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Research Article

Significance of Microglial Efferocytosis during Unraveling of Stroke Pathogenesis Unveiling its Masked Potential for Improving Clinical Outcomes in Stroke Disease Models

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Abstract

According to American Heart Association, Stroke is the 5th leading cause of death in the United States. Comparatively, neuronal cells are more liable to subtle variances in blood supply. The neuronal cell death that ensues secondary to ischemia related cellular stresses eventually culminates into apoptosis. Piled up apoptotic neuronal debris should be weeded out swiftly otherwise sooner or later they transform to undergo necrotic cell death. Sheltering necrotic cells in the neuronal milieu is an encumbrance as they spurt danger signals that can act upon TLR4 receptors to instigate inflammation, thus magnifying neuronal damage and tissue loss. Piled up apoptotic neurons are purged by migrating microglial cells by canonical efferocytosis through participation of machinery including find-me signals, eat-me signals, bridging molecules, and binding receptors. Nevertheless, neurons exposed sub-toxic concentrations of LPS are prone to be cleared by non-canonical efferocytosis. Studies reveal that, microglial efferocytosis in the ischemic brain is knackered due to decreased efferocytosis machinery, oxidative stress, metabolic dysregulation, astrocytic interference, and Eph-A mediated effects. On top of that, hegemony of non-canonical efferocytosis also contributes to supernumerary neuronal loss thereby amplifying neuronal loss in stroke. Therefore, research efforts need to be expended to rectify these distortions to ensure that canonical efferocytosis is perpetual while curbing non-canonical efferocytosis in the ischemic zones. Pertinent research areas include arginine metabolism, calcium signaling, polyamines, microglial migration, and defective efferocytosis. This will encourage prompt neural regeneration, healing that will ultimately transform into better neurological recovery and optimal clinical outcomes in stroke.

Keywords: Microglia; Efferocytosis; Neuronal death; Stroke; Amyloid beta; Necrosis; Apoptosis; Phagocytosis; Canonical; Non-canonical

Abbreviations

LDL: Low Density Lipoprotein; HMGB1: High Mobility Group Box1; MSU: Mono Sodium Urate; S100B: Alarmin; TLR4: Toll-Like Receptor-4; TLR2: Toll-Like Receptor-2; NALP3 NLR Family Pyrin Domain Containing 3; MHC Class II Complex: Major Histocompatibility Class II Complex; MCAO; Middle Cerebral Artery Occlusion; MMP-9: Matrix Metalloproteinase -9; ATP: Adenosine Triphosphate; S1P: Sphingosine-1-Phosphate; LPC: Lyso Phosphatidyl Choline; CX3C: Motif Chemokine Ligand 1; CX3CL1 Fractalkine; G2A: G-Protein Receptor 132; P2Y2: Purinergic Receptor; CX3CR: C-X3-C Chemokine Receptor; C1q: Complement 1q; C3b: Complement 3b; TIM (1, 3 & 4) Family: T-Cell Immunoglobulin Mucin Domain-1&4; TAM Family: Tyrso3 Axl & Mer); RAGE: Receptors For Advanced Glycation End Products; MFG-E8: Milk Fat Globule-EGF Factor 8 Protein; CCN1: Cellular Communication Network 1; GAS6: Growth Arrest Specific 6; DOCK180: CED-5/180 kDa Protein Downstream of Chicken Tumor Virus No. 10; ELMO: CED-12/Engulfment and Migration; RAC: Rac Family Small GTPase 1; LC3: Light Chain 3; SIGLEC-11: Sialic Acid Binding Ig Like Lectin 11; MERTK: MER Proto-Oncogene; CRT: Calreticulin; STAT-6: Signal Transducer and Activator of Transcription 6; PPARγ: Peroxisome Proliferator-Activated Receptors; ER: Endoplasmic Reticulum; TREM2: Triggering Receptor Expressed On Myeloid Cells 2; RXR: Retinoid X Receptor; NHE1: Na+/H+ Exchanger; SPP1: Secreted Phosphoprotein 1; ROS: Reactive Oxygen Species; ADAM17: A Disintegrin and Metalloproteinase17; cGAS: Cyclic GMP-AMP Synthase; STING: Stimulator for Interferon Genes; αvβ3: Integrin; EphA: Erythropoietin-Producing Hepatocellular Carcinoma Receptors Type A; ERK: Extracellular Signal Regulated Kinase; ATP: Adenosine Tri Phosphosphate; UTP: Uridine Tri Phosphate; Lyso-PC: Lyso-phosphatidylcholine; SIP: Sphingosine-1-phosphate; P2Y12: G-Inhibitory-Protein Receptor; EPO: Erythropoetin; NFAT: Nuclear Factor Activated T-cells; HIF1α: Hypoxia Inducing Factor-Alpha; ROCK1: Rho-Associated Coiled-Coil-Containing Protein Kinase 1; MLC: Myosin Light Kinase Phosphorylation; GPCR: G-Protein Coupled Receptors; CD47: High Affinity Receptor for Thrombospondin-1 (TSP-1); SIRP-α: Signal Regulatory Protein Alpha; Siglec: Sialic Acid Binding Ig-like Lectin; LXRα: Liver-X-Receptors Alpha; LXRβ: Liver-X-Receptors Beta; Dbl: GTP Exchange Factor; PLC: Phospho Lipase; IP3: Inositol Phosphate 3; IP3R: Inositol Phosphate 3 Receptor; PS: Phosphatidylm Choline; CRAI-1: Calcium Release Activated Calcium Channel Protein; STIM1: Stromal interaction molecule-1; SOCE: Store Operate Calcium Channel; Drp1: Dynamin Related Protein 1; MCU: Mitochondrial Calcium Uniporter; MLCK: Myosin Light Chain Kinase; PI3 kinase: IL- β: Interleukin Beta; COPD: Chronic Obstructive Pulmonary Disease

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Introduction

In the year 2020, the total number of deaths attributed to the stroke is estimated to be roughly 7.08 million. According to the American Heart Association, Stroke is the 5th leading cause of death accounting for at least 160, 264 deaths alone in the year 2020 in the United States [1]. Its incidence is regretfully high with approximately 795,000 people experiencing a new or recurrent stroke episode every year coupled with its prevalence around 3.3%. In the year 2020, the death rate of stroke is around 38.8 per 100,000 populations [1]. Atypical clinical presentation, low healthcare access, shorter timeframe for treatment effectiveness and lack of proper patient education are some of the factors accounting for increased mortality and morbidity of stroke in the elderly populations. In a study serially following up with patients getting discharge dafter a stroke episode, the 30-day hospital admission rate is around 12.5% among Medicare patients. In years 2018-2019, the health care expenditure related to the diagnosis, treatment, hospital admission, and rehabilitation of stroke is estimated to be $56.5 billion.

Prompt administration of anti-thrombotic therapy is critical following the onset of cerebral ischemia in stroke [2]. Even with timely therapy, the clinical recovery and duration of hospitalization varies from patient to patient. Some patients recover quickly after a few days after hospitalization whereas others have a protracted recovery with the onset of severe motor and clinical deficits. The clinical recovery of patients following ischemia induced neuronal insults is to a large extent contingent upon on the sturdiness of the innate immune defenses, which forms the supreme catalyst for mitigation of neuro inflammation as well as the inception and furtherance of neuronal healing pathways. These aforementioned events are vitally important and form the underlying deep-rooted basis for neurological recovery, resolution of patient symptoms, lessened clinical sequalae, and regaining of patient independence post stroke inception and evolution.

Therefore, understanding the cellular and sub-cellular events that crystallize following the onset of cerebral ischemia is considerably essential so that any vacillations of the innate immune defenses and their repercussions can be scrupulously studied, rigorously investigated and clearly fathomed. These brain immune defenses are mainly carried out by resident microglial cells by various mechanisms including secreting anti-inflammatory cytokines, neutrophic factors and removing dying neurons & neutrophils from the site of ischemic damage by the specialized and regulated process known as microglial efferocytosis [3]. In this regard, we would like to focus this review on the microglial efferocytosis, which is the main innate immune defense strategy responsible for purging accumulated dead cellular debris in the neuronal milieu during the unfolding of ischemia induced neuronal loss. This entails microglial migration towards the site of ischemic neuronal death for engulfing dying neurons, henceforth paving the way for neuronal healing and regeneration in stroke.

The review is discussed in the following subsections: types of neuronal cell death secondary to cerebral ischemia, stockpiling of the dead neuronal debris, microglial draining pathways including canonical and non-canonical efferocytosis, mechanisms of canonical and non-canonical microglial efferocytosis, research studies that symbolize the significance of canonical and non-canonical efferocytosis in the stroke models, flaws of microglial efferocytosis in the stroke models and lastly future research studies that are recommended to unravel the obscurities in this process during the transpiration of stroke pathogenesis and final conclusions.

Methods

We performed a PubMed search of the relevant articles that summarizes types of neuronal death in the ischemic zones of stroke, and consequential innate immune defenses particularly pertinency of microglial efferocytosis in clearing the accumulated neuronal debris during the evolution of stroke. We searched the PubMed using the inclusion criteria including microglial stimulation, efferocytosis, neuronal death, stroke & amyloid beta, necrosis, apoptosis, canonical efferocytosis, non-canonical efferocytosis, eat-me signals, find-me signals, bridging molecules, microglial receptors, Rho signaling pathway, LC3 associated phagocytosis, anti-inflammatory cytokines and tissue healing/regeneration in the stroke disease models. The exclusion criterion is microglial phagocytosis. We did not use MESH (Medical Subject Heading) in the literature search.

We succinctly summarized the modus-operandi of canonical and non-canonical microglial efferocytosis following neuronal cell death during cerebral ischemia. On top of that, we highlighted previous research studies that delved into and highlighted the significance of canonical and non-canonical microglial efferocytosis during the origin and materialization of neuronal apoptotic signaling pathways during stroke.

Remarkably, we searched the PubMed and detail enumerated the possible stumbling blocks that are commonly encountered during the deployment of these clearance pathways following the onset of cerebral ischemia. Furthermore, we investigated the research studies that pin-pointed the possible dysregulation and imbalances in both these processes which might further fan the flames for instigating height ended neuronal death and exacerbated brain tissue thinning in the stroke models. On the grounds of these flaws, one might expect numerous barriers which would potentially for stall the uninterrupted smooth sailing of these protective mechanisms, thus bringing for the scenario that sparks off a heaping of dead cellular debris in the neuronal milieu. This unattended piled up neuronal debris will eventually metamorphosize into necrotic mass, a ramification that subsequently provokes disastrous repercussions particularly with respect to neurological recovery, tissue healing, symptom severity and clinical outcomes in stroke.

Therefore, to rectify these fallacies, future research studies are warranted to comprehend these physiological protective elimination pathways in a crystal-clear manner. So, we specifically included a section on the future research and listed a few pertinent areas that need to be delved for assimilating the possible imperfections of microglial efferocytosis that might become operative during stroke.

This review addresses the important research topic of microglial efferocytosis and its fallacies in the stroke models, particularly focused upon the relevant investigations that should be launched towards exploring these processes. Pursuing research for unraveling these clearance processes might be beneficial in improving the clinical outcomes of stroke. Our review is a small step to focus the attention on this unacknowledged physiological process and the potential benefits that might be unlocked by straightening out this derailed pathway and keep it running in its full potential in the stroke models. This review adds value to the current literature by pertinently under scoring the significance and competence of microglial efferocytosis during stroke pathogenesis. By under lining its physiological contribution and implications noticeably at the cellular level, we tend to underscore the presumption that, reprioritizing the research efforts towards this

forgotten protective process might unveil novel molecular targets, which might potentially form the foundational basis for crafting new cellular therapies. These cellular therapies can be tested in the cellular models, animal models and eventually in the clinical trials for their therapeutic efficacy in hastening neurological recovery and optimizing clinical outcomes in the stroke models.

Discussion

Cerebral ischemia spurring necrotic and apoptotic cell death

Hypertension, high LDL cholesterol (Low Density Lipoprotein), cardiovascular disorders (arrhythmias), diabetes, smoking and diabetes are the most common risk factors that eventually increase the proclivity of developing stroke and related vascular disorders [4]. High-risk patients are prone to the vessel occlusion of cerebral circulation (internal carotid, middle cerebral, anterior cerebral, posterior cerebral and basilar artery) either by slowly enlarging atherosclerotic plaque, thrombus, or a systemic embolus migrating to the cerebral vasculature abruptly eventually leading to the ischemia stroke. Alternatively, rupture of cerebral vessels due to weakness or vascular remodeling can incite the onset of hemorrhagic stroke where there is seepage of blood into the brain tissues [4]. It is important to note that, ischemic stroke (75%) is more predominant than hemorrhagic stroke (25%) [5]. As brain cells are very sensitive to changes in the brain circulation, even subtle alterations for shorter periods of time can ultimately culminate in the neuronal cell death through apoptosis. Some of the prevalent and frequent sub-cellular downstream signaling events that instigate neuronal cell death during ischemia include free radical damage, mitochondrial stress, ER stress, oxidative stress, lipid peroxidation and inflammation. These alterations will sooner or later will give rise to additional changes including glial cell activation, leukocyte infiltration, glutamate excitotoxicity, blood-brain barrier dysfunction, complement activation, chemokine, and cytokine secretion [5]. As a result of these cellular and sub cellular aberrations, neurons neither undergo cell death by apoptosis, which will slowly progress and eventually culminates into necrosis if they are nor cleared in a time dependent manner. Compared to the central area, which is severely ischemic and undergoes necrotic cell type of cell death, the adjoining area encircling it is designated as a ischemic penumbra or peri-infarct zone (Figure 1) [5]. According to the literature, this peri-infarct region is less severely damaged, and cells present in this zone are either severely stressed with a possibility of reverting back to their normal physiological state or else surmised to undergo apoptotic cell death. Apparently, stressed neurons will be generated by exposure to the sub-lethal concentrations of inflammatory mediators such as Lipopolysaccharide (LPS) generated during the course of stroke pathogenesis. This subset of living and viable neurons can be delineated by being stressed, but without having any activation of sub-cellular apoptotic signaling mechanisms. These stressed and viable neurons in the peri-infarct can be discerned by their external exposure of eat-me signals like Phosphatidyl-Serine (PS) on their plasma membrane (Figure 1). It is important to underscore the fact that, just as these inflammatory mediators taper off and fade away, the viable but stressed neurons will revert back to their normal physiological state with internalization of eat-me signals, thus averting their potential removal by the wandering macrophages.

Apoptotic cell death is typically characterized by cellular shrinkage, pyknosis, karyorrhexis, intact cell membrane, cytoplasm retained in the apoptotic bodies and lack of inflammation [6]. In contrast, the necrotic type of cell death is symbolized by cellular swelling, karyolysis, pyknosis, karyorrhexis, organelle dysfunction, breakdown of the cellular membrane, exocytosis of cellular contents and coexistent inflammation (Figure 1) [6].

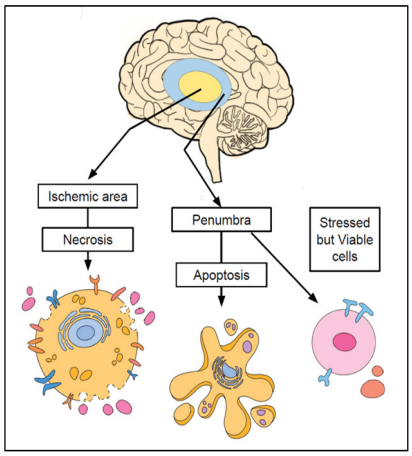

Figure 1: Types of cell death in the zones of cerebral ischemia: In the middle cerebral artery occlusion, the sector of neurological damage can be divided into zones namely central ischemic zone and peripheral per-infarct zone or the penumbra. The neurons housing in the central ischemic zone are subjected to severe ischemia related cellular stresses and thus are ill-fated to undergo necrotic type of cell death. Conversely, neurons residing in the peri-infarct region or penumbra would be prone to go through apoptotic cell death. Over and above that, neurons in the penumbra will be burdened by exposure to sub-lethal concentrations of cytotoxins, thus will live through a stressful but a viable phase. Prompt removal of dying neurons by the professional phagocytes (microglia) from the ischemic area is important as it accelerates neuronal healing and limits inflammation. It is important to perceive that fact the apoptotic cells are removed by canonical efferocytosis while stressed but viable cells are cleared by non-canonical efferocytosis.

Stockpiling of neuronal cellular debris: treacherous consequences

Due to above-mentioned types of cell death, there is a stockpiling of cellular debris in the neuronal milieu. This cellular debris becomes a liability for the surrounding neurons particularly when a larger proportion of dying neurons are destined to undergo necrosis as necrotic cells disintegrate and trickle danger signals in the neuronal interstitium, thus magnifying ongoing inflammation and further amplifying neuronal cell death, henceforth perpetuating the neurotoxic effects of cerebral ischemia (Figure 2) [7]. Even though apoptotic cells never release danger signals, their untimely removal, and prolonged accumulation in the neuronal niche provokes their progression towards necrosis. This transmutation will trigger the release of excessive danger signals, tissue factors and inflammatory cytokines into the neuronal environment [7]. Some of the pertinent and conventional danger signals discharged by the necrotic cells include HMGB1, uric acid (MSU; Mono Sodium Urate), galectins S100B (Alarmin), hyaluronic acid, heparan sulfate, heat shock proteins, defensin, and thioredoxin and cathelicidins. These danger signals will act upon the TLR2 (Toll-Like Receptor-2), TLR4 (Toll-Like Receptor-4) and NALP3 (NLR family pyrin domain containing 3) receptors and this will kick start the onset of neuro-inflammation during cerebral ischemia (Figure 2). As these danger signals are released, they are recognized, ingested, digested and processed and, thus assembling them for launching through MHC-class II complex (Major Histocompatibility Class II Complex). These processed danger signals will then be presented to the T-lymphocytes for driving the

innate immune responses. Since the brain does not have a lymphoid tissue, T-lymphocytes migrate into the brain tissues from the peripheral lymphoid nodes and systemic circulation via meninges, choroid plexus, and blood brain barrier [7,8]. In temporary and permanent MCAO (Middle Cerebral Artery Occlusion) models of stroke, T-cell infiltration has been demonstrated in the brain within 3-5 days and 7 days, respectively [9,10]. It is important to note that, regulatory CD4+ T lymphocytes offer neuro protection in the ischemic stroke models by immuno modulation (decreased cytokine & MMP-9 [Matrix Metalloproteinase -9] production and BBB [Blood brain barrier] protection and neuro repair (limiting astrogliosis, increasing oligodendrocyte replication and increasing neuronal stem cell populations) mechanisms [11-16].

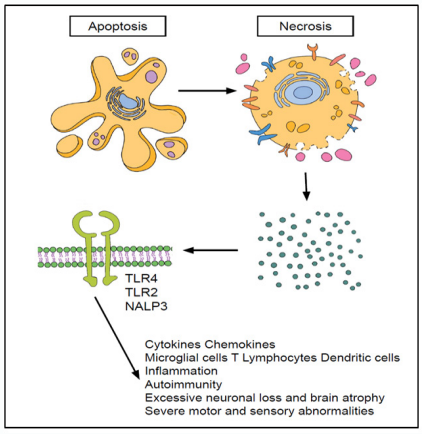

Figure 2: Pernicious consequences secondary to piling of dead neuronal debris: Accumulation of dead neurons in the vicinity of the ischemic areas of the brain is always considered a stumbling block as it perpetuates inflammation and represses neuronal regeneration/recovery. Untimely clearance of apoptotic neuronal debris will enkindle signaling pathways that will incite their progression to necrosis. Necrotic type of cell death is characterized by cellular enlargement, nuclear disintegration, swelling of organelles, Cytoplasmic vacuolation, and cell lysis. This will provoke trickling of cellular contents into the extracellular space. These extravasated cellular contents carry numerous danger signals such as HMGB1, heat-shock proteins, uric-acid, defensins, and hyaluronic acid. These danger signals will exploit and actionize on the TLR4/2 and NALP3 receptors of microglial cells to perpetrate ominous consequences including inflammation, cytokines & chemokine secretion, stirring up T-lymphocytes, dendritic cells, neutrophils, and microglial cells resettlement. Amalgamation of these cellular events will invoke redundant neuronal loss, and inflated brain atrophy, thereby triggering the onset of severe motor and sensory abnormalities. This will spark off protracted neuronal recovery, prolonged hospital stays, and heightened morbidity/mortality in the stroke patients.

As CD8 T-lymphocytes reach the site of injury, they exert their toxic effect via antigen dependent (Direct cytotoxicity via secretion of granzyme B and perforin secretion), antigen independent effects (Cytokine secretion including IL-17[Interleukin-17], IL-21 [Interleukin-21], TNFα [Tumor necrosis factor-alpha] & IFNγ [Interferon Gamma]) [8]. Furthermore, the apparent migration of T-lymphocytes also triggers the onset of thrombo-inflammation due to their interactions with the neighboring platelets. A possible hypothesis for the occurrence of this thrombo-inflammation include platelets escorting the T-lymphocytes to the site of neuronal damage and overseeing their attachment to the sub-endothelial matrix and exposed collagen so that they deploy their detrimental effects in a tremendous magnitude leading to the deleterious effects ranging from vascular inflammation and thrombosis [17,18].Thrombo-inflammation emanates secondary to the micro vascular dysfunction, and heightened inflammation, a development that elicits rapid and massive spreading of the brain infarction following ischemic stroke [19]. Although innate immune responses are considered to be protective, their counterstrike defenses will not be sufficient to avert the evolvement of ischemia induced neuronal damage.

In most instances, these immune responses underperform or else anarchic, disproportionate, and unrestrained counteroffensive defenses are responsible for stirring up profound neuronal damage as well as ballooning of infarct volume, thus heralding poor clinical outcomes, increased morbidity and mortality in the stroke models.

Microglial cells coming to the rescue to reverse the neuronal damage

Canonical efferocytosis or phagocytosis: Microglial cells are the primary glial cells that immigrate into the site of neuronal damage during the ischemic stroke to mitigate these effects via canonical efferocytosis [20]. In canonical efferocytosis, dying neuronal cells release find me signals such as nucleotides Adenosine Triphosphate (ATP) Sphingosine-1-Phosphate (S1P), Lysophosphotidylcholine (LPC) and CX3C motif chemokine ligand 1 (CX3CL1) and apoptotic cell derived micro particles [21,22] for attracting microglial cells towards them. These find me signals will bind to S1-P-R (Sphingosine-1-Phosphate Receptor), G2A (G-protein receptor 132), P2Y2 (Purinergic receptor) and CX3CR (C-X3-C chemokine receptor) receptors on the microglial membrane thus giving them the necessary impetus for their eventual journey towards the dying neurons (Figure 3) [21,22]. Upon reaching the site of neuronal injury, microglial recognition of dying apoptotic neurons materializes due to their apparent flashing of specific eat-me-signals such as phosphotidylserine, calreticulin and complement factors (C1q [Complement 1q] & C3b [Complement 3b]). These eat-me signals on the dying neurons interconnect with numerous microglial engulfment receptors ranging from MERTK, TIM (1,3&4) [T-cell immunoglobulin mucin domain-1&4], TAM family (Tyrso3, Axl & Mer), CD300 family, stabilin receptors and RAGE [Receptors for advanced glycation end products] (Figure 3) [20-22]. Alternatively, these eat-me signals will interact indirectly with bridging molecules such as MFG-E8 (Milk fat globule-EGF factor 8 protein), CCN1 C(Cellular communication network 1), GAS6 (Growth arrest specific 6)and Protein S before communicating with microglial receptors for initiating the subsequent steps for jumpstarting the neuronal removal [20-23]. Tight engagement between apoptotic neurons and microglial cells via receptors and bridging molecules will provoke the onset of downstream signaling mechanisms that will ultimately pave the way for the formation of phagocytic cup, engulfment, and eventual pulverization of dead apoptotic neurons within the microglial cells. The possible downstream signaling events activated within the microglial cells for efferocytosis include activation of ELMO1-DOCK complex [CED-5/180 kDa protein downstream of chicken tumor virus no. 10- CED-12/engulfment and migration], RAC1 [Rac Family Small GTPase 1] stimulation, actin remodeling and phagocytic cup formation (Figure 3) [24]. Once the dead neuron is successfully ingested, it will be digested within the phagolysosomes and through deploying a specialized process known as LC3 (Light chain 3) Associated Phagocytosis (LAP) [25-27]. In LC3-associated phagocytosis, LC3 proteins are conjugated to the phagolysosomes so that proficient cargo destruction, anti-inflammatory cytokine generation (IL-10 & IL-12) and immune silencing will transpire as

processing of dead neurons by microglial cells unfolds [25]. This will be followed by prompt onset of neuronal repair, regeneration and tissue healing endeavors, thus paving the way for negligible neuronal loss, fast-tracked neurological recovery, less severe motor/sensory deficits, and best-possible clinical outcomes in stroke.

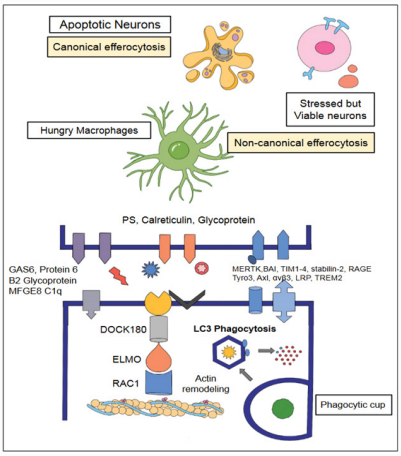

Figure 3: Apoptotic cells and stressed but viable cells are eradicated through canonical and non-canonical efferocytosis. Both these cells secrete find-me signals (Sphingosine-I-Phosphate & ATP etc.) which function as chemical messengers for attracting wandering microglial cells towards them. Over and above that, apoptotic/stressed cells make themselves conspicuous by exposing eat-me signals (PS & calreticulin) on their plasma membrane for their recognition by the incoming microglial cells. Upon their arrival, microglial cells interlink with these cells with the help of bridging molecules (MFG-E8 & Gas6 etc.) and membrane receptors (MERTK & TIM etc.). Stable interaction between microglial cells and dying/stressed neurons will arouse the stimulation of downstream signaling pathways (ELMO1-DOCK180-RAC1). This will evoke cytoskeletal rearrangement, actin remodeling and phagocytic cup formation. These above-mentioned morphological changes will be crucial for eventual microglial engulfment of dying/stressed neurons. Afterwards, they will be processed by lysosome digestion and LC3 phagocytosis. Efficacious pulverization of ingested neurons is imperative as it promotes tempering of surrounding inflammation by secretion of anti-inflammatory cytokines. Therefore, successful execution of this protective process is indispensable for not only clearing accumulated cellular debris but also for abating inflammation.

Non-canonical efferocytosis or phagoptosis: Alternatively, microglial cells remove viable but stressed neurons during unfolding of stroke pathogenesis through a process known as non-canonical efferocytosis or phagoptosis [28]. In this scenario, when live neurons are exposed to the sub-lethal concentrations of toxicants, they do not undergo cell death but rather become stressed and reciprocate by exteriorization of eat-me signals such as PS (Phosphatidyl Serine) albeit in a reversible manner. This exteriorization of eat-me signals in the stressed neurons erupts because the plasma membrane enzymes (Flippase & Scram blase) that are responsible for internalizing them are improperly dysregulated secondary to nitrate stress, reactive oxygen species overproduction, excess glutamate, and depletion of growth factors [28-31]. Apart from PS, other eat-me signals that are displayed by the stressed neurons include calreticulin and complement factors C1q & 3 [32-34]. Expression of Don't eat-me signals (CD47) on the neurons such as polysialic acid has been instrumental in inhibiting the phagocytosis of stressed neurons by interacting with SIGLEC-11(Sialic acid binding Ig like lectin 11) receptors on the microglial cells [35]. Stressed and viable neurons will be engulfed by the roaming neighboring macrophages because of their flashing eat-me signals expressed on their plasma membrane, which serves as a recognition symbol for their removal. Studies indicate that this process, when excessive, can be responsible for the exacerbated neuronal loss, neuro inflammation and brain atrophy during chronic hypoperfusion and focal brain ischemia [30,36]. In the *in vitro* studies, glutamate stressed neurons expose the PS and thus are efficiently engulfed and degraded by wandering microglial cells over expressing MGF-E8 and MerTK receptors. Thus, blocking microglial *MGF-E8* and *MerTK* (Mer protooncogene) prevented their engagement with the PS exposed and stressed neurons and prevented their removal. In the mixed neuronal-glial cerebral cultures, the addition of low concentrations of 250 nM Aβ1-42 (Amyloid beta) resulted in the exacerbated neuronal loss though activation of microglia as well as by instigating PS exposure on the stressed neurons [37]. In a similar manner, CRT (Calreticulin) exposure on the viable neurons can be sufficient for their removal by efferocytosis through microglial cells activated by LPS and non molar concentrations of Aβ1-42 *via* Lipoprotein Receptor Protein (LRP) receptors [34]. Bridging molecules (MFG-E8, protein S & GAS6), receptors (vitronectin receptors & MERTK), signaling mechanisms (ELMO-ROCK1-RAC1-actin) and processing (LC3 phagocytosis) are similar between both canonical and non-canonical efferocytosis.

Current studies showcasing the significance of efferocytosis in stroke models

Canonical efferocytosis in stroke models: According to Wei et al. STAT-6 (signal transducer and activator of transcription 6) is up regulated in the macrophage populations at the ischemic zones of the brain in the mouse models of stroke [38]. They demonstrate that, the STAT-6/arginine-1 signaling axis is imperative in boosting efferocytosis of the dead neurons in the ischemic penumbra thus enkindling reduced stroke volume, attenuated inflammation, and better clinical stroke outcomes [38]. Conceivably, STAT6 deficiency in the microglial cells was influential in triggering reduced dead neuronal clearance, expanding infarct volumes and widespread inflammation in the mouse models of stroke. Evidently in parallel to these findings, STAT6 and PPARγ (Peroxisome Proliferator Activator Receptor) were recognized to be supreme regulators of microglial efferocytosis in the mice models of stroke [39].

In another study, Sigma-1 receptors (Sig-1R) of the microglial cells were revealed to be indispensable for governing efferocytosis of dead/dying neurons in the stroke disease models [40]. Sigma-1 receptors (Sig-1R) in the ER (Endoplasmic Reticulum) related chaperone are predominantly involved in the direct activation of RAC1 mediated actin polymerization and phagocytic cup formation, which are crucial events necessary for the engulfment of dead neurons. Depletion of the Sig-1-Rfrom the macrophages instigated reduced clearance of dead neurons from the ischemia areas, which eventually set in motion deleterious consequences including enlarged infarct size, severe brain damage and worsened clinical neurological deficits in the mouse models of Transient Middle Artery Occlusion (TMCAO) [40]. Convincingly, adaptive transfer of Sig-1-R intact macrophages into the brain of the Sig-1R knockout mice contributed to better dead cell clearance, less neuro inflammation, small infarct volumes and better long-term clinical outcomes. Similarly, macrophage receptors, TREM2 (Triggering Receptor Expressed on Myeloid Cells 2) and Retinoid X Receptor (RXR) were crucial for carrying out phagocytosis of dead neurons from the vicinity of ischemic areas. Resultantly, their depletion was associated with decreased expression of genes associated with scavenging receptors scavenging deficits post-stroke recovery, heightened neuro inflammation, less effective neuronal regeneration, late clinical recovery and increased risk of developing

brain atrophy in the mice models of stroke [41,42].

Na+/H+ Exchanger isoform-1 (NHE1) in the microglial cells is primarily responsible for maintaining intracellular PH *via* permitting H+ efflux in exchange for Na+ influx [43]. This NHE1-mediated PH regulation is vital for maintaining the physiological equilibrium of the microglial cells, thus forming the underlying deep-rooted molecular basis for LPS and phoebe myristate acetate induced production of cytokines, superoxide anions and nitric oxide species [43]. That being the case, recent studies demonstrate that, Nhe1cKO were known to exhibit microglial anti-inflammatory phenotype, enhanced while matter myelination, decreased stroke volume, enhanced clinical recovery of motor & sensory functions, and increased survival rates following stroke [44]. Accordingly, Nhe1cKO mice revealed increased microglial transcriptomes for phagocytic genes such as P2RY12 which are principally involved in clearing the accumulating apoptotic cells following stroke and retinal development [45,46]. This translated into enhanced white matter myelination, increased oligodendrogenesis, and accelerated cognitive functional recovery in the Nhe1cKO mice as compared to control mice [46].

Non-canonical efferocytosis in stroke models: As discussed previously, neurons present in the peri-infarct region are less severely damaged and will be stressed but viable. These stressed neurons will display eat-me signals such as PS and calreticulin extracellularly due to the inactivation of plasma membrane enzymes in their plasma membrane [47]. Accordingly, microglial cells wandering nearby will look for these display signals and get attracted towards them. Recent studies demonstrate that, microglial cells roaming in the vicinity of peri-infarct regions were known to have increased expression of efferocytosis receptors (MER-TK) and bridging molecules (MFG-E8) so that they can accomplish the task of devouring eat-me signal displaying stressed neurons [28]. In the focal brain ischemia, microglial cells were shown to up regulate MERTK and MFG-E8 as early as 3 days for executing the task of clearing the stressed neurons from their vicinity and this translated into excessive brain atrophy, severe neurological deficits and poor clinical recovery [48]. Accordingly, mice lacking MFG-E8 or MERTK showed decreased brain atrophy, less motor deficits and prompt clinical recovery as compared to the control mice up to 4 weeks after being subjected to focal brain ischemia. Even in cell culture models, neurons stressed with glutamate were proficiently eradicated by wandering microglial cells in a MERTK and MFG-E8 dependent efferocytosis [48].

Is this microglial expunging is handicapped or dysregulated during the evolution of stroke: implications for clinical recovery: Despite microglial mediated efferocytosis being a paramount and highly valued innate defense during the unfolding of stroke pathogenesis, it might not commence and crystallize in a straightforward and uncomplicated manner. Few research studies highlight the fact that, this process is confronted with various encumbrances which might operate alone or synchronously, thus thwarting this protective endeavor from enforcing its full effect, thus inciting untoward consequences in the neuronal milieu following stroke.

In this section, the various distortions that can derail the process of microglial efferocytosis will be enumerated based on the previous research studies (Figure 4). Presumably, the strategy of neuronal clearance during stroke is hampered when the efferocytosis machinery including microglial receptors, bridging molecules, eat-me signals, and find-me signals are decreased thus spurring the stockpiling of neuronal debris with related untoward consequences. Middle Cerebral Artery Occlusion (MCAO) models performed so far revealed that, receptors associated with efferocytosis (P2RY12, CX3CR1, Siglec1&3, TREM2, SPP1 [Secreted phosphoprotein 1], AXL, & GPR34 [G-protein coupled receptor 34]) were down regulated, thus hindering the neuronal interaction necessary for dead neuronal ingestion and clearance during stroke recovery [49].

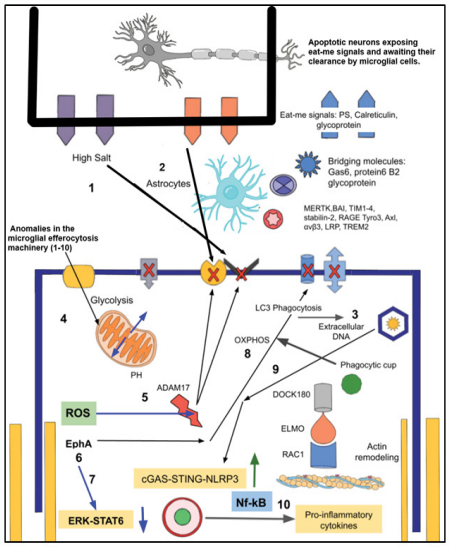

Figure 4: Few studies were performed previously to investigate the operational robustness of microglial efferocytosis in the stroke models. They came to know that there were numerous hindrances that impede the safe and unhampered deployment of this purging pathway during cerebral ischemia. Some of factors including high salt diet (1), astrocyte meddling (2), ADAM17 (3) metabolic disequilibrium (4), ROS accumulation (5), EphA upregulation (6) decreased STAT6 signaling pathway (7), destruction of MERTK (8), and extracellular DNA (9) and acting alone or colliding together synchronously tend to cripple this protective pathway. This will instigate the activation Nf-KB mediated secretion of pro-inflammatory cytokines (10) leading to amplification of inflammation during progression of stroke. On top of that, paralysis of this pathway will invoke the stock piling of dead neuronal cells with the resultant untoward consequences.

It is important to understand that; efferocytosis primarily depends on the metabolic regulation of the microglial cells including glycolysis, fatty acid β oxidation and oxidative phosphorylation [50]. Up regulation of microglial efferocytosis transpires in an atmosphere of increased oxidative phosphorylation, along with surplus supplementation of ketones, lactate, and pyruvate [50]. Continual removal of apoptotic cells during stroke is primarily dependent upon efferocytosis induced microglial glycolysis and uninterrupted supply of lactate which is mainly required for perceiving and juxta positioning with dying neurons in the surrounding vicinity [51]. In stroke, there is reduced blood supply with decreased supply of oxygen, and nutrients (glucose, lipids, ketone bodies, pyruvate, and lactate) to the brain tissues including neurons and microglial cells [52]. Due to the scarcity of essential nutrients the metabolic pathways in the microglial cells will be dysfunctional and chaotic, thus paving the way for the derailment of microglial efferocytosis. This culminates into a series of microglial aberrations including reduced motility, anomalous engulfment cup formation, lessened lysosomal number, increased PH, and crippled engulfment capacity. Research demonstrates that, microglial auto phagy which is primarily responsible for sustaining the survival and phagocytic function is a viable target and re-energizing this critical physiological process with rapamyc in can be a feasible

therapeutic strategy to restore the paralyzed microglial efferocytosis in the stroke models [52].

It has been previously shown that, oxidative stress and inflammation are very critical cellular events that play a pivotal role in the culmination of ischemic-reperfusion injury in the stroke models. ROS [Reactive Oxygen Species] released from these pathological cellular events have been shown to have baneful effects on cellular proteins, enzymes, mitochondrial respiratory chain and endoplasmic reticulum, thereby leading to neuronal apoptosis and necrosis [53]. Studies performed in COPD (Chronic Obstructive Pulmonary Disease) reveal that, oxidative stress curtails the efferocytosis proficiency in M2 macrophages, thus curbing their ability to purge piling cellular debris during evolving of COPD pathogenesis [54]. Recently, it was revealed that ROS was notorious for inflicting its pernicious effect on the microglial cells induced efferocytosis by provoking the cleavage and dismantling of MERTK receptor *via* activation of ADAM17 (A Dis Integrin and Metalloproteinase 17) [55]. With the down regulation of crucial efferocytosis receptors, removing apoptotic neurons through MERTK-Gas6 mediated signaling pathway from the ischemic neuronal milieu is hindered [55]. This instigates the heaping up of apoptotic neuronal debris along with hastening their progression towards necrotic type of cell death. As neurons undergo necrosis, they exude intracellular danger signals such as DNA into the extracellular space. This escaped extracellular DNA can act on the neighboring microglial cells and trigger the secretion of pro-inflammatory cytokines through cGAS (Cyclic GMP-AMP Synthase)-STING (Stimulator of Interferon Genes) and NLRP3 signaling pathway [55]. This perpetuates the vicious cycle of neuro inflammation, oxidative stress, excessive neuronal dismantling, increased brain atrophy and severe clinical sequalae in stroke patients.

As microglial cells lie in close juxtaposition to atrocities, their close coordination is indeed deemed necessary for the safe and effective removal of dead neuronal corpses from the ischemic brain [56]. A recent study demonstrated that, astrocytes release inhibitory signals into the extracellular space which can indirectly impact the phagocytic capacity of the microglial cells [57]. Although microglial efferocytosis is tightly regulated for boosting neuro-regeneration, tissue healing, inflammatory abatement, and clinical recovery, exacerbated and uncontrolled weeding out of dying as well as normal neurons can be counterproductive [38,39]. Few studies conducted unraveled that, overactive microglial cells execute unwarranted and unreasonable depopulation of neurons and oligodendrocytes following focal brain ischemia. This malfeasance thwarts the neuronal recovery and precipitates redundant brain tissue loss [30,58]. Lifestyle modifications including food habits are not only important for intercepting the risk factors for the causation of stroke but also can influence the neuronal recovery and healing process after the onset of stroke. A recent study unveiled that, a high-salt diet can indirectly down regulate the expression of microglial efferocytosis receptor TREM2, thus provoking deleterious aftereffects including decreased microglial efferocytosis, higher inflammatory cell migration, pro-inflammatory environment, massive infarct volume and severe neurological deficits [41].

EphA (Erythropoietin-Producing Hepatocellular Carcinoma Receptor Type A) is a tyrosine kinase receptor that is primarily expressed in the peripherally derived macrophages and brain resident microglial cells [59]. It has been demonstrated to be primarily involved in bolstering up neuro inflammatory environment following brain damage. EphA activation in the microglial cells leads to the modulation of phagocytic receptor expression (ERTK, Gas6, MFG-E8, and integrins like αvβ3), actin polymerization, cytoskeletal rearrangement, and production of anti-inflammatory cytokines through its action on ERK [Extracellular signal-regulated kinases]-STAT6-MERTK pathway. In a study by Soliman et al. EphA knock-out mice exhibited enhanced microglial efferocytosis, efficient dead cell removal and prompt resolution of inflammation due to increased expression of p-MERTK, p-ERK, and p-Stat6 in the damaged brain tissues [59].

Taken together, the conglomeration of these signaling events in the neuronal milieu of the ischemic areas will derail the continuous operation of canonical microglial efferocytosis during stroke related neuronal death. This will incite the stockpiling of necrotic and apoptotic neuronal cellular debris with the resultant sinister consequences including exacerbated inflammation, excessive neuronal loss, increased brain tissue loss and severe neurological sequalae.

Gaps in existing research, and the feasibility of translating preclinical findings to clinical practice: Most of the current studies on the canonical and non-canonical efferocytosis were performed on microglial receptors (MERTK, Sig-1-R and P2YR12) and bridging molecules (MFG-E8), looking into their potential role in clearing the piled up neuronal debris during stroke models. This might not provide a complete picture of integrity of efferocytosis happening in the neuronal milieu during stroke. Assessing the integrity of microglial efferocytosis should encompass investigating the signaling molecules and pathways that regulate dead neuronal recognition, ingestion, and processing inside the microglial cells. Furthermore, consequences of this dead cell digestion including growth factors and cytokine secretion and their end-target effects on neuronal healing should be assessed.

We hypothesize that; full-fledged operation of microglial efferocytosis is instrumental in facilitating the neuronal healing and regeneration following unfolding of neuronal death in the stroke models. Any stumbling blocks that restrain the materialization of this critical process will have repercussions including delayed clinical recovery and severe complications after stroke onset. Conceivably, this purging process forms the foundational basis for clinical recovery, regaining of patient independence and optimal clinical outcomes after a stroke episode. Research studies should be launched to grasp the vigourness of this clearance process and its effects on the aftermath of stroke onset. This would entail perceiving the expression of eat-me signals, find-me signals, bridging molecules, microglial receptors and signaling molecules/pathways that will ensure smooth sailing of this process after neuronal death in stroke. Studies need to perform to unravel the regulators of neuronal healing pathways particularly in the presence or absence of microglial efferocytosis in animal models of stroke. Conceivably, it should be worthwhile to assess whether energizing this microglial clearance process would be beneficial in kick starting and speeding up tissue regeneration pathways in stroke disease models. Pertinently, research should be geared towards understanding the thoroughness of LC3 phagocytosis in the microglial cells, a critical process instrumental for generation of end products that stimulate the nuclear receptors in the microglial cells. Stimulation of nuclear receptors such as PPAR gamma, LXR, & RXR facilitates in procreation of raw material (microglial receptors and bridging molecules), whose continuous supply ensures that there

will be uninterrupted maneuvering of microglial efferocytosis during stroke evolution. Efforts should be made to assess the expression of these nuclear receptors that play a vital role in energizing microglial efferocytosis in stroke. On top of that, after-effects of neuronal cell digestion including secretion of resolving and anti-inflammatory cytokines which play a crucial in shaping subsequent neuronal healing pathways should be delved in a thorough manner.

On top of that, efforts should be expended to ascertain the degree of neuro inflammation and accumulation of neuronal stimulants such as LPS, & TNF (Tumor Necrosis Alpha) during onset of stroke pathogenesis, a synergistic combination that can transfigure the normal living neurons into stressed ones. Gauging the amount of stressed but living neurons displaying eat-me signals PS (Phosphatidylserine) in the peri-infarct regions is critical as they can be eaten alive by neighboring wandering microglial cells during stroke. Nevertheless, with simultaneous transpiration of canonical and non-canonical efferocytosis in the neuronal milieu, sustaining a delicate balance between both forms of efferocytosis is very much essential, without which there will be untoward consequences in the neuronal terrain during evolution of stroke pathogenesis. Swaying the balance towards non-canonical efferocytosis can have disastrous clinical consequences during stroke, as in this clinical scenario, exacerbated removal of dying as well as living neurons from the brain tissues can be the ultimate outcome. This will lead to excessive brain tissue thinning, severe neurological deficits, long-drawn clinical recovery, and heightened morbidity & mortality in stroke, To avert this from happening, the first and foremost step is to ascertain the relationship between these two types of efferocytosis and their relative unfolding as stroke related neuronal death ensues. By comprehending the key signaling molecules and receptors that govern the relative evolvement of these two processes, future research studies can be drafted and therapeutic interventions that can be formulated to energize canonical efferocytosis and suppress the non-canonical efferocytosis for optimizing clinical outcomes in stroke.

As aforementioned research studies are conducted in stroke disease models, we might unveil the key signaling molecules (receptors, tyrosine kinases and nuclear factors) that are crucial in governing microglial efferocytosis during stroke. These molecular targets can utilize as a springboard for crafting novel cell-based therapies. These new therapies should be initially tested in the cell culture and animal models before they are incorporated into clinical trials for their clinical efficacy of modulating neuronal tissue healing in stroke. Conceivably, these novel cell-based therapies might modestly effective either alone in combination with currently evidence-based therapies for stroke models.

Future research is warranted to explore the gazillions of unknowns in the stroke models

Efferocytosis machinery: It is quite evident that, apoptotic and stressed neurons release find-me signals (ATP [Adenosine Triphosphosphate], UTP [Uridine Triphosphate], Lyso-PC [Lyso-Phosphatidylcholine] & S1P [Sphingosine-1-Phosphate]) and display eat-me signals such as PS and calreticulin to attract the neighboring wandering macrophages towards them [60]. Gauging the relative concentration of signals and their concomitant microglial receptors for recognizing them in the ischemic and peri-infarct regions might be prudent to understand the intensity of microglial efferocytosis and its putative significance in purging the dying neurons as well as facilitating tissue healing in the mice models of stroke. For example, under-release or sub-recognition of ATP/UTP secondary to altered expression of microglial receptors such as P2Y12 [G-inhibitory-protein receptor] [61,62] can precipitate their stock piling of dead neuronal debris in the ischemic areas with resultant deleterious consequences.

Find-me signal priming of microglial cells: It has been postulated that, dying apoptotic cells release find-me signals for not only attracting the microglial cells but also for fine-tuning, transmuting, and priming them for the consummating the task of dead cell clearance. As a matter of fact, they are responsible for kick-starting microglial signaling events that impels the synthesis of raw material required for clearing the piled up apoptotic cells. A recent study revealed that, SIP released by the apoptotic cells acts upon the microglial cells to launch NFAT [Nuclear Factor Activated T-Cells]-HIF1α [Hypoxia inducing factor-alpha] signaling events which will ultimately stir up the synthesis of Erythropoietin (EPO) [63]. This EPO will eventually escape extracellularly and act upon the EPO receptors of the macrophages and trigger ERK-PPARγ-RXR signaling pathways for spawning the raw material essential for efferocytosis (MFGE8, MERTK, GAS6 & CD36) [63]. Activating EPO signaling in the microglial cells can be beneficial as it amplifies the microglial phagocytosis of the dead cells from the ischemic zones. Further research should be warranted to investigate the importance of this microglial SIP-EPO signaling events and its aftereffects on the dead neuronal clearance in the cell culture and animal models of stroke.

Microglial and apoptotic neuron migration: It has been speculated that, dying apoptotic cells exhibit motility secondary to cytoskeleton changes. These changes entail caspase-3 induced cleavage of Rho-Associated Coiled-Coil-Containing Protein Kinase 1 (ROCK1) protein that precipitates MLC (Myosin Light-Chain Kinase) phosphorylation and cytoskeletal remodeling [64]. These sub-cellular events in the apoptotic cells from the foundational basis for their motility and aggregation at a focal point so that they can be proficiently cleared up by the migrating microglial cells [60]. On the other side of the coin, perception of find-me signals by GPCR (G-Protein Coupled Receptors) on the microglial cells induces the activation of RhoA (Ras homolog family member A; Guanosine triphosphatase hydrolase enzymes) and RAC1 mediated signaling events which energizes the microglial cells to gravitate towards the dying neurons. It has been forethought that, synchronous migration of both dying neurons and microglial cells is equally essential for the materialization of efferocytosis in the ischemic zones. It has been alleged that; these turns of events were supposed to increase the efficiency of microglial efferocytosis. Without this transpiration, microglial cells might be less effective in clearing the neuronal debris or else it stipulates the participation of a greater number of microglial cells for accomplishing the task of purging the accumulated neuronal debris during evolution of stroke. Consequently, evaluating the ischemic areas for focal agglomerations of apoptotic neuronal cells as well as microglial migration during ischemia induced neuronal loss would help us to comprehend whether microglial efferocytosis proceeds uninterrupted in an unequivocal manner in the ischemic zones of the brain during unfolding of stroke.

Eat-me signals and don't eat-me signals: Previous studies conducted indicate that the neurons when exposed to toxic stimulants will be stressed and exteriorize PS, and calreticulin on their outer leaflet of the plasma membrane [65,66]. Intracellular calcium elevation, ER stress and ROS accumulation are the important

factors that will facilitate the exposure of these eat-me signals. These cellular aberrations act through inactivation of enzymes in the plasma membrane which are primarily responsible of retaining them intracellular [65]. Wandering microglial cells will recognize these stressed but viable neurons with exposed PS/calreticulin and clear them *via* non-canonical efferocytosis. In some instances, even after exposure to eat-me signals, some cells will not be ready for the phagocytic meal because they require additional PS post-translational modification or due to the presence of don't eat-me signals. Don't eat-me signals such as CD47(High affinity receptor for thrombospondin-1 (TSP-1) and sialic acid present on the neuronal membrane can prevent efferocytosis by binding to SIRP-α (Signal Regulatory Protein Alpha) and Single (Sialic acid binding Ig-like lectin) on the microglial cells. Microglial removal of the stressed neurons is speculated to be dependent on the balance of these eat-me and don't eat-me signals during the onset of cerebral ischemia. It would be worthwhile to investigate the relative concentration of these eat-me and don't eat-me signals on the neurons stressed with oxidative stress, and inflammation. That being said, r induced downstream signaling events that will be operational during non-canonical efferocytosis in the cell culture and animal stroke models should shed light on the status quo of this unconventional process.

Metabolites from macerating apoptotic neurons: After microglial cells ingest apoptotic neurons, they are well pulverized by LC3 Associated Phagocytosis (LAP), and the metabolites brought into being will be crucial in shaping the further incremental effects on the future cycles of macrophage efferocytosis. These cellular metabolites will be sensed by nuclear receptors (LXRα [Liver-X-receptors alpha], LXRβ [Liver-X-Receptors Beta], PPARγ, PPARδ, RXRα), a culmination that will be beneficial by alleviating the inflammation as well as by reinforcing the future efferocytosis cycles [23]. Inflammation is toned down due to the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β [23]. At the same moment, efferocytosis is galvanized due to heightened synthesis of bridging molecules (GAS6 & MFGE8), and Microglial Receptors (MERTK). That being said, research efforts should be directed towards comprehending microglial cellular events post ingestion of dead neurons, particularly whether the metabolites produced are effective in stimulating the nuclear receptors of macrophages for kindling protective repercussions. This is important to decipher any fallacies in the microglial cellular signaling events post dead neuronal ingestion, specifically metabolites generated and their relationship to blunted efferocytosis in the stroke models.

Arginine metabolism: Recent studies reveal that, the metabolism of apoptotic cells produces arginine and ornithine which will be subsequently converted into putrescine [67]. This putrescine is responsible for the activation of Dbl (GTP exchange factor) and RAC1 downstream targets, which is the overarching signaling pathway for further rounds of efferocytosis [67]. A study by Cai et al. revealed that the Arginine1 pathway is essential for continuous efferocytosis and downgrading neuro inflammation in mouse models of stroke [38]. Therefore, it would be worthwhile to evaluate the true state of affairs of arginine metabolism in the microglial cells accumulating near the ischemic areas of stroke models. Additionally, it would be prudent to check whether bolstering arginine metabolism in the adjoining microglial cells would be beneficial in resolving inflammation, preventing neuronal death, and allaying neuronal loss in the stroke models.

Calcium signaling: Previous studies suggest that calcium signaling in the microglial cells plays a significant role in modulating the efferocytosis during ischemia-related neuronal death. Studies indicate that, calcium uprising in the microglial cells is one of the key cellular downstream events that preside over successful maneuvering of microglial engulfment during stroke. This calcium up regulation come to light because of the various signaling pathways operating synchronously to arouse the extravasation of calcium from the multiple sites including ER (Endoplasmic Reticulum), mitochondria and SOCE (Store Operated Calcium Channels) into the cytoplasm of the microglial cells. Interlocking of PS with MERTK receptors of microglial cells will invigorate PLC [Phospholipase]-IP3 [Inositol phosphate 3]-IP3R [Inositol Phosphate 3 Receptor] signaling axis and this will incite extravasation of calcium from the ER into the cytoplasm [68]. Over and above that, PS [Phosphatidylcholine]-MERTK binding energizes ORAI1 (Calcium release-activated calcium channel protein)-STIM1 (Stromal interaction molecule-1) interaction to give rise to SOCE (Store Calcium Channel) mediated calcium entry into the cytoplasm [69]. Besides that, Drp1 (Dynamic related protein 1) which is up regulated during efferocytosis was revealed to arouse mitochondrial fission, thereby dampening the calcium retro ceding into mitochondria through MCU (Mitochondrial Calcium Uniporter) [70]. Due to the aforementioned proceedings, uprising intracellular calcium provokes F-actin disassembly, meshing of calmodulin with MLCK (Myosin Light Chain Kinase), MLCK phosphorylation, phagocytic cup formation and apoptotic cell engagement [71]. Therefore, a close interplay of an amalgamation of events eventually culminates into calcium up regulation within the microglial cells linking apoptotic neurons. This skyrocketing of calcium sequentially incites chronological changes paramount for the engulfment and maceration of dead neuronal cells. That being said, it would be interesting to evaluate whether calcium related signaling events are transpiring in a systematic and orderly fashion in the microglial cells interacting with dead neurons accumulating in the ischemic areas. Over and above that, research should be geared towards looking into the therapeutic modalities that amplify the calcium mediated signaling events which might offer therapeutic benefit by reinforcing the efferocytosis potency of the microglial cells during stroke pathogenesis.

Polyamines: A recent study demonstrated that, microglial engulfment of apoptotic neurons incites increased intracellular accumulation of polyamines namely spermidine and spermine [72]. These piled up sperm dines within the apoptotic cells will be inclined to extravasate and aggregate in the extracellular milieu, a proceeding powered by caspase dependent signaling pathways within the apoptotic cells.

These trickled polyamines undergo endocytic transport into the microglial cells with the assistance of RAC1, actin and PI3 kinases [Phosphoinositide 3-kinases] following engulfment of neurons [73]. Thus, excess buildup of polyamines within the microglial cells transpires secondary to their percolation from the extracellular environment, but not due to heightened intracellular synthesis. These finding gains traction due to the fact that, these polyamines are influential in clamping down the excess production of pro-inflammatory cytokines such as IL-6 and IL-β within the microglial cells during stroke. As a matter of fact, perpetuation of pro-inflammatory backdrop during stroke is detrimental because it provokes excess neuronal demise in addition to triggering eat-me signal (PS & Calreticulin) exposure on the normal living neurons, thereby sparking off their removal through idiosyncratic non-canonical efferocytosis. Accordingly,

persistence of these neurotoxic cytokines during ischemia induced neuronal damage abets in the removal of dying as well as normal neurons, thus precipitating excess brain tissue thinning with resultant genesis of severe motor/sensory deficits following stroke. Thus, polyamines induced allying of inflammatory environment might be conducive to tissue healing and regeneration following stroke. Based on these findings, it might be useful to characterize the changes in the polyamines and their effects on neuro inflammation and efferocytosis in the stroke models.

Plausible hypothesis for defective efferocytosis in stroke models: lessons learned from atherosclerosis: A hand full of underlying mechanisms speculated for defective macrophage efferocytosis in the atherosclerosis models include less M2/M1 macrophage ratio, endothelial dysfunction, ER stress, ROS, Inflammation (TLR4, MMP & ADAM17) mediated annihilation of eat-me signals [MFG-E8, MERTK, LRP1], TNF-alpha-induced up regulation of Don't eat-me signals [CD47], oxidized LDL mediated secretion of auto-antibodies that mask eat-me signals on the apoptotic cells thus inciting their transmutation into poor meal substrates, and epigenetic regulation [74,75]. As a result, unfavorable consequences can be ensued ranging from plaque inflammation, release of cytokines & chemokines, foam cell accumulation, plaque vulnerability and acceleration of atherosclerosis [75]. Lessons learned from atherosclerosis research should be taken into consideration and appropriate research studies should be steered to evaluate whether these mechanisms are actually flourishing in the ischemic regions of stroke models. Exploring and comprehending the prevailing transgressions of microglial efferocytosis should be the initial step. This will shed light and enable us to craft novel cell-based therapeutic modalities to circumvent these divergent and flawed clearance pathways so that expedited dead cell depopulation and tissue healing can supervene.

Conclusions

This review summarizes the physiological and pathological relevance of canonical and non-canonical microglial efferocytosis in the stroke models. Canonical efferocytosis is an innate immune response by wandering professional phagocytes (microglial cells) that protects the ischemic brain tissues from unwanted piling up of apoptotic neuronal debris. However, multiple studies performed so far exposed the fact that there are numerous quagmires that will intercept the successful maneuvering of canonical efferocytosis after the inception of cerebral ischemia in the stroke models. To further fan the flames, coterminous on-going non-canonical efferocytosis of the stressed but viable neurons becomes a predicament as it propels supernumerary neuronal loss from the ischemic brain tissues. Evidently, extravagant, and disproportionate operation of both these processes is detrimental for the post-stroke recovery. As a result, further research is warranted to comprehend the underpinnings of the fallibilities in both these processes so that microglial efferocytosis is instrumental in the pertinent removal of amassed dead neurons from the ischemic zones for promoting tissue healing and regeneration. This will conceivably unearth viable molecular targets that can resurrect, and jump starts the modus-operandi of canonical microglial efferocytosis during the evolution of stroke. Simultaneously, research efforts should be expended towards repressing the removal of stressed but viable neuronal cells by non-canonical efferocytosis from the ischemic tissues.

Novel therapeutic interventions that can reasonably crank-up canonical efferocytosis and destabilize non-canonical efferocytosis should be discovered in the future and their effectiveness ought to be checked in the cellular and animal models of stroke. This will be the starting point for the execution of clinical trials to assess their clinical efficacy in the stroke patients. In this regard, the effectiveness of blocking agents to eat-me signals, find-me signals, bridging and microglial receptors should be considered. Therapeutic strategies should also be devised by altering the metabolic profile of the microglial cells which will transfigure them into proficient phagocytes that skillfully purge the accumulated neuronal debris during the brain ischemia. These novel cell-based interventions which will reinvigorate the apoptotic dead cell clearance during cerebral ischemia while muzzling overblown removal of stressed but viable neurons will pave the way for tissue healing, regeneration, better clinical recovery, and optimal clinical outcomes following stroke.

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