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Microglia-Regulated Activation of $CD4^+$ T Cells in the Development of Experimental Autoimmune Encephalomyelitis

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SUMMARY

Autoantigen-specific encephalitogenic T cells gaining access to the central nervous system (CNS) and leading to self-destructive inflammation are the critical driving force for multiple sclerosis (MS). It has been shown that peripherally primed T cells have to infiltrate into the CNS and to get reactivated by antigen-presenting cells (APCs) in order to induce inflammation in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. The contributions of macrophages and dendritic cells as APCs at the border between CNS and periphery were shown. However, antigen presentation in CNS remains largely undefined; especially the function of glia cells as APCs remains elusive.

Microglia are the only CNS-resident immune cells, thus their potential function in regulating T cell activation cannot be overlooked. In this study, we determined to understand the relationship between microglia and T cells. Live imaging revealed that naive microglia or LPS-stimulated microglia cannot induce the activation of NFAT-GFP-expressing MBP-specific T cells ($T_{MBP\text{-}NFAT\text{-}GFP}$ cells) and NFAT-GFP-expressing OVA-specific T cells ($T_{OVA\text{-}NFAT\text{-}GFP}$ cells), indicated by translocation of NFAT-GFP from cytosol into the nucleus. Interestingly, Interferon-γ (IFN-γ)-stimulated microglia significantly induced activation of both $T_{MBP\text{-}NFAT\text{-}GFP}$ cells and $T_{OVA\text{-}NFAT\text{-}GFP}$ cells even without the presence of cognate antigen, indicating antigen-independent T cell activation by microglia, which are regulated by IFN-γ. In addition, by comparing macrophages and microglia in the CNS of rats with EAE, we showed that macrophages were more efficient APCs than microglia. Interestingly, IFN-γ-stimulated microglia and microglia from EAE rats showed similar T cell stimulation capacity.

IFN-γ, a signature cytokine of T_H1 cells, was highly upregulated in both T_{MBP} cells and T_{OVA} cells after co-culturing with naive microglia even without the presence of cognate antigens, suggesting microglia regulate T cell activation antigen-independently. Furthermore, we evaluated different cytokine expression in T_{MBP} cells and T_{OVA} cells after co-culturing with microglia and found two innate cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), were highly upregulated. The induction of these cytokines required direct contact

SUMMARY

of T cells and microglia. Intriguingly, knockout TNF-α in T cells by using CRISPR/Cas gene editing showed that TNF signaling regulated IL-1β production. Moreover, IL-2R, OX-40 and major histocompatibility complex class II (MHCII) were highly upregulated on both T_{MBP} cells and T_{OVA} cells after co-culturing with microglia in absence of antigen, which further support the idea that microglia regulate T cell activation antigen-independently. Beside of microglia, we looked at whether astrocytes activate T cells as similar as microglia. However, astrocytes were not as strong as microglia in inducing T cell activation.

To characterize the transcriptome profile of microglia and macrophages at the early stage of EAE, we performed RNA-sequencing analysis. Compared to naive microglia, microglia from EAE rats showed strong enrichment of genes associated with IFN-γ signaling, suggesting that microglia are influenced by T_H1 cells. Moreover, microglia strongly upregulated chemokines such as *Cxcl9, Cxcl10, Cxcl11* and *Ccl5* which are important for T cell recruitment into the CNS. Although macrophages have a higher T cell stimulating capacity than microglia, at the very early stage of EAE, microglia are the main myeloid cells in the CNS that activate T cells before macrophages penetrate into the CNS.

In summary, our study showed that in addition to antigen-dependent manner, microglia can regulate T cell differentiation and activation antigen-independently. Although macrophages showed higher T cell activating capacity, their number is low at early stages of EAE. At that time point, microglia can stimulate T cells and induce production of inflammatory cytokines. In addition, as results of interaction with T cells, microglia produced chemokines, which could be important for the further recruitment of T cells and macrophages.

ZUSAMMENFASSUNG

Autoantigen-spezifische enzephalitogene T-Zellen, die in das Zentralnervensystem (ZNS) eindringen und eine selbstzerstörerische Entzündung auslösen, sind die treibende Kraft für Multiple Sklerose (MS). Mithilfe der experimentellen Autoimmunenzephalomyelitis (EAE), einem Tiermodell für MS, ist gezeigt worden, dass in der Peripherie stimulierte T-Zellen das ZNS infiltrieren und dort durch Antigen-präsentierende Zellen (APCs) reaktiviert werden müssen, um eine Entzündung auszulösen. Makrophagen und Dendritische Zellen sind an diesem Prozess an der Grenze zwischen ZNS und Peripherie beteiligt. Dennoch ist die Antigenpräsentation im ZNS noch weitestgehend unverstanden; im Besonderen die Funktion von Gliazellen als APCs ist noch ungeklärt. Da Mikroglia die einzigen residenten Immunzellen des ZNS sind, ist ihr möglicher Einfluss auf die T-Zell-Aktivierung im ZNS nicht zu vernachlässigen.

Das Ziel der vorliegenden Arbeit war, mithilfe des EAE-Modells in der Ratte die Beziehung zwischen Mikroglia und T-Zellen zu verstehen. Echtzeitmikroskopie von co-kultivierten T-Zellen und Mikroglia zeigte, dass weder naive noch mit LPS stimulierte Mikroglia $T_{MBP\text{-}NFAT\text{-}GFP}$- oder $T_{OVA\text{-}NFAT\text{-}GFP}$-Zell-Aktivierung, gekennzeichnet durch Translokation von NFAT-GFP in den Nukleus, induzieren können. Interessanterweise waren mit Interferon-γ (IFN-γ) stimulierte Mikroglia signifikant besser darin, sowohl $T_{MBP\text{-}NFAT\text{-}GFP}$-Zellen als auch $T_{OVA\text{-}NFAT\text{-}GFP}$-Zellen zu aktivieren, sogar in Abwesenheit von entsprechendem Antigen. Die Ergebnisse deuten auf einen Antigen-unabhängigen Mechanismus der T-Zell-Aktivierung durch IFN-γ-stimulierte Mikroglia hin. Desweiteren zeigte sich im Vergleich von Makrophagen und Mikroglia aus dem ZNS von Ratten mit EAE, dass Makrophagen effizientere APCs sind. Die Kapazität von Mikroglia von Ratten mit EAE, T-Zellen zu aktivieren, ähnelte der Aktivierungskapazität von IFN-γ-stimulierten Mikroglia aus naiven Tieren. Die Expression von IFN-γ, einem charakteristischen Zytokin der T_H1-Zellen, wurde sowohl in MBP- als auch in OVA-spezifischen T-Zellen durch die Co-Kultur mit naiven Mikroglia signifikant hochreguliert, selbst in Abwesenheit der entsprechenden Antigene. Das deutet stark darauf

hin, dass Mikroglia die T-Zell-Differenzierung durch einen antigen-unabhängigen Mechanismus regulieren. Die Messung weiterer Zytokine ergab eine Hochregulierung von zwei Zytokinen, Tumornekrosefaktor α (TNF-α) und Interleukin-1β (IL-1β), sowohl in T_{MBP}- als auch T_{OVA}-Zellen als Resultat aus der Co-Kultur mit Mikroglia. Die Induktion der Zytokine erforderte einen direkten Zell-Zell-Kontakt. Der Knockout von *Tnf* in T-Zellen mithilfe der CRISPR/Cas-Technologie zeigte, das die Expression von IL-1β durch TNF reguliert wird. Außerdem wurden IL-2R, OX-40 und MHCII sowohl in MBP- als auch OVA-spezifischen T-Zellen durch Co-Kultur mit Mikroglia in Abwesenheit von Antigen hochreguliert, wodurch die Theorie einer antigen-unabhängigen T-Zell-Aktivierung durch Mikroglia bestärkt wurde. Neben Mikroglia wurden auch Astrozyten auf ihre Fähigkeit, T-Zellen zu aktivieren, untersucht und als weniger potent identifiziert.

Durch Transkriptomanalysen wurden die Expressionsprofile von Mikroglia und Makrophagen im Anfangsstadium der EAE charakterisiert. Im Vergleich zu naiven Mikroglia exprimieren Mikroglia aus Ratten mit beginnender EAE in erhöhtem Maße Gene, die mit der Antwort auf IFN-γ assoziiert sind, was auf eine Kommunikation von Th1-Zellen mit Mikroglia hindeutet. Außerdem exprimieren Mikroglia aus Ratten mit EAE höhere Level verschiedener Chemokine, z.B. *Cxcl9*, *Cxcl10*, *Cxcl11* und *Ccl5*, die für die Rekrutierung von T-Zellen in das ZNS wichtig sind. Obwohl Makrophagen effizienter darin sind, T-Zellen zu stimulieren, sind Mikroglia zunächst die einzigen myeloiden Zellen im ZNS, die T-Zellen aktivieren können, bevor Makrophagen infiltrieren.

Diese Studie konnte zeigen, dass Mikroglia neben Antigen-abhängigen Prozessen auch Antigen-unabhängige Mechanismen nutzen, um die T-Zell-Differenzierung und –Aktivierung zu regulieren. Makrophagen haben zwar eine höhere Kapazität, T-Zellen zu aktivieren, infiltrieren das ZNS jedoch zu einem späteren Zeitpunkt als die ersten T-Zellen. Zu Beginn der EAE sind also nur Mikroglia anwesend, die die T-Zellen stimulieren und die Produktion proinflammatorischer Zytokine induzieren. Außerdem produzieren Mikroglia Chemokine, die für die Rekrutierung weiterer T-Zellen und Makrophagen wichtig sein könnten.

ABBREVIATIONS

APC	Antigen-presenting cell
BAMs	Border-associated macrophages
BBB	Blood-brain barrier
BMDCs	Bone marrow-derived DCs
Ca^{2+}	Calcium ion
cDCs	Conventional DCs
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CP	Choroid plexus
CSF	Cerebrospinal fluid
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
HLA	Human leucocyte antigen
i.p.	Intraperitoneal
i.v.	Intravenous
IB4	Isolectin GS-IB4
IFNs	Interferons
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
InsP3	Inositol-1,4,5-trisphosphate
IP-10	Interferon-γ-inducible protein of 10 kDa
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharides

ABBREVIATIONS

MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MHCII	Major histocompatibility complex class II
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MSigDB	Molecular Signature Database
NF-κB	Nuclear factor-κB
NFAT	Nuclear factor of activated T cells
OPCs	Oligodendrocyte precursor cells
OVA	Ovalbumin
PBS	Phosphate buffered saline
PLL	Poly-L-lysine hydrobromide
PLP	Myelin proteolipid protein
PMA	Phorbol 12-myristate 13-acetate
PPMS	Primary progressive MS
PS	Penicillin-Steptomycin
RNA-seq	RNA-sequencing
RRMS	Relapsing remitting MS
SPMS	Secondary progressive MS
TCR	T cells receptor
tEAE	Transfer EAE
TGF-β	Transforming growth factor β
T_H	T helper
TLRs	Toll-like receptors
TNFR	TNF receptor
TNF-α	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
VCAM-1	Vascular cell adhesion protein 1
VLA-4	Very-late antigen

1 INTRODUCTION

1.1 Multiple Sclerosis: the autoimmune disorder

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) with progressive demyelination and neurodegeneration. MS is one of the major causes of neurological disability, which affects more than two million individuals worldwide (Filippi, Bar-Or et al. 2018). Three different categories of MS have been defined in order to standardize the terminology used for describing the disease patterns of MS since Charcot firstly named MS in 1868, which include relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (Lublin and Reingold 1996, Lublin, Reingold et al. 2014). Patients with RRMS are clinically characterized by self-limited episodes of acute neurologic dysfunctions, such as sensory disturbances and optic neuritis (Baecher-Allan, Kaskow et al. 2018). However, if the disease of RRMS patients gets progressively worse, it leads to SPMS. In PPMS, the neurologic dysfunctions of MS patients get progressively worse from onset without distinct relapses (Lublin and Reingold 1996).

Although previous results showed that the risk for developing MS is partly determined by genetic factors such as variation at the loci of human leukocyte antigen (HLA) genes and interleukin-2 (IL-2) receptor alpha genes (Moutsianas, Jostins et al. 2015, Parnell and Booth 2017), and environmental factors such as smoking, lack of vitamin D and Epstein-Barr virus infections (Ascherio and Munger 2007), the exact cause for MS is unknown. The pathological process underlying MS includes breakdown of blood-brain barrier (BBB), mononuclear cell infiltration and following inflammation in the CNS, demyelination, axonal degeneration, oligodendrocyte loss, and reactive gliosis (Trapp and Nave 2008). However, the pathogenic mechanisms behind the progressive MS are still incompletely understood. The traditional view is that adaptive immune response plays a pivotal role in the pathogenesis of MS. It is suggested that myelin-specific autoreactive lymphocytes, mostly T helper (T_H) cells, are primed in the periphery and activated in the CNS after crossing the BBB which further leads to the formation of local inflammatory demyelinating lesions in

CNS (Stys, Zamponi et al. 2012, Ciccarelli, Barkhof et al. 2014). Indeed, a genome-wide association study on a big cohort of MS patients demonstrated that the majority of risk genes related to the disease are associated with T_H cells (Cotsapas and Mitrovic 2018). In contrast to $CD8^+$ T cells, T_H cells are unlikely to kill oligodendroglia directly or cause axonal damage autonomously. Instead, after breakthrough of the BBB, T_H cells secrete communicator cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) to recruit myeloid cells which cause the formation of the lesion in the CNS (Codarri, Greter et al. 2013). The dominant presence of myeloid cells in inflammatory lesions in both MS and experimental autoimmune encephalomyelitis (EAE) has been shown critical for the disease development (Huitinga, van Rooijen et al. 1990, Tran, Hoekstra et al. 1998, Trebst, Sorensen et al. 2001).

The active inflammatory demyelinating lesions in the CNS, which are populated by macrophages and microglia, are mainly seen in RRMS patients (Trapp, Bo et al. 1999). Both gray matter and white matter can be affected (Kutzelnigg and Lassmann 2005). Activated microglia are dominantly present at the edge of actively demyelinating lesions as well as in periplaque areas. The cluster of activated microglia in periplaque regions is frequently observed, where they partially surround myelin sheaths and degenerating axons. In addition, the engagement of microglia together with the complement component C3d in disrupted myelin segments has been proposed as the source of MS antigens (Prineas, Kwon et al. 2001). However, the majority of phagocytic cells presented in the demyelinating center of active lesions are macrophages (Lassmann 2018). Due to limited availability of sophisticated methods to distinguish between infiltrated macrophages and microglia, the exact function played by each cell type remains to be further defined. In general, in active MS lesions, phagocytes produce high amounts of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), chemokines and vasoactive substances to accelerate the progress of inflammation (Codarri, Greter et al. 2013). In addition, phagocytes can produce neurotoxic oxidation products such as reactive oxygen species and glutamate, which potentially contribute to demyelination and axon damage (Nikic, Merkler et al. 2011). In contrast to RRMS patients, the active inflammatory lesions are rarely observed in progressive MS patients, in which the subset of lesions varies among cases (Mahad, Trapp

et al. 2015). One characteristic pathological feature of progressive MS is diffuse injury, such as atrophy, microglia activation and axonal injury, in normal-appearing gray and white matter (Kutzelnigg, Lucchinetti et al. 2005). Due to a lack of suitable animal models for progressive MS and limited mechanistic understanding of it, the majority of current therapeutic methods are beneficial for RRMS patients not for patients with progressive MS (Mahad, Trapp et al. 2015).

1.2 Experimental Autoimmune Encephalomyelitis

It has been known that only humans can develop MS (Bove 2018), thereby no spontaneously developed diseases in other species resemble MS sufficiently to help in understanding such a disorder. However, CNS inflammation and neurodegeneration which resembles MS can be induced in animals. EAE has been most widely used as a model for MS in the laboratory. EAE was discovered by an accidental event, that patients who got rabies virus vaccine developed neuroparalytic complications. The inoculum for the rabies virus vaccination was prepared from rabbit spinal cord, which was contaminated with neural elements. Back then, it was not clear whether the rabies virus or the neural elements caused the neurological dysfunction. The issue was addressed later on after administrating homogenates containing neural elements similar to rabies vaccination into nonhuman primates. Such immunizations can produce acute encephalitis that resembles MS (Croxford, Kurschus et al. 2011, Ransohoff 2012). Since then, MS studies performed on EAE have been proceeded massively.

EAE can be induced in many species and strains by various ways. Immunization of rodents with myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or myelin proteolipid protein (PLP) emulsified in complete Freund's adjuvant (CFA) have been used to induce active EAE (Stromnes and Goverman 2006). By contrast, passive EAE can be induced by transferring autoantigen-reactive $CD4^+$ T cells, such as MBP-specific $CD4^+$ T cells (Ben-Nun, Wekerle et al. 1981). In the adoptive transfer EAE (tEAE), pathogenic $CD4^+$ T cells are the driving force for the disease progression. Manipulating the transcriptome of T cells *in vitro* before transfer enables investigators to characterize the function of certain cytokines

and biological factors in EAE (Constantinescu, Farooqi et al. 2011). The major common feature between active EAE and MS is the formation of demyelination, whereas passive EAE reproduces the neuroinflammatory features of MS patients including macrophage accumulation and $CD4^+$ T cell infiltration (Lassmann and Bradl 2017). The main difference between EAE and MS is that EAE requires an external trigger, such as immunization with myelin antigen or transfer of encephalitogenic T cells, to initiate the disease (Ransohoff 2012). In addition, in EAE the neuroinflammation mainly occurs in spinal cord, whereas in MS the neuroinflammation prominently present in cerebellar and cerebral cortex (Ransohoff 2012). Importantly, none of the EAE models can perfectly mimic all the courses of MS, which limits their usage in revealing the whole picture of the pathogenesis of MS. Nevertheless, it is a useful tool for developing preclinical therapies for MS by selecting subtypes of EAE since each EAE model reflects a part of the MS pathology. For example, PLP-induced active EAE in SJL/J mice shows a relapsing-remitting disease course (Mattner, Staykova et al. 2013). Indeed, studies performed in EAE have successfully helped to develop neurobiologically based therapies, such as Lamotrigine, or immunologically based therapies, such as Natalizumab (Constantinescu, Farooqi et al. 2011).

1.2.1 Transfer EAE in Lewis rats

Rats are commonly used laboratory animals for studying MS, among which Lewis rats were reported to be highly responsive to MBP immunization (Kibler, Fritz et al. 1977, Wekerle, Kojima et al. 1994). Adoptive transfer of MBP-specific T cells into Lewis rats induces clinical EAE. The rats show paralysis within 2-3 days, together with body weight loss. The disease reaches its peak 4-5 days after T cell transfer followed by recovery within 10 days (Fig. 1.2.1) (Ben-Nun, Wekerle et al. 1981, Flugel, Willem et al. 1999, Bartholomaus, Kawakami et al. 2009). The biggest advantages of this adoptive tEAE model lie in its high reproducibility, predictability and early onset.

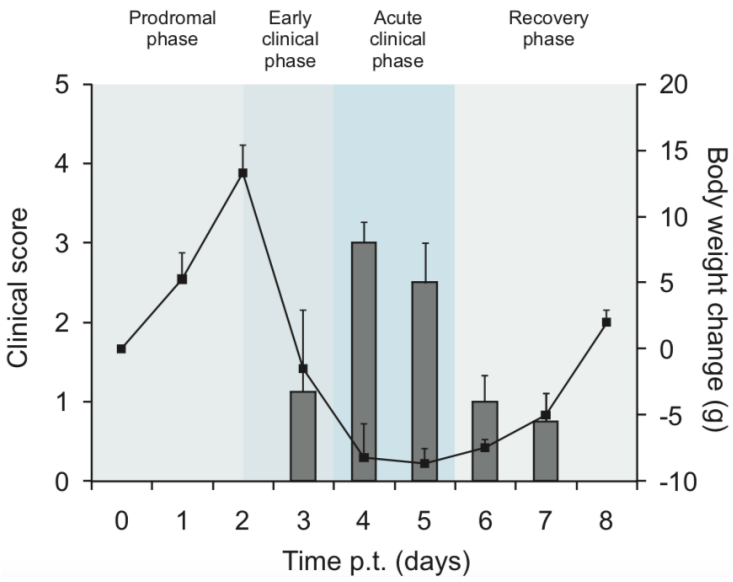

Fig. 1.2.1| Clinical score (bars) and body weight (line) after adoptive transfer of MBP-specific T cells by intravenous (i.v.) injection (Adopted from (Bartholomaus, Kawakami et al. 2009). License ID: 4997100447269).

The clinical process of this adoptive tEAE includes acute onset and spontaneous recovery, which enables to mimic the relapse of clinical symptoms in MS (Croxford, Kurschus et al. 2011). However, during the EAE process, the neuropathological lesions mainly exist in spinal cord and brain stem and are characterized by immune cell infiltration including $CD4^+$ T cells, macrophages, B cells and natural killer cells with little or no demyelination which limits its role in investigating myelin damage and remyelination (Schluesener, Sobel et al. 1987, Flugel, Berkowicz et al. 2001, Kapadia and Sakic 2011). Nevertheless, it has been successfully used for validating therapeutic compounds, such as Fingolimod (Fujino, Funeshima et al. 2003), and a very useful tool for investigating T cells' migrating behavior and trafficking path from periphery to CNS, their phenotype changes and the mechanism of acute inflammation formation in the CNS (Odoardi, Sie et al. 2012, Schlager, Korner et al. 2016).

1.3 Innate and adaptive immunity in the CNS

The immune system has long been viewed as body defense mechanism against pathogens whose primary function is to recognize self and non-self and thus respond accordingly. From the immunological point of view, the CNS is both structurally and functionally unique and

has been considered as immune-privileged organ. The main arguments in favor for this are that the BBB restricts pathogens and immune cells from entering the CNS and the lack of lymphatic drainage in the CNS parenchyma (Louveau, Herz et al. 2018). However, in the early 1990s, Matzinger proposed that the immune system does not only respond to the signals from pathogens outside of the body but also to the signals released from inside of damaged tissues, even under sterile injuries (Matzinger 1994). Of note, some researchers demonstrated that the immune response to CNS antigens does exist (Medawar 1948, Hasek, Chutna et al. 1977). More importantly, experimental evidence showed that proteins and tracers injected into CNS parenchyma can be found in the peripheral lymph nodes (Cserr, Harling-Berg et al. 1992, Kida, Pantazis et al. 1993, Louveau, Smirnov et al. 2015). *In vivo* two-photon imaging showed that intraparenchymal extracellular proteins exit the parenchyma through perivascular efflux into the cerebrospinal fluid (CSF) (Iliff, Wang et al. 2012), while the CSF was found to exit the CNS via arachnoid granulations into the sinus lumen (Go, Houthoff et al. 1986). Those studies suggested the existence of routes that permit the CNS to communicate with the peripheral immune system.

Recent studies showed that the CNS is not an immune-free organ. Single cell analyses revealed that a complex and abundant immune cell landscape exists in CNS border interfaces, which includes dendritic cells (DCs), border-associated macrophages (BAMs), innate lymphoid cells, monocytes, T cells and granulocytes (Goldmann, Wieghofer et al. 2016, Mrdjen, Pavlovic et al. 2018). Even though the immune cells under homeostatic conditions have limited access to the CNS parenchyma, the constant patrolling of those cells at the border is playing a pivotal role in providing immune surveillance and affects the CNS function (Kipnis 2016). However, when the CNS undergoes acute injury such as contusive spinal cord injury or is exposed to inflammatory factors such as nitric oxide or TNF caused by systemic inflammation, the homeostasis between CNS and periphery will be disturbed (Carson, Doose et al. 2006, Hazeldine, Lord et al. 2015) followed by BBB disruption. Once the integrity of the BBB is compromised, T cells gain access to the CNS parenchyma. The entry of activated encephalitogenic T cells could lead to the production of inflammatory cytokines, further disruption of the BBB and formation of MS plaques (Cross, Cannella et al. 1990, Engelhardt 2006).

1.3.1 T cells in MS/EAE

Encephalitogenic T cells that have lost the tolerance to CNS-specific antigen, and hence induce an immune response in the CNS as a driver of MS have been tremendously discussed. $CD4^+$ and $CD8^+$ T cells are found within the CNS lesions as well as in the CSF of MS patients (Traugott, Reinherz et al. 1983, Goverman, Hunkapiller et al. 1986). The involvement of $CD4^+$ T cells in MS progress is consistent with the genetic risk factors related to HLA class II molecules (Cotsapas and Mitrovic 2018). Uncontrolled autoimmune T cell responses may cause destructive outcomes. Therefore, the regulation of immune responses is decisively important to limit immune cell induced damage. Three steps are involved in initiating T cell-regulated neuroinflammation: (i) recognition of antigen and activation of T cells in the periphery; (ii) infiltration into the CNS; and (iii) inflammatory cascade initiation and recruitment of other leukocytes (Codarri, Greter et al. 2013).

It is not yet conclusive how neuroantigen-reactive $CD4^+$ T cells get activated and experienced with CNS antigens in the periphery. One possible explanation is that $CD4^+$ T cell activation is triggered while the body mounts an immune defense against pathogens, such as human herpesvirus and Epstein-Barr virus, which may have a structural similarity to neuronal antigens (Challoner, Smith et al. 1995, Wandinger, Jabs et al. 2000). Secondary lymphoid organs have been considered as the places where T_H cells encounter cognate antigens presented by antigen-presenting cells (APCs). Additionally, the T cell activation may occur in gut-associated lymphoid tissue in microbiota dependent manner (Berer, Mues et al. 2011). Upon activation, T_H cells could obtain the ability to cross the BBB (Codarri, Greter et al. 2013).

Under homeostatic conditions, T cells enter the CNS from circulatory system via three vascular routes, which are the capillary of parenchyma venules, vessels of choroid plexus (CP), and the leptomeninges (Mundt, Greter et al. 2019). However, without recognizing the cognate antigens, those T cells will undergo apoptosis or return to periphery through the lymphatics in meninges (Louveau, Smirnov et al. 2015, Louveau, Herz et al. 2018). The entry of T cells into CNS via leptomeninges has been shown relevance to EAE initiation. For example, intravital imaging of T cells in Lewis rats showed that the pathogenic effector T

cells can infiltrate the CNS via leptomeningeal instead of CP vessels in a very-late antigen (VLA)-4 and vascular cell adhesion protein 1 (VCAM-1) dependent manner (Vajkoczy, Laschinger et al. 2001, Bartholomaus, Kawakami et al. 2009, Schlager, Korner et al. 2016, Kyratsous, Bauer et al. 2017). In addition, the β-synuclein-specific T cells, which were found to induce autoimmune lesions in cortex, penetrate into the CNS by using this leptomeningeal route (Lodygin, Hermann et al. 2019). It has been proposed that the molecular mechanism behind T cell infiltration is depending on the nature of the lymphocyte (Engelhardt and Ransohoff 2012). For instance, in spinal cord microvessels of mice, interferon-γ (IFN-γ) producing T_H1 cells cross the BBB in a VLA-4 dependent way (Vajkoczy, Laschinger et al. 2001, Glatigny, Duhen et al. 2011, Rothhammer, Heink et al. 2011), while in the CP vessels IL-17-producing T_H17 cells enter the CNS in a lymphocyte function-associated antigen 1 (LFA-1) dependent manner (Reboldi, Coisne et al. 2009, Rothhammer, Heink et al. 2011).

Once neuroantigen-reactive T_H cells pass through the BBB, they may encounter their cognate antigen presented by local APCs, which further starts a process of immune cell activation and following inflammation. Depending on the subsets of T_H cells, the pathological characteristics may vary. It was considered that T_H1 cells, which predominantly secrete IFN-γ, TNF-α and IL-2, are pathogenic and responsible for the inflammation in MS (Coffman 2006, Gutcher and Becher 2007) given that T_H1 produced cytokines are present in the lesions of EAE and correlate to the severity of the disease (Merrill, Kono et al. 1992). This is further supported by a study using mouse EAE which showed silencing T_H1 cell polarizing cytokine IL-18 and IL-12 protected the mice from developing EAE (Leonard, Waldburger et al. 1995, Shi, Takeda et al. 2000). Moreover, adoptive transfer of T_H1 cells is sufficient to induce EAE (Domingues, Mues et al. 2010). Over decades, T_H1 cells have been considered as the main pathogenic T_H subtype, until paradoxical observation of IFN-γ function in EAE development steered the investigators' attention to another type of T_H cells, T_H17 cells. T_H17 cells are a distinct T_H subset and were firstly described by Langrish et al. after observing that IL-23 promotes an expansion of $CD4^+$ T cells which carries pathogenicity characterized by IL-17A, IL-17F, IL-21 and IL-22 production (Langrish, Chen et al. 2005, Korn, Bettelli et al. 2009). Although IL-23 knockout mice are resistant to EAE, none of these T_H17

signature cytokines alone is indispensable for the development of EAE (Kreymborg, Etzensperger et al. 2007, Sonderegger, Kisielow et al. 2008, Haak, Croxford et al. 2009). Nowadays it is accepted that multiple pathways participate in the development of neuroinflammation. Both T_H1 and T_H17 cells are able to initiate the encephalomyelitis with different symptoms depending on the location of lesion (Kroenke, Carlson et al. 2008, Stromnes, Cerretti et al. 2008). Nevertheless, in order to induce EAE, peripherally activated or *in vitro* polarized T cells have to be reactivated in the CNS. Since T cell activation related genes such as *Ifng*, *Tnf* and *Csf2* are dramatically upregulated in CNS parenchyma compared to those in blood, leptomeninges and CSF, discovering the mechanisms that are responsible for T cell reactivation in CNS parenchyma becomes critical (Schlager, Korner et al. 2016).

1.3.2 Antigen-presenting cells of the CNS

Beside of the fact that T_H cells have been accepted as key players in MS, results from EAE suggested that APCs are also critical for initiating and progressing the autoimmune responses (Mundt, Mrdjen et al. 2019). APCs can deliver three signals to T cells during immune reaction, which are important for antigen-specific T cell activation (Fig. 1.3.1). The first signal is that APCs present antigens through major histocompatibility complex class II (MHCII) to the T cell receptor (TCR) on T_H cells to facilitate their recognition of cognate antigens; the second signal is that APCs promote T cell activation and expansion through engagement of costimulatory molecules on APCs, such as CD86 and CD80, with CD28 on T cells; and the third signal is cytokines, such as IL-18 and IL-12, secreted by APCs that direct T cells to further differentiate into effector T subsets. These pathways work together and activate antigen-specific T cells to differentiate into effector T cells (Gutcher and Becher 2007).

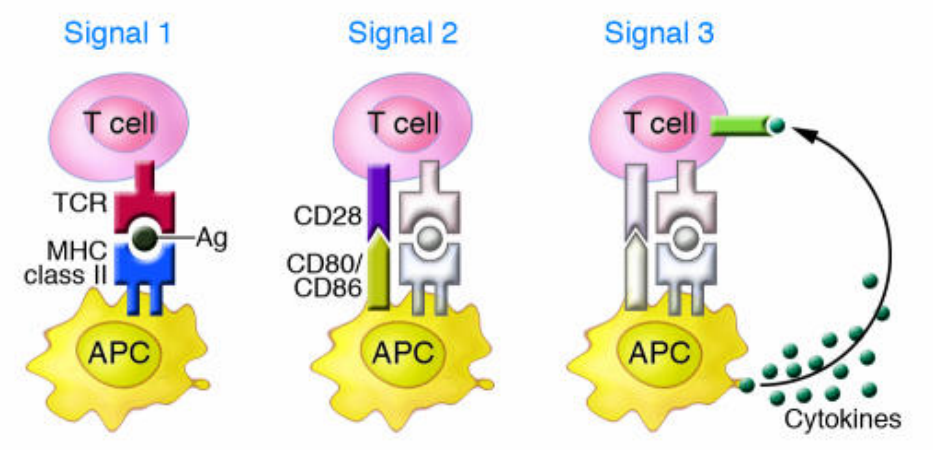

Fig. 1.3.1| Three signals delivered by APCs to activate T cells. Signal 1: APCs present antigen peptide to TCR via MHCII. Signal 2: costimulatory molecules CD86/CD80 on APCs interact with CD28 on T cells. Signal 3: cytokines produced by APCs polarize T cells to an effector subset. (Adopted from (Gutcher and Becher 2007). License ID: 1093573-1).

Many studies have been focused on identifying the APCs that associate with infiltrated T_H cells (Mundt, Greter et al. 2019), however the essential APCs in the CNS remain elusive. Among glia cells, oligodendrocytes do not express MHCII, therefore, are not able to present antigens to T cells. Although the oligodendrocyte precursor cells (OPCs) can upregulate the MHCII by IFN-γ (Kirby, Jin et al. 2019), whether OPCs activate $CD4^+$ T cells as APCs during EAE remains unclear. Other cells, such as astrocytes, neurons, endothelial cells, pericytes are speculated as potential nonimmune APCs given their ability in T cell reactivation. However, under homeostasis in the CNS, none of these cells express MHCII and their roles in the context of neuroinflammation are inconsistent (Becher, Bechmann et al. 2006, Waisman and Johann 2018, Mundt, Mrdjen et al. 2019).

Some researchers speculated that the encounter of T cells to antigens occurs at the border regions of the CNS with the help of MHCII expressing professional APCs. As described above regarding the immune cell landscape at the CNS borders (section 1.3), DCs and BAMs are potential APCs. However, single cell analysis showed that BAMs as well as microglia do not express MHCII under homeostatic conditions (Van Hove, Martens et al. 2019). Although mature macrophages express molecules required for antigen presentation, such as *Cd74*, *H2-Aa* and *H2-Ab1* encoded MHC II related proteins, and can interact with T cells,

conditional depletion of MHCII in CX3CR1^{+} macrophages showed no effects on EAE pathogenesis (Jordao, Sankowski et al. 2019, Mundt, Mrdjen et al. 2019). Therefore, they appear to have a dispensable function in antigen presentation and instructing T cells' entry to the CNS. One study determined that the MHCII expressing DCs are the principle APCs in the CNS interface, and CD11c^{+} DCs alone are enough to prime neuroantigen-reactive T_H cells to enable their invasion into CNS (Greter, Heppner et al. 2005). In addition, specific depletion of MHCII in conventional DCs (cDCs) prevented the development of autoimmune neuroinflammation in the *Zbtb46*^{Cre} *Iab*$^{fl/fl}$ mouse induced by myelin oligodendrocyte glycoprotein 35-55 epitope (MOG35-55) (Mundt, Mrdjen et al. 2019). Therefore, the cDCs, instead of BAMs, seem to be the principle cell type for antigen presentation and T cell reactivation in CNS interfaces. However, it is still a debate as to whether DCs participate in reactivating encephalitogenic T cells in the CNS, given that during neuroinflammation the complex myeloid landscape has led to confounding results in the identification of specific cell types, for example during neuroinflammation microglia can upregulate CD11c (Jordao, Sankowski et al. 2019).

1.3.3 Microglia in MS/EAE

Microglia are the only innate immune cells in CNS parenchyma under steady state conditions and constitute 5-10% of all brain cells (Aguzzi, Barres et al. 2013). It has been considered that microglia play a key role in maintaining the homeostasis of CNS by constantly surveying the microenvironment via dynamically retracting and extending the highly ramified processes (Nimmerjahn, Kirchhoff et al. 2005). Therefore, microglia can detect the subtle changes and thereby respond accordingly through a variety of cell surface receptors, such as fractalkine- and purino-receptors, Toll-like receptors (TLRs), phosphatidylserine and lipid receptors and triggering receptor expressed on myeloid cells 2 (TREM2) (Arcuri, Mecca et al. 2017). Activated microglia exert neuroprotective as well as neurotoxic functions in the CNS. Microglia exhibit neuroprotective roles by removing neuronal cell bodies, producing anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and IL-10 (Wolf, Boddeke et al. 2017). Unhinged activated microglia produce pro-inflammatory mediators such as TNF-α, IL-6, and IL-1β which contribute

essentially to brain disorders such as Parkinson's disease, Alzheimer's disease, traumatic brain injury, and the neuroinflammatory disease MS (Wolf, Boddeke et al. 2017).

Tremendous attention has been paid to discovering the role of microglia in EAE development, however with limited progress partly due to the challenges in distinguishing microglia from other myeloid cells. Fate mapping and single cell RNA-sequencing (RNA-seq) revealed that microglia have the same origin as meningeal and perivascular macrophages which is yolk sac progenitor cells. By contrast, all choroid plexus macrophages originate from embryonic progenitor cells (Ginhoux, Greter et al. 2010, Goldmann, Wieghofer et al. 2016, Prinz, Erny et al. 2017). Some studies used the combination of myeloablation and parabiosis to replace the circulating progenitors without disturbing CNS-resident microglia found that infiltrated monocytes do not contribute to the resident microglia pool (Ajami, Bennett et al. 2011), suggesting that microglia remain unique in their transcriptional and functional identity. The most commonly used marker for distinguishing microglia in the CNS parenchyma from the infiltrated monocytes are based on the expression of CD11b and CD45, that $CD11b^{+}CD45^{high}$ indicates monocytes and $CD11b^{+}CD45^{low}$ indicates microglia. However, due to lack of sophisticated methods to distinguish resident microglia from infiltrated macrophages *in vivo* and difficulty in culturing primary microglia, the early studies showed inconsistent results about their role in EAE. For example, Williams et al.'s *in vitro* study showed that microglia from allogeneic donors are capable of inducing T cell proliferation (Williams, Ulvestad et al. 1993), whereas Carson et al. showed that microglia cannot induce T cell proliferation (Carson, Sutcliffe et al. 1999).

Since microglia can uptake antigens, it can be speculated that they function as APCs. Therefore, other efforts have been devoted to discovering the antigen presentation ability of microglia, given that in some rodents like Lewis rats, up to 5% microglia express MHCII constitutively in normal CNS (Ford, Goodsall et al. 1995). In addition, microglia showed upregulated expression of MHCII and costimulatory molecules CD80, CD86 and CD40 in MS and EAE lesions (Fig. 1.3.2) (Dong and Yong 2019). Ford et al. firstly investigated the antigen presentation ability in microglia comparing to other CNS macrophages in EAE. In this study, Lewis rats got a lethal dose of irradiation before injection of congenic bone marrow cells to

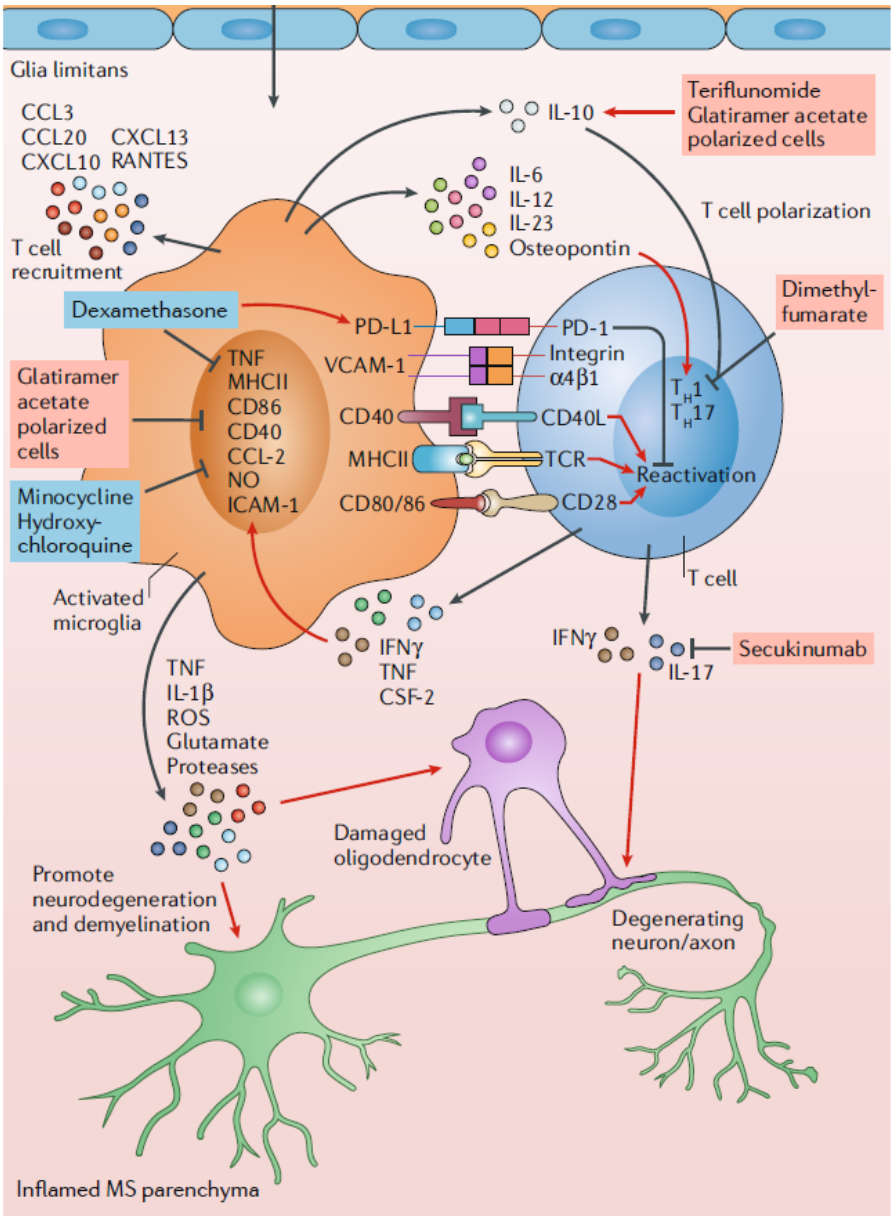

Fig. 1.3.2| Microglia interact with T cells in EAE and MS CNS parenchyma. Activated T cells infiltrated into perivascular compartment by using VCAM-1 and integrin α4β1. After traverse the BBB limits, T cells meet microglia and interact through receptors and cytokines, which further lead to neurodegeneration and demyelination. In the red and blue boxes listed currently approved therapeutic strategies that target at T cell and microglia interactions outside or inside the CNS. (Adopted from (Dong and Yong 2019). License ID: 4997551421262).

induce the engraftment of monocyte into CNS. Microglia and engrafted monocytes were sorted and co-cultured with MBP reactive T cells. The results showed that infiltrated monocytes instead of microglia are effective APCs (Ford, Goodsall et al. 1995). A later study

showed that depletion of MHCII on microglia has no impact on EAE severity in a MOG_{35-55} induced mouse EAE and on demyelination in a cuprizone model (Wolf, Shemer et al. 2018). Of note, in this study depletion of MHCII on microglia delayed the EAE onset for about four days (Wolf, Shemer et al. 2018). By contrast, in a Theiler's virus-induced MS animal model, microglia are activated and express a similar profile of effector molecules as infiltrated macrophages and also exhibit similar antigen presentation abilities (Mack, Vanderlugt-Castaneda et al. 2003). In addition, with the multistep activation by GM-CSF and IFN-γ, microglia acquire the APC function efficiently (Matyszak, Denis-Donini et al. 1999). Despite of controversial data on microglia functioning as APCs, microglia do interact with T cells and induce upregulation of IL-10 in both T cells and microglia, implying an anti-inflammatory role of microglia in MS patients (Chabot, Williams et al. 1999). However, microglia can also contribute to the inflammation. For example, MBP-primed T cells can markedly induce the expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide in microglia (Dasgupta, Jana et al. 2002), which further supports the idea that microglia crosstalk with T cells.

More studies on the relationship between microglia and T_H1 cells suggest that microglia play a role in amplifying neuroinflammation during EAE. For instance, IFN-γ can activate microglia which in turn recruit T cells into the CNS (Takeuchi, Wang et al. 2006). Gene analysis showed that IFN-γ induces the upregulation of IFN-γ signaling factors, MHC related genes, and chemokines in microglia (Rock, Hu et al. 2005), implicating that IFN-γ enhances the antigen presenting ability of microglia. Moreover, IFN-γ-activated microglia can damage the CNS cells through phagocytosis and production of cytotoxic factors such as nitric oxide, glutamate, pro-inflammatory cytokines, and superoxide (Kempermann and Neumann 2003, Platten and Steinman 2005). Therefore, during EAE development, after the peripherally primed T cells infiltrated into the CNS, microglia potentially receive the signals from T cells which contribute to the formation of the inflammatory cascade.

Finally, microglia depletion by using *CD11b-HSVTK* transgenic mice and bone marrow chimeras showed that microglia fuel the neuroinflammation and promote the mice to develop active EAE (Heppner, Greter et al. 2005), suggesting a detrimental role of microglia

in EAE development. However, depleting microglia in nonobese diabetic mice, a mouse model of SPMS, showed enhanced secondary progression of EAE and $CD4^+$ T cell infiltration, axonal degeneration and demyelination (Tanabe, Saitoh et al. 2019). In addition, Rubino et al. showed that ablating microglia before EAE induction do not affect the disease and T cell responses; ablating microglia at the peak of EAE exacerbate the disease severity without affecting the pathology of spinal cord; depleting microglia during the peak of EAE can lead to robust infiltration of inflammatory monocytes to the CNS (Rubino, Mayo et al. 2018). Those findings suggested a protective role of microglia in EAE development.

As described above, the role of microglia in MS/EAE is contradictory and still one of big remaining questions. It seems decisively important to understand the contribution of microglia in EAE development especially whether microglia instruct T cells to initiate the neuroinflammation.

1.3.4 Cytokine networks in MS/EAE

Cytokines, which include lymphokines, chemokines, growth factors and IFNs, enable the communication between different cells (Becher, Spath et al. 2017). Clinical studies revealed that the abnormal production of certain cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, GM-CSF, and the IFN-γ-inducible protein of 10 kDa (IP-10) correlates with the intensity of neuroinflammation in MS patients (Traugott and Lebon 1988, Maimone, Gregory et al. 1991, Sorensen, Tani et al. 1999, Galli, Hartmann et al. 2019). The use of EAE models further unveiled the function of these cytokines in neuroinflammatory disorders and the potential therapeutic effects by targeting their biological activity (Palle, Monaghan et al. 2017). Given the pleiotropic nature of cytokines, one cytokine can act differently on different cell types. For instance, an *in vitro* study showed that IL-6 can induce the proliferation of mesenchymal stromal cells; while engaging IL-6 receptor on T_H cells can drive the production of IL-17 (Kishimoto 2006, Dorronsoro, Lang et al. 2020). Especially, *in vivo* the complex network of different cytokines makes it challenging to unravelling the role of a given cytokine. In MS, invaded leukocytes are the main source of pro-inflammatory cytokines (Becher, Spath et al. 2017). Those cytokines delivered by CNS-invading cells are in general harmful to the tissue. The initial wave of cytokines secreted by leukocytes triggers the cascade of the complex

cytokine production by CNS-resident cells, which in turn induce more recruitment of leukocytes, thus fueling the formation of massive inflammatory reaction (Goverman 2009).

IL-6 and IL-1 are both important in generating the pathogenic function of T_H cells in the periphery as well as in the CNS. IL-6 promotes differentiating T_H17 cells to acquire pro-inflammatory effector functions and suppresses regulatory functions by preventing the expression of *FoxP3*, suggesting a crucial role of IL-6 for EAE development (Korn, Mitsdoerffer et al. 2008). Furthermore, IL-6 upregulates the expression of IL-1 receptor on T cells, thereby stabilizing T_H cell polarization by licensing them to sense IL-1 in CNS (Chung, Chang et al. 2009). The IL-1 family contains 11 members of cytokines and 10 members of receptors which are primarily engaged in innate immunity (Dinarello 2018). All innate immune cells express members of the IL-1 family and/or are regulated by them (Garlanda, Dinarello et al. 2013). IL-1β has been shown to mediate both innate and adaptive immunity by promoting inflammatory responses in acute phase of innate immune responses and by enhancing T cell differentiation and priming in adaptive immune responses (Zheng, Fletcher et al. 1995, Jain, Song et al. 2018). Abnormal production of IL-1β is involved in autoimmune disorders, such as EAE (Dinarello 2011). $CD4^+$ T cells deficient in IL-1 signaling were not able to induce EAE due to the compromised polarization and acquisition of encephalitogenicity (Chung, Chang et al. 2009). In addition, IL-1 signaling was shown to be critical for pre-committed T_H1, T_H17 and T_H2 lineage cells to obtain the capacity of effector cytokine production, for instance, blockade of IL-1R substantially reduces the production of IFN-γ, IL-17A and IL-13 (Jain, Song et al. 2018).

During EAE, multiple immune cell types contribute to the IL-1β production, such as bone marrow-derived DCs (BMDCs), macrophages, neutrophils and mast cells (Secor, Secor et al. 2000, Levesque, Pare et al. 2016, Jain, Irizarry-Caro et al. 2020). Recently the role of IL-1β produced by T cells has been noted. At an early stage of EAE, infiltrated $CD4^+$ T cells express pro-IL-1β in spinal cord and IL-1β knockout in $CD4^+$ T cells protected the mice from developing EAE (McCandless, Budde et al. 2009). Despite the significant insights of the pathogenic function of IL-1β provided by results from EAE, the exact contribution of IL-1β to MS remains largely undefined. Nevertheless, IL-1β is found in the CNS lesions and in the

CSF of MS patients, and the IL-1β level in the CSF correlates to the number of demyelinating lesions, suggesting IL-1β plays an important role in MS (Seppi, Puthenparampil et al. 2014).

The role of IFN-γ in exacerbating MS promoted researchers to investigate the contribution of IFN-γ to CNS autoimmune disease (Panitch, Hirsch et al. 1987). However, defining the role of IFN-γ has been challenging due to the pro- and anti-inflammatory attributes of this cytokine. On the one hand, administrating IFN-γ into CNS induced inflammation, on the other hand applying IFN-γ-blocking antibodies exacerbated the progress of EAE (Billiau, Heremans et al. 1988, Sethna and Lampson 1991). In addition, lack of IFN-γ and IFN-γ receptor expression also exacerbated the progress of EAE, suggesting a protective role of IFN-γ in autoimmune disease (Steinman 2007). The impacts of IFN-γ on EAE can be different depending on the time of injection. For example, injecting IFN-γ before onset exacerbated disease, while injecting during the effector stage attenuated the disease (Naves, Singh et al. 2013). Furthermore, IFN-γ regulates the production of IL-17 in T_H cells given that the number of IL-17 producing T_H cells is significantly increased with lack of IFN-γ signaling in CNS (Harrington, Hatton et al. 2005, Park, Li et al. 2005). Besides the impact of IFN-γ on T cells, it can also affect the EAE by regulating CNS-resident cells. Increase IFN-γ level before EAE onset in adult mice reduces the clinical score and prevents demyelination, oligodendrocyte loss and axonal damage by regulating the activation of integrated stress response in oligodendrocytes (Lin, Bailey et al. 2007). IFN-γ signaling in astrocytes also plays a role in EAE development. Silencing IFN-γ receptor on astrocytes protected the mice from developing EAE by reducing the production of chemokines such as CXCL-1, CXCL-2 and CXCL-9 and inhibiting infiltration of B cells, monocytes and neutrophil into CNS (Ding, Yan et al. 2015). IFN-γ attenuates EAE by promoting APCs to phagocytose myelin debris in CNS, which prevents the generation and accumulation of lipid peroxidation products (Sosa, Murphey et al. 2015). Finally, IFN-γ stabilizes the integrity of BBB by enhancing endothelial cells to express tight junction proteins (Ni, Wang et al. 2014).

As another pleiotropic cytokine, TNF belongs to a complex and large superfamily of soluble and membrane-bound ligands that bind to a variety of receptors. Most cell types can produce TNF, which is unlike IFN-γ that can be produced mainly by lymphocytes. Analysis

of blood samples showed enhanced expression of TNF and IFN-γ in MS patients (Beck, Rondot et al. 1988, Rieckmann, Albrecht et al. 1995). TNF can induce oligodendrocyte apoptosis and decrease glutamate uptake by astrocytes, which may contribute to oligodendrocyte damage (Hovelmeyer, Hao et al. 2005, Korn, Magnus et al. 2005). In addition, TNF induces the expression of VCAM-1 on endothelial cells as well as on astrocytes, which in turn promote T cells to infiltrate to CNS parenchyma (Rosenman, Shrikant et al. 1995). In spite of the pro-inflammatory effects of TNF, neutralization of TNF in MS patients resulted in severe relapses and higher relapse rate (1999). Moreover, neutralizing TNF in some patients who have inflammatory arthritis caused the formation of demyelinating lesions (Mohan, Edwards et al. 2001). The mechanism behind the TNF neutralizing treatment is not fully understood. One possible explanation is that TNF binds to different TNF receptors, TNFR1 and TNFR2 and result in adverse outcomes. EAE studies showed that TNFR1 signaling triggers oligodendrocyte death and TNFR1 deficient mice are resistant to EAE development (Hovelmeyer, Hao et al. 2005, Steeland, Van Ryckeghem et al. 2017), suggesting a pathogenic role of TNFR1. In contrast, TNFR2 signaling contributes to remyelination by regulating the differentiation and proliferation of oligodendrocyte progenitor cells in a cuprizone model of demyelination (Arnett, Mason et al. 2001), and TNFR2 deficiency exaggerated EAE development (Suvannavejh, Lee et al. 2000), suggesting a protective role of TNFR2. Taken together, TNF exerts complex effects in CNS autoimmunity that varies depending on the binding receptor.

GM-CSF was initially recognized as hematopoietic growth factor ascribed to its ability in inducing proliferation of bone marrow progenitor cells and their differentiation to form granulocyte and macrophage colonies *in vitro* (Burgess and Metcalf 1980). Later on, GM-CSF was found playing a role in inflammation by acting on mature myeloid cells, neutrophils and macrophages (Hamilton, Stanley et al. 1980). McQualter et al. first showed that GM-CSF knockout mice are resistant to EAE development with failure of immune cells to infiltrate the CNS and less proliferation of antigen-specific splenocytes (McQualter, Darwiche et al. 2001), thus revealing a crucial role of GM-CSF in CNS autoimmune disorders. Since then, tremendous attention has been paid to find out the GM-CSF network and its cellular sources. Lymphocytes, innate lymphoid cells, endothelial cells, fibroblasts, and

epithelial cells can produce GM-CSF, although they usually demand an activating stimulus (Hamilton 2020). Consistently, under steady state, only small amounts of GM-CSF can be detected and mainly confined to nonsterile tissues like gut, skin and lung (Hamilton and Achuthan 2013). However, it is still not fully understood which factors induce the production of GM-CSF in T_H cells. At least, it was shown that IL-23-stimulated T_H17 cells produce GM-CSF, thus GM-CSF was considered as a T_H17 related cytokine (El-Behi, Ciric et al. 2011). Later, fate-mapping of GM-CSF expression showed GM-CSF was not restricted to T_H17 cells. A discrete encephalitogenic T_H subset was shown to account for GM-CSF production in an IL-23 receptor and an IL-1 receptor signaling dependent manner, and the vast majority of those cells co-expressed TNF, IL-2 and IFN-γ instead of IL-17 (Komuczki, Tuzlak et al. 2019). Ablating GM-CSF-producing T_H cells attenuated EAE development without affecting IFN-γ^+ T cell and IL-17A$^+$ T cell infiltration into the CNS, which indicates that GM-CSF defines the pathogenicity of T_H cells (Galli, Hartmann et al. 2019, Komuczki, Tuzlak et al. 2019), which could contribute to the mechanism how CNS-infiltrating leukocytes damage the CNS in MS.

All myeloid cells express the receptor for GM-CSF (Croxford, Lanzinger et al. 2015), thus myeloid cells represent GM-CSF response candidates. Microglia (Ponomarev, Shriver et al. 2007), monocytes (Ko, Brady et al. 2014), neutrophils (Kroenke, Chensue et al. 2010), CNS-infiltrated monocyte-originated DCs (King, Dickendesher et al. 2009), and dermal CD103$^+$ DCs (King, Kroenke et al. 2010) have been implicated in playing a GM-CSF dependent pathogenic role in autoimmune disease. However, specific knockout of the GM-CSF receptor *Csf2rb* in cDCs, resident microglia, CNS-invading neutrophils, and CCR2$^+$Ly6C^{hi} monocytes showed that GM-CSF signaling in CCR2$^+$Ly6C^{hi} monocytes, but not in other cell types, is crucial for the development of EAE (Croxford, Lanzinger et al. 2015). Collectively, GM-CSF is critical in inducing the influx and activity of monocytes, which in turn damage the CNS. Therefore, it is important to understand the regulation of GM-CSF production.

1.4 T cell activation indicator: NFAT

As part of the adaptive immune responses, T cells get activated after recognizing their specific antigen via TCRs. Three main signaling pathways participate in TCR related T cell activation which are calcium, mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) pathways (Conley, Gallagher et al. 2016). Calcium ion (Ca^{2+}) concentrations in T cells control crucial and complex effector functions, such as differentiation, proliferation, metabolism, cytokine secretion and cytotoxicity (Trebak and Kinet 2019). Therefore, Ca^{2+} levels have been used for indicating T cell activation. For example, our lab has been using the fluorescence-based calcium biosensor, Twitch1, to study T cell activation during EAE (Kyratsous, Bauer et al. 2017).

Nuclear factor of activated T cells (NFAT) is the Ca^{2+} dependent transcription factor, which plays an important role in the T cell signaling cascade (Shaw, Utz et al. 1988). In resting T cells, NFAT remains as a highly phosphorylated form and locates in the cytosol. After antigen-dependent activation, TCR ligation triggers receptor-associated tyrosine kinase activation which further induces the activation of phospholipase C-γ. Activated phospholipase C-γ causes phosphatidylinositol-4,5-bisphosphate hydrolyzation to diacylglycerol and inositol-1,4,5-trisphosphate ($InsP_3$), which induces the release of Ca^{2+} from its intracellular store in endoplasmic reticulum (ER). The emptying of Ca^{2+} stores in the ER triggers the opening of Ca^{2+} channels in the plasma membrane, which induces the influx of extracellular Ca^{2+} resulting further increase of intracellular Ca^{2+} concentrations. The increased Ca^{2+} binds to calmodulin, which induces calcineurin activation. Activated calcineurin dephosphorylates NFAT and causes NFAT translocation into the nucleus, where NFAT regulates the expression of T cell activation related genes (Macian 2005).

A truncated NFAT fused to GFP (ΔNFAT-GFP), which keeps the function of calcineurin interaction and nucleus translocation but lacks its binding ability to DNA (Pesic, Bartholomaus et al. 2013). ΔNFAT-GFP lost the DNA binding ability, therefore does not regulate gene expression as well as does not interfere with endogenous NFAT, which enable it to be a useful tool to detect and study T cell activation. Unlike Twitch1 that can sense a

lower level of Ca^{2+} without requiring translocation, ΔNFAT-GFP reflects the gene expression changes following T cell activation (Mues, Bartholomaus et al. 2013). This activation sensor of T cells, ΔNFAT-GFP, was also introduced into the T cells by using retroviral gene transfer in our lab. The translocation of ΔNFAT-GFP from the cytoplasm to the nucleus happened within minutes after T cell activation, and has been successfully used in imaging T cell activation in real time (Pesic, Bartholomaus et al. 2013).

OBJECTIVES

After autoimmune T cells break the limits of BBB, they acquire further activation in the CNS parenchyma. Experimental studies showed that, T cell activation in CNS leads to the inflammatory cascade initiation and other leukocytes recruitment. Thus, it is important to understand how T cells get activated and the mechanism behind their pathogenicity formation in CNS. Since T cells entering the CNS ahead of peripheral macrophages, it suggests that CNS-resident cells potentially provide the signals for T cell activation. Among all the CNS-resident cells, microglia are the only innate immune cells in CNS parenchyma, which prioritize them as first candidate cell type in inducing T cell activation in CNS.

In this study, we will look at the interaction and activation between T cells and microglia. We hypothesize that before onset of EAE, microglia instruct T cells to acquire their pathogenicity, which is crucial for further infiltration of peripheral lymphocytes.

Firstly, we will establish primary adult microglia culture and T cell/microglia co-culture system. Then we will use live imaging to visualize whether microglia induce T cell activation *in vitro*. Here we will co-culture primary microglia with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells and $T_{OVA\text{-}NFAT\text{-}GFP}$ cells, which are engineered with T cell activation indicator. By using this method, the activation of T cells can be recorded and tracked in real time.

Secondly, we will evaluate the ability of phagocytes from EAE rats in inducing T cell activation and compare the differences between macrophages and microglia from EAE rats in activating T cells.

Thirdly, we will characterize the activation profile of MBP-specific and OVA-specific T cells after co-culturing with microglia by using qPCR and FACS staining. Therefore, to find out the potential pathways which are responsible for microglia and T cell interaction and their activation.

Lastly, we will perform transcriptome analysis of microglia and macrophages from early stage of EAE rats to identify the activation profile of microglia and macrophages.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Animals

Lewis rats were obtained from Charles River or Janvier Labs and were kept in the animal facility of the Biomedical center, LMU. All the animal experiments were approved by local authority (Regierung von Oberbayern) and were performed under the animal experimentation regulation of Bavarian state. No gender differences among the rats were noted in this study.

2.1.2 Reagents, Buffers and Media

Name	Ingredient	Amount	Company & Cat no.
EH	DMEM	97.5% Vol	Sigma, D5671
	HEPES, 1M	2.5% Vol	Sigma, H0887
DF12 + FBS + PS	DME/F-12	500 ml	Sigma, D8437
	FBS superior	50 ml	Biochrom, D12247
	Penicillin-Streptomycin 100×	5 ml	Sigma, P4333
TCM	DMEM	1 L	Sigma, D5671
	L-glutamine, 200 mM	2 mM	Sigma, G3126
	Penicillin-Streptomycin 100×	10 ml/L	Sigma, P4333
	L-asparagine monohydrate	0.036 g/L	Sigma, A4284
	Sodium pyruvate solution, 100 mM	1 mM	Sigma, S8636
	MEM Non-essential amino acid solution 100×	10 ml/L	Sigma, M7145
	2-Mercaptoethanol	4 µl/L	Merck, 8057400
RM	TCM	99% Vol	-
	Rat serum	1% Vol	-
TCGF	TCM	440 ml	-
	Horse serum	50 ml	PAA Laboratories, B15-027
	PMA stimulated EL4 IL2 supernatant	10 ml	-
TCM + FBS	TCM	90% Vol	-

	FBS superior	10% Vol	Biochrom, D12247
FM	Horse serum	50% Vol	PAA Laboratories, B15-027
	EH	40% Vol	-
	DMSO	10% Vol	Sigma, D4540
10× PBS (pH 7.4)	NaCl	1400 mM	VWR Chemicals, 27810.295
	KCl	27 mM	Merck, 104936
	Na_2HPO_4	100 mM	Sigma, 71640
	KH_2PO_4	18 mM	Sigma, P5655
	H_2O	1 L	-
PBS (pH 7.4)	10× PBS	10% Vol	-
	H_2O	90% Vol	-
FACS buffer	PBS	99% Vol	-
	Rat serum	1% Vol	-
	Sodium azide	0.05% Vol	Roth, K305.1
Sorting buffer	PBS	100 ml	-
	Rat serum	1 ml	-
	EDTA, 0.5M	0.4 ml	Merck, 108418
	HEPES, 1M	2.5 ml	Sigma, H0887
ACK buffer	$KHCO_3$	1 mM	Sigma, 431583
	NH_4Cl	150 mM	Merck, 1145
	EDTA	0.1 mM	Merck, 108418
	H_2O	500 ml	-
Stock isotonic Percoll (SIP)	Percoll	90% Vol	Sigma, GE17089101
	10X PBS	10% Vol	-
70% SIP	SIP	70% Vol	-
	PBS	30% Vol	-
37% SIP	SIP	37% Vol	-
	PBS	63% Vol	-
30% SIP	SIP	30% Vol	-
	PBS	70% Vol	-
Nycoprep (pH 7.0)	Nycodenz	28.2 g	Axis-shield, 1002424
	NaCl	0.6 g	VWR Chemicals, 27810.295
	Tricine, 100mM	10 ml	Sigma, T5816
	H_2O	200 ml	-
PLL	Poly-L-lysine hydrobromide	5 mg	Sigma, P6282
	H_2O	50 ml	-

Accutase® solution	-	-	Sigma, A6964

Table 2.1.1I Reagents, buffers and media for cell culture.

Name	Ingredient	Amount	Company & Cat no.
LB	Tryptone	10 g	Sigma, 107213
	Yeast extract	5 g	Sigma, 103753
	NaCl	5 g	VWR Chemicals, 27810.295
	H_2O	1 L	-
LB agar plate	LB	1 L	-
	Bacto agar	15 g	Fisher scientific, 10455513
	Ampicillin	100 mg/L	Sigma, A0166

Table 2.1.2I Reagents, buffers and media for bacteria culture, DNA extraction.

Name	Ingredient	Amount	Company & Cat no.
PBST	PBS	99.95% Vol	-
	Tween®20	0.05% Vol	Sigma, P1379
Blocking buffer	PBS	100 ml	-
	Bovine serum albumin (BSA)	1 g (1 %)	Sigma, A9647
PB	Permeabilization wash buffer 10×	10% Vol	Biolegend, 421002
	H_2O	90% Vol	-
2% PFA	PBS	1 L	-
	Paraformaldehyde	20 g	Merck, 104005
4% PFA	PBS	1 L	-
	Paraformaldehyde	40 g	Merck, 104005
TMB solution	-	-	eBioscience™, 00420156
10× TAE	Trizma® base	48.4 g	Merck, T1503
	EDTA	3.7 g	Merck, 108418
	Acetic acid	11.4 ml	Sigma, 33209
	H_2O	to 1 L	-
Agarose gel	Agarose	0.5 g	Biozym, 840004
	1× TAE	50 ml	-
	Midori green advance	5 µl	Nippon genetics, MG04

Table 2.1.3I Reagents and buffers for ELISA, intracellular staining, cloning and histology.

2.1.3 qPCR Primer

Gene symbol	Gene name	Gene ID	Accession no.	Sequence (5' to 3')
Il1b	*interleukin 1 beta*	24494	NM_031512.2	F: TAGCAGCTTTCGACAGTGAGG
				R: TCTGGACAGCCCAAGTCAAG
Il6	*interleukin 6*	24498	NM_012589.2	F: GTTTCTCTCCGCAAGAGACTT
				R: TGGTCTGTTGTGGGTGGTAT
Tnf	*tumor necrosis factor*	24835	NM_012675.3	F: ATGGGCTCCCTCTCATCAGT
				R: GCTTGGTGGTTTGCTACGAC
Ifng	*interferon gamma*	25712	NM_138880.2	F: TGTCATCGAATCGCACCTGA
				R: TGTGGGTTGTTCACCTCGAA
Il17a	*interleukin 17A*	301289	NM_001106897.1	F: GGAGAATTCCATCCATGTGCC
				R: ATGAGTACCGCTGCCTTCAC
Il23a	*interleukin 23 subunit alpha*	155140	NM_130410.2	F: CTCTGTAACTGCCTGCTTAGTC
				R: GCTTTGTCACAGGTCGGTAT
Il10	*interleukin 10*	25325	NM_012854.2	F: CCTCTGGATACAGCTGCGAC
				R: TAGACACCTTTGTCTTGGAGCTTA
Fas	*Fas cell surface death receptor*	246097	NM_139194.2	F: CCCGGACCCAGAATACCAAG
				R: TTCAAGTCCACACGAGGTGC
Fasl	*Fas ligand*	25385	NM_012908.1	F: TGGCCCACTTAACAGGGAAC
				R: CTCATTGATCACAAGGCCGC
Csf2	*colony stimulating factor 2*	116630	NM_053852.1	F: TAATGAGTTCTCCATCCAGAGGC
				R: CTGGTAGTGGCTGGCTATCA
C1qc	*complement C1q C chain*	362634	NM_001008524.1	F: CCCTACATGCTCAGGATGGTT
				R: GGAGCAGCAGCAGGTAGAG
LY86	*lymphocyte antigen 86*	291359	NM_001106128.1	F: GGACCAGCCCAAGTTCTCTTT
				R: AGAGCTGGTATTCTCCCTGT
B2m	*beta-2 microglobulin*	24223	NM_012512.2	F: CGGGGTGGTGATGAGAAGTT
				R: AAGGCTCCTTGTCCCTTGAC
Ppia	*peptidylprolyl isomerase A*	25508	NM_017101.1	F: CTGAGCACTGGGGAGAAAGG
				R: CACCCTGGCACATGAATCCT

Table 2.1.4I Primers used for qPCR.

2.1.4 sgRNA

Il1r1 (sgRNA1)	*interleukin 1 receptor type 1*	25663	NM_013123.3	Oligo1: CACCGCACGTACGGTTAGTATACCC

				Oligo2: AAACGGGTATACTAACCGTACGTGC
Il1r1 (sgRNA2)				Oligo1: CACCGTGCAAAGGATCCCTCTCCGT
				Oligo2: AAACACGGAGAGGGATCCTTTGCAC
Il1r1 (sgRNA3)				Oligo1: CACCGATGCACGGAGTATCCAAATG
				Oligo2: AAACCATTTGGATACTCCGTGCATC
Il1r1 (sgRNA4)				Oligo1: CACCGGTGTGTTATAGCACAAGCCA
				Oligo2: AAACTGGCTTGTGCTATAACACACC
Il1b (sgRNA1)	*interleukin 1 beta*	24494	NM_031512.2	Oligo1: CACCGCATCATCCCACGAGTCACAG
				Oligo2: AAACCTGTGACTCGTGGGATGATGC
Il1b (sgRNA2)	-	-	-	Oligo1: CACCGAAGCTCCACGGGCAAGACAT
				Oligo2: AAACATGTCTTGCCCGTGGAGCTTC
Il1b (sgRNA3)	-	-	-	Oligo1: CACCGCACATGGGTCAGACAGCACG
				Oligo2: AAACCGTGCTGTCTGACCCATGTGC
Tnf (sgRNA)	*tumor necrosis factor*	24835	NM_012675.3	Oligo1: CACCGGGTGATCGGTCCCAACAAGG
				Oligo2: AAACCCTTGTTGGGACCGATCACCC
NT	*Non-targeting*	-	-	GCTGCATGGGGCGCGAATCA

Table 2.1.5I sgRNA used for *Tnf, Il1b* and *Il1r1* knockout.

2.1.5 Antibodies, cell dyes, cytokines, stimulators, conjugates

Name	**Clone, isotype**	**Company, cat no.**	**Applications**
Mouse anti-rat Iba1	1022-5, IgG2b	Santa Cruz Biotechnology, sc-32725	Histology
Isolectin GS-IB4	-	ThermoFisher SCIENTIFIC, I21413	Cell labeling
Mouse anti-rat CD45	OX-1, IgG1κ	BioLegend, 202211	Flowcytometry
Mouse anti-rat CD11b/c,	OX-42, IgG2aκ	BioLegend, 201803	Flowcytometry
Mouse anti-rat MHCII	HIS19, IgG1	Invitrogen, 11092082	Flowcytometry
Mouse anti-rat, IFN-γ	DB1, IgG1κ	Invitrogen, 14731085	Flowcytometry ELISA

Mouse anti-rat CD134	OX-40, IgG2bκ	eBioscience™, 17134282	Flowcytometry
Mouse anti-rat CD25	OX-39, IgG1	Bio-Rad, MCA273R	Flowcytometry
Mouse anti-rat, αβTCR	R37, IgG1	Bio-Rad, MCA453G	Flowcytometry
Mouse anti-rat CD4	W3/25, IgG1	Bio-Rad, MCS55R	Flowcytometry
Armenian hamster anti-rat TNF-α	TN3-19.12, IgG	BioLegend, 506101	Flowcytometry
Rabbit anti-rat/mouse IL-1β	-, IgG	Rockland, 210401319	Flowcytometry
Mouse anti-rat GM-CSF	83308, IgG2b	R&Dsystems, MAB5181	Flowcytometry
Donkey anti-mouse APC	-, polyclonal	Jackson ImmunoResearch, 715136151	Flowcytometry
Goat anti-armenian hamster APC	-, polyclonal	Jackson ImmunoResearch, 127135160	Flowcytometry
Donkey anti-rabbit APC	-, polyclonal	Biolegend, 406414	Flowcytometry
Donkey anti-mouse PerCP	-, polyclonal	Jackson ImmunoResearch, 715126151	Flowcytometry
PerCP streptavidin	-	Jackson ImmunoResearch, 016120084	Flowcytometry
FITC mouse isotype	IgG1, κ	Biolegend, 400110	Flowcytometry
Goat anti-rat IFN-γ, Biotin	-, Ig	Biolegend, 518803	ELISA
Streptavidin-HRP	-	SouthernBiotech, 7105-05	ELISA
Brefeldin A	-	Sigma, B7651	Flowcytometry
Mouse anti-rat MHCI	OX-18	Purified in the lab	Live imaging
Mouse anti-rat MHCII	OX-6	Purified in the lab	Live imaging

Table 2.1.6I Antibodies, cell dyes, and conjugates used for histology, cell labeling, flowcytometry, ELISA and live imaging.

Name	**Reconstitution**	**Company, cat no.**	**Applications**
Rat IFN-γ	100 µg/ml; 0.1% rat serum, in PBS	Peprotech, 400-20	Cell culture
Rat IL-1β	20 µg/ml; in H_2O	Peprotech, 400-01B	Cell culture
Lipopolysaccharides (LPS)	1 mg/ml; in PBS	Sigma, L4391	Cell culture
Ionomycin	1 mM; in EtOH	Sigma, I0634	Cell culture
Phorbol 12-myristate 13-acetate (PMA)	1 mg/ml; in DMSO	Sigma, P8139	Cell culture

Rat IFN-γ	2000 pg/ml; in 1% rat serum, PBS	BioLegend, 580009	ELISA

Table 2.1.7I Cytokines and stimulators used for cell activation and ELISA standard.

2.1.6 Reagents, kits and plates

Name	company, cat no.	Applications
2 × qPCRBIO SyGreen Mix Separate-ROX	PCRbiosystems, PB20.14	qPCR
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad, 1725271	qPCR
Hard-Shell® 96-well PCR plates	Bio-Rad, HSP9655	qPCR
FastDigest BbsI	New England BioLabs, R3539S	Cloning
FastAP	ThermoFisher SCIENTIFIC, EF0654	Cloning
FastDigest buffer (10×)	ThermoFisher SCIENTIFIC, B64	Cloning
10× T4 Ligation buffer	New England BioLabs, B0202S	Cloning
T4 PNK	New England BioLabs, M0201S	Cloning
Quick Ligation™ Kit	New England BioLabs, M2200S	Cloning
Wizard® SV Gel and PCR Clean-Up System	Promega, A9282	DNA purification
RNeasy Plus Mini Kit	Qiagen, 74134	RNA extraction
RNeasy Plus Micro Kit	Qiagen, 74034	RNA extraction
cDNA synthesis kit	ThermoFisher SCIENTIFIC, K1632	cDNA synthesis
NucleoBond® Xtra Midi EF	Macherey-Nagel, 740420.50	Plasmid DNA purification
QIAprep Spin Miniprep Kit	Qiagen, 27104	Plasmid DNA purification
Agilent RNA 6000 Pico Reagents	Agilent Technologies, 5067-1513	RNA quality evaluation
Collibri™ 3’ mRNA Library Prep Kit	ThermoFisher SCIENTIFIC, A38110024	RNA sequencing
Agilent™ High Sensitivity DNA Kit	Agilent Technologies, 5067-4626	DNA quality evaluation

Table 2.1.8I Reagents, kits and plates used for real-time quantitative PCR (qPCR), cloning, RNA extraction, plasmid DNA purification, RNA sequencing and RNA/DNA quality evaluation.

2.1.7 Antigens

MBP was prepared by our colleagues. Ovalbumin (OVA) was purchased from Sigma.

2.2 Methods

2.2.1 Cell culture

Primary microglia culture

2 to 4 months old Lewis rats were used for microglia culture. Microglia were acquired from the brain and spinal cord of Lewis rats by using Percoll gradients as described previously (Cardona, Huang et al. 2006). Briefly, the brain and spinal cord were taken out of the rats followed by stripping off the meninges in cold PBS to avoid monocyte contamination. The tissue was dissociated mechanically in Falcon® 100 µm cell strainers in cold PBS by homogenizing continuously to obtain a single cell suspension. The cell suspension was centrifuged at 400 *g*, 4 °C for 5 min. The cell pellet was resuspended with 9 ml 37% SIP. Three 15 ml centrifuge tubes were used for setting up the Percoll gradient. For each tube, 3 ml cell suspension were overlaid on 3 ml 70% SIP followed by overlaying 3 ml 30% SIP and 2 ml PBS. The gradient was centrifuged at 300 *g*, 18 °C for 30 min with a minimal brake to avoid interphase disturbance. After centrifuge, the cell debris was removed carefully. 1 ml of the 70%-37% interphase from each 15 ml centrifuge tube was collected and transferred into one clean 15 ml centrifuge tube (Fig. 2.2.1). PBS was used to wash the cells. Cells were either used for experiments directly or inoculated in corresponding cell culture dishes and cultured in DF12 + FBS + PS in a humidified incubator at 5% CO_2 and 37 °C for 5 to 8 days. For the culture, cells acquired from brain and spinal cord of one rat were plated in one well of PLL pre-coated 6-well plate, two wells of PLL pre-coated 12-well plate or two Ibidi 4 well^{Ph+} µ-slides. After 5 to 8 days of culture, the density of the microglia is around 80%.

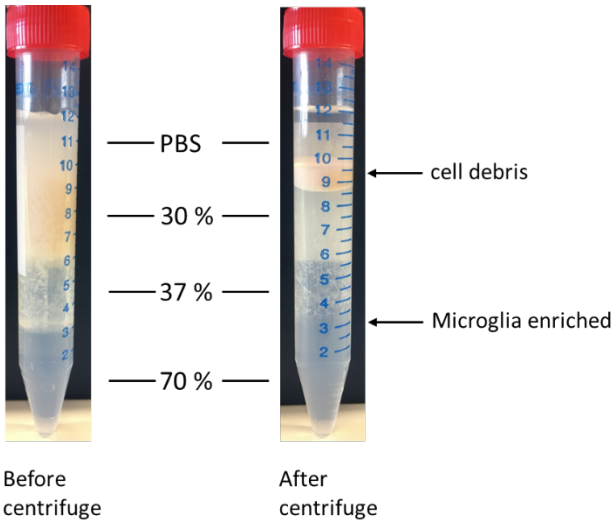

Fig. 2.2.1| Gradient set up. Percoll gradient before and after centrifuge. Microglia and cell debris were separated according to their density.

Primary astrocyte culture

Postnatal day 3 Lewis rats were used for astrocyte culture as described previously (Yan, Wang et al. 2020). Briefly, the brain was isolated and transferred into cold PBS followed by stripping off the meninges. The brain was homogenized on a Falcon® 100 µm cell strainer to obtain a single cell suspension. Cell suspension was centrifuged to pellet the cells. The cell pellet was resuspened in 10 ml DF12+FBS+PS and was plated in a PLL pre-coated 75 cm^2 flask. The cells were cultured in a humidified incubator at 5% CO_2, 37 °C. After 6 days of culture, the flask was shaken at 110 rpm for 18 hours (hrs) to detach the microglia whereas astrocytes remained attached to the flask. Astrocytes were collected by incubating with trypsin/EDTA for 8 min at 37 °C. Purified astrocytes were resuspended in DF12 + FBS + PS and were plated in PLL pre-coated 6-well plate at a density of 0.6×10^6 cells /well, 2 ml/well. The purified astrocytes were cultured for one day before further treatment.

Establishment and culture of $CD4^+$ T cell lines

MBP- or OVA-specific $CD4^+$ T cells, which are engineered by retroviral transduction to express ΔNFAT-GFP or EGFP, were established by our lab previously (denoted as $T_{MBP\text{-}NFAT\text{-}GFP}$, $T_{MBP\text{-}GFP}$, $T_{OVA\text{-}NFAT\text{-}GFP}$ and $T_{OVA\text{-}GFP}$) (Flugel, Willem et al. 1999, Pesic, Bartholomaus et al. 2013). In similar way, Cas9 expressing MBP-specific T cell ($T_{MBP\text{-}Cas9\text{-}EGFP}$ cell) line has been

generated by PD Dr. Naoto Kawakami. Established $CD4^+$ T cell lines in FM can be stored in liquid nitrogen and used after thawing.

Thymocyte isolation. The thymus was taken out of the naive Lewis rat and homogenized in 20 ml EH by forcing it through a cell strainer to obtain a single cell suspension. To pellet the cells, the cell suspension was centrifuged at 400 *g*, 4 °C for 5 min followed by resuspension in 10 ml fresh EH and irradiation with 50 Gy by an X-ray irradiator (Cellrad). Thymocytes were kept at 4 °C and could be used within 48 hrs.

Restimulation. Frozen stocks of resting $CD4^+$ T cells were taken out from the liquid nitrogen and were thawed quickly at 37 °C and resuspended in 20 ml EH medium. 3.5×10^6 $CD4^+$ T cells were mixed with 70 – 100 × 10^6 irradiated thymocytes. The mixed cells were centrifuged at 400 *g*, 4 °C for 5 min to pellet the cells. 5 ml RM was used for restimulation with the presence of 10 µg/ml cognate antigen (MBP or OVA). Cells were plated in a 60 mm cell culture dish and were cultured for one day or two days in a humidified incubator at 37 °C, 10% CO_2. On day one or day two, $CD4^+$ T cells were used for further experiments. Alternatively, on day two after restimulation, T cells were adoptively transferred to recipient rats or expanded in TCGF for another 4 days. On day six, $CD4^+$ T cells were either used for further restimulation or preserved in FM in liquid nitrogen.

Co-culture of microglia/astrocytes and $CD4^+$ T cells

Microglia were cultured for 5 to 8 days before co-culture with $CD4^+$ T cells. 0.5×10^6/well, 2×10^6/well or 1.5×10^6/well $CD4^+$ T cells were used for co-culturing with microglia in Ibidi 4 wellPh+ µ-slides, in a 12-well plate or in the upper layer of a 6-well transwell insert, respectively.

Purified astrocytes were plated on 6-well plates one day before starting co-culture with 1.5×10^6/well $CD4^+$ T cells.

2.2.2 EAE induction

To induce adoptive tEAE, 5 × 10^6 restimulated T_{MBP} cells were injected by i.v. or intraperitoneally (i.p.) into a Lewis rats. Body weight and clinical score were measured and

recorded daily. The clinical scores were assessed as follows: 0, no disease; 1, flaccid tail; 2, gait disturbance; 3, complete hind limb paralysis; 4, tetraparesis; and 5, death (Kyratsous, Bauer et al. 2017).

2.2.3 Immunofluorescence analysis

Microglia or astrocytes were cultured on PLL pre-coated 13 mm cover glasses in 24-well plates. The cells were fixed with 4% PFA for 30 min. PB was applied for permeabilization and blocking for 2 hrs at room temperature. Primary antibodies were applied at a dilution of 1:500 in PB and incubated overnight at 4 °C. Cells were washed three times with PB and incubated with secondary antibodies for 2 hrs at a 1:500 dilution in PB at room temperature followed by three washing steps with PBS. The cover glass was mounted with Fuoromount-G™ mounting medium (Invitrogen) on slides and the fluorescence was analyzed by microscopy (Leica microsystems GmbH, Leica DM IL LED).

2.2.4 Flowcytometry (FACS)

Splenocyte isolation

The spleen was taken out of the rat and was put into cold PBS. To obtain a single cell suspension, the spleen was homogenized on a cell strainer. Then the cell suspension was centrifuged in a 50 ml centrifuge tube at 400 *g*, 4 °C for 5 min to pellet the cells. After removing the supernatant, 5 ml ACK buffer were added to the cell pellet and was incubated on ice to lyse the red blood cells. After 5 min, cold PBS was used to wash the cells. The cells were kept in 20 ml PBS and were filtered with a 70 µm cell strainer before FACS staining.

Surface staining

A maximum 1 × 10^6 cells were transferred into a 96-well V-bottom plate and were centrifuged at 400 *g*, 4 °C for 3 min followed by washing with 100 µl/well FACS buffer. Primary antibodies were applied at a dilution of 1:100 in 100 µl FACS buffer and were incubated for 30 min on ice. Three washing steps with 100 µl/well FACS buffer were applied to remove the remaining primary antibody. Secondary antibodies or fluorescence-conjugated streptavidin in FACS buffer were applied at a dilution of 1:400 in 100 µl/well

FACS buffer and were incubated for 30 min on ice, followed by washing once with FACS buffer and twice with PBS. Cells were resuspended with 100 µl PBS and were analyzed by a BD FACSVerse™ flowcytometer.

Intracellular staining

To induce cytokine production, T cells were transferred into 96-well V-bottom plates and were incubated with ionomycin and PMA at a final concentration of 1 µM and 1 µg/ml diluted in TCM + FBS for 2.5 hrs in an incubator heated to 37 °C with 10% CO_2. Brefeldin A was added at a final concentration of 10 µg/ml for 2.5 hrs to stop the secretion of cytokines. T cells from day one or day two after restimulation and T cells that were co-cultured with microglia, were incubated with brefeldin A for 2.5 hrs. The cells were fixed by incubating with 150 µl/well 2% PFA for 15 min on ice. PB was applied to permeabilize the cells at 150 µl/well for 15 min on ice. Primary antibodies were applied at a dilution of 1:100 in PB using 100 µl/well for 30 min on ice. Three washing steps with PB were performed to remove the remaining primary antibodies. Secondary antibodies were applied at a dilution of 1:400 in PB using 100 µl/well for 30 min on ice. PBS was used to wash the cells for three times before analyzing with a BD FACSVerse™ flowcytometer.

All the data was analyzed by FlowJo software (FlowJo, LLC).

2.2.5 Live imaging

An inverted motorized live cell fluorescence microscope (Leica Microsystems GmbH, Leica DMi8, with 20× objective, NA. 0.55) with a 5% CO_2, 37 °C incubator was used for *in vitro* live imaging to detect T cell activation. The µ-slide 4 well Ph+ (Ibidi) was used as culture dish for imaging. Live imaging was performed at the Core Facility Bioimaging of the Biomedical Center, LMU Munich.

Imaging of microglia from naive rats and T cells

In some experiments, microglia prepared from naive rats were stimulated with IFN-γ (100 ng/ml) or LPS (100 µg/ml) for 24 hrs. Alternatively, microglia were incubated with MBP or OVA at a concentration of 10 µg/ml for 2 hrs before co-culture with $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}}$

$_{GFP}$ cells. After adding 0.5 × 10^6/well $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}GFP}$ cells to microglia, the slides were kept for 10 min for T cells to settle before live imaging. The interactions between microglia and $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}GFP}$ cells were recorded for 30 min with a 30 sec interval.

Imaging of CNS-infiltrated phagocytes and T cells

Imaging of phagocytes and $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}GFP}$ cells in a co-culture system. Phagocytes were isolated from the CNS of rats with tEAE on day 4 after transfer of T_{MBP} cells by running Percoll gradients in the same ways as for microglia isolation. The clinical scores of the EAE rats used for phagocytes isolation were between 2 and 3. The isolated phagocytes were directly plated on Ibidi 4 wellPh+ µ-slides and were kept in culture for 6 days as described for primary microglia culture (Section 2.2.1). After 6 days, $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}GFP}$ cells were added into the phagocyte culture. In some experiments, phagocytes were pretreated with MBP or OVA for 2 hrs. The interaction was recorded for 30 min as described before.

Imaging of microglia/infiltrated macrophages and $T_{MBP\text{-}NFAT\text{-}GFP}$ or $T_{OVA\text{-}NFAT\text{-}GFP}$ cells in a co-culture system. To separate infiltrated macrophages from resident microglia, the respective populations were sorted based on the CD45 and CD11b expression. Briefly, after running the Percoll gradients, the microglia/macrophage-enriched interphase was taken out and stained for CD45 and CD11b. 400 µl sorting buffer were used to resuspend the cells. $CD11b^+CD45^{high}$ (macrophage) and $CD11b^+CD45^{low}$ (microglia) populations were sorted by using a BD FACSAria IIIu cell sorter. Around 100,000 infiltrated macrophages or resident microglia were sorted from one EAE rat. Macrophages and microglia were plated on two wells of a 4 wellPh+ µ-slide, respectively, and were cultured in DF12 + FBS + PS in a humidified incubator with 5% CO_2 at 37 °C for 6 days before live imaging.

2.2.6 Quantification of IFN-γ by ELISA

ELISA was used to quantify the IFN-γ protein in the supernatants of the co-culture system of $CD4^+$ T cells and microglia. After 24 hrs of co-culture, the supernatant was collected and centrifuged at 2000 *g* for 5 min to remove cell debris. The supernatant was stored at -20 °C

for short time or was analyzed right away. Briefly, Nunc MaxiSorp^{TM} flat-bottom plates were coated with anti-IFN-γ capture antibodies at a concentration of 1 µg/ml in 100 µl/well blocking buffer at 4 °C overnight. The plate was washed with 300 µl/well PBST for three times. Blocking buffer was applied to block the nonspecific binding at 200 µl/well for 1 hr at room temperature. The plate was washed with 300 µl/well PBST for three times. Standards were prepared in blocking buffer by making a serial dilution at 0 pg/ml, 31.25 pg/ml, 62.5 pg/ml, 125 pg/ml, 250 pg/ml, 500 pg/ml, 1000 pg/ml and 2000 pg/ml. The samples were diluted with blocking buffer appropriately. 100 µl/well standards and samples were added into designated wells (in triplicates) and were incubated for 1 hr at room temperature. The plate was washed with 300 µl/well PBST for three times. Biotin-conjugated anti-rat IFN-γ antibody (detection antibody) was applied 1:2000 in blocking buffer, 100 µl/well for 2 hrs at room temperature. The plate was washed with 300 µl/well PBST for five times. Streptavidin-HRP was applied 1:4000 in blocking buffer, 100 µl/well for 30 min at room temperature. The plate was washed with 300 µl/well PBST for three times. 100 µl/well TMB substrate solution were added and incubated for 30 min in the dark at room temperature. To stop the reaction, 100 µl/well 3 M HCl were applied. The absorbance at 450 nm was measured by VICTOR 2 1420 Multilabel Counter (PerkinElmer) within 15 min after adding the stop solution.

2.2.7 Quantitative real-time-PCR

mRNA extraction

mRNA was extracted by using the RNeasy Plus Mini kit according to the manufacturer's instruction. The concentration of mRNA was evaluated by a NanoDropTM 2000/2000c Spectrophotometer (ThermoFisher SCIENTIFIC).

cDNA synthesis

cDNA synthesis was performed by using the cDNA synthesis kit according to manufacturer's instruction. Same amount of RNA template (decided by NanoDrop) from each sample was reverse transcripted.

Reaction 1 (per sample):

Template RNA	0.1 ng – 5 µg (depending on the yield of sample)
Oligo $(dT)_{18}$ primer	1 µl
Water, nuclease free	To 12 µl

The reaction was performed at 70 °C for 5 min.

Reaction 2: prepare the reaction mix as follows (per sample)

5× reaction buffer	4 µl
10 mM dNTP Mix	2 µl
RiboLock RNase Inhibitor (20 U/µl)	1 µl
Reverse transcriptase (200 U/µl)	1 µl

The reaction mix was added to the reaction 1 (final volume is 20 µl) followed by reaction at 37 °C for 5 min; 42 °C for 60 min; and 70 °C for 10 min. The first strand cDNA synthesis was diluted 1:10 with water.

qPCR

Prepare the reaction mix as follows:

qPCRBIO SyGreen mix (2×)	10 µl
Forward and reverse primer (10 µM each)	0.8 µl
Water	4.2 µl
cDNA template	5 µl
Total reaction volume	20 µl

Duplicate reaction for each sample was performed in Hard-shell® 96-well PCR plate as follows: denature at 95 °C for 3 min; amplify for 40 cycles at 95 °C for 10 sec, 60 °C for 30 sec; melt analyze at 95 °C for 10 sec followed by ramping down to 65 °C then go to 95 °C with a 0.5 °C increments at 5 sec/step. CFX Connect Real-Time PCR System (Bio-Rad) was used for this reaction.

2.2.8 Establishment and culture of TNF-α, IL1R1 and IL-1β knockout T cell lines

Resources and contributors

Plasmid pMSCV-sgNT-puro-BFP and plasmid pMSCV-sgTnf-puro-BFP were kindly provided by Arek Kendirli. Plasmid pCL-Eco was used as described in previous study (Mues, Bartholomaus et al. 2013). SgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells were kindly provided by PD Dr. Naoto Kawakami.

TNF-α, IL1R1 and IL-1β sgRNA cloning

SgRNAs for Il1r1 and Il1b were cloned into the backbone plasmid pMSCV-gRNA-puro-BFP as follows:

Step 1: Digestion and dephosphorylation of backbone vector

pMSCV-gRNA-puro-BFP	5 µg
FastAP	3 µl
FastDigest BbsI	2 µl
FastDigest buffer (10×)	5 µl
H_2O	To 50 µl

The plasmid was digested and dephosphorylated at 37 °C for 2 hrs.

Step 2: Backbone (vector) acquisition

Gel electrophoresis was used to separate different DNA segments of the products from step 1 according to their size. The interesting DNA segments were cut out of the gel on a UV transilluminator (UVT-22, Herolab). The DNA segments were purified from the gel by using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction.

Step 3: Phosphorylation and annealing of sgRNA coding inserts.

Oligo 1 (100 µM)	1 µl
Oligo 2 (100 µM)	1 µl
10× T4 Ligation buffer	1 µl

H_2O	6.5 µl
T4 PNK	0.5 µl

The reaction was performed at 37 °C for 1 min, 95 °C for 5 min followed by ramping down to 25 °C at 5 °C/min.

Step 4: Ligation

Oligo products from step 3 were diluted 1:200 with H_2O. Ligation mix was prepared as follows:

Diluted oligo products	1 µl
Backbone (vector), around 50 ng	1 µl
2× Quick Ligase Buffer	5 µl
H_2O	2 µl
Quick Ligase	1 µl

Ligation mix was incubated at room temperature for 5 to 8 min.

Step 5: Transformation

1 µl ligation products from step 4 were transformed into to 50 µl 5-alpha competent *E. Coli* (New England BioLabs, C2987I) by following the manufacturer's instruction. To the end, 1 ml of transformed *E. Coli* suspension was obtained.

Step 6: Sequencing

Plate 100 µl products from step 5 on a 100 mm agar plate which containing ampicillin, and culture overnight at 37 °C. Two single clones were picked up from the agar plate, and were cultured in 3 ml ampicillin LB medium overnight at 37 °C with shaking at 180 rpm. DNA was extracted by using QIAprep Spin Miniprep Kit according the manufacturer's instruction from 1.5 ml bacteria culture, and was sent for sequencing to make sure the sgRNA was cloned to the plasmid.

Step 7: Bacteria culturing and DNA purification

MATERIAL AND METHODS

In order to obtain high amount of DNA, bacteria were picked up and cultured in 3 ml ampicillin LB medium overnight at 37 °C with shaking at 180 rpm. Then bacteria were transferred into 300 ml ampicillin LB medium and cultured overnight at 37 °C with shaking at 180 rpm. DNA was extracted by using NucleoBond® Xtra Midi EF according to the manufacturer's instruction.

Retrovirus production

Culturing HEK cells

HEK cells were suspended in FM and stored in liquid nitrogen. To culture and expand HEK cells, the cells were taken out from nitrogen and thawed in a 37 °C water bath. 10 ml EH were used to resuspend the HEK cells. After pelleting the cells by centrifuging at 400 *g* for 5 min, the cell pellet was resuspended in TCM + FBS and cultured in a humidified incubator at 37 °C and 10% CO_2. The HEK cells were split every 2 or 3 days.

Transfecting HEK cells and collecting the retrovirus

On day 0, HEK cells were plated at a density of 1.2 – 1.5 × 10^6 cells/100 mm dish in 8 ml TCM + FBS. On day 1, the following mix per dish of HEK cells was prepared for transfection: mix A containing 500 µl TCM, 6 µg plasmid pMSCV-sgRNA-BFP and 3.5 µg pCL-ECO; mix B containing 500µl TCM and 20 µl PEI Max (2 mg/ml). Both mixes were incubated for 5 min at room temperature. Then mix B was added to mix A followed by vortex and incubation at room temperature for 20 min. The mix was added dropwise onto HEK cells. The HEK cells were cultured in a humidified incubator at 37 °C and 5% CO_2. On day 2, the supernatant was removed and 8 ml/dish fresh TCM + FBS was added. The cells were continued for culturing in a humidified incubator at 37 °C and 10% CO_2. On day 3, the supernatant was collected and centrifuged at 2000 *g* for 10 min in order to remove cell debris. The supernatant was stored at 4 °C and 8 ml fresh TCM + FBS was added to continue the culture. On day 4, the supernatant was collected again and centrifuged at 2000 *g* for 10 min to remove cell debris. All collected supernatant was filtered through a 0.45 µm filter. The supernatant was concentrated by centrifuging at 4000 *g*, room temperature for 20 min by using Amicon® Ultra – 15 centrifugal filters (cut off: 100K nominal molecular weight limit (NMWL), Merck

Millipore Ltd.). The concentrated retrovirus was aliquoted in cryotubes and stored in -80 °C for further use.

Retroviral transduction of T cells

$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells were restimulated for two days as described before (section 2.2.1). On day two, a Nycoprep gradient was applied to remove dead cells and thymocytes that had been used as APCs. Briefly, T cells were resuspended in 2 ml EH and then overlaid on 2 ml Nycoprep in a 15 ml Falcon tube followed by centrifuging at 675 *g* for 10 min with mild brake. The 2 ml interface between Nycoprep and EH were taken out and washed with 15 ml PBS. The cells were resuspended with appropriate amount of TCGF to make a concentration of 3×10^6 cells/ml. Polybrene (Sigma) was added at 8 µg/ml. The cells were plated in non-TC treated 12-well plates at 500 µl/well. Concentrated retrovirus was added onto T cells at 50 µl/well followed by centrifuging at 2000 *g*, for 90 min at room temperature. 1 ml/well TCGF was added and T cells were cultured in a humidified incubator at 37 °C with 10% CO_2. One day later, the cells were transferred into 100 mm dishes and 0.5 µg/ml puromycin (Santa Cruz) were applied to select the successfully transduced T cells for 3 days. FACS analysis was used for checking the transduction efficiency based on BFP expression in T cells.

2.2.9 RNA-Sequencing

Microglia and macrophages from the CNS of EAE rats (three days after adoptive transfer of 5×10^6 $T_{MBP\text{-}GFP}$ cells); microglia from naive rats; macrophages from the spleen of naive rats were sorted based on CD45 and CD11b expression by using a BD FACSAria IIIu cell sorter. Total RNA was extracted by using the RNeasy Plus Micro Kit according to the manufacturer's instruction. The concentration of the total RNA was evaluated by using the Agilent RNA 6000 Pico kit according to manufacturer's instruction on an Agilent 2100 bioanalyzer. mRNA library was prepared by using Collibri™ 3' mRNA library prep kit according to the manufacturer's instruction. The Agilent™ high sensitivity DNA kit was used to assess the library quality before submission to sequencing. Illumina HiSeq1500 sequencer was used for performing the sequencing at Gene Center Munich, LMU. Sequencing data was aligned

to rat genome by RNA STAR (Galaxy Version 2.7.2b). Gene Set Enrichment Analysis (GSEA) was used to identify the enriched gene sets in sequencing data by running against the Molecular Signature Database (MSigDB) (Subramanian, Tamayo et al. 2005).

2.2.10 Statistical analysis

All results were analyzed using GraphPad Prism 7. Data were shown as mean ± S.E.M. Significance was indicated according to *p*-value (**p* <0.05, ***p* <0.01, ****p* <0.001, ns = no significant difference).

3 RESULTS

3.1 Microglia are insufficient to activate CD4$^+$ T cells as APCs

3.1.1 Highly purified primary microglia

To study the function of microglia in activating CD4$^+$ T cells, we started with an *in vitro* approach to get first insights into whether microglia interact with CD4$^+$ T cells. Therefore, firstly we established primary microglia culture from brain and spinal cord of adult Lewis rats. As described in the methods (section 2.2.1), the meninges were mechanically removed and microglia were purified by using Percoll gradients. To check the purity of freshly isolated microglia from the CNS of naive Lewis rats, the cells were stained with anti-CD45 and anti-CD11b antibodies.

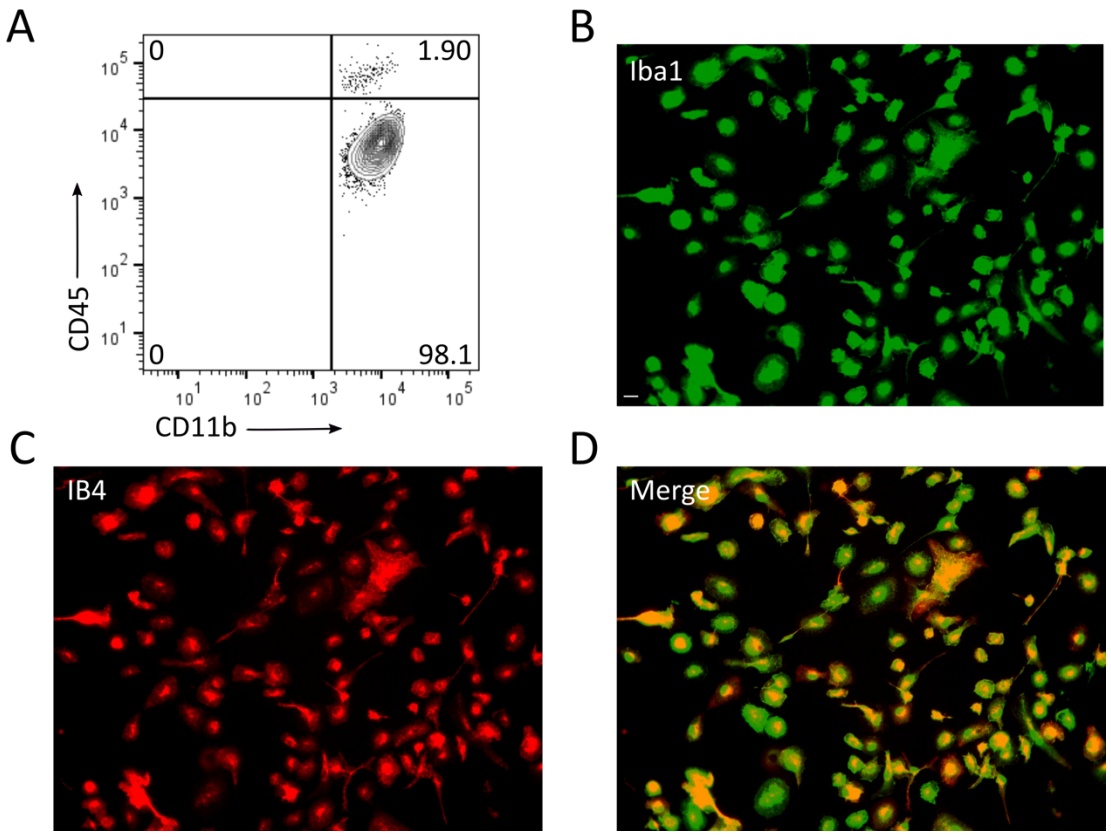

Fig. 3.1.1| Highly purified primary microglia. A. Freshly isolated cells from naive Lewis rat's CNS were stained with anti-CD45 and anti-CD11b antibodies. $CD11b^{+}CD45^{high}$ indicates macrophage; $CD11b^{+}CD45^{low}$ indicates microglia. B-D. Cells were cultured for 6 days and stained with anti-Iba1 antibody (B) and isolectin GS-IB4 (IB4) conjugated with Alexa Fluor™ 594 (C). Most of the cells were double-stained as shown in the merged image (D). Scale bar 20 µm. The results are representative of three independent experiments (A) or two independent experiments (B-D).

FACS analysis showed that a vast majority of cells were $CD11b^{+}CD45^{low}$ microglia with minor contamination of $CD11b^{+}CD45^{high}$ monocytes (Fig. 3.1.1 A). After culturing for 6 days, cells were stained with anti-Iba1 antibody and isolectin GS-IB4 (IB4). Although these reagents stain microglia, IB4 can also recognize endothelial cells (Alroy, Goyal et al. 1987) and anti-Iba1 antibody can recognize monocytes/macrophages. All cultured cells were double positive for Iba1 and IB4 (Fig. 3.1.1 B-D), indicating those cells are pure microglia. Taken together, microglia can be efficiently isolated from the CNS of adult rats and be cultured for *in vitro* studies.

3.1.2 The role of microglia in inducing $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation

The activation sensor NFAT-GFP in $CD4^{+}$ T cells translocates within a few minutes when T cells get stimulation and has been used to visualize the activation of T cells in real time (Pesic, Bartholomaus et al. 2013). In addition, GFP allows tracking of the T cells both *in vitro* and *in vivo*. Therefore, the NFAT-GFP activation sensor can be used for investigating whether microglia interact with $CD4^{+}$ T cells and induce activation of the T cells. To this end, resting MBP-specific T cells which express NFAT-GFP ($T_{MBP\text{-}NFAT\text{-}GFP}$ cells) were co-cultured with microglia and live imaging was performed to observe the activation of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells which is detected by NFAT-GFP translocation. In this co-culture system, microglia were labeled with Alexa Fluor 594 conjugated IB4 to distinguish them from $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. Microglia and $T_{MBP\text{-}NFAT\text{-}GFP}$ cells can be clearly detected and distinguished as shown in bright-field images and in images from the GFP/RFP channels (Fig. 3.1.2 A). Fig. 3.1.2 B shows an example of how the translocation of NFAT-GFP occurred in T cells. The time point when T cell and microglia interaction started was set as 0 min. Here, the sequential images showed that 2 min after the interaction had started, the GFP signal in the T cell translocated from

the cytosol to the nucleus, indicating activation of the T cell (Fig. 3.1.2 B). Therefore, this co-culture system can be efficiently used to investigate the interaction of T cells and microglia and T cell activation.

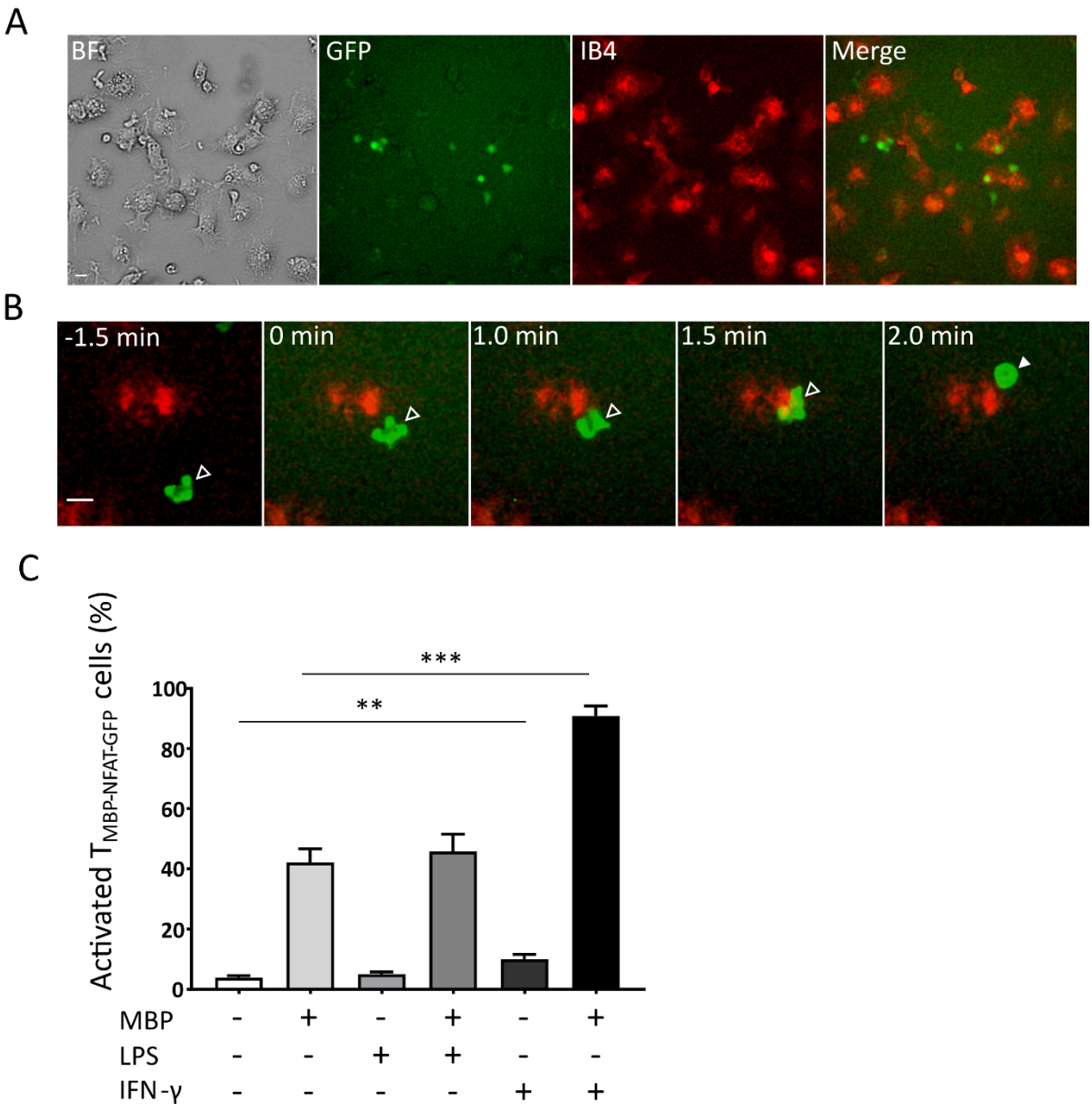

Fig. 3.1.2| *In vitro* co-culture of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells and microglia. A. Fluorescent images of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells (GFP) and IB4 stained microglia (IB4). Bright-field (BF). Scale bar 10 µm. B. An example of T cell and microglia interaction and the translocation of NFAT-GFP. Scale bar 10 µm. Inserted numbers indicate the time after contact starts. The time point when interaction starts is set as 0 min. Open arrowheads indicate non-activated T cells, filled arrowhead indicates an activated T cell. C. Quantification of microglia induced T cell activation by analyzing the translocation of NFAT-GFP in T

cells. In some experiments, microglia were pre-incubated with LPS (1 µg/ml), IFN-γ (100 ng/ml), MBP (10 µg/ml) or combination as indicated. The results are representative (A, B) or means +/- S.E.M of three independent experiments, $**p < 0.01$, $***p < 0.001$, unpaired t test.

By using this *in vitro* co-culture system, we evaluated the T cell activation capacity of microglia under different conditions. We imaged every 30 sec for 30min and quantified T cell activation. Around 4% $T_{MBP\text{-}NFAT\text{-}GFP}$ cells underwent NFAT-GFP translocation when they were co-cultured with non-treated microglia from naive rats, indicating that quiescent microglia alone hardly induced $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation (Fig. 3.1.2 C). Therefore, we asked whether activated microglia could induce T cell activation. First, we used LPS to induce microglia activation before co-culturing them with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. However, LPS-stimulated microglia did not show increased ability of T cell activation (Fig. 3.1.2 C).

Next we tested whether IFN-γ, a T_H1 cell signature cytokine, can enable microglia to obtain the capacity to activate $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. IFN-γ was applied to the primary microglia culture before co-culture with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. IFN-γ-stimulated microglia induced around 10% of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells activation which is significantly higher than naive microglia, suggesting that IFN-γ enhanced microglia's capacity to activate $T_{MBP\text{-}NFAT\text{-}GFP}$ cells (Fig. 3.1.2 C). Of note, microglia that had been pre-treated with LPS or IFN-γ were washed intensively before co-culturing with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells to rule out the side effects of those stimulations directly on $T_{MBP\text{-}NFAT\text{-}GFP}$ cells.

To evaluate whether microglia can activate $T_{MBP\text{-}NFAT\text{-}GFP}$ cells antigen-dependently, naive or LPS/IFN-γ-stimulated microglia were pre-incubated with MBP for 2 hrs before co-culturing with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. Interestingly, the results showed that MBP alone increased the proportion of activated T cells from 4% to 42% (Fig. 3.1.2 C). Moreover, the addition of MBP also enhanced the T cell activation capacity of LPS/IFN-γ-stimulated microglia. Among all conditions, MBP-treated IFN-γ-stimulated microglia acquired strongest capacity in activating $T_{MBP\text{-}NFAT\text{-}GFP}$ cells (Fig. 3.1.2 C). These results indicate that microglia can induce $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation antigen-dependently although microglia were not saturated by endogenous MBP.

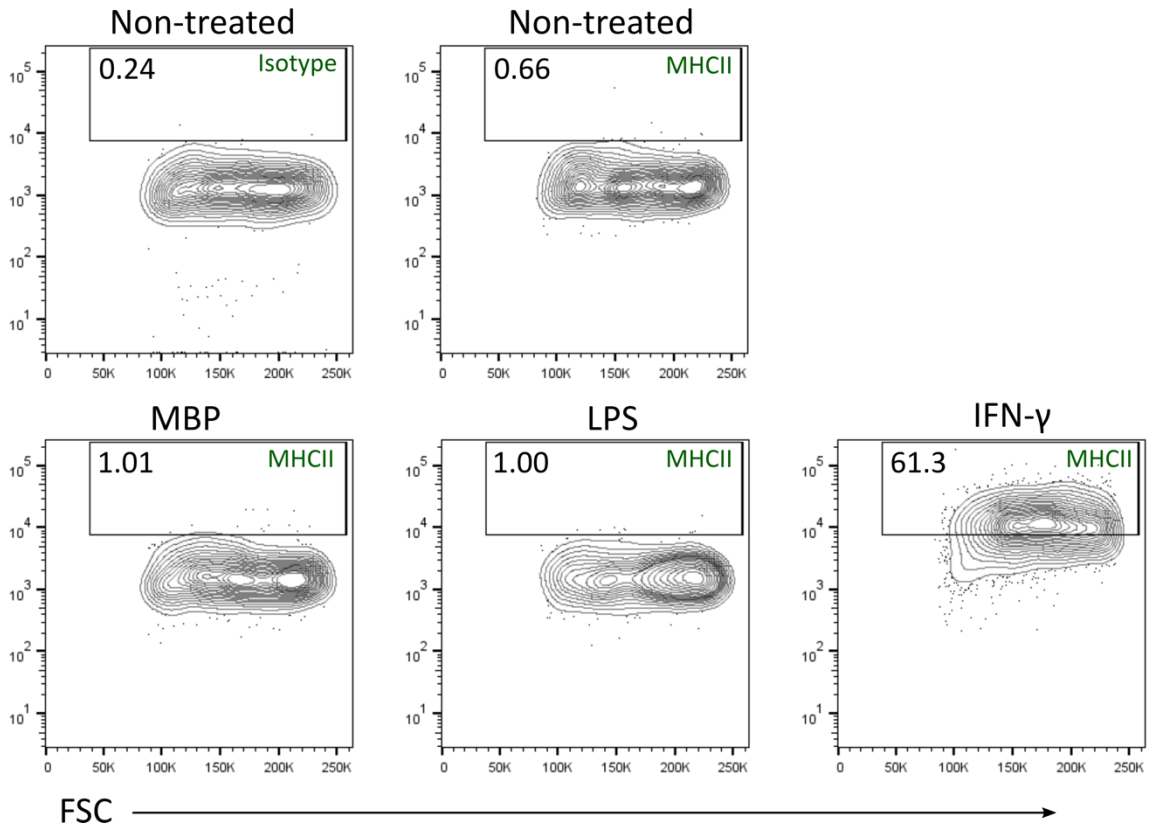

Fig. 3.1.3| MHCII expression on cultured microglia. Non-treated; MBP (10 µg/ml for 2 hrs); LPS (1 µg/ml for 24 hrs), or IFN-γ (100 ng/ml for 24 hrs) treated microglia were stained with anti-MHCII or isotype control antibodies. The results are representative of two independent experiments.

It has long been considered that antigen presented by APCs via MHCII regulates $CD4^+$ T cell activation (London, Lodge et al. 2000). To understand more in depth about T cell activation by microglia, MHCII expression on microglia was assessed. Flowcytometric analysis showed that MHCII expression on non-treated microglia was very low (Fig. 3.1.3). However, MBP and LPS treatments do not influence the MHCII expression on microglia (Fig. 3.1.3), although these treatments significantly increased T cell activation upon following co-culture (Fig. 3.1.2). This discrepancy is discussed later (section 4.1). In contrast, IFN-γ enhanced MHCII expression dramatically, which is in line with the T cell activation capacity of the pre-treated microglia. Taken together, quiescently cultured microglia cannot induce $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation. LPS stimulation was not enough to fulfill the capacity of microglia to activate $T_{MBP\text{-}NFAT\text{-}GFP}$ cells, whereas with the upregulation of MHCII by IFN-γ and additional antigen, microglia acquired strong ability to activate $T_{MBP\text{-}NFAT\text{-}GFP}$ cells.

3.1.3 The role of microglia in inducing $T_{OVA\text{-}NFAT\text{-}GFP}$ cell activation

Since microglia were prepared from the CNS parenchyma by using Percoll gradients after mechanical homogenization, we cannot exclude the contamination of endogenous MBP in the culture that may influence the results. In addition, as described above, microglia were not saturated with endogenous MBP. Still, to exclude this possibility, we looked at NFAT-GFP-expressing OVA-specific T cells ($T_{OVA\text{-}NFAT\text{-}GFP}$ cells) and their interaction with microglia which do not present cognate antigen for $T_{OVA\text{-}NFAT\text{-}GFP}$ cells. Surprisingly, IFN-γ-stimulated microglia alone significantly increased $T_{OVA\text{-}NFAT\text{-}GFP}$ cell activation to 19%, compared to 2% by non-treated microglia (Fig. 3.1.4). Since the percentages of activated $T_{MBP\text{-}NFAT\text{-}GFP}$ and

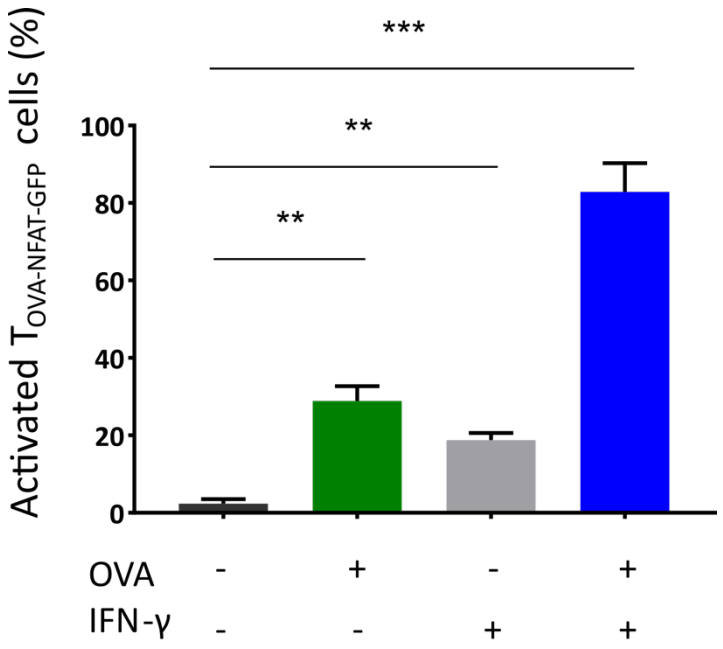

Fig. 3.1.4| *In vitro* co-culture of $T_{OVA\text{-}NFAT\text{-}GFP}$ cells and microglia. Quantification of activated T cells by analyzing translocation of NFAT-GFP in $T_{OVA\text{-}NFAT\text{-}GFP}$ cells induced by microglia with or without IFN-γ (100 ng/ml), OVA (10 µg/ml) or a combination of both. Means +/- S.E.M of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired t test.

$T_{OVA\text{-}NFAT\text{-}GFP}$ cells were similar, it is unlikely that contaminating MBP influenced T cell activation. More importantly, the results suggest the antigen-independent activation of T cells by microglia which is regulated by IFN-γ. Same as in the MBP condition, OVA treated microglia significantly increased the percentage of activated $T_{OVA\text{-}NFAT\text{-}GFP}$ cells to 30%,

indicating that microglia can activate T cells antigen dependently (Fig. 3.1.4). In addition, microglia treated with IFN-γ and OVA showed the highest potential in stimulating T cells (Fig. 3.1.4). Taken together, microglia can activate T cells via two different pathways, i.e. in an antigen-dependent manner and in an IFN-γ regulated antigen-independent mechanism, which are further discussed in section 4.1. These pathways can work together to activate T cells.

3.1.4 The role of phagocytes from the CNS of EAE rats in inducing T cell activation

Microglia pre-treated with IFN-γ, a T_H1 cell signature cytokine, activated both T_{MBP} cells and T_{OVA} cells more efficiently than non-treated microglia even without the presence of cognate antigens. Of note, during the acute phase of tEAE in Lewis rats, IFN-γ producing T_H1 cells were abundantly found in the CNS (Kawakami, Lassmann et al. 2004) where microglia exist. Therefore, we asked whether microglia from the inflamed CNS of EAE rats can activate T cells. We isolated phagocytes (containing microglia and macrophages) from EAE rats and co-cultured them with $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. Intriguingly, phagocytes alone strongly induced the activation of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells. Approximately 80% of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells were activated (Fig. 3.1.5 A), which was much higher than induced by IFN-γ-stimulated or MBP treated microglia (Fig. 3.1.2 C). This was possibly due to the fact that phagocytes from EAE rats were activated and were possibly loaded with endogenous cognate antigen during neuroinflammation and preparation of the primary culture. However, looking at $T_{OVA\text{-}NFAT\text{-}GFP}$ cells which were co-cultured with phagocytes from EAE rats revealed that around 40% of $T_{OVA\text{-}NFAT\text{-}GFP}$ cells were activated (Fig. 3.1.5 A), implying that there is an antigen-independent pathway of the T cell activation. With additional antigen, the proportion of activated T cells were increased and around 95% $T_{MBP\text{-}NFAT\text{-}GFP}$ cells and 90% $T_{OVA\text{-}NFAT\text{-}GFP}$ cells were activated (Fig. 3.1.5 A), indicating phagocytes can activate T cells antigen dependently.

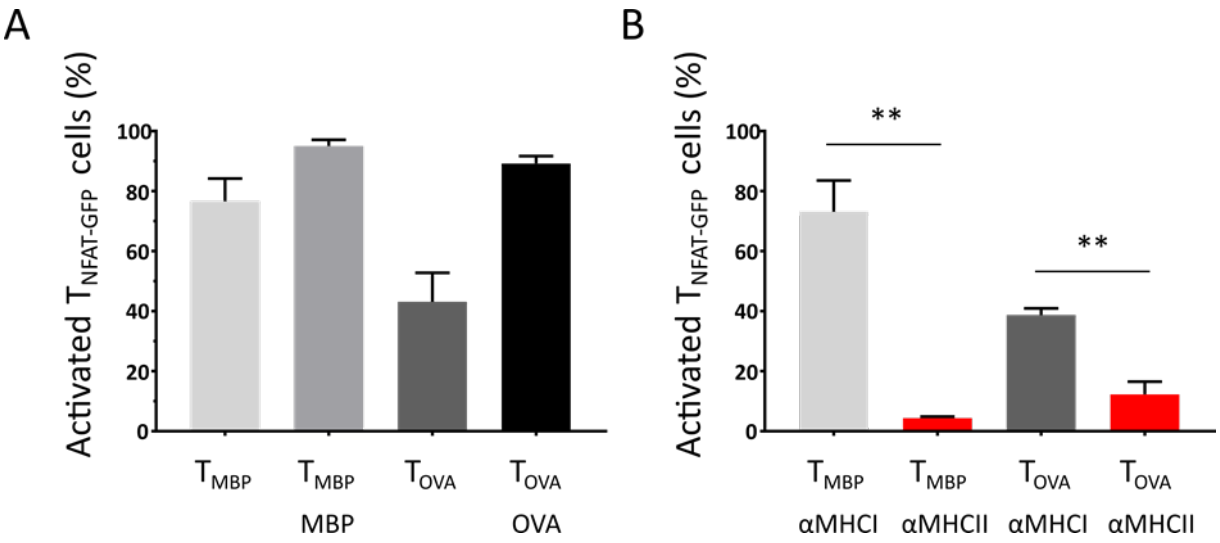

Fig. 3.1.5| Phagocytes from EAE rat induced T cell activation. A. Proportions of activated T cells in co-culture with phagocytes (microglia/macrophages). $T_{MBP\text{-}NFAT\text{-}GFP}$ cells/$T_{OVA\text{-}NFAT\text{-}GFP}$ cells co-cultured with or without antigen (MBP (10 µg/ml) or OVA (10 µg/ml)). B. Quantification of activated $T_{MBP\text{-}NFAT\text{-}GFP}$ cells/$T_{OVA\text{-}NFAT\text{-}GFP}$ cells in co-culture with phagocytes together with anti-MHCII blocking antibody (αMHCII) or control anti-MHCI antibody (αMHCI) (10 µg/ml). Means +/- S.E.M of three independent experiments, $^{**}p < 0.01$, unpaired t test.

To confirm whether T cell activation induced by phagocytes was regulated by MHCII, we applied the anti-MHCII blocking antibody to the phagocytes before co-culturing them with $T_{MBP\text{-}NFAT\text{-}GFP}$/$T_{OVA\text{-}NFAT\text{-}GFP}$ cells. The blocking of MHCII strongly but not completely prevented the activation of T cells (Fig. 3.1.5 B), suggesting that antigen independent T cell activation can be subdivided to MHC-dependent and -independent mechanisms.

3.1.5 Macrophages are more efficient APCs than microglia in EAE

Peripheral macrophages infiltrate the CNS during the acute phase of EAE, thereby cultured phagocytes from the CNS of EAE rats are a mixture of infiltrated macrophages and microglia. To assess the cell composition of the cultured phagocytes from EAE rats, we stained the cultured cells with anti-CD11b and anti-CD45 antibodies. Flowcytometry analysis showed that around 25% were macrophages and 57% were microglia in our *in vitro* culture (Fig. 3.1.6 A). To compare the ability of microglia and infiltrated macrophages to induce $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation, we sorted macrophages ($CD11b^{+}CD45^{high}$) and microglia ($CD11b^{+}CD45^{low}$)

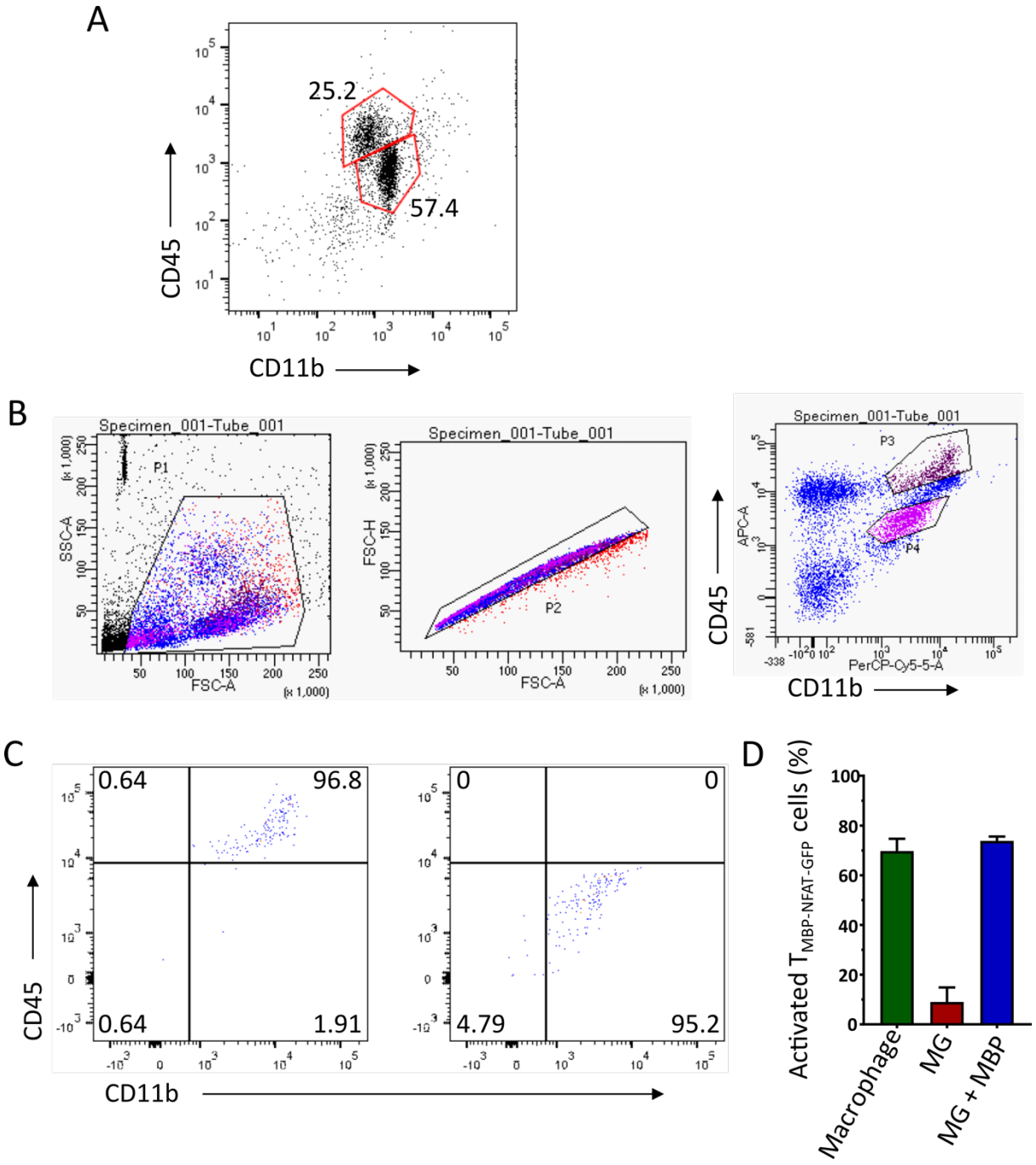

Fig. 3.1.6| Activation capacity of macrophages and microglia from EAE rats. A. Phagocytes from EAE rat were stained with anti-CD45 and anti-CD11b antibodies after culturing for 6 days. n = 1. B. Gating strategy to distinguish $CD11b^{+}CD45^{high}$ macrophages (P3) and $CD11b^{+}CD45^{low}$ microglia (P4). P3 and P4 were sorted separately for further culturing. C. The purity of sorted P3 and P4 population. D. Quantification of T cell activation induced by macrophages and microglia (MG) by analyzing the translocation of NFAT-GFP in T cells. MBP (10 µg/ml) was added to co-culture of microglia/T cell

(MG+MBP) directly. Representative data (B, C) and means +/- S.E.M (D) of three independent experiments.

(Fig. 3.1.6 B). The purity of microglia and macrophages was assessed by flowcytometry, which showed more than 95% purity in both microglia and macrophages (Fig. 3.1.6 C). The purified microglia and macrophages were further cultured for 6 days and were used for co-culture with T cells and following imaging (Fig. 3.1.6 D). Interestingly, in the absence of exogenous MBP, we found that macrophage alone activated 70% of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells, whereas only 9% of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells were activated by microglia (Fig. 3.1.6 D). The proportion of activated $T_{MBP\text{-}NFAT\text{-}GFP}$ cells by microglia was comparable to the one by IFN-γ-stimulated microglia (Fig. 3.1.2 C), suggesting that microglia from EAE rats were experienced with T_H1 cell cytokines. Moreover, after adding MBP into the co-culture, 74% of $T_{MBP\text{-}NFAT\text{-}GFP}$ cells were activated (Fig. 3.1.6 D), which further supports the idea that *ex vivo* microglia acquired from EAE rats remained activated and were able to activate T cells efficiently when antigen is provided. These findings are in line with the previous results that IFN-γ-stimulated microglia were potent activators of T cells when cognate antigen was present as shown in Figure 3.1.2. Taken together, in the acute phase of EAE, CNS-infiltrated macrophages instead of microglia are more efficient in activating $T_{MBP\text{-}NFAT\text{-}GFP}$ cells, although both cell types are able to stimulate T cells.

To evaluate the contribution of MHC-TCR interaction to the observed T cell activation, we analyzed the MHCII expression on macrophages and microglia from both naive and EAE rats. Flowcytometric analysis showed that in both naive and EAE rats, peripheral macrophages expressed MHCII (Fig. 3.1.7). In contrast, the macrophages and microglia in the CNS of naive rats expressed very low levels of MHCII (Fig. 3.1.7 A). Once the rats developed clinical EAE, both infiltrated macrophages and microglia upregulated MHCII (Fig. 3.1.7 B), although microglia upregulated MHCII not as high as in infiltrated macrophages. The expression of MHCII in macrophages and microglia in EAE rat correlated to their ability to activate T cells.

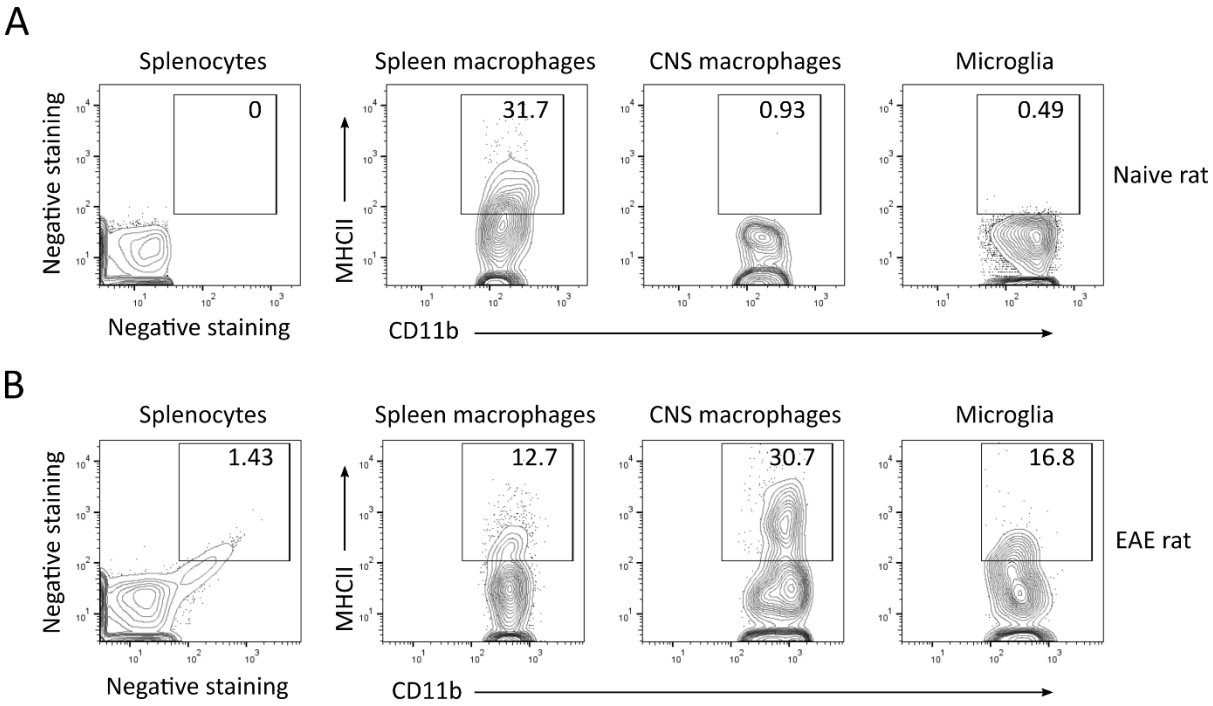

Fig. 3.1.7| MHCII expression profile on microglia and macrophages in naive and EAE rats. Microglia and macrophages from naive (A) and EAE rats (B) were stained with anti-MHCII antibody and were pre-gated on live $CD11b^{+}CD45^{high}$ (macrophages) and $CD11b^{+}CD45^{low}$ (microglia) cells. Splenocytes without staining were used for gating. The results are representative of two independent experiments.

3.2 Microglia differentiate $CD4^{+}$ T cells into T_H1 cells

For our live imaging experiments we co-cultured T cells and microglia for 30 min, and such short interactions may not be sufficient to induce detectable changes in gene expression in the T cells. Therefore, to understand whether T cell activation observed in imaging can change gene expression of T cells, we co-cultured T cells with microglia for 24 hrs and analyzed their RNA and protein expression profiles. At first, we looked at IFN-γ protein expression in the T cells, as a key cytokine which is expressed after T cell activation. Interestingly, we observed that microglia alone upregulated IFN-γ expression in GFP expressing MBP-specific T cells ($T_{MBP\text{-}GFP}$ cells). Approximately 40% of the T cells expressed IFN-γ by co-culturing with microglia whereas almost no T cells express this cytokine when the T cells were cultured alone (Fig. 3.2.1 A and B). Addition of exogenous MBP further

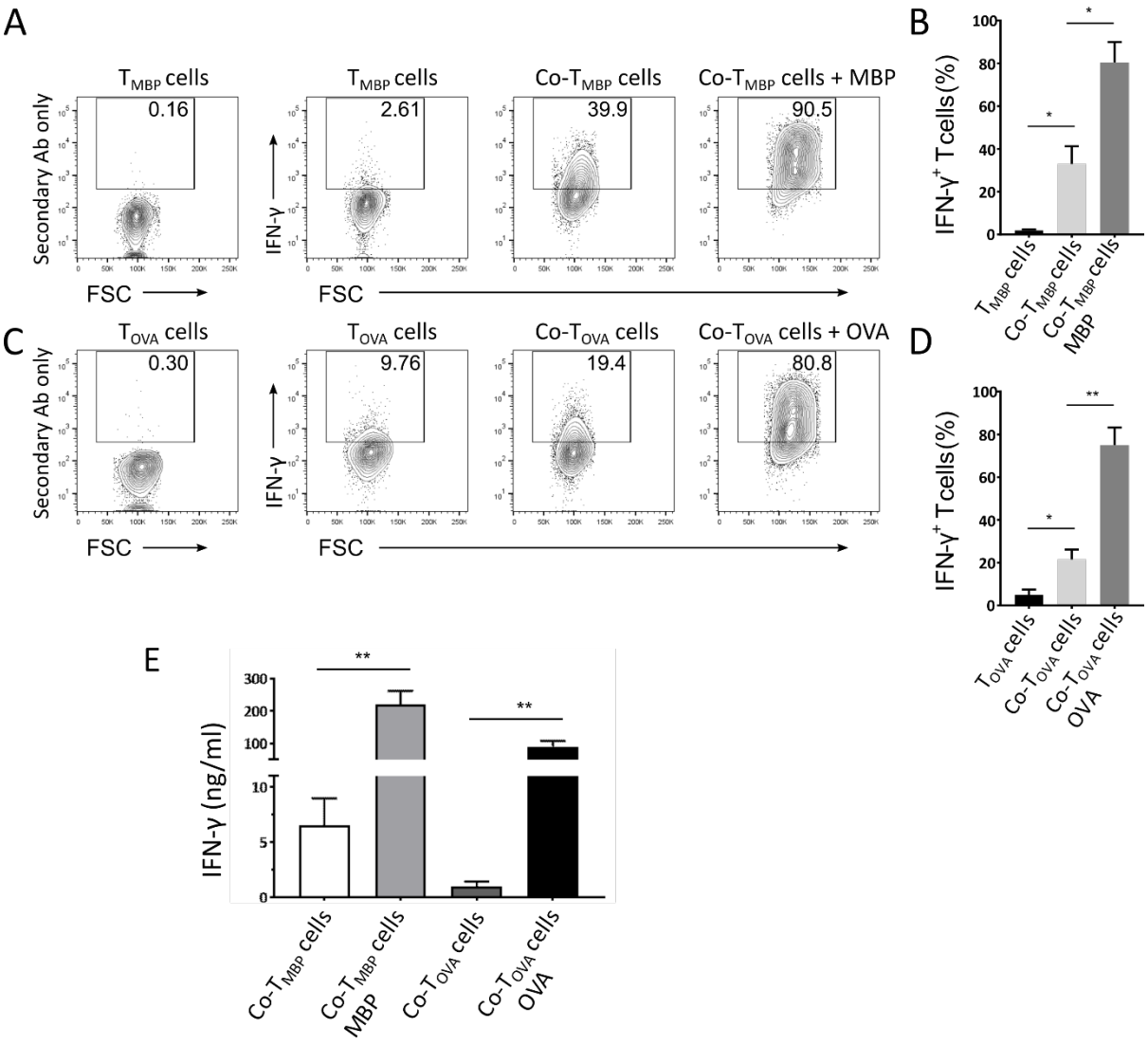

Fig. 3.2.1| Microglia induce IFN-γ in $CD4^+$ T cells. A and C. Microglia and $T_{MBP\text{-}GFP}$/$T_{OVA\text{-}GFP}$ cells were co-cultured for 24 hrs with or without MBP (10 µg/ml)/OVA (10 µg/ml), respectively (Co-$T_{MBP/OVA}$ cells + MBP/OVA or Co-$T_{MBP/OVA}$ cells). As control, $T_{MBP\text{-}GFP}$/$T_{OVA\text{-}GFP}$ cells were cultured alone ($T_{MBP/OVA}$ cells). IFN-γ was analyzed by intracellular staining. $T_{MBP\text{-}GFP}$/$T_{OVA\text{-}GFP}$ cells stained with secondary antibodies (Ab) only were used for gating. The results are representative of three independent experiments. B. D. Quantification of IFN-γ^+ T cells. Means +/- S.E.M from three independent experiments, *$p < 0.05$, **$p < 0.01$, unpaired t test. E. IFN-γ concentrations in supernatants of co-cultures were analyzed by ELISA. Means +/- S.E.M of three independent experiments, **$p < 0.01$, unpaired t test.

increased the proportion of IFN-γ expressing T cells and almost all T cells expressed IFN-γ (Fig. 3.2.1 A and B). To test the antigen dependency of IFN-γ induction by microglia, we

analyzed IFN-γ expression in GFP expressing OVA-specific T cells ($T_{OVA\text{-}GFP}$ cells) after co-culturing with microglia for 24 hrs with or without OVA. As similar as observed in $T_{MBP\text{-}GFP}$ cells, microglia induced IFN-γ expression in $T_{OVA\text{-}GFP}$ cells in the absence of OVA, but with lesser extent than in $T_{MBP\text{-}GFP}$ cells (Fig. 3.2.1 C and D), suggesting that microglia induced IFN-γ expression in $CD4^+$ T cells in an antigen-independent manner. Same as the $T_{MBP\text{-}GFP}$ cells, addition of cognate antigen to the co-culture induced IFN-γ expression in most of the T_{OVA} cells (Fig. 3.2.1 C and D). The induction of IFN-γ expression was confirmed in the supernatant of the co-culture, which is correlated with the results of intracellular staining (Fig. 3.2.1 E). Taken together, microglia can induce IFN-γ production in $CD4^+$ T cells in both antigen-dependent and antigen-independent manners.

3.3 Cytokine expression regulated by microglia and T cells interaction

3.3.1 Microglia regulate cytokine expression in T_{MBP} cells

In rat tEAE, activation related genes are strongly upregulated in T_{MBP} cells especially in the CNS parenchyma as compared to the T_{MBP} cells in the meninges, CSF and blood on day 3 after adoptive transfer (Schlager, Korner et al. 2016). Since this time point is before peripheral macrophages robustly invade into the CNS, the activation of T cells can be induced by microglia. In addition, our results from the *in vitro* co-culture system had shown that microglia induced IFN-γ in $CD4^+$ T cells. These findings promoted us to wonder whether local neuronal cells play an important role in changing the phenotype of infiltrating T cells during EAE development. We set out to investigate whether neuronal cells regulate T cell phenotypes by analyzing mRNA expression. Especially, we focused on the regulation of cytokines by microglia. Therefore, we sorted co-cultured T_{MBP} cells to separate them from microglia for qPCR analysis. We used restimulated T_{MBP} cells with irradiated thymocytes in presence of MBP as positive control and T cell which were cultured alone as negative control. Surprisingly, we found that after co-culturing with microglia in the absence of MBP for 24 hrs, T_{MBP} cells significantly upregulated pro-inflammatory cytokines, including *Ifng* 95 times

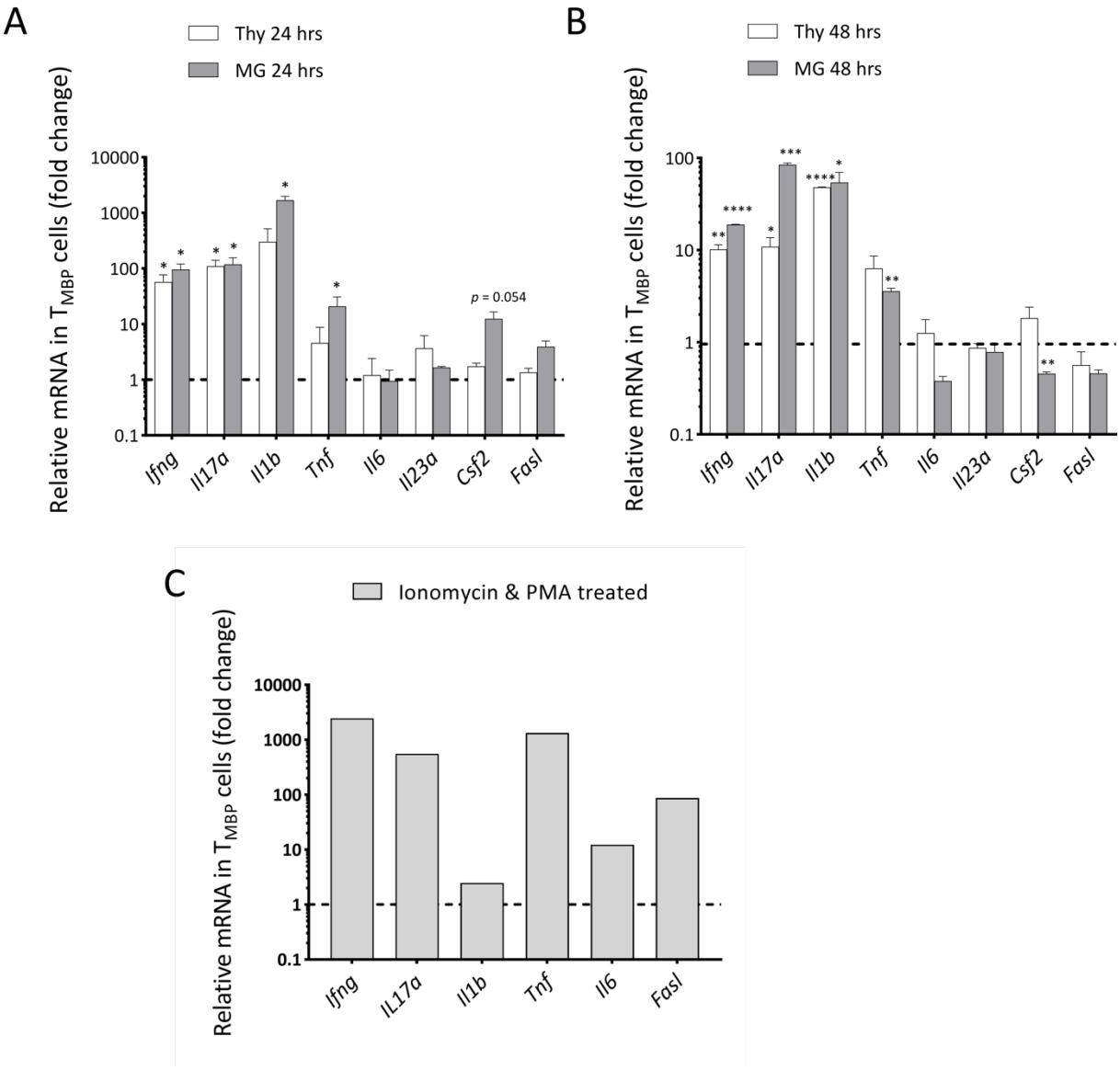

Fig. 3.3.1| Microglia induce pro-inflammatory cytokine expression in T_{MBP} cells. A, B. The mRNA levels of cytokines in T_{MBP} cells were analyzed by qPCR after co-culturing with microglia (MG); restimulated by thymocytes (Thy) for 24 hrs (A) or 48 hrs (B) respectively. As negative control, T cells were cultured alone for the same time periods. The mRNA expression is shown relative to negative control (dash line). Means +/- S.E.M of two to three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, unpaired t test, compared to negative control. C. The mRNA levels of cytokines were analyzed in non-stimulated and Ionomycin/PMA-stimulated T_{MBP} cells. The mRNA expression is shown relative to non-treated T cells (dash line). n = 1.

and *Tnf* 25 times higher compared to the negative control. Notably, microglia dramatically upregulated *Il1b* expression in T_{MBP} cells up to 1000 times compared to the negative control

(Fig. 3.3.1 A). After co-culturing with microglia for 48 hrs, T_{MBP} cells expressed around 18 times more *Ifng*, 2 times more *Tnf* and 50 times more *Il1b* as compared to the negative control (Fig. 3.3.1 B). Of note, after co-culturing with microglia for 48 hrs, although *Ifng, Il17a, Il1b and Tnf* remain significantly upregulated, the increased level was lower as compared to 24 hrs, suggesting that induction of these genes in T_{MBP} cells happened at a rather early phase and mRNA decreased quickly. More importantly, the upregulation of the cytokines by microglia in the absence of antigen was comparable to restimulated T_{MBP} cells by thymocytes in the presence of MBP (Fig. 3.3.1 A and B), suggesting that microglia have a high potential to stimulate T cells. In contrast to inflammatory cytokines, comparable expression levels of Treg or T_H17 cytokines, such as *Il6* and *Il23a*, were observed in all conditions, indicating that expression of these cytokines is not regulated by microglia. GM-CSF (encoded by *Csf2*) which is produced by pathogenic T cells has been recognized as critical cytokine for the recruitment of myeloid cells into the CNS during neuroinflammation (Komuczki, Tuzlak et al. 2019). There is a trend towards an increase of *Csf2* in T cells after co-culturing with microglia for 24 hrs, whereas *Csf2* was downregulated after 48 hrs of co-culture (Fig. 3.3.1 A and B). FasL is constitutively expressed on $CD4^+$ T cells and was found to regulate myeloid cells to produce IL-1β (Jain, Irizarry-Caro et al. 2020). We did not observe an upregulation of *Fasl* in T_{MBP} cells after co-culturing with microglia or thymocytes (Fig. 3.3.1 A and B). Taken together, microglia were strong regulators of inflammatory cytokines in T_{MBP} cells in an antigen-independent manner. These upregulated cytokines might play a role in promoting the inflammatory cascade at the early phase of EAE. In addition, we looked at cytokine levels in ionomycin/PMA-stimulated T_{MBP} cells, the preliminary results showed no upregulation of *Il1b* (Fig. 3.3.1 C), although other cytokines were upregulated as similar as in the T cells that were co-cultured with microglia. The result indicates a unique pathway regulates *Il1b* expression in T_{MBP} cells by microglia.

3.3.2 Microglia regulate cytokine expression in T_{OVA} cells

To further confirm the antigen-independent activation of T cells by microglia, we co-cultured T_{OVA} cells with microglia and cytokine profile of T cells were analyzed by qPCR. As similar as observed in T_{MBP} cells, T_{OVA} cells upregulated inflammatory cytokines when co-

cultured with microglia. For example, *Ifng, Il17a* and *Il1b* expression were significantly upregulated and a trend towards an increase of *Tnf* was observed (Fig. 3.3.2). Also, like in T_{MBP} cells, we did not observe upregulation of *Il6*, *Il23a*, *Csf2* and *Fasl* (Fig. 3.3.2). Taken together, microglia can induce cytokine expression in $CD4^+$ T cell without cognate antigens.

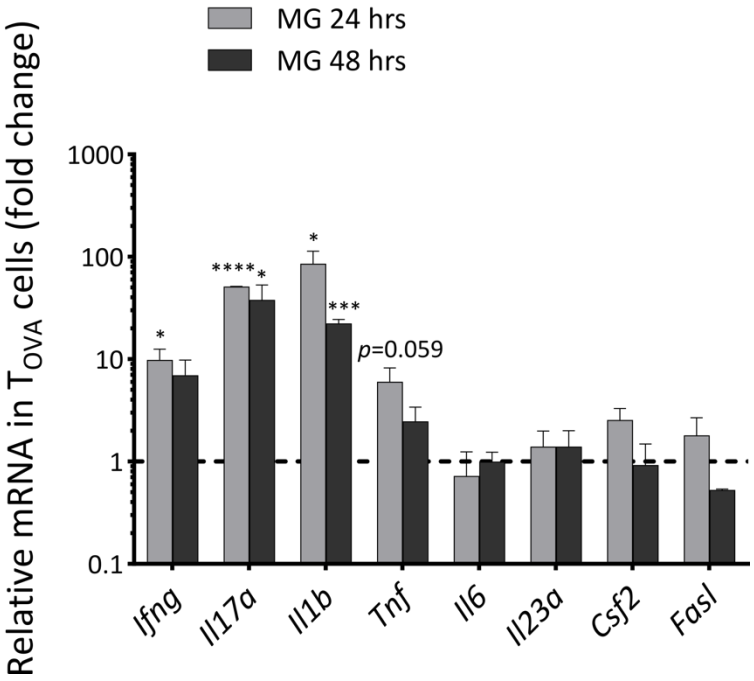

Fig. 3.3.2| Microglia induce pro-inflammatory cytokine expression in T_{OVA} cells. The mRNA levels of cytokines were analyzed in T_{OVA} cells by qPCR after co-culturing with microglia for 24 hrs or 48 hrs, respectively. As negative control, T cells were cultured alone for same time periods. The mRNA expression is shown relative to negative control (dash line). Means +/- S.E.M of two independent experiments, $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$, unpaired t test, compared to negative control.

3.3.3 T_{MBP} cells regulate gene expression in Microglia

As shown above, microglia induced cytokine expression in T cells. Therefore, we could hypothesize that T cells change microglia phenotype. To answer this question, we evaluated gene expression of *Il1b, Il6* and *Tnf* in microglia after co-culturing with T_{MBP} cells for 24 hrs and 48 hrs. Surprisingly, our preliminary results showed that T cells did not induce upregulation of the typical innate cytokines *Tnf* and *Il1b* in microglia, we solely observed an increase of *Il6* after co-culturing for 24 hrs (Fig. 3.3.3). A previous study showed that mouse myeloid cells produced IL-1β when co-cultured with $CD4^+$ T cells that were stimulated with

anti-CD3 and anti-CD28 antibodies (Jain, Irizarry-Caro et al. 2020). In contrast, microglia alone can induce IL-1β production by rat T cells but rat $CD4^+$ T cells do not induce the production of IL-1β in microglia, although microglia do induce in T cells (Fig. 3.3.1 and Fig. 3.3.3). These results imply the existence of distinct pathways for regulating the activation of microglia and $CD4^+$ T cells in different species.

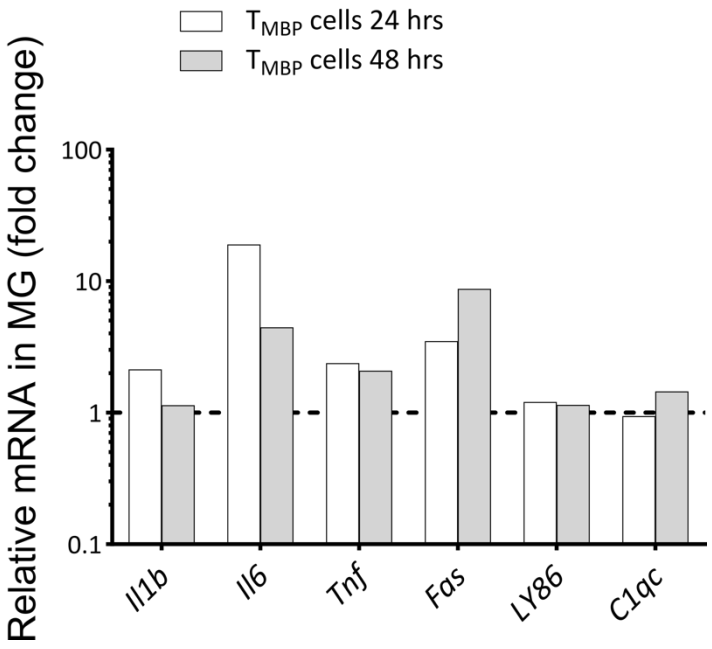

Fig. 3.3.3| Gene expression in microglia. Microglia were co-cultured with T_{MBP} cells for 24 hrs and 48 hrs before T cells were washed out by Accutase® solution and PBS. qPCR was used for analyzing gene expression in microglia. As negative control, microglia were cultured alone. The mRNA expression is shown relative to negative control (dash line). n = 1.

Fas signaling has been reported play a role in regulating IL-1β production in myeloid cells and in controlling $CD4^+$ T cell differentiation (Meyer Zu Horste, Przybylski et al. 2018, Jain, Irizarry-Caro et al. 2020). Therefore, we examined *Fas* expression in microglia and found that its expression is slightly increased after co-culturing with T cells (Fig. 3.3.3). MD-1, encoded by the gene *LY86*, forms a complex with radioprotective 105 kDa protein to regulate B cell survival and proliferation were found upregulated in microglia instead of macrophages in a mouse EAE model (Miura, Shimazu et al. 1998, Jordao, Sankowski et al. 2019). However, we did not observe any changes of *LY86* expression in microglia in our co-

culture system (Fig. 3.3.3). A complement encoding gene *C1qc,* which was upregulated in disease-associated microglia in a mouse EAE model (Jordao, Sankowski et al. 2019), was also not regulated in our setup (Fig. 3.3.3). Taken together, T cells did not influence the phenotype of microglia except for a minor induction of Il6. Therefore, microglia are more like a director which control T cell activation.

3.3.4 T cells and microglia activation requires direct interaction

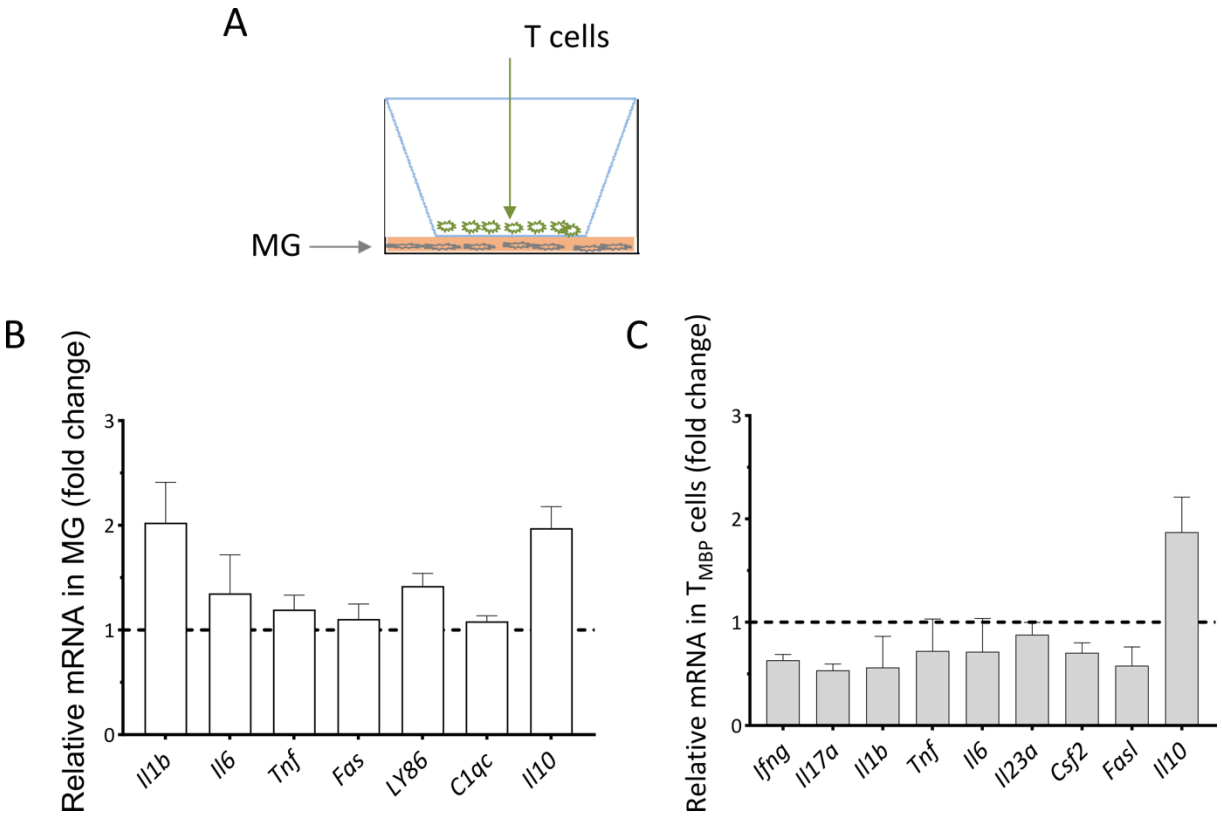

Fig. 3.3.4| T_{MBP} cell and microglia activation requires direct interaction. A. Scheme of transwell co-culture of T_{MBP} cells and microglia. T_{MBP} cells were cultured in the upper transwell insert, microglia were cultured in the lower wells of 6-well plates. B. and C. The mRNA expression of cytokines was analyzed by qPCR in microglia (B) and T_{MBP} cells (C) after 24 hrs of co-culture. Microglia and T cells which were cultured alone were used as negative control. The mRNA expression is shown relative to negative control (dash line). Means +/- S.E.M of three independent experiments.

To clarify whether activation of microglia and T_{MBP} cells was regulated by direct interaction or by soluble factors, we co-cultured T_{MBP} cells and microglia in a transwell system. In this

co-culture system, microglia and T_{MBP} cells were separated by a transwell insert (pore size 0.4 µm) and can only communicate through soluble factors (Fig. 3.3.4 A). After co-culturing for 24 hrs, the mRNA expression of selected genes was analyzed in both microglia and T_{MBP} cells. However, neither microglia (Fig. 3.3.4 B) nor T_{MBP} cells (Fig. 3.3.4 C) showed significant changes in a transwell co-culture system. Taken together, T_{MBP} cell and microglia activation require direct interaction, indicating that the ligand and receptor encounters play a crucial role.

3.4 Microglia induce membrane phenotype changes of T cells

In adoptive tEAE, *in vitro* restimulated T_{MBP} cells are maximally activated before injection and display upregulated activation markers, such as OX-40 and IL-2R (Flugel, Berkowicz et al. 2001). To interrogate whether microglia upregulate these markers on T_{MBP} cells, we co-cultured microglia with T_{MBP} cells for 24 hrs in the absence of MBP. In this experiment, we compared the expression of cell surface molecules, including TCR, OX-40, IL-2R and MHCII, with T cells which were fully restimulated with thymocytes in the presence of MBP for 24 hrs and 48 hrs. Strikingly, flowcytometry analysis showed that microglia substantially induced OX-40 and IL-2R expression on T_{MBP} cells to a comparable extent as fully restimulated T_{MBP} cells (Fig. 3.4.1). The results indicate that microglia can induce T cell activation in absence of antigen. As similar as previous experiments, microglia might uptake MBP during their preparation. To test whether microglia induced activation of T cells was due to the contamination of myelin, we looked at the OX-40 and IL-2R expression on T_{OVA} cells. Indeed, microglia upregulated OX-40 and IL-2R on T_{OVA} cells like T_{MBP} cells (Fig. 3.4.1). In addition, both T_{MBP} cells and T_{OVA} cells obtained MHCII expression (Fig. 3.4.1), which is considered to be expressed by acutely activated $CD4^+$ T cells (Reske, Mohle et al. 1987). Those results further support the idea that microglia can activate T cells in an antigen-independent manner.

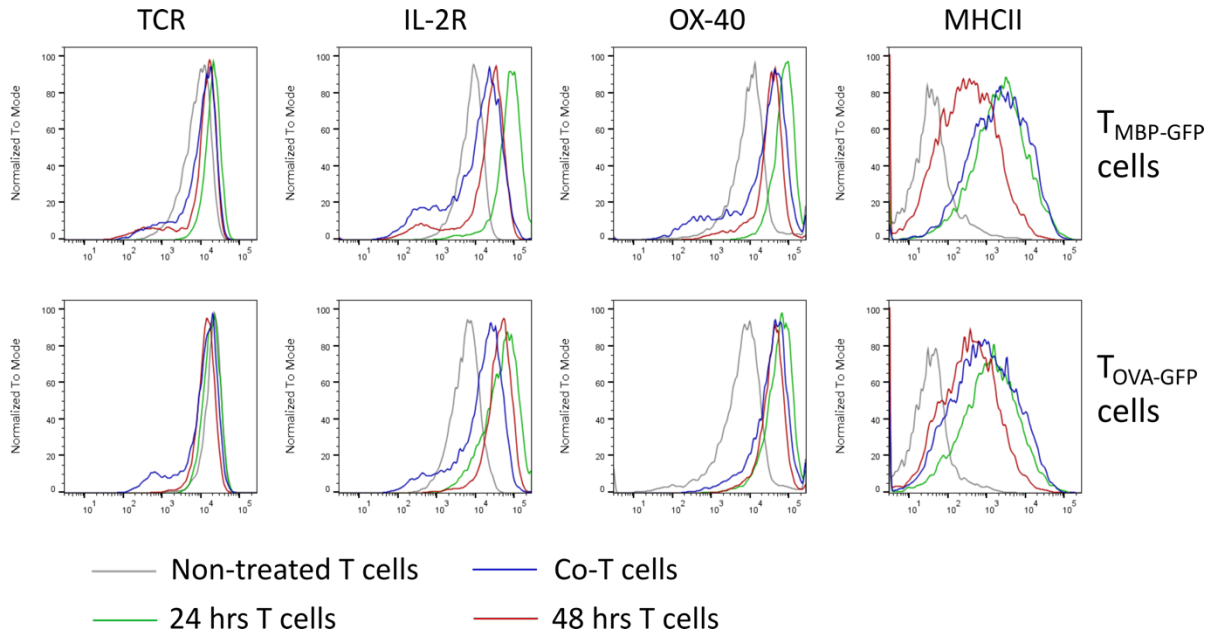

Fig. 3.4.1| Upregulation of activation markers on T cells after co-culturing with Microglia. $T_{MBP-GFP}$ cells/$T_{OVA-GFP}$ cells were restimuated by thymocytes for 24 hrs (24 hrs T cells) or 48 hrs (48 hrs T cells) in presence of exogenous MBP/OVA. $T_{MBP-GFP}$ cells/$T_{OVA-GFP}$ cells were co-cultured with microglia for 24 hrs (Co-T cells) in absence of exogenous MBP/OVA. Non-treated T cells indicate T cells which were cultured alone. The cells were stained for TCR, IL-2R, OX-40 and MHCII. Data are representative of three independent experiments.

3.5 Astrocytes are less effective in inducing T cell activation

Astrocytes are the most enriched neuronal cells in the CNS which were found play a key role in MS pathogenesis by enhancing the immune response and inhibiting remyelination (Nair, Frederick et al. 2008). A previous study showed that the communication between astrocytes and T_H1 and T_H17 cytokines define the entry site of T cells into the CNS (Williams, Manivasagam et al. 2020). Here, we investigated whether astrocytes interact with T_{MBP} cells and induce their activation. Firstly, we cultured primary astrocytes from neonatal rats. Anti-GFAP staining showed that the majority of cells were astrocytes in our primary culture (Fig. 3.5.1 A and B). Then we co-cultured primary astrocytes with T_{MBP} cells for 24 hrs followed by analyzing gene expression of cytokines in the T cells. Our results showed that *Il1b* was significantly upregulated, however no changes of *Ifng*, *Il17a*, *Tnf*, *Il6*, *Il23*, *Csf2, Il10* and *Fasl* expression were observed (Fig. 3.5.1 C). Although *Il1b* was significantly upregulated, the

increase was not as robust as when co-cultured with microglia (Fig. 3.3.1 A). Those results suggested that microglia instead of astrocytes were more efficient in regulating cytokine expression in $CD4^+$ T cells.

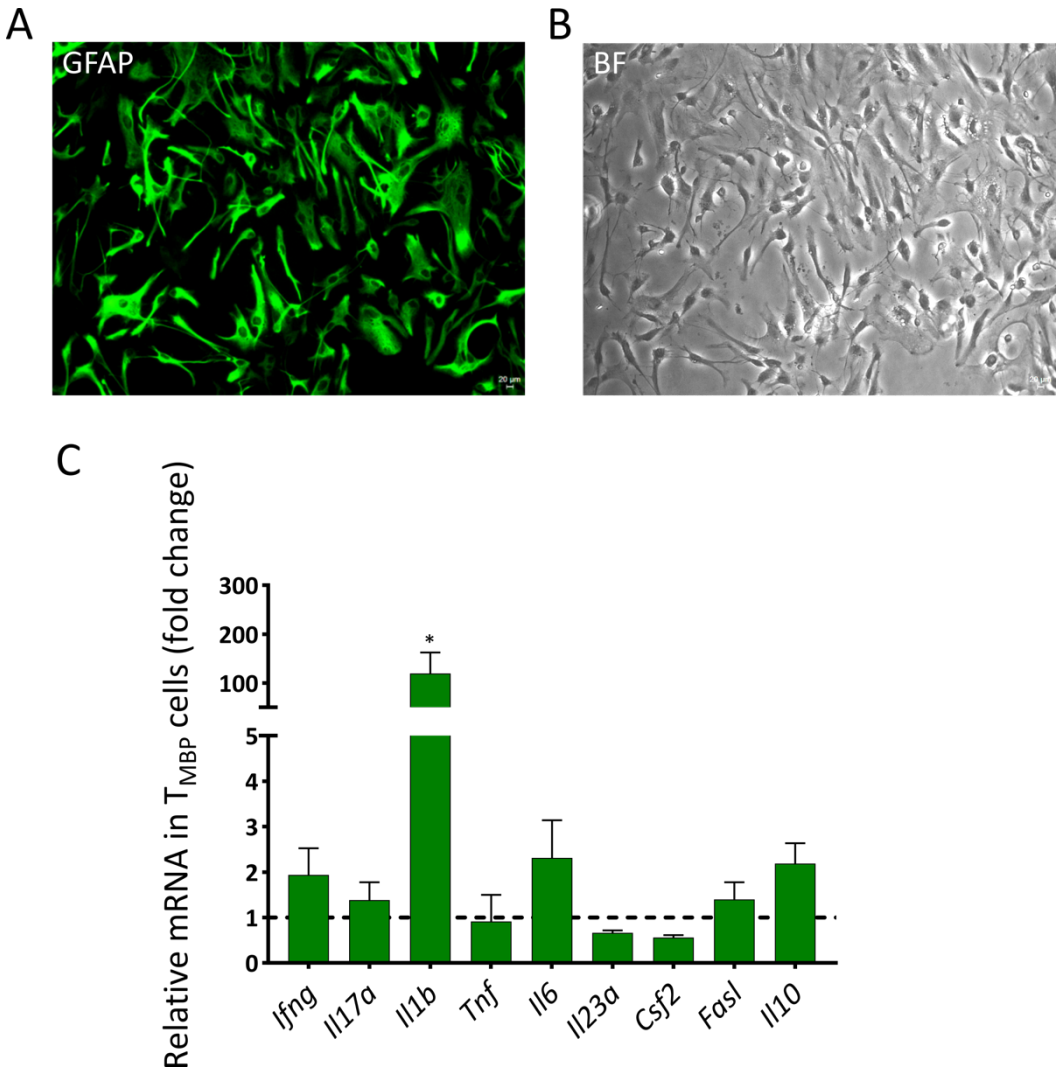

Fig. 3.5.1| Astrocytes induce T_{MBP} cell activation. Cultured astrocytes were stained with anti-GFAP antibody (green) and visualized by fluorescence microscopy. Fluorescence image (A) and bright-field (BF) images (B) are shown. Scale bar 20 μm. Representative pictures of two independent experiments. C. Astrocytes and $T_{MBP\text{-}GFP}$ cells were co-cultured for 24 hrs. T_{MBP} cells were collected and used for analyzing cytokine gene expression by qPCR. T cells which were cultured alone were used as control. The mRNA expression is shown relative to control (dash line). Means +/- S.E.M. of four independent experiments, $*p < 0.05$, unpaired t test, compared to control T cells.

3.6 Microglia-activated T_{MBP} cells alone are not enough to induce EAE

Since restimulated T_{MBP} cells and microglia-activated T cells showed similar activation phenotype (Fig. 3.3.1 and Fig. 3.4.1), we asked whether T_{MBP} cells activated by microglia were able to induce EAE. Therefore, we injected co-cultured $T_{MBP\text{-}GFP}$ cells into rats; here we used restimulated $T_{MBP\text{-}GFP}$ cells as positive control. However, we did not observe clinical signs from recipients of co-cultured T_{MBP} cells, indicating that the co-cultured T_{MBP} cells alone were not able to induce clinical EAE (Fig. 3.6.1 A). Although co-cultured T_{MBP} cells failed to induce EAE, we observed increased numbers of $CD4^+$ cells in the spinal cord parenchyma (Fig. 3.6.1 B). In EAE development, pathogenic T cells recruit peripheral macrophages into the CNS which further lead to the formation of local inflammation (Mundt, Greter et al. 2019). Therefore, we looked at the infiltration of macrophages in spinal cord parenchyma, however, we did not observe infiltration of peripheral macrophage into CNS of recipients of co-cultured T cells (Fig. 3.6.1 C). Although co-cultured T_{MBP} cells were activated by microglia, they were not experienced with antigen *in vitro* therefore they did not recognize the cognate antigen and accumulated in CNS which is possibly the reason why they were not able to induce EAE.

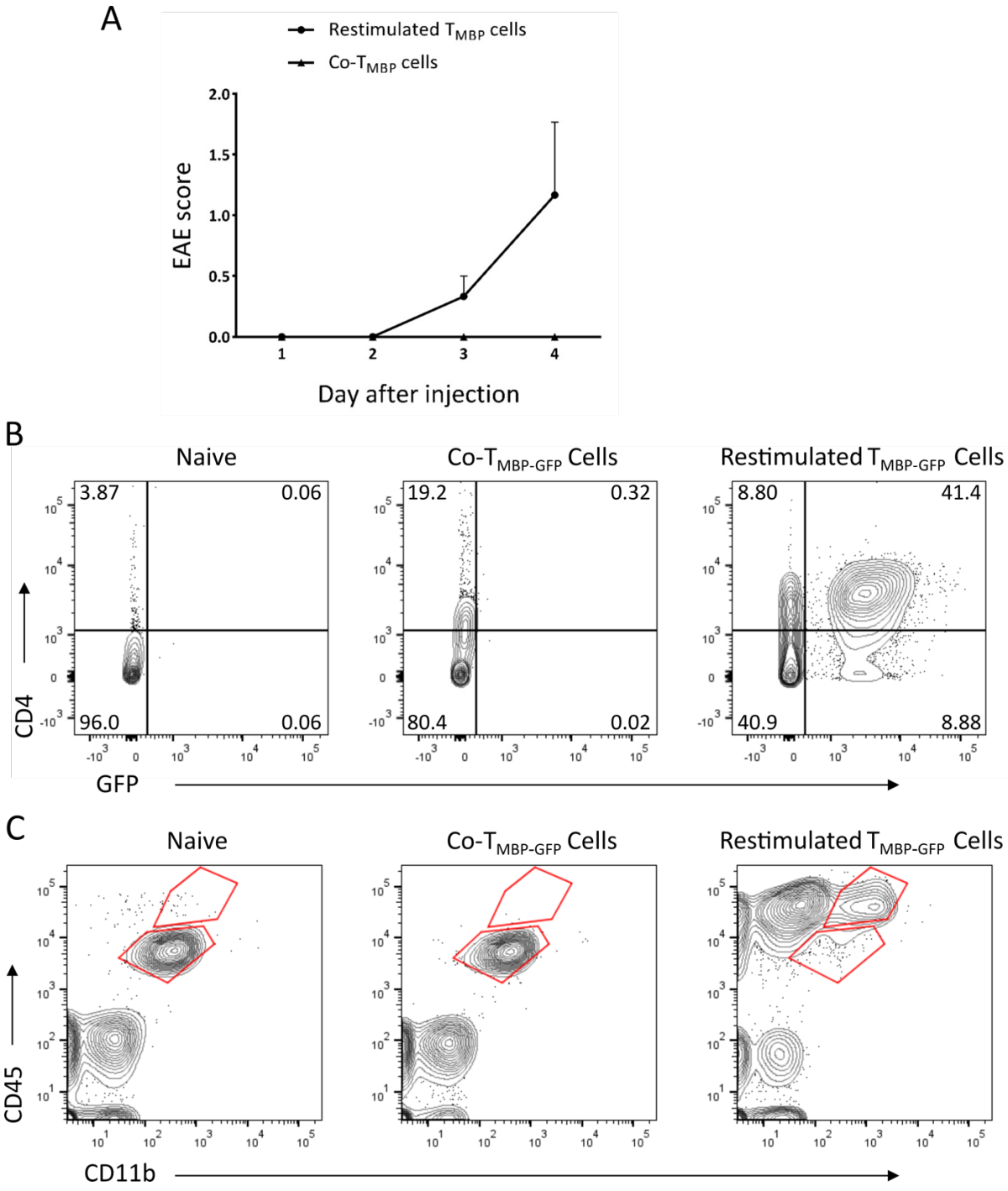

Fig. 3.6.1| T_{MBP} cells activated by microglia failed to induce EAE. A. EAE score of rats after transfer of 2 × 10^6 restimulated or co-cultured $T_{MBP\text{-}GFP}$ cells. One rat per group. Means +/- S.E.M. of three independent experiments. B. C. Mononuclear cells isolated from the CNS of naive rats and recipients of microglia co-cultured (Co-$T_{MBP\text{-}GFP}$ cells) and restimulated with thymocytes (Restimulated $T_{MBP\text{-}GFP}$ cells) were stained for CD4, CD45 and CD11b and analyzed by FACS. Representative results of three independent experiments.

3.7 TNF signaling in the regulation of T cell activation

3.7.1 TNF-α knockout decreased IFN-γ and IL-1β production

Our *in vitro* data showed that microglia can induce T cell activation even without antigen. Among the pronounced upregulated genes, two innate cytokines *Tnf* and *Il1b* drew our attention (Fig. 3.3.1 and Fig. 3.3.2). TNF signaling was found play an important role in regulating IFN-γ production in $CD4^+$ T cells and IL-1β production in myeloid cells (Chou, Tsai et al. 2001, Jain, Irizarry-Caro et al. 2020). Therefore, here we asked whether microglia regulated T cell activation via TNF-α, which acts on T cells in an autocrine or paracrine manner. In order to answer this question, we used TNF-α deficient T_{MBP} cells. We took advantage of CRISPR/Cas9 technology and genetically engineered *Tnf*-targeted sgRNA (sgTnf) into MBP-specific T cells which stably express the Cas9-EGFP fusion protein ($T_{MBP\text{-}Cas9\text{-}EGFP}$ cells) to knockout TNF-α. Non-targeted sgRNA (sgNT) was used as negative control. To check the knockout efficiency of TNF-α, sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells were incubated with ionomycin and PMA to induce cytokine expression. FACS staining showed that TNF-α was almost completely depleted with minor impact on IFN-γ production (Fig. 3.7.1).

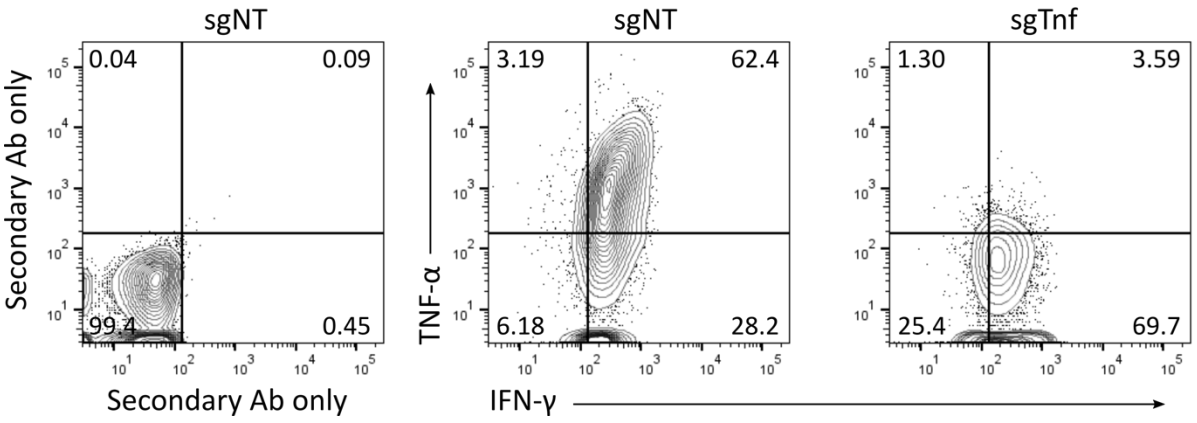

Fig. 3.7.1| TNF-α knockout efficiency in $T_{MBP\text{-}Cas9\text{-}EGFP}$ cells. sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ were stimulated with ionomycin and PMA. Intracellular staining of TNF-α and IFN-γ was

performed after stimulation. sgNT cells stained with secondary antibodies (Ab) only were used for gating. The results are representative of two independent experiments.

After successfully established TNF-α knockout T_{MBP} cell line, we evaluated the expression of TNF-α, IFN-γ and IL-1β both in sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells with or without co-culturing with microglia. The FACS analysis showed that without co-culture both sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells expressed small amount of TNF-α and IFN-γ and relatively higher amount of IL-1β (Fig. 3.7.2). Co-culturing with microglia, the expression of all those cytokines were dramatically upregulated in sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells (Fig. 3.7.2). Interestingly, the proportion of IFN-γ and IL-1β producing sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells were around half of control sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells (Fig. 3.7.2 B and C). Collectively, those results suggested that the microglia induced upregulation of cytokines in $CD4^+$ T cells was partially regulated by TNF signaling.

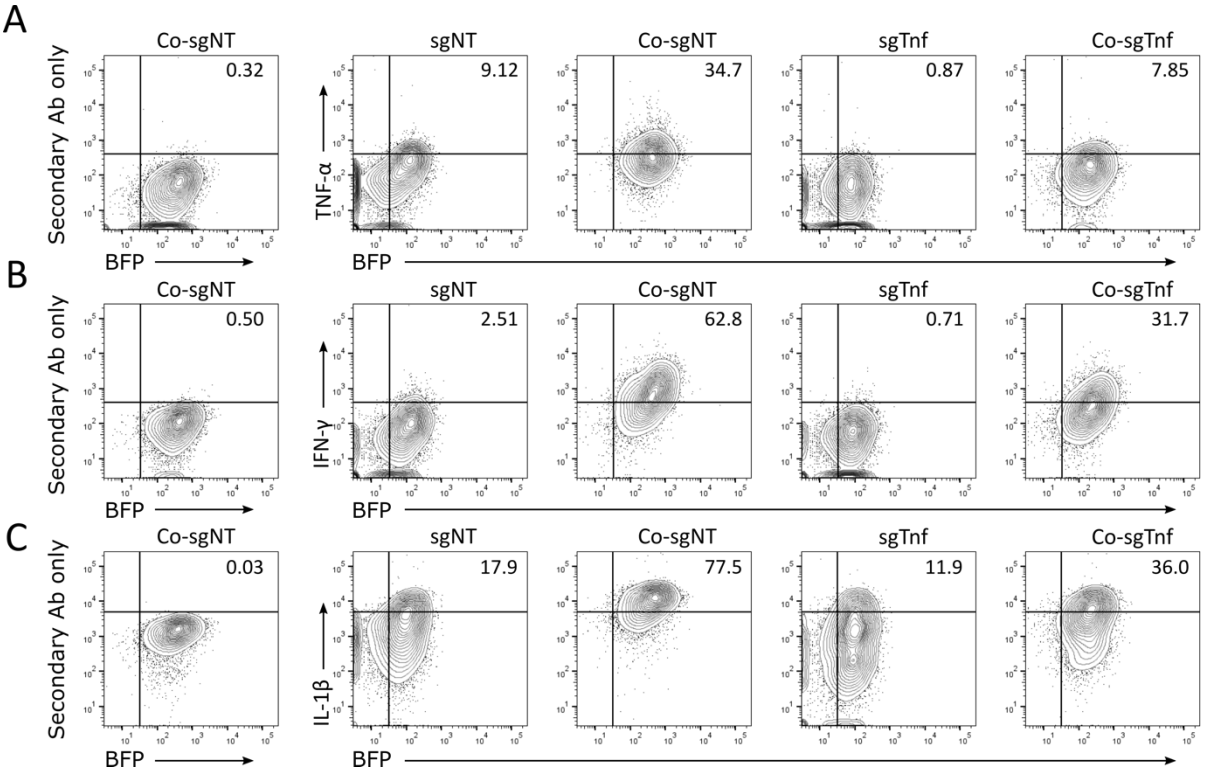

Fig. 3.7.2| TNF signaling regulates IFN-γ and IL-1β expression in $T_{MBP\text{-}Cas9\text{-}EGFP}$ cells. sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells (sgNT), sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ (sgTnf) without co-culture and sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells, sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ after co-culturing with microglia for 24 hrs (Co-sgNT; Co-sgTnf) were stained for TNF-α (A), IFN-

γ (B) and IL-1β (C). Co-sgNT cells stained with secondary antibody (Ab) only were used for gating. The results are representative of three independent experiments.

3.8 IL-1β signaling in regulating T cell activation

The innate cytokine IL-1β is critical for pre-committed T_H1 cells to produce IFN-γ (Jain, Song et al. 2018). Our study showed that microglia significantly induced IL-1β expression in T_{MBP} cells (Fig. 3.3.1), which is TNF-dependent (Fig. 3.7.2). We hypothesized that produced IL-1β can act on T cells and influence their phenotype. To this end, first we evaluated the IL-1β receptor, IL1R1, expression on T_{MBP} cells. The FACS analysis showed that T_{MBP} cells constitutively expressed IL1R1 and knockout TNF-α did not alter its expression (Fig. 3.8.1 A).

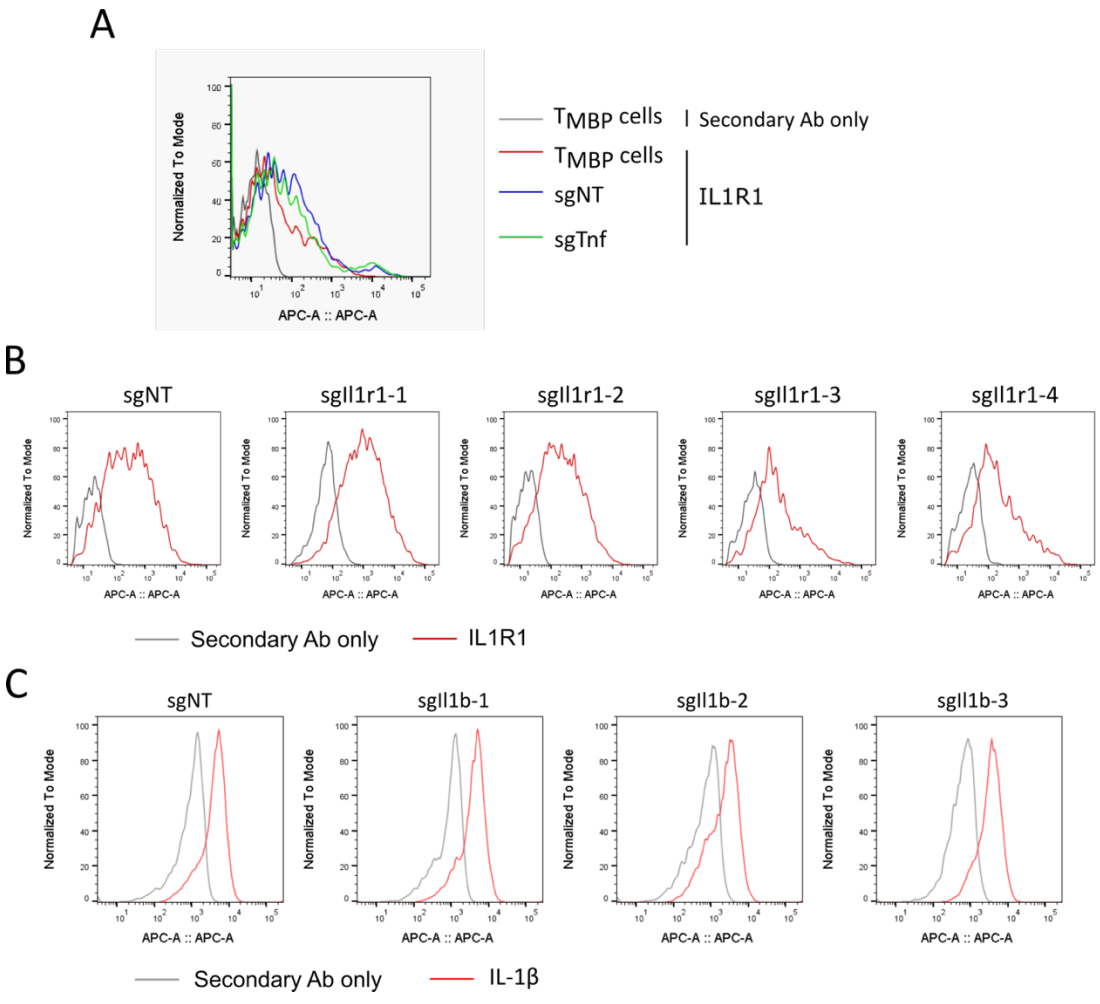

Fig. 3.8.1| IL1R1 expression on T_{MBP} cells; knockout efficiency of different sgRNAs targeting *Il1r1* and *Il1b*. A. T_{MBP} cells, sgNT-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells and sgTnf-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells were stained for IL1R1. B. sgIl1r1

was retrovirally delivered into $T_{MBP\text{-}Cas9\text{-}EGFP}$ cells. IL1R1 knockout efficiency of four sgRNAs was checked by IL1R1 staining. Cells were pre-gated on BFP-positive cells. C. sgIl1b was retrovirally delivered into $T_{MBP\text{-}Cas9\text{-}EGFP}$ cells. IL-1β knockout efficiency was checked on day 2 after restimulation by intracellular staining. Cells were pre-gated on BFP-positive cells. The results are representative of two independent experiments.

In order to further understand the function of IL-1β signaling, we applied four sgRNAs to $T_{MBP\text{-}Cas9\text{-}EGFP}$ cells to knockout IL1R1 expression. Unfortunately, none of those four sgRNAs successfully knocked out the expression of IL1R1 (Fig. 3.8.1 B). Given that in microglia/$CD4^+$ T cell co-culture system, $CD4^+$ T cells were the main producer of IL-1β instead of microglia (Fig. 3.3.3), depleting IL-1β in $CD4^+$ T cells could alternatively help us to understand the function of IL-1β signaling in the regulation of $CD4^+$ T cells by microglia. Hence, we tried to knockout IL-1β in $CD4^+$ T cells by generating sgIl1b-$T_{MBP\text{-}Cas9\text{-}EGFP}$ cells. Three different sgRNA targeting *Il1b* were evaluated in knocking out IL-1β expression. Unfortunately, none of them was efficient in knocking out IL-1β (Fig. 3.8.1 C). Further attempts need to be done to find a way to knockout IL-1 signaling in $CD4^+$ T cells.

3.9 RNA-sequencing of microglia and infiltrated macrophages in EAE

3.9.1 IFN-γ signaling is enriched in microglia from EAE rats

To gain insight into microglia activation profile in EAE development, we used RNA-seq to characterize the transcriptome changes of microglia and macrophages from EAE rats. T_{MBP} cells were adoptively transferred into Lewis rats and the rats were sacrificed on day three after T cell transfer, when the bulk of T cells started to penetrate into the CNS and created inflammatory lesions in the CNS (Odoardi, Sie et al. 2012). Microglia and macrophages were sorted based on their CD45 and CD11b expression (Fig. 3.9.1).

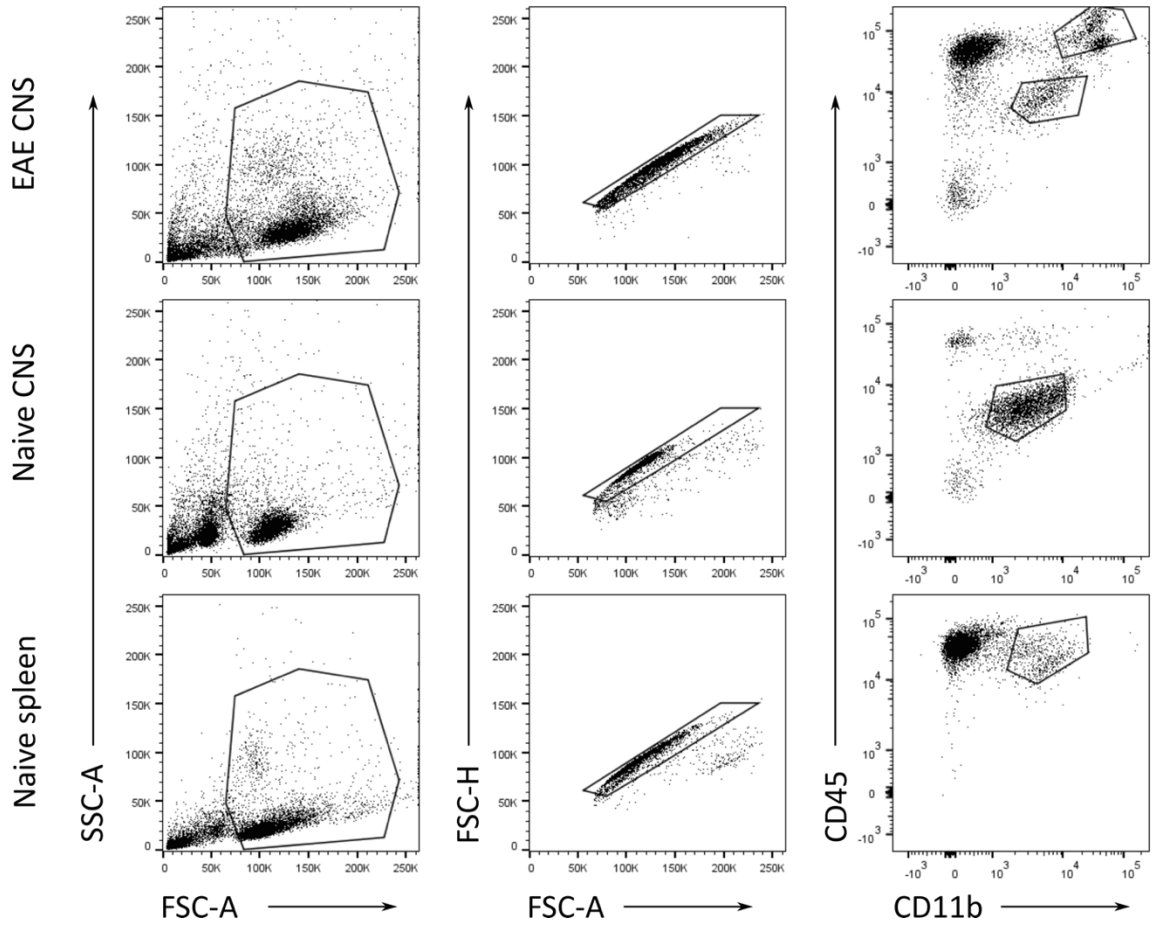

Fig. 3.9.1| Gating strategy for sorting of microglia and macrophages. The cells from the CNS of EAE rats and naive rats and from spleen of naive rats were stained for CD11b and CD45. Microglia ($CD11b^{+}CD45^{low}$) and macrophages ($CD11b^{+}CD45^{high}$) were sorted and used for RNA-seq. Representative FACS plots of two independent experiments are shown here.

Gene expression was compared between microglia from EAE rats and from naive rats as well as between macrophages from the CNS of EAE rats and from spleens of naive rats. Volcano plot displayed 1035 upregulated genes and 1035 downregulated genes among 9484 detected genes in microglia from EAE rats compared to microglia from naive rats; 914 upregulated genes and 714 downregulated genes among 8974 detected genes in macrophages from EAE rats compared to macrophages from the spleens of naive rats (cut off fold change 2, p value 0.01) (Fig. 3.9.2). The results showed that chemokines such as *Cxcl9* and *Cxcl11* were among the most upregulated genes in both microglia and macrophages in the inflamed CNS. In addition, IL-2 signaling genes such as *Il2rg* and *Il2rb*

were significantly upregulated in microglia from EAE rats (Fig. 3.9.2). The top regulated genes are shown in the supplementary tables 1-4.

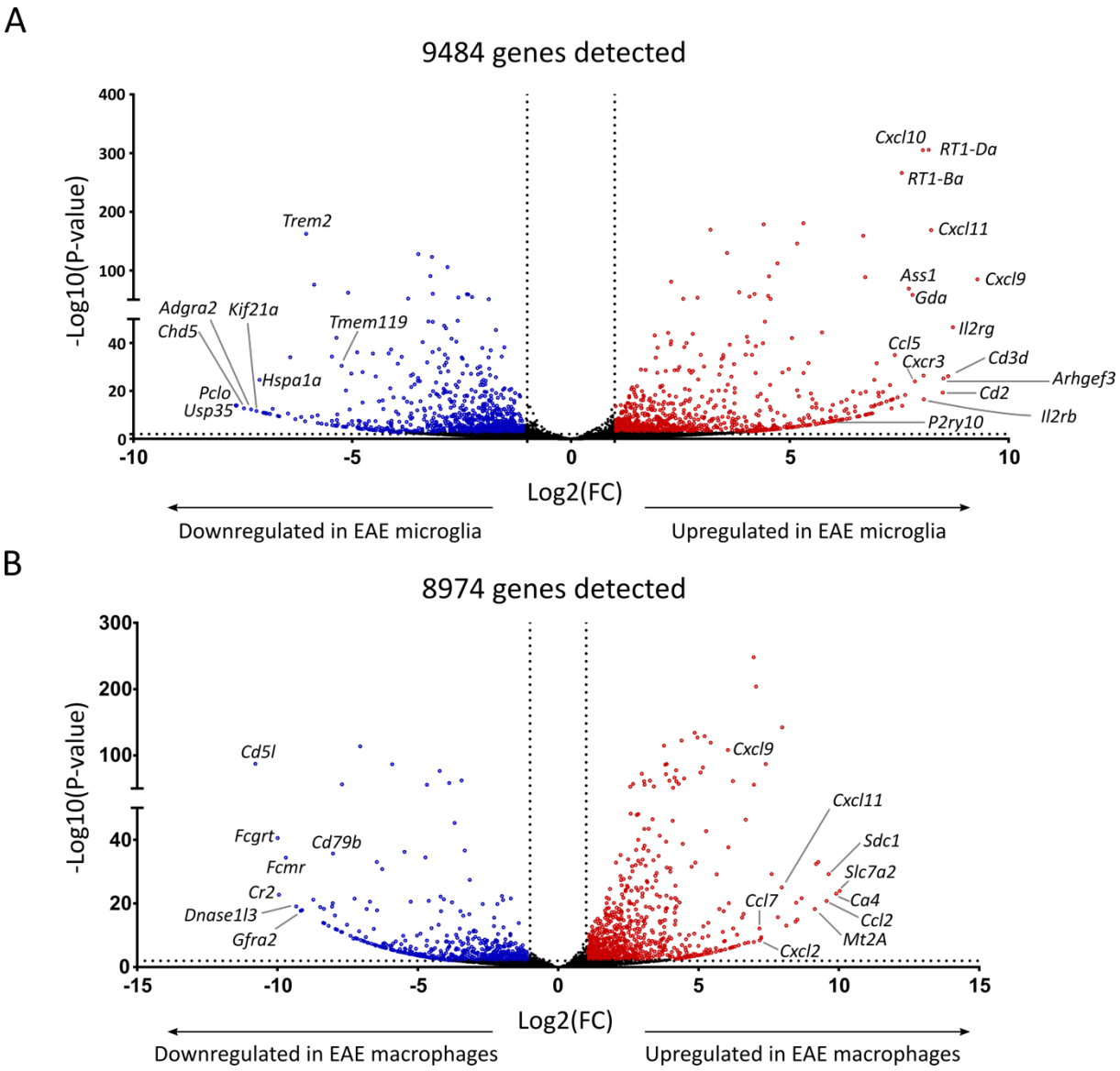

Fig. 3.9.2| Volcano plots show differently expressed genes in microglia and macrophages from EAE rats vs from naive rats. A. Downregulated genes (blue dots) and upregulated genes (red dots) among 9484 detected genes in microglia from EAE rats compared to microglia from naive rats. B. Downregulated genes (blue dots) and upregulated genes (red dots) among 8974 detected genes in macrophages from EAE rats as compared to macrophages from spleens of naive rats. Cut-off P-value is set to 0.01, cut-off fold change (FC) is set to 2. Cumulative results of two samples per group.

RESULTS

GSEA showed that microglia from EAE rats displayed significant enrichment of IFN-γ signaling compared to microglia from naive rats. Notably, a group of genes related to IFN-γ response (e.g., *Cxcl9-11*, *Cd74*, *Ccl5*, *Irf1* and *Vcam1*; Fig. 3.9.3 A) were significantly upregulated, indicating that T_H1 cells induce microglia activation during the early stage of EAE. In addition, GSEA identified enriched genes that participate in cytokine and receptor interaction, which may help us to understand the mechanism of T cell and microglia interaction. Of note, microglia significantly upregulated receptor *Il2rg* which is known important for maintaining T cell homeostasis (Fig. 3.9.3 B) (Rochman, Spolski et al. 2009). Furthermore, chemokines such as *Cxcl9, 10, 11* and *Ccl5* were significantly upregulated in microglia from EAE rats compared to microglia from naive rats. Those cytokines were important for recruiting T_{MBP} cells which express corresponding chemokine receptor such as CXCR3, CCR5 and CXCR4 into the CNS during EAE development (Schlager, Korner et al. 2016). This indicated that at early stage of EAE, T_H1 cells instruct microglia to secret chemokines which further recruit more T cells infiltration. Moreover, we looked at the top regulated cytokines and chemokines in microglia and compared the expression levels of those genes to infiltrated macrophages. The results showed that macrophages expressed higher levels of chemokines *Cxcl9, 10* and *11* compared to microglia (Fig. 3.9.3 C), suggesting that macrophages contribute to T cells recruitment as well. VCAM-1 and ICAM-1 binding to VLA-4/LFA-1 on T cells have been shown important for T cell trafficking (Schlager, Korner et al. 2016). Here the results showed that microglia expressed *Vcam1* to a more extent than macrophages, whereas both microglia and macrophages expressed high level of *Icam1* (Fig. 3.9.3 C), indicating that both microglia and macrophages regulate T cell trafficking in CNS parenchyma. Given that T cells can upregulate IL-1β in myeloid cells (Jain, Irizarry-Caro et al. 2020), here we looked at the *Il1b* level and found that macrophages were the prominent producer of *Il1b* compared to microglia in line with Fig. 3.3.3 which showed that microglia do not produce IL-1β. Next, we looked at the represented receptor expression in microglia and macrophages, and observed that both microglia and macrophages strongly expressed costimulatory molecules *CD40*, IL-2 signaling related receptors such as *Il2rg* and *Il2ra*, indicating that both microglia and macrophages can interact with T cells. The upregulation of *Tnfrsf1a* and *Tnfrsf1b* suggested enhanced TNF signaling in microglia and macrophages

(Fig. 3.9.3 D). In addition, *Il1r2* which can negatively regulate IL-1 signaling (Boraschi, Italiani et al. 2018) was significantly upregulated in microglia, albeit at a lower level compared to macrophages (Fig.3.9.3 D). Taken together, those results suggested that at the very early stage of EAE, microglia can be influenced by T_H1 cells which in turn upregulate chemokines to further recruit more T cells into the CNS parenchyma, which might play a pivotal role in promoting the formation of neuroinflammation.

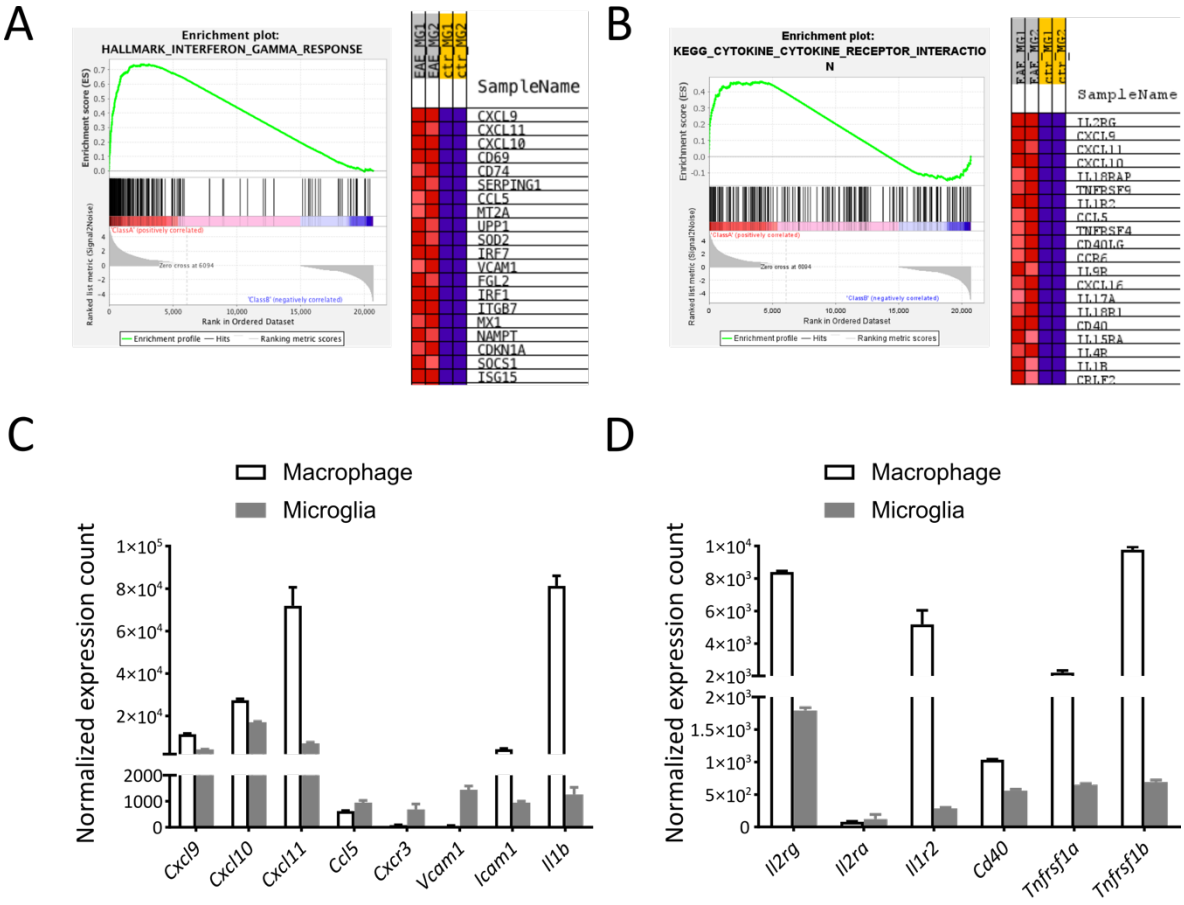

Fig. 3.9.3 Gene expression in microglia and macrophages from EAE rats. Differentially expressed genes of microglia from EAE rats were queried against hallmark gene sets (A) or c2. canonical pathway gene sets (B) in MSigDB database by using gene set enrichment analysis (GSEA). GSEA identified highly significant changes in IFN-γ response pathway with normalized enrichment score (NES): 2.17, false discovery rat (FDR) q value: 0.000 (A), and cytokine-cytokine receptor interaction pathway with NES: 1.38, FDR q value: 0.181 (B). Heat maps display the top 20 upregulated genes of the gene sets in the pathways (A and B). C. D. Top upregulated cytokines and receptors in microglia

(picked from data shown in fig. 3.9.2) in comparison to macrophages from EAE rats. Those data were processed from next-generation sequencing (NGS) results. Cumulative results of two samples per group.

4 DISCUSSION

4.1 Microglia, can you present antigen to $CD4^+$ T cells?

The search for the real APCs in the CNS during pathogenesis of MS/EAE has never stopped. Recently, Mundt et al. showed that depletion of MHCII on CD11c-expressing cells protected mice from EAE development, suggesting a critical role of cDCs, which are characterized by CD11c expression, in EAE pathogenesis (Mundt, Mrdjen et al. 2019). However, not only cDCs express CD11c but also microglia express CD11c during neuroinflammation (Wlodarczyk, Holtman et al. 2017). In addition, microglia are the principle immune cell in CNS and their antigen presenting function in MS/EAE has been frequently asked (Almolda, Gonzalez et al. 2011). Therefore, it is important to study the function of microglia as APCs. Here, we aimed to study the antigen presenting function of microglia by focusing on the interaction between microglia and encephalitogenic T cells during the development of CNS inflammation.

There are still some technical difficulties in identifying the critical APCs in the CNS during EAE development. Bone marrow chimeras are often used to replace a particular cell population in the CNS. However, due to irradiation, the tightness of BBB is not as same as it is in naive animals. The BBB often becomes leaky and thus more immune cells can penetrate into the CNS (Mildner, Schmidt et al. 2007). In addition, the immunization with CFA, which is often used to induce active EAE, also affects the landscape of the CNS (Raghavendra, Tanga et al. 2004). Therefore, these treatments may create difficulties in distinguishing the CNS-resident cells from the CNS-invading myeloid cells due to overlapping phenotypes and make it complicated to identify the APCs. Furthermore, conditional gene deletion, often performed by using the Cre-loxP system, cannot provide complete knockout of target genes in specific cell population and also potentially results in gene deletion in unwanted cell populations (Mundt, Mrdjen et al. 2019).

DISCUSSION

In vivo systems clearly have great advantages as they are closer to physiological condition. At the same time, it is also difficult to interpret the findings due to their complexity as described above. Therefore, in this study, we used an *in vitro* co-culture system with primary microglia and CNS antigen-specific $CD4^+$ T cells. Unlike the traditional way of culturing microglia, which is acquiring microglia from postnatal day 1 rats, we challenged ourselves to establish primary microglia from adult rats. In contrast to primary neonatal microglia, primary adult microglia were shown to respond to pro-inflammatory cytokines, such as nitric oxide (Brannan and Roberts 2004), thus adult microglia better reflect the *in vivo* situation since tEAE is induced in adult rats. Primary microglia were isolated from the brain and spinal cord of adult rats by Percoll gradients (Cardona, Huang et al. 2006). By this method, we acquired highly purified microglia with almost no contamination of peripheral macrophages (Fig. 3.1.1). The microglia can be kept in culture for days with ramified morphology and can be labeled with fluorescence-conjugated IB4, which enables to record their movement over time by fluorescence microscopy. After successfully established primary adult microglia culture, we evaluated their antigen presenting capacity to $CD4^+$ T cells by using *in vitro* live imaging. In this case, we used NFAT-GFP-expressing T_{MBP} cells and T_{OVA} cells. The translocation of NFAT indicates activation of the T cells as a result of strong elevation of intracellular calcium levels, which is often induced by antigen presentation by APCs (Kyratsous, Bauer et al. 2017).

4.1.1 Antigen-dependent T cell activation by microglia

By using our *in vitro* co-culture system, we examined T cell activation by microglia. The imaging data revealed that microglia from naive rats do not activate T cells. We further asked whether activated microglia were able to activate T_{MBP} cells. Here, LPS were used to induce microglia activation. Bacterial LPS are highly conserved among microbes and are recognized by TLR4 (Miller, Ernst et al. 2005). The binding of LPS to TLR4 induces the production of various pro-inflammatory mediators such as IL-1β, TNF-α and IL-6 (Papageorgiou, Lewen et al. 2016, Rathinam, Zhao et al. 2019). However, application of LPS did not change the T_{MBP} cell activation capacity of microglia.

In contrast to LPS, our imaging study showed that IFN-γ-activated microglia exhibited an enhanced ability to activate T_{MBP} cells. The enhanced ability was correlated with upregulation of MHCII on microglia, indicating that IFN-γ instead of LPS induces MHCII expression and thus the antigen presenting function of microglia which is in line with findings from a previous study (Rock, Hu et al. 2005). During the acute phase of tEAE in Lewis rats, which is induced by *in vitro* restimulated T_{MBP} cells and used in this study, IFN-γ producing T_H1 cells were abundantly found in the CNS parenchyma (Kawakami, Lassmann et al. 2004). During EAE pathogenesis, CNS-infiltrating T cells produced IFN-γ which has been shown to stimulate microglia and thus enables microglia to acquire higher antigen presenting capacities (Aloisi, Ria et al. 2000). Therefore, microglia can contribute to EAE pathogenesis, especially at a very early phase of EAE before peripheral macrophages infiltrate into the CNS.

During the process of microglia isolation, the CNS of the rat was mechanically homogenized which produced myelin debris. Therefore, microglia may uptake myelin and present myelin-derived antigens to T cells. To exclude this possibility, we co-cultured OVA-specific T cells with microglia and imaged T cell activation as for MBP-specific T cells. The imaging showed that microglia pre-activated by IFN-γ activated T_{OVA} cells in a similar way as T_{MBP} cells even without cognate antigens (Figs. 3.1.2 and 3.1.4). The results suggest that myelin debris does not influence the activation of T_{MBP} cells. More importantly, the activation of T_{OVA} cells by IFN-γ-stimulated microglia indicates that microglia can activate T cells in an antigen-independent manner, which is discussed more in detail in section 4.1.2.

Surprisingly, with the addition of cognate antigen, untreated microglia gained the capacity to activate T_{MBP} cells. In this case, microglia were pre-incubated with MBP for two hrs before co-culture with T_{MBP} cells. Correspondingly, pre-incubation of microglia with OVA activated T_{OVA} cells. These results indicate that MHCII on naive microglia is not saturated with antigens, which again support the exclusion of the possibility of MBP contamination during microglia preparation. As expected, we did not observe upregulation of MHCII by MBP. The observation of a significantly increased capacity of MBP/OVA treated microglia in activating T_{MBP}/T_{OVA} cells was rather confounding. One possibility is that high amounts of antigen

enable the limited MHCII on microglia to reach the full potential in presenting antigens and thereby lead to increased T cell activation. On the other hand, the MHCII expression on naive microglia was very low, therefore the T cell activation may not be entirely attributed to MHCII-mediated antigen presentation.

The antigen-dependent T cell activation by microglia can have important roles in the early phase of EAE. After adoptive transfer of T cells into recipient rats, the first few T cells penetrate into the CNS within some hrs (Hickey, Hsu et al. 1991, Odoardi, Sie et al. 2012). At this time point, the infiltration of peripheral macrophages has not started yet. A previous study from our lab showed that meningeal perivascular macrophages can present endogenous myelin antigens to infiltrated T cells in the leptomeninges (Bartholomaus, Kawakami et al. 2009). The present study indicates that microglia can do a similar job in the CNS parenchyma.

In the context of EAE, engrafted $CD4^+$ T cells become reactivated after encountering their cognate antigen presented by APCs in the CNS (Tompkins, Padilla et al. 2002). We tested whether phagocytes from the CNS of EAE rats can activate $CD4^+$ T cells *ex vivo*. Surprisingly, phagocytes substantially induced both T_{MBP} and T_{OVA} cell activation even without the addition of antigen. Anti-MHCII blocking antibodies reduced the proportion of activated T cells to a similar extent as observed by naive microglia (Figs. 3.1.2 C, 3.1.4 and 3.1.5 B). However, on the one hand, phagocytes activated T_{OVA} cells without presence of their cognate antigen; on the other hand, blocking MHCII reduced T_{OVA} cell activation, seems contradictory. In addition to the classical antigen presenting function, it has been speculated that MHCII can function as receptor to mediate cytokine production, cell adhesion and expression of costimulatory molecules (Liu, Zhan et al. 2011). Moreover, MHCII was shown to promote TLR signaling by regulating Bruton's tyrosine kinase activation via a costimulatory molecule CD40 and thus regulate innate immune responses (Liu, Zhan et al. 2011). Therefore, the observation that blocking MHCII reduced the T_{OVA} cell activation capacity of phagocytes from EAE rats could be due to a compromised immune response of phagocytes.

4.1.2 Antigen-independent T cell activation by microglia

Due to a lack of OVA antigens in the CNS, very few T_{OVA} cells alone penetrate into the CNS, but they do not get activated within the CNS and they cannot induce EAE. When co-transferred with T_{MBP} cells, which open the BBB, T_{OVA} cells can penetrate into the CNS in massive numbers. Still, T_{OVA} cells do not get activated (Flugel, Berkowicz et al. 2001). In addition, the imaging of T cells in acute spinal cord slices showed that compared to T_{MBP} cells, T_{OVA} cells moved in the spinal cord parenchyma with higher motility and lower meandering index, suggesting T_{OVA} cells do not stop in the proximity of APCs (Kawakami, Nagerl et al. 2005). By using the calcium sensing protein Twitch1, which fluorescently indicates changes of intracellular calcium concentration as a result of T cell activation, our lab showed that less than 1% of $T_{OVA\text{-}Twich1}$ cells emitted high-calcium signals at the leptomeninges of the spinal cord (Kyratsous, Bauer et al. 2017). All these results from previous studies pointed out that T_{OVA} cells do not find their cognate antigen in the CNS and, therefore, do not get activation.

In contrast, to our surprise, our *in vitro* co-culture experiments illustrated that 40% of T_{OVA} cells were activated as detected by NFAT-GFP translocation after co-culture with phagocytes from EAE rats (Fig. 3.1.5). Anti-MHCII blocking antibody treatment diminished T cell activation only partially, indicating that T cells can be activated in an antigen/MHCII-independent manner in the CNS. The antigen-independent activation of T cells was further confirmed by FACS analysis of surface markers (Fig. 3.4.1). The upregulation of IL-2R and OX-40 on T_{OVA} cells in Fig. 3.4.1 is contradictory to the previous observation that those markers were not upregulated (Flugel, Berkowicz et al. 2001). This might be explained by different experimental conditions. Whereas in the previous publication, T_{OVA} cells were analyzed after they had been freshly prepared from the CNS, we analyzed those cells from our *in vitro* co-culture system. Since the *in vitro* culture system contained only microglia and T cells, the contribution from other glial cells is missing. Although further analysis is needed, we show the possibility that microglia can activate T cells in an antigen-independent manner.

DISCUSSION

In this thesis, we showed that direct contact seems to be necessary (Fig. 3.3.4) and the contribution of MHCII (Fig. 3.1.5). The molecular mechanisms of antigen-independent activation need more studies, especially MHCII independent mechanisms. For example, costimulatory molecules provided by APCs were shown to be responsible for T cell activation (Gutcher and Becher 2007). Whether costimulatory molecules play a role in T cell activation induced by IFN-γ-stimulated microglia remains to be further defined. For future studies, the usage of the calcium sensing protein Twitch will be useful. In contrast to NFAT-GFP, which detects absolute T cell activation, Twitch can detect weaker stimulation which is not sufficient to induce NFAT translocation by itself, but sufficient to change T cell phenotypes (Kyratsous, Bauer et al. 2017). In addition, such weak signals can be accumulated to induce NFAT-GFP translocation. In combination, we could delete molecules which are speculated to be involved in the signaling cascade of T cell activation by using CRISPR gene editing. Although our trial for IL-1β and IL1R1 was unsuccessful, the CRISPR/Cas9 system is successfully being used in our lab to delete other specific molecules in rat T cells.

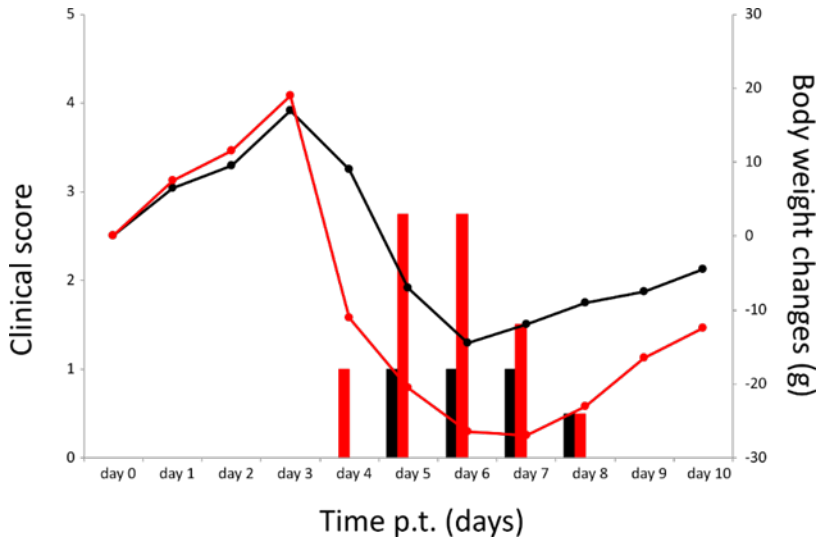

Fig. 4.1.1| Clinical score (bars) and body weight changes (lines) after adoptive transfer of T_{MBP} cells (black) or co-transfer of T_{MBP} cells and T_{OVA} cells (red). The result contains two rats per group. Representative results of three independent experiments. (Provided by PD Dr. Naoto Kawakami)

It is important to study whether the antigen-independent activation contributes to EAE pathogenesis. Our preliminary result showed that T_{OVA} cells enhanced clinical severity of EAE which is induced by T_{MBP} cells (Fig. 4.1.1), although T_{OVA} cells alone do not induce clinical EAE (Flugel, Berkowicz et al. 2001). This preliminary result strongly suggests that antigen-independent activation indeed contributes to development of EAE.

4.1.3 Encephalitogenicity T cells activated by microglia

Notably, the expression level of IL-2R and OX-40 on T_{MBP} cells after co-culture with microglia in absence of exogenous MBP was comparable to fully restimulated T_{MBP} cells in the presence of MBP. These molecules are considered as activation markers of $CD4^+$ T cells. In addition, OX-40 is a costimulatory molecule which belongs to the TNFR super family and is important for $CD4^+$ T cell differentiation, expansion and survival (Redmond, Ruby et al. 2009). Some evidences suggest that costimulatory signals alone can activate $CD4^+$ T cells (Ramming, Thummler et al. 2009). Therefore, it is possible that the upregulated costimulatory signals contributed to microglia induced $CD4^+$ T cell activation.

Since microglia induced membrane phenotype changes in T_{MBP} cells and upregulation of cytokines to an extent comparable to fully restimulated T_{MBP} cells, we asked whether those activated T_{MBP} cells were sufficient to induce EAE. However, we did not observe any clinical signs after adoptively transferred co-cultured T_{MBP} cells as well as no infiltrated co-cultured T_{MBP} cells in the CNS. Those co-cultured T_{MBP} cells were not experienced with CNS antigens during co-culture with microglia thus may have not acquired encephalitogenicity.

4.1.4 Microglia vs infiltrated macrophages

During EAE, peripheral macrophages infiltrate into the CNS, therefore the cultured phagocytes isolated from the CNS of EAE rats contain both infiltrated macrophages and microglia. Our results showed that microglia were less effective in inducing T_{MBP} cell activation compared to macrophages (Fig. 3.1.6). However, microglia do induce T cell activation and can contribute to T cell activation, which can be important in the early phase of EAE before peripheral macrophages infiltrate as discussed above. The differences between microglia and macrophages in activating T cells were correlated to MHCII

expression which is in line with a previous study showing that MHCII expression in microglia was less important than in circulating myeloid cells (Jordao, Sankowski et al. 2019). Notably, the proportion of activated $T_{MBP\text{-}NFAT\text{-}GFP}$ cells by microglia from EAE rats (Fig. 3.1.6) was comparable to the one induced by IFN-γ-stimulated cultured microglia from healthy rats (Fig. 3.1.2), implying that microglia induced $T_{MBP\text{-}NFAT\text{-}GFP}$ cell activation is possibly regulated by IFN-γ derived from T_H1 cells in EAE.

4.2 Innate immunity underlying microglia and $CD4^+$ T cell activation

In the live imaging experiments to observe $CD4^+$ T cells co-cultured with microglia, the recording time period per imaging window was 30 minutes after pooling the two cell populations. Such a short period is sufficient to detect the activation, which is indicated by NFAT-GFP translocation in an imaging setup, but may not be long enough to detect activation induced changes of gene expression. In addition, the co-culture imaging system is not suitable to show any changes in microglia. Therefore, to detect alterations in gene expression in T cells and microglia by transcriptome analysis, we co-cultured microglia with T_{MBP} cells or T_{OVA} cells for 24 hrs. The results showed that microglia significantly increased IFN-γ production in both T_{MBP} cells and T_{OVA} cells. The observation that microglia induced inflammatory cytokines in T_{OVA} cells in the absence of cognate antigen further strengthen the assumption of an antigen-independent pathway of T cell activation.

In addition to IFN-γ and IL-17, the innate cytokines TNF-α and IL-1β were also highly upregulated in T_{MBP} cells and T_{OVA} cells after co-culturing with microglia. By using a co-culture system of mouse $CD4^+$ T cells and BMDCs, Jain et al. showed that T cells instructed BMDCs to produce IL-1β via TNF signaling (Jain, Irizarry-Caro et al. 2020) and upregulated IL-1β in turn enabled the $CD4^+$ T cells to produce the effector cytokines IFN-γ and IL-17 (Jain, Song et al. 2018). This was not the case for microglia, which do not produce IL-1β in our co-culture system. This observation is in line with a previous study identifying infiltrated macrophages instead of microglia as the main producer of IL-1β (Vainchtein, Vinet et al. 2014). Interestingly, unlike in the co-culture of mouse $CD4^+$ T cells and BMDCs where

stimulating anti-CD3 antibody is needed for T cell activation, in our rat microglia and $CD4^+$ T cell co-culture system, microglia alone were able to activate $CD4^+$ T cells. Taken together, these results suggest that microglia have a distinct mechanism from BMDCs to activate T cells. Indeed, Jordão et al. showed nicely the different gene expression profiles of microglia and peripheral-derived macrophages by using advanced single-cell RNA-seq (Jordao, Sankowski et al. 2019).

Given that microglia upregulate the expression of TNF-α, IL-1β and IFN-γ in T_{MBP} cells, we wondered whether there is an interconnection among these cytokines. Our study showed that TNF-α knockout dramatically reduced both IL-1β and IFN-γ production (Fig. 3.7.2), indicating that TNF signaling participates in the microglia-regulated activation of T_{MBP} cells. We tried to deplete IL-1β or IL1R1 in T_{MBP} cells to evaluate whether IL-1 signaling regulates T_{MBP} cell differentiation. Unfortunately, none of the designed sgRNAs mediated an efficient knockout (Fig. 3.8.1). Therefore, further studies are necessary to illustrate the crosstalk of these cytokines. Of note, T_{MBP} cells expressed low levels of IL-1β in the absence of microglia (Fig. 3.7.2). IL-1β cannot bypass TCR signaling or costimulatory signals to regulate $CD4^+$ T cell effector functions (Jain, Song et al. 2018). Consistently, in our results, IL-1β alone was not able to differentiate T_{MBP} cells into T_H1 cells since IFN-γ expression remains at a very low level (Fig. 3.7.2). Collectively, T_{MBP} cell activation by microglia requires complex and direct receptor ligand interaction.

Astrocytes are the most enriched cell type in the CNS but their role in regulating $CD4^+$ T cell activity remains unclear. Astrocytes do not express MHCII under steady state conditions (Mundt, Greter et al. 2019). Although astrocytes can express MHCII following IFN-γ stimulation, they do not express costimulatory molecules, suggesting an incomplete function as APCs (Brambilla 2019). Consistently, we showed that astrocytes did not induce expression of IFN-γ, IL-17 and TNF-α in T_{MBP} cells, whereas IL-1β expression in T_{MBP} cells was significantly upregulated by astrocytes. As we discussed above, IL-1 signaling is important for pre-committed $CD4^+$ T cells to obtain effector function, but IL-1 alone has limited effect on T cells. These results indicate that during EAE, astrocytes do not influence as much as microglia do, but could enhance the effector function of $CD4^+$ T cells by upregulating IL-1β.

DISCUSSION

It is interesting to notice that microglia instructed $CD4^+$ T cells, whereas microglia themselves did not show dramatic changes upon co-culture. These results indicate that $CD4^+$ T cells, but not microglia, were the main producers of TNF-α and IL-1β in the co-culture system. $CD4^+$ T cells express receptors for both TNF-α and IL-1β (Croft 2009). Therefore, communications among T cells via cytokines were speculated. For example, TNF-α promotes the production of IL-4 and IFN-γ in T cells, thus controls the frequency of effector $CD4^+$ T cells (Croft 2009).

In order to find the mechanisms that potentially regulate the function of microglia and $CD4^+$ T cells, RNA-seq was applied to characterize the transcriptome profile of microglia from naive and EAE rats. The EAE rats we used here were three days post adoptive transfer of fully restimulated T_{MBP} cells. At this time point, the rats showed partial paralysis of hind limbs, indicating massive inflammation in the CNS. In this rat EAE model, T_{MBP} cells infiltrate into the CNS in two waves. The first wave happens within first few hrs after transfer. The T cells in the first wave do not have any clinical effects and do not recruit peripheral macrophages into the CNS but prepare the CNS milieu to a pro-inflammatory condition. The second wave, which is more aggressive, happens three to four days after transfer (Hickey, Hsu et al. 1991). In the second wave, massive amounts of T cells infiltrate into the CNS followed by macrophages (Kawakami, Lassmann et al. 2004). Indeed, the microglia from EAE rats were prepared after second wave of T cell infiltration. RNA-seq showed enriched genes in IFN-γ signaling pathways in microglia from EAE rats compared to those from naive rats, indicating that microglia also respond to T cell derived signals *in vivo*. As a result, microglia dramatically upregulated chemokines to further recruit more lymphocytes into the CNS (Rock, Hu et al. 2005). Our data identified *Cxcl9* as the most significantly upregulated gene, suggesting microglia attract lymphocytes into the CNS. Performing RNA-seq of microglia after the first but before the second wave of T cell infiltration may provide interesting results about the contribution of microglia during EAE development.

In summary, our study illustrates that microglia can activate $CD4^+$ T cells in both antigen-dependent and -independent manners. We identified two innate cytokines, TNF-α and IL-1β, which were highly upregulated in $CD4^+$ T cells upon co-culture with microglia. Knocking

out TNF-α in $CD4^+$ T cells dramatically reduced the capacity of microglia to activate $CD4^+$ T cells. Therefore, innate immunity plays an important role in the activation of $CD4^+$ T cells. Consistently, RNA-seq identified enriched TNF signaling in microglia at an early stage of EAE. Our findings thus provide new insights into the mechanism of microglia in activating $CD4^+$ T cells during EAE development.

5 SUPPLEMENTAL INFORMATION

Supplementary table 1: Upregulated genes in microglia from EAE rats compared to microglia from naive rats. Cut off FC is 20.

Gene Name	Log_2(FC)	$-Log_{10}$(P-value)	$-Log_{10}$(P-adj)
Cxcl9	9.28	84.64	81.95
Il2rg	8.73	46.59	44.26
Cd3d	8.62	26.15	24.28
Arhgef3	8.51	25.31	23.46
Cd2	8.50	19.31	17.62
Cxcl11	8.23	168.86	165.76
RT1-Da	8.17	305.65	301.94
Il2rb	8.06	16.54	14.95
Kcnn4	8.06	26.47	24.59
Cxcl10	8.04	305.23	301.70
Cxcr3	7.86	24.01	22.19
Gda	7.81	58.27	55.77
Ass1	7.72	69.11	66.49
Msr1	7.64	18.37	16.70
Cd247	7.57	13.83	12.33
RT1-Ba	7.56	266.36	262.96
Cnn3	7.46	17.34	15.72
Ccl5	7.40	34.96	32.87
Prr5l	7.36	16.66	15.06
Cd3e	7.34	16.35	14.76
Skap1	7.33	12.75	11.30
Fabp5	7.31	16.25	14.67
RT1-Bb	7.29	22.47	20.67
Il18rap	7.21	15.66	14.11
RT1-Db1	7.13	19.55	17.85
Trib3	7.09	14.90	13.36
Lck	7.05	14.58	13.07
Dusp2	7.03	17.77	16.13
Top2a	7.03	19.36	17.66
Socs1	6.98	31.54	29.52
Bcl3	6.95	14.07	12.56
Prf1	6.93	24.62	22.78
Adam19	6.92	13.68	12.20
Zbp1	6.89	10.69	9.32
Ubd	6.86	13.56	12.08
Iqgap1	6.85	10.59	9.24
Ctsw	6.80	10.37	9.03

Gimap4	6.75	10.14	8.81
Ciita	6.74	19.67	17.96
Apol3	6.73	10.06	8.73
Lgals3	6.72	88.44	85.74
Serping1	6.68	159.22	156.21
Mt1	6.61	18.93	17.25
Sh2d2a	6.59	9.38	8.09
Zap70	6.59	9.46	8.17
Bcl11b	6.58	9.43	8.14
RGD1564664	6.54	9.35	8.06
Gpr171	6.49	11.49	10.10
RT1-DOa	6.49	13.44	11.97
Nkg7	6.45	19.81	18.09
Cd38	6.37	8.63	7.38
Mt2A	6.34	10.72	9.35
Ube2c	6.30	8.33	7.09
Tgm2	6.30	16.31	14.72
Upp1	6.29	12.49	11.06
Flt1	6.25	11.79	10.39
Fam111a	6.21	8.13	6.91
Itk	6.18	8.03	6.81
Fam102a	6.18	15.33	13.78
Acod1	6.16	7.95	6.74
Psat1	6.09	7.66	6.47
Gimap9	6.08	7.68	6.49
Gimap7	6.05	9.31	8.02
Tnfrsf4	6.03	7.53	6.35
P2ry10	6.02	7.51	6.33
Sit1	6.01	7.41	6.24
Il1r2	5.98	14.21	12.70
Adgrg5	5.98	7.36	6.20
B4galnt1	5.95	8.71	7.46
Mthfd1l	5.93	8.87	7.60
Il2ra	5.93	7.12	5.96
Hopx	5.93	11.75	10.35
Ifi44l	5.88	7.06	5.90
Mybl2	5.80	6.81	5.67
Lat	5.78	6.74	5.61
Crispld2	5.75	7.78	6.58
Icos	5.74	7.96	6.75
Vcam1	5.74	44.43	42.12
Cox6a2	5.74	12.28	10.86
Tnfrsf18	5.73	6.59	5.46
Ptgs2	5.65	6.38	5.27
Pstpip2	5.61	13.00	11.54

Cdc42ep2	5.60	14.70	13.17
Pde4d	5.60	6.12	5.02
Lilrc2	5.55	7.36	6.20
Hk3	5.52	16.65	15.05
Cd44	5.51	13.18	11.72
Mcm5	5.48	19.01	17.32
Cd69	5.47	5.90	4.82
Olfm1	5.45	8.83	7.56
Ttr	5.44	5.79	4.72
LOC691141	5.43	31.62	29.60
Rftn1	5.42	9.98	8.66
Gng3	5.42	5.76	4.69
Ggt1	5.38	5.59	4.54
Patj	5.33	8.69	7.44
Diaph1	5.32	27.51	25.61
Ptges	5.32	6.46	5.35
Gbp2	5.31	180.58	177.27
Rora	5.29	5.45	4.42
Phgdh	5.28	7.25	6.09
Slc7a2	5.22	8.19	6.96
Gvin1	5.21	5.23	4.21
Tubb6	5.21	17.77	16.13
Il17re	5.20	5.23	4.21
Sod2	5.17	145.71	142.75
Ndrg1	5.17	20.30	18.56
Cdk1	5.15	5.12	4.11
Lgals1	5.09	4.97	3.98
Tnfrsf14	5.09	4.96	3.96
Csf2rb	5.07	4.91	3.92
Scimp	5.07	4.90	3.91
Spn	5.05	42.14	39.88
Tnfrsf9	5.03	4.83	3.85
LOC100359515	5.01	6.47	5.35
Ifi27l2b	4.98	29.11	27.15
Rasip1	4.96	4.69	3.72
Serpinb6b	4.95	6.08	4.99
Dok2	4.94	4.61	3.65
Slco3a1	4.93	4.62	3.66
Cyfip2	4.88	9.69	8.38
Tuba1c	4.87	5.12	4.11
Isg15	4.83	27.74	25.82
Cd3g	4.78	4.30	3.36
Ahnak	4.73	4.19	3.26
Ccr6	4.72	10.97	9.59
Prdx5	4.72	111.91	109.08

Prxl2a	4.72	4.80	3.82
Stk39	4.70	4.15	3.23
Noct	4.69	4.11	3.20
Ramp1	4.69	4.10	3.19
Jaml	4.65	4.03	3.13
Gata3	4.65	5.70	4.64
Etv6	4.64	4.04	3.14
Igf2r	4.63	4.01	3.12
Rab11fip1	4.62	5.61	4.56
Bspry	4.62	4.00	3.10
Xdh	4.61	7.92	6.72
Sfxn1	4.59	4.43	3.48
Wdhd1	4.58	3.92	3.03
Cish	4.57	3.90	3.02
Spp1	4.57	12.11	10.69
Cdkn1a	4.56	51.45	49.05
MGC105649	4.55	22.57	20.77
Crip1	4.54	13.40	11.93
Eml2	4.54	3.84	2.96
Slfn4	4.52	89.80	87.07
Psen2	4.51	56.40	53.92
Chchd10	4.47	16.14	14.57
Acap1	4.46	3.68	2.82
Ikzf3	4.46	3.68	2.82
Plvap	4.46	4.19	3.27
Fgl2	4.43	48.99	46.63
Birc3	4.43	4.11	3.20
Abitram	4.40	3.59	2.74
Irf1	4.40	178.60	175.38
Slc7a11	4.39	4.04	3.14
Ifitm1	4.39	40.00	37.79
Bcat1	4.37	4.87	3.88
Rell1	4.34	3.94	3.05
Cks1b	4.34	3.49	2.65
Irgm	4.33	18.79	17.11
Ptpn22	4.32	5.77	4.70

Supplementary table 2: Downregulated genes in microglia from EAE rats compared to microglia from naive rats. Cut off FC is 20.

Gene Name	**Log_2(FC)**	**-Log_{10}(P-value)**	**-Log_{10}(P-adj)**
Usp35	-7.66	13.97	12.47
Pclo	-7.65	13.79	12.30
LOC102551819	-7.64	13.99	12.49

Chd5	-7.47	12.78	11.33
Adgra2	-7.31	12.34	10.92
Kif21a	-7.19	11.68	10.28
Hspa1a	-7.12	24.68	22.83
Ankrd55	-7.03	10.90	9.53
Unc80	-7.00	10.85	9.48
Kalrn	-6.96	10.74	9.36
Shisa8	-6.96	10.62	9.26
Ston2	-6.88	10.38	9.03
Abca9	-6.88	10.42	9.07
Ednrb	-6.82	12.56	11.13
Ppfia4	-6.70	9.63	8.33
Ryr2	-6.67	9.37	8.08
Gnas	-6.46	10.55	9.20
Kcna2	-6.41	34.10	32.03
Tjp1	-6.30	8.21	6.98
Tsga10	-6.16	9.19	7.91
Nefh	-6.08	7.37	6.20
Trem2	-6.05	162.29	159.24
Myt1l	-5.92	9.22	7.94
Gal3st4	-5.87	75.76	73.12
Peg3	-5.79	9.89	8.57
Unc13b	-5.78	6.60	5.47
Dok5	-5.70	6.35	5.24
Enpp2	-5.59	6.05	4.96
Astn1	-5.46	34.38	32.30
Lef1	-5.46	7.56	6.38
Ppp1r12b	-5.46	5.71	4.65
Wdr66	-5.40	5.55	4.51
Dock4	-5.36	42.19	39.92
Serinc4	-5.36	5.48	4.44
Bnc2	-5.35	7.88	6.67
Cald1	-5.35	5.46	4.42
Numb	-5.34	10.19	8.86
Tek	-5.33	6.27	5.16
Tmem119	-5.24	30.46	28.47
Khdrbs2	-5.21	5.12	4.11
Ank2	-5.17	5.05	4.05
Fbxl20	-5.14	20.20	18.46
Msh5	-5.14	4.97	3.98
P2ry12	-5.10	61.95	59.38
Peli3	-5.08	6.19	5.09

Wdsub1	-5.06	12.29	10.87
Siglec5	-5.02	27.25	25.36
Xkrx	-5.01	7.41	6.24
Klhl30	-4.98	5.84	4.77
Klf4	-4.89	36.20	34.07
Zfp799	-4.88	4.81	3.83
Acvr2b	-4.86	4.39	3.45
Crispld1	-4.86	7.60	6.41
Pmp22	-4.86	4.34	3.40
Trim2	-4.85	5.81	4.74
Alpk2	-4.83	4.33	3.39
Itga9	-4.83	4.31	3.37
Cd84	-4.82	7.30	6.14
Syt1	-4.79	4.21	3.29
Treml1	-4.76	27.85	25.93
Camk2n1	-4.76	15.16	13.61
Brap	-4.75	4.16	3.24
Fmnl3	-4.71	6.46	5.35
Pamr1	-4.70	4.98	3.98
Rtn4rl1	-4.69	4.87	3.88
Ap3b2	-4.67	6.06	4.97
Gpbp1l1	-4.67	4.36	3.42
Col9a2	-4.62	3.91	3.03
Snhg11	-4.62	7.42	6.24
Cwh43	-4.61	3.90	3.01
Epb41l1	-4.59	3.88	3.00
Erp27	-4.57	4.95	3.95
Hcfc2	-4.54	3.79	2.91
Me3	-4.54	3.78	2.91
Ago3	-4.54	3.78	2.90
Med12l	-4.53	35.63	33.51
Col11a2	-4.51	3.73	2.86
Iqce	-4.51	3.72	2.85
Zfp579	-4.50	3.71	2.85
Slc18b1	-4.47	7.39	6.22
Dpp6	-4.46	26.09	24.23
Casc1	-4.46	3.59	2.74
Ttc26	-4.46	4.33	3.40
Dzip1	-4.39	4.98	3.99
Zmat1	-4.39	15.69	14.13
LOC103691310	-4.36	3.47	2.63
Coq8a	-4.34	3.44	2.60

Arhgap20	-4.33	3.44	2.60
Per3	-4.32	4.47	3.52

Supplementary table 3: Upregulated genes in macrophages from EAE rats compared to macrophages from naive rats. Cut off FC is 20.

Gene Name	**Log_2(FC)**	**$-Log_{10}$(P-value)**	**$-Log_{10}$(P-adj)**
Slc7a2	10.03	23.92	22.01
Ca4	9.91	23.10	21.20
Sdc1	9.64	29.20	27.19
Ccl2	9.56	20.78	18.95
A3galt2	9.27	33.00	30.89
Ptges	9.19	32.40	30.32
Mt2A	9.14	18.27	16.54
Cish	8.67	21.62	19.77
Lhfpl2	8.53	14.95	13.33
Egr2	8.48	20.20	18.40
Maff	8.46	14.36	12.77
Vdr	8.13	13.07	11.51
Fabp5	7.98	142.16	138.79
Cxcl11	7.96	25.01	23.07
Thbs1	7.83	15.70	14.05
Sdc4	7.61	29.23	27.20
Acod1	7.40	86.79	83.99
Chac1	7.24	9.18	7.77
Htra1	7.23	9.39	7.97
Cxcl2	7.18	8.40	7.05
Ccl7	7.17	12.17	10.64
Tgm2	7.05	203.96	200.46
Gpr84	6.98	55.85	53.43
Arg1	6.98	7.89	6.57
Ass1	6.96	248.12	244.44
Ccl12	6.77	7.77	6.46
Socs1	6.68	46.32	44.01
Mt1m	6.65	7.46	6.16
Bcl2l1	6.60	16.73	15.05
Ccl22	6.55	15.55	13.91
Mmp7	6.44	6.85	5.60
Serpine1	6.38	10.17	8.73
Csf2rb	6.31	6.49	5.27
Ch25h	6.23	12.13	10.61
Cnn3	6.22	61.19	58.68

Sema3c	6.22	6.27	5.06
Apol9a	6.21	6.26	5.06
Atxn1l	6.20	6.22	5.02
Cxcl3	6.18	6.16	4.96
Defb52	6.12	6.01	4.82
Scimp	6.08	20.07	18.29
Cd14	6.07	37.61	35.39
Cxcl9	6.05	108.06	105.17
Nrg1	5.95	5.61	4.45
Clic4	5.94	8.26	6.92
Glipr2	5.92	8.09	6.76
B3galnt1	5.90	17.61	15.91
Cdkn1a	5.87	11.09	9.61
Lyrm1	5.82	5.31	4.17
Ereg	5.77	5.19	4.06
Kcnab1	5.76	5.18	4.05
Cxcl10	5.75	18.28	16.55
Hk3	5.65	29.13	27.12
Jdp2	5.62	5.97	4.78
Cgas	5.62	4.86	3.77
Fcrlb	5.47	4.57	3.51
Upp1	5.44	119.19	116.17
Wsb2	5.42	4.47	3.42
LOC100359515	5.41	5.53	4.38
F3	5.38	4.34	3.29
Rnase10	5.36	4.35	3.31
Jag1	5.35	9.81	8.38
Klrk1	5.35	13.93	12.35
S100b	5.35	5.12	4.00
Il2ra	5.32	4.30	3.25
Tcp11	5.29	4.25	3.21
Bmf	5.28	5.61	4.45
Mmp14	5.28	42.75	40.48
Nampt	5.22	128.89	125.69
LOC102553386	5.22	4.78	3.69
Inhba	5.20	4.07	3.06
Limk2	5.17	12.87	11.32
Niban2	5.16	81.87	79.14
Bhlhe40	5.14	9.24	7.83
Ltb4r	5.11	3.92	2.92
C1qtnf1	5.08	3.86	2.88
Gbp5	5.07	74.40	71.75

Mogat2	5.06	3.84	2.85
Slc41a2	5.01	3.74	2.77
Enpp3	5.00	6.66	5.43
Kdelr3	4.98	3.66	2.70
Fcgr2b	4.96	126.97	123.84
Ggn	4.91	3.57	2.62
Aldoc	4.90	4.08	3.07
Crem	4.89	32.85	30.76
Gbp2	4.87	134.07	130.79
Mreg	4.86	3.51	2.57
Actg1	4.85	3.49	2.55
Ccl3	4.85	10.55	9.09
Xaf1	4.82	4.16	3.13
Rxra	4.77	3.37	2.44
Il23a	4.76	3.35	2.43
Trib3	4.76	31.53	29.47
Ado	4.70	3.69	2.72
Pam	4.70	3.25	2.34
Osm	4.69	6.74	5.50
Ccl17	4.66	10.06	8.62
RGD1307182	4.64	3.19	2.28
Gfap	4.62	3.53	2.59
Lss	4.60	3.13	2.23
Il17a	4.59	3.10	2.21
Olr1	4.58	4.63	3.56
C2	4.57	3.08	2.19
Dok2	4.56	8.79	7.42
Il1a	4.55	5.38	4.24
Prr15	4.54	3.04	2.16
Ptgs2	4.54	6.15	4.95
Tnfaip3	4.51	3.40	2.47
Cdc42ep2	4.49	65.17	62.59
Cxcl1	4.45	2.91	2.05
Tubb6	4.44	38.72	36.49
Phlda1	4.41	6.98	5.72
Bcat1	4.39	122.51	119.44
Wfdc21	4.38	2.83	1.98
Nfkbid	4.35	4.45	3.40
Cd33	4.35	2.78	1.94
Ckap2l	4.34	3.06	2.18
Dusp2	4.33	20.31	18.51
Pmepa1	4.33	10.25	8.80

Supplementary table 4: Downregulated genes in macrophages from EAE rats compared to macrophages from naive rats. Cut off FC is 20.

Gene Name	Log_2(FC)	-Log_{10}(P-value)	-Log_{10}(P-adj)
Cd5l	-10.78	87.42	84.55
Fcgrt	-9.99	40.52	38.27
Cr2	-9.94	22.75	20.86
Fcmr	-9.70	34.35	32.21
Dnase1l3	-9.33	19.08	17.32
Gfra2	-9.17	17.69	15.99
Hpgds	-9.13	17.90	16.19
Gzma	-8.72	21.15	19.32
Ms4a1	-8.48	18.88	17.12
Hap1	-8.37	13.92	12.34
Rtn1	-8.34	18.29	16.56
Fabp4	-8.33	13.78	12.20
LOC100361706	-8.18	12.95	11.40
Plcb2	-8.06	18.98	17.22
Clic2	-8.03	20.08	18.29
Cd79b	-8.02	35.68	33.51
Gzmm	-7.95	16.21	14.55
Mzb1	-7.93	11.96	10.44
Tnfrsf13c	-7.88	11.74	10.24
Padi4	-7.73	11.07	9.59
Nrxn2	-7.73	11.11	9.63
Itgb5	-7.70	56.21	53.77
Adgrg3	-7.70	10.99	9.51
Ebf1	-7.52	13.31	11.75
Rnase11	-7.40	9.85	8.42
Fads1	-7.40	9.85	8.42
Rpgrip1	-7.29	9.01	7.62
Kcna2	-7.25	21.54	19.70
Dpp4	-7.22	9.13	7.74
Afap1l1	-7.16	8.98	7.59
Acss1	-7.16	8.93	7.54
Cd24	-7.09	11.30	9.81
Cdh1	-7.06	113.65	110.71
Clec4m	-7.03	8.50	7.14
Pdzd2	-6.96	8.30	6.95
Il22ra2	-6.90	8.08	6.76
Fcrl1	-6.89	8.08	6.76

Slc9a9	-6.85	9.48	8.06
Gzmk	-6.82	18.23	16.51
Pitpnm2	-6.79	7.71	6.41
Dysf	-6.77	7.61	6.31
Igf1	-6.70	20.58	18.77
Hepacam2	-6.65	7.35	6.07
Agpat3	-6.56	12.03	10.51
Hba-a1	-6.55	7.05	5.79
Ccdc61	-6.51	6.81	5.56
Adora1	-6.47	6.86	5.60
Hbb-b1	-6.46	33.02	30.90
Ngfr	-6.45	17.82	16.11
Jmy	-6.36	6.53	5.30
Stk39	-6.33	6.47	5.24
Slamf7	-6.26	30.77	28.73
Slc45a3	-6.26	6.29	5.08
Slc8a1	-6.26	6.29	5.08
Tspan7	-6.23	7.36	6.07
Aebp1	-6.19	6.12	4.92
Frmd4b	-6.19	7.53	6.24
Trib2	-6.15	6.00	4.81
Zfp251	-6.14	5.98	4.79
Pxdc1	-6.13	5.96	4.78
Np4	-6.13	7.15	5.88
Cd27	-5.99	6.73	5.49
Mpped1	-5.96	5.56	4.41
Tnnt1	-5.94	6.56	5.33
Fancm	-5.93	5.42	4.28
Vcam1	-5.92	6.81	5.56
Hba1	-5.91	86.71	83.94
Rgs5	-5.91	5.40	4.26
Gcgr	-5.90	5.42	4.27
Gpr15	-5.89	5.36	4.22
Akap11	-5.88	7.39	6.10
Ust	-5.87	5.32	4.18
Bend5	-5.85	5.31	4.17
LOC100134871	-5.78	8.09	6.76
Madd	-5.73	4.98	3.87
Fcer2	-5.73	5.05	3.93
Unc79	-5.71	4.96	3.85
Cdip1	-5.71	5.00	3.90
Ncapd3	-5.67	4.88	3.78

Lysmd4	-5.64	4.86	3.77
Cep162	-5.64	5.67	4.51
Spib	-5.59	7.25	5.97
Galnt6	-5.57	4.72	3.64
Jakmip1	-5.54	4.66	3.59
Heph	-5.52	9.87	8.43
Pip5k1b	-5.52	4.62	3.55
Ackr3	-5.51	4.57	3.51
Ptch1	-5.48	4.54	3.48
Ccr3	-5.47	36.19	34.01
Dchs1	-5.47	4.49	3.43
Sacs	-5.46	5.85	4.67
Ppfia4	-5.45	8.34	7.00
Iba57	-5.44	5.64	4.48
Ice2	-5.41	4.41	3.36
Ngp	-5.40	4.41	3.36
Carmil2	-5.33	4.26	3.22
Notch4	-5.31	4.22	3.19
Kcnk1	-5.31	4.22	3.19
Fstl3	-5.29	4.19	3.15
Ccdc180	-5.29	5.51	4.36
Timeless	-5.28	4.17	3.14
Tnik	-5.28	4.93	3.83
Syn1	-5.27	4.15	3.12
Klhdc1	-5.25	4.71	3.63
Arhgap21	-5.25	6.46	5.24
Dst	-5.25	20.59	18.78
Tenm2	-5.24	4.10	3.08
Cyp46a1	-5.24	4.09	3.08
Efnb3	-5.22	4.64	3.57
Pde3b	-5.20	4.75	3.67
Tnfrsf13b	-5.16	3.96	2.96
Mns1	-5.13	3.90	2.91
RT1-M2	-5.12	3.91	2.91
Vsx1	-5.10	3.86	2.88
Gpr174	-5.07	3.82	2.84
Gimap6	-5.07	3.82	2.84
Ankrd44	-5.06	5.79	4.62
Usp46	-5.06	4.28	3.24
Mical3	-5.03	4.20	3.17
Nav1	-5.03	3.69	2.72
Epb41l3	-5.03	9.95	8.52

Art2b	-5.03	3.74	2.77
Reep6	-5.02	3.73	2.76
Lin9	-4.99	3.67	2.71
Togaram1	-4.97	3.62	2.66
Zfp397	-4.94	3.59	2.64
Ucp3	-4.93	4.16	3.13
Mks1	-4.93	3.56	2.61
S1pr3	-4.92	3.58	2.63
Cela1	-4.90	4.10	3.08
Slco2b1	-4.90	10.55	9.09
Cdca7l	-4.90	3.54	2.59
Cul9	-4.88	3.51	2.57
Arap2	-4.88	3.50	2.57
Cc2d2a	-4.86	3.48	2.54
Fcho1	-4.86	4.01	3.00
Kif5a	-4.86	3.42	2.49
Acy3	-4.84	3.44	2.51
Pgm2l1	-4.83	3.76	2.78
Lax1	-4.82	3.42	2.49
Niban3	-4.78	3.36	2.43
Zfp563	-4.77	3.70	2.73
Mtrf1l	-4.77	3.34	2.42
Defa5	-4.75	4.12	3.10
P2rx1	-4.75	3.32	2.40
RGD1306556	-4.73	3.28	2.36
Acp5	-4.73	34.44	32.29
Vsig8	-4.72	3.27	2.36
Ptbp2	-4.69	6.73	5.49
Txk	-4.68	3.22	2.31
Hba-a2	-4.68	55.60	53.20
Tmem209	-4.67	3.18	2.28
Rarres1	-4.67	8.35	7.00
Epcam	-4.67	3.19	2.28
Dennd2c	-4.67	3.19	2.28
Mmd	-4.62	3.12	2.22
Pla2g15	-4.61	20.80	18.98
Snap47	-4.61	3.10	2.21
Chaf1a	-4.60	4.66	3.59
Gimap8	-4.58	3.07	2.19
Bbs9	-4.57	3.06	2.17
Gse1	-4.54	2.97	2.09
Inf2	-4.52	5.14	4.01

Pou2af1	-4.52	5.67	4.50
Ccr6	-4.51	12.21	10.68
Meis3	-4.51	2.97	2.10
Mis18bp1	-4.51	3.64	2.68
Mmp12	-4.49	3.44	2.51
Cd163	-4.49	3.52	2.58
Zfp296	-4.48	2.94	2.07
Pdgfc	-4.48	2.94	2.07
Fgf11	-4.48	2.93	2.06
Atp1b2	-4.47	2.93	2.06
Tmem9	-4.46	5.50	4.36
Akr1e2	-4.45	2.90	2.03
Retnlg	-4.44	3.54	2.60
Kiaa0408L	-4.44	2.87	2.01
Elmo1	-4.41	2.85	1.99
Cdhr1	-4.41	2.85	1.99
Tspan18	-4.40	2.83	1.98
Itsn1	-4.39	5.98	4.79
LOC688754	-4.37	2.76	1.92
Clec4a	-4.36	2.79	1.94
Tmcc2	-4.34	2.75	1.91
Atp1b1	-4.34	3.06	2.18

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AFFIDAVIT

Affidavit

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Street

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Zip code, town, country

I hereby declare, that the submitted thesis entitled:

Microglia-Regulated Activation of CD4^{+} T Cells in the Development of Experimental Autoimmune Encephalomyelitis

……..

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Shanghai, 25.10.2021 — Cuilian Du

place, date — Signature doctoral candidate

LIST OF PUBLICATIONS

1. **Du C***, Wang Y, Zhang F, Yan S, Guan Y, Gong X, Zhang T, Cui X, Wang X, Zhang CX, (2017). Synaptotagmin-11 inhibits cytokine secretion and phagocytosis in microglia. *Glia*, DOI: 10.1002/glia.23186.

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3. Yan S, Wang Y, Zhang Y, Wang L, Zhao X, **Du C**, Gao P, Yan F, Liu F, Gong X, Guan Y, Cui X, Wang X, Xi Zhang C, (2020). Synaptotagmin-11 regulates the functions of caveolae and responds to mechanical stimuli in astrocytes. *FASEB J*, DOI: 10.1096/fj.201901715R.

4. **Du C***, Zhang F, Zhang CX (2018). Phagocytosis Assay for α-Synuclein Fibril Uptake by Mouse Primary Microglia. *Bio-protocol*, DOI: 10.21769/BioProtoc.2986