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Original Paper

Anti- Versus Pro-Inflammatory Metabololipidome Upon Cupping Treatment

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Key Words
Metabololipidome • Polyunsaturated fatty acids • Cupping • UPLC-MS/MS • Inflammation

Abstract
Background/Aims: This study aimed to explore the metabololipidome in mice upon cupping treatment. ***Methods:*** A nude mouse model mimicking the cupping treatment in humans was established by administrating four cupping sets on the back skin for 15 minutes. UPLC-MS/MS was performed to determine the PUFA metabolome in mice skin and blood before and after cupping treatment. The significantly changed lipids were administered in macrophages to assess the production of pro-inflammatory cytokines IL-6 and TNF-α by ELISA. ***Results:*** The anti-inflammatory lipids, *e.g.* PGE_1, 5,6-EET, 14,15-EET, 10S,17S-DiHDoHE, 17R-RvD1, RvD5 and 14S-HDoHE were significantly increased while pro-inflammatory lipids, *e.g.* 12-HETE and TXB_2 were deceased in the skin or plasma post cupping treatment. Cupping treatment reversed the LPS-stimulated IL-6 and TNF-α expression in mouse peritoneal exudates. Moreover, 5,6-EET, PGE_1 decreased the level of TNF-α, while 5,6-EET, 5,6-DHET downregulated IL-6 production in macrophages. Importantly, 14,15-EET and 14S-HDoHE inhibited both IL-6 and TNF-α induced by lipopolysaccharide (LPS). 17-RvD1, RvD5 and PGE_1 significantly reduced the LPS-initiated TNF-α, while TXB_2 and 12-HETE further upregulated the LPS-enhanced IL-6 and TNF-α expression in macrophages. ***Conclusion:*** Our results reveal the identities of anti-inflammatory *versus* pro-inflammatory metabolipidome and suggest the potential therapeutic mechanism of cupping treatment.

Introduction

Cupping, a physical therapy which creates a negative pressure on the skin and results in a local and visible hematoma, has been invented since thousands of years ago [1, 2]. In the general cupping procedures, the therapist places a cup at the treatment site with open

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face against the skin surface. Pumping out the inside air by either burning up oxygen or air vacuuming. The negative pressure will conduce to hyperemia and then form an ecchymosis in the targeted area of cupping. The local ecchymosis was reported to activate *Qi* (closely relevant to material energy) and adjust circulation in skin and muscles in the Chinese Medicine Theory [3]. Owing to its efficacy, convenience and low cost, cupping has now been widely applied to treat muscle pain, tendency and fatigue [4-8]. This treatment is also used to alleviate the pain symptoms of illness, such as post-herpes zoster neuralgia [9], post-operative nausea and vomiting [10] and cancer [6]. Moreover, cupping treatment has been drawn increasing attention including Olympic celebrities [11].

Previous researches mainly focused on its role in improving skin temperature [5], plasma pressure [5], heat effect [12], and plasma oxygen in local sites [7], and subjective human feeling indices (*e.g.*, pain scores [4], visual analogue scale [8], numerical rating scale [6]). Although these indices can quantify the therapeutic effect, the fundamental mechanism remains unclear.

Homeostasis is delicately regulated by pro- and anti-inflammatory lipids [13]. The temporal and differential levels of lipid mediators also represent the stage of inflammation [14]. The metabolites derived from ω-3, 6, and 9 polyunsaturated fatty acids (PUFAs; *e.g.* linoleic acid (LA), arachidonic acid (AA), eicosapntemacnioc acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA)) are essential lipid mediators involved in inflammation initiation and resolution. For example, ω-3 PUFAs potentially exert anti-inflammatory activities and have promising benefits in various inflammatory human diseases such as diabetes, atherosclerosis, asthma, and arthritis [15]. Deficiencies of ω-3 PUFAs contribute to several chronic inflammatory diseases, including obesity and diabetes [16]. Leukotriene B_4 (LTB_4), 5-HETE and prostaglandin E_2 (PGE_2) are pro-inflammatory, while lipoxin A_4 (LXA_4) and PGI_2 are anti-inflammatory metabolites derived from AA, an ω-6 PUFA [13, 17, 18]. These relationships between tissue homeostasis and lipid metabolism inspire us to address whether cupping treatment modulates the metabolic balance between pro- and anti-inflammatory PUFAs.

Here, we employed reversed-phase ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), a sensitive and powerful technique, which provides a platform to identify the PUFA metabolome in nude mice. Our results demonstrated that several anti-inflammatory lipids (e.g. PGE_1, 5, 6-EET, 14, 15-EET, 10S,17S-DiHDoHE, 17R-resolvin D1 (RvD1), RvD5 and 14S-HDoHE) were increased and many pro-inflammatory lipids (e.g. 12-HETE and Thromboxane B_2 (TXB_2)) were deceased in the skin and plasma post cupping treatment. Moreover, PGE_1, 5, 6-EET, 5, 6-DHET and 12-HETE differentially regulated interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) production from RAW264.7 macrophages. These findings identified the anti- *versus* pro-inflammatory metabololipidome upon cupping treatment, and suggested potential PUFA-derived lipid mediators that function as diganostic biomakers and therapeutics compounds in cupping treatment.

Materials and Methods

Mice

Nude and C57BL/6 mice were purchased from the Peking University Animal Center (Beijing, China) and were kept under specific pathogen-free conditions at the Animal Center of Xinqiao Hospital, Third Military Medical University. All animal experiments were approved by the ethics committee of Third Military Medical University. All methods were performed in accordance with the animal ethics guidelines and regulations of Third Military Medical University and complies with the Declaration of Helsinski. The cupping procedure details were described as in Fig. 1 and the Results section.

Chemicals and Reagents

Formic acid (>99%), methyl formate, hexane, 2-propanol, acetonitrile, chloroform and methanol (all HPLC-MS grade) were purchased from Honeywell (New Jersey, USA). SepPak C18 SPE Cartridges (500 mg,

6mL) were purchased from Waters (Hertsfordshire, UK). Lipid mediators including 12-HETE (12-hydroxy-5Z,8Z,10E,14Z -eicosatetraenoic acid), 14, 15-EET (14, 15-epoxy-5Z,8Z,11Z-eicosatrienoic acid), 5, 6-EET (5, 6-epoxy-8Z,11Z,14Z-eicosatrienoic acid), 5, 6-DiHET (5, 6-dihydroxy -8Z,11Z,14Z-eicosatrienoic acid), 10S,17S-DiHDoHE (10(S),17(S)-dihydroxy -4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid), 14(S)-HDoHE (14S-hydroxy -4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid), 17(R)-RvD1 (7S,8R,17R -trihydroxy-4Z,9E,11E,13Z,15E19Z-docosahexaenoic acid), RvD5 (7S,17S -dihydroxy-4Z,8E,10Z,13Z,15E,19Z-docosahexaenoic acid), PGE_1 (9-oxo-11α,15S -dihydroxy-prost-13E-en-1-oic acid), TXB_2 (9α,11, 15S-trihydroxythromba -5Z,13E-dien-1-oic acid), 20-HDoHE (20-hydroxy-4Z,7Z,10Z,13Z,16Z,18E -docosahexaenoic acid), and lipid standards were obtained from Cayman Chemicals (Ann Arbor, MI, USA). All the lipid standards were dissolved in methyl formate or methanol as a premixed solution and stored at -80°C in glass tubes.

UPLC-MS/MS

Lipid mediators were analyzed by UPLC-MS/MS as described previously [19, 20]. Prior to sample extraction, d_4-LTB_4, d_4-PGE_2 and d_8-HETE (500 pg each), were added to permit quantification. The lipid metabolites were isolated by solid phase extraction on a C18 column (6 mL, 500 mg, 37~55 μm particle, Waters). Samples were washed with 10 mL of water and 6 mL of n-hexane, dried and eluted by gravity with 8 mL of methyl formate. Extracted samples were separated by an Acquity UPLC I-Class system (Waters, MA, USA). The column (Acquity UPLC BEH C18, 2.1 × 100 mm; 1.7 μm; Waters) was eluted at a flow rate of 0.2ml/mim with MeOH/water/acetic acid (60/40/0.01, v/v/v) ramped to 80/20/0.01 (v/v/v) after 5 min, 95/5/0.01 (v/v/v) after 8 min and then to 100/0/0.01 (v/v/v) for the next 4min, subsequently returned to 60/40/0.01(v/v/v) and maintained for 5 min. A 10μL aliquot of each sample was injected onto the column. The column temperature was kept at 40°C. All samples were kept at 4°C throughout the analysis.

Mass spectrometry was performed on an AB Sciex 6500 QTRAP, triple quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source. Lipid mediators were detected in negative electrospray ion (ESI) mode. Curtain gas (CUR), nebulizer gas (GS1), and turbo-gas (GS2) were set at 10 psi, 30 psi, and 30 psi, respectively. The electrospray voltage was −4.5 kV, and the turboion spray source temperature was 550 ℃. Lipid mediators were analyzed using scheduled multiple reaction monitoring (MRM). Mass spectrometer parameters including the declustering potentials and collision energies were optimized for each analyze. Nitrogen was employed as the collision gas. Data acquisitions were performed using Analyst 1.6.2 software (Applied Biosystems, CA, USA). Multiquant software (Applied Biosystems) was used to quantify all metabolites.

Cytokine analysis in vivo and in vitro

After C57BL/6 mice were treated with or without cupping for 22 hrs, they were then intraperitoneally injected with or without lipopolysaccharide (LPS, 1ng/kg, Sigma-Aldrich, Steinheim, Germany) for 2 hs, the plasma and perotineal exudates were collected for IL-6 and TNF-α with ELISA as indicated below.

RAW264.7 cells were purchased from American Type Culture Collection and cultivated in DMEM medium with 5% fetal bovine serum. The supernatants were harvested for cytokine determination after cells were treated with or without indicated compounds for 12 hrs. The secretion of IL-6 and TNF-α from the compound treated or control RAW264.7 cells were accessed using the BD Cytometric Bead Array (CBA) according to the manufacturers protocol and as described previously [21]. In brief, The BD CBA Flex Set contains a bead population with distinct fluorescence intensity as well as the appropriate phycoerythrin (PE) detection reagents and standards. The bead population is coated with capture antibodies sensitive to IL-6 or TNF-α. The bead population was incubated with test samples to form specific complexes. After the addition of PE-conjugated detection antibodies, all samples were incubated again. The fluorescence of samples were measured in the FL-3 channel of an FACSCalibur flow cytometer (BD, NJ, USA). The results were analyzed by FCAP Array v3 Software (BD).

Bone marrow (BM) cells were extracted from the femurs and tibias of C57BL/6 mice by flushing with 1640 medium using a 1ml syringe. The flushing fluid were then passed through a 100-μm nylon cell strainer (FALCON, NY, USA). BM-derived macrophages were obtained as described previously [22]. BM-derived macrophages were treated with or without LPS (1ng/ml, Sigma-Aldrich, Steinheim, Germany) and indicated lipids (see results) for 12 hrs. The supernatant IL-6 and TNF-α levels of BM macrophages were identified by ELISA (ab100747 and ab100712, Abcam) according to the manufacturers protocol.

Data analysis

Statistical differences between groups were compared with GraphPad Prism 7 software (GraphPad, CA, USA) using one-way ANOVA. *P* values less than 0.05 was considered statistically significant. UPLC–MS/MS data of the PUFA metabolites in mice were subjected to Heatmaps and Principal Component Analysis (PCA) using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/).

Results

Cupping Treatment in nude mice

Nowadays, although cupping treatment is not indicated for use clinically in many countries, it has been widely applied for routine health care, especially in Asian countries. To reveal the underlying mechanism by which this physical therapy regulates the tissue homeostasis, we constructed a mouse model to mimic the operation in humans. We first designed a dry cupping set which was consisted of a 1000 μl pipetip connected with a sheared perfusion tube and a 5ml syringe (Fig. 1A). Since the nude mice are hairless and the skin is smooth and pink-white, we were able to vacuum the skin by using this device and observe the outward appearance. The nude mice were anesthetized by isoflurane and treated by four cupping sets on the back skin for 15 minutes. To guarantee the consistency, all the syringes were set at the scale of 2 cm (Fig. 1A). After cupping treatment, no skin lesion such as blister was observed, four dark red spots were left on the cupping sites, with a diameter ~5 mm each (Fig. 1B). The manifestation of the nude mice skin after cupping indicated that our experimental model could mimic the cupping treatment in humans.

Lipidomics analysis in mice before and after cupping treatment

To investigate the effect of cupping treatment on lipid metabolism, we used UPLC-MS/MS to analyze the PUFA metabolome in mice before and after cupping treatment. The skin and plasma from untreated nude mice were collected as control (Ctrl). The dark red spots (treated skin, TS) and adjacent skin (AS), as well as the plasma of the cupping treated nude mice were harvested as treatment group. 64 representative metabolites of AA, EPA, DHA and other PUFAs in mice were evaluated, with a total of 30 kinds of lipids including LTB_4, 11-HETE, 20-HDoHE, TXB_2, 5, 6-EET, 14, 15-DHET 5, 6-DHET, 12-HETE, 5-HETE, $PGF_{1\alpha}$, PGD_1, PGD_2, 11, 12-DHET, LXB_4, 14, 15-EET, 12-HEPE, 8S-HEPE, 15-HEPE, PGE_1, 4-HDoHE, 17R-RvD1, RvD5, 14S-HDoHE, 16-HDoHE, 17-HDoHE, 7-HDoHE, 10S,17S-DiHDoHE, PGE_2, 5S,15S-DiHETE, 15S-HETrE in skin and 24 kinds of lipids including LXB_4, 12-HETE, PGE_2, TXB2, 5, 6-EET, 5, 6-DHET, 14, 15-EET, 14, 15-DHET, 11, 12-DHET, 11-HETE, 5-HETE, 8, 9-DHET, 5-HEPE, 18-HEPE, 11-HEPE, 12-HEPE, 4-HDoHE, 7-HDoHE, 17-HDoHE, 20-HDoHE, 16-HDoHE, 13-HDoHE, 14S-HDoHE, and 9-HODE in plasma were

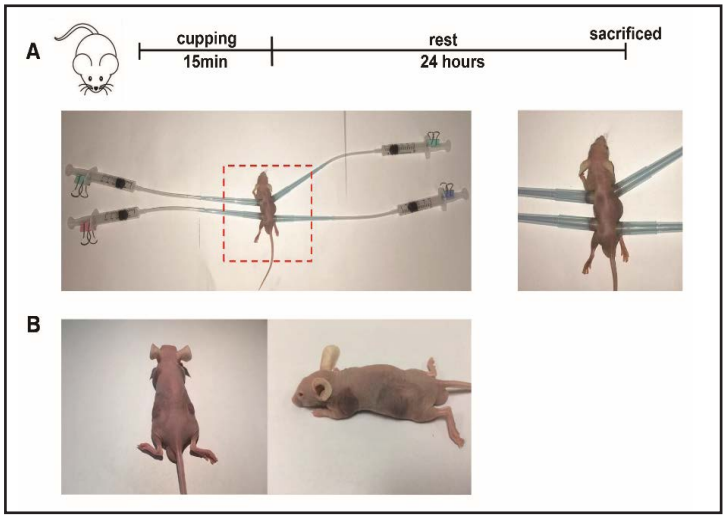

Fig. 1. The cupping experimental procedure. A. After treated with negative pressure aspiration (2ml each) on the back skin at 4 sites for 15 min and rest for 24 hrs, the nude mice were sacrificed for metabolipidomics analysis.B. The appearance of mice skin after cupping treatment.

Table 1. The levels of PUFA metabolites (ng/ml) in mice plasma before and after cupping treatment

PUFA metabolites	Ctrl	Ctrl	Ctrl	Ctrl	Mean±SEM	cupping	cupping	cupping	Mean±SEM
LXB4	8.76	7.89	8.13	7.36	8.03±0.29	8.36	7.49	10.77	8.87±0.98
12-HETE	468.04	397.4	382.15	448.17	423.94±20.38	325.38	388.74	359.8	357.97±18.31
PGE2	0.33	0.59	0.47	0.5	0.47±0.06	0.86	0.58	0.7	0.71±0.08
TXB2	14.84	15.75	13.93	11.68	14.05±0.87	5.47	5.7	7.11	6.09±0.51
5,6-EET	26.25	30.91	32.4	40	32.39±2.86	44.14	52.85	28.97	41.98±6.98
5,6-DHET	1.28	2.57	2.08	2	1.98±0.27	3.93	3.04	3.28	3.41±0.27
14,15-EET	6.73	6.75	4.77	4.22	5.62±0.66	14.24	12.4	13.96	13.53±0.57
14,15-DHET	3.93	6.27	8.56	10.41	7.29±1.41	5.47	4.03	9.79	6.43±1.73
11,12-DHET	1.97	3.36	2.27	2.35	2.48±0.3	3.15	2.47	3.53	3.05±0.31
11-HETE	2.5	5	6.5	12.75	6.69±2.18	3.64	4.42	3.79	3.95±0.24
5-HETE	2.22	2.91	4.21	4.41	3.43±0.52	6.09	6.32	6.54	6.32±0.13
8,9-DHET	1.93	2.33	2.19	3.23	2.42±0.28	2.23	2.47	2.84	2.51±0.18
5-HEPE	1.86	3.72	6.49	9.63	5.42±1.69	2.76	2.67	5.79	3.74±1.03
18-HEPE	8.91	13.77	10.61	13.93	11.8±1.23	16.78	14.3	12.14	14.41±1.34
11-HEPE	4.08	6.15	5.05	9.56	6.21±1.19	4.71	3.94	5.9	4.85±0.57
12-HEPE	26.71	81.85	66	56.4	57.74±11.6	102.66	107.63	63.17	91.15±14.06
4-HDoHE	1.31	1.3	3.32	1.09	1.76±0.52	2.89	1.13	2.38	2.13±0.52
7-HDoHE	5.43	7.26	7.35	11.18	7.8±1.21	5.25	3.67	7.47	5.46±1.1
17-HDoHE	9.3	9.51	16.2	34.9	17.48±6.02	15.98	13.79	10.69	13.48±1.54
20-HDoHE	43.13	69.31	74.69	31.21	54.59±10.4	11.49	7.61	5.65	8.25±1.72
16-HDoHE	11.32	10.43	10.5	11.43	10.92±0.26	10.77	11.43	11.33	11.17±0.21
13-HDoHE	1.14	1.62	1.29	1.61	1.41±0.12	3.39	2.61	2.73	2.91±0.24
14S-HDoHE	226.07	259.89	247.36	216.45	237.44±9.88	204.7	222.14	219.88	215.57±5.48
9-HODE	335.55	178.17	179.18	666.12	339.75±114.9	216.63	190.4	143.55	183.52±21.37

Table 2. The levels of PUFA metabolites (ng/mg protein) in control (Ctrl), cupping treated-(TS) and adjacent-skin (AS)

PUFA metabolites	Ctrl	Ctrl	Ctrl	Ctrl	Mean±SEM	TS	TS	TS	Mean±SEM	AS	AS	AS	Mean±SEM
LTB4	31.02	31.94	35.96	21.45	30.09±3.07	30.62	25.86	66.54	41.01±12.84	27.17	30.57	21.7	26.48±2.58
11-HETE	319.38	312	314.02	319.55	316.24±1.91	331.76	361.67	315.9	336.44±13.42	292.29	243.77	245.54	260.53±15.89
20-HDoHE	6.46	9.28	9	4.25	7.25±1.18	4.06	5.18	7.95	5.73±1.16	9.84	5	5.58	6.81±1.53
TXB2	41.62	32.84	42	29.67	36.53±3.12	35.42	34.44	44	37.95±3.04	30.22	33.06	29.38	30.89±1.11
5,6-EET	24.01	15.49	15.17	10.94	16.4±2.74	364.82	405.35	422.81	397.66±17.18	215.68	203.91	243.74	221.11±11.81
14,15-DHET	8.6	8.36	9.17	9.16	8.82±0.2	8.23	9.48	9.2	8.97±0.38	8.53	9.04	13.97	10.51±1.73
5,6-DHET	6.86	6.29	12.23	0	6.35±2.5	11.84	4.17	6.27	7.43±2.29	11.3	9.63	10.43	10.45±0.48
12-HETE	21.98	31.92	21.39	0	18.82±6.72	40.13	34.36	40.14	38.21±1.93	69.82	67.05	31.37	56.08±12.38
5-HETE	7.18	19.69	20.11	21.37	17.09±3.32	50	51.4	21.96	41.12±9.59	35.63	38.12	20.11	31.29±5.63
PGF1a	2.36	2.21	2.27	1.7	2.14±0.15	1.55	2.4	1.49	1.81±0.29	1.91	1.71	1.05	1.56±0.26
PGD1	5.95	2.89	5.82	4.28	4.74±0.72	9.7	3.06	12.83	8.53±2.88	8.94	6.9	5.16	7±1.09
PGD2	458.47	268.68	418.78	314.84	365.19±44.18	312.15	309.34	582.28	401.26±90.52	468.57	374.99	309.34	384.3±46.2
11,12-DHET	4.34	3.57	2.34	5.32	3.89±0.63	3.97	4.96	4.34	4.42±0.29	8.25	7.35	7.18	7.59±0.33
LXB4	32.23	22.83	43.39	25.86	31.08±4.55	78.87	42.11	32.47	51.15±14.14	25.92	28.35	25.05	26.44±0.99
14,15-EET	1.65	2.46	1.5	1.41	1.76±0.24	9.33	10.9	13.03	11.09±1.07	5.27	13.17	14.08	10.84±2.8
12-HEPE	26.68	40.07	33.32	40.53	35.15±3.27	50.93	20.27	28.02	33.07±9.2	37.73	83.18	31.51	50.81±16.29
8S-HEPE	2.1	1.52	1.72	0.98	1.58±0.23	3.59	3.83	1.1	2.84±0.87	3.78	3.74	2.1	3.21±0.55
15-HEPE	17.56	34.54	24.28	53.79	32.54±7.9	54.94	14.72	17.59	29.08±12.95	78.33	63.08	27.76	56.39±14.98
PGE1	22.94	23.64	8.35	6.05	15.25±4.67	19.41	7.79	7.04	11.41±4	16.16	8.91	10.03	11.7±2.25
4-HDoHE	3.92	0.85	1.84	1.58	2.05±0.66	4.3	4.82	2.88	4±0.58	3.09	2.97	2.68	2.91±0.12
17R-RvD1	2.69	5.13	3.12	0	2.74±1.06	19.66	32.73	9.05	20.48±6.85	6.43	6.86	6.89	6.73±0.15
RvD5	1.44	1.46	1.37	0	1.07±0.36	3.45	5.31	1.47	3.41±1.11	7	6.92	3.19	5.7±1.26
14S-HDoHE	9.32	6.06	7.61	5.34	7.08±0.88	285.62	257.77	285.32	276.24±9.23	186.41	117.35	115.96	139.91±23.26
16-HDoHE	10.51	4.26	3.37	7.24	6.35±1.62	9.05	9.54	12.48	10.36±1.07	8.4	5.46	8.1	7.32±0.93
17-HDoHE	31.22	69.51	25.91	20.47	36.78±11.13	395.59	94.84	113.73	201.39±97.25	50.67	36.07	84.41	57.05±14.31
7-HDoHE	4.3	4.82	8.88	3.92	5.48±1.15	9.13	6.93	4.25	6.77±1.41	9.7	7.56	4.01	7.09±1.66
10S,17S-DiHDoHE	0.23	0.18	0.11	0.35	0.22±0.05	2.73	2.47	6.81	4±1.41	5.82	9.09	6.33	7.08±1.02
PGE2	3.12	4.15	6.16	4.28	4.43±0.63	5.52	3.62	18.83	9.32±4.78	7.97	7.69	8.48	8.05±0.23
5S,15S-DiHETE	4.22	5.05	7.21	6.26	5.69±0.66	8.16	2.95	8.89	6.67±1.87	8.85	8.08	11.15	9.36±0.92
15S-HETrE	18.45	24.22	31.26	20.2	23.53±2.84	53.46	54.77	23.66	43.96±10.16	16.77	33.32	30.09	26.73±5.06

unambiguously identified and quantified in this study (Table 1 and 2, Fig. 2 and (for all online suppl. material, see www.karger.com/doi/10.1159/000487563) Fig. S1). The levels of PUFA metabolites were quantified and subjected to heatmaps by using MetaboAnalyst 3.0. The heatmaps showed that the levels of these PUFA metabolites were differentially changed in the skin and plasma of the nude mice after cupping treatment (Fig. 2B).

Anti- and pro-inflammatory lipids in skin after cupping treatment

To analyze the PUFA metabolites in mice skin tissue with or without cupping treatment, we further compared the above identified lipids in TS, AS and Ctrl. PCA was used to

distinguish the differences of PUFA metabolites in all analyzed mice skin samples in 2- and 3-dimensional scatter plots. The principal component 1 (PC1) was the axis, which contained the largest possible amount of information and PC2 was perpendicular to PC1. The principal components were orthogonal and linear combinations of the original variables. PCA score plots were used to reflect the relationship of PUFA metabolites in mice skins with different treatments. The score plots of PCA provided a clear discrimination of these three groups. PC1 and PC2 were able to describe respectively 65.8% and 19.3% of total variance. They accounted for 85.1% of total variance (Fig. 3A). Samples with cupping treatment, including treated skin and adjacent skin were grouped in small regions in the score plot. And the responses of controls are clustered away from those corresponding to the cupping treated groups.

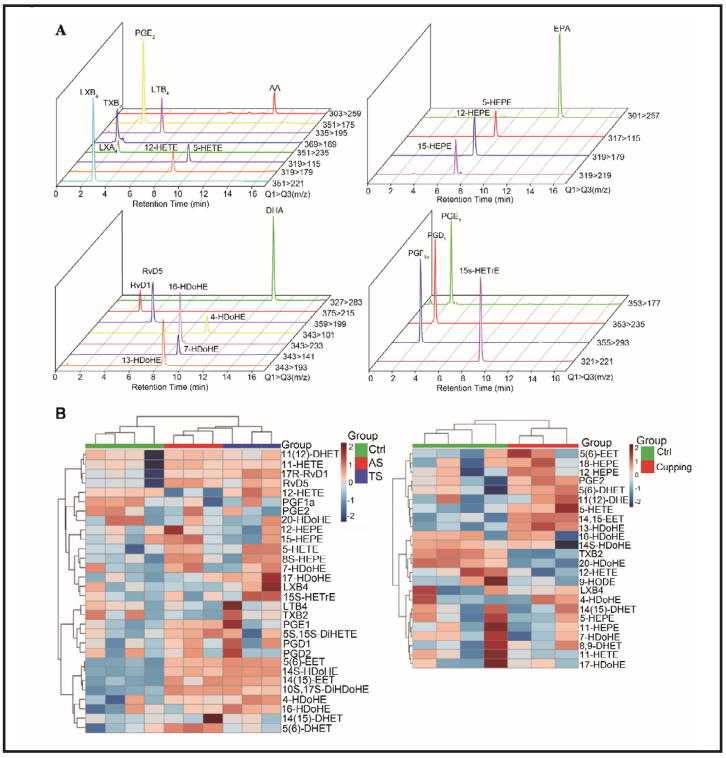

Fig. 2. The PUFA metabolome in mice skin and plasma before and after cupping treatment. A. Representative MRM chromatograms show the retention times for each identified bioactive LMs: Q1, M-H (parent ion); and Q3, diagnostic ion in the tandem mass spectrometry (MS/MS) (daughter ion). Representative metabolites of AA, EPA, DHA and other PUFAs. B. The heatmap of PUFA metabolome in mice skin (left panel) and plasma (right panel) with or without cupping treatment. Results were expressed as mean of n=5 mice each group.

We also analyzed the variables (PUFA metabolites contents) in our PCA. The PUFA metabolites with significantly different levels in each treatment were analyzed and scattered at the edges of the loading plot, whereas PUFA metabolites with similar levels in each treatment were gathered in the middle right part of the loading plot. Of note, the PUFA metabolite contents, such as the amounts of 12-HETE, 5, 6-DHET, 17R-RvD1, RvD5, 14, 15-EET, 5, 6-EET, 14S-HDoHE, PGE_1 and 10S,17S-DiHDoHE, were the most statistically significant variables (Fig. 3B).

The levels of PUFA metabolites in Ctrl, TS and AS were also accessed with GraphPad Prism 7 software using one-way ANOVA. In accordance with the results of PCA (Fig. 3A and 3B), various lipids increased in both TS and AS, including 5, 6-EET, 14, 15-EET, 10S,17S-DiHDoHE and 14S-HDoHE; while 5, 6-DHET, PGE_1, 17R-RvD1 and RvD5 were only up-regulated in AS, but not in TS. In contrast, 12-HETE was down-regulated in AS (Fig. 3C). It has been acknowledgeable that 14, 15-EET [23], 5, 6-EET [24], 5, 6-DHET [25], 10S,17S-DiHDoHE[26], 17R-RvD1 [27], RvD5 [27] and PGE_1 [28] are anti-inflammatory while 12-HETE is pro-inflammatory [29]. Together these results showed that cupping treatment increased anti-inflammatory lipids and decreased pro-inflammatory lipids.

Anti- and pro-inflammatory lipids in plasma after cupping treatment

Next we analyzed the PUFA metabolites in plasma before and after cupping treatment. The PCA of the first two PCs was performed. The two ellipses indicated 75.5% bivariate

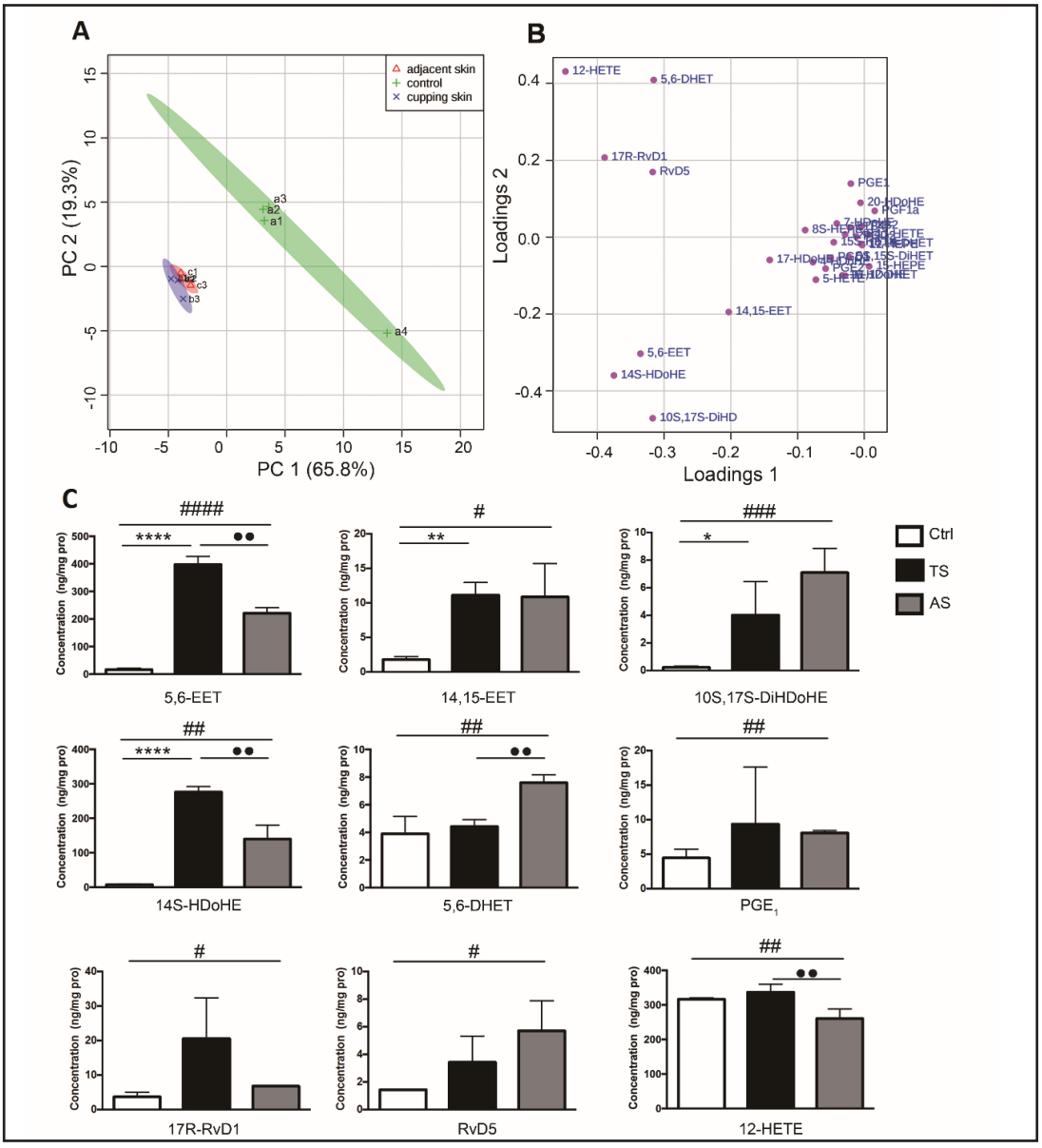

Fig. 3. The significantly altered PUFA metabolites in mice skin tissue with or without cupping treatment. A. Score plot of principal component analysis based on PUFA metabolites profiling analysis of all mice skin tissue samples (n=5). B. Projection of variables in a two-dimensional loading plot for all measured samples, showing the major variables representing PUFA metabolites concentrations. C. The levels of 12-HETE, 5,6-DHET, PGE1, 5(6)-EET, 14(15)-EET, 17-RvD1, RvD5, 14S-HDoHE and 10S, 17S-DiHDoHE in control (Ctrl), cupping treated-(TS) and adjacent-skin (AS). Results were expressed as mean ± SEM of n=5 mice each group. *P<0.05, **P<0.01, and ****P<0.0001, Ctrl vs TS; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, Ctrl vs AS; *P<0.05, **P<0.01, ***P<0.001, TS vs AS.

normal ellipses that summarized the distribution of the principal component scores for the cupping treatment. The clusters corresponding to mice plasma with and without the treatment showed that both PC1 and PC2 were separated clearly. These results demonstrated that PCA was also able to discriminate the PCs of PUFA metabolome in the plasma after cupping treatment (Fig. 4A).

The individual PUFA metabolite contents responsible for the variation of the first two eigenvalues (PC1 and PC2) were analyzed (Fig. 4B). The graphical representation of

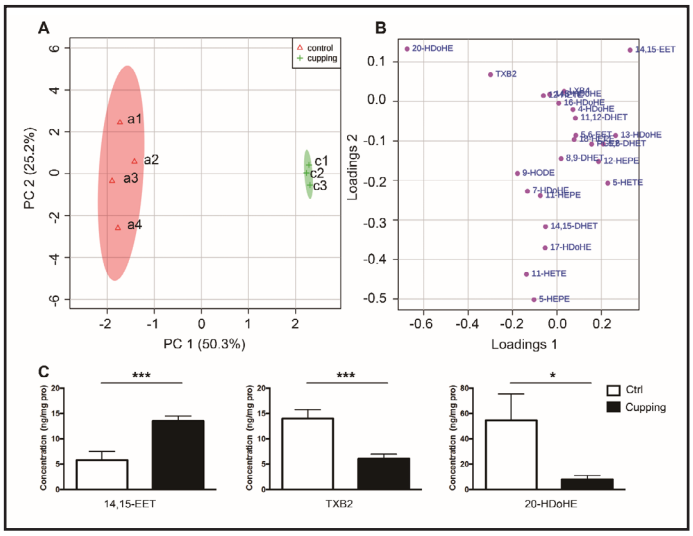

Fig. 4. The significantly altered PUFA metabolites in mice plasma with or without cupping treatment. A. Score plot of principal component analysis based on PUFA metabolites profiling analysis of plasma samples. B. Projection of variables in a two-dimensional loading plot for all measured samples, showing the major variables representing PUFA metabolites concentrations. C. The levels of 14(15)-EET, TXB_2, and 20-HDoHE in control (Ctrl), cupping treated plasma. Results were expressed as mean±SEM of n=5 mice each group. $^{*}P<0.05$, $^{***}P<0.001$, Ctrl vs Cupping.

the extent to which each factor accounted for the variance in the data and the relationship between the different PUFA metabolite variables indicated that the contents of 20-HDoHE, TXB_2 and 14, 15-EET in mice plasma with cupping treatment were significantly different compared with controls (Fig. 4B).

Although 20-HDoHE and TXB_2 were significantly decreased in the plasma, which was not shared in the skin. Intriguingly, 14, 15-EET was consistently up-regulated in both plasma and skin after cupping treatment (Fig. 3C and 4C). Since 14, 15-EET is anti-inflammatory [23] while TXB_2 is pro-inflammatory [30], these results demonstrated the efficiency of cupping treatment in regulating the balance between pro- and anti-inflammatory lipid profile in the plasma.

Cupping-triggered PUFA metabolome suppresses proinflammatory IL-6 and TNF- α

To further investigated roles of the cupping-triggered PUFA metabolites in inflammation, we administered these lipids to treat murine macrophages (RAW264.7) for 12 hrs and found that 4 of these lipids significantly regulated the production of pro-inflammatory cytokines including IL-6 and TNF-α. 5, 6-EET inhibited both the level of IL-6 and TNF-α. Administration of 12-HETE resulted in a significant increase of TNF-α but did not affect IL-6. 5, 6-DHET modestly increased TNF-α but decreased IL-6 (see online suppl. material, Fig. S2A and S2B). PGE_1 potently inhibited TNF-α (by~26 %) while increased IL-6 (~15 fold) (see online suppl. material, Fig. S2A and S2B). Of note, the level of TNF-α was at μg/ml range, whereas the level of IL-6 was at ng/ml range (see online suppl. material, Fig. S2C and S2D), suggesting that PGE1 showed a relative anti-inflammatory effect.

Then we assessed whether cupping treatment could restore tissue homeostasis *in vivo*, we injected low dosage of LPS intraperitoneally 22 hrs post cupping treatment and 2 hrs before sacrifice. The levels of IL-6 and TNF-α in plasma were undetected (data not shown). LPS administration *i.p.* significantly upregulated TNF-α, IL-6 in peritoneal exudates, which was potently rescued in by cupping treatment (Fig. 5A and 5B).

The BM-derived macrophages were also treated with cupping-triggered PUFA lipids for 12 hours to assess the alteration in IL-6 and TNF-α (Fig. 5C and 5D). 14, 15-EET and 14S-HDoHE inhibited both IL-6 and TNF-α induced by LPS. 17-RvD1 and RvD5 suppressed the LPS-enhanced TNF-α but not IL-6. PGE_1 significantly impaired the LPS-induced TNF-α while it modestly upregulate IL-6. In addition, TXB_2 and 12-HETE further boosted LPS-induced IL-6 and TNF-α.

Together these results indicated that cupping-altered PUFA metabolome showed an anti-inflammatory profile. These PUFA-derived lipid mediators triggered by cupping treatment

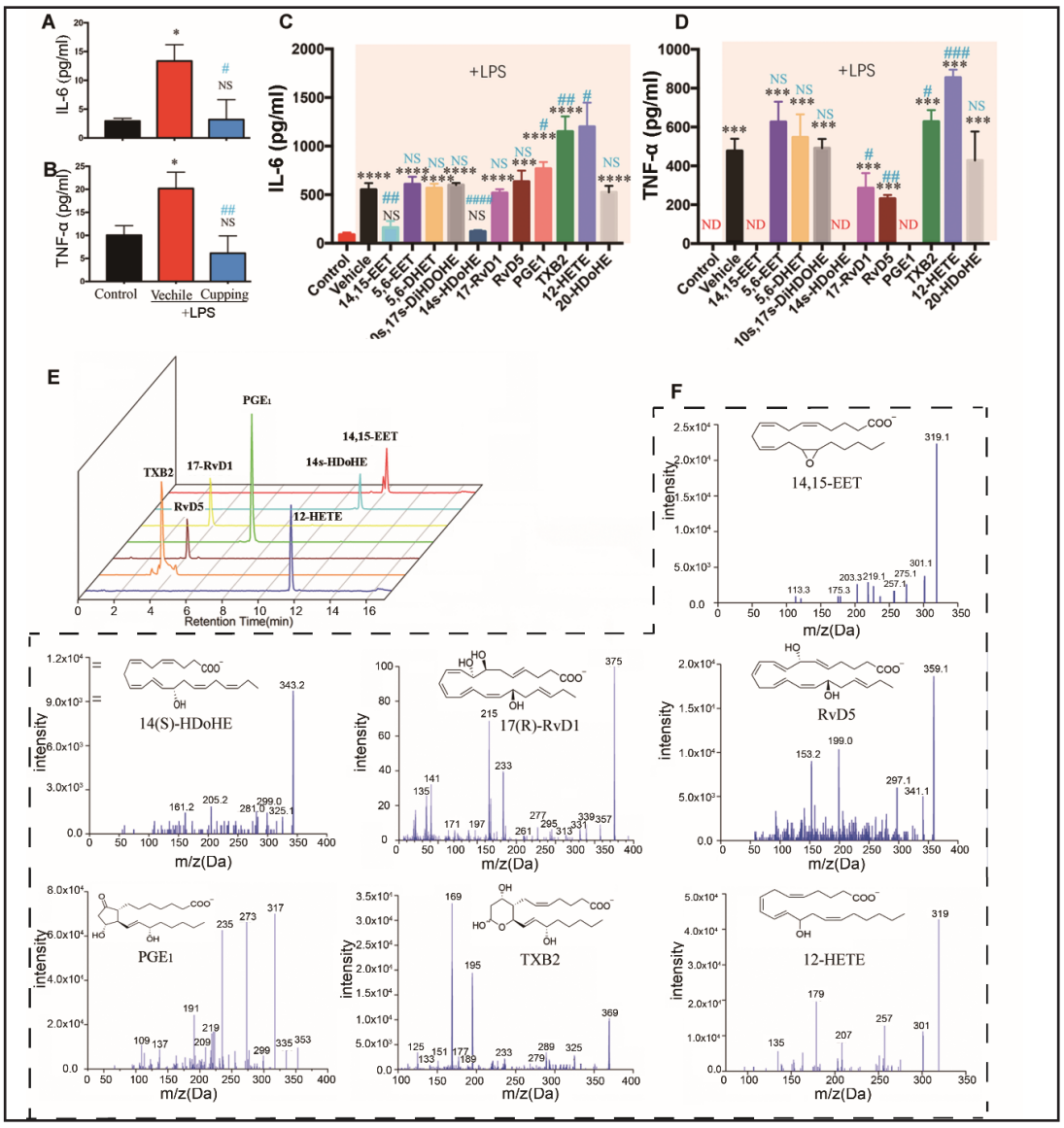

Fig. 5. Cupping derived PUFA metabolites regulate pro-inlfammatory cytokines in murine macrophages. A and B. After nude mice were treated with or without cupping for 22 hrs and then injected 1ng/kg LPS or saline (1ml) intraperitoneally for 2 hrs, the levels of IL-6 (A) and TNF-α (B) in peritoneal exudates were assessed with ELISA. Results were expressed as mean ± SEM of n=4 mice each group. *P<0.05 and nonsense (NS in black), compared with Control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with vehicle. C and D. IL-6 (A) and TNF-α (B) expression in BM-derived macrophages supernatants treated with or without LPS and indicated compounds (100nM of 14S-HDoHE, 100nM of 10S,17S-DiHDoHE, 100nM of 17R-RvD1, 100nM of RvD5, 1 μM of 14,15-EET, 1 μM of 5,6-EET, 1 μM of 5,6-DHET, 1 μM of PGE_1, 1 μM of TXB_2, 1 μM of 20-HDoHE, 1 μM of 12-HETE). Results were expressed as mean±SEM of n≥3 independent experiments. ND depicts non-detected. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and nonsense (NS in black) compared with Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 and nonsense (NS in blue) compared with Vehicle. E. MRM chromatograms of 14,15-EET, 14S-HDoHE, 17-RvD1, RvD5, PGE_1, TXB_2 and 12-HETE. F. MS/MS spectrum of 14,15-EET, 14S-HDoHE, 17-RvD1, RvD5, PGE_1, TXB_2 and 12-HETE.

could serve as diganostic biomakers and therapeutics compounds in cupping treatment (Fig. 5E and 5F).

Discussion

Cupping treatment is an ancient and traditional physical approach to improve health and maintain homeostasis [7]. To explore its underlying mechanism, we monitored the PUFA metabololipidome with a nude mice model. We quantified the levels of fatty acids in skin or plasma of nude mice before and after cupping treatment and found that numerous fatty acids were differentially regulated. Among these lipids, 14, 15-EET, 5, 6-EET, 5, 6-DHET, 14S-HDoHE 10S,17S-DiHDoHE, 17R-RvD1, RvD5 and PGE_1 were increased while 12-HETE was decreased in the skin. 14, 15-EET increased while TXB_2 and 20-HDoHE decreased in the plasma. Indeed, cupping treatment reduced the IL-6 and TNF-α production induced by LPS *in vivo*. We also identified 14, 15-EET, 14S-HDoHE, 17-RvD1, RvD5, PGE_1, TXB_2 and 12-HETE as potential biomarkers and therapeutic compounds in cupping treatment.

The homeostasis is governed by the balance between pro- and anti-inflammatory mediators [31]. In this study, we identified numerous significantly altered PUFA derived metabolites by cupping treatment. The increased lipids included 14, 15-EET, 10S,17S-DiHDoHE, 17R-RvD1, RvD5, 14S-HDoHE, 5, 6-EET, PGE_1, while the decreased lipids were 12-HETE and TXB_2. The biofunctions of most of these lipid mediators were investigated previously and introduced below.

14, 15-EET was reported to protect nucleus pulposus cells from death induced by TNF-α *in vitro* via the NF-κB pathway, and reduced a variety of pro-inflammatory cytokines (*e.g.*, TNF-α, IL-1, IL-6, IL-8) [32]. Furthermore, 14, 15-EET could stimulate the production of 15-epi LXA_4 [33], a dual anti-inflammatory and specialized pro-resolving mediator (SPM) that exert an essential role in inhibiting neutrophil activation and restoring homeostasis [13]. Although we did not observe the increase of lipoxins after cupping treatment for 24 hrs, 14, 15-EET significantly reduced IL-6 and TNF-α and in LPS-stimulated macrophages. The up-regulation of 14, 15-EET in both the skin and plasma might lead to their production at subsequent intervals.

10S,17S-DiHDoHE, 17R-RvD1 and RvD5 belong to SPM [26, 34] and 14S-HDoHE is a precursor to maresin 1, another SPM [35]. Although we did not observe significant change in IL-6 and TNF-α production from RAW264.7 cells after these SPM treatment for 12 hrs, 14S-HDoHE, 7R-RvD1 and RvD5 showed their anti-inflammatory function in LPS-stimulated BM macrophages. 14S-HDoHE could decrease the PMN infiltration into inflammatory sites [35]. 10S,17S-DiHDoHE was reported to reduce the severity of colitis *via* attenuating neutrophil infiltration and decreasing levels of pro-inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6)[26]. 17R-RvD1 is an aspirin-triggered epimer of RvD1 that reduces human PMN migration [36]. It shares the same function of RvD1 which was reported to target pro-inflammatory cytokines (IL-1β, IL-8 IL-6 and TNF-α) and genes [*i.e.*, Chemokine (C-X-C motif) ligand (CXCL9)] as well as persistent STAT3 activation in human inflamed adipose tissue [37]. RvD5 significantly reduced pro-inflammatory cytokines (keratinocyte chemoattractant and TNF-α) and enhanced the human macrophage phagocytosis of E. coli and bacterial killing in mice [34]. The increase of these above SPM delineated the beneficial actions of cupping treatment.

In addition, 5, 6-EET was reported to be anti-inflammatory, it suppressed various pro-inflammatory cytokines such as TNF-α [24]. PGE_1 was used to treat some chronic inflammatory diseases [38]. It was reported to protect cells from renal ischemia/reperfusion injury-induced oxidative stress and inflammation [39]. Consistently, in our study, 5, 6-EET and PGE_1 significantly suppressed TNF-α production in macrophages.

On the other hand, 12-HETE and TXB_2 are well-known pro-inflammatory lipid mediators [29, 30]. Our study showed that they both significantly promoted TNF-α and IL-6 production in macrophages. The reduction of them in the mice plasma suggested the anti-inflammation effect of cupping treatment.

The function of 20-HDoHE and 5, 6-DHET were not clearly elucidated yet. 20-HDoHE is biosynthesized from DHA and was increased during early period of oxidative stress *in vitro* [40] indicating that it probably played a pro-inflammatory role. It was reported that the

nonsteroidal anti-inflammatory drug (NSAID) diclofenac elevated the level of 5, 6-DHET in inflammatory status associated with obesity [25], suggesting the anti-inflammatory role of 5, 6-DHET. In our study, we found 5, 6-DHET decreased IL-6 in RAW264.7 macrophages *in vitro*, while 20-HDoHE did not significantly alter the levels of IL-6 and TNF-α.

Conclusion

In summation, we established a cupping mice model and utilized UPLC-MS/MS to assess the PUFA metabolome after cupping treatment. Although we did not perform cupping treatment in humans, our results indicated that the cupping treatment increased anti-inflammatory lipids and reduced pro-inflammatory lipids in mice skin and plasma. Since the lipid metabolism correlates with the physiological condition [41], the differential changes of PUFA derived metabolites in the local tissue or peripheral blood reflected an acceleration in homeostasis. Our findings explored the mechanism of cupping treatment from the new perspective of metabololipidome and suggested 14, 15-EET, 14S-HDoHE, 17-RvD1, RvD5, PGE_1, TXB_2 and 12-HETE as potential diganostic biomakers and therapeutics compounds in cupping treatment.

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YL conceived this topic and designed the study. QZ, XW, GY, JL, YZ, LW, TW, and XZ performed the experiments and analyzed the data. QZ, XW and YL drew the Figures and wrote the manuscript. All authors read and approved the manuscript.

Disclosure Statement

The authors have declared that no competing interest exists.

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