Myeloid cell responses after spinal cord injury

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8,800 words without references

30 pages without references

Total 41 pages

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1. Introduction

Spinal cord injury (SCI) results in control loss of motor, sensory and autonomic function below the level of lesion. This is due in large part to the inability of damaged axons to regenerate in the CNS environment and to the limited ability of the CNS to replace lost neurons. There are currently no effective treatments to improve neurological outcome after SCI in humans. As would be expected, much of the research effort has been devoted to studies to promote axon regeneration and re-establishment of damaged circuitry (Borton *et al.*, 2014; Filli & Schwab, 2015; Kadoya *et al.*, 2016; Lu *et al.*, 2017). In addition, attention has also focused on reducing secondary tissue damage that occurs in the days, weeks and months following injury (David *et al.*, 2012). Preventing secondary damage has been shown to improve histological and functional outcome in experimental animal models of SCI (Gris *et al.*, 2004; Ghasemlou *et al.*, 2010a; Ghasemlou *et al.*, 2010b; Lopez-Vales *et al.*, 2010; Lopez-Vales *et al.*, 2011). Although several factors, including hemorrhage and ischemia (Tator & Koyanagi, 1997; Rathore *et al.*, 2008; Kroner *et al.*, 2014) contribute to such damage, the inflammatory response is generally thought to contribute importantly to secondary damage after spinal cord trauma. Evidence that the inflammatory response is detrimental after SCI comes from experiments in which anti-inflammatory treatments show improvement (Mabon *et al.*, 2000; Wells *et al.*, 2003; Gris *et al.*, 2004; Stirling *et al.*, 2004; Fleming *et al.*, 2008; Kerr *et al.*, 2008; Lopez-Vales *et al.*, 2011). These detrimental effects are thought to be mediated in part by macrophages/microglia (Gris *et al.*, 2004; Kigerl *et al.*, 2009; Kroner *et al.*, 2014). However, macrophage/microglia are also known to have beneficial and pro-repair effects (Shechter *et al.*, 2009). Factors that influence the dual nature of these cells are now beginning to be understood. These include both extrinsic factors in the tissue environment (cytokines, growth factors, and others) and intrinsic factors

triggered by what these cells phagocytose that influence the activation or polarization state of these cells (David & Kroner, 2011; David *et al.*, 2015). However, the control of the plasticity of these cells is not yet fully understood. A better understanding of the factors that control the dual nature of these cells will help in the development of therapeutic strategies to reduce damage after spinal cord trauma.

Microglia and peripheral macrophages infiltrating into the CNS from the circulation arise from different myeloid origins. Microglia are the resident tissue macrophages of the CNS, which differ from macrophages that infiltrate into the CNS after injury or disease, and also from macrophages present in the normal CNS in border regions such as the meninges, perivascular spaces and choroid plexus (David & Kroner, 2011; Goldmann *et al.*, 2016; Korin *et al.*, 2017). In the subsequent section, we will discuss further the origins of the cells. Several recent studies have shown that these cell types have distinctly different gene expression signatures. These studies have led to the identification of several microglial-specific markers (e.g., P2ry12 and Tmem119, which will be discussed below), which are able to recognize microglia under normal homeostatic conditions. However, these markers generally get down-regulated after onset of inflammation. We will discuss what we have learned from these studies, and challenges and limitations in using these markers to study microglia and macrophage responses after CNS damage. We will also discuss the use of other labeling methods to identify peripheral monocyte-derived macrophages (MDMs) that have entered the CNS, and therefore study these MDMs and microglia in various neurological conditions. The early influx of neutrophils after tissue injury is a common feature of damage to the CNS as well as other tissues. The role of these neutrophils in the inflammatory response after CNS injury has received less attention in SCI. These myeloid cells get cleared by macrophages and in the process, have been shown to trigger the release of

pro-resolution lipids that actively resolve inflammation (Serhan, 2010). We will discuss the insights gained from recent findings about the expression and role of these bioactive lipid mediators after spinal cord injury. We have reviewed some of the literature on microglia specific gene expression but have not attempted to write a comprehensive review of all the published literature on gene expression of microglia and macrophages in the CNS. We will focus attention on work we have done in our lab and place it in the context of wider published work in the field.

2. Challenges in identifying and distinguishing different myeloid cell populations in SCI.

Microglia: When microglia become activated in regions of CNS damage, they withdraw their processes, acquire a rounded shape, and become amoeboid and phagocytic (Kreutzberg, 1996). In tissue sections, these microglia-derived macrophages cannot be distinguished from monocyte-derived macrophages (MDMs) infiltrating into the CNS from the peripheral circulation, based on their morphology and staining for markers such as CD11b (αM integrin) and Iba-1 (Ionized calcium binding adaptor molecule 1). These cells are therefore often referred to as microglia/macrophages. However, recent studies on gene expression profiling of microglia have led to the identification of several potential microglial markers that can distinguish them from peripheral MDMs and other tissue macrophages. However, there still are some limitations to their widespread use.

Microglia and infiltrating macrophages have different origins. Fate mapping studies reveal that erythromyeloid progenitor cells that give rise to microglia migrate from the yolk sac at embryonic day 8.5 to populate the developing CNS (Ginhoux *et al.*, 2010; Schulz *et al.*, 2012). This process is dependent on Csf1r, Pu.1 and Irf8 (Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013). Thereafter, microglial numbers are maintained throughout life by cell proliferation and are not

replenished from the circulation (Tay *et al.*, 2017). Monocytes and MDM found in the CNS arise from the bone marrow and are replenished throughout life (Ajami *et al.*, 2011; Mildner *et al.*, 2013; Yona *et al.*, 2013). Several microglia-specific genes have recently been identified that are selectively expressed in microglia compared to other tissue macrophages and leukocytes (Table 1). P2ry12 was first reported to be expressed by microglia but not macrophages (Hollopeter *et al.*, 2001; Sasaki *et al.*, 2003). One of the earliest transcriptome studies that looked for microglia selective genes, compared purified adult mouse microglia with macrophages from the spleen and monocytes (Bedard *et al.*, 2007). 127 potentially interesting genes were identified, of which a small subset of 11 genes coding for signaling molecules were selected for further analysis using *in situ* hybridization comparing tissue sections of brain versus spleen. This analysis identified 4 genes that were expressed in microglia-like cells but undetected in spleen (*P2y12* (*GPR12*), *GPR34*, *MSR2* and *F11R*), 4 others were enriched in microglia-like cells but also expressed in a subpopulation of spleen cells (*Siglec-H, Oflm13, Stab1 and P2y13 (GPR86*)). Analysis combining *in situ* hybridization combined with immunohistochemistry to identify the cell types expressing these genes in the brain and spleen showed that *P2Ry12, F11R Oflm13, GPR34, MSR2* and *Stab1* were enriched in microglia compared to spleen macrophages; and *Siglec-H* and *P2y13* being expressed equally in both. When brain and blood Ficoll-separated leukocytes were compared by qPCR, all except *F11R* and *GPR84* were expressed more abundantly in macrophages in the brain (these are likely to be macrophages associated with blood vessels, choroid plexus and meninges), with *MSR2*, *Oflm13* and *Stab1* being expressed selectively by such cells in the brain (Bedard *et al.*, 2007). Of the various markers noted in this study, P2ry12, Siglec-H and Oflml3 have been further substantiated in other studies as being useful as microglial markers. A crucial point to note here is that the specificity of microglial

markers identified in several studies is dependent on which cell type(s) they are being compared with. In the context of the normal CNS or in injury or disease, the need is to have markers that can distinguish microglia from monocyte-derived macrophages that have infiltrated into the CNS parenchyma.

In the past five years, several other transcriptome studies on microglia have provided additional insights and have led to the identification of unique signature genes. An early study characterized gene expression of tissue macrophages from various mouse organs (including microglia) that distinguished them from dendritic cells (Gautier *et al.*, 2012). This study showed that *MerTK, TLR4, TLR7, TLR8* and *TLR13,* were expressed equally in all types of tissue macrophages. Microglia were unique in that they expressed low levels of hundreds of transcripts that were expressed in other tissue macrophages (spleen, lung, peritoneum). Interestingly, 65 genes were expressed more than three-fold higher in microglia as compared to other tissue macrophages, including *Trem2, Tmem119, Fcrls, Olfml3, Gas6, Sall1, Cx3cr1, Hexb, Siglec-H* (Gautier *et al.*, 2012). Another study using deep RNA sequencing comparing purified microglia with other CNS cell types, and other myeloid cell types showed 29 highly specific markers for microglia (Chiu *et al.*, 2013). Of these, the three most highly enriched genes were *Tmem119, Siglec-H* and *Olfml3* (Chiu et al., 2013). This study also provided flow cytometry evidence that Siglec-H and Olfml3 are expressed by microglia but not peripheral macrophages. Recently, Siglec-H was also shown by immunofluorescence labeling to be a reliable microglia-specific marker in histological sections (Konishi *et al.*, 2017). It is expressed in almost all Iba1+ cells in the CNS parenchyma at all ages starting from E17 to adulthood (Konishi *et al.*, 2017). Using $CCr2^{RFP/+}$ mice, they also showed that Siglec-H was expressed in microglia but not infiltrating macrophages in the injured optic nerve and in the spinal cord of mice with Experimental

Autoimmune Encephalomyelitis (EAE) (Konishi *et al.*, 2017). In *CCr2*$^{RFP/+}$ mice, infiltrating macrophages are labeled with RFP and hence can be distinguished from microglia. RFP is also not expressed in macrophages in the injured peripheral nerve. These experiments were done 7 days after optic nerve injury or after onset of hind limb paralysis in EAE, so it is not known if Siglec-H expression is downregulated at later chronic time points.

Another direct RNA sequencing study showed that adult microglia express a unique set of genes coding for proteins required to 'sense' or recognize endogenous ligands and microbes (referred to as the "sensome") (Hickman *et al.*, 2013). Seven of the 25 most highly expressed "sensome" transcripts in microglia as compared to peripheral macrophages include *P2ry12, P2ry13, Tmem119, Gpr34, Siglec-H, Trem2* and *Cx3cr1* (Hickman et al., 2013). Importantly, 16 out of 22 microglia-selective sensome genes code for proteins that interact with endogenous ligands and not pathogens (Hickman *et al.*, 2013). A later study using RNA sequencing, microarray, and quantitative mass spectrometry found 106 genes that were enriched in microglia compared to neurons and other CNS glia (Butovsky *et al.*, 2014). This study found that *Fcrls, P2ry12, Tmem119, Olfml3, Hexb, Tgfbr1, Gpr34* and *Sall1* were highly expressed in mouse microglia compared to monocytes, other tissue macrophages and a wide range of immune cell types (Butovsky *et al.*, 2014). Sall1, a transcriptional regulator, is expressed only in microglia and no other CNS cell types or other mononuclear phagocytes (Buttgereit *et al.*, 2016). Genetic inactivation of Sall1 lead to conversion of resting microglia to an reactive phenotype (Buttgereit *et al.*, 2016). Fcrls which is highly expressed in mouse microglia, is not expressed in human microglia (Butovsky *et al.*, 2014) but is a good marker for studies in mice. Not only that, microglia from newborn mice and microglial cell lines (N9, BV2) which are widely used, do not express the adult microglia signature genes indicated above (Butovsky *et al.*, 2014). This

highlights the difficulties in extrapolating results from studies done with neonatal microglia and microglial cell lines, to what might be happening in the adult CNS, and the need to use adult microglia. In addition, monocytes recruited into the CNS in amyotrophic lateral sclerosis (ALS) or EAE also do not express microglia signature genes (Chiu *et al.*, 2013; Butovsky *et al.*, 2014). Another recent study also found seven genes (*Tmem119*, *Fcrls*, *P2ry12*, *P2ry13*, *Gpr34*, *Gpr84*, *Il1a*) highly expressed in microglia-enriched genes in $CD45^{+}$ cells purified from mouse brain (Bennett *et al.*, 2016). These findings confirm several earlier reports (discussed above). This study also found that Tmem119 was expressed in microglia but not other CNS macrophages (meninges, perivascular and choroid), and is a highly specific microglial marker (Bennett *et al.*, 2016). Expression of Tmem119 is developmentally regulated; it is not expressed until postnatal day 3-6 and reaches maximum levels by postnatal day 14 (Bennett *et al.*, 2016). *In vivo*, Tmem119 is microglia specific and stably expressed in the early phases of several inflammation models – in the spinal cord, 4 days after sciatic nerve injury; 1 and 3 days after intraperitoneal injection of LPS; and 7 days after optic nerve injury (Bennett *et al.*, 2016). Similarities (e.g., *Cx3cr1*, *Itgam* [CD11b], *P2ry12)* and differences (e.g., *Tal1 and Ifi16*) in microglial signature genes in human versus mouse has also been reported (Holtman *et al.*, 2015; Galatro *et al.*, 2017) and should be borne in mind when extrapolating data from murine models to study human CNS injury or pathology.

Taken together, these studies show that P2ry12, Fcrls, Tmem119, Olfml3 Siglec-H and Sall1 are microglia selective and could be potentially good, microglia specific markers. However, an important limitation to their wider use is the evidence that these microglial signature genes, which are expressed robustly in the normal CNS under homeostatic conditions, can be down-regulated in inflammatory/neurodegenerative states (Kassmann *et al.*, 2007; Chiu *et*

al., 2013; Butovsky *et al.*, 2014; Keren-Shaul *et al.*, 2017). P2ry12 labels normal resting microglia very well but its expression is reduced or lost after trauma-induced inflammation (Haynes *et al.*, 2006). This was first reported using neonatal brain slice cultures (postnatal day 4-7) mice in which P2ry12 staining was barely detectable after 24 hours, and this correlated with a change in morphology from process-bearing to amoeboid (Haynes *et al.*, 2006). The same paper also showed a complete loss of P2ry12 staining *in vivo* 4 days after LPS injection into the striatum in adult mice (Haynes *et al.*, 2006). Shorter survival times were not checked. We recently reported that P2ry12 labeling is detected in the injured spinal cord at 5 and 28 days after contusion injury (Greenhalgh *et al.*, 2016), although labeling was far less strong and ubiquitous in the microglial population than in uninjured animals. At the onset stage of EAE, activated microglia with short processes located adjacent to infiltrating immune lesions express P2ry12 (Greenhalgh *et al.*, 2016) but this staining is lost at the later peak stage of disease (our unpublished observations). P2ry12 staining of microglia is also lost in active and expanding lesions in multiple sclerosis (Zrzavy *et al.*, 2017). In cerebral ischemia in mice, we found process-bearing microglia in the peri-infarct region that were strongly P2ry12-positive (Zarruk *et al.*, 2017). Reduced P2ry12 staining of rounded, activated microglia was detected in the lesion core and lesion border at 72h after permanent middle cerebral artery occlusion (Zarruk *et al.*, 2017). In addition, some rounded microglia lacked P2ry12 staining, indicating that P2ry12 is down-regulated with inflammation in stroke (Zarruk *et al.*, 2017). In general, P2ry12 staining deceases in various inflammatory states, and the level of expression of P2ry12 may vary depending on the type and duration of the lesion and the region of the CNS involved.

Tmem119 is expressed in normal microglia and in microglia in the first 7 days after optic nerve (Bennett *et al.*, 2016) or SCI (our unpublished data). It appears to be down-regulated at

longer times after CNS injury (unpublished observation) but this needs to be clearly documented. We have also found FCRLS to be an excellent FACS marker for microglia in the normal and injured CNS, five days after SCI (Greenhalgh *et al.*, 2016) and 72 hours after cerebral ischemia (Zarruk *et al.*, 2017) but its expression after longer durations has yet to be established. The same is also the case with Siglec-H (Konishi *et al.*, 2017) as mentioned above. The loss of microglial homeostatic signature, including genes that are specific to or highly enriched in these cells (such as Tmem119 and P2ry12), is associated with neurodegenerative disease (Keren-Shaul *et al.*, 2017; Krasemann *et al.*, 2017), and referred to as 'DAMs' (Keren-Shaul *et al.*, 2017) or 'MGnDs' (Krasemann *et al.*, 2017). These studies highlight the plasticity of microglia in the context of disease and inflammation. Therefore, distinguishing activated microglia from MDMs at chronic time points after SCI or other forms of CNS damage or disease still poses a major challenge. Such tools are needed to identify and distinguish these cells in tissue sections, and to purify them by FACS to assess their gene and protein expression profiles. One way to get around this problem is to have more stable markers of peripheral MDMs.

Monocyte-derived macrophages: In the normal CNS, the meninges are a repository of infiltrating myeloid cells (Engelhardt *et al.*, 2017; Prinz & Priller, 2017), which recent mass cytometry CyTOF (cytometry by time of flight) studies have shown can comprise up to about 12% of the total leukocyte cell population associated with the normal, resting CNS (Korin *et al.*, 2017), and that 95% of all CNS leukocytes are in extra-vascular locations (Mrdjen *et al.*, 2018). Embryonically derived macrophages that have phenotypic features very similar to microglia are located in border regions such as meninges, choroid plexus, and perivascular spaces and referred to as border-associated macrophages (BAMs) (Goldmann *et al.*, 2016). Recently, mass cytometry and fluorescence cytometry analysis revealed that microglia and BAMs have distinct

expression profiles and lack Sall1 (microglial marker), Ly6C (monocyte) and CD11c (dendritic cell marker). Sall1 is a key marker that can distinguish between microglia and BAMs (Mrdjen *et al.*, 2018). This study also showed that Siglec-H distinguishes microglia from a population of macrophages in the CNS with closely related cell surface expression patterns (Mrdjen *et al.*, 2018).

Monocyte-derived macrophages (MDMs) from the circulation that arise from the bone marrow are recruited to the CNS after injury or disease (Ajami *et al.*, 2011; David & Kroner, 2011; Shechter *et al.*, 2013; Greenhalgh & David, 2014). There are no MDM-specific antibody markers that can be used to distinguish these cells from microglia. After SCI, two modes of macrophage entry to sites of CNS damage have been reported in mice, one route from the circulation via the meninges, and another from the choroid plexus via the central canal (Shechter *et al.*, 2013). The former have been reported to be pro-inflammatory while the latter have pro-repair properties (Shechter *et al.*, 2013).

Transcriptome analysis revealed three macrophage-enriched genes - fibronectin, Cxcl13, and endothelin B receptor (Hickman *et al.*, 2013). Macrophages also expressed significantly higher levels of *P2rx4*, *Ccr1*, *Cxcr7*, *Ifitm2*, *Ifitm3*, *Ifitm6* and *Tlr8* (Hickman *et al.*, 2013). In addition, some microglial sensome genes were expressed more highly in macrophages; these include *Itgam*, *Cd74*, *Emr1*, *Itgb2*, *Cd37*, *Clec7a*, *Cmklr1*, *Ifitm6*, *Pilra* and *Fcgr4* (Hickman *et al.*, 2013). At sites of CNS injury or disease, fibronectin is often expressed and localized to meningeal fibroblasts which infiltrate CNS lesions. It is therefore not a good marker to distinguish peripheral macrophages in CNS lesions. The other genes listed above need to be rigorously assessed to establish whether they can be used to distinguish MDMs from other cell types at sites of CNS damage.

Genetic reporter mice to study microglia and infiltrating macrophages after SCI

The study of microglia cells was revolutionized at the turn of the century by the creation of CX_3CR1-GFP reporter mice (Jung *et al.*, 2000). Microglia show high expression of the fractalkine receptor (CX_3CR1), which is involved in their interaction with neurons that express the CX3CL1 ligand (Cardona *et al.*, 2006). This reporter mouse was used in the seminal two-photon microscopy live imaging studies that revealed microglial surveillance and their response to injury (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Despite their importance to the field, CX3CR1-GFP mice do not distinguish between microglia and infiltrating monocyte-derived macrophages, as CX3CR1 is broadly expressed in circulating monocytes, subsets of peripheral mononuclear phagocytes and myeloid progenitors in the bone marrow (Yona *et al.*, 2013). Other strategies using genes common to microglia and macrophages include Csf1r-EGFP mice (Sasmono *et al.*, 2003). Therefore, the use of these mice to distinguish microglia and infiltrating macrophages after traumatic SCI is limited. To circumvent this, CX3CR1-GFP/+ reporter mice were crossed with CCR2-RFP/+ mice that labels circulating monocytes utilizing the CCR2 chemokine receptor which is thought to mediate the trafficking of $Ly6C^{hi}$ cells (Saederup *et al.*, 2010; Mizutani *et al.*, 2012). CCR2 is not expressed in resident microglial cells, however, upon infiltration of CCR2-rfp/+ monocytes into the CNS, the receptor can be downregulated, leading to a loss of reporter expression. This must be considered when investigating later time points after CNS injury.

Cx3cr1Cre:R26-yfp animals, harboring the latent Cre recombinase and exposed to tamoxifen at 5 to 7 weeks of age showed that almost 90% of microglia were YFP+ after two weeks, which was maintained for up to 14 weeks (Goldmann *et al.*, 2013). Importantly, four

weeks after the end of tamoxifen treatment, circulating monocytes were negative for YFP. This genetic approach, therefore, provides a window of time after tamoxifen treatment when the reporter is only expressed in resident microglia, distinguishing them from infiltrating MDMs (Goldmann *et al.*, 2013). The transcription factor Sall1 is expressed exclusively in microglia, and thus *Sall1*^{GFP} reporter mice are also a good tool to identify microglia (Buttgereit *et al.*, 2016; Mrdjen *et al.*, 2018).

Using a different approach, we and others have recently used the LysM-EGFP knock-in mice to identify MDMs. In these mice, EGFP is inserted into the lysozyme M locus (Faust *et al.*, 2000), and labels mature myelomonocytic cells that include macrophages and neutrophils but not microglia (Faust *et al.*, 2000; Mawhinney *et al.*, 2012; Fenrich *et al.*, 2013; Greenhalgh & David, 2014; Greenhalgh *et al.*, 2016). This system must not be confused with the LysM-Cre mouse, which uses the LysM promoter to target gene deletion in microglia (Goldmann *et al.*, 2013; Wang *et al.*, 2015a). Unlike the EGFP knock-in mice in which robust and continuous expression of the LysM promotor is required for EGFP expression, Cre mediated gene targeting requires only low-level transient expression. Unlike the robust expression in myelomonocytic cells, microglia express the LysM promoter partially, and at a low level (Lein *et al.*, 2007), including during development which results in Cre-mediated targeting of between 25–45% of microglia (Goldmann *et al.*, 2013; Wang *et al.*, 2015a). Such low-level expression is not sufficient to induce expression of EGFP in microglia. We assessed this rigorously by quantifying the expression of LysM-EGFP in microglial in mouse models of SCI, EAE and cerebral ischemia. In SCI, using flow cytometry, we found LysM-EGFP expressed in only about 2.5% of FCRLS+ microglial at 5 days post-injury (Greenhalgh *et al.*, 2016). In the permanent middle cerebral artery occlusion model, LysM-EGFP expression was seen in only 3% of P2ry12+ microglia in

tissue sections (Zarruk *et al.*, 2017). Also, very few P2ry12 microglia expressed LysM-EGFP in and around EAE lesions at the onset stage of the disease (Greenhalgh *et al.*, 2016). These studies show that LyM-EGFP is a very good tool to distinguish peripheral myeloid cells (MDMs and neutrophils) from microglia. Neutrophils which are also LysM-EGFP^{+} can be distinguished from MDMs easily in FACS by the expression of Ly6G, and in tissue sections by their characteristic polymorphonuclear morphology, and their small size (Greenhalgh *et al.*, 2016; Zarruk *et al.*, 2017). LysM-EGFP knock-in mice can therefore be used to separate MDMs from microglia and neutrophils by FACS for gene and protein expression profiling. We have shown that infiltrating MDMs can also be easily identified and distinguished from microglia using LysM-EGFP knock-in mice combined with one of the microglia-specific markers (P2ry12, Tmem119) in tissue sections and flow cytometry (Greenhalgh *et al.*, 2016; Zarruk *et al.*, 2017).

Differential expression of CD45 as assessed by FACS is still widely used in the field along with a panel of other antibodies to CD11b (myeloid cells), Ly6G (neutrophils) and Ly6C (monocytes) to isolate microglia. The key being that microglia express CD45 at a distinguishably lower level than macrophages. Caution is often needed when using such isolation as the expression of CD45 in microglia and MDMs may change in the inflamed CNS. However, we and others have found that CD11b+, CD45^{low}, Ly6G- Ly6C- represent the vast majority (>95%) of microglia when assessed alongside newer, more specific markers microglial, even after CNS injury (Greenhalgh & David, 2014; Zarruk *et al.*, 2017). These antibody tools are restricted to flow cytometry and may miss small subpopulations of cells. These are therefore valuable tools to identify and purify microglia until stable microglia-specific markers become available to identify microglia in acute and chronic inflammatory states.

3. Plasticity of microglia and macrophage phenotype after CNS injury.

Microglia and macrophages have a highly plastic phenotype that can change rapidly *in vitro* and *in vivo* in response to a variety of factors. These cells can transform from resting to pro-inflammatory or anti-inflammatory states, and from cytotoxic to pro-repair phenotypes and *vice versa*. After CNS injury microglia respond within 5-15 minutes via $P2Y_{12}$ receptor - ATP-dependent mechanisms to extend cytoplasmic processes towards CNS lesions (Davalos *et al.*, 2005; Hines *et al.*, 2009). Two photon-microscopy studies have shown that inactivating or ablating these microglia result in enlargement of the size of the lesion, suggesting that this early microglial response is protective (Hines *et al.*, 2009). Depending on the histological features of the region, microglial cell bodies can also translocate towards the lesion (Dibaj *et al.*, 2010). Microglia also proliferate in response to CNS perturbations. Damaged associated molecular patterns (DAMPs) released immediately at the site of injury stimulate microglia and other CNS cells (glia and neurons) via toll-like receptors to release chemokines, cytokines inducible nitric oxide synthase (iNOS) and matrix metalloproteinases (Pineau & Lacroix, 2007; Piccinini & Midwood, 2010; Heiman *et al.*, 2014; Katsumoto *et al.*, 2014) that act to recruit neutrophils and MDMs from the circulation into the CNS. The microglia and MDMs recruited to the site of injury are influenced by a variety of factors in their immediate environment (that include chemokines, cytokines, growth factors, bioactive lipids, etc) that are constantly changing with time after injury.

Extensive work done on macrophages *in vitro* has shown that stimulation with pro-inflammatory stimuli such as LPS or IFNγ induce a proinflammatory, cytotoxic phenotype, while stimulation with IL-4 or IL-13 induce an anti-inflammatory, pro-repair phenotype. These activation states are generally referred to as polarization states, with the former referred to as M1

and the latter anti-inflammatory state being referred to as M2 cells (with several additional M2 states) (Gordon, 2003; Martinez *et al.*, 2008; David & Kroner, 2011). However, it has become apparent that this simplified *in vitro* classification is inadequate to describe the activation states in many *in vivo* conditions (Murray *et al.*, 2014). *In vitro,* culture and stimulation conditions can be defined, however, many factors with antagonistic pro and anti-inflammatory properties act simultaneously on macrophages and microglia at any given time *in vivo*. Moreover, these factors and their concentrations change continually after injury. As a result, clean M1 and M2 phenotypes likely do not exist *in vivo*. Instead one sees mixed phenotypic states that may be skewed at any given time towards more pro-inflammatory, cytotoxic or anti-inflammatory, pro-repair states, depending on the ever-changing mix and concentrations of factors in the cellular environment. A network analysis of transcriptome data of macrophages stimulated *in vitro* with either IL-4 or IFN-γ results in a network existing along a somewhat bipolar axis; however, the network is oriented away from this axis when macrophages are stimulated with a variety of other factors (Xue *et al.*, 2014). This was further illustrated by the complex 3-dimensional matrix distribution of the network of the transcriptome data obtained from macrophages stimulated with a cocktail of factors (Xue *et al.*, 2014).

The heterogeneity in MDM and microglia populations at different times at the site of SCI can now be studied by single cell RNA-sequencing using FACS sorted cells. In addition, information at the protein level can also be obtained by mass cytometry CyTOF of several dozen markers (Korin *et al.*, 2017). For the latter, markers useful for mouse SCI analysis might include: P2ry12, FCRLS, Tmem119, Sal1, Siglec-H, MHC II TNF, IL-1β, IL-10, TGFβ, iNOS,STAT1, STAT6, STAT4, SOCS1, SOCS3, Arg-1, CD206, CD16/32, CD86, IL-12 (Kigerl *et al.*, 2009; Shechter *et al.*, 2013; Kroner *et al.*, 2014; David *et al.*, 2015; Korin *et al.*, 2017).

In addition to soluble factors, the activation or polarization state of macrophages and microglia is also modulated by phagocytosis. Among the debris and cells that macrophages and microglia phagocytose in the injured spinal cord is the large amount of myelin debris as well as red blood cells (RBCs) (resulting from hemorrhage) present in the tissue. We have shown by flow cytometry and Q-PCR analysis that macrophages and microglia stimulated *in vitro* with LPS and IFNγ to acquire a pro-inflammatory (M1-like) state, will switch to an anti-inflammatory, pro-repair (M2-like) state upon phagocytosis of myelin. This switch includes marked reduction in expression of TNFα, Ly6C and iNOS; and in addition, these cells produce soluble factors that promote increased neurite outgrowth and inhibit NF-κB signaling (Kroner *et al.*, 2014). Phagocytosis and internalization of myelin is essential for changes in expression of these molecules as determined by the uptake of pHrodo-red tagged myelin which fluoresces upon change in pH after entering into lysosomes (Kroner *et al.*, 2014). It was surprising therefore that despite microglia and macrophages in the injured spinal cord phagocytosing myelin in the first 7-10 days after injury (Greenhalgh & David, 2014) they still express a predominantly pro-inflammatory M1-like phenotype (Kigerl *et al.*, 2009; Kroner *et al.*, 2014). We questioned whether this lack of appearance of an anti-inflammatory phenotype after myelin phagocytosis *in vivo* in SCI might be do the presence of pro-inflammatory cytokines in the tissue. Several lines of evidence indicate that the presence of TNFα is likely to be the key factor in keeping the macrophages and microglia in a pro-inflammatory M1-like state in the first two weeks after SCI when myelin phagocytosis is occurring: (i) LPS stimulated macrophages that phagocytose myelin can be prevented from acquiring an anti-inflammatory and pro-repair M2-like phenotype by co-treatment with recombinant TNFα; (ii) LPS-stimulated BMDMs from TNF knockout mice showed reduced expression of M1 markers (CD86, iNOS and IL-12), which were

further markedly reduced by myelin phagocytosis; (iii) after SCI in TNF knockout mice there was greater expression of M2 markers, and a remarkable improvement in locomotor recovery (Kroner *et al.*, 2014).

In contrast to this, macrophages and microglia stimulated *in vitro* with IL-4 to acquire an anti-inflammatory phenotype will rapidly switch to a pro-inflammatory state upon phagocytosis of senescent RBCs, resulting in a rapid and marked increase in expression of TNFα and iNOS (Kroner *et al.*, 2014). RBCs likely mediate this effect in part by heme and the iron contained in heme, as each RBC contains over a billion atoms of iron. Interestingly, the uptake of iron-dextran by macrophages *in vitro* reduced expression of M2 markers (CD206, Ym1) but did not have an effect on M1 phenotype markers (TNFα, iNOS, CD16, CD86). However, after these cells were transplanted into the injured spinal cord macrophages that were fed iron-dextran showed a marked increase in expression of TNFα and CD16/32, similar to the levels seen in macrophages that phagocytosed RBCs. These studies indicate that factors in the injured CNS tissue can drive macrophages that have taken up iron-dextran to robustly acquire a pro-inflammatory state. In fact systemic injections of iron dextran into mice for 7 days after SCI led to a 3.5-fold increase in TNFα expression by ELISA and a worsening of locomotor recovery (Kroner *et al.*, 2014). In other work, we have also shown that insufficient levels of M2 factors, such as IL-4, in contused spinal cord tissue also hampers microglia and macrophages from acquiring an anti-inflammatory phenotype (Francos-Quijorna *et al.*, 2016). We found that administration of IL-4 at the lesion site resulted in increased numbers of microglia and macrophages expressing Arg1 and CD206. This is especially evident when IL-4 was injected at 2 days after injury, corresponding with the massive recruitment of MDMs into the lesioned cord. Importantly, administration of IL-4 markedly improved functional outcomes and reduces tissue

damage after contusion injury (Francos-Quijorna *et al.*, 2016). These studies show some of the reasons why macrophages and microglia in the injured spinal cord remain in a pro-inflammatory state for prolonged periods that can then impact on poor recovery. Differences in the polarization state of microglia and macrophages have also been reported to influence remyelination in the CNS. Both cell types (microglia and peripheral infiltrating macrophages) showed a switch from a M1 to a more M2-like state during remyelination following focal demyelination induced by injection of lysophosphatidylchoine (LPS) into the mouse corpus callosum (Miron *et al.*, 2013). Interestingly, M2 cell conditioned medium augmented oligodendrocyte differentiation *in vitro*; while depletion of M2 cells from corpus callosum lesions *in vivo* impaired oligodendrocyte differentiation (Miron *et al.*, 2013). Additionally, coupling of young and aged mice by parabiosis was able to rescue the remyelination defect in aged mice with concomitant increase in M2 cell density in demyelinated lesions. These experiments underscore the role of macrophage polarization on oligodendrocyte differentiation and remyelination in the adult CNS. These M2 cell mediated effects were shown to be mediated via activin-A - a member of the TGF-β superfamily (Miron *et al.*, 2013). These studies also highlight how the activation state on bipolar continuum of polarization, in restricted *in vitro* conditions, may be reversed by the addition of just one or two factors. This emphasizes the sensitivity of microglial cells to multiple, often antagonistic, stimuli acting concurrently, and reinforces the importance of complexity of their *in vivo* microenvironment in modulating their activation or polarization states (Ransohoff, 2016).

4. Heterogeneity of the response of microglia and infiltrating macrophages.

After SCI microglia respond immediately via P2ry12-ATP signaling and TLR signaling as mentioned above. The chemokines and cytokines produced (Pineau & Lacroix, 2007) recruit

peripheral circulating myeloid cells that include neutrophils and MDM after SCI (Fleming *et al.*, 2006; Kigerl *et al.*, 2006; Stirling & Yong, 2008; David *et al.*, 2012). Neutrophils are recruited within 6 hours and are found in the injured spinal cord for the first couple of weeks. On the other hand MDMs from the circulation begin to infiltrate into the spinal cord at about 3 days, reach peak numbers at 7-10 days after injury, and remain in the tissue for prolonged periods (Fleming *et al.*, 2006; Kigerl *et al.*, 2006; David *et al.*, 2012). It appears that several weeks after injury many of these cells in the core of the lesion show signs of decreased viability (Rathore *et al.*, 2008). Since microglia and macrophages are both found in the injured spinal cord, we assessed if there are differences in their phagocytic response. Using evidence of axonal or tissue debris we found that microglia contain phagocytic material in the first three days after injury, which is the time before the entry of MDMs from the circulation (Greenhalgh & David, 2014). Phagocytosis of axons was assessed by labeling the axons with Fluororuby. After the entry of MDMs, the proportion of microglia with phagocytic material decreases while increasing number of infiltrating peripheral macrophages identified using LyM-EGFP took over phagocytosis from microglia (examined for up to 42 days) (Greenhalgh & David, 2014). Interestingly, infiltrating macrophages are more susceptible to cell death after phagocytosis than are microglia. Moreover, microglia proliferate much more than infiltrating macrophages after SCI (Greenhalgh & David, 2014). These findings indicate that these two myeloid cell populations differ remarkably in their responses to CNS injury.

The time course of infiltration of peripheral circulating myeloid cells (neutrophil and MDMs) is also somewhat similar after permanent cerebral ischemic lesions as compared to SCI but slightly earlier. We used LysM-EGFP knock-in mice to study the expression profile of inflammatory genes after permanent middle cerebral artery occlusion. There was significant

infiltration of EGFP+ myeloid cells into the lesioned cortex by 72 hours (Zarruk *et al.*, 2017). Taking advantage of the EGFP labeling of myeloid cells in these mice, we used FACS to collect microglia (CD11b+, CD45lo, Ly6G-, LysM-EGFP-) and infiltrating macrophages (CD11b+, CD45hi, Ly6G-, LysM-EGFP+) at 72h post-lesion. The RNA obtained from these cells was used to screen a PCR array for changes in mRNA expression of 84 immunity and inflammation related genes. Of all the mRNAs that were changed, 12 were up-regulated in macrophages while only 3 were up-regulated in microglia; and only 1 down-regulated in macrophages while 7 down-regulated in microglia (Zarruk *et al.*, 2017). Genes that are highly upregulated in infiltrating macrophages include those involved in neutrophil and macrophage recruitment (Spp1 (osteopontin) and cxcl2), recruitment of monocytes and other immune cells (ccl2, ccr5, and il1b), and immune cell activation (tlr2, il1b) (Wolpe & Cerami, 1989; Diab *et al.*, 1999; Lund *et al.*, 2009; Conductier *et al.*, 2010; Rittling, 2011; Hammond *et al.*, 2014). On the other hand, microglia down regulated several key genes involved in inflammation (tlr4, tlr7, tlr9, and ccr5). This gene expression pattern in microglia suggests the acquisition of a quiescent phenotype. Infiltrating macrophages on the other hand express cytokines that recruit and direct the influxing cells (neutrophils and MDMs) into the core the lesion. The two cell types therefore can act to contain the infiltrating myeloid cells to the core of the lesion and thus prevent their spread to surrounding intact tissue. This may be a way to self-limit and contain the expansion of the lesion and contribute to the natural mechanisms to resolve inflammation in stroke. Our double immunofluorescence labeling showed a striking increase in expression of TNFα in microglia in the peri-infarct region. Further, our flow cytometry analysis showed that up to 22% of microglia express TNFα at 3 days after permanent cerebral ischemia (Zarruk *et al.*, 2017). Similar findings about microglial TNFα expression in other animal model systems have also been reported

previously at different time points after permanent cerebral ischemic lesions (Lambertsen *et al.*, 2009). Interestingly, the size of the stroke lesion is markedly increased (60%) in mice in which microglia lacked TNFα expression (Lambertsen *et al.*, 2009). The striking expression of TNFα we see in microglia in the peri-infarct region could therefore serve to protect neurons and glia from damage likely via TNFR2 and thus limit the expansion of the lesion.

A gene expression profiling of microglia and macrophages from mice with experimental autoimmune encephalomyelitis (EAE) showed that monocyte-derived macrophages are highly phagocytic and inflammatory (Yamasaki *et al.*, 2014). In contrast, microglia at onset of disease showed global suppression of cellular metabolism genes (Yamasaki *et al.*, 2014). Interestingly, electron microscopy revealed that unlike microglia, MDMs are located at nodes of Ranvier and where they may initiate demyelination (Yamasaki *et al.*, 2014). Recent evidence from work in experimental autoimmune encephalomyelitis (EAE) also show a dichotomy in the response of microglia and infiltrating macrophages (Gao *et al.*, 2017). These studies showed that deletion of TNF receptor 2 (TNFR2) in microglia leads to worsening of inflammation and EAE pathology and clinical symptoms, while ablating TNFR2 in monocytes/macrophages resulted in reduced inflammation and improved EAE (Gao *et al.*, 2017). There is therefore mounting evidence from a number of lesion models that after the acute phase after CNS damage, microglia have a protective anti-inflammatory phenotype, while infiltrating macrophages are more inflammatory. Whether such differences in microglia and macrophage functions also occur after SCI is not fully known at present. On the other hand, prolonged microglial neuroinflammation and dysregulation in chronic neurodegenerative disease are deleterious to the CNS (Colonna & Butovsky, 2017). Both beneficial and detrimental roles for MDMs have also been described. As the role of microglia and MDMs in spinal cord injury are considered critical to pathological processes, more

work into the kinetics of microglia and MDM responses need to be better understood in order to effectively target these cells to improve outcome after SCI.

5. Neutrophil-macrophage responses and the generation of Specialized Pro-Resolving

The early cellular and molecular events of the inflammatory response have been studied extensively and is characterized by plasma extravasation, infiltration of leukocytes, and production of different bioactive mediators. Acute inflammation is a fundamental physiological process to clear pathogens, dead cells and debris from damaged tissue, and also mediates tissue repair and paves the way for repair and restoration of tissue homeostasis. However, excess or uncontrolled inflammation leads to chronic changes and tissue damage (Serhan, 2014; Serhan *et al.*, 2015b). For many years, the termination of inflammation was thought to be a passive event associated with the dissipation of the inflammatory challenge and the reduction of factors involved in leukocyte tracking, such as chemoattractant mediators and cell adhesion molecules. It is now known that resolution of inflammation is an actively orchestrated process (Serhan, 2014; Serhan *et al.*, 2015a).

To our knowledge, the first study describing the resolution of inflammation was done by Eugene L. Opie (Opie, 1907). He showed that intrapleural injection of turpentine, a fluid obtained by the distillation of resin and highly irritating, caused infiltration of polymorphonuclear that peaked at 2-3 days, disappearing after 5 days. He also showed that the clearance of polymorphonuclear cells (PMN) coincided with the presence of macrophages, linking for the first time the clearance of the PMN cells and resolution of inflammation. PMN cells, also known as granulocytes, arise from granulo-monocytic progenitors in bone marrow

characterized by poly-lobulated nucleus and by their cytoplasmic granules. Commonly, the term PMN often refers specifically to neutrophil, the most abundant of the granulocytes in the injured spinal cord; the other types (eosinophils, basophils, and mast cells) are not noticeable (Neirinckx *et al.*, 2014). After infection or traumatic lesion, neutrophils migrate across endothelial barriers to reach the inflammatory site, being highly sensitive to chemoattractant signals such as CXCL12-CXCR4, CXCL1/2-CXCR2 and IFNγ. Indeed, neutrophils are the firsts circulating leukocytes that enter into the injured spinal cord, and reach maximal counts by 24 hours (Kigerl *et al.*, 2006; Stirling & Yong, 2008; Francos-Quijorna *et al.*, 2017). Once neutrophils reach the lesion site they release a variety of toxic mediators such as oxidative enzymes and proteases, to clear the lesioned tissue and/or microbes (Brinkmann *et al.*, 2004). These molecules released by neutrophils do not act specifically in microbes or cell debris, but also non-specifically on healthy neighboring cells. This is especially relevant in the CNS due to the failure of axons to regenerate and the poor capacity for replacement of dead neurons (David *et al.*, 2012). Neutrophils also release pro-inflammatory mediators such as cytokines and eicosanoid that are crucial for the recruitment of circulating MDMs into the lesion site, leading to greater accumulation of immune cells. This has led to neutrophils being thought of as unfavorable actors in the pathophysiology of SCI and other CNS conditions, and thus, different neuroprotective approaches have focused on targeting neutrophil migration into the spinal cord. Indeed, there is persistent presence of neutrophils in the injured spinal cord in mice up to at least 28 days (Francos-Quijorna *et al.*, 2017) that could lead to prolonged bystander effects.

Not all the products released by neutrophils have detrimental effects. Indeed, we previously described that neutrophils are the main cellular source of secretory leukocyte protease inhibitor (SLIPI), which mediates beneficial actions in SCI, in part, by decreasing inflammation

(Ghasemlou *et al.*, 2010a) but also by promoting axon regeneration (Hannila *et al.*, 2013). Moreover, they also release oncomodulin, a growth factor that induces axon regeneration (Kurimoto *et al.*, 2013). An interesting study revealed that depletion of circulating neutrophils by administration of the Gr1 blocking antibody increased neurological deficits after SCI in mice (Stirling *et al.*, 2009). This study also showed that with the lack of neutrophils there was greater levels of cytokines, such as CXCL1, CCL2 and CCL9, reduced expression of healing factors, and greater tissue scarring, a sign of greater resolution failure (Stirling *et al.*, 2009). These studies therefore suggested for that although neutrophils may release toxic factors, their presence in the injured spinal cord is crucial for the proper control of the inflammatory response and tissue repair.

Once the physiological function of neutrophils has been fulfilled in the inflamed tissues, they undergo spontaneous apoptosis (Fox *et al.*, 2010). This is a programmed cell death that occurs to preserve membrane integrity and prevent uncontrolled release of the harmful cell contents (Kolb *et al.*, 2017). Apoptotic cells are recognized by macrophages by "find-me' signals and "eat-me" signals that then lead to their phagocytosis (also known as efferocytosis) (Elliott *et al.*, 2017). In the injured spinal cord these apoptotic cells are phagocytosed by macrophages and microglia. We and other research groups have shown that LPS-stimulated macrophages switch off the release of pro-inflammatory cytokines (i.e TNFα and IL-12) and down-regulated the expression of M1 markers upon phagocytosis of apoptotic neutrophils (Kroner *et al.*, 2014). Others have shown that this is accompanied by the release of anti-inflammatory cytokines such as IL-10 and TGFβ (Byrne & Reen, 2002) and the up-regulation of arginase-1 and suppression of nitric oxide synthesis by macrophages (Freire-de-Lima *et al.*, 2006). These observations suggest

that engulfment of apoptotic neutrophils evokes different signaling changes in macrophages that results in their redirection towards an anti-inflammatory state.

One of the most important changes evoked by efferocytosis of neutrophils by macrophages is the production of bioactive lipids that induce resolution of inflammation. The family of these bioactive lipids, are also known as 'specialized pro-resolving mediators' (SPMs), that include: lipoxins, which are generated from arachidonic acid; E-series resolvins from eicosapentaenoic acid (EPA); and D-series resolvins, neuroprotectins, and maresins from docosahexaenoic acid (DHA) (Serhan, 2010; 2014; Serhan *et al.*, 2015a). SPM actively turn off the inflammatory response by acting on distinct G protein coupled receptors expressed on immune cells that activates dual anti-inflammatory and pro-resolution programs (Buckley *et al.*, 2014; Serhan, 2014; Serhan *et al.*, 2015a). Among the anti-inflammatory actions of SPMs include the induction of anti-inflammatory cytokines or inflammatory scavenging molecules such as IL-10, IL-1 decoy receptors and IL-1 receptor antagonists (Buckley *et al.*, 2014; Serhan, 2014). Importantly, SPMs activate specific mechanisms that trigger the resolution of inflammation, which include: (i) down-regulation of pro-inflammatory cytokines; (ii) abrogation of intracellular pathways that lead to inflammation; (iii) clearance of inflammatory cell detritus (such as apoptotic neutrophils) by macrophages and (iv) normalization of immune cells counts to basal levels also referred to as catabasis (Buckley *et al.*, 2014; Serhan, 2014; Serhan *et al.*, 2015a). Failure to produce adequate amounts of SPMs or failure to bind to their receptors could lead to the persistence of inflammation leading to chronic inflammation. Several reports have shown that there is defective synthesis of SPMs in the cerebrospinal fluid (CSF) of individual diagnosed with Alzheimer disease or multiple sclerosis (Pruss *et al.*, 2013; Wang *et al.*, 2015b). We have also reported defective production of SPMs in the spinal cord after contusion injury in

mice. It is therefore quite plausible that the defective synthesis of SPMS could contribute to the persistent inflammatory response after SCI.

We have recently reported lipidomics analysis showing delayed synthesis of SPMs in the injured spinal cord. In this study we assessed the effects of maresin-1 (a member of the SPM family) treatment given 1 hour after spinal cord contusion injury in mice. We assessed the effects of maresin-1 on inflammatory responses and recovery after SCI. We found that although exogenous administration of maresin-1 (MaR1) did not affect the recruitment of neutrophils and macrophages, it induced fast and enhanced neutrophil clearance from the lesioned spinal cord (Francos-Quijorna *et al.*, 2017). This included greater engulfment of neutrophils by macrophages, as well as, faster elimination of the inflammatory signals, as revealed by the reduction of pro-inflammatory cytokines (i.e. CXCL1, CXCL2, IL-6) and signaling pathways (JAK-STAT and MAPK) (Francos-Quijorna *et al.*, 2017). Interestingly, macrophages displayed less pro-inflammatory (M1-like) markers after MaR1 treatment, as revealed the reduced expression of LyC6 and iNOS (Francos-Quijorna *et al.*, 2017). These observations suggest that despite the enhanced phagocytic activity of macrophages they may be less cytotoxic. Importantly, the biological effects induced by MaR1 in SCI led to significant improvement in locomotor function and protection against secondary tissue damage (Francos-Quijorna *et al.*, 2017). The role of other SPMs in SCI needs to be examined. Similar protective actions have been also described after the administration of MaR1 and other SPMs such as Neuroprotectin D1 and Lipoxin A4 (LXA4) following cerebral ischemia (Marcheselli *et al.*, 2003; Wu *et al.*, 2010; Ye *et al.*, 2010). These finding suggest that inappropriate biosynthesis of SPMs, which it likely due to an inefficient crosstalk between neutrophils and macrophages and microglial can contribute to persistent inflammation after CNS trauma.

6. Challenges and future directions.

Enormous progress has been made in our understanding of microglia in the past two decades. These include an understanding from fate mapping studies of their origins from the yolk sac during early embryonic development, and their subsequent renewal by proliferation *in situ* throughout life. One of the major challenges in studying microglial responses in CNS injury and disease is being able to distinguish them from infiltrating MDM. This need led to a large number of transcriptome studies comparing microglial gene expression with macrophages from a variety of different sources. The results of these wide-ranging studies revealed a number of microglia-enriched genes (summarized in Table 1). Although many of these markers are useful in identifying resting microglia from MDMs, their expression is generally downregulated after onset of inflammation in injury or disease making them less useful to clearly distinguish microglia from infiltrating MDMs in the damaged CNS. Better markers are therefore needed to isolate and purify microglial populations from other myeloid cell derived macrophages to study their functional differences and potential interactions in injury and disease. There is now evidence that microglia display regional differences in gene expression profile indicating that their immediate cellular environment dictates their expression profile. It is therefore likely that the microglia and also macrophages infiltrating the site of injury and disease from the peripheral circulation may also show a range of differences in expression profile that reflect different functions within the injured tissue. Identifying such differences will require doing single cell RNA sequencing of microglia and macrophage populations. The heterogeneity of the microglia and macrophage populations in the injured spinal cord can then be evaluated and the changes in their profiles monitored over time after injury. We know already that microglia and peripheral

macrophages are highly plastic in their inflammatory profiles and that this is influenced by chemokines, cytokines and other factors including bioactive lipids, as well as by what they phagocytose. After injury, the entire microglia and macrophage populations at the site of injury are not going to change “en masse”, e.g., there will be subpopulation that phagocytose myelin that become anti-inflammatory, while other subgroups that phagocytose RBC would display a proinflammatory profile. In addition, the mix of bioactive factors that can modulate their expression and phenotype patterns change over time. The use of mass cytometry (CyTOF) (40+ heavy metal isotope tagged antibodies) and multi-color (20+ antibodies) fluorescence cytometry to study such changes at the protein level needs to be better exploited in the future. Implicit in this is the idea that microglia and macrophage may serve different roles and these roles may change over time after injury. The ultimate goal of these studies is to be able to modulate the heterogenous and plastic nature of microglia and macrophages in the injured CNS to reduce their cytotoxic functions while promoting their pro-repair properties.

Gene	Protein	Method	Species	Compared to	References
P2Ry12	purinergic receptor P2Y12	microarray mRNA sequencing mass spectrometry *in situ* hybridization	mouse	peripheral macrophages (including spleen), glial cells, neurons	(Bedard *et al.*, 2007) (Hickman *et al.*, 2013) (Bennett *et al.*, 2016) (Butovsky *et al.*, 2014)
Siglec-H	sialic acid binding Ig-like lectin	microarray mRNA sequencing *in situ* hybridization histology	mouse	peripheral macrophages (including spleen), myeloid cells	(Bedard *et al.*, 2007) (Gautier *et al.*, 2012) (Chiu *et al.*, 2013) (Hickman *et al.*, 2013) (Konishi *et al.*, 2017)
Oflm13	olfactomedin-like protein 3	microarray mRNA sequencing mass spectrometry	mouse	peripheral macrophages (including spleen), dendritic cells, glial cells, neurons	(Bedard *et al.*, 2007) (Gautier *et al.*, 2012) (Chiu *et al.*, 2013) (Butovsky *et al.*, 2014)

Tmem119	transmembrane protein 119	mRNA sequencing histology mass spectrometry microarray *in situ* hybridization	mouse	peripheral macrophages, dendritic cells, myeloid cells, glial cells, neurons	(Gautier *et al.*, 2012) (Chiu *et al.*, 2013) (Hickman *et al.*, 2013) Konishi *et al.*, 2017 (Butovsky *et al.*, 2014) (Bennett *et al.*, 2016)
Fcrls (Msr2)	Fc receptor-like S, scavenger receptor	mRNA sequencing mass spectrometry microarray *in situ* hybridization	mouse	peripheral macrophages (including spleen), dendritic cells, glial cells, neurons	(Gautier *et al.*, 2012) (Butovsky *et al.*, 2014) (Bennett *et al.*, 2016) (Bedard *et al.*, 2007)
GPR34	G protein-coupled receptor 34	microarray mRNA sequencing mass spectrometry *in situ* hybridization	mouse	peripheral macrophages (including spleen), glial cells, neurons	(Bedard *et al.*, 2007) (Hickman *et al.*, 2013) (Butovsky *et al.*, 2014) (Bennett *et al.*, 2016)
F11R	junctional adhesion molecule A	microarray *in situ* hybridization	mouse	spleen macrophages	(Bedard *et al.*, 2007)
Stab1	stabilin 1	microarray	mouse	spleen macrophages	(Bedard *et al.*, 2007)
P2y13 (GPR86)	purinergic receptor P2Y13	microarray	mouse	peripheral macrophages	(Bedard *et al.*, 2007)

				(including spleen),	(Hickman *et al.*, 2013) (Bennett *et al.*, 2016)
Trem2	triggering receptor expressed on myeloid cells 2	mRNA sequencing	mouse	various tissue macrophages, dendritic cells	(Gautier *et al.*, 2012) (Hickman *et al.*, 2013)
Gas6	growth arrest specific 6	mRNA sequencing	mouse	tissue macrophages, dendritic cells	(Gautier *et al.*, 2012)
Cx3cr1	chemokine (C-X3-C motif) receptor 1	mRNA sequencing	mouse	tissue macrophages, dendritic cells	(Gautier *et al.*, 2012) (Hickman *et al.*, 2013)
Sall1	spalt like transcription factor 1	mRNA sequencing mass spectrometry microarray	mouse	tissue macrophages, dendritic cells, glial cells, neurons	(Gautier *et al.*, 2012) (Butovsky *et al.*, 2014)
Hexb	hexosaminidase B	mRNA sequencing mass spectrometry microarray	mouse	tissue macrophages, dendritic cells, glial cells, neurons	(Gautier *et al.*, 2012) (Butovsky *et al.*, 2014)

Author declaration:

SD and RLV have patent applications pending for Maresin in SCI. All authors declare no other financial conflicts of interest. This manuscript has not been published previously in whole or in part, nor under consideration for publication elsewhere.

Acknowledgements:

The work done in the authors lab were supported by grants from the Canadian Institutes of Health Research (SD) and the Spanish Ministry of Economy and Competitiveness (SAF2016-79774-R), International Foundation for Research in Paraplegia, Wings for Life Foundation and by funds from the Fondo de Investigación Sanitaria of Spain (TERCEL and CIBERNED) (RLV). We wish to thank our collaborators on various projects including Juan G. Zarruk, Rosmarini Passos Dos Santos, Isaac Francos-Quijorna, Eva Santos-Nogueira, Karsten Gronert , Aaron B Sullivan , Marcel A Kopp, Benedikt Brommer, and Jan M Schwab.

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