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Immature monocytes recruited to the ischemic mouse brain differentiate into macrophages with features of alternative activation

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ABSTRACT

Acute stroke induces a local inflammatory reaction causing leukocyte infiltration. Circulating monocytes are recruited to the ischemic brain and become tissue macrophages morphologically indistinguishable from reactive microglia. However, monocytes are a heterogeneous population of cells with different functions. Herein, we investigated the infiltration and fate of the monocyte subsets in a mouse model of focal brain ischemia by permanent occlusion of the distal portion of the middle cerebral artery. We separated two main subtypes of $CD11b^{hi}$ monocytes according to their expression of the surface markers Ly6C and CD43. Using adoptive transfer of reporter monocytes and monocyte depletion, we identified the pro-inflammatory $Ly6C^{hi}CD43^{lo}CCR2^{+}$ subset as the predominant monocytes recruited to the ischemic tissue. Monocytes were seen in the leptomeninges from where they entered the cortex along the penetrating arterioles. Four days post-ischemia, they had invaded the infarcted core, where they were often located adjacent to blood vessels. At this time, Iba-1^{-} and Iba-1^{+} cells in the ischemic tissue incorporated BrdU, but BrdU incorporation was rare in the reporter monocytes. The monocyte phenotype progressively changed by down-regulating Ly6C, up-regulating F4/80, expressing low or intermediate levels of Iba-1, and developing macrophage morphology. Moreover, monocytes progressively acquired the expression of typical markers of alternatively activated macrophages, like arginase-1 and YM-1. Collectively, the results show that stroke mobilized immature pro-inflammatory $Ly6C^{hi}CD43^{lo}$ monocytes that acutely infiltrated the ischemic tissue reaching the core of the lesion. Monocytes differentiated to macrophages with features of alternative activation suggesting possible roles in tissue repair during the sub-acute phase of stroke.

1. Introduction

Brain ischemia induces neuronal cell death and inflammation that attracts circulating leukocytes to the injury site (Gelderblom et al., 2009; Chu et al., 2014). Several lines of evidence support that monocytes can promote further inflammation and exacerbate the brain lesion in acute stroke (Dimitrijevic et al., 2007). However, they seem to exert beneficial effects in the sub-acute phase of stroke by preventing hemorrhagic transformation (Gliem et al., 2012). Identification of monocytes infiltrated in the ischemic tissue is tricky since they become morphologically indistinguishable from the population of reactive resident microglia. Furthermore, circulating monocytes are a heterogeneous population of cells composed of various subsets with different phenotypes (Strauss-Ayali et al., 2007). In humans, the majority of monocytes are $CD14^{+}$ $CD16^{-}$, but there is a minority of monocytes expressing different levels of CD16 that change their proportions in response to acute stroke (Urra et al., 2009). Mouse monocytes express cell surface molecules that are different from those of human monocytes, but functional equivalences between mouse and human monocytes have been proposed making use of the mouse surface marker lymphocyte antigen 6 complex, locus C (Ly6C), and leukosialin, also known as sialophorin or cluster of differentiation 43 (CD43) (Geissmann et al., 2003; Strauss-Ayali et al., 2007; Sunderkötter et al., 2004; Ziegler-Heitbrock et al., 2010). Ly6C is anchored to the cell surface via a phosphatidylinositol moiety and upon

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cross-linking initiates cell stimulation (Bamezai et al., 1989). CD43 is a sialylated glycoprotein binding to CD54 (ICAM-1) and E-selectin (Merzaban et al., 2011; Zarbock et al., 2011), and playing complex roles in cell–cell and cell–endothelium interactions by exerting either pro-adhesive or anti-adhesive actions (Ostberg et al., 1998) and facilitating cell infiltration (Woodman et al., 1998). Mouse monocytes with high expression of CD43 are classified as 'non-classic' or 'intermediate' monocytes with functional equivalence to $CD16^+$ human monocytes (Ziegler-Heitbrock et al., 2010). Comparatively, the $Ly6C^{hi}CD43^{lo}$ monocytes are considered pro-inflammatory due to their high production of TNF-α and IL-1β (Ziegler-Heitbrock et al., 2010). In stroke patients, an increased proportion of classic $CD14^{hi}CD16^-$ circulating monocytes on admission was associated with early clinical worsening, poor outcome and mortality at day 90 (Urra et al., 2009), suggesting that the classic monocytes could play some deleterious role. However, the specific monocyte subtypes that infiltrate the ischemic brain, as well as their function and fate, are not well characterized after ischemia in the absence of reperfusion. Here we studied the features of circulating monocyte subsets after permanent brain ischemia, their infiltration to the ischemic brain tissue, and their fate.

2. Materials and methods

2.1. Animals

Animal work was performed according to our local regulations in compliance with the Spanish legislation (Real Decreto 53/2013) and European Community Directives, and following the ARRIVE guidelines. The Ethical Committee (CEEA) of the University of Barcelona approved the experimental procedures. Brain ischemia was induced in adult (3–4 months) male C57BL/6j mice (Charles River, Lyon, France). C57BL/6j mice carrying the CD45.1 allele (Ly5.1) (B6.SJL-Ptprc Pepc/BoyJ) and transgenic C57BL/6 mice expressing the red fluorescent protein DsRed under the control of the β-actin promoter were used to obtain reporter monocytes for adoptive transfer experiments.

2.2. Brain ischemia

Permanent distal occlusion of the right middle cerebral artery (MCAo) was carried out under isoflurane anaesthesia in 30% O_2 and 70% N_2O, as reported (Pérez-de-Puig et al., 2013). In brief, after drilling a small hole in the cranium at the level of the distal portion of the MCA, the artery was occluded by cauterization. Flow obstruction was visually verified. After surgery, animals were allowed to recover from the anaesthesia and were returned to their cages. None of the animals died after MCAo. In a group of mice, MRI ($T2_w$) was carried out in a 7.0T horizontal animal scanner (BioSpec, Bruker BioSpin, Ettlingen, Germany), equipped with a 12 cm inner diameter actively shielded gradient system (400 mT/m), as described (Pérez-de-Puig et al., 2013).

2.3. Isolation of blood leukocytes

In anesthetized animals, blood was extracted from the cava vein and collected in EDTA tubes (1.6 mg EDTA/mL blood; Micro tube 1.3 mL K3E, Sarstedt) for analysis. Five-hundred μL of total blood were incubated for 10 min with 5 mL of red blood cell (RBC) lysis solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Cells were centrifuged at 800×*g* for 5 min and washed in 10 mL of cold PBS. Leukocytes were finally collected in fluorescence-activated cell sorting (FACS) buffer (PBS, 2 mM EDTA, 2% FBS) for flow cytometry analysis.

2.4. Isolation of brain cells

Mice were anesthetized and perfused transcardially with 40 mL saline containing heparin (5 U/mL). The ischemic cortex (ipsilateral) and the corresponding region of the non-affected hemisphere (contralateral) were dissected out and analyzed separately. The tissue was incubated for 20 min at 37 °C in 2 mL of RPMI 1640 (Life Technologies S.A., Alcobendas, Madrid, Spain) containing 100 U/mL collagenase IV and 50 U/mL DNAse I. Brain tissue was passed through a tissue grinder and cells were recovered after centrifugation at 400×*g* for 10 min and separated from myelin and debris in 70% and 30% isotonic percoll gradient (GE Healthcare) in Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Samples were centrifuged at 1000×*g* for 30 min without acceleration or brake. Cells were collected from the interface, washed once with HBSS, and processed for flow cytometry.

2.5. Isolation of monocytes from the bone marrow

Monocytes were extracted from the CD45.1 mice or the transgenic DsRed mice. Mice were killed by isoflurane overdose, the femurs and tibiae from hindlimbs were removed, cleaned of all connective tissue and placed on 5 mL cold PBS. In sterilized conditions, bones were washed with 70% ethanol and placed in complete media, i.e. RPMI 1640 medium (Life Technologies S.A.), containing 10% FBS. The ends of each tibia and femur were clipped to expose the bone marrow, and bone marrow cells were flushed from bones into medium using a syringe with a 23-gauge needle. The cell suspension was passed through a 70 μm nylon mesh strainer and centrifuged at 300×*g* for 6 min. Monocytes were enriched by using the EasySep negative selection mouse monocyte enrichment kit (StemCell Technologies, Grenoble, France). Briefly, cells were resuspended at 1×10^8 cells/mL in FACS buffer with 5% rat serum and incubated for 15 min at 4 °C with EasySep® Mouse Enrichment Cocktail at 50 μL/mL. A wash was carried out with FACS Buffer and centrifuged at 300×*g* for 10 min. Cells were resuspended at 1×10^8 cells/mL in FACS buffer and incubated with EasySep®Biotin Cocktail at 60 μL/mL for 15 min at 4 °C. Cells were incubated for 10 min with EasySep® Magnetic Particles at 150 μL/mL. FACS buffer was added to a final volume of 2.5 mL and the sample tube was placed in the EasySep® magnet for 5 min. The desired fraction was poured off into a new tube and centrifuged 300×*g* for 10 min. Isolated monocytes expressed high levels of Ly6C ($Ly6C^{hi}$) and their purity was above 90%.

2.6. Flow cytometry

Isolated brain and blood cells were washed with FACS buffer, incubated at 4 °C for 10 min with FcBlock (1/200; Clone 2.4G2; BD Pharmingen), and incubated with primary antibodies in FACS buffer for 30 min at 4 °C. The antibodies used were rat anti-mouse CD11b (clone M1/70, Alexa Fluor 647, BD Pharmingen), CD45 (clone 30-F11, FITC, BD Pharmingen), Ly6G (clone 1A8, PE-Cy7, BD Pharmingen), CD45.1 (clone A20, V450, BD Horizon; or APC, Tonbo Biosciences), Ly6C (clone ER-MP20, FITC, Abcam; or clone HK1.4, eFluor450, eBioscience), CD43 (clone S7, PE, BD Pharmingen), CD11c (clone HL3, PE, BD Pharmingen), MHCII (clone M5/114.15.2, PerCP, Biolegend), and CCR2 (475301, fluorescein, R&D). For intracellular staining (ICS) in flow cytometry, after surface staining, cells were incubated in fixation buffer (Biolegend) at room temperature for 20 min, and permeabilized with permeabilization wash buffer (Biolegend) at room temperature for 20 min. Additional incubation with an antibody against arginase-1 (Arg-1) (sheep polyclonal, PE, R&D Systems) diluted in the permeabilization wash buffer was carried out for 30 min. Isotype controls were rat IgG2bκ (clone A95-1, Alexa Fluor 647