The Function of Cytokines in Ischemic Stroke

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1 The Complex Sequelae Resulting from Ischemic Stroke

Ischemic stroke produces a complex injury profile that is characterized by multiple, successive waves of tissue injury. The acute phase primarily involves energy failure and excitotoxic injury, which are hallmarks of most neurodegenerative diseases and set the stage for delayed cell death. During the acute phase, neural tissue most directly irrigated by the occluded vessel experiences rapid energy failure resulting from oxygen and glucose deprivation. The resulting failure of cellular respiration necessitates the switch to anaerobic energy production, leading to acidosis. This metabolic switch, coupled with excessive release of the neurotransmitter glutamate from injured neurons and neighboring astrocytes, promotes ionic instability that compromises cell membranes and facilitates neuronal cell death. In addition to free radical injury, which is achieved in large part through the production of superoxide and peroxynitrite radicals, the release of sequestered intracellular calcium stores also promotes apoptotic signaling and cellular oedema [1].

These mechanisms of ischemic stroke pathology are well documented and have provided the basis for several decades of stroke research, whereby the primary goal was to limit excitotoxicity as means of limiting these injurious responses.

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Unfortunately, this approach was met with very limited success [2, 3], as relatively few stroke patients are eligible for the thrombolytic therapies (i.e., tissue plasminogen activator) that must be administered within 3–6 h of stroke onset. With few viable treatment options, the majority of patients are limited to supportive care during the period of infarct expansion. During this period, the viable tissue surrounding the core infarct becomes compromised through perpetuation of the above mechanisms coupled with detachment-induced injury from loss of synapses and neuroglial support. Without recanalization and restoration of blood flow, neural injury will progress based upon the severity of the initial insult. This will continue until endogenous remodeling mechanisms, such as glial scarring, are successful in protecting healthy surrounding tissue from the inflammatory microenvironment present within the infarction.

The rapid progression of neuronal injury, as well as the inability to diagnose ischemic stroke patients in time to receive thrombolytic therapy, has prompted investigations into other therapeutic targets. Among these was a broad category of neuroinflammatory responses that encompasses a wide range of deleterious phenomena. For example, depending upon the specific context, neuroinflammation may include blood–brain barrier (BBB) permeability, glial scar formation, extravasation of peripheral immune cells, and/or activation of resident immune cells [4]. Additionally, and somewhat paradoxically, immune cell chemotaxis is accompanied by the upregulation and release of cytokines, chemokines, prostaglandins, proteases, and other proinflammatory molecules that perpetuate cellular disruption and tissue injury [5]. To complicate matters, these signals collectively demonstrate dual and often overlapping roles that cloud interpretation of their individual actions within the grand scheme of stroke pathology. For these reasons, the inflammation that ensues following ischemic stroke produces a complicated injury profile with multiple mechanisms that need to be addressed in order for any therapeutic approach to be successful.

A critical component of any therapeutic is the ability to dampen proinflammatory signaling that contributes to delayed cell death. This delayed wave of neural injury is facilitated by BBB disruption in response to the initial ischemic event. Within hours to days of stroke onset, matrix metalloproteinases (MMPs) cleave tight junction proteins involved with sealing the barrier. The result is a biphasic opening of the BBB that is initiated by MMP-2 and enhanced through the actions of MMP-9 [6]. Immune cell signaling exacerbates the inflammatory milieu within the core infarct. Microglia respond to resident death signals, while peripheral leukocytes are activated by CNS antigens that have leaked into the systemic circulation. The result is a feed-forward proinflammatory microenvironment that is modulated by resident glia and peripheral leukocytes through the upregulation and release of cytokines. Thus, cytokine signaling has profound effects on the ultimate fate of the injured brain and is regulated by activated immune cells both within and outside of the brain parenchyma.

2 Cytokines as Therapeutic Targets for Ischemic Stroke

Cytokines are involved in a wide variety of biological processes, including embryonic development, stem cell differentiation, innate and adaptive immunity, cognition, progressive neural injury and neurodegenerative disease pathology. The complexity of cytokine actions is reflected in the distinct families of cytokines, which include interleukins, chemokines, mesenchymal growth factors, adipokines, and the tumor necrosis factor family [7]. Depending upon the cytokine, these signals can influence cell signaling by cell surface receptor binding, antibody binding, and direct DNA binding. Thus, this interesting class of molecules has attracted much attention as researchers have searched for new therapeutic targets.

Ischemic stroke produces a biphasic injury profile that can be characterized in terms of an "acute" excitotoxic response followed by a "delayed" neuroinflammatory response. Indeed, cytokines have emerged as important signaling molecules that play key roles in both injury phases and are intimately connected with immune cell activation. To date, there have been a multitude of studies aimed at uncovering the precise mechanisms by which cytokines influence cellular responses to cerebral ischemia. Results from clinical studies and animal models have identified several key cytokines that can be targeted for the treatment of ischemic stroke.

The body of work regarding cellular immune responses to ischemic stroke is extensive and is discussed in great detail throughout this book. Although it was not historically the case, neuroinflammation is now widely regarded as a principle cause of delayed infarct expansion that is driven, in large part, by cytokine signaling [8, 9]. Both the acute and progressive phases of stroke injury have been linked to the upregulation and release of proinflammatory cytokines [4, 5]. Animal models have offered the means to conduct controlled studies designed to elucidate the mechanisms by which cytokines influence injury and remodeling of the ischemic brain. This section reviews some of the key findings related to cytokine production and signaling after ischemic stroke. Additionally, promising therapeutic approaches predicated on modulating cytokine signaling are discussed within the context of neuroinflammation and delayed infarct expansion.

2.1 Tumor Necrosis Factor Alpha Signaling and Expression

Tumor necrosis factor alpha (TNFα) has long been identified as a key proinflammatory effector of ischemic tissue. Although it is principally produced by activated macrophages, virtually all lymphocytes have the capability of producing TNFα as part of the systemic inflammatory response. This cytokine binds two distinct receptors, TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2), and TNFR signal transduction can have various consequences including induction of NF-κB and AP1 transcription factors, as well as initiation of apoptotic

signaling through caspase activation. To complicate matters, TNFR activation can also suppress inflammatory signaling and apoptosis.

The existence of distinct TNFRs and various intracellular signaling pathways coupled to TNFRs explains some of the inconsistencies from ischemic stroke studies. For example, activation of TNFRs can result in two antagonistic responses. Virtually all cells typically present within the brain after stroke express TNFR1. The intracellular death domain contained within TNFαR1 is linked to Fas-associated protein with a death domain (FADD) signaling, where activation can result in the induction of caspases and/or cytochrome c release. Alternatively, TNFα binding to TNFR2 can activate TNF receptor-associated factor 2 (TRAF2), which induces expression of cell survival-associated genes that are regulated by mitogen-activated protein kinase (MAPK), NF-κB and AP-1 transcription factors [10, 11]. Additionally, TNFR2 lacks an intracellular death domain and is expressed on the surfaces of cells present within the cerebral infarct, including resident glia (oligodendrocytes and astrocytes), cerebrovascular endothelial cells, T-cells, and monocytes. Thus, the dualistic nature of TNFα signaling can produce caveats concerning the measurement of cytokine levels in the brain. In fact, a recent study demonstrated that TNFα production increased in both microglia and monocytes between 24 and 72 h after transient middle cerebral artery occlusion (tMCAO). Additionally, this study showed a positive correlation between TNFα production and the phagocytic capacity of microglia [12], which might suggest a beneficial role in response to ischemia.

Since TNFR binding can result in a multitude of effects depending upon the receptor activated and the local environment (i.e., the cell types present and the extent of inflammatory signals), the timing of cytokine production and release has been a subject of great interest as researchers work to understand the functional consequences of TNFα signaling. Animal models of ischemia, particularly those investigating immunoinflammatory mechanisms, have been instrumental in characterizing protein and mRNA levels of various cytokines, including TNFα. Studies exploring ischemia/reperfusion injury in liver found that Kupffer cells and neutrophils increased expression of TNFα along with other inflammatory mediators including reactive oxygen species (ROS) and nitric oxide (NO) [13]. Importantly, elevated TNFα also correlates with damage to various other body tissues following ischemia [14].

Consistent with ischemic peripheral tissues, TNFα transcript and protein levels are consistently upregulated in the injured and ischemic brain. This cytokine is expressed at early time points following stroke and is a critically important facilitator of immune cell recruitment to the cerebral infarct. TNFα gene transcript was elevated as early as 6 h following tMCAO in mice and remained elevated at extended time points up to 96 h after stroke [8, 15, 16]. Furthermore, TNF-α was elevated in the systemic circulation 24 h after tMCAO in the same model system, but returned to normal levels by 96 h post-stroke [16].

A similar expression pattern was detected in the spleen and provides clues as to its role in progressive inflammation. The spleen is a reservoir of immune cells and plays a substantial role in systemic immune responses. There is now a body of work

implicating splenic signaling in progressive neural injury following various insults including ischemic stroke, hemorrhagic stroke, and traumatic brain injury (TBI). Because the BBB is breached as a result of these and many other insults, activated peripheral immune cells can exert effects both systemically and at the level of the brain. Mice subjected to tMCAO showed a splenic expression pattern where the expression of various cytokines, including TNFα, was increased between 1 and 2 days following stroke [15, 17, 18].

2.2 TNFα as a Therapeutic Target for Ischemic Stroke

Various experimental approaches have been used to determine whether TNFα is a viable target for the treatment of stroke. Some of these include exogenous administration and/or blockade of TNFα, the utilization of knockout mice lacking the TNFα gene ($TNF\alpha^{-/-}$) or key immune cell populations, and animals subjected to splenectomy prior to insult. These methodologies have provided valuable information as to the role of TNFα in proinflammatory signaling. Furthermore, data from these models provides insights into acute signals originating at the level of the brain as well as those that are regulated by the peripheral immune response.

Popular approaches to investigating cytokine signaling involve boosting levels of the protein, and conversely, deletion or blockade of the protein. Rodent stroke models, including tMCAO and permanent MCAO (pMCAO), have been useful in conducting such studies due to their cost-effectiveness and reproducibility. Studies show that the administration of TNFα increases infarct volume in these models [19], demonstrating a deleterious role for TNFα that is dose-dependent. Similarly, reducing TNFα by blocking the biosynthetic enzyme, TNFα converting enzyme (TACE), was effective in reducing infarct volume and functional deficits in rats subjected to pMCAO [20]. Consistent with these data, other data showed efficacy in reducing tMCAO injury following administration of TNFα neutralizing antibodies [21].

Indeed, this cytokine has traditionally been regarded as a proinflammatory mediator that exacerbates cellular injury due to its elevation in neurodegenerative disease and after brain injury. However, several lines of evidence indicate that TNFα may be at least moderately misunderstood since it also appears to play a beneficial role under certain conditions. Data showed that TNFα upregulates the antioxidant superoxide dismutase 2 (SOD2), which is important in converting superoxide into molecular oxygen or the less damaging radical hydrogen peroxide. Interestingly, SOD2 has been implicated in stroke preconditioning [11], which would suggest a protective role for TNFα. Consistent with a protective role for TNFα, mice lacking the TNFα gene actually demonstrated worse outcomes in terms of total infarct volume relative to control mice [22]. To further complicate the issue, deletion of the gene coding for TNFR1, but not TNFR2, protected mice against pMCAO [22]. These data are consistent with the pro-apoptotic and cell survival-associated roles of TNFR1 and TNFR2, respectively, and thus highlight the dual nature of TNFα.

From a strictly therapeutic perspective, there is evidence that selective targeting of TNFα itself or TNFRs may prove beneficial for the treatment of ischemic stroke. The promise of this approach comes from TNFR effects and altered TNF expression patterns in response to known neuroprotective treatments and strategies. For example, activation of TNFR2 triggered oligodendrocyte (OL) progenitor cell recruitment in a cuprizone-induced white matter injury model [23]. Since white matter injury is directly related to motor deficits in ischemic stroke pathology, this mechanism may prove beneficial to improve these outcomes. Other data showed that administration of cocaine- and amphetamine-regulated transcript (CART) decreased infarct volume at early and delayed time points following tMCAO. Furthermore, the observed neuroprotection was accompanied by reduced TNFα levels in the blood and a dampened proinflammatory T-cell response [16].

Interestingly, studies investigating immune cell function also point to a crucial role for TNFR signaling. Peripheral immune activation is now an emerging area of research due to a growing body of work demonstrating a clear link between exacerbation of brain injury and the activation of T-cells, monocytes, and peripheral macrophages. Removal of the spleen, a lymphoid organ that acts as a reservoir for peripheral immune cells, has been shown to elicit protection in models of stroke, TBI, and ischemia-reperfusion injury to peripheral body tissues [24–27]. Since splenectomized animals are protected against ischemia, both in peripheral tissues and at the level of the brain, researchers have also focused efforts on cytokine signaling in spleen-derived immune cells. One technique involves harvesting splenocytes from animals subjected to focal ischemia. These cells are then activated and assayed to determine the inflammatory profile, including cytokine production and release.

From splenectomy experiments and assays with cultured splenocytes from ischemic tissues, spleen-derived TNFα has emerged as a candidate target due to its response profile. For example, splenectomy caused reductions in hepatic injury and leukocyte infiltration that were accompanied by decreased TNFα release [25]. In addition, neuroprotection and improved spatial memory performance following splenectomy were associated with decreased TNFα in a model of TBI [27]. Splenocytes harvested from CART-protected animals showed a reduced capacity to produce proinflammatory cytokines, including TNFα [16]. In a model of tMCAO, splenocytes harvested from stroke mice showed upregulated TNFα expression after activation with CD3/CD28 antibodies [8]. These experiments were later replicated to show similar effects in splenocytes harvested up to 24 h following tMCAO, demonstrating a prolonged effect of TNFα production following ischemic stroke in which the cytokine was elevated as late as 96 h after the insult [16]. Alterations in splenic TNFα have also been observed in response to stem cell therapies. Treatment with hematopoetic stem cells (HSCs) blocked splenic TNFα gene transcript [17], while human umbilical cord blood (HUCB) cell treatment reduced the extent of concovalin A-induced TNF-α production and splenocyte proliferation [28].

To date, the various approaches to blocking TNFα signaling have been met with some success in animal models and as well as the clinical setting. Administration of MMP/TACE inhibitors has been effective in rodent models, with BB-94

demonstrating protection against transient global ischemia and KB-R7785 reducing neural injury following MCAO. In addition to inhibiting TACE, which is a key regulator of TNFα activation, another promising approach is blocking the soluble form of TNFα (sTNFα) and its receptors (sTNFRs). This method would act to blunt the effects of sTNFα binding, thus offering another level of regulation. Recently, the TNFα blocking compound etanercept has shown preclinical efficacy along with extended efficacy in reducing motoric and gait disturbances in a clinical trial [29, 30]. Although there is some promise in the clinic and various reports of preclinical efficacy, the utility of targeting TNFα remains debatable when considering its complex, dualistic role.

2.3 Interleukin-1 Beta (IL-1β) Signaling and Expression

Interleukin-1 beta (IL-1β) is another putative proinflammatory cytokine associated with exacerbated injury following ischemic stroke. A member of the IL-1 cytokine family, IL-1β is predominantly produced by macrophages as a proprotein that is proteolytically cleaved into its active form by caspase 1. In general, IL-1β functions in multiple capacities to increase cell proliferation and differentiation, facilitate immune cell chemotaxis through the activation of chemokines, and regulate apoptotic signaling pathways.

In human stroke patients, IL-1β is increased in plasma and cerebrospinal fluid during the acute phase [30, 31]. Together with TNFα and MMPs, IL-1β plays a key role in the BBB disruption that renders the brain susceptible to activated peripheral macrophages and monocytes. Perivascular astrocytes are a major source of neural IL-1β at this early time point [32]. In fact, a recent study demonstrated elevations in IL-1β as early as 1 h after tMCAO that appeared to be influenced by the activity of MMP-2 [33]. However, microglia begin to upregulate IL-1β as they transition from the early M2 phenotype, which is predominantly thought to be beneficial, to the putative M1 proinflammatory phenotype [34].

IL-1β has most frequently been examined by measuring mRNA content at selected time points following experimental stroke. Previous studies using the tMCAO model showed that IL-1β was elevated in ischemic brain at 6 h post-stroke and remained increased up to 96 h after insult [16].

2.4 IL-1β as a Therapeutic Target for Ischemic Stroke

Consistent with a proinflammatory role in disease pathology, animal studies showed that therapeutic doses of HSCs inhibited splenic chemokine and cytokine production, including IL-1β, when administered 24 h after tMCAO. Additionally, treatment reduced apoptotic neurons, activated macrophages/microglia, and T-cell infiltration [17]. Similarly, the deleterious effects of IL-1β signaling were demonstrated in a

model of neurodegenerative disease (ME7 prion disease) in which intrahippocampal injection of IL-1β induced astrocytic chemokine production and enhanced infiltration of T-cells, neutrophils, and monocytes into the brain parenchyma [35]. The recruitment of peripheral immune cells observed in this study is consistent with the IL-1β profile previously detected in the spleen following focal ischemia. For example, IL-1β gene transcript was elevated by 22 h post-reperfusion in a rodent model of tMCAO [15, 18] and remained elevated at 48 h after stroke [17]. Interestingly, the involvement of spleen-derived IL-1β in brain injury was also documented following TBI in rats. Removal of the spleen immediately following TBI reduced IL-1β production, and this effect occurred concomitantly with reductions in mortality and vasogenic oedema. Additionally, the same animals showed improved performance on the Morris Water Maze, demonstrating a spatial memory benefit that was directly correlated with reduced levels of Il-1β [27].

The preponderance of data demonstrating a role for IL-1β in perpetuating a proinflammatory microenvironment makes this cytokine an attractive target for stroke therapy. As such, many laboratories are investigating mechanisms of inhibiting IL-1β signaling. A recent study showed that mice deficient in lactadherin, an endogenous glycoprotein with IL-1β inhibitory actions, showed increased IL-1β production and greater infarct size following permanent distal MCAO relative to wildtype controls. Furthermore, the increase in cerebral infarction was negated in mice that were administered an IL-1R antagonist [36].

Despite these encouraging data, there are caveats associated with inhibiting IL-1β. This cytokine is a key modulator of immune cell function, and its complex regulation at the transcriptional level may pose problems in terms of selective targeting of the proinflammatory isoform. To the latter point, a recent study showed that a specific variant of the IL-1β gene produces a protein that was associated with higher recanalization rates in stroke patients following tPA administration [37]. In light of these data, it will be critical to understand the biological activity of all SNP variants to ensure that the appropriate IL-1β variant is being targeted.

2.5 Interleukin-6 Signaling and Expression

Interleukin-6 (IL-6) is another interesting cytokine in that experimental evidence suggests a dual nature, including proinflammatory and prosurvival effects. The cytokine has been implicated in the pathogenesis of many neurological diseases. Similar to the other cytokines discussed within this chapter, the purported involvement in ischemic injury (and therefore potential as a therapeutic target) has arisen largely from observed expression profiles in affected patients and experimental subjects.

One noteworthy distinction between IL-6 and many of the other cytokines investigated is that IL-6 serum levels have shown a positive correlation with negative outcomes and brain injury in human stroke patients [38]. In fact, of the clinical variables used to predict mortality, IL-6 remains among the most reliable [39].

Recent data from the Northern Manhattan Study (NOMAS) has expanded upon the predictive validity of IL-6 as a biomarker. The NOMAS study is an ongoing investigation of risk factors conducted using a multiethnic cohort living in the same community. In this study, researchers concluded that IL-6 was an accurate predictor of ischemic stroke when levels were higher than high-sensitivity C-reactive protein, another biomarker that has been useful as a stroke predictor [40].

Consistent with these clinical data, IL-6 gene transcript is also induced in mice after tMCAO [8], and the signaling pathways activated by IL-6 suggest that this cytokine may prove useful as a therapeutic target. IL-6 signaling is complex and signal transduction can result in very different effects on cells. The receptor complex that is activated by IL-6 is composed of IL-6R subunits alpha (IL-6Rα) and beta (gp130). Interestingly, the gp130 intracellular domain can initiate MAPK signaling or activate the Janus kinase/transducers and activators of transcription (Jak/STAT) pathway. Ultimately, these signal transduction pathways result in transcriptional regulation through binding to the promoters of genes driven by AP-1, NF-κB, and other regulatory elements.

The complexity of IL-6 signaling is mirrored by its diverse functions. This cytokine has been shown to play a role in normal physiological functions, including lipid and iron metabolism, osteoclast stimulation during bone remodeling, angiogenesis, and immune system regulation. In terms of the latter, IL-6 is involved in promoting neutrophil production and chemotaxis, enhancing proinflammatory Th17 cell production and inhibiting the putative proinflammatory cytokines TNFα and IL-1β [41]. As such, IL-6 has been implicated in various disease states including autoimmune disorders, diabetes, cancer, arthritis, Alzheimer's disease, and even some forms of psychopathology.

2.6 IL-6 as a Therapeutic Target for Ischemic Stroke

Data from experimental models has warranted a close look at IL-6 as researchers search for novel therapeutic targets. However, contradictory results have complicated the discussion as to whether the actions and effects of this cytokine can be modulated effectively. For example, one study using IL-6 knockout mice found that IL-6 gene deletion had no effect on cerebral infarction in the tMCAO model [42]. A separate study using IL-6 knockout mice in the same tMCAO model showed worsened long-term infarcts and functional outcomes relative to wildtype mice. This recent study attributed these effects to reduced capacity for angiogenesis and diminished STAT3 activation. Additionally, cultured neurons, endothelial cells, and mixed glia increased IL-6 gene transcript in response to IL-6 application, suggesting that resident cells of the brain are capable of upregulating IL-6 following exogenous administration [43].

Although these conflicting data highlight the difficulty in formulating conclusions from animal studies, other lines of evidence are consistent with the latter study in demonstrating benefits of IL-6 signaling. Intracerebroventricular (i.c.v.)

administration of recombinant IL-6 prior to pMCAO significantly decreased infarct volume in rats, suggesting that IL-6 is directly neuroprotective at the level of the brain [44]. These data are supported by another group that utilized a mouse model of tMCAO to demonstrate reduced infarct volume following i.c.v. administration of IL-6. Here, the afforded protection was linked to STAT3-mediated SOD2 production and this study included another group of IL-6R deficient mice that showed exacerbated neural injury relative to wildtype and IL-6-treated mice [45].

Successful cell therapies have also provided insights into mechanisms of IL-6 protection. For example, cultured NSCs that were preconditioned with IL-6 prior to transplantation effectively increased angiogenesis, reduced infarct volume, and improved functional outcomes in mice subjected to ischemic stroke. Once again, the protective effects in this study were associated with SOD2 induction [46]. In a separate study investigating the splenic contribution to ischemic stroke, NSCs blocked stroke-induced elevations in IL-6 at 24 h and were found to be in direct contact with $CD11b^+$ splenic macrophages at 3 days [18]. It is noteworthy that IL-6 is secreted by macrophages in response to pathogen-associated molecular patterns (PAMPs) and other inflammatory signals. Since IL-6 was upregulated prior to maximal infarct expansion and the blunted IL-6 production was linked to macrophages, it is conceivable that selective inhibition of macrophage-derived IL-6 could be a promising strategy. Interestingly, splenocytes harvested from mice 22 h after tMCAO, but not at other time points, increased IL-6 upon activation in vitro [8]. These data suggest that splenic immune cells might be preferentially responsive at a time point consistent with delayed BBB opening and infarct expansion. In this case, it may be possible to develop a more selective and/or time-sensitive approach to targeting IL-6 signaling.

2.7 Interleukin-10 Signaling and Expression

Interleukin-10 (IL-10) is widely considered anti-inflammatory and exerts its actions through multiple mechanisms including the suppression of proinflammatory cytokines and the direct activation of cell survival pathways [47]. This cytokine binds to IL-10R, which is composed of two IL-10R1 domains and two IL-10R2 domains. The IL-10 receptor is coupled to the Jak/STAT signal transduction pathway, which is activated when IL-10 binds to IL-10R1 and initiates the signal transduction pathway through the IL-10R2 subunits [48].

Interleukin-10 blocks proinflammatory signaling by macrophages/microglia, inhibits signaling pathways activated by death receptors, and is associated with the Th2 phenotype [48]. Importantly, Th2 cells have been identified and implicated as contributors to the protective effects of anti-inflammatory compounds targeting peripheral immune cell activation following ischemia. In agreement with its role as a protective cytokine, data from human patients showed that those with worsening scores (based upon the Canadian Stroke Scale) within 48 h of ischemic stroke also showed significantly lower levels of systemic IL-10 [49]. However, the clinical data

available is inconsistent. For example, a study examining cytokine levels before and after thrombolytic therapy found a reduction in IL-10 plasma levels in the acute phase of stroke but no significant change after tPA administration [31]. These data were similar to a previous study that documented reduced IL-10 serum levels after stroke, yet no significant relationship between IL-10 levels and clinical outcomes [50].

Although circumstantial, the clinical reduction in IL-10 suggests that this cytokine may be involved in stroke pathology. Furthermore, experimental data does mirror the clinical expression profile in some ways. Unfortunately, data from these models is inconsistent and therefore difficult to interpret. In the pMCAO model of ischemic stroke, IL-10 gene transcript was reduced in rats 12 h following occlusion [51]. Conversely, a separate study demonstrated elevations in IL-10 mRNA 6 h post-MCAO in rodents.

2.8 IL-10 as a Therapeutic Target for Ischemic Stroke

The protective role of IL-10 has been documented in several experimental ischemia models. For example, IL-10 knockout mice showed larger infarct volumes after permanent focal ischemia. In the same study, primary neuronal cultures from knockout animals showed heightened susceptibility to oxygen glucose deprivation (OGD), while application of recombinant IL-10 protected these neurons from OGD [52]. A later study replicated these deleterious outcomes in mice lacking the IL-10 gene [21] while a recent study showed that the beneficial effects of ischemic preconditioning were negated in mice treated with IL-10R blocking antibodies prior to myocardial infarction [53].

In contrast to the previously mentioned knockout study, a separate study showed only a modest effect on infarct volume in IL-10 knockout mice, with slight increases in infarct volume and neurological deficits. Interestingly, several proinflammatory cytokines were elevated 4 days after stroke but other putative anti-inflammatory proteins were increased between 4 and 7 days after ischemia [54]. Taken together, these data once again demonstrate the caveats associated with targeting cytokine signaling. In this case, it would appear that both compensatory responses and temporal effects are critical in cytokine regulation of the ischemic microenvironment.

Other recent studies have shown that regulatory immune cells, particularly T-regulatory (T-reg) and B-reg cells, may suppress proinflammatory pathways that exacerbate neural injury following stroke [15, 21, 55, 56]. B-cell-deficient mice showed increased neural injury and behavioral deficits relative to controls [57]. A follow-up study employed adoptive transfer of IL-10-enriched, splenic B-cells stimulated with LPS in vitro. These cells were administered intravenously to mice 1 day prior to tMCAO. Data showed reduced infarct volumes that were accompanied by reduced extravasation of T-cells and monocytes into the brain parenchyma [58].

These data are consistent with previous reports documenting the success of therapies boosting IL-10 signaling. For example, IL-10 overexpression in neuroglia and

brain endothelial cells reduced infarct volume in mice after pMCAO [59], and viral-mediated i.c.v. gene transfer of IL-10 reduced infarct volume after pMCAO and dampened hippocampal injury after global ischemia in rats [60]. Importantly, the selective targeting of IL-10 signaling may lie in the targeting of specific IL-10-expressing immune cell types, rather than the pan activation of IL-10 signaling pathways. Gene transcript for several cytokines discussed in this chapter, including IL-10, was downregulated within the ischemic brains of SCID mice that are deficient in functional lymphocytes [15]. A recent study using IL-10 knockout mice and Rag2-deficient mice, which lack mature lymphocytes, suggested that IL-10 is required for CD4+ T-cells to facilitate protection in a rat model of facial nerve axotomy [61]. The authors linked protective IL-10 signaling to Th2 cell activation based upon these data and a previous report demonstrating the neuroprotective effects of Th2-type CD4+ cells [62].

Although plausible, questions remain regarding the selective targeting of IL-10 and the immune cell phenotype(s) that should be targeted following cerebral ischemia. Since CD4+ T-cells include distinct T-reg phenotypes and also represent both Th1- and Th2-type lymphocytes, it is difficult to ascertain a precise cellular target from these studies alone. Instead, it may be more beneficial to target a specific time point following ischemia. The preponderance of evidence concerning IL-10, and in fact all cytokines that influence the fate of ischemic tissues, indicates that the timing after insult may hold the key to successful therapeutics.

2.9 Interferon-Gamma Signaling and Expression

Interferon-gamma (IFN-γ) is an important cytokine involved in innate immune responses and has also been identified as a potential therapeutic target for ischemic stroke treatment. Due to its potent proinflammatory effects, IFN-γ may be involved in several aspects of ischemic injury. The IFN-y receptor exhibits ubiquitous expression and is found on virtually all cell types. Similar to IL-10, the receptor complex consists of two IFNγR1 domains that confer IFN-γ binding and two IFNγR2 domains that mediate signal transduction through Jak/STAT phosphorylation. Lymphocytes are major producers of IFN-γ, which primarily targets macrophages to protect against pathogens through chemokine production and secretion [63].

In terms of regulation, IFN-γ production is increased through positive feedback by virtue of its own expression, as well as through the actions of IL-12. The net result of IFN-γ signaling can include a multipronged inflammatory milieu created through the induction of MCP chemokines, B-cell activation, and ROS production. These events, in turn, facilitate a switch in microglia/macrophages toward the M1 phenotype and priming of naïve T-cells toward the Th1 phenotype [60]. Since IFN-γ elicits such profound effects after insult, the expression levels of this cytokine have been investigated both clinically and in preclinical stroke paradigms.

Data from multiple studies has shown increased IFN-γ production at various time points following ischemic stroke. For example, IFN-γ mRNA was increased

2 days following tMCAO and remained elevated 6 days after stroke [51]. Another study found elevated plasma levels of IFN-γ 24 h after tMCAO that returned to control levels by the 4-day time point [16]. Consistent with a role in cerebral infarct expansion, IFN-y increased 72 h after stroke in the brains of rats subjected to pMCAO [64] and mice subjected to tMCAO [65]. Thus, a role for IFN-γ in post-stroke signaling is evident according to data from studies employing multiple models and species.

A hallmark of stroke, both clinically and in animal models, includes infiltration of peripheral leukocytes into ischemic brain tissue. The extravasation of peripheral immune cells results from several processes, including proteolytic cleavage of BBB proteins by MMPs, induction of endothelial adhesion molecules, recruitment of polymorphonuclear cells, and extravasation of other immune cell types through a compromised BBB [33]. With regard to immune cell populations, the spleen is a known mediator of systemic inflammation and has been shown to contribute to ischemic injury in multiple tissues, including brain. Indeed, splenic IFN-γ gene transcript was increased 22 h after focal ischemia in rodents [15].

One major source of IFN-γ is natural killer (NK) cells, which were aptly named due to their ability to destroy cells infected by pathogens. These immune cells were detected in the brains of ischemic stroke patients and peaked within 2–5 days after onset. In the same study, a mouse model of pMCAO was utilized to further examine NK cell infiltration and cytokine expression. These data showed that IFN-γ-expressing NK cells peaked in the ischemic mouse brain 12 h post-stroke, while the interferon-inducible chemokine IP-10 was detected at both acute and delayed time points after stroke [66]. IP-10 expression is induced by IFN-γ and facilitates the Th1 inflammatory response through dual actions, including the direct activation of the CXCR3 receptor on Th1 cells and antagonism of the CCR3 receptor present on Th2 cells. Thus, IP-10 induction through IFN-γ signaling can amplify the Th1 response and dampen the Th2 response simultaneously, resulting in perpetuated tissue inflammation [67]. Importantly, T-cells and NK cells upregulate IFN-γ in response to signals from activated macrophages. In ischemic tissues, these macrophages are activated through toll-like receptors (TLRs) seated on the plasma membrane. In addition to PAMPs, which are the typical TLR ligands in cases of infection, these receptors also bind proteolytic fragments and epitopes of intracellular proteins that are released following cellular necrosis. These signals trigger macrophage secretion of cytokines that ultimately drive the production of IFN-γ in NK cells and T-cells. Thus, there are multiple ways in which IFN-γ production is induced and perpetuated following brain ischemia.

2.10 IFN-γ as a Therapeutic Target for Ischemic Stroke

By virtue of the mechanisms through which IFN-γ is activated, the microenvironment resulting from cerebral ischemia is ripe for IFN-γ production and signaling. Likewise, the fact that IFN-γ is a major orchestrator of the proinflammatory Th1

response suggests that tissue injury resulting from peripheral immune cell actions may be due, in large part, to IFN-γ signaling. Interestingly, there is some clinical data that indirectly supports this mechanism. Patients that developed post-stroke infections exhibited worse outcomes than their non-infected counterparts regardless of stroke severity. These outcomes were also linked to Th1 responsiveness to the brain antigens myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP), which come into contact with T-cells as a consequence of BBB disruption, and patients with elevated Th1 responses 3 months post-stroke showed worsened outcomes [68]. Although these data are far from a smoking gun, they do support the notion that IFN-γ exerts actions involving immune cell modulation of ischemic tissue injury.

In terms of therapeutic potential, multiple lines of evidence support the selective targeting of IFN-γ as means of mitigating ischemic stroke injury. In one study, administration of simvastatin reduced infarct volume and IFN-γ levels in mice subjected to tMCAO. Interestingly, protection was also afforded by splenectomy, including reduced levels of IFN-γ in brain, and these effects were negated in splenectomized mice that received adoptive transfer of splenocytes [65]. These data linked neuroprotection with peripheral splenic immune cells and IFN-γ signaling, as did reports from other laboratories. For example, i.c.v. administration of IFN-γ blocking antibodies reduced infarct volume following tMCAO in mice. In this study, removal of protective T-reg cell populations augmented the activation of resident microglia and infiltration of T-cells, which produced IFN-γ [21].

Consistent with a peripheral source of IFN-γ, levels of this cytokine were elevated in the spleen 24 h following pMCAO while removal of the spleen reduced neural IFN-γ expression by 72 h [64]. Additionally, splenocytes harvested from mice subjected to focal ischemia demonstrated an increased capacity for IFN-γ production. In similar experiments involving harvested splenocytes but assessing the efficacy of HUCB cell therapy, data showed that protective doses of HUCB cells also reduced splenocyte proliferation and production of IFN-γ in vitro upon stimulation with concovalin A [28]. The ability of protective therapies to blunt IFN-γ signaling was also documented following administration of CART in mice subjected to tMCAO. Data showed that CART administration at the time of reperfusion reduced infarct volume and IFN-γ plasma levels 24 h after reperfusion. In addition, harvested splenocytes failed to produce IFN-γ following artificial stimulation in vitro [16].

Neuroprotection studies and in vitro assays have expanded our understanding of cytokine signaling and the potential for selective targeting in brain disease, including ischemic stroke. Knockout studies are another valuable tool for identifying mechanisms of action and have been utilized to explore the role of IFN-γ in cerebral ischemia. Mice lacking IFN-y have decreased infarcts compared to WT mice, consistent with a role in exacerbating neural injury. Interestingly, these knockout mice exhibit infarcts that are similar in magnitude to Rag1 knockout mice lacking T-cells and B-cells [69], suggesting a prominent role for peripheral immune cell-derived IFN-γ. In separate studies utilizing a variety of knockouts for IFN-γ and various immune cell populations, the protection afforded by deletion of RAG1 was reversed

by adoptive transfer of splenocytes from wildtype mice, but only partially restored upon transfer from IFN-$\gamma^{-/-}$ mice. Although none of the mice showed improvements in neurological scores [69], these experiments linked splenic IFN-γ to brain infarction following ischemic stroke. Another study utilizing SCID mice, a transgenic line similar to Rag1$^{-/-}$ in T- and B-cell deficiency, found reduced cortical infarction in SCID mice 22 h after tMCAO that was accompanied by elevations in splenic IFN-γ mRNA [15].

Taken together, these data provide strong support for selective targeting of IFN-γ and/or immune cell targets that are primarily responsible for IFN-γ-mediated infarct expansion. Although experimental models have improved our understanding of the key players regulating the proinflammatory microenvironment, the complexity of this environment mandates a cautious, deliberate approach. Future studies will determine whether IFN-γ can be modulated effectively to expand the therapeutic window for ischemic stroke treatment.

2.11 Leukemia Inhibitory Factor Signaling and Expression

Leukemia inhibitory factor (LIF), although far less studied compared to the other cytokines discussed in this chapter, may hold important therapeutic potential for the treatment of ischemic stroke and other neurological disorders. Under normal conditions, LIF is instrumental in facilitating cellular functions related to proliferation, differentiation, and cell survival. Under pathological conditions, however, LIF appears to be beneficial through its ability to induce antioxidant- and prosurvival-related gene expression [70]. This anti-inflammatory cytokine is a member of the IL-6 cytokine family [71] and binds to a heteromeric receptor dimer consisting of a LIF-binding chain (LIFR) and a converter subunit (gp130). Receptor activation by LIF can transduce signaling of multiple pathways, particularly those regulated by MAPK [72], phosphatidylinositol-3-kinase (PI3K)/Akt [73], and JAK/STAT [74, 75].

To date, few studies have conducted detailed investigations of LIF expression following brain ischemia. Nevertheless, LIF expression has been documented both clinically and experimentally to some degree. Postmortem tissues from human stroke patients revealed increased LIF expression in peri-infarct zones that localized to neurons and endothelial cells, while LIF plasma levels were found to be reduced within the first 6 h following ischemia. Alterations in LIF expression were also documented in rats after MCAO, with greatest levels detected 90 min post-stroke [76]. In response to experimental cortical lesion, rats showed a 30-fold increase in astrocytic LIF mRNA relative to controls. Specifically, LIF gene transcript was elevated as early as 6 h post-insult and reached its peak at the 24h time point. Closer examination revealed an expression pattern localized predominantly to astrocytes, along with a limited population of microglial cells [77].

The known protective role of astroglia following neural injury, coupled with the induction of LIF gene transcript, suggests that this cytokine may be instrumental in

the endogenous response to injury. The ability to upregulate antioxidant gene expression would be highly beneficial following brain ischemia, as free oxygen and nitrogen radicals are major contributors to the acute phase of injury. Similarly, the ability to bolster expression of prosurvival genes would be beneficial at both early and delayed time points following stroke. With this in mind, the following section summarizes key findings related to the therapeutic potential of targeting LIF for ischemic stroke treatment.

2.12 LIF as a Therapeutic Target for Ischemic Stroke

The prosurvival pathways elicited by LIF, particularly the PI3K/Akt pathway, are now regarded as promising targets for the treatment of any injury involving oxidative injury, tissue necrosis, and apoptotic signaling. The promise of LIF has been demonstrated in several experimental injury models, including those that produce white matter injury. Data showed that exogenous LIF administration reduced demyelinating injury in experimental autoimmune encephalomyelitis (EAE), a model that recapitulates white matter damage reminiscent of multiple sclerosis, by preserving oligodendrocytes (OLs) [78]. Additionally, LIF effectively improved outcomes following spinal cord transection in mice [79]. The protection afforded in the latter case was attributed to activation of JAK/STAT and Akt signaling and was also associated with the induction of anti-apoptotic proteins. Similarly, LIF reduced motor nerve degeneration in the SOD1 G93A murine model of familiar amyotrophic lateral sclerosis (ALS) [80].

Importantly, the benefits of LIF treatment observed in these earlier studies have now been extended to models of cerebral ischemia. In these recent studies, similar pathways appear to mediate the protective effects of LIF. Data showed that LIF effectively decreased infarct volume and white matter injury when administered to rats following pMCAO. In addition to mitigating neural injury, LIF treatment also improved functional outcomes. Further investigation revealed a mechanism operating through Akt-mediated induction of the antioxidant protein peroxiredoxin 4 (Prdx4), as LIF efficacy in protecting OL primary cultures from OGD was negated by co-incubation with Akt inhibitors and Prdx4 neutralizing antibodies [81]. Although neuronal cultures were not used in these studies, neurons and OLs are both susceptible to oxidative injury and therefore benefit from the induction of antioxidant proteins in the wake of ischemia. Likewise, stroke injury includes OL cell death and neuronal injury results, in part, from anoikis (detachment-induced cell death) following the disruption of astroglial junctions.

The therapeutic potential of LIF, although promising, lies in the ability to target LIF signaling at time points prior to maximal infarction. Since the infarct expands from hours to days following stroke onset, it is likely that the therapeutic window can be extended providing that the LIF safety profile is acceptable. To date, LIF has been used clinically for separate indications and has demonstrated a good safety profile. Nevertheless, a better option would be to stimulate the endogenous pathways

activated by LIF (i.e., Akt signaling) as means of circumventing potential issues with safety and dosing. Since LIF can be released by several cell types following injury [77, 82, 83], it will be crucial to characterize the sources of endogenous LIF signaling. Once the temporal and cell-specific responses are uncovered with regard to LIF signaling, there will be more therapeutic options to mimic the endogenous protection afforded by this cytokine.

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