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RESEARCH

Modulation of the Immunological Milieu in Acute Aneurysmal Subarachnoid Hemorrhage: The Potential Role of Monocytes Through CXCL10 Secretion

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Abstract

Emerging evidence indicates that aneurysmal subarachnoid hemorrhage (aSAH) elicits a response from both innate and adaptive immune systems. An upregulation of CD8 + CD161 + cells has been observed in the cerebrospinal fluid (CSF) after aSAH, yet the precise role of these cells in the context of aSAH is unkown. CSF samples from patients with aSAH and non-aneurysmal SAH (naSAH) were analyzed. Single-cell RNA sequencing (scRNAseq) was performed on CD8 + CD161 + sorted samples from aSAH patients. Cell populations were identified using "clustering." Gene expression levels of ten previously described genes involved in inflammation were quantified from aSAH and naSAH samples using RT-qPCR. The study focused on the following genes: CCL5, CCL7, APOE, SPP1, CXCL8, CXCL10, HMOX1, LTB, MAL, and HLA-DRB1. Gene clustering analysis revealed that monocytes, NK cells, and T cells expressed CD8 + CD161 + in the CSF of patients with aSAH. In comparison to naSAH samples, aSAH samples exhibited higher mRNA levels of CXCL10 (median, IQR = 90, 16–149 vs. 0.5, 0–6.75, $p = 0.02$). A trend towards higher HMOX1 levels was also observed in aSAH (median, IQR = 12.6, 9–17.6 vs. 2.55, 1.68–5.7, $p = 0.076$). Specifically, CXCL10 and HMOX1 were expressed by the monocyte subpopulation. Monocytes, NK cells, and T cells can potentially express CD8 + CD161 + in patients with aSAH. Notably, monocytes show high levels of CXCL10. The elevated expression of CXCL10 in aSAH compared to naSAH indicates its potential significance as a target for future studies.

Keywords Monocytes · Hemorrhage · Subarachnoid · Aneurysm

Introduction

Delayed cerebral ischemia (DCI) is a devastating complication that occurs in 14–16% of patients with aneurysmal subarachnoid hemorrhage (aSAH) [1]. Young age, female sex, clinical severity assessed using the World Federation of Neurosurgical scale, and hemorrhage volume measured with the modified Fisher scale appear to be risk factors for developing DCI [1]. DCI is a complex process involving the activation of the innate and adaptive immunological responses [2, 3]. Various cytokines such as CXCL-10 have been implicated in neurotoxicity after aSAH [4–6]. These mediators can promote the recruitment and activation of macrophages, monocytes, and neutrophils in the subarachnoid space [4, 5]. Ultimately, inflammation may result in vasospasm, ischemia, and neuronal damage [7].

In this prospective pilot study, our objective was to investigate potential elements of the immune response

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following aSAH. Our previous data suggested that CD8 + CD161 + cells increase in patients who developed vasospasm after aSAH. Moreover, confocal immunostaining of unruptured intracranial aneurysms demonstrated the presence of CD8 + CD161 + cells in the aneurysm wall [8]. The role of CD8 + CD161 + cells in the response that follows aSAH is unclear. In this study, we further characterized their possible role in aSAH. Single-cell RNA sequencing (scRNAseq) using human CSF samples was used to analyze the immunological response after aSAH. CD8 + CD161 + cells from aSAH samples were selected with flow cytometry. Messenger RNA levels of ten candidate genes previously associated with the immune response following aSAH were quantified using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) in samples from patients with aSAH and non-aneurysmal subarachnoid hemorrhage (naSAH). Our hypothesis centers around the potential role of CD8 + CD161 + cells in orchestrating immune mechanisms that could contribute to the DCI following aSAH.

Methods

Patient Population

After Institutional Review Board approval by the University of Iowa, patients with subarachnoid hemorrhage (SAH), or their legal representatives, provided written consent to participate in this study. SAH was confirmed by computed tomography. Subsequently, patients with suspicion of SAH underwent digital subtraction angiography to confirm the presence of an aneurysm. Both patients with aSAH and naSAH were included. Patients with active central nervous system infections and autoimmune disorders were excluded.

Experimental Approach

Two experiments were conducted to characterize the immunological response of patients with SAH (Fig. 1). The first experiment used scRNAseq to investigate the transcriptosome of different cellular populations in human CSF from patients with aSAH. The second experiment quantified messenger ribonucleic acid (mRNA) levels of aSAH and naSAH samples using reverse-transcriptase quantitative PCR (RT-qPCR).

CSF Processing

CSF samples were drawn from extra-ventricular drains of patients with SAH. Five milliliters of samples were centrifuged at 2000 rpm for 10 min. The supernatants were aliquoted into 1.5 mL tubes and stored at −80 °C.

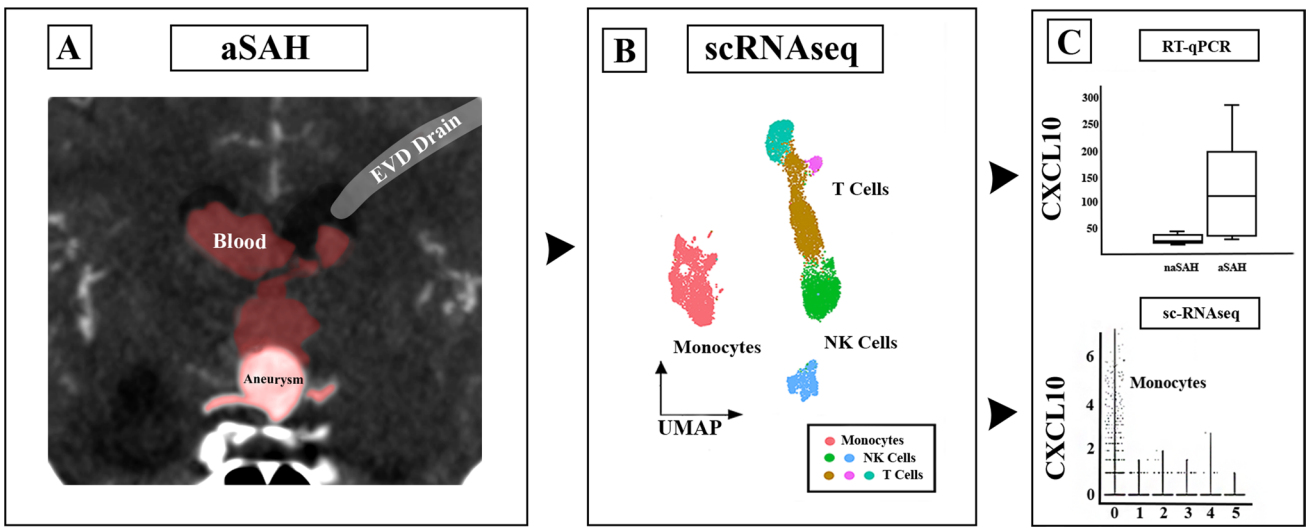

Fig. 1 The experimental approach in our study involved the following steps. **a** After aneurysmal subarachnoid hemorrhage (aSAH), an external ventricular device (EVD) was inserted to drain cerebrospinal fluid (CSF) from the ventricles. The CSF collected through this device contained blood due to the hemorrhage. **b** To investigate the cellular composition and gene expression patterns, single-cell RNA sequencing (scRNAseq) was performed on the collected CSF samples. Prior to sequencing, cells were sorted based on the expression of CD8 + CD161 + markers. These markers were particularly expressed in monocytes. Cell sorting allowed us to focus on a specific subset of cells for analysis. **c** We then performed RT-qPCR in samples from aSAH and naSAH. In this experiment, we observed elevated levels of CXCL10 in the CSF of patients with aSAH compared to individuals with naSAH. aSAH, aneurysmal subarachnoid hemorrhage. RT-qPCR, reverse transcriptase quantitative polymerase chain reaction. scRNAseq, single-cell RNA sequencing

Experiment 1: scRNA Analysis of aSAH and naSAH CD8 + 161 + Cells

aSAH and naSAH samples were sorted for CD8 + CD161 + cells. Details of the methods for sorting the samples are found in the supplementary methods. CD8 + CD161 + sorted cells were prepared as described above for scRNAseq with a 10X Chromium system (10XGenomics). The cells were partitioned into droplet-based Gel Bead-In-Emulsions for reverse transcription and to generate 10X barcoded cDNA libraries. These libraries were then sequenced with an Illumina NovaSeq 6000 at the Genomics Division of the Iowa Institute of Human Genetics at the University of Iowa. The read files for each sample were pre-processed with Cell Ranger Single-Cell Software (v.4.0.0), which was used to perform sample demultiplexing, barcode processing, read alignment, and single-cell counting (10X genome reference "refdata-gex-GRCh38-2020-A" was used for the reference alignment). Downstream analysis was performed in R using the "seurat" package [9, 10]. Quality control filtering of cells was done to exclude cells containing less than 500 or more than 2500 genes detected. Gene expression values were normalized and scaled. The nearest neighbor graph was computed with "FindNeighbors," and clusters were detected with "FindClusters" with resolution = 0.1. A Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction was calculated on the resulting seurat object using the calculated PCA components to obtain a two-dimensional representation of the cell states. Cluster markers were calculated with seurat "FindMarkers" using the "wilcoxon" test. Two different analyses were performed: whole CSF unsorted aSAH and naSAH samples and sorted CD8 + CD161 + aSAH samples. Cellular types were identified for each cluster classified using the PanglaoDB database [11]. Cell populations were identified based on the expression of the top 10 expressed cellular markers (supplementary Table 1).

After identifying the cell types, the expression of ten target genes of clinical interest in each cellular population was analyzed. The following genes were selected after a thorough review of the literature: CCL5 [6, 12, 13], CCL7 [14, 15], APOE [14, 15], SPP1 [16], CXCL8 [17], CXCL10 [18, 19], HMOX1 [18, 19], LTB [20], MAL [21], and HLA-DRB1 [22]. We compared the transcriptosomes of CD8 + CD161 + cells from two aSAH patients. These cells were sorted by flow cytometry based on a previous observation that CD8 + CD161 + cells are elevated during the acute phase of aSAH in patients who developed vasospasm [8]. Finally, the expression of previously described microglial markers was analyzed in the aSAH samples [23]. This was performed to analyze the origin of the cells that were clustered by scRNA analysis.

Experiment 2: RT-qPCR Analysis of Gene Targets

mRNA expression levels of these previously described 10 genes were quantified in 13 CSF samples of patients with aSAH and naSAH. Detailed primers are found in supplementary Table 2.

Statistical Analysis

Statistical analysis was performed using SPSS Version 27.0 (IBM, Armonk, NY). The Shapiro–Wilk test was used to evaluate normality in our sample. Normally distributed variables are reported as mean ± SD and non-normally distributed variables as median (IQR). The Mann–Whitney *U* or the Student's *t* test were used to compare variables depending upon distribution. Statistical significance is expressed by *p* values at an $\alpha = 0.05$.

Results

A total of 15 patients were included through all experiments conducted in this study (Table 1). Specific severity scales scores, and sample collection dates for experiment 1 are detailed in supplementary Table 3, and for experiment 2 in supplementary Table 4. The causes of SAH for each naSAH patient are described in supplementary Table 5.

Experiment 1: scRNAseq of CD8 + CD161 + aSAH CSF Samples

Two CD8 + CD161 + sorted aSAH samples were analyzed with scRNAseq. The first sample was collected on day 3 and the other on day 6. The total number of sorted cells was 3921 and 5738 CD8 + CD161 + cells. Distinct cell populations of NK cells, monocytes, and T cells were identified as expressing CD8 + CD161 + markers (Fig. 1). CCL5 was expressed throughout all cellular populations. CCL7, HLA-DRB1, APOE, SPP1, CXCL8, and HMOX1 were primarily expressed by monocytes (supplementary Fig. 1). Monocytes

Table 1 Patient demographics

	Aneurysmal SAH (*N* = 11)	Non-aneurysmal SAH (*N* = 4)
Age median (IQR)	65 (53, 67)	68 (55, 74)
Female % (*n*)	73 (8)	25 (1)
Sample day post-hemorrhage median (IQR)	3 (2, 5)	3 (2, 3)

IQR interquartile range; *SAH* subarachnoid hemorrhage

also were the main cell type expressing CXCL10 (supplementary Fig. 2). LTB and MAL were mainly expressed in T cells (supplementary Fig. 1).

Most of the monocytes co-expressed CD14 and CD16 suggesting that these cells were in an intermediate stage of maturation (Fig. 2). Additionally, these cells expressed peripheral monocyte markers such as CD14, FCER1G, CD86, CD68, TMEM 119, and CD44, suggesting a peripheral origin for these cells. Furthermore, the monocyte cluster in the CD8 + CD161 + aSAH samples had a very weak expression of microglial-specific markers such as TMEM119, CD80, and MYB (Fig. 3).

Experiment 2: RT-qPCR Analysis of Gene Targets

Thirteen CSF samples were analyzed using RT-qPCR: 9 patients with aSAH and 4 patients with naSAH. aSAH samples were collected at a median day 3 (1, 4), while naSAH samples were also collected at a median day 3 (2, 4). Of the 10 gene targets that were analyzed, CXCL10 levels were higher in aSAH samples than in naSAH (median, IQR = 90, 16–149 vs. 0.5, 0–6.75 $p = 0.02$) (Table 2). Similarly, patients with aSAH had higher levels of HMOX1 compared to patients with naSAH (median, IQR = 12.6, 9–17.6 vs. 2.55, 1.68–5.7, $p = 0.076$).

Discussion

We previously described that CD8 + CD161 + cells are elevated in the acute phase of aSAH in patients who developed DCI [8]. In the present study, we observed CD8 + CD161 + monocytes, NK cells, and T cells in aSAH samples. Among these cell types, we identified a subpopulation of monocytes that expressed high levels of CXCL10. Microglial marker analysis suggested a peripheral origin for this subpopulation. Furthermore, we observed that CXCL10-RNA was significantly elevated in aSAH compared to naSAH.

Aneurysmal rupture leads to the release of various signals that increase the permeability of the blood-brain barrier (BBB) by inducing endothelial injury [24]. A damaged BBB favors the migration of activated neutrophils, T cells, and monocytes into the central nervous system [25]. Hemoglobin deposition in the subarachnoid space promotes inflammation, increased BBB dysfunction, and activation of peripheral inflammatory cells and microglia [26]. scRNAseq of CD8 + CD161 + cells in the CSF of patients with aSAH led to the profiling of three different cell populations: monocytes, NK, and T cells. Monocytes are especially relevant to the immune response in aSAH as multiple studies have shown increased monocyte counts in the CSF of patients with aSAH [3]. The clinical significance of CD8 + CD161 + monocytes in aSAH CSF samples is unclear, but it is highly suggestive of monocyte activation.

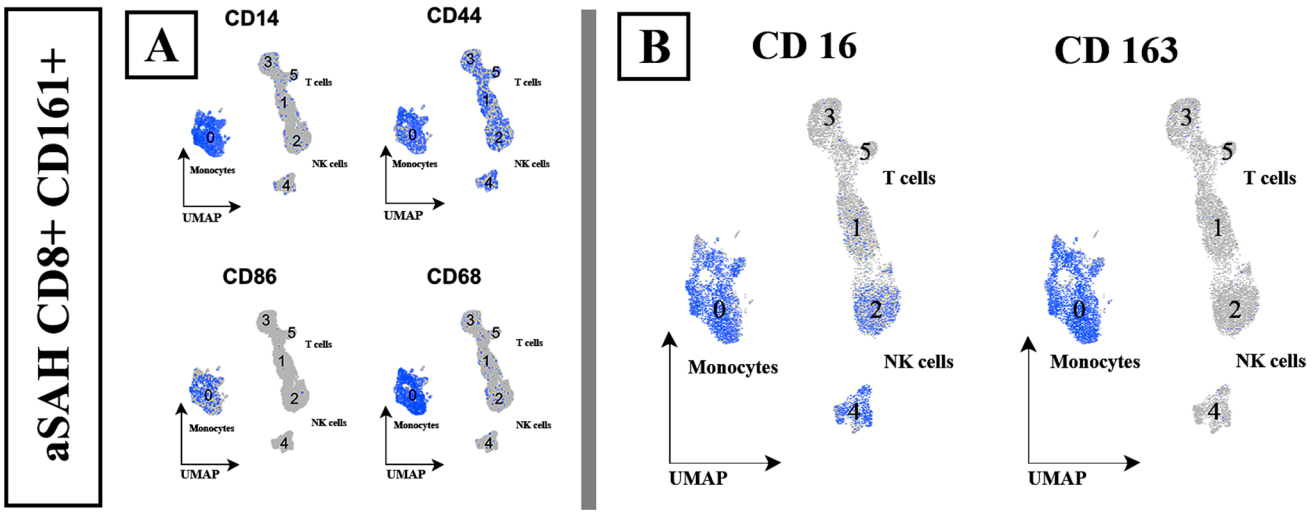

Fig. 2 Monocyte markers expressed in aSAH samples. (**a**) CD14, a marker commonly found on immature and intermediate monocytes, exhibited a strong signal within the monocyte population. Additionally, markers of monocyte activation, such as CD86 and CD68, showed significant expression within the monocyte cluster. The coexpression of these markers suggest an activated state. (**b**) Similarly, CD16 and CD163, although not limited to the monocyte cluster, were strongly expressed. This suggests that the intermediate phenotype of monocytes is predominant in our sample. aSAH, aneurysmal subarachnoid hemorrhage

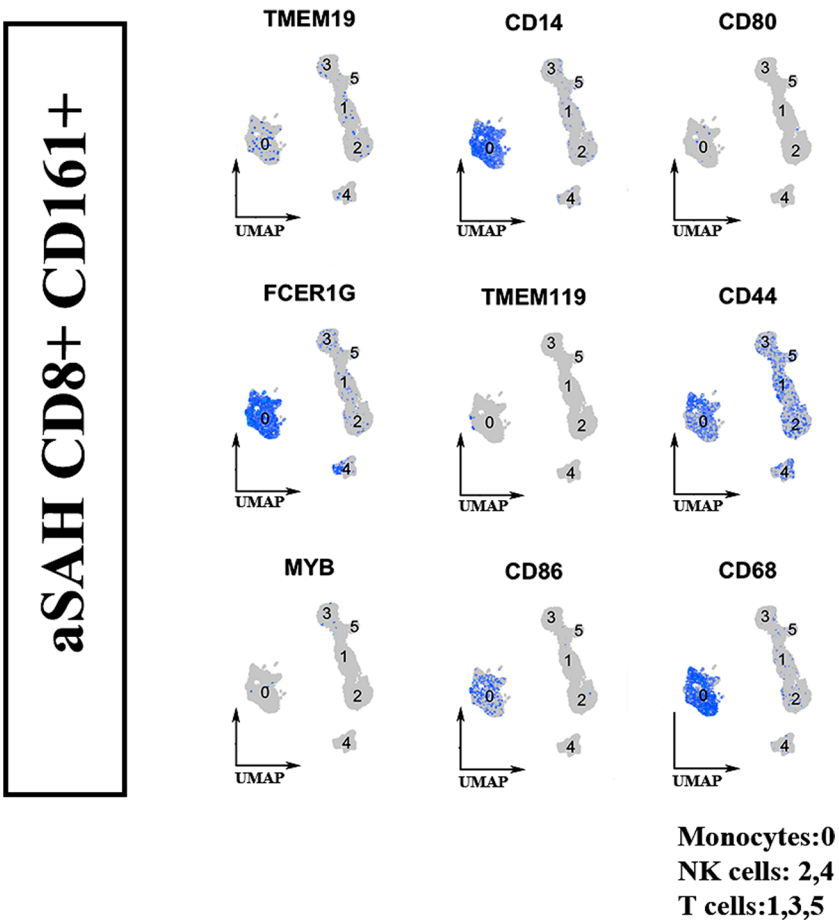

Fig. 3 Microglial markers in aSAH. Various shared markers of peripheral monocytes and microglial cells were expressed in monocytes in the CSF of patients with aSAH. Microglial-specific markers including TMEM119, MYB, and CD80 were not widely expressed suggesting a peripheral origin for monocytes. A peripheral origin is feasible in the setting of a damaged blood-brain barrier that favors the infiltration of peripheral cells that can modify the local immunological environment of aSAH. aSAH, aneurysmal subarachnoid hemorrhage

Table 2 Genes analyzed using RT-qPCR. RNA levels of ten genes of interest in the CSF of patients with aSAH (aneurysmal) and naSAH (non-aneurysmal). Data are represented as a median (IQR). *Indicates a significant difference between gene expression in the aSAH and naSAH groups (Mann–Whitney U; $p < 0.05$). Values in bold indicate statistical significance

Gene	Aneurysmal median (IQR)	Non-aneurysmal median (IQR)	p value
CCL5	4.76 (2.95, 6.8)	4.5 (2.95, 9)	1
CCL7	20 (10, 29.6)	19.2 (10, 34.1)	1
HLA-DRB1	9.89 (6.44, 11.11)	7.89 (6, 8.86)	0.604
APOE	9.02 (2.87, 18.42)	9.73 (2.87, 18.59)	0.825
SPP1	3.81 (1.13, 9.85)	5.52 (2.99, 7.84)	1
CXCL8	2.31 (1.15, 2.56)	1.19 (1.15, 3,46)	0.604
CXCL10	90 (16, 139)	0.5 (0, 6.75)	**0.02***
HMOX1	12.6 (9, 17.6)	2.55 (1.68, 5.7)	0.076
LTB	1.15 (1.04, 2.13)	1.08 (0.75, 1.89)	0.710
MAL	0.76 (0.68, 1.88)	1.23 (1.13, 1.41)	0.710

*$p < 0.05$

Monocytes can be activated through direct cellular interaction or by circulating cytokines [27]. An altered BBB may allow peripheral monocyte infiltration. CXCL10 production by infiltrating monocytes can recruit more monocytes and amplify the immune response. We observed that mRNA levels of CXCL10 were higher in aSAH compared to naSAH CSF. CXCL10 is a strong chemoattract for monocytes, NK, and T cells, leading to recruitment and migration [28, 29]. It has been postulated that increased leukocyte counts lead to late complications of aSAH [30, 31]. In our study, CXCL10 was mainly expressed in the CD8 + CD161 + cells. The main cell type expressing CXCL10 was monocytes. Although CXCL10 can be expressed by various cell types [32], previous studies have demonstrated secretion of CXCL10 mainly by human monocytes [28, 29]. In our samples, monocytes expressed markers that suggested a peripheral origin (Fig. 3). CD44 was widely expressed; this marker is known to be expressed in the peripheral leukocytes but not by the microglia [23]. Moreover, intermediate monocytes (CD14 + CD16+, Fig. 2), the main subtype present in our CD8 + CD161 + samples, actively secrete cytokines that favor inflammation [33]. Increased levels of intermediate monocytes have also been associated with worse neuropsychiatric outcomes during inflammatory states [34]. This finding supports the potential role of monocytes in generating signals for leukocyte recruitment in aSAH.

CXCL10 may also facilitate cellular injury through direct neurotoxicity. CXCL10 signaling through CXCR3 can induce apoptosis in fetal neurons through intracellular calcium dysregulation [35]. Moreover, CXCL10 blockage results in improved neurologic function and halting of disease progression in mice with induced multiple sclerosis [31]. However, further research is needed considering most studies are animal-based and were not done in aSAH.

CXCL10 may also mediate the cellular environment in the unruptured aneurysm. Prior to rupture, CXCL10 is elevated in the unruptured aneurysmal sac [4]. Moreover, CXCL10 was identified in a group of genes that predicted the presence of unruptured aneurysms [36]. CD8 + CD161 + cells are present in the aneurysmal wall of unruptured aneurysms [8]. It is unclear if CD14 + monocytes expressing CD8 + CD161 + may play a role in aneurysm formation. Further molecular profiling of CXCL10 in aSAH could help in the identification of potential therapeutic targets.

Limitations

The present study has several limitations that should be acknowledged:

1. Using naSAH CSF samples as a control may introduce bias to our results. In future studies, we will consider adding a non-hemorrhagic control group, such as normal pressure hydrocephalus patients.
2. The scRNAseq analysis in this study was conducted on a small sample size ($n = 2$). Although this sample size is typical for scRNAseq studies and it may be sufficient to provide valuable insights on the immunological response profile, it may limit the generalizability of the findings.
3. CSF samples were collected at different time points after hemorrhage. The study's focus was not on examining the chronological changes in the immune response over time but rather on providing a broad characterization of the role of CD8 + CD161 + cells. Moreover, vasospasm and DCI can manifest within a broad window, typically between days 3 and 14 post-aSAH, but these events can happen on day 1 and up to 20 days post-hemorrhage [3]. For consistency, future studies should collect CSF at determined timepoints.
4. Patients with different hemorrhage volumes and clinical presentations were included in this study. Our findings suggest the presence of a common mechanism in events triggered by aneurysm rupture, irrespective of hemorrhage severity and outcomes.
5. The study primarily focused on CD8 + CD161 + cells in aSAH, given previous research suggesting their activation. However, multiple cell lineages are involved in the immune response following aSAH, and the characterization of all these cell lineages was beyond the scope of this pilot study.
6. The analysis was limited to ten genes that were previously described in the literature as potential relevant genes in the immune response following aSAH. It is important to explore other genes that may involved in aSAH to gain a comprehensive understanding of the immunological response.
7. Patients developing vasospasm after SAH have not been included in the second experiment. We were interested in evaluating the inflammatory response profile irrespective of whether it resulted in vasospasm or DCI, given the difficulty in predicting these outcomes at the time of sample collection. The absence of any patients presenting with vasospasm provided an advantage in terms of having a homogeneous sample with similar outcomes. However, future and larger studies may include patients who develop vasospasm and DCI.

Conclusions

Monocytes, NK, and T cells express CD8 + CD161 + in patients with aSAH. CXCL10 was primarily expressed by monocytes. CXCL10 is a potential target for future studies due to its higher levels of expression in aSAH compared to naSAH patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12975-024-01259-4.

Author Contribution Supervision and concept of the study: EAS. Acquisition and analysis of data: MSC, YL, ES, AG, and CD. Manuscript drafting and final approval: all authors.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Ethics Approval The Institutional Review Board of the University of Iowa approved this study under IRASH (IRB ID number: 201902739).

Informed Consent Informed Consent was acquired for every patient under supervision of the Institutional Review Board of the University of Iowa.

Competing Interests The authors declare no competing interests.

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