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REGULATION OF MICROGLIA AND MONOCYTE FUNCTION BY THE CYTOKINE TGF-β

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ABSTRACT

It has been almost a century since Pío del Río-Hortega described the microglial cell, proposing it to be primarily occupied with phagocytosis of waste products. While this makes up one of its functions, we know today that microglia participate in numerous tasks and can interact with and regulate all other cell types of the brain. Given this knowledge, it is perhaps not surprising that microglia dysfunction has been implicated in a wide range of neurological disorders, including diseases of neuroinflammatory (multiple sclerosis), neurodevelopmental (schizophrenia) and neurodegenerative (amyotrophic lateral sclerosis and Alzheimer's disease) character.

Monocytes are produced in the bone marrow and are released into the bloodstream from where they can infiltrate virtually any tissue, either as a homeostatic process to replace the local macrophage pool, or as an inflammatory response to tissue damage. Monocytes can engraft the central nervous system under various conditions and are historically recognized as the main drivers of the demyelinating process that clinically manifests as multiple sclerosis. However, given the right signals, monocytes can also integrate into the neural network and become permanent residents of the brain. The work presented in this thesis explores how these distinct processes are regulated by the cytokine TGF-β.

Using conditional gene targeting approaches we investigated the role of TGF-β signaling in monocytes during autoimmune neuroinflammation as well as during homeostatic replacement of microglia after experimental depletion. We demonstrate that, when autoimmune paralysis is established, TGF-β suppresses monocyte effector functions including pro-inflammatory cytokine production and oxidative damage, which initiates remission of disease. Subsequently, in a setting of microglia loss, monocytes required TGF-β to colonize the microglial niche and to maintain microglia-like phenotype and function. Abrogation of TGF-β signaling in monocyte-derived microglial replacements resulted in the spontaneous initiation of demyelination and neuronal damage that clinically presented as a progressive and fatal motor disease.

In summary, our studies provide novel mechanisms by which TGF-β regulates brain homeostasis, which are likely deregulated during disease and that could be pharmacologically targeted.

LIST OF SCIENTIFIC PAPERS

I. Parsa R*, **Lund H***, Tosevski I*, Zhang XM, Malipiero U, Beckervordersandforth J, Merkler D, Prinz M, Gyllenberg A, James T, Warnecke A, Hillert J, Alfredsson L, Kockum I, Olsson T, Fontana A, Suter T* and Harris RA*. *TGFβ regulates persistent neuroinflammation by controlling Th1 polarization and ROS production via monocyte-derived dendritic cells.* **Glia**. 64(11), 1925-1937 (**2016**)

II. **Lund H**, Pieber M, Parsa R, Han J, Grommisch D, Ewing E, Kular L, Needhamsen M, Butovsky O, Jagodic M, Zhang XM, Harris RA. *Competitive repopulation of an empty microglial niche gives rise to functionally distinct subsets of microglia-like cells*. **Manuscript**

III. **Lund H**, Pieber M*, Parsa R*, Grommisch D, Ewing E, Kular L, Han J, Zhu K, Nijssen J, Hedlund E, Needhamsen M, Ruhrmann S, Guerreiro-Cacais AO, Berglund R, Forteza MJ, Ketelhuth DFJ, Butovsky O, Jagodic M, Zhang XM*, Harris RA*. *Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling.* **Nature Immunology.** 19(5), 1-7 (**2018**)

IV. **Lund H**, Zhu K, Pieber M, Ohshima M, Blomgren K, Zhang XM, Harris RA. *Long-term effects of microglial depletion on tau pathology and spatial memory.* **Manuscript**

*Equal contribution

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Zhang XM, **Lund H**, Mia S, Parsa R, Harris RA. Adoptive transfer of cytokine-induced *immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice.* **Glia**. 62(5), 804-17 (**2014**)

II. Parsa R, **Lund H**, Georgoudaki AM, Zhang XM, Guerreiro-Cacais AO, Grommisch D, Warnecke A, Croxford AL, Jagodic M, Becher B, Karlsson MC, Harris RA. *BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis.* **Journal of Experimental Medicine**. 213(8), 1537-53 (**2016**)

III. **Lund H**, Pieber M, Harris RA. *Lessons Learned about Neurodegeneration from Microglia and Monocyte Depletion Studies.* **Frontiers in Aging Neuroscience**. 9, 234 (**2017**)

IV. Sackmann V, Ansell A, Sackmann C, **Lund H**, Harris RA, Hallbeck M, Nilsberth C. *Anti-inflammatory (M2) macrophage media reduce transmission of oligomeric amyloid-beta in differentiated SH-SY5Y cells*. **Neurobiology of Aging**. 60, 173-182 (**2017**)

V. Needhamsen M, Ewing E, **Lund H**, Gomez-Cabrero D, Harris RA, Kular L, Jagodic M. *Usability of human Infinium MethylationEPIC BeadChip for mouse DNA methylation studies*. **BMC Bioinformatics**. 18, 486 (**2017**)

CONTENTS

LIST OF ABBREVIATIONS

BBB	Blood brain barrier
CLP	Common lymphocyte progenitor
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CSF1R	Colony stimulating factor 1 receptor
DC	Dendritic cell
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte-macrophage progenitor
HSC	Hematopoietic stem cell
HSVTK	Herpes simplex virus thymidine kinase
M-CSF	Macrophage colony stimulating factor
MDP	Monocyte-dendritic cell progenitor
MEP	Megakaryocyte-erythrocyte progenitor
moDC	Monocyte-derived dendritic cell
MPP	Multipotent progenitor
ROS	Reactive oxygen species
TGF-β	Transforming growth factor beta

1 INTRODUCTION

1.1 TISSUE-RESIDENT MACROPHAGES

These are exciting days to study macrophage biology! While originally recognized for their phagocytic properties (macrophage = big eater, Greek) – a discovery that earned Russian zoologist Ilya Mechnikov the 1908 Nobel Prize in Physiology or Medicine – we understand today that macrophages can have wide-ranging functions spanning from regulating wiring of the brain to controlling our bowel movements. Macrophages are present in virtually every organ of the body where they have fundamental functions tailored to the need of the specific tissue. A number of historic examples are illustrated in **Figure 1**.

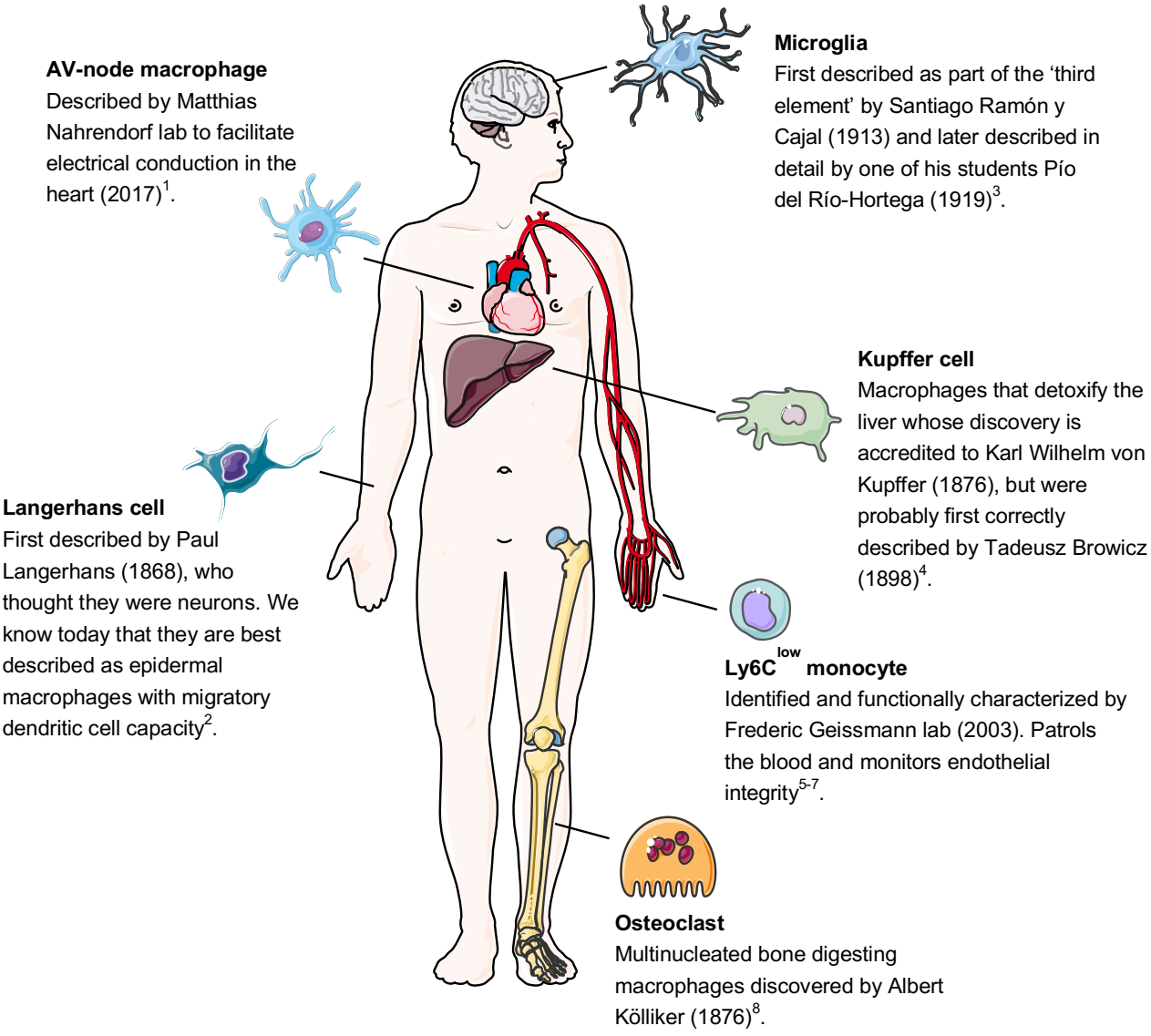

Figure 1: Some important examples of tissue-resident macrophage populations and their discovery or functional description.

1.2 DEVELOPMENT OF TISSUE-RESIDENT MACROPHAGES

Three successive waves of hematopoiesis occur during embryonic development and the mature circulating immune system is derived from the final *definitive* wave, which gives rise to hematopoietic stem cells (HSC). Although macrophages had been observed in the embryo before the generation of HSCs, these were not thought to contribute lastingly to adult macrophage populations. With the identification of several distinct organ-specific macrophage populations such as brain *microglia*, splenic *red pulp macrophages*, *Kupffer cells* in the liver, van Furth and colleagues instead proposed that all macrophage populations were continuously derived from circulating monocytes (definitive hematopoiesis in the bone marrow), a model they termed the 'mononuclear phagocyte system'[9]. This was an impressive model if we consider the knowledge available at that time. However, with the advent of advanced techniques to trace cellular lineages we have understood that most mature macrophage populations are in fact derived from embryonic precursors. Although the exact sequence of events is still a matter of research and debate, there is a consensus that the first tissue macrophages are derived from erythromyeloid progenitors that arise in the yolk sac at embryonic day 7 (E7.0)[10,11]. During the first wave of hematopoiesis, these yolk sac macrophages colonize the entire embryo, including the brain, to generate microglia and all other types of tissue-resident macrophages. According to one model, the erythromyeloid progenitors subsequently migrate to the fetal liver, give rise to fetal liver monocytes that later invade all organs except the brain and dilute the yolk sac derived tissue macrophages[12]. Concomitant with this, fetal HSCs in the aorta-gonad-mesonephros region colonize the fetal liver where they establish definitive hematopoiesis but will also seed the fetal bone marrow which will generate adult HSCs. One model suggest that these fetal HSCs are the precursors of most tissue-resident macrophages[13]. Another model suggests instead that all tissue resident macrophages are derived from yolk sac macrophages and that subsequent waves of fetal liver monocytes only minimally contribute to tissue-macrophage pools[14].

These models thus differ on the final composition of most peripheral tissue-resident macrophages, but agree that microglia derive only from yolk sac macrophages. This has been attributed to the closure of the blood brain barrier (BBB) which occurs before fetal liver monocyte colonization of the embryo and prevents their entry into the parenchyma[11,15]. After birth, adult HSC-derived monocytes from the bone marrow will displace embryonic macrophages in some organs, as further discussed below. The consequence of this process is ontogenic heterogeneity across organs[16,17], and the proposed physiological explanation is organ growth and accessibility. Organs that remain accessible through the blood will continuously recruit macrophage precursors throughout embryogenesis[10].

1.3 DEVELOPMENT AND TURNOVER OF MICROGLIA

In a series of papers published almost 100 years ago, Pío del Río-Hortega described microglia as a separate cellular entity from the previously defined neuroglia (astrocytes)[3]. Using only histological techniques Río-Hortega noticed that microglia precursors invaded the brain during embryonic development and proposed that they were of mesodermal origin[18]. Elegant studies in the 1990s further suggested that microglial progenitors originated in the yolk sac, penetrated the brain in the embryo and proliferated extensively to give rise to the microglial

pool[19]. Final proof was delivered by Florent Ginhoux and colleagues[20] in the form of fate-mapping experiments using *Runx1*^{CreER} mice to specifically label macrophages in the yolk sac at E7.5 which appeared in the brain rudiment at E9.5. This process required a functioning circulatory system and was dependent on CSF1R. A more detailed analysis revealed that microglia arise from a c-kit^{+} progenitor with both erythroid and myeloid potential in the yolk sac[21] (erythromyeloid progenitor). Microglia development was dependent on PU.1, a master myeloid transcription factor[22], as well as IRF8, and the cells invaded the brain in a manner dependent on matrix metalloproteinases but independently of chemokine signaling[21]. The development of adult microglia from erythromyeloid progenitors is a process occurring in at least three developmental stages, with each stage characterized by distinct transcriptomes driven by unique transcription factors[23,24]. This supports the idea of distinct functional roles of microglia during development vis-à-vis adult homeostasis, which has been experimentally observed (see HOMEOSTATIC FUNCTIONS OF MICROGLIA). It was recently demonstrated that male and female microglia display striking transcriptional differences, which are influenced both pre- and post-natally by the microbiome[24]. The importance of the microbiome for proper microglia development was demonstrated by Erny *et al.,* who found germ-free mice to have an immature microglia phenotype which could be restored by recolonization with complex microbiota, this being dependent on microbiota-derived short chain fatty acids[25].

If homeostatic conditions are maintained, the microglial pool is sustained throughout adult life with minimal or no input from definitive hematopoiesis, as demonstrated by long-term parabiosis experiments[20,26,27]. Microglia require constant signaling through the CSF1R for survival[28], and can use either of its ligands M-CSF[20] or IL-34[29,30] for their turnover. Several recent studies demonstrate that the microglial pool is maintained by coupled proliferation and apoptosis[31-33], meaning that when one microglial cell undergoes apoptosis, a nearby cell proliferates to compensate for the loss. The turnover rates appear to be variable in different brain regions, as Tay *et al.* reported that microglia in the olfactory bulb turn over fastest, completing a full cycle every 8 months as compared to 15 months for the hippocampus, and as long as 41 months for the cortex[32]. These slow turnover rates are challenged by another study using BrdU incorporation rates and live imaging of microglia that estimated that the entire microglial population turned over every 96 days[31]. A long-term imaging study of individual cortical microglia demonstrated that a minority of imaged microglia died over a 15-month period, indicating that as much as half of the cortical microglial pool may survive for the lifespan of a mouse[33]. Human microglia were estimated[34] to have an average life span of 4.2 years based on analysis of incorporation of atmospheric ^{14}C.

1.4 OTHER CNS-ASSOCIATED MACROPHAGES

Microglia are the predominant myeloid cells in the brain during steady-state conditions and reside in the parenchyma. However, CX3CR1^{+} macrophages also line non-parenchymal spaces such as the meninges, vasculature and choroid plexus in the adult brain[35]. Based on early bone marrow chimera experiments it was proposed that perivascular macrophages originated from adult hematopoiesis[36]. However, it has subsequently been demonstrated that irradiation sensitizes the brain for engraftment by peripheral myeloid cells[37], and recent fate

mapping and parabiosis studies have revealed that perivascular, meningeal and choroid plexus macrophages are all embryonically-derived[38]. Perivascular and meningeal macrophages ($CX3CR1^{+}CD45^{hi}CD206^{+}$) subsequently self-maintain, whereas choroid plexus macrophages ($CX3CR1^{+}CD45^{hi}$) have a slow turnover from CCR2-dependent peripheral monocytes[38]. These non-parenchymal macrophage populations were more recently shown to separate based on CCR2, CD38 and MHCII expression and were suggested to make up as much as 10% of the central nervous system (CNS) leukocyte pool, based on high dimensional mass cytometry[39]. The sensitivity of these 'border-associated macrophages' to intravenous delivery of a CSF1R-specific antibody further confirms their location outside of the BBB. In contrast, microglia that reside inside the BBB are not depleted by this treatment[39].

1.5 HOMEOSTATIC FUNCTIONS OF MICROGLIA

Microglia are integral components of the developing brain, being able to promote selective death or survival of specific neuronal populations. For example, microglia induce programmed death of hippocampal neurons[40] and Purkinje neurons[41] during development and orchestrate their phagocytic removal. Conversely, IGF1 production by microglia supports survival of layer V cortical neurons during postnatal development[42]. Embryonic microglia have also been shown to associate with dopaminergic axons and control their outgrowth to participate in correct layering of cortical interneurons[43]. During postnatal development microglia promote synaptic pruning[44], which is facilitated by the complement system[45,46]. Finally, BDNF production by microglia regulates dendritic spine formation and elimination postnatally, influencing hippocampal learning[47]. These findings identify microglia as a central player in sculpting neuronal circuits, failure of which is linked to neurodevelopmental disorders such as autism and schizophrenia[48].

Microglia in the uninjured CNS are traditionally described as being 'resting' or 'quiescent', considered silent sentinels waiting for immune activation or trauma. However, time-lapse two-photon imaging in the cerebral cortex of live mice has revealed that microglia are highly dynamic cells even in the naive brain[49]. While microglial cell bodies always remain stationary (even beyond a year of repeated imaging[33]), their processes are constantly extending and retracting[49], making transient contacts with synapses[50]. This motility requires ATP sensing by purinergic receptors[51], several of which are expressed in microglia[52].

1.6 MONOCYTE DEVELOPMENT IN THE BONE MARROW

The development of circulating monocytes occurs from HSCs in the bone marrow. Hematopoiesis is classically described as a stepwise process whereby highly proliferative and multipotent stem cells differentiate into cells with increasingly restricted lineage potential[53]. This is presented as a tree-model (**Figure 2**) starting with the HSC and branching out to form all the cellular lineages of the blood. According to this model, separation of blood lineages starts with the commitment to either *lymphoid* (common lymphoid progenitor, CLP) or *myeloid* (common myeloid progenitor, CMP) lineages. The myeloid lineage then divides into a platelet/erythrocyte-forming branch (megakaryocyte-erythrocyte progenitor, MEP) and a

neutrophil/macrophage/dendritic cell (DC) forming branch (granulocyte-macrophage progenitor, GMP), which further separates into downstream lineage-committed progenitors[54,55]. The existence of these distinct differentiation states is supported by cell transfer experiments. According to this model, circulating monocytes are formed downstream of the GMP via the monocyte-DC progenitors (MDP)[56] and the common monocyte progenitor (cMoP)[57]. However, recent single cell RNA-sequencing experiments suggest that commitment to distinct cellular fates already occurs at the multipotent progenitor (MPP) stage, and that intermediate differentiation states with multi-lineage potential are very rare[58]. This was confirmed *in vivo* by following the fate of individual transplanted hematopoietic progenitors, demonstrating that CMPs rarely gave rise to multiple lineages[59]. This data questions the classic tree-model and suggests that CMPs, rather than having multi-lineage capacity, are a collection of heterogeneous progenitors with pre-fixed states. Historically, CMPs and other progenitor populations have been studied as bulk-sorted populations, which could explain the conflicting results. New models incorporating this knowledge are being proposed[60].

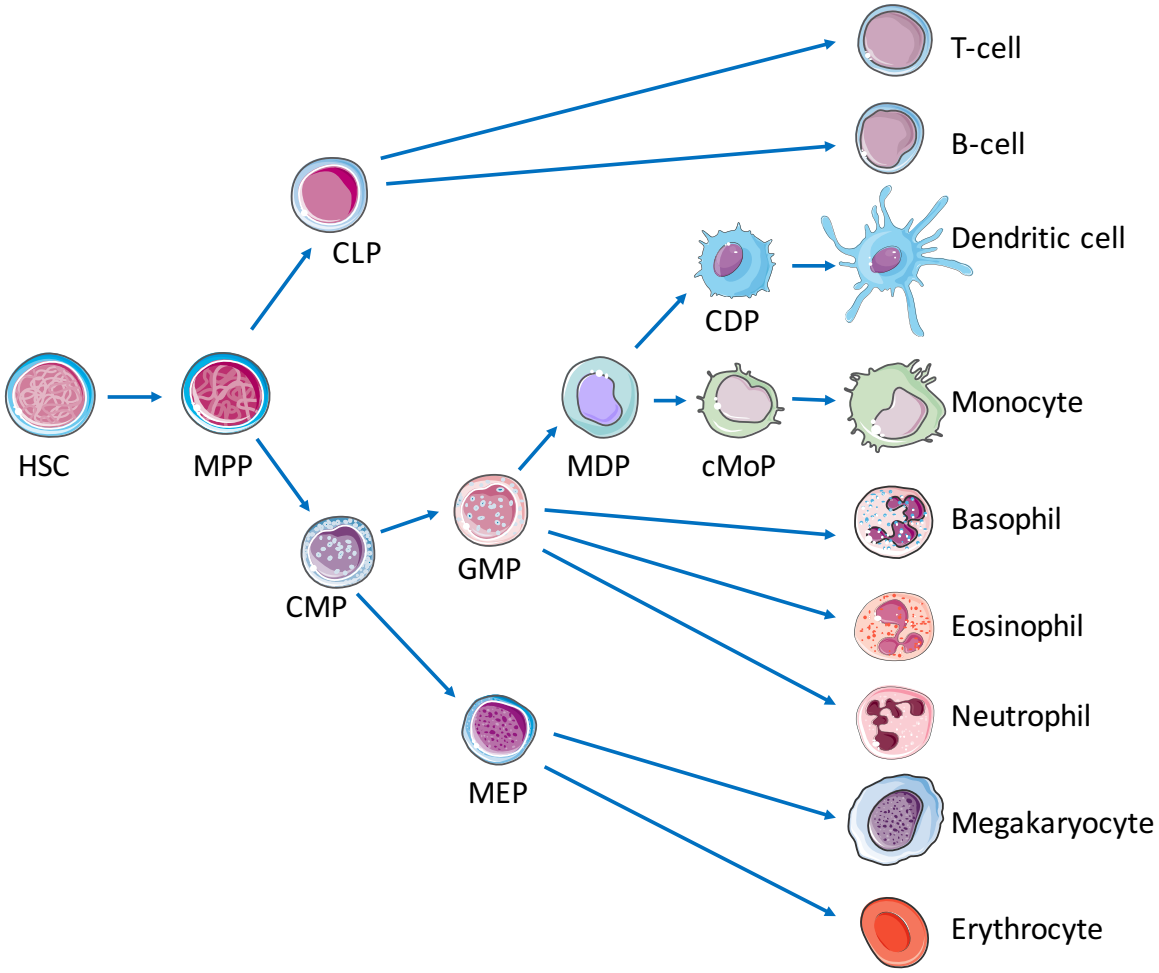

Figure 2: The classic model of hematopoiesis starting with a binary choice to commit to myeloid (CMP) or lymphoid (CLP) lineages. This model is being revised.

The commitment to a distinct cellular fate is believed to be determined by a unique combination of transcription factors[61]. For example, monocyte specification relies on high expression of the pioneer transcription factor PU.1 combined with the presence and absence of specific transcription factor partners, including IRF8, C/EBPα and GFI1. At lower doses, PU.1 instead promotes neutrophil development[62] and IRF8 and GFI1 at high doses promotes monocyte[63] or neutrophil[64] development, respectively. Co-expression of IRF8 and GFI1 arrests progenitor cells in a metastable state, each transcription factor promoting one cellular fate and inhibiting the other, until one factor prevails, inducing either monocyte or neutrophil development[65]. Individual myeloid lineages are enforced by the action of cytokines, including M-CSF and G-CSF, which act at the GMP stage to induce monocyte or granulocyte commitment, respectively[66]. Furthermore, deficiency in M-CSF or G-CSF results in loss of the monocyte/macrophage[67] and granulocyte[68] lineages, respectively. GM-CSF is a more promiscuous cytokine, which can support the growth and development of granulocyte, monocyte and conventional DC lineages[69,70]. However, GM-CSF deficiency results in no major alterations in the bone marrow, indicating that it is redundant during steady-state hematopoiesis[71]. M-CSF, G-CSF and in particular GM-CSF are significantly induced during settings of stress or infection to support the increased demand of mature circulating myeloid cells and to imbue them with effector functions. In these settings, the CSFs have been thought to act only on downstream progenitors or fully differentiated myeloid cells. However, M-CSF was recently shown to act already on HSCs to induce PU.1 expression and initiate myeloid lineage commitment and that this was potentiated by bacterial compounds[72].

Monocytes in the circulation exist in two main subsets described respectively as $CD14^{+}CD16^{-}$ and $CD14^{low}CD16^{+}$ in humans[73] and as $Ly6C^{hi}$ and $Ly6C^{low}$ in the mouse[5]. $Ly6C^{hi}$ monocytes are considered short-lived inflammatory cells with the capacity to infiltrate tissues upon inflammation. $Ly6C^{low}$ monocytes do not enter tissues but instead patrol the vessel lumen where they have scavenging functions in the steady-state, and upon inflammation they can recruit neutrophils to mediate endothelial cell disposal[6,7]. These observations concord with the idea that $Ly6C^{low}$ monocytes should be considered terminally-differentiated, blood-resident macrophages[74].

$Ly6C^{hi}$ monocytes express CCR2 highly, which is required for their exit from the bone marrow and entry into the circulation[75] in response to a CCL2 gradient produced by mesenchymal stem cells in proximity to the vascular sinuses of the bone marrow[76]. In the circulation the steady-state half-life of $Ly6C^{hi}$ monocytes is only 20 hours, and they subsequently downregulate Ly6C expression to give rise to terminally differentiated $Ly6C^{low}$ monocytes[77]. This differentiation process was recently characterized in detail using single cell RNA-sequencing, demonstrating this to be a C/EBPβ-dependent pathway occurring through a $Ly6C^{int}$ transitional state[78]. The gene signature of the $Ly6C^{int}$ population was characterized by high expression of *CD209a* and MHCII associated molecules, which overlaps with a recently identified $CD209a^{+}MHCII^{+}$ subpopulation of monocytes that preferentially gives rise to monocyte-derived dendritic cells (moDCs)[79]. This stood in contrast to a $CD209a^{-}MHCII^{-}$ $Ly6C^{hi}$ population of monocytes giving rise to $iNOS^{+}$ macrophages during microbial stimulation[79]. The potential heterogeneity of the monocyte pool has been further increased by the recent finding that a 'neutrophil-like' subtype of monocytes develop downstream of GMPs in an MDP/cMoP-independent manner. In this study, GMPs and MDPs independently

generated Ly6C^{hi} monocytes with distinct gene expression profiles which were differentially mobilized from the bone marrow in response to toll like receptor ligands[80]. Recently, another study identified a Ceacam1^{+}Msr1^{+}Ly6C^{-}F4/80^{-}Mac1^{+} subset of monocytes with bi-lobed nuclei that developed directly from GMPs and were specifically involved in fibrosis[81]. How this subset relates to the neutrophil-like monocyte above remains to be clarified.

In summary, the increased sensitivity of RNA sequencing techniques has revealed increased complexity in the monocyte compartment. Whether these subtypes truly represent distinct macrophage precursors with different downstream functional properties will require additional detailed investigation. At the time of writing, we are thus faced with the possibility that the diversity in monocyte progeny can be explained by the existence of distinct monocyte subsets with unique differentiation programs, transcription factors and functional capacities. Alternatively, and as discussed below, the monocyte compartment is homogenous and diversity is dependent on imprinting by the tissue environments. These hypotheses are not mutually exclusive.

1.7 MONOCYTE DIFFERENTIATION TO MATURE MACROPHAGE POPULATIONS

1.7.1 Steady-state macrophage populations

Under steady-state conditions most macrophage populations are sustained without input from mature circulating monocytes[27,77,82], but notable exceptions exist. Ly6C^{hi} monocytes constantly replenish the macrophage pool in the intestine[83,84]. Macrophages colonize the entire length of the intestine and are enriched in the lamina propria close to the epithelial layer[85] and are thus constantly exposed to commensal bacteria and orally acquired antigens. However, despite phagocytic and bacteriocidal ability, intestinal macrophages are unresponsive to toll like receptor stimulation in both mouse and Man[86,87]. This anti-inflammatory phenotype can be explained, at least in part, by constant IL-10R signaling in gut macrophages, abrogation of which leads to colitis[88]. Exposure to the microbiota, which is a defining feature of macrophages in the intestine, likely drives requirement for monocyte replenishment. This is supported by the fact that embryonic macrophages are displaced by monocyte-derived macrophages during the first weeks after birth, which coincides with the time of bacterial colonization of the gut. Furthermore, germ-free mice also have significantly fewer intestinal macrophages than do conventionally housed mice[84].

In addition to intestinal macrophages, circulating monocytes slowly replace macrophages in the dermis[89], the exocrine pancreas[90], the testicles[91], the peritoneal cavity[92] and the choroid plexus of the brain[38]. Replenishment of cardiac macrophages by monocytes has also been proposed[93] although this is a matter of controversy[94-96]. Interestingly, several of these monocyte-replenished macrophage pools have adjacently located macrophage populations which turnover independently of monocytes, including Langerhans cells in the epidermis[2], macrophages in pancreas endocrine islets[89], and microglia as well as perivascular and meningeal macrophages in the brain[20,38]. This proposes the existence of tightly controlled microenvironments regulating macrophage turnover in these organs.

1.7.2 Infection

During viral, bacterial, fungal and parasitic infections monocytes are recruited in high numbers to the affected organs from the circulation[97]. One well-studied example is *Listeria monocytogenes* infection, which recruits monocytes in a CCR2-dependent manner to the spleen where they differentiate into TNFα- and iNOS-producing macrophages (originally called Tip-DCs) that are crucial in combatting the infection[75]. In the liver, *Listeria* selectively infects Kupffer cells that die by necroptosis, leading to a dramatic drop in their numbers. The embryonic-derived Kupffer cell pool is completely replaced by recruitment of monocyte-derived macrophages and their M-CSF/IL-4 dependent local proliferation. During the early stage of infection infiltrating monocytes have an inflammatory profile characterized by high expression of IFN-γ that under the influence of IL-4 leads to monocytes adopting a Kupffer-cell like phenotype to promote restoration of tissue integrity[98]. γ-Herpes virus infection in the lung similarly leads to the selective death and complete replacement of embryonic-derived alveolar macrophages with monocyte-derived macrophages. These have a distinct transcriptional profile and subsequently protect mice from development of house dust mite allergy[99]. Taken together, these examples demonstrate that bacterial and viral infections can dramatically and permanently alter the macrophage landscape in distinct organs, giving long-lasting effects on tissue homeostasis.

1.7.3 Sterile tissue injury

Sterile tissue injury regularly results in monocyte invasion of the affected organ to assist in clean-up and to promote a return to homeostasis. For example, skeletal muscle injury causes infiltration of $Ly6C^{hi}$ monocytes that differentiate into $CX3CR1^{hi}$ anti-inflammatory macrophages that participate in muscle regeneration[100]. Paracetamol-induced liver injury similarly leads to $Ly6C^{hi}$ monocyte recruitment, occurring *en masse* within 24 hours of injury in a CCR2- and M-CSF-dependent manner. These monocytes differentiate into short-lived $CX3CR1^{+}$ macrophages (virtually gone by 120h), contribute to repair of liver damage and exhibit a gene-expression profile that is distinct from liver-resident $CX3CR1^{neg/low}$ Kupffer cells[101].

Monocytes also contribute to the cardiac macrophage pool during heart disease. An experimental model of myocardial infarction results in extensive CCL2-driven[102] recruitment of $Ly6C^{hi}$ monocytes to the heart[103,104]. The local ischemia causes apoptosis of virtually all $F4/80^{+}$ resident cardiac macrophages within 24 hours, which are quickly recovered (4 days) by the infiltrating monocytes[95]. Hypertension results in a similar but more limited expansion of the heart macrophage pool driven by monocyte infiltration, which can contribute to heart failure[105].

Ionizing irradiation is known to condition the brain for engraftment of $Ly6C^{hi}$ monocytes[37], which has confounded the interpretation of many neurological disease models[106]. However, more recently, it was reported that after irradiation, CNS-recruited monocytes integrate into neurogenic niches and participate in brain repair in a G-CSF-dependent manner[107]. Furthermore, a number of acute injuries to the brain cause a robust engraftment of monocytes, including stroke[108], epileptic seizures[109] and traumatic brain injury[110,111]. The role of monocytes in stroke and epileptic seizures appear to be mostly deleterious, although there are conflicting reports, as we have reviewed elsewhere[112]. Traumatic brain injury induced by

a controlled cortical impact results in transient accumulation of $CCR2^{+}CX3CR1^{+}$ monocytes/macrophages in the hippocampus that, if inhibited, reduces neuroinflammation and alleviates injury-induced cognitive impairment[110]. Spinal cord injury has been proposed to attract both $Ly6C^{hi}CX3CR1^{low}$ and $Ly6C^{low}CX3CR1^{hi}$ monocytes to the injury site through alternate routes which have deleterious or beneficial effects on wound healing and motor-function recovery, respectively[113]. In the CNS, whether monocytes contribute long-term to the microglial pool after resolution of these disease models is incompletely explored. Taken together, these studies indicate that monocytes can have both disease-promoting and -preventing functions during injury, even in the same organ.

1.7.4 Experimental depletion

Experimental depletion of distinct macrophage subsets represents a useful tool to study the capacity of local proliferation or monocyte recruitment to compensate for cellular loss. For example, when Kupffer cells are depleted in *Clec4f*-DTR mice, monocytes repopulate the liver in one wave, giving rise to fully functional, long-lived macrophages that are transcriptionally and phenotypically indistinguishable from normal Kupffer cells[114]. Similarly, cardiac macrophages are replenished completely by monocytes when depleted in CD11b-DTR mice[95]. Furthermore, an empty alveolar macrophage niche as achieved in $Csf2r^{-/-}$ mice, can be repopulated by adoptive transfer of purified yolk sac macrophages, fetal liver or adult monocytes without loss of function or ultimate differences in the transcriptomic signature[115].

Depletion of microglia has been achieved in a variety of ways. Using $Cx3cr1^{CreER}R26^{DTR}$ mice, Bruttger *et al.* achieved 80% reduction in the microglial pool, which was rapidly compensated for by local hyperproliferation[116]. A CSF1R antagonist results in even more efficient depletion (>99%), which is similarly recuperated by proliferation of the residual microglia[28,117]. If mice are exposed to lethal irradiation, however, the entire microglial pool is replenished by circulating precursors[116,118], likely $Ly6C^{hi}$ monocytes. While proliferating microglia adopt a transcriptional profile more similar to naive microglia, bone marrow-derived 'microglia' remain transcriptionally distinct[116]. Delivery of gangciclovir to CD11b-HSVTK mice via an intracerebroventricular cannula results in complete ablation of microglia as long as the drug is delivered[119]. Removal of the drug results in rapid repopulation of microglia by peripheral myeloid cells[120,121], which remain beyond 6 months and respond by extending processes toward ATP[120], indicating that peripherally-derived macrophages can adopt the microglial purinergic machinery.

1.8 TISSUE MICROENVIRONMENT CONTROL OF MACROPHAGE IDENTITY

All macrophage populations are seeded from a limited set of progenitors, yet they display great diversity both in phenotype and function. The current consensus is that the unique microenvironments present in individual tissue niches play a prevailing role in determining macrophage identity[16,122,123]. In fact, tissue-specific differentiation programs are initiated immediately after colonization of embryonic tissues by yolk sac-derived pre-macrophages[124]. Tissue-specific transcription factors have been identified for many macrophage subsets, including *Pparg* for alveolar macrophages[125], *Spic* for red pulp macrophages[126], *Id3* for

Kupffer cells[124], *Runx3* for Langerhans cells[127], *Gata6* for peritoneal macrophages[128,129] and *Sall1* for microglia[118]. Evidence of imprinting by the microenvironment was reported after adoptive transfer of bone marrow cells into irradiated mice, demonstrating that the tissue-specific chromatin states in the lung, liver, spleen and peritoneum are adopted by transplant-derived macrophages[130]. This study further demonstrated the power of the alveolar macrophage niche in reprogramming incoming macrophage precursors; Peritoneal macrophages transplanted into the lung were transcriptionally more similar to alveolar macrophages than their peritoneal counterparts when retrieved 15 days later[130]. This is further supported by the ability of yolk sac macrophages, fetal liver monocytes and adult bone marrow-derived monocytes to recapitulate alveolar macrophage transcription and function when transplanted into an empty alveolar macrophage niche[115]. These findings have spawned the recently proposed 'niche theory',[16] which suggests that the tissue microenvironment imprints functionality on the macrophage precursor independently of its origin, provided that the niche is accessible and that empty niches are available. Such imprinting would suggest the existence of 'niche signals' regulating tissue-specific macrophage identity[131], which have been resolved for some organs but remain the subject of ongoing research. For example, red pulp macrophage identity is driven by the transcription factor Spi-C[126], which is induced by heme, a by-product of splenic erythrocyte turnover[132]. Peritoneal macrophage-specific functions are controlled by retinoic acid, a metabolite of vitamin A produced in the omentum, the peritoneum-associated adipose tissue[128]. Alveolar macrophages require both GM-CSF[125,133] and TGF-β signaling[134] which drive PPARγ expression, promoting alveolar macrophage survival. TGF-β also maintains the microglia-specific transcriptome[135], probably by inducing *Sall1* transcription[118], as discussed in greater detail below.

1.9 TGF-β SIGNALING

The transforming growth factor (TGF)-β family in mammals includes three members, TGF-β1, 2 and 3. TGF-βs are synthesized as propeptides containing an N-terminal portion called the latency-associated protein (LAP) and a C-terminal domain that is the mature cytokine. These are cleaved from each other in the Golgi apparatus by the endopeptidase furin. After cleavage and assembly, LAP and the cytokine portion of TGF-β remain non-covalently attached. The crystal structure demonstrates that LAP wraps around TGF-β, covering all contact sites that could interact with receptors[136] and TGF-β can thus be stored inactive in the extracellular space. TGF-β can be activated by a number of mechanisms[137] of which thromospondin and integrins are important *in vivo.*

TGF-β signals via tetrameric receptors composed of two type I receptors (TGFβRI) and two type II receptors (TGFβRII), which are both serine/threonine kinases (**Figure 3**). Upon engagement of TGF-β, TGFβRII phosphorylates the cytoplasmic domain of TGFβRI, which triggers the recruitment of intracellular receptor Smad proteins (R-Smad) Smad2 and Smad3, leading to their phosphorylation. Phosphorylated Smad2/3 forms a trimeric complex with Smad4 and translocates to the nucleus to activate or repress gene expression by binding Smad-responsive elements[137]. Alternatively, TGFβRI can associate with the R-Smads Smad1 and Smad5. While classically involved in bone morphogenic protein signaling, Smad1/5 can be phosphorylated in macrophages to drive alternative gene expression and functional

outcomes[138,139]. In addition to classic Smad-mediated transcription, TGF-β can induce several Smad-independent signaling pathways, including MAP kinases, PI3 kinases and Rho GTPases[140]. TGF-β limits its own signaling by promoting transcription of Smad7, which blocks binding of R-Smads to TGFβRI or recruits ubiquitin E3 ligases to the receptor, degrading TGFβRI[141].

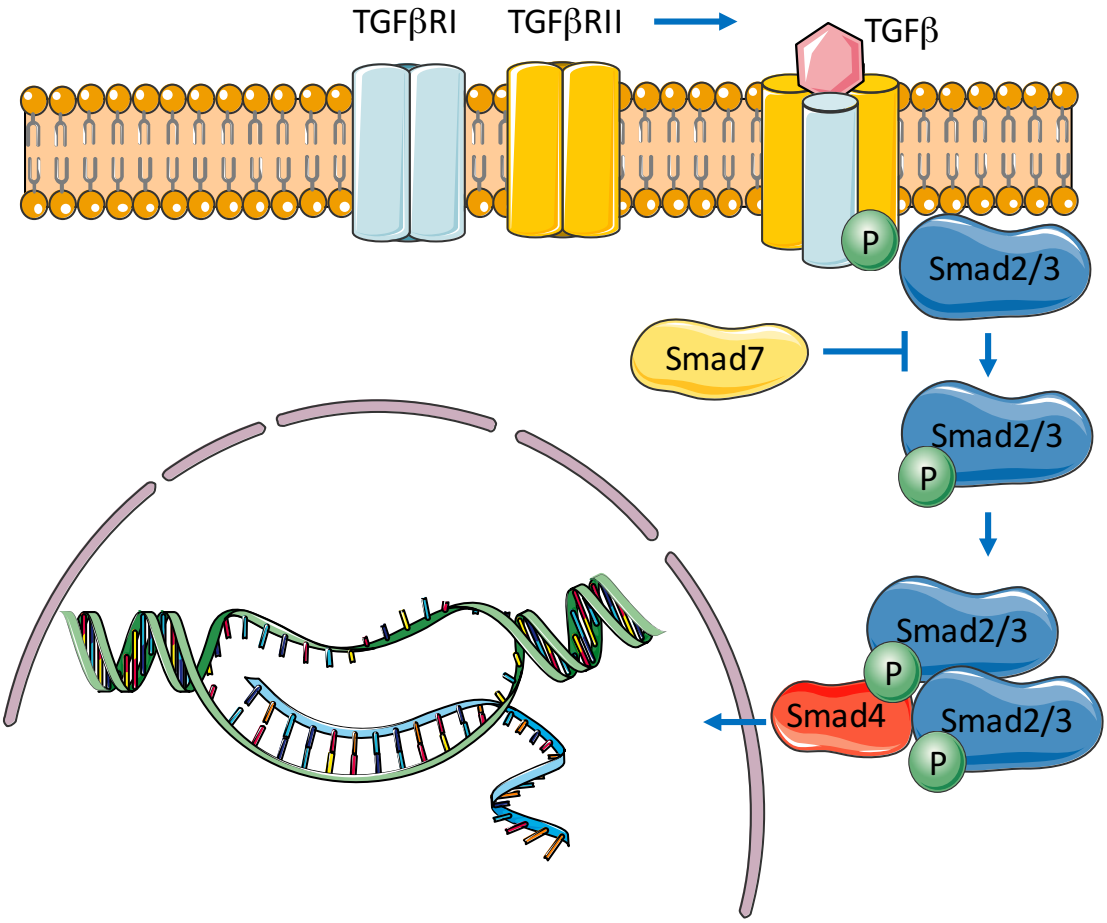

Figure 3: Smad-dependent TGF-β signaling.

TGF-β1, 2 and 3 have distinct properties *in vivo*[142], and TGF-β1 is the predominant isoform expressed in the immune system[137]. *Tgfb1*$^{-/-}$ mice either die in the embryo or after birth by multi-organ failure[143,144], highlighting a crucial role of this cytokine in controlling innate and adaptive immune responses. In the CNS, TGF-β1 has distinct effects on virtually all cell types, including microglia. When peripheral expression of TGF-β1 is rescued in *Tgfb1*$^{-/-}$ mice, the resulting CNS-conditional *Tgfb1*$^{-/-}$ mice do not succumb to multi-organ failure, but instead develop neurological deficits which manifest as motor impairments with onset at 4-6 months[135], however the exact mechanism of pathology was not elucidated.

In the same study, TGF-β1 was shown to be a major regulator of the homeostatic gene signature in microglia[135]. Microglia-specific enhancers are enriched in Smad-binding motifs which function in collaboration with the transcription factor PU.1 to drive microglia-specific gene expression[145]. In addition, the transcription factor *Sall1*, which is unique to microglia, is induced by TGF-β[118]. The importance of TGF-β in defining microglia identity is further

underscored by its ability to induce microglia gene expression *in vitro,* irrespective of macrophage type[135,145]. However, as opposed to alveolar macrophages[134] and Langerhans cells[146-148], microglia do not require TGF-β for development[135] or survival in the steady-state[118].

1.10 MICROGLIA AND MONOCYTE FUNCTIONS IN CNS AUTOIMMUNITY

Experimental autoimmune encephalomyelitis (EAE) is the prototypical model of neuroinflammation caused by CNS-infiltrating leukocytes, as evident in multiple sclerosis. EAE is induced by immunization with myelin antigens that drive the activation and expansion of self-reactive T-cells that subsequently migrate to the CNS. Encephalitogenic T-cells alone are capable of driving disease onset, as evidenced by their capacity to adoptively transfer disease to the recipient[149]. Inside the CNS antigenic stimulation by CNS-resident phagocytes drives reactivation of self-reactive T-cells. This role is normally assigned to DCs[150] or leptomeningeal macrophages[151] and not microglia[152]. Infiltrating T-cells produce an array of cytokines of which IFN-γ, IL-17 and GM-CSF are probably the most well studied examples. Their actions are complex and sometimes opposing, but include increasing the permeability of the BBB, activating astrocytes to produce cytokines and licensing phagocytes for tissue damage[153]. These events dramatically alter the immune cell composition in the CNS[39]. Monocytes and monocyte-derived cells are the most prevalent infiltrates at peak of disease[39], their numbers correlating with disease severity[154] and mice that lack circulating monocytes are resistant to EAE[155-157].

These studies highlight the non-redundant role of monocytes in initiating and driving tissue destruction during EAE. The phagocytic and tissue-destroying capacity of monocytes have been linked to the cytokine GM-CSF, since monocyte-specific disruption of the GM-CSF receptor protected mice from EAE[158]. Furthermore, overexpression of GM-CSF in $CD4^+$ T-cells alone results in myeloid cell infiltration of the CNS and development of fatal demyelination[159]. CNS-resident microglia express low levels of MHCII in the steady state and their contribution to EAE severity[160] has been attributed to production of inflammatory cytokines and reactive oxygen species (ROS)[152,161]. This is supported by a recent study demonstrating that microglia-specific abrogation of the protein kinase TAK1 dramatically suppressed EAE by cell-intrinsic downregulation of NFκB signaling[162]. These studies support a detrimental role of microglia during initiation and peak of disease. During the remission phase, however, microglia have been proposed to play a beneficial role. Using scanning electron microscopy, one study found that while monocytes initiated demyelination at the nodes of Ranvier, microglia appeared to scavenge myelin debris[163]. This is consistent with microglia being able to regulate myelination in the steady-state[164], during aging[165] and following demyelinating damage[166].

1.11 MICROGLIA AND MONOCYTE FUNCTIONS IN NEURODEGENERATION

Neurodegenerative diseases are characterized by the pathological accumulation of misfolded and aggregated proteins in the CNS. For a variety of neurodegenerative diseases, specific proteins misfold and aggregate in distinct brain regions, working as seeds that can structurally corrupt naive proteins[167]. Neuropathologically, this manifests as a progressive and predictable

spreading pattern of aggregates through the brain[168]. For Alzheimer's disease the neuropathology encompasses extracellular plaques containing amyloid beta protein and intracellular neurofibrillary tangles composed of the microtubule-associated protein tau. Both amyloid beta and tau aggregates display spreading behavior in Alzheimer's disease[169,170] and their aggregation is transmissible[171,172]. Glial reactions in Alzheimer's disease have been recognized since the original description of the disease[173], and microglia are now implicated in a number of neurodegenerative disorders[11]. More recently, microglia have been conclusively linked to Alzheimer's disease by the fact that a majority of the genetic risk loci are genes expressed in microglia[174]. The prevailing hypothesis suggests that microglia contribute to Alzheimer's disease pathology by virtue of their chronic activation and impaired clearance mechanisms[175]. However, complete absence of microglia for 4 weeks did not affect amyloid deposition[119], and neither did the enforced repopulation of the microglial niche by peripherally- derived myeloid cells[121,176]. Amyloid beta levels were similarly unaffected by microglial depletion in the amyloid depositing 5×FAD model, although it prevented neuronal loss and improved contextual memory[177]. The absence of any effect on amyloid beta burden by microglia depletion could be explained by recent results suggesting that the key function of amyloid-plaque associated microglia, rather than phagocytosis of the amyloid, is to form a barrier around it and to compact the amyloid fibrils into a dense plaque[178]. TREM2, haplodeficiency in which increases the risk of Alzheimer's disease by 4-fold[179,180], was found to orchestrate this response.

Research on microglia actions in Alzheimer's disease has mostly been focused on interactions with amyloid beta and not on tau aggregates. This reflects the recognition that amyloid beta deposits are extracellular, whereas tau deposits are intraneuronal and not normally accessible to microglia. However, activation of microglia in tau transgenic mice occurs[181-184] and can precede deposition of insoluble tau[182]. Phagocytosis of tau deposits by microglia has been described both *in vitro* and *in vivo*[185-187]. The subsequent release of tau-containing exosomes has been reported to orchestrate spatiotemporal progression of tau pathology via the perforant path[185]. Interestingly, microglia inflammasome activation has been shown to play an analogous role in seeding and spreading of amyloid beta pathology[188]. Reactive microgliosis, as observed in CX3CR1 deficient mice, can drive tau pathology and exacerbate cognitive decline[189,190]. In further support of this notion, peripheral lipopolysaccharide administration also significantly exacerbates tau pathology[183,191]. However, to what degree microglia activation occurs in human Alzheimer's disease brains is a matter of debate[192]. In fact, significant microglial loss is observed in brain areas affected by neurofibrillary tangles, and tau oligomers induce microglial apoptosis *in vitro*[193]. Furthermore, the progression of neurofibrillary pathology is accompanied by the appearance of microglia with dystrophic features, including deramification, fragmentation of processes and spheroid formation[194] which has been interpreted as a sign of cellular senescence[195]. In marmoset brains, which also accumulate fibrillar tau and dystrophic microglia with age, hyperphosphorylated tau was only detected in dystrophic but not normal microglia[196].

2 METHODS

2.1 CRE-FLOX SYSTEM

The Cre-flox system is a powerful tool which can be used to generate tissue-specific gene deletions, turn transgene expression on or off, track individual cells or lineages, report gene expression and deplete specific cell types. To achieve cell- or tissue-specific gene deletion, mice expressing loxP sites flanking a gene of interest are crossed with mice expressing Cre-recombinase under a cell-restricted promoter. The Cre-recombinase will then excise the DNA inside the loxP-sites. More recently, ligand-dependent chimeric Cre-recombinases (CreER recombinases) have been developed which allow Cre-activity to be turned on by the administration of tamoxifen[197]. I have used this system in my studies to delete *Tgfbr2* in distinct cell subsets (see GENE TARGETING) and to specifically deplete microglia.

2.2 MICROGLIA DEPLETION

Depletion of microglia has been widely used to address their role in different neuropathological contexts[112]. One of the main goals of my thesis work has been to produce a model system in which depletion of microglia could be accomplished with high efficiency and for longer periods of time, in order to study microglial functions in different neuroinflammatory and neurodegenerative settings.

Studying macrophage functions *in vivo* by their conditional depletion has a long history. Early experiments employed silica and asbestos to deplete macrophages[198]. The depletion technique became mainstream following the development of clodronate-encapsulated liposomes[199], which are still frequently used today. During the past decade this technique has been superseded by mice expressing the diphtheria toxin receptor (DTR) specifically in the cell type of interest[200]. Depletion can then be accomplished by administration of diphtheria toxin (DT).

We first used transgenic mice expressing DTR under the CD11b promoter (CD11b-DTR mice[201]) and administered DT intraperitoneally at doses that had previously been used to deplete peripheral macrophage populations, but failed to detect any signs of microglia depletion (**Figure 4A**). A study published at the same time accomplished only transient depletion (recovered in 36 hours) of microglia using peripheral administration of DT in CD11b-DTR mice[42]. We thus attempted to administer DT directly to the brain, which counterintuitively resulted in significantly increased numbers of microglia (**Figure 4B**). Continuous delivery of DT for 14 days using an intracerebroventricular cannula was equally ineffective (**Figure 4C**), despite having been reported to be efficient using gangciclovir in CD11b-HSVTK mice[119].

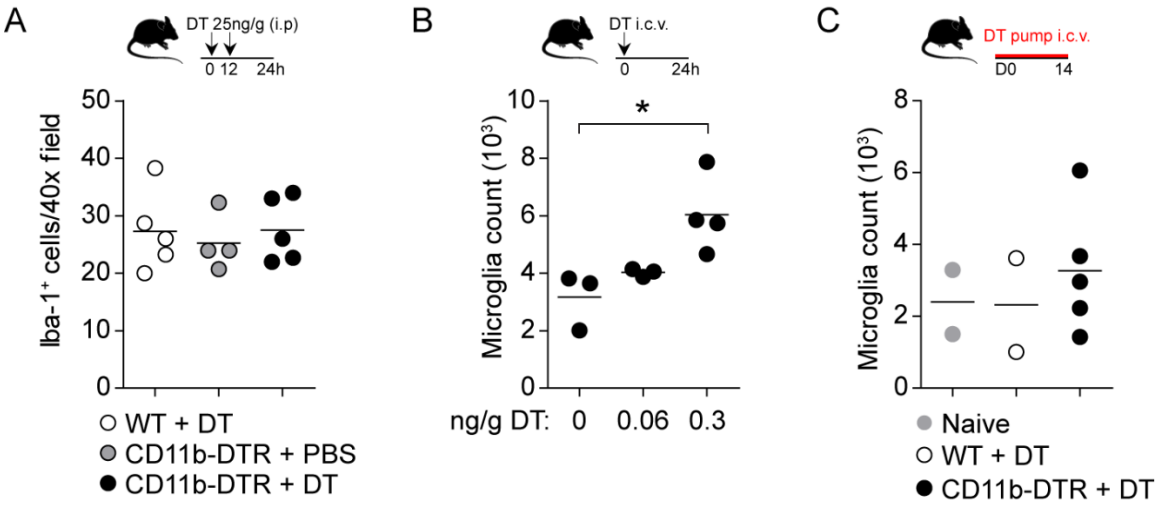

Figure 4: Attempted microglial depletion using CD11b-DTR mice and administration of DT by various routes; (A) two intraperitoneal doses (B) one intracerebroventricular dose or (C) 14 day continuous intracerebroventricular delivery through a cannula connected to an osmotic pump.

The development of *Cx3cr1*^{CreER} mice by two independent laboratories[47,162] permitted specific depletion of microglia when crossed with *R26*^{DTR} mice, which have the DTR inserted into the ubiquitously expressed Rosa26 locus[200]. Widespread expression of DTR is blocked by an upstream loxP-flanked STOP cassette. Tamoxifen administration in *Cx3cr1*$^{CreER/+}$*R26*$^{DTR/+}$ thus results in excision of the stop cassette, allowing transcription of DTR in microglia. DT administration in these mice initially appeared promising to us as we achieved approximately 80% depletion (**Figure 5A**). However, as later published[116], rapid microglial proliferation quickly compensated the cellular loss (**Figure 5A**). Additional cycles of TAM and DT could not meaningfully reduce the microglial pool (**Figure 5A**). To circumvent the need for DT to reach the brain, we crossed *Cx3cr1*^{CreER} mice with *R26*^{DTA} mice[202], in which tamoxifen administration resulted in almost complete eradication of microglia (**Figure 5B**). However, macrophage populations possess an inherent resistance against depletion and the microglial population could rebound even after >99% depletion (**Figure 5B**). As detailed in **Paper II**, the microglial niche was repopulated by a combined process of CNS-resident proliferation and infiltration of Ly6C^{hi} monocytes, generating F4/80^{low} and F4/80^{hi} cells, respectively. This model is useful to study the impact of microglia with short time frames (>90% depleted from day 2-10), as well as mechanisms of repopulation (**Paper II**).

To study microglial functions during longer time frames, as is relevant in neurodegenerative disease models, we continuously administered tamoxifen to *Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ mice over the course of one month. Repeated subcutaneous administration or continuous administration in the chow resulted in depletion efficiencies of 60-70% (**Figure 5C-D**) and this protocol was used in **Paper IV**.

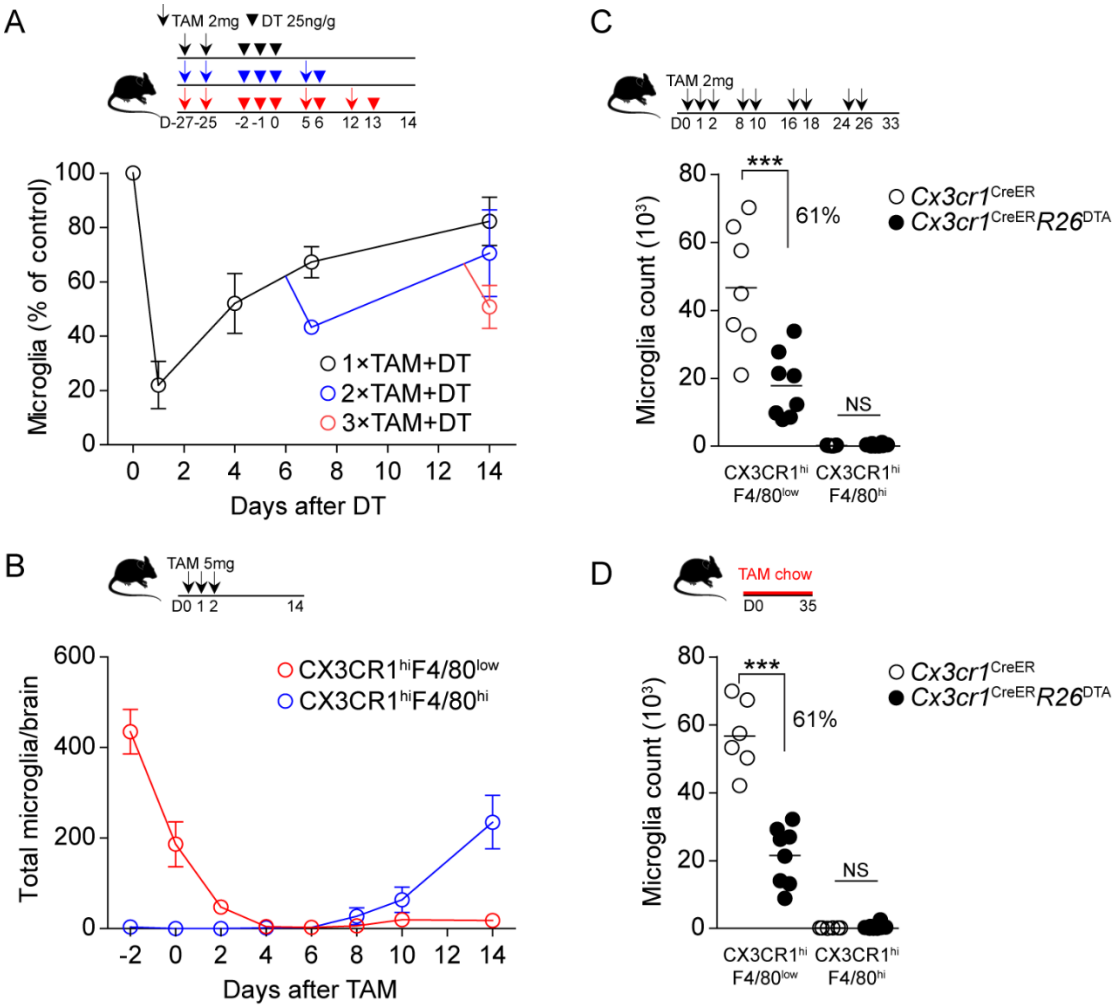

Figure 5: Depletion/repopulation kinetics in (A) *Cx3cr1*^{CreER}*R26*^{DTR} mice receiving one, two or three cycles of tamoxifen and DT or (B-D) *Cx3cr1*^{CreER}*R26*^{DTA} mice receiving (B) three subcutaneous doses of tamoxifen (C) repeated subcutaneous tamoxifen injections or (D) continuous delivery of tamoxifen in the chow.

2.3 GENE TARGETING

Deletion of *Tgfbr2* in specific monocyte/macrophage subsets was achieved by crossing *Tgfbr2*$^{fl/fl}$ (floxed) mice[203] with *LysM*^{Cre} mice[204] or *Cx3cr1*^{CreER} mice [47]. *LysM*^{Cre} mice efficiently deleted *Tgfbr2* in monocytes (**Paper III**) and monocyte-derived macrophages during EAE (**Paper I**), but not microglia (**Paper I**). *Cx3cr1*^{CreER} mice were used to target microglia (**Paper III**). Due to upregulation of CX3CR1 mRNA and protein during microglia niche colonization, monocyte-derived macrophages could be targeted using *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ chimeras.

2.4 MICROGLIA, MONOCYTE AND MACROPHAGE NOMENCLATURE

In this thesis I present studies related to microglia, monocytes and monocyte-derived cells and the nomenclature used can be both complicated and easily confused. The following

section aims to clarify the nomenclature and strategies employed to separate these populations.

Separation of microglia from infiltrating macrophages during neuroinflammatory conditions is complicated, if not impossible, using traditional techniques such as immunohistochemistry. The introduction of multi-color flow cytometry and production of reliable fluorophore-conjugated antibody clones have significantly simplified this task. More recently, the use of unsupervised high-dimensional cytometry has permitted excellent separation of the myeloid compartment in a range of tissues[205,206], including the CNS during both steady-state and neuroinflammatory conditions[39,207,208]. In our studies we have used a panel of up to eight markers for flow cytometry that has allowed us to adequately delineate the CNS myeloid compartment. In the naive brain, microglia dominate the myeloid compartment and are easily separated from blood-derived leukocytes based on their lower expression of CD45. During EAE, microglia become activated and alter their surface marker expression[39] yet remain distinguishable[209] as $CD45^{low}$. Simultaneously, a heterogeneous population of myeloid cells infiltrates the CNS, including but not limited to neutrophils, monocytes and conventional DCs. Ly6G expression readily distinguishes neutrophils. Monocytes give rise to inflammatory 'monocyte-derived' cells that are characterized as $MHCII^{hi}Ly6C^{+}CD11c^{+}$. They are the most abundant leukocytes infiltrating the parenchyma during EAE[153], but their nomenclature can be confusing. Some studies refer to them as *inflammatory monocyte-derived macrophages* or simply macrophages whereas the term *monocyte-derived dendritic cells* (moDC) is also widely used. This latter term can easily lead to confusion with conventional DCs, which make only a limited contribution to the CNS myeloid pool during EAE[39,209], and can be separated from the monocyte-derived pool based on F4/80 expression. In **Paper I** we have employed the term moDC for consistency with previous studies.

In **Paper II** we describe $CX3CR1^{+}F4/80^{hi}$ macrophages that arise from brain-infiltrating monocytes following microglial depletion. While these macrophages adopt a surface phenotype ($MHCII^{low}F4/80^{hi}CD11c^{low}Ly6C^{-}$) that is distinct from inflammatory moDCs, they also derive from the circulating $Ly6C^{hi}$ monocyte pool.

Non-parenchymal border-associated (meningeal, perivascular, choroid plexus) macrophages can comprise up to 10% of the CNS myeloid compartment during steady-state, do not expand during EAE but are difficult to separate from microglia and monocyte-derived cells using conventional flow cytometry markers[38,39]. We have not actively separated these cells from microglia or infiltrating leukocytes in our studies.

3 RESULTS AND DISCUSSION

3.1 PAPER I: TGF-β SIGNALING IN MONOCYTE-DERIVED CELLS INITIATES EAE REMISSION

Previous work in our research group described immunomodulatory effects of TGF-β on macrophages and microglia *in vitro* and when transferred into autoimmune disease models of type 1 diabetes[210] and multiple sclerosis[211]. In addition, work by other groups had demonstrated beneficial effects of phagocyte-specific abrogation of TGF-β signaling in settings of bacterial meningitis[212] and tumor growth[213,214]. This indicated that the immune-dampening effects of TGF-β on the myeloid compartment were deleterious during settings of cancer and infection, but beneficial during autoimmunity. Furthermore, while potent effects of administering or inhibiting TGF-β on the course of EAE had been described[215-218] the cellular targets of these therapies were not well defined. We knew that the spinal cord macrophage load correlated with disease severity[154,157] and that TGF-β production and signaling was at its strongest at the peak of EAE[218-220].

We thus **hypothesized that TGF-β signaling in myeloid cells would regulate autoimmune neuroinflammation**. Specifically, we thought that during the course of the inflammatory response TGF-β could work as a switch to control macrophage-induced spinal cord damage. To address this hypothesis, we crossed *LysM*^{Cre} and *Tgfbr2*$^{fl/fl}$ mice to specifically target TGF-β signaling in myeloid cells. Importantly, we did not detect spontaneous alterations in the immune compartments of the spleen or lymph nodes during steady state. Our lack of peripheral immune activation convinced us that we were not affecting TGF-β signaling in conventional DCs, an event which had been described to result in increased T-cell activation and spontaneous autoimmunity[221,222]. After immunization with myelin oligodendrocyte glycoprotein we observed no differences in the onset of disease, suggesting that T-cell priming and re-activation in the CNS were not affected. At peak of disease we observed no differences in cellular infiltrates or production of IL-17 or IFN-γ by T-cells retrieved from the CNS. Differences in the EAE score were apparent from day 16; *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$ mice did not enter remission, which normally occurs in WT mice. In fact we did not detect any improvement of EAE symptoms in *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$ mice throughout the study period (30 days). This chronic EAE stage was characterized by a selective retention of *Tgfbr2*$^{-/-}$ and MHCII^{hi} moDCs in the spinal cord and a concomitant switch in T-cell cytokine profile favoring IFN-γ production.

Subsequent sets of experiments provided a mechanism for the perpetuation of disease by demonstrating that: i) TGF-β could directly limit inflammation-induced IL-12, but not IL-23 production in macrophages, which (ii) could directly shift T-cell toward IFN-γ production. iii) *Tgfbr2*$^{-/-}$ moDCs were characterized by increased *Nox2* expression and ROS-production. iv) ROS-production in macrophages was TGF-β and IFN-γ dependent. This mechanism is illustrated in **Figure 6**. Taken together our data demonstrated that TGF-β signaling controlled

activation of moDCs in the spinal cord, which is crucial for the initiation of EAE remission. It also provides a (TGF-β dependent) mechanism for the observation that the monocyte-compartment contracts during EAE remission[154].

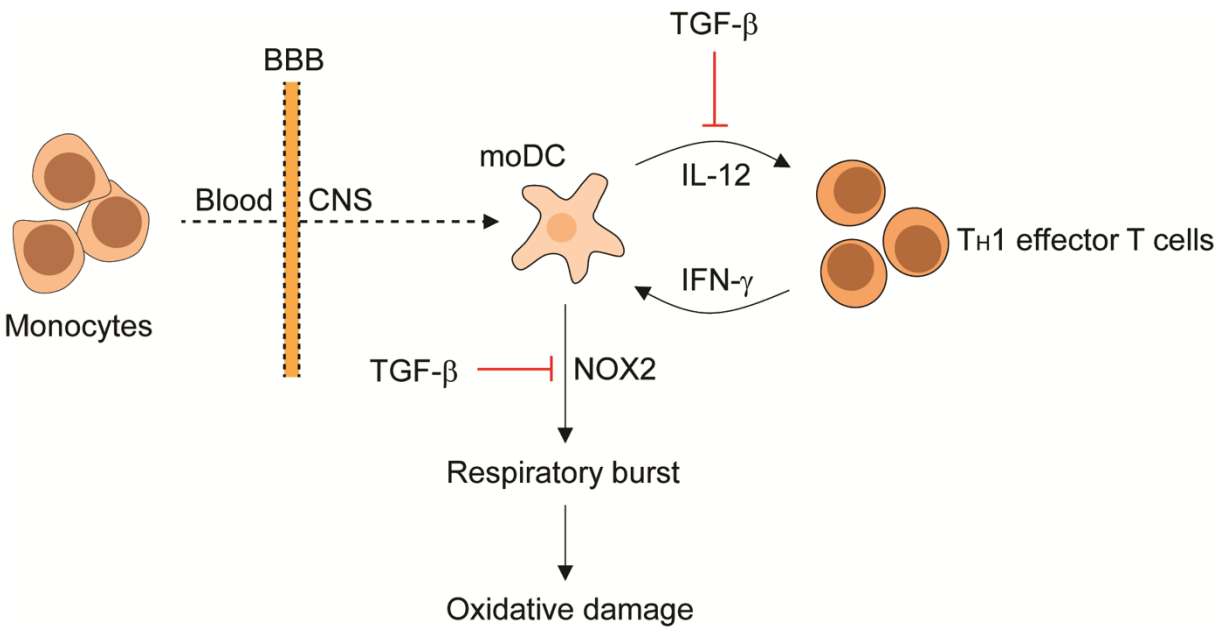

Figure 6: Suggested mechanism whereby TGF-β regulates chronic EAE

3.2 PAPER II: REPOPULATION OF THE MICROGLIAL NICHE BY MONOCYTE-DERIVED MACROPHAGES

In **Paper I** we addressed the role of TGF-β signaling in monocyte-derived cells during T-cell initiated neuroinflammatory disease. In the subsequent papers we developed the tools necessary (**Paper II**) to address the function of TGF-β (**Paper III**) in microglia and monocyte-derived macrophages during steady-state conditions.

In **Paper II** we provided a new model of microglia depletion by crossing *Cx3cr1*^{CreER} and *R26*^{DTA} mice and administered tamoxifen to the offspring, resulting in >95 % depletion of microglia. Subsequently, we observed that microglia were repopulated by a combined mechanism of intrinsic proliferation of surviving microglia and infiltration of monocytes. It should be noted that these two processes have been reported separately and are not novel *per se*[28,116,120]. However, repopulation by peripherally-derived myeloid cells was previously described to occur only under conditions in which the BBB is damaged, and is considered by many to be an experimental artifact[223]. In light of the results presented in **Paper II** as well as recently published data, I believe this concept needs revision. Below I discuss the factors regulating peripheral repopulation taking into account current and previous models of microglia depletion.

BBB disruption: *Cx3cr1*^{CreER}*R26*^{DTR} mice are repopulated by peripheral myeloid cells only after whole-body irradiation[116], which led authors to suggest that this was a prerequisite for repopulation. Furthermore, the peripheral repopulation observed by Varvel *et al.* in CD11b-HSVTK mice[120] required an intracerebroventricular cannula, and damage to the BBB can thus not be excluded. However, this conclusion was always speculative and not based on firm experimental data. As we observed in **Paper II**, BBB disruption did not occur after microglia depletion in *Cx3cr1*^{CreER}*R26*^{DTA} mice. Lack of BBB disruption was confirmed by a recent paper from Cronk *et al.* using *Cx3cr1*^{CreER}*Csf1r*$^{fl/fl}$ mice to partially deplete microglia for up to 12 weeks, resulting in repopulation by monocyte-derived cells[224].

Stem cells in the circulation: Since peripheral repopulation was enhanced under settings of irradiation and bone-marrow transplantation, where stem and progenitors cells are released into the circulation *en masse,* it has been suggested that peripheral 'microglia' arose from myeloid progenitors without passing through a monocyte intermediate[116,154]. Furthermore Bruttger *et al.* observed that a monocyte-depleting antibody did not impair peripheral repopulation in whole-body irradiated *Cx3cr1*^{CreER}*R26*^{DTR} mice[116]. However, we could reconstitute the microglial niche of *Cx3cr1*^{CreER}*R26*^{DTA} mice through adoptive transfer of purified Ly6C^{hi} monocytes. This was confirmed in *Cx3cr1*^{CreER}*Csf1r*$^{fl/fl}$ mice by Cronk *et al.* who additionally used parabiosis to conclusively show that monocytes can replace microglia under physiological conditions[224].

Brain inflammation: Does microglial death induce an inflammatory reaction in the brain that is sufficient to cause peripheral myeloid cells to engraft? We did observe a strong inflammatory response in the brain of *Cx3cr1*^{CreER}*R26*^{DTA} mice following depletion, as evidenced by astrocyte activation and cytokine production, although this had largely dissipated within two weeks. Importantly, however, Bruttger *et al.* also reported astrocytosis and production of pro-inflammatory cytokines in the brains of *Cx3cr1*^{CreER}*R26*^{DTR} mice whereby approximately 80% of microglia were depleted but no peripheral repopulation was observed[116]. Thus, 80% microglia death is not sufficient to cause any peripheral repopulation, yet as demonstrated by Cronk *et al.*, 25% sustained depletion is. Inflammation caused by primary microglia death alone is therefore unlikely to account for peripheral repopulation.

Microglia loss of proliferative activity: Our results support a model whereby impaired microglial proliferative capacity is an important factor regulating peripheral repopulation. Despite active proliferation following depletion in *Cx3cr1*^{CreER}*R26*^{DTA} mice, microglia do not recover the entire niche. By contrast, *Cx3cr1*^{CreER}*R26*^{DTR} mice recover their microglial loss in a matter of days through hyperproliferation of the surviving microglia[116], which could explain why monocytes are not given an advantage. This is further supported by the peripheral repopulation observed following chronic tamoxifen treatment in *Cx3cr1*^{CreER}*Csf1r*$^{fl/fl}$ mice[224], which likely results in permanent impairment of microglial proliferative capacity. This is of course contradicted by the observation that microglia can be virtually eradicated using a CSF1R antagonist and that when drug administration is ceased the few surviving microglia repopulate the niche without peripheral help[28,117]. However, in those experiments the chronic

inhibition of CSF1R likely simultaneously impairs monocytes from colonizing the niche. Furthermore, why monocytes are given an advantage over microglia following depletion in CD11b-HSVTK mice[120,121] remains to be elucidated.

Taken together, none of these factors alone can explain why monocytes repopulate the microglial niche. It is possible that a combination of these factors can synergize at a microcompartmental level not evident when the CNS is analyzed in bulk. Importantly, the conclusion from the studies mentioned above is that peripheral repopulation by the microglial niche can occur even under physiological conditions. As discussed in the *Introduction* there are certain conditions, such as infection or injury, that can lead to permanent replacement of alveolar macrophage or Kupffer cell pools with monocyte-derived macrophages. It would thus be of great interest to investigate whether survivable CNS infections or trauma would result in long-term integration of monocyte-derived macrophages into the parenchyma. Considering that humans live for many decades and may experience one or several CNS insults (i.e. concussion, stroke) throughout life, it is not inconceivable that the human microglial pool becomes diluted with monocyte-derived macrophages.

Another interesting aspect of our study is the identification of markers that can specifically distinguish monocyte-derived macrophages from microglia. Transcriptional profiling identified several candidates, including Clec12a and CXCR4, which were also upregulated at the protein level. In future studies, it would be interesting to apply these markers in various murine disease models or human brain samples where such discrimination is difficult. Of note, several genes of the *Ms4a* cluster on mouse chromosome 19 were among the most highly upregulated in monocyte-derived macrophages. Variants in MS4A genes have been identified in genome-wide association studies and are associated with risk of developing Alzheimer's disease, but the mechanism remains obscure[225]. Another interesting observation in this regard is the high overlap of our peripherally-derived macrophage gene signature with the one observed in microglia from *Sall1* deficient mice[118]. Consistently, *Sall1* and its homolog *Sall3,* which are microglia-specific transcription factors, were among the most significantly downregulated genes in monocyte-derived macrophages in our gene set. This indicates that monocytes utilize alternative transcription factors to obtain 'microglia-like' phenotype and function.

In conclusion, there are a number of outstanding questions that would be of great interest to investigate. In light of recently identified subsets of monocytes[80,81], is there a specific subset of Ly6C^{hi} monocytes that replaces microglia? Furthermore, type I and II interferon-signaling was highly enriched in peripherally derived microglia, therefore how do interferons regulate replacement of microglia by monocytes? Finally, given the recent observation that microglia have an epigenetic 'memory' of previous inflammatory stimuli[226], do monocyte-derived macrophages that repopulate the microglial niche contain an epigenetic record of experiences in the bone marrow or blood?

3.3 PAPER III: TGF-β REGULATES FUNCTIONAL COLONIZATION OF THE MICROGLIAL NICHE

Our results in **Paper II** demonstrated that monocytes could integrate long-term into the microglial niche and adopt microglia-gene expression and function. An obvious follow-up question was whether response to microglia niche factors was required for this integration. In the years leading up to the conception of this study, several articles were published which described the specific gene signature of microglia using microarray or RNA-seq technologies[227]. Subsequently, TGF-β was identified as as a key signal in the development and maintenance of this gene signature in microglia[135,228]. We thus **hypothesized that TGF-β signaling in monocytes was required for microglial niche colonization**. We thus produced *LysM*$^{Cre/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ chimeric mice, depleted microglia and sorted the newly repopulating macrophages. This demonstrated that monocytes lacking TGF-β signaling did not colonize the microglial niche which could reflect a requirement of TGF-β for CNS entry. Consistent with this, TGF-β can induce chemotaxis[229,230] and upregulate surface integrin expression[231] on human monocytes. However, this idea is strongly contradicted by our observations in **Paper I**, where *Tgfbr2* deficient monocytes were not impaired from entering the CNS during EAE. I would instead argue that under the circumstances present during microglial depletion, differentiation into CX3CR1^{+} 'microglia-like' cells requires active TGF-β signaling in monocytes. This further highlights that the process generating macrophages under neuroinflammatory conditions (**Paper I**) may be significantly different from that generating macrophages to replace microglia (**Paper III**). It could also indicate that these two macrophage populations are generated by different subsets of monocytes that are differentially dependent on TGF-β and should be further addressed.

Strikingly, when we instead abrogated TGF-β signaling in macrophages after colonization of the niche (using *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ chimeras), mice developed a progressive and fatal motor disease. Subsequent experiments demonstrated that macrophages were transcriptionally reprogrammed by the loss of *Tgfbr2* and spontaneously enacted demyelination in the spinal cord. Furthermore, using *Cx3cr1*$^{CreER/+}$*Tgfbr2*^{fl} mice we also demonstrated that microglia lacking TGF-β signaling developed a similar motor disease but with significantly slower pace. These mechanisms are depicted in **Figure 7**.

Our results provide a pathological mechanism for the observation by Butovsky *et al.* that *Tgfb1* deficient mice develop motor disease at 4-6 months of age[135]. No CNS lesions were described in that study, which made it difficult to dissect the loss of TGF-β signaling on individual immune cell subsets or even neurons or astrocytes. In addition, motor symptoms in CNS-*Tgfb1*$^{-/-}$ mice were accompanied by the appearance of Ly6C^{hi} cells in the CNS, which made it difficult to assign the pathology to resident or infiltrating subsets[135].

The relevance of the TGF-β pathway to neurological disease is further underscored by the recent observation that TGF-β signaling is suppressed in several neurodegenerative disease models, including Alzheimer's disease[232]. In that study neurodegenerative microglia were instead characterized by activation of pathways induced by TREM2 and APOE, genetic

variants in which significantly increase the risk of developing Alzheimer's disease[233]. Furthermore, a meta-study determined TGF-β1 to be the only cytokine upregulated in the cerebrospinal fluid of Alzheimer's disease patients[234], indicating that TGF-β signaling is dysregulated in this disease.

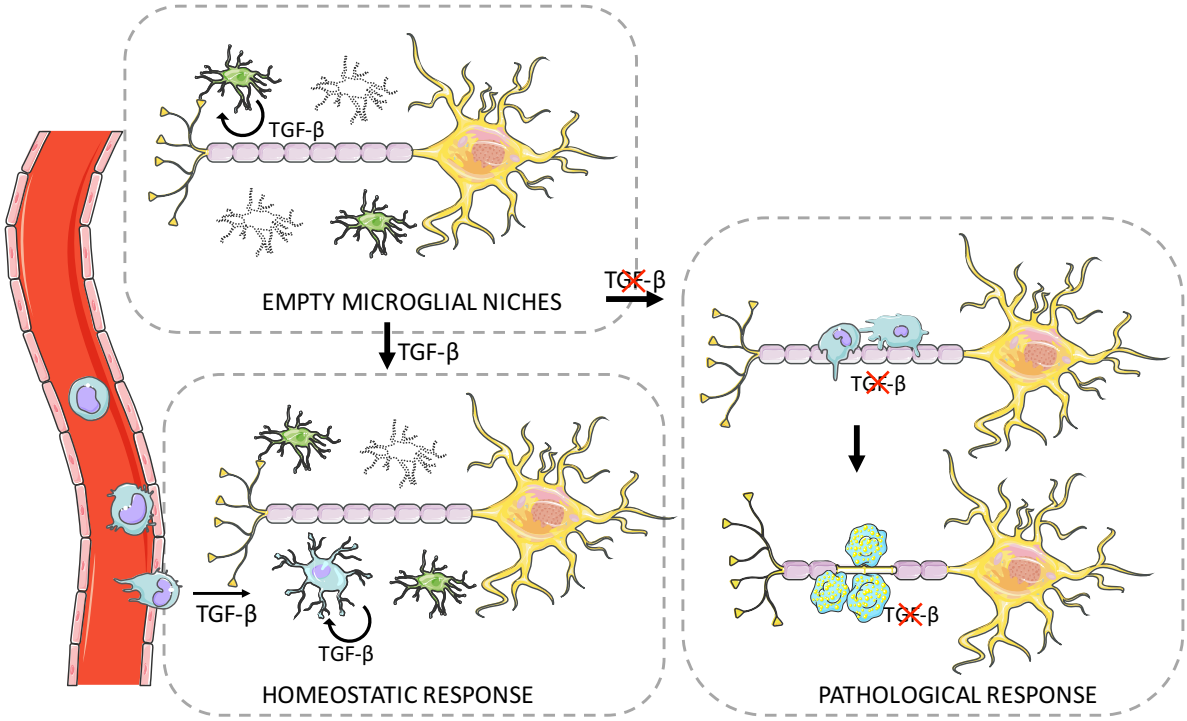

Figure 7: Mechanism whereby TGF-β regulates the integration of monocytes into empty microglial niches. Maintenance of the homeostatic microglia gene signature requires TGF-β (top left box). Colonization of microglial niches requires TGF-β signaling in monocytes. After integration into the niche TGF-β is required for homeostatic functions in monocyte-derived macrophages (bottom left box). In the absence of TGF-β signaling, monocyte-derived macrophages become licensed for tissue damage, attack axons and become giant myelin-filled cells (right box).

One observation that requires further investigation was the fact that chimeric *Cx3cr1*$^{CreER/+}$*Tgfbr2*$^{fl/fl}$→*Cx3cr1*$^{CreER/+}$*R26*$^{DTA/+}$ mice consistently developed lesions in the same anatomical areas. This was most predominant in the dorsal column of the spinal cord which was consistently degenerated at the end stage of disease, whereas the ventral columns were always untouched. Similarly, lesions in the thalamus were observed in all end-stage mice, but other areas of the cerebrum appeared normal. Microglia are known to display region-dependent gene expression[235], and our results thus suggest the existence of sub-compartment specific vulnerability to loss of TGF-β signaling.

3.4 PAPER IV: MICROGLIA REGULATION OF ALZHEIMER'S DISEASE TAU PATHOLOGY

In **Paper IV** we addressed the long-term consequences of microglial loss on tau pathology. Almost all transgenic mouse models of neurodegenerative disease share the common feature that the neuropathology is paralleled by robust microgliosis. This is also true for mouse models of tauopathy expressing the frontotemporal dementia-associated mutants P301S and P301L. In this study we used a mouse model (hTau mice) in which pathology is significantly less aggressive. Levels of insoluble tau are lower than in P301L mice[236] and neuronal loss develops only at an advanced age (15 months)[237]. Nevertheless, hTau mice develop hippocampal-dependent spatial memory deficits by 7-12 months of age[190,238,239]. In this study we first characterized the microglial phenotype and were surprised to discover an apparent lack of microglial activation, cytokine production and proliferation. Even at an advanced age (22 months) we did not detect altered gene expression in microglia as a consequence of tau pathology. hTau mice thus represent a mouse model in which microglial activation does not visibly contribute to disease progression. This is in contrast to most transgenic mouse models of proteinopathic neurodegenerative diseases, in which microglial depletion almost invariably leads to improvement of neuropathology, as we have previously reviewed[112]. In this context, **we thus hypothesized that microglial depletion in hTau mice would lead to an exacerbation of disease.** However, to our surprise we did not detect any impact on tau aggregation and associated spatial memory impairment as a consequence of chronic microglial depletion.

It is possible that the limited degree of depletion and the relatively mild pathology observed in hTau mice was not enough to yield robust differences in clinical and biochemical readouts. Indeed, hTau mice present with several-fold lower insoluble tau compared to P301L transgenic mice[236]. However, it could be argued that the extended depletion time used (3 months) in this study should compensate for the incomplete depletion. Additional experiments in older hTau mice with more robust pathology as well as using the CSF1R inhibitor that can produce >99% long-term depletion would address these concerns.

In summary, despite the lack of effect on tau pathology, the model presented in this manuscript represents a novel and useful tool to address microglial functions in neuropathological contexts.

4 FUTURE PERSPECTIVES

The past decade has produced a formidable body of literature placing microglia at the center stage of development, homeostasis and aging of the brain. Based on the results presented in this thesis, I propose three questions that should guide future research.

To what extent are peripherally-derived macrophages observed in the human brain? While this question has been pondered for many years, our studies and those of others have now identified markers that should be further explored in developing reagents that can specifically label central vs peripheral 'microglia'. Such studies are already underway[240].

What are the tissue specific cues that regulate microglia disease states? Microglia activation states were for a long time oversimplified, characterized as a dichotomy of 'good' and 'bad' based on only a limited set of markers[241]. New studies have demonstrated that macrophages have complex states that are often guided by tissue-specific cues. Each tissue-resident macrophage population is probably guided by a collection of distinct cues that creates tissue-specific contexts. As our study shows, identification of these tissue-specific contexts is vital for understanding disease.

Which pathways in microglia drive neurodegeneration and can be pharmacologically targeted? A growing number of studies have reported neurological deficits similar to those identified in our study, as a direct consequence of dysregulation of signaling pathways in CNS macrophages. This includes mice with overexpression of GM-CSF[159], deficiency in NRROS[242], mutations in BRAF[243] or constitutive activation of NF-κB[244]. This indicates that several pathways could converge to create 'common' neurodegenerative signatures in microglia and some candidates have already been proposed[232,245]. Targeting these pathways pharmacologically may represent avenues for therapy.

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