Regenerative Therapy 24 (2023) 54–63

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Clodronate liposomes may biases MSC differentiation toward adipogenesis through activation of NLRP3

Shuang Ding [b, 1], Yuhan Wang [a, c, 1], Zhiting Liu [b, c], Yuwei Du [a, c], Yi Zhou [a, c], Yahui Liu [a, c], Jingfang Sun [b], Yue Li [c], Lingyu Zeng [a, b, c, *]

[a] Department of Hematology, The Affiliated Hospital of Xuzhou Medical University, Xuzhou, China
[b] The Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu Province, China
[c] Xuzhou Medical University, China

ARTICLE INFO

Article history:
Received 3 March 2023
Received in revised form
26 April 2023
Accepted 20 May 2023

Keywords:
Clodronate-liposomes
HSCT
NLRP3
Adipocytes
MSC

ABSTRACT

Introduction: Clodronate-Liposomes (Clod-Lipo) injection after hematopoietic stem cell transplantation (HSCT) has been shown to be detrimental to hematopoietic reconstitution after transplantation, and our previous study showed that Clod-Lipo injection after HSCT increased adipocytes in the bone marrow cavity of mice after HSCT, but the reason for the large increase in adipocytes has not been clearly explained. The aim of this study was to investigate the source and mechanism of bone marrow cavity adipocytes after HSCT injection of Clod-Lipo.

Methods: BALB/c mice received 7.5 Gy of total body irradiation followed by infusion of 5x106 bone marrow mononuclear cells from C57BL/6 via the tail vein. Clod-Lipo were injected through the tail vein on the first day after HSCT and every 5 days for the rest of the day. BALB/c mice were then divided into three groups: BMT, BMT + Clodronate-Liposomes (BMT + Clod-Lipo), and BMT + PBS-Liposomes (BMT + PBS-Lipo). Bone marrow pathological changes were detected by H&E staining, Western blot was used to detect the expression of NLRP3 and Caspase-1 in mouse bone marrow cells, and RT-qPCR was used to detect the expression levels of the key transcription factors peroxisome proliferator-activated receptor γ (PPAR-γ) and CCAAT/enhancer binding protein (C/EBPα) mRNA in bone marrow cells. Mouse mesenchymal stem cells (MSC) cultured in vitro were identified by flow cytometry, and adipocyte induction assays were performed using Clod-Lipo action for 24 h, Oil red staining was used to identify adipogenesis. Western blot was performed to detect NLRP3 and caspase-1 expression in MSC after Clod-Lipo action. Caspase-1 was blocked with Ac-YVAD-cmk (Ac-YV), followed by adipogenesis assay after 24 h of Clod-Lipo action to observe the change in the amount of adipogenesis.

Results: Compared with the other two groups, a significant increase in adipocytes was found in the Clod-Lipo group by HE staining, and increased expression of NLRP3 and Caspase-1 in mouse bone marrow cells was found by western Blot. By culturing MSC in vitro and performing adipogenesis assay after 24 h of Clod-Lipo action, it was found that adipogenesis was increased in the Clod-Lipo group, while the expression of NLRP3 and Caspase-1 was increased in MSCs, and adipogenesis assay was performed after 2 h of action using Caspase-1 inhibitor, and it was found that adipocytes was reduced.

Conclusions: The results of this study suggest that MSC are biased towards adipocyte generation in response to Clod-Lipo, a process that may be associated with activation of the NLRP3/caspase-1 pathway.
© 2023, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Abbreviations: Clod-Lipo, Clodronate-Liposomes; PBS-Lipo, PBS-Liposomes; MSC, mesenchymal stem cell; HSCT, hematopoietic stem cell transplantation; HSC, hematopoietic stem cell.

* Corresponding author. The Affiliated Hospital of Xuzhou Medical University, China. Tel.: +86 51683262588.
E-mail address: zengly2000@163.com (L. Zeng).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.reth.2023.05.001
2352-3204/© 2023, The Japanese Society for Regenerative Medicine. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

HSCT is an effective treatment for hematologic malignant blood disorders [1]. The interaction of hematopoietic stem cells (HSCs) with cells and cytokines in the hematopoietic microenvironment upon entry into the organism is a key factor in the success of HSCT, and the ability of HSCs to homing and implantation directly affects hematopoietic reconstitution as well as patient prognosis. Clod-Lipo, a macrophage scavenger, mediates macrophage clearance to attenuate multiple myeloma tumor development in vivo [2], and Clod-Lipo is used clinically to treat liver fibrosis, breast cancer metastasis [3,4], and also to attenuate pulmonary ischemia-reperfusion-induced pulmonary edema [5]. It was shown that after HSCT, injection of Clod-Lipo caused severe damage to the bone marrow cavity and a large increase in adipocytes in mice [6], but the reason for the increase in adipocytes is not clear. MSC are precursor cells for adipocytes, and we suspect that Clod-Lipo biases MSC toward adipogenesis.

MSC have the ability to differentiate into three types of cells: chondrocytes, osteoblasts and adipocytes [7]. MSC maintain a certain balance of adipogenesis and osteogenesis, and studies have shown that adipogenic factors inhibit osteogenesis and similarly osteoinductive factors inhibit adipogenesis [8]. For example, PPARγ is a major inducer of adipogenesis and inhibits bone production [9]. Bone marrow adipocytes originate from MSC in the bone marrow [10], and they are thought to be negative regulators of hematopoiesis and are associated with impaired hematopoiesis [11]. Adipocytes in the bone marrow increase as the organism ages, but the function of hematopoietic stem cells decreases instead, and in fat-free mice, the implantation of hematopoietic stem cells accelerates, indicating that adipocytes are detrimental to hematopoiesis [12].

The bone marrow microenvironment is a three-dimensional environment that supports and regulates production [13]. Its structural and functional integrity is an important link to ensure normal hematopoietic function. The bone marrow hematopoietic microenvironment is composed of complex cell populations, including osteoblasts, osteoclasts and adipocytes, which all play essential regulatory and supportive roles in bone marrow hematopoiesis; various non-cytokines expressed by these cells, such as OPN, TPO, VEGF, TGF-β, contribute to the hematopoietic regulation of the hematopoietic microenvironment [14]. In our previous study, we found that various inflammatory complexes such as NLRP1, NLRP3, NLRP6 and NLRC4 were expressed in bone marrow cells after hematopoietic stem cell transplantation and that the expression of Caspase-1, IL-1β and IL-18 was increased, suggesting that NLRP3/Caspase-1 plays an important role in inflammatory bone marrow injury after transplantation [15].

NLRP3/Caspase-1 pathway is a key pathway in the inflammatory response [16], and NLRP3 inflammatory vesicle activation promotes Caspase-1 expression. NLRP3 inflammatory vesicles are composed of NLRP3, ASC, Pro-caspase-1 [17], Caspase-1 plays an important role in the control of apoptosis and cellular inflammation, where Caspase-1 is mainly involved in the inflammatory response of cells. NLRP3 inflammatory vesicles are located in the cytoplasm in an inactive form, and proper expression of NLRP3 is essential for hematopoietic stem cell transplantation, homing, and hematopoietic reconstitution [18]. It was shown that NLRP3 is involved in hematopoietic regulation, and the knockdown of NLRP3 inflammatory vesicles in mice is not conducive to hematopoiesis, but promotes apoptosis of hematopoietic stem/progenitor cells [19]. It is thus clear that proper activation of NLRP3 after HSCT facilitates hematopoietic recovery, but whether NLRP3 activation using Clod-Liposomes will further promote hematopoietic reconstitution after HSCT is not known.

We have previously shown that Clod-Lipo injection increases “fat vacuoles” in the bone marrow lumen. We examined the mRNA expression of adipose-related transcription factors in bone marrow cells and found increased expression of both PPAR-γ and C/EBPα, indicating an increase in adipocytes in the bone marrow lumen. In addition, increased expression of NLRP3 and Caspase-1 in bone marrow cells was also found, whether these phenomena were caused by Clod-Lipo needs further explanation. The aim of this paper is to show that Clod-Lipo contributes to the bias of MSCs toward adipocyte production through activation of NLPR3.

2. Materials and methods

2.1. Materials

Rabbit anti-mouse NLRP3 antibody (CatNo.15101) was purchased from Cell Signaling. Rabbit polyclonal Caspase-1 antibody (CatNo.22915-1-AP) was purchased from Proteintech. FITC-conjugated anti-mouse CD11b antibody (CatNo.101206) 、APC-conjugated anti-mouse F4/80 antibody (CatNo.123116) 、PE-conjugated anti-mouse CD90.2 antibody (CatNo.105307) and PE/Cyanine7-conjugated anti-mouse CD45 antibody (CatNo.103114) were purchased from BioLegend. FITC-conjugated anti-mouse CD29 antibody (CatNo.102221) was purchased from Invitrogen. Clodronate-Liposomes and PBS-Liposomes (CatNo.CP-005-005) were purchased from Liposoma B.V. Ac-YVAD-cmk (CatNo.178603-78-6) was purchased from SigMA.

2.2. Animals and treatment

Females C57BL/6 and males BALB/c mice, aged 6–8 weeks and weighed 20 g, were purchased from the Beijing Vital River Laboratory Animal Technology Co Ltd. The mice were housed in a specific-pathogen-free (SPF) animal Center of Xuzhou Medical University (Quan-shan, Xuzhou, China) under a stable temperature (22 °C) and humidity (60%). All animal care and procedures were approved by the Ethics Committee of Xuzhou Medical University (Xuzhou, China).

C57BL/6 female mice were used as donors, while BALB/c male mice were used as recipients. BALB/c mice were divided into three groups: BMT, BMT + Clodronate liposomes (Clod-Lipo), and BMT + PBS-liposomes (PBS-Lipo), 6 mice per group. The BALB/c recipients received a total body irradiation of 7.5 Gy at a dose rate of 1.92 Gy/min using GSR C1 gamma irradiator (Gamma-Service Medical, Bautzner, Germany), followed by infusion of 5×10^6 bone marrow mononuclear cells from the C57BL/6 donors through tail vein. The day of transplantation was day 0, and Clod-Lipo 200ul was injected through the tail vein on the first, sixth and eleventh days after transplantation. Bone marrow cell specimens were obtained at post-transplantation day 14.

2.3. H&E staining

Day 14 after transplantation, mice were sacrificed and femurs were isolated, fixed in paraformaldehyde solution for 48 h, then decalcified in 0.5 mM EDTA for two weeks, dehydrated, paraffin-embedded, and cut into 4 um slices, then stained with eosin and Hematoxylin solution. Then the film is dehydrated, transparent and sealed. The films were left to dry overnight in a fume hood and photographed and recorded under a light microscope the next day.

2.4. Western blot

On day 14 after transplantation, mice were executed and mouse bone marrow cell suspensions were obtained. Extraction of

Table 1
List of primer sequence.

Gene	Forward	Reverse
PPAR-γ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
C/EBPα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
β-actin	CTGAGAGGGAAATCGTGCGT	AACCGCTCGTTGCCAATAGT

proteins from mouse bone marrow cells or primary cultured mesenchymal stem cells. Bone marrow cells were lysed in a lysis solution consisting of RIPA and PMSF for 15 min, shaken every 5 min, on ice throughout, then centrifuged at 15,000 rpm for 15 min to remove the supernatant, and finally added 1/4 5xloading, in a metal bath for 15 min. Proteins separated on 10% SDS-PAGE, and transferred to an NC membrane. The membranes were incubated with rabbit anti-mouse NLRP3 antibody and Caspase-1 antibody overnight at 4 °C and then incubated with HRP-conjugated anti-rabbit secondary antibody for 1 h at room temperature. Membranes were visualized using enhanced chemiluminescence. GAPDH was used as a control. Protein expression was quantified using Image J software.

2.5. Real-time quantitative PCR(RT-qPCR)

Total RNA was isolated from bone marrow cells of mice at day 14 after transplantation or out of MSC cells with TRIZOL and cDNA samples were synthesized from 500 ng isolated RNA samples using Primer Script RT Reagent Kit from Takara. Expression of target genes was detected by quantitative PCR which was performed on LightCycler480 (Roche Diagnostics Ltd., Switzerland) by using Platinum SYBR Green qPCR Super Mix-UDG kit (Life Technologies, Massachusetts, USA) according to manufacturer's instructions.

β-actin was used as an internal control. PCR product specificity was confirmed by a melting-curve analysis. The relative mRNA expression was determined by using 2−ΔΔCt method. All primer sequences used in this study are listed in Table 1.

2.6. Flow cytometry

At the indicated time points, we isolated single-cell suspensions of bone marrow cells from femurs and tibias. Macrophage were incubated with CD11b and F4/80 antibodies for 30 min on ice. Then 3 mL of PBS buffer was added for termination, the supernatant was removed by centrifugation, and 100 ul of PBS buffer was added to the machine for detection.

Primary cultured MSCs were collected and incubated with CD45, CD29 and CD90.2 for 30min on ice. Then 3 mL of PBS buffer was added for termination, and the supernatant was removed by centrifugation, and 100 ul of PBS buffer was added on the machine to detect the expression of MSC markers.

2.7. Isolation, culture and identification of bone marrow mesenchymal stem cells

Normal male BALB/c mice were dislocated and executed at the cervical vertebrae, soaked in 75% alcohol for 5 min, and the tibia and femur bones were aseptically removed, crushed using a mortar and pestle, and rinsed in PBS to obtain bone marrow cell suspension. After centrifugation, the bone marrow mononuclear cells were cultured in BALB/c mouse mesenchymal stem cell complete medium containing 10% FBS and 1% glutamine. Incubation in an incubator with 5% CO2, after 72 h, replace with new MSC complete medium. On the fifth day, when the primary cells reach 80%–90% fusion, use trypsin digestion for 2 min and observe the cells

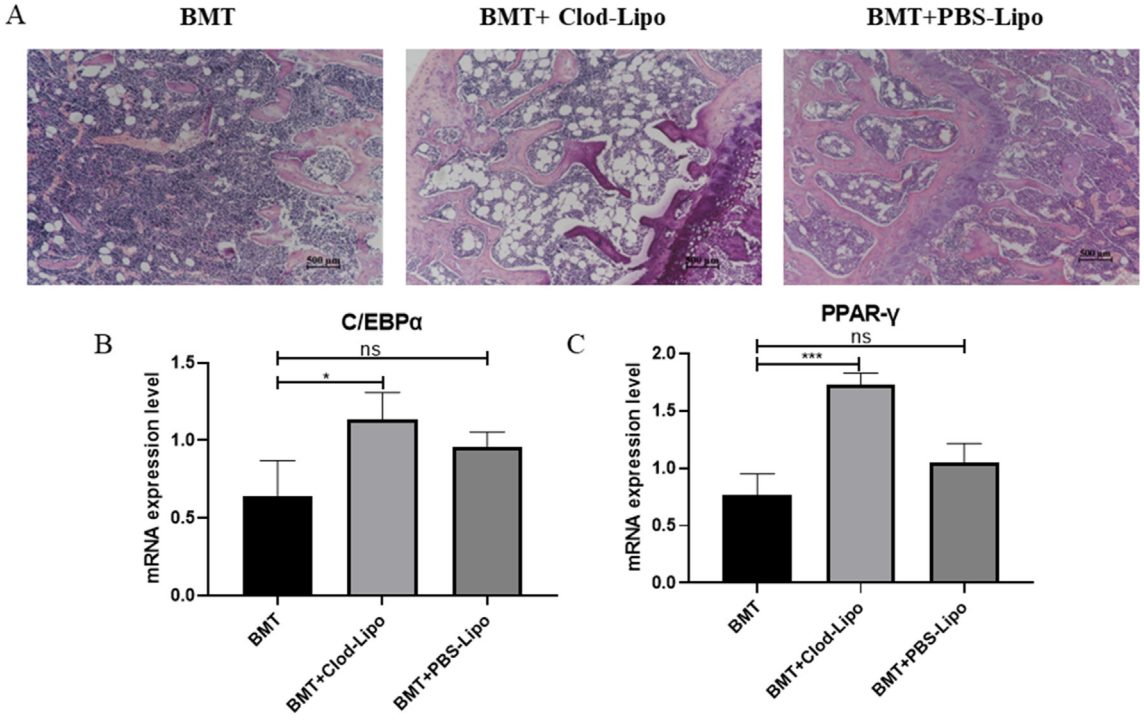

Fig. 1. The effect of Clodronate-Liposomes on the bone marrow lumen of mice after HSCT. (A) H&E staining of bone marrow in Clodronate-Liposomes and PBS-liposomes treated mice 14days after HSCT (magnification, x200). PPAR-γ and C/EBPα mRNA level in Bone marrow cells were analyzed by RT-qPCR in Clodronate-Liposomes and PBS- Liposomes treated mice at 14 days after HSCT, mice that were not treated after transplantation served as the control group. Values represent mean ± SD of at least 3 mice of each group. (B) Gene expression of C/EBPα; Gene expression of PPAR-γ (C); HSCT: hematopoietic stem cell transplantation; *P < 0.05 (n = 3) and **P < 0.01 compared with BMT; NS, not significant.

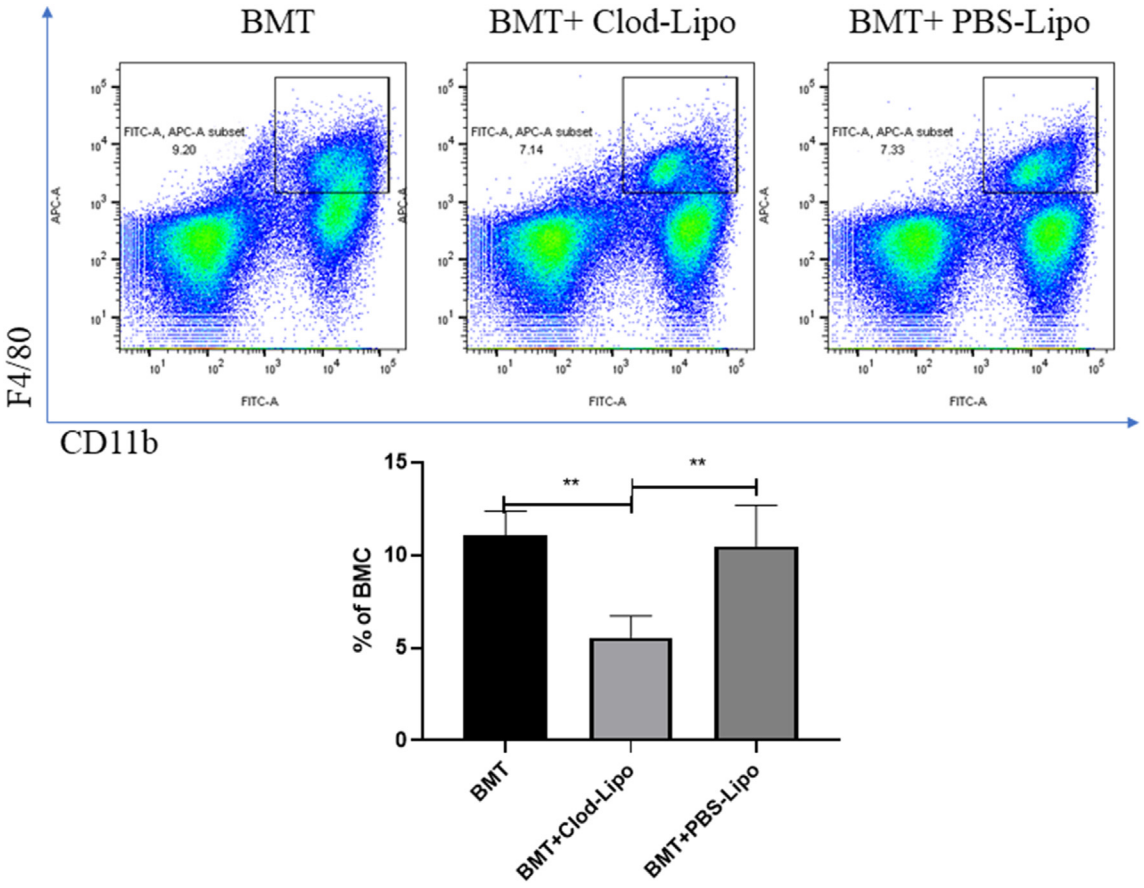

Fig. 2. The effect of Clodronate-Liposomes on the bone marrow macrophage. The percentage of macrophages in the bone marrow cells of clodronate-liposome and PBS-liposome-treated mice was analyzed by flow cytometry on day 14 after hematopoietic stem cell transplantation.

rounding under the microscope, add 3 times the volume of fresh medium for termination, follow 1:3 passages for culture, and after passing to the fourth generation, collect the cells to mark cell surface markers for 30 min and identify them by flow cytometry.

2.8. Oil red staining of bone marrow mesenchymal stem cells after induction of adipogenesis

Cells of the fourth generation were spread on six-well plates with 4×10^5 cells per well, and when cell fusion reached 80%–90%, Clod-Lipo and PBS-Lipo were added for induction for 24 h. The control group was left untreated. After 24 h, all cells were replaced with BALB/c mouse complete adipogenic differentiation medium induction solution A and after 3 days with maintenance solution B. After repeated induction for 3–5 times, the culture medium was discarded and fixed with 4% paraformaldehyde at room temperature for 15 min under an inverted microscope according to the cell status and the size of lipid droplet formation, rinsed twice in PBS buffer, and then stained with Oil Red O for 30 min to observe the adipogenesis under the microscope. The area of oil-red staining was semi-quantitatively analyzed after photographing using IMAGE J software.

2.9. Use of Caspase-1 inhibitors

Fourth-generation cells cultured in vitro were incubated with medium containing Ac-YV for 2 h. The control group added the same amount of DMSO to the medium. 2 h later, all MSC were re-cultured with medium containing Clod-Lipo for 24 h. Adipogenesis experiments were then performed following the same steps as described above.

2.10. Statistical analyses

Data were analyzed by GraphPad Prism software (version 8.0) and represented as mean ± SD. One-way ANOVA followed by Newman–Keuls multiple comparisons was used for the comparison of one parameter in different groups. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Clodronate liposomes increased adipocytes in the bone marrow lumen and decreased the proportion of macrophages in the bone marrow

Our previous study found that Clod-Lipo injection after HSCT caused structural disorders, inflammatory cell infiltration and increased adipose vacuoles (Fig. 1A) in the bone marrow lumen of mice, suggesting that Clod-Lipo may be associated with increased adipocytes. The mechanism of adipocyte production in the bone marrow cavity is not clear. We first examined the mRNA expression of two transcription factors, PPAR-γ and C/EBPα, in bone marrow cells and found that C/EBPα (Fig. 1B) and PPAR-γ (Fig. 1C) expression was increased in the BMT + Clod-Lipo group compared to the BMT and BMT + PBS-Lipo groups, and PPAR-γ was increased more significantly. PPAR-γ and C/EBPα are key regulators of adipocyte production, and the increased

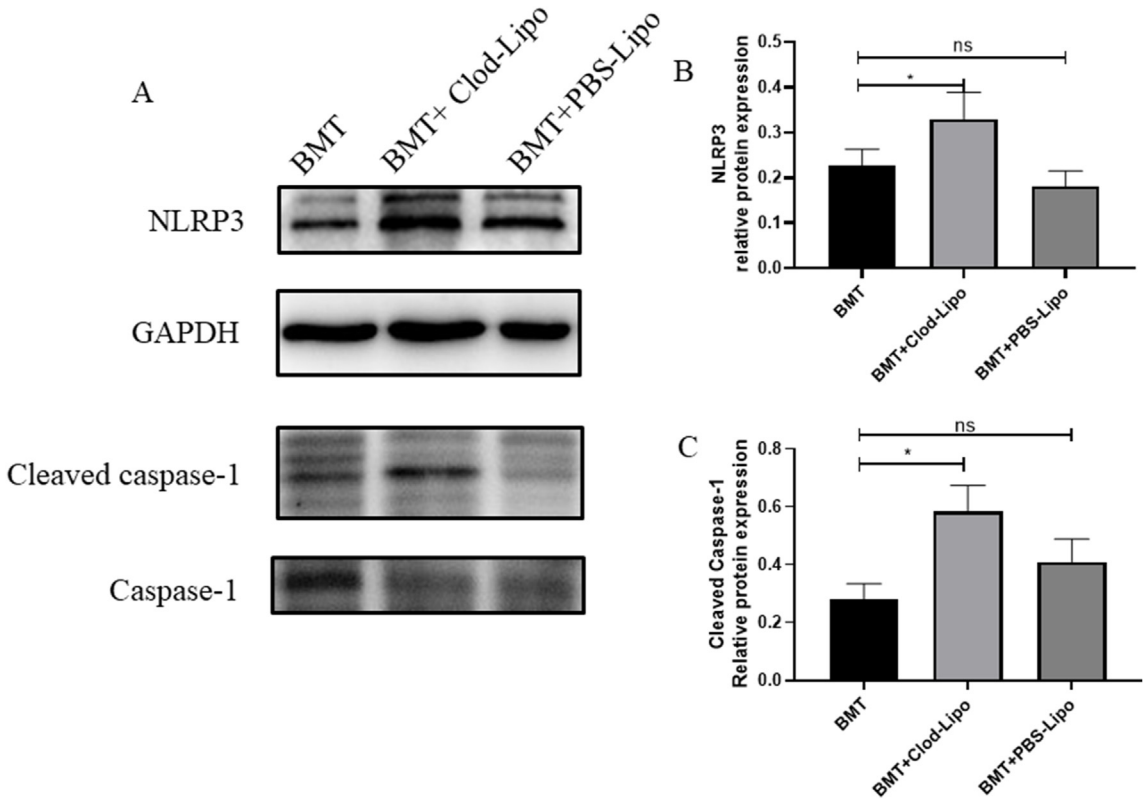

Fig. 3. The effect of Clodronate-Liposomes on NLRP3 and Cleaved Caspase-1 in Bone marrow Cells. (A) Bone marrow cells were isolated for analysis of NLRP3 and Cleaved Caspase-1 expression by Western blot in Clodronate-Liposomes and PBS- Liposomes treated mice at 14 days after HSCT, mice that were not treated after transplantation served as the control group. NLRP3 protein expression quantification (B) and Cleaved Caspase-1 protein expression quantification(C). Compared with BMT, *P < 0.05 (n = 3) and **P < 0.01, NS, not significant.

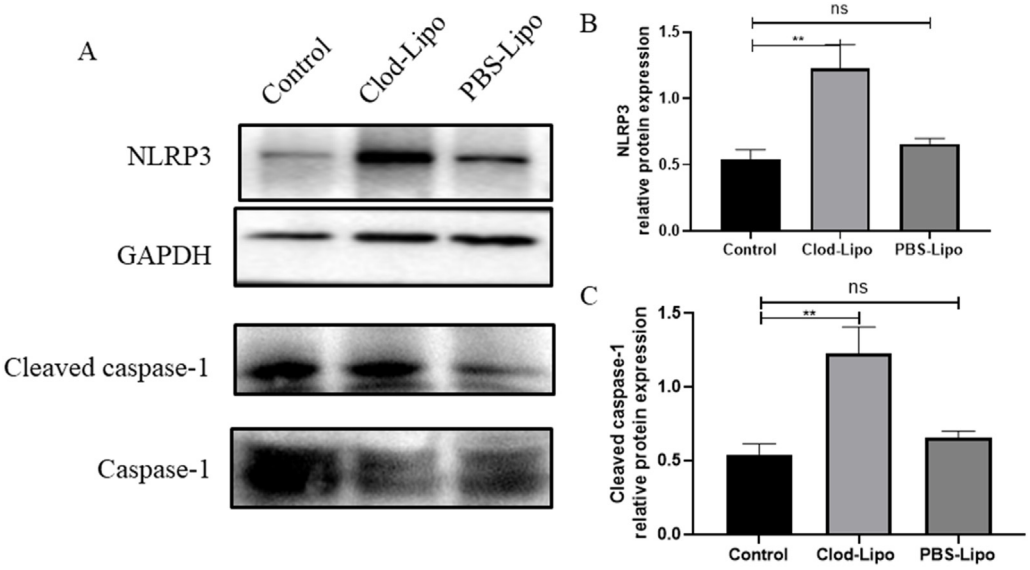

Fig. 4. The effect of Clodronate-Liposomes on NLRP3 and Cleaved Caspase-1 in Mesenchymal Stem Cells Cultured in Vitro. (A) In vitro cultured to fourth generation MSC were collected after treatment with Clodronate-Liposomes and PBS-Liposomes for 24 h. NLRP3 and Cleaved Caspase-1 protein expression levels were analyzed by Western blot. NLRP3 protein expression quantification (B) and Cleaved Caspase-1 protein expression quantification(C). Compared with control, *P < 0.05 (n = 3) and **P < 0.01, NS, not significant.

mRNA levels are further evidence of increased adipocytes in the bone marrow lumen. The percentage of macrophages was found to be lower in the BMT + Clod-Lipo group compared to the BMT and BMT + PBS-Lipo groups by flow cytometry (Fig. 2).

3.2. Clodronate liposomes activate the NLRP3/caspase-1 pathway

Bone marrow cells of mice were collected after HSCT, and increased expression levels of inflammatory pathway-related proteins NLRP3 and Cleaved Caspase-1 were found by Western

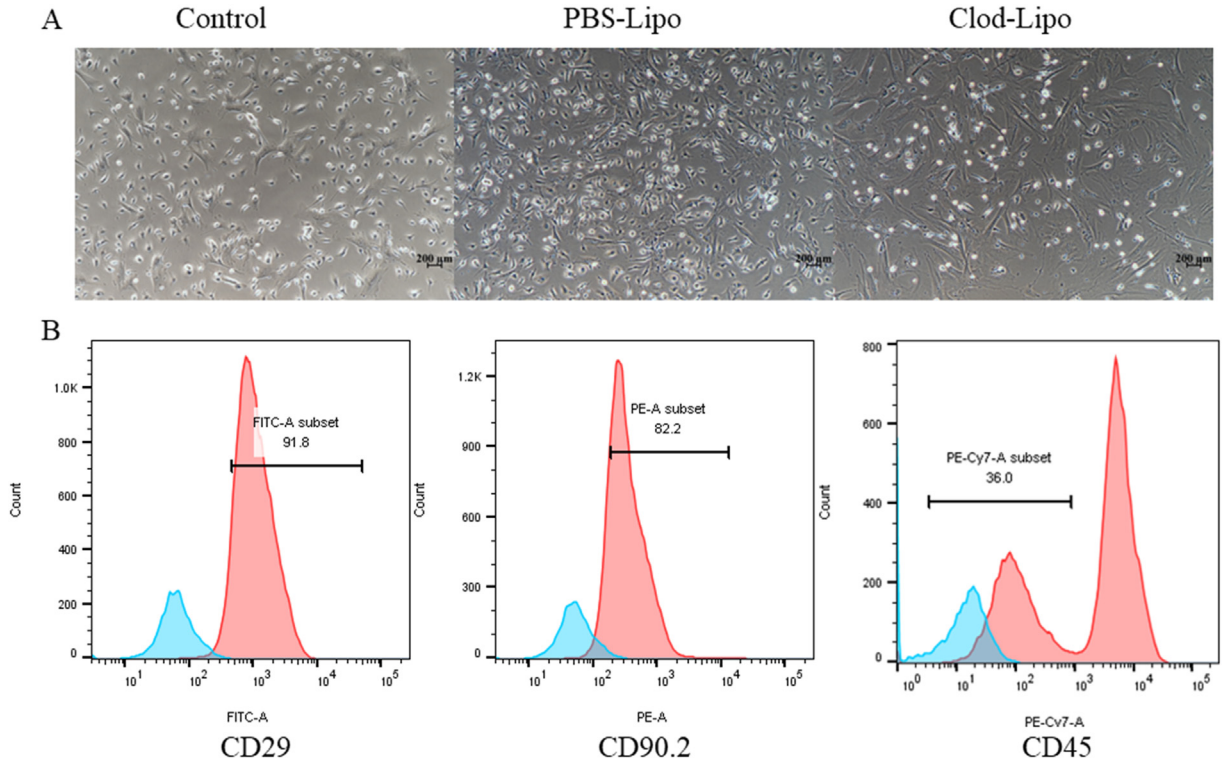

Fig. 5. Morphology and identification of mesenchymal stem cells. (A) (left) Mesenchymal stem cells cultured in vitro up to the fourth generation (middle) mesenchymal stem cells cultured in vitro to the fourth generation after treatment with Clodronate-Liposomes or (right) mesenchymal stem cells cultured in vitro to the fourth generation after treatment with PBS-Liposomes (magnification, x200) (B) Cell surface markers of mouse mesenchymal stem cells.

blot (Fig. 3A). Meanwhile, we collected Clod-Lipo-treated cells and detected increased expression of NLRP3 and Cleaved Caspase-1 proteins, indicating that Clod-Lipo activated the NLRP3/Caspase-1 inflammatory pathway (Fig. 4A). Three independent experiments were performed, and each time the target protein was compared to the internal reference protein for quantitative analysis, and the results obtained in a histogram for statistical analysis (Fig. 3B–C, Fig. 4B–C). Bone marrow inflammation is triggered after HSCT, NLRP3 is an important pathway of inflammatory injury, and NLRP3 inflammatory vesicle activation promotes Caspase-1 expression. It indicates that severe inflammation may occur on day 14, which is consistent with the pathological analysis of severe bone marrow severity damage on day 14.

3.3. Morphology and identification of mouse mesenchymal stem cells in primary culture in vitro

MSC are the precursor cells of adipocytes, and to explore the mechanism of adipocyte origin, we examined the effect of Clod-Lipo on adipogenesis of MSC. We isolated primary MSCs from mice for passaging culture, and when MSC were passed to the fourth generation, the morphology of the cells was homogeneous with a typical long shuttle shape. After 24 h of Clod-Lipo treatment, the cells became longer and grew in a “pine needle-like” elongated manner. In contrast, there was no significant change in the cells after the addition of PBS-Lipo (Fig. 5A). We collected fourth generation MSCs and identified them by flow cytometry and found that MSC surface markers showed high expression of CD29, CD90.2 and low expression of CD45 (Fig. 5B).

3.4. The effect of clodronate-liposomes on primary cultured mesenchymal stem cells

To investigate the effect of Clod-Lipo on primary cultured MSCs, Clod-Lipo or PBS-Lipo was added when the cells reached 90% growth, and after 24 h of action, the cells were collected for flow assay. It was found that the addition of Clod-Lipo increased the percentage of CD45-negative, indicating that Clod resulted in a higher purity of primary cultured MSCs (Fig. 6). Consistent with the results of related literature. The differences were statistically significant.

3.5. Clodronate-liposomes induce bias of in vitro cultured mesenchymal stem cells toward adipocytes

To investigate the effect of Clod-Lipo on adipogenesis of MSC, we plated cells cultured up to the fourth generation. When cell fusion reached more than 90%, we changed the medium containing Clod-Lipo to act for 24 h. After 24 h, the medium was replaced with a special medium for the induction of adipogenesis in mice, and after several inductions, oil red O staining was performed when the lipid droplets reached a certain level. It was found that there were more red lipid droplets after the addition of Clod-Lipo effect compared to the other two groups (Fig. 7A). This phenomenon was also demonstrated by semi-quantitative analysis using IMAGE J software (Fig. 7B). In addition, the expression of C/EBPα (Fig. 7C) and PPAR-γ (Fig. 7D) was found to be increased in the Clod-Lipo group compared with the Control and PBS-Lipo groups by RT-qpcr.

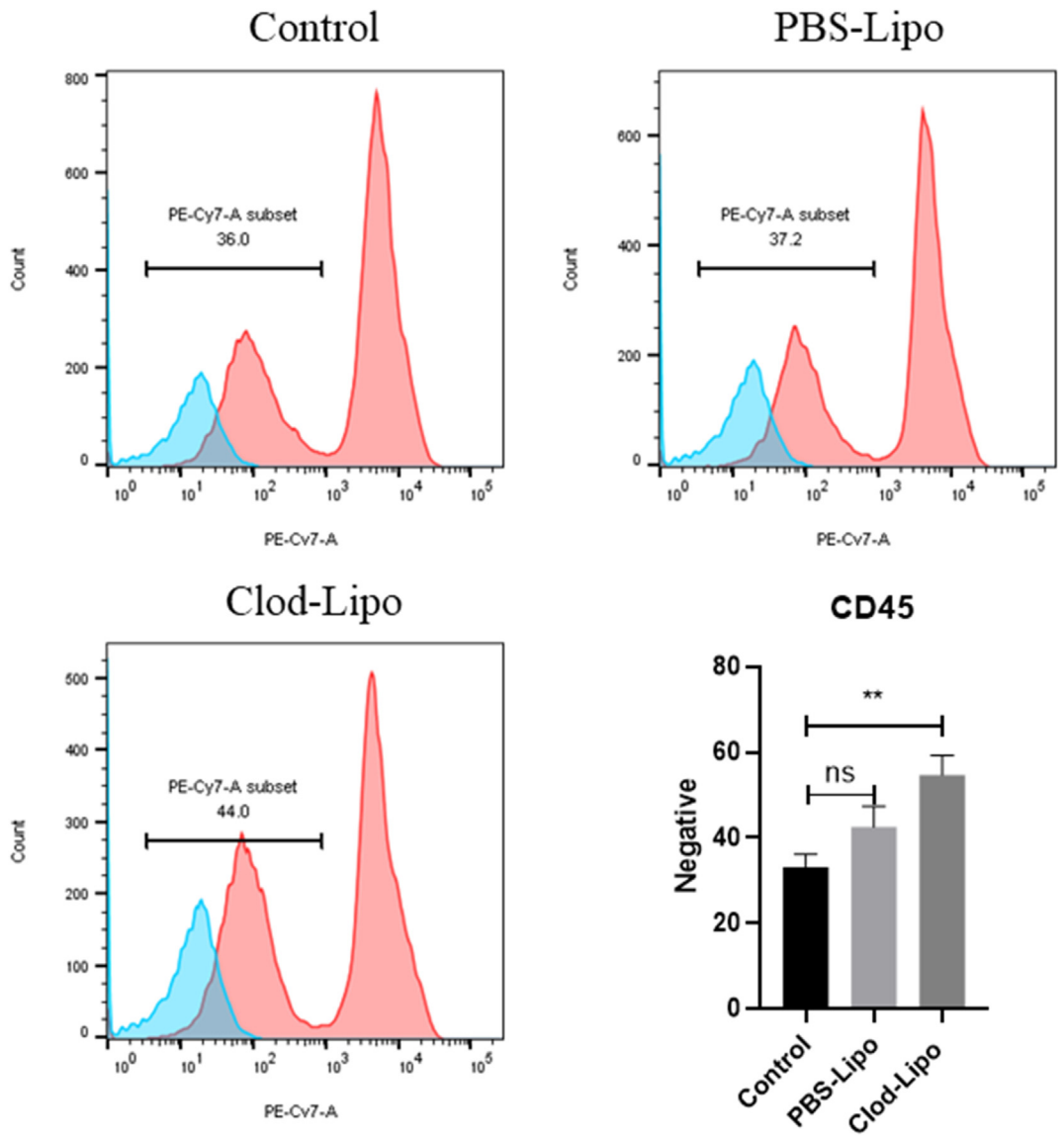

Fig. 6. The effect of Clodronate-liposomes on primary cultured mesenchymal stem cells. CD45 expression levels in different groups and statistical analysis. N = 3, three independent, *P < 0.05, NS, not significant.

3.6. Caspase-1 inhibitors are detrimental to in vitro cultured mesenchymal stem cell to adipocyte formation

To demonstrate that Clod-Lipo can induce MSCs to be biased towards adipocyte production, this process may be related to Clod-Lipo activation of NLRP3. We inhibited Caspase-1 using Ac-YVAD-cmk before the action of Clod-Lipo, blocking this pathway of NLRP3/Caspase-1. The results showed that blocking the pathway, followed by Clod-Lipo action followed by adipogenesis experiments, did not result in increased adipocyte production (Fig. 8A and B). Also, the expression of C/EBPα (Fig. 8C) and PPAR-γ (Fig. 8D) was found to be decreased in the Ac-Yv group compared to the control group by RT-qpcr.

4. Discussion

Studies have shown that Clod-Lipo injection after HSCT leads to a significant increase in adipocytes in the bone marrow cavity of mice, but the source of adipocytes and the mechanisms of adipocyte formation have not been extensively studied. It is known that HSCT is a potential treatment for certain malignant and non-malignant diseases [20]. The bone marrow tissue of mice after HSCT was severely damaged, and mice were subjected to gamma irradiation for marrow clearance before transplantation, and mice transplanted after ionizing radiation showed a decrease in bone marrow cells due to the toxic effects of irradiation [21].

The findings revealed that Clod-Lipo injection after HSCT caused a significant increase in adipose vacuoles in the bone marrow lumen of mice and an increased mRNA expression of PPARγ and C/EBPα, key transcription factors for adiposity in whole bone marrow cells, and these data suggest that Clod-Lipo injection after HSCT promotes adipocytosis in the bone marrow lumen. There is no clear explanation for the promotion of adipocytosis. Considering the inflammation occurring after transplantation, we examined the expression of NLRP3/Caspase-1 inflammatory signaling pathway and found that NLRP3 and activated Caspase-1 expression was increased in the Clod-Lipo group. We speculate that the increase in adipocytes may be related to the activation of the NLRP3/Caspase-1 pathway. Post-

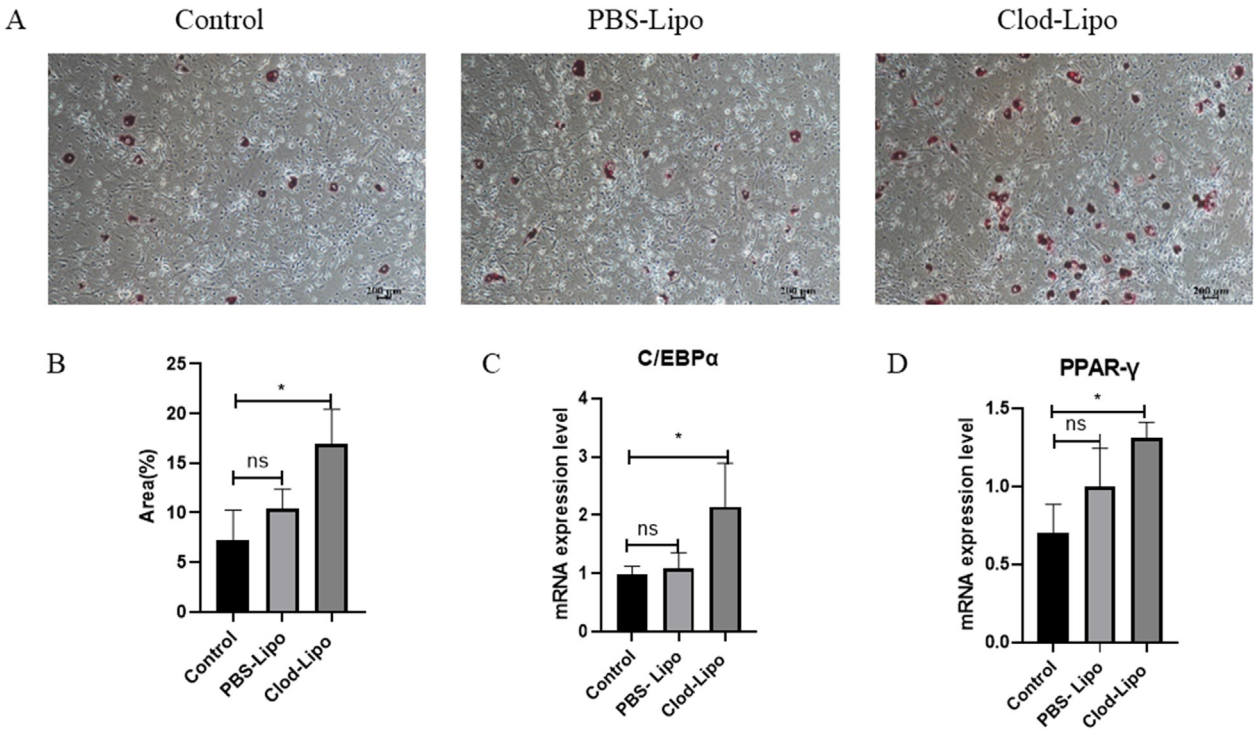

Fig. 7. The effect of Clodronate-Liposomes on the induction of adipogenesis in mesenchymal stem cells cultured in vitro. (A) Oil red O staining of the fourth generation MSC after adipogenic induction. Left: Oil Red O staining of fourth generation MSC without any treatment. Middle: Oil Red O staining of fourth generation MSC treated with Clodronate-Liposomes for 24 h. Right: Oil Red O staining of fourth generation MSC treated with PBS-Liposomes for 24 h (B) Semi-quantitative analysis using IMAGE J software. (C) Gene expression of C/EBPα; Gene expression of PPAR-γ (D). N = 3, three independent, *P < 0.05, NS, not significant.

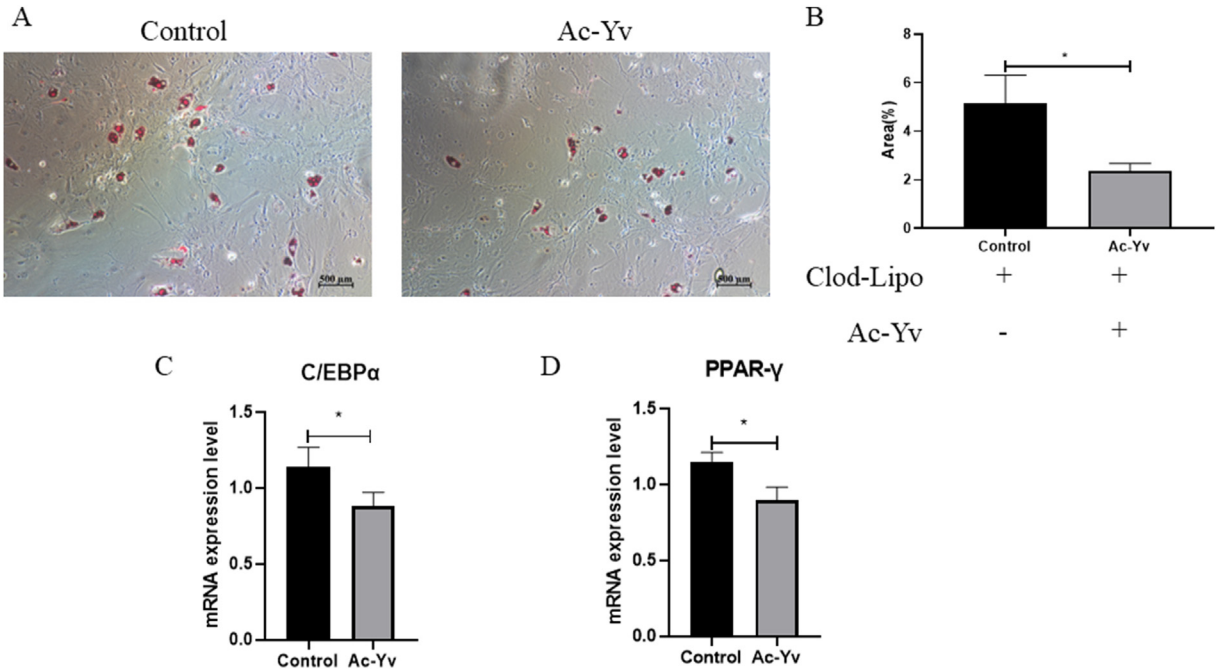

Fig. 8. The effect of Caspase-1 inhibitors on the induction of adipogenesis in mesenchymal stem cells cultured in vitro. (A) Oil Red O staining of fourth-generation mesenchymal stem cells treated with Caspase-1 inhibitor Ac-YVAD-cmk for 2 h, followed by Clod-Liposomes for 24 h. Left: Oil Red O staining of fourth generation mesenchymal stem cells after 24 h of treatment with Clod-Liposomes only. Right: Oil Red O staining of fourth generation MSCs after treatment with Ac-YVAD-cmk for 2 h and then Clod-Liposomes for 24 h (B) Semi-quantitative analysis using IMAGE J software. (C) Gene expression of C/EBPα; Gene expression of PPAR-γ (D). N = 3, three independent, *P < 0.05, NS, not significant.

transplant recipients are prone to complications, and NLRP3 activation plays a key role in the development of transplant complications [22]. Inflammasomes are large multimeric protein complexes that mediate rapid immune responses to pathogen infection and tissue injury and are composed of NLRP1, NLRP2, NLRP3, NLRP6 and NLRC4, with NLRP3 being one of the most studied inflammasomes in the field of immunometabolism [23]. The NLRP3/Caspase-1 pathway is a key pathway in the inflammatory response that plays a role in development, and inflammation is a defense mechanism, but excessive activation of inflammation has deleterious effects on hematopoietic stem cells, leading to bone marrow failure [24]. Uncontrolled inflammation can also exacerbate tissue and organ damage [25].

MSC are precursors of adipocytes, an important component of the bone marrow microenvironment, and play an important role in supporting hematopoiesis [26]. MSC therapy prevents and treats graft-versus-host disease that occurs in hematopoietic stem cell transplantation, while facilitating the implantation of hematopoietic stem cells [27]. Bone marrow adipose tissue is a unique adipose tissue and adipocytes have a complex role in hematopoiesis in the bone marrow microenvironment and are considered as “space fillers” for bone marrow cells with negative hematopoietic functions [28]. Our study found that the adipocytes in the bone marrow cavity were significantly increased by Clod-Lipo injection after HSCT, and we suspected that the increase of adipocytes in the bone marrow cavity of mice was related to MSC. The addition of Clod to primary MSC culture results in selective phagocytosis by macrophages in heterogeneous cell populations, resulting in effective removal of macrophages without affecting MSC and increasing MSC purity [29]. By culturing primary MSC in vitro followed by passaging, the fourth generation cells were identified as MSC by surface markers, and lipogenesis assay was performed after Clod-Lipo action, and the increased formation of lipid droplets was found by Oil Red O staining, which indicated that Clod-Lipo would induce MSC to bias towards adipogenesis.

To demonstrate that this phenomenon is associated with the NLRP3/Caspase-1 pathway, we collected MSC after Clod-Lipo action and detected protein expression by Western blot, and found increased expression of NLRP3 and activated caspase-1. We inhibited caspase-1 with Ac-YV and then induced adipogenesis with Clod-Lipo and found that MSC adipogenesis was indeed reduced compared to the group without the inhibitor, suggesting that Clod-Lipo prompted MSC to favor adipogenesis possibly associated with activation of the NLRP3/Caspase-1 pathway.

The limitation of this study is the lack of in vivo experimental validation of the NLRP3/Caspase-1 pathway in mice and the lack of applying the inhibitor to mice or directly using knockout mice to observe the changes of damage in the bone marrow cavity of mice. We plan to conduct the next study at a later stage.

5. Conclusion

The present results indicate that MSC are biased towards adipocyte generation in response to Clod-Lipo, a process that may be associated with activation of the NLRP3/caspase-1 pathway.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement.

This research was supported by National Youth Natural Science Foundation of China (grant no.81900106), and postgraduate research and practice innovation in Jiangsu Province (KYCX21-2738).

References

[1] Apperley J, Niederwieser D, Huang XJ, Nagler A, Fuchs E, Szer J, et al. Haploidentical hematopoietic stem cell transplantation: a global overview comparing asia, the European union, and the United States. Biol Blood Marrow Transplant 2016;22(1):23–6.
[2] Opperman KS, Vandyke K, Clark KC, Coulter EA, Hewett DR, Mrozik KM, et al. Clodronate-liposome mediated macrophage depletion abrogates multiple myeloma tumor establishment in vivo. Neoplasia 2019;21(8):777–87.
[3] Han J, Zhang X, Lau JK, Fu K, Lau HC, Xu W, et al. Bone marrow-derived macrophage contributes to fibrosing steatohepatitis through activating hepatic stellate cells. J Pathol 2019;248(4):488–500.
[4] Tacconi C, Commerford CD, Dieterich LC, Schwager S, He Y, Ikenberg K, et al. CD169(+) lymph node macrophages have protective functions in mouse breast cancer metastasis. Cell Rep 2021;35(2):108993.
[5] Zhou P, Guo H, Li Y, Liu Q, Qiao X, Lu Y, et al. Monocytes promote pyroptosis of endothelial cells during lung ischemia-reperfusion via IL-1R/NF-kappaB/NLRP3 signaling. Life Sci 2021;276:119402.
[6] Qiao J, Liu L, Xia Y, Ju W, Zhao P, Jiang Y, et al. Macrophages ameliorate bone marrow inflammatory injury and promote hematopoiesis in mice following hematopoietic stem cell transplantation. Exp Ther Med 2018;16(2):567–72.
[7] Andrzejewska A, Lukomska B, Janowski M. Concise review: mesenchymal stem cells: from roots to boost. Stem Cell 2019;37(7):855–64.
[8] Atashi F, Modarressi A, Pepper MS. The role of reactive oxygen species in mesenchymal stem cell adipogenic and osteogenic differentiation: a review. Stem Cell Dev 2015;24(10):1150–63.
[9] Li Y, Jin D, Xie W, Wen L, Chen W, Xu J, et al. PPAR-Gamma and wnt regulate the differentiation of MSCs into adipocytes and osteoblasts respectively. Curr Stem Cell Res Ther 2018;13(3):185–92.
[10] Sebo ZL, Rendina-Ruedy E, Ables GP, Lindskog DM, Rodeheffer MS, Fazeli PK, et al. Bone marrow adiposity: basic and clinical implications. Endocr Rev 2019;40(5):1187–206.
[11] Shin E, Koo JS. The role of adipokines and bone marrow adipocytes in breast cancer bone metastasis. Int J Mol Sci 2020;21(14).
[12] Yu VW, Scadden DT. Hematopoietic stem cell and its bone marrow niche. Curr Top Dev Biol 2016;118:21–44.
[13] Johnson CB, Zhang J, Lucas D. The role of the bone marrow microenvironment in the response to infection. Front Immunol 2020;11:585402.
[14] Sendker S, Waack K, Reinhardt D. Far from health: the bone marrow microenvironment in AML, A leukemia supportive shelter. Children 2021;8(5).
[15] Hong F, Chen Y, Gao H, Shi J, Lu W, Ju W, et al. NLRP1 in bone marrow microenvironment controls hematopoietic reconstitution after transplantation. Transplant Cell Ther 2021;27(11):908 e901–e908 e911.
[16] Tao J, Xia LZ, Liang L, Chen Y, Wei D, Meng J, et al. MiR-124-3p promotes trophoblast cell HTR-8/SVneo pyroptosis by targeting placental growth factor. Placenta 2020;101:176–84.
[17] Zhu S, Shi P, Lv C, Li H, Pan B, Chen W, et al. Loss of NLRP3 function alleviates murine hepatic graft-versus-host disease. Biol Blood Marrow Transplant 2018;24(12):2409–17.
[18] Adamiak M, Abdel-Latif A, Bujko K, Thapa A, Anusz K, Tracz M, et al. Nlrp3 inflammasome signaling regulates the homing and engraftment of hematopoietic stem cells (HSPCs) by enhancing incorporation of CXCR4 receptor into membrane lipid rafts. Stem Cell Rev Rep 2020;16(5):954–67.
[19] Zhang J, Chen Y, Yin D, Feng F, An Q, Liu Z, et al. Caspase-3/NLRP3 signaling in the mesenchymal stromal niche regulates myeloid-biased hematopoiesis. Stem Cell Res Ther 2021;12(1):579.
[20] McDaniel Mims B, Enriquez J, Pires Dos Santos A, Jones-Hall Y, Dowd S, Furr KL, et al. Antibiotic administration exacerbates acute graft vs. host disease-induced bone marrow and spleen damage in lymphopenic mice. PLoS One 2021;16(8):e0254845.
[21] Batey K, Kim J, Brinster L, Gonzalez-Matias G, Wu Z, Solorzano S, et al. Residual effects of busulfan and irradiation on murine hematopoietic stem and progenitor cells. Exp Hematol 2022;105:22–31.
[22] Espinoza JL, Kamio K, Lam VQ, Takami A. The impact of NLRP3 activation on hematopoietic stem cell transplantation. Int J Mol Sci 2021;22(21).
[23] Unamuno X, Gomez-Ambrosi J, Ramirez B, Rodriguez A, Becerril S, Valenti V, et al. NLRP3 inflammasome blockade reduces adipose tissue inflammation and extracellular matrix remodeling. Cell Mol Immunol 2021;18(4):1045–57.

[24] Leimkühler NB, Schneider RK. Inflammatory bone marrow microenvironment. Hematol Am Soc Hematol Educ Program 2019-12-06;2019(1):294–302.
[25] Shin JN, Rao L, Sha Y, Abdel Fattah E, Hyser J, Eissa NT. p38 MAPK activity is required to prevent hyperactivation of NLRP3 inflammasome. J Immunol 2021;207(2):661–70.
[26] Xia C, Wang T, Cheng H, Dong Y, Weng Q, Sun G, et al. Mesenchymal stem cells suppress leukemia via macrophage-mediated functional restoration of bone marrow microenvironment. Leukemia 2020;34(9):2375–83.
[27] Batsali AK, Georgopoulou A, Mavroudi I, Matheakakis A, Pontikoglou CG, Papadaki HA. The role of bone marrow mesenchymal stem cell derived extracellular vesicles (MSC-EVs) in normal and abnormal hematopoiesis and their therapeutic potential. J Clin Med 2020;9(3).
[28] Hawkes CP, Mostoufi-Moab S. Fat-bone interaction within the bone marrow milieu: impact on hematopoiesis and systemic energy metabolism. Bone 2019;119:57–64.
[29] Song JH, Kim JW, Lee MN, Oh SH, Piao X, Wang Z, et al. Isolation of high purity mouse mesenchymal stem cells through depleting macrophages using liposomal clodronate. Tissue Eng Regen Med 2022;19(3):565–75.