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Review Article

Microglia and macrophages differ in their inflammatory profile after permanent brain ischemia

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ABSTRACT

We studied the expression of pro- and anti-inflammatory molecules in microglia and infiltrating monocyte-derived macrophages after permanent Middle Cerebral Artery Occlusion (pMCAO). LysM-EGFP knock-in mice were used to distinguish between these two cell types, as peripheral myeloid cells are LysM-EGFP$^+$, while microglia are not. This was confirmed with P2ry12 (a microglial specific marker), Iba-1 and EGFP immunostaining. The peak of LysM-EGFP$^+$ myeloid cell infiltration was 72 h post-ischemia, and were distributed evenly in the lesion core, surrounded by a dense region of microglia. Flow cytometry showed that a higher percentage of microglia expressed TNF-α at 3 (24.3% *vs* 1.4%) and 7 (18.8% *vs* 3.4%) days post-pMCAO as compared to infiltrating macrophages. Microglia and macrophages were purified by fluorescence activated cell sorting 72 h post-ischemia to assess the mRNA expression of inflammatory markers. Macrophages upregulated expression of mRNA for arginase-1 (Arg-1) by 1000-fold, and IL-1β by 90-fold as compared to microglia. At the protein level, a significantly number of macrophages expressed Arg-1, while few if any microglia expressed Arg-1. However, IL-1β protein was not detected in macrophages by flow cytometry or immunofluorescence labeling of tissue sections. It was, however, detected in astrocytes along the lesion border. A PCR-array screen of 84 inflammatory genes revealed that pro-inflammatory chemokines and cytokines were predominantly upregulated in macrophages but down-regulated in microglia in the ischemic brain. Our results show clear differences in the inflammatory expression profiles between microglia and macrophages 72 h post-ischemia which may shape repair and pro-regenerative mechanisms after stroke.

1. Introduction

The acute response after cerebral ischemic injury is mainly an innate immune response to damage that is triggered by resident CNS cells, in particular microglia (Schilling et al., 2003). This as well as other factors lead to the recruitment of neutrophils and monocyte-derived macrophages (MDMs) from the circulation over a period of 7 days (Chu et al., 2014; Gelderblom et al., 2009; Schilling et al., 2003). Microglia and MDMs are thought to contribute importantly to the inflammatory response during the acute phase (Benakis et al., 2014; Iadecola and Anrather, 2011a). At sites of cerebral ischemia, some aspects of the inflammatory response contribute to tissue damage and neurological deficits, while other aspects are beneficial (Denes et al., 2011; Kamel and Iadecola, 2012). There is increasing evidence that MDMs and microglia are highly plastic cells whose phenotype is influenced by their environment and what they phagocytose (Benakis et al., 2014; ElAli and Jean LeBlanc, 2016). Microglia respond within minutes to ATP released at the site of injury to extend processes that form a dense meshwork surrounding the lesion (Davalos et al., 2005; Hines et al., 2009). Depletion of microglia using a variety of methods results in significant expansion of the lesion or infarct size (Lalancette-Hebert et al., 2007; Montero et al., 2009; Szalay et al., 2016). On the other hand, continued microglial or MDM activation leads to cytokine production and generation of reactive oxygen species that can induce secondary damage, which leads to worse outcome (Cuartero et al., 2015; Iadecola and Anrather, 2011a). Several recent studies have therefore sought to characterize the phenotype and functional contributions of microglia and MDMs in various models of cerebral ischemia (Denes et al., 2011; Hu et al., 2012; Miro-Mur et al., 2016; Perego et al., 2011; Schilling et al., 2005).

An important challenge is to distinguish between microglia and MDMs that enter the CNS after ischemic injury. Microglia retract their cytoplasmic processes when they become phagocytic and are difficult to distinguish from MDMs based on their morphology and antigenic phenotype in tissue sections. Novel markers specific or highly enriched in microglia have recently been discovered (Bennett et al., 2016;

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Butovsky et al., 2014, Buttgereit et al., 2016) but antibodies have yet to become commercially available. In flow cytometry, these cells can be differentiated by the expression level of CD45 and Ly6C (Ritzel et al., 2015). However, MDMs down-regulate expression of Ly6C after entry into the CNS (Garcia-Bonilla et al., 2016). Adoptive transfer of CD45.1 + monocytes into host mice expressing the CD45.2 allele, or adoptive transfer of DsRed (red fluorescent reporter) expressing monocytes, in the absence of myelobalation of host cells, has been used effectively to characterize the phenotype of macrophages entering the ischemic cortex (Miro-Mur et al., 2016). The transplanted cells, however, comprise a very small proportion of the myeloid cells that enter the ischemic area and so are not ideal for studies of entire cell populations. Carboxyfluorescein succinimidyl ester (CFSE) labeling of blood derived cells has also been used effectively to distinguish between microglia and infiltrating blood-derived cells (Denes et al., 2011) but this labels all proliferating blood cells and may not be ideal for purifying cells for gene expression analysis. The LysM-EGFP knock-in transgenic mouse in which EGFP is inserted into the lysozyme M locus, labels hematogenous macrophages and neutrophils but not microglia (Faust et al., 2000; Greenhalgh and David, 2014; Greenhalgh et al., 2016; Mawhinney et al., 2012). This EGFP labeling helps to distinguish MDMs that have entered the damaged CNS from microglia that are LysM-EGFP-negative and was confirmed using microglia specific antibodies (Greenhalgh et al., 2016). Neutrophils can be distinguished from MDMs easily in fluorescence activated cell sorting (FACS) by the expression of Ly6G, and in tissue sections by their characteristic polymorphonuclear morphology and with antibody markers. LysM-EGFP knock-in mice can therefore be used to separate MDMs from microglia by FACS and the purified cell populations used for expression profiling. This is the first use of LysM-EGFP knock-in mice in stroke to distinguish between infiltrating macrophages and resident microglia and to separate these two cell types for expression analysis.

In this study, we have performed permanent middle cerebral artery occlusion (pMCAO) in LysM-EGFP knock-in mice to study the inflammatory profile of microglia and MDMs using a variety of techniques. This analysis reveals unique features of these two cell types in the cortex after pMCAO.

2. Materials and methods

2.1. Animals

Male heterozygote LysM-EGFP knock-in mice (generated by Thomas Graf), 8–12 weeks of age were used for all *in vivo* experiments. All procedures were approved by the Animal Care Committee of the Research Institute of the McGill University Health Centre and followed the guidelines of the Canadian Council on Animal Care, and the ARRIVE guidelines for reporting animal research (Kilkenny et al., 2010).

2.2. Induction of permanent focal cerebral ischemia

Surgery leading to focal cerebral ischemia was conducted as described previously (Zarruk et al., 2012) and a variant of a model described earlier (Chen et al., 1986; Liu et al., 1989). In brief, animals were put under anesthesia with isoflurane in O_2 (0.5 L/min) during the whole procedure. During surgery, body temperature was maintained at 37.0 ± 0.5 °C using a homeothermic system with a rectal probe (Harvard apparatus). A small craniotomy was made over the trunk of the left middle cerebral artery (MCA) and above the rhinal fissure. The permanent middle cerebral artery occlusion (pMCAO) was done by ligature of the trunk of the MCA just before its bifurcation between the frontal and parietal branches with a 9-0 suture. Complete interruption of blood flow was confirmed under the operating microscope. Additionally, the left common carotid artery was then occluded. Mice in which the MCA was exposed but not occluded served as sham-operated controls (sham). After surgery, animals were returned to their cages with free access to water and food. No spontaneous mortality occurred after pMCAO.

2.3. Immunofluorescence and confocal microscopy

Cryostat sections of the brain (14 µm thick) from 4% paraformaldehyde perfused-fixed LysM-EGFP mice at different time points after pMCAO were used for immunofluorescence labeling. Tissue sections were first incubated with 0.3% TritonX-100 (Sigma-Aldrich Inc.), 5% normal goat serum (Jackson ImmunoResearch Inc.), 2% ovalbumin (Sigma-Aldrich Inc.) in phosphate buffer saline (PBS) for 3 h at room temperature (RT) to block non-specific binding of antibodies. Sections were then incubated overnight at 4 °C with the following primary antibodies goat anti-arginase-1 (1:200; Santa Cruz; SC-18354), goat anti-IL-1β (1:200 R & D Systems; AF-401-NA) chicken anti-GFP (1:500; Abcam; ab13970), mouse anti-TNF-α (1:100; Abcam; ab1793); combined with the following cell type specific antibodies: rabbit anti-Iba1 (1:500; Wako; 019-19741 for microglia/macrophages), rat anti-CD11b (1:300; Serotec; MCA711 for macrophages/microglia), guinea pig anti-glial fibrillary acidic protein (GFAP) (1:500; Synaptic Systems cat No. 173004 for astrocytes), rabbit anti-P2ry12 (1:500; from Dr. Oleg Butovsky, for microglia). Sections were washed 3 times, 15 min each in 90 ml of 0.05% Tween-20 - PBS solution and similarly in PBS and incubated with the appropriate fluorescent-conjugated secondary antibodies - anti-rabbit Alexa Fluor 647, anti-rat Alexa Fluor 568, anti-guinea pig Alexa Fluor 647, anti-mouse Fab-fragment Alexa Fluor 594 or anti-goat Alexa 568 (Invitrogen). Tissue sections were viewed with a confocal laser scanning microscope (FluoView FV1000, Olympus) and micrographs taken with the FV10-ASW 3.0 software (Olympus). The various parameters for taking images on the confocal microscope (Analog PMT Offset; Excitation Out Put Level; PMT Voltage; and Gain) were set at the same setting for images taken for comparisons.

2.4. Fluorescence-Activated Cell Sorting (FACS)

After intra-cardiac perfusion with PBS the whole ipsilateral infarcted hemisphere of pMCAO and sham control ipsilateral hemisphere from LysM-EGFP mice were dissected, dissociated and stained as previously described (Greenhalgh et al., 2016). Cell fractions were stained using the following antibodies: rat anti-mouse CD11b (BD Horizon V450) (1:300), rat anti-mouse CD45 PE-Cy7 (1:300) (BD Pharmingen), rat anti-mouse Ly6G PE (1:300) (BD Pharmingen), rat anti-mouse Ly6G PerCP 5.5 (1:300) (BD Pharmingen), rat anti-mouse Ly6C APC-Cy7 (1:300) (BD Pharmingen), rat anti-mouse TNF-α PE (1:100) (BD Pharmingen), IL-1β APC (1:200) (BD Pharmingen) and fixable viability dye eFluor 780 (eBioscience). Cells were acquired using a FACS CantoII and analyzed using FlowJo software.

2.5. Purification of microglia and macrophages

To characterize the inflammatory profile of microglia and macrophages, the cells were FACS sorted using FACSAria Fusion (BD Biosciences). The ipsilateral hemisphere of pMCAO and sham LysM-EGFP was dissected after intra-cardiac perfusion with PBS and cells were extracted and stained as was done for FACS experiments described above. Two hemispheres were pooled for each experiment (n = 3). To separate microglia from macrophages, the following gating strategy was used: $CD11b^+$, $CD45^+$, $Ly6G^-$, LysM-$EGFP^-$ cells were sorted as microglia, and $CD11b^+$, $CD45^+$, $Ly6G^-$, LysM-$EGFP^+$ were sorted as macrophages. For these experiments, ipsilateral pMCAO microglia were compared to ipsilateral microglia from sham control animals.

Bone marrow monocytes (BMm) were used as the control cells to compare against MDMs from the ipsilateral pMCAO hemisphere. Spleen macrophages were also used in some experiments as an additional group for comparison. To extract the BMm, naïve LysM-EGFP mice were deeply anesthetized (ketamine 50 mg/kg; xylazine 5 mg/kg;