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# Leukocyte inflammatory phenotype and function in migraine patients compared with matched non-migraine volunteers: a pilot study

Hongtao Li<sup>1,2\*</sup>, Qiang Fu<sup>3†</sup>, Kamaira Philips<sup>4</sup>, Yufei Sun<sup>3</sup>, Keturah R. Faurot<sup>2</sup>, Susan A. Gaylord<sup>2</sup> and John Douglas Mann<sup>2,5</sup>

## Abstract

**Background:** Migraine is a neurological condition characterized by chronic inflammation. However, not much is known about the potential role of peripheral blood immune cells in the pathophysiology of migraine.

**Methods:** We investigated the status of peripheral blood immune cells of 15 adults with frequent episodic or chronic migraine recruited chronologically from a randomized clinical trial (RCT) on Nutrition for Migraine (NCCIH 5R01AT007813-05) and 15 non-migraine, healthy volunteers (control) matched by age, gender, and Body Mass Index (BMI).

Continuous variables were presented as means  $\pm$  standard deviation as well as medians, and comparisons between patients and healthy volunteers were performed with non-parametric Wilcoxon signed rank tests. Statistical analysis was performed using Stata (StataCorp. 2019. Stata Statistical Software). Fluorescence-Activated Cell Sorting (FACS) data were processed using FlowJo software (Ashland, OR: Becton, Dickinson and Company; 2019).

**Results:** We observed that migraineurs had a significantly lower percentage of non-classical monocytes (CD14<sup>+</sup>CD16<sup>++</sup>) in blood circulation, compared to the control group. In addition, Migraineurs also showed a significantly lower percentage of blood CD3<sup>+</sup>CD4<sup>+</sup> helper T cells and CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, compared to controls. Differences in leukocyte surface markers between chronic migraine patients and their matched controls were more prominent than those between episodic migraine patients and their matched controls.

**Conclusions:** Our results suggest that migraine is associated with dysregulated peripheral immune homeostasis and that inflammation and autoimmunity may play a role in its pathophysiology.

**Keywords:** Migraine, Inflammation, Autoimmunity, Monocytes, T cells

## Summary sentence

Migraineurs' peripheral blood shows altered monocyte and T cell subpopulation compared with the control group, suggesting migraine is potentially associated with inflammation and autoimmune processes.

<sup>†</sup>Hongtao Li and Qiang Fu Both authors contributed equally to this work.

\*Correspondence: hongtaoli66@gmail.com

<sup>1</sup> Department of Science and Mathematics, Hulman Hall Room 116, Saint Mary-of-the-Woods College, 1 St Mary of Woods Coll, IN 47876, USA  
Full list of author information is available at the end of the article



## Background

Migraine is a major public health problem afflicting over 16% of women, 7% of men, and 12% of the total adult US population and costing billions in health care utilization [1, 2]. Inflammation has long been considered a risk factor in migraine pathogenesis [3, 4]. Examples of elevated inflammatory biomarkers, including fibrinogen and C reactive protein (CRP), have been reported in migraine pathology [5–7]. In 2001, Kemper et al. reviewed 45 clinical studies from 1966 to 1999 and found differences in serum levels of complement, immunoglobulin, histamine, cytokines, and immune cells (monocyte and polymorphonuclear leukocytes) between migraineurs and volunteers without migraine. However, the findings in the various studies were inconsistent and conflicting with each other in many cases [8, 9], indicating a need for further investigation into the role of immune system dysfunction in migraineurs.

Given this emerging link between inflammation and migraine pathogenesis, identification of leukocyte surface antigens could potentially serve as biomarkers to help with diagnosis. In 2006, Du and colleagues reported that the genes significantly up-regulated by migraine were mostly from platelet/monocytes, while others were from PMNs, CD4<sup>+</sup>, CD8<sup>+</sup> T cells, and NK cells [10]. Research has also shown that lymphotoxin alpha (or tumor necrosis factor-beta) and  $\alpha$ -fodrin are among the seven-upregulated genes in migraine with aura, compared with healthy controls [11]. Recently, a genomic-wide analysis using whole blood of 83 migraine cases and 83 age and gender-matched non-migraine controls revealed that multiple immune-inflammatory pathways, such as functional categories of HECS, Microglia, RACTOME, and Gene ontology biological processes, were underlying the pathophysiology of the disorder [12].

Arumugam and Parthasarathy (2016) studied the autoimmune biomarkers CD4<sup>+</sup>CD25<sup>+</sup> population, helper and suppressor T cell populations, observing a significantly higher CD4<sup>+</sup> population and lower CD8<sup>+</sup>T cell population in migraineurs, compared to healthy volunteers. Furthermore, the CD4<sup>+</sup>CD25<sup>+</sup> population was significantly lower in migraine patients compared to healthy volunteers [13], suggesting that migraine may be related to autoimmunity.

Monocytes comprise a heterogeneous population that plays a vital role in immune surveillance of the central nervous system (CNS). There are at least three different subsets: classical monocytes (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate monocytes (CD14<sup>++</sup>CD16<sup>+</sup>) and non-classical monocytes (CD14<sup>+</sup>CD16<sup>++</sup>) [14]. Waschbisch and colleagues reported that monocytes expressing Fc $\gamma$ RIII, or CD16<sup>+</sup>, which include both intermediate and non-classical monocytes together, were reduced in the peripheral

blood and migrated to the sites of inflammation, contributing to the injury of the blood–brain barrier (BBB) in CNS autoimmune diseases such as multiple sclerosis (MS) [15]. The research literature has reported the patrolling behavior of the inflammatory non-classical monocyte subset [16–19] and the activation of genes associated with cytoskeleton mobility [16]. CD16<sup>+</sup> monocytes have been reported migrating to the central nervous system in MS, HIV associated neurocognitive disorder, and giant cell arteritis [15, 20, 21]. To our knowledge, there is no report showing that CD16<sup>+</sup> monocytes could migrate to the CNS, thereby decreasing the population percentage in migraineurs' peripheral blood.

T cell surface integrins can play an important role in cellular adhesion to extracellular matrix and cell signaling. Lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4) changes have been linked to migraine attacks [22, 23]. LFA-1 is an integrin and belongs to the integrin superfamily of adhesion molecules. It has been reported that T cell surface  $\beta$ 2 subunit or CD18 is required for trafficking to the intestinal tissue during an intestinal immune responses [24].

The objective of the present study was to characterize migraineurs' peripheral leukocyte surface biomarkers and ex vivo functions to determine whether our findings indicate that migraine is related to neuroinflammation and autoimmunity. We used pre-intervention/baseline samples collected from a parent RCT entitled "Clinical and Metabolic Effects of Altering Omega-3 and Omega-6 Fatty Acids in Migraine" funded by an NIH R01-AT007813 (2013–2018) awarded to investigators at the University of North Carolina (UNC) at Chapel Hill [2]. We proposed to examine the leukocyte phenotype profile and cell type function between matched non-migraine volunteers and the migraineurs' baseline data. This comparison may provide mechanistic insights and initial therapeutic strategies for targeting inflammation in migraine.

## Materials and methods

### Participants and ethical clearance

Fifteen consecutive migraine patients who entered the parent R01 RCT study during 2015 and 2016 were selected to be involved in this study. Both studies received approval from UNC's Institutional Review Board (IRB) and the Office of Human Research Ethics and all participants provided informed consent. These subjects were matched with fifteen healthy controls who were recruited to the study from the same population from which the migraineurs were drawn, through university advertising (e.g., mass email). Ascertained via telephone screening and health history questionnaire, eligible volunteers were 18 years of age or older; without chronic pain, pregnancy,

or major medical/psychiatric illness; non-smokers; not taking omega-3 supplements; and without concurrent illness. Volunteers were matched to migraine participants by age (10-year categories), sex, and Body Mass Index (BMI) (<20, 20–24.9, 25–29.9, 30–34.9, >35). Migraine patients provided samples on the day of randomization before receiving the intervention (or the enrollment visit). If they were having a migraine attack, this visit was postponed. Both migraine patients and control group volunteers had fasted for 8 or more hours prior to phlebotomy. Samples were processed immediately.

**Peripheral whole blood staining method using BD bioscience protocol**

Following BD whole blood staining protocols [25], we chose three panels of antibodies, with each panel containing eight antibodies. Table 1 shows the experimental design with the intention to investigate different peripheral leukocyte populations. The antibodies used in this study included: anti-CD3 (APC-H7), anti-CD36 (PerCP-Cy5.5), anti-CD14 (V500), anti-CD16 (BV421), anti-HLA-DR (BB515), anti-CD56 (PE –Cy7), anti-CD86 (APC) (BioLegend, San Diego, CA), anti-CD163 (PE) (BioLegend, San Diego, CA), anti-CD8 (v500), anti-CD4 (PerCP-Cy5.5) (BioLegend, San Diego, CA), anti-CD25 (BB515), anti-CD18 (PE), anti-CD49d (APC), anti-CD11c (BV510), anti-CD123 (BV421), anti-CD19 (PE-Cy7), and anti-CD80 (PE). All antibodies were purchased from BD Bioscience except where stated otherwise. Briefly, appropriate volumes of fluorochrome-conjugated antibody were added to 100 µL of whole blood and incubated 15 to 30 min at room temperature. Then, the erythrocytes were lysed, and the samples were washed, fixed, and

analyzed within about 24 h. Data were collected with FACS CANTO II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Peripheral blood mononuclear cells (PBMC) isolation, stimulation, and supernatant collection**

PBMCs were isolated from freshly collected human blood samples following density gradient centrifugation over Ficoll-Hypaque density gradient. Briefly, a diluted cell suspension was carefully layered over Ficoll-Hypaque in a conical tube without disturbing the interface, centrifuged at 1500 rpm for 30 min at room temperature with the brake off. The mononuclear cell layer was at the interphase. The RBC and platelets were washed and a cell counting procedure was performed by TC20™ Automated Cell Counter (BIO-RAD, Hercules, CA).

In vitro stimulations of one million PBMCs per well with 100 ng/ml lipopolysaccharide (LPS) for 24 h were set up in a 6-well culture plate. After stimulation, supernatants were collected for cytokines analysis.

**Multiplex measurement of cytokines**

To investigate the cytokine production by ex vivo LPS, nine cytokine (TNF-α, IL-6, IL-10, IL-1β, IFN-γ, IL-4, IL-17a, IL-12 p70, and IL-21) levels in PBMC supernatants from pre- and post-LPS 24 h of stimulations were analyzed using R&D multiplex analysis system at the university's Cytokine & Biomarker Analysis Facility Center.

**Cell gating strategies**

For monocyte gating, neutrophils, NK cells, B cells, and T cells were successively excluded following the strategy by Mukherjee et al. [19]. For T cell gating, subsets were chosen based on conventional bivariate scatterplots of side scatter signal and CD3<sup>+</sup>, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup>CD25<sup>+</sup> T cells subsets.

**Statistical analysis**

This study presented continuous variables as means ± standard deviations and medians. Due to the small sample sizes, comparisons between patients and healthy volunteers were performed with Wilcoxon signed rank tests. An exploratory sensitivity analysis was undertaken using a hierarchical linear model using random effects of the matched pair. For distributions that were non-normal, estimates were checked using transformations. If interpretation of the results did not change, the original model results were reported. Results are provided for unadjusted differences in means between groups as well as estimates adjusted for chronic migraine and meeting criteria for medication overuse headache. Our statistical analysis was performed using Stata (Stata-Corp. 2019. Stata Statistical Software: Release 16. College

**Table 1** The three flow cytometry panels of antibodies with the intention of different population screening. Following BD whole blood staining protocols, we chose three panels of antibodies, with each panel containing eight antibodies as shown in the table.

Monocyte, its subsets and activation markers		NK, T cells, their subsets and activation markers		B, Dendritic cells, their subsets and activation markers	
CD3	APC-H7	CD8	V500	CD3	APC-H7
CD36	PerCP-Cy5.5	CD4	PerCP-Cy5.5	CD4	PerCP-Cy5.5
CD14	V500	CD3	APC-H7	CD11c	BV510
CD16	BV421	CD16	BV421	HLA-DR	BB515
HLD-DR	BB515	CD25	BB515	CD123	BV421
CD56	PE-Cy7	CD18	PE	CD86	APC
CD86	APC	CD56	PE-Cy7	CD19	PE-Cy7
CD163	PE	CD49d	APC	CD80	PE

Station, TX: StataCorp LLC.). Fluorescence-Activated Cell Sorting (FACS) data were processed using FlowJo software (Ashland, OR: Becton, Dickinson and Company; 2019). Cytokine multiplex data were processed using Stata (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.).

## Results

### Descriptive statistics

A total of 30 subjects – 15 migraine patients and 15 matched control individuals – participated in this study. Their demographic comparisons are listed in Table 2. As expected, 80% were female, and the mean ( $\pm$  standard deviation) age was  $39.3 \pm 12.2$  in the migraine sample, and  $39.7 \pm 12.2$  in the controls. BMI means were  $27.2 \pm 4.6$  and  $27.4 \pm 4.8$  in migraine and control groups, respectively.

**Table 2** Demographic comparison between migraine and its matched control group

	Migraine sample	Controls
Male gender <sup>a</sup>	3 (20%)	3 (20%)
Age <sup>a</sup>	39.3 (12.2)	39.7 (12.2)
Body Mass Index <sup>a</sup>	27.2 (4.6)	27.4 (4.8)
Race/ethnicity		
NonHispanic White	12 (80%)	10 (67%)
NonHispanic Black	2 (13%)	2 (13%)
Other	1 (7%)	3 (20%)
Education		
High school or less	3 (20%)	0 (0%)
Some college	2 (13%)	5 (38%)
Bachelor's degree	5 (33%)	4 (31%)
Advanced degree	5 (33%)	4 (31%)
Income		
\$20,000 or less	1 (7%)	0 (0%)
\$21,000—40,000	1 (7%)	4 (29%)
\$41,000—60,000	2 (13%)	4 (29%)
\$61,000—80,000	2 (13%)	3 (21%)
More than \$80,000	7 (47%)	3 (21%)
Relationship status		
Living with partner	12 (80%)	7 (50%)
Not living with partner	3 (20%)	7 (50%)
Smoking status		
Current smoker	1 (7%)	0 (0%)
Former smoker	4 (27%)	3 (21%)
Nonsmoker	10 (67%)	11 (79%)
Alcohol intake		
None or rare	13 (87%)	10 (71%)
Occasional	2 (13%)	4 (29%)

Continuous variables presented as mean (standard deviation)

Categorical variables presented as n(%)

<sup>a</sup> Matching variables

Migraine has many comorbidities, and people with migraines were significantly more likely to report comorbidities related to inflammation among other conditions [22]. The 15 migraine patients' pain information, including headache, comorbid pain, with or without aura, whether it meets chronic migraine criteria or not, and medication overuse information are provided in the Table 3. The number of migraines in 30 days was  $11.2 \pm 5.7$  days, and the number of headaches in 30 days was  $18.6 \pm 7.7$  days. About 53% of the patients met chronic migraine criteria, based on the most recent diagnostic criteria (ICHD-3), i.e., headache occurring on 15 or more days/month for more than three months, which, on at least eight days/month, has the features of migraine headache. Eight out of 15 patients (53%) met the criteria for overuse of any pain medication.

### Comparison of CD14<sup>+</sup>CD16<sup>++</sup> monocytes and CD14<sup>++</sup>CD16<sup>-</sup> monocytes

To examine whether migraine patients have altered blood monocytes, we analyzed the peripheral blood classic and non-classic monocytes in migraineurs and matched control group using flow cytometry. In PBMCs single cells population, we removed NK and T cells, and then divided the HLA-DR<sup>+</sup> population into three subgroups and analyzed by CD14 and CD16 expression strength (Fig. 1A). We found that the non-classical monocyte percentage ( $11.9 \pm 10.5$ ) (Table 4 and Fig. 1B) was lower in the migraine group compared with the control group, ( $33.9 \pm 21.4$ ) ( $p=0.005$ ). In contrast, the percentage of classical monocytes (CD14<sup>++</sup>CD16<sup>-</sup>) was  $49.9 \pm 27.6\%$  in the control group and  $72.3 \pm 18.1\%$  in the migraine group ( $p=0.06$ ) (Table 4 and Fig. 1B). Supplemental Table 1 also provides estimates comparing individuals with chronic migraine and medication overuse headache (MOH) to controls. These estimates should be interpreted with caution due to the extremely small sample sizes.

In exploratory sensitivity analyses using hierarchical linear models, we found significant differences at the  $p=0.05$  level between patients and controls comparing non-classical (mean difference[MD] -21.65; 95% confidence interval [CI] (-38.88, -4.42)) and classical monocytes (MD26.5; 95% CI: (3.04, 50.12)) in models adjusted for chronic migraine and MOH (Supplemental Table 2).

### Percentage of CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells in blood

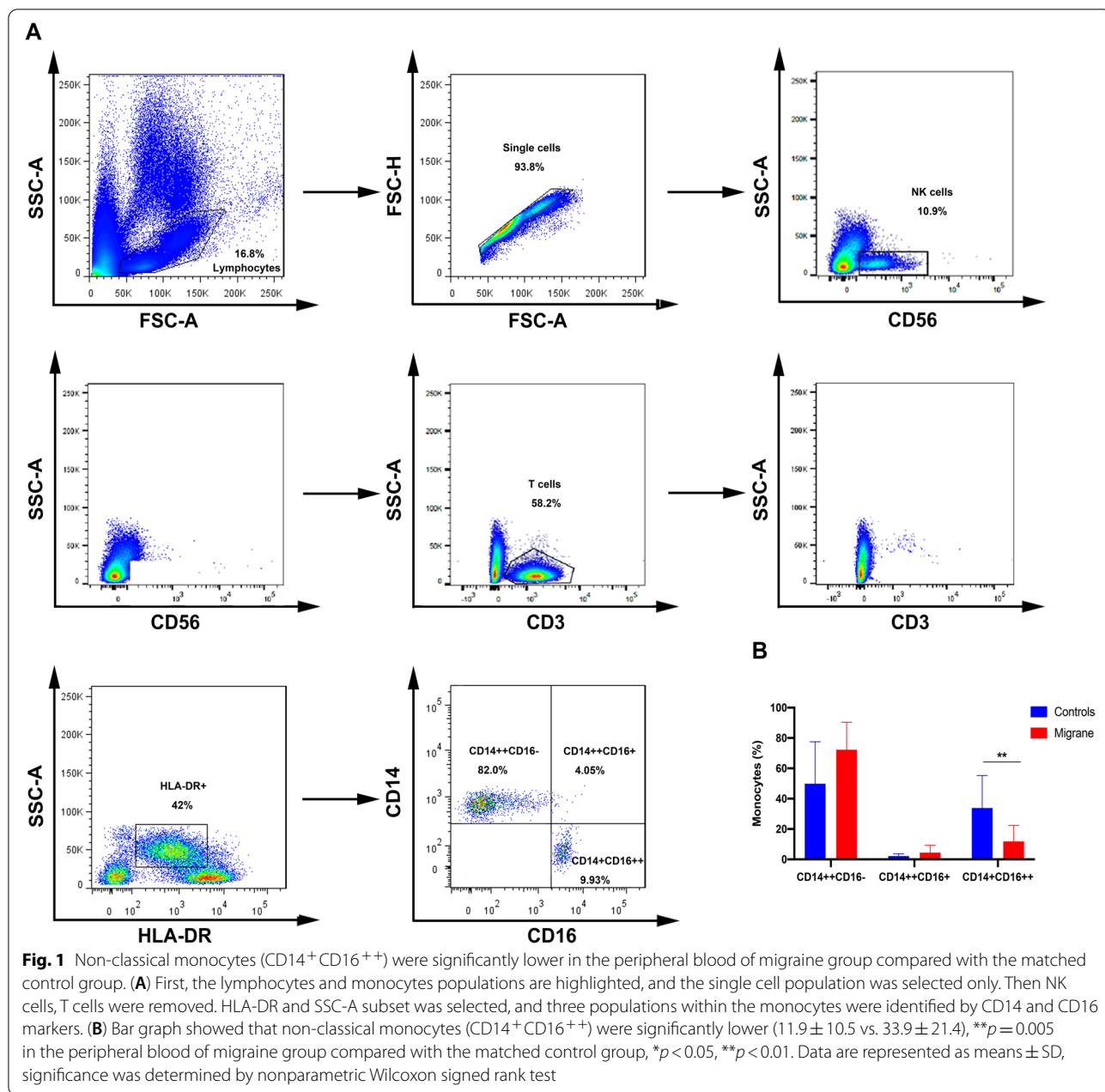
In our migraine group, CD4<sup>+</sup>T cells were significantly lower ( $64 \pm 5.45$  vs.  $70.6 \pm 8.92$ ) ( $p=0.035$ ) and CD8<sup>+</sup>T cells were slightly higher ( $p=0.3$ ) in the migraine group compared with the control group (Table 4 and Fig. 2). However, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio difference is not significant ( $p=0.09$ ). (Table 4).

**Table 3** Migraine, headache (HA), pain information and overuse of pain medication

Study ID	Number of HA per 30 days	Number of migraines per 30 days	Comorbid pain	Aura	Meets chronic migraine criteria	Percent of days with HA (A)	Percent of days with Migraine (B)	Percent of headaches that are migraines (A/B)	Meets criteria for overuse of any pain medication	Triptan overuse	NSAID /ASA overuse	Overuse of opioids	Overuse multiple drug classes
M1133	9	9	TMJ	1	0	31%	29%	91%	0	0	0	0	0
M1139	26	20	None	1	1	87%	65%	75%	1	0	1	0	1
M1140	30	19	IBS	0	1	100%	62%	62%	0	0	0	0	0
M1142	30	20	Fibromyalgia	0	1	100%	68%	68%	1	0	0	0	1
M1143	30	20	Fibromyalgia	0	1	100%	68%	68%	0	0	0	0	0
M1144	17	7	IBS	0	0	57%	22%	38%	0	0	0	0	0
M1145	24	9	Back pain	0	1	81%	29%	35%	1	0	0	1	1
M1149	10	6	None	0	0	34%	21%	60%	1	0	0	0	1
M1152	9	6	None	1	0	29%	20%	70%	0	0	0	0	0
M1153	19	12	None	0	1	64%	41%	64%	1	0	0	0	1
M1154	17	6	Arthritis	1	0	55%	21%	38%	1	0	0	0	1
M1155	16	8	Back pain	0	1	52%	28%	54%	0	0	0	0	0
M1156	13	5	None	0	0	43%	18%	42%	1	0	0	0	1
M1158	12	11	Arthritis	0	0	41%	38%	92%	1	0	0	0	1
M1159	16	10	None	1	1	55%	32%	58%	0	0	0	0	0
Mean	18.6	11.2	Sum	5	8				8				
SD	7.7	5.7	Percent	33%	53%				53%				

Definition of chronic migraine: Headache occurring on 15 or more days/month for more than 3 months, which, on at least 8 days/month, has the features of migraine headache





We identified the CD4<sup>+</sup>CD25<sup>+</sup> population in this study and display the results in Fig. 3A. Compared with the control group, the migraine group had a significantly lower percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells (Table 4 and Fig. 3B). CD4<sup>+</sup>CD25<sup>+</sup> T cell percentages in matched control and migraine groups were about 8.3% and 5.7% of CD3<sup>+</sup> T cells respectively (*p* = 0.001). Significant differences between groups persisted after adjustment for chronic migraine and MOH (MD -2.52; 95% CI: -4.46, -0.57)(Supplemental Table 2).

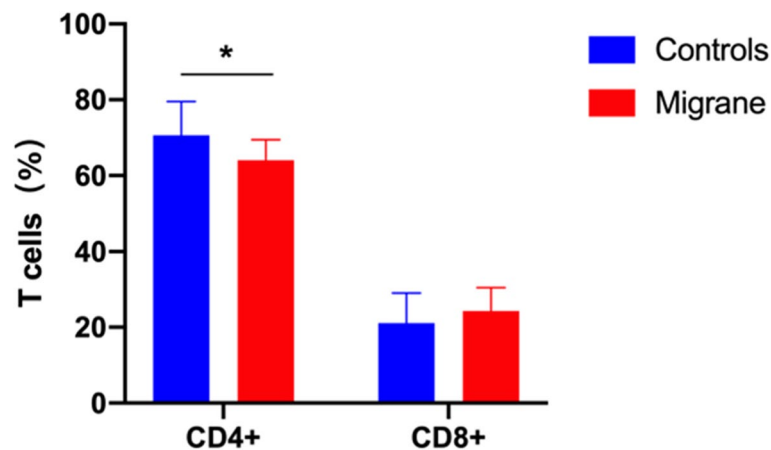
#### Integrin CD18 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells

Compared with the matched control group, migraine patients' CD18, which is part of the LFA-1, were lower in expression levels measured by mean fluorescence intensity (MFI) on both CD4<sup>+</sup> T and CD8<sup>+</sup> T cells, as shown in Table 4 and Fig. 4. The CD18 MFI on CD4<sup>+</sup> helper T cells in matched control and migraine group were 735 ± 139 and 619 ± 135, respectively (*p* = 0.06). The CD18 MFI on CD8<sup>+</sup> killer T cells in matched control and migraine groups were 924 ± 239 and 771 ± 250,

**Table 4** Means, standard deviations, and medians for groups defined by control and migraine status

Variable	Controls		Migraine		p value*
	n = 15 Mean (SD)	Median	n = 15 Mean (SD)	Median	
Monocytes (%)					
Classical (CD14 <sup>++</sup> CD16 <sup>-</sup> )	49.9 (27.6)	48.7	72.3 (18.1)	78.5	0.06
Intermediate (CD14 <sup>++</sup> CD16 <sup>+</sup> )	2.23 (1.51)	1.86	4.44 (4.88)	3.44	0.09
Nonclassical (CD14 <sup>+</sup> CD16 <sup>++</sup> )	33.9 (21.4)	33.2	11.9 (10.5)	9.16	0.005
T cells (%)					
CD4 <sup>+</sup>	70.6 (8.92)	66.3	64.0 (5.45)	63.3	0.035
CD8 <sup>+</sup>	21.1 (7.92)	22.3	24.3 (6.16)	22.9	0.3
CD4 <sup>+</sup> /CD8 <sup>+</sup>	4.02 (2.10)	3.23	2.80 (0.75)	2.83	0.09
CD18(MFI) CD4 <sup>+</sup>	735 (139)	730	619 (135)	608	0.06
CD18(MFI) CD8 <sup>+</sup>	924 (239)	938	771 (250)	715	0.09
CD49(MFI) CD4 <sup>+</sup>	1247 (240)	1275	1195 (341)	1047	0.9
CD49(MFI) CD8 <sup>+</sup>	1468 (321)	1424	1327 (332)	1358	0.4
CD36	352 (255)	242	272 (191)	224	0.4
CD4 <sup>+</sup> CD25 <sup>+</sup>	8.29 (2.31)	7.69	5.66 (1.35)	5.08	0.001

\* p values in this table are based on comparisons with the controls based on the nonparametric Wilcoxon signed rank test

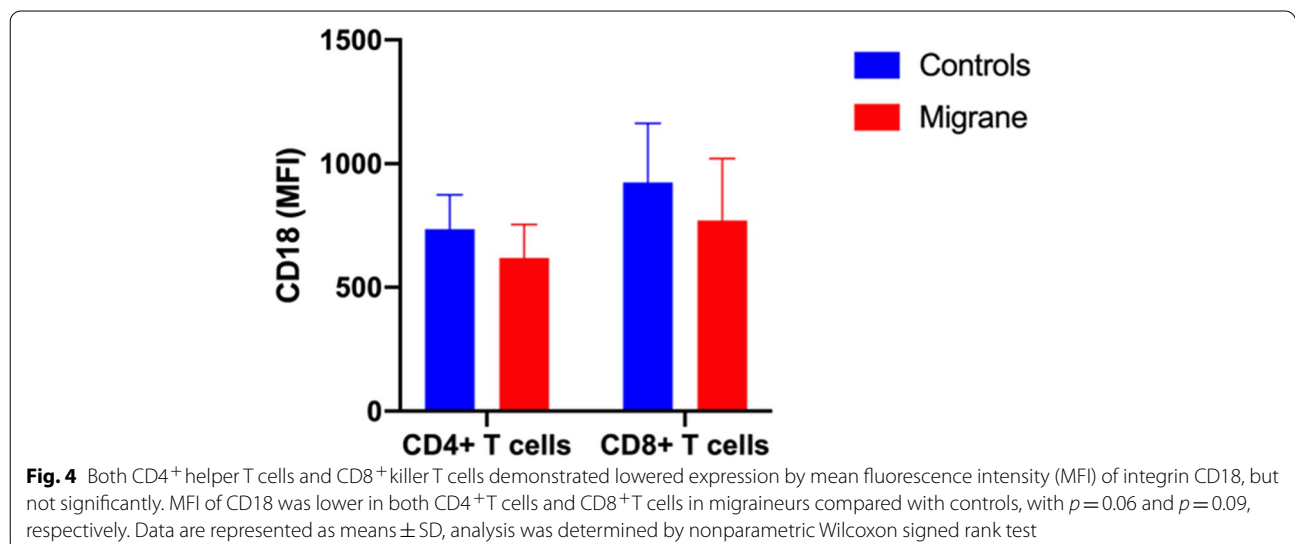
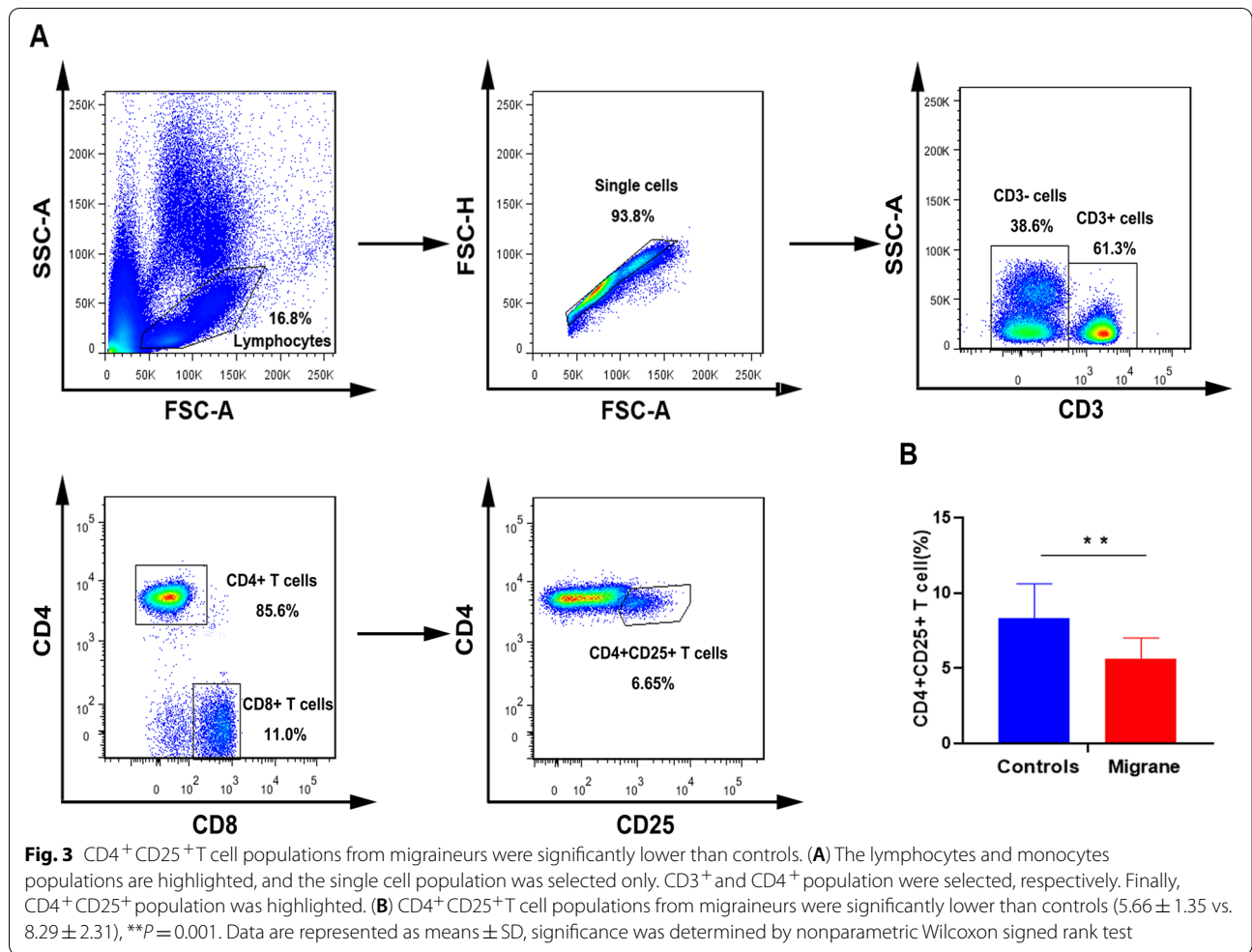


**Fig. 2** CD4 T cell populations from migraineurs were significantly lower than controls. (A) CD4<sup>+</sup> T cell populations from migraineurs were significantly lower than controls (64.0 ± 5.45 vs. 70.6 ± 8.92), \**p* = 0.035. (B) CD4<sup>+</sup>/CD8<sup>+</sup> ratio was lowered in the migraineurs, yet the change was not significant (not shown). \**p* < 0.05, \*\**p* < 0.01. Data are represented as means ± SD, significance was determined by nonparametric Wilcoxon signed rank test

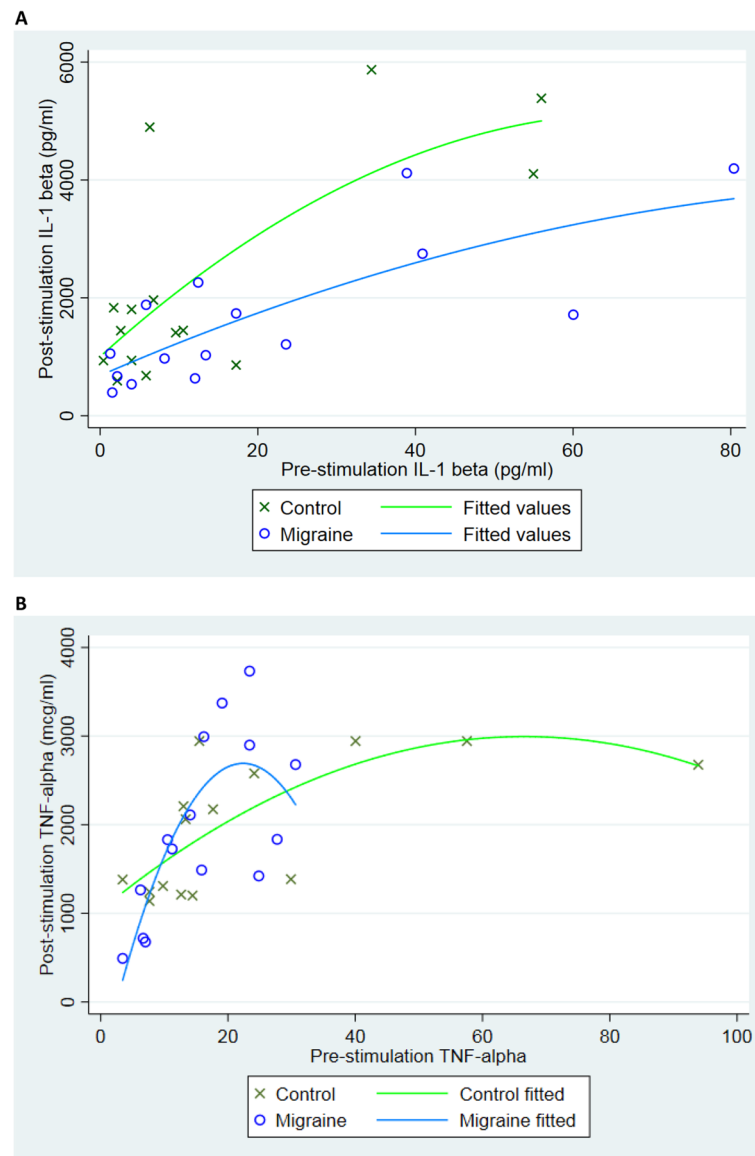
respectively (*p* = 0.09) (Table 4 and Fig. 4). Adjusting for chronic migraine and MOH attenuated the differences between the two groups. We found no significant difference in peripheral blood CD49d expression between migraineurs and healthy controls and controlling for chronic migraine and MOH reversed the direction of the difference. (Table 4 and Supplemental Table 1).

#### Cytokine analysis after ex vivo stimulation

We stimulated PBMCs from the recruited patients and control volunteers ex vivo with LPS; nine cytokines from supernatant were measured using the R&D multiplex system. Out of the nine cytokines (TNF- $\alpha$ , IL-6, IL-10, IL-1 $\beta$ , IFN- $\gamma$ , IL-4, IL-17a, IL-12 p70, and IL-21) studied, only IL-1  $\beta$  and TNF- $\alpha$  were in the readable ranges. No significant differences were detected. (Fig. 5A and B).







**Fig. 5** Cytokine analysis with 24-h LPS stimulation revealed no significant difference in inflammatory cytokine levels (IL-1 beta and TNF- $\alpha$ ) between migraineurs and controls. Pre-stimulation and post-stimulation of IL-1 $\beta$  (Fig. 5A) and TNF- $\alpha$  (Fig. 5B) were processed using Stata software, no significant changes were found

### Other cell populations

Other than the monocytes and T cell populations, we did not find differences in B cells, NK cells, or dendritic cells between the migraineurs and the control group.

### Discussion

Using peripheral whole blood staining methodology, we found that migraineurs had both myeloid and lymphoid leukocyte surface marker changes compared with the matched control group, which supports the neurogenic inflammatory and autoimmune nature of migraine.

We demonstrated that peripheral blood non-classical monocytes (CD14<sup>+</sup>CD16<sup>++</sup>) in migraine patients were lower than controls. In migraine, triggering factors such as chronic stress, certain foods, hormonal fluctuation, depression and other unknown causes can generate sterile inflammation in CNS and activation of nociceptors [23]. In the presence of these inflammatory stimuli, circulating inflammatory monocytes can quickly migrate into affected tissues, where they differentiate into macrophages and dendritic cell subsets [26–28]. The patrolling monocytes localize to the endothelium of vascular inflammatory tissues

and release inflammatory mediators [26, 29]. Patrolling CD16<sup>+</sup> monocytes exhibit long-range crawling over the endothelium of vascular tissues [17, 18], and may contribute to the pathogenesis of a wide range of chronic inflammatory diseases, such as obesity, diabetes, atherosclerosis, Chronic Obstructive Pulmonary Disease lung fibrosis, lung cancer, and Alzheimer's disease [30–32]. This population of monocytes can be detected in perivascular circulation; studies have demonstrated decreased levels of nonclassical monocytes in the peripheral blood, as reported in severe forms of lupus nephritis and MS [15, 33].

Current migraine research has provided some evidence of transmigration of non-classical monocytes to the CNS. Nagata et al. searched biomarkers for migraines using microarray analysis in 2009 [11]. In their report, the expression of 15 genes were altered in migraineurs compared to controls, and one-fifth of these genes were associated with cytoskeleton proteins. It has been reported that human non-classical monocytes exhibit crawling behavior on the endothelium both in vivo and in vitro [16–18, 26, 30]. Non-classical monocytes highly expressed genes associated with cytoskeleton mobility, including Rho GTPase, RHOC and RHOE, and some upstream Rho activators and downstream effectors [16]. These findings are consistent with the Waschbisch clinical study regarding CD16<sup>+</sup> monocytes migrating to the CNS in MS [15]. Tension-type headache and migraine without aura are among the most common primary headaches in MS patients [34]. CD16<sup>+</sup> monocytes play a pivotal role in immune-surveillance of the CNS, and they could migrate to the site of inflammation and contribute to the dysfunction of the BBB and CNS autoimmune disease [15] or NeuroAIDS [35]. Both the monocyte chemokine receptor CXCR7 [35, 36] and the integrity of the BBB play roles in inflammatory migraine pathophysiology [37, 38]. Due to limitations imposed by the parent RCT, and the small sample size of this affiliated pilot study, we have not investigated the profile of cerebrospinal fluid (CSF) samples. Immune cell profiling in CSF in future studies could provide additional insights into migraine pathogenesis.

Our study also indicated the potential association between migraine and failure of immunoregulation. We detected a significantly lower peripheral blood CD4<sup>+</sup> and its subgroup CD4<sup>+</sup>CD25<sup>+</sup>T cells in the migraine group compared to the matched healthy controls even after controlling for covariates (See Fig. 2 and 3). The finding of relatively lower CD4<sup>+</sup>CD25<sup>+</sup>T cells in migraineurs is consistent with a previous migraine study [13]. The difference between ours and Arumugam's study are in the T cells subsets CD4 and CD8 cells. In our migraine group, CD4<sup>+</sup>T cells were significantly lower and CD8<sup>+</sup>T cells were slightly higher in the migraine group compared with the control group (Fig. 3A). However, the differences in CD4<sup>+</sup>/CD8<sup>+</sup> ratio was small. (Fig. 3B) and appears to be largely due to lower ratio levels

in the chronic migraine group (Table 4). These results suggest that failure of immunoregulation could play a role in migraine pathophysiology. In an experimental mouse model of autoimmune encephalomyelitis, non-classical monocytes were shown to be potent suppressors of T cells [39]. Loss of quantity and/or suppressive function of non-classic monocytes may contribute to the development of autoimmune disease. Interestingly, a recent in vitro experiment showed that the CD16<sup>+</sup> monocytes, not CD16<sup>-</sup> classical monocyte, promoted CD4<sup>+</sup>T cell trafficking via the endothelial barrier [15]. Provided the CD16<sup>+</sup> monocytes did migrate to the CNS, they then may enhance T cell entry into the CNS, the latter further facilitating CD16<sup>+</sup> monocytes' attachment to the microvasculature and contributing to the breakdown of the BBB. This could explain why peripheral blood CD4<sup>+</sup>T cell numbers were lower in our study.

Considering that about half of the recruited patients also fall into chronic migraine or suffer from overuse of any pain medication, we did further analysis based on these two categories themselves. The results are in supplemental Table 1. We found that chronic migraine patients demonstrated the most significant changes compared with their matched volunteers, including non-classical monocytes, CD4<sup>+</sup>T cells, CD4<sup>+</sup>/CD8<sup>+</sup> ratio, CD18 MFI on CD4<sup>+</sup> and CD8<sup>+</sup>T cells, CD49 MFI on CD4<sup>+</sup>T cells, CD36, and CD4<sup>+</sup>CD25<sup>+</sup>T cell populations. We realized that the sample size was even smaller, and the pathophysiology of the transformation from episodic to chronic migraine can not be concluded at this time.

CD36 is a transmembrane protein expressed in various types of cells, including adipocytes, monocytes, macrophages, platelets, endothelial, and muscle cells [40]. This protein belongs to the scavenger receptor [40, 41] and binds to lipoproteins, apoptotic cells, and long-chain fatty acids; thus, CD36 is also known as fatty acid translocase [42]. Coburn et al. demonstrated that long chain fatty acid uptake and utilization have been defective in CD36 knockout mice [43]. Our team reported that a dietary intervention with increased omega-3 fatty acids helped to reduce headache pain [44, 45], and it has been known that omega-3 could induce the CD36 mRNA expression in an animal model [46, 47]. CD36 can bind to three classes of ligands including modified phospholipids, long chain fatty acids and domains of thrombospondin homologs [40]. Broad expression of CD36 on different cells suggests this multifunctional receptor could be involved in a broad spectrum of diseases [40, 48–50]. The effects of high dietary omega-3 and low omega-6 targeted alteration to treat migraine in the parent randomized clinical trial suggested that the binding of long chain fatty acid to CD36 might play vital roles in chronic headache [44] and possibly for migraine [45]. Indeed, predominant monocytes and platelets gene up-regulations are characteristic for migraine [10]. CD36

binding to domains of thrombospondin homologs and the downstream inflammation activation might play a role in migraine pathophysiology, but the details are still elusive.

T-cell subset and expression of integrins are reduced to potentially facilitate the lymphocyte transmigration to the CNS. LFA-1 is expressed on white blood cells including lymphocytes and other leukocytes and plays a crucial role in the emigration of leukocytes, leaving the bloodstream to enter the tissues. It is a heterodimeric glycoprotein with non-covalently linked subunits alpha (CD11a) and beta (CD18). Empl et al.'s study reported that migraine-with-aura patients had significantly lower LFA-1 expression on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells than that of controls [51]. Sarchielli and colleagues found that LFA-1 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells were progressively down-regulated at two and four hours after migraine attack onset [52].

Previous research has reported that elevated levels of biomarkers of inflammation are linked to migraine [5, 53, 54]. In the early phase of inflammation, the release of excessive amounts of pro-inflammatory cytokines and lipid mediators is tied to the pathogenesis of organ dysfunction. Cytokines and chemokines are essential molecular and pain mediators in neurovascular inflammation. Some proinflammatory cytokines levels increased after migraine attacks [23, 26, 27]. In addition, cytokines might play a critical role in the initiation and persistence of pain by activating nociceptive sensory neurons [55, 56]. The most relevant cytokines related to migraines include IL-1, IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , and TGF- $\beta$  [9, 57–59]. Recent studies investigating neuroinflammation in migraine reveal the role of inflammasome via inflammasome complex players including IL-1 $\beta$  and IL-18 [60]. In this study, we did not find significant changes of inflammatory cytokines from serum (data not provided) or ex vivo LPS stimulated PBMCs between migraine patients and matched control groups. Although IL-6 has been reported with higher production from this population in the Koon's study [61], we found a reduced non-classical monocyte population in the peripheral blood, and this is likely to be a reason for not finding significantly increased IL-6 production. Moreover, condition of ex vivo LPS stimulation needs to be investigated in the future.

There are some limitations in this pilot study. For instance, the sample size was relatively small, and the ex vivo LPS stimulation of PBMC for cytokine production used only a single 24-h stimulation time point. Because of limited patient samples available from the clinical parent study, CSF samples were not examined.

In summary, we observed that the percentage of peripheral non-classical monocyte (CD14<sup>+</sup>CD16<sup>++</sup>) in migraine is lower than in controls, suggesting possible migration of the CD14<sup>+</sup>CD16<sup>++</sup> population into the endothelium of cranial vessels. This migration potentially plays a role in releasing inflammatory mediators, leading to migraine

pathogenesis. Future research regarding the levels of non-classic monocyte population in the CNS (for example, in cerebrospinal fluid) would be of interest to confirm the potential migration of the CD16<sup>+</sup> monocyte population. Migraine appears to be an inflammatory disease with a lowered CD4<sup>+</sup>T cell population, specifically a lower CD4<sup>+</sup>CD25<sup>+</sup> T cell population. These preliminary findings need to be confirmed with studies conducted on a larger sample size of patients with migraine.

If future studies can directly confirm the migration of the non-classical monocytes in the CNS of migraine patients, potential therapeutic strategies may be broadened. For example, since CD16<sup>+</sup> monocytes express CXCR7 on cell surface; using CXCR7 antagonist might help reduce the non-classical monocytes transmigration across the BBB [35]. This study also provides additional evidence for the use of targeted alterations in dietary linolenic acid and n-3 EPA<sup>+</sup>DHA in chronic migraineurs [44, 45]. Neuroinflammation pathways, specifically those involving inflammasome proteins, such as IL-1 $\beta$ , IL-18, and caspase-1, seem promising candidates as biomarkers or treatment targets in migraine [60], providing some interesting direction for further study.

## Conclusions

Our results suggest that migraine is associated with dysregulated peripheral immune homeostasis and that neuroinflammation and autoimmune may play a role in its pathophysiology.

## Abbreviations

BBB: Blood brain barrier; BMI: Body mass index; CNS: Central nervous system; LFA-1: Lymphocyte function-associated antigen 1; MFI: Mean fluorescence intensity; MS: Multiple sclerosis; PBMC: Peripheral blood mononuclear cells; PMNs: Polymorphonuclear leukocytes; VLA-4: Very late antigen-4; n-3: Omega-3.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12883-022-02781-4>.

**Additional file 1. Supplemental Table 1.1** Means, standard deviations, and medians for groups defined by control and migraine status, sub-setting by chronic vs episodic and medication overuse. **Supplemental Table 1.2** Means, standard deviations, and medians for groups defined by control and migraine status, sub-setting by chronic vs episodic and medication overuse. **Supplemental Table 1.3** Means, standard deviations, and medians for groups defined by control and migraine status, sub-setting by chronic vs episodic and medication overuse.

**Additional file 2. Supplemental Table 2.** Differences between migraines and controls adjusted for MOH and Chronic Migraine

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#### Authors' contributions

H.L. designed the experiments, performed the experiments, and was the primary author of this manuscript. Q.F. contributed to experimental design, data processing, and statistical analysis. K.P. recruited and consented the control group, performed experiments with H.L., and critically revised this manuscript. Y.S. processed the flow cytometry raw data, statistical analysis, and generated related graphs. K.R.F. was a Co-I on the parent R01, and was involved with the NC TraCS 2 k grant application (protocol), data analysis and editing the manuscript as the senior author. S.G. and J.D.M. participated in editing the manuscript and management of the NIH T-32 fellowship grant NCCIH 5T32AT003378, as well as participating as Co-I and PI on the parent R01. The author(s) read and approved the final manuscript. The authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets used and/or analysed during the current study are not able to share due to ethical/legal restrictions. UNC Health/CDWH only permits data use on a project-by-project basis (i.e., a given IRB) and any disclosures outside of UNC require a data use agreement.

#### Declarations

##### Ethics approval and consent to participate

This study was carried out according to the guidelines and regulations for human research ethics and good clinical and lab practice. The Institutional Review Board of the University of North Carolina at Chapel Hill reviewed and approved the experimental protocols (Study # 15–1139). Participants in the study gave informed consent for their participation, including completing questionnaires and providing blood specimens for analysis.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare no conflict of interest. The contents of this publication are solely the authors' responsibility and so do not represent the official views and opinions of the N.I.H.

##### Author details

<sup>1</sup>Department of Science and Mathematics, Hulman Hall Room 116, Saint Mary-of-the-Woods College, 1 St Mary of Woods Coll, IN 47876, USA. <sup>2</sup>Department of Physical Medicine and Rehabilitation Program On Integrative Medicine, University of North Carolina, Chapel Hill, NC, USA. <sup>3</sup>School of Pharmacology, Institute of Aging Medicine, Binzhou Medical University, Yantai 264003, People's Republic of China. <sup>4</sup>Division of Comprehensive Oral Health, Adams School of Dentistry, University of North Carolina, Chapel Hill, NC, USA. <sup>5</sup>Department of Neurology, University of North Carolina, Chapel Hill, NC, USA.

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