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Original Research

GLUT1 Mediates the Metabolic Reprogramming and Inflammation of CCR2$^+$ Monocytes/Macrophages from Patients with DCM

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Abstract

Background: Macrophages expressing CC chemokine receptor 2 (CCR2) possess characteristics and performance akin to M1 polarized macrophages, which promote inflammation. Advanced heart failure (HF) patients with higher abundance of CCR2$^+$ macrophages are more likely to experience adverse remodeling. The precise mechanism of CCR2$^+$ macrophages in how they affect the progression of dilated cardiomyopathy remains unknown. **Methods**: Cardiac biopsy samples from dilated cardiomyopathy patients (DCM) were used for immunohistochemistry and immunofluorescence staining. PCR is employed to identify the *IL-1β*, *IL-6*, *TNF-α*, *TGF-β*, *MMP2*, *MMP9*, *PKM1*, *PKM1*, *GLUT1*, *GLUT2*, *GLUT3*, *GLUT4*, *PDK1*, *PFKFB3*, *PFK1* and *HK2* mRNA expression of CCR2$^+$ monocytes/macrophages from the peripheral blood of DCM patients. Seahorse was used to evaluate the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of CCR2$^+$ monocytes/macrophages. 2-DG was used to simulate a lack of glucose. Lentivirus containing GLUT1 inhibitory sequence was used to knockdown GLUT1 gene expression of CCR2$^+$ monocytes/macrophages. Western Blot and immunofluorescence staining was used to evaluate the expression of NLRP3. **Results**: Immunostaining results of cardiac biopsy tissue from dilated cardiomyopathy (DCM) patients demonstrated that the progression to HF was associated with an increase in the number of CCR2$^+$ macrophages. PCR results demonstrated that CCR2 monocytes and macrophages derived from the blood of DCM patients expressed elevated levels of inflammatory factors and up regulation of glycolysis related genes. In addition, OCR and glucose uptake experiments confirmed that increased glucose uptake of these cells was associated with greater inflammation and correlated with a worsening of cardiac function. limiting the glucose supply to CCR2$^+$ monocytes and macrophages, or suppressing the activity of glucose transporter 1 (GLUT1) could reduce inflammation levels. **Conclusions**: These results suggest that CCR2$^+$ monocytes and macrophages rely on metabolic reprogramming to trigger inflammatory response and contribute to myocardial injury and the progression of DCM.

Keywords: macrophages; GLUT1; CCR2; dilated cardiomyopathy; metabolic reprogrammings

1. Introduction

Dilated cardiomyopathy (DCM) is a myocardial disease characterized by ventricular dilation and impaired cardiac contractility (systolic and diastolic function), which is a leading contributor to heart failure (HF) with reduced ejection fraction [1,2]. Despite the considerable advances in the treatment of DCM, some individuals remain at risk of sudden cardiac death and intractable heart failure, which necessitates cardiac transplantation or mechanical circulation support.

Pathological changes associated with inflammation and autoimmune reactions have been demonstrated to be pivotal in the development and advancement of DCM, yet the exact mechanism remains unknown [3]. Macrophages may be involved as immune cells that oversee the progression of myocarditis to DCM [4]. Yang *et al*. [5] conducted an evaluation on the expression level of 22 different types of immune cells in patients with DCM. This research found that the expression of M1 macrophages was significantly higher compared to normal heart tissue in the DCM group. CCR2$^+$ macrophages, with features that are similar to the traditionally identified M1 macrophages, secret inflammatory cytokines, which can result in myocardial damage and an unfavorable remodeling process, which can accelerate the development of heart failure [6]. Research has demonstrated that the presence of CCR2$^+$ macrophages is a predictor of unfavorable remodeling in individuals with advanced HF [7].

Macrophage metabolic reprogramming has been demonstrated to be strongly associated with cardiovascular diseases, potentially being the key factor in initiating macrophage inflammatory response [8]. It has been ob-

Table 1. Dilated cardiomyopathy (DCM, administered cardiac biopsies) patient information.

	Patient 1	Patient 2	Patient 3	Patient 4
Clinical features				
Gender	Male	Male	Female	Male
Age, years	47	25	52	47
Symptoms				
Cardiopalmus	No	No	No	Yes
Pectoralgia	No	No	Yes	No
Expiratory dyspnea	No	Yes	Yes	Yes
Experiencing HF symptoms	No	No	Yes	Yes
NYHA classification	2	2	3	3
Laboratory examination				
hs-TnT, ng/mL	0.014	0.009	0.009	0.212
BNP, pg/mL	26.49	<10	1388.56	356.68
hs-CRP, mg/L	3.09	3.32	10.47	6.45
CMR				
LA APD × LA TD, mm × mm	42 × 61	48 × 58	43 × 68	83 × 57
LVTD, mm	42	65	68	70
RA APD × RA TD, mm × mm	60 × 48	52 × 38	45 × 34	43 × 67
RV TD, mm	38	32	17	27
LV EF, %	60	34	23	17
LV CO, L/min	5.0	5.5	4.6	2.2
LV EDV, ml	144.7	258.9	208.7	217.5

APD, anterior posterior diameter; BNP, brain natriuretic peptide; CMR, cardiac magnetic resonance; CO, cardiac output; EDV, end-diastolic volume; EF, ejection fraction; HF, heart failure; hs-CRP, high sensitivity c reactive protein; hs-TNT, hypersensitive troponin T; LA, left atrium; LV, left ventricular; NYHA, New York Heart Association; RV, right ventricular; TD, transverse diameter.

served that the circulating monocytes of individuals affected by cardiovascular illness exhibit elevated aerobic glycolysis and retain this pattern of metabolism even after they transform into cardiac macrophages [9,10]. Following an acute myocardial infarction in mice, a significant up-regulation of glycolytic related genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cardiac macrophages is observed, which promotes glucose uptake and lactate production [11]. Under hypoxic conditions, the activation of hypoxia-inducible factor 1α (HIF-1α) in macrophages promotes glycolysis, interrupts the process of tricarboxylic acid (TCA) and oxidative phosphorylation, and increases the production of citric acid and succinic acid, which results in the accumulation of lactic acid [8,12]. This further triggers the release of inflammatory factors such as reactive oxygen species (ROS) and activates the NF- κB pathway, leading to the up-regulation of IL-1 β, IL-6, IL-18 and TNF-α [8]. The secretion of inflammatory factors contributes to the stabilization of HIF-1α, thus setting up a negative feedback loop [12]. The metabolic reprogramming of macrophages has an impact on the inflammatory response in ischemic heart disease, which is a result of the balance between the M1 and M2 macrophages in the acute and recovery stages [8].

In this investigation, we aim to explore a connection between the metabolic reprogramming of CCR2$^+$ monocytes/macrophages from the DCM patients and the presence of chronic inflammation as well as the relationship of glucose metabolism to the process. Such work can provide an uncharted peak checkpoint that establishes a link between the overconsumption of glucose and the functioning of inflammatory effectors. This can underscore the potential of metabolic reprogramming of macrophages as a novel therapeutic target for addressing the inflammatory response observed in cases of DCM.

2. Methods

2.1 Patients and Controls

In this investigation, 4 individuals afflicted with HF (with patients 2–4 specifically presenting with DCM) were subjected to a trans jugular interventricular septum myocardial biopsy. The cardiac biopsy samples were utilized for pathological evaluation, whereas biopsy samples of HF patients with non-DCM were employed as a control group. Table 1 provides the demographic characteristics of the 4 DCM patients. Incorporated into this investigation of cell sorting *in vitro* were 23 individuals who had been clinically diagnosed with DCM, as well as 14 healthy individuals who served as controls. Table 2 provides an overview

Table 2. DCM patient information.

	DCM (n = 23)	Control (n = 14)	t/Z	*p*
Clinical features				
Gender, n	Male: 17; Female: 6	Male: 3; Female: 11		0.003
Age, yrs	64.70 ± 11.95	63.07 ± 3.79	0.592	0.558
Smoking, n	7	4	-	1.000
Drinking, n	5	3	-	1.000
NYHA classification, n	IV: 3; III: 20	II: 6; I: 6; 0:2	-	<0.001
Diabetes, n	6	6	-	0.470
Hypertension, n	13	9	-	0.738
Ischemic etiology, n	6	1	-	0.217
COPD, n	3	0	-	0.275
Chronic kidney disease, n	6	0	-	0.065
AF, n	11	1	-	0.013
Stoke/TIA, n	7	3	-	0.710
Laboratory examination and echocardiography				
LVEF	0.34 ± 0.11	0.63 ± 0.03	–11.932	<0.001
NTproBNP, pg/mL	4561.50 (14443.00)	67.24 (73.48)	–4.998	<0.001
hs-TnT, ng/mL	0.039 (0.061)	0.007 (0.004)	–4.863	<0.001
hs-CRP, mg/L	5.700 (10.260)	0.995 (2.360)	–3.476	0.001
Fasting blood sugar, mmol/L	5.46 (1.68)	5.60 (1.41)	–0.783	0.434
HbA1c, %	7.00 ± 1.13	6.94 ± 0.83	0.111	0.913
Serum creatinine, µmol/L	87.00 (31.00)	66.50 (14.00)	–4.060	<0.001
Serum total bilirubin, µmol/L	14.90 (22.30)	9.15 (8.60)	–2.928	0.003
Total cholesterol, mmol/L	3.76 ± 1.01	4.86 ± 1.21	–2.953	0.006
Free fatty acids, mmol/L	0.51 ± 0.21	0.39 ± 0.12	1.929	0.063
Medical history				
ARNI/ACEI/ARB, n	16	4	-	0.021
Beta Blocker, n	18	5	-	0.015
Spironolactone, n	13	0	-	<0.001
Statins, n	13	11	-	0.288
Nitrates, n	9	3	-	0.306
Diuretic agent, n	20	0	-	<0.001
Digoxin, n	8	0	-	0.007

ACEI, angiotensin converting enzyme inhibitor; AF, Atrial fibrillation/ Atrial flutter; ARNI, Angiotensin receptor-neprilysin inhibition; ARB, angiotensin receptor antagonist; COPD, chronic obstructive pulmonary disease; HbA1c, hemoglobin A1c; hs-CRP, high sensitivity c reactive protein; hs-TNT, hypersensitive troponin T; LVEF, Left ventricular ejection fraction; NTproBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; TIA, transient ischemic attacks.

of the patients' general information. The exclusion criteria were as follows: myocarditis, pericardial disease, acute cerebrovascular disease, moderate or severe liver dysfunction, severe infection, severe lung disease, severe renal dysfunction (estimated glomerular filtration rate [eGFR] <15 mL/min/1.73 m^2, calculated by using the CKD-EPI formula), history of malignant tumors, thyroid disorders, autoimmune diseases, hemopathy and recently experienced trauma or surgery. The study was approved by the Ethics Committee of Tianjin Chest Hospital and written informed consent was obtained from all participants.

2.2 Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy and DCM donors using density centrifugation with Lymphoprep (STEMCELL Technologies, Vancouver, Canada). From the PBMCs fraction, $CCR2^+$ monocytes were isolated by magnetic-activated cell sorting (MACS) using a magnetic pole (EasySepTM #18000, Miltenyi, Cologne, Germany), EasySep™ Release Human PE Positive Selection Kit (#17654, STEMCELL Technologies, Vancouver, Canada) and CD192 (CCR2) and Antibody #130-118-338 (Miltenyi, Cologne, Germany). In order to generate $CCR2^+$ bone marrow-derived macrophages (BMDMs), 10^7/mL $CCR2^+$ PBMCs were seeded into a six well plate and cultured for

a period of 5 days in RPMI 1640 medium (#31870074, Life Technologies, Carlsbad, CA, USA) supplemented with 20 ng/ml of M-CSF (#14-8789-80, eBioscience, San Diego, CA, USA) and 10% of FBS (Lonza, Basel, Switzerland). On the third day, the medium was replaced to ensure optimal growth conditions. $CCR2^+$ BMDMs attached to plates were detached using StemPro Accutase Cell Dissociation Reagent (Lonza, Basel, Switzerland). Cell activity was assessed using MTT assay (MTT kit, #C0009S, Beyotime Biotech. Inc., Beijing, China).

2.3 Mitochondrial Respiration and Glycolysis

The Seahorse XF24 analyzer (Seahorse Bioscience, USA) was utilized to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in accordance with our previously described method [13]. $CCR2^+$ BMDMs (5×10^6 cells/mL) were grown in 24-well plates with 1 µM oligomycin, 1 µM trifluoromethoxy carbonyl cyanide phenylhydrazone, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1 µM rotenone together with 1 µM antimycin A were added in sequence. The Seahorse analyzer software was utilized to calculate OCR and ECAR.

2.4 Measurement of Glucose Uptake

$CCR2^+$ BMDMs (5×10^6 cells/mL) were placed in a glucose-free RPMI medium with 5 µM of fluorescent D-glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (Cayman Chemical, Ann Arbor, MI, USA) and incubated for an hour at 37 °C. The Lionheart FX automated imaging system (Bio Tek, Winooski, VT, USA) was employed to analyze the fluorescent intensities.

2.5 Measurement of mtROS

Isolation of intact mitochondria from macrophages was conducted in accordance a commercially available mitochondrial extraction kit (Solarbio, Beijing, China) as per the manufacturer's instructions. For the purpose of measuring intramitochondrial ROS level, isolated mitochondria were transferred to a 96-well flat-bottomed plate and a 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe detection kit (#C2938, Thermo Fisher Scientific, Waltham, CA, USA) together with a Lionheart FX automated imaging system (Bio Tek, USA) was used.

2.6 RT-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was conducted using the mRNA reverse transcription kit (Roche, Basle, Switzerland) as per the manufacturer's instructions. SYBR Green PCR master mix (Roche, Basle, Switzerland) was employed in RT-PCR, which was conducted using a CFX96TM PCR detection system (BioRad, Redmond, WA, USA). The primer sequence is provided in Table 3.

Table 3. Primer sequence.

Target gene		Primer sequence (5′→ 3′)
PKM1	Forward:	CGAGCCTCAAGTCACTCCAC
	Reverse:	GTGAGCAGACCTGCCAGACT
PKM2	Forward:	ATTATTTGAGGAACTCCGCCGCCT
	Reverse:	ATTCCGGGTCACAGCAATGATGG
GLUT1	Forward:	TATGTGGAGCAACTGTGTGGT
	Reverse:	TCCGGCCTTTAGTCTCAGGA
GLUT2	Forward:	CGGCTGGTATCAGCAAACCT
	Reverse:	AGAAAGAGAGAACGTCGCCC
GLUT3	Forward:	GTCATGATCCCAGCGAGACC
	Reverse:	CTGGGGTGACCTTCTGTGTC
GLUT4	Forward:	TAGGCTCCGAAGATGGGGAA
	Reverse:	CCCAGCCACGTCTCATTGTA
PDK1	Forward:	AGTGCCTCTGGCTGGTTTTG
	Reverse:	GCATCTGTCCCGTAACCCTC
PFKFB3	Forward:	CTTGTCGCTGATCAAGGTGA
	Reverse:	TTCTGCTCCTCCACGAACTT
PFK1	Forward:	CTGTACTCATCAGAGGGCAAG
	Reverse:	TGCCAGCATCTTCAGCATGAG
HK2	Forward:	ACGGAGCTCAACCATGACCAA
	Reverse:	AAGATCCAGAGCCAGGAACTC
IL-6	Forward:	AGTTCCTGCAGAAAAAGGCAAAG
	Reverse:	ATTTGCCGAAGAGCCCTCAG
IL-1β	Forward:	CAGGCTGCTCTGGGATTCTC
	Reverse:	GTCCTGGAAGGAGCACTTCAT
TNF-α	Forward:	GCTGCACTTTGGAGTGATCG
	Reverse:	GCTTGAGGGTTTGCTACAACA

2.7 Cell Transfection

$CCR2^+$ PBMCs and BMDMs were transfected with GLUT1 inhibitory sequence ($GLUT1^{-/-}$) containing lentivirus (Genechem, Shanghai, China) or lentivirus containing scrambled control sequences. Then, 5×10^6 cells/mL $CCR2^+$ PBMCs and BMDMs were inoculated into a 6-well plate. The infection reagent and lentivirus were added to $CCR2^+$ PBMCs and BMDMs according to the manufacturer's instructions. Following a 72-hour infection period, the identification of GLUT1 mRNA expression in $CCR2^+$ PBMCs and BMDMs was conducted through the utilization of RT-PCR.

2.8 Immunostaining

The heart tissues were fixed in 4% paraformaldehyde for 72 hours, following which they were embedded in paraffin and cut into 4 µm thick sections. The sections were then subjected to hematoxylin/eosin staining (H&E). The tyramide signal amplification plus multiplex fluorescence staining kit (#G1236-100T, Servicebio, Wuhan, Hubei, China) was used for staining CCR2 (#ab254375, Abcam, Cambridge, UK), CX3CR1 (#ab167571, Abcam, Cambridge, UK) and α-Actin (#ab11003, Abcam, Cambridge,

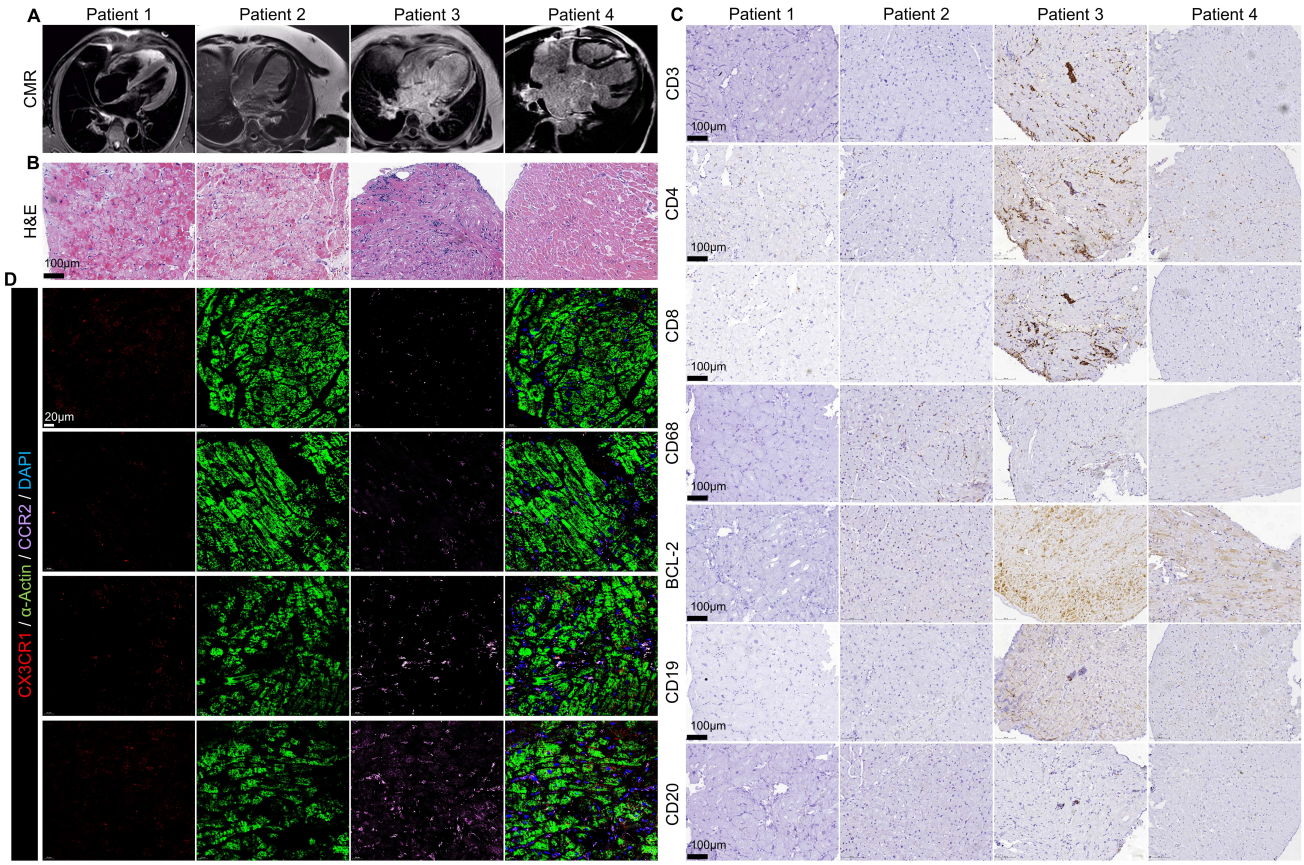

Fig. 1. Patient 1-4 CMR histological examination. (A) Typical CMR images of patient 1–4. (B) H&E staining of patient 1–4 (scale bar = 100 μm). (C) Immunohistochemical staining of CD3, CD4, CD8, CD68, BCL-2, CD19 and CD20 (scale bar = 100 μm). (D) Patient 1-4 immunostaining of CX3CR1 (Red), α- Actin (green), CCR2 (Pink) (scale bar = 20 μm).

UK) according to the manufacturer's protocol. Following washing with PBS, the sections were counterstained with DAPI and observed through a fluorescence microscope and digital camera (Axio Observer Al, Carl Zeiss, Germany). Immunohistochemical staining of CD3 (total T cells), CD4 (helper T cells), CD8 (cytotoxic T cells), CD68 (macrophages), BCL-2 (proteins marker of apoptosis), CD19 (B cells) and CD20 (B cells) was entrusted to the Pathology Department of Tianjin Chest Hospital.

2.9 Western Blot

Western blot analysis was performed to determine the NLRP3 (#ab263899, Abcam, Cambridge, UK) expression in CCR2$^+$ macrophages. The relative values were adjusted to GAPDH expression levels and normalized relative to the baseline controls.

2.10 Statistical Analysis

Data analysis was conducted using SPSS software version 24 (v24, IBM Corp., Chicago, IL, USA). The Shapiro-Wilk test was performed to determine the normality of continuous variables. Normally distributed continuous variables were presented as mean and standard deviation ($\bar{x} \pm$ s), and Intergroup comparisons were done using independent two-tailed Student's *t*-tests. For differences across multiple groups with one variable, one-way analysis of variance (ANOVA) was utilized, and for groups with multiple variables, a two-way ANOVA was applied. Non-normally distributed continuous variables were presented as median and interquartile interval (M(Q)), and the Wilcoxon Mann-Whitney test was used to compare different groups. Categorical variables were expressed as frequencies and compared using Fisher's exact test. A p value < 0.05 was considered statistically significant. All experimental n numbers are provided in the figure legends.

3. Results

3.1 Correlation Analysis between the Number of CCR2$^+$ Cells in the Heart of DCM Patients and the Degree of their Heart Failure

From July to October 2021, the Cardiac intensive care unit of Tianjin Chest Hospital administered cardiac biopsies on four patients that had been clinically diagnosed with cardiomyopathy; these results showed that three of the cases correlated with the clinical manifestations of heart failure resulting from dilated cardiomyopathy, and they were consequently included in the study. Patients 2–4 were clinically identified as having DCM, Patients 3 and 4 were identified

as having heart failure (DCM/HF), as demonstrated by the clinical information. Relevant examinations and tests are provided in Table 2. Fig. 1A provides typical cardiac magnetic resonance (CMR) images of these patients.

Histological examination (H&E staining) revealed that the myocardium of DCM patients suffered from severe edema and vacuolar degeneration, with leukocyte infiltration between the myocardium The degree of injury increased as the left ventricular ejection fraction decreased (Fig. 1B). Immunohistochemical staining revealed that CD19 and CD20 were not present in the myocardium of the patients, whereas CD68, CD3, CD4, CD8 and BCL2 were expressed (Fig. 1C).

The connection between macrophages and myocardial injury was assessed through co-localization immunofluorescence staining (Fig. 1D, CX3CR1: cardiac resident macrophage marker, CCR2: myeloid proinflammatory macrophage marker, α- Actin: myocardial skeleton protein). Results indicated that CX3CR1$^+$ cells (i.e., cardiac resident macrophages, Red) were exhausted in both DCM and DCM/HF patients, and as DCM progressed to HF, the number of proinflammatory macrophages (i.e., circulating infiltrating macrophages, CCR2$^+$ cells, purple) significantly increased (Fig. 1D). The findings revealed that the increased number of CCR2$^+$ macrophages was associated with the myocardial injury of DCM. This was further substantiated by the CMR examination results (CMR, Fig. 1A), which confirmed the positive correlation between the degree of CCR2$^+$ macrophage cardiac injury and DCM heart.

3.2 The Level of Inflammation in CCR2$^+$ Monocytes and Macrophages of Patients with DCM

To verify the link between CCR2$^+$ macrophages and DCM, a study was conducted on CCR2$^+$ monocytes isolated from peripheral blood of 23 DCM patients and 14 control patients. The patient information is provided in Table 3. Briefly, the DCM group and the control group exhibited considerable differences in assessing the primary indicators of heart failure such as NYHA classification, LVEF%, and the level of NT-proBNP and hs-TnT; No substantial divergence was observed between the two groups in terms of the prevalence of hypertension and coronary artery stenosis; The usage of anti-heart-failure-related drugs such as angiotensin converting enzyme inhibitor (ACEI)/ angiotensin receptor antagonist (ARB)/ angiotensin receptor enkephalinase inhibition (ARNI), β blockers, spironolactone, diuretics, and digoxin in the DCM group was significantly higher than that in the control group; and the proportion of patients in the two groups who had ingested ivabradine, statins, nitrates and anticoagulants was not significantly different.

Isolated CCR2$^+$ monocytes were induced into macrophages *in vitro* and the mRNA expression analyzed for inflammation-related genes (*IL-1*β, *IL-6* and *TNF-*α) in CCR2$^+$ monocytes and CCR2$^+$ macrophages by RT-PCR. The results revealed that the mRNA expression of inflammation-associated genes *IL-1*β, *IL-6* and *TNF-*α in CCR2$^+$ monocytes and macrophages from the DCM group was significantly higher than that of the control group (Fig. 2A–C). There was a positive correlation to the New York Heart Association (NYHA) classification (Fig. 2D–F). It was observed that with the deterioration of cardiac function, the expression of inflammatory related mRNA in CCR2$^+$ monocytes and macrophages from peripheral blood of patients went higher (Fig. 2D–F).

The mRNA expression of the related genes of extracellular matrix remodeling such as transforming growth factor-β (TGF-β), matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) in CCR2$^+$ macrophages were examined using RT-PCR. Compared to the control group, the mRNA expression of TGF-β, MMP2 and MMP9 of CCR2$^+$ macrophages in the DCM group were significantly higher (Fig. 2G–I) . There was a positive correlation between the mRNA expression and the worsening of NYHA cardiac function grading (Fig. 2J–L).

Utilizing the prior medication history of patients in the DCM group, consideration was given to possible effects of statins, β-receptor blockers, ARNI, and direct oral anticoagulants (Rivaroxaban/Dabigatran) — all of which are commonly prescribed for patients with heart failure. Statins had a slight effect on decreasing the *IL-1*β and *IL-6* mRNA expression of CCR2$^+$ monocytes, though without any statistical significance (Fig. 2M–N). On the other hand, neither β receptor blockers, ARNI, nor rivaroxaban/dabigatran had any effect on *IL-1*β mRNA expression (Fig. 2N). Furthermore, statins, ARNI, and rivaroxaban/dabigatran had a slight effect on reducing the *TGF-*β of CCR2$^+$ macrophages mRNA expression, though without any statistical significance, while β receptor blockers had no effect (Fig. 2O).

3.3 Metabolic Reprogramming of CCR2$^+$ Monocytes/Macropssshages in DCM Patients

CCR2$^+$ monocyte and macrophage oxygen consumption rate (OCR) did not differ statistically between the two groups (Fig. 3A,C), but extraceullular acidification rate (ECAR) was significantly higher between the two groups (Fig. 3B,D).

RT-PCR analysis revealed an upregulation of CCR2$^+$ monocyte and macrophage glycolysis related genes, such as pyruvate kinase isoform M1 (*PKM1*), pyruvate kinase isoform M2 (*PKM2*), *GLUT1*, *GLUT2*, *GLUT3*, *GLUT4*, phosphoinositol dependent protein kinase 1 (*PDK1*), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases 3 (*PFKFB3*), phosphofructokinase 1 (*PFK1*), and hexokinase 2 (*HK2*), in DCM patients (Fig. 3E,F). Quantitative analysis further revealed that the expression of *GLUT1* in CCR2$^+$ monocytes and macrophages of DCM patients was significantly higher than that of the control group, with statistical significance, while

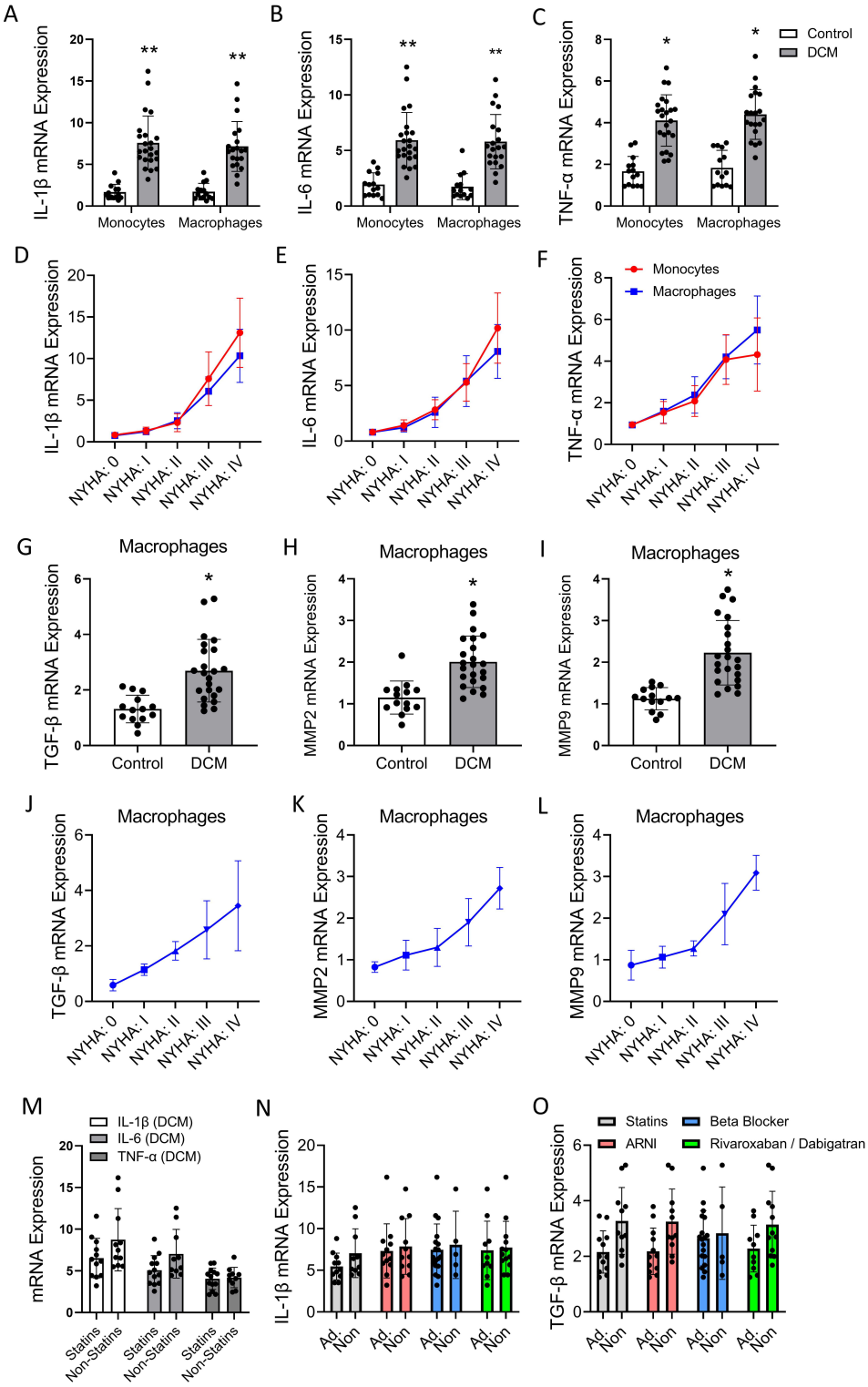

Fig. 2. The level of inflammation in CCR2$^+$ monocytes and macrophages of patients with dilated cardiomyopathy. mRNA expression of inflammation-associated genes *IL-1β* (A), *IL-6* (B) and *TNF-α* (C) in CCR2$^+$ monocytes and macrophages. NYHA classification is associated with *IL-1β* (D), *IL-6* (E) and *TNF-α* (F) mRNA expression. mRNA expression of inflammation-associated genes *TGF-β* (G), *MMP2 (H)* and *MMP9* (I) in CCR2$^+$ macrophages. NYHA classification is associated with *TGF-β* (J), *MMP2* (K) and *MMP9* (L) mRNA expression. (M) Relationship between statins used by DCM and *IL-1β*, *IL-6* and *TNF-α*. (N) Relationship between *β*-receptor blockers, ARNI, and Rivaroxaban/Dabigatran used by DCM and *IL-1β*. (O) Relationship between *β*-receptor blockers, ARNI, and Rivaroxaban/Dabigatran used by DCM and *TGF-β*. All data are presented as the mean ± SD. Statistical significance is indicated as: $*p < 0.05$, $**p < 0.01$ compared with the control group.

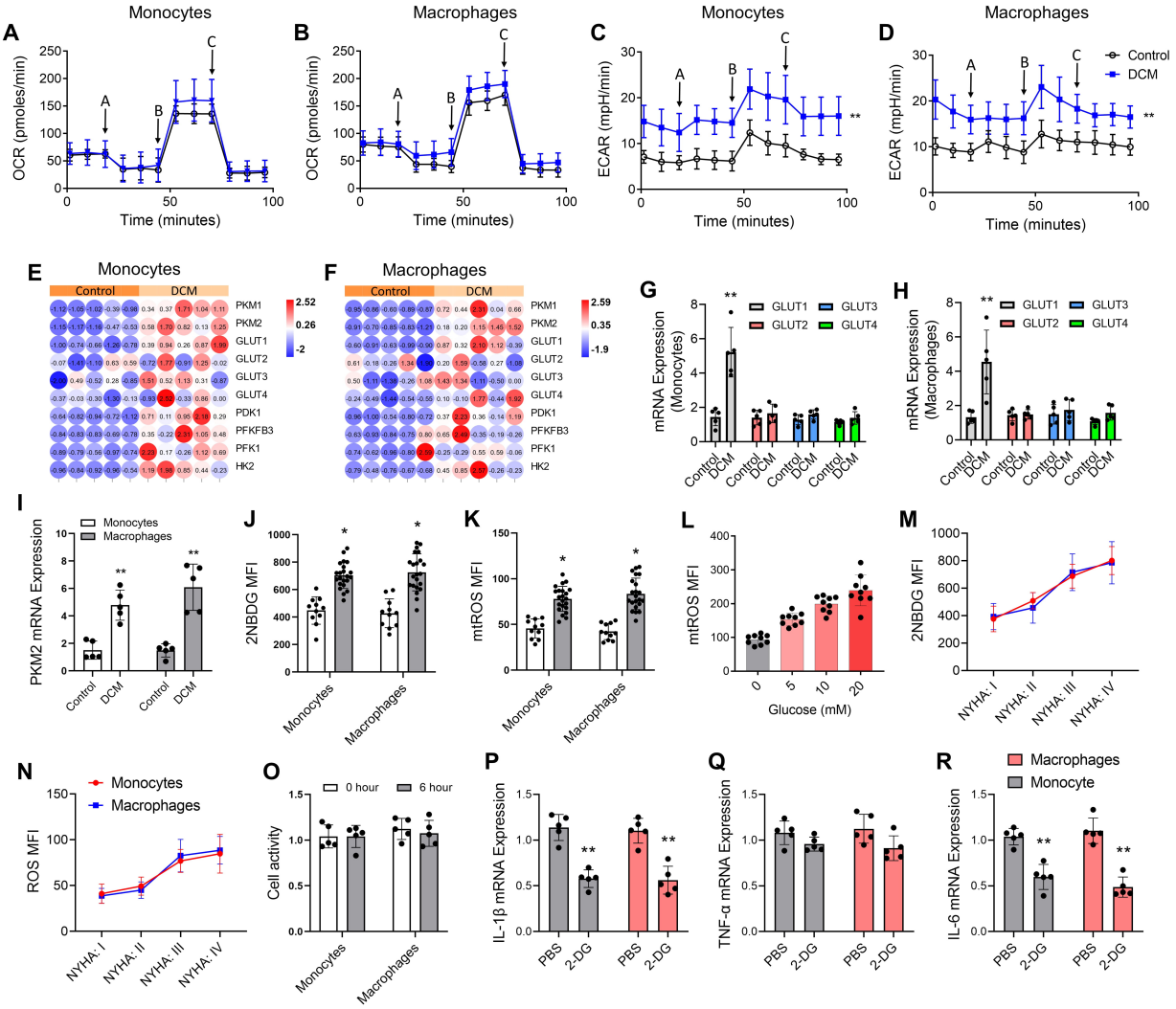

Fig. 3. Metabolic reprogramming of CCR2$^+$ monocytes and macrophages in DCM patients. oxygen consumption rate (OCR) (A,C) and extracellular acidification rate (ECAR) (B,D) in CCR2$^+$ monocytes and macrophages measured using a Seahorse Bioscience XF24 analyzer (n = 4). Probes were done with serial addition of A: oligomycin, B: FCCP, and C: antimycin A/rotenone as indicated (E,F) are heat maps displaying the mRNA expression of genes related to glycolysis in CCR2$^+$ monocytes and macrophages (n = 5). Also shown are the mRNA expression of *GLUT* 1–4 in CCR2$^+$monocytes (G) and macrophages (H), n = 5. (I) mRNA expression of *PKM2* in CCR2$^+$ monocytes and macrophages, n = 5. (J) Glucose uptake in CCR2$^+$ monocytes and macrophages were measured using the fluorescence-labeled glucose analogue (2-NBDG) by mean fluorescence intensity (MFI). (K) reactive oxygen species (ROS) levels in CCR2$^+$ monocytes and macrophages using MitoSOX fluorescent probe. (L) ROS levels in CCR2$^+$ macrophages exposed to different concentrations of glucose. NYHA classification is associated with 2-NBDG MFI (M) and mtROS MFI (N). (O) the activity of CCR2$^+$ monocytes and macrophages, n = 5. *IL-1β* (P), *TNF-α* (Q) and *IL-6* (R) mRNA expression level after administering 2-DG, n = 5. All data are presented as the mean ± SD. Statistical significance is indicated as: $*p < 0.05$, $**p < 0.01$ compared with the control group.

the expression of *GLUT2*, *GLUT3* and *GLUT4* had no statistical difference between the two groups (Fig. 3G,H).The enzyme *PKM2*, a pivotal component of glycolysis, displayed a similar up-regulation trend as the other enzymes, with a statistically significant variation between the two groups (Fig. 3I).

3.4 The Uptake of Glucose by CCR2$^+$ Monocytes/Macrophages and Inflammation.

CCR2$^+$ monocytes and macrophages of DCM patients had high glucose uptake and ROS levels in comparison to the control group (Fig. 3J,K). This increase correlated with a worsening of cardiac function (NYHA classification, Fig. 3M,N). The mitochondrial ROS level of CCR2$^+$ monocytes and macrophages of DCM patients was found to be positively correlated with glucose concentration (Fig. 3L).

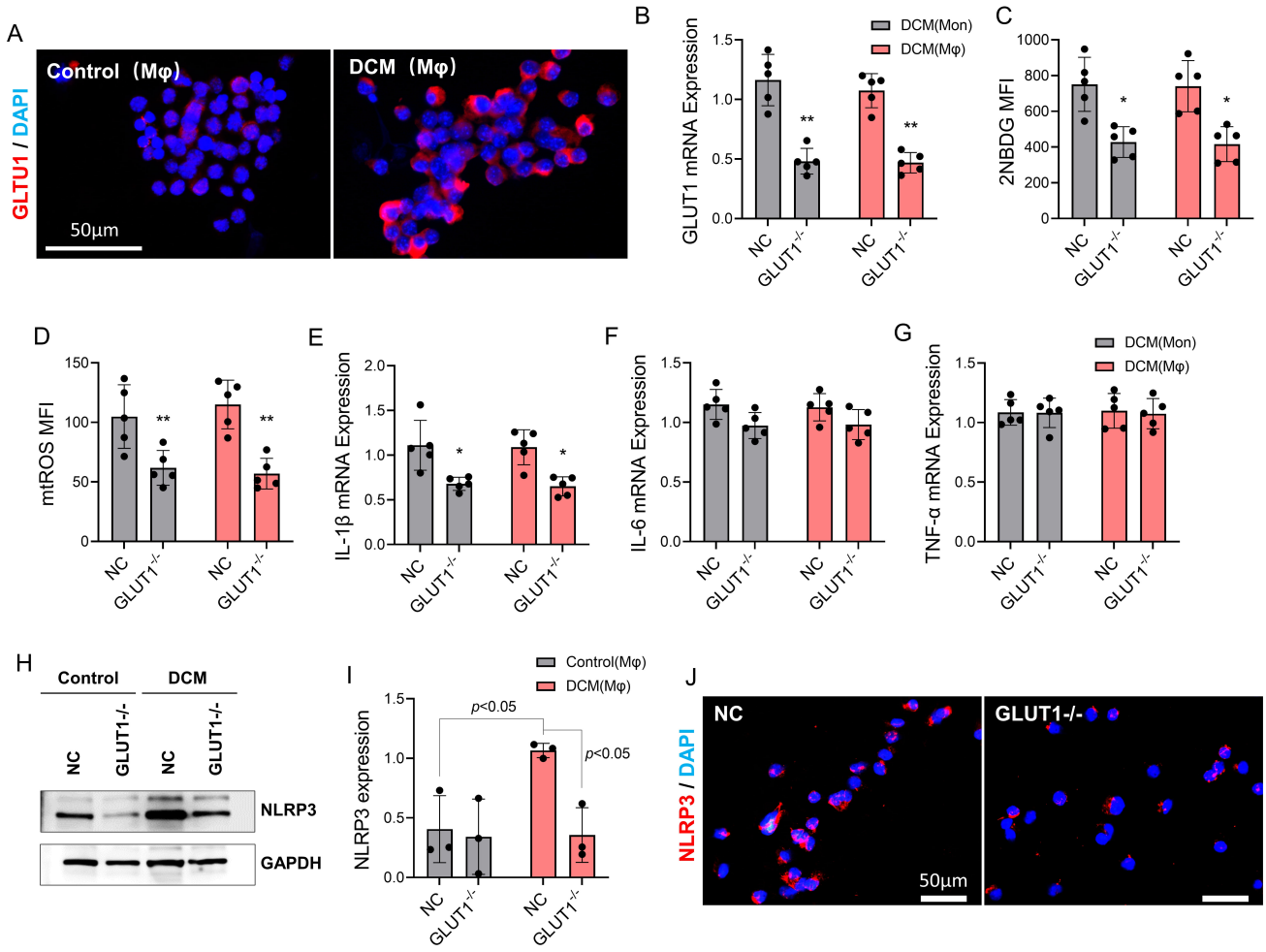

Fig. 4. GLUT1 regulates the metabolism reprogramming of CCR2$^+$ macrophages in DCM patients to promote inflammatory response. (A) Immunostaining of *GLUT1* in CCR2$^+$ macrophages. (B) mRNA expression of GLUT1 in DCM CCR2$^+$ monocytes and macrophages under GLUT1$^{-/-}$, n = 5. The level of 2NBDG MFI (C) and mtROS MFI (D) in DCM CCR2$^+$ monocytes and macrophages under GLUT1$^{-/-}$, n = 5. *IL-1β* (E), *IL-6* (F) and *TGF-β* (G) mRNA expression level in DCM CCR2$^+$ monocytes and macrophages under GLUT1$^{-/-}$, n = 5. (H,I) Protein expression of NLRP3 in CCR2$^+$ macrophages under GLUT1$^{-/-}$, n = 3. (J) Immunostaining of NLRP3 in DCM CCR2$^+$ macrophages. All data are presented as the mean ± SD. Statistical significance is indicated as: $*p < 0.05$, $**p < 0.01$ compared with the control group.

CCR2$^+$ monocytes and macrophages from DCM patients were exposed to 2-DG, an artificial glucose analogue that can simulate a lack of glucose for 6 hours. The activity of CCR2$^+$ monocytes and macrophages remained unchanged (Fig. 3O), but IL-6 and IL-1β mRNA expression level decreased significantly (Fig. 3P,R). There was no major fluctuation in the TNF-α mRNA expression (Fig. 3Q).

3.5 GLUT1 Regulation of the Metabolism Reprogramming of CCR2$^+$ Macrophages in DCM Patients to Promote Inflammatory Response

To further investigate the potential mechanism of CCR2$^+$ monocytes and macrophages inflammation and glucose uptake, shRNA was employed to suppress the mRNA expression of GLUT1 in CCR2$^+$ monocytes and macrophages from DCM patients. Immunofluorescence staining revealed that CCR2$^+$ macrophages in DCM patients expressed GLUT1 at a higher level than those in the control group (Fig. 4A). Furthermore, silencing CCR2$^+$ monocytes and macrophages in DCM patients with shRNA resulted in a decrease in the mRNA expression of *GLUT1* (GLUT1$^{-/-}$, Fig. 4B). Following the silencing of *GLUT1*, the glucose uptake capacity of CCR2$^+$ monocytes and macrophages in the DCM group was significantly decreased (Fig. 4C), the level of mitochondrial ROS was significantly decreased (Fig. 4D), and the expression of the inflammatory factor *IL-1β* was significantly reduced (Fig. 4E). However, silencing *GLUT1* had no significant effect on the mRNA expression of *IL-6* and *TNF-α* (Fig. 4F,G). In addition, for NOD-like receptor protein 3 (NLRP3) under GLUT1$^{-/-}$, the results revealed that the NLRP3 expression in CCR2$^+$ macrophages from the pe-

ripheral blood of DCM group were significantly higher than that of the control group. Also, the NLRP3 expression significantly decreased after GLUT1 was silenced (Fig. 4H,I). Furthermore, NLRP3 immunofluorescence staining also confirmed this result (Fig. 4J).

4. Discussion

It has been established that inflammatory processes and autoimmune reactions have a significant impact on the development of DCM [14]. However, the precise mechanism of this is yet to be determined. In cardiac biopsy samples of patients with DCM, it is often observed that there is a continuous infiltration of inflammatory cells, such as $CD4^+$ and $CD8^+$ T lymphocytes and M1 macrophages, suggesting a correlation between inflammation and cardiac dysfunction [5]. $CCR2^+$ monocytes demonstrate pro-inflammatory activity and can differentiate into $CCR2^+$ macrophages with features that are similar to the traditionally identified M1 macrophages. In this study, immunofluorescence staining and CMRI of patients revealed that, as myocardial fibrosis worsened and LVEF decreased, the number of $CCR2^+$ cells in myocardium increased, signifying an infiltration of inflammatory macrophages. We observed that in the vicinity of the $CCR2^+$ cells aggregation, the concentration of α-actin decreased, which is a major element of sarcomere filaments and is responsible for the formation of the myocardial cells' cytoskeleton and the excitation-contraction coupling of myocardia. This evidence suggests that $CCR2^+$ monocyte and macrophage may contribute to the myocardial damage associated with DCM.

Clinical studies have verified that the IL-6 concentration in cardiac tissue of individuals suffering from advanced heart failure is higher than that of those with ischemic cardiomyopathy [15–17]. The direct injury effect of IL-6 on cardiac cells, its ability to inhibit the excitation-contraction coupling of cardiac cells, and its involvement in the onset of HF are all established [15,16]. IL-1β has been shown to induce the production of chemokines, facilitate the adherence and infiltration of inflammatory cells, and further stimulate the proliferation of fibroblasts, which thus contribute to myocardial fibrosis [18]. TNF-α can increase the presence of proteins in the interstitial tissue of the heart, thus stimulating myocardial fibrosis and causing apoptosis of the cardiac cells, which leads to their death [19]. In the current study, subgroup analysis of DCM revealed that the mRNA expression of *IL-1*β, *IL-6* and *TNF-*α corresponded with the decline in cardiac function grading, which is in agreement with the findings of Parthenakis [20]. The new results further demonstrate the detrimental effects of *IL-1*β, *IL-6* and *TNF-*α on myocardial tissue. Additionally, in comparison to the healthy control group, the mRNA expression of *MMP2* and *MMP9* in the DCM group was significantly higher, indicating that DCM patients may experience augmented extracellular matrix remodeling, which is associated with cardiac fibrosis. Results from subgroup analysis indicated that *TGF-*β, *MMP2*, and *MMP9* increased as cardiac function grade increased. This suggests an augmentation of extracellular matrix remodeling. Examining the prior medication history of DCM patients further studied the impact of typical CHF drugs on the characteristics of $CCR2^+$ monocytes and macrophages. The results indicated that statins could possibly reduce inflammation and extracellular matrix remodeling due to $CCR2^+$monocytes and macrophages, while ARNI and direct oral anticoagulants (Rivaroxaban /Dabigatran) may act to counteract the extracellular matrix remodeling caused by $CCR2^+$ macrophages.

Previous research has verified that the mononuclear cells of CAD patients have a high glycolysis rate, which causes a high production of ROS and intensifies their pro-inflammatory activity [9]. The new results presented here indicates that $CCR2^+$ monocytes and macrophages from peripheral blood of DCM patients demonstrate high expression of inflammation-related factors, and the magnitude of these inflammatory factors may be associated with ROS level and glycolysis. Results also indicate that $CCR2^+$ monocyte and macrophage of DCM require more energy to sustain their life activities than in a steady-state. However, this increased energy is not obtained through aerobic respiration and oxidative phosphorylation, but rather from the up-regulation of cell glycolysis, which implies that metabolic reprogramming has occurred. It is generally accepted that oxidative phosphorylation of cells is hindered during aerobic glycolysis. Yet in this study, a reduction in OCR in DCM patients was not seen. This necessitatates further investigation into the underlying reason and mechanism. Upon assessing the subgroups of individuals with DCM, the glucose uptake and mitochondrial ROS level of $CCR2^+$ monocytes and macrophages increased in proportion to the cardiac function grading of NAHY. It was hypothesized that as DCM progressed, the inflammatory response and glycolysis of $CCR2^+$ monocytes and macrophages would also increase. Treatment of $CCR2^+$ monocytes and macrophages with 2-DG instead of glucose resulted in significant decreases in the level of ROS and mRNA expression of *IL-6* and *IL-1*β, following glucose deprivation. It was evident that metabolic reprogramming of $CCR2^+$ monocytes and macrophages strongly correlated with the secretion of inflammatory substances, thus leading to a pro-inflammatory effect. Inhibiting its metabolic reprogramming can decrease its inflammatory response.

The GLUTs protein family is responsible for the control of glucose uptake and metabolism in adipose tissue, skeletal muscle, and liver. Generally, GLUTs are responsible for the transport of glucose in and out of the cells which maintains the balance of blood glucose [21,22]. Data presented here has verified that $CCR2^+$ monocytes and macrophages derived from peripheral blood of those with DCM show a heightened expression of *GLUT1*. *GLUT1*

may be the crucial enzyme that facilitates the entry of glucose into $CCR2^+$ monocytes and macrophages for further glycolysis. Following the silencing of *GLUT1* mRNA expression in $CCR2^+$ monocytes and macrophages from DCM patients via shRNA, the glucose uptake capacity of $CCR2^+$ monocytes and macrophages was significantly reduced. In addition, the level of mitochondrial ROS was significantly decreased and the expression of inflammatory factors was also diminished.

A constraint of the present investigation is the limited number of research samples, which impedes our ability to accurately elucidate the impact of drugs on macrophage metabolic reprogramming and its role in mediating inflammatory response. Furthermore, owing to the limited number of $CCR2^+$ macrophages obtained during the cultivation process *in vitro*, then current examination of inflammatory factors and extracellular matrix-related proteins was restricted to mRNA levels. This did not allow accurate discernment of variations in protein expression of these factors and proteins. The findings of our study may have aramifications for the management of DCM and its associated inflammatory conditions. Results from our study showed that $CCR2^+$ monocytes and macrophages from the peripheral blood of DCM patients had undergone metabolic reprogramming leading to alterations in their inflammatory phenotype. Analysis of $CCR2^+$ monocytes and macrophages of DCM patients revealed that the expression of *GLUT1* had an effect on the inflammatory phenotype of these cells when they underwent metabolic reprogramming. By suppressing the expression of *GLUT1*, it is possible to decrease the amount of mitochondrial ROS and the expression of inflammatory factors in $CCR2^+$ monocytes and macrophages, which can lead to a restriction in the progression of DCM. Our research can aid in the discovery of fresh therapeutic objectives for dilated cardiomyopathy, and can lead to innovative methodologies to decelerate the progression of cardiac remodeling in individuals with this ailment.

5. Conclusions

$CCR2^+$ monocytes and macrophages from the peripheral blood of DCM patients had experienced a metabolic transformation, resulting in changes to their inflammatory characteristics. By inhibiting GLUT1, the production of mitochondrial ROS and the expression of inflammatory factors in $CCR2^+$ monocytes and macrophages can be limited, thus slowing the advancement of DCM.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CF: Data curation, Investigation, Methodology, Writing — original draft; HJ: Data curation, Investigation, Methodology; XY: Data curation, Investigation, Methodology; HC: Conceptualization, Project administration; LL: Conceptualization, Project administration, Writing — original draft; JF: Conceptualization, Project administration, Funding acquisition, Validation. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The studies involving human participants were reviewed and approved by the Ethics Committee of Tianjin chest hospital (IRB-SOP-016(F)-001-02). The patients/participants provided their written informed consent to participate in this study.

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Conflict of Interest

The authors declare no conflict of interest.

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