CCL2 and CXCL10 are associated with poor outcome after intracerebral hemorrhage

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Abstract

Objective: Intracerebral hemorrhage carries a high mortality and survivors are frequently left with significant disability. Immunological mechanisms may play an important role in hemorrhage-induced brain injury, however, research linking these mechanisms with clinical outcome remains limited. We aim to identify serum inflammatory mediators that are associated with outcome after intracerebral hemorrhage in order to translate data from experimental models to a patient cohort and identify potential targets worthy of reverse translation.

Methods: A prospective cohort study at two comprehensive stroke centers enrolled patients with spontaneous intracerebral hemorrhage. Peripheral blood was collected at 6, 24, and 72 h from onset. Functional outcome was assessed at 90 days using the modified Rankin Scale (mRS). Serum inflammatory mediators were measured using multiplex ELISA. Multivariable modeling identified serum biomarkers independently associated with functional outcome at 90 days.

Results: 115 patients completed the study. At 6 h after onset, patients with elevated CCL2 had worse mRS score at day 90 (OR 4.07, 95% CI 1.27–13.10, P = 0.02) after adjusting for age, gender, ICH volume, IVH, infratentorial location and NIHSS score. At 24 and 72 h after onset, elevation in CXCL10 was independently associated with worse 90 days mRS score (24 h: OR 8.08, 95% CI 2.69–24.30, P < 0.001; 72 h: OR 3.89, 95% CI 1.12–13.49, P = 0.03).

Interpretation: Acute and subacute elevations in specific immune factors are associated with poor outcome, highlighting potential pathways that may contribute to ongoing brain injury in patients with intracerebral hemorrhage.

Introduction

Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes and is associated with the highest morbidity and mortality of all stroke types.1,2 To date, there is no effective medical treatment for ICH. Much of the primary tissue damage in ICH is caused by mechanical injury to tissues adjacent to the hematoma. Further injury follows from the release of blood components into the parenchyma, which can activate resident immune cells, exacerbate blood-brain barrier disruption, worsen edema, and trigger cellular necrosis and apoptosis.3–5 This cascade of events is broadly referred to as secondary injury in ICH, and inflammation appears to play an important role.6,7

Inflammation is initiated by numerous factors after ICH. Although the process remains incompletely understood, components of the hematoma—such as heme, clotting factors, and complement—activate resident microglia that then release chemokines and pro-inflammatory cytokines into the parenchyma.8 This inflammatory milieu triggers an increase in the permeability of the blood-brain barrier and peripheral leukocyte infiltration. Neutrophils and monocyte-derived macrophages are the first cell types to arrive at the site of injury and are the predominate contributors of early inflammation.9,10 These cells secrete additional cytokines and other inflammatory mediators in response to stimuli in the perihematoma...
region and can modulate the progression of tissue injury and cell death.10–12

Cytokines regulate local and systemic inflammation, as well as cell growth, proliferation, and differentiation. Chemotactic cytokines—or chemokines—induce the migration of leukocytes throughout the body. In the perihematoma tissue of ICH patients, cytokines, chemokines, and growth factors are some of the most highly expressed gene types.13–15 Therefore, we chose to examine the association between elevations of these molecules in peripheral blood and functional outcome after ICH. We measured a broad panel of inflammatory and anti-inflammatory cytokines, chemokines and growth factors at three time points after ICH onset, with the hope of identifying pathways of interest in patients with ICH for both forward and reverse translation.

**Methods**

**Patient enrollment**

Patients were prospectively enrolled from the Hospital of the University of Pennsylvania and Hartford Hospital from July of 2008 to June of 2013. All patients aged ≥18 years who presented within 24 h of spontaneous ICH were approached for enrollment. Patients with known underlying vascular lesions (AVM, AVF, aneurysm, venous sinus thrombosis), traumatic brain injury, systemic malignancy, immunosuppression, or autoimmune disease were excluded. Patients with significant pre-stroke disability, defined as modified Rankin scale score greater than 2, were excluded from the analysis of functional outcomes. The study was approved by the institutional review boards at both institutions and informed consent was obtained for all subjects. Patients were managed by stroke or neurocritical care specialists according to standard guidelines.16

**Data collection**

Baseline demographic information (age, gender, past medical history, medication use), laboratory data (complete blood count, electrolyte panel, and coagulation testing), NIH stroke scale score, Glasgow coma score, ICH volume (measured by ABC/2)17 and location, and incidence of fever, infections, and surgical interventions, were prospectively collected for each subject.

**Outcome assessment**

The modified Rankin Scale (mRS) was assessed by a certified member of the research team at 90 days either at an outpatient follow-up visit or by a structured telephone interview.18 All clinical data, including outcome, were assessed blinded to serum cytokine/chemokine results.

**Blood collection**

The time periods for sample collection were predefined. Peripheral venipuncture was performed for blood collection at 6 ± 6 h, 24 ± 12 h and 72 ± 12 h after symptom onset. When the onset of symptoms was uncertain, the time the patient was last known normal was used. Blood was collected into BD vacutainer serum separator tubes, inverted five times, allowed to clot for 20 min, and then centrifuged at 1000 g for 10 min for serum collection. Serum was frozen at −80°C in 500 μL aliquots until analysis.

**Cytokine/chemokine analysis**

Serum was analyzed using a multiplex human cytokine panel (human cytokine magnetic kit, Millipore, Billerica, MA) for IL-1β, IL-1ra, IL-4, IL-6, IL-8, IL-10, CX3CL1 (fractalkine), G-CSF, GM-CSF, CXCL10 (IP10), CCL2 (MCP1), CCL7 (MCP3), CCL22 (MDC), and TNF, allowing for multiple simultaneous cytokine analyses and decreasing sample processing time. All samples were analyzed in a single batch according to manufacturers instructions and read on a Luminex 200 instrument in a clinical and translational research core facility by dedicated laboratory staff. Samples were analyzed in duplicate and any sample with greater than 20% coefficient of variation within an analyte was excluded. No samples exceeded this threshold.

**Statistics**

Univariate descriptive statistics were performed on the clinical characteristics of the cohort and each serum factor. Each serum factor was analyzed for overall distribution, changes over time, and associations with age, gender, prehospital functional status (mRS), ICH volume, intraventricular hemorrhage (IVH), infratentorial location, National Institutes of Health Stroke Scale (NIHSS) score, Glasgow Coma Scale (GCS) score, external ventricular drain (EVD) placement, surgical evacuation, infections, and functional outcome (mRS at 90 days). The full scale for the NIHSS, GCS, and mRS scores was used in all analyses. Serum cytokine/chemokine levels were not normally distributed and most did not become normally distributed after logarithmic transformation. Thus, the upper quartile was defined as elevated for analyses and compared to the lower three quartiles in analyses. Multi-variable analyses were conducted using ordinal logistic regression to determine the clinical variables that were
independently associated with poor outcome across the entire mRS scale in our cohort. Each serum cytokine/chemokine level was then added to the multivariable model to determine its independent contribution to outcome. We did not adjust for multiple comparisons, consistent with the recommendations of statistical experts,\textsuperscript{19} in order to not inflate the likelihood of Type II errors in this exploratory study. Rather we provided P values to facilitate interpretation of the strength of the association and the likelihood of Type I errors (false positives). The proportional odds assumption was upheld. Statistics were performed using Stata/IC v11 (StataCorp, College Station, TX).

Results

The study enrolled 128 subjects, including 59 at the Hospital of the University of Pennsylvania and 69 at Hartford Hospital. There were no differences in clinical characteristics, outcome or inflammatory mediator expression of the patients from the two hospitals. Of the 128 subjects, 13 were lost to follow up and were excluded from the analysis (Fig. S1). Subjects that were lost to follow-up had significantly lower pre-ICH mRS (P = 0.004), lower NIHSS scores (P = 0.025), and fewer infections (P = 0.026), but no difference in serum cytokine levels at any time point. The final study population consisted of 115 subjects with complete outcome data. The characteristics of the study cohort are shown in Table 1.

Seventy-six subjects presented to the hospital within 12 h and had samples drawn during the earliest time window (median 5.8 h [interquartile range (IQR) 3.6–9.1] from onset), of which 36 had samples collected at all three time points, 24 had samples collected in the first two time points, 2 had samples collected in the first and last time points, and 14 had a sample collected during the first time point only. The most common reasons for missing later sample collections were death and discharge.

Fifty subjects were enrolled in the second time window (median 22.5 h, (IQR) 19.5–23.8) from onset, of which 25 had samples collected in the second and third time window and 25 had a sample collected during the second time window only. Two subjects were enrolled in the third time window (median 68.5 h from onset).

As the numbers of subjects in each time window differed, associations between clinical factors, cytokine levels, and the subset of subjects enrolled in each set of time points were explored for possible bias. Subjects that had only a 12-h sample collected did have significantly higher in-hospital mortality than other groups (P < 0.001), however these subjects accounted for fewer than 20% of all samples collected at the 12 h time point. There were no significant differences in other clinical factors or cytokine expression among groups of subjects contributing data at each time point.

Summary statistics for each inflammatory mediator at each time point are shown in Table 2, and the threshold considered elevated is defined by the upper quartile. The thresholds established for defining elevation of each cytokine/chemokine were higher than reported for healthy subjects.\textsuperscript{20} Scatterplots of the distributions of each factor at each time point are presented in Figs. S2–S15.

Univariate analyses were performed to detect associations of elevations in each inflammatory mediator with these clinical variables. These results are shown in Table 3.

A multivariable model was created to determine clinical factors independently associated with outcome in the cohort. Components of the ICH score, including age, ICH volume, presence of intraventricular hemorrhage, infratentorial location, and Glasgow Coma Scale score, as well as gender, site of enrollment, and NIHSS score were initially included. In our cohort, age, gender, ICH volume, IVH, infratentorial location, and NIHSS score were associated with outcome. These variables were therefore included in the multivariable analyses of each serum inflammatory mediator and outcome. The results of the multivariable analyses are shown in Table 4. Elevated CCL2 levels at 6 h and elevated CXCL10 levels at 24 and 72 h were independently associated with poor outcome at 90 days. No other inflammatory mediators had an association with outcome after adjusting for these clinical predictors of outcome (Tables S1–S3).

The temporal pattern of expression of each biomarker was identified for subjects with data from all 3 collection times (n = 36). The pattern assignments are shown in Figure 1. The temporal pattern variable was added to the multivariate model to explore associations between

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Table 1. Characteristics of the cohort.

| Characteristic                  | Value         |
|--------------------------------|---------------|
| Age (years)                    | 67.5 [57.5–78.0] |
| Male                           | 60.9%         |
| Pre-ICH functional status (mRS)| 0 [0–0]       |
| ICH volume (mL)                | 16.8 [5.6–40.0] |
| Initial NIHSS score            | 15 [5–24]     |
| Initial GCS score              | 14 [9–15]     |
| Infratentorial location        | 12.2%         |
| Intraventricular hemorrhage    | 45.2%         |
| EVF placed                     | 17.9%         |
| Surgical evacuation            | 17.0%         |
| Length of hospitalization (days)| 7.2 [3.6–19.3] |
| In-hospital mortality          | 23.5%         |
| Functional outcome at 90 days (mRS)| 4 [2–6]     |
| Poor outcome (mRS 4–6) at 90 days| 54.8%       |

Data are presented as median [Interquartile range] or percent. n = 115.
Two patterns of expression of CCL2 were associated with outcome at 90 days after adjusting for clinical predictors of outcome (Table 5). Early elevation in CCL2 with decreasing levels over time was associated with poor outcome, while increasing levels of CCL2 over time was associated with improved outcome.

**Discussion**

The main objective of our study was to identify innate immune factors that are independently associated with clinical outcome in ICH. We adopted a relatively unbiased approach and surveyed a broad panel of cytokines, chemokines, and growth factors at 6, 24, and 72 h after ICH. We also collected a comprehensive set of clinical data from the subjects in our study and determined that age, gender, ICH volume, IVH, infratentorial location, and NIHSS score were associated with functional outcome at day 90, consistent with previous work. We then included these variables in our multivariable analyses assessing for independent contributions of inflammatory markers to poor functional outcome.

After controlling for these factors, we found that very early elevation in serum CCL2 was associated with worse functional outcome at 90 days. CCL2 is a potent chemokine for circulating monocytes as well as memory T-cells and dendritic cells. By binding to its receptor CCR2, CCL2 induces the migration of these cell types to sites of injury and infection. It is primarily produced by monocytes and macrophages during inflammation. In addition to its chemotactic role, CCL2 increases the permeability of the blood-brain-barrier.

Previous human studies have indicated an association between CCL2 expression and outcome after stroke. In one ischemic stroke study, elevated CCL2 plasma expression 7 days after onset was associated with a higher mRS score at 90 days post stroke in patients with a National Institute of Health Stroke Scale (NIHSS) score greater than 12. Another ischemic stroke study found that elevated expression of CCL2 in the serum at 24, 48, and 72 h after onset was associated with worse outcome after 28 days. An earlier analysis of this cohort found a similar association between CCL2 levels at 24 h after ICH onset and outcome at day 7, but long-term outcome was not assessed. Consistent with our CCL2 findings, others have reported that early elevations in monocyte counts are associated with fatality after ICH.

In murine models of ICH, results have indicated that suppression of CCL2, or its receptor CCR2, has time-dependent effects on outcome. In one study, CCL2 & CCR2 mice demonstrated delayed hematoma expansion and clearance, supporting the proposed function of this cytokine-receptor axis as a mediator of vascular integrity in the brain. Another study confirmed that CCR2 mice, as well as wild-type mice that have been monocyte depleted, exhibit better motor functioning during the first few days after ICH compared to wild-type controls. Together these experimental studies support a deleterious role for the CCR2-CCL2 axis early after ICH, which is consistent with our results in humans.

Through temporal exploration of biomarker expression patterns, we found additional evidence to support this time-dependent effect of CCL2. Interestingly, in our cohort, two patterns of CCL2 expression were associated
with outcome. Subjects with an acute elevation in CCL2 after ICH that then decreased by 72 h after ICH had worse outcomes at 90 days. Conversely, those with low early CCL2 that then increased by 72 h after ICH had better outcomes at 90 days. Preclinical work has identified important contributions of macrophages to recovery in ICH, ischemic stroke, and mild traumatic brain injury through mechanisms such as phagocytosis of cellular debris, promotion of angiogenesis, and secretion of growth factors. These temporal patterns are consistent with the 6 h data and also provide clinical evidence for potential later beneficial effects of monocyte-macrophage recruitment in patients. No other temporal biomarker patterns were associated with outcome.

The second major finding of this work is that subacute elevations in serum CXCL10 are independently associated with worse long-term clinical outcome. CXCL10 is a chemokine that is secreted by a variety of immune and non-immune cell types in response to IFN-γ as well as Toll-like receptor ligands, TNF, and other inflammatory stimuli. It functions as a chemoattractant for activated T cells, B cells, macrophages and NK cells. CXCL10 and its cognate receptor CXCR3 facilitate the migration of lymphocytes into target tissues. In non-immune cells, the CXCL10-CXCR3 axis appears to play an important role in angiostasis, wound repair and tissue remodeling, and cellular apoptosis.

Numerous murine ischemic stroke studies have noted that CXCL10 is significantly upregulated within the ischemic region after both transient and permanent middle cerebral artery occlusion. In one of these studies, inhibition of IFN-γ signaling prior to middle cerebral artery occlusion blocked induction of CXCL10 and reduced infarct volume, T-cell infiltration, and neurodegeneration. One experimental hemorrhagic stroke study found elevated levels of CXCL10 in the ipsilateral hemisphere 12 h after ICH, but associations between expression of this chemokine and outcome after ICH were not explored. Interestingly, CXCL10 release has been demonstrated in response to thrombin and fibrinogen, confirming the relevance of this cytokine to ICH pathology.

In humans, CXCL10 was found to be similarly upregulated after ischemic stroke and significantly more NK cells were observed in the ischemic hemisphere than the nonischemic hemisphere. Interestingly, the PRIME study demonstrated an association between elevated systemic CXCL10 and risk of ischemic stroke in asymptomatic males. While studies have shown the deleterious effect of CXCL10 in various systemic inflammatory diseases, to our knowledge, no human hemorrhagic stroke studies have examined an association between CXCL10 and clinical outcome.

Of the fourteen biomarkers examined in our cohort, no other immune factor at any time point demonstrated an association with outcome 90 days after ICH once we controlled for age, gender, ICH volume, IVH, infratentorial location, and NIHSS score. In fact, the majority of factors we attempted to measure were below the limit of detection of our assay in most patients. The distributions of most factors were remarkably skewed, with only a subset of patients showing elevations at any time point. Though it remains unclear whether the concentration of cytokines and chemokines in the serum is correlated with that in the CNS after ICH, these results have important implications for future studies, including trials that aim to explore biomarkers as either patient selection tools or intermediate endpoints for immunomodulatory agents. Given the burgeoning interest in immune biomarkers after ICH, we provide the distributions for all analytes at all time points to inform the planning of future studies.

### Table 3. Univariate associations between clinical factors and elevations in inflammatory mediators.

| Inflammatory mediator | Clinical factor | Odds ratio | Confidence interval | P  |
|-----------------------|----------------|------------|---------------------|----|
| 6 h                   |                |            |                     |    |
| IL-10                 | Age, per year  | 0.87       | 0.76–0.99           | 0.04 |
| IL-10                 | Gender (Male) | 0.31       | 0.10–0.96           | 0.04 |
| IL-10                 | IVH            | 4.42       | 1.50–12.98          | <0.01 |
| IL-1β                 | ICH volume, per mL | 0.97 | 0.94–1.00 | 0.03 |
| IL-4                  | Age, per year  | 0.93       | 0.88–0.99           | 0.02 |
| TNF                   | NIHSS, per point | 1.05 | 1.00–1.11 | 0.05 |
| TNF                   | Initial GCS, per point | 0.87 | 0.76–0.99 | 0.04 |
| 24 h                  |                |            |                     |    |
| CCL2                  | Initial GCS, per point | 0.88 | 0.79–0.99 | 0.04 |
| G-CSF                 | ICH volume, per mL | 1.02 | 1.00–1.04 | 0.02 |
| G-CSF                 | NIHSS, per point | 1.05       | 1.01–1.10           | 0.03 |
| G-CSF                 | Initial GCS, per point | 0.87 | 0.78–0.98 | 0.02 |
| G-CSF                 | Evacuation     | 3.11       | 1.07–9.02           | 0.04 |
| IL-6                  | Initial GCS, per point | 0.87 | 0.77–0.97 | 0.02 |
| IL-6                  | Evacuation     | 4.50       | 1.54–13.13          | 0.01 |
| IL-8                  | Initial GCS, per point | 0.86 | 0.76–0.97 | 0.01 |
| IL-8                  | IVH            | 2.75       | 1.07–7.02           | 0.04 |
| IL-8                  | Evacuation     | 3.11       | 1.07–9.02           | 0.04 |
| IL-10                 | Evacuation     | 5.63       | 1.92–16.50          | 0.01 |
| 72 h                  |                |            |                     |    |
| IL-10                 | IVH            | 4.38       | 1.18–16.18          | 0.03 |
| G-CSF                 | EVD            | 9.40       | 1.86–47.23          | 0.01 |
| CCL22                 | Age, per year  | 0.92       | 0.87–0.98           | 0.01 |
| IL-1β                 | NIHSS, per point | 0.92 | 0.85–1.00 | 0.04 |
| IL-1β                 | Gender (Male) | 0.24       | 0.08–0.72           | 0.01 |
| IL-4                  | NIHSS, per point | 0.89 | 0.80–1.00 | 0.04 |
| IL-4                  | Gender (Male) | 0.20       | 0.05–0.80           | 0.02 |
| IL-6                  | EVD            | 5.70       | 1.19–27.35          | 0.03 |
| IL-8                  | EVD            | 6.36       | 1.31–30.83          | 0.02 |
| IL-8                  | Age, per year  | 0.95       | 0.90–1.00           | 0.05 |

The Odds Ratios for an elevated inflammatory mediator for each presenting clinical factor are listed.
Although the findings could be due to chance due to multiple testing in this exploratory analysis, the effect size was large with these two factors (and nonexistent for the others), there is biologic plausibility for these factors in the pathophysiology of the inflammatory response, and both CCL2 and CXCL10 elevations are consistent with preclinical studies. However, given the multiple factors tested in our cohort, the results would be enhanced by replication in other cohorts with highly sensitive assays.

Summary and Conclusions

In conclusion, after controlling for clinical variables known to influence outcome, elevated serum CCL2 concentration 6 h after ICH and elevated serum CXCL10 concentration 24 and 72 h after ICH were associated with worse functional outcome at 90 days after ICH onset.
These results suggest that serum concentrations of both CCL2 and CXCL10 may be useful prognostic indicators at certain time points after ICH and may play a direct role in the progression of secondary injury. Further research is needed to explore the possible mechanisms underlying the described associations and to discern whether there is therapeutic potential in altering the expression of these proteins at specific time points after ICH.

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Conflicts of Interest

The authors report no conflicts of interest.

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Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article

Figure S1. Enrollment flow chart by time point of entry into the study.
Figure S2. CCL2 levels by time point.
Figure S3. G-CSF levels by time point.
Figure S4. GM-CSF levels by time point.
Figure S5. CX3CL1 levels by time point.
Figure S6. IL-10 levels by time point.
Figure S7. CCL7 levels by time point.
Figure S8. CCL22 levels by time point.
Figure S9. IL-1ra levels by time point.
Figure S10. IL-1β levels by time point.
Figure S11. IL-4 levels by time point.
Figure S12. IL-6 levels by time point.
Figure S13. IL-8 levels by time point.
Figure S14. CXCL10 levels by time point.
Figure S15. TNF levels by time point.
Table S1. Association of serum levels of each inflammatory mediator at 6 h with poor functional outcome at 90 days.
Table S2. Association of serum levels of each inflammatory mediator at 24 h with poor functional outcome at 90 days.
Table S3. Association of serum levels of each inflammatory mediator at 72 h with poor functional outcome at 90 days.