Macrophage CD163 expression in cerebrospinal fluid: association with subarachnoid hemorrhage outcome

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OBJECTIVE Even though heme-induced cerebral inflammation contributes to many of the adverse sequelae seen in patients with subarachnoid hemorrhage (SAH), little is known about the mechanism; mouse models have shown a critical role for macrophages/microglia. Macrophage CD163 is a hemoglobin scavenger receptor involved in blood clearance after SAH. The authors hypothesized that the modified Fisher score is independently associated with cerebrospinal fluid (CSF) macrophage CD163 expression on postictal day 1, and that CSF macrophage CD163 expression is associated with 1-month neurological outcome.

METHODS CSF macrophages from 21 SAH and 28 unruptured aneurysm patients (control) were analyzed for CD163 expression using flow cytometry and confocal microscopy on postictal day 1. Significant associations with modified Fisher scale grades or modified Rankin Scale scores were determined using linear regression and a matched case control analysis.

RESULTS CSF macrophage CD163 expression was significantly increased in SAH patients compared with controls ($p < 0.001$). The modified Fisher scale (mF) grades ($\beta = 0.407$, $p = 0.005$) and CSF bilirubin concentrations ($\beta = 0.311$, $p = 0.015$) were positively and independently associated with CSF macrophage CD163 expression when the analysis was controlled for age and sex. CSF macrophages from an SAH patient with a high mF grade had increased co-localization of CD163 and glycophorin A (CD235a, an erythrocyte marker) compared with those from an SAH patient with a low mF grade. The controls had no co-localization. CSF macrophage CD163 expression ($p = 0.003$) was inversely associated with 1-month neurological outcome, when SAH patients were matched based on mF grade.

CONCLUSIONS This early study suggests that CSF macrophage CD163 expression, as measured by flow cytometry, may have some neuroprotective function given its inverse association with outcome and provides unique insights into the neuroinflammatory process after SAH.

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KEYWORDS subarachnoid hemorrhage; aneurysm; outcome; macrophage; CD163; vascular disorders

NEARLY half of all subarachnoid hemorrhage (SAH) patients die within 6–12 months postictus, and approximately 50% of survivors develop long-term neurological deficits.^{4,20,30} Heme-induced cerebral inflammation is a contributing factor to the high morbidity and mortality rates seen in SAH.^{5,28,32} Biomarker studies relating to hematoma resolution in SAH patients are limited to serum and cerebrospinal fluid (CSF) protein concentration, and none have examined the CSF innate immune cell

populations and their contribution to hematoma burden or patient outcome.^{10,11,16,21} Utilizing flow cytometry to evaluate CSF macrophage populations in SAH patients may provide new insight into the association between heme-induced cerebral inflammation, hematoma burden, and patient outcome.

Cluster of differentiation 163 (CD163) is of particular interest in hemorrhagic stroke, due to the fact that it has been identified as a hemoglobin-haptoglobin (Hb-Hp)

ABBREVIATIONS BIDMC = Beth Israel Deaconess Medical Center; BSA = bovine serum albumin; CD163 = cluster of differentiation 163; CSF = cerebrospinal fluid; CT = computed tomography; DCI = delayed cerebral ischemia; EVD = external ventriculostomy drain; Hb = hemoglobin; HO = heme oxygenase; Hp = haptoglobin; ICH = intracerebral hemorrhage; IQR = interquartile range; LAR = legally authorized representative; mF = modified Fisher scale; mRS = modified Rankin Scale; neuro-ICU = neurological intensive care unit; PBS = phosphate-buffered saline; SAH = subarachnoid hemorrhage; sCD163 = soluble form of CD163.

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scavenger receptor on macrophages.¹⁵ Both animal and human studies suggest that the CD163 scavenging system is critical for Hb clearance and attenuating heme-induced inflammation in SAH,¹⁰ traumatic brain injury,²² and intracerebral hemorrhage (ICH).^{6,12}

Our study is the first to identify an independent association between CSF macrophage CD163 expression after SAH and patient outcome as measured by the modified Rankin Scale (mRS).

We undertook the current set of experiments 1) to investigate whether there was an independent association between modified Fisher scale (mF) grade and CSF macrophage CD163 expression as measured by flow cytometry and 2) to see whether CSF macrophage CD163 expression was associated with mRS score. Our study demonstrates that CSF macrophage CD163 expression could be an early biomarker of neuroprotection as it is inversely associated with patient outcome.

Methods

Study Population

This study is an investigator-initiated prospective observational cohort study of aneurysmal SAH patients and patients with unruptured cerebral aneurysms admitted to the neurological intensive care unit (neuro-ICU) at the Beth Israel Deaconess Medical Center (BIDMC) in Boston, Massachusetts, between 2015 and 2016. With informed consent from the patients or their legally authorized representative (LAR), CSF was collected from patients who had external ventriculostomy drains (EVDs) placed for clinical reasons within 48 hours of ictus. Informed consent was also obtained to acquire CSF from patients with unruptured aneurysms in the operating room during open surgery for aneurysm clipping. The diagnosis of SAH was established by computed tomography (CT) or xanthochromia of the CSF if the CT was negative. Patients were not enrolled if: 1) they were less than 18 years old, 2) they were pregnant, 3) more than 48 hours had elapsed since ictus, or 4) they, their families, or LARs did not provide consent for participation in the study. Diagnosis of unruptured cerebral aneurysms occurred incidentally by CT angiogram or MR angiography in an outpatient setting. All patients who qualified for the study and provided consent for participation or had such consent provided by their LARs were included. All patient eligibility requirements, consent methodology, and enrollment protocols, as well as sample collection, processing, and storage procedures were approved by an institutional review board of BIDMC.

General Management

Care for patients with SAH as well as patients with unruptured cerebral aneurysms conformed to guidelines established by the American Heart Association.⁴

Clinical and Radiographic Data Collection

Clinical variables such as Hunt and Hess grade,¹⁴ general demographic characteristics, patient history, laboratory values, and medications were recorded on admission to the BIDMC neuro-ICU. Admission CT scans were inde-

pendently evaluated by a study neurointensivist (K.A.H.) for the presence and quantity of blood in the subarachnoid space and the intraventricular space to determine the mF grade.⁸ Patients with an unruptured aneurysm were assigned an mF grade of 0.

Determination of Delayed Cerebral Ischemia

Delayed cerebral ischemia (DCI) was defined by 2 criteria. First, the cerebral infarction must not be evident on the admission CT scan. Second, it must not be a result of a surgical complication. CT scans obtained at 7 days after admission were evaluated for any evidence of ischemic stroke. Ultimately, DCI was treated as a categorical variable, so if there was any evidence of an ischemic stroke that fulfilled DCI criteria, the CT scan was interpreted as positive.

CSF Collection and Cell Pellet Harvest

Twenty milliliters of CSF was collected from each SAH patient in a sterile manner via the most distal EVD port within 48 hours of ictus; 20 ml of CSF was collected from patients with unruptured aneurysms in the operating room during elective open cerebrovascular clipping surgery. In both cases, the collected CSF was transported on ice and immediately processed. The CSF was centrifuged at 500g for 5 minutes; the cell-free spinal fluid was stored at -80°C for future analysis, while the cell pellet was immediately processed as explained in the *Flow Cytometry* methods section below.

Flow Cytometry of CSF Cell Population

Acquisition of cells was performed on a FACSAria II flow cytometer (BD Biosciences), and analysis was completed using FlowJo software (Tree Star). The cell pellet harvested from CSF was incubated with red blood cell lysis buffer (BioLegend) for 5 minutes on ice. Cells were washed and resuspended in FACS buffer (1% bovine serum albumin [BSA], 2-mM ethylenediaminetetraacetic acid, and 0.05% NaN₃ in phosphate-buffered saline [PBS]). The cells were incubated with human TruStain FcX Fc-receptor blocker (5:100, BioLegend) to block unspecific sites. Subsequently, cells were stained with the following fluorescent-tagged antibodies: PE anti-human CD163, PE/Cy7 anti-human CD15, and APC anti-human CD14 (5:100, BioLegend). After standard gating off of the parent forward and side scatter gate, CSF macrophages were classified¹ as CD14⁺/CD15⁻ and were analyzed for their CD163 expression.

Confocal Microscopy of CSF Macrophages

Sorted CSF macrophages were washed after collection and incubated on a 0.1% poly-D-lysine-coated 6-well cell culture dish for 12 hours at 37°C in a humidified atmosphere with 5% CO₂, with cell-free CSF from the patient supplemented with 10% BSA. Subsequently, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were then incubated with a blocking buffer consisting of 3% BSA, 10% goat serum, and 0.1% Tween 20 (Sigma-Aldrich) in PBS for 30 minutes. The following primary antibodies were applied:

rabbit anti-human CD163 (1:100, Bioss) and mouse anti-human CD235a (1:1000, BioLegend). Then, the following secondary antibodies were applied: goat anti-rabbit 568 and goat anti-mouse 488 (1:500, Thermo Fisher Scientific).

Multiwavelength Analysis

Bilirubin, hemoglobin, and protein content in the cell-free CSF of patients was analyzed via ultraviolet-visible spectroscopy as described previously.^{27,28} In brief, absorbance (OD [optical density]) was measured at wavelengths of 340 nm (A_{340}), 415 nm (A_{415}), and 460 nm (A_{460}), and bilirubin (c1), hemoglobin (c2), and protein (c3) concentration were calculated using the following formulas:

- $A_{340} = 0.012 \cdot c1 + 0.0015 \cdot c2 + 0.000053 \cdot c3$
- $A_{415} = 0.049 \cdot c1 + 0.0069 \cdot c2 + 0.000016 \cdot c3$
- $A_{460} = 0.083 \cdot c1 + 0.0006 \cdot c2 + 0.000007 \cdot c3$

A total of 3 readings per CSF sample were performed, and the average OD at each measured wavelength was used to determine concentrations in the equations above. Because the bilirubin concentration of individual's sample can be influenced by the CSF flow rate, a ratio of bilirubin (mg/L) to total protein (mg/L) was determined for use in the logistical regression.

Statistical Analysis

Continuous variables were assessed for normality with skewness and kurtosis. Data that were not normally distributed were reported as medians with interquartile ranges (IQRs). Data that were normally distributed were reported with means and standard deviations. Categorical variables were reported as counts and proportions in each group. Grouping was based on clinical significance or median versus mean, depending on normality. To study associations between CSF macrophage CD163 expression and other variables, linear regression was performed. To study associations between mRS score and other variables a matched case control analysis was performed. To study the independent associations with these dependent variables, we first performed a univariate chi-square analysis for categorical variables and a Student t-test for continuous variables. Significant independent associations ($p < 0.05$) from the univariate analysis, as well as variables with biological plausibility, were entered into the regressions, as well as a priori chosen flow cytometry covariates, in a forward, stepwise manner using SPSS version 19 (IBM Corp.). Matched case control analysis was also performed using SPSS, where case and control were defined by dichotomized mRS outcome, matched based on mF grade, and statistically analyzed based on CSF macrophage CD163 expression. The outputs yielded standardized β coefficients with 95% confidence intervals for each of the independent variables along with their significance for regression and p values for all other tests. Only variables with $p < 0.05$ were considered to be statistically significant.

Results

Study Cohorts

A total of 49 patients were enrolled in this study. The

median ages for female and male SAH patients were 59 and 54 years old, respectively; the median ages for female and male control patients were 59 and 65 years old, respectively. The median Hunt and Hess grade for SAH patients was 3 for females and 4 for males; a grade of 0 was assigned to all unruptured aneurysm patients. The median mF grade was 3 for female SAH patients and 4 for male SAH patients; an mF grade of 0 was assigned to all unruptured aneurysm patients. The median mRS score was 2 for female SAH patients and 3 for male SAH patients (Table 1).

Modified Fisher Scale Grade and Bilirubin Concentration Are Independently Associated With Expression of CD163 in CSF Macrophages of SAH Patients

There was a significant increase in the CSF macrophage (CD14⁺/CD15⁻) population in the SAH patient group compared with controls (independent t-test; $p < 0.05$, Fig. 1B), with gating strategy shown (Fig. 1A). CD163 expression was significantly higher in CSF macrophages from SAH patients compared with those from control patients (independent t-test, $p < 0.05$, Fig. 1C).

We performed a univariate analysis against CSF macrophage CD163 expression with known risk factors for SAH, including age ($p = 0.285$), sex ($p = 0.433$), history of smoking ($p = 0.431$), hypertension ($p = 0.431$), and alcohol use ($p = 0.431$), as well as clinical characteristics such as Hunt and Hess grade ($p = 0.399$), mF grade ($p = 0.406$), DCI ($p = 0.431$), and CSF bilirubin concentration ($p = 0.238$). We next performed a multivariate regression analysis and found that the mF grade ($\beta = 0.407$, $p = 0.005$) and CSF bilirubin concentration ($\beta = 0.311$, $p = 0.015$) were positively and independently associated with CSF macrophage

TABLE 1. Patient characteristics

Characteristic	SAH	Control
No. of patients	21	28
Age in yrs, median (IQR)		
Male	55 (52–66)	65 (60–67)
Female	60 (51–64)	59 (53–64)
Male sex	12 (57%)	6 (21%)
mF grade, median (IQR)		
Male	4 (2–4)	0
Female	3 (2–3)	0
Hunt & Hess grade, median (IQR)		
Male	4 (2.75–5)	0
Female	3 (2–3)	0
mRS score, median (IQR)		
Male	3.5 (2–5)	1
Female	2 (1–2)	1
DCI	4 (19%)	NA
History of smoking	16 (76.2%)	18 (64.3%)
Hypertension	8 (38.1%)	19 (67.9%)
Alcohol use	16 (76.2%)	13 (46.4%)

NA = not applicable.

Data are presented as number of patients (%) unless otherwise indicated.

FIG. 1. A–C: CSF macrophage CD163 expression in patients with unruptured aneurysms (controls) and patients with SAH as determined by flow cytometry. Flow cytometry gating strategy for measurement of CD163 expression level in CD14⁺/CD15[−] CSF macrophages (A). The percentage of CD14⁺/CD15[−] CSF macrophages in control and SAH patients (independent t-test, **p* < 0.05) (B). The percentage of CD14⁺/CD15[−]/CD163⁺ CSF macrophages in control and SAH patients (independent t-test, **p* < 0.05) (C). **D:** Representative confocal images of sorted CD14⁺/CD15[−]/CD163⁺ CSF macrophages from a control patient, an SAH patient with a modified Fisher scale (mF) grade of 1, and an SAH patient with an mF grade of 4, stained with CD235a (green) and CD163 (red), illustrating the increased co-localization of CD235a and CD163 (orange) that was found in CSF macrophages from SAH patients with higher mF grades. Scale bar = 20 μ m.

CD163 expression, when controlling for age and sex. CSF bilirubin concentration controlled for hemoglobin degradation, which may vary between patients (Table 2).

Lastly, we performed confocal microscopy on sorted CSF macrophages from 2 SAH patients with mF grades of 1 and 4 as well as 1 control patient. The z-stacked confocal images of these CSF macrophages showed the highest intracellular co-localization of CD163 and glycophorin A (CD235a, an erythrocyte marker) in macrophages from the SAH patient with an mF grade of 4, followed by macrophages from the SAH patient with an mF grade of 1; minimal co-localization occurred in macrophages from the control patient (Fig. 1D).

CSF Macrophage CD163 Expression in SAH Patients is Inversely Associated With Modified Rankin Score

Because mF grade is independently associated with CSF macrophage CD163 expression, we performed a matched case control analysis of SAH patients only, to determine the relationship between CSF macrophage CD163 expression and mRS score, with mF grade being the matched variable. The mRS score was dichotomized into good (1–3) and poor (4–6) outcomes. One-to-one matching allowed for 18 SAH patients to be included in this analysis, with each bar representing 3 patients in Fig. 2. The *p* value for CD163 compared between the good and poor outcome groups, with mF grade matching, was

TABLE 2. Univariate and multivariate analysis for independent association with CSF macrophage CD163 expression

Variable	Chi-Square Univariate p Value (n = 49)	Multivariate (n = 49)	
		β Coefficient	p Value
Age	0.285	0.051	0.655
Sex	0.433	0.007	0.956
Hunt & Hess grade	0.399		
mF grade	0.406	0.407	0.005
DCI	0.431		
History of smoking	0.431		
Hypertension	0.431		
Alcohol use	0.431		
CSF bilirubin concentration	0.238	0.311	0.015

Boldface type indicates statistical significance.

statistically significant ($p < 0.003$). Furthermore, CD163 had an inverse relationship with mRS score; that is, higher CD163 expression on CSF macrophages was associated with lower mRS scores or better outcomes.

Discussion

This is the first study examining cell-specific CD163 expression in the CSF of patients with subarachnoid hemorrhage (SAH). Previous clinical studies on CD163 focused on the soluble form of CD163 (sCD163) in CSF and serum.^{10,12,22} Most investigators suspected that the source of sCD163 in patient CSF was glial cells or macrophages; however, recent studies showed that neurons also express CD163.^{7,13,19} Using flow cytometry, we identified the CD163⁺ macrophage population in the CSF of SAH patients, and showed an independent association between CSF macrophage CD163 expression with the modified Fisher scale (mF) grade as well as 1-month modified Rankin Scale (mRS) score. Furthermore, immunofluorescent microscopy of sorted CSF macrophages showed increased co-localization of CD163 and erythrocyte membrane marker (CD235a) with increasing blood burden as measured by mF grade, lending support to our flow cytometry results.

In our multivariate analysis, the mF grade was positively and independently associated with CSF macrophage CD163 expression, when controlling for age, sex, and CSF bilirubin concentration (Table 2). CSF bilirubin concentration was included in the multivariate analysis in order to control for hemoglobin degradation, via the heme oxygenase (HO) pathway. These results are not surprising, as the CD163 scavenging system plays a role in hematoma resolution after hemorrhagic stroke.^{6,17,18,25,31} Since the mF is a radiological characterization of the blood burden in SAH, the direct and independent association of mF grade with CSF macrophage CD163 expression seems logical.

One potential explanation for the apparent neuroprotective effect of CSF macrophage CD163 in SAH patients may be explained by the CD163-HO-1 scavenging system. Membrane-bound CD163-mediated endocytosis of extravasated Hb and Hp-Hb complexes have been shown

FIG. 2. Graphical representation of 1:1 matched case control analysis of 18 SAH patients, 3 per bar. Patients matched with mF grade, case and control defined by dichotomized outcome, and CD163 expression analyzed. * $p < 0.003$.

to upregulate expression of HO-1, the inducible enzyme required for the breakdown of heme into iron, carbon monoxide, and biliverdin; biliverdin is then converted into bilirubin by biliverdin reductase.^{24–26}

Our previous studies in a murine SAH model also supported a neuroprotective function for microglial/macrophage HO-1.^{27,28} If CD163 functions as the membrane receptor for HO-1, then increased CD163 expression should likely be neuroprotective in hemorrhagic stroke. Preclinical evidence for the neuroprotective effect of the CD163-HO-1 scavenging system was also provided by a murine intracerebral hemorrhage (ICH) model in which CD163 was genetically deleted, resulting in decreased hematoma resolution and worsened cognitive function.¹⁷ Clinical evidence exists as well. Brain biopsies from patients with ICH demonstrated a positive correlation between glial CD163 and HO-1 expression, but an inverse correlation between glial CD163 and the pro-inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)- α .¹⁸ It is possible that the increase in CSF macrophage CD163 expression seen in our patient population is indicative of higher HO-1 expression. Therefore, patients with increased CSF macrophage CD163 expression are better able to resolve their hematoma burden, thus contributing to an improved outcome.

However, conflicting evidence exists for the roles of CD163 and macrophages. A recent clinical study showed that serum sCD163 in ICH patients was positively and independently associated with mRS score.¹² In addition, 2 ICH animal studies revealed that hematoma resolution by CD163-expressing macrophages and neurons led to poor neurological outcome.^{6,19} Therefore, dichotomous roles for CD163 may exist, depending on cell type and disease model; further studies will be necessary to test this hypothesis.

With respect to the role of macrophages, most preclinical studies in mouse models of SAH demonstrate that macrophage recruitment to the aneurysm site facilitates

growth and rupture of the aneurysm. These same studies show evidence that reducing the macrophage population resulted in a reduced rupture rate.^{2,3,29} Therefore, it is curious that our results in SAH patients suggest that an increased population of CD163-expressing macrophages in the CSF is associated with an improved outcome (Fig. 2). These two seemingly disparate observations can be married with the realization that the role of macrophages before aneurysm rupture may be entirely different from their role after aneurysm rupture. Additionally, CD163 is generally thought to be an M2 marker or a marker indicating that the macrophage is more reparative and less inflammatory; others have demonstrated this anti-inflammatory property of monocyte CD163 as well, albeit not in a cerebral hemorrhage model.^{9,23,25,26,31} Finally, our preclinical work in a mouse model of SAH demonstrated that microglial HO-1 was critical to hematoma resolution, mirroring our results in this SAH population (Table 2). In fact, when we selectively deleted microglial HO-1 in mice subjected to SAH, these mice performed worse on cognitive assays, suggesting a protective role for microglia after SAH.²⁸ Together these works indicate that further research is necessary to define the complex role of the spectrum of macrophage function involved in aneurysm formation and rupture in SAH.

This study lays the groundwork for further exploration of the role of CSF macrophage CD163 expression in neuroinflammation after SAH. That being said there are limitations to this study, among them being the retrospective nature and the small sample size as well as the differing methods and times of CSF collection, which may result in confounding.

Conclusions

Our results indicate that CSF macrophage CD163 expression, measured by flow cytometry, could provide insight into the underlying heme-induced cerebral inflammatory response.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Hanafy. Acquisition of data: Thomas, Ogilvy, Griessenauer. Analysis and interpretation of data: Thomas. Drafting the article: Hanafy. Critically revising the article: Hanafy. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Hanafy. Statistical analysis: Hanafy.

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