The role of myeloid cells in neurodegenerative diseases. Studies on cellular phenotype and communication.

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The role of myeloid cells in neurodegenerative diseases

Studies on cellular phenotype and communication

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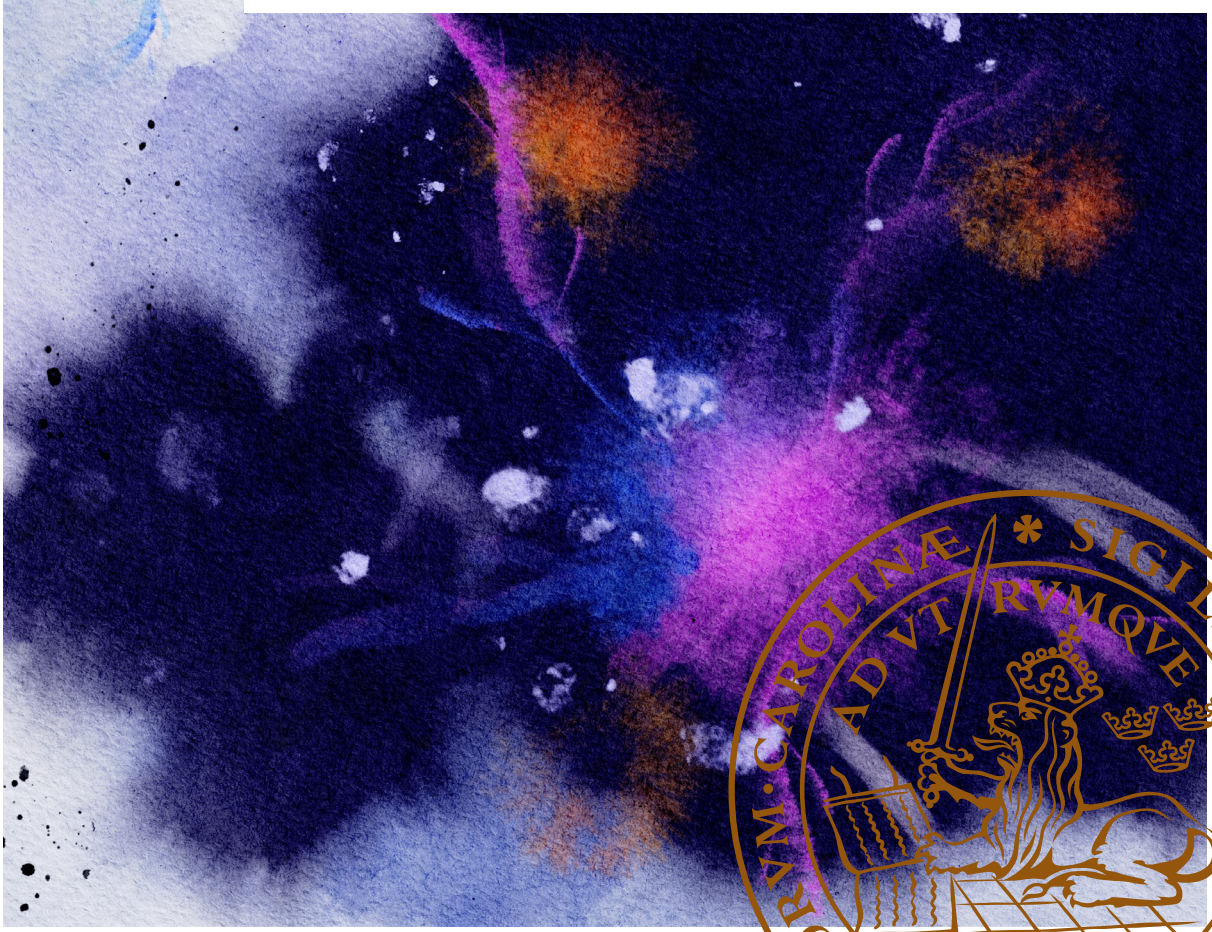

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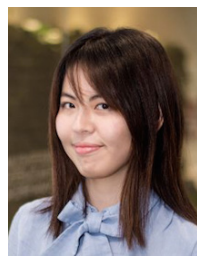

Born and raised in China. I moved to Sweden in 2012 for the master's program in biotechnology at Lund University and started my PhD study with neuroscience in 2015. My research is about the characterization of microglia in neurodegenerative diseases, such as Alzheimer's disease (AD). I am also interested in the cross-talk between microglia and the peripheral immune system. The value of my work may provide potential molecular targets for early diagnostics and pharmacological intervention to prevent the progression of AD.

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Yiyi Yang

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Abstract

Neurological disorders are listed as the second leading cause of death worldwide. Stroke and Alzheimer's disease (AD) are the major contributors to neurological disorders of deaths. Every year, around 14 million new people are affected by stoke and nearly 10 million new cases of AD are diagonosed worldwide. The number of deaths and disabilities due to these disorders have increased considerably. Neuroinflammation is a major pathological feature appearing in the central nervous system (CNS) in these disorders. The central cellular components for neuroinflammation in CNS is microglia. The multifaceted roles of microglia under pathological conditions have been investigated by several research groups. Even if the knowledge about these cells is extensive, many aspects of their role in diseases have not been well elucidated. One of them is how microglia contribute to the pathology. Thus, I investigated how microglia communicate with other cells through extracellular vesicles (EVs) and how microglia cross-talk with the periphery under AD pathology.

Firstly, upon LPS activation of microglia, we found that larger sizes of EVs were released and the levels of proinflammatory cytokines, TNF and IL-6, were increased in EVs. The proteomic profile was shifted to ribosomal assembly and translation. Complete ablation of TNF expression alleviated the inflammation and lead to reduced EVs secretion in a mouse model of stroke, as well as *in vitro* with LPS stimulation.

Microglia are highly dynamic cells and can alter their phenotypes in response to diverse conditions. Therefore, we wanted to elucidate the activation profile of microglia under pathogenic conditions. In the early phase of AD pathogenesis, there is little known about how microglia contribute to the pathology. Thus, the 5xFAD mouse model of AD was used to investigate the effects before the formation of amyloid-β (Aβ) plaques in the CNS. Using a proteomic approach, early inflammatory activation of microglia was found before the formation of plaques.

Last but not least, we studied the cross-talk between CNS and periphery under Aβ pathology. There are conflicting findings from animal studies on the contribution of peripheral inflammation on AD due to the variations in the degree of inflammation, timepoint of the stimulation, and the duration. Therefore, we designed our study to have two timepoints wherein we modulate the innate immunity, one was at the pre-plaque stage and the other was at the postnatal stage. Strikingly, a reduced number of plaques was found in hippocampus accompanied by less microglial activation in 5xFAD mice 4.5 months after the peripheral LPS challenge during the pre-plaque state. Subsequently, single-cell sequencing demonstrated that the early inflammation at the postnatal phase affected microglia and bone marrow resident monocytes (BM-Mo) similar to how AD affects these cell types. Interestingly, unique subpopulations of microglia were identified that were enriched in genes associated with lysosomal function (Lyz2) and a detrimental inflammatory marker (Galectin-3). These particular subsets carried a BM-Mo-like phenotype and appeared in response to acute peripheral inflammation and chronic inflammation caused by AD. We also observed that some subsets of myeloid cells were provoked by Aβ pathology but not systemic inflammation and vice versa.

To summarize, microglia have multiple ways of contributing to the pathology of AD. Systemic inflammatory challenge can have pronounced effects on innate immunity in the CNS and the underlying mechanism remains to be elucidated. The findings of this thesis also provide potential candidates for use in early diagnostics and pharmacological targets to prevent the progression of AD.

Key words
Microglia, Neuroinflammation, Alzherimer's disease, Stroke, Innate immunity, Systemic inflammation, Monocytes, Single-cell sequencing

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Dynamic alterations of microglia (in dual colors) under β-amyloid pathology. β-amyloid plaques are in orange. The transition of microglia from resting to activation is indicated by changes from blue and green colors to pink and red colors.

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致我亲爱的女儿和我的家人

To my little daughter, Wei-yi Xu, and my family

路漫漫其修远兮，吾将上下而求索。——屈原《离骚》

I sit at my window this morning where the world
like a passer-by stops for a moment, nods to me and goes.
Rabindranath Tagore, Stray Birds

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List of publications

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Manuscript

IV. **Postnatal LPS injection induces a unique phenotype of myeloid cells in the adulthood associated with amyloid-beta pathology.** Yiyi Yang, Sara Bachiller, Bazhena Bahatyrevich-Kharitonik, Roy Francis, Tomas Deierborg.
Manuscript

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*Equal contribution to the paper

Abbreviations

AD	Alzheimer's disease
CNS	central nervous system
BM-Mo	bone marrow resident monocytes
MRI	resonance imaging
CT	computed tomography
NO	nitric oxide
APP	amyloid precursor protein
Aβ	amyloid-β
NFT	neurofibrillary tangles
AICD	APP intracellular domain
PET	positron emission tomography
PSEN1	presenilin-1
PSEN2	presenilin-2
APOE	apolipoprotein E
TREM2	triggering receptor expressed on myeloid cells 2
MCI	mild cognitive impairment
CVD	cerebrovascular disease
NK	natural killer
ROS	reactive oxygen species
TNF	tumor necrosis factor
MS	multiple sclerosis
ALS	amyotrophic lateral sclerosis
EMP	erythromyeloid progenitor
CSF1R	macrophage colony-stimulating factor 1 receptor
TFG-β	transforming growth factor- β
IL	interleukin
CX3CL1	CX3C chemokine ligand 1
CX3CR1	CX3C chemokine receptor 1
CD	Cluster of differentiation
PRRs	pathogen recognition receptors
DAMPs	danger associated molecular patterns
PAMPs	pathogen associated molecular patterns
TLRs	Toll-like receptors
NF-κB	nuclear factor κB

IRFs	interferon regulatory factor
EVs	extracellular vesicles
ILVs	intraluminal vesicles
MVB	multivesicular body
ESCRT	endosomal sorting complex required for transport
MHC	major histocompatibility class
iNOS	inducible nitric oxide synthase
IGF-1	insulin-like growth factor 1
MS4A	membrane-spanning 4-domains subfamily A
CAM	CNS-associated macrophages
CLEC7A	C-Type Lectin Domain Containing 7A
SPP1	secreted Phosphoprotein 1
DAM	disease-associated microglia’
MGnD	microglia neurodegenerative phenotype
TMEM 119	transmembrane protein 119
P2RY12	purinergic receptor P2Y
HEXB	hexosaminidase subunit β
LPL	lipoprotein lipase
ITGAX	complement component 3 receptor 4 subunit
TYROBP	transmembrane immune signaling adaptor
CTSD	cathepsin D
HIF1	hypoxia-inducible factor 1
CMP	common myeloid progenitor
BM	bone marrow
cMoP	common monocyte progenitor
CXCR4	C-X-C motif chemokine receptor 4
CCR2	C-C motif chemokine receptor 2
BBB	blood-brain-barrier
ICAM	intercellular adhesion molecule
LPS	lipopolysaccharides
CR1	complement C3b/C4b receptor 1
PLCG2	phospholipase C gamma 2
BIN1	bridging integrator 1
RD1	retinal degeneration mutation 1
pMCAO	permanent middle cerebral artery occlusion
scRNA-seq	Single-cell sequencing
IHC	Immunohistochemistry
RSC	retrosplenial cortex
DG	dentate gyrus
solTNF	soluble TNF
tmTNF	transmembrane TNF

Popular summary

Brain disorders are the second leading cause of death worldwide. Among them, stroke and Alzheimer's disease (AD) are the main contributors. Every year, around 14 million new people are affected by stroke and nearly 10 million new cases of AD are diagnosed (WHO 2020). The number of deaths and disabilities due to these disorders have increased considerably in the past decades and it is estimated to continuously rise globally. Therefore, it is urgent to develop effective therapies for these diseases, especially AD as there is currently no effective treatment available.

Neuroinflammation is an inflammation in the brain. It is an important feature of these brain diseases. Microglia play a central role in neuroinflammation. They are the only resident immune cells in the brain and act as guards, taking care of the brain under healthy conditions. However, these allies can become enemies in the brain as they are over-activated under disease conditions. The reactions of these harmful forms of microglia can start neuroinflammation. Likewise, over-activation of immune cells in the body may cause peripheral inflammation.

To be able to therapeutically treat these diseases, it is important to understand how neuroinflammation is amplified and triggered in the brain. Firstly, we investigated a previously undiscovered way of cell-cell communication via extracellular vesicles (EVs) in microglia. EVs are small vesicles spontaneously released by all types of cells. They can amplify the signals of the diseases and transport them over long distance. We found that under inflammatory conditions, larger sized EVs were released from microglia and the levels of inflammatory mediators were increased in those EVs. A better understanding of EVs could provide potential biomarkers for early diagnosis of AD. They also have the potential to be a means to specifically deliver drugs.

Secondly, to know when neuroinflammation is triggered in AD, we investigated the protein-profile of microglia before and after amyloid-β (Aβ) deposition. Aβ deposition is one of the hallmarkers in the AD brain causing neuroinflammation. We chose to use an AD mouse model (5xFAD) with high production of Aβ to study the starting point of neuroinflammation. We found the appearance of activated microglia was even before the presence of Aβ deposition.

Finally, we wanted to investigate how to prime the microglia in AD through a more convenient way as an alternative therapy. Microglia have the capability to respond faster and more effectively when they meet the stimuli a second time. This

phenomenon is defined as priming. The LPS, a major component on the cellular wall of bacteria, was introduced to the body of the mice as the first stimuli to prime microglia. And Aβ is regarded as the second stimuli. Two time-points were decided to prime the microglia, the postnatal stage and the adulthood before the appearance of Aβ deposition. Following, induction of acute peripheral inflammation by LPS in adult mice, we found a long-lasting influence on microglia. When we injected LPS in 5xFAD mice, we found a reduced activation of microglia and a decreased amount of Aβ load. Moreover, induction of an even earlier challenge with systemic LPS injection in the postnatal stage caused a stronger long-term effect on microglia. Transcriptomic analysis at single-cell level revealed a great diversity within the microglial population. After LPS induction, a unique group of microglia carried a similar signature as those affected by AD was found. This group of microglia may have a detrimental role in the progression of AD. More studies will be required to further verify the function of this microglial subpopulation.

Taken together, microglia seem to retain an immunological memory that may reduce the pathology but may also participate in the development of AD. These findings will help us to modulate microglia in a beneficial way to prevent the disease. More studies are required to validate our experimental findings in humans. A better understanding of the different functions of microglia in AD may provide potential molecular targets for early diagnostics and pharmacological intervention to prevent the progression of AD.

Populärvetenskaplig sammanfattning

Hjärnsjukdomar är den andra vanligaste dödsorsaken i världen. Bland dessa utgör stroke och Alzheimers sjukdom flest dödsfall. Årligen drabbas runt 14 miljoner personer av stroke och 10 miljoner insjuknar i Alzheimers sjukdom (WHO 2020). Antalet dödsfall och invalidiserade på grund av dessa sjukdomar har ökat de senaste decennierna. Detta gäller i synnerhet Alzheimers där framgångsrika behandlingar ännu saknas.

Neuroinflammation betyder inflammation i hjärnan. Tillståndet är ett viktigt inslag vid stroke och Alzheimers. Mikroglia är hjärnans främsta immuncell och de spelar en central roll för neuroinflammationen. Dessa celler är nervcellernas livvakter. Under friska förhållanden stöttar mikrogliacellerna hjärnans funktioner. Men vid sjukdom kan dessa livvakter överaktiveras och förvandlas till nervcellernas fiender. Tillsammans med andra sjukliga förändringar kan överaktiverade mikroglia orsaka neuroinflammation. Detsamma gäller även immunceller i övriga kroppen som kan orsaka perifer inflammation.

För att behandla dessa sjukdomar är det viktigt att förstå hur neuroinflammationen utlöses och sprids i hjärnan. Därför undersökte jag kommunikationen mellan celler via extracellulära vesiklar (EV) i mikroglia. EVs är som små bubblor som frigörs spontant av alla typer av celler. Dessa bubblor kan bära på molekyler som signalerar sjukdom när de sprids i kroppen. Vi upptäckte att större storlekar av EVs utsöndrades under inflammatoriska tillstånd. Dessutom bar dessa vesiklar på ökade nivåer av inflammatoriska signalmolekyler. En bättre förståelse för EVs skulle kunna ge potentiella biomarkörer för tidig diagnos av AD. De kan också användas som budbärare för att leverera läkemedel till specifika platser i kroppen.

För att ta reda på när neuroinflammationen startar vid Alzheimers undersökte vi proteinsammansättningen i mikroglia före och efter amyloid-beta(Aβ) -plack bildats i hjärnan. Ansamling av ihopklumpade så kallade Aβ-plack är typiskt hos patienter med sjukdomen och leder till neuroinflammation. För detta användes musmodellen 5xFAD. Dessa möss bär på fem mutationer som leder till högre nivåer av proteinet Aβ. I musmodellen aktiverades mikrogliacellerna redan innan dessa plack bildades.

När mikroglia stöter på samma hotfulla stimuli igen reagerar den snabbare, ett fenomen som kallas primning. Detta skulle kunna användas som en alternativ behandling vid Alzheimers sjukdom genom att mikrogliaceller triggas i ett tidigt skede för att sedan lättare aktiveras och skydda hjärnan när sjukliga Aβ-plack

bildats. Men att ingripa direkt i hjärnan är komplicerat. Därför ville jag i avhandlingens sista del undersöka om aktiveringen av mikroglia kan modifieras via det perifera immunsystemet i resten av kroppen. Lipopolysackarid (LPS) är en del av bakteriers cellvägg och kan användas som ett första stimuli för att trigga mikroglia. Därefter kan Aβ fungera som ett andra stimuli för att återaktivera mikroglia. Detta undersöktes genom att inducera inflammation i kroppen med LPS vid två olika tillfällen hos möss. I ena gruppen framkallades inflammationen redan när mössen var ungar och i den andra hos vuxna möss som ännu inte utvecklat Aβ-plack. Om inflammationen orsakades i vuxna möss ledde det till långvarig påverkan på mikrogliacellerna i hjärnan. Dessutom minskade mängden Aβ i musmodellen av Alzheimers sjukdom. I de fall där inflammationen inducerades redan hos ungarna påverkades mikroglia långvarigt i ännu större utsträckning, något som också ses i Alzheimersmössen. Nivåerna av olika genuttryck mättes i enskilda mikrogliaceller och visade att många olika typer av mikrogliaceller fanns i hjärnproverna från mössen. De möss som haft en kroppslig inflammation orsakad av LPS hade en speciell typ av mikrogliaceller som också fanns hos Alzheimersmössen. Denna typ av mikroglia kan ha en skadlig roll vid utvecklandet av Alzheimers. Därför behöver funktionen hos denna typ av mikrogliaceller undersökas vidare i framtida studier.

Sammantaget verkar mikroglia primas av LPS för att minska sjukliga förändringar och påverka utvecklingen av Alzheimers. Dessa resultat kan hjälpa oss att modulera immunsystemet på ett fördelaktigt sätt för att förhindra eller lindra sjukdomen. Fler studier krävs för att säkerställa att samma gäller hos människor. En bättre förståelse för olika funktioner hos mikroglia vid Alzheimers sjukdom kan ge potentiella kandidater för tidigare diagnos och farmakologiska angreppspunkter för att förhindra sjukdomsutvecklingen.

论文简介

脑部疾病（brain disorders）是全球第二大死因。其中，中风和阿尔茨海默症（又称老年痴呆症）占据了重大比例。据世界卫生组织的报告，全球每年仍有近 1400 万中风和 1000 万阿尔茨海默症新增病例，而这一数字还有持续上升的趋势。不幸的是，目前医学界对这些疾病尚无有效的治疗方法。

脑部疾病的一个重要特征是神经系统炎症 (Neuroinflammation)。作为大脑中唯一驻留的免疫细胞，小胶质细胞 (microglia) 在神经炎症中起到了重要的作用。在健康状态下，这些细胞充当守卫，维护着大脑的正常运作。然而在病理情况下，这些过度活跃的“守卫”，就会变为大脑的敌人，引起神经炎症。同样的，体内其他免疫细胞的过度活化也可能导致大脑以外部分的炎症。

了解神经炎症的传播机制与触发对治疗此类疾病尤为重要。首先，我们对胞外小泡（extracellular vesicles，EVs）在小胶质细胞中的作用进行了研究。胞外小泡是最近被发现的胞间通讯的新途径之一。任何类型的细胞都可自发释放胞外小泡。它们可携带疾病信号，并在体内远距离、扩大传播。我们发现在炎症作用下相对较大的胞外小泡会从小胶质细胞中释放，并且这些小泡中炎症因子的水平也有所增加。对胞外小泡的深入研究可以为阿尔茨海默症的早期诊断提供生物标志物。而且胞外小泡还具有成为靶向运送药物载体的潜力。

其次，为了研究神经炎症是如何触发的，我们分析了 β 淀粉样蛋白沉积前后小胶质细胞的蛋白图谱。β 淀粉样蛋白的沉积是阿尔茨海默症中引起神经炎症的典型标志之一。我们决定采用可产生大量β淀粉样蛋白的小鼠模型（5xFAD）作为研究对象，并观察到在β淀粉样蛋白沉积之前，小胶质细胞中就已出现了大量炎症相关蛋白的表达。

最后，我们阐述了如何通过外周免疫系统调节小胶质细胞的活化，作为更为简单方便的阿尔兹海默症疗法。我们了解到当小胶质细胞在第二次遇到相同或类似的刺激时能够做出更快更有效的免疫反应，被称为启动效应（Priming）。我们通过对 5xFAD 老鼠模型注射 LPS (一种存在于革兰氏阴性细菌外膜的脂多糖)来触发启动效应，用以观察小胶质细胞对 β 淀粉样蛋白吞噬作用的影响。我们选择了两个不同的时间点对 5xFAD 小鼠进行测试，即

幼儿期和β淀粉样蛋白未沉积时的成年期。四个半月后，在注射了脂多糖的成年 5xFAD 小鼠中，我们发现小胶质细胞的炎症反应降低了并伴随着β淀粉样蛋白积累的减少。此外，在六个月大的 5xFAD 小鼠中，我们发现更为早期的 LPS 诱导对小胶质细胞的影响更大。单细胞高通量转录组分析显示大脑中的小胶质细胞存在着丰富的多样性。我们发现了一组独特的小胶质细胞，这组小胶质细胞出现在幼儿期被 LPS 诱导后的成年老鼠中。它们与未经诱导的阿尔茨海默症成年小鼠中的一组小胶质细胞有相似特征，这些特征可能使其对阿尔茨海默症的发展具有推动作用。我们计划在之后的实验中继续验证这组小胶质细胞的功能。

综上所述，小胶质细胞可通过启动效应减少β淀粉样蛋白的沉积。这些发现将有助于我们通过调节小胶质细胞，来达到治疗疾病的目的。深入了解小胶质细胞在阿尔兹海默症中的不同功能，可为阿尔兹海默症的早期诊断提供新的思路，并为延缓疾病进展提供潜在的药物靶点。

Context of this thesis

I came to Sweden in August 2012 for the master's program in biotechnology at Lund University. At that time, I was a student at Faculty of Engineer (LTH). Afterwards, I became interested in medical research generally and was enrolled in a Ph.D. preparatory program for stem cell biology, at Faculty of Medicine in my second year of the master's education. It provided me a great opportunity to learn flow cytometry, which later was extensively applied in the studies included in this thesis.

I started my Ph.D. project with my supervisor, Tomas Deierborg, in 2015 to characterize the different phenotypes of microglia upon inflammatory stimulation. We wanted to investigate the role of microglia in neurodegenerative diseases as a broad topic with specific aims related to how microglia communicate with other cells.

In the first study, as EVs emerged as a novel pathway for cellular communication and were demonstrated to be involved in the aggregation of misfolded proteins and spreading of the pathology, in collaboration with Christopher Dunning, we investigated the effects of inflammation on the dynamics of EVs released from microglia (Paper I). Firstly, I tried to isolate EVs by a novel label-free technology developed by Thomas Laurell and his group, called acoustophoresis and acoustic trapping. However, due to the low reproducibility and still ongoing development of the technology, we decided to use a traditional ultracentrifugation instead. And LPS was applied as a standard method to activated the microglia to study certain aspects.

Next, we wanted to have more clinically relevant studies related to neuroinflammation, so we focused on Alzheimer's disease (AD) as it is one of the major neurodegenerative diseases and involves neuroinflammation. A mouse model of AD is a suitable choice to study experimentally different aspects of microglial activation. With this, we sought to elucidate the pathogenesis of sporadic form of AD and how the neuroinflammation contributes to it. Thus, we initiated a study looking at early alterations of microglia in a mouse model of AD (Paper II). In parallel, I got the chance to work with my colleague Antonio on a project using the same model to look at how galectin-3 (Gal-3) expressed in microglia contributed to the AD pathology. As a result, I gained more experience with experimental skills and a better understanding of the pathology in this model. And Gal-3 was later used as a marker for the activation of microglia (**Paper II and III**).

These findings made us to think that the early alterations in microglia may initiate detrimental effects in the central nervous system (CNS) and periphery and may even accelerate the progression of the diseases. To answer this question, the following two studies were proposed to evaluate the effects of LPS-raised inflammation at different stage of life on myeloid cells. There are conflicting findings from animal studies on the contribution of peripheral inflammation on AD due to different experimental setups. Therefore, we designed the study to have two timepoints for modulating innate immunity, one was at the pre-plaque stage (**Paper III**) and the other was at the postnatal stage (**Paper IV**).

Overall, these findings in my thesis can be viewed as an effort to investigate the inflammatory response of microglia under different aspects-one is LPS-triggered inflammation and the other is Aβ-caused chronic inflammation. The general aims of my work were to promote a better understanding of the different roles of microglia and monocytes in the progression of AD and to help develop a potential treatment for neurological disorders.

Introduction

In this part, I will first describe the pathology of neurological disorders, including ischemic stroke (Paper I) and Alzheimer's disease (AD) (Papers II, III, and IV), that I investigated in my projects, of which AD will be emphasized. Following this, I will focus on myeloid cells, microglia from brain and monocytes from periphery as key players in AD.

Neurological disorders

Neurological disorders are diseases that affect the central nervous system (CNS) and peripheral nervous system. The symptoms of these disorders vary significantly, ranging from emotional disorders, such as depression and delusions, to physical disability, including paralysis, seizures, muscle weakness, as well as loss of memory and sensation. The number of deaths and the number of people with a disability due to these disorders have increased considerably in the past decades, and it is estimated to continue to rise globally. Neurological disorders were listed as the second leading cause of death worldwide[1]. Among the different disorders, stroke was the major contributor to neurological causes of death, accounting for 64.4% of deaths, followed by Alzheimer's disease and other dementias (27.8%)[2].

Ischemic stroke

Ischemic stroke, the most common form of stroke, has been estimated to have around 10 million new cases each year with about 50% mortality rate[3–5] Men have a higher risk (52% of all strokes) than women (48% of all strokes) experiencing ischemic stroke[6]. A comprehensive epidemiological study across different countries implicated several potential modifiable risk factors, such as hypertension, physical training, smoking, alcohol consumption and diabetes[7]. Ischemic stroke is caused by a reduction of blood flow to a region of the brain, leading to the activation of various cascades and neuronal damage[8]. In just a few hours after onset, patients with ischemic stroke often manifest an infarct core, an irreversible impaired area, surrounded by a possibly recovered, damaged area called the penumbra[9]. Magnetic resonance imaging (MRI) and computed tomography (CT) have been developed to better assess the lesion in ischemic patients[10].

The underlying mechanisms behind ischemic stroke injury have been extensively studied. Various components are involved in the injury, including excitotoxicity, oxidative stress and inflammation[11]. Excitotoxicity is caused by the elevation of excitatory neurotransmitters, such as glutamate, which initiates over-activation of N-methyl-d-aspartate receptors, α-amino-3-hydroxy-5-methyl-4-propionate receptors or kainite receptors[11]. These receptors then facilitate a large influx of Ca^{2+} and Na^{+}, causing cellular death[12,13]. The process of restoring blood flow after the event, referred to ischemia-reperfusion, triggers the generation of superoxide and nitric oxide (NO) in the impaired neurons[14]. Last but not least, inflammation takes place due to dying cells and debris. Inflammation in ischemic stroke is mainly mediated by the activation of innate immunity in the CNS[15], which will be described in detail in the **Innate immunity** section. Meanwhile, inflammatory molecules released in response to cerebral ischemia disrupt the blood-brain barrier. This allows for the infiltration of myeloid cells and lymphocytes into the brain, which then participate in the pathological alterations[16,17]. Understanding the role of inflammation behind ischemic stroke may reveal essential targets for the development of an effective treatment.

Alzheimer's disease

Dementia is characterized by symptoms including memory loss, language disability and cognitive impairment. It has been considered by the World Health Organization (WHO) as a public health priority. Dementia encompasses various neurological disorders, such as Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies and frontotemporal dementia. In 2015, 46.8 million people globally were estimated to suffer from dementia, and the numbers were calculated to reach 74.7 million by 2030 and 152 million by 2050[1,18]. The economic burden of dementia was estimated to reach one trillion US dollars by 2018 and is expected to double by 2030[18].

Alzheimer's disease was first reported in 1906 by Alois Alzheimer at a meeting in Tübingen, Germany. The symptoms and analysis were noted for his patient Auguste Deter who displayed, as known today, histological AD features: the substantial loss of neurons and the appearance of amyloid plaques and neurofibrillary tangles (Figure 1)[19]. At that time, nobody realized the importance of this discovery. However, Alzheimer continued studying his observations and published the findings in 1911, which initiated the first of now numerous studies and publications on one of the most pressing diseases in modern times.

AD is the most common cause of dementia, accounting for 60–80% of cases[20]. The primary risk factor for AD is ageing. The number of AD patients is expected to grow exponentially worldwide due to longer life expectancy and population ageing. Patients with AD present with memory decline that gradually worsens, followed by other motor deficits, emotional disturbances and sleeping disorders[21]. These

symptoms affect patients' daily life to a large degree and, by extension, their family members since the main burden of caring for AD patients is often carried by families rather than healthcare systems, especially in low-income and lower-middle-income countries[20]. AD has become one of the leading causes of disability and death for the elderly[22].

Epidemiology of Alzheimer's disease

The number of AD patients is estimated to increase by more than 5 million cases each year[21]. The prevalence of AD is around 0.5 % in the population aged 65-69 years old, which then increase to 12-fold higher in the elderly aged 85 years old and older[23], as age is the predominant risk factor for AD. In other words, the incidence of AD increases significantly with age, and the pattern is the same across different ethnicities [24–26]. The progression of the disease usually takes between 8 to 10 years ranging from the second to twenty-fifth year after diagnosis[27]. Moreover, gender is another risk factor for developing AD. Epidemiological studies have revealed that women have a double likelihood of developing AD than men[1,28]. This is not simply due to the longer life span of women, but also other factors, such as hormone effects and the different cellular functions of microglia in women compared to men[28–31].

There are two forms of AD according to the age of onset. The majority of AD patients develop the disease after the age of 65 years, and these cases are considered as late-onset AD. However, 1-6% of AD patients present with the disease at an age younger than 65 years old, so-called early-onset AD. Nearly 60% of early-onset AD cases have a family history of AD, and 13% of familial cases are caused by autosomal mutations[32]. These cases due to autosomal mutations are classified as familial AD, which constitutes only 5% of all AD cases[33]. The other 95% of AD patients are diagnosed with sporadic AD, which has a late-onset and appears to be a multifaceted disease involving a combination of multiple susceptibility genetic factors and environmental risk factors[34].

Pathophysiology of Alzheimer's disease

One hundred years after the discovery of the disease, the pathogenesis of AD is still not clear. The two main pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles (NFT)[35–37], illustrated in Figure 1. Amyloid plaques are primarily comprised of amyloid-β (Aβ) and are retained in the extracellular space[38,39]. Aβ is derived from the cleavage of the amyloid precursor protein (APP) by secretases. APP is located on chromosome 21 and appears to be a transmembrane protein in many tissues but is heavily expressed in neurons[40–42]. Several important functions modulated by APP include neural plasticity and synapse formation[43,44]. APP and its proteolytic product, Aβ, have also been reported to translocate into mitochondria, causing dysregulation of cellular bioenergetics[45,46]. There are three different secretases involved in the metabolism of APP: α-secretase, β-secretase and γ-secretase [47]. APP is firstly cleaved either by α-secretase or β-secretase and then

mediated by γ-secretase [48]. Evidence shows that BACE1 but not BACE2 is the major β-secretase for producing Aβ peptides in neurons[49]. Soluble APPβ, AICD and different isoforms of Aβ, mainly Aβ40 and Aβ42, are generated[50]. Aβ40 is the most dominant form of Aβ in humans, while Aβ42 is less abundant and more prone to aggregate as oligomers and fibrils[50,51]. Therefore, the ratio of Aβ40 to Aβ42 is critical in AD. The production of Aβ is a normal physiological process, but the function of it remains elusive. Aβ42 is a fundamental molecular blocks, building up into amyloid deposits in the brain under the time course of AD[52,53].

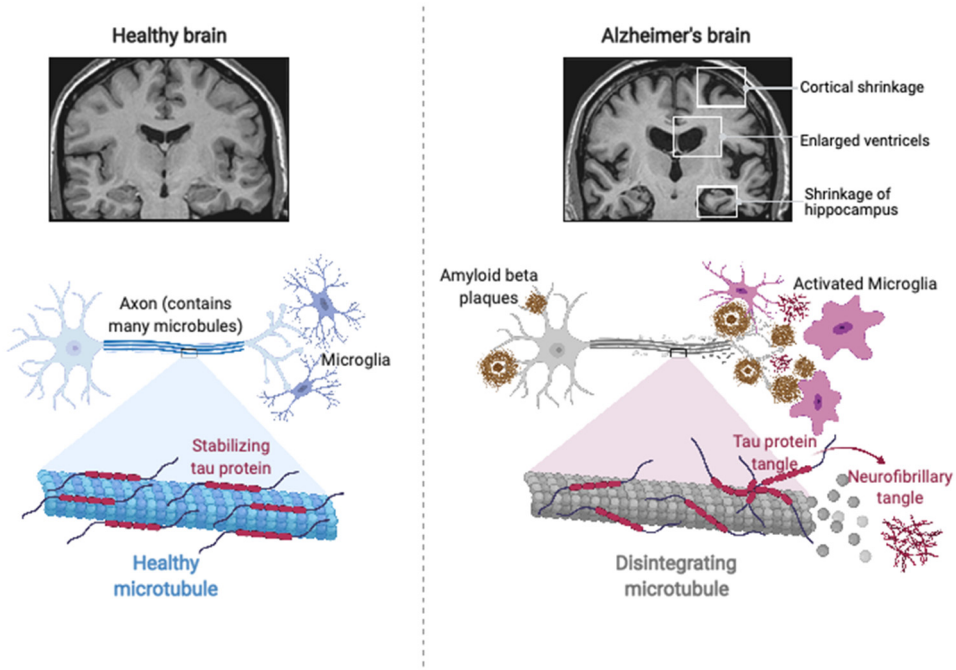

Figure 1 Pathology of Alzheimer's disease
The two main pathological hallmarks of Alzheimer's disease (AD) are amyloid plaques and neurofibrillary tangles. These features also activate microglia surrounding them. AD patients also have brain atrophy with enlarged ventricles and shrinkage of hippocampus (created with BioRender.com).

Another hallmark of AD is intracellular deposition of NFT in neurons, formed by hyperphosphorylated tau protein[54,55]. Tau is a microtubule-associated protein and genetically located on chromosome 17[56,57]. It is found almost exclusively in neurons and plays a critical role in the stabilization of microtubules in neuronal axons and is involved in axonal transport. During the development of AD, tau dissociates from microtubules, leading to the translocation of tau from the axon to other cellular compartments and causing an abundance of unbound tau in the cytosol[57]. Subsequently, aggregates of tau protein form and further build up into NFT as seen

in AD[58,59]. The appearance of NFT seems to more thoroughly reflect the severity of the disease and is more closely linked with cognitive impairment than amyloid plaque deposition in the brain[56,60].

These two hallmarks are not unique to AD. Aβ plaques are found on the walls of cerebral blood vessels in patients with cerebral amyloid angiopathy[61]. NFT are also seen in other neurological disorders, such as frontotemporal dementia and Down syndrome (trisomy 21)[40,62]. Nevertheless, a stereotypical pathology pattern was established for AD using positron emission tomography (PET) and post-mortem tissue in order to better distinguish AD from other disorders[63,64]. It has been revealed that hyperphosphorylated tau aggregates are first present in the locus coeruleus and then spread out to the entorhinal cortex, subsequently developing in hippocampus and eventually in neocortex. In contrast, the distribution of Aβ plaques in AD progresses in the opposite direction, starting in neocortex and then progressing to allocortical, diencephalic and basal ganglial structures. The reasons why these two pathological features have different tracks of dissemination are not known completely. Moreover, post-mortem analyses suggest that there are limitations in the use of these two hallmarks for the identification of the disease in the population[65,66]. Other potential factors also contribute to the clinical presence of AD.

Genetic risk factors

It is estimated that around 70% of the risk of developing AD can be attributed to genetics[67,68]. Several mutations in genes of APP and subunits of γ-secretase, including presenilin-1 (PSEN1) and presenilin-2 (PSEN2), have been found to be directly linked with early-onset AD[69–71]. The hallmarks in familial forms of AD are shared by some cases of sporadic AD, so studying these autosomal mutations are important for understanding the pathogenesis of AD, even though they only account for 1-5% of total AD cases[72]. Over 32 different APP variants have been identified in AD patients[27]. It has been found that these mutations are located in or nearby either the APP transmembrane domain or secretase cleavage sites. Most of the pathogenic APP variants affect the processing of APP by increasing Aβ production and the ratio of Aβ42 to Aβ40[27]. Furthermore, missense PSEN1 mutations are the most common cause of early-onset AD cases[73]. Dysfunction of PSEN1 leads to the earliest appearance of AD at 30 years old. To date, more than 170 mutations in the PSEN1 gene were discovered at chromosome 14 in 400 families[27]. The mutations in PSEN1 disrupt the proper function of γ-secretase, resulting in an increased ratio of Aβ42 to Aβ40[74]. In comparison, mutations in PSEN2 are an infrequent cause of early-onset AD[75]. It has been reported that the onset of AD related to PSEN2 may be attributed to other genetic or environmental factors[75]. Overall, these mutations favor the amyloidogenic pathway of APP and increase Aβ deposition in the brain.

95% of AD cases appear to be sporadic and have a late-onset age, ranging from 60 to 65 years old. Late-onset AD is believed to be heterogeneous and influenced by various genes and environmental factors. The strongest genetic risk factor studied

so far for late-onset AD is apolipoprotein E (APOE), specifically the ε4 allele[76,77]. APOE has another two alleles, ε2 and ε3. ε3 is the most common allele, occurring in 65-70% of the population, whereas ε2 has the lowest frequency, around 5-10% of the population[78]. However, there is a large variation in the distribution of these three alleles within different ethnic populations[79]. APOE is known to be involved in the metabolism of cholesterol and triglycerides. ε2 and ε3 encoded isoforms favourably bind to high-density lipoproteins, while the ε4 isoform (apoE4) preferentially binds to very low-density lipoproteins[80]. Carriers of one allele of ε4 have about three-times higher risk of developing AD[80,81]. This risk significantly increases to about 12-fold if ε4 comprises both alleles[80,81]. In contrast, the ε2 polymorphism decreases the risk of developing AD[82].

Moreover, gender has been found to have a modifying effect on the risk of developing AD. Women who are ε3/ε4 heterozygotes have double the risk of developing AD compared to those who are ε3 homozygotes. However, men who are ε3/ε4 heterozygotes have a similar risk of developing AD as men who are ε3 homozygotes, which is a significantly reduced risk compared to men who are ε4 homozygotes[82–85]. Nonetheless, APOE ε4 only contributes about 27% to the heritability of AD[86].

Researchers have made a great effort to identify other genetic risk factors using high-throughput genotyping and next-generation sequencing. Several genome-wide association studies (GWAS) have successfully pinpointed additional genes linked to AD. For example, variants found in triggering receptor expressed on myeloid cells 2 (TREM2) genes raised the risk of AD by 2.9%[87,88]. However, these variants appeared to be population-specific as no correlation was observed in Chinese, Japanese or Iranian cohorts[89]. The mechanism behind why the defects in TREM2 contribute to a higher risk of AD needs to be elucidated. TREM2 is highly expressed on the cellular surface of microglia. Animal experiments have demonstrated that phagocytosis is impaired when expression of TREM2 is abolished[90], and that deficiency of TREM2 induces a higher Aβ burden[91–93]. Taken together, these findings implicate microglia and neuroinflammation as playing an important role in the pathogenesis of AD[94–96].

Modifiable risk factors

AD progresses during a long preclinical period. This preclinical stage may last decades prior to diagnosis of the disease. During this long period, many factors have been assessed to answer the question of whether the onset of symptoms is associated with early-life or midlife exposures or exposures just before the onset of the disease. As a result, various modifiable risk factors have been identified for AD (summarized in Table 1).

Several studies have indicated there may be a relationship between vascular disease and cognitive decline and dementia[97,98]. Emerging evidence shows that vascular risk

factors play important roles in the transformation from mild cognitive impairment (MCI) to AD[99,100]. Particularly, cerebrovascular disease (CVD) has been suggested to have a stronger link to AD than the effect of ageing[101], as CVD and AD share many risk factors, such as hypertension[102], atrial fibrillation[103], atherosclerosis[104], obesity[105], type 2 diabetes[106] and APOE ε4 genotype[101,107].

Tabel 1. Risk factors for Alzheimer's disease
Besides ageing, various genetic and modifiable risk factors have been identified for Alzheimer's disease.

Different types of risk factors	
Genetic factors	Genetic mutations related to APP, PSEN1, and PSEN2 genes, Tau, APOE ε4[101,107], Myeloid-related genes (e.g., CR1[108–110], TREM2[111], MS4A[110], CD33[108–110],CLU[112]), PICALM[113], other susceptibility genes
Comorbidity	Cerebrovascular disease[102], Type 2 diabetes[114], Atherosclerosis[104], Obesity[115], Midlife hypertension[102], and Atrial fibrillation[102]
Lifestyle factors	Physical exercise[116], Low educational attainment[117–119], Heavy alcohol consumption[120], Depression[121,122], Smoking[123], and High-fat diet[124]
Other factos	Traumatic brain injury[125,126], Epilepsy[127–129], Stress[130], Inadequate sleep[131,132], and Infections[133]

Other factors related to lifestyle have also been suggested to play a role in AD. These factors include physical exercise, education level, alcohol consumption, depression, smoking and diet. The potential to prevent AD through these modifiable risk factors would be huge but has not yet been well elucidated. Clinical studies suggest that physical activity could be neuroprotective for AD patients as it is associated with larger brain volume in hippocampus and increased cognitive reserves and neuroplasticity[134,135]. Another factor that could promote cognitive preservation is education level. One study by Stern et al. showed that people with high educational attainment and high professional success had low incidence and a lower risk of developing AD[117]. Moreover, it is under debate whether depression is a consequence of AD or a risk factor for the disease. However, recent clinical studies support the idea that depression is strongly related to AD onset[121,122]. Individuals with MCI diagnosed with depression had a higher Aβ load and quicker conversion to AD than non-depressed MCI patients[136].

Several other factors may contribute to impairment in the brain. One is heavy alcohol intake, which induces a decline in cognitive performance[120] as well as loss of cholinergic neurons that may initiate AD development or worsen disease manifestation [137,138]. In contrast, mild to moderate alcohol consumption (1-2 drinks per day) has been reported to lower the risk for AD[139]. Another risk factor is smoking since it has negative effect on CVD, which mentioned before, is a risk factor for AD. Several animal studies have demonstrated that nicotine can exacerbate AD pathology, including tau phosphorylation, neuroinflammation and amyloid pathology[140–142]. In humans, populations with habits of both smoking and heavy alcohol drinking have a higher rate of AD incidence than populations with neither of these habits[123]. Last but not least, a high-fat diet may increase the risk of AD by

increasing the risk of obesity and diabetes, which may facilitate cognitive impairment[124]. Compelling evidence suggests that it is beneficial to have a balanced diet (e.g., Mediterranean diet) comprised of fruits, vegetables, fish and meat that is high in good fats (e.g., omega-3 fatty acid) and low in sugar[143,144]. Sufficient intake of docosahexaenoic acid, vitamins B6, B12, C and E, and folate are crucial since these contribute to the synthesis of phospholipids, an important compound in neuronal membranes[144]. It has been estimated that addressing a combination of seven modifiable risk factors would decrease AD cases around 10-20% every decade relative to the estimated trend for AD cases by 2050[145].

Alzheimer's disease hypothesis and therapy

While there is no effective treatment for AD, several important hypotheses have been proposed to provide potential pharmaceutical targets. The first drug approved for the treatment of AD was tacrine in 1995, developed based on the neurotransmitter hypothesis[146]. This hypothesis is based on findings of significantly reduced activity of choline acetyltransferase in several brain regions in AD patients [147]. Lower activity of this enzyme results in lower amount of acetylcholine [148,149]. Thus, the inhibition of acetylcholinesterase was introduced as an AD treatment to decrease the degradation of acetylcholine[150–152]. Newer generations of acetylcholinesterase inhibitors are widely used now for treating AD, such as donepezil which has fewer side effects and higher specificity[153,154]. Still, this treatment only ameliorates the symptoms for a short period of time rather than stops the progression of AD[155,156].

Among all of the drugs tested in clinical trials, therapeutics that address the amyloid hypothesis are the most frequently tested, accounting for 22.3%[157]. People with Down syndrome develop dementia with a similar appearance to AD, including memory loss and Aβ plaques[158]. From this, it has been claimed that the causative genetic factor for AD is located on chromosome 21[40]. The amyloid cascade hypothesis was first proposed in 1992, supported by evidence from familial forms of AD, which are AD cases with an autosomal dominant mutation in APP, PSEN1 or PSEN2[159]. This hypothesis states that the formation of Aβ plaques causes neuronal loss and dysfunction, leading to the formation of NFTs, activation of microglia and development of neurodegeneration[159]. However, a great number of findings question this hypothesis as the main event in AD pathogenesis. One piece of evidence is that the same number of amyloid plaques can be observed in post-mortem brain samples from people who died without cognitive impairment as from patients with sporadic AD[160,161]. It is consistent with findings from PET scan studies that show that many people have significant amyloid plaque load without any symptoms of memory deficits[160,162,163]. Another finding shows that Aβ deposition is not associated with neuronal loss and cognitive decline[164–166]. Although several anti-Aβ antibodies (solanezumab from Eli Lilly, gantenerumab and crenezumab from Roche/Genentech and aducanumab from Biogen Idec) have been developed to

reduce the Aβ burden, none of them have successfully made it through clinical trials[167–170]. This suggests that it is better to consider Aβ as a pathological feature instead of as a key consequence of the disease.

Conversely, tau pathology correlates better with memory decline and severity of AD than Aβ does in PET and CSF analyses[171–173]. Since tau phosphorylation causes destabilization of microtubules and promotes NFT formation, this would eventually trigger neuronal loss and synaptic damage[165,174]. Neuropathological studies show that tau pathology can be found about 10 years before the formation of Aβ plaques[175]. Hence, the tau hypothesis postulates that tau pathology increases the production of Aβ and is the central cause of neurodegeneration in sporadic AD. However, most of the drugs and treatments developed in response that have aimed at preventing tau aggregation and phosphorylation failed in clinical trials[176–178]. There are still several treatments undergoing clinical trials, such as a tau aggregation inhibitor (TRx0237 from TauRx Therapeutics)[179] and a vaccine targeting hyperphosphorylated tau ACI-35 (AC immune/Janssen)[180] and AADvac1 (Axon Neuroscience SE)[181].

AD is a multifaceted disease that is difficult to model simply. Another important factor in AD that has been established is the role of microglia, which will be introduced in the second part of the introduction. Moreover, various genetic and environmental factors in combination could drive the development of the disease. More studies are required to understand the fundamental rules of AD. Nevertheless, combinations of different treatments, perhaps, may be the best therapy for AD patients.

Innate immunity

The immune system

The immune system was developed to maintain homeostasis and protect the body from a wide range of invaders and stimuli, such as bacteria, viruses, parasites as well as cancer cells and traumatic shock. The immune response is well orchestrated and conducted mainly by two different components, innate immunity and adaptive immunity. Innate immunity, involving monocytes, macrophages, and natural killer (NK) cells, primitively provides immediate response to pathogens and functions as the first line of defense. However, the adaptive immune system reacts more slowly and is regulated more specifically by lymphocytes and antibody release. Moreover, the adaptive immune system also mounts a stronger immune response and retains a memory signature of the antigen from pathogens. When inflammation occurs in the periphery, the two components work cooperatively to solve the problem. However, there are substantial differences when it comes to the inflammatory response in the CNS.

Firstly, I will focus mainly on innate immunity in my thesis as the studies I present here have investigated the role of microglia in different aspects, including extracellular vesicles in Paper I and anti- and pro-inflammatory function in Paper II, III and IV. Secondly, I would like to introduce monocytes as a way to understand the function of systemic inflammation in the periphery on the progression of AD (Paper III and IV). Last but not least, the concept of innate immunity memory will be discussed to better explain the results from the studies (Paper III and IV).

Neuroinflammation

Neuroinflammation is described as an inflammatory response in the brain or spinal cord. As it is one of the major pathological features that starts to appear in the brain during the progression of AD, inflammation in CNS will be addressed in this section. The central cellular components involved in CNS neuroinflammation are resident glia, including microglia and astrocytes, endothelial cells and peripheral immune cells[182]. These diverse cells can release cytokines, chemokines, reactive oxygen species (ROS) and secondary messengers (such as prostaglandins) in response to inflammation[183].

The brain was previously considered to be an immune-privileged place. However, emerging evidence shows, in fact, that the CNS takes advantages not only of resident immune cells, but also of the cross-talk with the systemic immune system[184–187]. Due to the complexity of neuroinflammation, it is difficult to identify the causes and consequences of it. The consequences of an inflammatory response depend very much on the context, time and cause of the initial insult. On one hand, inflammation can be beneficial as it stimulates phagocytosis of dysfunctional cells and aggregated misfolded proteins (e.g., Aβ and NFTs)[188–190]. Meanwhile, the systemic immune system is also involved in the mitigation of CNS inflammation. For instance, T cells and monocytes are recruited to eliminate cytotoxicity in the CNS[191–193]. On the other hand, overactivation of the immune system can trigger the production of pro-inflammatory cytokines, such as nitric oxide (NO), tumor necrosis factor (TNF)-α and superoxide, leading to cerebrovascular lesions and neuronal death or damage[194–197]. Neuroinflammation is associated with the pathology and disease progression of AD in various ways. Common inflammatory responses are observed in different neurodegenerative diseases (e.g., AD, Parkinson's disease, Huntington's disease, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS)), but each disease also has its particular inflammatory response that contributes to the pathology[198].

Microglia

Microglia, the resident immune cells in the CNS, are believed to be key players in neuroinflammation as well as maintenance of homeostasis. Microglia were first

identified as the third element, together with oligodendrocytes in the CNS by Santiago Ramón y Cajal in 1913. It was not until 1919 when a Spanish scientist, Pío Del Río-Hortega, named this cell type in the brain parenchyma "microglia" [199].

Around 10% of the cells in the brain are microglia, with variable density across different brain regions[200]. Recently, convincing findings have indicated that microglia are differentiated erythromyeloid progenitors (EMPs) that have migrated from the yolk sac in the embryonic stage[201,202]. These precursors have also been seen giving rise to progenitors in bone marrow, which later become macrophages in the periphery. Therefore, the ontogeny of microglia is distinct from monocytes in the periphery (Figure 2). Under normal conditions, the pool of microglia in CNS is minimally comprised of bone marrow-derived monocytes, and the self-renewal of microglia has been demonstrated to have as low as an average turnover rate of 28% each year[203]. The transcription factor PU.1 and macrophage colony-stimulating factor 1 receptor (CSF1R) signaling are curial for myeloid-lineage manipulation and microglial survival[204,205]. Several other environmental factors, such as CSF1, transforming growth factor- β (TGF-β) and interleukin (IL)-34, are involved in furthering the maturation of microglia[201,206].

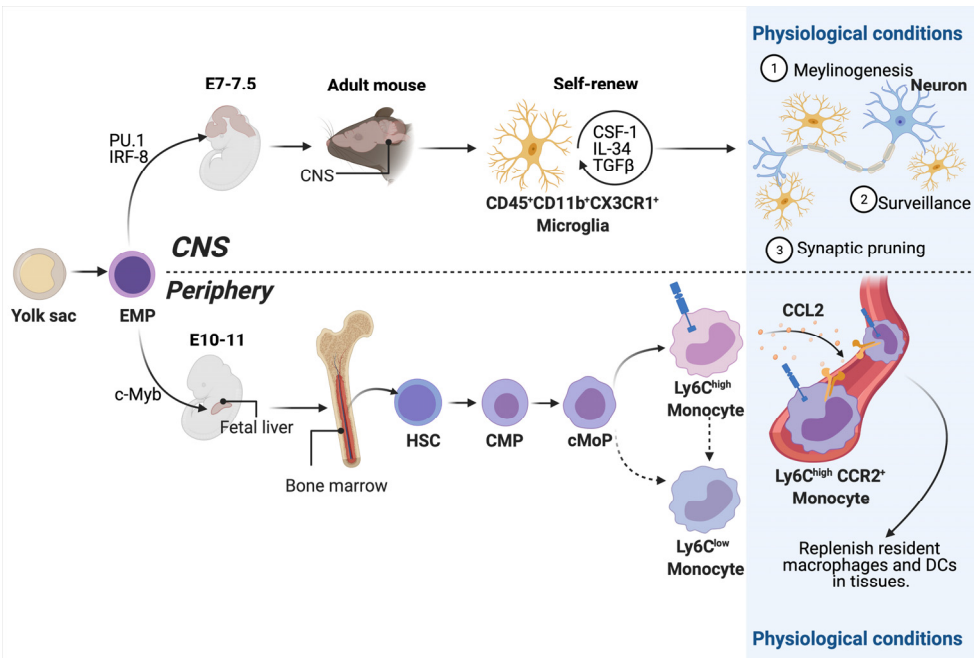

Figure 2 Development of microglia and monocytes from periphry

Microglia are differentiated erythromyeloid progenitors (EMPs) that have migrated from the yolk sac in the embryonic stage. Several transcription factors are important for microglia migration and survival, including PU.1 and IRF-8 for migration, CSF1, TGF-β, and IL-34 for survival (created with BioRender.com).

With advances in live imaging tools, the historical view of microglia has changed from static bystanders with a minimal homeostatic occupation in the CNS to a more complex and active role. In fact, under normal physiological conditions, microglia are constantly surveying and monitoring brain activity by expanding and withdrawing their highly dynamic processes[207,208]. The highly dynamic actions of microglia are essential components of normal brain function and tissue integrity. For instance, microglia are able to shape presynaptic and postsynaptic structures[209–211], phagocytose cell debris[212], produce a wide range of soluble factors in response to different stimuli[213,214] and travel towards injured areas for repair[215]. In addition, increasing evidence has provided insight into microglial function in learning and memory formation[216,217]. Moreover, as microglial precursors come to the brain at the same timepoint when neurons appear before the presence of astrocytes and oligodendrocytes, microglia participate in many developmental events in the CNS. These roles include supporting and regulating neurogenesis at the postnatal stage[218,219], promoting neuronal death[220,221], pruning synapses through the complement system[222–224] and developing neural circuits[225–228].

In order to fully carry out these crucial functions and more, numerous receptors are expressed on microglia. Bidirectional communication between microglia and neurons is delicately modulated by the CX3C chemokine ligand 1 (CX3CL1)-CX3C chemokine receptor 1 (CX3CR1) and Cluster of differentiation (CD) 200-CD200 receptor signaling pathways[229,230]. These two receptors, CX3CR1 and CD200R, are critical to sustain the microglia population. Current animal experiments show that microglia are more active in anesthetized mice. It has been further illustrated that neuronal activity during wakefulness controls suppression of microglia surveillance through norepinephrine signaling[231,232].

As microglia are key members of the innate immune system in CNS, they have a wide range of receptors called pathogen recognition receptors (PRRs) that can quickly recognize and bind soluble pathological molecules. These signals are either sterile stimuli (e.g., protein aggregates) or danger associated molecular patterns (DAMPs) from an internal tissue insult. Extensive studies have indicated that fibrillar Aβ in AD is one of the DAMPs that can activate microglia. However, dying neurons also release DAMPs, such as ATP[233], high mobility group box protein 1 (HMGB1) and lysophosphatidylcholine[234,235]. Moreover, microglia can directly detect pathogen associated molecular patterns (PAMPs) from external invading pathogens via PRRs. Well-known examples of PRRs in infection and neurodegeneration are Toll-like receptors (TLRs) and inflammasomes.

Lipopolysaccharide (LPS) is a molecule from gram-negative bacteria that binds to TLR4, leading to the recruitment of the adaptor protein MyD88 and the activation the downstream cascades[236]. The cascades then initiate nuclear translocation of transcription factors, including nuclear factor κB (NF-κB) and members of the interferon regulatory factor (IRFs) family, and induce proinflammatory cytokines[236]. LPS is also used as a standard method to induce infection or neuronal

degeneration in animals to mimic severe neuroinflammatory conditions that occur in neurological disorders[237]. Previous studies from our laboratory have demonstrated that galectin-3, a member of the β-galactosidase binding protein family, also acts as a ligand to TLR4 and negatively correlates with Aβ deposition in mice[238,239]. Inflammasomes are another important sensor in innate immunity that regulate the activation of caspase-1 and production of IL-1β and IL-18. Several studies from the Heneka group have also suggested there may be an association between the inflammasome and Aβ pathology[240–242].

New way to communicate: extracellular vesicles

While signaling typically occurs via autocrine, paracrine, and endocrine processes, extracellular vesicles (EVs) have recently been proposed to be a non-canonical pathway to transport signals between cells[243]. EVs were first reported as a way for cells to get rid themselves of unwanted components[244]. Besides particular secretory cells, which transport neurotransmitters and hormones, many types of cells are able to release EVs, ranging from bacteria to plants and humans[245–247]. EVs are highly diverse in terms of size, content and function. Based on how they are generated, EVs are generally divided into two categories: exosomes and microvesicles. Exosomes are intraluminal vesicles (ILVs) generated by the fusion of multivesicular body (MVB) with the plasma membrane[248,249]. The biogenesis and secretion of EVs are demonstrated briefly in Figure 3. Exosomes about 50-150 nm in diameter have primarily been found to be released by reticulocytes[244], B cells[250] and dendritic cells[70]. Currently, the ability to release exosomes has been found in different types of cells[251], and the role of exosomes under normal and disease conditions increasingly draws attention in the cell-cell communication field.

Microvesicles were previously defined as platelet dust as they were discovered in the plasma and sera from healthy individuals[252]. Subsequently, a process called ectocytosis was characterized in activated neutrophils that secreted vesicles composed of plasma membrane. Microvesicles are derived directly from the outward budding of the plasma membrane[253]. The size of microvesicles is normally from 100 nm to 1000 nm in diameter. However, tumor cells can secrete much larger vesicles (maximum to 10 μm). Microvesicles have been extensively studied for their functions in blood coagulation[254,255]. Recently, several studies have investigated the role of microvesicles in the cross-talk between different cells under pathological situations, such as cancer[256] and neurogenerative diseases[257,258].

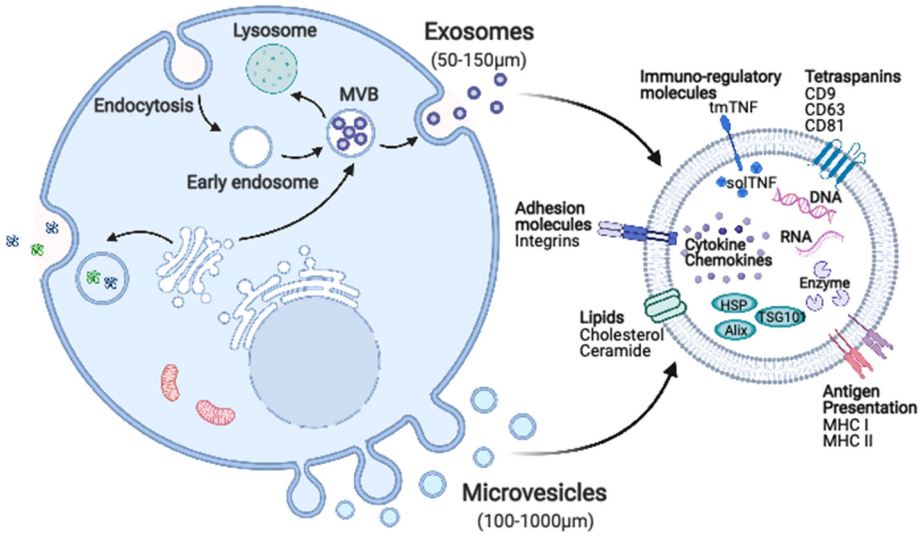

Figure 3 Biogenesis, secretion, and the content of extracellular vesicles
Extracellular vesicles (EVs) are divided into two categories: exosomes (50-150 nm in diameter) and microvesicles (100-1000 nm in diameter). Exosomes are intraluminal vesicles (ILVs) generated by the fusion of multivesicular body (MVB) with the plasma membrane. Microvesicles are derived directly from the outward budding of the plasma membrane. They appear to have similar compositions and overlapping sizes that make it difficult to know their origin after they have been isolated. The contents of EVs vary and can include proteins, RNA, DNA and lipids, which help determine the recipients of EVs. Alix, ALG-2 interacting protein X; HSP, heat shock protein; TSG101, tumor susceptibility gene 101 protein; transmembrane form of TNF; solTNF, soluble form of TNF; MHC, major histocompatibility complex (created with BioRender.com).

The formation of exosomes and microvesicles is similar as they share intracellular sorting machineries and even budding sites (Figure 3). Therefore, it is sometimes difficult to differentiate the two subpopulations of EVs. The mechanisms underlying exosome biogenesis have been well studied with the discovery of the endosomal sorting complex required for transport (ESCRT)[259]. However, exosomes can also be generated in an ESCRT-independent manner[260,261]. In contrast, the regulatory pathways involved in the biogenesis of microvesicles are still under investigation. Once EVs are released into the extracellular space, they can travel to their recipient and deliver their cargo, initiating changes within the recipient. There are several ways EVs can bind to target cells. One is through recognition by cellular receptors in order to dock at the plasma membrane. Others are fusion with the target cell or internalization by endocytosis. These processes are strictly modulated and complex and seem to be target- and origin-specific[262].

The contents of EVs vary and can include proteins, RNA, DNA and lipids, which can help determine the recipients of EVs. One study showed that a subpopulation of exosomes carrying APP bound specifically to neurons rather than glia[263]. As microglia are the key players in neuroinflammation, microglia-derived EVs have attracted growing interest. Microglia-derived EVs have also been found in

significantly high levels in plasma from patients with MCI and AD[264]. These EVs have been demonstrated to promote neurotoxicity by increasing soluble Aβ species[264]. Moreover, several microRNAs, such as let-7g-5p, miR126-3p, miR142-3p and miR146a-5p, have been found in EVs and their levels are associated with the severity of AD[265]. This goes hand-in-hand with other evidence showing a role of EVs in the regulation of inflammation and propagation of neurological diseases such as AD and PD[266]. Taken together, microglia-derived EVs seems to play a critical role in the promotion and spread of pathogenesis. However, the crucial role of EVs has been underestimated. In addition, several clinical trials using EVs to deliver drugs are ongoing [267]. More knowledge on EVs may shed light on possible therapeutic approaches for early disease intervention and disease diagnosis.

The heterogeneity of microglia: beyond the M1 and M2 classification

Microglia transform when they become activated. The morphology of microglia shifts from a ramified shape to a more ameboid form. The phenotype changes as a result of signaling pathways. To better understand the different states of microglia, a classical classification of microglia (M1 and M2) was proposed according to peripheral macrophage studies[268]. M1 refers to the pro-inflammatory profile, and M2 is defined by anti-inflammatory activation[269]. The M1 phenotype induces neurotoxicity by releasing pro-inflammatory cytokines (e.g., IL-1, TNF-α and IL-6) and ROS[270,271]. Upregulation of major histocompatibility class (MHC) II, CD86 and inducible nitric oxide synthase (iNOS) can be seen in M1 microglia[272]. In contrast, the M2 profile is considered neuroprotective due to the production of neurotrophic factors, such as insulin-like growth factor-1 (IGF-1), and anti-inflammatory cytokines, such as IL-10[271,273]. Other markers, such as arginase 1 and Ym1, are used to characterize the M2 microglia population [274–276]. It also has been shown that these microglia are more capable of phagocytosising[269]. Due to the simplicity of the model, it is believed that the categories of M1 and M2 are insufficient for reflecting the plasticity and dynamics of microglia in CNS[277].

An increasing body of studies analyzing transcriptomic and proteomic profiles have revealed conserved transcriptional signatures from microglia between different species as well as considerable heterogeneity in an area- and disease stage-specific manner[278–280]. Bulk analysis of microglia in the CNS has indicated a higher expression of F4/80 in microglia from cerebellum[200], and various CD68 expression levels have been reported in the brains of rodents and humans[281–283]. Moreover, TNF-encoding TNF-α and CD32 were highly enriched in the hippocampus[284], and microglia localized in white matter exhibited higher expression of ubiquitin specific peptidase 18 than cortical microglia[285]. Recent comprehensive single-cell sequencing has provided deeper insight into the transcriptional landscape of microglia during development and progression of diseases. For example, a distinct subpopulation of microglia was identified in murine embryonic brain that upregulated membrane-spanning 4-domains subfamily A (MS4A) family proteins,

which had been known to be expressed by CNS-associated macrophages (CAMs)[286]. CAMs are resident in the interfaces between brain parenchyma and periphery, such as the perivascular space, meninges and choroid plexus[287,288]. However, the function of this subset of microglia remains to be elucidated. Interestingly, a unique subset of microglia was characterized with elevated levels of C-Type Lectin Domain Containing 7A

(CLEC7A), secreted Phosphoprotein 1 (SPP1), LGALS3 (encoding galectin-3) and IGF-1 in early postnatal brain but then disappeared during adulthood[280,289,290]. This subpopulation shares a large similarity with disease-associated microglia (DAM) and microglia neurodegenerative phenotype (MGnD) found in animal models of AD, ALS and MS[291,292].

Specific cluster of microglia in neurological disorders

Activation of microglia under pathology, the homeostatic microglia enriched with transmembrane protein (TMEM)119, purinergic receptor P2Y (P2RY12) and hexosaminidase subunit β (HEXB) are disturbed[286,293,294]. DAM carry the signature of high expression levels of lipoprotein lipase (LPL), complement component 3 receptor 4 subunit (ITGAX (CD11c)), APOE, CD63, CD9 and CLEC7A combined with lower expression levels of homeostatic genes (Figure 4)[291,292]. DAM have anti- and pro-inflammatory phenotypes regulated by different genes (see Figure 4)[295]. Additionally, DAM have been histologically localized around Aβ plaques in AD patients[291]. Furthermore, the APOE-TREM2 signaling cascade has been suggested to drive the presentation of DAM[91,292,296]. In addition, DAM have also been shown to exist in different mouse models with demyelination. However, the features of DAM in different models are diverse[280,286]. Some models may involve the recruitment of peripheral cells while others may not. This may be explained by microglial alteration of phenotype in a context-dependent manner. In a mouse model involving systemic infection with LPS, microglia show a unique profile with reduced expression of TREM2, transmembrane immune signaling adaptor (TYROBP), gene of cathepsin D (CTSD) and hypoxia-inducible factor 1

(HIF1)-α, which is the opposite in DAM[297]. These findings suggest that the phenotypic profiles of microglia are dependent on inflammatory environment. Taken together, microglia respond specifically to various environmental stimuli. To determine whether the various phenotypes of microglia are beneficial or detrimental to pathogenesis still requires clarification.

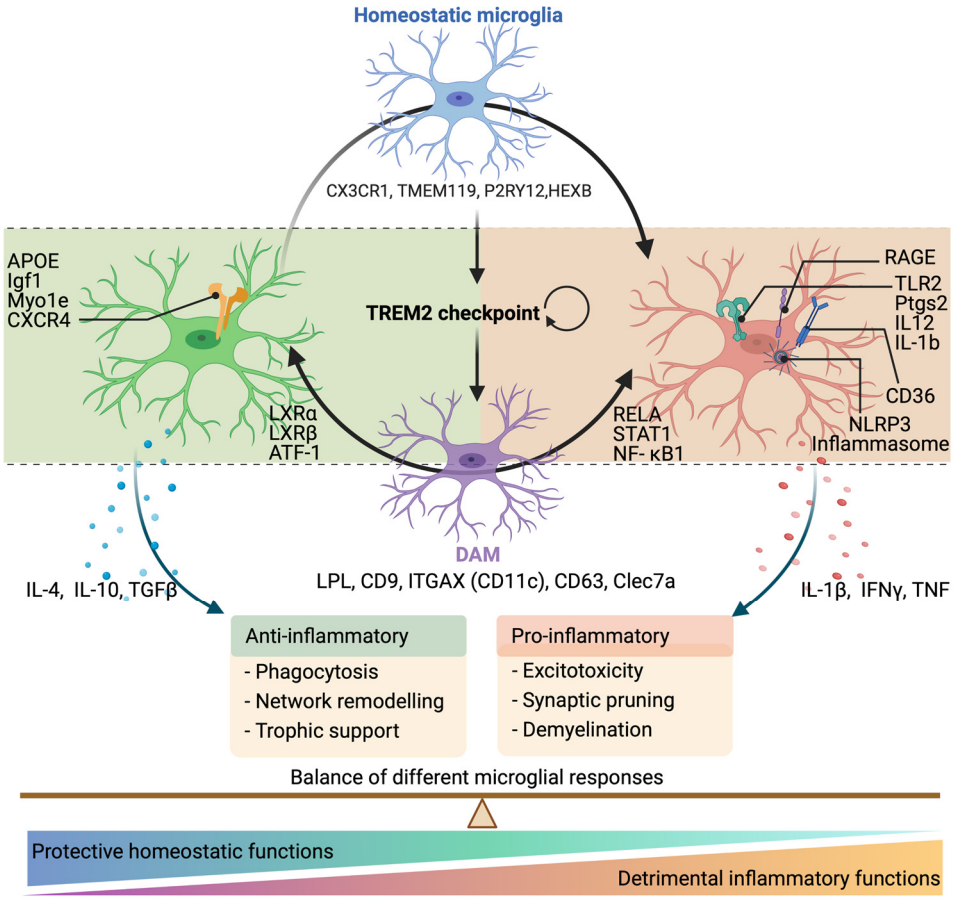

Figure 4 Modulation of disease-associated microglia (DAM) between phenotypes
DAM have anti- (green) and pro-inflammatory (orange) phenotypes regulated by different genes. The consequences of microglial activation are proposed as the balance of these two phenotypes. DAM, disease-associated microglia (created with BioRender.com).

Monocytes

Similar to microglia in CNS, the key player in innate immunity in the periphery is monocytes. Monocytes are derived from a common myeloid progenitor (CMP) and settle down in the bone marrow (BM), where CMPs also give rise to erythrocytes, platelets, dendritic cells and granulocytes[298]. From CMPs, monocytes emerge from a unipotent common monocyte progenitor (cMoP), which express Ly6C in mice and CD14 in humans (Figure 2) [298–300]. cMoPs have a high rate of proliferation and temporally express C-X-C motif chemokine receptor 4 (CXCR4) within BM[300]. After 24 hours, CXCR4+ pre-monocytes further differentiate into CXCR4^{-} Ly6C^{Hi} monocytes, which then enter into the peripheral blood by C-C motif chemokine receptor 2 (CCR2) regulation (Figure 2)[301]. However, the function of CCR2 in

emigration of monocytes is unknown in humans. Bone marrow is thought to be the *bona fide* reservoir for monocyte generation in adulthood. However, the spleen has also been found to be a great reservoir for monocytic precursors [298]. Circulating monocytes, accounting for 5-10% of leukocytes in humans and about 4% in mice, can further differentiate into macrophage and dendritic cells[302]. After travelling to tissue, monocytes differentiate into tissue-specific macrophages for replenishing the population in tissue-specific niches or in response to insults. Studies using antibodies specific for different surface receptors and antigens have elucidated a substantial heterogeneity of monocytes, which possibly reflects cellular responses specific to their microenvironments.

Under normal conditions, monocytes have little to no engraftment in the CNS due to intact blood-brain-barrier (BBB)[201,303]. Under pathological conditions, such as AD and sepsis (overwhelming systemic infection), monocytes can be recruited into the CNS with other lymphocytes as the integrity of the BBB is disrupted and permeabilized (see Figure 5)[304–306]. The participation of monocytes in AD cannot be neglected as there is clear evidence from patient studies and preclinical data from animal experiments. In AD patients, circulating monocytes were found to have shorter telomeres in an age-dependent manner[307]. Another analysis identified a negative correlation involving the ratio of intercellular adhesion molecule (ICAM)-3/CD14 in peripheral-blood monocytes isolated from MCI and AD patients. In an AD animal model, circulating monocytes were observed by *in vivo* imaging clearing up the Aβ in veins but not in arteries[308]. The role of monocytes in the clearance of Aβ in CNS is controversial as recent evidence showed that resident microglia are the only immune cells around plaques with no contribution from peripheral monocytes[309]. In contrast, a study reported that CCR2-deficient mice developed AD earlier due to a lack of monocyte infiltration into CNS[191]. Moreover, another research group demonstrated that monocyte-derived microglia have a higher capability to phagocytose Aβ than resident microglia in the CNS[310]. Besides the role of phagocytosis to sustain homeostasis, monocytes also release different cytokines and inflammatory mediators to initiate inflammation and recruit other cells. These findings highlight the multifaceted role of monocytes, which are not simply precursors, but also give rise to macrophages in tissue and perform specific functions under different circumstances. Furthermore, monocytes are also considered a potential target for AD diagnosis and treatment.

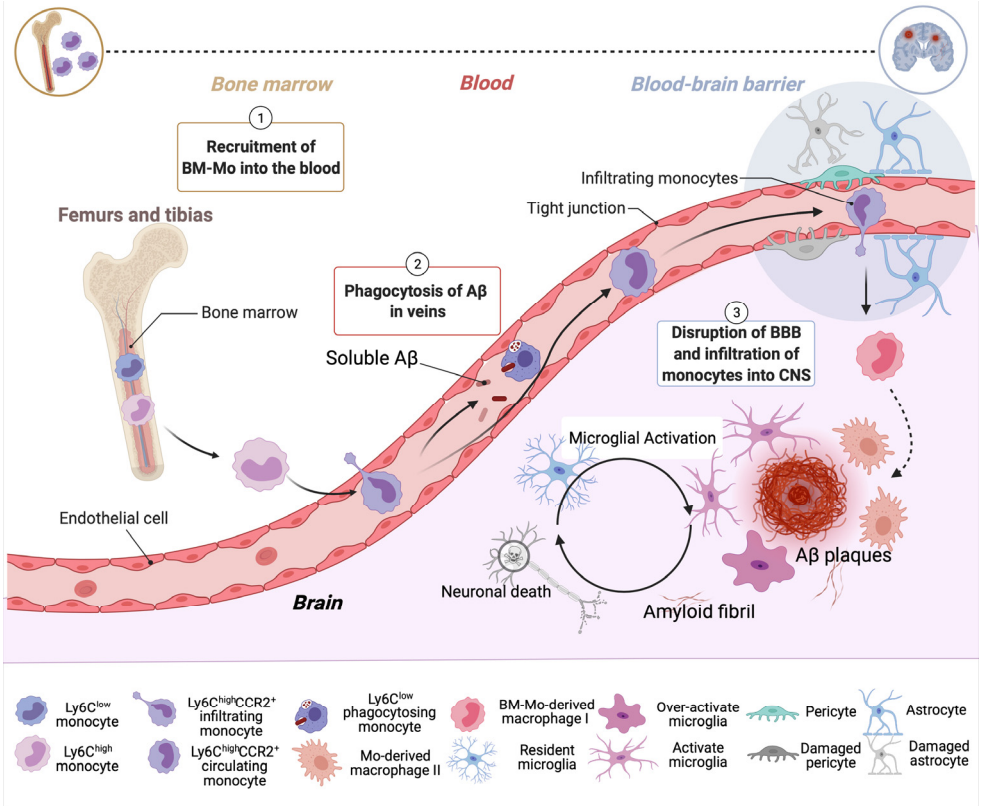

Figure 5 Infiltration of peripheral monocyte into central nervous system under Alzherimer's disease
Ly6C^{high} Monocytes from bone marrow give rise to Ly6C^{low} monocytes remaining inside the bone marrow. Under the pathology of Alzheimer's disease (AD), Ly6C^{high} monocytes enter the peripheral blood by regulation of CCR2. Ly6C^{high} CCR2^{+} monocytes in the veins can phagocytose soluble amyloid-β in the blood. Subsequently, Ly6C^{high} CCR2^{+} monocytes infiltrate the central nervous system (CNS) as the integrity of the blood-brain barrier (BBB) is disrupted and permeabilized. The role of these infiltrating monocytes is still under debate (created with BioRender.com).

Innate immunity memory

Increasing evidence has indicated that immunological memory or trained immunity is no longer a trait exclusive to the adaptive immune system, but it is also exhibited by myeloid cells of the innate immune system, such as monocytes, macrophages, and nature killer cells[311,312]. The concept of trained immunity in the innate immune system is defined as an enhanced immune response to protect the host from pathogens after vaccination or infection, which is modulated by metabolic, epigenetic and transcriptional alterations of specific innate cell populations rather than antibodies and lymphocytes. The first evidence of this was found in plants as a phenomenon denoted as systemic acquired resistance[313]. Following this, more evidence suggested that trained immunity can occur in invertebrate animals as well[314–316]. These findings lead to the question of whether a similar process can take

place in vertebrates. Consequently, researchers revealed that mice primed with microbial ligands of PRRs were spared from lethal inflammatory reactions from the second exposure of infection[317,318]. The central regulatory element in innate immunity is epigenetic reprogramming[311].

Microglia are the main component of innate immunity in the CNS. They are the first line of defense once pathological signs occur. Emerging evidence shows that microglia are able to be primed and attain innate memory, but the reported consequences are conflicting. Two groups demonstrated that a low dose of lipopolysaccharides (LPS) by intracerebral administration alleviated AD pathology [319,320]. However, other studies found overactivated microglia, elevated neuronal loss and impaired cognition in AD transgenic mice after systemic or intracerebral infection[321,322]. Recently, consecutive challenges with LPS in the periphery have been found to mitigate the pathology, whereas a single dose aggravated the amyloidosis in mice with AD pathology[323]. Taken together, the innate immunity in CNS can be modified by stimuli, provoking different consequences that strongly depend on the concentration of the stimulus, timepoint of intervention and duration after the intervention. Innate immune memory may be exploitable and has great potential in the design of a therapeutic intervention for AD.

To conclude, several variants in genes, such as complement C3b/C4b receptor 1 (CR1)[108–110], TREM2[111], MS4A[110], phospholipase C gamma 2 (PLCG2)[324] and bridging integrator 1 (BIN1)[110], expressed in myeloid cells can raise the risk of developing AD as revealed by recent GWAS studies. This highlights the essential role of microglia and monocytes during the progression of AD, and the dysfunction of myeloid cells can help determine the ultimate consequence of the disease. The response of the innate immune system in AD is complex and is hard to label as just detrimental or beneficial. Taken together, increasing knowledge of the innate immune system in AD provides a novel direction for further understanding and preventing the disease.

Rationale

When I started my PhD education in 2015, neuroinflammation was suggested as an important component underlying the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD). However, it was under debate whether the neuroinflammation is a causative factor in or a the consequence of the diseases.

The multifaceted roles of microglia under pathological conditions is implicated by several studies. However many other aspects have not been well studied. One of them is how microglia contribute to the pathology. Thus, we performed these four studies in this thesis to investigate how microglia communicate with other cells through extracellular vesicles and how microglia cross-talk with the periphery under AD pathology.

Moreover, microglia are commonly characterized into the 'M1' and 'M2' phenotypes used for peripheral macrophage identification. Ambiguous markers between monocytes and microglia impede understanding of the different roles of innate immunity in AD. Therefore, another aim of my thesis was to better characterize microglia and monocytes under different conditions.

The findings of this thesis could provide potential candidates for early diagnostics and pharmacological targets to prevent the progression of AD. As several anti-neuroinflammatory drugs have failed in the clinical trials, these studies will also help with finding a suitable time window for the intervention in AD.

Aims

The aims are listed below for each study:

To investigate the inflammatory response of microglia.

- Paper I:
  To investigate the effects of inflammation on extracellular vesicles (EVs) derived from microglia.
- Paper II:
  To elucidate early alterations in the innate immune response of the microglia in the 5xFAD mouse model of Alzheimer's disease.

To investigate the potential approaches to modulate innate immunity.

- Paper III:
  To clarify how acute inflammation in the periphery at the pre-plaque stage could lead to the long-term effects on innate immunity in the brains of 5xFAD mice.
- Paper IV:
  To investigate the long-term effects of acute inflammation at the postnatal stage on amyloid-β pathology and the heterogeneity of microglia and bone marrow resident monocytes.

Methods

Here, I will to summarize the methodologies applied in my studies. I also want to discuss the considerations and importance of why we chose these particular methods. Detailed descriptions of the different methods are provided in the materials and methods part of each paper.

Cell culture and treatment

Cell culture (Paper I)

Cell lines are less time-consuming, labor-intensive and cheaper models compared to primary cultures. There are many microglia cell lines available, such as HAPI from rats, BV2 and N9 from mice and HMC3 from human. BV2 cells express similar surface markers as primary microglia, including ionized calcium binding adaptor molecule 1 (IBA1) and CD11b[325]. Several studies have shown that several proinflammatory cytokines are released by BV2 cells with stimulation of LPS[325]. Increased phagocytosis was also demonstrated in BV2 microglia after treatment with Aβ (1-42) fibrils[326]. BV2 microglia have many similarities to primary microglia[327]. Furthermore, proinflammatory profiles have been well established in our laboratory using LPS stimulus. We have examined the inflammatory effects of LPS at different time points (3, 6,12 and 24 h). We found that the expression level of iNOS peaked at 12 h and declined by 24 h using western blot. Thus, we utilized BV2 cells for studying inflammatory activation. This was done by treating with 1 μg / ml LPS (Sigma-Aldrich, Colony 0127-B8) for 12 h and then changing to serum-free media for 12 h to collect EVs released due to inflammatory activation. It is important to note that the BV2 cell line was developed by introducing oncogenes into mouse microglia that allow them to proliferate at a higher rate and to have an exaggerated reaction when stimulated. Therefore, we limited the passage number to 20 for all experiments to avoid confounding results. Nevertheless, the findings from the cell line study should be carefully conducted and validated *in vivo*.

Extracellular vesicle isolation procedure (Paper I)

A traditional method, centrifugation, was used to isolate EVs. Conditioned media was collected from cell cultures and subjected to a series of low-speed centrifugation steps (500×g for 10 min, 2000×g for 10 min, and 10,000×g for 30 min) at 4 °C to remove cells and cellular debris. The supernatant was then spun down with ultracentrifugation at 100,000×g for 70 min. Compared to using kits from companies, isolation by centrifugation takes a longer time but is a less irritable process. EVs isolated this way contain natively expressed proteins and are usable for further proteomic analysis. The biggest drawback of this method that I suffered from the most is the low yield and recovery of EVs. The recovery was calculated, ranging from 5% to 20% with large variations within the same batch. To harvest enough samples to run the following experiment, I had to culture BV2 cells in T175 flasks instead of T75 flasks.

Measurement of extracellular vesicle size by nanoparticle tracking analysis (NTA) (Paper I)

The size and total number of EVs were measured using a NanoSight LM10 (Malvern, UK) that could do Nanoparticle Tracking Analysis (NTA). This technology is based on Brownian motion together with light scattering properties, and the size distribution and concentration of EVs samples can be obtained. It is an excellent and efficient method. However, the limitation is that it is not sensitive enough to detect particles smaller than 100 nm and larger than 600 nm. Thus, there is a possibility that some subpopulations of vesicles may not be present or accurately measured.

Animal models

TNF knockout mice (Paper I)

Adult male C57/BL6 mice (between 7 and 8 weeks of age, n = 20) (Taconic Ltd. Ry, Denmark) and TNF knockout (TNF-KO) breeding couples (The Jackson Laboratory) were transferred to the Laboratory of Biomedicine, University of Southern Denmark. This animal experiment followed the relevant guidelines and regulations approved by the Danish Animal Ethical Committee (numbers 2011/561-1950 and 2013-15-2934-00924). This was a collaboration with a Danish group to further study the role of TNF in EVs trafficking *in vivo*. It was a great opportunity for us to validate our *in vitro* results in animals.

5xFAD mice (Paper II, III, and IV)

Transgenic 5xFAD mice on a C57/BL6-SJL background (The Jackson Laboratory) were used to study the pathology of AD. These mice carry human APP and PSEN1 with three mutations in the human APP transgene (the Swedish mutation, K670N/M671L; the Florida mutation, V716V; and the London mutation, V717I) and two mutations in the human PSEN1 transgene (M146L/L286V). These mutations are related to familial forms of AD. Animal housing, handling and experiments were approved by the Malmö/Lund animal ethics committee (M30-16, Dnr5.8.18-01107/2018).

This model is one of several human APP and PSEN1 double-mutant (APP+PSEN1) mouse models. The mice express intracellular Aβ42 at 1.5 months old, display amyloid plaques by 2 months, and develop cognitive deficits earlier than other APP+PSEN1 models at around 4 months[328]. Neuronal loss and synaptic dysfunction can also be found by 4 months[329]. This is a rather more aggressive AD mouse model compared to the APP+PSEN1and 2xKI AD mouse models. We chose this model due to early activation of microgliosis as well as early cognitive impairment and late motor dysfunction, which can be detected at 9 months of age[326,330]. This model allowed us to evaluate the effects of systemic infection not only on microglial activation, but also on Aβ deposition and cognition in the CNS at 6 months of age, which was done in **Paper III and IV**.

However, one big drawback to this model is the lack of Tau pathology, another hallmark of AD, suggesting limitations in our reflections of the complications of the disease and in our results which may be less clinically relevant. Another disadvantage is the overwhelming Aβ expression, which may produce some artifacts not linked to AD pathology in humans. Here, we have considered both sides of using this mouse model. Overall, the 5xFAD model is still a valuable model to study, specifically, the microglial response towards Aβ under systemic inflammation. Certainly, we need to further validate our findings in humans using AD patient brain sections and blood samples from septic patients.

Genotyping (Paper II, III, and IV)

5xFAD mice are heterozygous and carry the retinal degeneration mutation 1 (RD1) from the original breeding. We performed PCR to genotype the 5xFAD and WT mice. Mice with RD1 were excluded due to visual deficits, which could influence the results from cognition behavioral tests.

Animal surgery, treatment, and behavioral tests

Induction of experimental stroke, permanent middle cerebral artery occlusion (pMCAO) (Paper I)

The distal part of the left middle cerebral artery was permanently occluded. Mice were allowed to survive for one day (immunofluorescent staining and cytokine measurement) or five days (EV analysis) post-occlusion, whereafter they were sacrificed to collect blood and brains for further analysis. pMCAO is a commonly used model for stroke. Since we studied EVs secreted from BV2 cells upon LPS stimulation, this inflammatory mouse model was highly appreciated for confirming what we found *in vitro*. Rats and mice subjected to pMCAO are known to developed neuroinflammation in the brain[331,332] and an altered inflammatory response in the periphery[333,334].

Intraperitoneal injection of LPS (Paper III and IV)

To mimic the physiological condition of mild sepsis, we decided to intraperitoneally inject LPS in mice of different ages. Before conducting the full experiment, we did a pilot study to titrate and determine the most effective concentration of LPS (1mg/kg, 5mg/kg, and 10mg/kg) that led to no mortality so that we could study the long-term effects of systemic infection in the CNS and periphery. Thus, when we ran the full experiment, mice were subject to i.p. injection at postnatal day 9 and at 6 weeks old of either lipopolysaccharide (1mg/kg, L4516-from *Escherichia coli* O127:B8, Sigma-Aldrich) or an equal amount of saline for control. All of the mice in this project were induced using the same batch of LPS.

The limitations of using LPS are that the inflammation triggered neglects the effects of gram-positive organisms and polymicrobes[335] and that the response initiated by LPS is restricted to just one PAMP molecule, so this model resembles only a small subset of human sepsis patients[336]. Moreover, human sepsis has a lower expression level of inflammatory cytokines in plasma and reaches the peak more slowly than LPS-induced sepsis in animals[337,338]. However, LPS replicates many aspects of the progression of sepsis and simplifies explanations of the underlying mechanism behind the effects seen. It has been utilized as a standard method in the field. Compared with other models, such as the cecal ligation and puncture model, LPS administration is much easier to control and perform on mice[335].

Novel object recognition test (Paper III and IV)

It has been reported that the 5xFAD mouse model develops cognitive deficits at 4 months of age using the novel object recognition test[339,340]. This mouse model also

shows motor dysfunction at 9 months old[340]. Thus, we performed several different behavioral tests in the pilot study to assess the effects of LPS on the progression of the pathology. First, mice were allowed to explore the empty arena for 5 minutes to measure possible effects from the treatment on locomotion. Following this, two identical objects were placed in the center of the arena for 15 minutes. Retention tests were then performed 1 h and 24 h after the training session to assess short-term memory and long-term memory, respectively. Mice tend to be more curious towards a novel object. However, 5xFAD mice exhibit impaired memory and do not have a preference for the objects. Moreover, we analyzed depressive-like behavior, spatial memory impairment, and motor deficits at 6 months of age, but we did not find any effect of LPS on 5xFAD mice in the forced swimming test, tail suspension test, nor in the Y-maze test. Therefore, the novel object recognition test was used to analyze the effect of LPS on behavior in these mice.

Isolation of monocytes and microglia

Cell enrichment using magnetic beads (Paper II, III and IV)

CD11b magnetic beads from Miltenyi Biotech were used for microglia isolation. The purity of the cell population was verified by flow cytometry. The enrichment was around 95% as identified by two markers, CD45 and CD11b. However, a large amount of debris was observed from these samples. A few reasons could be that myelin remained from the brain disassociation procedure or that dying microglia were present during the whole process. Therefore, further purification of the cell population was required for downstream analysis.

Monocytes were isolated using the same method after being flushed out from murine femurs and tibias. Negative selection was made to obtain a pure monocyte population. In general, the enrichment of monocytes was quite successful without as much debris as the microglia isolation. This method gave a fast, effective and reproducible means to enrich the cells for other analyses. However, further characterization was needed to identify particular cell types.

Flow cytometry (Paper II, III, and IV)

Flow cytometry is commonly exploited in hematopoietic research for the identification of cellular markers, relative valuation of intracellular and surface proteins and measurement of physical features. Nowadays, it is increasingly implemented in the neuroscience field. This is a powerful tool allowing us to purify specific cell subpopulations, characterize interesting cell subsets and assess the expression of proteins in a much faster and more effective way than western blot

and immunostaining. Furthermore, it opens up 6-8 or more channels for a diverse combination of antibodies. Limitations of this method are the difficulty of isolating cells from low-input samples (less than 10,000 cells) and the requirement for antibodies to target antigens. In addition, since the samples need to be in a single cell suspension, spatial information of the location of the cells in brain tissue is lost.

Transcriptomic and proteomic analysis

Single-cell sequencing (Paper IV)

Single-cell sequencing (scRNA-seq) is a recent, advanced next-generation technology to analyze the genome or transcriptome in individual cells to elucidate heterogeneity of cell populations. Unlike traditional sequencing methods measuring the average expression of genes for many cells, scRNA-seq provides the potential to find a rare subpopulation of cells that may be missed in a bulk analysis. This technique has grown in interest and has been implemented extensively in various research fields since it was first published in 2009 for the identification of cells from early developmental stages[341]. Here, we obtain a high-throughput single-cell transcriptome library (3' ends of RNA) through a Chromium system from 10× Genomics. This method helped us to gain a better and deeper understanding of complex cell populations and identify cellular markers to distinguish microglia from infiltrating monocytes and perivascular macrophages, which have highly similar physiological functions, surface receptors, and genetic profiles to microglia. It also allowed us to map the genotypes of microglia and monocytes to their phenotypes, which has been one of the long-lasting challenges in innate immunity research. The drawbacks of this approach are the high cost for each run and the requirement for a well-established pipeline to analyze a huge amount of data from scRNA-seq. These limit the universal use in exploring heterogeneity under different conditions. Moreover, the individual signatures were lost by pooling the samples together from each condition to reduce the expense. Overall, this technology gave a substantial opportunity to our study to reveal alterations in myeloid cells upon different stimuli.

Immunohistochemistry (Paper II and III)

Immunohistochemistry (IHC) is a historical and traditional approach for protein expression in fixed tissue. Immunohistological analysis was done to quantify the Aβ load and number of microglia to estimate the neuroinflammatory status in **Paper II and III**. Two brain regions, the retrosplenial cortex (RSC) and dentate gyrus (DG) were analyzed. The RSC has been found to have the highest burden of β-amyloid plaques,[339] and the DG is a memory-related brain region that is impaired by 6

months of age in 5xFAD-model mice. The biggest advantage of using IHC was the ability to localize specific cells that presented proteins of interest in the tissue. Especially for the studies on the brain, this information provides crucial clues for understanding the underlying molecular mechanisms and physiological role of cells in a specific brain region. However, IHC lacks a high sensitivity of detection due to the low specific-binding of antibodies and high background noise depending on how the tissue is prepared and the condition of the animals. For example, auto-fluorescence is usually higher in aged animal brains than young ones because of the variety of cell types and the accumulation of lipofuscin, a pigment that gathers in neurons with aging[342]. Brain tissue also has numerous lipid bilayers, resulting in light scattering and auto-fluorescence. Thus, to mitigate these effects, we performed an antigen retrieval step to expose epitopes before the first blocking step and an additional blocking step before secondary staining. Another drawback of this method is the number of antibodies that can be used for a given sample as only 3 or 4 antibodies can be used, which is based on the laser channels available for a given microscope. Moreover, the whole procedure takes a relatively long time and requires careful perfusion and fixation of the brain samples.

Western blot analysis (Paper I and II)

Western blot is a well-established and extensively used method to relatively quantify the expression level of protein. It was applied to relatively quantify target proteins, including markers for EVs (Alix and Flotillin-1) and inflammatory proteins (iNOS, NLRP3, and Pro-caspase-1) in **Paper I** and different species of amyloid aggregates (Aβ40 and Aβ42) in **Paper II**. Actin was used as a housekeeping protein for cell lysates. However, whether actin is expressed consistently in EVs is unknown. To better normalize EV release, we normalized Alix and Flotillin-1 levels with the total protein amount detected in the cell lysates.

Multiplex cytokine enzyme-linked immunosorbent assay (ELISA) (Paper I, II, III, and IV)

The concentrations of different cytokines in EVs and media as well as serum and brain homogenate from mice were measured with different Mesoscale Discovery plates using a QuickPlex SQ120 Plate Reader (Mesoscale Discovery, Rockville, USA) according to the manufacturer's instructions. The different cytokines are detailed in the papers. The Mesoscale Discovery system utilizes a multiplex ELISA to immediately analyze up to 10 different analytes, e.g. metabolites, and various Aβ isoforms. The sensitivity of detection of various molecules is relatively high. The lowest level of cytokine that can be accurately measured by the pro-inflammatory panel in **Paper I** is listed here: IFN-γ, 0.04 pg/mL; IL-1β, 0.11 pg/mL; IL-2, 0.22 pg/mL; IL-4, 0.11 pg/mL; IL-5, 0.06 pg/mL; IL-6, 0.61 pg/mL; KC/GRO, 0.24

pg/mL; IL-10, 0.94 pg/mL; IL-12p70, 9.95 pg/mL and TNF-α, 0.13 pg/mL. However, a few samples that were out of the detection range were either removed due to low concentration or diluted later reanalysis. Due to the lack of housekeeping proteins in the assay for brain homogenates, we normalized the samples by loading the same amount of total protein in **Paper IV**.

Mass spectrometry (Paper I, II, and III)

Mass spectrometry (MS) is a comprehensive technique to identify and quantify different proteins. We utilized qualitative (**Paper I and II)** and quantitative analysis (**Paper III)** from the Proteomics Core Facility at Sahlgrenska Academy at the University of Gothenburg. As mentioned before in the EV Isolation Procedure section, the amount of EVs was not sufficient to run quantitative MS, so while **Paper II** aimed to compare different proteins expressed in microglia before and after amyloid plaque deposition, the qualitative method was selected to identify proteins in these samples instead.

Microscopy

Confocal microscopy (Paper II, III, and IV)

Confocal microscopy has several benefits over conventional epi-fluorescence microscopy, such as control over the depth of field and elimination of background noise. It also has fewer spillover effects between channels as the images are taken using the lasers one by one. The largest disadvantage that I have encountered so far is the long preparation time needed to set up all of the parameters that are suitable for the different conditions. In addition, the life expectancy of the specimen is shortened due to bleaching during the process.

Transmission electron microscopy (TEM) (Paper I)

TEM offers an excellent opportunity to visualize EVs ranging from 50nm to 1000nm in diameter, which are too small to see with conventional microscopy. The analysis was carried out at the Lund University Bioimaging Center using fresh samples. Since the protein content of the EVs had already been identified by western blot and MS, a general picture of the EVs derived from BV2 microglial cells was taken without any antibody labeling.

Data Analysis

Bioinformatic analysis

Gene Ontology (GO) enrichment analysis was carried out by different databases according to sample specificity. In short, Funrich software was used for **Paper I**, and the PANTHER^{TM} online database was applied in **Paper II**. Quantitative MS data was analyzed using the Perseus platform in **Paper III**. An extensive computational pipeline and analysis were established by the National Bioinformatics Infrastructure Sweden in **Paper IV**.

Statistical analysis

Data were analyzed in GraphPad Prism (Version 8 or 9) as described in the original papers. Briefly, as the data followed a normal distribution, parametric tests were applied, including Student's t-test, one-way ANOVA, and two-way ANOVA followed by Tukey's test for multiple comparisons.

Results

Inflammatory response of microglia upon different stimuli (Paper I and Paper II)

Highlights

- LPS stimulation on BV2 microglia gave rise to larger extracellular vesicles (EVs) containing increased levels of proinflammatory cytokines in vitro.
- The proteomic profile of EVs shifted to ribosomal assembly and translation in the presence of LPS in vitro.
- Complete ablation of TNF expression alleviated the inflammation with reduced amount of EVs secretion in vivo and in vitro.
- In young 5xFAD mice (6 weeks old), inflammation can be detected in the microglia.

Modulation of innate immunity by systemic inflammation (Paper III and Paper IV)

Highlights

Following peripheral LPS challenge at 6 weeks of age in 5xFAD mice

- Suppression of microglial activation at 6-month-old 5xFAD mice.
- Less plaque load in hippocampus in 6-months-old 5xFAD mice.
- Monocytes responded differently than microglia.

Following peripheral LPS challenge at postnatal day 9 in WT and 5xFAD mice

- 6 months later, unique subpopulations of microglia were found in WT mice. These subpopulations diminished in 5xFAD mice by 6 months of age.
- One subpopulation of monocytes was significantly reduced in 6-month-old WT but not in 5xFAD mice.

Paper I

Inflammation leads to distinct populations of microglial extracellular vesicles.

Main findings

LPS stimulation had the following effects on microglia-derived extracellular vesicles (EVs) *in vitro*.

- The levels of TNF and IL-6 were increased in EVs
- Larger size of EVs were released, particularly in the range of 200-300nm, with a higher ratio of Flotillin-1 and CD63,
- Higher number of EVs was secreted.
- With the inhibition of TNF, the number of EVs produced by microglia in the presence of LPS was attenuated down to control level.
- Proteomic profile was altered with more proteins associated with RNA binding and translocation from gene ontology analysis.

Permanent middle cerebral artery occlusion (pMACO), a focal cerebral ischemia mouse model, triggered the following events in wild type (WT) and TNF-knockout (TNF-KO) mice *in vivo*.

- One day after pMCAO, the systemic inflammation occurred in the periphery with the listed consequences.
    - Upregulation of TNF, IL-5, and IL-12p70 in WT, but not TNF-KO.
    - Increased release of IL-1β, IL-6, and KC/GRO (CXCL1) in WT and TNF-KO, but such increases were attenuated in TNF-KO.
    - Increased number of EVs in both genotypes.
- Five days after pMCAO, EVs counts reduced significantly in TNF-KO compared to WT.

Conclusion

Our data showed altered EV production in BV2 microglial cells and altered EV cytokine levels and protein composition in response to LPS challenge.

Impact

Our findings provide new insights into the potential roles of EVs that could be related to the pathogenesis in neuroinflammatory-affected diseases.

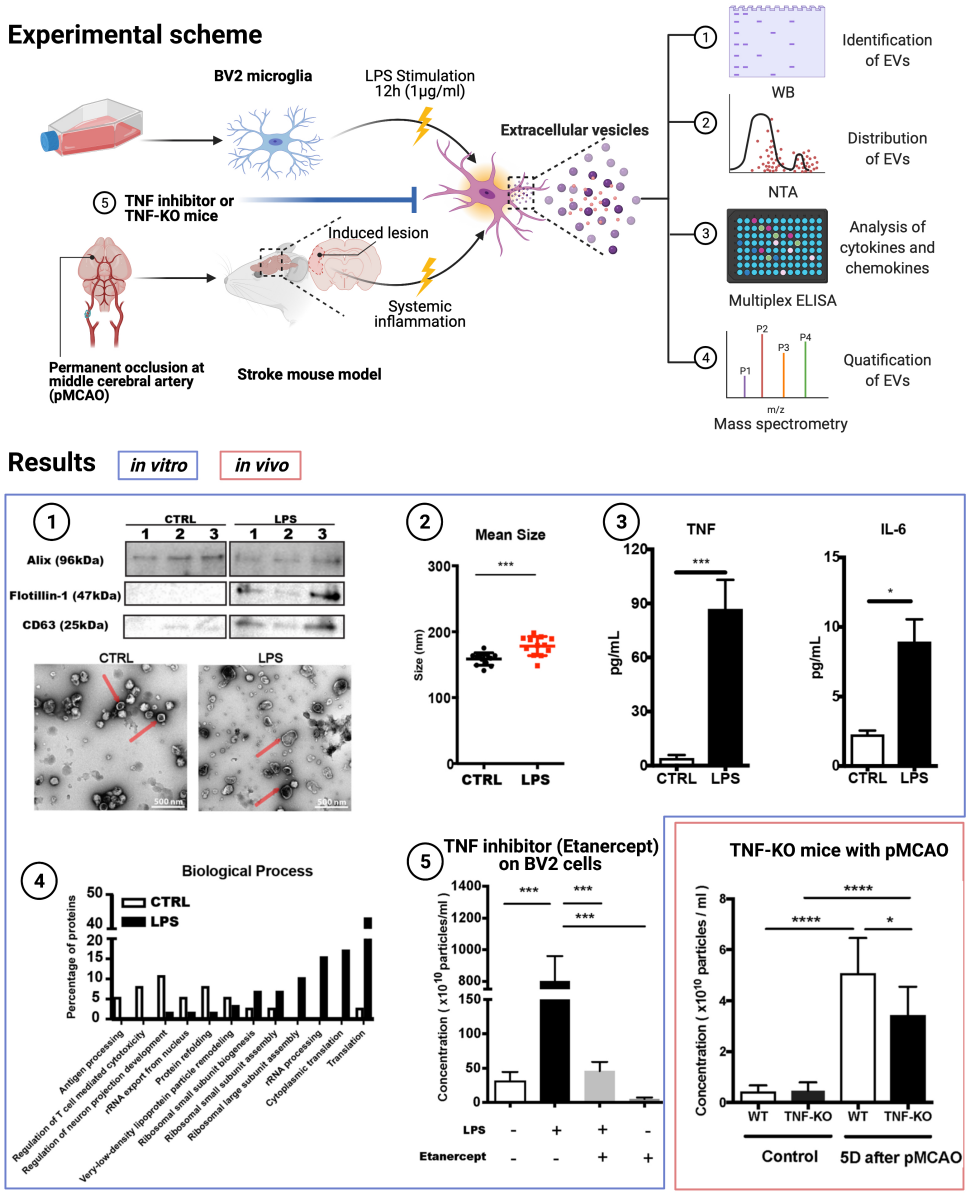

Figure 6 Experimental scheme and main findings of Paper I, "Inflammation leads to distinct populations of extracellular vesicles from microglia"(created with BioRender.com).
The results are ordered according to the experimental scheme.

Paper II

Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD.

Main findings

Inflammatory effects before (2 and 6 weeks old) and after Aβ deposition (10 weeks old) in 5xFAD mice.

- Pro- and anti-inflammatory cytokines were specifically changed at different ages of mice in the brain.
    - At ages of 6 and 10 weeks, a trend of reduced IL-12 appeared.
    - At 10 weeks of age, when Aβ deposition had already begun, IL-1β and IL-10 were significantly increased.
- Distinct alterations in protein expression in microglia was revealed by gene ontology analysis.
    - At 6 weeks of age, the top three signaling pathways in microglia annotated were JAK/STAT, PDGF, and p38 MAPK cascades.
    - At 10 weeks of age, the top pathways were synaptic vesicle trafficking, glutamate receptor signaling and adrenergic receptor signaling.
    - 10-week-old mice had significant upregulation of several innate immunity-related signaling pathways, including IFN-γ, MAPK cascade, and interleukin pathways, compared to 6-week-old mice.

Quantification of microglial activation and Aβ deposition in 5xFAD mice.

- At 6 weeks of age, prior to Aβ plaque deposition, higher levels of soluble Aβ42 were found in brain.
- At 6 weeks, galectin-3 positive microglia were observed in hippocampal CA1 in the pyramidal layer near APP-positive neurons.
- At 10 weeks of age, Aβ plaques were first seen in subiculum.

Conclusion

Early inflammation occurs in microglia even before the accumulation of Aβ.

Impact

Targeting early microglial alteration may be crucial for pre-clinical AD trials.

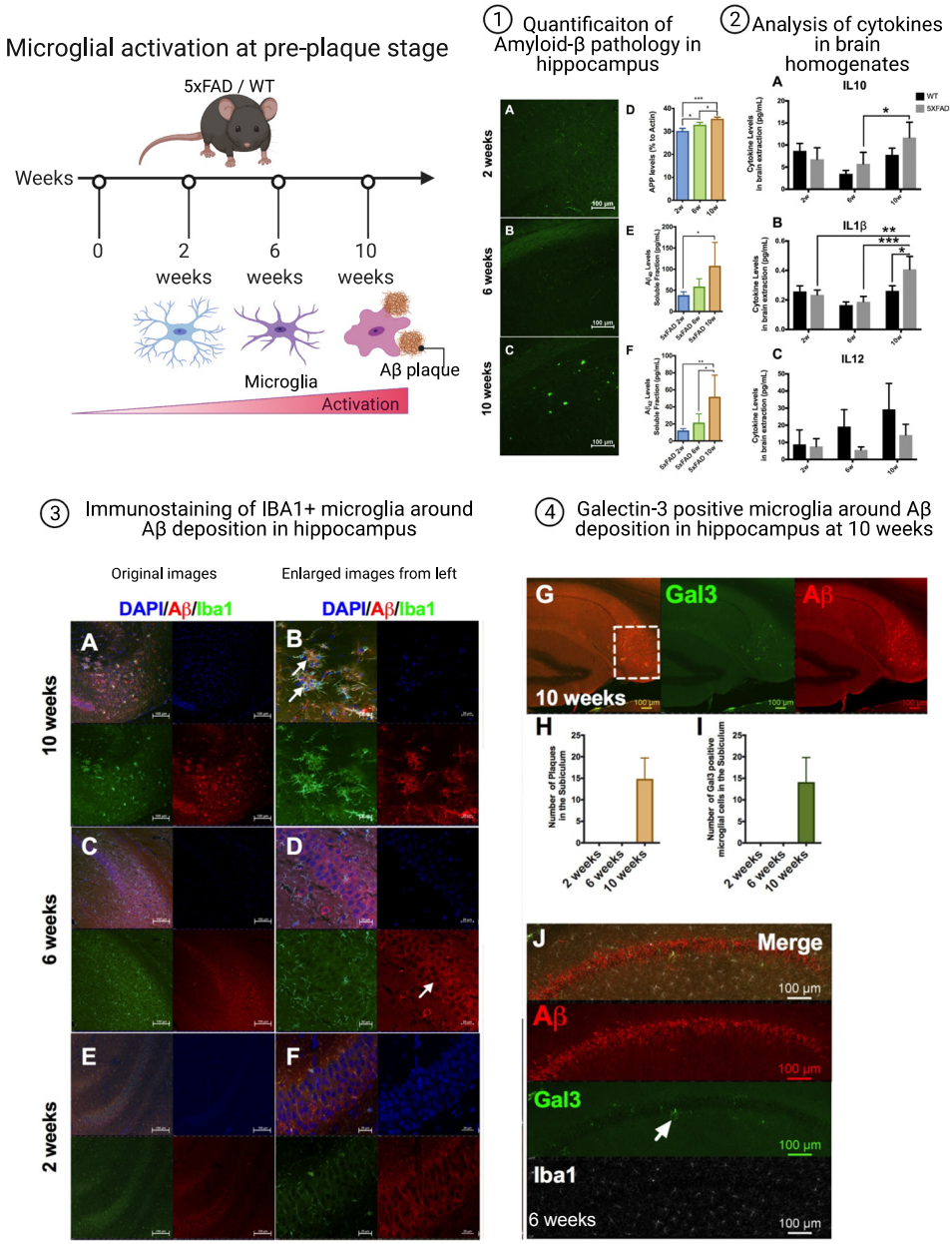

Figure 7 Experimental scheme and main findings of Paper II, "Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD"(created with BioRender.com).

Paper III

Long-term effects of acute systemic inflammation on myeloid cells persist into adulthood in the 5xFAD mouse model of Alzheimer's disease.

Main findings

Long-term effects of LPS induced systemic inflammation and Aβ pathology on myeloid cells.

- Microglia from CNS:
  - Periphery LPS challenge was able to induce a similar effect as Aβ pathology, such as a lower expression of Cacybp, S100a9, and Fabp4.
- Monocytes from bone marrow:
  - WT-LPS mice demonstrated upregulation of apoptosis and PDGF cascades compared to WT
  - 5xFAD-LPS vs 5xFAD showed increased expression in the Wnt pathway and downregulation of PDGF signaling compared to 5xFAD only.
  - Aβ pathology in CNS caused a larger expression of Alzheimer's disease-related proteins, including APP, CLU, and APOE.

Quantification of microglial activation and Aβ deposition in 5xFAD mice 4.5 months after LPS administration.

- Significantly reduced number of microglia around plaques in dentate gyrus (DG).
- Significantly fewer galectin-3-positive microglia in retrosplenial cortex (RSC) and DG.
- Reduced plaque burden was found in DG but not in RSC.

Conclusion

Our findings emphasize the potential long-term effects of early systemic inflammation which may contribute to mitigate Aβ load as a consequence.

Impact

It provides a potential approach to modulate innate immunity for beneficial effects.

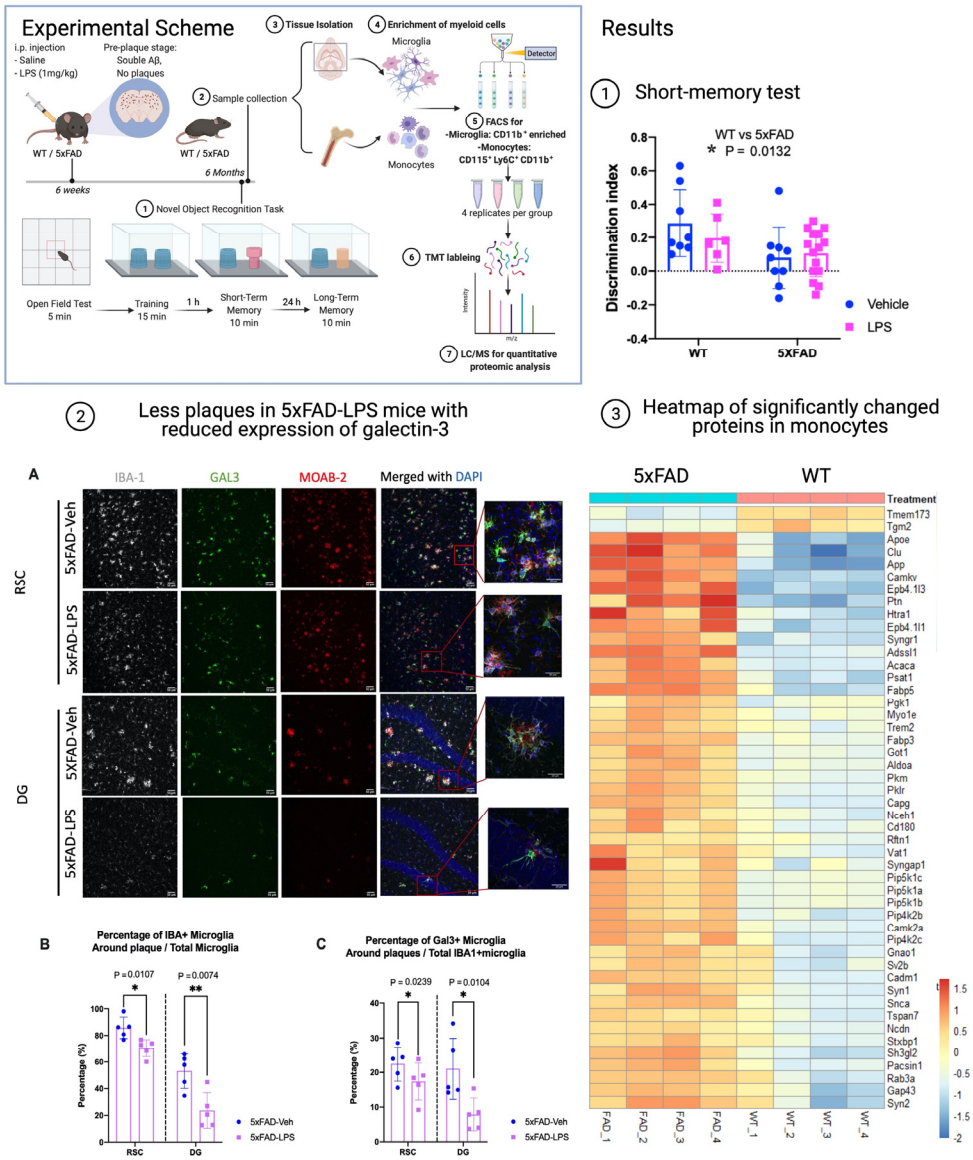

Figure 8 Experimental scheme and main findings of Paper III, "Long-term effects of acute systemic inflammation in adulthood on myeloid cells in the 5xFAD mouse of Alzheimer's disease"(created with BioRender.com).

Paper IV

Postnatal LPS injection induces a unique phenotype in myeloid cells in the adulthood that is associated with amyloid-beta pathology.

Main findings

Long-term effects of LPS challenge at postnatal day 9 in WT and 5xFAD mice

- Single-cell sequencing revealed 19 different clusters for two cell types.
    - Homeostatic microglia were identified in cluster 1. Disease-associated microglia (DAM) were mapped at cluster 5. Perivascular macrophages were located at cluster 8.
    - Monocytes resided mostly in cluster 9 to 19.
- Unique subpopulations of microglia were found in WT, but were diminished in 5xFAD mice.
    - These unique subpopulations adopted a monocyte-like phenotype, with high expression of Lyz2, Tmsb10, Lgals1, and Lgals3.
- Several cluster-specific genes were identified: Fos in cluster 3, Lpl, Itgax and Ank in cluster 5, Mif in cluster 6, and Mrc1 and CD74 in cluster 8.

Cytokine and chemokines analysis on the long-term effects in the brain region of hippocampus and the periphery.

- In hippocampus: GM-CSF and TNF-α were significantly ameliorated due to the Aβ pathology. MIP-1α was increased in an Aβ dependent manner. MIP-1β (CCL4) was significantly reduced in 5xFAD-vehicle but increased with LPS stimulation in 5xFAD mice.
- In the periphery: MMP-9 and TNF-α were significantly increased in the serum in an Aβ dependent fashion.

Conclusion

We found long-lasting effects from early systemic inflammation, including activation of inflammation through alteration of transcriptomics profiles and modulation of different inflammatory mediators on myeloid cells.

Impact

An improved characterization of myeloid cells may shed the light on the pathogenesis and give alternatively approaches for early diagnosis, treatment, and eventually prevention of the disease.

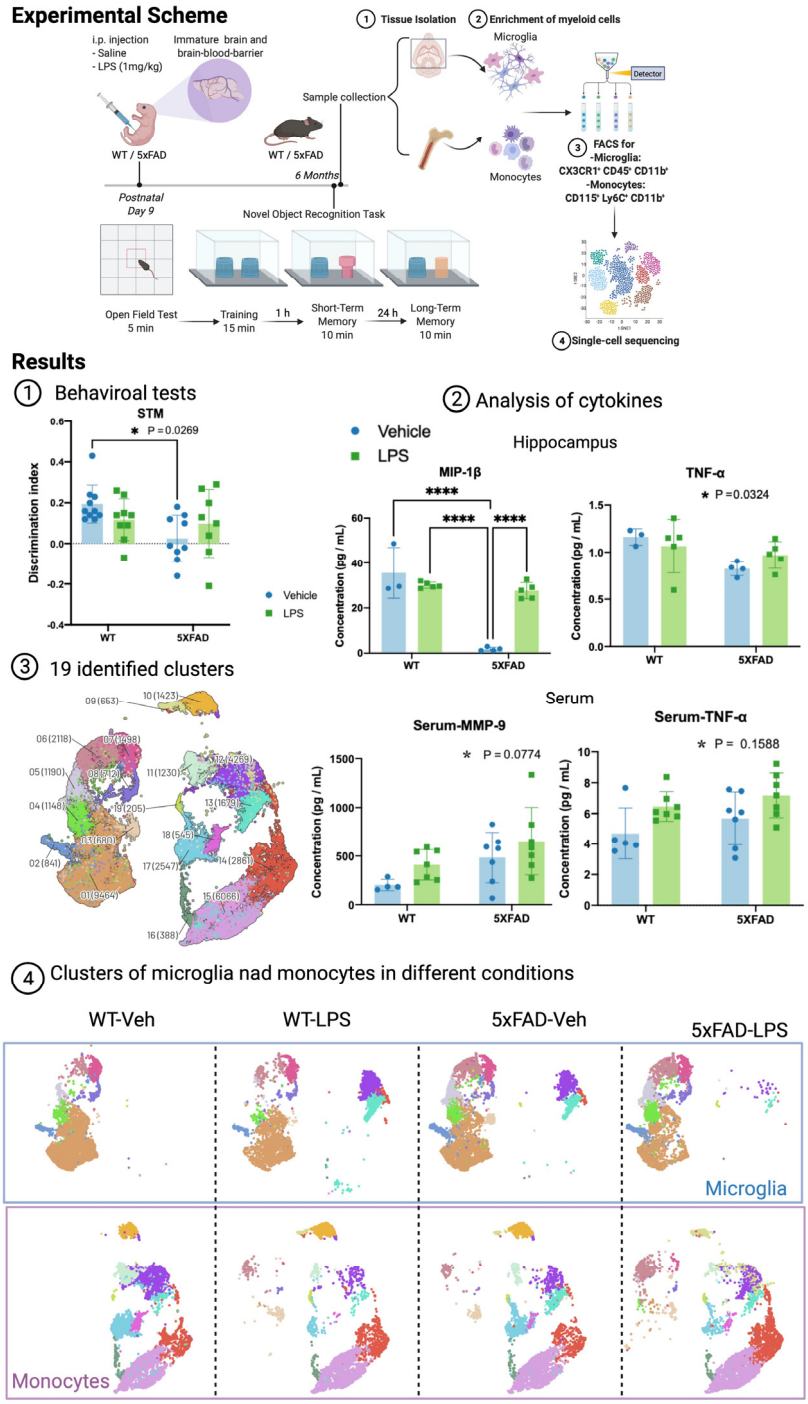

Figure 9 Experimental scheme and main findings of Paper IV, "Postnatal LPS injection induces a unique phenotype of myeloid cells in the adulthood associated with amyloid-beta pathology"(created with BioRender.com).

Discussion

Inflammatory response of microglia upon different stimuli

As mentioned in the introduction, activation of peripheral innate immunity and neuroinflammation play crucial roles in neurodegenerative diseases. Microglia as a key element of innate immunity in the central nervous system (CNS). The activation of microglia is initiated by different intracellular mechanisms, resulting in the release of proinflammatory mediators, which promote the propagation of inflammation[264,343,344].

TNF- cytoprotective or cytotoxic upon LPS activation

In this thesis, we investigated a non-canonical pathway that could spread these mediators, which was through extracellular vesicles (EVs). Other groups have shown that EVs play a role in the regulation of cytokines and have an association with aggregation of pathogenic proteins (ref). We found that inflammation was closely connected to EV production and secretion in Paper I. By using tumour necrosis factor (TNF) inhibitor *in vitro* and TNF-knockout mice (*in vivo)* to reduce inflammation, we observed a reduction in the release of pro-inflammatory cytokines and of the total number of EVs released. However, the EVs collected from plasma originated mainly from cells in the periphery and not directly from microglia in CNS. Nevertheless, these results suggest that TNF could be a universal signalling pathway that can decrease inflammation[345].

In fact, there are two forms of TNF–soluble TNF (solTNF) and TNF (tmTNF)– which have contradictory functions [346,347]. solTNF is related to neurotoxicity and inflammation[348–350], while the transmembrane form is involved in functional recovery and neuroprotection[346,351]. Hence, the specific form of TNF carried in microglial EVs or the expression of a particular form of TNF in the brain and serum will probably determine whether the outcome is neuroprotective or neurotoxic. One piece of evidence from Paper IV showed that TNF-α was reduced in hippocampus due to the overexpression of Aβ. However, it was increased in the serum from the same mice. One reasonable explanation could be that more tmTNF was released by tissue homogenization. In contrast, a higher level of solTNF was released in the

serum. In our studies, we measured total TNF in EVs and conditioned medium (Paper I), serum (Paper III and IV), and brain homogenate (Paper II, IV). More studies investigating the specific form of TNF could provide a fuller picture of inflammation in the CNS and periphery.

Early activation of microglia in the 5xFAD mouse model

We studied inflammatory alterations related to microglia in 5xFAD mice. Early activation of microglia was observed at 6 weeks of age, even before the appearance of plaques. APP-positive neurons and soluble Aβ42 were also found at 6 weeks of age, possibly leading to the alterations in microglia. In Paper II, we found that the JAK-STAT, MAPK and Interleukins pathways were highly upregulated in microglia isolated from 6-week-old 5xFAD mice. As the pathology grows exponentially in this model, microglia likely became overactivated and played a detrimental role in the CNS (studied in Paper III and IV). This study pointed out an intervention time window for Paper III. Galectin-3 (Gal3) expression was previously demonstrated in the microglia surrounding Aβ plaques in 5xFAD mice[239]. Intensive studies from our group have revealed the critical role of Gal3 in microglial activation in neurodegenerative diseases[238,239,352]. In line with earlier findings, Gal3-positive microglia were present around APP-positive neurons at 6 weeks of age and by the Aβ plaques in the subiculum by 10 weeks of age. Whether these microglia have detrimental or beneficial effects at the pre-plaque stage is not clear. Although a detrimental role of Gal3 was illustrated in the 5xFAD model at 6 months and 18 months of age[239], different subpopulations of Gal3-positive microglia were not revealed there. Thus, we further investigated this in Paper III and IV.

Modulation of innate immunity by systemic inflammation

Here, I will focus on the different effects of peripheral LPS challenge and Aβ pathology on microglia and monocytes (Paper III and IV). The potential to prime the innate immune system may provide an opportunity to halt the progression of AD. Moreover, we attempted to map the phenotypes of myeloid cells to functional profiles in Paper IV.

Trained innate immunity and immune tolerance

The concept of innate immunity memory was introduced earlier in this thesis, defined as the ability of microglia to be primed and retain the innate memory. Trained innate immunity results in a quick response with an enhanced immune

reaction when a previously known pathogen or stimulus reappears[311,312]. Conversely, innate tolerance is a compensatory regulation to limit the extent of hyperactivation and tissue damage in repetitive or chronic infection[311,316,353]. Studies conflict on the consequences of innate tolerance [312,319,320]. Innate immunity in CNS can be modified to a certain extent, and this strongly depends on the concentration of the stimulus, the timepoint of the intervention and the duration after the intervention. Many studies have addressed the acute effects of inflammation in CNS[241,321,322]. The long-term alterations in microglia under Aβ pathology, however, are not as clear . Thus, we looked at the long-term effects of systemic inflammation in 5xFAD mice at different time points.

In Paper III, we primed the microglia in adult mice with a single dose of LPS given before plaque deposition. We stimulated the mice with peripheral LPS injection, resulting in fewer Aβ plaques in the dentate gyrus (DG) at 6 months old of age compared to age-matched 5xFAD mice. These findings suggest a beneficial effect on microglia of LPS priming, and we found that the hippocampus may be more sensitive to this than the retrosplenial cortex (RSC). It is possible that the over-loading amount of Aβ in RSC was beyond the clearance capability of microglia. Interestingly, expression of Gal-3 in microglia seemed to be suppressed in DG and RSC along with reduction of MHC I. Proteomic analysis showed an indication of upregulation of EIF4EBP1 and downregulation of Rel in microglia from LPS-primed 5xFAD mice compared to 5xFAD. EIF4EBP1 is a downstream protein in the mTOR cascade related to cell proliferation, survival, and autophagy[354,355]. Dephosphorylation of EIF4EBP1 initiates a cap-dependent pathway for protein translation. Increased levels of EIF4EBP1 are related to activation of mTOR upon LPS stimulation. Still, the consequences of this phenomenon are not clear and could possibly include increasing autophagy to clear up Aβ or promote cell proliferation and survival[356]. More studies are required, and more on this topic will be mentioned in **Future Perspectives**. In addition, Rel is one of the NF-κB subunits associated with epigenetic modification in innate immunity and can persist for a long period of time[357]. Studies have reported that primed microglia have a higher phagocytic capacity and can increase the amount of repressive histone modifications, H3K9me2, in the promoters for the IL-1β and TNF-α genes[358–360]. Such alterations in the expression of these genes are consistent with our hypothesis and current findings. Therefore, we would like to further study the role of Gal-3 in the NF-κB signaling pathway, which has not been well elucidated before.

In Paper IV, we chose to prime microglia in postnatal mice as the blood-brain barrier (BBB) and brain are not yet fully developed then. Intriguingly, we saw more Gal3-positive microglia in 5xFAD and 5xFAD-LPS mice after the early intervention compared to the later intervention in adult 6-week-old mice. This suggests that postnatal mice are more vulnerable to triggers that activate microglia than adult mice when inflammation occurs in the periphery. Since this is an ongoing project, more

histological labelling is needed to verify the consequences, such as labelling Claudin-5 for integrity of BBB, CD68 for phagocytosis, and CCL4 for inflammatory response.

No effect on behavioural deficits in 5xFAD subject to LPS

Administration of a peripheral LPS challenge, either in adulthood or at the postnatal stage, did not induce cognitive impairment in WT nor worsen the cognition in 5xFAD mice. This may be due to that a single dose of LPS (1μg/kg) may be insufficient to impair memory-related brain regions. Another reason may be that the timepoint when we looked for behavioral deficits may have been too late. Neither STM nor LTM tests showed memory alterations due to LPS treatment. However, our work is limited by the low number of mice in each group (at least 6 animals per group) since large variations existed within the groups. More mice are needed per group for the statistical tests.

The heterogeneity of microglia and monocytes

An immune response and their related pathways are frequently elicited in AD and other neurodegenerative diseases. With an increasing number of researchers using single cell RNA-seq as a means to study this question, the heterogeneity of microglia and monocytes has been uncovered and can be defined by diverse markers with potential roles in different signaling cascades[291,361–363]. In Paper IV, we compared the immune response of monocytes to that of microglia in mice that experienced acute systemic inflammation as well as developed AD pathology as a chronic inflammation in the brain. Our results suggest these two conditions triggered different immune responses in microglia and monocytes. Several previously identified phenotypes were also confirmed in this study, such as homeostatic microglia (Cluster 1) and DAM (Cluster 5). DAM have been observed in the vicinity of plaques and can phagocytose β-amyloid in CNS[291]. These findings highlight the protective role of DAM in AD. Interestingly, this cluster appears primarily in 5xFAD mice with some DAM in WT as well, but very few appeared in the WT-LPS group. This implies that DAM may be more responsive to events requiring phagocytosis, such as plaques, and may not respond effectively to LPS stimulation. Further comparisons of monocytes are needed to investigate the different responses in the periphery.

Detrimental role of microglia

We found a novel subtype of microglia with a monocyte-like transcriptomic profile and investigated their reaction to different inflammatory stimuli. This distinct

subtype exists in both WT mice after early-life peripheral LPS stimulation and 5xFAD mice without early-life inflammation. In other words, this subtype only appeared under conditions when only one stimulus challenged the immune system. Further investigation into this subtype revealed potential markers, including Lyz2, S100a6, Lgals3 (Gal-3), and Lgals1 (Gal-1), as labels that could be used for immunostaining or qPCR to further validate and examine the functionality of this subpopulation in the brain. Lyz2 is primarily involved in the clearance of bacteria, and S100a6 may lead to apoptosis through activation of the RAGE signaling pathway[364]. In line with previous studies from our group, this subtype of microglia may have detrimental effects in CNS under Aβ pathology[239]. It is highly likely that these microglia come from the periphery as they localized closely with BM-Mo in the transcriptomic mapping. However, this speculation needs evidence from further animal studies.

General conclusions

Overall, the studies in this thesis imply the following:

- LPS activation leads to unique populations of EVs from microglia.
- LPS stimulates microglia to release larger extracellular vesicles (EVs) containing increased levels of pro-inflammatory cytokines.
- Complete ablation of TNF expression alleviates inflammation with reduced EV secretion *in vivo* and *in vitro*.
- In 5xFAD mice, microglial inflammation starts as early as at 6 weeks of age, prior to Aβ plaque appearance.
- Systemic inflammation in adulthood induces long-term alterations in innate immunity, such as repression of activated microglia and promotion of apoptosis in monocytes from bone marrow.
- Peripheral LPS challenge at the pre-plaque stage triggers a long-lasting effect on microglia, leading to a reduction of Aβ burden in the hippocampi of 5xFAD mice.
- Early systemic inflammation and Aβ pathology induce distinct subpopulations of microglia that upregulate Lyz2 and Lgals3 (encodes galectin-3).

Future Perspectives

The importance of inflammation in the development of neurological disorders has been intensively investigated and elucidated in recent years. Questions on the molecular mechanisms behind these diseases are still awaiting an answer. In this thesis, I focused mainly on the role of innate immunity, microglia in the brain and monocytes located in the bone marrow, all under the pathology of Alzheimer's disease (AD). The early activation of microglia and the potential modulation of these cells by systemic inflammation are demonstrated in the papers included in this thesis. However, several fundamental questions remain to be solved, such as when and how neuroinflammation occurs before the manifestation of clinical symptoms, what factors and pathways are involved in the initiation of detrimental microglial activation, and how innate immunity contributes to the pathology. With emerging advanced technologies, there are great opportunities to answer these questions.

Challenges of studying microglia

I have studied the effects of different stimuli on microglia *in vitro* and *in vivo*: a murine microglial cell line and 5xFAD mice were used to address specific questions. Here, I would like to discuss the challenges of studying microglia. As microglia are highly dynamic cells in the brain and respond immediately to the environment, one of the complications is that cultured microglial cell lines do not reflect the physiological complexity in the human brain. Efforts have been made to establish human iPSC-derived microglial lines and three-dimensional cellular culture systems, but the full recapitulation of microglia *in vivo* is still lacking.

Another challenge is how to maintain microglial phenotypes after the isolation and purification processes. One of the approaches could be *in vivo* live imaging using specific reporter mice. However, this tool also has limitations as evidence shows that microglia in anaesthetised mice are more active than those from awake mice[365,366]. Moreover, any type of manipulation on the brain, such as cranial window surgery or perfusion, can injure the brain and activate microglia. Therefore, these problems should always be taken into consideration when designing an experiment to study microglia under physiological conditions.

The last challenge that I realized in my studies is the large overlap in markers between microglia and myeloid cells in the periphery. This made it difficult to distinguish microglia from infiltrating myeloid cells during inflammation as our goal was to elucidate the roles of microglia and monocytes in the progression of AD. The use of a reporter mouse with inducible fluorescent labelling linked to genes exclusively expressed either in microglia or monocytes may have been a better choice, such as tamoxifen-inducible $CX3CR1^{CreER}$ mice crossed with $R26^{Reporter}$ for microglia and $CCR2^{CreER}$×Rosa26 mice for monocytes[309] since CX3CR1 is generally expressed in peripheral monocytes, dendritic cells, and NK cells as well. Other reporter mice have been developed to have more precise microglia labelling, such as $TMEM119^{TdTomato}$ and $Hexb^{TdTomato}$[367] mice.

Shared responses between systemic inflammation and Alzheimer's disease

We observed a long-lasting effect of systemic inflammation on microglia and bone marrow-resident monocytes. The signaling pathways triggered under systemic inflammation and AD shared commonalities to some extent. In **Paper III,** systemic inflammation reduced plaque deposition in the hippocampus but not in retrosplenial cortex. It is possible that hippocampus is more vulnerable towards inflammation. If so, microglia would respond differently in these two brain regions. Thus, it would be interesting to validate the findings obtained from proteomic analysis by histology. For example, MHC I was downregulated in microglia from LPS-administered WT and 5xFAD mice. Localizing MHC I positive microglia in the brain may provide evidence of region-specific innate responses. I propose the following experiments to elucidate how LPS primes the immune system under AD pathology through two signalling cascades: One is to investigate how LPS and Aβ affect the translocation of Rel in the NF-kb pathway, leading to manipulation of gal-3 expression. The other one is to study the mTOR pathway due to significantly high expression of EIF4EBP1 in LPS-injected 5xFAD mice. Inhibitors of Rel (IT-603 and IT-901) and EIF4EBP1 (Rapamycin) would be tested in a human microglial cell line to have higher clinical relevance.

Validation of the single-cell sequencing data

Single-cell RNA sequencing (scRNA-seq) allows us to explore gene expression at the single-cell level, which has accelerated the revolution in transcriptomics. The heterogeneity of microglia and BM-Mo under pathological conditions was revealed in **Paper IV**.

One drawback of this technology is that it does not directly indicate the expression level of proteins, which are the functional molecules in signalling pathways. Thus, specific clusters or genes found in scRNA-seq must be validated *in vivo* by histological staining or western blot. As we saw region-specific effects from LPS injection in adulthood in Paper III, it would be interesting to study the spatial characteristics of various subpopulations, which is another drawback of scRNA-seq. Thus, I would suggest performing immunohistochemistry on brain sections as a remedy. I would also propose to apply qPCR to identify interesting gene expression from brain tissues from two regions: hippocampus and prefrontal cortex.

Furthermore, the cellular markers of myeloid cells in mice and humans are not consistent. Therefore, it is necessary to validate the findings in AD or septic patients. Given the fact that spatial transcriptomics and single-cell proteomics are available, they can give a deeper insight into the cellular changes in a particular area, such as in the vicinity of Aβ plaques[368]. With studies incorporating these technologies and more, increasing knowledge may one day elucidate the pathogenesis of AD and other neurological disorders.

Modulation of innate immunity

In this thesis, Aβ pathology is the central pathological feature for the activation of microglia and chronic inflammation in AD. In sporadic forms of AD, there are various genetic risk factors and associated comorbidities affecting the manifestation of the disease. More translational models are necessary to better reflect the various pathological aspects of AD in humans. Of note, the overexaggerated pathological damage in the 5xFAD model can cause over-activation of microglia and mask the period with beneficial microglial functions in the human brain. Therefore, to better mimic the preclinical situation, less aggressive AD mouse models are required to study neuroinflammation, such as the APP knock-in mice developed by the Saido group[369,370] and a new APP knock-in mice established this year by the Sanchez group[371]. Given the evidence that cognitively normal people have Aβ deposition in the brain, the ability to maintain homeostasis during aging is individually specific[372]. Thus, it is crucial to investigate potential factors related to the capability of microglia in the brain since microglia carry out the inflammation initiated by pathological damage, such as in response to misfolded proteins (Aβ and Tau), cellular death, and neuronal impairment[373]. Moreover, more support for the immune system in CNS is available from the periphery as infiltrating immune cells also contribute to homeostatic maintenance. The long-lasting effects on myeloid cells from a single dose of LPS seemed to reduce Aβ load in my studies. However, more studies are needed to show how the peripheral immune system contributes to pathology. Nevertheless, modulation of the peripheral immune system may be an alternative approach for pharmaceuticals and therapeutics for neurological diseases.

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Paper I

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Inflammation leads to distinct populations of extracellular vesicles from microglia

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Abstract

Background: Activated microglia play an essential role in inflammatory responses elicited in the central nervous system (CNS). Microglia-derived extracellular vesicles (EVs) are suggested to be involved in propagation of inflammatory signals and in the modulation of cell-to-cell communication. However, there is a lack of knowledge on the regulation of EVs and how this in turn facilitates the communication between cells in the brain. Here, we characterized microglial EVs under inflammatory conditions and investigated the effects of inflammation on the EV size, quantity, and protein content.

Methods: We have utilized western blot, nanoparticle tracking analysis (NTA), and mass spectrometry to characterize EVs and examine the alterations of secreted EVs from a microglial cell line (BV2) following lipopolysaccharide (LPS) and tumor necrosis factor (TNF) inhibitor (etanercept) treatments, or either alone. The inflammatory responses were measured with multiplex cytokine ELISA and western blot. We also subjected TNF knockout mice to experimental stroke (permanent middle cerebral artery occlusion) and validated the effect of TNF inhibition on EV release.

Results: Our analysis of EVs originating from activated BV2 microglia revealed a significant increase in the intravesicular levels of TNF and interleukin (IL)-6. We also observed that the number of EVs released was reduced both in vitro and in vivo when inflammation was inhibited via the TNF pathway. Finally, via mass spectrometry, we identified 49 unique proteins in EVs released from LPS-activated microglia compared to control EVs (58 proteins in EVs released from LPS-activated microglia and 37 from control EVs). According to Gene Ontology (GO) analysis, we found a large increase of proteins related to translation and transcription in EVs from LPS. Importantly, we showed a distinct profile of proteins found in EVs released from LPS treated cells compared to control.

Conclusions: We demonstrate altered EV production in BV2 microglial cells and altered cytokine levels and protein composition carried by EVs in response to LPS challenge. Our findings provide new insights into the potential roles of EVs that could be related to the pathogenesis in neuroinflammatory diseases.

Keywords: Microglia, Extracellular vesicles (EVs), Neuroinflammation, TNF

Background

Microglia are considered the main innate-immune cells of the central nervous system (CNS). They continuously survey their microenvironment and have the ability to interact with neurons to regulate their activity [1]. In the healthy brain, microglia continuously survey their surroundings with highly dynamic processes [2] and become activated in response to injury, infection or neurodegenerative processes [3]. Emerging evidence has shown that microglia are key causative players in neuroinflammation, which in turn is believed to play a major role in neurodegenerative diseases [4].

Microglia are highly dynamic cells with the ability to transform their morphology from ramified to amoeboid and alter their phenotypes corresponding to diverse conditions. Traditionally, macrophages and microglial cells are classified into two different phenotypes, M1 and M2. M1-microglia are proinflammatory, secreting inflammatory cytokines, chemokines, and nitric oxide (NO), which is believed to result in neuronal dysfunction and

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accelerate the progression of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). In contrast, M2-microglia are believed to have neuroprotective functions, including increased production of interleukin (IL)-4 and neurotrophic factors, along with an increase in phagocytosis which in turn leads to clearance of cell debris and tissue damage [3, 5]. However, there is a wide spectrum of microglial activation between the two defined phenotypes [6], and microglia might even have specific neuronal functions beyond typical pro-/anti-inflammatory responses [7]. A better understanding of the interactions between microglia and other cells in the brain is therefore needed in order to design therapies to ameliorate the detrimental effects of microglial reactions in brain diseases.

The main interaction between cells occur through cellular signaling pathways including autocrine, paracrine, and endocrine processes [8] and extracellular vesicles (EVs) can be important to transport signals between cells. In fact, increasing evidence has shown EVs are considered one of the main participants in cell-to-cell communication along with having a proposed role in the spread of pathology in neurodegenerative disease [9, 10]. These vesicles are able to carry pathogen-associated and damage-associated molecular patterns that act as signals to regulate and propagate the inflammatory response [11–13]. Hence, investigation of EV trafficking under inflammatory conditions may broaden our understanding of the roles of microglia in neurodegenerative diseases, as well as their potential in therapeutic manipulation.

The secretion of EVs is a highly conserved process [14]. However, a number of studies using proteomic analysis of EVs released by various cell types, including microglia, have revealed a diverse range of markers and alteration of protein composition [15, 16]. The lack of knowledge on EV's regulation in vitro and in vivo halts a clear understanding of EV functions in cell-to-cell communication. It is likely that different subsets of EVs have different functional properties, and trafficking of EVs is most likely modulated by specific signaling pathways.

Although consensus within the field is being reached, the classification of EVs is not easy. While different subsets of EVs are being described with increasing rate, for simplicity, we will focus on two different classes of EVs of different sizes and origins. EVs shed directly from the plasma membrane are characterized as microvesicles or ectosomes ranging from 100 to 1000 nm. Exosomes are generated within the endosomal pathway and terminate at the multivesicular endosomal body (MVB), whereby they are released upon the MVB fusing with the plasma membrane. Generally, the size of exosomes is smaller than microvesicles and below 100 nm.

A recent study has shown that the size distribution and protein composition of EVs in macrophages can be changed after bacterial infection [17]. Thus, there is a critical need for both identification of specific markers and particular signaling pathways controlling EV trafficking. The mechanism of action of EVs in microglial communication is poorly understood. In this study, we hypothesized that activation of microglia can secrete a distinct population of EV through modulation of specific signaling pathways. We investigated the dynamics of EVs from activated microglial (BV2) cells subjected to lipopolysaccharide (LPS) stimulation. We used differential ultracentrifugation to isolate EVs, including microvesicles and exosomes. EVs were then characterized in terms of size and concentration by nanoparticle tracking analysis (NTA), while the origin of EVs was indicated by western blotting using antibodies against CD63, flotillin-1, and Alix. Importantly, we also analyzed the levels of inflammatory cytokines in EVs. Secretion of EVs was altered by suppression of inflammation in microglia via inhibition of tumor necrosis factor (TNF) signaling in vivo and in vitro. Subsequently, qualitative proteomic analysis was performed to reveal a different protein composition of EVs in response to LPS challenge. Taken together, our findings provide new insights into the role of EVs in regulating microglial cell communication.

Methods

Cell culture

BV2, an immortalized murine microglial cell line, was cultured in growing medium containing Dulbecco's modified Eagle medium (DMEM) (Gibco™GlutaMAX™, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Fisher Scientific) in 5% CO_2 in air at 37 °C in a humidified incubator. Cells were re-cultured every 2 days starting at a concentration of 2×10^5 cells/ml in T75 flask (Sarstedt). For a large scale of EV collection, microglia were plated in T175 flask (Sarstedt). For inflammatory activation, cells were challenged with 1 μg/ml LPS (Sigma-Aldrich, Clony 0127-B8) for 12 h and then grown for 12 h in serum-free media prior to collection of EVs. For TNF inhibition experiment, microglia were plated in growing medium either with 1 μg/ml LPS, 200 ng/ml etanercept, or both for 12 h. EVs were collected from serum-free media 12 h after treatment.

Animals

Adult male C57BL/6 mice (between 7 and 8 weeks of age, $n = 20$) were purchased from Taconic Ltd. (Ry, Denmark) and transferred to the Laboratory of Biomedicine, University of Southern Denmark, where they were allowed to acclimatize for 7 days prior to surgery. TNF knockout (TNF-KO) breeding couples were originally purchased from The Jackson Laboratory and transferred to the Laboratory of Biomedicine where they were

established as a colony. Animals were housed under diurnal lighting conditions and given free access to food and water [18]. All animal experiments were performed in accordance with the relevant guidelines and regulations approved by the Danish Animal Ethical Committee (numbers 2011/561-1950 and 2013-15-2934-00924).

Induction of experimental stroke, permanent middle cerebral artery occlusion (pMCAO)

The distal part of the left middle cerebral artery was permanently occluded under Hypnorm and Dormicum anesthesia (fentanyl citrate (0.315 mg/ml; Jansen-Cilag) and fluanisone (10 mg/ml; Jansen-Cilag, Birkerød, Denmark), and midazolam (5 mg/ml; Hoffmann- La Roche, Hvidovre, Denmark)), respectively. After surgery, mice were injected subcutaneously with 1 ml of 0.9% saline and allowed to recover in a 25 °C controlled environment. Mice surviving for 5 days were returned to the conventional animal facility after 24 h. For post-surgical analgesia, mice were treated with 0.001 mg/20 g buprenorphine hydrochloride (Temgesic, Schering-Plough, Ballerup, Denmark) three times at 8-h intervals, starting immediately prior to surgery. Mice were allowed to survive for 1 day (immunofluorescent staining and cytokine measurement) or 5 days (EV analysis) whereafter they were killed using either an overdose of pentobarbital (200 mg/ml) containing lidocaine (20 mg/ml) (Glostrup Apotek, Glostrup, Denmark) and perfused through the left ventricle using 4% paraformaldehyde (PFA) or killed by cervical dislocation. The blood and brains were collected for further analysis.

Extracellular vesicle isolation procedure and transmission electron microscopy (TEM)

For isolation of EVs, cells were cultivated in growing medium DMEM and then deprivation of serum for a period of 12 h. The media was then collected and subjected to a series of low-speed centrifugation steps (500×*g* for 10 min, 2000×*g* for 10 min, and 10,000×*g* for 30 min) at 4 °C in order to remove cells and cellular debris. The supernatant was then collected in centrifuge tubes (Beckman Coulter) and spun at 100,000×*g* for 70 min before the resultant EV pellet was washed in a large volume of phosphate-buffered saline (PBS) before repeating the 100,000×*g* spin. The pellets containing EVs were resuspended in 20 μl of PBS and stored at 4 °C or long term at − 20 °C. For electron microscopic analysis, samples of EVs were fixed with an equal volume of 2% PFA and loaded onto Formvar/carbon-coated electron microscopic grids. EVs were observed under TEM at 80 kV. TEM was carried out at Lund University Bioimaging Center.

Measurement of extracellular vesicles size by nanoparticle tracking analysis (NTA)

The size and total number of EVs were measured by using NanoSight LM10 (Malvern, UK) with the technology of Nanoparticle Tracking Analysis (NTA). In liquid suspension, particles undergo Brownian motion together with light scattering properties, the size distribution and concentration of EVs samples can be obtained [17]. Samples were diluted with distilled water to obtain optimal concentration for detection (10^6–10^9 particles/ml) and injected with a continuous syringe system for 30 s × 5 times at speed 50 μl/min. Data acquisition was undertaken at ambient temperature and measured 5 times by NTA. Data were analyzed with NTA 2.2 software (Malvern, UK) with minimum expected particle size 10 nm.

Western blot analysis

Cell pellets and EVs were lysed in RIPA buffer (Sigma-Aldrich) supplemented with proteinase inhibitors (Thermo Scientific) and PhosphoStop (Roche Diagnostics GmbH). The concentration of cell lysates was determined using bicinchoninic acid assay (BCA) (Thermo Scientific), while concentrations obtained using NanoSight were utilized to ensure even loading of EVs. Samples were loaded onto 4–20% Mini-Protean TGX Precast Gels (Bio-Rad) and then transferred to Nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo System (Bio-Rad). Membranes were incubated with following primary antibodies: Alix (Cell Signaling; 1:1000), flotillin-1 (Cell Signaling; 1:1000), CD63 (Santa Cruz Biotechnology; 1:1000), inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnology; 1:3000), NLRP3 (Adipogen; 1:1000) and pro-caspase1 (Adipogen; 1:1000). All secondary antibodies were horse-radish protein (HRP) conjugated (Vector; 1:5000 or 1:10000). Protein bands were detected using Clarity Western ECL Substrate (Bio-Rad) or Pierce™ ECL Western Blotting Substrate (ThermoFisher), and imaged on Bio-Rad ChemiDoc XRS+. Protein levels were normalized to beta-actin (Sigma-Aldrich; 1:15,000). Image lab™ software (Bio-Rad) was used to analyze the results.

Multiplex cytokine enzyme-linked immunosorbent assay (ELISA)

The concentrations of different cytokines in EVs and in isolated media as well as serum from mice were measured with the MSD Mouse Proinflammatory V-Plex Plus Kit (Interferonγ (IFNγ), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF, C-X-C motif chemokine ligand 1 (KC/GRO), Mesoscale) using a QuickPlex SQ120 Plate Reader (Mesoscale Discovery, Rockville, USA) according to the manufacturer's instructions. The data was analyzed with MSD Discovery Workbench software. The levels of cytokines in EVs and media were normalized to each samples total protein content in cell lysates. In

total, 6 independent EV samples were analyzed; however, those samples that were under the lowest detection limit were removed from the statistical analysis.

Immunohistochemistry

Immunofluorescent double labeling for TNF and CD11b was performed on 16-μm thick, cryostat-cut tissue sections from C57BL/6 mice with 1-day survival after pMCAO as previously described in detail [18, 19].

Extracellular vesicle fluorescent labeling

Following isolation, EVs were labeled with PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane Labeling (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, EVs were resuspended in 1 ml PBS before 1 ml of Diluent C supplemented 4 μl PKH67 dye. Samples were incubated at room temperature for 4 min prior to the addition of 2 ml of 1% bovine serum albumin (BSA) (VWR International) to bind excess dye. Samples were then supplemented with 5 ml PBS and placed in 300 kDa Vivaspin filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany), prior to centrifugation for 5 min at 4000×*g* to remove excess dye. This process was repeated a further two times, followed by a further two washes in a clean filter with DMEM (Thermo Fisher Scientific) in place of PBS. The same procedure minus EVs was carried out as control.

TNF inhibition on dynamics of extracellular vesicle trafficking

PKH67-labeled EVs (2×10^{10} particles/ml) were incubated with BV2 cells as indicated previously. After 12 h incubation, cells were washed three times with PBS and one time with 1 M NaCl prior to fixation with 4% PFA for 20 min on ice. Cells were then imaged by fluorescence microscope (Olympus IX71) at × 20 magnification and images processed using Cellsens Standard version 1.6 software (Olympus). Vesicle uptake was analyzed by measuring fluorescent intensity using ImageJ software (National Institutes of Health).

Mass spectrometry

Mass spectrometry was carried out on an Orbitrap Fusion Tribrid MS system (Thermo Scientific) equipped with a Proxeon Easy-nLC 1000 (Thermo Fisher). Injected peptides were trapped on an Acclaim PepMap C18 column (3-μm particle size, 75-μm inner diameter × 20 mm length). After trapping, gradient elution of peptides was performed on an Acclaim PepMap C18 column (100 Å 3 μm, 150 mm, 75 μm). The outlet of the analytical column was coupled directly to the mass spectrometer using a Proxeon nanospray source. The mobile phases for liquid chromatography (LC) separation were 0.1% (*v*/*v*) formic acid in LC-mass spectrometry grade water (solvent A) and 0.1% (*v*/*v*) formic acid in acetonitrile (solvent B). Peptides were first loaded with a constant pressure mode with a flow rate of solvent A onto the trapping column. Subsequently, peptides were eluted via the analytical column at a constant flow of 300 nl/min. During the elution step, the percentage of solvent B increased from 5 to 22% in the first 20 min, then increased to 32% in 5 min and finally to 98% in a further 2 min and was keeping it for 8 min. The peptides were introduced into the mass spectrometer via a Stainless steel emitter 40 mm (Thermo Fisher) and a spray voltage of 1.9 kV was applied. The capillary temperature was set at 275 °C.

Data acquisition was carried out using a top N-based data-dependent method with cycle time of 3 s. The master scan was performed in the Orbitrap in the range of 350–1500 mass to charge ratio (*m/z*) at a resolution of 60,000 full width at half-maximum (FWHM). The filling time was set at maximum of 50 ms with limitation of 4×10^5 ions. In a second stage of tandem mass spectrometry (MS/MS) ion trap collision-induced dissociation was acquired using parallel mode, filling time maximum 300 ms with limitation of 2×10^3 ions, a precursor ion isolation width of 1.6 *m/z* and resolution of 15,000 FWHM. Normalized collision energy was set to 35%. Only multiply charged (2^+ to 5^+) precursor ions were selected for MS/MS. The dynamic exclusion list was set to 30 s and relative mass window of 5 ppm.

Bioinformatic analysis

Gene Ontology (GO) classifications and enrichments were performed using FunRich [20]. The identified proteins were compared with web tool Exocarta database and also with the Top100 exosomal proteins from the database [21].

Data analysis

MS/MS data were searched with PEAKS (7.5). UniProt *Mus musculus* (house mouse, including 16,792 sequences) was used with non-tryptic specificity allowing up to 3 missed cleavages. A 15 ppm precursor tolerance and a 0.1 Da fragment tolerance were used. Oxidation (M) and deamidation (NQ) were treated as dynamic modification and carbamidomethylation (C) as a fixed modification. Maximum post-translational modification per peptide was 2. Search results were filtered by using 1% false discovery rate and 2 unique peptides.

The rest was evaluated using either unpaired *t* test or one-way ANOVA followed by Tukey's test for multiple comparisons. All statistical analysis was done using the GraphPad Prism 7.0 software for Macintosh (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SD. A confidence interval of 95% was set as

significant. The exact P values are given in the figure legends. Figures were organized using Adobe Illustrator.

Results

Proinflammatory responses from LPS-stimulated BV2 microglial cells

First, we examined the activation of BV2 cells after 12 h culture in the presence of 1 μg/ml LPS followed by deprivation of serum for 12 h to elicit a strong inflammatory reaction. The inflammatory enzyme iNOS is expressed by activated microglia [22] and as expected, the level of iNOS was significantly increased in cells upon LPS stimulation (Fig. 1a). Moreover, the protein levels of other important inflammatory mediators, NLRP3 (Nod-like receptor protein 3) and pro-caspase1 (involved in the maturation, production, and release of IL-1β and IL-18 [23]) were found to be elevated considerably (Fig. 1b, c). According to our previous studies, the viability of BV2 cells is not affected by LPS activation [22, 24]. These results suggest that an activated pro-inflammatory status of microglia remained over the 12 h EV collection period following LPS treatment.

Changes in EV size distribution in response to LPS activation

Transmission electron microscopy (TEM) was performed to visualize microglial-derived EVs (Fig. 1d). Images from control condition and LPS treatment revealed heterogeneous populations of EVs from microglia in the range between 100 and 1000 nm in diameter. LPS treatment seemed to induce microglial release of larger EVs, around 200–300 nm in diameter.

To further characterize EVs released under pro-inflammatory condition by LPS treatment, we compared size distribution of EVs derived from non-activated and LPS-activated microglia using Nanoparticle Tracking Analysis (NTA). The range of EVs detected with NTA was from 50 to 700 nm. Different size subpopulations of EVs were observed in EVs released from activated and non-activated microglial cells (Fig. 1f). According to the diameter of EVs, we can classify them into two subpopulations: one ranging from 50 to 100 nm can be considered as exosomes and another subpopulation with size exceeding 100 nm can be regarded as microvesicles (MVs). As can be seen in Fig. 1f, the population of MVs ranging from 300 to 400 nm has a higher frequency in the LPS-activated EVs. We found EVs released from LPS-activated cells to be significantly larger (178 ± 5.66 nm) compared to the size of EVs from control cultures (159 ± 4.95 nm) (Fig. 1f; $p < 0.001$). D90 measurement shows the upper limit of 90% measured particles, and in control condition, the D90 value for EVs was 205 ± 3.61 nm, whereas the value was 254 ± 11.06 nm in LPS-activated samples (Fig. 1f; $p < 0.001$). These results revealed that LPS-stimulated microglia release larger EV populations compared to control (Fig. 1g). EV samples were blotted for different EV markers including a marker for plasma membrane (flotillin-1) and an endosomal marker (Alix) as well as the EV marker CD63 for MVB (Fig. 1e) to elucidate the subcellular origin of the EVs [25]. We observed that in EVs released from microglia after LPS activation had a higher ratio of flotillin-1 and CD63 when compared to those from non-activated cells when loading equal amounts of EVs in each lane, suggesting altered EV biogenesis and release after an inflammatory stimulus.

Increased production of TNF and IL-6 in EVs upon LPS activation

Next, we studied the cytokine release from LPS-activated BV2 microglia to evaluate the free concentration of released cytokines and the cytokine concentration in EVs. Culture medium from activated and non-activated microglia was collected, and EVs were isolated from equal amounts of medium. EVs, along with the EV-depleted media, were then subjected to analysis by multiplex ELISA. Out of ten inflammatory cytokines analyzed, the levels of two pro-inflammatory cytokines, TNF and IL-6, were found to be significantly increased in EVs from activated microglia (Fig. 2a, b). TNF and IL-6 are two representative pro-inflammatory cytokines produced by microglia related to neurodegenerative diseases [3]. Notably, there was also a significant upregulation in the concentration of these two cytokines in medium (Additional file 1). However, other pro-inflammatory cytokines such as IL-5 and IL-1β were found to be increased only in the medium, but not in EVs (Additional files 1 and 2). Importantly, the level of TNF was much higher increased, 22-fold, compared to 5-fold in IL-6. Thus, we further investigated the effect of TNF in regulation of EV release in the following study.

Reduction of EVs by inhibition of inflammation via TNF pathway

In view of the specific increase of TNF in the EVs after LPS-stimulation, we set out to further characterize the role of TNF in the microglial release of EV. To investigate the mechanistic basis for EV regulation on LPS signaling in microglial cells, we quantified the number of EVs released from microglia after TNF inhibition with etanercept upon LPS activation. Notably, activated microglia secreted a 30-fold increase in the number of EVs under LPS stimulation (Fig. 3a). In contrast, the effect of LPS on EV release was completely attenuated down to control levels using etanercept (200 ng/ml), a TNF inhibitor that blocks both soluble and transmembrane forms of TNF (Fig. 3a). In the presence of the

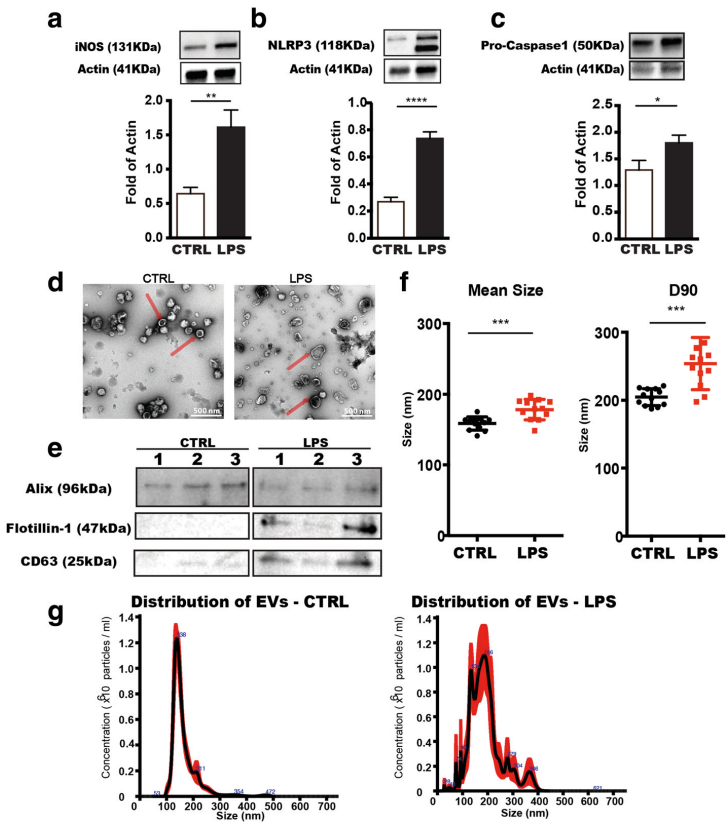

Fig. 1 Characterization of microglia-derived extracellular vesicles. Microglia (BV2) were activated by treatment with LPS for 12 h before extracellular vesicles were isolated from the media of both LPS-treated (LPS) or control (CTRL) cells. **a** Western blot analysis of protein expression levels of iNOS in cell lysates from control and activated microglia with LPS stimulation (Mean ± SD, $n = 5$). **b**, **c** Components of the inflammasome, NLRP3 and pro-Caspase 1, were measured by western blot in cell lysates with representative pictures of blots (Mean ± SD, $n = 5$). **d** Representative TEM imaging of extracellular vesicle populations from CTRL and LPS-derived microglia. The imaging illustrates heterogeneity and sphere structure of extracellular vesicles. Typical microvesicles are pointed with red arrows. Scale bars: 500 nm. **e** Western blots showed alterations of expression levels of vesicle markers indicated different origins of extracellular vesicles from CTRL and LPS. Three biological independent samples were blotted in each condition. **f** The size of extracellular vesicles was determined in diameter from CTRL and LPS-treated microglia. The mean size shows the average diameter of extracellular vesicles in samples ($n = 12$). D90 demonstrates the upper limit of extracellular vesicles size in 90% of the population ($n = 12$). **g** Representative histograms of extracellular vesicles size distributions collected from CTRL and LPS conditions. Sample from LPS condition was diluted 25 times more than control to obtain similar concentration of EVs to demonstrate size distribution. Concentrations ($\times 10^6$ particles/ml) by size (nm) of recorded extracellular vesicles are showed. The major subpopulations in EV samples are indicated with digitals showing the mean diameter of extracellular vesicles. Histograms were generated from five independent measurements by NTA 2.2 software (Unpaired t test, $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$)

TNF inhibitor, the amount of EVs released from cells was also reduced compared with control condition (Fig. 3a). Next, we wanted to understand whether the reduction of EV is caused by decreased EV uptake through inhibition of TNF. Thus, we assessed the capability of EV uptake under different conditions as indicated above. We found no significant difference between the conditions on internalization of PHK76-labeled EVs, indicating the reduction of EVs was due to TNF treatment on EV release not by affecting EV uptake/turnover (Additional file 3).

Next, we evaluated the degree of inflammatory status in microglia in relation to the amount of EVs secreted. To that aim, the level of iNOS was analyzed by western blot on cells previously treated in different conditions. We found two-fold reduction in iNOS levels following

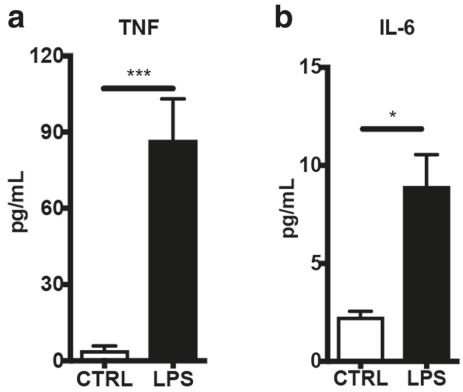

Fig. 2 Increased levels of proinflammatroy cytokines in microglia-derived extracellular vesicles upon LPS activation. The levels of cytokines were analyzed by multiplex ELISA plate. **a** Bar graph shows a significant upregulation of TNF ($n = 3$). **b** Bar graph shows a significant upregulation of IL-6 ($n = 6$)(Mean ± SD, Unpaired t test, $^*P < 0.05$; $^{***}P < 0.001$)

TNF inhibition. Interestingly, the reduction of EVs released upon TNF inhibition was reduced 16-fold, suggesting that TNF signaling is particularly important when it comes to reducing the number of EV released in proinflammatory activation of microglia (Fig. 3a). Together, these results implicate that the secretion of EVs is dramatically impeded by TNF inhibition in LPS-activated microglia, which is only partly associated to an overall reduction in the inflammatory status.

Evaluation of systemic inflammation in mice after focal cerebral ischemia

As the level of TNF has shown to be upregulated in EVs under LPS activation in vitro, we next studied whether the secretion of EVs in vivo was affected by complete ablation of TNF signaling in a strong neuroinflammatory situation. To this purpose, we chose an experimental stroke model, permanent middle cerebral artery occlusion (pMCAO), as an in vivo inflammatory model. Experimental stroke in rat and mouse is known to induce neuroinflammation in the brain [26, 27] as well as alter the inflammatory response in the periphery [28, 29]. Our earlier study has revealed significant increase of TNF receptors, toll-like receptor (TLR) 2 and IL-1β at mRNA levels in wild type (WT) and TNF-KO mice 1 day after pMCAO, indicating inflammation occurred in the brain [18]. We also wanted to assess systemic inflammatory response in mice after pMCAO. Therefore, we measured levels of different cytokines in serum using Multiplex ELISA from WT and TNF-KO mice without manipulation and 1 day after pMCAO. TNF expression levels were statistically elevated in WT at day 1 and unchanged in TNF-KO mice (Additional file 4). IL-5 and IL-12p70 were also found statistically elevated in WT mice 1 day after manipulation, but not in TNF-KO (Fig. 4a, c). While in the case of IL-1β, IL-6, and KC/GRO, expression levels were considerably increased 1 day after pMCAO in both types of mice compared with unmanipulated mice (Fig. 4b, d, f). Notably, such increases induced by pMCAO were significantly attenuated by deficiency of TNF in mice. Levels of IL-10 were remarkably lower in TNF-KO mice compared with WT mice after pMCAO, but not before (Fig. 4e). Levels of IL2, IL-4, and IFNγ were remained at baseline levels at day 1 in both types of mice subjected to pMCAO (Additional file 4). In conclusion, we found clear evidence of significant upregulation of proinflammatory cytokines at day 1 after pMCAO in both types of mice. However, in TNF-KO mice, such inflammation induced by pMCAO was remarkably attenuated at day 1 after stroke.

Decreased EVs in TNF knockout mice after focal cerebral ischemia

Given the impact of pMCAO on systemic inflammation in WT and TNF-KO mice, we examined microglia activation in the brain. We observed that TNF co-localized with the microglial marker, CD11b, in the peri-infarct area 1 day after pMCAO (Fig. 3d), which shows that focal cerebral ischemia could induce an initial phase of microglia activation involving TNF signaling and tissue injury, as we have shown before [30]. Moreover, the volume of infarct was also assessed in WT mice and TNF-KO mice 5 days after pMCAO in the previous study, which has shown that the injury was significantly larger in TNF-KO mice than WT mice [18]. Previously, we have shown that the infarct volume correlates with the number of EVs in plasma after this stroke model [31]. Thus, we analyzed the number of EVs in the plasma of TNF-KO mice after pMCAO. The production of EVs increased from both genotypes 5 days after pMCAO indicating inflammation occurred, which in line with our previous findings in vitro (Fig. 3a, c). Importantly, we found here that a complete ablation of TNF successfully reduced the counts of EVs in the plasma of TNF-KO mice 5 days after induction of permanent focal cerebral ischemia (Fig. 3c), but not at day 1 (Additional file 5). These results are consistent with our in vitro data and indicate the number of EVs is related to an inflammatory event and that TNF signaling is important in the mechanism regulating EV release.

Identification of microglial EVs proteins

To further elucidate cellular communication by EVs under inflammation, we set out to identify proteins in association to EVs released by BV2 cells. To this aim,

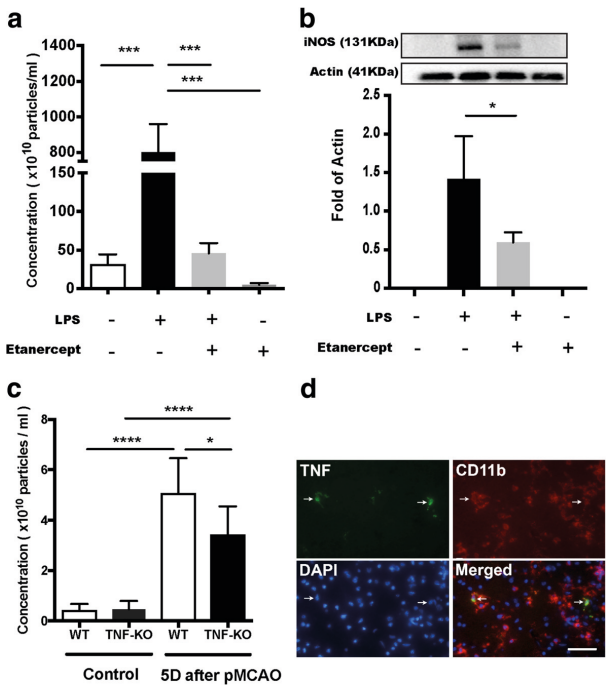

Fig. 3 Reduction in the number of microglia-derived extracellular vesicles by inhibition of TNF signaling pathway. Extracellular vesicles were visualized by TEM. Concentrations of extracellular vesicles in conditional media and plasma from mice were measure by NTA. **a** Comparison of extracellular vesicles concentrations in different conditional media. BV2 microglia cell line was treated with either LPS (1 μg/ml) or etanercept (200 ng/ml) or in the presence of both. Control (CTRL) was cells without any treatment (Mean ± SD, one-way ANOVA, ***$P < 0.001$, $n = 3$). **b** Expression levels of iNOS in BV2 cells previously treated with different conditions were analyzed by western blot. iNOS bands were not able to be visualized in the conditions of control and etanercept due to out of the detection limit (Mean ± SD, Unpaired t-test, *$P < 0.05$, $n = 4$). **c** Bar graph shows a comparison of the amount of extracellular vesicles in plasma from mice (WT, n = 6; TNF-KO, n = 6) without surgery and mice (WT, $n = 9$; TNF-KO, $n = 8$) 5 days after a stroke model, permanent middle cerebral artery occlusion (pMCAO) (Mean ± SD, one-way ANOVA followed by Tukey's test for multiple comparisons, *$P < 0.05$, ***$P < 0.001$). **d** The brain section in area of infarct from C57BL/6 mouse subjected to pMCAO stained with anti-TNF (green) and anti-CD11b (red), nuclei stained with DAPI. TNF+ cells are also stained for CD11b indicated with arrow; scale bar: 20 μm

proteomic analysis was performed using mass spectrometry on EV samples from LPS-activated and control microglia. Biological independent duplicates of EVs were pooled together and analyzed. In total, 86 proteins were identified with two peptides confirmed and high confidence, as -10lgP is set for 20 as threshold, using PEAKS analysis program (Tables 1 and 2) [32]. In total, 37 proteins from control and 58 proteins from LPS-stimulated microglia were successfully mapped with UniProt database. Among them, we found 9 proteins in common (Table 3) and 49 specific proteins present in LPS condition. The analysis showed enzymes, chaperones, ribosomal structure proteins, and membrane receptors previously reported in other immune cells in our samples (Fig. 5 d, e). Of these, the majority of the identified proteins were associated with RNA binding, and more than half of them had Gene Ontology annotations related to membrane and extracellular exosome (Fig. 5c, d). The identified proteins were also compared with the ExoCarta database, which has exosomal proteins identified from previous publications [21]. In our samples, only one protein has not been reported in ExoCarta database (Fig. 5a). Compared with top 100-ranked proteins presented in the database, we identified 17 of them in our study (Fig. 5a). Notably, 89.5% proteins from this study have not been reported before and first identified in microglia (Table 4), suggesting that these cells are releasing specific EVs.

Functional profiles of the quantified microglia EVs proteins

Next, we wanted to know the functions of the quantified proteins differentially secreted in EVs under LPS activation. The 86 quantified proteins in the microglial-derived

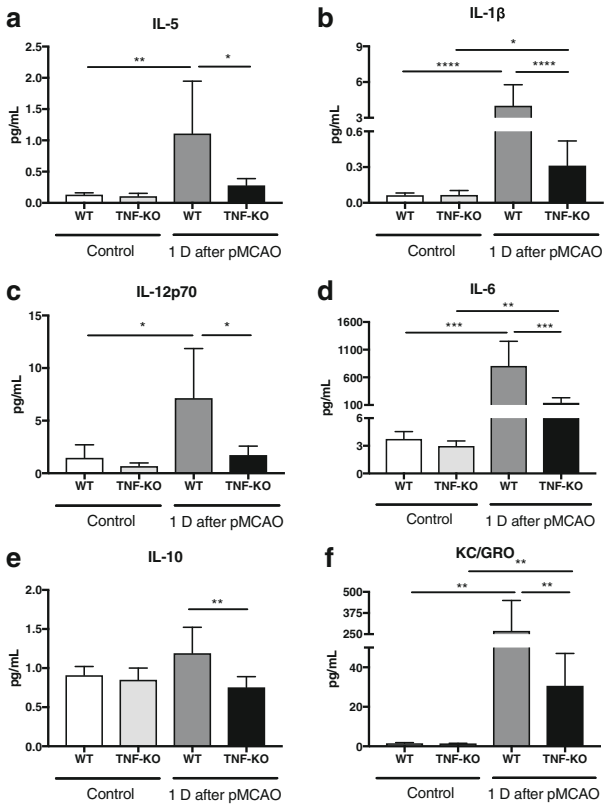

Fig. 4 Systemic inflammation in mice after focal cerebral ischemia. The levels of cytokines in serum were analyzed by multiplex ELISA from WT and TNF-KO mice without manipulation and 1 day after focal cerebral ischemia. **a** and **c** Bar graphs show significant upregulation of IL-5 and IL-12p70 in WT mice after pMCAO. **b**, **d**, **f** Bar graphs show significant upregulation of IL-1β, IL-6, and KC/GRO in both types of mice subjected to pMCAO. **e** Bar graph shows remarkable reduction of IL-10 in TNF-KO mice compared with WT mice 1 day after pMCAO. (Mean ± SD, one-way ANOVA followed by Tukey's test for multiple comparisons, $n = 3–6$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$)

EVs were analyzed using FunRich software [21] to validate our data referred to the Vesiclepedia Exosome Database [33]. The analysis was based on their cellular compartments to which they belong, their molecular function and the biological process in which they are involved. In the analysis, some of the proteins were annotated in more than one cellular component, molecular function, and biological process.

Firstly, enrichment in pathways from membrane and extracellular exosome was observed for GO analysis on cellular component (Fig. 5d). There were large increases in proteins from LPS-stimulated EVs from ribosome, focal adhesion, extracellular matrix, and membrane (Fig. 5d). In contrast, a reduction of proteins associated with extracellular exosome was found in LPS-stimulated EVs, which is also confirmed by western blot with increased ratio of flotillin-1 and CD63 indicating more MVs released. In the categories of molecular function, the majority of the proteins were annotated to RNA binding, 38.9% in control and 72.4% in LPS (Fig. 5c). Importantly, the proteins contributed to ribosome function raised from 2.8 to 56.9% after LPS challenge (Fig. 5c). The profile of proteins in molecular function was dramatically altered after inflammatory stimulation. It is likely that microglia released a distinct population of EVs related to transduction and translation after activation of LPS. Intriguingly, the profile of EVs was peculiarly different after activation according to classification of biological process (Fig. 5e). EVs were found involved in regulation of neuron projection development at "rest" status (control), however, with activation the proteins identified in EVs shifted to ribosomal assembly and translation. The role of microglia was changed

Table 1 List of proteins identified in control EV samples

Protein ID	Gene name	Protein name	Score
P08226	APOE	Apolipoprotein E	506
P21956	MFGE8	Lactadherin	136
P21460	CST3	Cystatin-C	123
Q9WU78	PDCD6IP	Programmed cell death 6-interacting protein	121
P11152	LPL	Lipoprotein lipase	107
Q8VDN2	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	105
P62737	ACTA2	Actin, aortic smooth muscle	84
Q61753	PHGDH	D-3-phosphoglycerate dehydrogenase	80
P07901	HSP90AA1	Heat shock protein HSP 90-alpha	80
P10605	CTSB	Cathepsin B	75
P01942	HBA	Hemoglobin subunit alpha	73
P63017	HSPA8	Heat shock protein 8	71
Q62419	SH3GL1	Endophilin-A2	70
Q8R366	IGSF8	Immunoglobulin superfamily member 8	69
Q68FD5	CLTC	Clathrin heavy chain 1	67
P10923	SPP1	Osteopontin	64
Q61207	PSAP	Prosaposin	64
P06869	PLAU	Urokinase-type plasminogen activator	62
Q8CGP5	HIST1H2AF	Histone H2A type 1-F	61
P52480	PKM	Pyruvate kinase	59
P17182	ENO1	Alpha-enolase	56
P04104	KRT1	Keratin, type II cytoskeletal 1	50
P68369	TUBA1A	Tubulin alpha-1A chain	47
P01887	B2M	Beta-2-microglobulin	45
P09405	NCL	Nucleolin	44
P09055	ITGB1	Integrin beta-1	42
P01901	H2-K1	H-2 class I histocompatibility antigen, K-B alpha chain	41
P01899	H2-D1	H-2 class I histocompatibility antigen, D-B alpha chain	36
P29341	PABPC1	Polyadenylate-binding protein 1	41
P06797	CTSL	Cathepsin L1	38
P10852	SLC3A2	4F2 cell-surface antigen heavy chain	37
P08905	LYZ2	Lysozyme C-2	34
P10126	EEF1A1	Elongation factor 1-alpha 1	28
Q61937	NPM1	Nucleophosmin	27
Q9DBJ1	PGAM1	Phosphoglycerate mutase 1	25
P28798	GRN	Granulins	24
P14206	RPSA	40S ribosomal protein SA	24

Proteins retrieved in control extracellular vesicle samples from mass spectrometry. Protein ID and gene name are according to Uniprot Knowledgebase. Score values were obtained using MASCOT. The Score shows how well the observed protein matches to the stated protein in the database. Only protein identifications supported by at least two high confident peptides (confidence > 95%) were considered

from "rest" state to "activated" state by not only production of cytokine and chemokine, but also with altered secretion of EVs, which could be directly linked to detrimental inflammatory responses related to neurodegenerative diseases.

In larger proportion, 49 proteins were exclusively present in EVs samples from LPS activation, including some cytoskeleton proteins and ribosomal proteins. Importantly, three of them were associated with inflammatory and neuropathological pathways: tubulin beta 4B

Table 2 List of proteins identified in LPS EV samples

Protein ID	Gene name	Protein name	Score
P68372	TUBB4B	Tubulin beta-4B chain	258
P62242	RPS8	40S ribosomal protein S8	201
P47963	RPL13	60S ribosomal protein L13	181
Q9CZX8	RPS19	40S ribosomal protein S19	178
P15864	HIST1H1C	Histone H1.2	172
Q8VEK3	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	148
Q9CR57	RPL14	60S ribosomal protein L14	146
P63276	RPS17	40S ribosomal protein S17	138
P11152	LPL	Lipoprotein lipase	130
P16858	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	128
P68369	TUBA1A	Tubulin alpha-1A chain	123
P62082	RPS7	40S ribosomal protein S7	106
Q9D8E6	RPL4	60S ribosomal protein L4	101
P35979	RPL12	60S ribosomal protein L12	101
P14131	RPS16	40S ribosomal protein S16	100
P70696	HIST1H2BA	Histone H2B type 1-A	100
P12970	RPL7A	60S ribosomal protein L7a	98
P60710	ACTB	Actin, cytoplasmic 1	94
Q9CXW4	RPL11	60S ribosomal protein L11	85
Q9CZM2	RPL15	60S ribosomal protein L15	84
P02301	H3F3C	Histone H3.3C	83
P47911	RPL6	60S ribosomal protein L6	83
P43276	HIST1H1B	Histone H1.5	83
Q8CGP5	HIST1H2AF	Histone H2A type 1-F	82
P14115	RPL27A	60S ribosomal protein L27a	74
P27659	RPL3	60S ribosomal protein L3	72
O55142	RPL35A	60S ribosomal protein L35a	70
P25206	MCM3	DNA replication licensing factor MCM3	68
P62702	RPS4X	40S ribosomal protein S4, X isoform	63
P10126	EEF1A1	Elongation factor 1-alpha 1	63
Q8BP67	RPL24	60S ribosomal protein L24	62
P14206	RPSA	40S ribosomal protein SA	62
P11499	HSP90AB1	Heat shock protein HSP 90-beta	60
P08226	APOE	Apolipoprotein E	60
P35980	RPL18	60S ribosomal protein L18	59
P63017	HSPA8	Heat shock protein 8	59
P80318	CCT3	T-complex protein 1 subunit gamma	58
P62918	RPL8	60S ribosomal protein L8	57
O08585	CLTA	Clathrin light chain A	55
Q9Z1Q9	VARS	Valine-tRNA ligase	54
P01942	HBA	Hemoglobin subunit alpha	51
P97351	RPS3A	40S ribosomal protein S3a	50
P62806	HIST1H4A	Histone H4	48
P62270	RPS18	40S ribosomal protein S18	44

Table 2 List of proteins identified in LPS EV samples *(Continued)*

Protein ID	Gene name	Protein name	Score
P62911	RPL32	60S ribosomal protein L32	43
P14869	RPLP0	60S acidic ribosomal protein P0	41
Q9JIK5	DDX21	Nucleolar RNA helicase 2	41
P62281	RPS11	40S ribosomal protein S11	41
Q68FD5	CLTC	Clathrin heavy chain 1	40
P62717	RPL18A	60S ribosomal protein L18a	39
P86048	RPL10L	60S ribosomal protein L10-like	39
P62267	RPS23	40S ribosomal protein S23	37
P25444	RPS2	40S ribosomal protein S2	36
P14148	RPL7	60S ribosomal protein L7	31
P53026	RPL10A	60S ribosomal protein L10a	28
P62264	RPS14	40S ribosomal protein S14	28
P62908	RPS3	40S ribosomal protein S3	23
P04918	SAA3	Serum amyloid A-3 protein	23

Proteins retrieved in LPS extracellular vesicles samples from mass spectrometry. Protein ID and gene name are according to Uniprot Knowledgebase. Score values were obtained using MASCOT. The score shows how well the observed protein matches to the stated protein in the database. Only protein identifications supported by at least two high confident peptides (confidence > 95%) were considered

(TUBB4B), heterogeneous nuclear ribonucleoprotein U (HNRNPU) and serum amyloid A 3 (SAA3). Nine proteins were shared in samples from control and LPS conditions (Fig. 5b). Proteins related to immune system process and stimuli response were detected in both samples: lipoprotein lipase (LPL), apolipoprotein E (APOE), and heat shock protein 8 (HSPA8). Notably, APOE was particular with high score in the EVs before and after LPS activation. Variants of APOE (APOE4) is known as the strongest risk factor for late onset Alzheimer's disease [34] and has also been suggested to have a proinflammatory effect on microglia [35].

Discussion

Compelling evidence has suggested that the activation of innate immunity and neuroinflammation play crucial roles in neurodegenerative diseases [36–38]. Microglia are an essential component in innate immunity in the CNS. Activation of microglia is followed by the initiation of intracellular machinery that leads to production of cytotoxic and proinflammatory cytokines and chemokines, which promote progression of inflammation and affect neighboring cells through different mechanisms. The secretion of proinflammatory signals can be conducted in classic secretion manner or non-canonical manner via vesicles. Previous studies have shown the important role of EVs in regulation of cytokine production on recipient cells and propagation of pathogenic proteins [11, 39].

Table 3 The common proteins shared in CTRL and LPS EV samples

Protein ID	Gene name	Protein name
P08226	APOE	Apolipoprotein E
P11152	LPL	Lipoprotein lipase
P01942	HBA	Hemoglobin subunit alpha
P63017	HSPA8	Heat shock protein 8
Q68FD5	CLTC	Clathrin heavy chain 1
Q8CGP5	HIST1H2AF	Histone H2A type 1-F
P68369	TUBA1A	Tubulin alpha-1A chain
P10126	EEF1A1	Elongation factor 1-alpha 1
P14206	RPSA	40S ribosomal protein SA

Proteins retrieved in extracellular vesicle samples from mass spectrometry. Protein ID and gene name are according to Uniprot Knowledgebase

The functions of microglia in the brain are diverse depending on stimulus and different brain regions. In response to different inflammatory/homeostatic conditions, they can be either beneficial or detrimental. Increasing evidence has suggested that there is a spectrum of activation in microglia based on the profile of secreted molecules [3]. However, the importance of EV released by microglia has not been well characterized. Thus, in the present study, we directly evaluated effects of LPS-stimulation on EVs derived from BV2 microglia, in terms of physical and biological properties. Our results demonstrate that (i) upon LPS-activation, the size distribution of EVs released from microglia increases in size, indicating larger vesicles are released under inflammatory conditions; (ii) IL-6 and in particular TNF are increasingly secreted in microglia-derived EVs after LPS challenge; (iii) inhibiting the TNF signaling pathway resulted in a robust reduction in the number of vesicles released from LPS-treated microglia and in

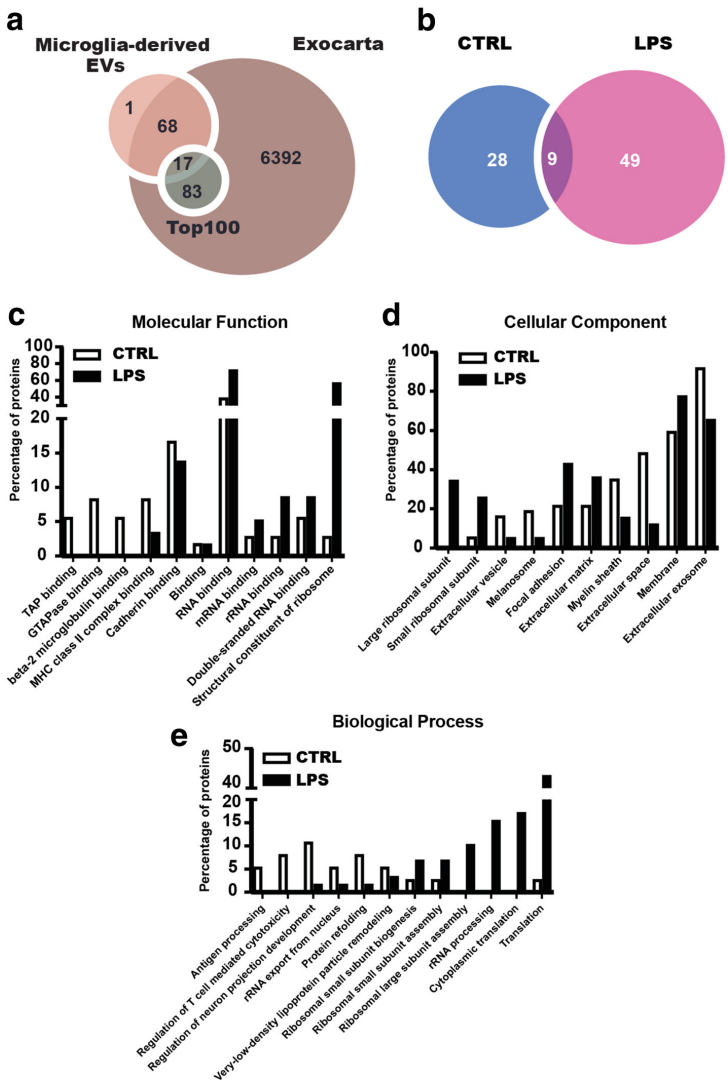

Fig. 5 Bioinformatic analysis of the identified proteins in BV2 microglia-derived extracellular vesicles. Gene ontology (GO) analysis of the identified proteins was performed using Exocarta based software FunRich. The comparison was carried on proteins expressed exclusively in each condition. **a** Comparison of 86 identified proteins from microglial extracellular vesicles with online database Exocarta and the top 100 proteins commonly reported in the same database. **b** Comparison of the number of proteins identified and quantified in control microglia and LPS-activated microglia derived extracellular vesicles samples. **c** Bioinformatic analysis from FunRich shows comparison of GO analysis on Molecular Function on proteins from CTRL and LPS-activated microglia released extracellular vesicles (GO terms are with $P < 0.01$). **d** Bioinformatic analysis from FunRich shows comparison of GO analysis on Cellular Component on proteins from CTRL and LPS-activated microglia released extracellular vesicles (GO terms are with $P < 0.01$). **e** Bioinformatic analysis from FunRich shows comparison of GO analysis on Biological Process on proteins from CTRL and LPS-activated microglia released extracellular vesicles (GO terms are with $P < 0.01$)

Table 4 New proteins found in present microglia-derived EV samples

Protein ID	Gene name	Protein name
P08226	APOE	Apolipoprotein E
P21460	CST3	Cystatin-C
Q8VDN2	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1
P62737	ACTA2	Actin, aortic smooth muscle
Q61753	PHGDH	D-3-phosphoglycerate dehydrogenase
P10605	CTSB	Cathepsin B
P01942	HBA	Hemoglobin subunit alpha
Q62419	SH3GL1	Endophilin-A2
Q8R366	IGSF8	Immunoglobulin superfamily member 8
Q68FD5	CLTC	Clathrin heavy chain 1
P10923	SPP1	Osteopontin
Q61207	PSAP	Prosaposin
P06869	PLAU	Urokinase-type plasminogen activator
Q8CGP5	HIST1H2AF	Histone H2A type 1-F
P04104	KRT1	Keratin, type II cytoskeletal 1
P68369	TUBA1A	Tubulin alpha-1A chain
P01887	B2M	Beta-2-microglobulin
P09405	NCL	Nucleolin
P09055	ITGB1	Integrin beta-1
P01901	H2-K1	H-2 class I histocompatibility antigen, K-B alpha chain
P01899	H2-D1	H-2 class I histocompatibility antigen, D-B alpha chain
P29341	PABPC1	Polyadenylate-binding protein 1
P06797	CTSL	Cathepsin L1
P10852	SLC3A2	4F2 cell-surface antigen heavy chain
P08905	LYZ2	Lysozyme C-2
P10126	EEF1A1	Elongation factor 1-alpha 1
Q61937	NPM1	Nucleophosmin
P28798	GRN	Granulins
P14206	RPSA	40S ribosomal protein SA
P68372	TUBB4B	Tubulin beta-4B chain
P62242	RPS8	40S ribosomal protein S8
P47963	RPL13	60S ribosomal protein L13
Q9CZX8	RPS19	40S ribosomal protein S19
P15864	HIST1H1C	Histone H1.2
Q8VEK3	HNRNPU	Heterogeneous nuclear ribonucleoprotein U
Q9CR57	RPL14	60S ribosomal protein L14
P63276	RPS17	40S ribosomal protein S17
P62082	RPS7	40S ribosomal protein S7
Q9D8E6	RPL4	60S ribosomal protein L4
P35979	RPL12	60S ribosomal protein L12
P14131	RPS16	40S ribosomal protein S16
P70696	HIST1H2BA	Histone H2B type 1-A
P12970	RPL7A	60S ribosomal protein L7a
P60710	ACTB	Actin, cytoplasmic 1

Table 4 New proteins found in present microglia-derived EV samples *(Continued)*

Protein ID	Gene name	Protein name
Q9CXW4	RPL11	60S ribosomal protein L11
Q9CZM2	RPL15	60S ribosomal protein L15
P02301	H3F3C	Histone H3.3C
P47911	RPL6	60S ribosomal protein L6
P43276	HIST1H1B	Histone H1.5
Q8CGP5	HIST1H2AF	Histone H2A type 1-F
P14115	RPL27A	60S ribosomal protein L27a
P27659	RPL3	60S ribosomal protein L3
O55142	RPL35A	60S ribosomal protein L35a
P25206	MCM3	DNA replication licensing factor MCM3
P62702	RPS4X	40S ribosomal protein S4, X isoform
P10126	EEF1A1	Elongation factor 1-alpha 1
Q8BP67	RPL24	60S ribosomal protein L24
P14206	RPSA	40S ribosomal protein SA
P11499	HSP90AB1	Heat shock protein HSP 90-beta
P08226	APOE	Apolipoprotein E
P35980	RPL18	60S ribosomal protein L18
P63017	HSPA8	Heat shock protein 8
P80318	CCT3	T-complex protein 1 subunit gamma
P62918	RPL8	60S ribosomal protein L8
P62911	RPL32	60S ribosomal protein L32
P14869	RPLP0	60S acidic ribosomal protein P0
Q9JIK5	DDX21	Nucleolar RNA helicase 2
P62281	RPS11	40S ribosomal protein S11
P62717	RPL18A	60S ribosomal protein L18a
P86048	RPL10L	60S ribosomal protein L10-like
P62267	RPS23	40S ribosomal protein S23
P25444	RPS2	40S ribosomal protein S2
P14148	RPL7	60S ribosomal protein L7
P53026	RPL10A	60S ribosomal protein L10a
P62264	RPS14	40S ribosomal protein S14
P62908	RPS3	40S ribosomal protein S3
P04918	SAA3	Serum amyloid A-3 protein

Proteins identified in present study were compared with proteins uploaded in Exocarta database from microglial origin. Protein ID and gene name are according to Uniprot Knowledgebase

mice subjected to pMCAO; and (iv) in response to LPS, BV2 microglia release EVs with a distinct proteomic profile related to transcription and translation.

EVs can either enhance or suppress inflammation and act as main factors to regulate inflammation and immunity [40, 41]. There is evidence showing microglia can release IL-1β, upon exposure to ATP derived from astrocytes, by shedding MVs which contain the entire machinery important for processing of it including the P2X7 receptors [42]. This is consistent with our findings that EVs contain more molecules related to transcription and translation in activated state and include a particular population of MVs budding from the plasma membrane. The mechanism underlying release of EV is still not clear. TNF can induce neurotoxicity by modulating glutamate production that results in excitotoxic neuronal death [43]. One study conducted by Wang et al. has shown that TNF promotes the release of EVs from astrocytes through increased expression of glutaminase, which convert glutamine to glutamate [44]. TNF can also induce extensively production of glutamate from microglia in an autocrine manner to cause excitotoxicity

and contribute to neuronal damage [45]. Thereby, these findings together with our results suggest that microglial EV release could be potentially modulated by TNF through specifically regulated mechanisms.

Our data suggests that inhibition of TNF signaling in microglia may impact on inflammation, with an observed reduction both in the release of different cytokines and in the total number of EVs released, implying that the release of EVs in activated microglia seems to be regulated. This idea is further supported by data from our experimental stroke model that showed altered EV counts in plasma from TNF-KO mice compared to WT. These results contradict previous work that showed an increase in the amount of MVs in mice treated with TNF inhibitor for 5 days after pMCAO compared to saline-treated mice [31]. It is therefore reasonable to assume that the difference we observed is due to the different experimental set-up. A systemic administration of TNF inhibitor in the mice 30 min after surgery could merely have a transient effect on the TNF signaling pathway, whereas using the TNF-KO model is expected to be more stable to investigate and characterize long-term effects of EVs release.

From our previous study, pMCAO could initiate microglia activation and inflammation in the brain [18, 28]. Therefore, we also evaluated systemic inflammation in mice subjected to pMCAO. Although several inflammatory factors, such as IL-1β and IL-6, were significantly increased in both types of mice at day 1 after manipulation, such induction was attenuated in TNF-KO mice. Another study has shown that EVs derived from macrophages stimulated with bacterial infection are able to increase secretion of proinflammatory cytokines in recipient cells, including TNF [17]. Taken together, we can speculate that inflammatory propagation can be mitigated by a reduction in the number of microglia-derived EVs, thus halting inflammation via TNF signaling. However, this is complicated by the fact that there are two forms of TNF, soluble TNF (solTNF), which is related to neurotoxicity and inflammation, while the transmembrane TNF (tmTNF) is involved in functional recovery and neuroprotection [18, 46]. Hence, both the cellular contribution to TNF signaling and specifically the form of TNF carried in microglial EVs is important to evaluate final outcome of experiment, neuroprotective or neurotoxic. In our study, we measured total TNF in EVs including tmTNF and solTNF. However, the specific form of TNF is likely to be important for the inflammatory and cytoprotective outcome. We believe such studies investigating the specific form of TNF carried in EVs are important in future studies.

We also studied the protein composition to further characterize the EVs released under control or inflammatory conditions. Using western blot analysis, we looked for changes in the levels of either plasma membrane or endosomal markers to elucidate where these vesicles had originated. Indeed, we observed an increase in signal from plasma membrane markers when compared to endosomal markers upon LPS activation. When combined with the increase in overall size observed with NanoSight, this supports our theory that more MVs are released under inflammatory conditions.

Furthermore, our proteomic analysis indicated that the two populations of EVs were dramatically different in categories of molecular function and biological process under GO analysis. From the qualitative proteomic analysis, 86 proteins were identified with high accuracy. Compared to Exocarta database, most of the proteins have been reported in previous studies in exosomes from other cell types, where 89.5% of the quantified proteins were firstly identified in microglial EVs. The small overlap with previous studies is most likely due to a lack of studies utilizing microglia-derived EVs, with a sole study performed on EVs from the N9 microglial cell line responsible for all microglial proteins present in ExoCarta [15]. It was also clear from the FunRich analysis [20] that the proteins detected in EVs from LPS-treated cells had different functions to those from control cells, with proteins involved in RNA binding and structural components of the ribosome more prevalent in LPS-derived EVs. While the analysis on the cellular origin of the EV proteins revealed that the extracellular exosome and membrane were dominant from both conditions, it is of interest to note that EVs isolated from inflammatory condition were detected with more membrane and less exosome proteins, consistent with a shift towards MV release rather than exosome.

For the first time, our data indicates that microglia change its EVs releasing machinery after LPS activation with an increase in the overall number of EVs, and more EVs budding from the membrane. It is tempting to speculate that non-activated microglia have an expedient and controlled release of EVs, whereas in an inflammatory condition microglia release a large variation of EVs that will perturb the normal homeostatic function of microglia. We also think that, upon LPS-stimulation, microglia respond to release populations of extracellular cargoes with a unique proteomic profile related to RNA transcription and translation. The existence of various RNA molecules in EVs is well-established including mRNA and microRNA. In fact, microRNAs can function as ligands for TLRs and induce immune responses or inhibit activation by suppressing TLR signaling [13, 47]. These RNAs are selectively sorted to EVs under different mechanisms [47]. However, the mechanisms responsible for this packaging are not clear. Thus, our study implies proteins potentially involved in such mechanisms that are increased when inflammation takes place. According

to our proteomic data, we speculate that EVs could actually carry the whole machinery not only RNAs to recipient cells and surrounding tissue.

Finally, by comparing the protein cargoes from LPS-activated and non-activated BV2 microglia, we were able to identify potential candidates likely to be involved in the communication of microglial cells with other effector cells under inflammation. We found 49 proteins exclusively present in EVs in the presence of LPS. Among them, TUBB4B, HNRNPU, and SAA3 are proteins related to inflammation and neuropathology. Indeed, TUBB4B is a member of tubulin family and known as a component of cytoskeleton [48]. Liu X et al, suggested that TUBB4B may be a part of the same disease pathway as leucine-rich repeat kinase 2 (LRRK2), which is a crucial factor to understand the etiology of Parkinson's disease (PD) [49]. HNRNPU acts as a key factor to maintain 3D structure of chromatin and has been reported as a posttranscriptional regulator for NF-κB inflammatory pathway [50]. In addition, SAA3 is a member of serum amyloid A (SAA) and acute phase protein accompanying with other inflammatory cytokines and chemokines [51]. It has been implied to play a role in the inflammatory processes occurring in Alzheimer's disease (AD) and multiple sclerosis (MS) [51]. In addition to those proteins found exclusively in LPS EVs, we identified APOE in EVs from both LPS and control conditions. This protein is commonly present in membranes and is considered one of the most important lipoproteins involved in cholesterol shuttling between astrocytes and neurons along with being involved in remodeling and reorganization of neuronal networks after injury [34]. It is also one of the major genetic risk factors for late onset sporadic AD and can function as a ligand in receptor-mediated endocytosis with extracellular β-amyloid [34, 52].

Conclusions

The present data show that upon activation by LPS, BV2 microglia release EVs with a distinct proteomic profile compared to control. Our data suggests that under these inflammatory conditions, MVs are the predominate EV, containing increased levels of TNF in particular, and to a lesser degree IL-6. We further provide evidence in vitro and in vivo that TNF signaling is important in quantitatively controlling EV release. Furthermore, through proteomic analysis, we are able to provide lists of proteins with the potential to modulate EV trafficking in microglia, in particular a change in EV proteins related to neuronal maintenance and protein translation after LPS activation. We believe that EV regulation in microglia and its specific role in neuroinflammation will be important to fully understand the inflammatory pathogenesis in neurodegenerative diseases.

Additional files

Additional file 1: Supplementary figures for cytokines in conditioned medium from microglia show significant upregulations of TNF ($n = 7$), IL-1β ($n = 7$), IL5 ($n = 7$), and IL-6 ($n = 3$). Measured by multiplex ELISA (Unpaired t test, $^{*}P < 0.05$; $^{***}P < 0.001$). (PDF 201 kb)

Additional file 2: Supplementary figures for cytokines in microglia-derived extracellular vesicles not altered after LPS treatment. Measured by multiplex ELISA (Unpaired t test, $^{*}P < 0.05$; $^{***}P < 0.001$). (PDF 180 kb)

Additional file 3: Supplementary figures for the effect of TNF inhibition on dynamics of EV trafficking. Images were taken and then measured for fluorescent intensity. A) Representative images of BV2 cells cultured with PHK76-labeled EVs 12 h after different treatments, including pre-treatment of cells with either LPS (1 μg/ml) or etanercept (200 ng/ml) or in presence of both. Control (CTRL) was cells without any treatment. Cells without EV were regarded as baseline. Merged images of the indicated areas show PHK76 internalized cells (Scale bar, 50 μm). B) Comparison of total fluorescent intensity (IntDen) in BV2 cells after incubation of dye-labeled EVs. No significant differences were found between the conditions (one-way ANOVA, $n = 3$). (PDF 9799 kb)

Additional file 4: Supplementary figures for cytokines in serum from WT and TNF-KO mice before and 1 day after pMCAO. Measured by multiplex ELISA (one-way ANOVA followed by Tukey's test for multiple comparisons, $n = 3$–6, $^{**}P < 0.01$). (PDF 30 kb)

Additional file 5: Supplementary figures for quantification of extracellular vesicles in plasma from WT and TNF-KO mice subjected to pMCAO (Unpaired t test, $n = 3$). (PDF 18 kb)

Abbreviations

AD: Alzheimer's disease; APOE: Apolipoprotein E; BCA: Bicinchoninic acid assay; BSA: Bovine serum albumin; CNS: Central nervous system; DMEM: Dulbecco's modified Eagle medium; ELISA: Enzyme-linked immunosorbent assay; EVs: Extracellular vesicles; FBS: Fetal bovine serum; FWHM: Full-width at half-maximum; GO: Gene Ontology; HNRNPU: Heterogeneous nuclear ribonucleoprotein U; HRP: Horse-radish protein; HSP8: Heat shock protein 8; IFNγ: Interferony; IL: Interleukin; iNOS: Inducible nitric oxide synthase; KC/GRO: C-X-C motif chemokine ligand 1; LC: Liquid chromatography; LPL: Lipoprotein lipase; LPS: Lipopolysaccharide; LRRK2: Leucine-rich repeat kinase 2; m/z: Mass to charge ratio; MS: Multiple sclerosis; MS/MS: Tandem mass spectrometry; MVB: Multivesicular endosomal compartments; MVs: Microvesicles; NLRP3: Nod-like receptor protein 3; NO: Nitric oxide; NTA: Nanoparticle tracking analysis; PBS: Phosphate-buffered saline; PD: Parkinson's disease; PFA: Paraformaldehyde; pMCAO: Permanent middle cerebral artery occlusion; SAA3: Amyloid A-3 protein; solTNF: Soluble form of tumor necrosis factor; TEM: Transmission electron microscopy; TLR: Toll-like receptor; tmTNF: Transmembrane form of tumor necrosis factor; TNF: Tumor necrosis factor; TNF-KO: Tumor necrosis factor knockout; TUBB4B: Tubulin beta 4B

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY and TD designed the studies and participated in the data analysis, interpretation, and writing of the manuscript. BHC contributed to cytokine analysis from mice. KLL contributed to all surgical and histological aspects of the study including the analysis of tissue specimens. YY performed most experiments and analyzed and interpreted data. ABS helped to interpret data and participate in the design of the study. CD assisted with extracellular vesicle experiment and provided useful input to the drafting of the paper. All authors have read and approved the final manuscript.

Ethics approval

All animal experiments were performed in accordance with the relevant guidelines and regulations approved by the Danish Animal Ethical Committee (numbers 2011/561-1950 and 2013-15-2934-00924).

Competing interests

The authors declare that they have no competing interests.

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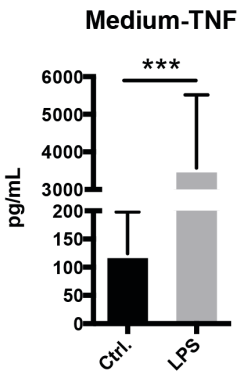
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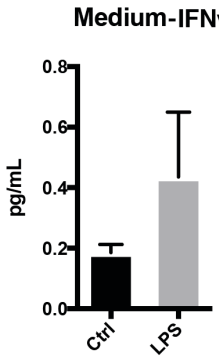
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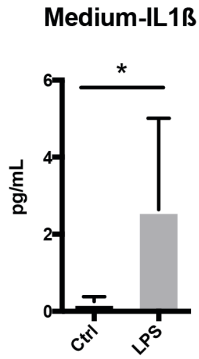
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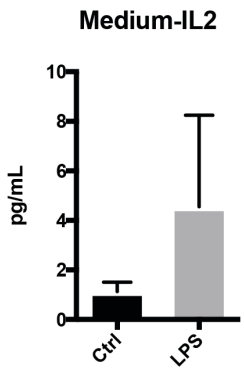
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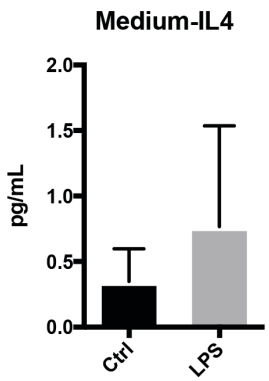
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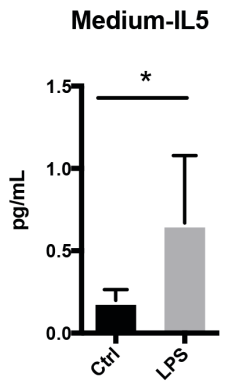
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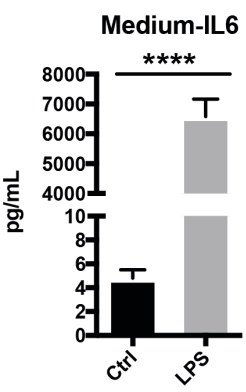
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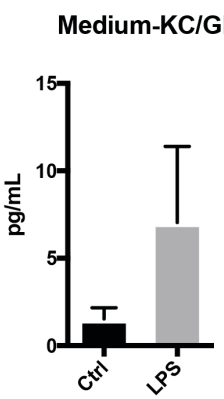
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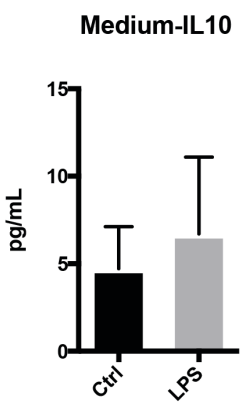
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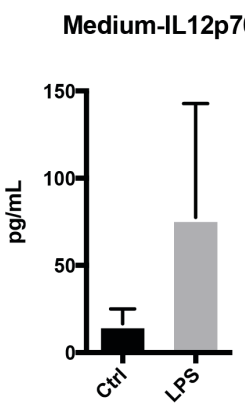
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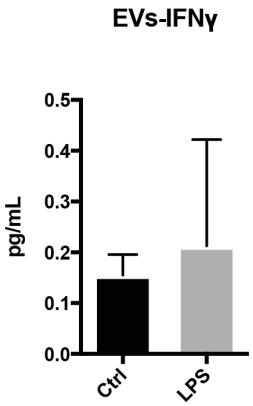
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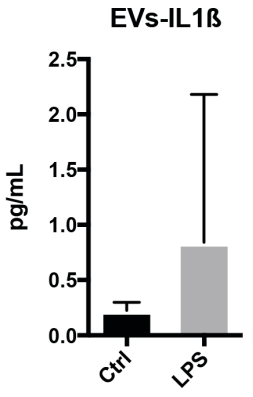
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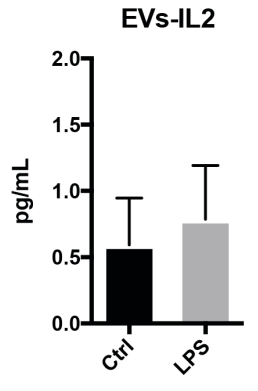
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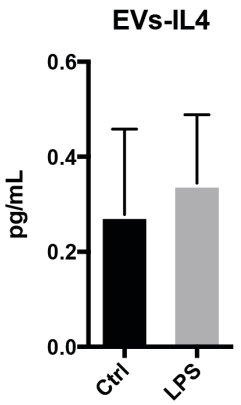
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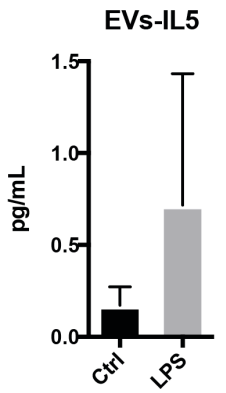
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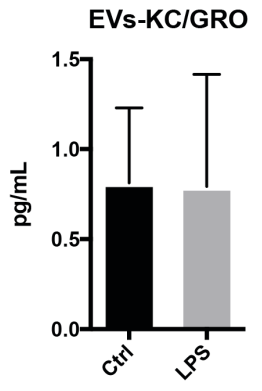
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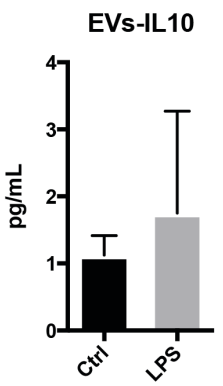
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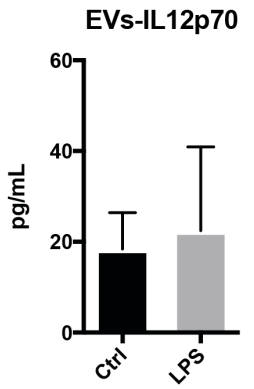
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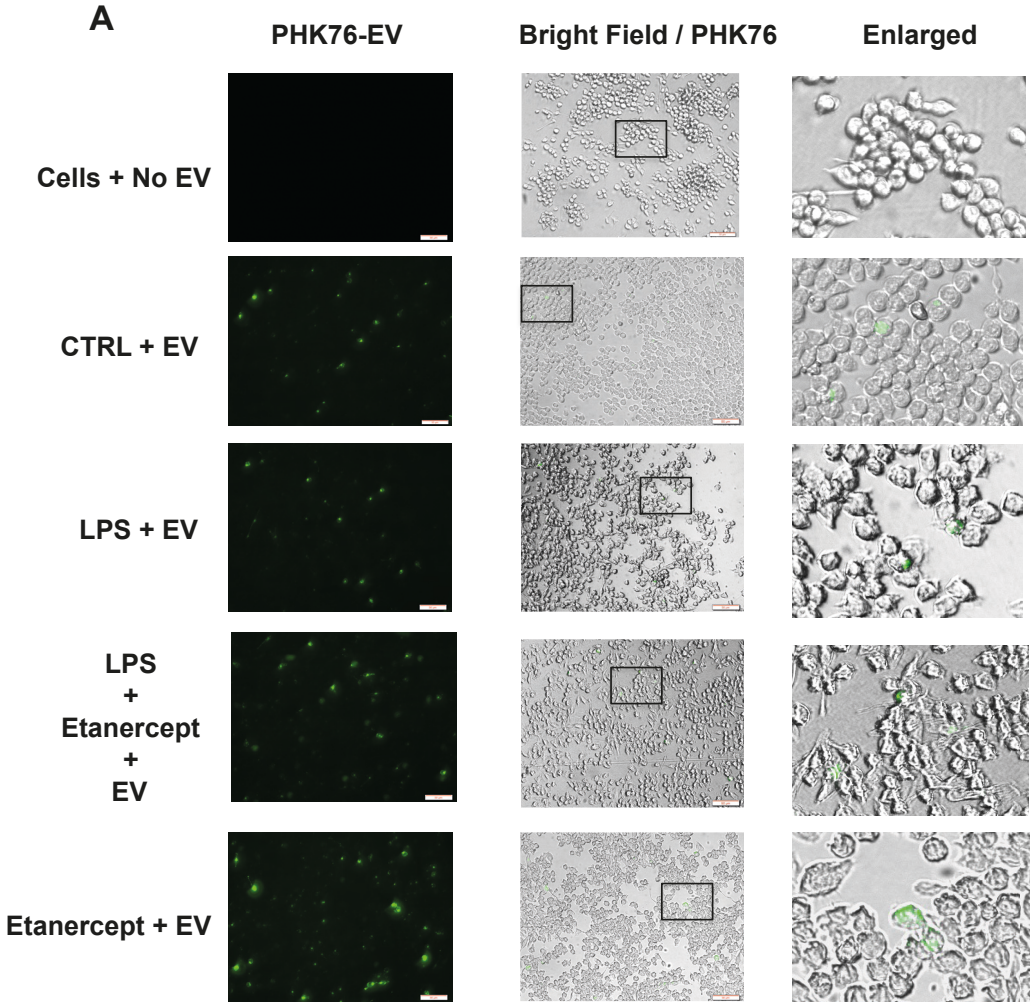
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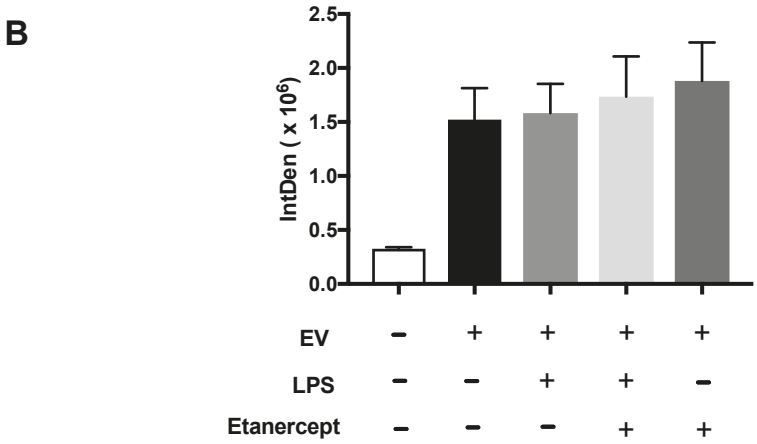
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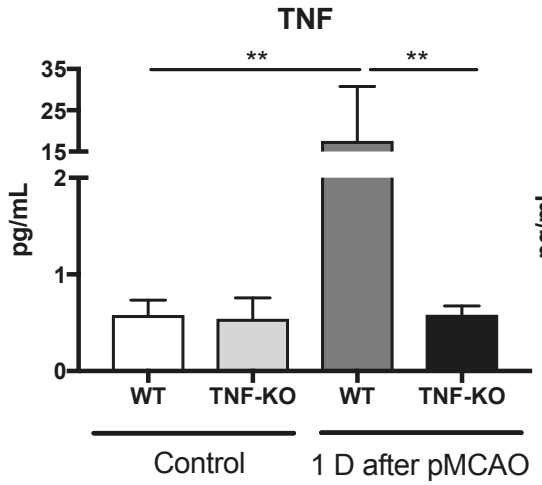
TNF
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pg/mL
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TNF-KO
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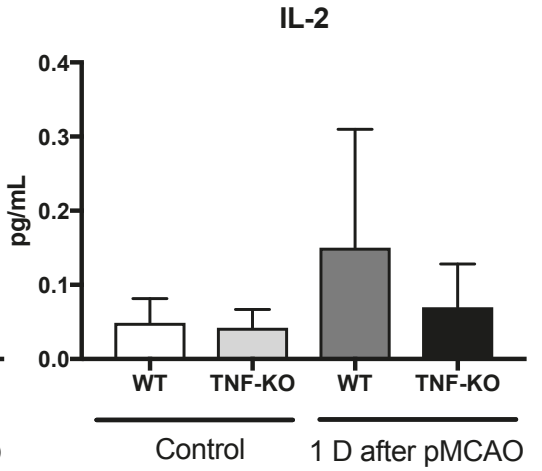
Control
1 D after pMCAO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WT
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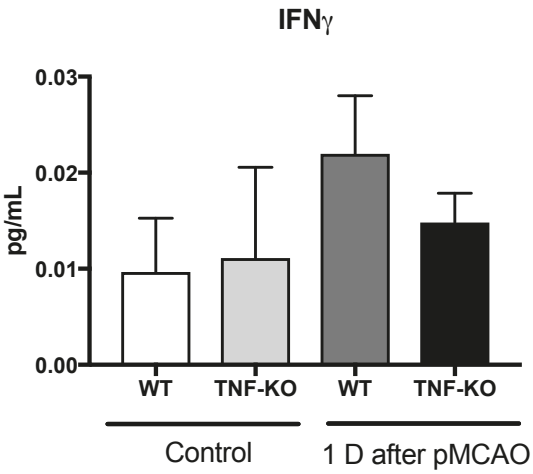
WT
TNF-KO
Control
1 D after pMCAO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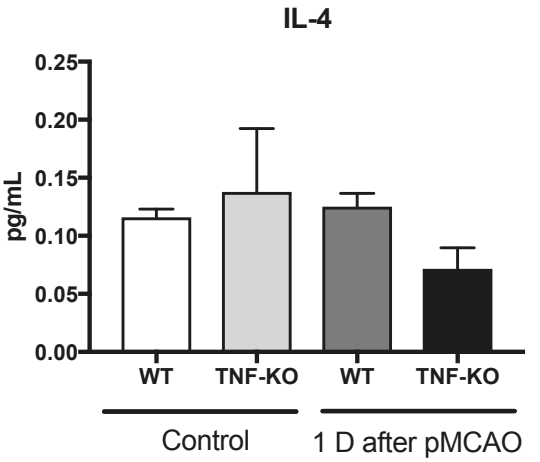
WT
TNF-KO
WT
TNF-KO
Control
1 D after pMCAO


IL-4
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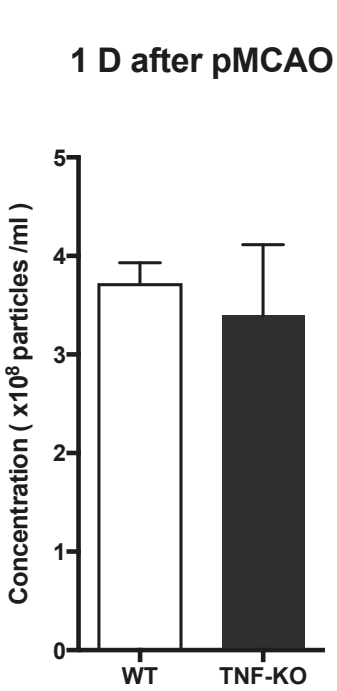
WT
TNF-KO
WT
TNF-KO
Control
1 D after pMCAO


1 D after pMCAO
Concentration (x10^8 particles /ml)
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WT
TNF-KO

Paper II

SCIENTIFIC REPORTS

OPEN

Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD

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Antonio Boza-Serrano, Yiyi Yang, Agnes Paulus & Tomas Deierborg

Alzheimer's disease (AD) is the most common form of dementia characterized by the formation of amyloid plaques (Aβ). Over the last decade, the important role of the innate immune system for the disease development has been established. Chronic activation of microglial cells creates a proinflammatory environment, which is believed to be central for the development of the disease as well as its progression. We used the AD mouse model 5xFAD to investigate if inflammatory alterations are present in microglial cells before plaque deposition. We applied mass spectrometry and bioinformation analysis to elucidate early microglial alterations. Interestingly, we found the cytokines IL1β and IL10 to be elevated in the 5xFAD brain after the formation of Aβ plaque at 10 weeks only. Using mass spectrometry analysis of microglial cells with bioinformation analysis, we found JAK/STAT, p38 MAPK and Interleukin pathways affected in microglial cells before plaque deposition at 6 weeks. At 10 weeks, GO analysis showed affected pathways related to interferon-gamma regulation and MAPK pathways. Our study points toward early inflammatory changes in microglial cells even before the accumulation of Aβ.

Alzheimer's disease (AD) is a neurodegenerative disease affecting more than 24 million people worldwide and future projections predicts almost 50 millions people affected by 2030[1]. The two main hallmarks of the disease are the amyloid plaques (Aβ) and the intra-neuronal phospho-Tau aggregation called neurofibrillary tangles (NFT). Tangles are linked to the late stage of the pathology, where symptoms to a large degree are non-reversible whereas plaques build up occurs earlier in the disease and primarily before the onset of cognitive symptoms[2]. Future therapeutic approaches are aiming for early treatment, even presymptomatic, to effectively fight the disease progression. For this reason, it is important to study how the disease progresses before the appearance of the first plaques.

Over the last decade, the important role of the innate immune system in development of AD pathology has been well-established[3]. In relation to the innate-immune system, the microglial cells are crucial for the homeostatic clearance of Aβ, synaptic formation modulation and innate immune response, which all are suggested to be affected in the AD disease pathogenesis[4]. The inflammatory response has received special attention in AD, based on recent genome-wide association studies[5]. The inflammatory response elicited in AD by microglial cells has been linked to their activation by different Aβ species[6–8]. This chronic activation of microglial cells and the proinflammatory environment created due to microglial activity is suggested to be decisive for AD pathogenesis. It is plausible that early microglial activation can be one of the first pathological processes involved in the initiation of the disease due to the capacity of lower molecular Aβ species to activate microglial cells *in vitro*[9]. These Aβ molecules can be found in the extracellular environment, before the typical plaque deposition and by that activate/alter microglial function. This process can potentially initiate and drive the progression of the disease; not only

Experimental Neuroinflammation Laboratory, Department of Experimental Medical Science, Lund University, Sölvegatan 19, BMC B11, 221 84, Lund, Sweden. Antonio Boza-Serrano and Yiyi Yang contributed equally to this work. Correspondence and requests for materials should be addressed to A.B.-S. (email: antonio.boza_serrano@med.lu.se) or T.D. (email: tomas.deierborg@med.lu.se)

by inducing proinflammatory microglial activation, but also by affecting the homeostatic function of microglia[10], thereby affecting other cells in the brain such as neurons and astrocytes.

In view of recent failures of clinical drug trials when patients with established AD has been enrolled, the research community is searching for early pathological events important for the disease development. Therefore, elucidation of molecular pathways involved in the early phase in AD pathogenesis is necessary. In the present study, we aim to investigate alterations of the innate immune response in the microglial cell population in 5xFAD mouse model of AD. 5xFAD mouse model carries five different mutations to strongly upregulate the human amyloid metabolism, which is known to induce a strong microglial response[11]. We studied the progression of the pathology in the 5xFAD mice at 2, 6 and 10 weeks of age and related the readout to age-matched wild-type mice. By using mass spectrometry, together with immunohistochemistry and ELISA, we performed a proteomic analysis and identified the protein profile of microglial cells linked to the early stage of microglia activation in the pathology.

We found specific changes in isolated microglia before the plaque deposition at 10 weeks of age. Our study reveals a number of different pathways related with the innate immune system response, potentially involved in the progression of the disease, even before the first plaque deposition. Our results encourage a focus on early disease treatment and suggest further investigation of early microglial alteration in prodromal stages of AD.

Materials and Methods

Animals. We used male and female, same number of each, 5xFAD mice obtained originally from Jackson Laboratories, aged 2–10 weeks and weighting 14–20 g when starting the experiment. WT and 5xFAD were divided in groups depending on their age: 2 weeks, 6 weeks or 10 weeks. Each group contains 8 animals. The mice were bred and housed in standard laboratory cages with sawdust bedding and free access to water and food. The holding room had a 12:12 h light-dark cycle. The mice were randomly assigned to different experimental groups (5xFAD and WT) with no differences in body weight and age between the groups.

All animal experiments were performed in accordance with the animal research regulations (RD53/2013 and 2010/63/UE) in Sweden and European Union, and with the approval of the Committee of Animal Research at the University of Lund (Ethical permit number: M29-16).

Genotyping. The genotypes of 5xFAD mice were determined using an integrated extraction and amplification kit (Extract-N-Amp™, Sigma-Aldrich). First, the samples were incubated at 94 °C for 5 mins, followed by 40 cycles with denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 1.5 mins. The following primers (CyberGene, Solna, Sweden) were used: For the 5xFAD the primers (5′ to 3′) used are listed below: APP Forward AGGACTGACCACTCGACCAG, APP Reverse CGGGGGTCTAGTTCTGCAT, PSN1 Forward AATAGAGAACGGCAGGAGCA, PSN1 Reverse GCCATGAGGGCACTAATCAT, WT APP Forward CTAGGCCACAGAATTGAAAGATCT, WTT APP Reverse GTAGGTGGAAATTCTAGCATCATCC, RD1, RD2 and RD3 AAGCTAGCTGCAGTAACGCCATTT,ACCTGCATGTGAACCCAGTATTCTATC, CTACAGCCCCTCTCCAAGGTTTATAG. The PCR products were separated by gel electrophoresis labelled with ethidium bromide and visualized using a CCD camera (SONY, Tokyo, Japan).

Sequential Protein extraction. The protein extraction was performed in brain samples from 5xFAD at 2, 6 and 10 weeks and WT mice at 2 and 10 weeks. Mice were transcardially perfused under deep anesthesia with PBS, pH 7.4. Then, the mice were decapitated and the brains were removed. One brain hemisphere was snap frozen in dry ice and the other one was fixed in paraformaldehyde (4%) as described below. Soluble protein fractions were obtained from the whole cortex (mice) using sequential protein extraction. Fractions were obtained by homogenization of the cortex with a dounce homogenizer in the presence of PBS (1 mL/100 μg of tissue). After centrifugation for 1h at 43.000 rpm in special tubes for high-speed centrifugation from Beckman-Coulter (#357448, 1,5 ml tubes) the supernatants, were obtained and aliquoted and stored at −80 °C (S1 fraction). The pellets were extracted in RIPA buffer (Sigma-Aldrich, Germany), ultracentrifuge at 30.000 rpm and supernatants, S2 fractions (intracellular particulate proteins), were aliquoted and stored. PBS and RIPA solution were prepared using a protein inhibitor (Protein Inhibitor Cocktail, ThermoScientific) to prevent protein degradation and to inhibit the enzymatic activity of phosphatases (PhosphoStop, Roche).

ELISA. A Mesoscale Discovery platform (MSD, from Rockville, Maryland, USA) was used to evaluate the cytokine levels by a proinflammatory reading plate for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 and TNF-α. To measure the cytokines in mouse brain soluble fractions, we pulled together 50 μg of the S1 and S2 fraction. Moreover, MSD plates were used to test the levels of human $A\beta_{40}$ and $A\beta_{42}$ in the soluble fraction of the brain extraction from WT and 5xFAD at different ages, 2, 6 and 10 weeks. Serial dilution of the soluble fraction was tested in order to obtain an accurate measure of the protein levels. The plates were developed using the 4× reading buffer diluted 1 time with distilled water and the plates were read using the QuickPlex Q120 reader from Mesoscale. The detection range of the different cytokines presented is the next (IL1β 1670-0,408 pg/mL: IL4, 1660-0,405 pg/mL; IL12, 32200-7,86; IL10, 3410-0,833). Other cytokines evaluated not included but not included in the results: IFN-γ (938-0,229 pg/mL), IL2 (2630-0,642 pg/mL), IL5 (967-0,236 pg/mL), IL6 (5720-1,40), KC/GRO (1980-0,483 pg/mL), TNF-α (627-0,153). $A\beta_{40}$ detection range (15100-3,69 pg/mL), $A\beta_{42}$ detection range (2280-0.557 pg/mL).

Western blot. Western blotting was used to measure APP levels in brain extractions. The proteins were extracted from the cortex using PBS or RIPA buffer (Sigma-Aldrich, Germany) with proteinase and phosphatase inhibitors. The blots were run using pre-casted gels (4–20% from Bio-Rad) in TGS buffer (Bio-Rad). The proteins were transferred to nitrocellulose membranes (Bio-Rad) using the TransBlot turbo system from BioRad. The membranes were blocked for 1h with skim milk at 3% in PBS, then washed 3 × 10 mins in PBS and Tween-20

at 0,1%, after which they were incubated with primary and secondary antibodies diluted in PBS-Tween 20 at 0.1%. To develop the blot we use ECL Clarity (Bio-Rad) according to the manufacturer's protocol and ChemiBlot XRS + system from Bio-Rad.

Tissue processing and immunohistochemistry. Mice were transcardially perfused under deep anesthesia (5% isoflurane in oxygen) with PBS, pH 7.4. The brains were removed and fixed in 4% PFA for 24h, then, washed in PBS 3 times and then fixed in 30% sucrose solution for 3 days. Brains were cut at a 40 μm thickness in the sagittal plane on a microtome and collected in 6 series in cold PBS and 0.02% sodiumazide. Sections from control and 5xFAD mice were processed in parallel for light microscopy immunostaining using the same batches of reagents/solutions. Brief protocol: We rinsed 3 × 5 mins in PBS, pre-incubation with 10% NDS (normal donkey serum) in 0.25% Tween-20 in PBS (blocking solution) 1h in room temperature. Primary antibody staining (mouse 6E10 Covance 803002, 1:500; rabbit Iba1 Wako ref 019-19741, 1:500) was performed in 10% blocking solution (NDS, 0.25% Tween-20) and incubated overnight in room temperarture. The day after, we rinsed 3 × 5 mins in Tween-PBS (0.25%) and 1 × 5 mins in 10% blocking solution (NDS, 0.25% Tween-20). Secondary antibody staining (anti-mouse 555 Alexa Fluor and anti-rabbit 488 Alexa Fluor were used at 1:500) in 10% blocking solution for 2h in room temperature (dark conditions) was used followed by DAPI 1:1000 for 5 mins in 0.25% Tween X-100 in PBS and subsequent rinsing 3 × 5 mins PBS. Next, the sections were coverslipped with Pro-LongTM Diamond Antifade Mountant (Invitrogen) and dried in dark conditions in room temperature for 24 hours. For staining with Thioflavin-S (T1892, Sigma Aldrich) sections were first washed 3 × 5 mins in 0.25% Tween-20 in PBS and then incubated for 5 mins with 0.5% Thioflavin-S. Next, we washed sections 3 × 10 mins in 0.25% Tween-20 in PBS, mounted with Pro-LongTM Diamond Antifade Mountant (Invitrogen) and dried in dark conditions for 24 hours. Nikon Confocal A1+ microscope was used to take the pictures; 20× and 60× pictures where taken in the same area using NIS elements in-house software.

Plaque quantification/microglial cells activation in the subiculum. Plaque load was defined as the number of Thioflavin-S positive Aβ plaques in the subiculum of 5xFAD mice. The number of labelled gal3/iba1 positive cells were quantified in the subiculum and CA1 pyramidal layer in the brains of 5xFAD. Images were acquired with a Nikon DS-5M high-resolution digital camera connected to a Nikon Eclipse 80i microscope. The camera settings were adjusted at the start of the experiment and maintained for uniformity. Digital 10× images from 2, 6 and 10 weeks old 5xFAD mice were used for plaque and microglial quantification. 6 sections/mouse; n = 4/age/genotype. Analysis of the fluorescent labelled structures was performed with Fiji ImageJ Software (W. Rasband, National Institutes of Health). The colour images for plaques were converted to binary images. Plaques and activated microglial cells were counted manually after setting the brightness threshold. Quantitative comparisons were carried out on sections processed at the same time with same batches of solutions. Pictures were retrieved from the same brain area in the different experimental groups.

Isolation of microglial cells. Mice were transcardially perfused, under deep isoflurane anesthesia, with PBS pH 7.4 in order to remove any peripheral cells from the vasculature in the brain. Then, brains were removed from the cranium. Microglial cells from 5xFAD and WT brains were isolated from the right hemisphere using magnetic beads for CD11b surface marker (Miltenyi, Germany) along with isolation columns from Miltenyi Biomarkers. Briefly, the right hemisphere was dissected in small pieces and store in PBS. The suspension was spinned down and the tissue was processed using the Neuronal Tissue Dissociation Kit from Miltenyi (130-092-628, Germany) following the manufacturer's protocol. The cell suspension was filtered using a 70 μM cell strainer. Next, we incubated our cell suspension with CD11b magnetic beads from Miltenyi for 30 mins at 4 °C and isolated the cells using the Miltenyi Isolation Columns. Once we isolated the cells, we added RIPA buffer (R0278, Sigma Aldrich) with phosphatase (04906837001, Roche) and proteinase inhibitors (04693159001, Roche) to avoid protein degradation.

Flow cytometry. Microglia isolated by CD11b microbeads (Miltenyi Biotec) were analyzed for microglial cell surface antigens by flow cytometry. Briefly cells were incubated with anti-CD16/CD32 antibody (BD Bioscience, 1:100) to block Fc receptors. Samples were then incubated with anti-CD11b-APC (Biolegend, 1:800) and anti-CD45-PE (BD Bioscience; 1:400) to confirm purity of the microglial population. Gating was determined by proper negative isotype stained controls and compensation was made with single stainings. A viability staining, 7-aminoactinomycin D (7AAD)(BD Bioscience) was used to exclude dead cells. Flow cytometry was perfomed in a FACSArial III cytometer (BD Biosciences) and FACS Diva software (BD Biosciences). Ten thousand events were recorded and microglia were identified by $CD11b^+$ and $CD45^+$ expression[12]. Data analysis was made using FlowJo 10.3 software (Three Star, Inc). Confirmed the enrichment of microglial cells by flow cytometry using CD11b and CD45 microglial markers (Supp. Figure 1).

Sample Preparation for proteomic analysis. Protein concentration was measured for each sample using Pierce™ BCA Protein Assay (Thermo Scientific, Rockford, USA) and the Benchmark Plus microplate reader (Bio-Rad Laboratories, Hercules, USA) with BSA solutions as standards. Aliquots of 1 or 4 μg of each protein extract sample were mixed into the pooled reference sample. Aliquots containing 10 μg of each experimental sample or reference sample were digested with trypsin using the filter-aided sample preparation (FASP) method[13]. Briefly, protein samples were reduced with 100 mM dithiothreitol at 60 °C for 30 mins, transferred on 30 kDa MWCO Nanosep centrifugal filters (Pall Life Sciences, Ann Arbor, USA), washed with 8 M urea solution and alkylated with 10 mM methyl methanethiosulfonate in 50 mM TEAB and 1% sodium deoxycholate. Digestion was performed in 50 mM TEAB, 1% sodium deoxycholate at 37 °C in two stages: the samples were incubated with 100 ng of Pierce MS-grade trypsin (Thermo Scientific, Rockford, USA) for 3 h, then 100 ng more of trypsin was added and the digestion was performed overnight. The peptides were collected by centrifugation labelled using

TMT 10-plex isobaric mass tagging reagents (Thermo Scientific) according to the manufacturer's instructions. The labelled samples from brain were mixed into corresponding sets, sodium deoxycholate was removed by acidification with 10% TFA.

The mixed labelled samples were subjected to the reversed-phase high pH fractionation on the AKTA chromatography system (GE Healthcare Life Sciences, Sweden) using the XBridge C18 3.5 μm, 3.0 × 150 mm column (Waters Corporation, Milford, USA) and 25 mins gradient from 7% to 40% solvent B at the flow rate of 0.4 ml/min; solvent A was 10 mM ammonium formate in water at pH 10.00, solvent B was 90% acetonitrile, 10% 10 mM ammonium formate in water at pH 10.00. The initial 31 fraction was combined into 15 pooled fractions in the order 2 + 17, 3 + 18, 4 + 19 etc. The pooled fractions were dried on Speedvac and reconstituted in 20 μl of 3% acetonitrile, 0.1% formic acid for analysis.

LC-MS/MS Analysis. Each fraction was analyzed on Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced with Thermo Easy-nLC 1200 nanoflow liquid chromatography system (Thermo Fisher Scientific, Odense, Denmark). Peptides were trapped on the C18 trap column (100 μm × 3 cm, particle size 3 μm) separated on the home-packed C18 analytical column (75 μm × 30 cm) packed with 3 μm Reprosil-Pur C18-AQ particles (Dr. Maisch, Germany) using the gradient from 6% to 32% B in 70 min, from 32% to 50% B in 5 min, from 50% to 100% B in 5 min; solvent A was 0.2% formic acid and solvent B was 80% acetonitrile, 0.2% formic acid. Precursor ion mass spectra were recorded at 70 000 resolution. The 10 most intense precursor ions were selected with the isolation window of 1.6, fragmented using HCD at stepped collision energy of 27, 35 and 47 and the MS^2 spectra were recorded at a resolution 35 000. Charge states 2 to 6 were selected for fragmentation, dynamic exclusion was set to 30 s.

Protein Evaluation. Data analysis was performed using Proteome Discoverer version 1.4 (Thermo Fisher Scientific, Waltham, USA). The protein database for *Mus musculus* (February 2017, 16854 sequences) was downloaded from Swissprot. Mascot 2.5.1 (Matrix Science) was used as a search engine with precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, one missed tryptic cleavage was accepted. Mono-oxidation on methionine was set as a variable modification, methylthiolation on cysteine and TMT-6 reagent modification on lysine and peptide N-terminus were set as a fixed modification. Percolator was used for the validation of identificated results; target false discovery rate of 1% was used as a threshold to filter confident peptide identifications.

Reporter ion intensities were quantified in MS3 spectra using Proteome Discoverer 1.4 at 0.003 Da mass tolerances with reporter absolute intensity threshold of 2000. The resulting ratios were normalized on the median protein value of 1.0 in each sample.

Protein bioinformatics analysis. Proteins profile similarities were analysed by using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html) among the different analyzed groups. Then, Gene Ontology (GO) analysis and Panther pathways analysis database were used to analyse the data from 2 different approaches. Individual groups were analysed followed with group comparison. The first 200 of the most abundant proteins form each individual group (5xFAD 2, 6 and 10 weeks and WT 2 and 10 weeks) were used to evaluate the main pathways affected by EnrichR (Panther data base). Then, GO analysis was performed using STRAP software. After the GO analysis, the resulted top 20 upregulated and downregulated proteins selected from group comparison (5xFAD 2w vs 5xFAD 6w, 5xFAD 10w vs 5xFAD 6w, 5xFAD 10w vs WT12w and 5xFAD 2w vs WT 2w) were used to further analyze the main pathways involved. Briefly, to select the proteins for the GO analysis we did the following: Step 1; for each protein, calculate group means, fold changes and perform t-tests to get p-values, filter on fold change > 0 (to picks only up-regulated proteins) and sort on p-values. To get downregulated proteins we only change step 2; to filter on fold change < 0. The pathway analysis was performed using EnrichR (Panther database for pathways). EnrichR implements four scores to report enrichment results: p-value, q-value, rank (Z-score), and combined score.

The p-value is computed using a standard statistical method used by most enrichment analysis tools: Fisher's exact test or the hypergeometric test. This is a binomial proportion test that assumes a binomial distribution and independence for probability of any gene belonging to any set.

The q-value is an adjusted p-value using the Benjamini-Hochberg method for correction for multiple hypotheses testing. The rank score or z-score is computed using a modification to Fisher's exact test in which we compute a z-score for deviation from an expected rank.

Finally, the combined score is a combination of the p-value and z-score calculated by multiplying the two scores as follows:

$$c = log(p) * z$$

where c is the combined score, p is the p-value computed using Fisher's exact test, and z is the z-score computed to assess the deviation from the expected rank.

PCA (principal analysis component) analysis was performed using Ingenuity Pathway Analysis (IPA) from Qiagen. PCA is a quality controls analysis and it was performed in order to detect outliers and to evaluate the variability within each group.

Statistical analysis. The differences between experimental groups were analysed with one-way ANOVA with Tukey's post hoc test or two-way ANOVA with Bonferroni post hoc test correction. $P < 0.05$ was considered as statistically significant. We used the statistical software GraphPad PRISM 7.0 (San Diego, CA, USA). Data is represented as mean ± S.D.

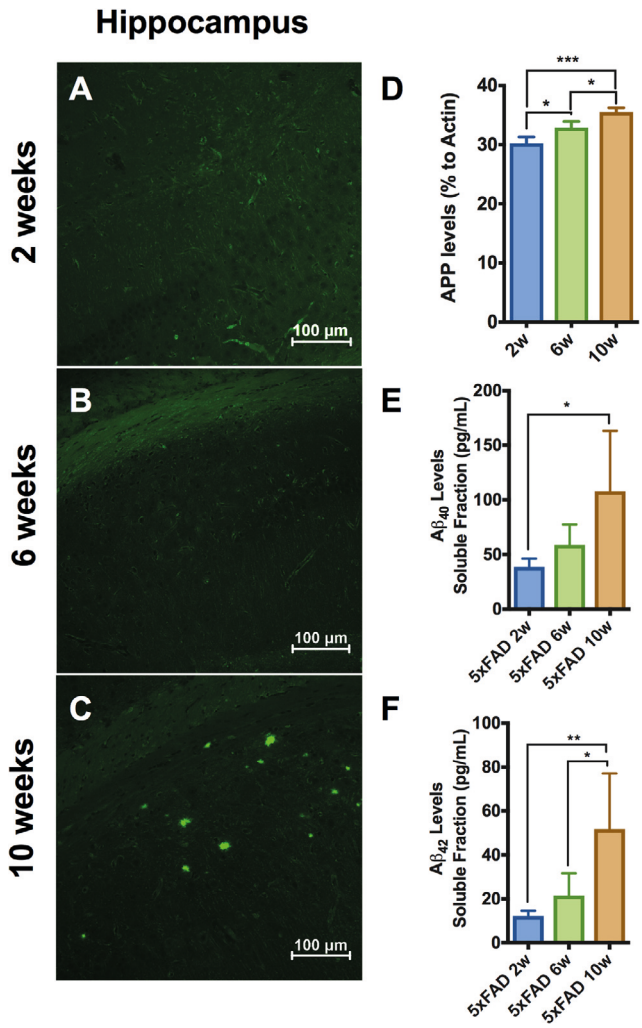

Figure 1. Characterization of AD brain pathology in 5xFAD mice. (**A**–**C**) Thioflavin-S staining on 5xFAD mice at 2 (**A**), 6 (**B**) and 10 (**C**) weeks. Only in 5xFAD mice at 10 weeks amyloid beta aggregates in the subiculum were present. n = 4 per group. (**D**) Human APP levels measured by western blot showed a significant increased in a time-dependent fashion from 2 weeks to 10 weeks in 5xFAD mice. (**E**, **F**) Aβ40 and Aβ42 levels were measured in brain homogenates soluble fraction by ELISA. (**E**) Aβ40 levels were significantly increased at 10 weeks compared to 2 weeks in 5xFAD mice. (**F**) Aβ42 levels were significantly increased in a time-dependent fashion in 5xFAD mice. n = 4 per group; *p < 0,05; **p < 0,005; One-Way ANOVA. Mean ± S.D.

Results

Characterization of the disease progression in 5xFAD mice. We evaluated the age-dependent plaque deposition in hippocampus, the APP production and the brain extracellular Aβ levels in the 5xFAD mice to confirm the age-dependent Aβ production as part of the pathology. First, we stained our sections with thioflavin-S to visualize fibrillar plaques to verify the deposition of Aβ plaques. Thioflavin-S positive aggregates were found only in 10 week-old 5xFAD mice in the subiculum (Fig. 1C) but could not be detected at younger ages, 2 and 6 weeks

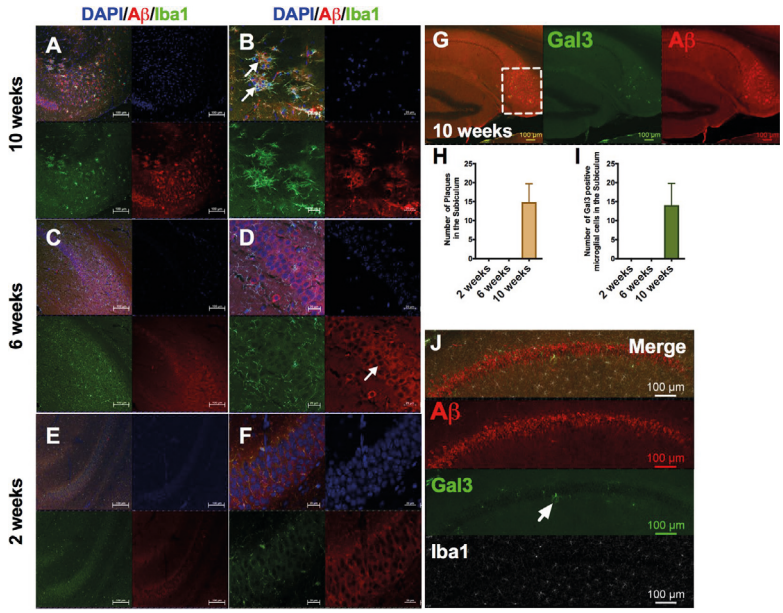

Figure 2. First plaque deposits appear at 10 weeks in 5xFAD mice in the subiculum subregion of hippocampus. Evaluation of the plaque deposition in 5xFAD from 2 to 10 weeks (**A**–**F**). (**A**, **B**) The first plaque deposits were present at 10 weeks. These plaque deposits were markedly surrounded by Iba1 positive cells (Details in B). Arrows indicated plaque deposits and Iba1 positive cells besides APP positive neurons (**B**). (**C**–**F**) At 2 and 6 weeks the plaque deposits were undetectable. At 6 weeks the APP positive neurons (white arrow in **D**) were more evident compared to 2 weeks (**E**, **F**). (**G**) Amyloid plaque depostis were found in the subiculum at 10 weeks. The squares indicates the area analyzed in the subiculum. (**H**) Aβ plaques quantification in the subiculum of 5xFAD mice from 2 to 10 weeks. (**I**) Gal3 positive microglial cells in the subiculum of 5xFAD from 2 to 10 weeks. Gal3 positive cells were Iba1 positive. (**J**) Gal3 positive microglial cells were found in the molecular layer in the hippocampus around APP positive cells at 6 weeks. DAPI: blue; 6E10 (Aβ): red; Iba1: Green. n = 3–4 per group.

(Fig. 1A, B) or in the WT mice (data not shown), which is in line with previous reports studying Aβ plaques in the 5xFAD mice[11,14]. Then, we used western blot to study the protein levels of the human amyloid precursor protein (APP) by using 6E10 antibody recognizing amino acid residues 1–16 in Aβ (Fig. 1D). We found a significant age-dependent increase of APP in the brain of 5xFAD mice from 2, 6 to 10 weeks of age (Fig. 1D). As expected, human APP was not detectable in WT brains (data not shown). Next, we evaluated by ELISA the human $A\beta_{40}$ and $A\beta_{42}$ levels in the soluble fraction from brain homogenates in the 5xFAD mice. We found a significant time-dependent increase in soluble $A\beta_{40}$ and $A\beta_{42}$ levels from 2 to 10 weeks of age (Fig. 1E, F). Previous studies have shown the ability of the soluble fraction to activate microglia[15,16]. $A\beta_{40}$ or $A\beta_{42}$, was not detected in WT mice (data not shown).

To further confirm the plaque deposition, we stained our brain sections from 5xFAD mice with human anti-beta amyloid (6E10) antibody together with the microglial marker Iba1 (ionized calcium binding adaptor molecule 1) to evaluate the presence of microglial cells around plaques, a characteristic hallmark of the plaque deposition (Fig. 2). In line with our thioflavin-S results, we were able to confirm plaque deposits at 10 weeks in our 5xFAD mice in the subiculum (Fig. 2A, B, G and H), but not before that time-point in 5xFAD mice (6 and 2 weeks, Fig. 2C–F) or in WT mice (data not shown). Importantly, at 10 weeks we found the typical plaque-associated microglial cells (Iba1+) around the plaques (Fig. 2B). Then, we searched for activated microglial cells using galectin-3, which has been associated to proinflammatory microglial cells around Aβ plaque in 5xFAD brains[17], and which we have found to be expressed in strongly activated microglial cells[17,18] as a TLR4 ligand[19]. Galectin-3 positive cells could only be detected at 10 weeks and then in association with the plaque in subiculum (Fig. 2G and I). Interestingly, already at 6 weeks of age we found galectin-3 positive microglial cells in the CA1 pyramidal layer of the hippocampus around APP expressing neurons (Fig. 2J). This potential microglial activation at 6 weeks in the hippocampus might be related to the soluble Aβ released by APP positive neurons[7,15]. This finding is in line with our hypothesis about microglial activation before plaque deposition due to neuronal release of Aβ soluble molecules.

Altered inflammatory cytokines in 5xFAD before plaque formation. The presence of soluble $A\beta_{40}$ and $A\beta_{42}$ at early time points in the 5xFAD mice (2 and 6 week, Fig. 1E, F), despite absence of plaque, spurs the question if we still can detect early inflammatory changes. When analysing the brain tissue levels of the anti-inflammatory cytokine IL10 we found a significant time-dependent difference (Two-way ANOVA, $p > 0.01$) with a significantly upregulated by 41,6% at 10 weeks compared to 6 weeks in 5xFAD mice (Fig. 3A, $p > 0.05$). Similar results were obtained for the proinflammatory cytokine IL1β (Fig. 3B). Two-way ANOVA analysis showed a significant effect of both time and genotype ($p > 0.001$). Post hoc analysis revealed IL1β to be significantly upregulated with 60% at 10 weeks in 5xFAD mice compared to control mice, as well as 6 and 10 weeks in 5xFAD where it was increased by 54%, and compared to 2 weeks increased by 42,4% (Fig. 3B). The elevated levels of IL10 and IL1β at 10 weeks, the time point where plaques appears surrounded by activated microglial cells, could indicate that these two cytokines are related to the immune reactions elicited by the Aβ plaque. Next, we studied the cytokine IL12 and found significant time- and genotype-dependent differences ($p > 0.05$, two-way ANOVA). However, we failed to find any significant differences in post hoc analyses (Fig. 3C).

The last cytokine we found to be significantly altered between 5xFAD and WT, among all the pro- and anti-inflammatory cytokines measured (see material and methods), was the anti-inflammatory cytokine IL4 (Fig. 3D). We found IL4 to be downregulated in the brain of 5xFAD mice at all time points together compared to control animals (Fig. 3D, two-way ANOVA, $p > 0.05$), albeit independent time point differences were not significant in posthoc analysis (one-way ANOVA). The apparent early impact of IL4 level at 2 weeks in the 5xFAD mice suggest that APP expression and/or Aβ products can, at a very early age, alter IL4 levels in the brain.

Bioinformatics analysis showed inflammatory pathways upregulated in 5xFAD microglial cells before plaque deposition. We used mass spectrometry in order to study protein alterations in microglia, qualitatively and quantitatively, in the WT and 5xFAD at 2 weeks and 6 weeks of age, before the formation of Aβ plaque, and at 10 weeks. Microglial cells were isolated from brains using CD11b magnetics beads (MACS).

Using a bioinformatic approach to understand the altered molecular and cellular processes, we analysed the 200 most abundant proteins at different time points and in the experimental groups. The protein profiles of 2 and 6 weeks 5xFAD microglia shared only 12,4% of the proteins (Fig. 4, analysed by software Veny 2.1 by CSIC). This difference was even larger when comparing 5xFAD at 6 and 10 weeks where only 1,6% of the proteins were the same, which could be related to the development of the Aβ plaque pathology. Surprisingly, the similarity between 2 and 10 weeks were larger with 4,5% similarity in the most 200 abundant proteins (Fig. 4A) which could indicate that early developmental programs at 2 weeks are also active at 10 weeks of age but in a pathological context. Comparing microglial proteins between 2 weeks, 6 weeks and 10 weeks, 5xFAD mice showed that 1,4% of the proteins were common in microglial cells from all of the time points studied (Fig. 4A). Interestingly, there were more proteins in common between 2 weeks old WT and 2 weeks old 5xFAD mice (66%) (Fig. 4B) than between 10 weeks WT and 10 weeks 5xFAD mice (7,3%) (Fig. 4C), indicating that microglial cells change their identity in response to the Aβ plaque deposition. Changes in microglial profile due to Aβ plaque formation have been shown in different mouse model and in human[20–22]. Moreover, as a data validation step, PCA analysis was performed to evaluate individual data point cluster in each group. Notably, no outliers were present and all groups formed clusters with some clusters overlapping each other (Fig. 4D).

Next, we analysed the protein profiles for the different experimental groups in terms of molecular pathways affected. The same 200 most abundant proteins were selected as before to analyse the main altered pathways in the 5xFAD mice at 2, 6 and 10 weeks of age. At 2 weeks in 5xFAD mice the main affected pathways were related to the RNA polymerase transcription and FAS ligand pathways (Table 1). Notably, already at 6 weeks we found inflammatory-related pathways affected. In fact, inflammatory pathways such as JAK/STAT, p38 MAPK and Interleukin pathways were among the most affected (Table 2). At 10 weeks in our 5xFAD mice, we found the vesicles trafficking as the most affect pathway followed by metabotropic and adrenergic regulation (Table 3), which is an interesting finding in this microglial cell population. The pathway analysis of the control groups (WT) did not show any inflammatory process affected at any time point (data not shown).

For the Gene Ontology (GO) analysis, we studied the molecular function, cellular component and biological process between different groups. We first investigated the very first age-related changes linked to AD pathogenesis by making a comparison between 5xFAD 2 weeks and 5xFAD 6 weeks. The molecular function was mostly affected in regards to the catalytic activity and the binding (Fig. 5A). Notably, both catalytic and binding activity were upregulated in a time-dependent manner in 5xFAD from 2 to 10 weeks and in comparison to WT (Fig. 5A) Next, we studied subcellular components related to the protein alterations in microglial cells. We found the endoplasmatic reticulum (ER), the cytoplasmatic compartment and the nucleus to be the main cellular components affected at 6 weeks compared to 2 weeks of age in the 5xFAD mice in relation to other affected cellular components (Fig. 5B). For the biological processes: cell regulation and cellular processes were clearly affected in 5xFAD at 6 weeks compared to 5xFAD at 2 weeks (Fig. 5C). Notably, the GO for the biological processes showed enrichment of the proteins related with immune system processes in the 5xFAD at 10 weeks compared to 5xFAD 6 weeks (Fig. 5C), in line with our hypothesis of inflammation development over time. The main cellular components altered at 10 vs 6 weeks in the 5xFAD microglial cells were the endosome and the mitochondria, in relation to other affected cellular components (Fig. 5B). Finally, we analysed the pathways affected for each compared group by using the top 20 proteins upregulated resulting from the GO analysis for both 5xFAD 10 and 6 weeks (Table 4) and 5xFAD 6 and 2 weeks (Table 5). Interestingly, the comparison between 6 and 2 weeks 5xFAD showed an increasing regulation of the FAS ligand pathways at 6 weeks old 5xFAD (Table 6B). The pathways analysis outcome between 10 weeks and 6 weeks old 5xFAD mice showed a clear upregulation of immune system related pathways such as interferon-gamma signalling pathways and a strong trend for altered B and T cell activation pathways and interleukin pathways in the 10 weeks old 5xFAD mice ($p = 0.055$, $p = 0.071$, respectively) which goes along with the alteration of cytokines we detected in 10-week old 5xFAD brains. Importantly,

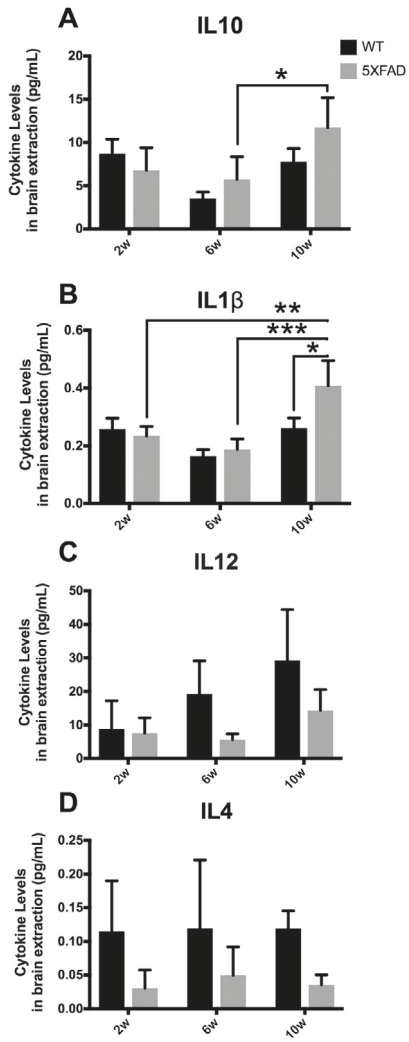

Figure 3. Altered cytokine pattern in brain homogenates from 5xFAD mice. ELISA evaluation of inflammatory cytokines from brain homogenates. (**A**) IL10, differences. (**B**) IL12, differences. (**C**) IL1β, differences. (**D**) IL4, differences. 5xFAD mice n = 5 for each 5xFAD group; WT n = 3 for each group. Mean ± S.D. mean. *$p < 0,05$; **$p < 0,005$; ***$p < 0.001$. Two-way ANOVA and One-way ANOVA with Tukey's post correction for multiple comparisons between groups.

we found a strong trend in Alzheimer's disease related-pathways such as the amyloid-secretase pathway to be upregulated at 10 weeks in microglial cells from the 5xFAD ($p = 0.055$, Table 6A).

Discussion

In this study, we investigated if altered inflammatory pattern in the commonly used AD mouse model 5xFAD could be found before plaque deposition. We studied inflammatory alterations related to microglial cells and we found a clear inflammatory activation in microglial cells isolated before Aβ plaque deposition with altered

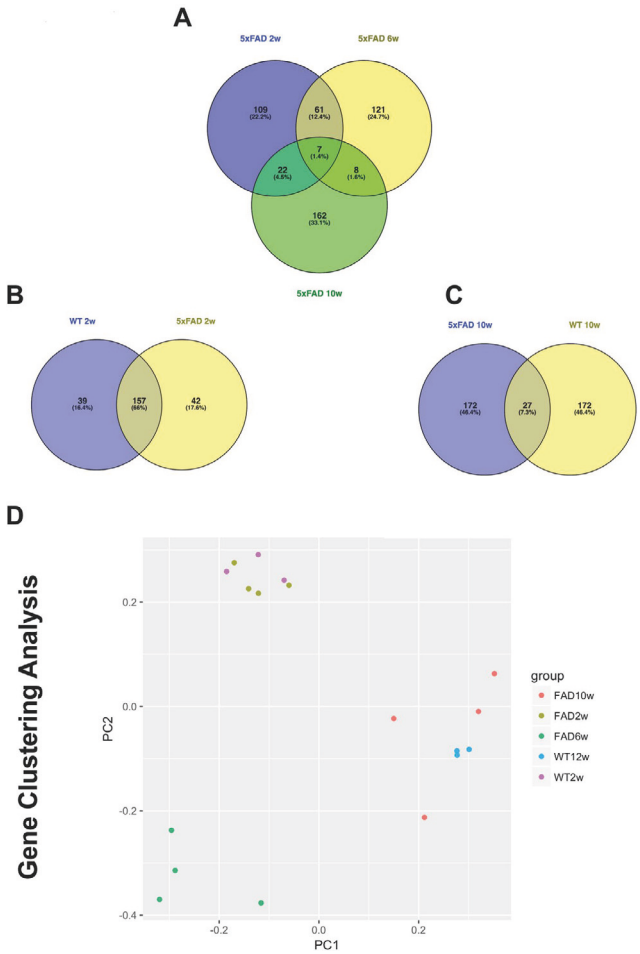

Figure 4. Protein profiles and PCA cluster analysis. (**A**) Comparison of the protein identity among the 200 most expressed proteins in 5xFAD. Among 2 and 6 weeks only 12.4% of proteins were the same. Among 6 and 10 weeks only 1.4% were the same. Among 2 and 10 weeks just 4.5% were the same. (**B**) Comparison between 2 weeks 5xFAD and 2 weeks WT mice results in 66% similarities in protein identity. (**C**) Comparison between 10 weeks 5xFAD and 10 weeks WT mice results in 7.3% similarities in protein identity. (**D**) Proteins cluster analysis (PCA). Proteins from the same group present a similar cluster. Analysis was performed using Ingenuity Pathways Analysis (IPA) from Quiagen.

cytokine levels in the 5xFAD brain and upregulation of proteins related to JAK-STAT, MAPK and Interleukins pathways along with a significant increase in the soluble Aβ levels. Moreover, at 10 weeks of age, the inflammatory pathways highlighted were linked to vesicles metabolism, which has been recently related to amyloid toxicity and plaque formation[23]. Moreover, at 6 and 10 weeks we found gal3 positive microglial cells around APP neurons and Aβ, respectively. Gal3 has been shown to be involved in the proinflammatory activation of microglial cells and it is expressed in microglial cells around Aβ plaques in 5xFAD mice[17,19]. The data obtained in this study demonstrate the role of microglial cells in the inflammatory response and the dynamics of the response from pre-plaque stages to the appearance of the first plaques.

This early aspect of microglial activation has received a lot of attention recently, and there are many studies trying to elucidate the dynamics of the inflammatory response in the context of AD. At present, the majority of

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	General transcription by RNA polymerase I	0.0004042	0.01213	−1.25	5.51
2	FAS signaling pathway	0.003552	0.05329	−1.31	3.84
3	Transcription regulation by bZIP transcription factor	0.06544	0.3410	−1.05	1.13
4	Ubiquitin proteasome pathway	0.06819	0.3410	−0.83	0.89
5	CCKR signaling map ST	0.8093	0.8093	0.98	−0.21
6	Wnt signaling pathway	0.7668	0.7932	0.92	−0.21
7	PDGF signaling pathway	0.3066	0.4379	0.26	−0.21
8	Opioid prodynorphin pathway	0.1648	0.3709	0.23	−0.23
9	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	0.3610	0.4709	0.35	−0.26
10	5HT4 type receptor mediated signaling	0.1479	0.3709	0.26	−0.26

Table 1. Main affected pathways in 5xFAD at 2 weeks (top 200 most abundant proteins).

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	JAK/STAT signaling pathway	0.0003162	0.01138	−1.44	6.43
2	PDGF signaling pathway	0.005052	0.06063	−1.46	4.09
3	p38 MAPK pathway	0.003783	0.06063	−1.40	3.94
4	General transcription regulation	0.03085	0.1915	−0.65	1.07
5	Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	0.03292	0.1915	−0.64	1.05
6	FAS signaling pathway	0.03723	0.1915	−0.54	0.89
7	Transcription regulation by bZIP transcription factor	0.06429	0.2893	−0.69	0.86
8	General transcription by RNA polymerase I	0.009316	0.08384	−0.31	0.76
9	Ras Pathway	0.1480	0.3674	−0.54	0.54
10	Interleukin signaling pathway	0.2078	0.4280	−0.07	0.06

Table 2. Main affected pathways in 5xFAD at 6 weeks (top 200 most abundant proteins).

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	Synaptic vesicle trafficking	0.000002697	0.00009360	−1.42	13.19
2	Metabotropic glutamate receptor group II pathway	0.00001080	0.0002267	−1.47	12.34
3	Beta3 adrenergic receptor signaling pathway	0.000002971	0.00009360	−1.12	10.42
4	Opioid prodynorphin pathway	0.00002609	0.0002348	−0.92	7.72
5	5HT4 type receptor mediated signaling pathway	0.00001576	0.0002348	−0.91	7.63
6	Opioid proopiomelanocortin	0.00002609	0.0002348	−0.85	7.07
7	Muscarinic acetylcholine receptor 2 and 4 signaling	0.00004056	0.0003194	−0.79	6.38
8	Muscarinic acetylcholine receptor 1 and 3 signaling	0.00005849	0.0004095	−0.76	5.93
9	Opioid proenkephalin	0.00002609	0.0002348	−0.65	5.44
10	Metabotropic glutamate receptor group III pathway	0.0001974	0.0009568	−0.39	2.68

Table 3. Main affected pathways in 5xFAD at 10 weeks (top 200 most abundant proteins).

studies have used APP-PS1 models in adult or aged mice, or even APP rats[24], with a wide range of interesting approaches, from studying complement system and synapsis loss[20] to autophagy in microglial cells[25]. For instance, López-Gonzaléz *et al.*, highlighted the upregulation of proinflammatory related genes in APP-PS1 mice in adult and aged mice (from 3 to 12 months)[26]. Our approach is focusing on very early alterations before the typical disease manifestation, from 2 weeks to 10 weeks, in the 5xFAD mouse model, using mass spectrometry to explore the protein expression in isolated microglial cells.

In the present study, we first determined when and where the Aβ deposition takes place. By using thioflavin-S and Aβ staining, we found Aβ deposits at 10 weeks restricted to the subiculum, a medial subregion of the hippocampus (Figs 1C and 2A). We found soluble levels of $A\beta_{40}$ and $A\beta_{42}$ to be increased before the plaque deposition (Fig. 1E,F).

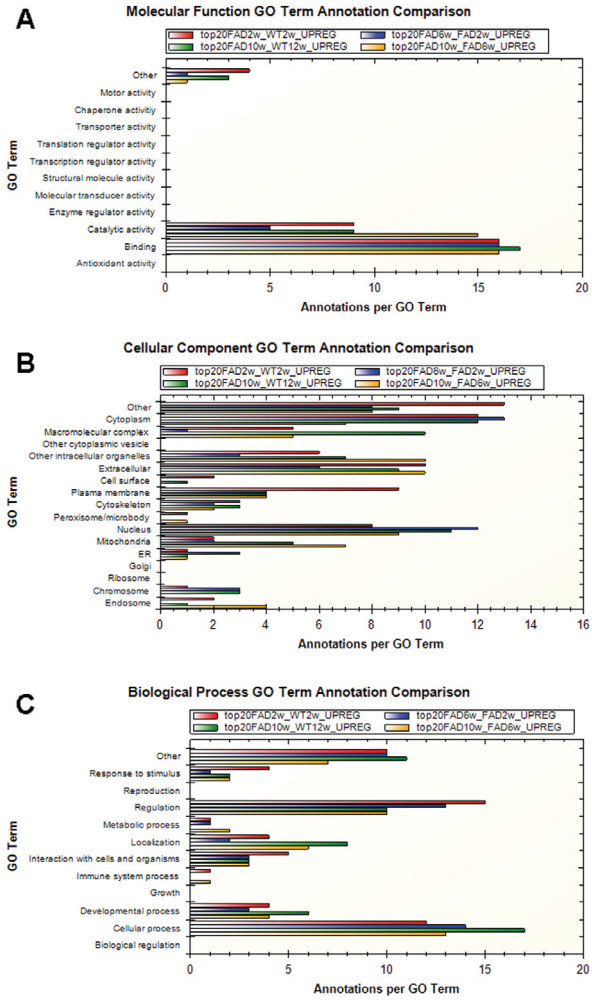

Figure 5. Gene Ontology Analysis. (**A**) Molecular function analysis on the top 20 upregulated proteins among different groups. (**B**) Cellular Component analysis on the top 20 upregulated proteins among different groups. (**C**) Biological Processes analysis on the top 20 upregulated proteins among different groups. Red, 5xFAD 2 weeks vs WT 2 weeks; Blue, 5xFAD 6 weeks vs 5xFAD 2 weeks; Green, 5xFAD 10 weeks vs WT 10 weeks; Yellow, 5xFAD 10 weeks vs 5xFAD 6 weeks. STRAP software was used to perform the GO analysis.

Microglial activation is one of the key events in AD progression. We evaluated the microglial activation based on gal3 expression, a marker of proinflammatory microglial activity[19], and we found a clear microglial activation in microglial cells at 10 weeks around Aβ plaques (Fig. 2G) in the subiculum and sparsely around APP neurons at 6 weeks. (Fig. 2J), before any plaque deposit can be found in the brain. The gal3 immunoreactivity at 6 weeks of age in the molecular layer of hippocampus is especially interesting due to the role of gal3 positive microglial cells in the inflammatory response. Previously, we have found a detrimental role of gal3 in microglial activation in model of α-synuclein-induced microglial activation related to Parkinson's disease pathogenesis[18]. In line with our inflammatory hypothesis, our present data demonstrate the presence of activated microglial cells in the hippocampus of 5xFAD in time points before plaque deposits.

Proteins	Name	Location
Ppp1r21	Protein phosphatase 1 regulatory subunit 21	2p16.3
Gtf3c5	General transcription factor IIIC subunit 5	9q34.13
Smchd1	Structural maintenance of chromosomes flexible hinge domain containing 1	18p11.32
Tdrd7	tudor domain containing 7	9q22.33
Sugp1	SURP and G-patch domain containing 1	19p13
Sh3glb1	SH3 domain containing GRB2 like, endophilin B1	1p22.3
Cherp	Calcium homeostasis endoplasmic reticulum protein	19p13.11
Fam129a	Family with sequence similarity 129 member A	1q25.3
Cenpv	Centromere protein V	17p11.2
Coasy	Coenzyme A synthase	17q21.2
Ndrg3	NDRG family member 3	20q11.23
Plek	pleckstrin	2p14
Prpf6	pre-mRNA processing factor 6	20q13.33
Fmnl3	formin like 3	12q13.12
Mad1l1	MAD1 mitotic arrest deficient like 1	7p22.3
Anxa7	annexin A7	10q22.2
Cmtr1	cap methyltransferase 1	6p21.2
Gvin1	GTPase, very large interferon inducible pseudogene 1	11p15.4
Parp2	Poly(ADP-ribose) polymerase 2	14q11.2
Gnl3	G protein nucleolar 3	3p21.1

Table 4. Gene Ontology Top 20 5xFAD 10 weeks upregulated proteins compared to 5xFAD 6 weeks.

Proteins	Name	Location
Mapk1	Mitogen-activated protein kinase 1	22q11.22
Cul3	cullin 3	2q36.2
Rragc	Ras related GTP binding C	1p34.3
Mfge8	Milk fat globule-EGF factor 8 protein	15q26.1
Acad8	Acyl-CoA dehydrogenase family member 8	11q25
Acads	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	12q24.31
Colgalt1	Collagen beta(1-O)galactosyltransferase 1	19p13.11
Dak	triokinase and FMN cyclase	11q12.2
Cobll1	Cordon-bleu WH2 repeat protein like 1	2q24.3
Uba5	Ubiquitin like modifier activating enzyme 5	3q22.1
Scamp2	Secretory carrier membrane protein 2	15q24.1
Rab5c	RAB5C, member RAS oncogene family	17q21.2
Mccc2	methylcrotonoyl-CoA carboxylase 2	5q13.2
Eci2	enoyl-CoA delta isomerase 2	6p25.2
Clybl	citrate lyase beta like	13q32.3
Ttr	transthyretin	18q12.1
Rab4a	RAB4A, member RAS oncogene family	1q42.13
Wdr7	WD repeat domain 7	18q21.31
Ppt1	palmitoyl-protein thioesterase 1	1p34.2
Clpp	caseinolytic mitochondrial matrix peptidase proteolytic subunit	19p13.3

Table 5. Gene Ontology Top 20 5xFAD 6 weeks upregulated proteins compared to 5xFAD 2 weeks.

Over the last decade, a large interest in the contribution of the innate immune system and its role in AD have been incurred. Especially, the microglial cells, have received a lot of attention. Understanding how the inflammatory response contributes to AD can be important to halt the progression of the disease. Hence, we aim to elucidate early inflammatory events related to Aβ immune reactions.

Here, we show an altered cytokine pattern in 5xFAD mice compared to control mice. In our study, protein levels of the cytokines IL10, IL12, IL1β and IL4 (Fig. 3) were altered in the early phase of the pathology in 5xFAD mice. When we further analysed cytokine levels, we found IL10 and IL1β to be significantly elevated in 5xFAD mice first at 10 weeks whereas IL12 showed a strong trend towards reduction starting at 6 weeks in 5xFAD mice compared to control animals, *i.e.* before the formation of plaque, albeit not significant. Interestingly, the anti-inflammatory cytokine IL-4 was overall reduced in the brain of 5xFAD mice at 2, 6 and 10 weeks of age.

Pathway 5xFAD 10w vs 6w	P-value	Adjusted p-value	Z-score	Combined score
Interferon-gamma signaling pathway	0.02764	0.1262	−1.45	3.01
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	0.02862	0.1262	−1.25	2.59
B cell activation	0.05551	0.1262	−1.15	2.38
Alzheimer disease-amyloid secretase pathway	0.05456	0.1262	−1.13	2.34
Ras Pathway	0.06682	0.1262	−1.05	2.18
T cell activation	0.07056	0.1262	−0.90	1.87
Interleukin signaling pathway	0.08261	0.1262	−0.65	1.34
Pathways 5xFAD 6w vs 2w	**P-value**	**Adjusted p-value**	**Z-score**	**Combined score**
FAS signaling pathway	0.03056	0.03056	−1.46	5.10

Table 6. Main innate-related pathways resulted from top 20 most upregulated proteins from the Gene Ontology.

IL10 is an anti-inflammatory cytokine known to cease a proinflammatory response[27], presenting a significant up regulation from 6 weeks (pre-plaque stage) to 10 weeks of age in the brains of 5xFAD mice (Fig. 3A), suggesting IL10 to be related to the pathology starting at the time/or due to plaque formation. The function of IL10 and its effects on AD pathogenesis has been studied recently. For instance, Guillot-Sestier and colleagues found IL10 deficiency to reduce the disease progression in APP-PS1 crossed with IL10 knockout mice[28]. They found a preserved synaptic integrity and altered regulation of innate immune genes with increased Aβ phagocytosis. Using a different approach, the role of IL10 was investigated and the outcome basically the same in a work where Chakrabarty *et al*., demonstrated how the up regulation of IL10, performed by the injection of an AAV2/1-*IL-10 expressing viral vector* increased the amyloid deposition and worsen the cognitive behaviour in an AD mouse model[29]. Taken together, these reports suggest IL10 to be involved in AD pathology, in line with our IL10 cytokine increase, at the time of plaque formation. The overall role of IL10 in the human brain for disease outcome and association to Aβ microgliosis is still unresolved.

Further, we found the pivotal proinflammatory cytokine IL1β to be elevated in the brains of 5xFAD mice at the time of plaque deposition at 10 weeks of age (Fig. 3B). IL1β has also been studied in experimental AD models, APPswe/PS-1dE9, where overexpression of IL1β resulted in altered plaque pathology[30]. IL1β has also been genetically related to AD, where polymorphism in the IL1β gene can be related to AD progression and sleep disorders[31]. Moreover, elevated levels of IL1β were found in the cerebrospinal fluid of AD patients indicating clinical relevance for mechanisms involving this cytokine in AD development.

Another cytokine that we found a genotype-related difference (overall effect, 2-way ANOVA) was IL12 (Fig. 3C). IL12 is suggested to be involved in the progression of AD pathology. In fact, IL12 inhibition reduces AD pathology in APP/PS1 mice by reducing plaque formation and cognitive deficits after intra-cerebroventricular injection of antibodies inhibiting IL12/IL23 signaling[32]. Clinically, elevated levels of IL12 subunit p40 has been found in CSF from AD patients[32]. However, a dual role of IL12 in the inflammatory response has been described, by regulation of IL10 by the activation of Th1 cells[33,34], showing the complex immune interplay involving IL12. Finally, we found one of the principal anti-inflammatory cytokine, IL4, to be reduced in 5xFAD mice compared to WT (Fig. 4D, overall effect, 2-way ANOVA). This downregulation of IL4 suggests that this cytokine is sensitive in early stages of the pathology. IL4 is considered an anti-inflammatory cytokine and may play a role in microglial activation[35].

To further elucidate the microglial phenotype, we isolated microglial cells from 5xFAD and WT brains and performed bioinformatic analysis of microglia protein profile generated by mass spectrometry. Mass spectrometry allowed us to precisely quantify and identify the proteins expressed in microglial cells from 5xFAD and WT mice. It is important to mention that at 10 weeks, the Aβ plaque is only present in the subiculum and it is only a very limited number of cells directly in contact with the plaques compared to the number of microglial cells isolated from the whole cortex and hippocampus (more than 250.000 microglial cells/brain analysed).

At week 2, we found processes linked to RNA transcription by RNA polymerase I and FAS signaling pathway affected (Table 1). FAS ligand signalling is an innate immune pathway related to apoptotic mechanisms and inflammatory response. For instance, the highlight of the apoptotic pathways can be linked to brain development connected to TNF-α expression[36].

Remarkably, at 6 weeks, we detected altered innate-immune related pathways in our microglial cells isolated from the 5xFAD mice (Table 2). These pathways were: JAK/STAT, p38 MAPK and Interleukin related pathways. All of them are directly involved in the inflammatory response elicited by microglial cells. For instance, p38 MAPK protein has been linked with microglial response by directly promoting the expression of inflammatory cytokine such as TNF-α, IL6 or IL-1β via TLR's ligands or in the presence of Aβ[37]. Moreover, p38 MAPK has been considered a suitable target for AD[38,39]. The role of interleukins in the inflammatory response has been widely studied and it's implication in AD progression discussed above[32,40–43]. Remarkably, at 10 weeks, we found synaptic vesicle trafficking as the most altered pathway in our analysis. We did not found the previous proinflammatory pathways up regulated among the main pathways affected at 10 weeks compared to the pathways described at 6 weeks. However, individual proinflammatory proteins found at 6 weeks ranked higher at 10 weeks

(see supplementary excel file). The prevalence of vesicles trafficking pathways over proinflammatory pathways at 10 weeks is likely related with the beginning of the plaque formation and deposition, as we found in the subiculum. Furthermore, the synaptic vesicle trafficking has been linked to the inflammatory response[23]. This data highlight the sensitivity of our approach, being able to address minor and important changes in the dynamic of the innate immune response. For instance, vesicles-like structures, *i.e*. exosomes and microvesicles are suggested to be involved in brain disorders such as Parkinson's disease disease and AD[23,44,45]. In line with the role of microvesicles in AD, a recent paper published by Verderio's lab, shown that microvesicles released by microglial cells are linked to Aβ aggregation and neurotoxicity[23].

To further analyse our proteomic data, we performed a GO analysis to establish the main molecular function, cellular component and biological processes affected. Regarding the molecular function, the most striking difference was found when comparing 5xFAD 6 and 2 weeks of age (Fig. 5A). The catalytic activity was upregulated at 6 weeks compared to 2 weeks 5xFAD mice. Among the proteins with a catalytic activity mainly upregulated in our analysis, we found protein phosphatase 1 regulatory subunit 21 (Ppp1r21) and GTPase protein, very large interferon inducible 1 (Gvin1) (Table 4). Phosphatases such as serine/threonine and tyrosine have been linked with AD pathology[46]. Gvin1, which is related with inflammatory response is upregulated in our 5xFAD in line with other studies[11].

Notably, the biological processes affected between 5xFAD at 10 and 6 weeks of age pointed out significant differences in the innate immune response (Fig. 5C). In fact, proteins such as Mapk1, Rragc and Dak were among the top 20 upregulated (Table 5). Mapk1, mitogen-activated protein kinase 1, is a protein belonging to the MAPK protein family. MAPK proteins have been widely studied and linked to microglial activation and IL1β production in AD[47]. For instance, microglial p38α MAPK is a regulator of proinflammatory cytokine expression induced by TLR ligands and Aβ[37].

The comparison from 5xFAD 6 weeks vs 2 weeks confirmed FAS ligand signalling pathway as the main up regulated pathway at 2 weeks of age (Table 5). Then, we made a comparison between 5xFAD at 10 and 6 weeks and we detected a number of different innate-immune related pathway affected such as: interferon gamma regulation, interleukins-related pathways and B and T cells activation pathways, which were upregulated in 5xFAD at 10 weeks compared to 6 weeks (Table 6). Our data demonstrate the implication of the innate immune response from 6 to 10 weeks based on the cytokine levels and especially due to the innate immune pathways upregulated at 6 weeks (MAPK p38 kinase, JAK-STAT or interleukin pathways) and 10 weeks (synaptic vesicles and metabolic alterations), as well as, microglial cells expressing gal3 around APP positive neurons in the hippocampus before plaque deposition. Future analysis of the proteomic of single cells, especially in relation to Aβ plaque, using CyTOF mass cytometry[48] or single cell RNAseq[49] can be useful to pin-point the detrimental signaling in microglia that contribute to the neuroinflammatory pathogenesis in AD.

Conclusion

In this study, we describe microglial changes in the very early phase of AD pathogenesis before plaque deposition in the brain of 5xFAD mice. We believe this approach can be useful to better understand the cellular pathways involved in the early disease progression, before any clinical manifestation of the disease. By performing proteomic analysis, and using bioinformation analysis to interpret the data, we were able to describe the dynamics of the inflammatory response from 2 to 10 weeks in 5xFAD AD mouse model. First, we detected important inflammatory pathways up regulated at 6 weeks (MAPK, JAK/STAT and Interleukin pathways) and then, at 10 weeks of age, we found a shift in the microglial profile with a predominant action of vesicle trafficking. Moreover, using ELISA we were able to validate our mass spectrometry data analysis and found innate immune alterations in important neuroinflammatory cytokines, IL10 and IL1β related to the time point of plaque deposition at 10 weeks. We believe that therapeutic approaches, where early microglial alteration can be targeted, are crucial in future pre-clinical AD trials.

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Author Contributions

A.B.S. and T.D. design the experiments and wrote the paper. A.B.S., Y.Y. and A.P. contributed to perform the experiments. A.B.S. and T.D. performed statistical analysis. A.B.S. perfomed the bioinformatic analysis along with Quantitative proteomic Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University.

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Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD

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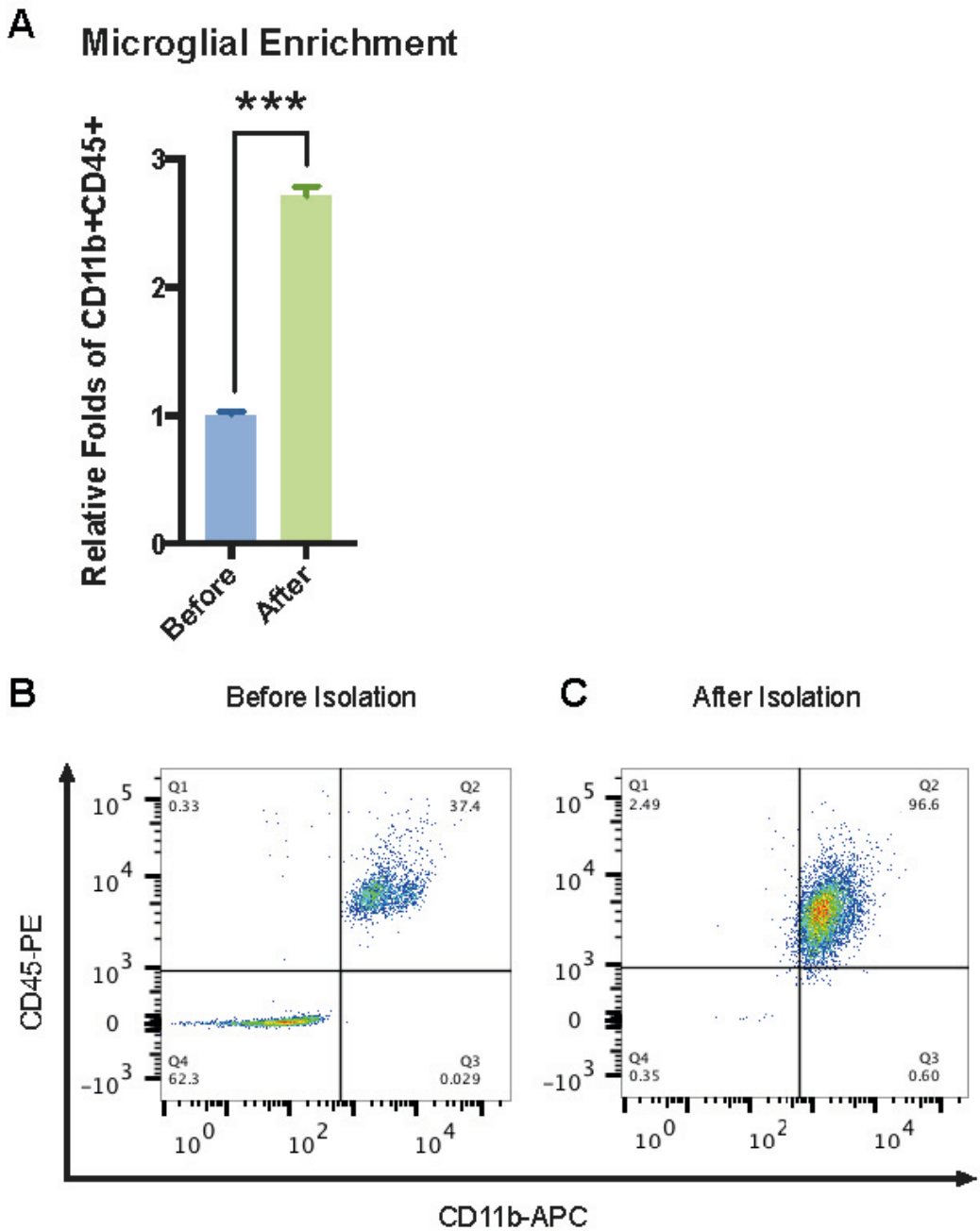
Supplementary Figure 1

Enrichment of microglia after CD11b microbeads isolation

A) Bars show a relative comparison of microglia that were $CD11b^+$ and $CD45^+$ before and after CD11b microbeads isolation. There is a significant enrichment of microglia population after the isolation compared to before (Paired t-test, $n = 4$, $P < 0.05$, error bars with mean ± SEM).

(B-C) Representative contour plots of CD11b microbeads isolated cells stained with anti-CD11b-APC and anti-CD45-PE antibodies. Microglia were identified by double positive for CD11b and CD45 staining. Only single and live cells were taken into consideration for the gating.


A
Microglial Enrichment
***
Relative Folds of CD11b+CD45+
3
2
1
0
Before
After
B
Before Isolation
C
After Isolation
CD45-PE
CD11b-APC
Q1
0.33
Q2
37.4
Q4
62.3
Q3
0.029
Q1
2.49
Q2
96.6
Q4
0.35
Q3
0.60