

Original Research

Inhibition of Galectin-3 Expression Could Preserve the Immunoregulatory Function of Mesenchymal Stem Cells During Cell Passaging

Minghui Wang^{1,†}, Yan Jiang^{1,†}, Jiangbo Wan¹, Difan Zhang¹, Fang Huang^{1,*}, Siguo Hao^{1,*}

¹Department of Hematology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 200090 Shanghai, China

*Correspondence: huangfang335@163.com (Fang Huang); haosiguo@xinhumed.com.cn (Siguo Hao)

†These authors contributed equally.

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Abstract

Background: Mesenchymal stem cells (MSCs) are one of the effective treatments for acute graft-versus-host disease (aGVHD) following allogeneic hematopoietic stem cell transplantation (allo-HSCT) due to their potent immunoregulatory function. Given the limited quantity and high heterogeneity of freshly isolated MSCs, extensive *in vitro* expansion is essential for clinical application. Prolonged passaging of MSCs leads to a decline in therapeutic efficacy, with the underlying mechanisms remaining unclear. This study aimed to explore the mechanism and intervention strategies of immunoregulatory dysfunction of MSCs during passaging. **Methods:** We compared the therapeutic effects of MSCs at early passages (passage 6, P6-MSCs) and later passages (passage 12, P12-MSCs) in a mouse GVHD model. We also analyzed the expression of Gal-3 in MSCs at different passages and its role in macrophage polarization. Additionally, the selective Gal-3 inhibitor TD139 was evaluated for its effects on Gal-3 expression and the immunoregulatory function of MSCs. **Results:** Our data showed an inverse correlation between passage number and therapeutic efficacy in MSCs. Late-passage MSCs (P12) exhibited significantly reduced efficacy in alleviating GVHD compared to early-passage MSCs (P6). The expression of Gal-3 was markedly upregulated in late-passage MSCs (P12), and it was found to directly inhibit anti-inflammatory M2-like macrophage polarization. Our research demonstrated that TD139 dose-dependently suppresses Gal-3 expression in MSCs and restores their immunoregulatory function. **Conclusion:** Gal-3 contributes to the decline in the immunomodulatory capabilities of MSCs. TD139, a Gal-3 inhibitor, has a potentially positive effect on rescuing the immunoregulation dysfunction of MSCs in late-passage and may represent a novel strategy to enhance the therapeutic potential of late-passage MSCs for GVHD treatment.

Keywords: mesenchymal stem cells; galectin-3; GB-0139 (TD139); graft vs host disease

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is widely utilized for the treatment of various hematologic malignancies, demonstrating significant immunotherapeutic potential [1]. However, it is constrained by severe graft-versus-host-disease (GVHD), which is a life-threatening complication [2]. The conditioning regimen in allo-HSCT activates antigen-presenting cells (APCs)—predominantly macrophages and dendritic cells—that present host antigens to donor T cells and initiate a robust cytokine response. Effector T cells, macrophages and pro-inflammatory cytokines target skin, lungs, gut and liver, leading to organ damage, which is a clinical manifestation of aGVHD [3].

Mesenchymal stem cells (MSCs) represent a unique population of multipotent precursor cells, defined by their capacities for self-renewal and differentiation into a range of cell lineages, including chondrocytes, osteoblasts, adipocytes, cardiomyocytes, and neurons [4,5]. Furthermore, MSCs exhibit potent immunoregulatory properties,

modulating both innate and adaptive immune responses. This immunoregulatory effect is primarily induced by inflammatory cytokines within the inflammatory microenvironment [6,7]. MSCs have been demonstrated to facilitate the shift of macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, while simultaneously inhibiting macrophage infiltration at inflammatory sites [8]. Additionally, MSCs directly inhibit the activation of cytotoxic CD8⁺ T cells and the differentiation of T helper 1 (Th1) and Th17 cells [9]. Simultaneously, they facilitate the differentiation and activity of CD4⁺CD25⁺FOXP3⁺ Treg cells [10,11]. This combined capacity for self-renewal and multilineage differentiation, coupled with their immunoregulatory function, underscores the potential of MSCs as a viable therapeutic modality.

MSCs are isolatable from a range of tissue sources, including bone marrow, adipose tissue, umbilical cord, and peripheral blood [12,13]. Given the limited abundance of MSCs in these tissues, extensive *ex vivo* expansion is required to achieve the sufficient cell numbers necessary for



in vivo application [14]. However, multiple studies have shown that MSCs may undergo a decline in their immunosuppressive and regenerative capacities after extended passages [15–18].

Galectin-3 (Gal-3, gene name: *Lgals3*), a β -galactoside-binding lectin, is widely expressed in various tissues and by most cell types, emerging as a crucial factor in processes such as cell proliferation, apoptosis, inflammation, and fibrosis. It exerts regulatory effects on both chronic and acute inflammatory conditions across different tissues [19,20]. Several investigations have revealed that Gal-3 exerts a notable influence on pro-inflammatory processes by inhibiting M2 macrophage polarization [21–23]. Furthermore, downregulation of Gal-3 has been shown to attenuate inflammation [24]. It has been shown that Gal-3 suppresses T cell activation by inhibitory receptors that negatively regulate T cell activation and responses [25].

Selective galectin inhibitors (TAZTDG, TD139, GB0139) modulate multiple pathophysiological processes, demonstrating significant potential as therapeutic candidates [26]. Among them, TD139 is a small-molecule inhibitor that selectively targets Gal3 by disrupting its β -galactoside-binding activity [27]. This compound has demonstrated efficacy in preclinical models of fibrosis and inflammation and is currently being evaluated in Phase II clinical trials for idiopathic pulmonary fibrosis and platelet activation [28–30].

Macrophages constitute a vital element of the innate immune system and mediate critical functions in both inflammatory responses and host defense [31]. In response to differing microenvironments, macrophages adopt distinct functional phenotypes: classically activated macrophages (M1-like) and alternatively activated macrophages (M2-like) [32]. M1-like macrophages are activated by lipopolysaccharide (LPS), resulting in the release of pro-inflammatory cytokines. These macrophages primarily mediate inflammatory responses and contribute to tissue damage. In contrast, M2-like macrophages are activated by interleukin-4 (IL-4), triggering the secretion of anti-inflammatory cytokines. These macrophages predominantly participate in anti-inflammatory responses and tissue repair [33].

The precise mechanism underlying the decline in the immunoregulatory effects of MSCs after multiple passages remains to be elucidated and constituted the primary focus of this study. Our results demonstrated that the effect of late-passage MSCs on M2-like macrophage polarization was diminished both *in vivo* and *in vitro*. Additionally, Galectin-3 (Gal-3) was significantly upregulated in late-passage MSCs compared to early-passage MSCs. Furthermore, Gal-3 directly inhibited M2-like macrophage polarization. Therefore, we hypothesized that the attenuation of the immunoregulatory function of MSCs was attributed to the overexpression of Gal-3 in late-passage MSCs. Notably, TD139, a selective Gal-3 inhibitor, dose-dependently

suppressed Gal-3 expression in MSCs, particularly in late-passage MSCs. More importantly, TD139 enhances the immunoregulatory potential of late-passage MSCs by promoting M2 macrophage polarization. Based on these findings, the combination of TD139 and MSCs may represent a novel therapeutic strategy for GVHD and other diseases.

2. Materials and Methods

2.1 Reagents and Animals

Fetal bovine serum (FBS), penicillin-streptomycin (P/S), trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). CD4 and CD8 MicroBeads and the AutoMACS system were purchased from Miltenyi Biotec. (Shanghai, China). Biolegend (San Diego, CA, USA) -provided antibodies included: APC-conjugated CD29 (303008), CD19 (115512), and F4/80 (123115); PE-conjugated CD11c (117307), Sca1 (108107), CD206 (141705), and inducible nitric oxide synthase (iNOS) (696805); with PE-Cy7-CD44 (103029) and FITC-CD11b (101205). Hieff qPCR SYBR Green Master Mix was acquired from Yeasen (Shanghai, China). FOXP3/Transcription Factor Staining Buffer set was purchased from eBioscience (San Diego, CA, USA). All primers were obtained from Sangon Biotech (Shanghai, China). TD139, Galectin-3 were purchased from Selleck&Bimake (Selleck&Bimake, Shanghai, China).

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice (10–12 weeks old) were obtained from Shanghai Laboratory Animal Center (SLAC, Shanghai, China) and maintained in specific pathogen-free (SPF) conditions with autoclaved water and standard chow. All animal tests were conducted in compliance with the guidelines established by the Ethics Committee of Xinhua Hospital Affiliated with the Shanghai Jiao Tong University School of Medicine (Ethics Approval No. XHEC-F-2022-076). All mice in this study were euthanized by cervical dislocation, in strict accordance with the guidelines outlined by the Ethics Committee of Xinhua Hospital Affiliated with the Shanghai Jiao Tong University School of Medicine (Shanghai, China).

2.2 Cell Culture

MSCs isolated from bone marrow of C57BL/6 mice were cultured in Dulbecco modified Eagle medium-low glucose (DMEM-L; Hyclone, Shanghai, China) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (10,000 U/mL penicillin, 10,000 μ g/mL streptomycin) (1% P/S; Gibco, USA) at 37 °C in a humidified atmosphere of 5% CO₂. MSCs at passage 2 (P2- MSCs) were passaged by 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco, Burlington, Canada) at a 1:3 ratio when they were 80%–90% confluent, up to P12. Here, the MSCs were classified as early passages of MSCs (passage 4 to 6-MSCs) and late passages (passage 7 to 12-MSCs). MSCs exhibited typical fibroblast-like morphology, characterized by long, polyg-

Table 1. Sequences of primers designed to measure the gene expression by qRT-PCR from MSCs or Raw 264.7 cells.

Gene	Forward Primers (5'-3')	Reverse Primers (5'-3')
<i>β-actin</i>	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG
<i>Galectin-3 (LGALS3)</i>	AGACAGCTTTTCGCTTAACGA	GGGTAGGCACTAGGAGGAGC
<i>TGF-β1</i>	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
<i>IL-10</i>	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
<i>Arg</i>	AGCACTGAGGAAAGCTGGTC	TACGTCTCGCAAGCCAATGT
<i>Ym1</i>	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
<i>CD206 (MR)</i>	AAACACAGACTGACCCTTCCC	GTTAGTGTAACCGCACCTCC
<i>IL-1β</i>	GTCACAAGAAACCATGGCACAT	GCCCATCAGAGGCAAGGA
<i>IL-6</i>	GAACAACGATGATGCACTTGC	CTTCATGTACTCCAGGTAGCTATGGT
<i>TNF-α</i>	CTTCTGTCTACTGAACTTCGGG	CACTTGGTGGTTTGCTACGAC
<i>COX2</i>	GCCTACTACAAGTGTCTTTTGTGCA	CATTTTGTGTTGATTGTTACACCAT
<i>CCL2</i>	AGGTGTCCCAAAGAAGCTGTA	ATGTCTGGACCCATTCTTCT
<i>CCL3</i>	TGACACTCTGCAACCAAGTCTTC	AACGATGAATTGGCGTGGA
<i>CCL4</i>	TTCTCTTACACCTCCCGGCAG	GTACTCAGTGACCCAGGGCTCA
<i>iNOS</i>	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
<i>Galectin-1 (Lgals1)</i>	AACCTGGGGAATGTCTCAAAGT	GGTGATGCACACCTCTGTGA

qRT-PCR, Quantitative Real-time PCR; MSCs, Mesenchymal stem cells; *TGF-β1*, Transforming growth factor-beta 1; *IL-10*, interleukin-10; *Arg*, Arginase; *IL-1β*, interleukin-1beta; *IL-6*, interleukin-6; *TNF-α*, Tumor necrosis factor-α; *COX2*, cyclooxygenase 2; *CCL2*, CC-chemokine ligand 2; *CCL3*, CC-chemokine ligand 3; *CCL4*, CC-chemokine ligand 4; *iNOS*, inducible nitric oxide synthase.

onal, plastic-adherent cells, and display the typical immunophenotypes, including Sca1 (+), CD29 (+), CD44 (+), CD19 (–), and CD11b (–) (**Supplementary Fig. 1A,B**).

The Raw264.7 cells, a macrophage cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco modified Eagle medium glucose (DMEM; Hyclone, China) containing 10% FBS and 1% P/S at 37 °C in a humidified atmosphere of 5% CO₂.

All cell lines (including Raw264.7) were validated by STR profiling and tested negative for mycoplasma contamination.

2.3 Quantitative Real-time PCR (qRT-PCR)

Firstly, total RNA was extracted from cells using EZ-press RNA Purification Kit (EZBioscience, CA, USA) according to manufacturer's protocol. Total RNA meeting purity criteria (A260/A280 ratio 1.8–2.0) was converted to complementary deoxyribonucleic acid (cDNA) with PrimerScript™ RT Master Mix (Takara, Shiga, Japan) per manufacturer's instructions. qRT-PCR was performed using relevant gene-specific primers and Hieff qPCR SYBR Green Master Mix (Yeasen, China) on Step One Plus Real-time PCR System (Life Technologies, CA, USA). The messenger ribonucleic acid (mRNA) expression levels of target genes were normalized to *β-actin* gene and calculated according to the 2^{–ΔΔCT} method. All primers were obtained from Sangon Biotech. Primer sequences are shown in Table 1.

2.4 Flow Cytometry Analysis

Fluorescein conjugated monoclonal antibodies (mAbs) used for experiments include phycoerythrin (PE)-conjugated mAbs to mouse, Fluorescein isothiocyanate (FITC)-conjugated mAbs to mouse, *allophycocyanin* (APC)-conjugated mAbs to mouse and R-Phycoerythrin-Cyanine 7 (PE-Cy7)-conjugated mAbs to mouse. Antibody information are provided in the Reagents and Animals section. In brief, for cell surface antigen staining, cells were collected, washed with phosphate buffered solution (PBS) and incubated with relevant fluorescein-conjugated mAb in the dark for 30 minutes at 4 °C. For cell nuclear/intracellular antigen staining, after stained with cell surface antigen, cells were fixed and permeabilized with eBioscience™ FOXP3/Transcription Factor Staining Buffer set (Invitrogen, CA, USA) according to the manufacture's protocol followed by intracellular staining with relevant fluorescein-conjugated mAb in the dark for 30 minutes at room temperature. Finally, cells were washed with PBS and performed flow cytometric analysis. Flow cytometry (FCM) was performed by using BD FACS CantoII (Becton-Dickinson Bioscience, Franklin Lakes, NJ, USA) and analysis was performed by using FlowJo software (Version 10.8.1; BD Biosciences, Ashland, OR, USA).

2.5 Establishment and Assessment of Acute GVHD Mice Model

Mice underwent bone marrow (BM) transplantation (BMT) according to the protocol described previously [34]. Briefly, recipient mice (BALB/c, male, 10–12 weeks) were

randomly divided into three groups as follows: the T cell depleted-bone marrow cells (TCD-BMC) + T cells + vehicle group, the TCD-BMC + T cells + P6-MSCs group and the TCD-BMC + T cells + P12-MSCs group. At day 0, after BALB/c recipients received 7.0 Gy ($^{137}\text{Cesium}$ source at 100 cGy/min) total body irradiation (TBI), TCD-BMC (5×10^6 cells/mouse) together with splenic CD3⁺ T cells (2×10^6 cells/mouse) from allogeneic donor mice (C57BL/6) were collected and co-injected into BALB/c recipients via tail vein. The recipients were transfused with vehicle, P6-MSCs (3×10^5 cells/mouse) or P12-MSCs (3×10^5 cells/mouse) intravenously at day 1, 3, 5 according to groups they were assigned to. The severity of GVHD was evaluated every 2 days post-transplantation using a standardized clinical scoring system, based on five parameters: weight loss, posture, activity, fur texture, and skin integrity. Scoring criteria conformed to established protocols as previously described [35]. The mice were euthanized via cervical dislocation on day 21 post-TBI without prior anesthesia, following which peritoneal cavity-derived macrophages were collected for subsequent analysis. Target organs (liver, lung, small intestine) were collected from per experimental group, fixed in 10% formalin, paraffin-embedded, hematoxylin and eosin (H&E)-stained, and blindly evaluated for GVHD pathology.

2.6 Isolation of T-cells

Spleens harvested from C57BL/6 mice were grinded through 40- μm nylon cell strainers and obtained splenic cell suspension. CD4⁺/CD8⁺ T cell isolation or depletion was performed using CD4 and CD8a MicroBeads and the AutoMACS system (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Briefly, 10 μL of CD4 MicroBeads and 10 μL of CD8a MicroBeads were added per 1×10^7 cells, and the mixture was incubated for 15 minutes at 4 °C in the dark. The cell suspension was then passed through a pre-rinsed LS column mounted on the AutoMACS Pro Separator to retain T cells (CD4⁺ and CD8⁺) via magnetic separation.

2.7 Generation of T Cell Depleted-bone Marrow Cells

Bone marrow cells (BMC) were isolated from the femurs and tibias of C57BL/6 mice following humane euthanasia. The marrow cavity was flushed with ice-cold DMEM-complete medium using a 25-gauge needle to obtain a single-cell suspension, which was sequentially filtered through a 40- μm nylon cell strainer to remove debris. Red blood cells in the suspension were lysed with ACK lysis buffer, and the resulting nucleated cell pellet was resuspended in MACS Buffer at a concentration of 1×10^8 cells/mL. T cell depletion (TCD) was performed using CD4 and CD8a MicroBeads according to the manufacturer's protocol. The efficiency of T cell depletion was validated by flow cytometry using anti-mouse CD3-PE antibody. Only TCD-BMC preparations with residual CD3⁺ T cells $\leq 1\%$

(consistent with purity validation in **Supplementary Fig. 2**) were used for transplantation experiments.

2.8 Preparation and Analysis of Macrophages

Macrophages were harvested from the peritoneal lavage of recipient mice at day 21 after BMT. After intraperitoneal injection of pre-cooled PBS, gentle abdominal massage was performed prior to peritoneal lavage fluid collection. Finally, the harvested cells were stained for flow cytometric analysis using anti-mouse CD11b-FITC, anti-mouse F4/80-APC and anti-mouse CD206-PE and anti-mouse iNOS-PE antibodies. CD11b⁺F4/80⁺ double-positive cells were identified as peritoneal macrophages (**Supplementary Fig. 3**).

2.9 Effects of MSCs on Macrophages

To investigate the effects of MSCs on macrophages at different passages, P6-MSCs and P12-MSCs were seeded into the lower chamber of transwell (Corning, USA, catalog number 3412, pore diameter 0.4 μm , polycarbonate membrane) at a density of 2×10^5 per well. When they reached approximately 70% confluence, Raw 264.7 cells were seeded into the upper chamber of transwell at a density of 1×10^7 per well and co-cultured with P6-MSCs or P12-MSCs for 48 hours. Besides, the co-culture system was treated with vehicle, Gal-3 (500 ng/mL), TD139 (100 μM), LPS (100 ng/mL) + vehicle, LPS (100 ng/mL) + galectin-3 (500 ng/mL), or LPS (100 ng/mL) + TD139 (100 μM). Finally, Raw264.7 cells were collected and stained with PE-conjugated anti-mouse CD206 or iNOS antibodies for flow cytometry.

2.10 Effects of Gal-3 on Macrophages

Raw264.7 cells were seeded into 24-well plates at a density of 3×10^6 per well and treated with vehicle, galectin-3 (100 ng/mL), galectin-3 (500 ng/mL) for 48 hours. Galectin-3 was dissolved in ddH₂O. Raw264.7 cells were collected and stained with anti-mouse CD206-PE or anti-mouse iNOS-PE antibodies for flow cytometry. Besides, expression of macrophage polarization-related mediator (iNOS, interleukin-1 β (IL-1 β), interleukin-6 (IL-6), Tumor necrosis factor- α (TNF- α), CC-chemokine ligand 2 (CCL2), CC-chemokine ligand 3 (CCL3), CC-chemokine ligand 4 (CCL4), cyclooxygenase 2 (COX2), interleukin-10 (IL-10), YM1, Transforming growth factor-beta1 (TGF- β 1), Arginase (Arg) and CD206) in Raw264.7 cells were determined by qPCR across experimental groups.

2.11 Cell Proliferation Assay

MSCs at different passages were seeded in 96-well cell culture plates at 3×10^3 per well. The cells were treated with vehicle or TD139 at 100 μM concentration for 72 h. The absorbance at 450 nm was measured every 24 h with a microplate reader (Thermo Fisher) according to the instructions of the CCK-8 kit (cell counting kit-8, Beyotime,

China). The total numbers of migrated cells were counted to evaluate cell proliferation.

2.12 Effects of TD139 on Macrophages

Raw 264.7 macrophages were seeded in 24-well cell culture plates at 1.5×10^6 per well. TD139 was dissolved in DMSO. The cells were treated with vehicle or TD139 (100 μ M) for 48 h. Then, the Raw 264.7 macrophages were harvested and stained for flow cytometric analysis using anti-mouse CD206-PE or anti-mouse iNOS-PE antibodies.

2.13 Statistical Analysis

All experiments were independently repeated a minimum of two to three times. The figure legends provide detailed information on reproducibility and sample sizes. Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA) or Student's *t*-test with GraphPad Prism software. Non-parametric murine survival data were analyzed using the log-rank test. All *p* values were two-sided, with *p* < 0.05 considered statistically significant.

3. Results

3.1 Late-passage MSCs Poorly Alleviated the Severity of GVHD

To determine whether passage number of MSCs would affect the therapeutic efficacy of MSCs in GVHD, recipient mice were received MSCs at different passages after BMT (Fig. 1A). Although there were no significant differences in survival of mice among three groups (Fig. 1B), the clinical GVHD scores were significantly higher in recipients treated with P12-MSCs compared to those treated with P6-MSCs (Fig. 1C, *p* < 0.0001). To confirm these results, histopathological examination was performed at day 21 post-BMT. As shown in Fig. 1D, the histopathological images showed more severe tissue damage in GVHD-target organs including small intestine, liver, lungs, in P12-MSCs-treated recipients compared to those treated with P6-MSCs. Taken together, these results suggest that MSCs at late passage (P12) are less effective in alleviating the severity of acute GVHD (aGVHD) compared to MSCs at early passage (P6).

3.2 The Effect of Late-passage MSCs (P12) on M2-like Macrophages Polarization was Weakened

Next, we sought to explore the potential mechanisms underlying the variation in MSCs at different passages in the context of GVHD. Given the powerful immunoregulatory effects of MSCs on macrophages, we next assessed the polarization of macrophages isolated from peritoneal lavage liquid of GVHD mice model treated with MSCs at different passages. Compared to the P6-MSCs-treated mice, the proportion of F4/80⁺ CD11b⁺ CD206⁺ M2-like macrophages was lower in the P12-MSCs-treated mice (Fig. 2A, *p* < 0.0001). Meanwhile, we utilized Raw246.7

cell line to investigate the effects of MSCs at different passages on macrophages *in vitro*. After co-culturing with MSCs at different passages, the proportion of CD206⁺ M2-like macrophages was also lower in P12-MSCs-treated group than that in P6-MSCs-treated group (Fig. 2B, *p* < 0.05). Moreover, the proportion of iNOS⁺ M1-like macrophages in Raw264.7 cells was higher in P12-MSCs-treated group than in P6-MSCs-treated group (data not shown). Collectively, our results indicated that MSCs at later passages exhibit a significantly reduced ability to induce M2 macrophage polarization.

To simulate the *in vivo* inflammatory environment, we supplemented the MSCs-Raw264.7 macrophage co-culture system with LPS. Although the proportion of CD206⁺ M2-like macrophages and iNOS⁺ M1-like macrophages did not differ significantly between P6-MSCs-treated and P12-MSCs-treated groups, mRNA expression of M2-associated anti-inflammatory mediators, such as IL-10 was reduced, while the mRNA expression of M1-associated pro-inflammatory mediators, including L-6, CCL4, and COX2, was elevated in the P12-MSCs-treated group compared to the P6-MSCs-treated group (Fig. 3A,B). Our results indicated that the therapeutic efficacy of MSCs at different passages for acute GVHD could be related to macrophage polarization and the effect of MSCs at late passage on M2-like macrophages polarization was notably weakened.

3.3 Galectin-3 Directly Suppressed M2-like Macrophages Polarization

Previous studies have demonstrated that the activation of Gal-3 enhances inflammation [23], whereas the downregulation of Gal-3 can suppress inflammatory responses [24]. Several studies have indicated that Gal-3 promotes a pro-inflammatory response by inhibiting M2 macrophage polarization and inducing apoptosis [21,22]. MSCs constitutively express Gal-3 at both the mRNA and protein levels, thereby exerting immunomodulatory effect via galectins [36]. Moreover, Gal-3 is a new informative model to predict aGVHD development [37]. Based on this, we inferred that Gal-3 expression may be linked to the effects of MSCs on macrophages, to verify this point, we first analyzed the expression of Gal-3 in MSCs at different passages. As shown in Fig. 4, Gal-3 expression escalated starting at passage 10 and markedly higher in MSCs at later passages compared to those at earlier passages. Then, we assessed the characteristics of Raw 264.7 macrophages in the presence of Gal-3. As shown in Fig. 5A, the proportion of iNOS⁺ M1-like macrophages notably increased in Gal-3-treated Raw 264.7 cells in a dose-dependent manner after 48 h. The M1-like macrophage phenotype is characterized by the expression of chemokines such as iNOS, CCL2, CCL3, CCL4 and the induction of pro-inflammatory mediators including IL-1 β , IL-6, TNF- α , COX2. Our data showed that, in addition to IL-1 β ,

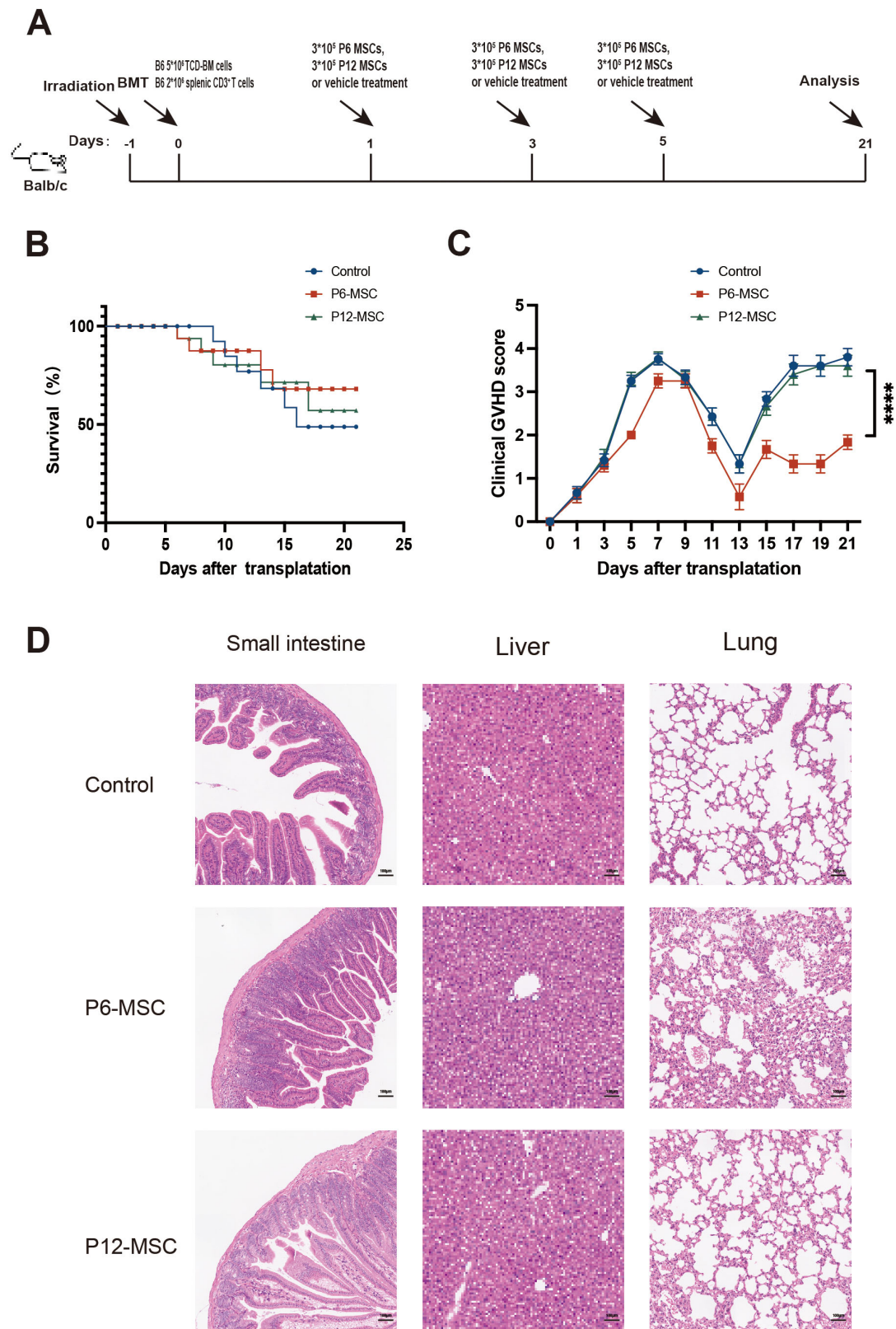


Fig. 1. Effects of MSCs at different passages on the GVHD mice. (A) Experimental scheme for GVHD model establishment and treatment. (B) Kaplan-Meier survival curve of GVHD mice treated with PBS, P6-MSCs, P12-MSCs. (C) Clinical GVHD score of GVHD mice treated with PBS, P6-MSCs, P12-MSCs. (D) Representative histopathology images of liver, lung and small intestine from GVHD mice treated with PBS, P6-MSC and P12-MSC on day 21 after transplantation. Scale bar, 100 μ m. Data were pooled from 3 independent experiments. $N = 3-5$ in each group. **** $p < 0.0001$. GVHD, graft-versus-host-disease; PBS, phosphate buffered solution.

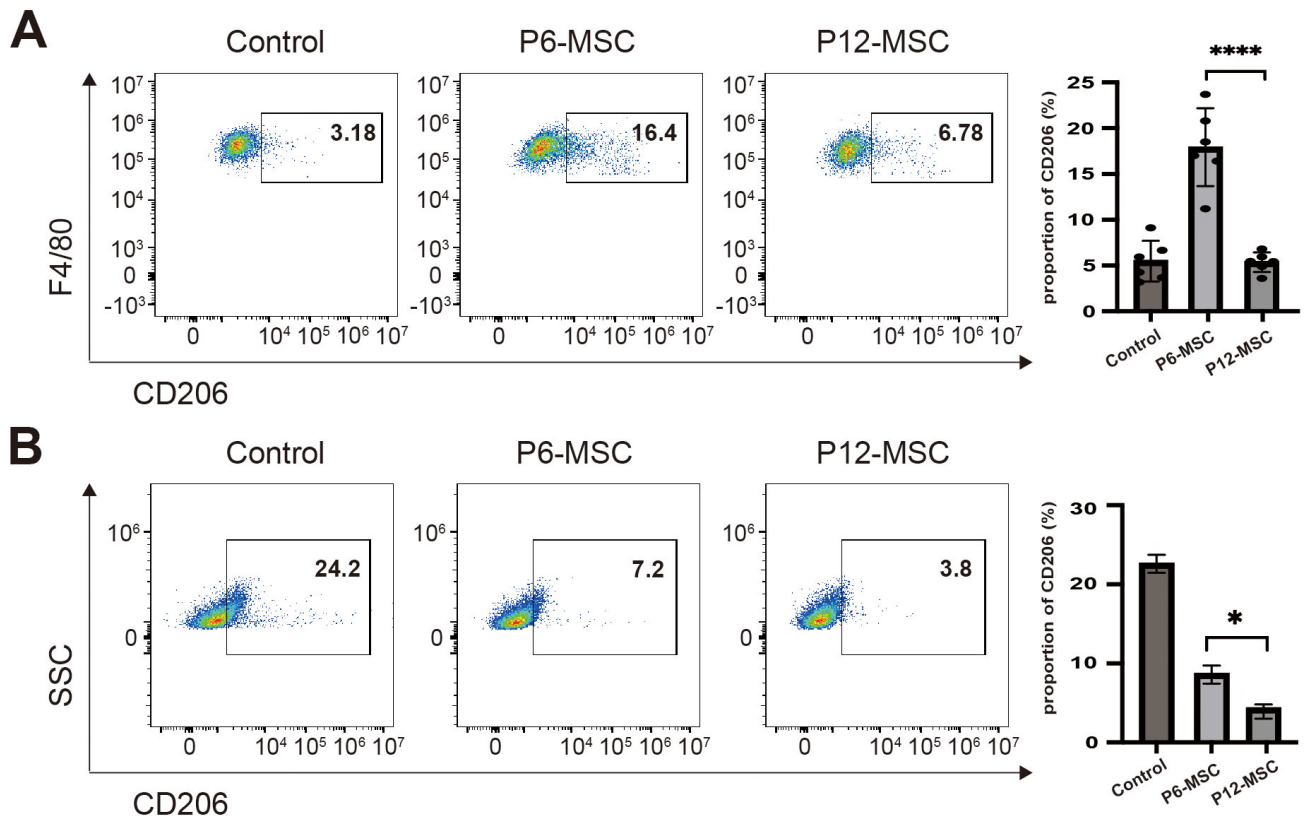


Fig. 2. The effect of MSCs on macrophage polarization both *in vivo* and *in vitro*. (A) Flow cytometric analysis of CD206⁺ M2-like macrophage proportion within F4/80⁺CD11b⁺ peritoneal macrophages from GVHD mice treated with PBS, P6-MSCs or P12-MSCs on day 21 post-transplantation. (B) Flow cytometric analysis of CD206⁺ M2-like macrophages percentages in Raw264.7 cells co-cultured with vehicle control (culture medium alone), P6-MSCs, or P12-MSCs without LPS stimulation for 48 hours. Data were pooled from 3 independent experiments. * $p < 0.05$, **** $p < 0.0001$. LPS, lipopolysaccharide.

the mRNA expression of these M1-associated mediators was significantly upregulated in Raw 264.7 macrophages treated with high doses of Gal-3 (Fig. 5B, $p < 0.01$). Furthermore, the proportion of CD206⁺ M2-like macrophages in Gal-3 treated group was significant decreased in a dose-dependent manner compared to the control groups (Fig. 5C, $p < 0.01$). Meanwhile, anti-inflammatory mediators associated with the M2 macrophage phenotype, including IL-10, TGF- β 1, Arg, and Ym1, were decreased in Gal-3-treated Raw264.7 macrophages compared to the control groups (Fig. 5D, $p < 0.0001$). All these data suggested that the differential effects of MSCs at different passages on macrophage polarization may be related to Gal-3. As MSCs progress through passages, they express higher levels of Gal-3, which could concentration-dependently induce macrophage polarization toward the iNOS⁺ M1 phenotype while suppressing CD206⁺ M2 macrophage polarization.

3.4 TD139 Could Suppress Gal-3 Expression in MSCs and Save the Immunoregulatory Function of MSCs at Later Passages

Based on the above data, it is evident that elevated expression of Gal-3 in MSCs at later passages results in

a diminished effect of MSCs on M2-like macrophage polarization. In this study, we sought to assess the potential applications of Gal-3 inhibitors. While prior studies have focused on Gal-3, our data demonstrated that Gal-1 is also expressed in MSC (Supplementary Fig. 4A). TD139, a selective small-molecule inhibitor of Gal-3, with no impact on Gal-1 (Supplementary Fig. 4B), and is currently undergoing Phase II clinical trials for idiopathic pulmonary fibrosis [28,38]. Treatment with TD139 at concentrations ranging from 10–100 μ M for 48 hours led to a significant dose-dependent reduction of Gal-3 expression in P12-MSCs (Fig. 6A). Based on this observation, a concentration of 100 μ M TD139 was selected for subsequent experiments. Similarly, Gal-3 expression was also inhibited in P6-MSCs following high-dose TD139 treatment (Fig. 6B, $p < 0.0001$). In addition, our data showed that TD139 treatment did not alter MSCs proliferation and viability (Supplementary Fig. 5).

To determine whether TD139 affect other profiles of MSCs, we also examined the immunophenotype of MSCs in presence of TD139. The results revealed that MSCs cultured with or without TD139 exhibited typical fibroblast-like morphology, characterized by long, polygonal, plastic-

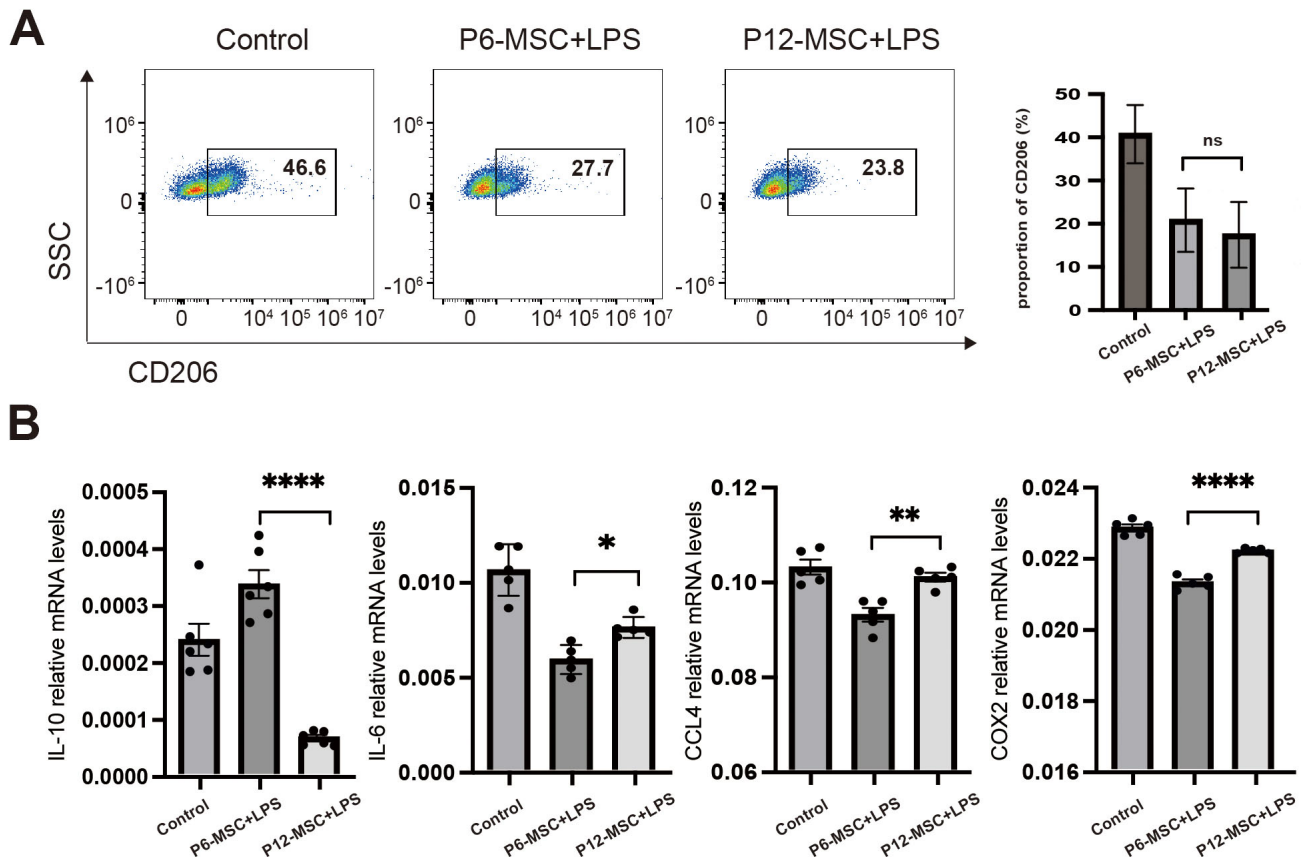


Fig. 3. The effect of MSCs on macrophage polarization in the presence of LPS. Raw264.7 cells were cocultured with vehicle control (culture medium alone), P6-MSCs or P12-MSCs in the presence of 100 ng/mL LPS for 48 h, distinct from Fig. 2 (no LPS). (A) Flow cytometric analysis of CD206⁺ cell percentages in Raw264.7 macrophages. (B) qPCR analysis of macrophage polarization-related mediator expression (IL-10, IL-6, CCL4, and COX2) in Raw264.7 macrophages. Data were pooled from 3 independent experiments. Data are presented as the mean $\pm$ SD. ns ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. SSC, Side Scatter.

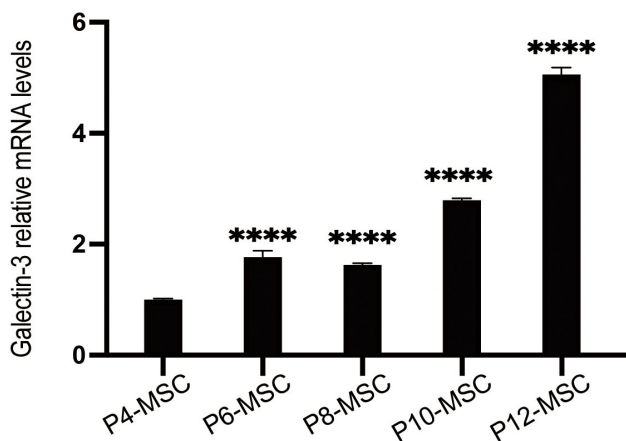


Fig. 4. The mRNA expression of Galectin-3 in MSCs at different passages by qPCR. Data were pooled from 3 independent experiments. Data are presented as the mean $\pm$ SD. **** $p < 0.0001$.

adherent cells, and displayed the expected immunophenotypes of MSCs, including Sca1 (+), CD29 (+), CD44 (–), CD19 (–), and CD11b (–) (data not shown). These find-

ings indicated that TD139 effectively inhibits Gal-3 expression in MSCs without altering their fundamental biological properties, including morphology and immunophenotype.

Since TD139 inhibits Gal-3 expression in MSCs, we inferred that TD139 could improve the immunoregulatory function of MSCs. In this study, we investigated the impact of TD139 on macrophage polarization induced by MSCs. As shown in Fig. 6, after incubation with TD139 at a concentration of 100 μ M, the proportion of CD206⁺ M2-like macrophages relative to Raw264.7 macrophages was significantly increased in the TD139-treated P6-MSCs group compared to the vehicle-treated P6-MSCs group (Fig. 6C, $p < 0.05$). Besides, the proportion of CD206⁺ M2-like macrophages relative to Raw264.7 macrophages was also increased in the TD139-treated P12-MSCs group compared to the vehicle-treated P12-MSCs group (Fig. 6D, $p < 0.001$). Moreover, although no significant differences were observed among groups containing P6-MSCs, the percentage of iNOS⁺ M1-like macrophages was decreased in the TD139-treated P12-MSCs group compared to the vehicle-treated P12-MSCs group (data not shown). Taken together, these results demonstrated that TD139

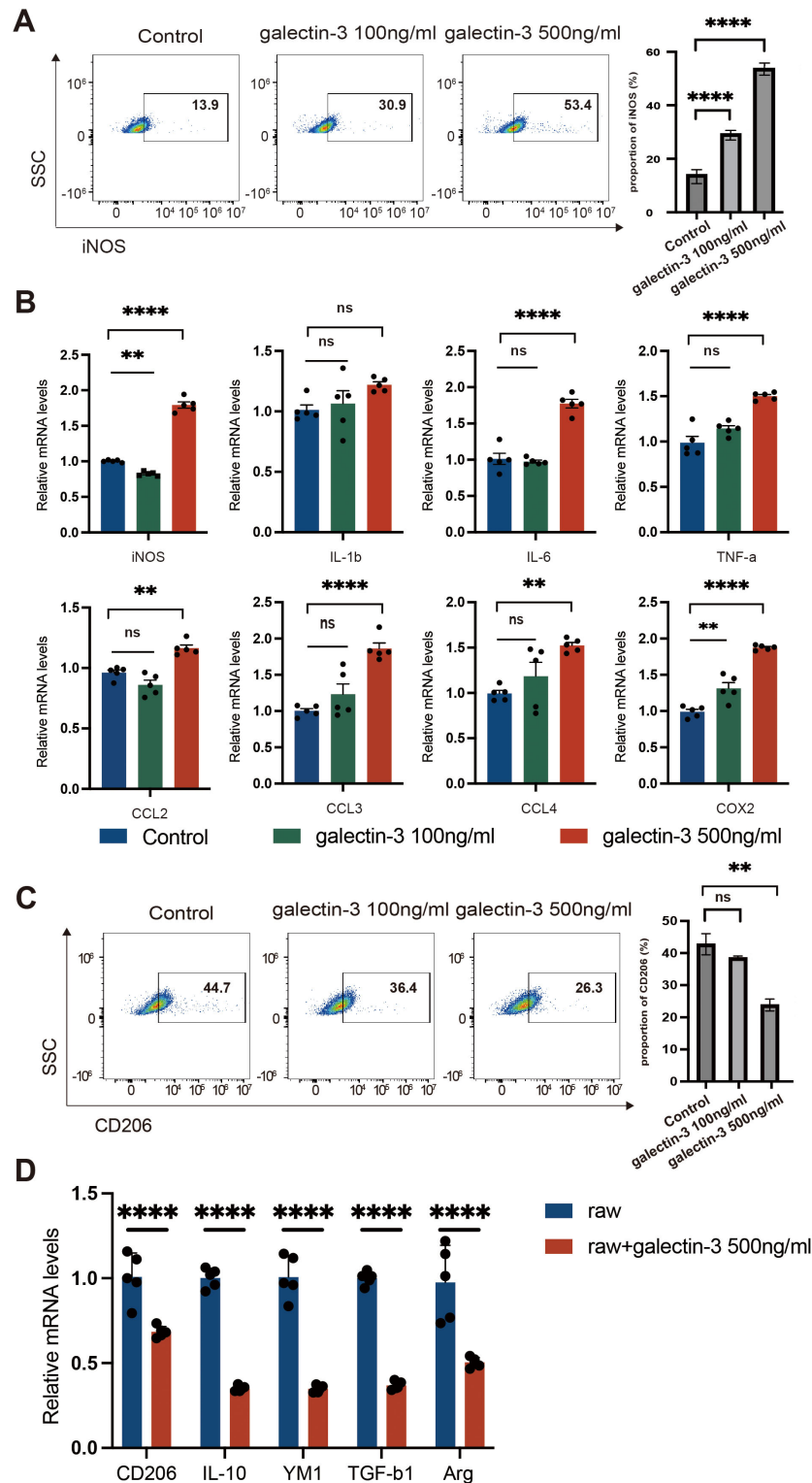


Fig. 5. The effect of Gal-3 on macrophage polarization. Raw264.7 cells were treated with vehicle, Gal-3 (100 ng/mL) or Gal-3 (500 ng/mL) for 48 h. (A) Flow cytometry analysis of iNOS⁺ M1 macrophage proportion in Raw264.7 macrophages. (B) qPCR analysis of iNOS⁺ M1 macrophage polarization-related mediator expression (iNOS, IL-1 β , IL-6, TNF- α , CCL2, CCL3, CCL4 and COX2) in Raw264.7 macrophages. (C) Flow cytometry analysis of CD206⁺ M2 macrophage proportion in Raw264.7 macrophages. (D) qPCR analysis of CD206⁺ M2 macrophage polarization-related mediator expression (IL-10, YM1, TGF- β 1, Arg and CD206) in Raw264.7 macrophages. Data were pooled from 3 independent experiments. Data are presented as the mean $\pm$ SD. ns ($p > 0.05$), ** $p < 0.01$, **** $p < 0.0001$. Gal-3, Galectin-3.

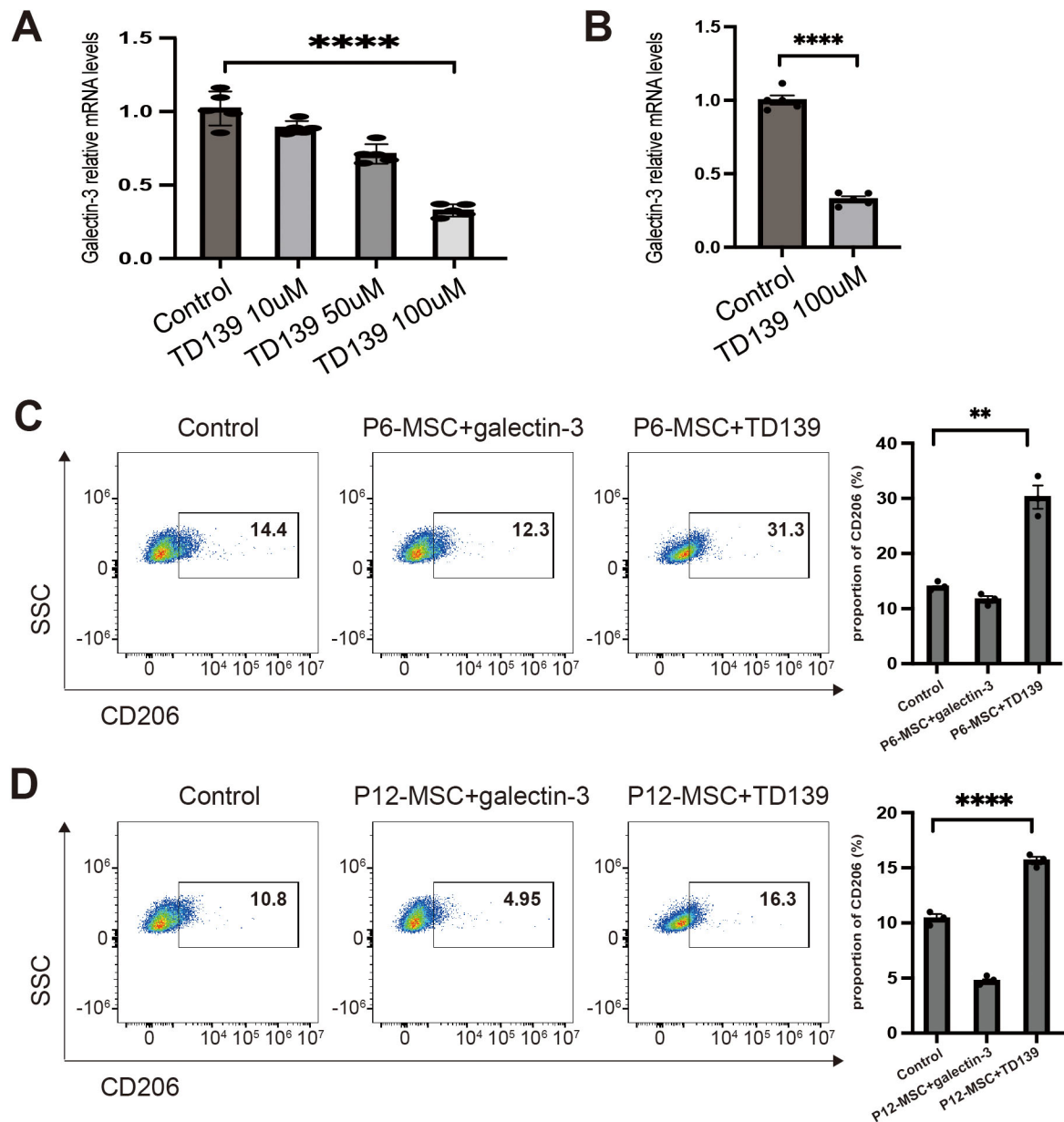


Fig. 6. The effect of TD139 on Gal-3 expression of MSCs and macrophage polarization induced by MSCs. MSCs at different passages were treated with DMSO, TD139 (10 μ M), TD139 (50 μ M) or TD139 (100 μ M) for 48 h (A,B). MSCs at different passages cocultured with Raw264.7 macrophages were treated with vehicle, Gal-3 (500 ng/mL) or TD139 (100 μ M) for 48 h (C,D). (A) qPCR analysis of Gal-3 expression in P12-MSCs. (B) qPCR analysis of Gal-3 expression in P6-MSCs. (C) Flow cytometry analysis of CD206⁺ M2 macrophage proportion in Raw264.7 macrophages cocultured with P6-MSCs. (D) Flow cytometry analysis of CD206⁺ M2 macrophage proportion in Raw264.7 macrophages cocultured with P12-MSCs. Data were pooled from 3 independent experiments. Data are presented as the mean $\pm$ SD. ** p < 0.01, **** p < 0.0001.

enhances the immunoregulatory effect of MSCs at early passages on macrophages by promoting CD206⁺ M2-like macrophages polarization. Furthermore, TD139 also saves the immunoregulatory function of MSCs at later passages via inducing CD206⁺ M2-like macrophages polarization and suppressing iNOS⁺ M1-like macrophages polarization. Additionally, both the proportion of CD206⁺ M2-like macrophages and iNOS⁺ M1-like macrophages rela-

tive to Raw264.7 macrophages in the TD139 treated P12-MSCs group was closely resembled the results observed in the TD139-treated P6-MSCs group. This indicated that TD139 treatment can restore the immunoregulatory capacity of MSCs at later passages to levels comparable to those at early passages, further confirming that TD139 can preserve the immunoregulatory function of MSCs at late passages.

4. Discussion

In the past few years, due to its immunosuppressive ability, MSCs have been applied for the treatment of GVHD after allo-HSCT, including gut GVHD, as well as for the management of refractory Crohn's disease [4,39–41]. MSCs derived from various biological sources usually require *ex vivo* expansion to achieve sufficient cell numbers for *in vivo* applications [14]. However, repeated *in vitro* passaging negatively affects the biological characteristics of MSCs, leading to a decline in their immunosuppressive and regenerative potential with each passage [16–18]. Therefore, it is necessary to develop strategies to save the immunosuppressive capability of MSCs during passaging, particularly for MSCs at later passage.

Several preclinical and clinical studies have documented the advantageous impacts of MSCs on GVHD [42–44]. Nevertheless, other studies have shown that *in vitro* aging adversely affects the immunosuppressive function of MSCs. Gao *et al.*'s study [45] elucidated that MSCs undergo a progressive aging process *in vitro*, and the cellular senescence influences their immunosuppressive activity. Von Bahr *et al.* [46] reported that in GVHD patients, 1-year survival was 75% in patients who received early-passage MSCs (from passages 1 to 2) in contrast to 21% in those who using later passage MSCs (from passages 3 to 4). In this study, we compared the therapeutic efficacy of MSCs at different passages in aGVHD mice model. Our data showed that compared to the control group, the severity of GVHD and tissue damage were not significantly different in the late-passage MSC group, whereas they were notably decreased in the early-passage MSC group, indicating that the therapeutic efficacy of MSCs diminishes with increasing passage.

In the pathogenesis of aGVHD, conditioning regimens in allo-HSCT initially activate host antigen-presenting cells, mainly macrophages and dendritic cells, thereby positioning macrophages as a central focus of research [34]. Previous studies have shown that with the progressive expansion *in vitro*, MSCs have undergone a phenotypic transformation from anti-inflammatory to pro-inflammatory, which leads to the deterioration of their therapeutic effects [47–49]. Thus, in this study, we examined peritoneal macrophages derived from aGVHD mice under various treatment. Consistent with previous studies, M2-like macrophages were increased in aGVHD mice treated with early-passage MSCs [50–52]. However, in contrast to the early-passage MSC treatment group, the number of M2-like macrophages was significantly reduced in the late-passage MSC group, suggesting that the variability in therapeutic efficacy of MSCs at different passages is associated with macrophage polarization. Subsequently, we conducted experiments with the Raw264.7 macrophage cell line, and the results showed that the effect of late-passage MSCs on M2-like macrophages polarization was notably weakened compared to early-passage MSCs. Taken together,

these results underscored the relationship between the immunomodulatory efficient of MSCs at different passages and macrophages polarization.

Gal-3 belongs to the beta-galactoside-binding animal lectin family, manifesting pleiotropic functions such as mediating cell–cell interactions, cell-matrix adhesion, and transmembrane signaling. It is produced by MSCs and exerts immunomodulatory effects [53]. Accumulating evidences have revealed that Gal-3 plays a pivotal role in macrophage polarization and the pathogenesis of aGVHD [37,54]. Li H *et al.* [54] provided evidence indicating that Gal-3 enhances inflammatory response by directly binding to NLRP3 in RAW264.7 macrophages. Therefore, we inferred that there may be an association between Gal-3, MSCs and macrophages. In the present study, we observed that Gal-3 expression was significantly increased in late-passage MSCs. Moreover, Gal-3 promoted an inflammatory response in Raw264.7 macrophages, notably by increasing M1-like macrophage polarization and expression of iNOS, IL-6, TNF- α , CCL2, CCL3, CCL4, COX2, while decreasing M2-like macrophage polarization and expression of CD206, IL-10, TGF- β 1, Arg, Ym1. Collectively, these findings suggested that the reduced therapeutic efficacy of MSCs at late passages was related to the upregulation of Gal-3 expression. While telomere attrition and DNA damage are established drivers of replicative senescence in passaged MSCs, Gal-3 emerges as a distinct senescence inducer that functions independently of both telomeric erosion and DNA repair machinery [55–59].

TD139 has emerged as the foremost small molecule inhibitor targeting Gal-3 and has been extensively investigated in various experimental studies for its potential applications [60]. Currently, it represents a promising therapeutic agent for idiopathic pulmonary fibrosis in the phase II clinical trial [28]. Previous studies have underscored the therapeutic potential in lung fibrosis, cancer metastasis and hepatitis [61–63]. Here, we investigated the therapeutic role of TD139 in GVHD mice and its effects on MSCs for the first time. As shown in **Supplementary Fig. 6**, TD139 treatment in Raw 264.7 cells reduced the iNOS⁺ M1 macrophage population while increasing the CD206⁺ M2 macrophage subset. This data indicated that TD139 directly inhibits M1-like macrophage polarization while promoting M2-like polarization. Nevertheless, as shown in Fig. 6, our results strongly indicated that TD139 dose-dependently suppressed the expression of Gal-3 in MSCs without affecting their biological properties. Inhibition of Gal-3 by TD139 significantly promoted M2-like macrophage polarization in both the P6-MSCs-Raw264.7 and P12-MSCs-Raw264.7 coculture systems, suggesting that TD139 enhanced the effect of MSCs on macrophages by inducing M2-like polarization. More importantly, in the presence of TD139, the proportion of M2-like macrophages was higher in Raw264.7 cells cocultured with P12-MSCs than in those cocultured with P6-MSCs. Although TD139 exhibited di-

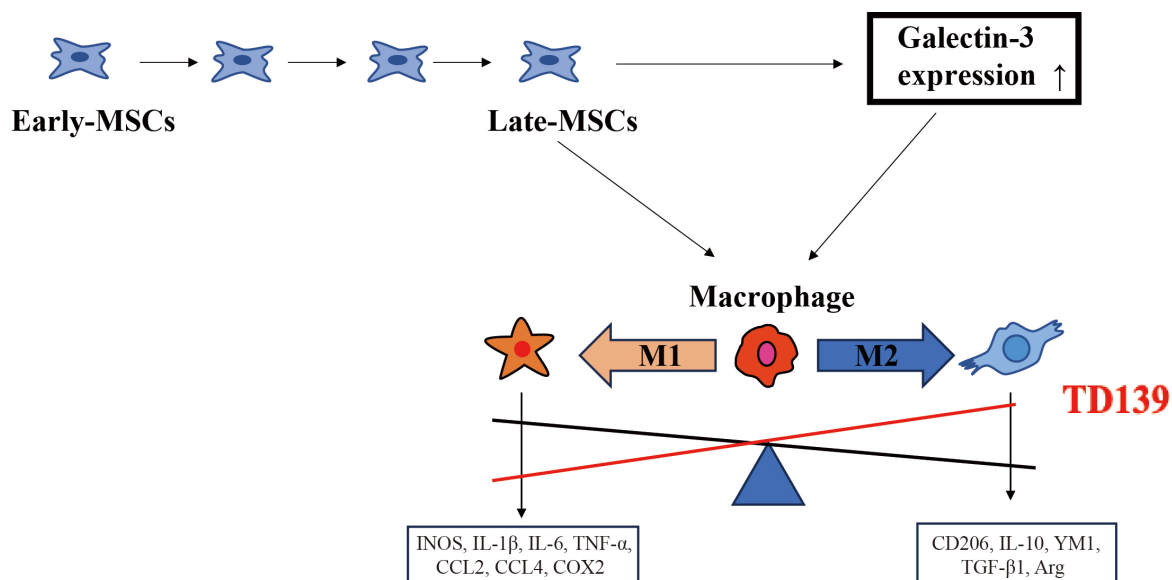


Fig. 7. The diagram of main results. MSCs, mesenchymal stem cells; IL-1 β , interleukin 1b; IL-10, interleukin 10; IL-6, interleukin 6; COX2, cyclooxygenase 2; CCL2, CC-chemokine ligand 2; CCL4, CC-chemokine ligand 4; TGF- β 1, Transforming growth factor-beta1; Arg, Arginase; TNF- α , Tumor necrosis factor- α ; iNOS, inducible nitric oxide synthase; $\uparrow$, the upward arrow indicated increased expression of galectin-3.

rect effects on macrophage polarization, its efficacy was substantially potentiated with the present of MSCs, suggesting that TD139 partially restored the immunomodulatory function of MSCs. Taken together, these findings suggested that TD139 can save the diminished immunoregulatory function of MSCs, particularly late-passage MSCs, enabling more MSCs to be obtained through passaging while preserving their immunoregulatory capabilities.

Although TD139-pretreated MSCs is a promising strategy for GVHD therapy, several challenges remain in their clinical application. We attempted to apply TD139 for pretreating MSCs and subsequently inject TD139-pretreated MSCs into GVHD mice. However, the results were not satisfactory (data not shown). In our opinion, the following factors may contribute to this outcome: First, TD139 may not fully inhibit the expression of Gal-3 in MSCs. Second, after TD139 pretreatment of MSCs *in vitro*, the expression of Gal-3 in MSCs may recover *in vivo*. To address these issues, we plan to use small interfering RNA (siRNA) to inhibit the expression of Gal-3 in MSCs for a prolonged period in future studies. Additionally, we aim to apply TD139 and MSCs simultaneously, rather than pretreating MSCs with TD139. In addition, our data demonstrated that Gal-3 inhibited M2-like macrophage polarization, mechanistic details require further study. Prior evidence implicated Gal-3 in regulating macrophage function through NF- κ B [64], TLR4/MyD88 [65], and CD98-PI3K pathways [66]. These pathways possibly mediate the M2-like macrophage suppression, future work will include co-immunoprecipitation for receptor interactions and pathway-specific inhibitors to elucidate signaling roles. Besides,

TD139's high polarity results in poor intestinal absorption necessitating alternative delivery routes [67]. Inhaled delivery of TD139, faces limitations in systemic diseases such as GVHD due to its dependency on patient adherence and inability to address extra-pulmonary manifestations [28]. To overcome these constraints, the next-generation galectin-3 inhibitor GB1211 was engineered for oral bioavailability, which maintains target affinity while enabling systemic exposure critical for GVHD therapy [67].

In conclusion, as shown in Fig. 7, the reduced therapeutic effect of late passage MSCs in treating GVHD is related to their impaired capacity to induce M2 macrophage polarization. Our study showed that the expression of Gal-3 increased in late passage MSCs, and that Gal-3 can suppress M2 macrophage polarization. Therefore, it is likely that Gal-3 plays a pivotal role in mediating the ability of MSCs to regulate macrophage polarization. Notably, treatment with TD139, a selective Gal-3 inhibitor, can restore the immunoregulatory function of late passage MSCs *in vitro*.

5. Conclusion

In summary, our study demonstrated that passaging *in vitro* impairs the therapeutic efficacy of MSCs in GVHD due to elevated Gal-3 expression in late-passage MSCs. Gal-3 directly suppressed M2-like macrophage polarization, thereby attenuating the immunoregulatory function of late-passage MSCs. TD139, a selective Gal-3 inhibitor, could restores the immunoregulatory function of MSCs, facilitating the clinical application of expanded MSCs for GVHD treatment.

Abbreviations

MSCs, mesenchymal stem cells; allo-HSCT, Allogeneic hematopoietic stem cell transplantation; GVHD, graft versus host diseases; APCs, antigen-presenting cells; Gal-3, Galectin-3; LPS, lipopolysaccharide; IL-10, interleukin 10; IL-6, interleukin 6; COX2, cyclooxygenase 2; CCL2, CC-chemokine ligand 2; CCL3, CC-chemokine ligand 3; CCL4, CC-chemokine ligand 4; Arg, Arginase; IL-1 β , interleukin 1 β ; TNF- α , Tumor necrosis factor- α ; TGF- β 1, Transforming growth factor-beta1; Th1, T helper 1; Th17, T helper 17; DMEM-L, Dulbecco modified Eagle medium-low glucose; DMEM, Dulbecco modified Eagle medium glucose; FBS, fetal bovine serum; P/S, penicillin-streptomycin; EDTA, ethylenediaminetetraacetic acid; Sca1, spinocerebellar ataxia type 1; FCM, flow cytometry; mAbs, monoclonal antibodies; PE, phycoerythrin; APC, *allophycocyanin*; FITC, Fluorescein isothiocyanate; PE-Cy7, R-Phycoerythrin-Cyanine 7; iNOS, inducible nitric oxide synthase; PBS, phosphate buffered solution; BM, bone marrow; BMT, bone marrow transplantation; TCD-BMC, T cell depleted- bone marrow cells; H&E, hematoxylin and eosin; CCK-8, Cell counting kit 8.

Availability of Data and Materials

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author Contributions

The concept was conceived by SGH. The overall study design was developed by MHW, JBW and SGH. The experiments were performed by MHW, FH, YJ, DFZ, and JBW. The data analysis was performed by MHW and YJ. The manuscript was written by MHW, JBW and SGH. The research was supervised by JBW and SGH. All authors contributed to editorial changes in the manuscript. All authors read and approved the final 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 animal study was approved by the Ethics Committee of Xinhua Hospital Affiliated to the Shanghai Jiao Tong University School of Medicine (Ethics Approval No. XHEC-F-2022-076). All experimental procedures were performed in accordance with the 3R principle (Replacement, Reduction, and Refinement) for animal research, aiming to minimize animal suffering and optimize experimental design.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL39918>.

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