Luckow, T. Brocker, K. Debowski, G. Fritz, G. Opdenakker, A. Diefenbach, K. Biber, M. Heikenwalder, F. Geissmann, F. Rosenbauer, and M. Prinz. 2013. 'Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways', *Nat Neurosci*, 16: 273-280.

Koellhoffer, E. C., L. D. McCullough, and R. M. Ritzel. 2017. 'Old Maids: Aging and Its Impact on Microglia Function', *Int J Mol Sci*, 18.

Kooner, A. S., H. Yu, and X. Chen. 2019. 'Synthesis of N-Glycolylneuraminic Acid (Neu5Gc) and Its Glycosides', *Front Immunol*, 10: 2004.

Kouser, L., S. P. Madhukaran, A. Shastri, A. Saraon, J. Ferluga, M. Al-Mozaini, and U. Kishore. 2015. 'Emerging and Novel Functions of Complement Protein C1q', *Front Immunol*, 6: 317.

Krstic, D., and I. Knuesel. 2013. 'Deciphering the mechanism underlying late-onset Alzheimer disease', *Nat Rev Neurol*, 9: 25-34.

Lawson, L. J., V. H. Perry, P. Dri, and S. Gordon. 1990. 'Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain', *Neuroscience*, 39: 151-170.

Lee, K. Y., J. Y. Kang, J. I. Yun, J. Y. Chung, I. K. Hwang, M. H. Won, and J. H. Choi. 2017. 'Age-related change of Iba-1 immunoreactivity in the adult and aged gerbil spinal cord', *Anat Cell Biol*, 50: 135-142.

Lehnardt, S. 2010. 'Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury', *Glia*, 58: 253-63.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. 'The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults', *Cell*, 86: 973-983.

Liao, H., J. Winkler, J. Wissfeld, A. Shahraz, C. Klaus, and H. Neumann. 2021. 'Low molecular weight polysialic acid prevents lipopolysaccharide-induced inflammatory dopaminergic neurodegeneration in humanized SIGLEC11 transgenic mice', *Glia*, 69: 2845-2862.

Lin, C. H., Y. C. Yeh, and K. D. Yang. 2021. 'Functions and therapeutic targets of Siglec-mediated infections, inflammations and cancers', *J Formos Med Assoc*, 120: 5-24.

Linnartz-Gerlach, B., L. G. Bodea, C. Klaus, A. Ginolhac, R. Halder, L. Sinkkonen, J. Walter, M. Colonna, and H. Neumann. 2019. 'TREM2 triggers microglial density and age-related neuronal loss', *Glia*, 67: 539-550.

Linnartz-Gerlach, B., J. Kopatz, and H. Neumann. 2014. 'Siglec functions of microglia', *Glycobiology*, 24: 794-799.

Lively, Starlee, and Lyanne C. Schlichter. 2018. 'Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNγ+TNFα) and Reprogramming by Resolving Cytokines (IL-4, IL-10)', *Frontiers in Cellular Neuroscience*, 12.

Loera-Valencia, R., A. Cedazo-Minguez, P. A. Kenigsberg, G. Page, A. I. Duarte, P. Giusti, M. Zusso, P. Robert, G. B. Frisoni, A. Cattaneo, M. Zille, J. Boltze, N. Cartier, L. Buee, G. Johansson, and B. Winblad. 2019. 'Current and emerging avenues for Alzheimer's disease drug targets', *J Intern Med*, 286: 398-437.

Lubbers, J., E. Rodriguez, and Y. van Kooyk. 2018. 'Modulation of Immune Tolerance via Siglec-Sialic Acid Interactions', *Front Immunol*, 9: 2807.

Machado, V., T. Zoller, A. Attaai, and B. Spittau. 2016. 'Microglia-Mediated Neuroinflammation and Neurotrophic Factor-Induced Protection in the MPTP Mouse Model of Parkinson's Disease-Lessons from Transgenic Mice', *Int J Mol Sci*, 17.

Marin-Teva, J. L., I. Dusart, C. Colin, A. Gervais, N. van Rooijen, and M. Mallat. 2004. 'Microglia promote the death of developing Purkinje cells', *Neuron*, 41: 535-47.

Marsters, C. M., D. Nesan, R. Far, N. Klenin, Q. J. Pittman, and D. M. Kurrasch. 2020. 'Embryonic microglia influence developing hypothalamic glial populations', *J Neuroinflammation*, 17: 146.

Mattson, M. P., and T. Magnus. 2006. 'Ageing and neuronal vulnerability', *Nat Rev Neurosci*, 7: 278-294.

McGrath, L. T., B. M. McGleenon, S. Brennan, D. McColl, ILroy S. Mc, and A. P. Passmore. 2001. 'Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde', *QJM*, 94: 485-90.

McWilliams, J. R., and G. Lynch. 1984. 'Synaptic density and axonal sprouting in rat hippocampus: stability in adulthood and decline in late adulthood', *Brain Res*, 294: 152-156.

Metzler-Baddeley, C., J. P. Mole, R. Sims, F. Fasano, J. Evans, D. K. Jones, J. P. Aggleton, and R. J. Baddeley. 2019. 'Fornix white matter glia damage causes hippocampal gray matter damage during age-dependent limbic decline', *Sci Rep*, 9: 1060.

Miller, R. J., W. Rostene, E. Apartis, G. Banisadr, K. Biber, E. D. Milligan, F. A. White, and J. Zhang. 2008. 'Chemokine action in the nervous system', *J Neurosci*, 28: 11792-11795.

Ming, G. L., and H. Song. 2011. 'Adult neurogenesis in the mammalian brain: significant answers and significant questions', *Neuron*, 70: 687-702.

Mócsai, Attila, Clare L. Abram, Zoltán Jakus, Yongmei Hu, Lewis L. Lanier, and Clifford A. Lowell. 2006. 'Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs', *Nature Immunology*, 7: 1326-1333.

Monje, M. L., H. Toda, and T. D. Palmer. 2003. 'Inflammatory blockade restores adult hippocampal neurogenesis', *Science*, 302: 1760-1765.

Moore, Z., J. M. Taylor, and P. J. Crack. 2019. 'The involvement of microglia in Alzheimer's disease: a new dog in the fight', *Br J Pharmacol*, 176: 3533-3543.

Moreno-Garcia, A., A. Kun, O. Calero, M. Medina, and M. Calero. 2018. 'An Overview of the Role of Lipofuscin in Age-Related Neurodegeneration', *Front Neurosci*, 12: 464.

Mori, T., A. Buffo, and M. Gotz. 2005. 'The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis', *Curr Top Dev Biol*, 69: 67-99.

Morisato, D., and K. V. Anderson. 1994. 'The spatzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo', *Cell*, 76: 677-688.

Morrison, J. H., and M. G. Baxter. 2012. 'The ageing cortical synapse: hallmarks and implications for cognitive decline', *Nat Rev Neurosci*, 13: 240-250.

Mortera, P., and S. Herculano-Houzel. 2012. 'Age-related neuronal loss in the rat brain starts at the end of adolescence', *Front Neuroanat*, 6: 45.

Mosher, K. I., and T. Wyss-Coray. 2014. 'Microglial dysfunction in brain aging and Alzheimer's disease', *Biochem Pharmacol*, 88: 594-604.

Nemeth, M. J., A. P. Cline, S. M. Anderson, L. J. Garrett-Beal, and D. M. Bodine. 2005. 'Hmgb3 deficiency deregulates proliferation and differentiation of common lymphoid and myeloid progenitors', *Blood*, 105: 627-634.

Nemeth, M. J., D. J. Curtis, M. R. Kirby, L. J. Garrett-Beal, N. E. Seidel, A. P. Cline, and D. M. Bodine. 2003. 'Hmgb3: an HMG-box family member expressed in primitive hematopoietic cells that inhibits myeloid and B-cell differentiation', *Blood*, 102: 1298-1306.

Ng, H. H., P. Jeppesen, and A. Bird. 2000. 'Active repression of methylated genes by the chromosomal protein MBD1', *Mol Cell Biol*, 20: 1394-1406.

Niki, E. 2009. 'Lipid peroxidation: physiological levels and dual biological effects', *Free Radic Biol Med*, 47: 469-484.

Nishida, N., T. Walz, and T. A. Springer. 2006. 'Structural transitions of complement component C3 and its activation products', *Proc Natl Acad Sci U S A*, 103: 19737-19742.

Noda, S., S. Sato, T. Fukuda, N. Tada, and N. Hattori. 2020. 'Aging-related motor function and dopaminergic neuronal loss in C57BL/6 mice', *Mol Brain*, 13: 46.

Norden, D. M., and J. P. Godbout. 2013. 'Review: microglia of the aged brain: primed to be activated and resistant to regulation', *Neuropathol Appl Neurobiol*, 39: 19-34.

O'Neil, S. M., K. G. Witcher, D. B. McKim, and J. P. Godbout. 2018. 'Forced turnover of aged microglia induces an intermediate phenotype but does not rebalance CNS

environmental cues driving priming to immune challenge', *Acta Neuropathol Commun*, 6: 129.

O'Neill, A. S., T. K. van den Berg, and G. E. Mullen. 2013. 'Sialoadhesin - a macrophage-restricted marker of immunoregulation and inflammation', *Immunology*, 138: 198-207.

Olson, J. K., and S. D. Miller. 2004. 'Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs', *J Immunol*, 173: 3916-3924.

Paasila, P. J., D. S. Davies, G. T. Sutherland, and C. Goldsbury. 2020. 'Clustering of activated microglia occurs before the formation of dystrophic neurites in the evolution of Abeta plaques in Alzheimer's disease', *Free Neuropathol*, 1.

Pakkenberg, B., and H. J. Gundersen. 1997. 'Neocortical neuron number in humans: effect of sex and age', *J Comp Neurol*, 384: 312-320.

Panchision, D. M., J. M. Pickel, L. Studer, S. H. Lee, P. A. Turner, T. G. Hazel, and R. D. McKay. 2001. 'Sequential actions of BMP receptors control neural precursor cell production and fate', *Genes Dev*, 15: 2094-2110.

Pandya, V. A., and R. Patani. 2021. 'Region-specific vulnerability in neurodegeneration: lessons from normal ageing', *Ageing Res Rev*, 67: 101311.

Paolicelli, R. C., G. Bolasco, F. Pagani, L. Maggi, M. Scianni, P. Panzanelli, M. Giustetto, T. A. Ferreira, E. Guiducci, L. Dumas, D. Ragozzino, and C. T. Gross. 2011. 'Synaptic pruning by microglia is necessary for normal brain development', *Science*, 333: 1456-1458.

Parakalan, R., B. Jiang, B. Nimmi, M. Janani, M. Jayapal, J. Lu, S. S. Tay, E. A. Ling, and S. T. Dheen. 2012. 'Transcriptome analysis of amoeboid and ramified microglia isolated from the corpus callosum of rat brain', *BMC Neurosci*, 13: 64.

Parhizkar, S., T. Arzberger, M. Brendel, G. Kleinberger, M. Deussing, C. Focke, B. Nuscher, M. Xiong, A. Ghasemigharagoz, N. Katzmarski, S. Krasemann, S. F. Lichtenthaler, S. A. Muller, A. Colombo, L. S. Monasor, S. Tahirovic, J. Herms, M. Willem, N. Pettkus, O. Butovsky, P. Bartenstein, D. Edbauer, A. Rominger, A. Erturk, S. A. Grathwohl, J. J. Neher, D. M. Holtzman, M. Meyer-Luehmann, and C. Haass. 2019. 'Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE', *Nat Neurosci*, 22: 191-204.

Perdiguero, E. G., K. Klapproth, C. Schulz, K. Busch, M. de Bruijn, H. R. Rodewald, and F. Geissmann. 2015. 'The Origin of Tissue-Resident Macrophages: When an Erythro-myeloid Progenitor Is an Erythro-myeloid Progenitor', *Immunity*, 43: 1023-1024.

Perry, V. H., C. Cunningham, and C. Holmes. 2007. 'Systemic infections and inflammation affect chronic neurodegeneration', *Nat Rev Immunol*, 7: 161-167.

Perry, V. H., M. K. Matyszak, and S. Fearn. 1993. 'Altered antigen expression of microglia in the aged rodent CNS', *Glia*, 7: 60-67.

Pillai, S., I. A. Netravali, A. Cariappa, and H. Mattoo. 2012. 'Siglecs and immune regulation', *Annu Rev Immunol*, 30: 357-392.

Pocock, J. M., and H. Kettenmann. 2007. 'Neurotransmitter receptors on microglia', *Trends Neurosci*, 30: 527-535.

Raj, T., K. J. Ryan, J. M. Replogle, L. B. Chibnik, L. Rosenkrantz, A. Tang, K. Rothamel, B. E. Stranger, D. A. Bennett, D. A. Evans, P. L. De Jager, and E. M. Bradshaw. 2014.

'CD33: increased inclusion of exon 2 implicates the Ig V-set domain in Alzheimer's disease susceptibility', *Hum Mol Genet*, 23: 2729-2736.

Ravetch, J. V., and L. L. Lanier. 2000. 'Immune inhibitory receptors', *Science*, 290: 84-9.

Rawal, P., and L. Zhao. 2021. 'Sialometabolism in Brain Health and Alzheimer's Disease', *Front Neurosci*, 15: 648617.

Reichwald, J., S. Danner, K. H. Wiederhold, and M. Staufenbiel. 2009. 'Expression of complement system components during aging and amyloid deposition in APP transgenic mice', *J Neuroinflammation*, 6: 35.

Rio-Hortega, PioDel. 1939. 'THE MICROGLIA', *The Lancet*, 233: 1023-1026.

Rogers, J., R. Li, D. Mastroeni, A. Grover, B. Leonard, G. Ahern, P. Cao, H. Kolody, L. Vedders, W. P. Kolb, and M. Sabbagh. 2006. 'Peripheral clearance of amyloid beta peptide by complement C3-dependent adherence to erythrocytes', *Neurobiol Aging*, 27: 1733-1739.

Rubovitch, V., Y. Zilberstein, J. Chapman, S. Schreiber, and C. G. Pick. 2017. 'Restoring GM1 ganglioside expression ameliorates axonal outgrowth inhibition and cognitive impairments induced by blast traumatic brain injury', *Sci Rep*, 7: 41269.

Safaiyan, S., N. Kannaiyan, N. Snaidero, S. Brioschi, K. Biber, S. Yona, A. L. Edinger, S. Jung, M. J. Rossner, and M. Simons. 2016. 'Age-related myelin degradation burdens the clearance function of microglia during aging', *Nat Neurosci*, 19: 995-8.

Saijo, K., and C. K. Glass. 2011. 'Microglial cell origin and phenotypes in health and disease', *Nat Rev Immunol*, 11: 775-787.

Salminen, A., and K. Kaarniranta. 2009. 'Siglec receptors and hiding plaques in Alzheimer's disease', *J Mol Med (Berl)*, 87: 697-701.

Schafer, D. P., E. K. Lehrman, A. G. Kautzman, R. Koyama, A. R. Mardinly, R. Yamasaki, R. M. Ransohoff, M. E. Greenberg, B. A. Barres, and B. Stevens. 2012. 'Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner', *Neuron*, 74: 691-705.

Schafer, D. P., and B. Stevens. 2013. 'Phagocytic glial cells: sculpting synaptic circuits in the developing nervous system', *Curr Opin Neurobiol*, 23: 1034-1040.

Schartz, N. D., and A. J. Tenner. 2020. 'The good, the bad, and the opportunities of the complement system in neurodegenerative disease', *J Neuroinflammation*, 17: 354.

Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S. E. Jacobsen, J. W. Pollard, J. Frampton, K. J. Liu, and F. Geissmann. 2012. 'A lineage of myeloid cells independent of Myb and hematopoietic stem cells', *Science*, 336: 86-90.

Schwarz, F., C. S. Landig, S. Siddiqui, I. Secundino, J. Olson, N. Varki, V. Nizet, and A. Varki. 2017. 'Paired Siglec receptors generate opposite inflammatory responses to a human-specific pathogen', *EMBO J*, 36: 751-760.

Schwarz, F., O. M. Pearce, X. Wang, A. N. Samraj, H. Laubli, J. O. Garcia, H. Lin, X. Fu, A. Garcia-Bingman, P. Secrest, C. E. Romanoski, C. Heyser, C. K. Glass, S. L. Hazen, N. Varki, A. Varki, and P. Gagneux. 2015. 'Siglec receptors impact mammalian lifespan by modulating oxidative stress', *Elife*, 4.

Sessa, G., P. Podini, M. Mariani, A. Meroni, R. Spreafico, F. Sinigaglia, M. Colonna, P. Panina, and J. Meldolesi. 2004. 'Distribution and signaling of TREM2/DAP12, the

receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia', *Eur J Neurosci*, 20: 2617-2628.

Siddiqui, S. S., R. Matar, M. Merheb, R. Hodeify, C. G. Vazhappilly, J. Marton, S. A. Shamsuddin, and H. Al Zouabi. 2019. 'Siglecs in Brain Function and Neurological Disorders', *Cells*, 8.

Sierra, A., A. C. Gottfried-Blackmore, B. S. McEwen, and K. Bulloch. 2007. 'Microglia derived from aging mice exhibit an altered inflammatory profile', *Glia*, 55: 412-424.

Simpson, D. S. A., and P. L. Oliver. 2020. 'ROS Generation in Microglia: Understanding Oxidative Stress and Inflammation in Neurodegenerative Disease', *Antioxidants (Basel)*, 9.

Singh Kushwaha, S., N. Patro, and I. Kumar Patro. 2018. 'A Sequential Study of Age-Related Lipofuscin Accumulation in Hippocampus and Striate Cortex of Rats', *Ann Neurosci*, 25: 223-233.

Sipione, S., J. Monyror, D. Galleguillos, N. Steinberg, and V. Kadam. 2020. 'Gangliosides in the Brain: Physiology, Pathophysiology and Therapeutic Applications', *Front Neurosci*, 14: 572965.

Sosinowski, T., A. Pandey, V. M. Dixit, and A. Weiss. 2000. 'Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling', *J Exp Med*, 191: 463-474.

Stratoulias, V., J. L. Venero, M. E. Tremblay, and B. Joseph. 2019. 'Microglial subtypes: diversity within the microglial community', *EMBO J*, 38: e101997.

Streit, W. J. 2002. 'Microglia as neuroprotective, immunocompetent cells of the CNS', *Glia*, 40: 133-139.

Streit, W. J., N. W. Sammons, A. J. Kuhns, and D. L. Sparks. 2004. 'Dystrophic microglia in the aging human brain', *Glia*, 45: 208-212.

Strohmeyer, R., M. Ramirez, G. J. Cole, K. Mueller, and J. Rogers. 2002. 'Association of factor H of the alternative pathway of complement with agrin and complement receptor 3 in the Alzheimer's disease brain', *J Neuroimmunol*, 131: 135-146.

Sudmant, P. H., H. Lee, D. Dominguez, M. Heiman, and C. B. Burge. 2018. 'Widespread Accumulation of Ribosome-Associated Isolated 3' UTRs in Neuronal Cell Populations of the Aging Brain', *Cell Rep*, 25: 2447-2456 e4.

Szymanski, C. M., R. L. Schnaar, and M. Aebi. 2015. 'Bacterial and Viral Infections.' in rd, A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger (eds.), *Essentials of Glycobiology* (Cold Spring Harbor (NY)).

Takahashi, K., C. D. Rochford, and H. Neumann. 2005. 'Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2', *J Exp Med*, 201: 647-657.

Takamiya, R., K. Ohtsubo, S. Takamatsu, N. Taniguchi, and T. Angata. 2013. 'The interaction between Siglec-15 and tumor-associated sialyl-Tn antigen enhances TGF-beta secretion from monocytes/macrophages through the DAP12-Syk pathway', *Glycobiology*, 23: 178-187.

Tay, T. L., D. Mai, J. Dautzenberg, F. Fernandez-Klett, G. Lin, Sagar, M. Datta, A. Drougard, T. Stempfl, A. Ardura-Fabregat, O. Staszewski, A. Margineanu, A. Sporbert, L. M. Steinmetz, J. A. Pospisilik, S. Jung, J. Priller, D. Grun, O. Ronneberger, and M. Prinz.

2017. 'A new fate mapping system reveals context-dependent random or clonal expansion of microglia', *Nat Neurosci*, 20: 793-803.

Tay, T. L., J. C. Savage, C. W. Hui, K. Bisht, and M. E. Tremblay. 2017. 'Microglia across the lifespan: from origin to function in brain development, plasticity and cognition', *J Physiol*, 595: 1929-1945.

Tezel, G., X. Yang, C. Luo, A. D. Kain, D. W. Powell, M. H. Kuehn, and H. J. Kaplan. 2010. 'Oxidative stress and the regulation of complement activation in human glaucoma', *Invest Ophthalmol Vis Sci*, 51: 5071-5082.

Tran, P. B., and R. J. Miller. 2003. 'Chemokine receptors: signposts to brain development and disease', *Nat Rev Neurosci*, 4: 444-455.

Tremblay, M. E., R. L. Lowery, and A. K. Majewska. 2010. 'Microglial interactions with synapses are modulated by visual experience', *PLoS Biol*, 8: e1000527.

Trouw, L. A., A. M. Blom, and P. Gasque. 2008. 'Role of complement and complement regulators in the removal of apoptotic cells', *Mol Immunol*, 45: 1199-1207.

Vaillancourt, D. E., M. B. Spraker, J. Prodoehl, X. J. Zhou, and D. M. Little. 2012. 'Effects of aging on the ventral and dorsal substantia nigra using diffusion tensor imaging', *Neurobiol Aging*, 33: 35-42.

Varki, A., and T. Angata. 2006. 'Siglecs--the major subfamily of I-type lectins', *Glycobiology*, 16: 1R-27R.

Varki, A., and P. Gagneux. 2015. 'Biological Functions of Glycans.' in rd, A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger (eds.), *Essentials of Glycobiology* (Cold Spring Harbor (NY)).

Varki, A., R. Kannagi, B. Toole, and P. Stanley. 2015. 'Glycosylation Changes in Cancer.' in rd, A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger (eds.), *Essentials of Glycobiology* (Cold Spring Harbor (NY)).

Varki, A., R. L. Schnaar, and R. Schauer. 2015. 'Sialic Acids and Other Nonulosonic Acids.' in rd, A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar and P. H. Seeberger (eds.), *Essentials of Glycobiology* (Cold Spring Harbor (NY)).

von Bernhardi, R., L. Eugenin-von Bernhardi, and J. Eugenin. 2015. 'Microglial cell dysregulation in brain aging and neurodegeneration', *Front Aging Neurosci*, 7: 124.

von Leden, R. E., G. Khayrullina, K. E. Moritz, and K. R. Byrnes. 2017. 'Age exacerbates microglial activation, oxidative stress, inflammatory and NOX2 gene expression, and delays functional recovery in a middle-aged rodent model of spinal cord injury', *J Neuroinflammation*, 14: 161.

Wake, H., A. J. Moorhouse, and J. Nabekura. 2011. 'Functions of microglia in the central nervous system--beyond the immune response', *Neuron Glia Biol*, 7: 47-53.

Waller, R., L. Baxter, D. J. Fillingham, S. Coelho, J. M. Pozo, M. Mozumder, A. F. Frangi, P. G. Ince, J. E. Simpson, and J. R. Highley. 2019. 'Iba-1-/CD68+ microglia are a prominent feature of age-associated deep subcortical white matter lesions', *PLoS One*, 14: e0210888.

Wang, L. H., and S. M. Strittmatter. 1996. 'A family of rat CRMP genes is differentially expressed in the nervous system', *J Neurosci*, 16: 6197-6207.

Wang, X., and E. K. Michaelis. 2010. 'Selective neuronal vulnerability to oxidative stress in the brain', *Front Aging Neurosci*, 2: 12.

Wang, X., N. Mitra, P. Cruz, L. Deng, Nisc Comparative Sequencing Program, N. Varki, T. Angata, E. D. Green, J. Mullikin, T. Hayakawa, and A. Varki. 2012. 'Evolution of siglec-11 and siglec-16 genes in hominins', *Mol Biol Evol*, 29: 2073-86.

Wang, Y., and H. Neumann. 2010. 'Alleviation of neurotoxicity by microglial human Siglec-11', *J Neurosci*, 30: 3482-3488.

Warwick, C. A., A. L. Keyes, T. M. Woodruff, and Y. M. Usachev. 2021. 'The complement cascade in the regulation of neuroinflammation, nociceptive sensitization, and pain', *J Biol Chem*, 297: 101085.

West, M. J., P. D. Coleman, D. G. Flood, and J. C. Troncoso. 1994. 'Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease', *Lancet*, 344: 769-772.

Whitney, G., S. Wang, H. Chang, K. Y. Cheng, P. Lu, X. D. Zhou, W. P. Yang, M. McKinnon, and M. Longphre. 2001. 'A new siglec family member, siglec-10, is expressed in cells of the immune system and has signaling properties similar to CD33', *Eur J Biochem*, 268: 6083-6096.

Wissfeld, J., I. Nozaki, M. Mathews, T. Raschka, C. Ebeling, V. Hornung, O. Brustle, and H. Neumann. 2021. 'Deletion of Alzheimer's disease-associated CD33 results in an inflammatory human microglia phenotype', *Glia*, 69: 1393-1412.

Wong, A. M., N. V. Patel, N. K. Patel, M. Wei, T. E. Morgan, M. C. de Beer, W. J. de Villiers, and C. E. Finch. 2005. 'Macrosialin increases during normal brain aging are attenuated by caloric restriction', *Neurosci Lett*, 390: 76-80.

Yanguas-Casas, N., A. Crespo-Castrillo, M. A. Arevalo, and L. M. Garcia-Segura. 2020. 'Aging and sex: Impact on microglia phagocytosis', *Aging Cell*, 19: e13182.

Yousef, G. M., M. H. Ordon, G. Foussias, and E. P. Diamandis. 2002. 'Genomic organization of the siglec gene locus on chromosome 19q13.4 and cloning of two new siglec pseudogenes', *Gene*, 286: 259-270.

Zabel, M. K., and W. M. Kirsch. 2013. 'From development to dysfunction: microglia and the complement cascade in CNS homeostasis', *Ageing Res Rev*, 12: 749-756.

Zanetta, J. P., A. Pons, M. Iwersen, C. Mariller, Y. Leroy, P. Timmerman, and R. Schauer. 2001. 'Diversity of sialic acids revealed using gas chromatography/mass spectrometry of heptafluorobutyrate derivatives', *Glycobiology*, 11: 663-676.

Zhang, H., and H. J. Forman. 2017. '4-hydroxynonenal-mediated signaling and aging', *Free Radic Biol Med*, 111: 219-225.

Zhang, M., T. Angata, J. Y. Cho, M. Miller, D. H. Broide, and A. Varki. 2007. 'Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils', *Blood*, 109: 4280-7.

Zhou, X., G. Yang, and F. Guan. 2020. 'Biological Functions and Analytical Strategies of Sialic Acids in Tumor', *Cells*, 9.

Zoller, T., A. Attaai, P. S. Potru, T. Russ, and B. Spittau. 2018. 'Aged Mouse Cortical Microglia Display an Activation Profile Suggesting Immunotolerogenic Functions', *Int J Mol Sci*, 19.

Zou, W., H. Kitaura, J. Reeve, F. Long, V. L. Tybulewicz, S. J. Shattil, M. H. Ginsberg, F. P. Ross, and S. L. Teitelbaum. 2007. 'Syk, c-Src, the alphavbeta3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption', *J Cell Biol*, 176: 877-888.

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