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Title:

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Date:

2021-10-01

Citation:

Wanrooy, B. J., Wen, S. W. & Wong, C. H. Y. (2021). Dynamic roles of neutrophils in post-stroke neuroinflammation. *Immunology and Cell Biology*, 99 (9), pp.924-935. <https://doi.org/10.1111/imcb.12463>.

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## SPECIAL FEATURE REVIEW

# Dynamic roles of neutrophils in post-stroke neuroinflammation

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**Keywords**

heterogeneity, ischemia, neuroinflammation, neutrophils, stroke

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Received 15 February 2021;

Revised 12 April 2021;

Accepted 20 April 2021

doi: 10.1111/imcb.12463

*Immunology & Cell Biology* 2021; **99**: 924–935**Abstract**

Clinical trials involving the blockage of peripheral inflammatory leukocyte recruitment into the brain have puzzlingly led to either no significant improvement in stroke outcome, or even worsened outcomes and increased mortality, prompting a re-evaluation of our understanding into the neuroinflammatory processes after stroke. Whilst traditionally understood as simple effectors of the innate immune system, emerging research in vascular disease biology has redefined the neutrophil as a specialized and highly specific cell type with dynamic functional capacity. Indeed, emerging experimental evidence indicates that neutrophils display diverse roles in the acute stages of ischemic stroke with the ability to elicit both pro-inflammatory and anti-inflammatory effects. Currently, there is some uncertainty as to whether neutrophil diversity is beneficial or harmful in stroke as their interactions with the resident cells of the brain, such as microglia and neurons, would potentially elicit heterogeneous outcomes. Current treatments for patients with stroke aim to remove the vascular blockage and to restore blood flow, but there are currently no drug treatments for managing the loss of functional brain tissue nor restoration of microglial and neuronal damage. If these hypothesized wound-healing functions of neutrophils can be validated in a stroke setting, promoting the recruitment of this type of neutrophils into the injured brain tissue may form a promising therapeutic target for the majority of stroke patients currently without treatment. In this review, we will provide an update on recent research that has explored neutrophil heterogeneity in the neuroinflammatory cascade after ischemic stroke.

**INTRODUCTION**

Traditionally, neutrophils are considered as short-lived, non-specific granulocytes of the innate immune system, where their egress from the bone marrow into the bloodstream is restricted through the actions of CXC chemokine receptor 2 (CXCR2) and CXCR4.<sup>1</sup> Neutrophils are typically thought of as being terminally differentiated, as a pool of immature neutrophils develop in the bone marrow, and once mature egress into the bloodstream ready to phagocytose, degranulate and respond to inflammatory stimuli. However, as neutrophils respond rapidly to even minor alterations in the microenvironment and are found throughout a

plethora of body systems, functional similarities in homology and morphology make it difficult to fully categorize both immature and mature cells.<sup>2</sup> Indeed, despite originating from the granulocyte monocyte progenitor (GMP), several new subsets of neutrophils have been purported under both steady state and pathology, each with their own distinct transcriptomic profiles.<sup>2</sup> A recent subset of committed unipotential neutrophil progenitors was recently coined NeP. When NeP were transferred to murine models of B16F10 melanoma, these cells had enhanced pro-tumor proliferation *in vivo*. Moreover, a human form of hNeP was elevated in patients with melanoma, but not in healthy patients.<sup>3</sup> These studies challenge the notion that

neutrophils are fully immature prior to a specific stimulus and suggest that neutrophil activation states may be pre-programmed even before an inflammatory (or anti-inflammatory) insult, which may in part explain the heterogeneous outcomes seen in unsuccessful clinical trials for stroke therapy.

Accumulating experimental and clinical evidence over the past decade has suggested that neutrophils exhibit functional heterogeneity depending on the disease and stage of inflammation.<sup>4–7</sup> Our knowledge of neutrophils displaying anything other than conventional inflammatory functions stems largely from work in cancer and tumor immunology,<sup>5</sup> with the adoption and classification as such from early work on macrophage heterogeneity.<sup>8</sup> Indeed, markers for distinct subsets of neutrophils have not been fully elucidated but are purported by previous research on M1/M2 monocytes/macrophages.<sup>9</sup> Pro-inflammatory or ‘N1’ neutrophils are characterized by the predominate surface expression of Ly6G, CD11b and cluster of differentiation 86 (CD86), and produce cytokines and effector molecules including interleukin (IL)-1 $\beta$ , IL-12, tumor necrosis factor (TNF) $\alpha$ , nitrous oxide (NO) and hydrogen peroxide.<sup>10,11</sup> These cells are short-lived, highly cytotoxic and function to clear micro-colonies of tumor cells but often at the collateral expense of worsening inflammatory damage. In contrast, ‘N2’ neutrophils are long-lived, anti-inflammatory and even immunosuppressive. These cells express Ly6G and CD11b in addition to CD206, Arg1 and YM-1.<sup>9,12,13</sup> The N2 cells produce anti-inflammatory cytokines including transforming growth factor beta (TGF $\beta$ ), IL-10 and vascular endothelial growth factor (VEGF),<sup>13</sup> and ultimately function to limit inflammation, confer neuroprotection, and contribute to tissue remodeling and wound healing.<sup>14</sup> In the setting of cancer, tumors can produce cytokines described above to directly control the peripheral recruitment and ‘terminal’ differentiation of neutrophil activatory states *in situ*.<sup>10</sup> Indeed, N2 neutrophils or tumor-associated neutrophils (TANs) have been shown to be induced by direct release of TGF $\beta$  by tumors.<sup>5</sup> In turn, N2 neutrophils promote pro-tumor growth factors often in conjunction with other leukocytes via the secretion of factors to promote angiogenesis, tissue remodeling and pro-tumor survival. An investigation into the origins of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) with pro- or anti-tumor activity revealed that CD14 may predict the lineage from neutrophils isolated from tumors of lung carcinoma mice. Indeed, CD14<sup>high</sup> tumor PMNs had significant elevation of Arginase 1 and NO.<sup>15</sup> These foundational findings provide insights into the possible functional overlap between N1 and N2 neutrophils in

cancer, and other neutrophil-mediated diseases such as ischemic stroke.

It is important to note that while convenient for conceptual understanding, these classifications likely oversimplify the complex nature of neutrophil activatory states, as neutrophils have been shown to encompass phenotypic diversity even under homeostasis. Recent phenotypic and transcriptomic characterization of neutrophil subsets provide important forward perspectives of neutrophil biology in the settings of tumor and myocardial infarction.<sup>16,17</sup> Indeed, it remains unknown whether this recently described neutrophil diversity is beneficial or harmful in the context of stroke as their interactions with resident cells in the ischemic brain may elicit heterogeneous outcomes. In this review, we will use the simplified terminologies N1 and N2 and discuss recent research that has explored neutrophil heterogeneity in the neuroinflammatory cascade after ischemic stroke.

## THE ROLE OF NEUTROPHILS IN ISCHEMIC STROKE

Neuroinflammation describes the process of inflammation that occurs exclusively in the brain, spinal and peripheral nervous tissue. The degree of neuroinflammation depends on the cause and nature of the insult, whether this be through infection, trauma, or sterile inflammation. Common to these states are the recruitment of peripheral leukocytes, edema, endothelial activation and degradation, cytokine production and blood–brain barrier (BBB) disruption.<sup>18–20</sup> The initial ischemic phase of stroke injury induces oxygen-glucose deprivation of neurons and the supporting parenchyma resulting in the release of their cell debris in the form of damage associated molecular patterns (DAMPs).<sup>21</sup> Purines including adenosine triphosphate (ATP) and high-mobility group box 1 (HMGB1) proteins are released from the mitochondria of dying cells and are some of the more potent DAMPs in triggering local inflammation.<sup>21</sup> Indeed, high levels of serum HMGB1 correlate with the severity of infarct size in patients with ischemia,<sup>22</sup> and the release of ATP stimulates excitatory neurotransmitter release causing calcium-mediated neuronal death.<sup>23</sup> This in turn releases IL-1, TNF $\alpha$ , IL-6 and inducible nitric oxide synthase (iNOS) from neurons, glial and parenchymal cells surrounding the ischemic core, promoting the activation of endothelium to upregulate adhesion molecules that aid in the recruitment of neutrophils and other peripheral leukocytes from the bloodstream into the brain. Of increasing importance, are the mechanisms of neutrophil recruitment and NETosis for the degree of stroke pathogenesis.

### Recruitment of neutrophils and the neurovascular network

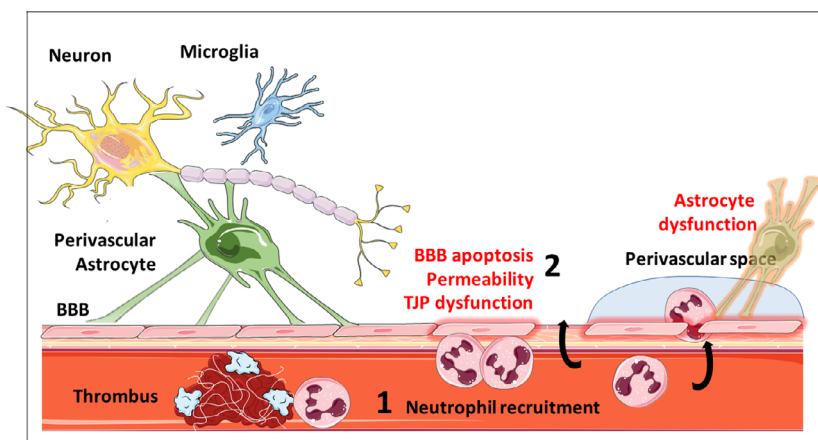
Upon recruitment from the periphery, neutrophils adhere to the cerebral endothelium surrounding the developing infarct within minutes, and peak at 2–3 days after ischemia onset.<sup>24–27</sup> Once recruited into the ischemic brain tissue, neutrophils contribute to endothelial and BBB degradation through the degranulation of primary proteases, including elastase, matrix metalloproteinase (MMP) –2 and –9.<sup>28</sup> In post-mortem brain tissue from patients with ischemic stroke, the circulating level of MMP-9 was associated with positive neutrophil infiltration into the microvessels of cerebral tissue with concomitant type IV collagen degradation, as an indicator of BBB damage.<sup>29</sup> In addition, the acute release of pro-inflammatory cytokines in the brain tissue following ischemia influences the expression and arrangement of tight junction proteins, as TNF $\alpha$  and IL-1 $\beta$  cytokine release has been shown to disrupt the expression of primary endothelial tight junction proteins including occludin and zona-occludens 1 (ZO1).<sup>30</sup> Furthermore, due to a lack of smooth muscle in the cerebral microvasculature, a type of astrocyte called perivascular astrocytes exist to regulate blood flow and to maintain the BBB integrity. Perivascular astrocytes cover the entirety of the transendothelial space in highly branched end-foot processes and respond to dynamic neuronal activation to facilitate the targeted delivery of oxygen and glucose.<sup>31</sup> During ischemia, the viability of astrocytes near the infarct decreases, impairs BBB integrity<sup>32</sup> and releases metalloproteinases, which further aid in degrading the neurovascular matrix.<sup>33,34</sup> This overall breakdown increases permeability and may provide the entry point for neutrophils into the parenchyma, as penetrance of neutrophils between tight junctions of endothelium has previously been demonstrated on porcine-derived endothelial and BBB cells *in vitro*.<sup>35</sup> Therefore, the combined focal glial cell death and ischemic endothelial damage set up the initial foundations for leukocyte recruitment into the parenchyma, and further expands the lesion core via neuroinflammation with the intimate involvement of neutrophils.

To date, it remains unclear whether neutrophils transmigrate into the brain parenchyma at the site of the ischemic infarct core after stroke, or whether they remain adherent to the cerebrovascular lumen of the adjacent penumbra tissue and influence the neuroinflammatory component from afar (Figure 1).<sup>36</sup> In a mouse model of transient (reperfused) stroke, it was found that neutrophils are more prevalent in the leptomeninges and perivascular spaces rather than in the parenchyma, and that neutrophil

transmigration was observed more markedly at 48–96 h rather than at 14 h.<sup>37</sup> This finding may be the result of the severity of the stroke induced, as a transient stroke often results in smaller infarcts compared with those as a result of a permanent (non-reperfused) stroke, where the size of infarct has been correlated previously to the clinical elevation of circulating neutrophils.<sup>38</sup> Foundational work by Enzmann *et al.*<sup>39</sup> first identified the presence of neutrophils in only the transendothelial space in mice. However, other studies continue to show conflicting results depending on the stroke model with some showing only the adherence of neutrophils to the luminal cerebrovascular vessels, but not transmigration.<sup>37</sup> Using a photothrombotic model of ischemic stroke, Neumann *et al.*<sup>40</sup> recently investigated neutrophil transmigration using a neutrophil-specific (Ly6G) tdTomato reporter mice that distinguished from eGFP<sup>+</sup> microglia and macrophages. Intravital 2-photon microscopy revealed that neutrophils were distinctly present and motile in the parenchyma 24 h after stroke, distinctly separated from the perivascular space. Moreover, neutrophils were categorized as either showing adherence to the luminal endothelium with normal sphericity, or elongated within the parenchyma consistent with neutrophil morphology under the activated state.<sup>40</sup> The discrepancy in findings regarding neutrophil transmigration after stroke is likely due to several factors including the model of stroke performed and the severity of brain damage. Moreover, it remains difficult to provide a clear picture from post-mortem human samples with various studies using inconsistent, and sometimes non-specific neutrophil markers in an attempt to identify the localization of neutrophils in post-stroke brain tissue.

### Presence of nets in stroke

Much like an immunological spider-web, neutrophil extracellular traps, or NETs, are web-like structures composed of chromatin, citrullinated histone complexes and chromosomal DNA.<sup>41</sup> Conventionally released by neutrophils to entrap extracellular microbes, NETs can also be triggered by danger signals that result from ischemic cell death, including extracellular ATP.<sup>42</sup> The NETs-releasing histone proteins are naturally cytotoxic due to their ability to disrupt cell membrane permeability<sup>43</sup> and thus the release of NETs following neutrophil activation is tightly regulated. During neutrophil activation, protein-arginine deaminase type 4 (PAD4) promotes chromatin decondensation, and disrupts the cell membrane to facilitate NETs in conjunction with granule proteins to be released via exocytosis into the extracellular space.<sup>44</sup> In the context of stroke, NETs have been detected in post-mortem brain tissue from patients with stroke



**Figure 1.** The compromised neurovascular unit during ischemia. (1) Upon lodgement of the thrombus in the cerebrovascular system, circulating neutrophils are recruited from the periphery and adhere to the cerebral endothelium adjacent to the thrombus. (2) Blood-brain barrier (BBB) degradation, tight junction (TJP)-dysfunction, increased vascular permeability and astrocyte dysfunction all allow for the possible transmigration of neutrophils into the perivascular spaces and brain parenchyma. Authors' own image, created using art obtained from Servier Medical Art licensed under a Creative Commons Attribution 3.0 Unported License.

surrounding the infarct core and in the luminal spaces.<sup>45</sup> In a rat model of experimental stroke, the level of citrullinated histone 3 (H3Cit) was associated with decreased endothelial viability, where viability was restored upon PAD4 inhibition,<sup>46</sup> suggesting the release of NETs following neutrophil activation in stroke contributes to cerebrovascular damage.

High-mobility group box 1 was also reported to be a potent activator of NET formation. In a mouse model of transient middle cerebral artery occlusion (MCAO) with anti-HMGB1 treatment, neutrophil transmigration was ablated, with a marked decrease in citrulline positive NET formation.<sup>46</sup> Furthermore, in another study, the experimental inhibition of NET formation in post-stroke mice was shown to decrease significantly the associated vascular damage and to promote angiogenesis.<sup>47</sup> The findings of these studies are suggestive of HMGB1 being a potent activator of NET formation, and that NET formation is detrimental to the cerebral tissue and neurovascular network after stroke. Whether neutrophils die upon release of NET is unclear. *In vivo* imaging revealed that at least some NET-releasing neutrophils in an infection setting retain their ability to migrate and perform phagocytosis<sup>48</sup>; however, chromatin-NET-like release is also associated with neutrophil necroptosis.<sup>49</sup> A clinical study assessing circulating neutrophil toxicity during stroke indicates that compared with healthy controls, stroke patients present a higher ratio of necrotic neutrophils to apoptotic neutrophils in circulation.<sup>50</sup> As necrotic neutrophils die without specific packaging of cellular contents, this unregulated release of intracellular ROS by dying neutrophils is a potent source of oxidative stressors.<sup>50</sup> Due to this largely non-specific release of

NETs and ROS, healthy cells are thus also subject to the cytotoxic effects of these molecules and arguably contribute to furthering neuroinflammation. Thus, a cycle of DAMP-release enhanced neutrophil recruitment, NETs release and impaired neurovascular unit contribute to expanding the infarct area and possibly worsening the damage during the acute stage of stroke.<sup>45,51</sup>

## NEUTROPHIL HETEROGENEITY IN STROKE

There is accumulating experimental and clinical evidence that suggests neutrophils exhibit functional heterogeneity, but our understanding of this notion in the context of stroke is only in its infancy. Experimental research examining the role of anti-inflammatory or N2 neutrophils in stroke is not extensive. The first substantive work of N2 neutrophils in stroke pathogenesis was reported by the seminal work of Cuartero *et al.* in 2013. The authors examined the role of rosiglitazone (RSG) on neutrophils after stroke,<sup>7</sup> an agonist well identified for its ability to confer macrophage class switching from M1 to M2.<sup>52,53</sup> The authors explored repurposing the treatment of RSG on the neutrophil population in mice that underwent permanent mid-cerebral artery occlusion (pMCAO), a non-reperfused model of ischemic stroke. The authors demonstrated that treatment with RSG significantly decreased the size of the infarct, which was concomitant with an increase in NIMPR-14<sup>+</sup> (Ly6G<sup>+</sup> and Ly6C<sup>+</sup> complex) cell numbers to the ischemic core at 24 h after stroke onset.<sup>7</sup> When neutrophils were depleted from post-stroke mice treated with RSG, the infarct size did not further decrease

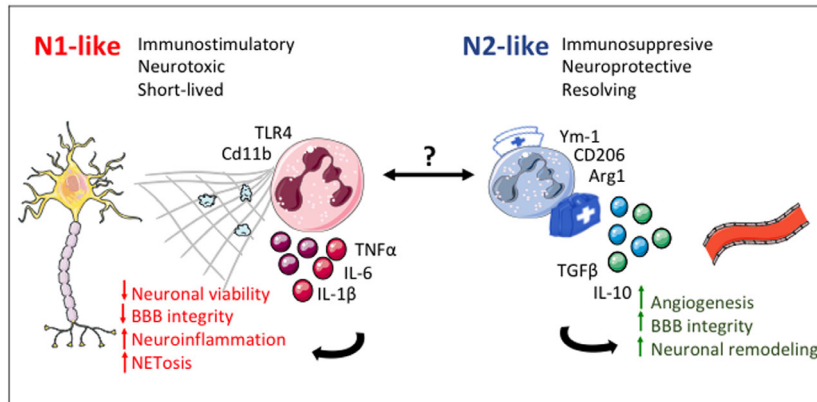
compared with neutrophil-sufficient mice, thus the authors concluded that it was neutrophils that exclusively conferred the neuroprotective role of RSG. While concluding that neutrophils were exclusively responsible for these effects, it is important to note that NIMPR14 is a marker for the Ly6G/Ly6C antibody complex, which would also include the detection of monocytes.<sup>54</sup> Thus, it cannot be concluded that the neuroprotective effects were solely contributed by neutrophils, as the antibody used would also label monocytes. Nevertheless, Cuartero *et al.* then assessed the neutrophil phenotype by measuring the surface expression of CD206 and Ym-1 (key M2 markers used classically for the monocyte/macrophage activation paradigm) via flow cytometry. The authors noted the presence of cells with co-localization of CD206 and Ly6G (neutrophil marker) positive cells via immunohistochemistry. Additionally, there was an increase in CD206<sup>+</sup>Ym-1<sup>+</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils in the brain of post-stroke mice treated with RSG via flow cytometry. Given the historical use of RSG for monocyte and macrophage switching, it would be valuable to evaluate its influence on these populations also. In any case, this study was the first to suggest the presence of N2 neutrophils in the post-stroke brain and their positive association with the reduction of infarct size after stroke.

Building on work by Cuartero *et al.*, Hou *et al.* examined the effects of both N1 and N2 neutrophils on their ability to influence recovery during reperfusion injury,<sup>55</sup> which is more closely aligned to the 13% of patients with stroke who receive thrombolytic intervention or tissue plasminogen activator (tPA) treatment.<sup>56</sup> Using 12 h IL-4 or lipopolysaccharide (LPS) stimulation, the authors differentiated primary rat neutrophils into N1 or N2 subsets confirmed by flow cytometry and gene expression. Specifically, IL-4 stimulation increased the expression of N2 markers including IL-4 and arginase 1 (Arg1), which mimicked previous research with IL-4-induced class switching in macrophages,<sup>57</sup> while LPS stimulation increased pro-inflammatory markers TNF $\alpha$  and IL-1. In a co-culture system, stimulation of neutrophils with IL-4 to the N2 phenotype provided neuroprotective effects to neurons that were temporarily deprived of oxygen and glucose (OGD) *in vitro*. In contrast, stimulation of rat-derived neutrophils with LPS toward an N1 phenotype saw decreased neuronal viability and more pronounced shortening of processes than untreated neurons exposed to OGD, thus suggesting the notion that continued N1 activation is detrimental to the survivability of neurons after stroke. These data are amongst the first of the kind to provide direct evidence for a neuroprotective role of N2 neutrophils. However, these *in vitro* experiments do not consider the *in vivo* microenvironment or consider the physical separation conferred by the BBB that prevents the

direct interaction between neutrophils and neurons. Human studies of BBB integrity after stroke indicate that permeability is evident and reversible after 1–2 h of ischemia and reperfusion, but not beyond 12 h,<sup>58</sup> which can accurately be reflected by the pathology of non-reperfused models but not in reperfusion injury. Currently, the spatiotemporal frequency of N1 and N2 neutrophils in the ischemic brain, possible mediators that modulate this factor and the associated function in experimental stroke models with or without reperfusion remains unknown. There are evidently increased efforts in the field to examine the role of neutrophils in stroke-mediated neuroinflammation, and the presence of neuroprotective N2 neutrophils is an exciting notion that may facilitate potential therapeutic targeting in the future. It is important to make clear, however, that while the labeling of N1 and N2 neutrophils as phenotypically separate populations is convenient for conceptual understanding, limited evidence at present suggests that neutrophils do not easily fall into these categories. Most likely, is the case that these cells can transiently switch between states depending on activatory signals to facilitate their role in resolving chronic inflammation and/or by promoting wound-healing (Figure 2). While the pro-inflammatory markers of N1 neutrophils including TNF $\alpha$ , IL- $\beta$  and ROS have been thoroughly elucidated in the literature, the attribution of N2 markers CD206, Arg1 and Ym-1 are often explored experimentally without sufficient justification or reasoning. Here, we briefly review the relevance and function of these markers for the designation of anti-inflammatory neutrophils.

### Arginase 1

Arginase 1 is a manganese-containing enzyme found most notably in myeloid-derived suppressor cells (MDSCs) and macrophages.<sup>59</sup> The primary catalytic function of Arg1 is to hydrolyze L-arginine into urea and L-ornithine, which are precursors to biomolecules including prolines and glutamines important for collagen matrix and neurotransmitter synthesis.<sup>60,61</sup> M2-like macrophages that aggregate during wound-healing express Arg1 constitutively, yet the enzyme is also found in neutrophils. In humans, Arg1 is constitutively expressed in circulating neutrophils in granular compartments.<sup>62</sup> In the setting of stroke, Arg1 has seemingly opposing functions depending on the stage of stroke.<sup>63</sup> During the acute stage of ischemic stroke, nitric oxide release by neutrophils and surrounding endothelia increases cerebral blood flow and decreases vascular resistance,<sup>64</sup> possibly to counter the glucose/oxygen deprivation caused by the cessation of perfusion. However, the production of NO released by dying



**Figure 2.** N1 and N2 neutrophils in ischemic stroke. Pro-inflammatory or 'N1' neutrophils are characterized by surface expression of TLR-4 and produce cytokines and inflammatory mediators including tumor necrosis factor alpha (TNF $\alpha$ ), interleukin (IL) -6 and IL-1 $\beta$ , as well as induce NETosis. These cells are purported to be neurotoxic and damaging to the BBB and neuronal populations. Conversely, 'N2' neutrophils are characterized by surface expression of Ym-1, CD206 and Arg1. These cells produce anti-inflammatory mediators including TGF $\beta$ , and IL-10, are purported to suppress neuroinflammation, promote angiogenesis, contribute to neuronal remodeling and restore BBB integrity after stroke. Authors' own image, created using art obtained from Servier Medical Art licensed under a Creative Commons Attribution 3.0 Unported License.

neurons in the parenchyma is highly inflammatory and also correlates with infarct severity.<sup>65</sup> In a small sample of patients with ischemic stroke, the serum levels of Arg1 were correlated to the level of stroke severity and neutrophil-to-lymphocyte ratio.<sup>66</sup> Interestingly, Arg1 competes with iNOS to degrade L-arginine to L-ornithine, thus the upregulation of Arg1 following stroke is associated with the decreased production of NO.<sup>67</sup> In a rat model of photothrombotic stroke, Arg1 staining was strongly expressed in the lesion between 8 and 30 days after stroke, but not at an earlier timepoint, indicating the expression of this N2/M2 marker is consistent with the resolution of neuroinflammation, although the study did not examine whether this elevated expression was specific in neutrophils.<sup>68</sup> In another study, the expression of Arg1 mRNA was significantly upregulated in Ly6G<sup>+</sup> neutrophils of post-stroke mice that were treated with the potent immunomodulator all-*trans* retinoic acid (atRA), with concomitant expression of IL-10 and CD206, and a decrease in NETosis and stroke pathology.<sup>69</sup> While the full function of Arg1 in the setting of stroke remains to be fully elucidated, evidence suggests that this marker has foundational basis for the injury resolution component of the neuroinflammatory cascade.

### CD206

CD206 is a C-type lectin carbohydrate binding mannose receptor expressed by tissue macrophages and some endothelial cells, and it functions as an anchorage point for the phagocytosis of mannoglycoproteins.<sup>70</sup> Traditionally, the receptor exists to bind pathogenic microbes with mannose-containing structures, which

makes its appearance in sterile inflammation and stroke pathology somewhat unusual.<sup>70</sup> The vast number of apoptotic and necrotic neutrophils during neuroinflammation after stroke likely contributes significantly to the circulating pool of glycoproteins not normally found during homeostatic conditions and outside the CNS. As glycoproteins are capable of widespread immune activation and host-tissue damage, it is plausible that the expression of CD206 functions to clear the initial, pro-inflammatory and uncoordinated release of glycoproteins in the brain following ischemic stroke. Indeed, early research has identified the presence of circulating CD206<sup>+</sup> neutrophils infiltrating the ischemic penumbra at 48–96 h after ischemic stroke during injury resolution, but not during the peak of neuroinflammatory processes at 24–48 after stroke onset.<sup>7</sup> In a post-mortem analysis of brain tissue from patients with ischemic stroke, it was found that CD206 expression was significantly upregulated in perivascular macrophages. Moreover, CD206 expression was significantly higher throughout the infarct core, but not in perilesional areas which were more concentrated with inflammatory markers.<sup>71</sup> The findings from these studies highlight that CD206 upregulation is concomitant during the resolution of neuroinflammation, although the full function and degree of involvement during stroke remains largely unknown.

### Ym-1

Ym-1 or chitinase 3-like protein is a secretory protein which functions to bind heparin and heparin sulfate. Originally characterized for its upregulation in

alternatively activated macrophages or M2 macrophages during instances of wound-healing, Ym-1 is also abundantly expressed in neutrophils.<sup>72</sup> The molecule contains a separate secretory signal peptide of unknown function that is phagocytosed by macrophages during inflammatory conditions, stimulating its polarization to the alternative phenotype.<sup>72</sup> In work by Cuartero *et al.* ~30% of neutrophils that had infiltrated the ischemic core in post-stroke mice were Ym-1 positive. When treated with RSG, the population of neutrophils that expressed Ym-1 was proportionally increased, suggesting a correlation of elevated Ym-1 positive neutrophils with a neuroprotective effect of RSG.<sup>7</sup> Interestingly, neutrophils isolated from the infarct core of post-stroke mice treated with another immunomodulatory molecule atRA had significant upregulation of Ym-1, which was associated with significant gene upregulation of Arg1, CD206 and IL-10.<sup>55</sup> Moreover, these neutrophils were found to be preferentially phagocytosed by macrophages in the infarct lesion. The current, yet limited, evidence suggests that while Ym-1 may be produced by neutrophils and is associated with anti-inflammatory neutrophil activation, the secretory molecule could also be released from neutrophils to mediate phenotype switching in macrophages from M1 to M2 in a paracrine manner after stroke. Indeed, infiltrating neutrophils are never alone in the cerebral microenvironment and their interactions with the resident cells in the brain need to be considered.

## NEUTROPHIL–MICROGLIA INTERACTIONS IN STROKE

The brain houses a plethora of specialized immune cells including microglia and astrocytes.<sup>73</sup> Microglia function similarly to peripheral macrophages and monocytes by clearing cellular debris through phagocytosis. Under homeostatic conditions, microglia are the primary immune surveyors of the brain and perform wide-ranging functions including monitoring the health of neurons, synaptic pruning and genesis. Microglia can produce and respond to pro-inflammatory cytokines such as IL-1, IL-2 and TNF $\alpha$ .<sup>74</sup> The dysfunction of microglia leads to a wide variety of neurodegenerative diseases including multiple sclerosis,<sup>75</sup> Parkinson's,<sup>76</sup> Huntington's<sup>77</sup> and Alzheimer's disease.<sup>78</sup> In addition, microglia also exhibit distinct interactions with neutrophils during the neuroinflammatory process after stroke.

The microglial–neutrophil relationship in stroke was largely characterized by Neumann *et al.* who observed that following ischemic damage in mice, neurons were protected from neutrophil-driven inflammation by microglial-mediated phagocytosis of neutrophils.<sup>79</sup> Recently, Neumann *et al.* used *in vivo* imaging to reveal

the direct internalization of neutrophils by microglia in the brains of post-stroke mice,<sup>40</sup> noting that neutrophils were entirely stationary during the point of contact which is consistent with the phagocytosis of neurons.<sup>80</sup> However, no assessments of the neutrophil N1/N2 phenotype were performed in the study, and the potential neutrophil characteristics that trigger microglia phagocytosis is uncertain. Nevertheless, the internalization of neutrophils by microglia in the post-stroke brain was also observed by Cuartero *et al.* but with a twist. The authors found that neutrophils positive for the Ym-1 were preferentially engulfed by microglia compared with Ym-1 negative neutrophils.<sup>7</sup> Of important note was the absence of positive staining for other N2 markers such as CD206, Arg1 and IL-10, which once again highlights the difficulty of classifying these neutrophil phenotype subsets and begs the question as to whether these cell types exist as concrete subtypes or merely exist transiently. Using a non-reperused model of experimental stroke, another study investigated whether microglial modulation or depletion of microglia influenced neutrophil recruitment to the ischemic lesion. In mice treated with anti-CSF1R, which resulted in marked impairments of microglial phagocytosis, neutrophil infiltration into the ischemic core was significantly elevated. Interestingly, the authors also noted that engulfment of neutrophils by mechanisms of phagocytosis by microglia at the perivascular spaces even prior to neutrophil transmigration into the post-stroke parenchyma.<sup>51</sup> While the relationship between microglia and neutrophils after cerebral ischemia is not complete, the current evidence suggests microglia serve as the ultimate protectors of the CNS, functioning to engulf pro-inflammatory neutrophils with an aim to restore homeostasis and retain neuronal function and survivability after stroke.

## IMPACT OF NEUTROPHILS ON NEURONAL DAMAGE IN STROKE

After stroke, neuronal death is primarily caused by oxygen and glucose deprivation which result in the development of the ischemic infarct. Neurons die through a step-wise process of ATP depletion, toxic glutamate accumulation and intracellular calcium influx leading to swelling of the organelles, and membrane disruption, all of which contribute to the recruitment of neutrophils.<sup>81</sup> The damaging impact of neutrophils on the neurovascular unit after stroke is more widely explored than the direct detriment of neutrophils on neurons and supporting glia, especially in diseases such as Alzheimer's, and TBI. Current understanding of neutrophil–neuronal interactions stem mostly from

*in vitro* experiments as there is little consistency across the animal models of ischemic stroke as to whether neutrophils do indeed transmigrate from the vasculature to reach the ischemic tissue. Indeed, the inflammatory effects of neutrophils on neurons are thought to be primarily mediated through degranulation and the release of inflammatory mediators including ROS, IL-1 $\beta$ , IL-6 and TNF $\alpha$ , as well as the release of NETs. Indeed, co-culture of rat peripheral neutrophils with primary hippocampal neurons resulted in dramatic neuronal death in the absence of any specific chemical insult, but could be averted with the use of protease inhibitors,<sup>82</sup> thus suggesting neurotoxicity of neutrophils is mediated through the release of proteases. However, a previous study showed that while neutrophils are not inherently toxic to neurons, their transendothelial migration across an IL-1-stimulated brain endothelium triggers neutrophils to acquire a neurotoxic phenotype that causes neuronal cell death.<sup>82</sup> Specifically, neutrophil migration was significantly reduced in IL-1-deficient mice compared with wildtype mice in an experimental model of stroke. Furthermore, it was found that transmigrated neutrophils significantly decreased the viability of cultured neurons, whereas the co-culture of non-migratory neutrophils with non-activated endothelium did not affect neuronal viability, indicating that only neutrophils that transmigrate across stimulated endothelium in an IL-1-dependent manner result in a neurotoxic phenotype and death of neurons.

In an *in vivo* mouse model of induced ocular nerve crush (OCN), Sas *et al.* used antiserum specific for CXCR2 to prevent the entry of mature neutrophils into the vitreous humor (VH) following zymosan mitigated OCN, resulting in the unexpected yet enhanced survival of retinal-ganglionic-cells (RGC) and axonal regeneration. The authors noted that neutrophil accumulation to the VH was delayed but not ablated, with concomitant accumulation of CD11b<sup>+</sup>Ly6G<sup>lo</sup>CD45<sup>hi</sup> immature neutrophils. The authors noted that both Ly6G<sup>lo</sup> and Ly6G<sup>hi</sup> populations had comparable levels of elastase and myeloperoxidase; however, Ly6G<sup>lo</sup> cells bore transcripts for *Arg1* and *Tgfb1*, which were further expanded with aCXCR2 treatment.<sup>83</sup> These results indicate that Ly6G<sup>lo</sup> immature neutrophils may have a supportive role in neuronal and axonal regeneration.

At sites of neurodegeneration after ischemic stroke in mice, neutrophils along with monocytes were found to be increased in the ipsilateral thalamic region, with a significant reduction in NeuN<sup>+</sup> neuronal cells.<sup>84</sup> To specifically observe physical interactions between neutrophils and neurons, Mai *et al.*, combined real-time microscopy to polymorphonuclear leukocyte (PMN)-cortical neuronal co-culture. In unstimulated conditions,

neutrophils made physical contact with neuronal cell body and dendrites resulting in disruptions to neurite outbranching and fragmentation. When undergoing OGD, the neurite length significantly decreased, indicating that neutrophils were detrimental to neuronal morphology and survivability under normo- or hypoxic conditions.<sup>85</sup> Although no assessments of neutrophil phenotype were made in these earlier *in vitro* studies, these results indicate that neutrophils may have an inherent toxicity toward neurons, which may be amplified during cerebral ischemia. Despite this, the direct neutrophil-neuronal interactions may only be possible after infiltrating neutrophils that have transmigrated through the activated endothelium and gained access to the brain parenchyma in *in vivo* settings.

## NEUTROPHILS AS TARGETS FOR STROKE THERAPY

With the traditionally described damaging roles of neutrophils in the neuroinflammatory cascade, many studies have revolved around either blocking or depleting neutrophils to prevent their entry into the brain and CNS in a number of diseases. Indeed, the restoration of functional tissue after cerebral ischemia has been the primary goal in the development of stroke therapy. However, there has been an undesirable, yet common, theme of promising experimental interventions that limit infarct volume and improve stroke outcome in animal models but have fallen short of translating to expected clinical outcomes.<sup>36</sup> Indeed, foundational experimental work prior to the Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN) phase II clinical trial of 966 patients in 2003, which involved the usage of a recombinant neutrophil inhibitory factor (CD11b/CD18 receptor block) demonstrated almost complete amelioration of infarct volume in rats following experimental stroke. However, this promising result was not seen clinically, with the trial being terminated early due to no observable changes after 90 days post-stroke.<sup>86</sup> A separate phase III trial using monoclonal antibody therapy (Hu23F2G HALT stroke trial) against humanized CD11b/CD18 receptor IV to block neutrophil adhesion after the initial cerebral ischemia was terminated with undisclosed results.<sup>87</sup> Similarly, a phase II trial testing the use of murine anti-ICAM-1 (Enlimomab) mAb found that stroke outcome in patients treated with Enolimomab was worsened compared with placebo at 90 days after treatment, with a subsequent rise in infection and fever.<sup>88</sup> These bench-to-bedside translation failures have largely prompted our re-examination of the role of infiltrating immune cells in post-stroke neuroinflammation. It is a pertinent reminder that experimental models of stroke

often do not integrate the common comorbidities of stroke, which includes obesity, cardiovascular disease and diabetes, all of which have several important influences on the immune system. The evidence of neutrophils displaying a variety of diverse roles in experimental stroke models may be due to the inherent nature of the model of stroke, or the fact that these comorbidities influence certain aspects of the host immune system, and thus ultimately affect the development of post-ischemic brain injury. Additionally, whether neutrophils fully transmigrate into the ischemic penumbra and the impact of their interactions with microglia are likely determining factors in whether they are providing a neuroprotective (N2) or neurodestructive (N1) role in stroke pathology.

## CONCLUSIONS

Emerging evidence in recent years suggests that neutrophils have complex roles to play besides simply engulfing bacteria and promote local acute inflammation. In the settings of stroke, whether N2 neutrophils bearing markers akin to M2 macrophages are of their own distinct phenotype with true anti-inflammatory functions remains to be further elucidated. Research examining markers associated with neutrophil polarity and recognizing their diverse phenotypes is indeed highly relevant. However, without a joint examination of function, the significance of these cell types cannot be fully appreciated. Indeed, whether neutrophil polarity is directly influenced by the transient neuroinflammatory environment after stroke or is pre-determined after their egress from the bone marrow has yet to be determined. To better our understanding of neutrophils in stroke, a number of factors will need to be considered in future studies; choosing accurate markers that identify distinct cellular populations, use of clinically relevant models of experimental stroke, and the utilization of appropriate animal strains and species. Experimental stroke models are generally well-designed to recapitulate the clinical host response, yet it is becoming increasingly clear that revealing underlying mechanisms with experiments involving the non-specific or global knock-down, inactivation or depletion of immune cells are unlikely to be the cure-all for limiting the neuroinflammatory cascade to save functional tissue after ischemic stroke. The future of stroke treatments will potentially entail a combination of therapeutics, which may involve skewing neutrophils toward the N2 phenotype in order to minimize the pro-inflammatory component of stroke and improve functional and neurological outcome. Current stroke treatments only address removing the blockage and restoring blood flow, but there are currently no treatments for managing the loss of functional brain

tissue. If these proposed anti-inflammatory and wound-healing functions of neutrophils could be validated in a clinical stroke setting, this could form the foundation for a therapeutic basis for many patients with stroke who currently are without treatment.

## ACKNOWLEDGMENTS

BJW is supported by the Research Training Programme Scholarship by the Australian Government. CHYW is supported by the CSL Centenary Fellowship. The financial support for the authors had no role in the preparation of this work.

## AUTHOR CONTRIBUTIONS

**Brooke Wanrooy:** Conceptualization; Investigation; Writing-original draft; Writing-review & editing. **Shu Wen Wen:** Supervision; Writing-review & editing. **Connie Wong:** Conceptualization; Funding acquisition; Supervision; Writing-original draft; Writing-review & editing.

## CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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