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The age-dependent immune response to ischemic stroke

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Abstract

Stroke is a devastating cause of global morbidity and mortality. Ischemic brain injury triggers a profound local and systemic immune response that participates in stroke pathophysiology. In turn, this immune response has emerged as a potential therapeutic target. In order to maximize its therapeutic potential, it is critical to understand how the immune response to ischemic brain injury is affected by age - the strongest non-modifiable risk factor for stroke. The development of multi-omics and single cell technologies has provided a more comprehensive characterization of transcriptional and cellular changes that occur during aging. In this review, we summarize recent advances in our understanding of how age-related immune alterations shape differential stroke outcomes in older versus younger organisms, highlighting studies in both experimental mouse models and patient cohorts. Wherever possible, we emphasize outstanding questions that present important avenues for future investigation with therapeutic value for the aging population.

INTRODUCTION

Stroke is currently the second leading cause of death and third leading cause of disability worldwide [1]. The incidence of ischemic stroke, and its associated mortality and morbidity, increase markedly with age [2–5]. This trend persists despite similar rates in recanalization and hemorrhagic transformation among young and old patients that undergo intravenous thrombolysis and mechanical thrombectomy [6, 7]. Recent insights from experimental

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COMPETING INTERESTS

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mouse models of ischemic stroke have shown that the immune response to brain ischemia actively participates in stroke pathophysiology and impacts outcomes [8–10]. How aging alters the immune response to ischemic brain injury and how this response impacts functional outcomes remains incompletely understood. Furthermore, the extent to which age-related changes in the immune response after ischemic stroke are cell-intrinsic, versus patterned by extrinsic signals derived from the brain and the peripheral microenvironment, remains unclear. Elucidating how neuroimmune interactions evolve with age is critical to effectively harness the immune system as a therapeutic tool in order to reduce injury propagation, enhance tissue repair, and maximize longitudinal functional recovery after ischemic brain injury. Here, we provide a brief overview of recent developments in our understanding of the age-related immunological response to stroke and highlight some open questions for future investigation.

Peripheral factors and resident CNS cells contribute to the proinflammatory signature of the aged brain

Aging is associated with a series of changes in the innate and adaptive immune system that manifest as a decline in immune function - termed “immunosenescence” - and an accumulation of inflammatory factors - termed “inflammaging” [11, 12]. Although our understanding of how immunosenescence and inflammaging impact brain physiology is in its infancy, aging is characterized by a progressive increase in neuroinflammation [13] (Figure 1). Single-cell transcriptomic studies of the central nervous system (CNS) suggest that this signature is largely driven by an expansion of pro-inflammatory subpopulations of both activated microglia and reactive astrocytes (Figure 1C, 1D) [13–17]. However, this signature is not cell-intrinsic. Metabolic restoration of peripheral myeloid cells in aged mice is sufficient to reduce age-related CNS inflammation and memory deficits [18]. Moreover, infusion of young blood and cerebrospinal fluid into aged animals reduces the pro-inflammatory signature of the aging brain and can restore synaptic function [19–21]. Together, these studies demonstrate that peripheral extrinsic factors instruct CNS resident cells to drive maladaptive age-related brain inflammation. Since endothelial cells of the cerebral vasculature and epithelial cells of the choroid plexus form the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) respectively, they have emerged as critical components that can either impede or propagate peripheral inflammatory cues [22–24]. In particular, age-related endothelial cell inflammation (Figure 1A) can provoke vascular dysfunction and contribute to the pathogenesis of cerebrovascular disease, such as ischemic stroke.

Transcriptomic characterization of age-related immune responses in ischemic stroke

Ischemic stroke triggers upregulation of selectins on brain endothelial cells [BECs; e.g. Vascular Cell Adhesion Molecule (VCAM)], which promote leukocyte adhesion (Figure 2; red cells) [8]. Ischemia-related BBB disruption allows danger associated molecular patterns (DAMPs), released from necrotic neurons, to enter the bloodstream and cerebrospinal fluid [25, 26]. DAMPs can further recruit immune cells directly through binding to pattern recognition receptors in the periphery, or indirectly through local activation and subsequent secretion of cytokines from CNS resident cells (Figure 2). Following this initial phase, there is T cell apoptosis and immunosuppression which predisposes patients to systemic infection

[27, 28]. Thus, ischemic brain injury initiates profound changes in both local and systemic immune function.

Recent transcriptomic studies in animal models have provided quantitative characterization of cell types and immune signaling pathways that are differentially affected by aging after ischemic stroke (Figure 2). A recent study using single cell RNA sequencing characterized the immune landscape in the aged mouse brain 72 hours after transient middle cerebral artery occlusion (t-MCAO), a mouse model for ischemic stroke [29]. Similar to findings reported in young animals after stroke, the frequency of aged microglia expressing homeostatic genes decreases after stroke and the aged microglia shift toward a highly proliferative state [29, 30]. However, aged microglia (Figure 2, light blue cells) are less responsive to ischemic injury, showing only 18 upregulated and 40 downregulated genes, as compared to young microglia which upregulated 250 and downregulated 21 genes after ischemic stroke [31, 32]. This lack of transcriptional plasticity may be a consequence of downregulation of purinergic receptors on aged microglia which normally respond to purines released after neuronal injury in ischemic stroke [33]. Yet, the functional implications of a dampened microglia response to injury are challenging to interpret given that resting aged microglia exhibit a heightened proinflammatory transcriptional signature compared to their young counterparts, thus resembling more “activated” cells (Figure 1C) [14, 31]. Do age-related inflammatory changes “prime” microglia prior to ischemic insult, or do aged microglia exhibit a senescence-associated phenotype that disrupts restoration of tissue homeostasis after ischemic injury? Single cell RNA sequencing studies have revealed that genes related not only to inflammatory processes, but also cell-cell communication, angiogenesis, and extracellular matrix (ECM) regulation are significantly upregulated in young compared to aged microglia after ischemic stroke [31]. Changes in microglia-mediated ECM remodeling may provide mechanistic insight into why ischemic injury preferentially pushes the aged brain towards fibrotic rather than regenerative processes (Figure 2) [34]. Studies in young mice have further demonstrated that upregulation of genes related to microglial phagocytosis and lipid metabolism modulate the clearance of lipid-rich tissue debris after ischemic stroke which is critical for functional recovery [35–37]. A recent study identified the presence of lipid droplet-accumulating microglia (LDAM) in the resting aged brain, which are defective in phagocytosis and secrete higher levels of proinflammatory cytokines and reactive oxygen species (ROS; Figure 1C) [16]. Future pharmacological and genetic ablation experiments will be useful to determine how LDAMs contribute to the blunted microglial response observed in aged animals after ischemic brain injury.

A striking hallmark of the transcriptional response to ischemic stroke in aged animals is the upregulation of type I interferon signaling (IFN1) in microglia (Figure 2, light blue cells) and oligodendrocytes [38]. This IFN1 response differs from that of young animals in both its magnitude and temporal profile, with persistent elevation in aged animals 14 days after stroke. Although the functional implications of this response remain to be determined, these findings align with previous data which identified chronically elevated IFN1 signaling in the aged brain (Figure 1C) [22, 39]. Importantly, blocking IFN1 signaling in aged mice (either pharmacologically or through ablation of the IFN1 receptor on microglia) attenuates maladaptive neuroinflammation and cognitive decline associated with normal aging [22, 39]. Moreover, the persistent elevation of IFNI signaling in the aged brain after ischemic insult

suggests that the therapeutic window for immunomodulation after ischemic stroke may be age-dependent. Elongation of the therapeutic window in older patients has massive clinical implications; yet the majority of rodent studies focus on the first 72 hours after stroke. It will be crucial for future studies to precisely map how acute age-related alterations in immune function evolve after ischemic stroke and how this trajectory impacts longitudinal restoration of brain homeostasis. Longitudinal studies in aged animals are of particular relevance given that in the first year after ischemic stroke elderly patients show an initial improvement in cognition that is then followed by a significant decline in cognitive function [40]. Critically, older age and recurrent stroke (rather than stroke severity or other vascular risk factors) are associated with an increased risk and accelerated presentation of cognitive decline [40].

Age-related immune alterations contribute to differential stroke outcomes

It is challenging to bridge the mechanistic gap between RNA sequencing data and ischemic stroke outcomes. Furthermore, cells (particularly of the innate immune system) are exceedingly sensitive to *in vitro* artifacts induced during tissue dissociation and cell isolation [41]. This *ex vivo* gene expression signature can distort the interpretation of functional responses and obscure age-related changes. Therefore, following identification of age-related transcriptional alterations after ischemic stroke, it is necessary to validate them *in vivo* and obtain mechanistic insight to establish their role in disease pathogenesis. To what degree does the immune alteration reflect a cell-intrinsic change due to aging versus a consequence of extrinsic factors in the aging microenvironment? Does the immune alteration give rise to differential functional outcomes after ischemic stroke? Elucidating these questions is critical for identifying points of therapeutic intervention. Recent studies using experimental animal models have begun to examine these questions and demonstrate that age-related immune alterations after ischemic stroke 1) represent changes in both cell-intrinsic and extrinsic properties and 2) have a causal role in functional outcomes.

The peripheral environment modulates age-related immune response to ischemic stroke

Transplantation of young bone marrow into aged mice reduces the number of brain-infiltrating neutrophils, occurrence of hemorrhagic transformation and severity of behavioral deficits after t-MCAO [32]. Conversely, transplantation of old bone marrow into young mice increases the number of brain-infiltrating neutrophils, reduces microglia phagocytosis and exacerbates behavioral deficits after t-MCAO. A similar rejuvenating effect can be recapitulated by transfusion of young blood and splenectomy [42, 43]. Replacement of aged blood 7 hours after t- MCAO with whole blood obtained from young mice results in a significant reduction in neurological deficits, peripheral neutrophilia, brain infiltrating neutrophils, and a decrease in matrix metalloproteinase-9 (MMP-9) levels [43]. The rescue effect of young blood is diminished upon the addition of MMP-9 which is highly expressed by brain-infiltrating neutrophils in aged mice (Figure 2) and has been associated with an increased risk of morbidity and mortality in ischemic stroke patients [29, 32, 43, 44]. Transfusion of young blood further: a) reduces circulating DAMPs released by the ischemic brain; b) removes activated aged leukocytes and other deleterious signals such as cytokines; and c) introduces young soluble factors that bathe barrier compartments – all of which may contribute to its overall neuroprotective effect.

Gut microbiome abnormalities and autonomic nervous system (ANS) dysfunction contribute to age-related systemic inflammation and stroke outcomes [45–48]. Importantly, the transfer of young doner microbiota to aged mice decreases systemic inflammation, morbidity, and mortality after t-MCAO [49]. Peripheral exosomes have also emerged as novel regulators of the age-related immune response to ischemic stroke. A recent study found that levels of serum exosomal complement components (C1q, C3a, and C3b) are increased with age and can cross the BBB, accumulate in the penumbra, and drive worse functional outcomes after ischemic stroke [50]. Complement present in aged exosomes exacerbates microglial activation and triggers excessive synapse phagocytosis (Figure 2). Strikingly, the delivery of young exosomes into aged rats after ischemic stroke significantly reduces infarct volume size, cognitive deficits, and sensorimotor deficits. Interestingly, C3a has further been implicated as a mediator of age-related vascular inflammation, lymphocyte infiltration, and BBB permeability through C3a receptor signaling on brain endothelial cells (Figure 1A) [51].

Together, these studies demonstrate that rejuvenation of the systemic environment can rescue maladaptive features of both chronic age-related neuroinflammation (discussed under “Peripheral factors and resident CNS cells contribute to the proinflammatory signature of the aged brain”) and the acute age-related immune response to ischemic brain injury. These findings have important therapeutic implications. Although the Plasma for Alzheimer Symptom Amelioration (PLASMA) Study has demonstrated the tolerability and safety of repetitive plasma transfusions, a clinical trial examining the therapeutic benefit of young plasma transfusion after ischemic stroke has not been performed [52]. Furthermore, it remains to be determined how age-related changes in other border compartments such as the meninges and cerebrospinal fluid prime the inflammatory response to ischemic injury in the aged brain [37].

The number and function of brain infiltrating immune cells impacts age-related stroke outcomes

The cellular immune response to ischemic stroke changes significantly with aging. Whereas monocytes make up a significantly larger proportion of immune cells migrating into the young brain after ischemic stroke, neutrophils (Figure 2, pink cells), known to produce high levels of ROS and MMPs, dominate the cellular response to ischemic stroke in the aged brain [29, 32]. Neutrophil depletion through anti-Ly6G treatment significantly improves long-term functional outcomes after ischemic stroke in aged, but not young, mice [53]. These findings suggest a unique age-dependent pathogenicity of the neutrophil response to ischemic stroke which may be caused by increased levels of the neutrophil activating cytokine, IL-6, or decreased levels of the bone marrow retention chemokine, CXCL12 [53]. Neutrophil accumulation may be amplified by age-associated microglia dysfunction, as microglial phagocytic activity in young mice controls the expansion of neutrophils beyond the infarct core [54]. Moreover, increased production of MMP-9 (Figure 2, pink pentagons) by aged neutrophils exacerbates BBB damage and may be responsible for worse age-related functional outcomes [32, 55]. However, brain infiltrating pro-inflammatory monocytes also play a crucial role in post-ischemic angiogenesis and tissue remodeling [56, 57]. Therefore, the skewed neutrophil to monocyte ratio in aged animals may contribute to failed

functional recovery through promotion of pro-fibrotic processes (Figure 2). Understanding the mechanisms regulating fibrotic scar tissue formation after CNS ischemic injury and its impact on functional recovery will be an important point of future investigation in the aged brain [58–60].

Consistent with findings in young mice, microglia depletion prior to t-MCAO results in an increased number of brain-infiltrating immune cells and larger infarction size in aged mice [61]. Therefore, despite their unique age-dependent transcriptional profile, microglia beneficially contribute to ischemic stroke outcomes in both young and aged animals. A recent study using conditional phagocytic receptor knockout mice reveals that while microglia phagocytosis mediates the beneficial removal of debris after ischemic stroke, it also results in the detrimental removal of synapses [62]. These findings emphasize the need for future studies to use genetic strategies to assess the contribution of specific signaling pathways in aged microglia after ischemic brain injury.

Resident lymphocytes have emerged as another immune population that modulates functional outcomes after ischemic stroke [63, 64]. Aging is associated with an increased number of CNS resident memory $CD8^+$ T cells ($CD44^+$) and dural antigen experienced B cells (Figure 1B) [63, 65, 66]. This expanded population of $CD8^+$ T cells (Figure 2, orange cells) amplifies ischemic brain injury through IL-15 mediated production of pro-inflammatory cytokines (IFNγ, TNFα, and IL-17) which increase peripheral immune cell recruitment [63, 65]. Both depletion of $CD8^+$ T cells and ablation of IL-15 in astrocytes (Figure 2, green cells) attenuates ischemic brain damage in aged mice and improves functional outcomes [67]. However, it is unclear how an accumulation of $CD8^+$ T cells and B cells in the aged brain may longitudinally promote or suppress auto-reactive responses to self-antigens following ischemic tissue injury. Moreover, it remains to be determined whether the persistent accumulation of $CD8^+$ T cells in the aged brain after ischemic injury represents an increase in recruitment, or local proliferation of an already expanded resident population. In contrast, recruitment of regulatory T cells to sites of tissue damage is severely dampened with age [68, 69]. This age-related decrease in recruitment is of particular interest given that regulatory T cells in young mice promote functional recovery after ischemic stroke by enhancing microglia-mediated white matter repair and suppressing neurotoxic astrogliosis [70, 71].

Overall, these studies demonstrate that age-related immune alterations contribute to differential experimental stroke outcomes that can be modulated through targeting of distinct immune components. It will be important for future studies to further investigate the potential immunomodulatory functions of non-classical immune cells. For example, emerging evidence supports the capacity of endothelial cells to facilitate immune homeostasis beyond regulation of immune cell recruitment [72]. Age-associated changes in brain endothelial cell inflammatory signaling suggest a potential age-specific immunomodulatory role of the neurovasculature that may be of therapeutic importance for ischemic stroke risk and functional outcomes [23, 24].

Identifying functionally-relevant age-dependent therapeutic targets in stroke patients

Animal models have been indispensable for elucidating how the immune response to brain ischemia has a causal role in ischemic stroke outcomes. However, many immunomodulatory drugs found to be effective in experimental stroke have failed in clinical trials [8]. The predominance of young animals in preclinical studies has likely contributed to the lack of clinical translation for a disease that occurs largely in an aged population. Furthermore, there are significant drawbacks of studying neuroimmune interactions in mice that have an underdeveloped immune system due to clean pathogen-free living conditions in the laboratory [73, 74]. Lastly, poor functional outcomes in older patients with ischemic stroke can be partially explained by age-related comorbidities such as hyperlipidemia, diabetes mellitus, and hypertension [75–77]. These comorbidities have not been widely incorporated in preclinical ischemic stroke models; thus, it is critical that experimental findings are validated in patient cohorts [78, 79]

Recent cohort studies have characterized the acute and longitudinal peripheral immune response to ischemic stroke. The magnitude of the innate immune response in the acute period is associated with poorer cognitive outcomes, while an increase in peripheral regulatory T cells is associated with improved functional outcomes and smaller infarction size [80, 81]. Older age is associated with an increase in peripheral IL-6, soluble tumor necrosis factor receptor I, and neutrophil-lymphocyte ratio in patients with both ST-segment-elevation myocardial infarction and acute ischemic stroke [82]. This data suggests a potentially unified systemic immune response to ischemic tissue injury that is age-dependent. Transcriptomic data further supports age-related changes in lymphocyte function after ischemic stroke and suggests that B cells may play a unique role in age-related stroke outcomes [83, 84].

It is necessary to distinguish between chronological (time passed since birth) and biological (level of physiological functioning) aging [85]. While the chronological age of women at stroke onset is older than men, DNA methylation profiles at time of stroke onset do not differ [86]. Understanding how sex-specific epigenetic aging impacts downstream physiology, such as the immune response to ischemic brain injury, will be of significant therapeutic value. It will also be important to investigate how epigenetic changes mediated by psychosocial factors modify the process of aging itself and the risk for age-related disease [87]. Recent studies highlight the complexity of “aging” which differs across individuals and within different cells of a single individual [88]. They emphasize the need for future studies to incorporate multidimensional and longitudinal immune profiling after ischemic stroke to validate age-dependent signatures. Incorporating this depth and temporal dimension with stroke outcomes in individuals of different ages with different genotypes, comorbidities, and environmental exposures will best inform risk prediction and drug discovery.

CONCLUDING REMARKS

Considerable progress has been made in identifying how aging alters the immune response to ischemic stroke. An improved understanding of how age-related immune alterations causally influence stroke risk and functional outcomes will have a substantial clinical impact. Recent multi-omics profiling studies demonstrate the complexity by which the

immune system remodels during aging. Leveraging the data derived from patient cohorts as testable hypotheses in experimental animal models will elucidate to what degree age-related immune alterations represent correlative or causative regulators of stroke pathophysiology. This future work is needed to establish a robust framework for the development of effective immunotherapeutic strategies in stroke.

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HIGHLIGHTS

- Omics approaches have expanded our understanding of immune aging and the immune response to ischemic stroke
- Age-related intrinsic and extrinsic changes shape the immune response to ischemic stroke
- The immune response to ischemic stroke contributes to differential outcomes in young and old hosts
- Targeting age-dependent immune alterations after ischemic stroke has therapeutic potential

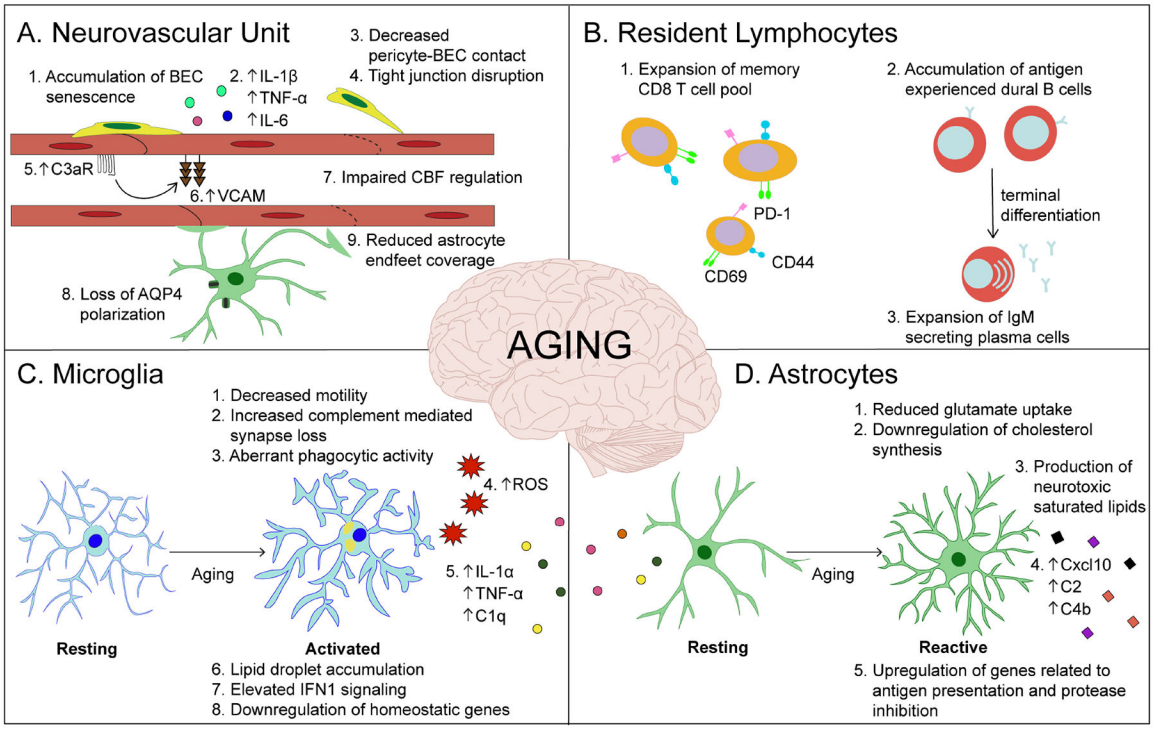

Figure 1. Age-related changes in neuro-immune crosstalk.

The aging brain shows increased levels of inflammation. **A)** Aging results in vascular inflammation (red), which is characterized by increased expression of cell adhesion molecules (e.g. VCAM-1), cytokine production, blood-brain barrier permeability and dysregulation of vascular tone - all of which contribute to age-related atherosclerosis, hypertension, and stroke. **B)** There is a prominent increase in activated memory $CD8^+$ T cells (orange) in the aged brain. Aged dura shows a distinct population of antigen experienced B cells (red) with an expansion of IgM plasma cells. **C)** Aged microglia (light blue) upregulate expression of proinflammatory cytokines and type one interferon (IFN1) signaling, downregulate homeostatic genes and accumulate lipid droplets. Functionally, aged microglia exhibit a reduced phagocytic capacity and motility. Microglia-derived C3 complement component mediates age-dependent synapse loss in neurons. Activated microglia further produce IL-1α, TNFα, and C1q, which are necessary and sufficient to induce a neuroinflammatory reactive astrocyte phenotype (green) exacerbated in the aged brain. **D)** Reactive astrocytes (green) in the aged brain significantly upregulate C3, C4b, Cxcl10, GFAP, Serpina3n and major histocompatibility complex (MHC) class I genes. The production of complement and saturated lipids by reactive astrocytes, as well as a downregulation of cholesterol synthesis, favor synapse elimination in the aged brain.

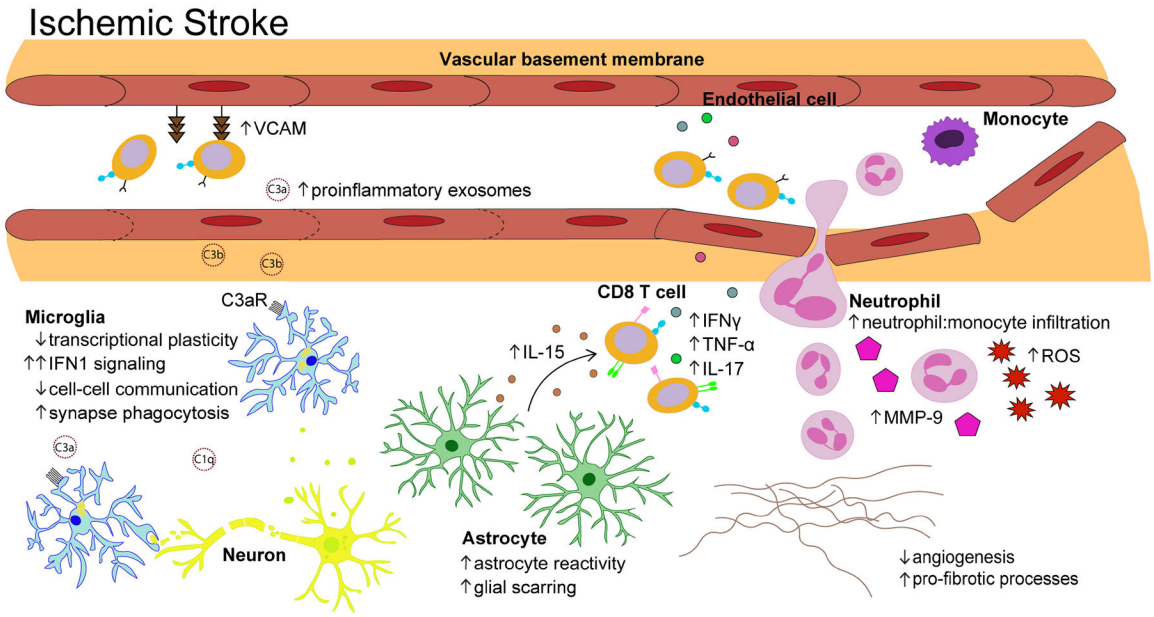

Figure 2. The immune response to ischemic stroke in the aged brain.

Following ischemic stroke, aged microglia (light blue) demonstrate a blunted transcriptional response dominated by genes associated with IFN1 signaling. The complement components that infiltrate the brain from serum exosomes bind to C3a receptors on aged microglia and trigger excessive synapse phagocytosis. Astrocyte reactivity (green) is exacerbated after ischemic stroke, leading to accelerated glial scar formation. Ischemic injury triggers aged astrocytes to significantly upregulate IL-15 which promotes further accumulation of $CD8^+$ T cells (orange) that recruit other peripheral immune cells through secretion of proinflammatory cytokines. The ratio of neutrophils (pink) to monocytes (purple) is significantly increased in the aged brain after ischemic stroke and infiltrating neutrophils are highly metabolically active, secreting large amounts of matrix metalloproteinase (MMP)-9 and reactive oxygen species (ROS). Genes related to angiogenesis and extracellular matrix (ECM) remodeling (e.g. Col3a1, Col6a1, Pdgfrb, Lox, Angptl4, Ecm, Mmp12, Eln) are significantly downregulated in the aged brain relative to that observed in young animals, indicative of reduced vascular remodeling.