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Antimicrobial Protein REG3A and Signaling Networks are Predictive of Stroke Outcomes

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Abstract

Regenerating Family Member 3 Alpha (REG3A) is a multifunctional protein with antimicrobial activity, and primarily secreted by the intestine and pancreas. Studies have shown an increased expression of REG3A in systemic inflammatory responses to acute injury and infection, but studies investigating REG3A during the pathogenesis of ischemic stroke are limited. The aims of this study were to examine the associations between arterial expression of REG3A and other arterial inflammatory proteins implicated in stroke pathogenesis, as well as associations between REG3A and markers of poor outcome for ischemic stroke. The University of Kentucky Blood and Clot Thrombectomy Registry and Collaboration (BACTRAC) protocol (clinicaltrials.gov NCT03153683) utilizes thrombectomy to isolate intracranial arterial blood (i.e. distal to thrombus)

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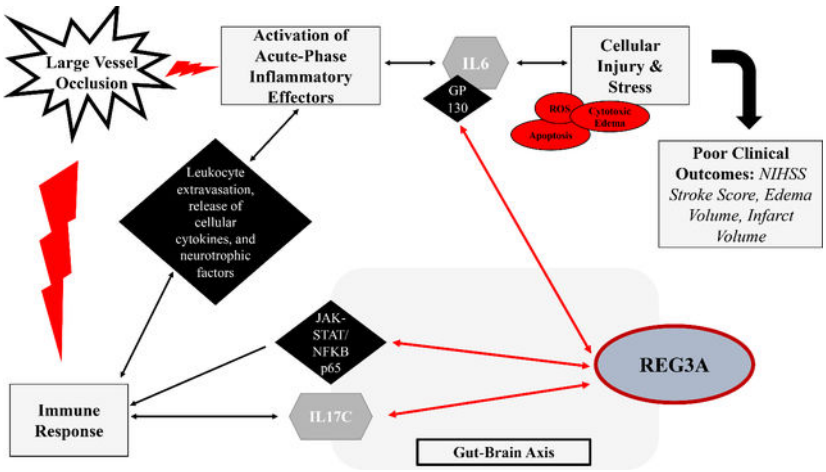
Disclosures
Drs. Keith Pennypacker, Justin Fraser, and Ann Stowe are co-owners of Cerelux, LLC. Dr. Fraser is a consultant with Stream Biomedical, Penumbra, and Medtronoic. Dr. Fraser is an equity holder of Fawkes Biotechnology.

and systemic arterial blood (i.e. carotid). Samples were analyzed by Olink Proteomics for N=42 subjects. Statistical analyses of plasma proteins included 2-sample t-tests, spearman and biserial correlations, and robust regression models to elucidate network signaling and association to clinical outcomes. Results indicated that levels of systemic REG3A were positively correlated with inflammatory proteins interleukin IL6 (R=0.344, p=0.030) and IL17C (R=0.468, p=0.002). 2-sided t- tests examining differences of systemic REG3A within quartiles of NIHSS admission score depicted significant differences between quartiles. Those with NIHSS scores corresponding to moderate and moderate-severe neurofunctional deficits had significantly higher levels of systemic REG3A compared to those with NIHSS scores corresponding to mild and mild- moderate neurofunctional deficits (p=0.016). STRING analyses of proteins in each robust regression model demonstrated substantial networking between REG3A and other systemic proteins highly relevant to ischemic stroke. The present study provides novel data on systemic REG3A in the context of ischemic stroke. These results demonstrate the influential role of REG3A regarding surrogate functional and radiographic outcomes of stroke severity. Additionally, they provide novel insight into the role of REG3A and related proteins during the complex neuroinflammatory process of ischemic stroke. These data provide a foundation for future studies to investigate REG3A and related networking proteins as potential biomarkers with prognostic potential, as well as potential therapeutic targets.

Graphical Abstract



We identified multiple mechanisms by which anti-microbial protein Regenerating Family Member 3 Alpha (REG3A) participates in the pathophysiologic response to large-vessel ischemic stroke. REG3A interacts with inflammatory cytokine Interleukin-6 (IL6) and immunomodulatory protein Interleukin-17C (IL17C) to induce other extracellular and intracellular effectors responsible for both driving and attenuating inflammation. These novel findings contribute to the ongoing investigation of inflammation and therapeutic strategies sequela of ischemic stroke.

Introduction

Stroke is a leading cause of death in the United States, of which 87% are ischemic (Virani, Alonso et al. 2020). Emergent large vessel occlusions (ELVOs) comprise 24%−46% of

all acute ischemic strokes (AIS), and are a cause of high morbidity and mortality if not urgently treated (Ramiro, Simats et al. 2018, Lövblad, Bouchez et al. 2019). Despite many advancements to elucidate stroke pathology, diagnostic and therapeutic options remain limited.

Studies have shown a complex dynamic between the brain and the gastrointestinal system in the pathogenesis of stroke (Selvaraj and Stowe 2017, Krishnan and Lawrence 2019). A common thread between the gut-brain, immune, and brain inflammatory responses are the acute-phase proteins, chemokines, and cytokines introduced at the time of stroke (Selvaraj and Stowe 2017, Krishnan and Lawrence 2019). One such molecule, Regenerating Family Member 3 Alpha (REG3A), also referred to as Pancreatitis Associated Protein (PAP1/PAP) and Hepatocarcinoma-Intestine- Pancreatic Protein (HIP), is an acute-phase inflammatory and antimicrobial protein that was previously reported to be elevated systemically in arterial blood sampled during mechanical thrombectomy (MT) of BACTRAC subjects (Maglinger, Frank et al. 2020). In non-pathologic states, REG3A is produced within the entero-hepatic system and is variable expressed within central nervous tissue (Uhlen, Fagerberg et al. 2015). There is limited information on REG3A in stroke pathology, which is further confounded with inconsistent nomenclature (Liu and Cui 2007, Harari and Liao 2010, Chen, Downing et al. 2019). Although limited, there is strong data that used rodents to model central nervous system (CNS) injury, such as traumatic brain injury (TBI), and have found that REG3A was upregulated at the site of injury. Consequent upregulation was associated with neuroprotective effects by the mechanism of attenuating excitotoxicity and oxidative stress(Haldipur, Dupuis et al. 2014, Chen, Downing et al. 2019). Although REG3A has not been discussed in context of human stroke, REG3A is associated with cellular mediators, IL6 and the IL17 family, which are known drivers of neuroinflammation in stroke pathogenesis (Vila, Castillo et al. 2000, Ampo, Suzuki et al. 2009, Harari and Liao 2010, Hilz, Moeller et al. 2011, Chen, Downing et al. 2019, Xie, Li et al. 2019). Both IL6 and IL17 directly interact with and upregulate the activity of REG3A (Bordon 2011, Chen, Downing et al. 2019). As such, investigating associations between key neuroinflammatory drivers and REG3A can provide insight into signaling networks between peripheral blood and the brain to discern both positive and negative effectors of stroke outcomes. In the current study, we examined REG3A in relationship with cytokine responses in systemic plasma samples as well as to the clinical outcomes of infarct volume and edema, and neurologic impairment.

Methods

Measures

The current study received ethics approval per IRB protocol number 48831 and was not pre-registered. Similar to the study design published by Maglinger, Frank et al. 2020, this study adopted a prospective study design and was exploratory in nature. No randomization procedures were applied and since the study cohort was derived from all enrolled subjects within the BACTRAC biobank, no sample calculation was performed. The image interpreter for measuring infarct and edema volume was blinded to all patient data besides diagnostic CT-head imaging leading up to mechanical thrombectomy. Inclusion criteria were patients

undergoing mechanical thrombectomy for large-vessel occlusion stroke. Exclusion criteria included prisoners, pregnant individuals, subjects <18 years-old, and patients with a past medical history of Moyamoya disease. Demographic data including age, sex, body mass index (BMI), hypertension (HTN), Type 2 Diabetes Mellitus (DM2), hyperlipidemia, and smoking status were collected on each subject. Normalized protein expression (NPX) was the unit of measurement for protein levels per which is based on a Log2 scale (Olink Proteomics, Boston, MA). Computed tomography angiography (CTA) scores were assigned during imaging analysis and indicated the level of perfusion associated with the occluded vessel. A score of 0 represents 0% perfusion of vessel; a score of 1 represents <50% perfusion of vessel; a score of 2 represents ≥ 50% perfusion.

Statistical Analyses

The infarct volume, infarct edema volume, mortality, the NIHSS Score on admission, CT angiography score (CTA score), and time from last known normal till thrombectomy (LKN) were analyzed as the main outcomes. Data diagnostics were performed examining all 42 subjects. Two subjects had significantly smaller infarct volumes (448mm^3 and 560mm^3 compared to a mean of 59,739 ± 74,880 mm^3) and edema volumes (0mm^3 and 220mm^3 compared to a mean of 60,529mm^3). These two patients were excluded from 2-sided t-tests, spearman correlations, and pairwise comparisons due to being extreme outliers, (N=40). However, these subjects were retained while performing robust methods since the weight of skew is considered during analysis. This is discussed in detail later in the methods.

The relationship between systemic REG3A and clinical outcomes such as infarct and edema volume, protein expression of systemic IL6 and IL17C, and potential confounders (e.g., sex, last known normal (LKN), CTA, etc.) were examined using spearman correlations. For analytic purposes, the NIHSS admission scores were broken down into statistical quartiles and were labeled as ‘minor stroke,’ moderate stroke,’ ‘moderate to severe stroke,’ and ‘severe stroke.’ Statistical quartiles were then analyzed between groups using 2-sided t-tests to identify inter-group differences. Quartiles were also examined by combining ‘minor stroke’ and ‘minor to moderate stroke’ and quartiles corresponding to ‘moderate to severe stroke,’ and ‘severe stroke’. Since multiple studies have demonstrated a positive correlation between inflammatory proteins and age (Weimar, Konig et al. 2004, Saposnik, Guzik et al. 2013), 2-sided t-tests were used to examine differences of age between NIHSS score quartiles.

To hypothesize potential proteins REG3A may network with in the context of stroke outcomes, a linear regression analysis was utilized. Firstly, regression diagnostics were performed. The presence of several outliers for infarct and edema volume, as well as high leverage values for some of the proteins were identified. Variables with non-normal distribution were log-2 transformed (Shapiro-Wilk; $p < .05$). Due to the presence of several outliers, robust regression analyses were utilized to examine the associations between REG3A, proteins, and infarct and edema volume. The advantage of using robust regressions is that the approach accommodates the inclusion of outliers with high leverage values. As such, all subjects (N=42) were considered in the robust analyses. Given these results, it was determined that a robust regression with MM (maximum) estimation would be used. In this

method, observations with high outliers and leverage values are given less weight in the analysis. Additionally, there are concerns that the number of subjects per variable within a linear regression model should be between five and twenty(Austin and Steyerberg 2015). However, a recent study found that low bias and accurately standard error estimates are achievable with as little as two subjects per variable (Austin and Steyerberg 2015). For the current study, a subject per variable ratio of over 4 was determined to diminish the risk of overfitting. In order to determine the set of proteins accounting for the most variability in infarct and edema volume, a forward selection approach was used. In the first step, REG3A was entered as a predictor. In the subsequent steps, the protein with the next strongest association with infarct or edema volume was entered, until 10 additional proteins were selected. A final model using REG3A and the additional 10 proteins was then used as input for protein-protein interaction analysis. For this analysis, the Benjamini-Hochberg procedure was used to control the false discovery rate at .05. For all other analyses, a p-value less than .05 was considered statistically significant. Proteins within the final model were entered into STRING V11 (https://string-db.org/) to generate protein-protein interaction networks. Networks were expanded as needed to observe downstream protein neighbors. Data analyses were performed using IBM SPSS Statistics Version 27 2020, and SAS v 9.4 (SAS Institute Inc, Cary, NC).

Results

Patient Demographics

40 subjects, 24 (60%) were female, were included in the study with a median age of 67 (25–96). 17 subjects were overweight and 7 were obese. Per category guidelines of the NIHSS, 1 (3%) patient had a minor stroke, 15 (38%) patients had scores corresponding to a moderate stroke, 11 (28%) were considered to have a moderate to severe stroke, and 12 (31%) had a severe stroke on admission. NIHSS statistical quartile and combined quartile descriptions are provided in Table 1-2. The mean LKN to thrombectomy completion time was 639 ± 367 minutes and the mean infarct volume was 59,739 ± 74,880 mm^3. Results of the findings and baseline characteristics are provided in Table 1-1.

REG3A versus Point-of-Care Variables

We examined the proteomic expression of arterial REG3A in context to sex, CTA score, and LKN as potential confounders. OLINK protein levels were reported as an arbitrary value designated as NPX. Results of 2-sided t-tests examining confounders demonstrated no significant differences in the levels of arterial REG3A between sexes ($p=0.865$, $df=38$, $t=0.171$) T-tests demonstrated no significant difference in REG3A levels and those with 0% and <50% perfusion and 0% perfusion and ≥ 50% perfusion, ($p=0.199$, $df=36$, $t=-1.31$). However, when examining the results of the biserial correlation, levels of REG3A were weakly correlated with degree of perfusion ($R= -0.316$, $p=0.053$). Spearman correlation examining the association between REG3A expression levels and LKN demonstrated no significant association ($R = -0.050$, $p > 0.05$)

REG3A and NIHSS Score

The NIHSS Score on admission was examined in multiple ways. Firstly, systemic REG3A was examined between NIHSS Admission score quartiles. T-tests demonstrated no inter-quartile differences of systemic REG3A between subjects with 'Minor' and 'Moderate-Severe' scores ($p=0.643$, $df=18$, $t=-0.471$). However, those with scores corresponding to 'Minor Stroke' had significantly lower levels compared to those weight scores corresponding to 'Moderate Stroke' ($p=.014$, $df=17$, $t=-2.73$). A near significant difference was observed between subjects with scores corresponding to 'Minor Stroke' and 'Severe' strokes. Those with scores corresponding to 'Minor Stroke' had near significantly lower levels of REG3A compared to those with scores corresponding to 'Severe Stroke' ($p=0.055$, $df=11$, $t=-2.15$). In examining the association between quartiled NIHSS Admission scores and average age between quartiles, there were no significant inter-quartile differences between 'Minor' and 'Moderate' ($p=0.238$, $df=17$, $t=-1.22$), 'Minor' and 'Moderate-Severe' ($p=0.494$, $df=18$, $t=-0.699$), and 'Minor' and 'Severe' ($p=0.067$, $df=18$, $t=-1.951$). These data are illustrated in Figure 2A. NIHSS Scores on admission were also examined as two groups by combining 'Mild' and 'Mild-Moderate' quartiles, and combining 'Moderate-Severe' and 'Severe' quartiles. Results indicate that subjects within the combined 'Moderate-Severe and Severe' quartiles had significantly higher levels of systemic REG3A compared to subjects who fell within the 'mild and mild-moderate' NIHSS score quartile ($p=0.016$, $df=34$, $t=2.54$). These results are illustrated in Figure 2B.

REG3A, Inflammatory Proteins and Stroke Outcomes

Spearman correlations examining levels of systemic IL6 and IL17C with REG3A are shown in Figure 1. Levels of REG3A, IL6, and IL17C were reported in arbitrary units NPX. This figure demonstrates a positive correlation with systemic levels of IL6 and IL17C with REG3A ($R=0.344$, $p=0.030$; $R=0.468$, $p=0.002$, respectively). Spearman correlations examining associations between levels of systemic REG3A, infarct volume, and edema volume found that REG3A did not indicate any significant correlation with either infarct or edema volume ($R=0.226$, $p=0.160$; $R=0.176$, $p=0.276$, respectively).

REG3A and Network Proteins

Robust regressions were used to determine associated network proteins, that, when analyzed along with REG3A, were shown to be predictive of infarct and edema volume. Table 2-1 lists the top ten proteins determined by robust regression analysis to be most predictive of each outcome in the context of REG3A. The predictive model of infarct volume had an $R^2= 0.592$, $p < 0.05$ and the predictive model for edema volume had an $R^2= 0.673$, $p < 0.05$. The final model using REG3A and the additional 10 proteins was used as input for protein-protein interaction analysis (Table 2-2). The final model representing infarct volume shows that REG3A does not significantly contribute to predicting infarct volume when controlling for multiple comparisons, however, its significance within the model for edema volume is retained ($R^2= 0.592$, $p < 0.05$, and $R^2= 0.673$, $p < 0.05$ respectively). Since the relationships provided by the regression were statistically derived, proteins were input into STRING to examine the existence of functional relationships. Additionally, to elucidate potential intracellular mechanisms by which REG3A may mediate neuroinflammation, we examined

the intracellular pathways listed with STRING. In looking at the STRING diagram, a line between two protein nodes represents a functional relationship between each protein as reported in the literature. Protein names and functions as reported in the literature reported from STRING networks are reported in Table 3. A map of protein-protein interactions for each outcome and the top ten biologic functions for each network is shown in Figure 3. Functions highlighted from these analyses include ‘inflammatory response,’ ‘leukocyte migration,’ ‘immune response,’ and ‘defense response.’ Intracellular pathways such as JAK/STAT, EGFR, PI3K/AKT, MAPK, and ERK were listed as signal pathways in both models. Collectively, these data depict inter-networking relationships with REG3A and other arterial proteins at the time of stroke. To our knowledge, we are the first to report on the role of REG3A in the context of ischemic stroke. This study is an example of how modern neurointerventional procedures coupled with advanced statistical and analytical methods may be used to improve our understanding of the biological and cellular changes occurring during ischemic stroke in human patients. Our main focus was to investigate the involvement of REG3A in proteomic networks and how functional relationships and related proteins drive the pathophysiology of ischemic stroke. First, we report that possible confounders such as sex and time, did not significantly contribute to the relationships between outcomes and REG3A. However, we observed that higher vessel perfusion is associated with lower levels of systemic REG3A. To create a foundation for understanding REG3A in context of stroke, we selected two prototypical inflammatory cytokines known to be important markers in stroke outcome, IL6 and IL17, which have been reported to interact with REG3A (Haldipur, Dupuis et al. 2014, Girijala, Sohrabji et al. 2017, Li, Zhu et al. 2018, Chen, Downing et al. 2019). We found that levels of REG3A-IL6, and REG3A-IL17 were positively correlated with one another. In examining REG3A and the NIHSS Score on admission, we found that REG3A levels were significantly higher in those within the moderate- severe category compared to the mild category. Interestingly, levels of arterial REG3A and infarct or edema volume were not significantly associated but, when in a network of proteins, REG3A was strongly predictive of both outcomes. REG3A is well-studied in its regulatory role of immune cells and release of inflammatory cytokines in carcinogenesis (Hans, Kossmann et al. 1999, Haldipur, Dupuis et al. 2014, Zhang, Wang et al. 2019). This shows that REG3A is involved in both inflammatory and cell survival processes. However, few studies report functions of REG3A within the nervous system or during CNS injury. Originally, Ampo et al. reported that the family of pancreatitis associated proteins including REG3A responded to TBI and neuroinflammation (Ampo, Suzuki et al. 2009). In complimenting studies, REG3A was found expressed within neurons of the forebrain, midbrain, and hindbrain in rodents and in post-mortem human brain tissue from patients who died from severe TBI (Collins 2012, Haldipur, Dupuis et al. 2014, Li, Zhu et al. 2018). These studies conducted by Haldipur et. al. and Marz-Weiss et. al. demonstrated a positive association between increased levels of REG3A with neuron survival and regrowth in subjects with CNS injury. These effects were hypothesized to result from REG3A-mediated diminution of reactive oxygen species (ROS), release of pro-inflammatory cytokines, and capacity to act as a long-lasting neurotrophic factor for central neurons (Marz-Weiss, Kunz et al. 2011, Haldipur, Dupuis et al. 2014). These studies indicate that REG3A, irrespective of the discussed pathologies, promoted cellular protection and survival in diverse contexts of inflammation and cellular injury.

Sex differences are known to be important in stroke (Girijala, Sohrabji et al. 2017); however, we did not find any significant associations between sex and levels of systemic REG3A. Since REG3A is considered to be an acute-phase inflammatory protein (Chen, Downing et al. 2019), we also wanted to determine if time from LKN to thrombectomy influenced levels of systemic REG3A. Our results demonstrate that time was not a significant factor associated with systemic levels of REG3A. In addition to time, the level of vessel perfusion was important to examine since REG3A is mainly found and produced within the entero-hepatic system. Individuals with higher levels of vessel perfusion could have a systemic/arterial decrease in REG3A compared to those with lower vessel perfusion. This could be possible if there's a lesser degree of vessel perfusion, permitting more blood flow into the intracranial space. Results of the t-test comparison demonstrated no significant difference in levels of systemic REG3A based on CTA scores. Yet, results of the correlation demonstrated a decrease of arterial REG3A as vessel perfusion increased. Although this is a weak relationship, this highlights a possible mode by which entero-hepatically expressed REG3A could relocate into the intracranial space and influence neuroinflammatory processes during stroke.

The NIHSS Score is a tool utilized to assess functional and neurologic impairment of individuals with suspected stroke (Hilz, Moeller et al. 2011). Additionally, NIHSS Scores serve as a surrogate marker for functional outcomes after stroke (Hilz, Moeller et al. 2011). In examining the NIHSS Score on admission, we found that levels of systemic REG3A were significantly higher in those within the moderate-severe category compared to the mild category indicating systemic levels of REG3A sampled during MT parallel NIHSS Scores surveyed upon admission. In turn, higher levels of REG3A appear to be associated with increased functional deficits, and increased stroke severity compared to those with lower circulating levels. As such, systemic levels of REG3A within hours of a stroke has the potential as a predictive biomarker for functional recovery. Although NIHSS Scores are associated with infarct volume (Girijala, Sohrabji et al. 2017), REG3A alone was not significantly associated with infarct or edema volume. Considering how REG3A mainly acts within the entero-hepatic system, we hypothesized that REG3A may be predictive of these outcomes by networking with other systemic proteins, such as IL6 and IL17. A previous study found that IL6 and IL17 were upregulated in BACTRAC subjects (Fraser, Collier et al. 2019, Maglinger, Frank et al. 2020). In ischemic stroke, IL6 is a biomarker for poor outcomes, such as increased infarct and edema volume (Vila, Castillo et al. 2000). With these observations, we set out to examine the relationship between IL6 and REG3A in our cohort. Previous reports demonstrated that REG3A and IL6 reciprocally upregulate each other, which was found to drive the chronic inflammation observed in inflammatory pancreatic cancer (Hans, Kossmann et al. 1999, Chen, Downing et al. 2019, Zhang, Wang et al. 2019). Synergy between IL6 and REG3A has also been reported for inflammatory carcinogenesis (Zhang, Zhou et al. 2019). Similar studies examining REG3A in inflammatory carcinogenesis found that the gp130 subunit of IL6 can act as a receptor for REG3A. In turn, REG3A moderated signaling of IL6 and IL6- mediated cell signaling cascades (Hans, Kossmann et al. 1999, Chen, Downing et al. 2019, Zhang, Wang et al. 2019). Our results indicate an association between IL6 and REG3A suggesting the likelihood of IL6-REG3A interactions having a role in stroke pathology.

The cytokine family interlukin-17 (IL17) is functionally related to REG3A and IL6 (Collins 2012, Ha, Wang et al. 2014, Chen, Downing et al. 2019). IL17 helps bridge acute and long-term immune processes and shifts immune phenotypes toward T-helper cell type 17(TH17). TH17 cells produce inflammatory molecules, and in stroke pathology aids the disruption of the blood brain barrier (Li, Zhu et al. 2018). Accordingly, IL17C can exacerbate inflammation and negatively contribute to the overall neuroinflammatory phenotype in stroke (Bordon 2011, Xie, Li et al. 2019). IL17 expression is increased through IL6 intracellular signaling and in turn its activation of intracellular transduction pathways can directly induce the expression of REG3A (Ha, Wang et al. 2014, Chen, Downing et al. 2019). Lai et al. found that IL17-induced REG3A led to keratinocyte hyperproliferation and cell survival in psoriasis-like phenotypes by signaling through cell survival pathways (Lai, Li et al. 2012). Our data show that systemic REG3A expression was associated with increased levels of IL17C. The functional interactions of REG3A, IL6, and IL17 appear consistent across modes of inflammation as reported in the literature (Bordon 2011, Li, Zhu et al. 2018, Chen, Downing et al. 2019). Together, these data support established associations between REG3A and IL17, and support multiple modes by which REG3A may partake in extracellular processes that mediate neuroinflammation during the early stroke response in human patients.

To better understand the function of REG3A within signaling networks, we performed a robust regression and entered those outputs into STRING. STRING constructs protein-protein networks by applying genomic and proteomic integration algorithms that determine the significance of biological functions based on existing literature (Austin and Steyerberg 2015). These STRING outputs provide biologic snapshots of REG3A in the acute period during stroke, and specifically, the REG3A signaling network that is involved in the response to this pathological event. When we examined the functions of these proteins in this network, REG3A was found to be associated with themes such as neuroprotection and leukocyte chemotaxis. For example, CXCL5, CXCL11, CCL5, ICAM1, and MCP3 are proteins involved in leukocyte, and particularly neutrophil, chemotaxis and TH-1 inflammatory phenotype in stroke (Wang, Li et al. 1999, Denes, Humphreys et al. 2010, Rodrigues and Granger 2015, Wang, Tu et al. 2016, Koper, Kaminska et al. 2018). A study examining anti- inflammatory effects of REG3A in acute pancreatitis found that REG3A attenuated migration of PMNs (Heller, Fiedler et al. 1999). These data indicate that REG3A could be mediating early leukocyte chemotaxis to the site of infarct. CX3CL1, MBL2, F7, CSF-1, and IL13 have been reported to perform neuroprotective functions in stroke (Osthoff, Katan et al. 2011, Limatola and Ransohoff 2014, Kolosowska, Keuters et al. 2019, Hu, Li et al. 2020, Noh, Ahn et al. 2020). Together, these data show the complexity of REG3A involvement in the immune/inflammatory molecular signaling initiated by ischemic stroke.

To elucidate potential intracellular mechanisms by which REG3A may mediate neuroinflammation, we examined the intracellular pathways listed with STRING. Intracellular pathways such as JAK/STAT, EGFR, PI3K/AKT, MAPK, and ERK were listed as signal pathways in both models. REG3A has been reported to be associated with dose-dependent changes in the production of inflammatory mediators, gene transcription, and cellular proliferation (Viterbo, Bluth et al. 2008, Parikh, Stephan et al. 2012, Wang, Zhou et al. 2014, Chen, Downing et al. 2019, Zhang, Wang et al. 2019). For example, a

study reported by Wang et. al. used ingenuity network analysis (IPA) to examine signaling profiles of REG3A. Their group found that REG3A- mediated signaling through JAK/STAT, NF-κB, and PI3K/AKT signal pathways led to reduced apoptosis and increased cellular proliferation in hepatocellular carcinoma (Wang, Cheng et al. 2019). Others found that molecular effectors of intracellular signaling through IL6/JAK/STAT were upregulated during acute inflammation, but administration of REG3A dose-dependently attenuated the activity of IL6/JAK/STAT and decreased the overall inflammatory response (Viterbo, Bluth et al. 2008, Parikh, Stephan et al. 2012, Wang, Zhou et al. 2014, Chen, Downing et al. 2019, Zhang, Wang et al. 2019). Instead, REG3A signaling through JAK/STAT in epithelial cells enhanced cell survival and proliferation (Denes, Humphreys et al. 2010, Zhang, Wang et al. 2019). The findings support the likelihood that REG3A utilizes these intracellular signal pathways to modulate gene expression during acute stroke.

There are several limitations to this study. Outlier diagnostics led to the removal of 2 outliers from correlation analyses. These patients had infarct and edema volumes significantly below the median and mean. Although smaller infarcts may present with less immediate implications, removal of these data points subtracts from clinical reality. In the development of biomarkers, it would be important to consider the extremes since the ideal biomarker should be generalizable across a population. In future studies, we intend to utilize our continuously enrolling tissue bank to add patients and samples to increase the cohort size. We understand that our demographic distribution is restricted by our geographic location, but we are developing national and international collaborations to increase heterogeneity of the patient population included in our data sets. Another limitation in the interpretation of these results includes the exclusion of comorbid conditions from the analyses. A follow-up to this correspondence will examine the association and implication of REG3A to comorbidities such as hypertension, diabetes, and hypercholesterolemia. Sampling of systemic and intracranial arterial blood during mechanical thrombectomy provides a glimpse of the pathophysiologic response during ischemic stroke. Moreover, utilizing proteomics and advanced statistical modeling will identify neuroinflammatory regulators unique to the stroke microenvironment. In turn, this information can be used to identify biomarkers prognostic of stroke outcomes. Since levels of systemic REG3A were related with NIHSS on admission, future studies will further investigate this relationship within our expanding cohort.

Conclusion

In 2015, MT emerged as a new standard for treating ischemic stroke (Wardlaw, Murray et al. 2014). In addition to reducing morbidity and mortality related to stroke, this procedure has allowed for critical research into the molecular biology occurring at the time of infarct. Here, we utilized mechanical thrombectomy to sample systemic arterial plasma from patients during ELVO. To our knowledge, we are the first group to examine REG3A specifically in context of ischemic stroke. We report novel data on the relationships between REG3A and well-known inflammatory cytokines such as IL6 and IL17. Utilizing advanced proteomic network mapping, we were able to validate and identifying protein networks associated with REG3A that collectively predicted infarct and edema volume. Although preliminary, our study demonstrates REG3A and associated proteins provide starting points for biomarker

discovery, reverse translation of human data to animal studies, and the development of novel therapeutics for a devastating pathology.

Acknowledgements

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Abbreviations

ELVO	Emergent Large Vessel Occlusion
MT	Mechanical Thrombectomy
BACTRAC	Blood and Clot Thrombectomy Registry and Collabortation
REG3a	Regenerating Family Member 3 Alpha
PAP1/PAP	Pancreatitis Associated Protein
TBI	Traumatic Brain Injury
NPX	Normalized Protein Expression
BMI	Body Mass Index
HTN	Hypertension
DM2	Type 2 Diabetes
LKN	Last Known Normal
NIHSS	National Institutes of Health Stroke Score

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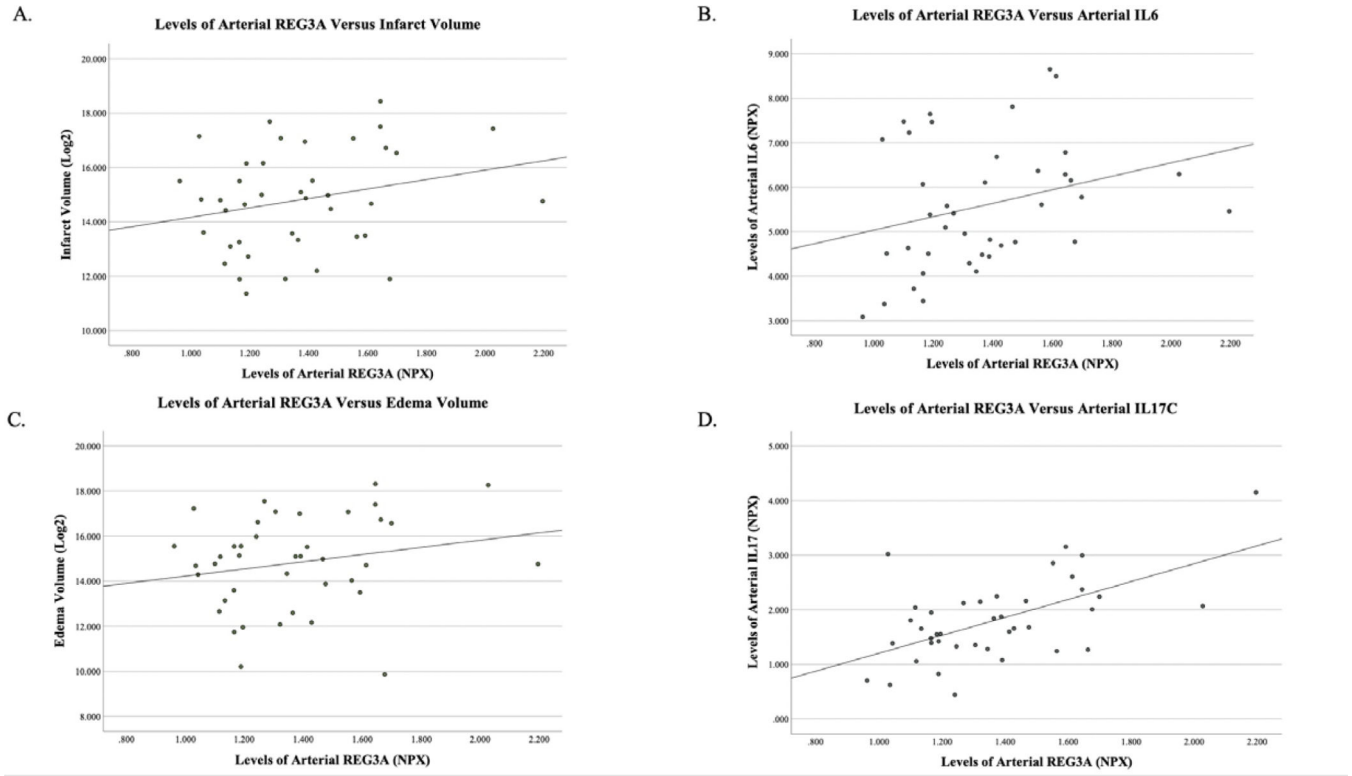

Figure 1:

Spearman Correlations of arterial REG3A versus: **(A)** Infarct volume(R=0.226, p=0.160); **(B)** Edema volume (R=0.176, p=0.276); (C) IL6 (R =0.344, p=0.030); (D) IL17C (R=0.468, p=0.002).

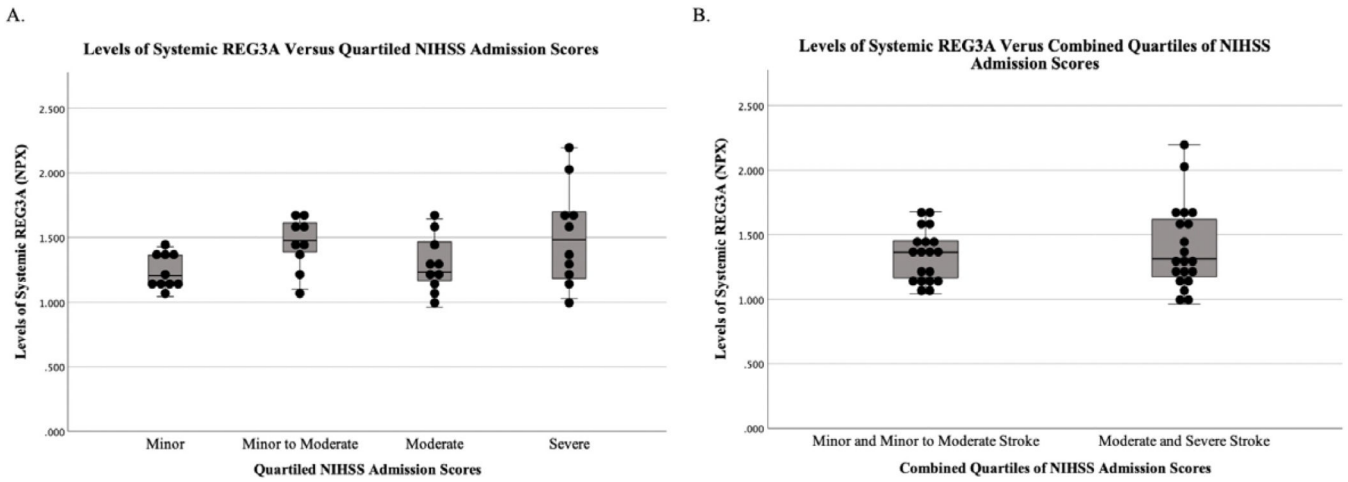

Figure 2:

Systemic levels of REG3A versus Quartiled NIHSS Scores on admission. Error bars represent standard deviation. **(A)** Illustrates statistical quartiles of NIHSS Admission Scores. T-tests found significantly lower levels of systemic REG3A in those with 'Minor' strokes compared to 'Minor and Moderate' strokes ($p=0.04$); Those with scores corresponding to 'Minor Stroke' had near significantly lower levels of REG3A compared to those with scores corresponding to 'Severe' stroke ($p=0.055$). **(B)** NIHSS Scores on admission were also examined as two groups by combining 'Minor and 'Minor-Moderate' quartiles, and combining 'Moderate' and 'Severe' quartiles. Subjects within the combined 'Moderate and Severe' quartiles had significantly higher levels of systemic REG3A compared to subjects who fell within the 'Minor-Moderate' NIHSS score quartile ($p=0.016$).

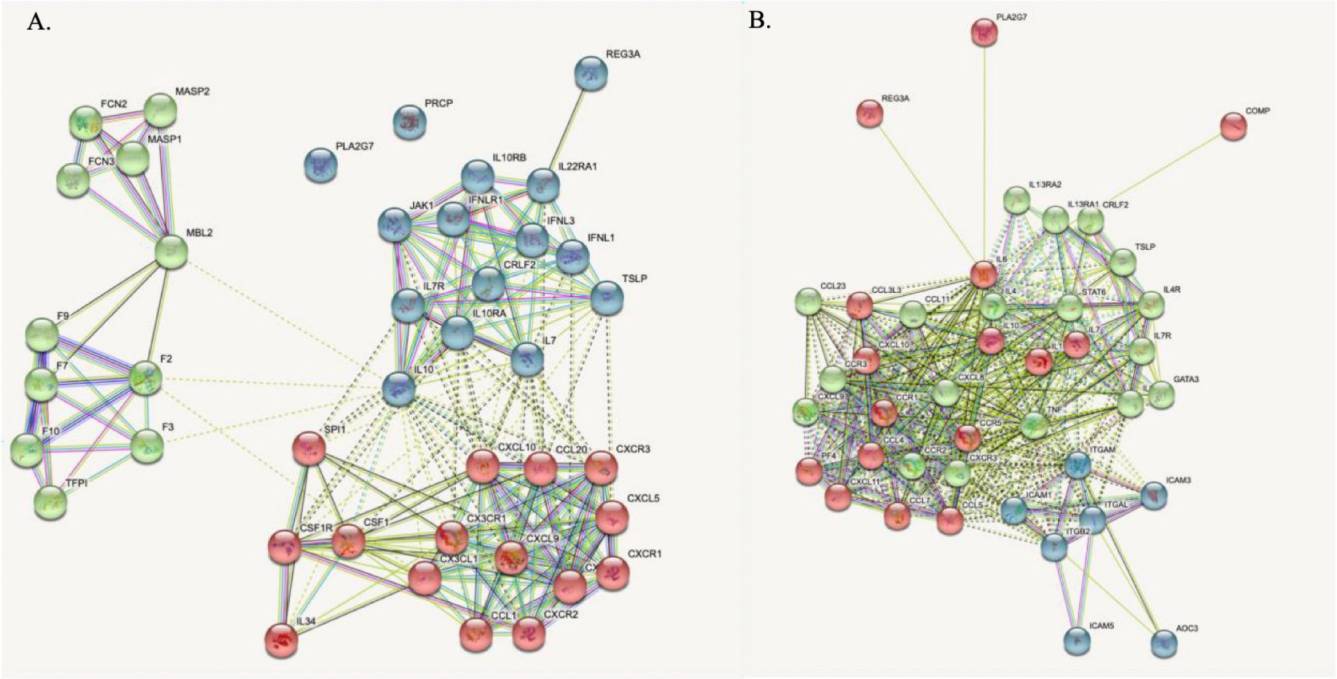

Figure 3:

STRING protein-protein network analyses for **(A)** Systemic REG3A and Infarct volume, **(B)** Systemic REG3A and Edema volume.

Table 1.

(1–1): Demographics and Characteristics for Thrombectomy Subjects.

Table (1–1)	
	Value (%)
Age (median; range)	67 (25–96)
Sex	
Female	24 (60)
Male	16 (40)
BM1	
<24.9	16 (40)
25–29.9	17 (42.5)
30–39.9	7(17.5)
>40	0(0)
Comorbidities	
Hypertension	28 (70)
Diabetes Mellitus II	9(22.5)
Hyperlipidemia	11 (27.5)
Previous Stroke	8 (20)
** **Smoking Status**	
Never	22 (58)
Currently	10 (26)
Previously (> 6 months)	6(16)
NIHSS Scores on Admission *	
Minor Stroke (1–4)	1(3)
Moderate Stroke (5–15)	15 (38)
Moderate/Severe (16–20)	11 (28)
Severe Stroke (≥ 21)	12 (31)
NIHSS Scores at Discharge **	
Minor Stroke (1–4)	13 (34)
Moderate Stroke (5–15)	17 (45)
Moderate/Severe (16–20)	5(13)
Severe Stroke (≥ 21)	3(8)
TICI Score	
2A = < 50% Perfusion	1 (2.5)
2B = > 50% Perfusion	20 (50)
3 = Full Perfusion	19(47.5)
LKN to Thrombectomy	

Completion Time (minutes) **	639.87 ± 367
Infarct Volume (mm^3)	59,739 ± 74,880
Edema Volume (mm^3)	63,549 ± 78,901
CTA Collateral Score **	
0	8(21)
1	24 (63)
2	5(13)
3	1(3)

Table (1–2)

Combined NIHSS Scores on Admission: Statistical Quartiles *	Value	Average Age	Age Range	NIHSS Scores on Admission: Statistical Quartiles *	Value	Average Age	Age Range
Minor and Minor to Moderate Stroke (1–16)	19 (47%)	63	27–87	**Minor Stroke (1–10)**	10 (25%)	61	27–87
				Moderate Stroke (1–16)	9 (23%)	64	46–83
Moderate-Severe and Severe Stroke (≥ 17)	20 (50%)	70	25–96	**Moderate/ Severe (18–21)**	10 (25)	62	35–77
				Severe Stroke (≥ 22)	10 (25%)	77	25–96

Values are median with range, mean ± SD, or (%); N=40 patients

*1 patient's data missing (n=39)

**2 patient's data missing (n=38). (1–2) NIHSS Statistical quartile and combined quartile breakdown with average age per quartile.

Table 2:

(Table 2–1) Robust regression provided top ten proteins with the highest predictive value for infarct volume or edema volume in context of REG3A. For directionality, a (+) indicates higher expression levels of that protein are associated with higher infarct/edema volume; a (-) indicates higher levels of that protein are associated with lower infarct/edema volume. R2 values for each predictive model are provided at the top of each table. **(Table 2–2)** Protein-protein interaction analysis of the top ten proteins were provided to control for multiple comparisons.

(2–1)			
Robust Regression			
Systemic blood and infarct volume R^1=0.5917			
Protein	P-value	Directionality	
CXCL11	<0.0001	(+)	
PLA2G7	<0.0001	(−)	
IL10RB	<0.0001	(+)	
PRCP	0.0002	(−)	
TSLP	0.0002	(+)	
F7	0.0002	(−)	
MBL2	0.0005	(−)	
CSF1	0.1806	(+)	
CX3CL1	<0.0001	(−)	
CXCL5	0.0026	(−)	
(2–2)			
Systemic blood and edema volume R^1=0.6725			
CXCL11	<0.0001	(+)	
PLA2G7	<0.0001	(−)	
CCL5	<0.0001	(−)	
CCL23	<0.0001	(−)	
ICAM1	0.2401	(+)	
TSLP	<0.0001	(+)	
MCP3	0.0213	(−)	
AOC3	<0.0001	(−)	
IL13	0.0002	(+)	
COMP	0.0025	(−)	
Protein-Protein Interaction			
Systemic Blood and Infarct Volume R^1 = 0.5917			
Protein	P-value	Directionality	FDR Significant?
IL 10RB	<0001	(+)	Yes
CXCL11	<0001	(+)	Yes
PLA2G7	<0001	(−)	Yes
CX3CL1	<0001	(−)	Yes

F7	0.0002	(−)	Yes
PRCP	0.0002	(−)	Yes
TSLP	0.0002	(+)	Yes
MBL2	0.0005	(−)	Yes
CXCL5	0.0026	(−)	No
REG3A	0.0094	(+)	No
CSF1	0.1806	(+)	No
Systemic blood and edema volume R^1= 0.6725			
REG3A	<0001	(+)	Yes
PLA2G7	<0001	(−)	Yes
CCL5	<0001	(−)	Yes
TSLP	<0001	(+)	Yes
CCL23	<0001	(−)	Yes
CXCL11	<0001	(−)	Yes
AOC3	<0001	(−)	Yes
IL13	0.0002	(−)	Yes
COMP	0.0025	(−)	No
MCP3	0.0213	(−)	No
ICAM1	0.2401	(−)	No

Table 3:

STRING Term ID is listed in the first column. STRING output provides a list of functions for the protein-protein interactions within each network. The top 10 biological functions are listed within the table per the smallest false discovery rate (FDR). Analysis strength and FDR and are listed in columns 3 and 4, respectively. Column 5 lists the proteins that are involved with the listed biologic function. The table is organized by network: **(3–1)** Systemic REG3A and Infarct Volume Network, **(3–2)** Systemic REG3A and Edema Volume Network.

Table 3–1. Systemic REG3A and Infarct Volume Network

Term ID	Biological Process	Strength	False Discovery Rate	Proteins Affiliated with Network
GO:0019221	Cytokine-mediated signaling pathway	1.29	7.92E-27	**CX3CL1, CCL1, IL10RA, IL7, IL22RA1, CSF1R, IL10RB, CXCR1, CXCL5, CXCL10, IL7R, CXCL11, CXCR2, CSF1, IFNLR1, IFNL1, F3, TSLP, JAK1, CX3CR1, CCL20, CXCL9, CXCR3, CRLF2, IL34, IFNL3, IL10**
GO:0006952	Defense response	1.06	1.55E-24	**CX3CL1, CCL1, IL22RA1, FCN3, CSF1R, IL10RB, FCN2, CXCL5, MASP1, CXCL10, CXCL11, F2, CXCR2, CSF1, IFNLR1, IFNL1, F3, TSLP, JAK1, CX3CR1, CCL20, CXCL9, CXCR3, MBL2, PRCP, REG3A, MASP2, IL34, IFNL3, IL10**
GO:0071345	Cellular response to cytokine stimulus	1.15	1.55E-24	**CX3CL1, CCL1, IL10RA, TFPI, IL7, IL22RA1, CSF1R, IL10RB, CXCR1, CXCL5, CXCL10, IL7R, CXCL11, CXCR2, CSF1, IFNLR1, IFNL1, F3, TSLP, JAK1, CX3CR1, CCL20, CXCL9, CXCR3, CRLF2, IL34, IFNL3, IL10**
GO:0051707	Response to other organism	1.06	1.88E-22	**CX3CL1, CCL1, IL10RA, TFPI, IL22RA1, FCN3, CSF1R, IL10RB, FCN2, CXCL5, MASP1, CXCL10, CXCL11, F2, CSF1, IFNLR1, IFNL1, TSLP, JAK1, CX3CR1, CCL20, CXCL9, MBL2, REG3A, MASP2, IL34, IFNL3, IL10**
GO:0006955	Immune response	0.96	3.36E-22	**CX3CL1, CCL1, IL7, FCN3, CSF1R, IL10RB, FCN2, CXCR1, CXCL5, MASP1, CXCL10, IL7R, CXCL11, F2, CXCR2, CSF1, IFNLR1, IFNL1, TSLP, JAK1, CX3CR1, CCL20, CXCL9, MBL2, PRCP, REG3A, MASP2, IL34, IFNL3, IL10**
GO:0009605	Response to external stimulus	0.85	5.17E-21	**CX3CL1, CCL1, IL10RA, TFPI, IL22RA1, FCN3, CSF1R, IL10RB, FCN2, CXCR1, CXCL5, MASP1, CXCL10, CXCL11, F2, CXCR2, CSF1, IFNLR1, IFNL1, TSLP, JAK1, CX3CR1, CCL20, CXCL9, CXCR3, MBL2, F7, REG3A, MASP2, IL34, IFNL3, IL10**
GO:0002682	Regulation of immune system process	0.98	9.07E-21	**CCL1, SPI1, IL7, FCN3, PLA2G7, FCN2, CXCL5, MASP1, CXCL10, IL7R, CXCL11, F2, CXCR2, CSF1, IFNLR1, IFNL1, TSLP, JAK1, CCL20, CXCL9, CXCR3, MBL2, F7, CRLF2, MASP2, IL34, IFNL3, IL10**
GO:0002376	Immune system process	0.81	8.13E-20	**CX3CL1, CCL1, SPI1, IL7, FCN3, CSF1R, IL10RB, FCN2, CXCR1, CXCL5, MASP1, CXCL10, IL7R, CXCL11, F2, CXCR2, CSF1, IFNLR1, IFNL1, TSLP, JAK1, CX3CR1, CCL20, CXCL9, CXCR3, MBL2, PRCP, REG3A, MASP2, IL34, IFNL3, IL10**
GO:0006954	Inflammatory response	1.3	1.95E-19	**CX3CL1, CCL1, CSF1R, IL10RB, CXCL5, CXCL10, CXCL11, F2, CXCR2, CSF1, F3, CX3CR1, CCL20, CXCL9, CXCR3, MBL2, PRCP, REG3A, IL34, IL10**
GO:0002684	Positive regulation of immune system process	1.09	1.45E-18	**CCL1, IL7, FCN3, PLA2G7, FCN2, CXCL5, MASP1, CXCL10, IL7R, CXCL11, CXCR2, CSF1, IFNL1, TSLP, CCL20, CXCL9, MBL2, F7, CRLF2, MASP2, IL34, IFNL3, IL10**

Table 3–2. Systemic REG3A and Edema Volume Network

Term ID	Biological Process	Strength	False Discovery Rate	Proteins Affiliated with Network

GO:0007166	Cell surface receptor signaling pathway	0.89	1.23E-26	**ICAM3, IL4, IL7, ICAM1, CCR2, CCR5, PF4, CCR1, STAT6, CCL11, IL13, CXCL10, IL7R, CXCL8, CXCL11, TSLP, ITGAL, CXCL9, IL13RA1, IL13RA2, CXCR3, IL2RG, CCL7, GATA3, IL4R, ITGB2, CRLF2, IL6, TNF, IL10, CCR3, ITGAM, CCL5, CCL3L3, CCL23, CCL4**
GO:0002684	Positive regulation of immune system process	1.18	9.01E-26	**ICAM3, IL4, IL7, ICAM1, PLA2G7, CCR2, PF4, CCR1, STAT6, IL13, CXCL10, IL7R, CXCL8, CXCL11, TSLP, CXCL9, IL13RA2, CCL7, GATA3, IL4R, ITGB2, CRLF2, IL6, TNF, IL10, ITGAM, CCL5, CCL4**
GO:0019221	Cytokine-mediated signaling pathway	1.39	5.96E-40	**IL4, IL7, ICAM1, CCR2, CCR5, PF4, CCR1, STAT6, CCL11, IL13, CXCL10, IL7R, CXCL8, CXCL11, TSLP, CXCL9, IL13RA1, IL13RA2, CXCR3, IL2RG, CCL7, GATA3, IL4R, ITGB2, CRLF2, IL6, TNF, IL10, CCR3, ITGAM, CCL5, CCL3L3, CCL23, CCL4**
GO:0006954	Inflammatory response	1.44	1.34E-32	**ICAM1, CCR2, CCR5, PF4, CCR1, CCL11, IL13, CXCL10, CXCL8, CXCL11, AOC3, ITGAL, CXCL9, CXCR3, CCL7, GATA3, REG3A, IL4R, ITGB2, IL6, TNF, IL10, CCR3, ITGAM, CCL5, CCL3L3, CCL23, CCL4**
GO:0050900	Leukocyte migration	1.57	2.09E-28	**ICAM1, CCR2, CCR5, PF4, CCR1, CCL11, CXCL10, CXCL8, CXCL11, ITGAL, CXCL9, CXCR3, CCL7, GATA3, ITGB2, IL6, TNF, IL10, ITGAM, CCL5, CCL3L3, CCL23, CCL4**
GO:0030595	Leukocyte chemotaxis	1.82	9.76E-26	**CCR2, CCR5, PF4, CCR1, CCL11, CXCL10, CXCL8, CXCL11, CXCL9, CXCR3, CCL7, ITGB2, IL6, IL10, CCL5, CCL3L3, CCL23, CCL4**
GO:0070098	Chemokine- mediated signaling pathway	2.01	2.47E-25	**CCR2, CCR5, PF4, CCR1, CCL11, CXCL10, CXCL8, CXCL11, CXCL9, CXCR3, CCL7, CCR3, CCL5, CCL3L3, CCL23, CCL4**
GO:0006955	Immune response	0.99	3.34E-25	**IL4, IL7, ICAM1, CCR2, CCR5, PF4, CCR1, STAT6, CCL11, IL13, CXCL10, IL7R, CXCL8, CXCL11, TSLP, ITGAL, CXCL9, IL13RA2, IL2RG, CCL7, GATA3, REG3A, IL4R, ITGB2, IL6, TNF, IL10, ITGAM, CCL5, CCL3L3, CCL23, CCL4**
GO:0002682	Regulation of immune system process	1.03	3.58E-25	**ICAM3, ICAM5, IL4, IL7, ICAM1, PLA2G7, CCR2, PF4, CCR1, STAT6, IL13, CXCL10, IL7R, CXCL8, CXCL11, TSLP, ITGAL, CXCL9, IL13RA2, CXCR3, CCL7, GATA3, IL4R, ITGB2, CRLF2, IL6, TNF, IL10, ITGAM, CCL5, CCL4**
GO:0006952	Defense response	1.05	1.16E-23	**ICAM1, CCR2, CCR5, PF4, CCR1, CCL11, IL13, CXCL10, CXCL8, CXCL11, AOC3, TSLP, ITGAL, CXCL9, CXCR3, CCL7, GATA3, REG3A, IL4R, ITGB2, IL6, TNF, IL10, CCR3, ITGAM, CCL5, CCL3L3, CCL23, CCL4**