Programmed Cell Death Ligand 1-Transfected Mouse Bone Marrow Mesenchymal Stem Cells as Targeted Therapy for Rheumatoid Arthritis

Qiong-ying Hu, Yun Yuan, Yu-chen Li, Lu-yao Yang, Xiang-yu Zhou, Da-qian Xiong, and Zi-yi Zhao

1Department of Laboratory Medicine, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072, China
2College of Medical Technology, Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072, China
3Department of Thyroid and Vascular Surgery, The Affiliated Hospital of Southwest University of Medical Sciences, Luzhou 646000, China
4Central Laboratory, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072, China
5TCM Regulating Metabolic Diseases Key Laboratory of Sichuan Province, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072, China

Correspondence should be addressed to Da-qian Xiong; 705006714@qq.com and Zi-yi Zhao; zhaoziyi925@163.com

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Programmed cell death ligand 1 (PD-L1) and its receptor (PD-1) are key molecules for immunoregulation and immunotherapy. PD-L1 binding PD-1 is an effective way to regulate T or B cell immunity in autoimmune diseases such as rheumatoid arthritis (RA). In our study, we overexpressed PD-L1 by constructing a recombinant of PD-L1-lentiviral vector, which was subsequently used to transfected mouse bone marrow mesenchymal stem cells (MBMMSCs) and significantly suppressed the development of collagen-induced arthritis (CIA) in DBA/1j mice. In addition, PD-L1-transfected MBMMSCs (PD-L1-MBMMSCs) ameliorated joint damage, reduced proinflammatory cytokine expression, and inhibited T and B cell activation. Furthermore, PD-L1-MBMMSCs decreased the number of dendritic cells and increased the numbers of regulatory T cells and regulatory B cells in joints of CIA mice. In conclusion, our results provided a potential therapeutic strategy for RA treatment with PD-L1-MBMMSC-targeted therapy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease characterized by synovitis in small- and medium-sized joints and joint damage and destruction that affects 1-2% of adults, and it is estimated that approximately 23.7 million people live with RA worldwide [1]. The current theory is that RA is involved in cross-talk between multiple systems and multiple cell types and results from interactions between activated Th1 and Th17 cells, the secretion of proinflammatory cytokines such as tumor necrosis factor (TNF) or interleukin (IL) by infiltrated macrophages, and B cell production of antibodies [2, 3]. These mediators or factors are potential targets for immunomodulatory or immunotherapy. With advances of immunosuppressants and biological drugs, patients with RA have relatively increased remission rates, although despite remission in 30–40% of patients, joint destruction still occurs in RA patients, and fundamental treatments for RA are still insufficient [4]. In addition to TNF inhibitors and other standard therapies, RA treatment involves targeting pathways, such as B7 family members (programmed cell death 1 ligand (PD-L1)) [5]. Preliminary studies confirmed that in mouse RA models, PD-1 or PD-L1 deficiency exacerbated RA [6,
2. Materials and Methods

2.1. Mice. Male 8-week-old DBA/1j mice were purchased from Beijing Hua Fu Kang Biological Technology Co., Ltd. (Beijing, China) and maintained in a pathogen-free animal care facility. All animal care and experimental procedures were performed according to the regulations of the Animal Care Committee of Chengdu University of Traditional Chinese Medicine.

2.2. Cell Lines. MBMMSCs were isolated from DBA/1j mice. Long bones of 4–6 weeks male mice were collected to harvest bone marrow. The hindlimbs of male mice were split by cutting off the knee. And the ends of bone were cut with a sharp pair of scissors. Be careful not to splinter the bones during the cutting process. Then, the bone marrow was washed with 1 ml syringe, and the washing solution was culture medium α-MEM. The cells were filtered through a 70μm strainer and collected with a 50 ml centrifuge tube. Bone marrow cells are diluted in complete culture medium (α-MEM, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) and cultured at 37°C with 5% CO₂. To confirm the MSCs, FACS were conducted to detect the feature of MSCs, such as CD105⁺ (MJ7/18, BD Biosciences, USA), CD73⁺ (TY/23, BD Biosciences, USA), CD90⁺ (55-2.1, BD Biosciences, USA), CD34⁻ (RAM34, BD Biosciences, USA), and CD11b⁻ (M1/70, BD Biosciences, USA) in Figure S1. Rat IgG2a κ Isotype (R35-95) and Rat IgG2b κ Isotype (A95-1) were also bought from BD Biosciences. MBMMSCs were maintained in UltraCULTURE™ medium (Lonza, USA).

2.3. Construction of the PD-L1-Lentiviral Vector. To amplify PD-L1 sequence, two pairs of primers were designed and synthesized based on the PD-L1 cDNA sequence (NM-00111283.2). The sequences both contained XbaI and NotI restriction enzyme site.

2.4. Virus Production. The PD-L1 gene was cloned into the lentiviral shuttle plasmid PCDH and 293T cells were cotransfected with the recombinant plasmid and the packaging plasmids PMD2.G and psPAX2 using the standard calcium phosphate precipitation method according to the Calcium Phosphate Cell Transfection Kit (Beyotime Bio-technology, China).

2.5. Transduction of MBMMSCs. MBMMSCs (1.0 × 10⁶) were seeded in a 6-well plate and incubated overnight. On the following day, the culture media was removed, and the MBMMSCs were transduced with 1 ml recombinant PD-L1-lentivirus at a multiplicity of infection (MOI) of 10 at 37°C, 5% CO₂. After 8 hours, the transduction media was replaced with fresh DMEM supplemented with 10% FBS at 37°C and 5% CO₂. After 24 hours, puromycin (4 μg/ml) was added to each well to screen the transduced MBMMSCs. After 3 days of screening, the growth morphology of the MBMMSCs was observed. The overexpression of PD-L1 was confirmed with anti-PD-L1 (MIH5, BD Biosciences, USA) by flow cytometry.

2.6. Animal Experiment. DBA/1j mice were immunized with bovine CII (Chondrex, Seattle, WA, USA) to establish a CIA model. When most of the mice showed features of CIA, the mice were randomly assigned to four groups as follows: (a) PBS (treated with 100 μl PBS), (b) MBMMSCs (treated with...
2.9. Real-Time PCR. The concentrations of these cytokines were calculated using the Rad CFX Connect platform using the SYBR Fast qPCR Mix according to the manufacturer’s instructions. The mouse primers (forward primer, 5′-TAGAATGAGGATATTTGCTGGC; reverse primer, 5′-TAGAATGAGGATATTTGCTGGC) to measure the expression of cytokines in joints. The primers were designed to target the specific regions of the cytokines of interest.

2.8. ELISA. The levels of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), interleukin 17a (IL-17a), and interleukin 17f (IL-17f) in mouse serum were measured by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). The levels of these cytokines were determined using a plate reader (Bio-Rad). The data are shown as the mean ± SEMs.

2.10. Histological Assessment. Joint tissues were obtained after sacrifice. Joint tissues were fixed in formalin/PBS for 24 hours and embedded in paraffin after decalcification.

3. Results

3.1. Construction and Characterization of PD-L1-Lentiviral Vector. PD-L1 (873 bp) was successfully cloned into the PCDH vector (7384 bp), and the recombinant lentiviral vector was named PCDH-PD-L1. The promoter was CMV/7 promoter and enzyme cleavage sites were XbaI and NotI (Figure 1(a)). FACS analysis was conducted to confirm the expression of PD-L1 in transfected MBMMSCs, and data showed that more than 90% of the cells were able to successfully express PD-L1 (Figure 1(b)).

3.2. PD-L1-MBMMSCs Alleviated Disease Progression in the CIA Model. We induced the CIA model as described previously, and a schematic diagram of CIA model establishment and treatment is shown in Figure 2(a). Joint clinical score was recorded to elucidate the effects of PD-L1-MBMMSCs. Compared with MBMMSC and Vector-MBMMSC treatment, PD-L1-MBMMSC treatment significantly inhibited the development of CIA and ameliorated the clinical score (P < 0.05) (Figure 2(b)). The photographs clearly showed that paws of the mice treated with PD-L1-MBMMSCs were less swollen than the paws of other mice on day 42 (Figure 2(c)). In addition, paw swelling was measured by slide gauge during the experiment. Compared with that in the PBS group, the thickness of the forelimb and hindlimb in the PD-L1-MBMMSC treatment group was significantly reduced (P < 0.01) (Figure S2A-B). At day 42 after treatment, the joints were collected to obtain ankle sections, which were then stained with H&E. The results showed limited cartilage damage, synovial hyperplasia, inflammatory cell infiltration, and bone erosion in the PD-L1-MBMMSC treatment group after CIA induction (Figure S2C-D). These findings reveal that both MBMMSCs and PD-L1-MBMMSCs can suppress the development of CIA and alleviate disease severity and that PD-L1-MBMMSCs are more effective than MBMMSCs.

3.3. PD-L1-MBMMSCs Regulated Immune Cells in the Spleen. Immune cells play vital roles in the pathogenesis of arthritis. To investigate the effect of PD-L1-MBMMSCs on
**Figure 1:** Generation and characterization of the PD-L1-lentiviral vector. (a) Schematic diagrams of the PD-L1-lentiviral vector containing PCDH cDNA and the therapeutic gene PD-L1. (b) FACS analysis of PD-L1 expression in MBMMSCs after transduction.

**Figure 2:** The effect of PD-L1-MBMMSCs on the development and pathological damage of CIA. (a) Schematic diagram of CIA model establishment and treatment. (b) Clinical scores of CIA mice after treatment with PBS, MBMMSCs, Vector-MBMMSC, and PD-L1-MBMMSCs. The data are shown as the means ± SEM (n = 10). *P < 0.05. (c) Joint appearance on day 42.
immune cells in the spleen, T cells, B cells, macrophages, and dendritic cells (DCs) in the spleen of CIA mice were analyzed by FACS. First, we measured total T cells, and there were no significant differences in the numbers of CD3+ T cells between the groups (Figures 3(a) and 3(b)). CD4+ T cells are essential for CIA induction and development. FACS analysis showed that the numbers of CD4+IFN-γ+ Th1 cells were decreased in the MBMMSC and PD-L1-MBMMSC groups (Figures 3(c) and 3(d)). Furthermore, CD4+IL-17a+ Th17 cells were reduced after PD-L1-MBMMSC treatment (Figures 3(e) and 3(f)). Regulatory T (Treg) cells are a class of T cells with immunomodulatory effects. In our study, the numbers of splenic CD4+FoxP3+ Treg cells in the PD-L1-MBMMSC-treated group were significantly higher than those in the other groups (Figures 3(g) and 3(h)). In addition to T cells, we also examined other lymphocytes, including B cells, macrophages, and DCs. CIA mice showed no significant decreases in active CD19+ B cells (Figures 4(a) and 4(b)).

Figure 3: PD-L1-MBMMSCs induce changes in T cells. FACS analyses of (a, b) CD3+ T cells, (c, d) CD4+IFN-γ+ Th1 cells, (e, f) CD4+IL-17a+ Th17 cells, and (g, h) CD4+FOXP3+ Treg cells in the spleen of CIA mice (n = 3). The data are shown as the means ± SEM (n = 3). * P < 0.05 and ** P < 0.01 versus PBS.
4(b)). The number of CD19^+IL10^+ regulatory B (Breg) cells in the spleen was similar to that of splenic Treg cells. Compared with PBS-treated mice, MBMMSC-treated mice and PD-L1-MBMMSC-treated mice showed dramatic recovery of the number of Breg cells (Figures 4(c) and 4(d)). No changes in macrophages were observed (Figures 4(e) and 4(f)), whereas PD-L1-MBMMSCs markedly decreased the number of CD11c^+ DCs (Figures 4(g) and 4(h)). These results suggest that PD-L1-MBMMSCs inhibit CIA development by regulating the numbers of T cells, B cells, and DCs in the spleen.

3.4. PD-L1-MBMMSCs Regulated Inflammatory Cytokine Production. Lymphocytes can secrete various cytokines, which have also been showed to have important roles in CIA pathogenesis. To assess whether PD-L1-MBMMSCs affected lymphocyte production of inflammatory cytokines, we examined serum expression levels of IFN-γ, TNF-α, IL-

![Graphs showing FACS analyses of CD19^+ B cells, CD19^+IL10^+ Breg cells, CD11b^+F4/80^+ macrophages, and CD11c^+ DCs in the spleen of CIA mice.](image-url)
1β, IL-6, IL-17α, and IL-10 each week. Proinflammatory factors (IFN-γ, TNF-α, IL-1β, IL-6, and IL-17α) were gradually reduced in the sera of mice treated with PD-L1-MBMMSCs compared with those of control-treated mice, and the levels of proinflammatory factors in the PD-L1-MBMMSC group were lower than those in the other groups (Figures 5(a)–5(e)). In contrast, the anti-inflammatory cytokine IL-10 was increased after PD-L1-MBMMSC treatment (Figure 5(f)). Cytokine levels were also measured in joint tissues by real-time PCR. Consistent with the serum analysis results, proinflammatory cytokine gene expression in joints was downregulated and anti-inflammatory cytokine gene expression was upregulated on day 42 in the PD-L1-MBMMSC group (Figures 6(a)–6(i)). These results indicate that PD-L1-MBMMSCs regulate inflammatory cytokines production in serum and joints.

4. Discussion

RA is a chronic autoimmune and systemic disease that mainly affects the joints. Long-term treatment results in approximately 40% of RA patients becoming resistant and
Figure 6: Continued.
developed in recent years [23, 24]. In this study, we modified a method for the treatment of arthritis that has been further developed recently [23, 24]. In this study, we modified MSCs with PD-L1 and demonstrated that PD-L1-expressing MSCs inhibited the development of arthritis in mice more effectively than unmodified MSCs.

MSCs isolated from the bone marrow, umbilical cord, or adipose tissue are pluripotent progenitors that can differentiate into cells that can form tissues, such as bone and cartilage. Studies have shown that mesenchymal stem cells can differentiate into osteoblasts during isolation and culture in vitro [25]. Moreover, MSCs exhibit immunosuppressive activity due to paracrine actions and interactions with different immune cells; in addition to their multilineage differentiation potential [26]. Our study showed that MBMMSCs could regulate immune cells in CIA mice, including T cells, B cells, and DCs. However, the regulatory effect of MBMMSCs was mainly associated with Th1 cells, Breg cells, and DCs. Additionally, PD-L1-MBMMSCs could affect Th17 cells and Treg cells at the same time. The levels of cytokines in serum and joint tissue were also assessed. MBMMSCs downregulated the levels of IFN-γ, TNF-α, IL-1β, IL-6, and IL-17a and upregulated the expression of IL-10. However, PD-L1-MBMMSCs exhibited a more significant regulatory effect on these cytokines than MBMMSCs, suggesting that the anti-inflammatory effect of MBMMSCs on CIA mice was enhanced after PD-L1 transfection.

Most autoimmune diseases are caused by dysfunction in the complex immune tolerance system. PD-1 is one aspect of this system [27]. It has been reported that PD-1 inhibits T cell proliferation and that PD-L1 and/or PD-L2 expressed by a variety of malignant tumor cells can mediate escape from host immunity by regulating T cells [28, 29]. A previous study confirmed that the synovium in RA can express PD-L1, PD-L2, B7-H3, and B7-H4 [30]. PD-L1 Fc administration increased PD-1 activity and inhibited T cell proliferation, leading to a reduction in arthritis. Consistent with previous research, our study proved that PD-L1 could decrease the numbers of Th1 and Th7 cells and upregulate the number of Treg in CIA mice. In addition to T cells, PD-1/PD-L1 also affects B cells and monocytes [31]. PD-L1-MBMMSC treatment resulted in a decrease in activated B cells and upregulation of Breg cells in this study. DCs were also suppressed by PD-L1 in CIA mice. Moreover, PD-L1 could significantly regulate the expression of cytokines in the serum and joints of CIA mice. Therefore, the PD-L1 pathway is a promising therapeutic target in RA.

In summary, we demonstrated that the administration of genetically modified MBMMSCs overexpressing PD-L1 improves the severity of experimental arthritis by not only suppressing autoimmune response to lymphocytes but also regulating cytokine production. Although MBMMSCs alone showed anti-inflammatory activity, their effects were weaker than those of PD-L1-MBMMSCs. Moreover, the antiarthritic effects on mice treated with PD-L1-MBMMSCs seem to be caused by the cumulative effects of the MBMMSCs themselves and PD-L1 secretion. These data provide new insights into the advantages of MBMMSCs as anti-inflammatory cells in RA therapy that can suppress the autoimmune response and deliver desirable genes, such as PD-L1, which is an effective strategy for RA treatment.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| PD-L1:       | Programmed cell death 1 ligand |
| PD-1:        | Programmed cell death 1 receptor |
| RA:          | Rheumatoid arthritis |
| MBMMSCs:     | Mouse bone marrow mesenchymal stem cells |
| CIA:         | Collagen-induced arthritis |
| PD-L1-MBMMSCs: | PD-L1-transfected MBMMSCs |
| TNF:         | Tumor necrosis factor |
| IL:          | Interleukin |
| ITSM:        | Tyrosine-based switch motif |
| Syk:         | Spleen tyrosine kinase |
| PI3K:        | Phosphatidylinositol 3-kinase |
| MSCs:        | Mesenchymal stem cells |
Data Availability

The initial data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

X.D., Z.Z., and Z.X. contributed in study design. H.Q. contributed in performing the experiment, and Y.Y. contributed in figure collection. Y.L. and L.Y. contributed in literature search.

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Supplementary Materials

Figure S1: the detection of the features of MSCs. FACS analysis of CD105^+, CD73^+, CD90^+, CD34^−, and CD11b^−. Figure S2: the state of the damage to the joint. (A, B) Forelimb and hindlimb paw thickness in the different groups. The data are shown as the means ± SEM (n = 10). **P < 0.01. (C) H&E staining of knee joint. The magnification is ×40. The scale bar is 200 μm. (D) Pathological scores of the knee joint. The data are shown as the means ± SEM (n = 3). *P < 0.05 and **P < 0.01 versus PBS. Supplementary Table 1: primer sequences for real-time PCR. (Supplementary Materials)

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