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Peripheral immune cells infiltrate into sites of secondary neurodegeneration after ischemic stroke

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ABSTRACT

Experimental stroke leads to microglia activation and progressive neuronal loss at sites of secondary neurodegeneration (SND). These lesions are remote from, but synaptically connected to, primary infarction sites. Previous studies have demonstrated that immune cells are present in sites of infarction in the first hours and days after stroke, and are associated with increased neurodegeneration in peri-infarct regions. However, it is not known whether immune cells are also present in more distal sites where SND occurs. Our study aimed to investigate whether immune cells are present in sites of SND and, if so, how these cell populations compare to those in the peri-infarct zone. Cells were isolated from the thalamus, the main site of SND, and remaining brain tissue 14 days post-stroke. Analysis was performed using flow cytometry to quantify microglia, myeloid cell and lymphocyte numbers. We identified a substantial infiltration of immune cells in the ipsilateral (stroked) compared to the contralateral (control) thalamus, with a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells. This result was further quantified using immunofluorescent labelling of fixed tissue. In the remaining ipsilateral hemisphere tissue, there were significant increases in the frequency of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, $Ly6G^+$ neutrophils and both $Ly6G^-Ly6C^{LO}$ and $Ly6G^-Ly6C^{HI}$ monocytes. Our results indicate that infiltrating immune cells persist in ischemic tissue after the acute ischemic phase, and are increased in sites of SND. Importantly, immune cells have been shown to play pivotal roles in both damage and repair processes after stroke. Our findings indicate that immune cells may also be involved in the pathogenesis of SND and further clinical studies are warranted to characterise the nature of inflammatory cell infiltrates in human disease.

1. Introduction

Ischemic stroke is characterized by significant cell death and a marked inflammatory response at the infarct site. The response in this region involves rapid activation of microglia which are key immune cells within the central nervous system (CNS) (Morioka et al., 1993; Davies et al., 1998). Microglia are often referred to as the resident macrophages of the brain due to a number of shared morphological characteristics and functions. For example, microglia, similarly to macrophages, respond to signals of injury, infection or cell impairment by adopting an 'active' phenotype whereby they modulate repair and recovery processes via actions such as phagocytosis of apoptotic cells and cellular debris, and the release of anti-inflammatory substances and neurotrophic factors (Nimmerjahn et al., 2005; Davalos et al., 2005; Wake et al., 2009). Microglia-specific functions include the surveillance of the local microenvironment for signals of disturbance to brain homeostasis such as cytokines, complement or abnormal protein aggregates (Hanisch and Kettenmann, 2007). Microglia activation has also been associated with tissue damage, via, for example, secretion of neurotoxic agents such as IFNγ, IL-1β, TNFα, IL-6, CXCL10, ROS and NO as well as proteolytic enzymes that act on the extra-cellular matrix leading to blood brain barrier (BBB) breakdown (del Zoppo et al., 2007; Boche et al., 2013). Further, treating animals with minocycline (a tetracycline that inhibits microglial activation), reduced infarct volume and increased neurogenesis when administered after experimental stroke (Liu et al., 2007).

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Importantly, in the event of ischemic stroke, the inflammatory response is accompanied by decreased integrity of the BBB (Ronaldson et al., 2012; Ilzecka, 1996). As a result, immune cells from the peripheral circulation infiltrate the parenchyma at the primary ischemic site. Peripheral immune cells have been reported to be present in primary sites of infarction in the first hours and up-to days after stroke in both human (Lindsberg et al., 1996; Price et al., 2004) and animal studies (Tanaka et al., 2003; Schilling et al., 2003). A large body of work now indicates that infiltrating immune cells contribute to increased neurodegeneration in the area immediately adjacent to the ischemic core, known as the peri-infarct region (Jin et al., 2010; Amantea et al., 2009). For example, studies blocking the ICAM-1 receptor, thereby inhibiting neutrophil adhesion and migration across the BBB, found reductions in post-stroke brain damage in rats (Zhang et al., 1995; Clark et al., 1991). Moreover, experimental studies in lymphocyte-deficient mice (SCID and Rag−/−) have shown reductions in infarct size, and improved neurological deficit suggesting that B and T cells may play a key role in the pathogenesis of stroke (Hurn et al., 2007; Yilmaz et al., 2006).

Recently, several studies have suggested that peripheral immune cells may also be beneficial to ischemic tissue repair processes. For example, regulatory T cells (Tregs), have been shown to play a role in limiting ischemic damage (Liesz et al., 2009; Gill and Veltkamp, 2016).

While the dynamics of immune cell infiltration in the peri-infarct zone during the acute phase after stroke are well characterized (Price et al., 2004), much less is known about the nature and role of inflammation during secondary neurodegeneration (SND). SND describes a chronic neuroinflammatory response to traumatic brain events, in areas remote from, but synaptically connected to, the primary site (Dihné et al., 2002; Iizuka et al., 1990). This inflammation is associated with neuron loss and subsequent neurological deficits (e.g. impaired verbal fluency) and delayed functional recovery (e.g. poor motor recovery) in affected patients (Ogawa et al., 1997; Forno, 1983; Schmitt et al., 1998; Schmitt et al., 2000; Gerhard et al., 2005; Pappata et al., 2000; Binkofski et al., 1996; Fernández-Andújar et al., 2014) and in animal models of stroke (Dihné et al., 2002; Iizuka et al., 1990; Jones et al., 2015). As in primary infarct sites, SND has been associated with pronounced microglial activation (Jones et al., 2015). This observation has been largely based on studies measuring the expression levels of ionized-calcium-binding-adapter-molecule-1 (Iba-1), complement receptor-3 (CD11b) or Cluster of Differentiation 68 (CD68) in SND sites after stroke. Iba-1, CD11b and CD68 expression are commonly used as markers of microglia activation in morphological studies. However, these markers are also present on circulating immune cells (Jeong et al., 2013). Iba-1 is expressed by blood-borne monocytes and tissue-dwelling macrophages (Umekawa et al., 2015; Imai and Kohsaka, 2002; Jeong et al., 2010), and CD11b and CD68 are present on neutrophils and monocytes (Jeong et al., 2010; Matsumoto et al., 2007). Thus, while increased expression of these molecules has been often interpreted as increased microglial activation, these findings may also indicate the presence of other immune cells at SND sites.

Morphologically, microglia are indistinguishable from macrophages derived from infiltrating monocytes, as both cells adopt either a ramified or amoeboid morphology depending on the surrounding environmental stimulus (Schilling et al., 2003). To differentiate between microglia and monocytes/macrophages, flow cytometry has proven to be a useful tool. Indeed, flow cytometry has now been used extensively to characterize the immune cells present in the peri-infarct region (Price et al., 2004). However, to our knowledge, no such study has been undertaken in areas of SND. In this study, we aim to quantify the inflammatory cell population specifically in sites of SND, with particular focus on distinguishing resident microglia from infiltrating immune cells.

In the current study, we employed a photothrombotic (PT) model of vascular occlusion to induce a highly localised cortical infarct within the somatosensory cortex of adult mice. This approach is well recognised to induce robust SND, particularly within the ipsilateral thalamus (Jones et al., 2015; Schroeter et al., 2006). PT stroke was favoured over other commonly employed methods of stroke inductions as it results in highly reproducible infarct sizes and locations (Chen et al., 2014; Watson et al., 1985). We performed flow cytometric analysis of tissue from the thalamus of stroked animals, quantifying the type and number of infiltrating immune cell ($CD45^+$) populations. Our results show for the first time that there is substantial infiltration of immune cells into the ipsilateral thalamus, with a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells in this region compared to the contralateral thalamus. In the remaining ipsilateral hemisphere tissue (non-thalamic tissue including the infarct region) there were also significant increases in the frequency of $CD4^+$ and $CD8^+$ T, as well as $B220^+$ B lymphocytes, myeloid cells ($CD11b^+CD45^{HI}$), $Ly6G^+$ neutrophils and both $Ly6G^-Ly6C^{LO}$ and $Ly6G^-Ly6C^{HI}$ monocytes. Our results indicate that infiltrating immune cells persist in ischemic tissue after the acute ischemic phase, and also infiltrate sites of SND.

2. Methods

2.1. Animals

C57BL/6 adult (10 weeks) male mice were obtained from the Animal Services Unit at the University of Newcastle. Heterozygous $Cx3CR1^{GFP/+}$ mice were obtained from Jackson Laboratories and housed at the Garvan Institute of Medical Research and Australian BioResources, Sydney. Animals were maintained in a temperature and humidity controlled environment with food and water available *ad libitum*. Lighting was on a 12:12 h reverse light–dark cycle with all procedures conducted in the dark phase under low-level red lighting. All experiments were approved by the University of Newcastle Animal Care and Ethics Committee, and conducted in accordance with the New South Wales Animals Research Act (1985) and the Australian Code of Practice for the use of animals for scientific purposes.

2.2. Photothrombotic occlusion

PT stroke was performed as described in (Jones et al., 2015). Briefly, vascular occlusion was achieved by intraperitoneal injection of mice with 0.2 mL of 10 mg/mL of rose bengal 8 min prior to 15 min of illumination using a cold light source with a fibre optic end of 4.5 mm diameter placed 2.2 mm lateral of bregma onto the exposed skull.

2.3. Single cell enrichment

For flow cytometry, 14 days post-stroke, animals were perfused transcardially with PBS. Brains were removed, the cerebellum discarded, and dissected laterally into two hemispheres. The thalamus were dissected from each hemisphere and transferred into ice cold PBS. The remaining hemisphere tissues were transferred into separate tubes. Each sample was homogenized in potter homogenizer and centrifuged. Supernatant was removed and the cell pellet resuspended in 35% percoll. This was overlayed onto PBS and centrifuged. Supernatant was decanted and the resulting single cell enriched sample was prepared for flow cytometry staining.

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