Stem Cells International

Stem Cell Therapy for Treating Autoimmune Diseases

Lead Guest Editor: Anand S. Srivastava
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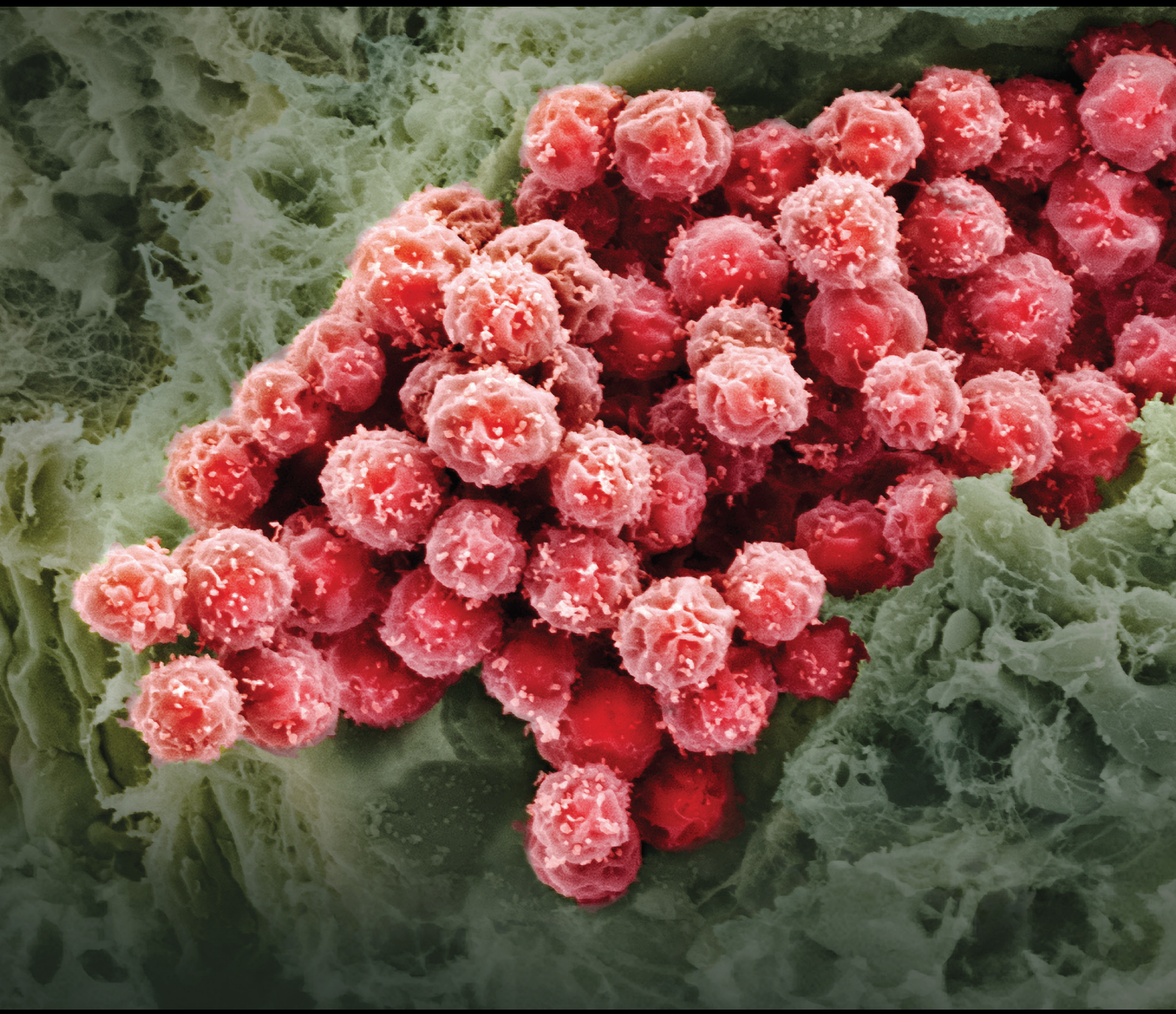

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Review Article

Stem Cell Therapy and Innate Lymphoid Cells

Divya Verma,[1] **Mukesh Verma**,[1] **and Rangnath Mishra**[2]

[1]*Division of Allergy & Immunology, Department of Medicine, National Jewish Health, Denver, USA*
[2]*Global Institute of Stem Cell Research and Therapy, San Diego, USA*

Correspondence should be addressed to Rangnath Mishra; rangnath@giostar.com

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Innate lymphoid cells have the capability to communicate with other immune cell types to coordinate the immune system functioning during homeostasis and inflammation. However, these cells behave differently at the functional level, unlike T cells, these cells do not need antigen receptors for activation because they are activated by the interaction of their receptor ligation. In hematopoietic stem cell transplantation (HSCT), T cells and NK cells have been extensively studied but very few studies are available on ILCs. In this review, an attempt has been made to provide current information related to NK and ILCs cell-based stem cell therapies and role of the stem cells in the regulation of ILCs as well. Also, the latest information on the differentiation of NK cells and ILCs from CD34+ hematopoietic stem cells is covered in the article.

1. Introduction

The innate immune responses are the first line of defense against invading pathogens and then initiate specific adaptive immune responses. These responses rely on the body's ability to recognize conserved features of pathogens even though those are not previously exposed to the host. This preliminary evolutionary defense strategy, relatively speaking, is the dominant immune response found even in plants, fungi, insects, and primitive multicellular organisms [1]. The major functions of the vertebrate innate immune system are for recruiting immune cells to the sites of infection through the production of chemical factors including specialized chemical mediators allied cytokines, activating the complement cascade to identify bacteria, activating cells to promote clearance of antibody complexes or dead cells, and identify and remove foreign substances present in organs, tissues, blood, and lymph, by specialized white blood cells. Functions like activation of the adaptive immune system through antigen presentation and acting as a physical and chemical barrier to infectious agents are also carried out by this system [2].

Innate lymphoid cells (ILCs), the major functional active component of the innate immune system, play important roles in fending off infections, and thereby, in controlling inflammation and diseases [2, 3]. ILCs, which lack Rag- (recombination-activating gene-) mediated antigen receptors' recombination ability [4, 5], are globally distributed throughout the body but more in the mucosal surface tissues [6]. These cells have the capability to communicate with other cell types of the body to coordinate the immune system functioning during homeostasis and during inflammation [4, 5, 7].

ILCs develop from the hematopoietic stem cells (HSCs) in several regulated steps (Figure 1). HSCs first differentiate into hematopoietic multipotent progenitors including common myeloid progenitors and lymphoid primed multipotent progenitors (LMPPs) [8, 9]. Next, LMPPs differentiate into common lymphoid progenitors (CLPs) and early innate lymphoid progenitors (EILPs). CLPs give rise to B cell and T cell precursors while EILPs give rise to natural killer cell precursors (NKPs) and to common helper ILC precursors (ChILPs) [10, 11]. CHILPs give rise to promyelocytic leukemia zinc finger (PLZF) expressing ILCPs [12, 13] which give rise to all ILC groups except NK cells or lymphoid tissue inducer (LTi) cells. ILCs can be recognized by their classical lymphoid cell-like morphology and by other biochemical characters which include none of the major surface molecules, designated as cell lineage marker negative (Lin^-); it can vary from species to species. In humans, the cell surface

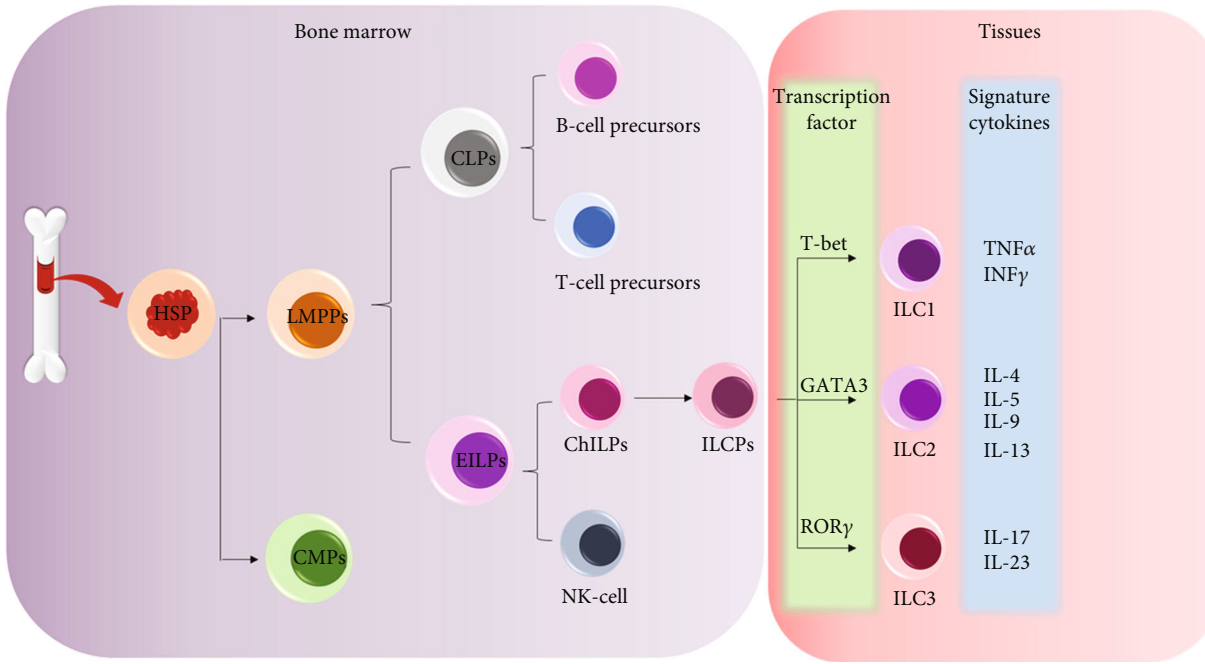

Figure 1: Differentiation of ILCs from hematopoietic stem cells (HSCs): HSCs differentiate into lymphoid-primed multipotent progenitors (LMPPs) and common myeloid progenitors (CMPs). LMPPs further differentiate into early innate lymphoid progenitors (EILP) and common lymphoid progenitors (CLPs). CLPs give rise to T and B cells whereas ELPs differentiate into common helper ILC progenitors (CHILPs) and NK cells. CHILPs further differentiate to produce PLZF-dependent ILC1s, ILC2s, and ILC3s.

markers are CD3, CD19, CD56, CD68, CD205, and FceR1 while in mice, these are CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/erythroid cells, NK1.1, and FceR1a. These cell surface molecules also help to recognize other immune cell types [5, 14, 15]. Based on the expressed cytokines and transcription factors, these cells are divided into three different ILC subsets [16, 17]. ILC1s are characterized by the expression of transcription factor T-bet and by the secretion of cytokines Th1 and IFN-γ. ILC2s, which express the transcription factor GATA-3 and produce Th2-type cytokines, respond to enormous extracellular pathogens. ILC3s, which express transcription factor RoRyt and produce Th17- and Th22-like cytokines, are required for host defense against extracellular bacteria and fungi. Though all ILCs are derived from HSCs but little is known about the role of ILCs in stem cell therapy and regulation of ILCs by stem cells during diseases.

2. NK Cells and Stem Cells

Natural killer (NK) cells, also known as large granulocytes, are specialized immune effector cells that play a critical role in immune activation against abnormal cells are also important for the immune surveillance. NK cells, which can be used for NK cell-based immunotherapy in cases of cancer, can be differentiated from stem cells from several sources like peripheral blood cells (PBCs), umbilical cord blood (UCB), embryonic stem cells (ESCs), CD34+ hematopoietic stem cells (HSCs), and induced pluripotent stem cells (iPSCs) [18]. However, the cells of different origins have their own limitations. For example, the abilities of the NK cells generated using allogeneic peripheral blood (PB) are donor-dependent and have heterogeneous activities indicating that the activities differ from donor to donor. UCB-derived NK cells also have donor-specific activities and are needed to be expanded before use. Recently, Goldenson et al. have reported that UMB and iPSC-derived natural killer cells have differences in their cytotoxic activity and KIR profiles [19], which is a very important information for the selection of the most effective NK cell populations for therapeutic purposes. iPSCs are also efficiently differentiated to produce mature NK cells (iPSC-NK cells) [20, 21], and these can be easily engineered by existing genome editing tools. Thus, iPSCs provide an important platform to produce NK cells with an improved population of homogenous cells having anti-tumor activity, a property extremely desired for treating solid tumors [21–23]. Studies have identified novel NK cell-specific chimeric antigen receptors (CARs) that mediate improved killing of ovarian cancer cells both in vitro and in vivo [21]. iPSCs used to create an unending source of induced pluripotent stem cell-derived NK (hnCD16-iNK) cells of human origins which have improved antibody-dependent cell-mediated cytotoxicity (ADCC) and are efficient in the killing of cells of solid tumors as well as those causing hematologic malignancies (Figure 2) [23]. CD16, a molecule of the immunoglobulin superfamily (IgSF) involved in antibody-dependent cellular cytotoxicity (ADCC), is found on the surface of natural killer cells. However, recently, some researchers generated a triple-gene-edited induced pluripotent stem cell (iPSC) [24]. They engineered a clonal iPSC line to express a high-affinity, non-cleavable version of the Fc receptor CD16a and a membrane-bound interleukin- (IL-) 15/IL-15R fusion protein. Their third edit was a knockout of the ectoenzyme

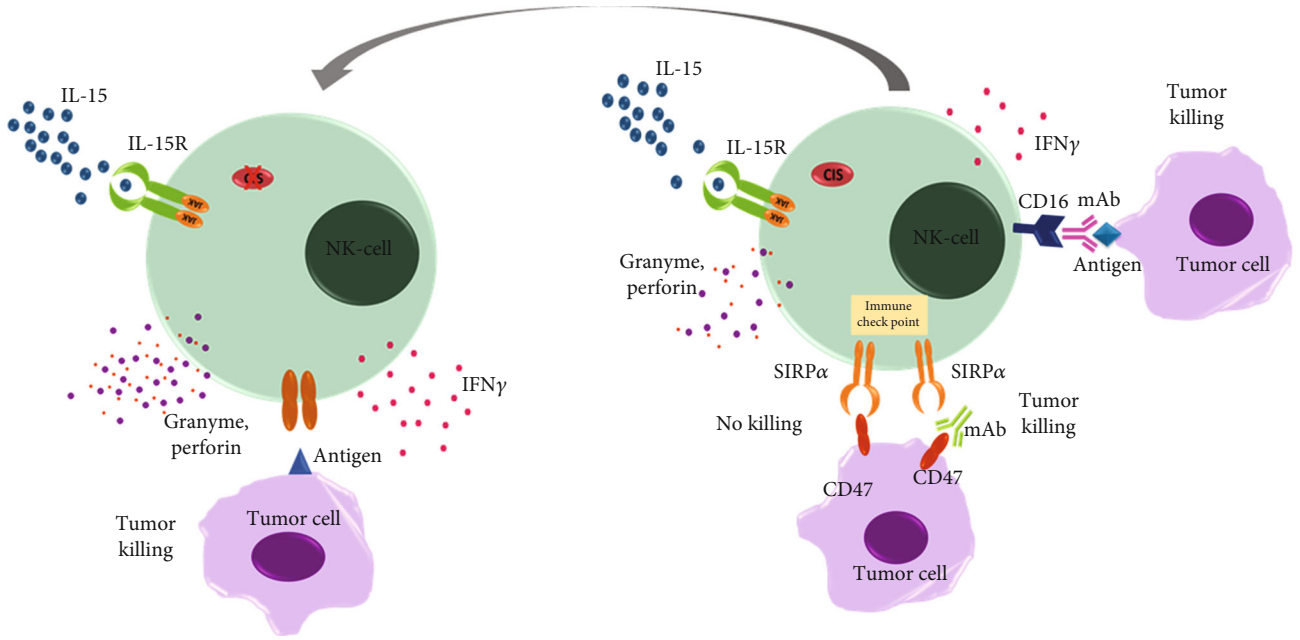

FIGURE 2: NK cell-mediated cell toxicity: NK cells can be derived from different sources like PB, UCB, ESCs, and CD34+ HSCs, and also from induced pluripotent stem cells (iPSCs). In the presence of CIS, NK cell cytotoxicity is blocked by inhibition of IL-15 and JAK/STAT signaling pathways. In the absence of CIS, NK cells produce more granzyme B and IFN-γ which are involved in tumor eradication. Antibody-mediated blocking of CD47 and CD16 is important for tumor killing; otherwise, they interact with their receptors and send "do not eat me" messages to NK cells and tumors continue to grow.

CD38, which hydrolyzes NAD+. Natural killer (NK) cells derived from these uniformly engineered iPSCs, termed iADAPT, displayed metabolic features and gene expression profiles mirroring those of cytomegalovirus-induced adaptive NK cells. iADAPT NK cells persisted in vivo in the absence of exogenous cytokine and elicited superior antitumor activity [24]. These findings suggest that unique subsets of the immune system can be modeled through iPSC technology for the effective treatment of patients with advanced cancer [1]. Although a lot of studies have been done related to the efficacy and generation of more potent NK cells, very few studies pointed to the role of NK cells in hematopoietic stem cell transplantation (HSCT). A recent study suggested that NK cells negatively regulate the number and function of transplanted HSCs in both humans and mice, and it works in a dose-dependent manner which is mediated by IFN-γ. In the same study, it was demonstrated that this negative effect of NK cells was restored by depletion of NK cells or by blocking the IFN-γ signaling [2, 25]. Zhu et al. reported that deletion of cytokine-inducible SH2-containing protein (CIS; encoded by the gene CISH) in human iPSC-derived NK cells promoted the expansion of NK cells and increased the cytotoxic activity against multiple tumor cell lines when maintained at low concentrations of cytokine interleukin-15 (IL-15) (Figure 2) [26]. Higher concentrations of IL-15 itself are known to cause toxicities [27, 28]. CIS, belonging to the suppressor of cytokine signaling (SOCS) protein family (other members: SOCS 1–7) [29], negatively regulates IL-15 signaling in NK cells and acts as a checkpoint to regulate NK cell-mediated antitumor immunity in mice [30]. IL-15 is well known to stimulate several functions of the NK cells like differentiation, proliferation, activation, and survival [26, 31–33] and cell metabolism [34–36]. Deletion of CISH in human iPSC-NK cells has been reported to improve metabolic fitness, especially glucose-driven glycolysis and oxidative metabolism which is required for NK cell antitumor and antiviral effector functions [37–40]. However, a study showed that transplantation of mesenchymal stem cells (MSCs) inhibited NK cell proliferation, as well as their cytotoxicity and cytokine production [41].

Signal regulatory protein α (SIRPα), a regulatory membrane glycoprotein belonging to the SIRP family, acts as an inhibitory receptor. It is mainly expressed by myeloid, neurons, and stem cells, and interacts with a transmembrane protein CD47 which is also known to send the "do not eat me" signal. SIRPα and CD47 interactions negatively regulate the effector function of innate immune cells such as host cell phagocytosis. The available body of literature suggests that NK cells do not have SIRPα checkpoint in normal healthy individuals. However, Deuse et al. reported that cells from patients had a higher expression of SIRPα on NK cells. SIRPα, upregulated by IL-2 stimulation, interacts with target cell CD47 in a threshold-dependent manner and counters other stimulatory signals by a number of molecules including IL-2, CD16, or NKG2D. Deletion of SIRPα or antibody-mediated blockade augmented the killing capacity of NK cells (Figure 2). They also observed SIRPα–CD47 was highly species specific because the overexpression of rhesus monkey CD47 in human MHC-deficient cells prevented cytotoxicity by rhesus NK cells in a xenogeneic setting. This result suggested that elevated expression of CD47 may prevent NK cell–mediated killings cell death in allogeneic and xenogeneic tissues [42].

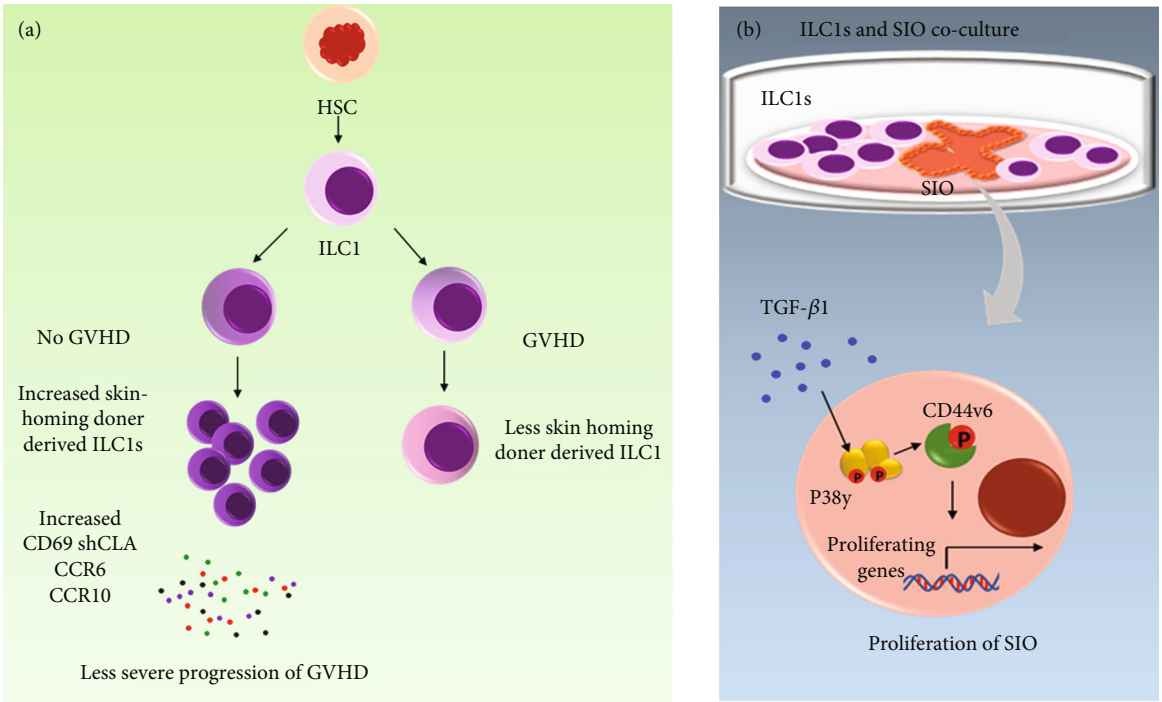

Figure 3: ILC1s in graft versus host disease (GvHD) and in small intestine organoids (SIO): (a) Increase in the expression profiles of ILC1 derived homing markers is an indication of no GvHD whereas their low expression is a marker of progression of severe GvHD. (b) In the coculture conditions, the ILC1s drive expansion of the intestinal epithelial stem cells through TGFb1-p38γ-induced phosphorylation, induce CD44v6 expression, and thereby, SIO proliferation.

3. ILC1 and Stem Cells

Both ILC1 and NK cells originate from the same cell lineage, and later, they diverge early in their developmental course. These cells can be differentiated on the basis of the expressed transcription factors, their cytotoxicities, and by the expression of their resident markers. Unlike NK cells, which are cytotoxic, circulating in the bloodstream, killing virus-infected cells, and tumor cells, ILC1s are noncytotoxic or weakly cytotoxic tissue resident cells which function in the defense against infections like viruses and certain bacteria. Being tissue resident, these cells play a major role in graft versus host disease (GVHD) also. The role of ILC1s in GVHD after hematopoietic stem cell transplantation (HSCT) was first time reported by Munneke et al., in 2014 [43]. They monitored the reconstitution of the ILC population after HSCT in 51 acute myeloid leukemia (AML) patients. Their findings suggest that patients who did not develop GVHD had an increased level of skin-homing of donor-derived ILC1s after the HSCs transplantation in PB in comparison to those who developed GVHD. It was further associated with increased expression of activation marker CD69, skin homing CLA (cutaneous leucocyte-associated antigen), and chemokine receptors CCR6 and CCR10 which correlated with less severe progression of GVHD (Figure 3(a)) [43]. However, the functional role of ILC1s could not be ascertained in this study. In 2021. Piperoglou et al. studied the ILC population reconstitution after HSCT in adult and young patients [44]. They found that ILC1 levels in adult patients before the HSCT were the same as that in the healthy controls [44]. However, it increased after the transplant and took one year to come back to the normal range in recipients with the non-GVHD group, whereas in the children it took only 6 months. They have also found a similar association with the homing markers as above mentioned [43, 44]. Both studies suggested that ILC1 population reconstitution was not affected by graft origin or by the amount of CD34 stem cells in the graft. The decrease in the level of circulating ILC1 was associated with the occurrence of GVHD, so that monitoring of ILC1 before HSCT and after reconstitution might be a useful prognostic marker for assessing the patient's risk of developing GVHD. ILC1 accumulation in IBD patients has been reported by Bernink et al. [45]. Jowett et al. studied the distribution of ILC1 in intestinal epithelium in inflammatory bowel disease (IBD) patients. For determining the role of ILC1s, they developed a reductionist coculture system with murine small intestine organoids (SIO). Findings from these coculture conditions revealed that ILC1s drive expansion of the intestinal epithelial stem cells through p38γ phosphorylation, which onwards induced CD44v6 expression and SIO proliferation. p38y phosphorylation was induced by TGF-1 secreted by ILC1s (Figure 3(b)) [46]. This finding suggests that ILC1s play a role in intestinal remodeling, which could exacerbate IBD-associated comorbidities when enriched in inflamed intestines. Recently, Bai et al. established the adult mouse liver like fetal liver contained Lin- Sca-1$^+$Mac-1$^+$ (LSM) cells, which were in significantly higher frequencies than in adult bone marrow (BM), PB, and small intestine lamina propria (siLP). They showed that adult liver

containing Lin–Sca-1^+Mac-1^+ hematopoietic stem cell (LSM HSC) population was capable to differentiate into tissue-resident liver ILC1s. The study also indicated that IFN-γ produced by mature ILC1s also promoted the expansion and differentiation of LSM HSCs into ILC1s but not into NK cells [47]. Further studies are needed to explain why IFN-γ derived from these ILC1s support LSM HSC differentiation into ILC1s but not into NK cells. This finding reveals the involvement of extramedullary hematopoiesis to a distinctive regional immune feature within the liver. Though it is not directly related to transplantation, but it is important to know that other groups have been identified umbilical cord blood-derived ILC1-like cells constitute a novel precursor for mature KIR+ NKG2A- NK cells [48]. NK cells derived from ILC1-like cells exhibited key NK cell effector functions including mobilization of cytotoxic granules, the killing of the HLA-deficient target cells, and CD16-mediated ADCC. This finding indicates that this novel ILC1 might be a useful target for NK cell-based stem cell therapy.

4. ILC2 and Stem Cells

ILC2s are tissue resident cells involved in the innate response to parasites, such as helminths, and help repair tissue damages. These cells, abundant in tissues like the skin, lung, liver, and gut, are characterized by the production of amphiregulin, and type 2 cytokines including IL-4, IL-5, and IL-13 in response to IL-25, TSLP, and IL-33. Because of their specific cytokine signature, they are considered the innate counterparts of Th2 cells. These cells express characteristic surface markers and receptors for chemokines, which are involved in the distribution of lymphoid cells to specific organ sites. The humans ILC2s expresses CRTH2, ICOS, KLRG1, ST2, CD161, CD127, and CD25 and requires IL-7 for their development [49] and activation of the fundamental transcription factors RORα and GATA3 for the maintenance of their functions. On the other hand, GATA3 deprivation inhibits the development and function of these cells. ILC2s are further classified into subpopulations named natural ILC2s (nILC2s) and inflammatory ILC2s (iILC2s), according to their patterns of response to IL-33 and IL-25 [50]. nILC2s respond to IL-33 in tissues in a natural immune state, while iILC2s respond to IL-25 or to the helminth parasites. Further, nILC2s express more Thy1 and ST2 and have reduced levels of KLRG1 [51]. On the other hand, iILC2s express more KLRG1 and have reduced levels of Thy1 and ST2. In addition, another subpopulation of ILC2s, named as the $ILC2^{10}$ cells, is characterized by their ability to produce IL-10 [52]. ILC2s are also characterized as memory ILC2s which generated a memory response after re-exposure to the allergens and produced type 2 cytokines as well as inflammation [15].

Allogeneic stem cell transplant (allo-SCT) has been used to provide curative therapies for patients with lymphoid malignancies (LM), high-risk acute leukemia, and other malignant diseases [53–55]. Despite the improvements in HLA typing and stem cell donor choices, GVHD remains the major complication of allo-SCT in 30% to 80% of transplanted recipients [56, 57]. Both preclinical transplant models and clinical transplant studies have focused on role for T cells in the pathophysiology of GVHD. Studies on the role of ILCs demonstrated that reduced numbers of circulating $CD69^+$ ILC2s were associated with the increased risk of a GVHD in AML patients (22). Bruce et al. studied behavior and role of the ILC2s in the GI track. Their findings demonstrated that when ILC2s were distributed in the GI tract but not in the lung the patients were highly sensitive to conditioning therapies (both chemotherapy and radiation) prior to allo-SCT. More importantly, the study demonstrated that there was a quite limited repopulation of ILC2s from the donor bone marrow in the GI tract. They examined the effects of chemotherapy on ILC2s and found that these were significantly reduced within 24 hours of cyclophosphamide treatment in the LP and MLN. Subsequently, they evaluated the reconstitution of donor and recipient ILC2s for 4 weeks after the allo-SCT and recorded a very reduced number of donor and/or recipient ILC2s in the LP of recipient mice who received irradiation and the bone marrow without donor T cells. The decrease in ILC2s in the LP at day 28 was more as compared to day 1. Cotransplantation of activated ILC2s reduces GVHD and increases recipients' survival due to the production of Th2-cytokine IL13 which suppresses the production of donor T cell proinflammatory cytokines IFN-γ and IL17A. These studies strongly suggest that expanded ILC2s may be a potent cellular therapy for the treatment of the lower GI tract GVHD [58]. Recently, it has been reported that third-party ILC2s prevent and treat gastrointestinal (GI) tract GvHD [3, 59]. Previously, it has been reported that an elevated level of soluble ST2 (IL33 receptor) in patients was associated with insufficiency of therapeutic response for GVHD on day 28 and increased mortality after 6 months following the allogeneic stem cell transplantation [60, 61]. The increased levels of ST2 were due to the loss of ILC2s in the GI tract, and it would respond to IL-33 caused inflammations. Cotransplantation of ILC2 decreases the ST2 level in the GI tract as well as the accumulation of IFN-γ– and IL-17–producing T cells and diminishes pro-inflammatory environment [58]. Another study reported that ILC2s promote the self-renewal of intestinal stem cells through IL-13 secretion which initiated the expression of Foxp1 and activated the β-catenin pathway [62]. This finding suggests that ILC2s, analogous to ILC3s, may also contribute to epithelial regeneration in the gut and GvHD prevention.

Chemotherapy-induced cell cycle distress cusses' activation of hematopoietic stem and progenitor cells (HPSCs) and promotes bone marrow (BM) regeneration. The role of ILC2 in the recovery of HSPCs from 5-fluorouracil– (5-FU–) induced stress was unknown. However, now we know that this is due to the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) by ILC2s. GM-CSF knockout mice treated with 5-FU failed to recover the population of HSPCs which causes severe loss of myeloid lineage cells and lethality, these mice were rescued by transferring BM ILC2s from wild-type mice. This finding might be useful for patients who have recovery problems related to HSPCs during chemotherapy [63].

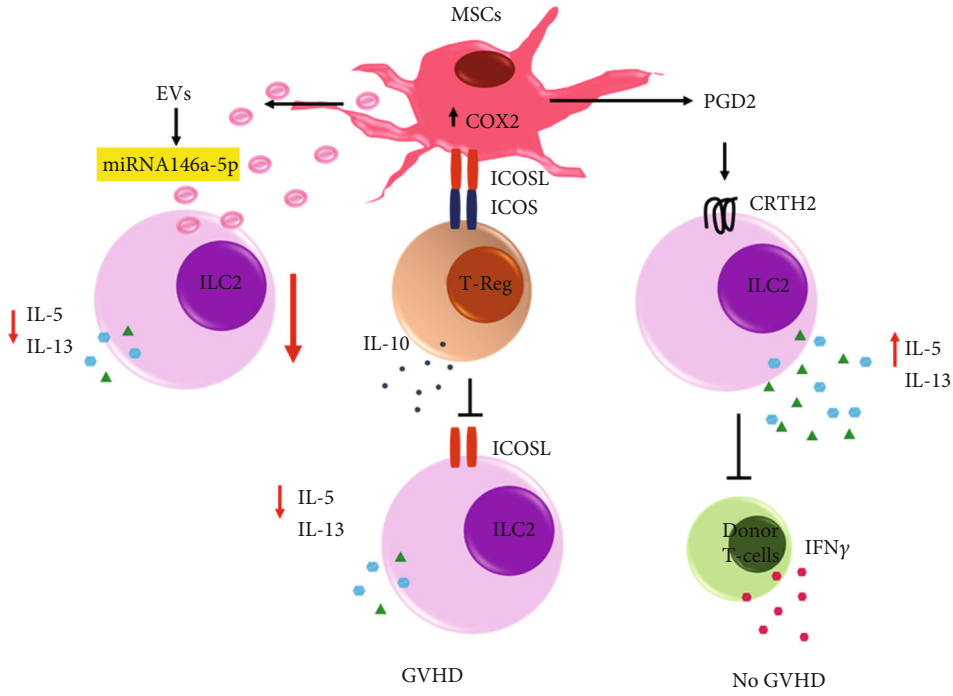

Figure 4: ILC2s and mesenchymal stem cells (MSCs) in GvHD and inflammation: MSCs express cyclooxygenase and prostaglandin D2 (PGD2), a ligand for prostaglandin D2 receptor 2, which binds to ILC2 and induces production of type 2 cytokines and inhibits IFN-γ production. MSCs also bind with Treg and induce the expression of IL10 which inhibits ILC2 and progression of GvHD. MSC-derived small extracellular vesicles (sEV) express miR146a-5p which inhibits ILC2 and production of type 2 cytokines.

Stem cell factor (SCF), a cytokine which binds to the receptor tyrosine kinase (c-Kit), is expressed on several myeloid and lymphoid cell types including Type 2 innate lymphoid cells (ILC2). SCF has been known to play roles in the survival of HSCs as in vivo it contributes to the self-renewal and maintenance of HSCs [64]. The importance of the SCF/c-Kit interaction in ILC has been further reported by Fonseca et al., and they found significantly increased SCF in the serum of both asthmatic patients as well as in the mouse model of the disease in comparison to the non-asthmatic controls. Further analysis revealed that the expression of SCF248 isoform was overexpressed in the lungs of allergic mice, whereas the expression level of the SCF220 isoform was unaltered when compared to naive mice [65]. Therefore, they concluded that the higher circulating levels of SCF in the serum were due to the increased expression of SCF248 in the lungs. Mice treated with SCF248 monoclonal antibody had mitigated the development of chronic asthmatic symptoms by decreasing the number of mast cells, ILC2s, and eosinophils, as well as by reducing the associated pathogenic cytokine responses [65]. To know the effects of SCF on the ILC2s, they stimulated the sorted ILC2 cells with recombinant murine SCF (rSCF). They observed that the cells stimulated with SCF had significantly increased expression of inhibitor of DNA binding 2 (ID2) and GATA3 [65]. Both transcription factors are important for the maintenance and differentiation of the ILC2 cells. This data suggested that the SCF is contributing to the development and maintenance of ILC2 phenotypes. Next, they studied the activation role of SCF on ILC2 effector functions and found that rSCF-stimulated sorted cells had increased levels of type 2 cytokines as well as of ILC2 expressed receptors IL9R and IL17RB (IL25R) [65].

Recent publications show that stem cells play an important role in the regulation of ILC2s. Findings on ILC2s isolated from both healthy or allergic rhinitis patients cocultured with pluripotent stem cell-derived MSCs (iPSC-MSCs) in the presence of IL25+IL33 showed decreased levels of type 2 cytokines IL5+, IL13+, and IL9 + demonstrate that stem cells inhibit the effector functions of ILC2s (Figure 4). In a subsequent study, they found that this inhibitory effect of MSCs on ILC2s was mediated by the regulatory T (Treg) cells which were regulated through ICOS-ICOSL interactions (Figure 4) [66]. Previously, it has been reported that MSCs from acute myeloid leukemia (AML) patients or from normal individuals overexpressing COX2 had elevated secretion of prostaglandin D2 (PGD2). PGD2 is a ligand for chemoattractant receptor homologous (CRTH2) receptor which is expressed by the ILC2 and Th2 cells. PGD2-CRTH2 interaction is important for the activation and function of ILC2s and production of type 2 cytokines. Type 2 cytokines are important for the inhibition of donor IFN-γ which produce by the donor Th1 cells and causes GVHDs (Figure 4). PGD-mediated activation of the ILC2-Treg axis is involved in the proliferation of normal and malignant HSPCs [66]. Recently, it has been reported that neuromesenchymal stem cells regulate ILC2 function and activation in obesity via a brain–adipose circuit [4, 67].

Unusual or abnormal actions of ILC2s have been recorded in certain pathological conditions. Stem cells or derived small extracellular vesicles are being tried to counteract certain ILC2-mediated pathological conditions. The effectiveness of small extracellular vesicles (sEV) derived from iPSC-MSCs on patients with allergic rhinitis and in mouse ILC2-dominant asthma model was reported by Fang et al. [68]. They developed a standardized scalable protocol of anion exchange chromatography for isolation of MSC-sEV and identified MSC-sEV by flow cytometry which expressed surface markers CD9/CD63/CD81 but did not express the general markers of MSCs like CD44, CD146, CD73, CD90, and CD105 [68]. To evaluate the effects of MSC-sEV on the functions of ILC2s, they isolated human PBMCs from patients with allergic rhinitis, cultured, and then activated them with IL-2/25/33 in the presence or absence of MSC-sEV. Cultured of PBMCs or sorted ILCs in presence of MSC-sEV had reduced expression of IL9+ and IL13+ ILC2 as compared to the control. Administration of MSC-sEV in a mouse model of asthma showed reduced levels of ILC2, type 2 cytokine expression, inflammatory cell infiltration, and mucus production in the lung and had alleviation in the airway hyperresponsiveness [68]. RNA sequencing analysis revealed that MSC-sEV had highly enriched miR-146a-5p, known to inhibit the function of activated ILC2 [69, 70]. So, they concluded that the inhibition of ILC2s, type 2 cytokine production, and airway inflammation in presence of MSc-sEV was mediated by miR-146a-5p (Figure 4). The findings suggest that MSC-sEV could be a novel cell-free strategy for the treatment of allergic, inflammatory, and asthmatic disease. Recently two subsets of ILC2s were identified in islet allografts of IL-33-treated mice: IL-10 producing ILC2s ($ILC2^{10}$) and non-IL-10 producing ILC2s (non-ILC^{10}). Intravenous transfer of $ILC2^{10}$ cells, but not non-ILC^{10}, prolonged islet allograft survival in an IL-10-dependent manner. Locally transferred $ILC2^{10}$ cells led to long-term islet graft survival, suggesting that $ILC2^{10}$ cells are required within the allograft for maximal suppressive effect and graft protection. This study has uncovered a major protective role of $ILC2^{10}$ in islet transplantation which could have the potential to be used as a therapeutic strategy [71].

5. ILC3 and Stem Cells

ILC3s constitute a group of cells imparting innate immunity which participates in defense mechanisms to mucous membranes or mucosa and represents a defense mechanism against extracellular parasites, bacteria, and fungi. These cells are involved in the maintenance of intestinal microorganisms-host homeostasis and in the regulation of host-commensal mutualism. ILC3s generally are characterized by the presence of surface markers Lin- CD127+ CD3-, transcription factor RoRyt, signature cytokines IL-17 (Th17) and IL22 (Th22 cells), and chemokine receptor CCR6. Initially, ILC3s were divided into two subsets: lymphoid tissue inducer cells- (LTi-) like ILC3s and natural cytotoxicity receptor (NCR) ILC3s (NKp46 in mice and NKp44 in humans) which are developmentally, phenotypically, and functionally different [4, 5, 16]. The sets of surface markers in different sets of ILC3s are species-specific. Lti-like ILC3 express LIN-, CD1a-, CD11c-, CD34-, CD123-, BDCA2-, FcεRI-, TCR$\alpha\beta$-, TCR$\gamma\delta$-, IL-7Rαhi, CD45int, RORγt (also CD4-, CD94-, CD7, nd CD161) in humans, and produce signature cytokines LTα, LTβ, IL-17A, IL-22 [4, 13, 72]. NCR+ ILC3 cells are further divided into two subtypes, i.e., NKp46+ ILC3 and NKp44. NCR+ ILC3 express LIN-, CD1a-, CD11c-, CD34-, CD123-, BDCA2-, FcεRI-, TCR$\alpha\beta$-, TCR$\gamma\delta$-, CD56, IL-7Rα, NKp30, NKp44, NKp46, AHR, and RORγt and produce cytokine IL22 [4, 13, 72]. On the other hand, NCR-ILC3 express CCR6, CD4-, CD16-, CD94-,c-kit, IL-7Rα, NKG2D-, NKp44lo/-, NKp46-, RANKL, AHR, and RORγt and cytokines IFN-γ, IL-17A, IL-22, and TNF-α [4, 13, 72]. The ability of ILC3 cells to promote tissue repair, maintain the tissue integrity, and defend against pathogens could be useful in patients receiving HSCT or those having severe GVHD. It has been reported that a transient increase in the levels of circulating NCR^+ ILC3 correlates well with reduced incidences of GvHD [43]. It has been shown that $CD34^+$ cells, used as a source of hematopoietic precursors in HSCT, derived from different sources including BM, PB of G-CSF-mobilized in donors or from umbilical cord blood (UCB), generate NK and ILC3 cells in different proportions. ILC3s are more represented in the lymphoid progenies of $CD34^+$ precursors derived from UCB or BM. In addition, a negative effect on the ILC3 generation is exerted by G-CSF, as shown by *in vitro* studies [73]. IL-1β has been shown to affect the differentiation of $CD34^+$ precursors towards ILC3, favoring NK cell development, suggesting that inflammatory responses may interfere with ILC3 generation [74, 75].

ILC3s regulate intestinal stem cell maintenance and subsequently help tissue repair in cases of acute insults. ILC3-produced IL-22 was considered to be important for stem cell protection [76]. However, it was recently reported that ILC3-driven epithelial proliferation and tissue regeneration are independent of IL-22. It has been demonstrated that ILC3s amplify the magnitude of Hippo-Yap1 signaling in intestinal crypt cells, ensuring adequate initiation of tissue repair, and preventing excessive pathology. These findings reveal that ILC3-driven intestinal repair entails distinct transcriptional networks to control stem cell maintenance and epithelial regeneration, which implies that tissue repair and crypt proliferation can be influenced by targeting innate immune cells in addition to the well-established effects of IL-22. The ILC3-driven tissue repair is Stat3 independent which involves activation of Src family kinases [77].

Kang et al. in 2020 reported the protective role of type 3 $NKp44^+$ILCs (ILC3s) which are significantly diminished in newly transplanted allografts in comparison to allografts after 6 months, where pro-inflammatory type 1 $NKp44^-$ILCs (ILC1s) were higher in intestinal transplants [78]. Moreover, serial immune monitoring revealed that in healthy allografts, protective ILC3s repopulate between 2 and 4 weeks postoperatively, but in allografts being rejected they remain diminished. $NKp44^+$ILC3 cells produce protective interleukin-22 (IL-22), whereas ILC1s produce proinflammatory interferon-gamma (IFN-γ) and tumor necrosis

factor-alpha (TNF-α). Intestinal grafts carry a large donor lymphoid load that is replaced by the cells of the recipient. The dynamics of this process may influence the tolerance, rejection, or graft-versus-host disease. Gómez-Massa et al. analyzed the distribution and turnover of T and B (Lin+) lymphocytes, natural killer (NK), and helper innate lymphoid cells (hILC) in the intestinal epithelium (IEp) and lamina propia (LP) from a long-term cohort of eight intestinal recipients and from a single patient monitored closely during the first 8 months posttransplant (posTx) [79]. Long-term intestinal grafts showed significantly higher %hILC than native bowels in IEp and LP until 10 years posTx and recovery to normal levels was observed afterward. They also observed an imbalance between hILC subsets in IEp (increase in ILC1s and decrease in ILC3s) that persisted along posTx time even when %hILC was like native bowels. Regarding hILC origin, they detected the presence of donor cells even 13 years posTx. However, this chimerism was significantly lower than in Lin+ and NK populations. The findings based on the observations from the patients monitored in the early posTx period showed that recipient hILC repopulate earlier and faster than Lin+ cells. The increases in ILC1s are often related to rejection and infection episodes [79]. This finding shows that ILCs might play a key role in the regulation of intestinal transplant graft homeostasis and could serve as sentinels for early recognition of allograft rejection and, therefore, could be a target for future therapies.

6. The Generation of NK Cells and ILCs from CD34+HSC

The most intriguing aspect of cancer therapy has been the resistance developed in the cancer cells to the drugs. Recent advances in the field of immunotherapy, in which NK cells play a very important role, have offered hope to cancer patients. This innovative approach is based on the idea of harnessing specific cells of the immune system to target tumor cells. The NK cell-based immunotherapy has emerged as a promising therapeutic approach against solid tumors and hematological malignancies. These cells are innate lymphocytes with an array of functional competencies, including anticancer, antiviral, and antigraft-vs.-host disease potentials. For this purpose, the NK cells are isolated and propagated. To achieve this, $CD34^+$ cells isolated from BM, PB, or UCB are cultured with SCF, FMS-like tyrosine kinase ligand (Flt3-L), IL-7, and IL-15 Cytokine-Mix (hereinafter, referred to as c-Mix), and analyzed by flow cytometry (FC) at different time intervals [80]. It has been noticed that the differentiation pattern of the cell population varies according to the source tissues they are isolated from. Generally, in these cells, CD34 expression progressively decreases during the in vitro culture. Oberoi et al. reported a refined approach based on ex vivo culture of PB-$CD34^+$ cells with optimized cytokine cocktails that reliably generate functionally mature NK cells, as assessed by analyzing NK-cell-associated surface markers and by cytotoxic activity. To further enhance NK cell expansion, they generated K562 feeder cells coexpressing 4-1BB ligand and membrane-anchored IL-15 and IL-21. Coculture of PB-derived NK cells and ex vivo differentiated from HSCs NK cells with these feeder cells had dramatically improved NK cell expansion [81]. This finding suggested mobilized PB-$CD34^+$ cells expanded and differentiated according to this two-step protocol as a promising source for the generation of allogeneic NK cells for adoptive cancer immunotherapy.

In stem cells of BM origin, $CD19^+$ cells are present while no $CD3^+$ cells are detected. At day (d) 0, PB HSCs have $CD33^+$, $CD13^+$, and $CD115^+$, suggesting their commitment towards the myeloid lineage. However, after 10 days, cells from all sources in cultures contain $CD33^+CD14^+$ cells which become predominant by d 30. Around day 10, lineage $(lin)^-CD56^+CD161^+$ ILCs are detectable only in BM cultures which increase up to 50% of cells by day 30, while they appear in PB and UCB cultures only at later time points. Notably, although the percentages of ILCs are significantly higher in BM cultures, BM HSCs display the lowest expansion rate. Thus, when considering the absolute numbers of $CD56^+CD161^+$ ILCs, UCB cultures give the highest recovery. Further, analysis of expressed transcription factors on CD56 and CD161 antigens expressing cells, the antigens expressed by both NK cells and ILC3s, demonstrate that the majority of $CD56^+CD161^+$ cells expressed receptor activator of nuclear factor kappa-B ligand (RANKL) and RORγt, thus representing ILC3s [82].

Upon stimulation, the $CD56^+CD161^+$ cells mainly produce ILC3s while the percentage of IFN-γ-producing cells, i.e., NK cells, remains low. Thus, $CD34^+$ cells from BM and UCB HSCs display a better capability of in vitro differentiation towards ILCs than those from PB. It is conceivable that the preferential myeloid commitment of PB $CD34^+$ cells may be a consequence of G-CSF-induced mobilization [73]. NK cells, also a component of the innate immunity system, are innate lymphocytes characterized by the expression of nuclear factor interleukin 3 regulated (NFIL3 or E4BP4), eomesodermin (EOMES) transcription factors (TFs), and by the ability to exert cytolytic activity, and also by the ability to release IFN-γ. In the haploidentical hematopoietic stem cell transplantation (haplo-HSCT) settings, donor-derived CD34+ NK cells play a major role in the control of leukemic relapses. Therefore, it is of utmost importance to define the cytokines that influence NK cell differentiation from CD34+ precursors. A recent study on the analysis of the effects of IL-1β on NK-cell differentiation of umbilical cord blood (UCB) CD34+ cells revealed that while IL-1β inhibited CD161+CD56+ cell proliferation, it increased expression of LFA-1, CD94/NKG2A, KIRs, and perforin in CD56+ cells. In addition, within the CD161+CD56+IL-1RI+LFA-1 cell fraction (representing group 3 innate lymphoid cells, ILC3-like cells), a significant increase in the levels of EOMES, NKp46, and CD94/NKG2A receptors, cytolytic granules, and IFN-γ was detected. This increase paralleled the decreases in the levels of related orphan receptors (RORγt) TF, NKp44 expression, and IL-22 production. The data suggest that IL-1β inhibits ILC3s while favoring NK cell maturation. Since in haplo-HSCT conditioning regimen, infections, or residual leukemia cells may induce

IL-1β production, this may influence the NK/ILC3 development from donor-derived CD34+ cells [74]. Shokouhifar et al. found that the differentiation of ex vivo expanded CD34+ cells through manipulation of RAS/MAPK, IGF-1R, and TGF-β signaling pathways is an efficient approach for generating functional NK cells that can be used for cancer immunotherapy [83]. $CD34^+$-HSPC cultured in the absence or in the presence of the EZH1/2 inhibitor UNC1999 and EZH2 inhibitor GSK126 showed that UNC1999 and GSK126 increased $CD56^+$ cell proliferation in comparison to the control. However, UNC1999 and GSK 126 favored the proliferation of no-cytotoxic $CD56^+$ILC3, evident by the early expression of the aryl hydrocarbon receptor (AHR) and ROR-γt transcription factors [84]. These results indicate towards novel epigenetic mechanisms involved in the modulation of NK cell maturation that may provide new tools for designing NK cell-based immunotherapy [84].

Human cytomegalovirus (HCMV) is highly prevalent in most populations worldwide which has a major influence on shaping the human immune system. In a recent report, the system was modified by infecting MSCs with HCMV to study the effect of virus infection on NK/ILC development. The report demonstrated that cord blood-derived hematopoietic progenitor cells were successfully differentiated into mature CD56+CD94+NKG2A+ NK cells from HCMV-infected MSCs having significantly higher antiviral cytokine production ability in comparison to NK cells developing from noninfected MSCs. Furthermore, the generation of ILC3s, characterized by expression of the signature transcription factor RAR-related orphan receptor gamma (RORγt) and by the production of IL-22, was strongly impaired by HCMV infection. These observations are of significant clinical relevance given that ILC3s are associated with protection from graft-versus-host disease (GvHD) following stem cell transplantation. Also, the finding demonstrates that HCMV-mediated reactivation could be associated with increased incidences of GvHD [85]. A recent report demonstrates that the differentiation of hematopoietic stem cells towards NK cells and away from common ILC precursors can be achieved by glucocorticoids, a finding that can be used for favoring the production of NK cells [86].

7. Conclusion

The stem cell therapy industry is growing at a very fast pace because it is playing a very important role in offering hope to a section of patients who have lost hope from existing medical knowledge. In past decades, several studies have been carried out with T and NK cell-based immunotherapy, but very little information is available regarding the ILC1, ILC2, and ILC3 as this is a very new field. These cells are known for maintaining homeostasis, but no mechanistic study is available regarding their role during stem cell therapy. Production and targeting the NK cells using stem cells have very high potentials to be used in the field of cancer immunotherapy.

Data Availability

Not applicable since it is a review article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Divya Verma and Mukesh Verma contributed equally to this work.

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Review Article

Evaluation of the Therapeutic Potential of Mesenchymal Stem Cells (MSCs) in Preclinical Models of Autoimmune Diseases

Radha Krishan Shandil, Saumya Dhup, and Shridhar Narayanan

Foundation for Neglected Disease Research (FNDR), 20A, KIADB Industrial Area, Doddaballapur, 561203, Bangalore, Karnataka, India

Correspondence should be addressed to Radha Krishan Shandil; rk.shandil@fndr.in
and Shridhar Narayanan; shridhar.narayanan@fndr.in

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Autoimmune diseases, chronic in nature, are generally hard to alleviate. Present long-term treatments with available drugs such as steroids, immune-suppressive drugs, or antibodies have several debilitating side effects. Therefore, new treatment options are urgently needed. Stem cells, in general, have the potential to reduce immune-mediated damage through immunomodulation and T cell regulation (T regs) by inhibiting the proliferation of dendritic cells and T and B cells and reducing inflammation through the generation of immunosuppressive biomolecules like interleukin 10 (IL-10), transforming growth factor-β (TGF-β), nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2). Many stem cell-based therapeutics have been evaluated in the clinic, but the overall clinical outcomes in terms of efficacy and the longevity of therapeutic benefits seem to be variable and inconsistent with the postulated benefits. This emphasizes a greater need for building robust preclinical models and models that can better predict the clinical translation of stem cell-based therapeutics. Stem cell therapy based on MSCs having the definitive potential to regulate the immune system and control inflammation is emerging as a promising tool for the treatment of autoimmune disorders while promoting tissue regeneration. MSCs, derived from bone marrow, umbilical cord, and adipose tissue, have been shown to be highly immunomodulatory and anti-inflammatory and shown to enhance tissue repair and regeneration in preclinical models as well as in clinical settings. In this article, a review on the status of MSC-based preclinical disease models with emphasis on understanding disease mechanisms in chronic inflammatory disorders caused by exaggerated host immune response in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) was carried out. We also emphasized various factors that better predict the translation of stem cell therapeutic outcomes from preclinical disease models to human patients.

1. Introduction

Stem cell transplantation is an emerging alternate treatment option for chronic autoimmune disorders. There are dozens of clinically observed autoimmune diseases that affect nearly 5% of the global population [1, 2]. Autoimmune disorders could be systemic or organ-specific. These diseases are caused by "the host immune system attacking itself." The nature and severity of these disorders depend on the complexity of the immune system and the type of immune response (innate, humoral, or cellular) involved. Major factors underlying autoimmune diseases are environment, lifestyle, genetic disposition, exaggerated immune reactivity, and inflammation and hormones in some cases [3–6]. Autoimmune diseases are typically chronic illnesses, which are difficult to ameliorate and treat. Maintaining a delicate balance between effector and regulatory immune function is required for avoiding autoimmune disorders [5–8]. There are approximately 80 autoimmune diseases known to scientists of which rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), inflammatory bowel syndrome (IBS), Crohn's disease (CD), and type 1

diabetes are well studied both in preclinical models and clinical studies [1, 3, 4, 9, 10].

MSCs are nonhematopoietic, multipotent cells and can differentiate into many cell types, including adipocytes, neuronal cells, and osteoclasts [9, 11, 12]. MSCs can be derived from human bone marrow, adipose tissue, umbilical cord, and dental pulp and can be cultured and expanded to large numbers in vitro to meet the large-scale need for clinical trials and therapy [7, 8, 11, 13–19]. MSCs can home into the injured and inflamed area/capillary beds, where they can sense and communicate with the local cell milieu, collect local data signals, and then intelligently release drug-like substances/growth factors that heal different organs like the liver, lung, bones, knee, and diabetic foot [12, 19, 20]. MSCs have immense immunomodulatory and regenerative capacity, which enables tissue and organ repair and regeneration, a feature distinct from conventional therapeutics. Hence, MSCs have been successfully used to treat inflammation, autoimmune diseases like arthritis and systemic lupus erythematosus, Crohn disease, diabetes, irritable bowel syndrome, the heart, spine injuries, and other organs by transplantation and even infections [11, 12, 19, 21–24].

Conventional therapies for autoimmune diseases have relied on globally dampening the immune responses and arresting the inflammation cycle by using immunosuppressive medications such as steroids, methotrexate, tacrolimus, and antibodies like infliximab and anti-TNF-α (Humira) to nonspecifically reduce antibody production or using prostaglandin-cyclo-oxygenase pathway inhibitors [13, 25]. These therapies remain the current standard of care (SOC) and are efficacious in most patients. The caveat is the need for high doses required for longer periods, leaving the patient susceptible to life-threatening opportunistic infections such as tuberculosis in RA patients treated with TNF-α antibodies and long-term risk of malignancy [25]. However, many of the treatment modalities currently being investigated lack specificity [13, 25]. While these treatment regimens mitigate the symptoms to a good extent, many benefits are counter balanced by associated toxicity and potentially serious side effects [26, 27]. Thus, developing more target-specific treatment options with reduced risk of systemic immune suppression and reduced toxicity and side effects is the need of the hour [25, 26].

The immunomodulatory properties of MSCs allow downregulation of chronic inflammatory processes. MSCs have been widely studied for immunomodulatory effects using bone marrow, umbilical cord, and adipose tissues to regulate the immune system in experimental models [13, 28–30] and human clinical studies [22–24, 31, 32]. Human clinical trial data show that MSC treatments are well tolerated. Kabat et al. [10] in a comprehensive review summarised results from 914 clinical trials using MSCs, distributed over 14 diseases, including autoimmune diseases. Bone marrow-derived MSCs were most commonly used, and an almost equal number of trials used autologous and allogeneic MSCs, and the median effective dose (MED) suggested was 100 million MSCs/human dose [10]. There are dozens of current ongoing trials reported at http://www.clinicaltrials.gov., encouraging MSC therapeutic development, including for autoimmune diseases. Various studies have demonstrated that adult MSCs can affect the immune T and B cell response by inhibiting the functions of dendritic cells, B cells, and T cells but enhancing the functions of regulatory T cells (T regs) by producing immunoregulatory molecules, thus making them good therapeutic candidates for autoimmune disease treatment [7, 13, 29, 31, 33, 34]. Hence, in this review, we highlight the therapeutic potential of MSC by evaluating their immunomodulatory potential in animal models of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and summarise the underlying immunomodulatory mechanisms specific to these diseases. In addition, the review will also highlight the recent progress made in the clinical translation of MSCs for autoimmune diseases.

2. Preclinical Animal Models of Autoimmune Diseases

2.1. Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a common chronic autoimmune disease that affects joints and connective tissues leading to chronic joint inflammation, stiffness, pain, and loss of mobility. It is often associated with vascular, metabolic, bone, and psychological comorbidities. Progression of RA is characterized by dysfunction of innate and adaptive immunity, including dysregulated cytokine networks which result in an inflammatory milieu progressively damaging the joint and surrounding tissues [35–37]. Current treatment options are limited, and some patients do not respond well in terms of efficacy, while others may experience detrimental side effects [38]. The most widely used treatment for RA includes corticosteroids [31], nonsteroidal [39] anti-inflammatory drugs (NSAIDs), nonbiologic [40] disease-modifying antirheumatic drugs (DMARDs), and biologic DMARDs [41, 42] like anti-TNF-α monoclonal antibodies [42], which are aimed at reducing the symptoms and gradually alleviating the disease pathogenesis.

Since the pathogenesis and clinical presentation of RA are diverse, a significant effort has been made in understanding its aetiology [43, 44], underlying mechanisms, and inflammatory [44, 45] and immunoregulatory pathways [33, 34, 46] to facilitate the development of newer and better therapies [46, 47].

There are nearly one hundred studies published on the development and validation of preclinical disease models of RA, which exhibit promising trends for the clinical application of MSC-based therapeutics. MSC-based studies have demonstrated a reduction of arthritis progression in the majority of preclinical models. Thus, MSC-based therapy may provide relief to patients not responding well to standard of care (SOC) drug-based treatments.

The collagen-induced arthritis model (CIA) in DBA/1 mice and BB, Brown-Norway rats is well validated for studying disease resolution with drugs and MSCs [28, 32, 33, 45–49]. Stem cells injected intravenously or parenterally specifically home into the inflamed arthritic tissues and concomitantly reduce pathogenic cytokines and disease severity scores [8]. The CIA shares many similarities with RA, such as the involvement of Th1 and Th17 cells in disease

progression and the presence of autoantibodies; thus, it best represents the systemic immune responses in human RA [46]. Augello et al. injected allogeneic bone-marrow MSCs (BM-MSCs) in DBA/1 mice intraperitoneally (IP). A single injection of these cells significantly reduced inflammatory cytokines, antigen-specific regulatory T cells (Tregs), and disease severity [48].

Similarly, Liu and his colleagues evaluated a single intravenous infusion of human umbilical cord MSCs (hUC-MSCs) on CIA in DBA/1 mice. They again reported an association between decreased proinflammatory cytokines and alleviation of RA symptoms [38]. Liu et al. also demonstrated the therapeutic potential of hUC-MSCs on CIA in DBA/1 mice and showed that upon injecting the cells through IP, a reduction in the severity of arthritis was observed with reduced levels of proinflammatory cytokines and chemokines (TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP1)), induction of Tregs, and increased levels of IL-10 [49]. Similar results were observed when Zhou et al. intravenously (IV) injected human adipose-derived mesenchymal stem cells (hA-MSCs) in a mouse model of CIA. The production of various inflammatory mediators was inhibited upon treatment with hA-MSCs, along with decreased antigen-specific Th1/Th17 cell expansion. Induction in the production of anti-inflammatory cytokine interleukin-10 was also observed.

Moreover, hA-MSCs could induce the generation of antigen-specific Treg cells with the capacity to suppress collagen-specific T cell responses [50]. A robust meta-analysis study [51] that evaluated the utility of MSC therapeutics in preclinical RA models (variables like donor species, tissue of origin, route of administration, and type of transplant—autologous, allogeneic, and xenogeneic) from 1995 to 2019 concluded that MSC therapeutics are good candidates because of their ability to attenuate exacerbated pathogenic immune response in RA and restore the balance between dysregulated proinflammatory and anti-inflammatory cell populations. They postulated that the efficacy of MSC-based therapeutics and amelioration of RA pathogenesis in experimental models could translate to RA in humans [51].

Alternatively, many investigators have employed adjuvant-induced arthritis (AIA) and spontaneous (K/BxN) arthritis models instead of CIA. In an AIA model, MSCs are injected directly into inflamed joints to reduce inflammation [52, 53]. Many studies postulated that higher efficacy with MSCs can be achieved with the infusion of stem cells before disease onset or during the early phase of the disease. Recently, Sampath et al. [54] reported a novel therapeutic combination of placental-derived MSCs (hPMSCs) with stigmasterol, a plant-derived sterol in the monosodium-iodoacetate osteoarthritis (OA) rat model. The authors found beneficial effects on intra-articular administration of hPMSCs with stigmasterol and demonstrated accelerated cartilage repair and regeneration using computerised tomography (micro-CT) and histopathology. This cellular therapy attenuated osteoarthritis lesions with concomitant cartilage repair and regeneration [54].

Numerous studies have been carried out to understand how MSCs decrease proinflammatory cytokines such as TNF-α [55, 56] or IL-6 and increase the anti-inflammatory cytokine IL-10, IFN-γ-induced protein 10 (IP-10), and/or chemokine receptor 3-alternative (CXCR3) anti-inflammatory cytokine levels in serum and synovium. The precise mechanistic network through which RA is inhibited by MSCs is still being evaluated and emerging. Recently, MSC-derived extracellular vesicles (EVs)/exosomes have emerged as key paracrine messengers that can exert a strong immunomodulatory and therapeutic effect on preclinical models of RA [57].

Though there are convincing preclinical studies on the therapeutic benefits of MSCs in RA, their translation into human RA treatment is an ongoing debate [10, 22]. At present, there is no so-called ideal protocol for MSC-based therapy for RA treatment in human patients [10]. A few clinical studies are available in the public domain (http://www.clinicaltrials.gov.) that evaluate the safety and efficacy of MSC-based therapy in RA, and some are still ongoing [10, 58–61]. So far, these proof-of-concept clinical translation studies have demonstrated promising results in terms of pathology resolution, safety, and efficacy with no to limited adverse events in patients with long histories of RA and particularly in refractory RA patients [59, 61].

2.2. Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE) is a severe autoimmune disease characterized by widespread tissue inflammation and damage to the affected organs. The autoimmune-mediated inflammatory responses in SLE are characterized by the production of autoantibodies/antinuclear antibodies; formation of immune complexes leading to autoantigen accumulation in various tissues including the kidneys, joints, vasculature, and skin; and secretion of proinflammatory cytokines that result in activation of cells of both the innate and adaptive immune systems [62, 63]. Pathogenesis of SLE is multifactorial and is driven by genetics, hormonal factors, immune dysregulation, and immune-mediated inflammatory injury [29, 64].

Current treatment strategies to manage SLE primarily include antimalarials (hydroxychloroquine (HCQ), quinacrine, corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), immunosuppressive drug cyclosporine A (CsA), azathioprine (AZA), methotrexate (MTX), tacrolimus (TAC), and cyclophosphamide (CTX)), mycophenolate mofetil (MMF), and biological agents (rituximab (RTX), belimumab) [24, 65, 66]. However, prolonged use of these drugs leads to toxicity and adverse events, which may promote secondary infections or malignant tumours [24–29, 31–66]. Hence, in severe conditions, alternatives like plasma exchange, high-dose immunoglobulin, or MSC treatment are preferred [67]. Thus, there is an urgent need to develop novel therapeutics for SLE with improved efficacy and reduced toxicity.

MSCs have the potential to regulate the immune system and control inflammation by inhibiting the activation of NF-κB and JACK/STAT and Akt/GSK3β signalling pathways to ameliorate SLE lesions [67]. Hence, MSCs have been extensively explored for the treatment of SLE in experimental animals [29, 68] and human patients [2, 8]. MSCs exert immunosuppressive effects through proinflammatory cytokine secretion and inhibiting lymphocyte activation and

proliferation. Several studies on the immunomodulatory effects of MSCs on preclinical models of SLE demonstrated the beneficial effect [32, 69–73]. SLE was associated with the activation and proliferation of autoreactive B cells and certain subtypes of T cells [74]. Moreover, deficiency in anti-inflammatory (Treg, Th2) and proinflammatory (Th17, Th1) subsets is one of the crucial factors in the pathogenesis of SLE, leading to tissue inflammation, immune dysfunction, and multiorgan failure [75]. Thus, to understand the mechanism of MSCs as therapeutic for SLE, the best-suited animal model is the Fas mutated MRL/lpr and NZB/W F1 mice, which develop lupus-like syndrome very similar to human SLE and have been widely used to study the mechanism of MSC therapy [76–78]. Jang et al. investigated the effect of hBM-MSCs on the pathogenesis of SLE in female NZB/W mice. They observed that the hBM-MSCs exhibited a protective effect associated with a reduction in autoantibodies, follicular T helper (Tfh) cells, proteinuria, and humoral immune components [79]. A subsequent report by Chang et al. showed that treatment of NZB/W F1 mice with umbilical cord MSCs (hUMSCs) inhibited the pathogenic and inflammatory immune response of SLE and evidently delayed lupus autoimmunity by modulating T cell differentiation and shifting Th1 to Th2 polarization and reducing levels of proinflammatory (TNF-α, IL-6, and IL-12) cytokines [29]. In addition, a study conducted by Liu et al. demonstrated that upon xenogeneic transplantation of human placenta-derived MSCs (hP-MSCs) in MRL/lpr mice, a decrease in levels of anti-dsDNA antibodies, NF-κB signalling pathway, expression of TNF-α, ICAM-1, plasminogen activator inhibitor-1 (PAI-1), and proteinuria level was observed [80]. Similar results were reported by Zhou et al., whereupon intravenous injection of hBM-MSC in MRL/lpr mice reduced serum levels of anti-dsDNA antibodies, proteinuria, and proliferation of T cells as observed. They also demonstrated a decrease in Th17 cell proportion, IL-17 concentration, and anti-dsDNA antibodies when human Early Embryonic MSCs (hEE-MSCs) were injected in MRL/lpr mice [81]. Park et al. observed similar results with hA-MSC transplantation in Roquin$^{san/san}$ mice. They observed that hA-MSCs markedly ameliorated autoimmunity in a murine model of SLE by decreasing the anti-dsDNA-ICOS+CD44+ follicular helper T cells, Th1, and Th17 and increasing the expression of interleukin-10-producing regulatory B cells [82]. These studies unequivocally demonstrated the immunoregulatory effects of MSCs on T cell populations. Ma et al. also demonstrated downregulation of B cell maturation and differentiation in mice following administration of allogeneic BM-MSCs in MRL/lpr mice [83].

Experimental studies [81–83] in genetically engineered mouse models (MRL/lpr and NZB/W F1) have created compelling evidence that MSC treatment can benefit and ameliorate SLE. These findings supported the exploration of MSC therapy in humans. However, heterogeneous presentation of lupus in humans presents a challenge, and mouse models may not represent a complete spectrum of pathogenesis but only a subset of changes observed in the human population [84]. A comprehensive review of the safety, efficacy, and signal pathways of stem cell therapy of SLE [68] suggested an immense potential for clinical applications. Allogenic MSC transplantation with peripheral blood MSCs in three lupus nephritis patients resulted in decreased serum creatinine levels. Leng et al. [85] assessed the efficacy of autologous peripheral blood-derived MSCs in a 10-year follow-up study in 24 SLE patients and showed a decline in median proteinuria from 4 gm/24 hr to zero levels in follow-up studies after 5 years of transplantation. Liang et al. [86] measured the efficacy of bone marrow MSCs in 15 refractory patients of SLE; 11/15 patients had decreased anti-dsDNA antibodies below baseline during a 12-month follow-up. The major bottleneck in the clinical application of autologous MSC-based therapy in SLE patients was functional abnormalities like cytoskeleton alteration, aberrant cytokine production, impaired phenotype, proliferation, and defective hemopoiesis [83, 87–89]. Due to this malfunctioning, the use of allogenic MSC was preferred for transplantation in SLE patients [30]. Clinical studies with allogeneic MSCs derived from bone marrow and umbilical cord suppressed autoimmunity and restored renal function in patients by reestablishing a balance between Th1- and Th2-related cytokines, decreasing IL-4 levels, and increasing the numbers of Treg cells, TGF-β, and IFN-γ[30, 32, 88].

2.2.1. Emerging Concepts. Additionally, many new cutting-edge concepts and technologies like exosomes and novel polyherbal drug formulations are emerging that are expected to revolutionize the use of MSC therapeutics in the near future. The use of MSC-secreted exosomes is believed to facilitate communication between cells and their microenvironments by transferring information via their cargo, including the proteins, lipids, and RNAs, gaining momentum as cell-free therapeutics [90]. These exosomes reduced myocardial ischemia/reperfusion injury in mouse models [90]. There are no studies known to our knowledge that have examined this exosomes-based approach from MSCs to treat RA and SLE in animal models or humans that may offer a novel approach in general to treat tissue injury and promote repair in autoimmune disorders [91]. Recently, Renganathan et al. [92] developed a plant-derived ayurvedic polyherbal formulation Dhanwantaram kashayam that regulated lipid metabolism and scavenged oxidative radicals in diabetic rats. This formulation enhanced the proliferation, mobilisation, and homing of stem cells and ameliorated diabetic conditions in experimental rats. These new approaches and formulations need rapid clinical testing and may provide a tangible MSC-based alternative for difficult-to-treat autoimmune diseases. MSC treatments have evoked great expectations, and their wide applicability to many more autoimmune diseases like type 1 diabetes [93] and type 2 diabetes [94] is expected to bring a turning point in modern medicine. Recently, Kotikalapudi et al. in an elegant report published significant success of P-MSC therapy in the control of experimental obesity-associated insulin resistance (IR), regulation of underlying mechanisms, and amelioration of diabetes [94]. They demonstrated homing of intramuscularly injected fluorescent labelled P-MSCs to the visceral region of the adipose tissue by in vivo imaging,

activated PI3K-Akt signalling, regulated glucose homeostasis, and insulin sensitivity in dysregulated adipocytes of WNIN/GR-Ob (Ob-T2D) rats. These preclinical findings suggest a potential of P-MSCs in the amelioration and management of type 2 diabetes.

3. Conclusions

Stem cell/MSC transplantation treatment is emerging as a rational and alternative therapeutic option for chronic autoimmune disorders like RA, SLE, and type 1 and type 2 diabetes. Undoubtedly, preclinical models of RA and SLE have played a significant role in deciphering underlying immune mechanisms, pathologies, and evaluation of the therapeutic potential of MSCs.

Collagen-induced arthritis models mimic human disease, and the associated markers and events reasonably predict human disease. Though there are convincing preclinical studies on the therapeutic benefits of MSCs in RA, their complete translation into human RA treatment is an ongoing debate. Recent experimental and human studies based on MSC-derived exosome research have yielded exciting results. Further exploration of exosome products in the resolution of RA and regeneration in autoimmune disorders may provide new treatment options.

On the other hand, the pathogenesis of human SLE is complex and varies from patient to patient and even at different times in the same patient. Animal models mimic many of these events but cannot cover the full heterogeneity of human lupus SLE. Nevertheless, animal models have greatly benefited the understanding of complex immunologic and pathologic mechanisms. MSC treatments have been well tolerated in human SLE patients. MSC therapeutics have shown good responses, and findings have translated reasonably to humans as well. Markers like serum autoantibodies (anti-dsDNA, ANA), creatinine, proteinuria, reduction in nephritis and inflammation, and alleviation of SLE lesions seem to well translate into human patients. Unfortunately, large animal models of RA and SLE in canines, sheep, pigs, etc., are lacking that could probably cover the spectrum of pathophysiology better and increase the translation of MSC therapeutics in these chronic ailments.

In summary, human clinical trial data in autoimmune disorders have shown benefit with little or no serious adverse events. There is a linear increase in stem cell human clinical trial registration totalling 914 MSC trials for 14 diseases, including autoimmune diseases, from 2004 to 2018 [10]. There are dozens of currently ongoing clinical trials at http://www.clinicaltrials.gov. which is encouraging for MSC therapeutic development including for many autoimmune diseases.

Abbreviations

MSCs: Mesenchymal stem cells
BM-MSCs: Bone marrow-derived mesenchymal stem cells
hUC-MSCs: Human umbilical cord-derived mesenchymal stem cells
RA: Rheumatoid arthritis
SLE: Systemic lupus erythematosus
NSAIDs: Nonsteroidal anti-inflammatory drugs
DMARDs: Disease-modifying antirheumatic drugs
SOC: Standard of care
CIA: Collagen-induced arthritis
EVs: Extracellular vesicles
IL: Interleukins
TNF-α: Tumour necrosis factor alpha
IFN-γ: Interferon gamma
TGF-β: Transforming growth factor beta
CXCR3: C-X-C chemokine receptor 3
PGE2: Prostaglandin E2.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

RK and SD searched and evaluated the concerned literature and wrote the review. SN critically reviewed the content and suggested relevant changes to finalize the review. The authors have read and approved the final version of the manuscript.

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Research Article

Gingiva-Derived Mesenchymal Stem Cells Attenuate Imiquimod-(IMQ-) Induced Murine Psoriasis-Like Skin Inflammation

Ziyu Ye,[1,2] Yanfang Liang,[3] Bihua Lin,[1,4,5] Yanyun Li,[1] Xingxing Chai,[1] Jiachun Lian,[1,6] Xueying Zhang,[1,6] Zhengping Che,[1,6] and Jincheng Zeng[1,2,4,5]

[1]*Dongguan Key Laboratory of Medical Bioactive Molecular Developmental and Translational Research, Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, Guangdong Medical University, Dongguan 523808, China*
[2]*Guangdong Xinghai Institute of Cell, Dongguan 523808, China*
[3]*Department of Pathology, Dongguan Hospital Affiliated to Jinan University, Binhaiwan Central Hospital of Dongguan, Dongguan 523000, China*
[4]*Key Laboratory of Medical Bioactive Molecular Research for Department of Education of Guangdong Province, School of Basic Medicine, Guangdong Medical University, Dongguan 523808, China*
[5]*Collaborative Innovation Center for Antitumor Active Substance Research and Development, Department of Biochemistry and Molecular Biology, School of Basic Medicine, Guangdong Medical University, Zhanjiang 524023, China*
[6]*Institute of Laboratory Medicine, School of Medical Technology, Guangdong Medical University, Dongguan 523808, China*

Correspondence should be addressed to Jincheng Zeng; zengjc@gdmu.edu.cn

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Human gingiva-derived mesenchymal stem cells (GMSCs) are isolated from the gingival propria with promising regenerative, immunomodulatory, and anti-inflammatory properties. Recently, several studies, including ours, have found that GMSCs have the therapeutic potentials of nerve regeneration and skin disorders in various types such as the cell itself, cell-free conditioned medium, or extracellular vesicles (EVs). However, the mechanobiological behavior of GMSCs is closely related to the culture conditions. Therefore, the purpose of this study was to evaluate the function of human GMSCs on imiquimod- (IMQ-) induced murine psoriasis-like skin inflammation in two-dimensional (2D) and three-dimensional (3D) culture conditions. Here, we isolated and characterized GMSCs in 2D and 3D culture conditions and found that GMSCs in 2D and 3D infusion can significantly ameliorate the IMQ-induced murine psoriasis-like skin inflammation, reduce the levels of Th1- and Th17-related cytokines IFN-γ, TNF-α, IL-6, IL-17A, IL-17F, IL-21, and IL-22, and upregulate the percentage of spleen $CD25^+CD3^+$ T cells while downregulate the percentage of spleen $IL\text{-}17^+CD3^+$ T cells. In summary, our novel findings reveal that GMSCs in 2D and 3D infusion may possess therapeutic effects in the treatment of psoriasis.

1. Introduction

Mesenchymal stem cells (MSCs) are multilineage cells with self-renewal and multipotent differentiation, and immunomodulatory/anti-inflammatory properties play a vital role in tissue repair and regeneration [1, 2]. They are present in almost all tissues, including adipose tissue, bone marrow, umbilical cord, synovium, skeletal muscles, dental pulp, gingival, amnion, placenta, and skin [3–5]. MSCs commonly express similar cell surface molecules, such as CD29, CD44, CD73, CD90, and CD105, but typically lack hematopoietic cell markers, such as CD14, CD19, CD34, and CD45 [2, 6]. Human gingiva-derived mesenchymal stem cells (GMSCs) are isolated from the gingival propria with promising regenerative, immunomodulatory, and anti-inflammatory properties. Similar properties shared with other MSCs, including BMSCs, UMSCs and ADMSCs, GMSCs have several unique characteristics, specially their high proliferative capacity. In addition, GMSCs can keep MSC characteristics and show stable morphology and maintain telomerase activity under long-term culture conditions. Besides the differentiation potentials (osteocytes and adipocytes), GMSCs possess the

potential to transdifferentiate into neural cells, endothelial cells, keratinocytes, and odontogenic cells under different induction conditions. Recently, several studies, including ours, have found that GMSCs have the therapeutic potentials of nerve regeneration and skin disorders in various types such as the cell itself, cell-free conditioned medium, or extracellular vesicles (EVs) [7–9]. A number of studies have found that the mechanobiological behavior of MSCs is closely related to the culture conditions. Most recently, some studies found that 3D spheroid GMSCs significantly increased the stem cell properties and therapeutic effects. In comparison to the 2D cultured GMSCs, 3D spheroid GMSCs showed enhanced multipotency and secreted an increased level of several chemokines and cytokines related with cell migration, proliferation, and angiogenesis; in vivo, 3D spheroid cultures of GMSCs improved mitigation of oral mucositis. The purpose of this study was to evaluate the function of human GMSCs on imiquimod- (IMQ-) induced murine psoriasis-like skin inflammation in two-dimensional (2D) and three-dimensional (3D) culture conditions.

Psoriasis is a T cell-mediated inflammatory autoimmune skin disease, with an imbalance between Th2 and Th1/Th17 cytokines [10]. MSCs could alleviate psoriasis skin lesions by suppressing the local levels of angiogenic and proinflammatory mediators and inhibiting activation and differentiation of DC-mediated $CD4^+$ T cells [11, 12]. More recently, some studies showed that MSCs could be an effective treatment for psoriasis [11–17]. Up to date, there are nine clinical trials on MSC-based therapy of psoriasis. [2]. At present, this is due to the easy accessibility, genomic stability, the highly proliferative activity, less morbidity of harvesting, the potent immunomodulatory, and regenerative potentials, as well as well tolerated by all recipient hosts without any obvious systemic adverse effects [18]. GMSCs have become an attractive source of adult stem cells for regenerative therapy and tissue engineering.

However, whether GMSCs also has an effective treatment for psoriasis and whether this effect is related to the different mechanobiological behavior of GMSCs is unknown. To date, some studies have reported that aggregation of MSCs in 3D spheroid culture can significantly enhance their multipotent differentiation, anti-inflammatory properties, and angiogenic and tissue regenerative effects [19]. In this study, we isolated and characterized GMSCs in 2D and 3D culture conditions and found that GMSCs in 2D and 3D infusion can significantly ameliorate the IMQ-induced murine psoriasis-like skin inflammation, reduce the levels of Th1- and Th17-related cytokines IFN-γ, TNF-α, IL-6, IL-17A, IL-17F, IL-21, and IL-22, and upregulate the percentage of spleen $CD25^+CD3^+$ T cells while downregulate the percentage of spleen IL-17^+CD3^+ T cells. Our novel findings reveal that GMSCs in 2D and 3D infusion may possess therapeutic effects in the treatment of psoriasis.

2. Materials and Methods

2.1. GMSC Isolation and Culture. Approved by the Ethics Committee of Binhaiwan Central Hospital of Dongguan, human gingival tissue samples were collected from clinically healthy patients without history of periodontal disease. The isolation of human GMSCs was described previously [3]. The gingival tissues were treated aseptically and washed several times with phosphate buffered saline (PBS). Then, the tissues were minced into small fragments (1-3 mm^3) and digested with collagenase IV (Sigma) solution at 37°C for 1 h and centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The cells were suspended in complete minimum essential medium α (α-MEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml), then placed into 10 cm cell culture dish, and maintained at 37°C and 5% CO_2 in a humidified incubator. The medium was refreshed every three days. After reaching 80% confluence, cells were digested with trypsin-EDTA solution (0.25%). Cells of passages 3-8 were used for the present experiments.

2.2. Multipotent Differentiation of GMSCs

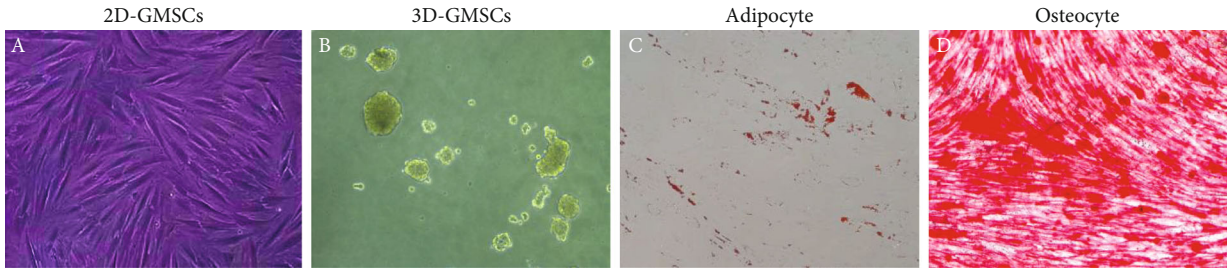
2.2.1. Osteogenic Differentiation. The GMSCs were seeded in 6-well plates (5×10^5 cells/well) and incubated with α-MEM, allowed to adhere overnight, and replaced with osteogenic induction medium (Scien Cell) every 3 days. Four weeks later, in vitro mineralization was assayed by Alizarin Red S staining.

2.2.2. Adipogenic Differentiation. As described above, the GMSCs were cultured in adipogenic differentiation medium (Scien Cell). The medium was refreshed every 3 days. Two weeks later, the cells were fixed and assessed by Oil Red O staining.

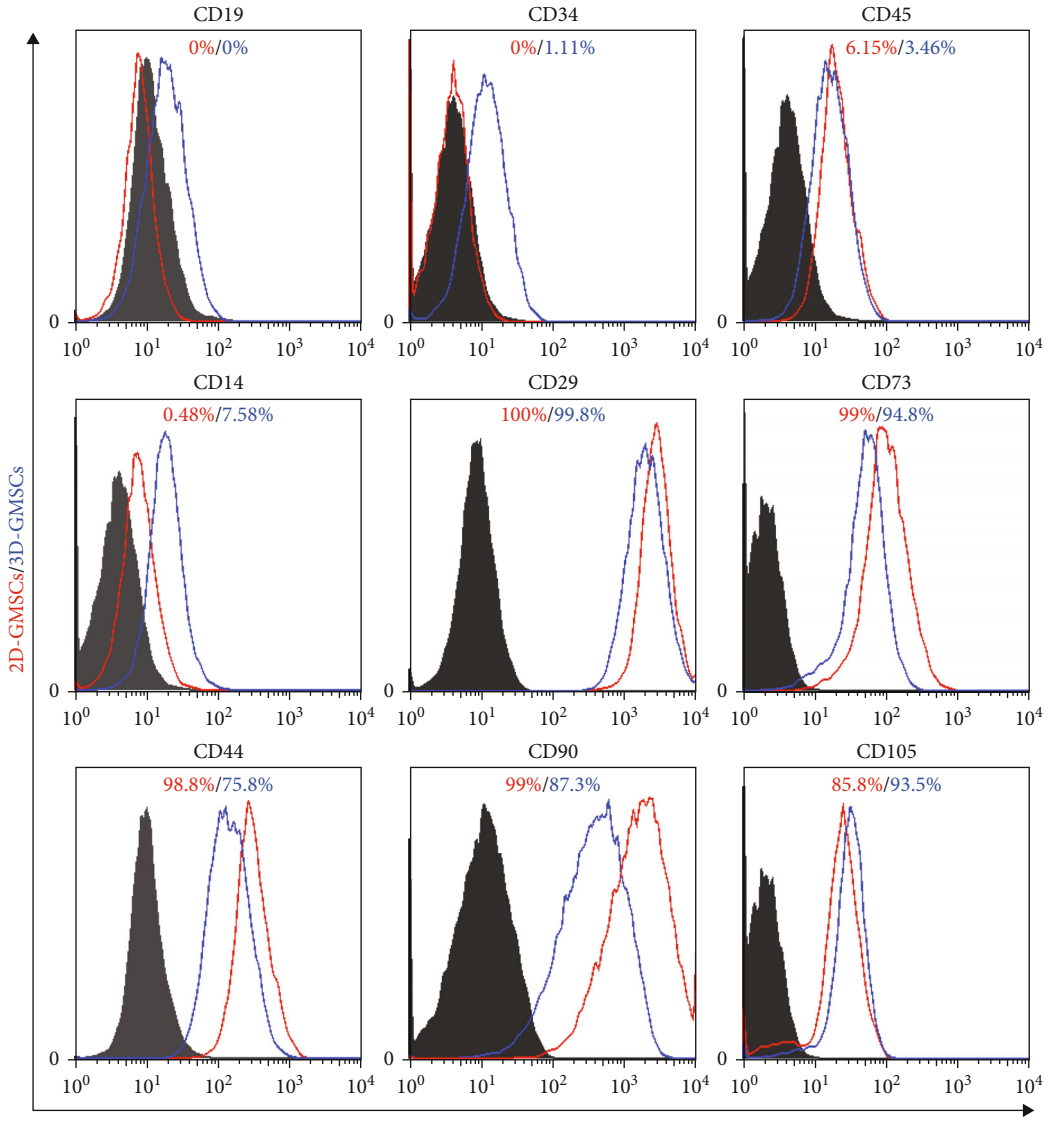
2.3. Spheroid Generation and Dissociation. GMSCs were seeded into ultralow attachment dishes (2×10^5/ml) and incubated with complete α-MEM to allow 3D spheroid formation for up to 3 days. To acquire spheroid-derived GMSCs, spheroids were incubated with 0.25% trypsin at 37°C for 15 min while pipetting every 5 min; the single cells were collected by centrifugation.

2.4. Cell Proliferation Analysis. CCK-8 (Dojindo) assay was used to measure the viability of GMSCs in 2D (2D-GMSCs) and GMSCs in 3D (3D-GMSCs) culture conditions on a normal culture plate. Briefly, a total of 1000 cells per well were cultured in five replicate wells in a 96-well plate. Then, 10 μl CCK-8 reagent was added to each well and cultured for 2 h. The absorbance was measured at 450 nm using a microplate reader. We performed this assay on day 1, day 3, day 5, and day 7.

2.5. Flow Cytometry. GMSCs were collected and suspended in cell staining buffer (0.5% BSA in PBS with 2 mM EDTA) followed by incubation with CD14 (PE), CD19 (PerCP-Cy5.5), CD29 (APC), CD34 (PE), CD44 (FITC), CD45 (PE), CD73 (PE), CD90 (FITC), and CD105 (PE) antibodies (Biolegend) in the dark at room temperature for 30 min. For intracellular staining, splenocytes from mice were first stained with CD3 (APC/Cy7), CD4 (FITC), and CD25 (APC) antibodies (Biolegend) and then fixed, permeabilized, and stained intracellularly for IL-4 (PE/Cy7) and IL-17 (PE).



(a)



(b)

Figure 1: Continued.

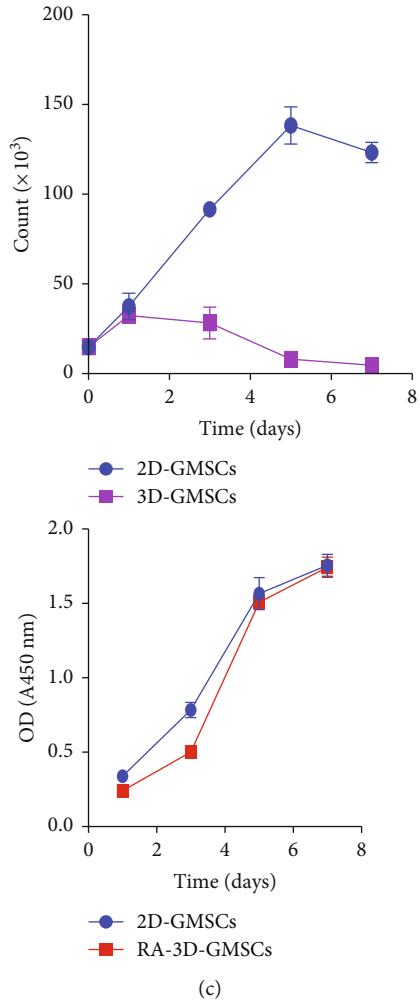


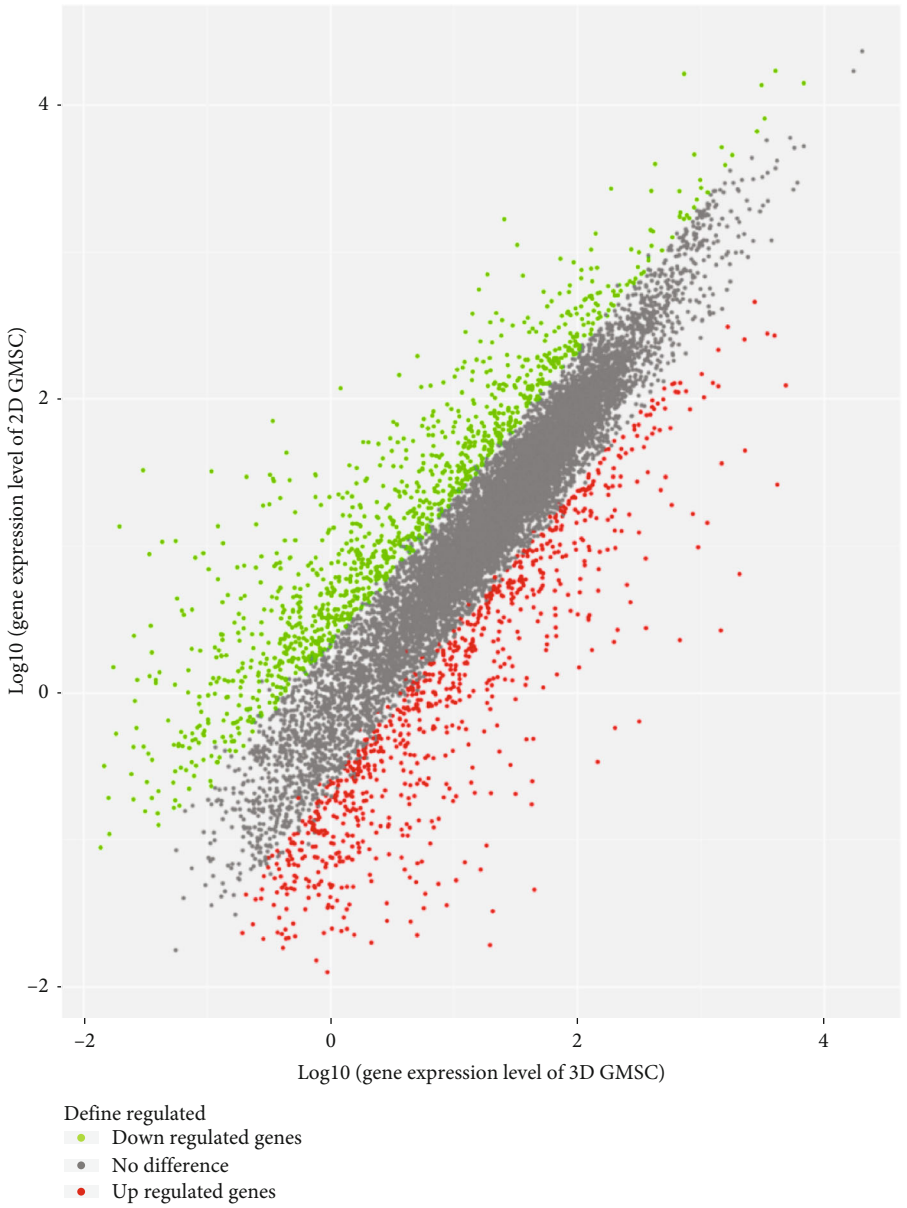
Figure 1: Characterization of GMSCs. (a) Representative images of 2D cultured GMSCs (A). Morphology of GMSCs cultured on a low attachment culture dish (B). Representative images of adipogenesis (C) and osteogenesis (D). (b) Flow cytometric analysis of surface markers in GMSCs under 2D or 3D conditions. (c) Proliferation assay for GMSCs cultured on a low attachment culture dish.

After staining, cells were washed twice with PBS and submitted to flow cytometric analysis (BD). Data were analyzed using the FlowJo 7.6 software.

2.6. Cytokine Analysis. For tissue cytokine analysis, the protein was extracted from skin tissue, then homogenate was centrifuged at 10,000 × g for 10 min at 4°C, and supernatant was collected. Protein concentration was determined using BCA protein Assay Kit. Cytokines (IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, TNF-α, and IFN-γ) in the serum and skin lysate were measured by the LEGENDplex Multi-Analyte Flow Assay kit (Biolegend, 740749) following the manufacturer's instructions. Briefly, 25 μl of the standard, serum or skin lysate, and buffer solutions was added to the wells. To each well, 25 μl of mixed beads was added. Then, the plate was covered with a plate sealer and shook at 500 rpm for 2 h at room temperature. After 2 washes, 25 μl of biotinylated detection antibodies was added to each well. The plate was then covered with a plate sealer and shook at 500 rpm for 1 h at room temperature. Subsequently, 25 μl of Streptavidin-phycoerythrin was added to each well, and the plate was covered with a plate sealer and shook at 500 rpm for 30 min at room temperature. After 2 washes, the samples were tested on a flow cytometer. The results were analyzed using the LEGENDplex data analysis software. The concentration of each analyte was quantified in pictograms per milliliter.

2.7. RNA Sequencing. The cells were collected and lysed by TRIzol, and total RNA was extracted according to the manufacturer's instructions (Invitrogen). RNA was quantified using Nanodrop spectrophotometer (Thermo Scientific). RNA sequencing was carried out by the Guangdong Longsee Biomedical Co., Ltd following standard protocols. Standard bioinformatics analysis was performed by the Guangdong Longsee Biomedical Co., Ltd.

2.8. Animals. Female C57BL/6 mice weighing 20 g (8 weeks old) were purchased from the Guangdong Medical Laboratory Animal Center (Foshan, China). Animal experiments in the study were approved by the Animal Experimental Ethics Committee of Guangdong Medical University in



(a)

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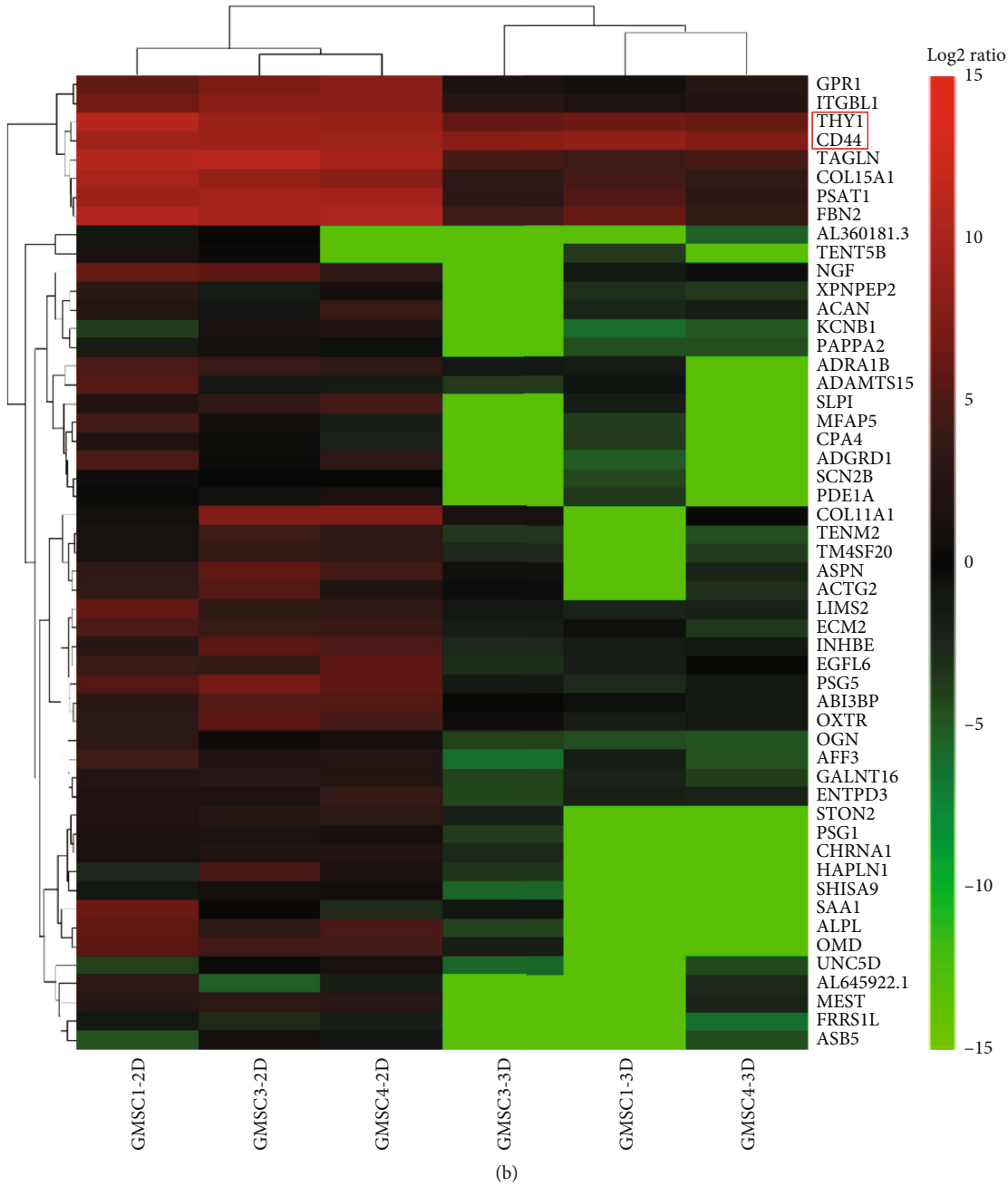

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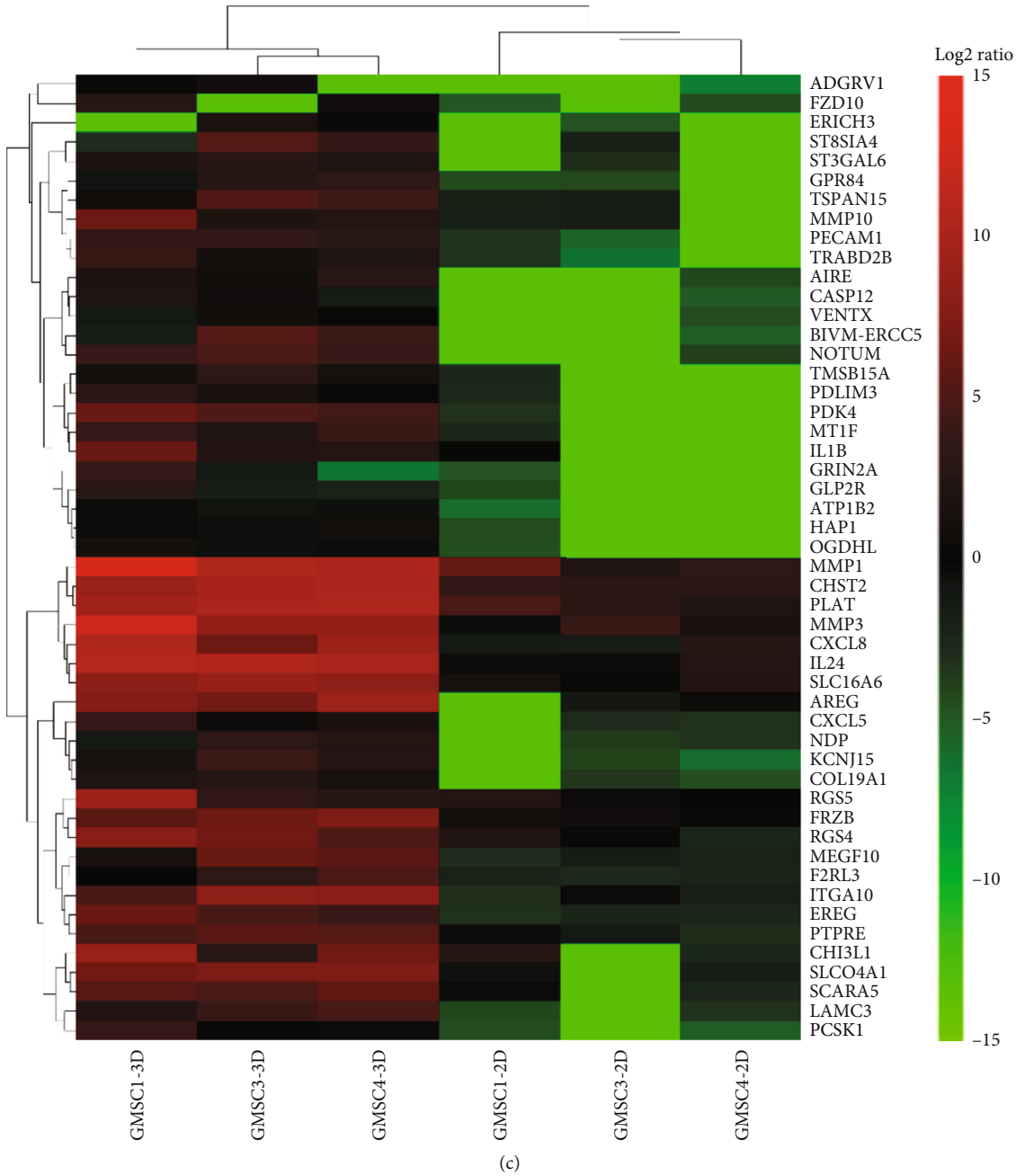

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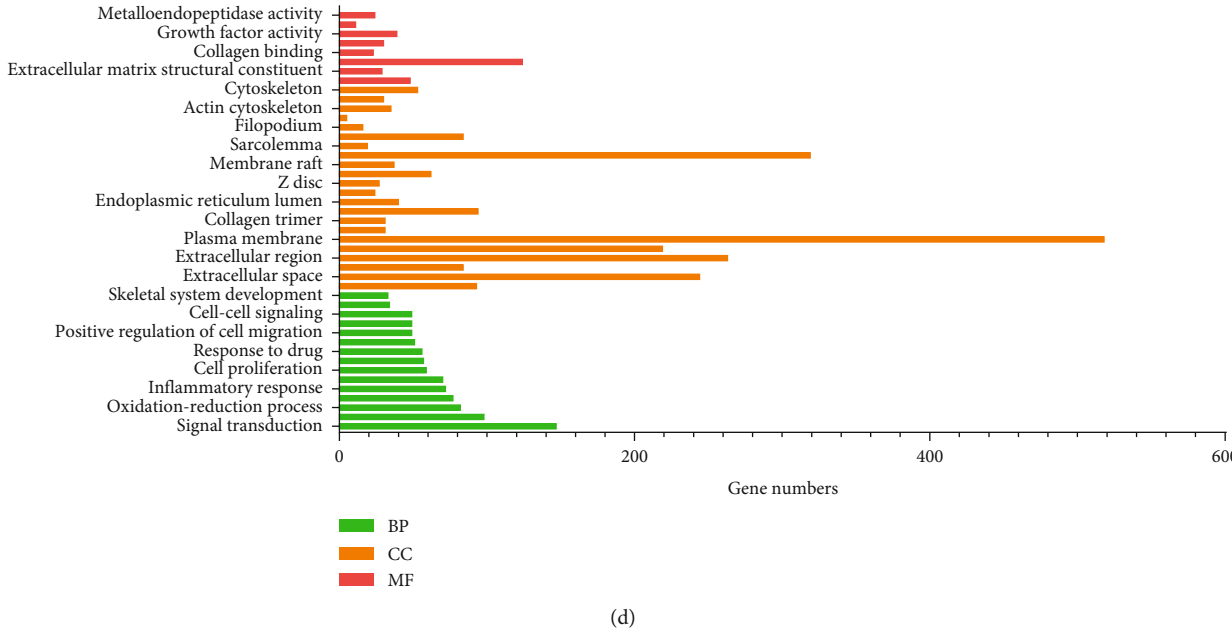


(d)

Figure 2: The 3D culture of GMSCs caused significant alterations in gene expression. (a) The difference of gene expression between 2D-GMSCs and 3D-GMSCs was analyzed by RNA sequencing. (b, c) The representative global view of gene expression changes between 2D-GMSCs and 3D-GMSCs. (d) Gene ontology analysis of differentially expressed genes. BP: biological process; CC: cellular component; MF: molecular function.

compliance with the National Guidelines for the Care and Use of Animals. Mice were group-housed in polycarbonate cages in the animal facilities with controlled temperature (23°C ± 2°C), 40%-65% humidity, and a 12-hour light/dark cycle. Mice were acclimatized for at least 1 week before the study, fed with a standard laboratory diet, and allowed free access to drinking water.

2.9. Establishment and Treatment of IMQ-Induced Murine Psoriasis-Like Skin Inflammation. Fifteen mice were randomly divided into three groups, with five mice in each, as follows: the IMQ control group, 2D-GMSC treatment group, and 3D-GMSC treatment group. On the day 0, the backs of mice were shaved using depilatory machine and cream. 5% imiquimod (IMQ) cream (Sichuan Mingxin) was used to induce psoriasis-like skin inflammation with a daily dose of 62.5 mg from day 1 to day 7, consecutively.

The effects of GMSCs were tested by administration of 2×10^6 2D-GMSCs or 3D-GMSCs in 200 μl PBS via the mouse tail vein on day 1 and day 4 consecutive IMQ treat. The IMQ control group received an intravenous injection of 200 μl PBS via the tail vein on day 1 and day 4. The severity of the inflammation of the psoriatic skin was assessed using the Psoriasis Area Severity Index (PASI). The degree of erythema, scaling, or thickening was each scored on a scale from 0 to 4, as follows: 0, none; 1, slight; 2, moderate; 3, marked; and 4, severe. The cumulative PASI scores (erythema+scaling+thickening) were calculated to reflex the severity of inflammation. All mice were sacrificed on day 8, and blood, spleen, and skin samples were collected for further studies.

2.10. Histology and Immunohistochemical Analysis. The back skin of all the mice was fixed with 10% Paraformaldehyde solution (PFA). For histological study, paraffin-embedded sections were stained with hematoxylin-eosin (HE) staining. For immunohistochemical studies, the paraffin-embedded sections (4 μm) were deparaffinized with xylene, rehydrated with graded ethanol, and heated in 10 mmol/L sodium citrate buffer (pH 6.0) for antigen retrieval. After blocking with 2.5% goat serum in PBS, the sections were incubated overnight at 4°C with primary antibodies (TNF-α, IL-6, IFN-γ, and IL-17A) and then detected using the universal immunoperoxidase ABC kit. All the sections were counterstained with hematoxylin. Images were captured using a light microscope (Olympus).

2.11. Statistical Analysis. All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between experimental and control groups were analyzed by a two-tailed unpaired Student's *t*-test using the GraphPad Prism 7 software. A value of less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of GMSCs under 2D and 3D Culture Conditions. As shown in Figure 1(a), we have successfully isolated GMSCs from gingival tissues and the cells exhibited a spindle-like morphology in 2D culture conditions (Figure 1(a), A) and spontaneously aggregated into 3D spheroids under growth condition of ultralow attachment (Figure 1(a), B). The results of osteogenic and adipogenic

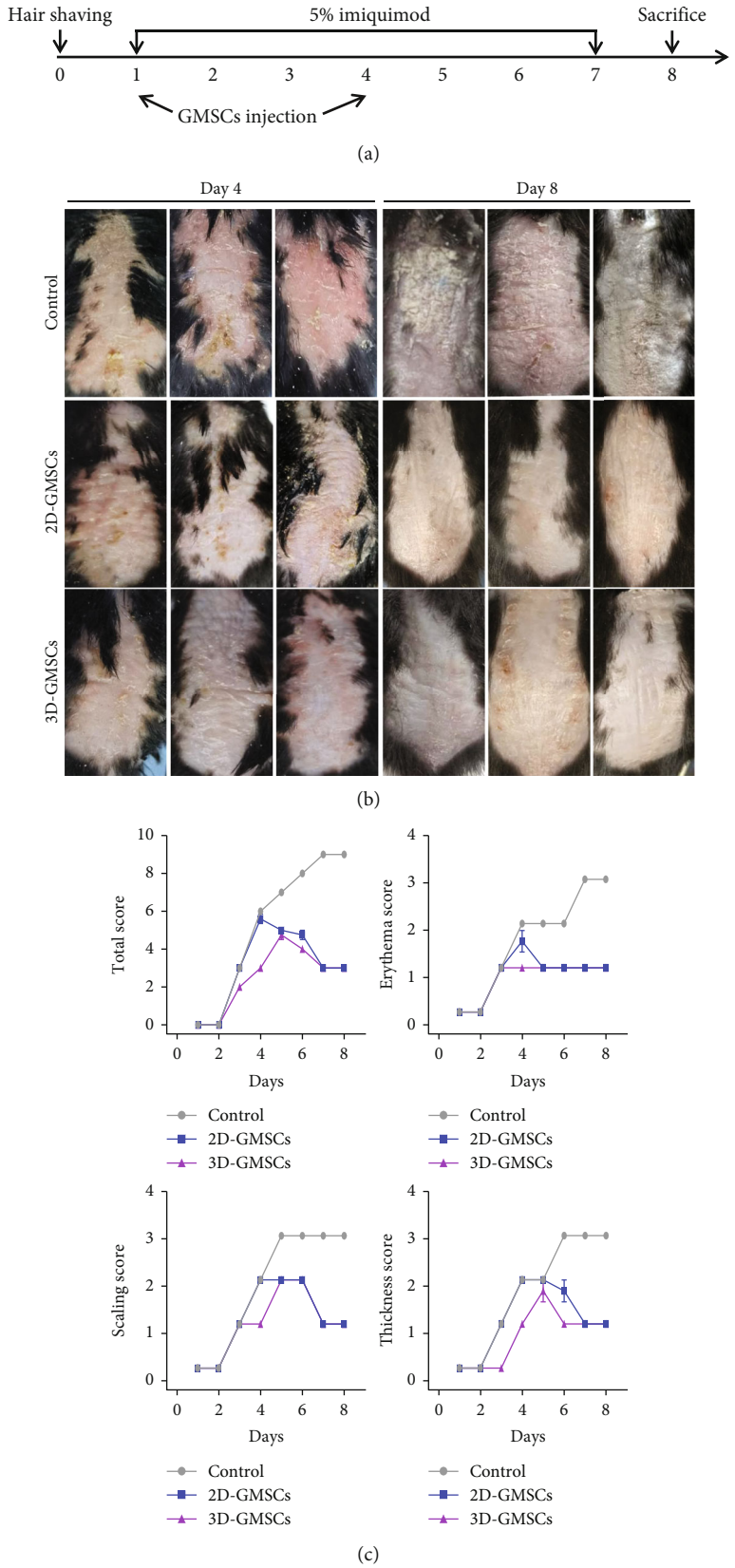


Figure 3: 2D-GMSCs and 3D-GMSCs significantly ameliorated psoriatic symptoms in IMQ-induced mice. MSC infusion ameliorated psoriatic symptoms in IMQ-induced mice. (a) Experimental protocol showing treatment regimens using 2D-GMSCs and 3D-GMSCs in psoriatic mice. (b) Typical presentation of the mouse back skin from IMQ, 2D-GMSCs, and 3D-GMSCs group on day 4 and day 8 was, respectively, shown. (c) Different levels of erythema, scales, thickness of back skin, and total score were scored daily.

differentiation experiments demonstrated that GMSCs have multiple differentiation capabilities (Figure 1(a), C and D). Flow cytometry analysis showed that GMSC cells were strongly positive for MSC markers CD29, CD44, CD73, CD90, and CD105, but negative for hematopoietic cell markers CD14, CD19, CD34, and CD45 (Figure 1(b)). These results were consistent with our previous studies [3]. The results of flow cytometry analysis showed that 3D-GMSCs also negatively expressed CD14, CD19, CD34, and CD45 (Figure 1(b)). And the expression of CD29, CD44, CD73, CD90, and CD105 was decreased, compared with 2D-GMSCs (Figure 1(b)). The cell proliferation results showed that the proliferation rate of GMSCs under 3D culture conditions (3D-GMSCs) was reduced compared with GMSCs under 2D culture conditions (2D-GMSCs) (Figure 1(c)). It is worth noting that when the 3D-GMSCs were replanted in the 2D culture conditions, the cell proliferation rate of replanted 3D-GMSCs (RA-3D-GMSCs) was restored, similar to 2D-GMSCs (Figure 1(c)). The RNA sequencing results showed that among the 13844 screened genes, altogether 1312 genes were significantly upregulated and 1022 genes were significantly downregulated in 3D cultured GMSCs compared with 2D cultured GMSCs ($|\log 2(\text{fold of gene expression change})| \geq 1$) (Figures 2(a)–2(c)). Gene ontology analysis of differentially expressed genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool. The results of the GO analysis found that alterations of biological processes (BPs) in the differentially expressed genes were significantly changed in the signal transduction, inflammatory response and oxidation-reduction process (Figure 2(d)). The differentially expressed genes' alterations in the cell component (CC) were mainly located in plasma membrane, extracellular exosome, and extracellular region (Figure 2(d)). The alterations in gene functionality at the molecular level (MF) were mainly associated with extracellular matrix structural constituent, collagen binding and growth factor activity (Figure 2(d)).

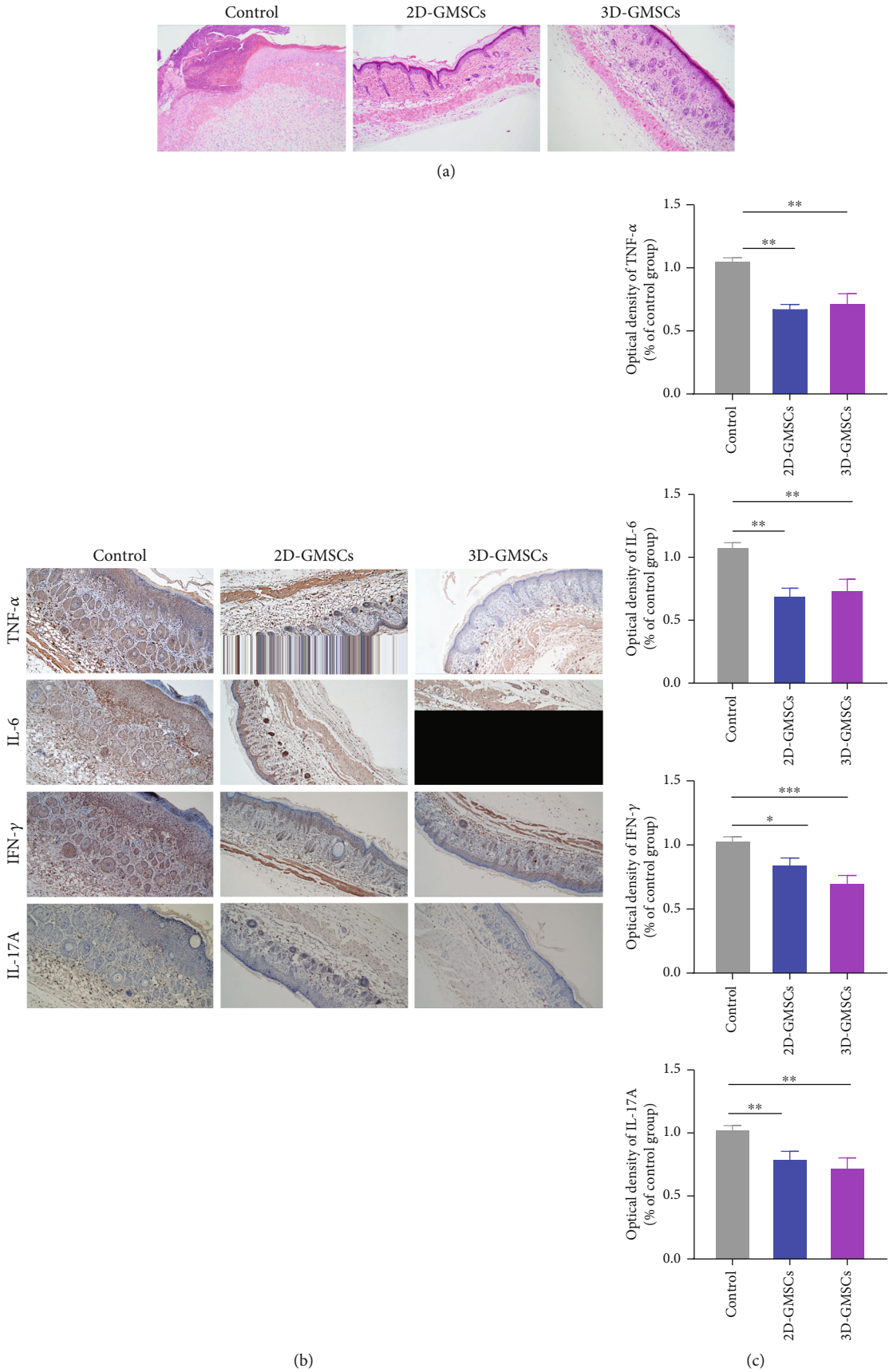
3.2. GMSCs Attenuate the Symptoms of IMQ-Induced Murine Psoriasis-Like Skin Inflammation under 2D and 3D Culture Conditions. To determine whether GMSCs also have an effective treatment for psoriasis, and whether this effect is related to the different mechanobiological behavior of GMSCs, we established a murine model of IMQ-induced psoriasis-like skin inflammation that was injected with 2D-GMSCs and 3D-GMSCs. The intervention strategy is shown in Figure 3(a). The morphological observation of the back skin is shown in Figure 3(b). IMQ control group mice exhibited the most serious symptoms of erythema, scaling, and thickness, which continuously increased in severity up to the end of IMQ application on day 7. However, 2D-GMSC and 3D-GMSC treatment significantly reduced the severity of skin lesions in mice. We also assessed the severity of psoriasis-like skin and total scores of skin lesions on days 1-8 via PASI scoring system. The PASI scores were gradually increased in IMQ control group mice. Interestingly, 2D-GMSC and 3D-GMSC treatment significantly decreased the PASI score (Figure 3(c)). These results suggest that GMSCs attenuate the symptoms of IMQ-induced murine psoriasis-like skin inflammation under 2D and 3D culture conditions.

3.3. GMSCs Attenuate the Skin Inflammation of IMQ-Induced Murine Psoriasis-Like Skin Inflammation under 2D and 3D Culture Conditions. HE staining further confirmed that 2D-GMSCs and 3D-GMSCs significantly decreased epidermal thicknesses (Figure 4(a)). Immunohistochemical studies demonstrated that treatment with 2D-GMSCs and 3D-GMSCs inhibited the expression of TNF-α, IL-6, IFN-γ, and IL-17A (Figures 4(b) and 4(c)). We also analyzed the levels of IL-6, IL-17A, IL-17F, IL-21, IL-22, TNF-α, and IFN-γ in the skin lysate. 2D-GMSC and 3D-GMSC treatment significantly reduced the levels of IL-6, TNF-α, IL-17A, IL-17F, and IL-21 and increased the level of IL-10 in the skin compared to that in control (Figure 4(d)). These results suggest that GMSCs significantly reduced the proinflammatory response, while upregulating the anti-inflammatory response of IMQ-induced murine psoriasis-like skin inflammation under 2D and 3D culture conditions.

3.4. GMSCs Strongly Influence the Expression of Inflammatory Mediators and Spleen $CD25^+CD3^+$ and $IL\text{-}17^+CD3^+$ T Cell Responses of IMQ-Induced Murine Psoriasis-Like Skin Inflammation under 2D and 3D Culture Conditions. Additionally, the mouse spleen volumes and spleen/body weight ratio in the 2D-GMSC and 3D-GMSC group were reduced compared to the IMQ control group (Figure 5(a)). Furthermore, we determined the percentage of Th17 and Treg cells in the spleen of mice, and splenocytes from all the mice were stained for CD3, CD25, and IL-17A. After 2D-GMSC and 3D-GMSC treatment, the percentage of $IL\text{-}17A^+CD3^+$ T cells was notably reduced, while the percentage of $CD25^+CD3^+$ T cells was significantly increased compared with the IMQ control group (Figures 5(b) and 5(c)). These data suggested that GMSCs could modulate spleen Th17 and Treg responses of IMQ-induced murine psoriasis-like skin inflammation under 2D and 3D culture conditions. We also analyzed the levels of IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, TNF-α, and IFN-γ in the serum. As shown in Figure 5(d), the levels of IL-6, TNF-α, IL-10, IL-17A, and IL-22 in the serum of mice were significantly decreased in the 2D-GMSC and 3D-GMSC group compared with the IMQ control group (Figure 5(d)). These data suggested that GMSCs could modulate the expression of serum inflammatory mediators of IMQ-induced murine psoriasis-like skin inflammation under 2D and 3D culture conditions.

4. Discussion

In the present study, we successfully isolated GMSCs from human gingival tissue, and these cells showed osteogenic and adipogenic differentiation capabilities. Additionally, the isolated GMSCs positively expressed CD29, CD44, CD73, CD90, and CD105 but did not express hematopoietic stem cell markers such as CD14, CD19, CD34, and CD45. Traditionally, 2D culture conditions have been used as a standard technique for in vitro expansion of MSCs. Compared with



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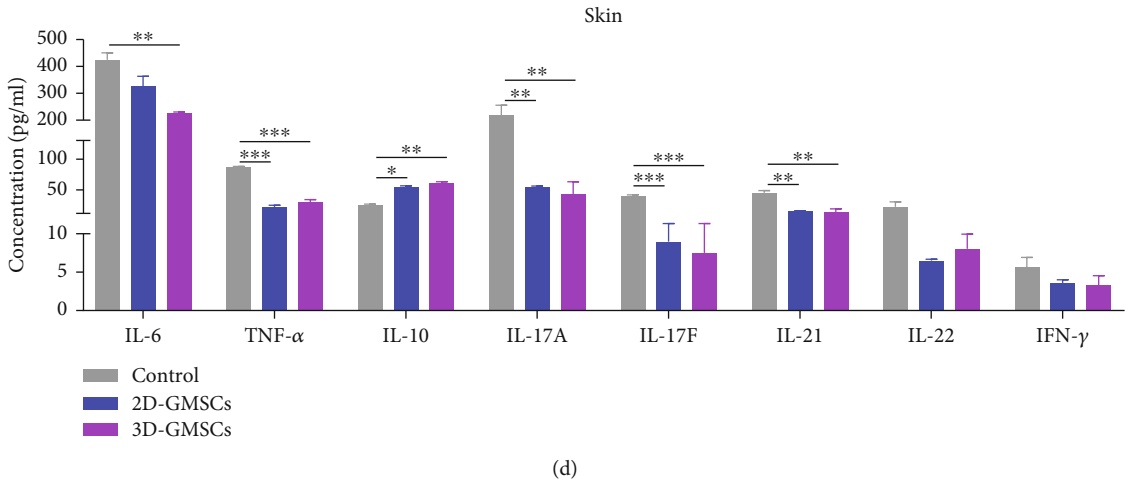


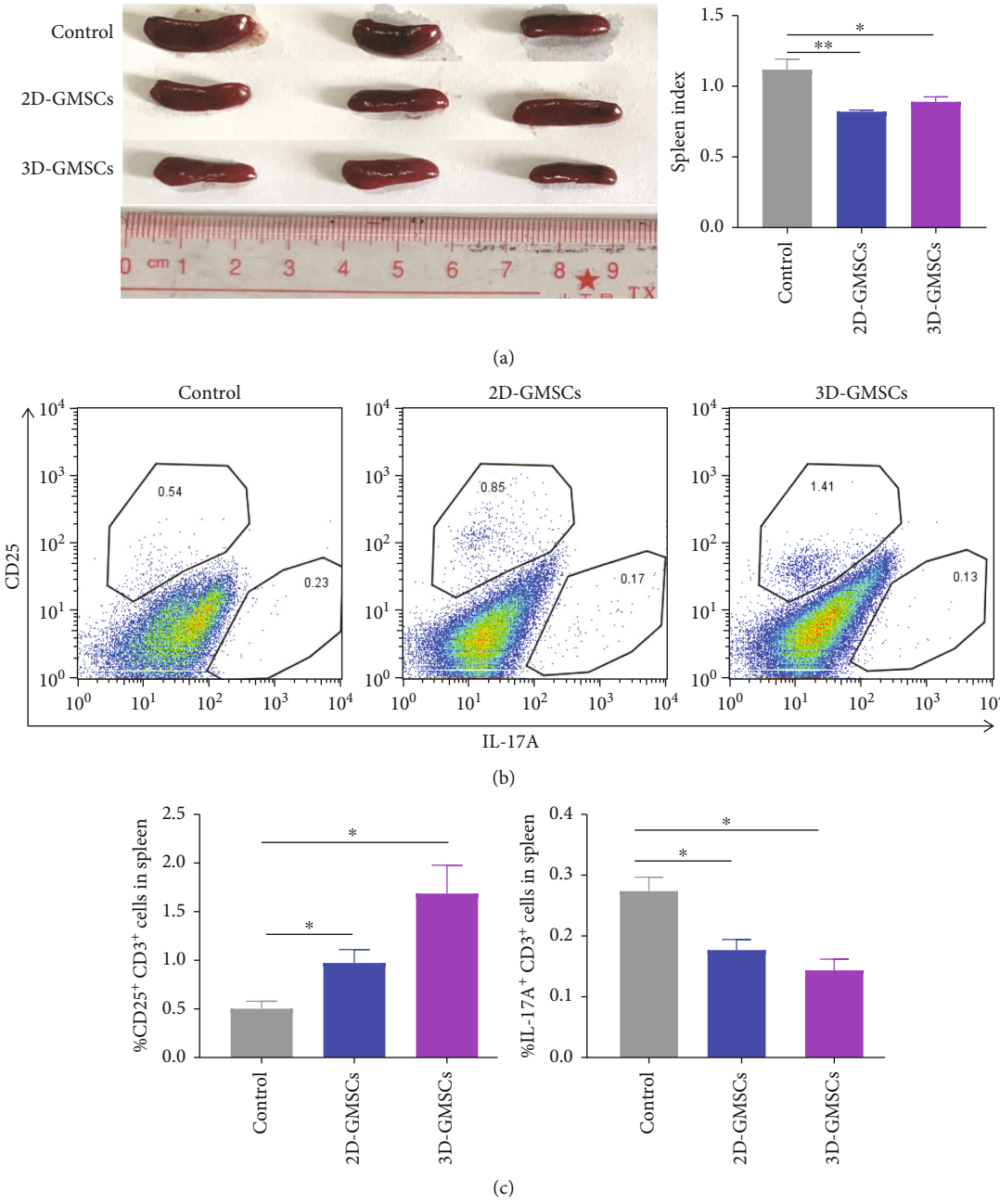
Figure 4: 2D-GMSCs and 3D-GMSCs significantly inhibited the skin inflammatory response in IMQ-induced mice. (a) Representative HE staining of mouse back skin. (b) Immunohistochemical studies were performed using antibodies for mouse TNF-α, IL-6, IFN-γ, and IL-17A. (c) The relative expression levels of TNF-α, IL-6, IFN-γ, and IL-17A were analyzed by pathological score (PS). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (d) The protein expression level of inflammatory cytokines in the skin tissue lysate evaluated via multiplex LEGENDplex analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

2D cell culture, 3D culture was regarded as more physiological with the characteristics better reserved [20]. In our study, we firstly observed the growth of GMSCs under 3D culture conditions. They spontaneously aggregated into spheroids under condition of ultralow attachment. In addition, the lower proliferation rate showed that 3D culture affected the growth characteristics of GMSCs. Next, the RNA sequencing analysis showed that a lot of genes involved in ossification, cytokine, mesenchymal cell differentiation, and chemotaxis were differentially regulated in 3D-GMSCs compared with 2D-GMSCs. Some previous studies on UC-MSCs and human bone marrow- and adipose tissue-derived MSCs revealed that 3D culture caused a significant upregulation of angiogenetic genes and promoted expression of proinflammatory and anti-inflammatory genes at the transcription level [21].

After RNA sequencing analysis, we focused on the effect of 3D culture on the immunophenotype of 3D-GMSCs through flow cytometry. Similar MSC marker expression was observed in 3D-GMSCs and 2D-GMSCs, whereas interestingly, the expression of CD44 and CD90 was downregulated in 3D-GMSCs. CD73 (Ecto-5′-nucleotidase, e-5′NT), a rate-limiting enzyme in the extracellular metabolism of ATP, can convert ATP to immunosuppressive adenosine; therefore, it is considered an important mediator of immunity [22]. Some studies demonstrated that CD73 expression was decreased in 3D spheroid-derived MSCs compared to the 2D cultured MSCs [23, 24]. Consistently, our results also showed the CD73 expression was downregulated at protein level in 3D spheroid-derived MSCs. CD90 (Thy1) is a glycophosphatidylinositol-anchored membrane protein highly expressed by MSCs [25]. According to reports, in vivo and in vitro, periosteum-derived cells sorted with CD90 have higher osteogenic potential than unsorted cells [26]. Recently, a study showed that compared with MSCs from wild-type mice, the osteogenic differentiation ability of MSCs from Thy1 knockout mice was reduced [27]. Together, these findings showed that CD90 was related with osteogenic differentiation. Most recently, studies have shown that 3D spheroids from AMSCs and WJ-MSCs showed higher expression of the osteogenic markers Runx2, osteopontin, and ALP at mRNA level than 2D cultured cells [28, 29]. Our present study showed that 3D-GMSCs exerted lower expression of the CD90 at mRNA and protein level than 2D-GMSCs.

Psoriasis is a chronic, Th1/Th17-mediated inflammatory disease, which is related to the excessive proliferation and differentiation of abnormal keratinocytes, resulting in erythema, thickness, and scaly plaques [30]. It affects an estimated 125 million people worldwide [31]. Initially, Th1 cells and the cytokines produced by these cells, such as TNF-α and IFN-γ, were associated with psoriasis [32]. Numerous studies showed that Th17 cells and their inflammatory mediators play an important role in the pathogenesis of psoriasis [33]. Th17 cytokines, such as IL-6, IL-17A, IL-17F, IL-21, and IL22, act on keratinocytes, leading to their activation and overproliferation [34, 35]. The activated keratinocytes in turn promote the recruitment of inflammatory cells [34, 35]. The current clinical treatment of psoriasis completely involves topical drugs, including vitamin D3 analogues, topical corticosteroids, calcineurin inhibitors, keratolytics, and biologics that inhibit TNF-α, IL-12, IL-13, IL-17, and IL-23 [31].

IMQ is an agonist of Toll-like receptors (TLR) 7 and 8, used to treat warts, and has been widely used to induce psoriasis-like skin inflammation [36]. Several studies have shown that MSCs and exosomes derived from MSCs can effectively improve psoriasis-like skin lesions in mouse models [37–39]. Here, this is the first study to investigate the therapeutic potential of 2D cultured GMSCs and spheroid-derived GMSCs in psoriasis-like lesions induced by IMQ administration in a mouse model. Our results



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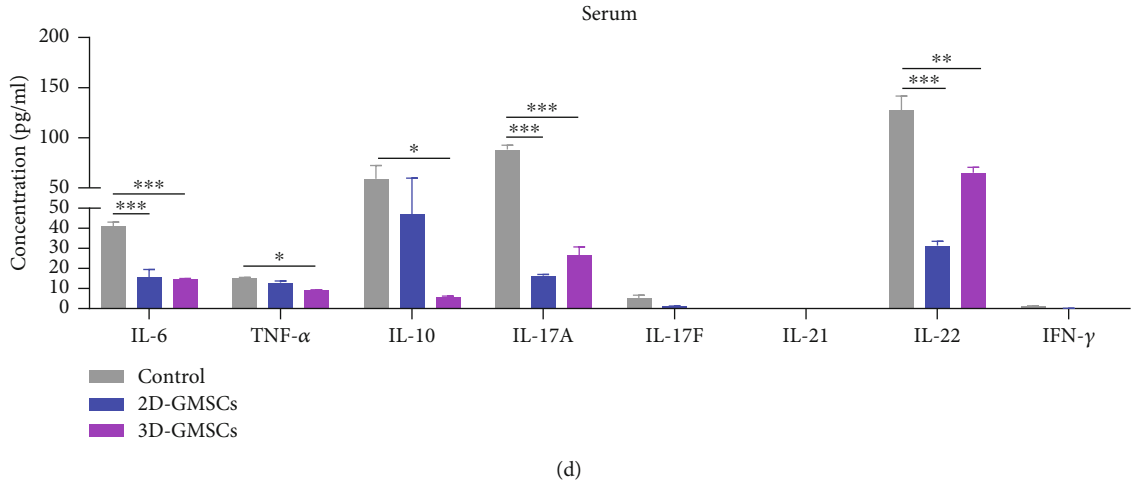

(d)

Figure 5: GMSCs strongly influence the expression of serum inflammatory mediators and spleen $CD25^+CD3^+$ and $IL\text{-}17^+CD3^+$ T cell responses. (a) Representative photographs of spleen and the ratio of spleen weight to bodyweight in different groups. $^*p < 0.05$; $^{**}p < 0.01$. (b) Representative flow cytometry staining for CD25 and IL-17A in $CD3^+$ T cells from mouse spleen. (c) Quantification of percentage of $CD25^+CD3^+$ T cells and $IL\text{-}17A^+CD3^+$ T cells from flow cytometry data. $^*p < 0.05$. (d) The protein expression level of inflammatory cytokines in the serum evaluated via multiplex LEGENDplex analysis. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

showed that GMSCs reduced erythema, skin thickness, and scaling exerted protective effects against psoriasis-like skin inflammation induced by IMQ under 2D and 3D culture conditions. Furthermore, HE staining confirmed that 2D-GMSCs and 3D-GMSCs could prevent the proliferation and abnormal differentiation of keratinocytes. The spleen is a major organ of the immune system and secreting a variety of immune-active cytokines; therefore, it plays an important role in immune activities. In this study, we found that 2D-GMSCs and 3D-GMSCs significantly inhibited the ratio of spleen to body weight, indicating that GMSCs under 2D and 3D culture conditions can regulate the inflammatory immune cells of the spleen to produce an inflammatory immune response with a systemic antipsoriatic effect. GMSCs cultured in 3D and 2D have similar effects. We found that 2D-GMSCs and 3D-GMSCs can inhibit IMQ-induced inflammation in psoriasis-like mouse models. 2D-GMSCs and 3D-GMSCs significantly reduced the serum levels of Th1 cytokines (TNF-α and IL-6), Th17 cytokines (IL-17A and IL-22), and IL-10, which means intravenous injection 2D-GMSCs and 3D-GMSCs inhibit IMQ-induced inflammation. Consistently, these results were observed in the skin; conversely, the level of IL-10 was increased. These results indicate that 2D-GMSCs and 3D-GMSCs inhibit IMQ-induced Th1/Th17 cytokine and psoriasis skin changes. However, further research is needed to determine the exact molecular mechanism of GMSC's anti-inflammatory effects. Finally, we measured the percentage of Treg and Th17 in the mouse spleen. We found that the ratio of Treg cells increased after treatment with 2D-GMSCs and 3D-GMSCs, while the ratio of Th17 cells decreased, showing that 2D-GMSCs and 3D-GMSCs exert immunomodulatory and anti-inflammatory properties. In summary, we revealed that transplantation of 2D-GMSCs and 3D-GMSCs has therapeutic potential for the treatment of psoriasis-like skin lesions.

Data Availability

All of the data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Ziyu Ye and Yanfang Liang performed the collection of data and data analysis and wrote the manuscript. Bihua Lin and Yanyun Li performed the collection of data and data analysis and reviewed and checked the article. Xingxing Chai and Jiachun Lian performed the collection of data. Xueying Zhang and Zhengping Che performed the data analysis. Jincheng Zeng performed the conception and design, financial support, data analysis and interpretation, and manuscript writing. The authors have read and approved the final manuscript. Ziyu Ye, Yanfang Liang, and Bihua Lin should be regarded as joint first authors.

Acknowledgments

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Research Article

Mesenchymal Stem Cells Ameliorate Hyperglycemia in Type I Diabetic Developing Male Rats

Hany N. Yousef, Samia M. Sakr, and Sahar A. Sabry

Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Cairo 11566, Egypt

Correspondence should be addressed to Hany N. Yousef; hany_barsoum@edu.asu.edu.eg

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One of the most promising treatments for diabetes mellitus (DM) is stem cell therapy. This study is aimed at elucidating the antidiabetic effect of mesenchymal stem cells (MSCs) on streptozotocin- (STZ-) induced DM in developing male rats. Twenty-four male albino rats (4 weeks old) were divided into control, diabetic, diabetic+MSCs1 (received MSCs one week after STZ treatment), and diabetic+MSCs2 (received MSCs 4 weeks after STZ treatment). Diabetic rats showed marked impairment ($p < 0.05$) in serum levels of glucose, insulin, C-peptide, glycosylated hemoglobin (HbA1c), malondialdehyde (MDA), total antioxidant status (TAS), and total oxidant status (TOS) in addition to disruption of the calculated values of homeostatic model assessment of insulin resistance (HOMA-IR), pancreatic β cell function (HOMA-β), and oxidative stress index (OSI). These biochemical alterations were confirmed by the histopathological and ultrastructural assessments which showed marked destructive effect on pancreatic islet cells. MSC therapy in an early stage reversed most of the biochemical, histological, and ultrastructural alterations in the STZ-induced diabetic model and restored the normal cellular population of most acinar cells and islet of Langerhans. These results indicate that MSC therapy of STZ-induced diabetic developing rats during an early stage has the capacity of β cell restoration and the control of blood glycemic homeostasis.

1. Introduction

Diabetes mellitus (DM) is among the most important endocrine disorders and public healthcare problems worldwide characterized by hyperglycemia as its hallmark feature. This disease results either from a loss of insulin-producing pancreatic β cells which is referred to as type I diabetes mellitus (T1DM) or through the mislaying of hormone responsiveness in its target tissues like adipose tissues and muscles which is known as type II diabetes mellitus (T2DM) [1]. The stubborn blood sugar concentration resulting as a consequence of diabetes generates free radicals causing oxidative stress that plays a substantial role in the pathophysiology of diabetes. Patients with DM have a high risk for many serious health disorders and may be subject to the irreversible injury, malfunction, and defect of many organs, including the skin, kidney, eye, nerves, and heart [2].

Due to the adverse effects caused by hyperglycemia on the different body organs, much research adopted various trails to overcome its pathological impacts. Patients with T1DM depend upon daily insulin injections; however, exogenous uptake of insulin can never be as accurate and dynamic like insulin secretion from endogenous β cells and therefore can only partially diminish the risk of the development of the disease complications [3].

So far, the trials to develop efficient immunosuppressive remediation to restrain β cell loss before DM onset had limited success [4]. Consequently, the reestablishment of endogenous insulin secretion represents a very important goal to maintain the blood sugar concentration within its normal range as well as to lessen or avoid complications of hyperglycemia and the patient's need for the self-management of blood glucose by exogenous insulin administration. Recent progress in regenerative medicine has capitalized on employing stem cells for the repair and regeneration of different cells and tissues. In this context, the transplantation of insulin-secreting cells obtained from human embryonic stem cells (hESCs) has been suggested as a therapeutic regimen for DM [5]. Nonetheless, the ethical

quandary surrounding the destruction of a human embryo was and continues to be a major impediment to the development of therapeutic medicines based on human embryonic stem cells (hESCs) [6]. Individual attitudes are so firmly embedded in basic moral values that constructing a definite policy that everyone can agree on appears implausible. This ethical quandary is reflected in many pieces of laws governing hESC research across the world [7].

Mesenchymal stem cells (MSCs) have emerged as hopeful candidates to cure many diseases due to their distinctive characteristic properties like self-renewal, immune-suppressive potential, and capability to transdifferentiate. They are easily obtained from varied tissue sources, like umbilical cord, bone marrow (BM), and adipose tissues, with the minimal risk of rejection, and many previous studies have provided evidences of their valuable roles. MSCs are employed in dermal repair and regeneration in severe burning and diabetic wounds [8], besides their role in restoring and regenerating the β cells of the pancreas [9, 10]. To the best of our knowledge, very few trials have been conducted on the beneficial role of MSCs in ameliorating T1DM in developing mammals. Therefore, the aim of the current work was to elucidate the potential of MSC infusion in ameliorating hyperglycemia in streptozotocin- (STZ-) induced diabetic developing male rats, monitored by biochemical, histological, and ultrastructural approaches.

2. Materials and Methods

2.1. Experimental Animals. Twenty-four male developing albino rats (4 weeks old) with body weights ranging from 60 to 80 g and at the age of one month were obtained from the Egyptian Organization for Biological Products and Vaccines, Giza, Egypt. The animals were housed in clear plastic cages (2 animals/cage) with wood chips as bedding in a room with controlled conditions (temperature range of $25 \pm 2^{\circ}$C, relative humidity of $55 \pm 5\%$, and a 12 h light/dark cycle). The animals were fed ad libitum with a standard diet and acclimatized in the laboratory for one week (from postnatal day 28 to postnatal day 35) prior to experimentation. The experiments were performed parallel to the ethical standards and according to the guide approved by the Local Institutional Animal Ethics Committee of Ain Shams University.

2.2. Induction of Experimental Diabetes. Rats fasted for 12 h have been rendered diabetic by a single intraperitoneal (IP) injection of freshly prepared STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 45 mg/kg of body weight dissolved in ice-cold saline (pH = 4.5) in a volume of 1 ml/kg of body weight [11]. To avoid hypoglycemia and mortality, rats were permitted to drink 5% glucose solution ad libitum overnight after STZ injection. Blood samples were taken from the tail vein 72 h after STZ administration, and the fasting blood glucose (FBG) concentration was determined by means of one touch ultra-glucometer and compatible blood glucose strips. Rats exhibiting FBG $\geq$ 250 mg/dl were considered diabetic and were selected for the experiments. Control rats were injected with normal saline solution parallel to the treated groups throughout the course of the study.

2.3. Preparation and Isolation of Bone Marrow- (BM-) Derived Mesenchymal Stem Cells (MSCs). Bone marrow was extracted from 10 adult male white albino rats' tibiae and femurs by flushing them with Dulbecco's modified Eagle's medium (DMEM) combined with 10% foetal bovine serum. A density gradient was used to separate nucleated cells, which were then resuspended in a complete culture medium supplemented with 1% penicillin–streptomycin. As a primary culture or upon formation of large colonies, cells were incubated at 37°C in 5% humidified CO_2 for 12–14 days. As large colonies were formed (80–90% confluence), the cultures were washed twice with phosphate buffer saline (pH 7) and trypsinized for 5 minutes at 37°C with 0.25% trypsin in 1 mM EDTA. Cells were resuspended in serum-augmented medium after centrifugation and incubated in 50 cm^2 culture flasks (Falcon). The resulting cultures were considered as first-passage cultures according to Alhadlaq and Mao [12]. MSCs are defined by their fusiform outline and adhesiveness [13].

Cells were resuspended in a wash buffer (BD Biosciences, Germany) after a short centrifugation. For 45 minutes at room temperature, 300 ml of cell suspension was incubated with antibodies against CD29, CD105, CD34, and CD90 conjugated with allophycocyanin (APC), cyanine 5 (CY5), phycoerythrin (PE), and fluorescein isothiocyanate (FITC) dyes, respectively. A FACSCalibur (BD Biosciences, Germany) was used for flow cytometry, and Cell Quest software was used for analysis.

2.4. Experimental Design. The rats were divided into four groups (6 animals each) as follows.

Group I (control group): healthy rats received normal saline solution parallel to the treated groups throughout the course of the study.

Group II (diabetic group): STZ-induced diabetic rats.

Group III (diabetic+MSCs1 group): a single dose of MSCs (0.5 ml containing 2×10^6 cells/rat) was injected via the tail vein [14] in rats with STZ-induced diabetes. This dose was conducted during the early phase (performed one week after induction of diabetes, i.e., one-week diabetic developing rats).

Group IV (diabetic+MSCs2 group): a single dose of MSCs (0.5 ml containing 2×10^6 cells/rat) was injected via the tail vein (as in group III) into diabetic rats during the late phase (4 weeks after induction of diabetes, i.e., 4-week diabetic developing rats).

2.5. Collection of Sera and Tissue Samples. After 7 days of MSC injection, animals were fasted overnight and afterwards anesthetized under light ether anesthesia. Blood samples were gathered by cardiac puncture and then centrifuged at $1500 \times g$ for 10 min at 4°C to get sera that were immediately stored at -80°C until use. Pancreatic tissue samples were also taken from rats of all groups for histological and ultrastructural studies.

2.6. Serum Biochemical Analysis

2.6.1. Glucose, Insulin, C-Peptide, and Glycosylated Hemoglobin. Serum glucose concentrations were assayed following glucose oxidase method [15], whereas rat-specific Enzyme-Linked ImmunoSorbent Assay kits were used to

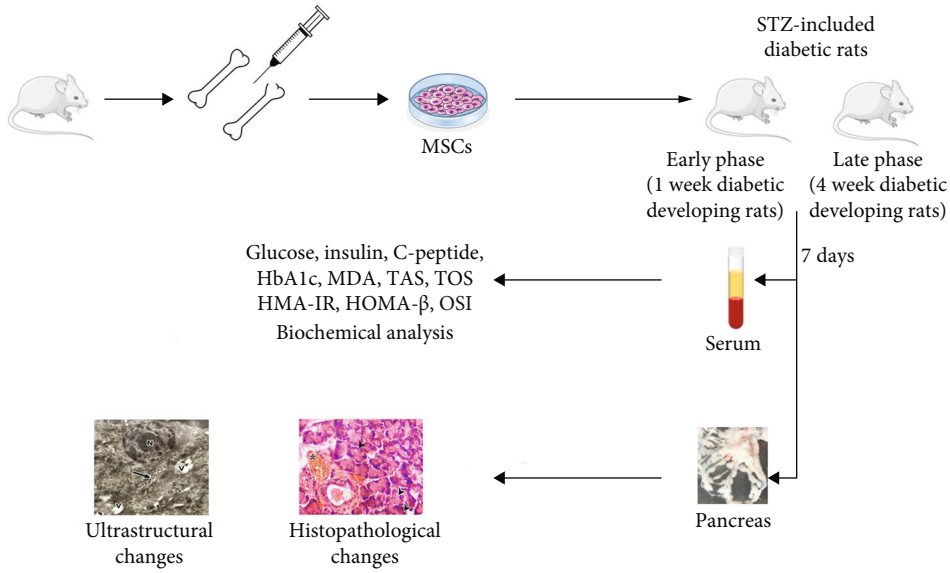


Figure 1: The isolation of MSC and the schematic representation of the study design.

quantify serum levels of insulin (ELISA, Crystal Chem, Elk Grove Village, IL, USA) and C-peptide (ELISA, DRG Instruments, GmbH, Marburg, Germany). The levels of glycosylated hemoglobin (HbA1c) were estimated in sera according to the method previously described by Nayak and Pattabiraman [16].

2.6.2. Homeostatic Model Assessment. Homeostatic model assessment of insulin resistance (HOMA-IR) and pancreatic β cell function (HOMA-β) were calculated using fasting serum insulin and fasting serum glucose concentrations according to the following equations [17]: HOMA-IR = fasting serum insulin (μIU/ml) × fasting serum glucose (mmol/l)/22.5; HOMA-β = fasting serum insulin (μIU/ml) × 20/fasting serum glucose (mmol/l) − 3.5.

2.6.3. Oxidative Stress Parameters. Lipid peroxidation (LP) was measured following the method of Draper and Hadley [18]. This method depends on the ability of malondialdehyde (MDA), a secondary product of lipid peroxidation, to react with thiobarbituric acid reactive substances (TBARS). The absorbance of the resulting colored product was measured spectrophotometrically at 532 nm.

Serum total antioxidant status (TAS) levels were determined following Erel's method [19], which is based on bleaching the green-bluish color of 2,2′-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) (ABTS) radical cation by sample antioxidants. The assay was calibrated using a stable antioxidant standard solution known as Trolox equivalent (Trolox Eq), and the results were expressed in mmol Trolox Eq/l.

Serum total oxidant status (TOS) levels were measured according to the method described by Erel [20]. This method is based on oxidation of ferrous ion–chelator complex into ferric ion by the various oxidants in the sample under acidic conditions. The assay was calibrated using hydrogen peroxide, and the results were expressed in μmol H_2O_2 Eq/l.

Values of oxidative stress index (OSI) were calculated using the following formula: OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/L)/TAS (μmol Trolox Eq/L) × 100 [21].

2.7. Histological and Ultrastructural Preparations. Small pieces of the pancreas were immediately fixed in aqueous Bouin's solution for 24 h. Paraffin-embedded sections (5 μm thickness) were stained with hematoxylin and eosin [22], microscopically examined, and photomicrographs were made as required. As for the electron microscopic preparations as described by Dykstra et al. [23], freshly excised pancreatic tissue samples were cut into very small pieces and fixed in 2.5% glutaraldehyde for 4 h and 2% paraformaldehyde in 0.1 mol cacodylate buffer (pH: 7.4). The samples were postfixed in buffered solution of 1% osmium tetroxide at 4°C for 1-5 h. This was followed by dehydration in ascending grades of ethyl alcohol, clearing in propylene oxide for two changes, 5 min each, and embedded in EPON epoxy resin. Semithin sections of 1 μm thickness were stained with toluidine blue and investigated under a bright field light microscope. Ultrathin sections were cut, mounted on form var-coated grids, and stained with uranyl acetate and lead citrate [24]. Sections were examined and photographed on a Jeol transmission electron microscope (JEOL Inc., Peabody, MA, USA) in the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

Figure 1 displays a visual summary of the followed methodology and main findings.

2.8. Statistical Analysis. All the biochemical data were expressed as mean ± SEM (6 rats/group). The statistical variations between the treatments were evaluated by one-way analysis of variance (ANOVA) using the SPSS/20.0 software

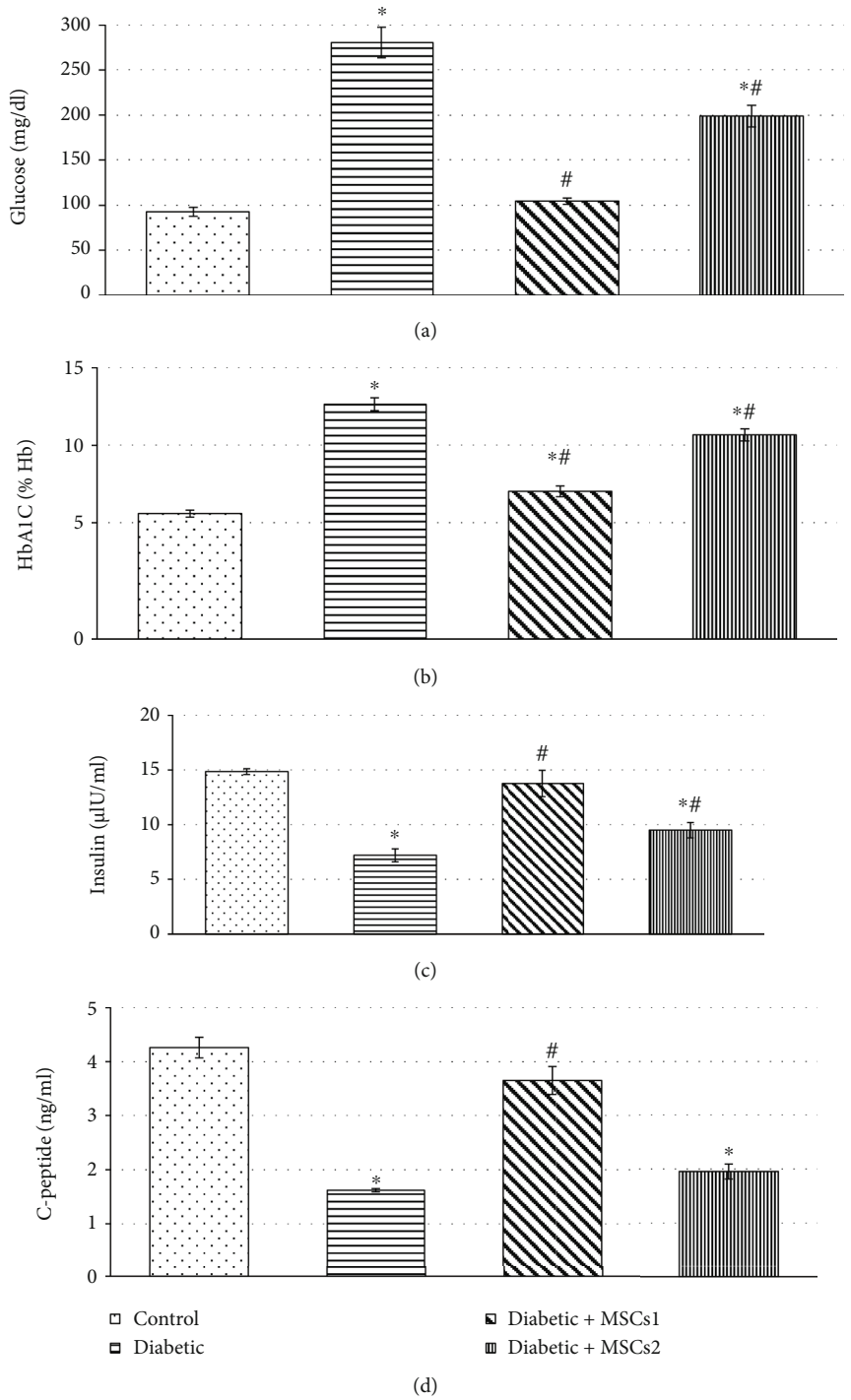


Figure 2: Effect of mesenchymal stem cell transplantation on serum levels of (a) glucose, (b) HbA1c, (c) insulin, and (d) C-peptide of the control and experimental groups of developing diabetic rats. Values are expressed as mean ± SEM ($n = 6$). Comparisons are $^{*}p \leq 0.05$ significantly different from the control group and $^{\#}p \leq 0.05$ significantly different from the diabetic group.

followed by Tukey post hoc test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Biochemical Analysis. Serum levels of glucose, HbA1c, insulin, and C-peptide were assessed to monitor the glycemic state of the control and experimental groups of developing rats. The data in Figure 2 show incredible increase ($p < 0.05$) in serum glucose and HbA1c levels accompanied by marked decline in levels of insulin and C-peptide in the STZ-induced diabetic group as compared with the control group. Meanwhile, the treatment of the diabetic developing rats with a single dose of MSCs during the early phase

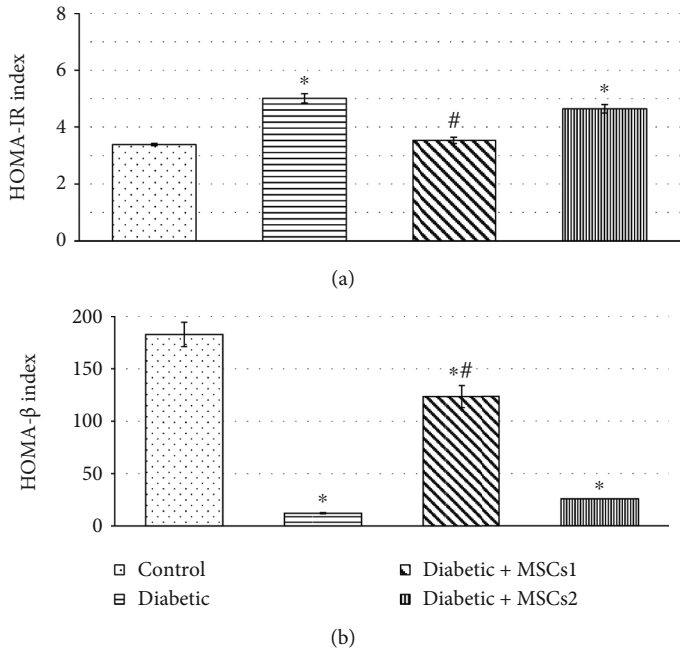


Figure 3: (a) Homeostatic model assessment of insulin resistance (HOMA-IR) and (b) homeostatic model assessment of pancreatic β cell function (HOMA-β) of the control and experimental groups of developing diabetic rats. Values are expressed as mean ± SEM ($n = 6$). Comparisons are $^{*}p \leq 0.05$ significantly different from the control group and $^{\#}p \leq 0.05$ significantly different from the diabetic group.

(diabetic+MSCs1 group) alleviated all the measured glycemic state indices. However, the treatment of diabetic developing rats with a single dose of MSCs during the late phase (diabetic+MSCs2 group) improved the values of these markers relative to STZ-induced diabetic developing rats but these values were still significantly different ($p \leq 0.05$) compared to the control animals.

Figure 3 depicts the impact of MSC administration to developing diabetic rats on insulin sensitivity and pancreatic β cell function using two simple mathematical indices (HOMA-IR and HOMA-β). Under the current experimental conditions, STZ-induced diabetic developing rats showed higher insulin resistance (HOMA-IR values 5.01 ± 0.16 vs. 3.39 ± 0.04, $p \leq 0.05$) and lower pancreatic β cell function (HOMA-β values 12.12 ± 0.55 vs. 182.81 ± 11.64, $p \leq 0.05$). The supplementation of the diabetic developing rats with MSCs during the late phase caused slight modulation of these parameters; however, they are still significantly different ($p \leq 0.05$) relative to the control values. On the other hand, the treatment of diabetic developing rats with MSCs during the early phase returned the values of insulin resistance to be very close to those of the control animals (HOMA-IR values 3.54 ± 0.11 vs. $3.39 \pm 0.04 \pm 0.04$, $p \geq 0.05$) and improved the pancreatic β cell function, but the values of HOMA-β were still significantly different ($p \leq 0.05$) from the control ones.

To evaluate the potential effects of intravenous injection of MSC transplantation on oxidative stress indices, the levels of MDA, TAS, TOS, and OSI were determined in sera of the control and experimental groups of developing rats. The obtained results (Figure 4) demonstrated that STZ-induced diabetic developing rats were subjected to oxidative stress which was confirmed by significant reduction of TAS accompanied by marked elevation in levels of MDA, TOS, and OSI compared with the control group. Slight modulation in the values of these oxidative stress indices was recorded in diabetic rats treated with MSCs during the late phase, but these markers are still significantly different ($p \leq 0.05$) when compared with the corresponding controls. Meanwhile, the treatment of the diabetic developing rats with MSCs during the early phase evoked significant alleviation ($p \leq 0.05$) in all the measured oxidative stress indices.

3.2. Histological and Histopathological Observations. The pancreas of a control developing rat is covered by a thin capsule of connective tissue that sends septa into it, separating the pancreatic lobules. The acini are surrounded by a basal lamina supported by a delicate sheath of reticular fibers. It has a rich capillary network. The pancreas is a mixed exocrine and endocrine gland. The exocrine portion is composed of the acinar cells which are pyramidal in shape with basal nuclei and possess granules at their apex. The intercalated duct penetrates partially into the acini forming centroacinar cells that constitute the intra-acinar portion of the intercalated duct. The endocrine portion of the pancreas is called the islets of Langerhans which dispersed randomly throughout the exocrine portion of the pancreas. The numbers of endocrine cells vary in the islets. Each islet is surrounded by a fine capsule of reticular fibers and consists of polygonal or rounded cells stain by hematoxylin and eosin lighter than (or not heavier than) pancreatic acinar cells and arranged in cords which are separated by sinusoid (Figures 5(a) and 5(b)). There are three different types of cells, but they are hardly differentiated by light microscope.

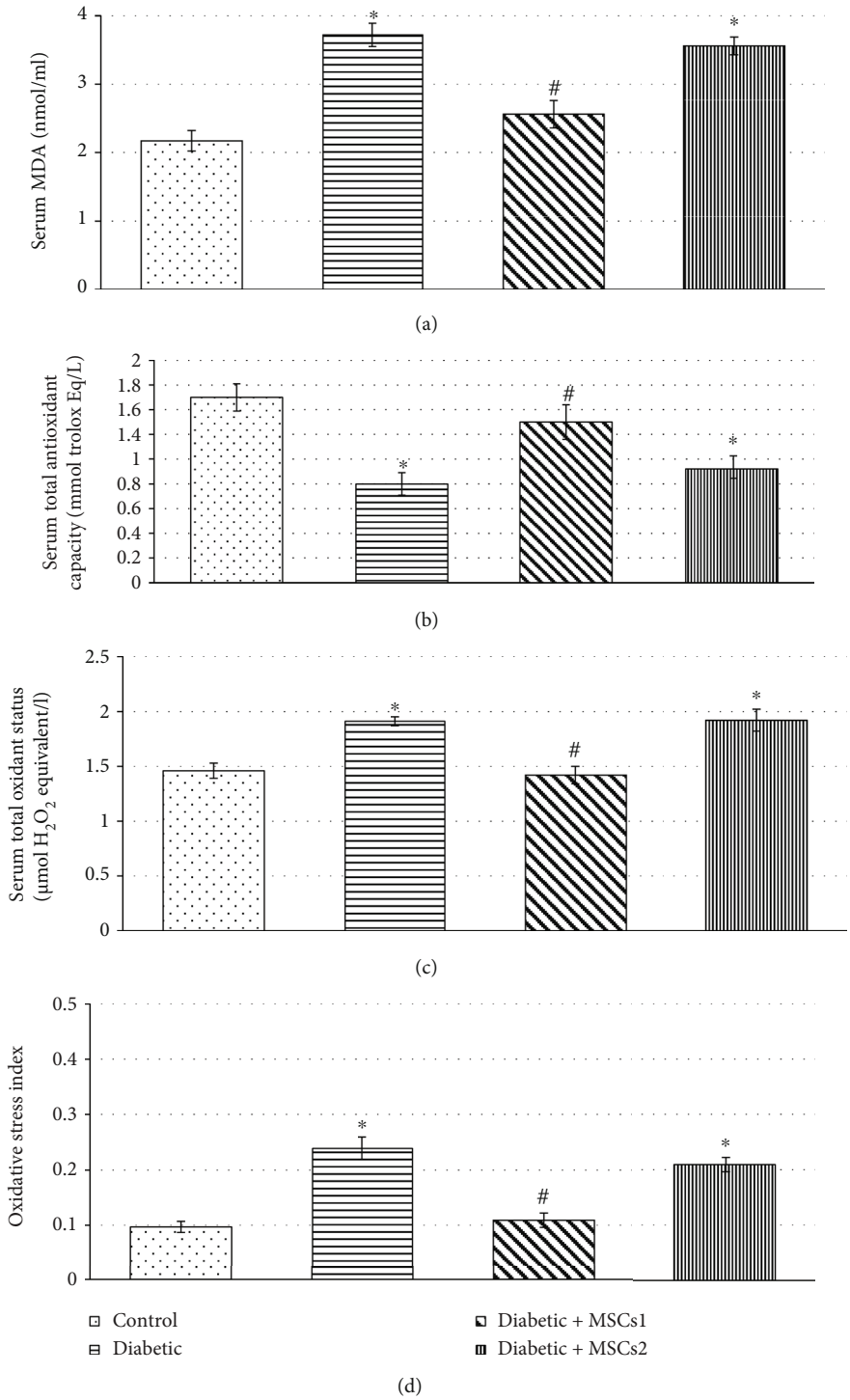
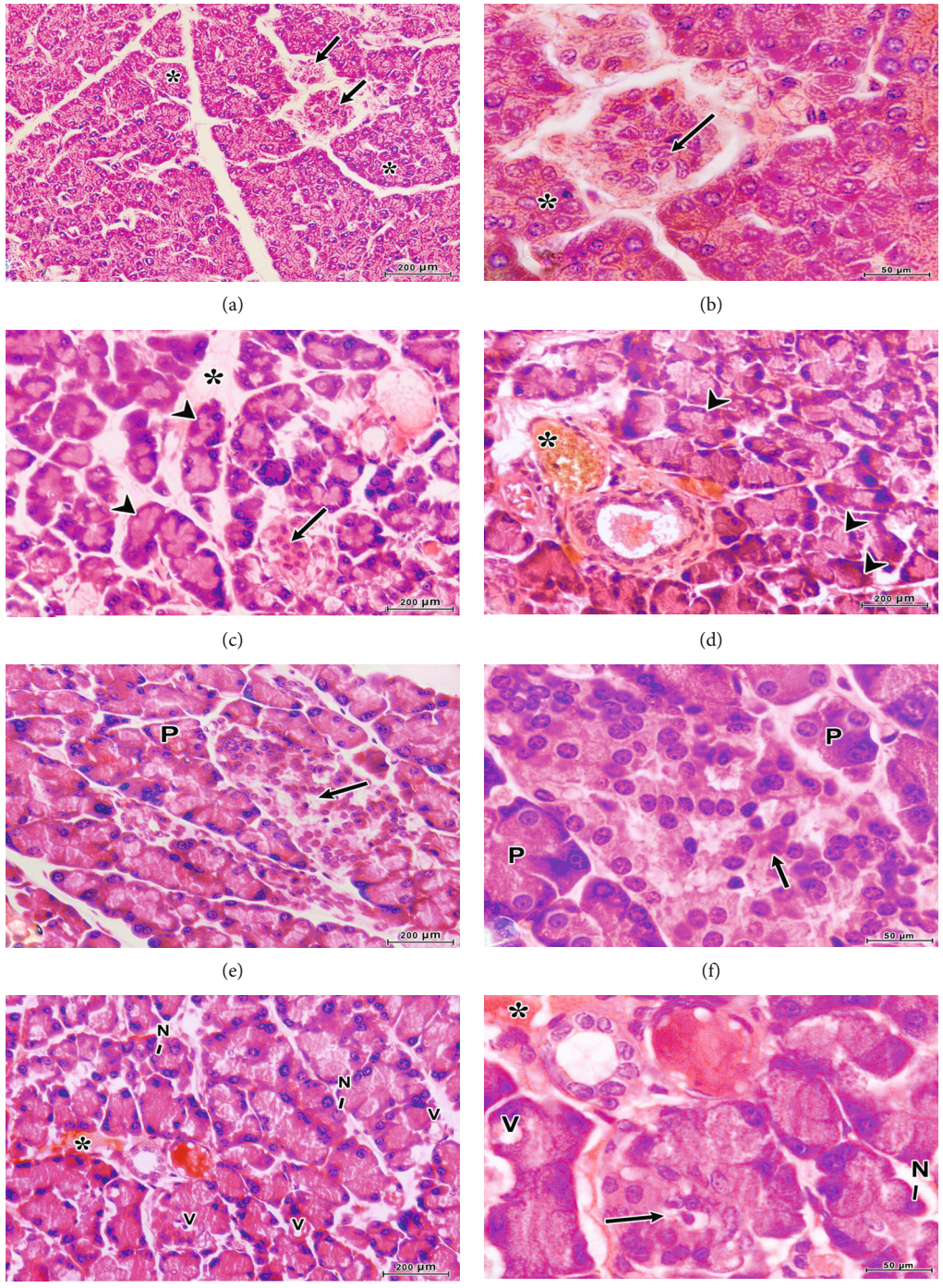


Figure 4: Effect of mesenchymal stem cell transplantation on serum levels of oxidative stress parameters: (a) malondialdehyde (MDA), (b) total antioxidant status (TAS), (c) total oxidant status (TOS), and (d) oxidative stress index (OSI) of the control and experimental groups of developing rats. Values are expressed as mean ± SEM ($n = 6$). Comparisons are $^{*}p \leq 0.05$ significantly different from the control group and $^{\#}p \leq 0.05$ significantly different from the diabetic group.

The histological examination of sections of the pancreas of STZ-induced diabetic developing rats illustrated pathological changes of both exocrine and endocrine parts of the pancreas. The pancreatic acini revealed focal acinar damage ranged from cytoplasmic vacuolation and pyknotic nuclei to acinar necrosis of some acinar cells with extravasations of blood from the damaged blood capillaries forming hemorrhagic appearance (Figures 5(c) and 5(d)). The endocrine part showed extensive damage of the islets of Langerhans which appeared to be irregular in shape with reduction of their size.



(a) (b) (c) (d) (e) (f) (g) (h)

Figure 5: (a, b) Photomicrographs of sections of the pancreas of a control developing rat illustrate closely packed lobules of normal pancreatic acini (∗) and endocrine islets (arrow). The acini are formed of pyramidal cells with basal nuclei and apical acidophilic cytoplasm. (c, d) Photomicrographs of sections of the pancreas of a diabetic developing rat show severe reduction and shrinkage of islets of Langerhans (arrow) with degeneration and necrosis of the acini components (arrowheads) and extravasations of blood from the damaged blood capillaries forming hemorrhagic appearance (∗). (e, f) Photomicrographs of sections of the pancreas of a diabetic developing rat treated with mesenchymal stem cells during early phase (diabetic+MSCs1 group) show normal pancreatic acini (P) and some acinar cells with cytoplasmic degeneration. Note the increase in density (arrow) and size of islet in this group. (g, h) Photomicrographs of sections of the pancreas of a diabetic developing rats, treated with mesenchymal stem cells during late phase (diabetic+MSCs2 group), show no significant recovery. The focal acinar damage represented by cytoplasmic vacuolation (V) and the nuclei (N) exhibited karyolysis of some acinar cells with shrinkage of islets of Langerhans (arrow).

The histological structure of the pancreatic tissues of most rats of the diabetic+MSCs1 group displayed normal built-up both in acinar cells and islets of Langerhans except the presence of few vacuoles between the cells of some islets of Langerhans (Figures 5(e) and 5(f)).

Although the pancreatic tissue of some rats in the diabetic+MSCs2 group revealed mild recovery, but most acinar cells exhibited cellular vacuolation with karyolysed nuclei. Damaged blood vessels with extensive edema are also observed (Figure 5(g)), and the reduction of size of islets of Langerhans is also noted (Figure 5(h)).

3.3. Ultrastructural Observations. Electron microscopic examination of the pancreas of the control developing rats revealed the fine structure of the acinar cells and islets of Langerhans. The cytoplasm of acinar cells contains numerous arrays of well-developed cisternae of rough endoplasmic reticula, mitochondria, and numerous electron dense secretory granules of variable sizes in the apical part (Figure 6(a)). The nuclei of these cells are basally located and spherical in shape, ensheathed by a nuclear envelope and possessing nucleoli, peripheral dense heterochromatin, and homogenous euchromatin material. The islets of Langerhans of the pancreas of the control developing rats are formed mainly of β cells. Their cytoplasm contains numerous electron dense secretory granules surrounded by wide lucent halo and rounded euchromatic nucleus (Figures 6(b) and 6(c)).

Electron microscopic examination of the pancreas of the diabetic group showed marked changes in pancreatic acini represented by dilated and fragmented rough endoplasmic reticula, decrease of secretory granules, vacuolated mitochondria, in addition to shrinkage and pyknotic nuclei. Also, the blood sinusoid was congested by hemolysed blood cells (Figures 6(d) and 6(e)). The β cells of the pancreas of diabetic rats showed obvious vacuolation and decrease of secretory granules, fusion of some granules, and pyknotic nuclei (Figure 6(f)).

Marked improvement in pancreatic acini after early treatment with MSCs (diabetic+MSCs1 group) represented by increase in zymogen granules, regular nuclear envelope and flattened rough endoplasmic reticula, and less affected mitochondria (Figure 6(g)). Treatment in the early phase (one-week diabetic developing rats) with MSCs revealed euchromatic nuclei, few vacuoles in β cells, and increase of secretory granules compared with the diabetic ones, and the mitochondria appeared nearly normal except that few of them appeared vacuolized (Figures 6(h) and 6(i)).

Electron microscopic examination of the pancreas of diabetic developing rats treated with MSCs (diabetic+MSCs2 group) in late phase (4-week diabetic developing rats) showed conspicuous degeneration in pancreatic acini represented by dilated rough endoplasmic reticula, decrease of secretory granules, degeneration of the mitochondria, in addition to the shrank nucleus which was surrounded by an irregular nuclear envelope. Congested blood sinusoid accompanied with hemolysed blood cells was also seen (Figures 6(j) and 6(k)). β cells showed obvious cytoplasmic vacuolation, severe degenerated mitochondria, decrease of secretory granules with fusion of some granules, and pyknotic nuclei (Figure 6(l)).

4. Discussion

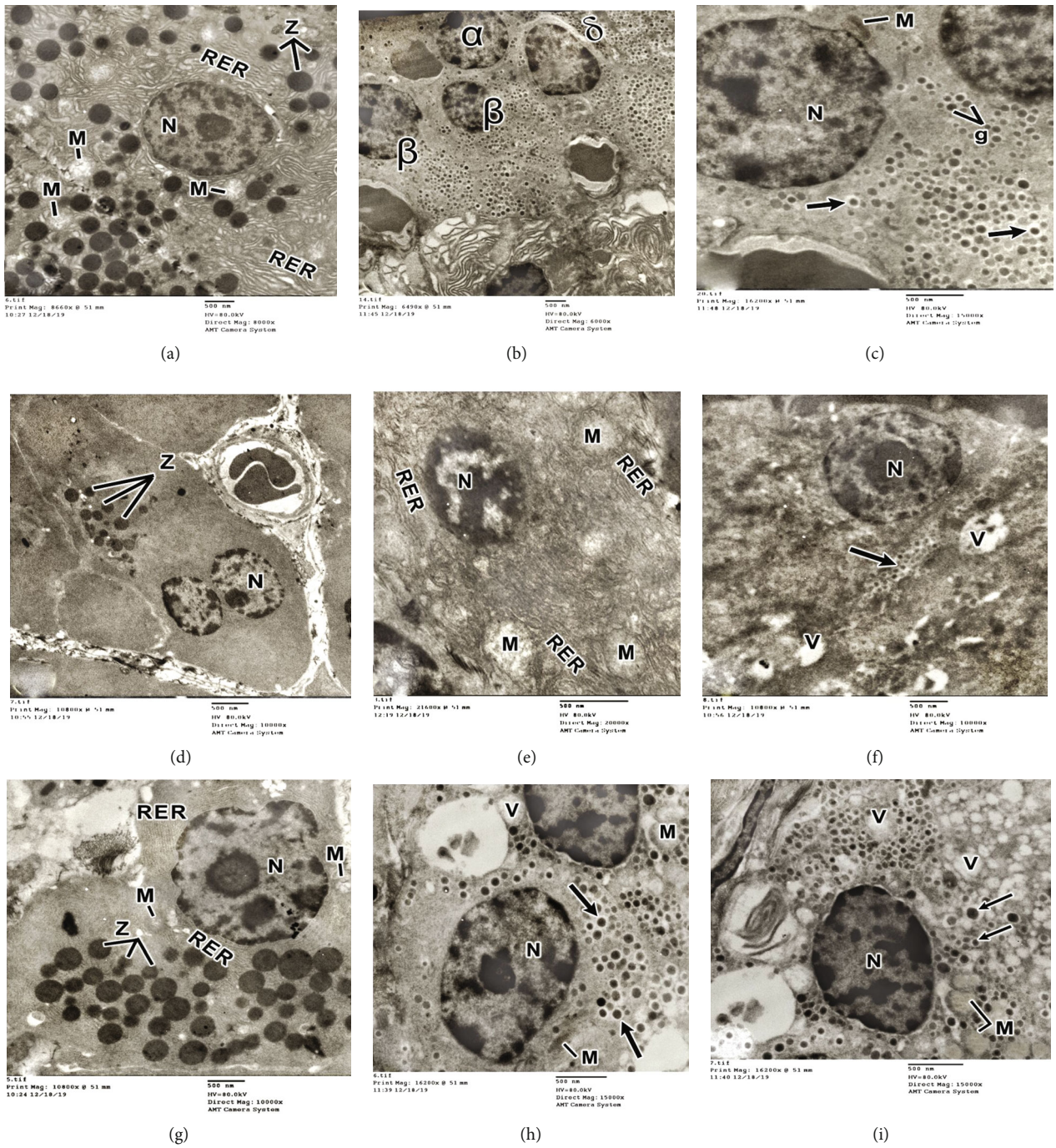
DM has been ranked as the fifth leading cause of death in the world [25]. The effective role of MSCs in the treatment of T1DM has been reported by several researchers. This is supported with the capacity of MSCs to differentiate into insulin-producing cells that can be used to replace damaged pancreatic β cells [26, 27]. In this context, the current investigation was designed to shed light on the potential of MSCs in ameliorating hyperglycemia in STZ-induced diabetic developing male rats, following biochemical, histological, and ultrastructural approaches.

In the present study, T1DM induction was confirmed by the obtained significant increase in serum levels of glucose and HbA1c which was accompanied by marked decline in the levels of insulin and C-peptide in sera of STZ-treated developing rats compared with the control values. Furthermore, STZ injection has resulted in marked elevation in insulin resistance indicated by an increase in HOMA-IR, with a significant decline in β cell function indicated by a decrease in HOMA-β. These findings are consistent with other studies [28, 29] showing that STZ administration induced progressive β cell dysfunction in rats.

STZ is a cytotoxic glucose analogue that penetrates β cells via glucose 2 transporter in the plasma membrane [30]. Inside β cells, biochemical pathways are induced by STZ causing DNA fragmentation and cell death: (i) DNA methylation through production of carbonium ions which activate the nuclear enzyme poly ADP-ribose synthetase and consequently lead to NAD^+ depletion; (ii) nitric oxide production, (iii) free radicals' generation, and (iv) altering NF-κB-based cell signaling [31, 32].

Serum C-peptide content is a real predictor of any change in the level of insulin since it is cosecreted by pancreatic cells with insulin as a by-product of the enzymatic cleavage of proinsulin to insulin [33]. HOMA-IR has been shown to be a robust method for the surrogate evaluation of insulin resistance [34]. In the current research, the high values obtained for HOMA-IR in diabetic rats are consistent with Rossetti et al. [35] who proved that high glucose levels induce the development of insulin resistance in peripheral tissues due to impairment of insulin secretion and insulin sensitivity. The biochemical basis for hyperglycemia-induced insulin resistance is still unclear. It may be attributed to modifications in the structure of insulin receptors and the glucose delivery system, resulting in disrupted signal propagation [36].

The observations obtained from the present light and electron microscopical studies clearly demonstrated that the administration of streptozotocin induced various histological and ultrastructural alterations in pancreatic tissues of developing rats. These histopathological changes included degeneration and necrosis of pancreatic acinar cells, with severe damage of the islets of Langerhans. The pancreatic acini revealed focal acinar damage accompanied with extravasations of blood from the damaged blood capillaries (Figures 5(c) and 5(d)). In addition, the pancreatic β cells displayed reduction in their size and number as well as marked cellular degeneration represented by cytoplasmic

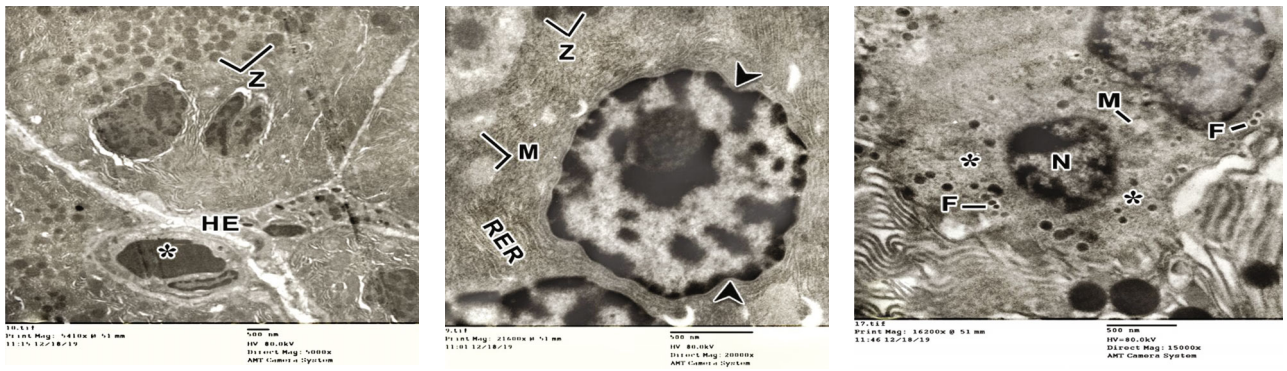


(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

Figure 6: Continued.



(j) (k) (l)

Figure 6: Electron micrographs of a pancreatic acinar cell of control developing rat demonstrating (a) zymogen (Z) granules, arrays of rough endoplasmic reticulum (RER), and mitochondria (M) with basally located nucleus (N); (b) parts of β, α, and δ cells; (c) parts of two β cells with spherical-shaped nuclei (N). β granules (g) have dark central core surrounded by an electron lucent halo (arrows). Electron micrographs of pancreas acinar cells of diabetic developing rat showing (d) decrease of the zymogen (Z) granules; (e) shrinkage and pyknotic nucleus (N), vacuolized mitochondria (M), and extensive fragmented arrays of rough endoplasmic reticulum (RER); (f) β cell in Langerhans islet of the pancreas displaying cytoplasmic vacuolation (V), obvious decrease of secretory granules (arrow), and pyknotic nucleus (N). Electron micrograph of pancreatic acinar cells of a diabetic developing rat treated with mesenchymal stem cells during early period (diabetic+MSCs1 group) illustrating (g) the acinar cells appeared nearly similar to the normal form with basally located normal nuclei (N) and normal aggregation of zymogen (Z) granules with few degenerated mitochondria (M) and arrays of rough endoplasmic reticulum (RER). (h) Aggregation of β cells which appeared nearly like the control group, euchromatic oval nucleus (N); few vacuoles in β cells and increase of the secretory granules (arrows) surrounded by electron lucent halo; (i) oval nucleus (N); increase in the secretory granules (arrows) with some vacuolized mitochondria (M). Few vacuoles are also seen (V). Electron micrographs of (j, k) pancreatic acinar cells and (l) β cell in Langerhans islet of a diabetic developing rat treated with mesenchymal stem cells in a late period (diabetic+MSCs2 group) showing (a) hemorrhage edema (HE) among the acini with congestion in the blood vessels (∗). (b) Damaged mitochondria (M); dilated rough endoplasmic reticulum (RER), zymogenic granules (Z), and an irregular nuclear envelope (arrowheads). (c) Vacuolized mitochondria (M) lost most their cristae and a scarcity of β granules in some β cells (∗). Fusion of some granules (F) is also seen. The nucleus (N) of islet β cell appeared with an irregular shape.

vacuolations and nuclear pyknosis (Figure 6(f)). These results were compatible and confirming the findings of pervious investigators [37–40] in their studies on the pancreas of alloxan-induced diabetic rats. Extravasations of blood from the damaged blood vessels cause the problem that not enough oxygen reaches the pancreatic tissue which leads to tissue degeneration and necrosis [39, 41].

Along the same line, electron microscopic observations of the pancreatic cells of diabetic rats and those of animals treated with MSCs in a late period (4-week diabetic developing rats) exhibited marked alterations in pancreatic acini concentrated in vacuolation and degeneration of the mitochondria, dilation of rough endoplasmic reticula, and minor appearance for the secretory granules (Figures 6(d), 6(e), 6(j), and 6(k)). Also, β cells showed reduction in their secretory granules and their nuclei manifested pyknotic appearance (Figures 6(f) and 6(l)). Similar degenerative features were observed in β cells of streptozotocin-diabetic adult rats treated with acarbose and *Rumex patientia* L. [42]. It has been reported that mitochondrial dysfunction can lead to cellular damage and apoptosis [43]. In this respect, Bogolepov [44] stated that tissue vacuolation is considered as a structural indication for disturbance in the permeability of the membranes that leads to the transport of water and electrocytes into the cell which may lead to cellular degeneration.

The present investigation showed that infusion of MSCs substantially suppressed damage of β cells and enhanced their repair as indicated by the improvement of glucose, insulin, C-peptide, insulin resistance, and β cell function. These outcomes are in concurrence with those obtained by Mansor et al. [45] and Hussien et al. [46] who demonstrated that MSCs possess tissue repair and/or cytoprotective potential due to their preferential homing property to damaged pancreatic tissues with significant islet reconstruction. In addition, treatment with a single dose of MSCs has greatly improved insulin sensitivity in diabetic developing male rats. The obtained improvements were most prominent during the early phase (performed one week after induction of diabetes) as compared with the late phase (performed 4 weeks after induction of diabetes). The aforementioned results are confirmed through the histological and ultrastructural observations on diabetic developing rats treated with MSCs in the early period (diabetic+MSCs1 group) which displayed restoration of normal cellular population of the acinar cells and islet of Langerhans (Figures 5(e), 5(f), and 6(g)–6(i)).

Repair of the acinar cells may be due to the rapid differentiation of MSCs into normal functional acinar cells since the presence of immature cells with damage cells help in tissue and cell regeneration [47]. In this respect, several authors suggested the following modes for regeneration. First, the new acinar cells were proliferated from preexisting acinar cells. Second, the degranulated and duct-like acinar cells redifferentiate to a normal acinar state [48–50].

Considering the mechanisms of MSC interactions with the immune response, MSCs possess a wide range of

immunoregulatory capabilities that result in immunosuppression of numerous effector functions through interacting with immune cells in both the innate and adaptive immune systems, including B cells, T cells, dendritic cells, natural killer cells, neutrophil, and macrophages [51]. MSCs also promote metabolism by secreting a wide range of chemokines, growth factors, and cytokines, as well as producing a vast array of secretomes and proteomes. These bioactive molecules are involved in immunomodulatory actions, mediating hematopoietic stem cell engraftment, MSC differentiation, and controlling angiogenesis and apoptosis [52, 53].

It has been well documented that MSCs have arisen as helpful means for the treatment of many diseases due to their ability to transdifferentiate in spite of their properties as self-renewal and immunosuppressive potential. In 2013, Wei et al. [54], Dimarino et al. [9], and Patel et al. [10] reported that MSCs could exert their actions through paracrine mechanisms and lead to cellular repairing from damage. Different reports on adult experimental mammals had revealed that there is no evidence for β cell regeneration following chemical ablation [55, 56]. Meanwhile, in young rodents, the rate of β cell proliferation is quite high and rapidly decreases with age [57–59].

In the current investigation, it was noticed that in the pancreas of developing rats of the third group (diabetic+MSCs1; one-week diabetic rats), the most affected cells were β cells than δ and α cells. This may reveal that a single intravenous injection of MSCs into developing rats at an early age has resulted in the emergence of β cells from δ and α cells. So, early treatment of diabetic rats with MSCs can play an important role in the restriction of type 1 diabetes at an early age. In this regard, Thorel et al. [60] and Chera et al. [61] reported that extreme loss of β cell accelerates the conversion of pancreatic δ and α cells into β cells. On the other hand, the extent of such improvement and recovery appears to be reduced greatly in the pancreas of animals of the fourth group (diabetic+MSCs2, i.e., 4-week diabetic developing rats) which means that as the rats became older, the chance for regeneration of acinar cells and β cells became insignificant, and this agrees with the explanation reported by Kassem et al. [62], Meier et al. [63], Köhler et al. [64], and Gregg et al. [65] who stated that proliferation of β cells in humans occurs only during early childhood.

Finally, as a consequence of our research, we advocate expanding stem cell treatment, particularly in cases of diabetes in children, based on ethical precautions in stem cell sources and in accordance with religion and law. The next step might be to experiment with different stem cell sources and dosages to see how effective they are in curing patients.

5. Conclusions

Based on the findings of this study, we can conclude that more MSC therapy at the early stage of STZ-induced diabetic rats and at a young age increases the chance of recovery compared to injury at a later stage and at an older age, and these return to the capacity of cells in the first state in blood glycemic homeostasis restoration and control.

Data Availability

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Efficacy and Safety of Mesenchymal Stem Cell Transplantation in the Treatment of Autoimmune Diseases (Rheumatoid Arthritis, Systemic Lupus Erythematosus, Inflammatory Bowel Disease, Multiple Sclerosis, and Ankylosing Spondylitis): A Systematic Review and Meta-Analysis of Randomized Controlled Trial

Liuting Zeng,[1] **Ganpeng Yu**,[2] **Kailin Yang**,[3] **Wang Xiang**,[4] **Jun Li**,[2] **and Hua Chen**[1]

[1]*Department of Rheumatology and Clinical Immunology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China*
[2]*People's Hospital of Ningxiang City, Ningxiang city, Hunan Province, China*
[3]*Beijing Anzhen Hospital, Capital Medical University, Beijing, China*
[4]*Zhangjiajie City People's Hospital, Zhangjiajie, Hunan, China*

Correspondence should be addressed to Liuting Zeng; zltab2016@hotmail.com, Ganpeng Yu; yuganpeng.guke@hotmail.com, Kailin Yang; yang.cardiology@ccmu.edu.cn, Jun Li; lijun.guke@hotmail.com, and Hua Chen; chhuaa332211@163.com

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Objective. To evaluate the efficacy and safety of mesenchymal stem cell (MSC) transplantation in the treatment of autoimmune diseases. *Methods*. The Chinese and English databases were searched for clinical research on the treatment of autoimmune diseases with mesenchymal stem cells. The search time range is from a self-built database to October 1, 2021. Two reviewers independently screened the literature according to the inclusion and exclusion criteria, extracted data, and evaluated the bias of the included studies. RevMan 5.3 analysis software was used for meta-analysis. *Results*. A total of 18 RCTs involving 5 autoimmune diseases were included. The 5 autoimmune disease were rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease, ankylosing spondylitis, and multiple sclerosis. For RA, the current randomized controlled trials (RCTs) still believe that stem cell transplantation may reduce disease activity, improve the clinical symptoms (such as DAS28), and the percentage of CD4+CD 25+Foxp3+Tregs in the response group increased and the percentage of CD4+IL-17A +Th17 cells decreased. The total clinical effective rate of RA is 54%. For SLE, the results showed that mesenchymal stem cell transplantation may improve SLEDAI [-2.18 (-3.62, -0.75), $P = 0.003$], urine protein [-0.93 (-1.04, -0.81), $P < 0.00001$], and complement C3 [0.31 (0.19, 0.42), $P < 0.00001$]. For inflammatory bowel disease, the results showed that mesenchymal stem cell transplantation may improve clinical efficacy [2.50 (1.07, 5.84), $P = 0.03$]. For ankylosing spondylitis, MSC treatment for 6 months may increase the total effective rate; reduce erythrocyte sedimentation rate, intercellular adhesion molecules, and serum TNF-α; and improve pain and activity. For multiple sclerosis, the current research results are still controversial, so more RCTs are needed to amend or confirm the conclusions. No obvious adverse events of mesenchymal stem cell transplantation were found in all RCTs. *Conclusion*. MSCs have a certain effect on different autoimmune diseases, but more RCTs are needed to further modify or confirm the conclusion.

1. Introduction

Autoimmune diseases are a series of diseases caused by the immune system's response to self-antigens, resulting in self-tissue damage or dysfunction [1]. It mainly includes systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome, polymyositis and dermatomyositis [1, 2]. Many autoimmune diseases are characterized by the production of autoantibodies, which bind to the host's own proteins or form immune complexes and deposit in tissues. Any organ of the body may become a target organ for autoimmunity, including skin, joints, kidneys, and blood vessels. The inflammatory effect caused by autoantibodies is mediated by binding to Fc receptors on leukocytes, which is an important cause of downstream tissue damage [3, 4]. Meanwhile, autoantibodies can also directly mediate tissue damage in diseases through complement activation [5]. In the development stage of the disease, genetic factors and environmental factors may interact in turn to promote the development of autoimmunity and ultimately lead to tissue inflammation and damage, becoming a chronic disease with multiple organs and multiple system damage [6, 7].

Clinically, once diagnosed, patients should be treated with medication in time to avoid further development of the disease causing damage to organs or systems such as the liver and kidney [8]. Clinically, commonly used glucocorticoids and traditional disease-improving antirheumatic drugs (DMARDs) have good anti-inflammatory, pain-relieving, and improving or delaying disease progression effects and are still used as the first-line choice for clinical treatment of rheumatic immune diseases [9, 10]. However, for first-line treatments with single or combined regimens that do not respond well or cannot tolerate them, other treatment options with potential curative effects need to be considered [11–13]. For example, stem cell transplantation, biological preparations, or new botanical preparations, as well as some antirheumatic drug candidates that may modulate or suppress immunity, in order to alleviate the condition of patients with refractory rheumatism and improve their quality of life. Among them, mesenchymal stem cells are one of the most promising therapeutic strategies [14].

Since the discovery of mesenchymal stem cells (MSC), the understanding of them has continued to deepen. Because of its proliferation and differentiation ability, the medical community expects it to be used in the treatment of clinical diseases [14]. In the past few decades, the initial research on MSC focused on its differentiation ability, but with the discovery of its immunomodulatory function, the direction of MSC-based therapeutic research has changed from the initial regenerative medicine to autoimmune diseases [15]. So far, there have been many reports in the literature on the treatment of autoimmune diseases with MSC [16, 17], and there are more and more researches on the application of MSC in clinical trials. One-third of the clinical studies focused on the treatment of autoimmune diseases by MSC [18]. Studies have found that MSCs are weakly immunogenic and did not express major histocompatibility complex (MHC) class II molecules, apoptotic gene ligands, and T cell costimulatory molecules (B7-1, B7-2, CD40, and CD40L). It did not express or express MHC class I molecules at very low levels, did not induce an immune response in vitro, and also had an immunosuppressive effect. These studies have laid the theoretical foundation for the transplantation of allogeneic bone marrow MSCs to treat autoimmune diseases [19]. For some autoimmune diseases (such as rheumatoid arthritis (RA) and SLE, Crohn's disease, primary Sjogren's syndrome, systemic sclerosis, dermatomyositis, ankylosing spondylitis, psoriasis, multiple sclerosis), animal experiments and clinical controlled trials have also shown that MSC transplantation can improve the clinical symptoms of the above diseases [18–20]. Due to the relative uncontrollability of cell culture used in these clinical randomized controlled trials, the complexity of clinical trial design, and the implementation factors of effective evaluation measures, there is an urgent need for a comprehensive systematic review and meta-analysis of the clinical controlled trials of mesenchymal stem cells for the treatment of autoimmune diseases. Therefore, this study registered the protocol on PROSEPRO in order to provide a complete and comprehensive evaluation and provide new evidence for clinical practice.

2. Materials and Methods

2.1. Protocol. This systematic review and meta-analysis were conducted strictly in accordance with the protocol registered in PROSPERO (CRD42021277144) and PRISMA guidelines (see supplementary materials) [21].

2.2. Research Databases. Chinese databases (including CNKI, VIP database, Wanfang Database, and Sinomed) and English databases (including Embase, Medline, PubMed, and Web of Science) were searched from the establishment of the database to Oct. 1st, 2021. Cochrane Library and ClinicalTrials.gov were also searched. The research retrieval strategy of Embase and PubMed were shown in Table S1.

2.3. Search Criteria

2.3.1. Participants. Patients who have been diagnosed with any kind of autoimmune disease according to authoritatively recognized standards were included. Patients had no restrictions on gender, age, region, etc.

2.3.2. Intervention Methods. The intervention of the experimental group was mesenchymal stem cell (MSC), which can be used alone or in combination with other therapies. The intervention measures of the control group were non-MSC therapy, which could be traditional therapy, placebo, etc.

2.3.3. Outcomes. Outcomes were the efficacy and safety indicators of the corresponding disease.

2.3.4. Study design. The randomized controlled trial (RCT) without any restrictions was selected.

2.3.5. Exclusion Criteria. Exclusion criteria include (1) animal experiments, (2) basic research, (3) not RCT, and (4) the intervention of the control group was MSC transplantation.

2.4. Research Screening, Extraction, and Quality Assessment. First, we deduplicate the literature according to the title, author, year of publication, etc. Then, we read the abstract for further screening and finally read the full text and further screened according to the literature screening criteria and extracted data from the included RCTs [22]. The risk of bias was independently assessed by two researchers in accordance with the Cochrane Risk Bias Assessment Form [23] provided by the Cochrane Collaboration. Two researchers independently screened the literature, extracted data, and assessed the quality of RCTs. If there is a disagreement, they will negotiate with the third researcher.

2.5. Statistical Analysis. Revnan5.3 was used for meta-analysis [22]. Relative risk (RR) and mean difference (MD) are used as the combined effect size of dichotomous variables (such as adverse events and effective rate) and continuous variables (such as SLEDAI score), respectively. The heterogeneity between RCTs was tested by chi-square test, and the test standard was $P < 0.1$. The degree of heterogeneity was judged based on I^2. When $I^2 > 50\%$, it indicated that there is high heterogeneity, and a random effect model was established. On the contrary, when $I^2 < 50\%$, a fixed effect model was established.

3. Results

3.1. Search Results. A total of 1109 records were retrieved initially, and 23 records were left for further screening after preliminary screening. Finally, 18 RCTs were included for they meet the search criteria, while 5 records were excluded [24–28]. The literature screening process is shown in Figure 1.

3.2. Description of Included Trials. The included RCTs involved a total of 5 autoimmune diseases: rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, ankylosing spondylitis, and multiple sclerosis. The studies of Fernández et al. (2018) and Lublin et al. (2014) were divided into 2 subgroups according to the dose, and the study of Petrou et al. (2020) was divided into 2 subgroups according to the route of administration. The included study characteristics were shown in Table 1.

3.3. Risk of Bias Assessment. The summary and graph of risk of bias were shown in Figures 2 and 3.

3.3.1. Random Sequence Generation and Allocation Concealment. Nine RCTs [30, 34, 38, 39, 41–43, 45, 46] described the random sequence generation methods and were rated as low risk of bias. Other RCTs did not describe the random sequence generation method and were assessed as unclear risk of bias. Four RCTs [30, 36, 42, 45] describe allocation concealment methods and were assessed as low risk of bias. Panés et al. [38] did not perform allocation concealment and was assessed as high risk of bias. Other RCTs did not describe the allocation concealment methods and were assessed as unclear risk of bias.

3.3.2. Blinding, Incomplete Outcome Data, and Selective Reporting. Six RCTs [34, 38, 39, 42, 45, 46] describe the specific methods of blind implementation and were assessed as low risk. Álvaro-Gracia et al. [31] and Tang et al. [32] claimed to use the blind method but did not describe the implementation process; Shadmanfar et al. [30] did not mention whether to use blinding; therefore, they were rated as unclear risk of bias. Other RCTs did not use blinding and their outcomes were subjective indicators; hence, they were rated as high risk of bias. Six RCTs [29–31, 33, 36, 44] have missing data and did not use appropriate statistical treatment method; hence, they were rated as unclear risk of bias.

3.4. Other Potential Bias. Other sources of bias were not observed, and they were rated as low risk of bias.

3.5. Rheumatoid Arthritis. RA often manifests as joint swelling, joint stiffness, and tenderness in the morning. It is mainly due to the invasion and damage of the cartilage and bone due to synovial hyperplasia, which involves a variety of immune cells and mediated inflammation. Three RCTs reported MSC treatment of RA. However, due to their different data presentation methods, a systematic review was conducted. Among all RCTs, the use of bone marrow mesenchymal stem cells is generally safe and tolerable. Yang et al. [29] showed that after MSC treatment, the disease activity was weakened and the clinical symptoms (including DAS28) were improved. The improvement of most patients' condition lasts for 12 months, and the total clinical effective rate is 54%. Two patients in the response group had pain and swelling at 24 weeks, and their ESR and CRP levels increased. It is also found that the dosage of prednisone acetate in 23 patients in the experimental group gradually decreased after the intervention. For the immune response, it found that the percentage of CD4+CD 25+Foxp3+Tregs in the response group increased and the percentage of CD4 +IL-17A+Th17 cells decreased; and the levels of IL-6 and TNF-α decreased significantly.

Shadmanfar et al. [30] shows that MSC may improve the patient's standing time and WOMAC total score and reduce the use of methotrexate and prednisolone. It also showed that patients with knee involvement found that knee pain was reduced by more than 50%. Álvaro-Gracia et al. [31] showed that a moderate proportion of patients meets the comprehensive measure of ACR 20/50/70 response, but fewer patients achieve an improvement of 50% or 70%.

3.6. Systemic Lupus Erythematosus. SLE mainly manifests as specific skin lesions, fatigue, weakness, fever, and weight loss and other inflammatory symptoms (such as decreased serum C3). The symptoms of multiple organs are related to the involvement of organs. It was mainly evaluated by SLEDAI. If the kidney is involved, urine protein would be used to assess the kidney involvement.

3.6.1. SLEDAI. Four RCTs reported SLEDAI [32–35]. The heterogeneity test showed that $I^2 = 52\%$, $P = 0.10$, considering the moderate heterogeneity among RCTs. Therefore, the random effects model is used for data analysis. The results show that the SLEDAI in the experimental group was lower than that in the control group (-2.18 (-3.62, -0.75), $P = 0.003$) (Figure 4).

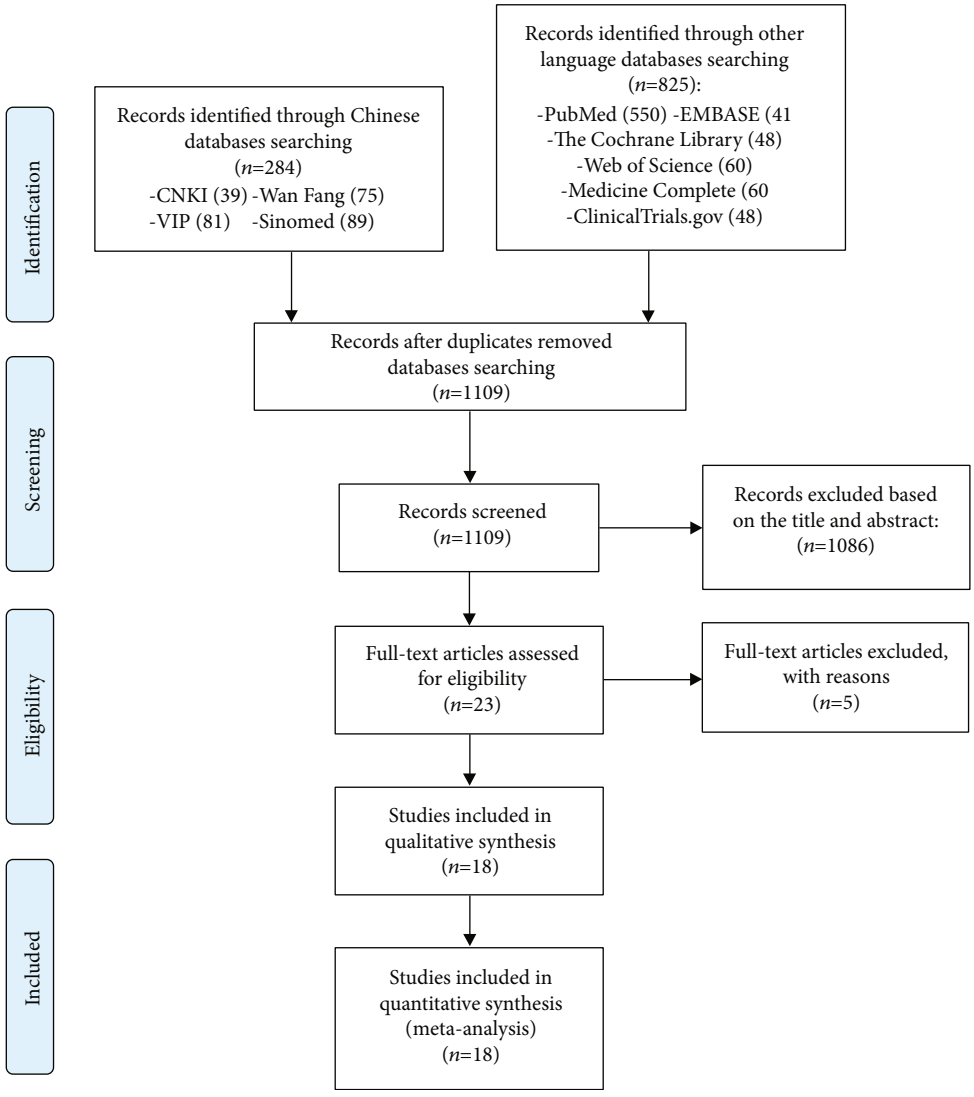

Figure 1: Flow diagram of research screening (CNKI: China National Knowledge Infrastructure).

3.6.2. Urine Protein. Four RCTs reported urine protein [32–35]. The heterogeneity test showed that $I^2 = 0\%$, $P = 0.72$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the urine protein in the experimental group was lower than that in the control group (-0.93 (-1.04, -0.81), $P < 0.00001$) (Figure 5).

3.6.3. Serum C3. Three RCTs reported serum C3 [33–35]. The heterogeneity test showed that $I^2 = 22\%$, $P = 0.28$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the serum C3 in the experimental group was higher than that in the control group (0.31 (0.19, 0.42), $P < 0.00001$) (Figure 6).

3.6.4. Adverse Events. Three RCTs reported serum adverse events [32, 34, 35]. The heterogeneity test showed that $I^2 = 0\%$, $P = 0.74$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the incidence of adverse events between two groups was of no statistical significance (0.87 (0.33, 2.29), $P = 0.79$) (Figure 7).

3.7. Inflammatory Bowel Disease. Inflammatory bowel disease is a chronic nonspecific gastrointestinal disease, which is disabling, can seriously affect all aspects of patients' lives, and also causes a heavy burden on the health care system and society. It mainly includes Crohn's disease and ulcerative colitis. Crohn's disease is an inflammatory bowel disease characterized by chronic inflammation of any part of the gastrointestinal tract, with a progressive and destructive course. The clinical symptoms are mainly diarrhea, abdominal pain, blood in the stool, fever, and fatigue. Ulcerative colitis mainly manifests as abdominal pain, rectal pain, bleeding, difficulty in defecation, fever, and fatigue.

3.7.1. Clinical Efficacy. A total of 4 RCTs were included [36–39]. The heterogeneity test showed that $I^2 = 74\%$, $P = 0.009$, considering the high heterogeneity among RCTs.

Table 1: The characteristics of the included studies.

Disease	Study	Trial registration number	Country	Sample size (female/male)		Intervention		Dosage	Route of medication	Relevant outcomes	Mean age (years)		Disease duration (years)		Duration
				Trial group	Control group	Trial group	Control group				Trial group	Control group	Trial group	Control group	
Rheumatoid arthritis	Yang et al. 2018 [29]	ChiCTR-ONC-16008770	China	52 (40/12)	53 (43/10)	MSC injection	1% albumin in physiological saline as the treatment or 50 mL of 1% albumin in normal saline without UCMSCs	$1*10^6$ cells/kg	Intravenous infusion	Disease activity, adverse events	Response: 50.7; nonresponse: 51.2	49.8	Response: 4 ± 2.75; nonresponse: 3.94 ± 2.79	3.89 ± 2.52	48 weeks
	Shadmanfar et al. 2018 [30]	NCT01873625	Iran	13 (13/0)	15 (13/2)	MSC injection	Normal saline	Not known	Intra-articular injection	Disease activity, adverse events	50.4 ± 8.5	48.1 ± 10.8	—	—	48 weeks
	Álvaro-Gracia et al. 2017 [31]	NCT01663116	Spain	46 (42/4)	7 (6/1)	MSC injection	Ringer's lactate solution	$1*10^7$ cell or 2∗10^7 cell or 3∗10^7 cell	Intravenous infusion	Disease activity, adverse events	54.15 ± 7.79	58.3 ± 14.25	14.36 ± 6.60	22.73 ± 22.65	24 weeks
Systemic lupus erythematosus	Tang et al. 2016 [32]	—	China	12 (11/1)		MSC injection +prednisolone +cyclophosphamide +mycophenolate	Prednisolone +cyclophosphamide +mycophenolate	$5*10^7$ cells	Intravenous infusion	Systemic lupus erythematosus disease activity index (SLEDAI), urine protein, adverse events	19-44		0.2-14		24 weeks
	Zeng et al. 2016 [33]	—	China	7 (not known)	8 (not known)	MSC injection +glucocorticoid +mycophenolate mofetil	Glucocorticoid +mycophenolate mofetil	$1*10^6$ cells	Renal artery	SLEDAI, urine protein, C3	—	—	—	—	48 weeks
	Deng et al. 2017 [34]	NCT01539902	China	12 (11/1)	6 (6/0)	MSC injection	Placebo	$2*10^8$ cells	Intravenous infusion	SLEDAI, urine protein, C3, adverse events	29 ± 10	29 ± 7	4.92 ± 3.67	7.83 ± 4.58	48 weeks
	Yang et al. 2014 [35]	—	China	17 (15/2)	20 (20/0)	MSC injection +glucocorticoid +cyclophosphamide	Glucocorticoid +cyclophosphamide	$3*10^7$ cells	Intravenous infusion	SLEDAI, urine protein, C3, adverse events	35.22 ± 10.13	36.23 ± 10.67	4.01 ± 2.97	4.31 ± 3.77	48 weeks
	Hu et al. 2016 [36]	NCT01221428	China	34 (13/21)	36 (14/22)	Mesenchymal stem cell (MSC) infusions twice besides the base treatment with a 7 day interval	Normal saline infusions twice besides the base treatment with a 7-day interval	$3.8 \pm 1.6*10^7$ cell	Intravenous infusion	Clinical efficacy (based on Mayo scores), Mayo score and IBDQ score, adverse events	42.9 ± 23.1	43.7 ± 28.7	5.6 ± 4.2	6.1 ± 4.9	24 weeks
Inflammatory bowel disease	Garcia-Olmo et al. 2009 [37]	—	Spain	14 (11/3)		Adipose-derived stem cells+fibrin glue	Fibrin glue only	$1*10^7$ cell/mL	Local injection	Clinical efficacy (healing of a complex perianal fistula), quality of life score (SF-12), adverse events	43.99 ± 8.97		—	—	8 weeks
	Panés et al. 2016 [38]	NCT01541579	Seven European countries and Israel	107(47/60)	105(49/56)	MSC injection	Normal saline injection	$1.2*10^7$ cell	Local injection	Clinical efficacy, adverse events	39.0 ± 13.1	37.6 ± 13.1	12.1 ± 10.0	11.3 ± 8.9	24 weeks
	Molendijk et al. 2015 [39]	NCT01144962	Netherlands	15 (6/9)	6 (3/3)	MSC injection	Normal saline+human albumin injection	$1*10^7$ cell or $3*10^7$ cell or $9*10^7$ cell	Local injection	Clinical efficacy (healing of a perianal fistula), adverse events	21-54	27-49	5-28	1-20	12 weeks
Ankylosing spondylitis	Su et al. 2020 [40]	—	China	20(8/12)	20(7/13)	MSC injection	Fliximab injection	$1*10^6$ cells/kg	Intravenous infusion	Clinical efficacy, immune index, and adverse events	32.15 ± 2.33	32.12 ± 2.31	—	—	24 weeks

Table 1: Continued.

Disease	Study	Trial registration number	Country	Sample size (female/male)		Intervention		Dosage	Route of medication	Relevant outcomes	Mean age (years)		Disease duration (years)		Duration
				Trial group	Control group	Trial group	Control group				Trial group	Control group	Trial group	Control group	
Multiple sclerosis	Ji et al. 2013 [41]	—	China	60 (39/19)		MSC injection +rituximab	Glucocorticoid	$5*10^7$ cells	Intravenous infusion and oral	Progression-free survival (PFS) rate, number of episodes, ability of daily living (ADL) scale, number of lesion, volume of lesion (cm^3), adverse events	28.3 ± 4.5		2.93 ± 0.13		96 weeks
	Fernández et al. 2018 [42]	NCT01056471	Spain	19 (13/6)	11 (8/3)	MSC injection low and high dose	Ringer's lactate	$1*10^6$ cells/kg or 4*10^6 cells/kg	Intravenous infusion	Expanded disability status scale (EDSS), number of lesion, volume of lesion (cm^3), adverse events	Low dose: 44.8 ± 8.0; high dose: 47.8 ± 9.7	46.3 ± 8.9	Low dose: 15.4 ± 6.1; high dose: 18.7 ± 8.7	18.9 ± 7.3	24 weeks
	Petrou et al. 2020 [43]	NCT02166021	Israel	32 (16/16)	16(4/12)	MSC injection	Normal saline	$1 * 10^6$ cells/kg	Intrathecal injection or intravenous infusion	EDSS, adverse events	Intravenous infusion: 49.05 ± 7.2; intrathecal injection: 47.42 ± 10.4	45.89 ± 10.9	Intravenous infusion: 10.28 ± 4.48; intrathecal injection: 12.90 ± 8.74	14.94 ± 8.27	24 weeks
	Li et al. 2014 [44]	—	China	13 (9/4)	10 (7/3)	MSC injection	No normal saline	$4*10^6$ cells/kg	Intravenous infusion	EDSS, cytokine	41.7 ± 5.6		2.90 ± 0.9		6 weeks
	Lublin et al. 2014 [45]	—	The United States and Canada	12(9/3)	4 (2/2)	MSC injection	Placebo	$1.5*10^8$ cells or $6*10^8$ cells	Intravenous infusion	EDSS, adverse events	36-58	40-52	—	—	48 weeks
	Llufriu et al. 2014 [46]	NCT01228266	Spain	9(7/2)		MSC injection	Suspension media	$1 - 2*10^6$ cells/kg	Intravenous infusion	EDSS, number of lesion, volume of lesion (cm^3), adverse events	36.8 ± 8.4		8.1 ± 2.15		24 weeks

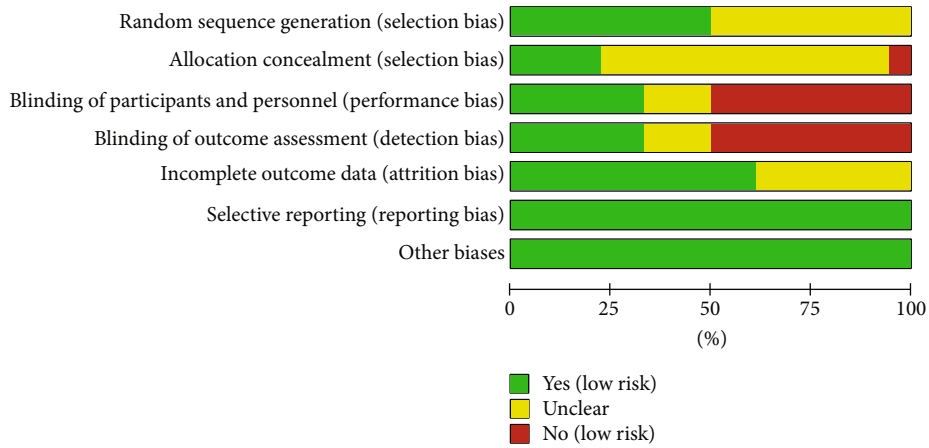

Figure 2: Risk of bias graph.

Therefore, the random effects model is used for data analysis. The results show that the clinical efficacy of the experimental group is better than that of the control group (2.50 (1.07, 5.84), $P = 0.03$) (Figure 8).

3.7.2. Adverse Events. A total of 4 RCTs were included [36–39]. The heterogeneity test showed that $I^2 = 0\%$, $P = 0.52$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the incidence of adverse events between two groups were of no statistical significance (0.99 (0.81, 1.22), $P = 0.96$) (Figure 9).

3.8. Ankylosing Spondylitis. Ankylosing spondylitis mainly manifests as chronic back pain and stiffness. It may be due to erosion, bone growth and vertebral fusion, and inflammatory damage involving Th1/17 and related cytokines. Only one RCT reported the treatment of ankylosing spondylitis with MSC. Su et al. [40] found that compared with the flximab group (control group), MSC treatment for 6 months may increase the total effective rate; reduce erythrocyte sedimentation rate, intercellular adhesion molecules, and serum TNF-α; and improve pain and activity.

3.9. Multiple Sclerosis. Multiple sclerosis is an immune disease characterized by chronic demyelination of the central nervous system. In multiple sclerosis patients, monocytes infiltrate into the perivascular space between the arteries and veins and pia mater, axon myelin sheath is lost and destroyed, and glial cell immunoreactivity changes lead to the formation of plaques in multiple parts of the central nervous system. Su et al. [40] found that the progression-free survival (PFS) rate, total number of episodes, and average number of episodes each year in the experimental group were lower than the glucocorticoid group (control group), while the quality of life in the experimental group was higher. Li et al. [44] also showed that compared with the control group, the overall symptoms of MSC-treated patients improved, and the EDSS and recurrence rate were reduced. However, the summary of other outcomes showed different results.

3.9.1. Number of Lesions and Volume of Lesions. Three RCTs reported number and volume of lesions [41, 42, 46]. For a number of lesions, the heterogeneity test showed that $I^2 = 0\%$, $P = 0.62$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the number of lesions between two groups were of no statistical significance (-1.13 (-3.80, 1.55), $P = 0.41$) (Figure 10).

For the volume of lesions, the heterogeneity test showed that $I^2 = 75\%$, $P = 0.007$, considering the high heterogeneity among RCTs. Therefore, the random effects model is used for data analysis. The results show that the volume of lesions between two groups were of no statistical significance (-5.08 (-11.33, 1.17), $P = 0.11$) (Figure 11).

3.9.2. Expanded Disability Status Scale. Three RCTs reported comparable data of EDSS [42, 43, 46]. The heterogeneity test showed that $I^2 = 85\%$, $P < 0.0001$, considering the high heterogeneity among RCTs. Therefore, the random effects model is used for data analysis. The results show that the EDSS between two groups were of no statistical significance (0.12 (-1.18, 1.43), $P = 0.85$) (Figure 12).

3.9.3. Adverse Events. Two RCTs reported adverse events [42, 45]. The heterogeneity test showed that $I^2 = 0\%$, $P = 0.56$, considering the low heterogeneity among RCTs. Therefore, the fixed effects model is used for data analysis. The results show that the adverse events between two groups were of no statistical significance (1.12 (0.81, 1.53), $P = 0.50$) (Figure 13).

4. Discussion

MSCs are a kind of adult stem cells that mainly exist in the bone marrow and have multidifferentiation potential, low immunogenicity, and immunomodulatory properties. In addition to the bone marrow, it can also be isolated and cultured from almost all adult tissues such as the placenta, umbilical cord, cord blood, and adipose tissue. MSCs have powerful immune regulation functions, can induce immune tolerance, and promote hematopoiesis and tissue repair.

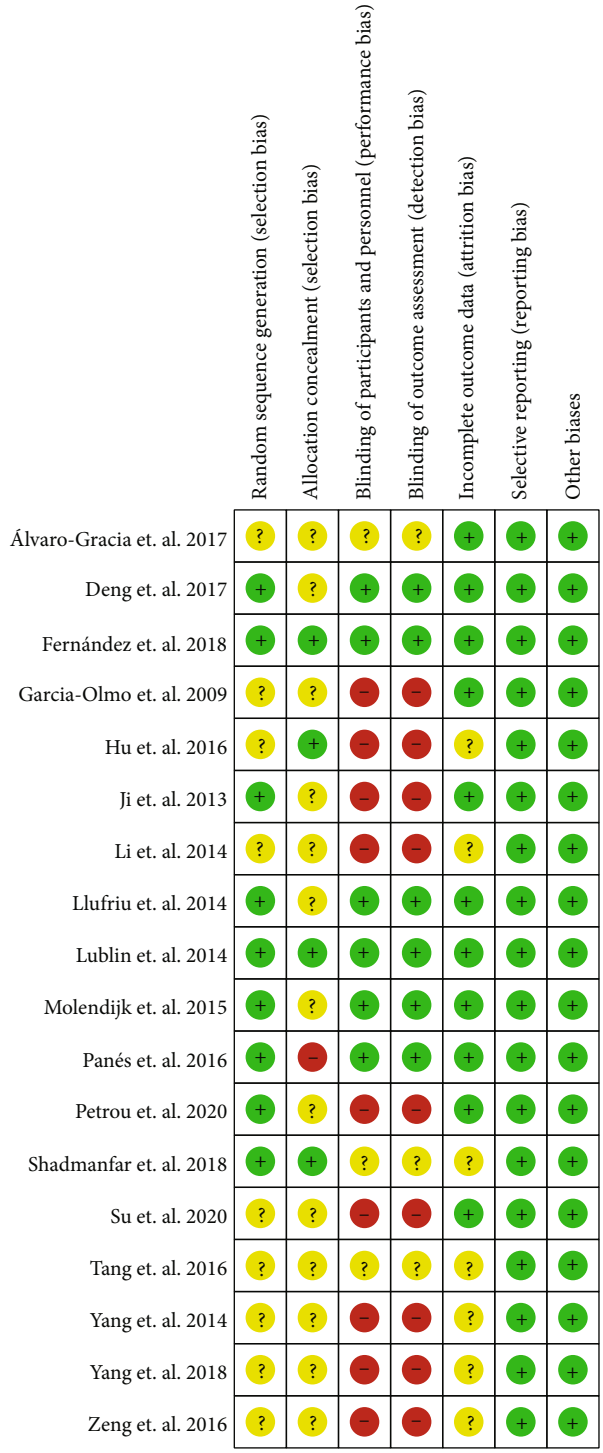

Figure 3: Risk of bias summary.

Studies showed that MSCs have the following characteristics: (1) inhibiting the proliferation of a variety of immune cells including T and B lymphocytes [47], (2) influencing the secretion of cytokines of immune cells to induce their anti-inflammatory effects [48], and (3) it may also release soluble factors and participate in the regulation of rabbit disease [49]. In addition, MSCs do not express major histocompatibility complex (MHC) class I molecules, but mainly express MHC class I molecules, which makes them have low immunogenicity [50]. Due to its multidirectional differentiation potential, immune regulation, hematopoietic support, low immunogenicity, and no immune rejection,, MSCs have been used in the treatment of refractory and severe autoimmune diseases in recent years, providing patients with safe and effective new treatment options.

4.1. The Molecular Mechanism of MSC Transplantation in the Treatment of Autoimmune Diseases. MSCs can exert their immunomodulatory properties by inhibiting the proliferation and activation of T lymphocytes, B lymphocytes, natural killer cells (NKs), and dendritic cells (DCs) [51, 52]. Studies have found that the MSCS of patients with autoimmune diseases has many problems such as changes in the number, abnormal cytoskeleton, decreased migration ability, abnormal multidirectional differentiation potential, and abnormal secretion of basic cytokines [53, 54]. It is currently believed that MSCs can inhibit the proliferation of multiple types of allogeneic immune cells [47] and exert immunoregulatory functions on T lymphocytes, B lymphocytes, macrophages, DCs, and NKs [17]. In addition, MSCs may exert immunomodulatory effects by secreting a variety of regulatory cytokines, such as interleukin- (IL-) 4, IL-7, IL-10, γ-interferon (IFN-γ), and prostaglandin E2 (PGE2) [55].

4.1.1. MSC's Immunomodulatory Effect on T cells. T cells mainly migrate into the thymus from pluripotent stem cells and pre-T cells in the bone marrow, differentiate into mature T cells under the induction of thymus hormone, and then play a series of immune functions. It has the characteristics of participating in delayed-type allergic reactions, regulating transplantation immunity, promoting the formation of precursor cells to produce antibodies, and regulating cellular immunity by secreting a variety of cytokines. It also has different subtypes such as helper, inhibitory, effector, and cytotoxic T cells [56, 57]. Current research has shown that various types of T cells are disordered in patients with autoimmune diseases, and intervention strategies for autoimmune diseases mediated by T cells have become the main direction of new drug development. MSC may secrete a variety of soluble cytokines through paracrine pathways, such as nitric oxide (NO), PGE2, and lumbromine 2,3 dioxygenase (IDO) and nutritional factors such as transforming growth factor (TGF)-β3 and tumor necrosis factor (TNF)-α [58, 59], to inhibit the proliferation of T lymphocytes [60–63]. This thereby affects the expression of cell surface markers, specific proliferation, the formation of cytotoxic T lymphocytes, Th1 type cell production of INF-γ, and Th2 type cell production of IL-4 [64, 65]. Glennie et al. found that MSC may suppress T cells in the G0/G1 phase of the proliferation cycle, downregulate cyclin 22 (an important conversion protein in the G1/S phase), and inhibit the p27Kipl protein, thereby causing a series of changes in the secretion of soluble cytokines, and ultimately inhibiting the activity of T lymphocytes [66]. In addition to cytokines, MSC may also exert an inhibitory effect through direct contact with T lymphocytes. MSC expresses PD-1 ligand 1 (PD-L1) and PD-L2 molecules that bind to programmed death protein 1 (PD-1) on the surface of T lymphocytes, causing the activity

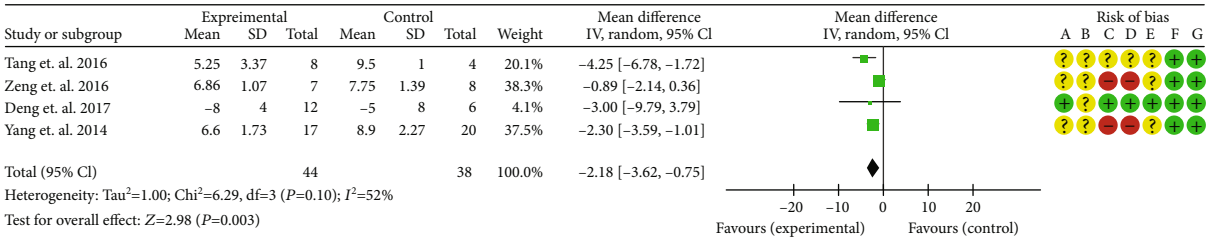

Figure 4: SLEDAI (CI: confidence interval).

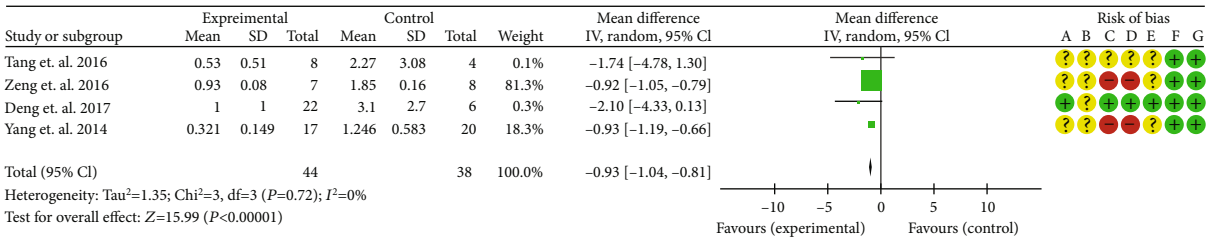

Figure 5: Urine protein (CI: confidence interval).

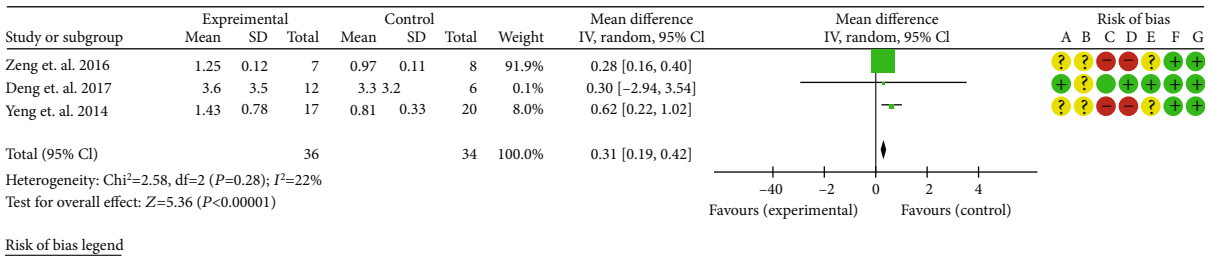

Figure 6: Serum C3 (CI: confidence interval).

of T lymphocytes to be inhibited and hindering their proliferation. These effects may only be exerted when the MSC is in direct contact with T lymphocytes [67]. It can be seen that MSC can affect the immune function of T cells through a variety of mechanisms, and the occurrence and development of a variety of diseases involve abnormal immune regulation of T cells. Therefore, clarifying the immune regulation of MSC to T cells can not only provide a theoretical basis for analyzing its specific mechanism of action in diseases but also provide new ideas for the treatment of immune-related diseases.

4.1.2. MSC's Immunomodulatory Effect on B Cells. When B cells bind to antigens, they activate and proliferate. On the one hand, plasma cells and memory B cells are produced

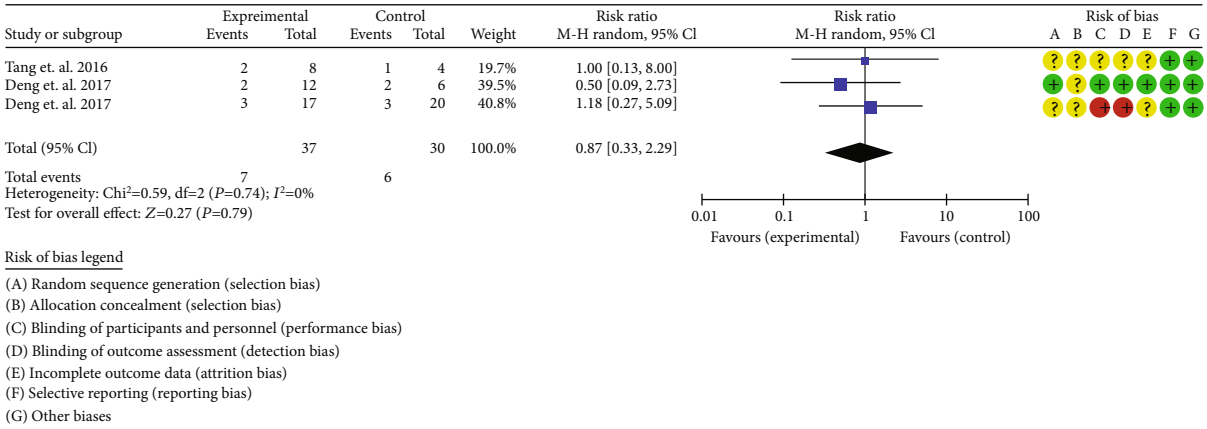

Figure 7: Adverse events of SLE (CI: confidence interval).

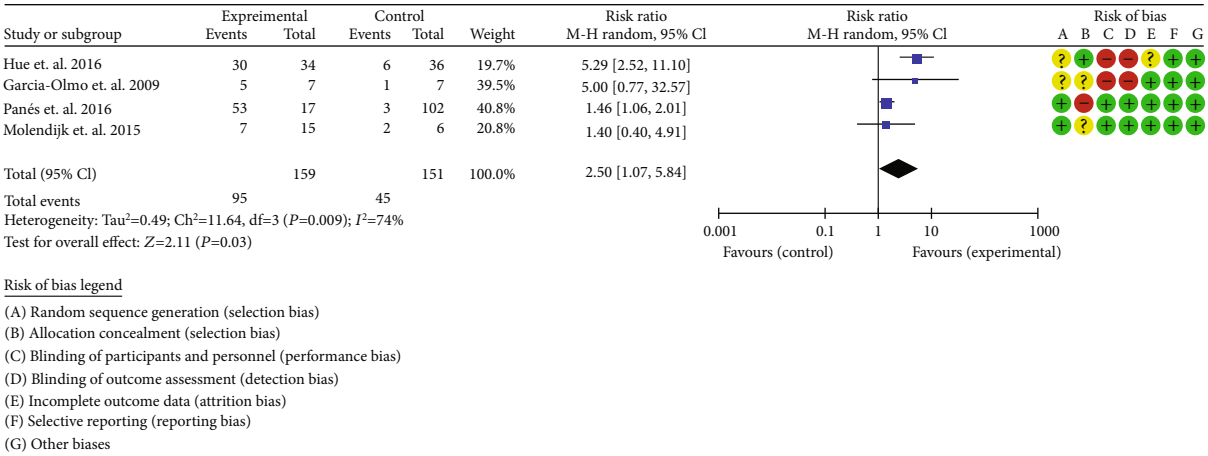

Figure 8: Clinical efficacy (CI: confidence interval).

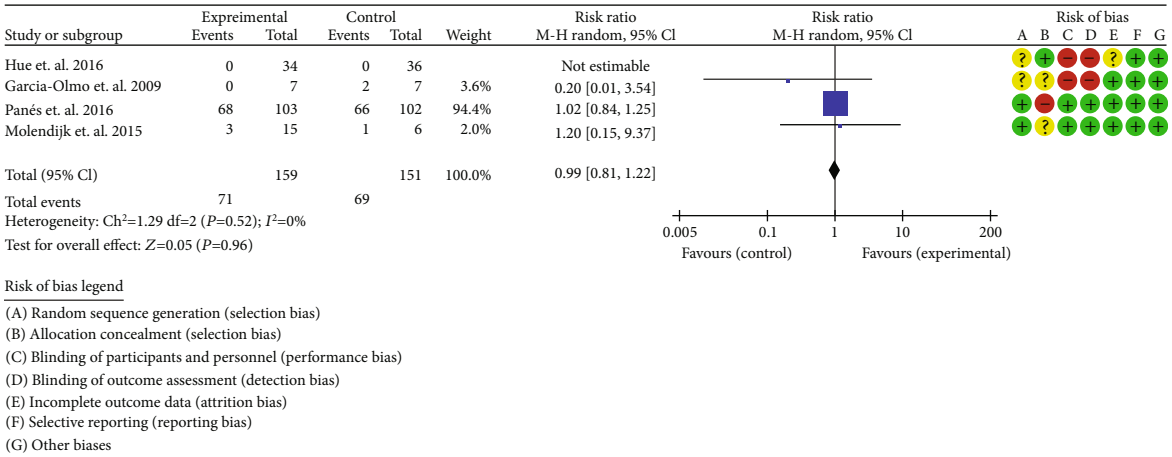

Figure 9: Adverse events of inflammatory bowel disease (CI: confidence interval).

in the germinal center. On the other hand, activating and proliferating B cells cause somatic hypermutation in the variable region of the B cell antigen receptor (BCR), leading to maturation of BCR and antibody affinity, and antibody class switching. This produces plasma cells and memory B cells, which in turn participate in a variety of immune responses [68]. Current research shows that B cells in autoimmune diseases are the main link in the production of autoantibodies, which is the main direction of drug research and development [69]. The negative regulatory effect of MSC on B

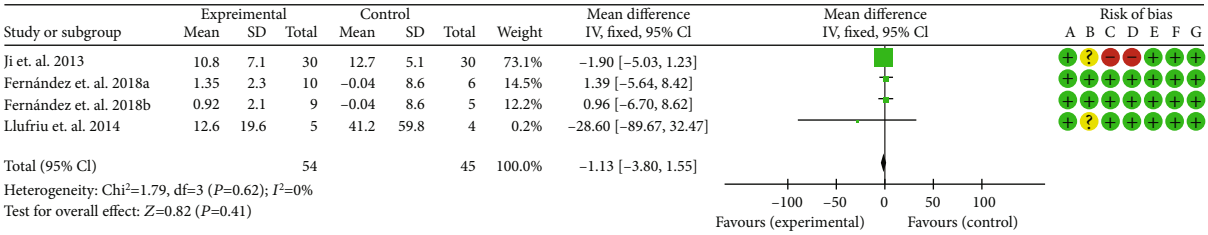

Figure 10: Number of lesions (CI: confidence interval).

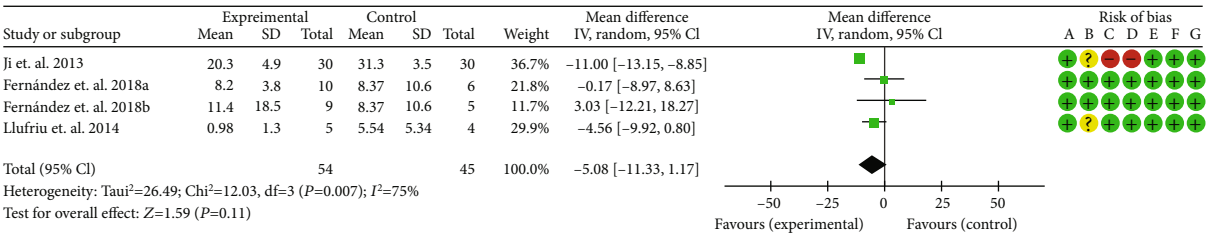

Figure 11: Volume of lesions (CI: confidence interval).

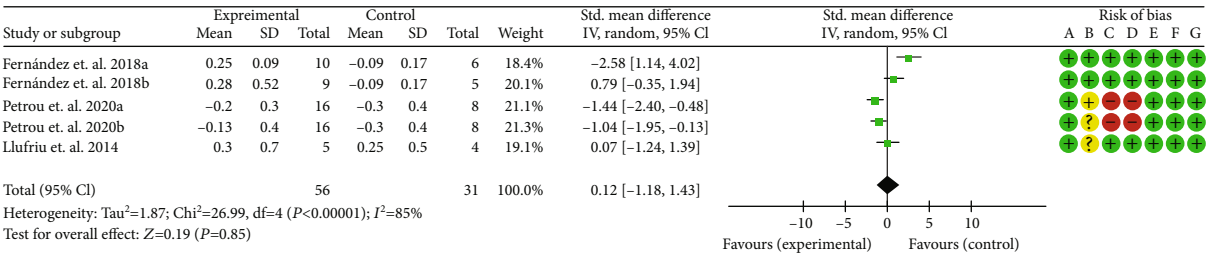

Figure 12: Expanded disability status scale (CI: confidence interval).

lymphocytes may be caused by direct contact with B cells to produce a series of cytokines and directly secrete some soluble cytokines to act on B cells. This in turn inhibits the proliferation of B cells and reduces the production of plasma cells and memory B cells, resulting in the reduction of B cells secreting antibodies, cytokines, and chemokines [70]. MSC can also promote the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) through the participation of stem cell antigen 1/lymphocyte antigen 6AIE protein and inhibit the maturation of B lymphocytes. TGF-β secreted by MSC participates in the inhibition of B lymphocytes by downregulating or blocking IL-7 derived from stromal cells. MSC can also inhibit B cell secretion of Ig A, lg G, and lg M [71] and downregulate the production

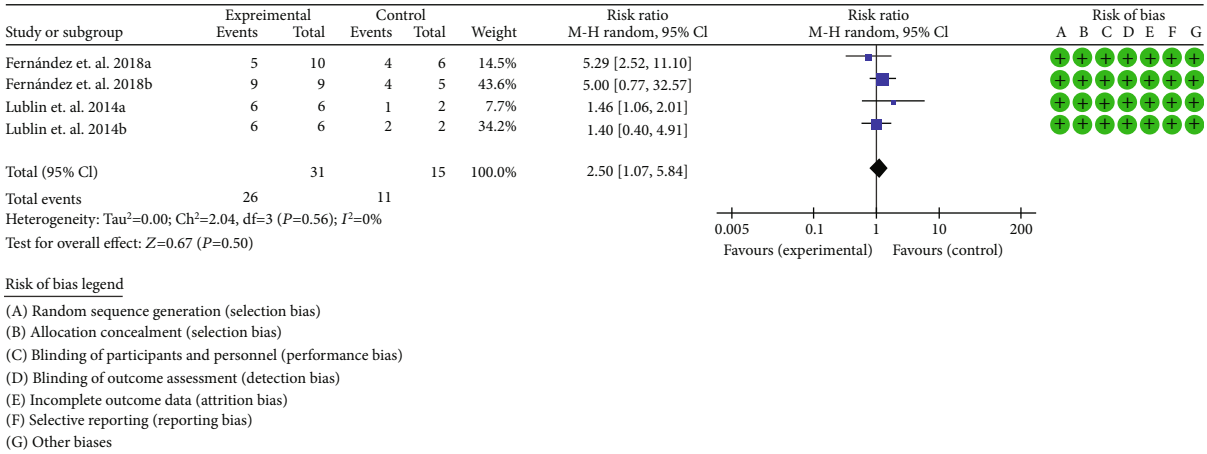

Figure 13: Adverse events of multiple sclerosis (CI: confidence interval).

of C-x-c motfreceptor 4 (CXCR4) and CXCL13 to inhibit B cell differentiation [70, 72]. Hermankova et al. found that in the presence of IFN-γ, MSC may inhibit the proliferation of B lymphocytes by expressing IDO [73].

Therefore, similar to T cells, MSC may regulate the immune function of B cells through a variety of mechanisms and play different roles in a variety of autoimmune diseases.

4.1.3. MSC's Immunomodulatory Effect on Immune Dendritic Cells (DC). DC can efficiently ingest, process, and present antigens and is the body's strongest antigen-presenting cell. Mature DC can activate initial T cells and then initiate, regulate, and maintain immune response, while immature DC has strong migration ability, can quickly migrate to the lesion site, and participates in the immune response [74]. The change of DC may damage the immune regulation mechanism, break the balance of natural immune tolerance, and cause autoimmune diseases. In addition, the activation of T cells and B cells by DC is also closely related to the occurrence of autoimmune diseases. Therefore, it is believed that DC is the hub of the pathological pathway of autoimmune diseases. The related research of DC on the treatment of autoimmune diseases illustrates the close relationship between DC and autoimmune diseases from another angle [75, 76].

When MSC and DC are cocultured, it can inhibit the differentiation of monocytes into DC by downregulating the expression of CD1a, CD86, and HLA-DR of MHC class II molecules. It also inhibits the expression of CD83, inhibits the secretion of TNF-4 from DC1 cells, and enhances the secretion of IL-10 from DC2 [77], thereby changing the DC phenotype from mature to immature stage, leading to immune silence [78]. MSC can significantly inhibit the transformation of GM-CSF and L-4 leads from CD14+ monocytes to DC. Djouad et al. found that MSC can secrete IL-6 and downregulate the expression of MHC I molecules, CD40, and CD86 on the surface of mature DC, or by secreting TGF-β, PGE2 and other cytokines, inhibit the activity of DC, and cause DC to differentiate into immature phenotype [79]. MSC affects the maturation of DC through a variety of ways, including the expression of antigen and costimulatory molecules, changes in antigen presentation and migration ability, maintaining the expression of cadherin, and inhibiting the expression of Cc motfireceptor 7 (CCR7) and Cc motiligand 19 (CCL19), thereby inhibiting the migration of DC and so on [72]. Therefore, MSC may inhibit the generation, proliferation, antigen presentation, migration, and deformation ability of DC and participate in the differentiation and maturation of DC.

In summary, it is currently believed that MSCs exert their immune regulation function mainly by inhibiting the proliferation of T lymphocytes, inhibiting the proliferation and differentiation of B lymphocytes, regulating the activity of NKs, and preventing the maturation of DCs. In the future, more MSCs' immune regulation mechanisms would be revealed.

4.2. Clinical Evidence of MSC Transplantation in the Treatment of Autoimmune Diseases

4.2.1. RA. Animal studies have shown that intraperitoneal injection of MSCs can effectively alleviate the symptoms of arthritis in mice [80]. A variety of MSC transplantation treatments, such as bone marrow source, fat source, and cord blood source, can effectively alleviate the symptoms of RA model mice [81, 82]. Previous studies have suggested that due to lack of immunogenicity and significant local immunosuppressive ability, MSCs from umbilical cord matrix tissue can be used more safely in allogeneic transplantation and can exert their immunomodulatory effects in the body without prior induction and activation and has gradually replaced bone marrow-derived MSCs [83]. The specificity of umbilical cord MSCs may be due to differences in gene and protein expression profiles, that is, increased expression of immunomodulatory surface proteins, such as CD200, CD273, and CD274, and cytokines such as IL-1β, IL-8, leukemia inhibitory factor, and TGF-β2 [84]. MSCs inhibit the proliferation of T lymphocytes and reduce the expression levels of INF-γ and TNF-α, thereby improving the clinical symptoms of autoimmune encephalomyelitis model mice. In addition, MSCs can accumulate in peripheral immune organs, causing immune tolerance to peripheral T

lymphocytes [85, 86]. Studies have found that TGF-β and IL-4 are also involved in the immune regulation of MS by MSCs [87, 88].

The 3 RCTs included in this systematic review showed the therapeutic effect of MSC transplantation on RA. Yang et al. (2018) showed that after MSC treatment, the disease activity was weakened and the clinical symptoms (including DAS28) were improved. It also found that the dosage of prednisone acetate in 23 patients in the experimental group gradually decreased after the intervention. For the immune response, it found that the percentage of CD4+CD 25 +Foxp3+Tregs in the response group increased and the percentage of CD4+IL-17A+Th17 cells decreased; and the levels of IL-6 and TNF-α decreased significantly. Shadmanfar et al. (2018) shows that MSC may improve the patient's standing time and WOMAC total score and reduce the use of methotrexate and prednisolone. Álvaro-Gracia et al. (2017) showed that a moderate proportion of patients meets the comprehensive measure of ACR 20/50/70 response, but fewer patients achieve an improvement of 50% or 70%. In addition, the combination therapy of mesenchymal stem cells and other cytokines will become a new mesenchymal stem cell combination strategy in the future. He et al. through intravenous injection of IFN-γ to patients, "emerging" mesenchymal stem cells, forming an immune microenvironment that is conducive to mesenchymal stem cells to exert their anti-inflammatory and immune regulation functions, to treat autoimmune inflammatory diseases such as RA [89]. Compared with the treatment of mesenchymal stem cell transplantation alone, during the three-month clinical observation period, the effective rate of "empowering" mesenchymal stem cell transplantation in the treatment of rheumatoid arthritis has been significantly improved, from 53.3% to 93.3%. This research had become an important advancement in the field of mesenchymal stem cell treatment of rheumatoid arthritis in recent years [89]. At present, the team is conducting a multicenter clinical randomized trial to prove the effect of the therapy in the treatment of diseases such as RA and SLE.

For dosage and infusion method, Yang et al. (2018) and Álvaro-Gracia et al. (2017) use intravenous infusion, and the dose of MSC is different. Shadmanfar et al. (2018) used the intra-articular injection method but did not describe the specific dosage. All three have curative effects, but because the same indicators are not reported, they cannot be combined for meta-analysis. And because the RCTs with the same dose and infusion methods were few, subgroup analysis was hard to perform. Therefore, it is not yet known which dose and which intervention method works best. We may only speculate based on current evidence that 1 to 3^*10^7 cells (or 1^*10^6 cells/kg dose) may achieve therapeutic effects through intravenous infusion or intra-articular injection.

4.2.2. SLE. SLE is an autoimmune disease that mainly manifests itself in the formation of autoantibodies and involves multiple organs and multiple systems. SLE is common in women of childbearing age, and its clinical manifestations are complex and diverse, and the exact pathogenesis has not been confirmed. At present, the main treatment options for SLE are glucocorticoids and immunosuppressive agents. This program has poor curative effect on some patients with refractory lupus and has many adverse reactions, which has a greater impact on the quality of life of patients. Animal studies have shown that the MRL/lpr effect of MSC alone or combined with cyclophosphamide in the treatment of SLE model mice is better than cyclophosphamide alone, which is shown in reversing multiple organ dysfunction in lupus mice and improving proteinuria and renal pathological damage [90, 91]. In addition, studies have confirmed that MSCs from different sources can control disease progression and improve disease performance in lupus model mice. Cord blood-derived MSCs can also effectively relieve the condition of lupus model mice [92], and fat-derived MSCs can improve the immune system damage caused by lupus to a certain extent and can reduce kidney damage [93].

This meta-analysis showed that the SLEDAI and urine protein in the experimental group was lower than that in the control group. The serum C3 in the MSC group was also higher than that in control group. In terms of safety, there was no statistical difference in the incidence of adverse events between the MSC group and the control group. It can be considered that the safety of MSC and the control group (placebo or traditional therapy) is equivalent. Other clinical trials also showed that MSC transplantation has significant clinical therapeutic effects, which can stabilize the patient's condition and reduce the recurrence of the patient's condition. The patients received MSC transplantation without rejection, and MSCs have good safety [90, 94]. Through a multicenter clinical study on MSC transplantation for the treatment of SLE, a total of 40 patients from 4 centers were enrolled. The results of the study showed that the overall survival rate after transplantation was 92.5%, and no serious transplant-related adverse reactions occurred [95]. Long-term follow-up of 9 patients with refractory SLE for up to 6 years showed that there was no increase in serum tumor markers before and 6 years after MSC infusion [95]. It shows that in these patients, allogeneic umbilical cord-derived MSC transplantation has good safety. In summary, combined with single-arm clinical trials and RCTs, for refractory SLE, MSC transplantation treatment has good safety.

For dosage and infusion method, except for the renal artery method used by Zeng et al. (2016), the intravenous infusion method is used for other RCTs. And these RCTs use different doses (from 1^*10^6 cells to 2^*10^8 cells). Therefore, it is difficult to evaluate which dose and method of administration are better. We may only speculate based on current evidence that 1^*10^6 cells to 2^*10^8 cells MSC transplantation may achieve therapeutic effects through intravenous infusion or renal artery.

4.2.3. Inflammatory Bowel Disease. Immune dysfunction is believed to play a key role in the occurrence and development of ulcerative colitis. Research suggests that mesenchymal stem cells may help tissue regeneration by suppressing inappropriate immune responses and providing various cytokines instead of directly restoring damaged cells [96]. The pathogenesis of ulcerative colitis is unclear. Studies have found that in the intestinal mucosa of patients with active

ulcerative colitis, there is a cytokine storm, especially IL 17 levels are significantly increased [97]. The imbalance in the ratio of regulatory T cells (Tregs)/helper T cells 17 (Th17) may be related to the occurrence and development of ulcerative colitis. Only CD4+CD25+regulatory T cells expressed by Foxp3 have immunomodulatory effects. The combination of Foxp3 and nuclear receptors can significantly inhibit the transcription of interleukin 17, thereby affecting the differentiation of Th17 cells [98]. Studies have found that Rab27A and Rab27B are GTPases related to exosomes, which are related to the secretion of exosomes and their docking in the plasma membrane of various cells. Compared with the healthy control group, a significant increase in the number of Rab27A+ or Rab27B+ intestinal immune cells can be observed in the colonic mucosa of the active ulcerative colitis group. This indicates that the immune response mediated by exosomes plays an important role in the pathogenesis of ulcerative colitis [99]. MSC can induce the apoptosis of T lymphocytes by secreting exosomes, stimulate monocytes to secrete IL10 and TGF β, promote the upregulation of CD4+ CD25+ Foxp3+ regulatory T cells, reduce the level of inflammatory factor IL 4, and increase the level of anti-inflammatory factor IL 10 to regulate the immune response. Anti-inflammatory factors such as TGF β and IL 10 can stimulate mesenchymal stem cells in vitro to secrete exosomes more effectively, which in turn promotes the upregulation of regulatory T cells, reduces intestinal inflammation, and promotes the repair and regeneration of damaged tissues [100, 101]. In addition, animal experiments have shown that mesenchymal stem cells can migrate to the colon and differentiate into vascular endothelial cells to promote the formation of new blood vessels in damaged parts [102–104], promote the reconstruction of microcirculation, and thus facilitate the repair of colonic mucosal inflammation. The number of directional migration of stem cells is related to the degree of tissue damage. With the aggravation of the damage, the migration rate of mesenchymal stem cells increases, and the number in the recovery period decreases significantly [105, 106]. When inflammation occurs in the intestine, mesenchymal stem cells can migrate in the body and settle on the surface of the intestinal mucosa and proliferate and differentiate into new colonic mucosal epithelial cells to repair the injured site [107]. Brittan et al. found that MSC transplantation can colonize and differentiate into intestinal subepithelial myofibroblasts after transplantation and promote intestinal mucosal repair and neovascularization by improving the intestinal microenvironment [108]. In the human body, whether mesenchymal stem cells differentiate directly into intestinal mucosal epithelial cells or myofibroblasts and promote intestinal epithelial cell repair and angiogenesis by improving the intestinal microenvironment still needs further research to confirm. This meta-analysis found that it can improve clinical efficacy. The incidence of adverse events between two groups was of no statistical significance.

For the dosage and infusion method, Garcia-Olmo et al. (2009), Panés et al. (2016), and Molendijk et al. (2015) used local injection methods, while Hu et al. (2016) used intravenous infusion. As intravenous infusion administration methods are reported less, it is not known which route of administration is better. And since the doses administered are also diverse, it is not known which method of administration is better. We may only speculate based on current evidence that 1 to $5^{*}10^{7}$ cells for MSC transplantation may achieve therapeutic effects through intravenous infusion or local injection.

4.2.4. Multiple Sclerosis. Ji et al. (2013) found that the progression-free survival (PFS) rate, total number of episodes, and average number of episodes each year in the experimental group were lower than that in the glucocorticoid group (control group), while the quality of life in the experimental group was higher. However, the summary of other outcomes showed that the number and volume of lesions and EDSS between the experimental group and control group was of no statistical significance. This controversial result is interesting, so more relevant research is needed in the future to amend or confirm the conclusion. However, basic research has found that MSC may have the effect of treating multiple sclerosis.

Multiple sclerosis is a chronic inflammatory demyelinating disease that mainly affects the central nervous system. Its pathological characteristics are mainly manifested by cell infiltration of myelin-specific autoreactive T cells and subsequent neuroinflammatory response, demyelination response, and neuronal cell damage. The destruction of axon integrity and the accumulation of irreversible sclerosis are the main causes of irreversible neurological damage [109, 110]. The pathogenesis of multiple sclerosis involves a variety of cells in innate immunity, such as Th17 helper T cells 1, Treg, microglia, dendritic cells, and macrophages. The destruction of the balance between helper T cells 1 and helper T cells 17 is considered to be an important mechanism leading to the pathogenesis of multiple sclerosis, and regulatory T cells are considered to be a key regulator of the adaptive immune response of multiple sclerosis [111, 112].

Although there are many kinds of drugs that can be used for the treatment of multiple sclerosis, most of them can only control the progression of the disease and improve the clinical symptoms of patients, but they cannot completely cure the disease. Once the patient's clinical manifestations develop into progressive disability, there is no effective way to protect, repair, and regenerate nerve tissue to restore the patient's nerve function. Therefore, myelin and nerve cell regeneration are still the main obstacles to the treatment of multiple sclerosis [113, 114]. In the past 20 years, stem cell transplantation has been considered a potentially effective treatment for invasive multiple sclerosis [115], and different types of stem cells, even stem cells of the same type but from different sources, have their unique characteristics.

Mesenchymal stem cells exert their therapeutic effects on multiple sclerosis mainly by regulating the immune response and promoting nerve repair. The regulation effect of rabbit disease is manifested by inhibiting innate and adaptive immune response, inhibiting the proliferation of pathogenic effect CD4+ T cells and B cells, regulating CD8+ T cell

subsets, inducing the generation of regulatory T cells, and affecting the functions of dendritic cells and natural killer cells. The nerve repair function is produced by secreting a variety of neurotrophic factors, affecting the differentiation of neural stem cells, and promoting remyelination and axon survival [115, 116]. Barati et al. found that promoting the production of M2 type microglia and inhibiting the expression of proinflammatory cytokines may be the mechanism for mesenchymal stem cells to treat demyelinating diseases [117]. Bone marrow mesenchymal stem cells can improve the symptoms of patients with multiple sclerosis by inhibiting the inflammatory response in the central nervous system, regulating the expression of interleukin 6, stimulating the production of nerve growth factor, and protecting axons [118]. Wang et al. showed that the supernatant of bone marrow mesenchymal stem cells can affect the function of CD4+ T cells [64]. It thereby inhibits the secretion of inflammatory factors in the peripheral blood of experimental autoimmune encephalomyelitis and reduces the degree of demyelination in the central nervous system of mice with experimental autoimmune encephalomyelitis.

Compared with human bone marrow mesenchymal stem cells, mesenchymal stem cells derived from human embryonic stem cells can significantly reduce the clinical symptoms of experimental autoimmune encephalomyelitis and more effectively prevent demyelination. This difference may be related to the high permeability of mesenchymal stem cells derived from human embryonic stem cells [64]. In addition to bone marrow mesenchymal stem cells, adipose-derived mesenchymal stem cells are also commonly used to treat multiple sclerosis and experimental autoimmune encephalomyelitis. Adipose-derived mesenchymal stem cells can pass through the blood-brain barrier and reduce the infiltration of brain B cells, T cells, and macrophages. In experimental autoimmune encephalomyelitis mice treated with adipose-derived mesenchymal stem cells, human leukocyte antigen G is one of the main factors to reduce the severity of the disease [119]. In addition, Li et al. [120] found that adipose-derived mesenchymal stem cells can also reduce the Th17/Treg ratio by releasing leukemia inhibitory factors and reduce the degree of disability in experimental autoimmune encephalomyelitis. Kurte et al. [121] observed that transplantation of mesenchymal stem cells before the onset of the disease in experimental autoimmune encephalomyelitis mice or at the peak of the disease has the best therapeutic effect. The findings of Strong et al. [122] emphasize the importance of choosing a donor. They injected adipose-derived mesenchymal stem cells from obese and wasting donors into mice with experimental autoimmune encephalomyelitis by intraperitoneal injection. The results showed that adipose-derived mesenchymal stem cells from obese donors failed to inhibit inflammation and clinical symptoms, and adipose-derived mesenchymal stem cells from obese donors increased the secretion of proinflammatory cytokines. Cell transplantation through intravenous injection is usually the preferred injection method in experiments, but intranasal administration can bypass the blood-brain barrier and directly enter the brain through the olfactory and trigeminal nerve pathways, which also provides researchers with another option [123].

For the dosage and infusion method, RCTs use different doses (from 5^*10^7 cells to 6^*10^8 cells). Therefore, it is difficult to evaluate which dose and method of administration are better. We may only speculate based on current evidence that 5^*10^7 cells to 6^*10^8 cells MSC transplantation may achieve therapeutic effects through intrathecal injection or intravenous infusion.

4.2.5. Ankylosing Spondylitis. Ankylosing spondylitis is an autoimmune disease mediated by immune complexes. The main symptom is a chronic progressive inflammatory disease that invades the spine and affects the patient's sacroiliac joints and surrounding joint tissues. It has a high clinical morbidity and disability rate. The study found that, compared with healthy donors, although bone MSCs (BMSCs) obtained from patients showed normal proliferation, cell viability, surface markers, and multiple differentiation characteristics, and their immunomodulatory ability was significantly reduced [124]. Xie et al. [125] found that because BMSCs secreted more bone morphogenetic protein 2 (BMP2) and less noggin (NOG), BMSCs of AS patients had stronger osteogenic differentiation ability than BMSCs of normal donors. This state may contribute to the underlying pathological osteogenesis in AS. Animal studies have shown that after MSCs are injected into mice, Th17 cells are inhibited and the percentage of CD4+-CD25+-Foxp3 +-Treg cells increases [126]. In addition, AS patients have a low number of Treg cells, a low B cell level, and abnormal function [127]. Studies have shown that MSCs can differentiate T cells into Th2 phenotype and inhibit the differentiation of Th17 cells, thereby reducing the cytokine levels of Th17 cells and promoting the regeneration process of subsequent tissue damage [128]. Clinical trials have shown that MSCs may help relieve the symptoms of AS patients [127–129]. Wang et al. [130] found that the Bath ankylosing spondylitis disease activity index (BASDAI), night pain score (VAS) and Bath ankylosing spondylitis functional index (BASFI) improved. Wang et al. [130] found that the Bath ankylosing spondylitis disease activity index (BASDAI), night pain score (VAS), and Bath ankylosing spondylitis functional index (BASFI) improved. Patients' ESR and immunoglobulin G decreased significantly at 3, 6, and 12 months after stem cell transplantation. This systematic review only found one RCT related to the treatment of ankylosing spondylitis with MSC. It is found that compared with the fliximab group, MSC treatment for 6 months may increase the total effective rate; reduce erythrocyte sedimentation rate, intercellular adhesion molecules, and serum TNF-α; and improve pain and activity.

For the dosage and infusion method, one RCT is injected with 1^*10^6 cells/kg through intravenous infusion, and it has a certain effect. For multiple sclerosis, the administration methods and dosages of each RCTs are varied, and the summary results have no significant curative effect compared with the control group. Therefore, the optimal dosage and route of administration are not yet known. We may only speculate based on current evidence that 1^*10^6 cells/kg dose of MSC transplanted by the intravenous infusion method has not been able to observe the therapeutic effect.

Nevertheless, more RCTs are still needed to further determine the key points of MSCs in the treatment of ankylosing spondylitis, such as cell source, dosage, route of drug administration, and especially intervention in the most ideal disease stage (early or late).

5. Conclusion

This systematic review and meta-analysis summarized the safety and effectiveness of MSC in the treatment of autoimmune diseases (RA, SLE, inflammatory bowel disease, multiple sclerosis, and ankylosing spondylitis) and provides relevant evidence for the future clinical research design (such as dose and disease severity) of clinical trials for MSC treatment of autoimmune diseases (such as rheumatoid arthritis, SLE, inflammatory bowel disease, multiple sclerosis, and ankylosing spondylitis).

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

Our study did not require an ethical board approval because it is a systematic review and meta-analysis.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

Ganpeng Yu, Liuting Zeng and Jun Li should be co-first authors.

Supplementary Materials

PRISMA 2020 checklist: checklist. Table S1: search strategies for PubMed and Embase. *(Supplementary Materials)*

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Research Article

Tanshinone *IIA*-Incubated Mesenchymal Stem Cells Inhibit Lipopolysaccharide-Induced Inflammation of N9 Cells through TREM2 Signaling Pathway

Nanqu Huang,[1] **Juan Huang**,[2] **Fei Feng**,[3] **Zhisheng Ba**,[1] **Yuanyuan Li**,[1] **and Yong Luo**[3]

[1]*National Drug Clinical Trial Institution, Third Affiliated Hospital of Zunyi Medical University (The First People's Hospital of Zunyi), Zunyi, Guizhou, China*
[2]*Department of Pharmacology and Chemical Biology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China*
[3]*Department of Neurology, Third Affiliated Hospital of Zunyi Medical University (The First People's Hospital of Zunyi), Zunyi, Guizhou, China*

Correspondence should be addressed to Yong Luo; luoyong@zmu.edu.cn

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Our previous study found that incubating mesenchymal stem cells (MSC) with tanshinone IIA (TIIA) before transplantation could significantly increase the inhibitory effect of MSC on neuroinflammation. Here, we investigated the possible mechanism of this effect. N9 cells and MSC were inoculated at a ratio of 1 : 1 into a Transwell coculture system. MSC were inoculated into the upper chamber, and N9 cells were inoculated into the lower chamber. In this experiment, N9 cells were treated with 1 μg/mL lipopolysaccharide (LPS) for 24 hours to induce inflammation, MSC were treated with 10 μM TIIA for 48 hours to prepare TIIA-incubated MSC (TIIA-MSC), and TREM2 siRNA was used to silence the *TREM2* gene in MSC. The changes in IL-1β, IL-6, and TNF-α were evaluated by Western blotting. We found that LPS significantly increased the levels of IL-1β, IL-6, and TNF-α. While both MSC and TIIA-MSC downregulated the levels of ($P = 0.092$, $P = 0.002$), IL-6 ($P = 0.014$, $P < 0.001$), and TNF-α ($P = 0.044$, $P = 0.003$), TIIA-MSC downregulated IL-6 more significantly ($P = 0.026$). In addition, silencing TREM2 reduced the ability of TIIA-MSC to attenuate IL-6 ($P = 0.005$) and TNF-α ($P = 0.033$). These data suggest that the enhanced anti-inflammatory effect of TIIA-MSC on LPS-induced N9 cells may be mediated through the TREM2 signaling pathway.

1. Introduction

Alzheimer's disease (AD) is a common degenerative disease of the central nervous system characterized by progressive cognitive dysfunction and behavioral impairment. Active investigation of the pathogenesis and treatment of AD has extremely important value and significance. In recent years, a large number of studies have shown that mesenchymal stem cells (MSC) have great potential in the treatment of AD. There are many possible mechanisms how MSC function in the treatment of AD, but they are largely related to the regulation of neuroinflammation by MSC: when inflammation is weak, MSC promote the immune response; when inflammation is too strong, MSC inhibit the immune response [1]. In addition, MSC can migrate toward injured tissue [2]. Based on these effects, MSC can be a promising treatment modality because of their anti-AD neuroinflammatory activity. Our previous research found that MSC are effective in improving the learning and memory ability of AD model rats by blocking neuronal apoptosis and reducing the neuroinflammation-related cytokines interleukin 1β (IL-1β), IL-6, and tumor necrosis factor (TNF-α) [3]. However, the application of MSC still faces problems and challenges. MSC are prone to aging after *in vitro* expansion, and the environmental factors may suppress their ability to differentiate into specific mature cells and regulate inflammation [4].

To solve these problems, our research group originally used the biologically active ingredient tanshinone IIA (TIIA) isolated from the traditional Chinese medicine *Salvia miltiorrhiza* Bunge (Danshen) before MSC transplantation. Danshen is a well-known medicinal herb with a long history of clinical application for treating cardiovascular diseases, cancer, and osteoporosis [5, 6]. TIIA is the main active component of Danshen and is known to significantly improve blood circulation and delay tumor progression [7]. Our previous research found that pretreatment with TIIA can improve the damage to hippocampal neurons in rats caused by oxygen-glucose deprivation (OGD) [8]. OGD mimics the environmental changes MSC are exposed to during expansion and transplantation. Therefore, we decided to test TIIA to solve this problem.

We found that TIIA enhanced the inhibition of neuroinflammation by MSC and that TIIA-treated MSC (TIIA-MSC) exerted better neuroprotection [3]. However, the specific mechanism is not clear. Interestingly, studies have shown that TREM2-modified MSC have better neuroprotective effects [9]. TREM2 is a type of immunoglobulin-like receptor that is highly expressed on microglia. After binding to ligands, its signal is mediated by dead cell activation receptor-related protein (DAP12), which plays a negative regulatory role in autoimmunity and inflammation [10]. TREM2 has two physiological functions, inhibiting inflammation and promoting the phagocytosis of apoptotic neurons [11]. However, this effect is complicated. Studies have shown the deleterious role of TREM2 deficiency in the development of AD [12]. However, there are also opinions that with the progression of amyloid pathology, the upregulation of TREM2 reduces inflammation, promotes immune tolerance, and promotes microglia to clear Aβ [13]. Upregulation of TREM2 may help prevent AD, and downregulation of TREM2 without affecting the physiological function of TREM2 may be a strategy for the treatment of AD [13]. Therefore, we speculated that the enhanced activity of TIIA-MSC against neuroinflammation may also be through the regulation of TREM2. In this study, we used a Transwell coculture system and TREM2 siRNA to further explore the possible mechanisms of the elevated anti-neuroinflammation activity of TIIA-MSC.

2. Materials and Methods

2.1. Materials. N9 cells, which are a microglial cell line derived from the mouse brain and have many phenotypic characteristics similar to those of primary mouse microglial cells [14], were purchased from Beijing Zhongke Quality Inspection Biotechnology (ZKCC-X1877). TIIA (purity > 99.37%) was obtained from MedChemExpress. Following reagents and instruments were obtained from indicated sources: LPS (L4391, Sigma), RPMI 1640 (61870036, Gibco), medium for mesenchymal stem cells (mubmx-03011-440, Cyagen), DMEM (10566016, Gibco), trypsin-EDTA (T1300, Solarbio), PBS (KGB5001, Keygenbio), FBS (E600001-0500, BBI Life Sciences), Penicillin–Streptomycin Liquid (P1400, Solarbio), OPTI-MEM® (31985-062, Gibco), Lipofectamine™3000 (L3000015, Invitrogen™), TREM2 RNA interference vector and negative control siRNA vector (Generalbiol), CD45 PerCP (103129, Biolegend), CD29 PE (102207, Biolegend), CD90 PE-CY7 (105325, Biolegend), NovoCyte™ Flow Cytometer (NovoCyte 2060R, ACEA BIO Co., LTD), automatic microplate reader (WD-2102B, Beijing Liuyi Biotechnology Co., Ltd.), TRIzol Reagent (CW0580S, Cwbiotech), HiFiScript 1st Strand cDNA Synthesis Kit (CW2569M, Cwbiotech), UltraSYBR Mixture (CW0957M, Cwbiotech), Real-Time Fluorescence PCR (CFX Connect™, Bio-Rad Laboratories), RIPA (C1053, Applygen Technologies), BCA Protein Assay Kit (CW0014S, Cwbiotech), Marker (#26617, Thermo Scientific), PVDF (IPVH00010, Merck), skimmed milk powder (P1622, Applygen Technologies), Mouse Monoclonal Anti-GAPDH (1/2000, TA-08, Zsbio), Rabbit Anti TREM2 (1/1000, DF12529, Affinity), Rabbit Anti IL-1β (1/1000, bs-0812R, Bioss), Rabbit Anti TNF-α (1/500, AF7014, Affinity), goat anti-mouse immunoglobulin (Ig)G H&L (1/2000, ZB-2305, Zsbio), and goat anti-rabbit immunoglobulin (Ig)G H&L (1/2000, ZB-2301, Zsbio).

2.2. Preparation and Identification of MSC. C57BL/6 mice were purchased from ZHBY Biotech, Jiangxi, China (grade: specific pathogen-free, SCXK 2019-0004) and housed at 22-23°C with a 12-hour light/dark cycle. The mice were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 minutes. The tibia and humerus of mice were removed under aseptic conditions. The periosteum and muscle tissue were dissected, and the tibia and humerus were washed repeatedly with DPBS containing 1% penicillin-streptomycin. Next, the medullary cavity was flushed thoroughly with DMEM complete medium to harvest bone marrow cells. Collected cells were centrifuged, the supernatant was discarded after centrifugation (1200 rpm, 5 min, 4°C), and the pellet was resuspended in DMEM complete medium. The bone marrow cells were spread evenly on plates and cultured in a humidified atmosphere with 5% CO_2 at 37°C [15]. All procedures were performed with the approval of the Animal Experimental Ethical Committee of Zunyi Medical University (no. (2019)2-231, 11 Mar 2019).

MSC were identified by flow cytometry using antibodies against CD29, CD45, and CD90. Briefly, the MSC were washed twice with ice-cold PBS. Subsequently, $1 - 3 \times 10^6$ cells were mixed with the antibodies CD45 PerCP, CD29 PE, and CD90 PE-CY7. The cell suspensions were incubated at room temperature for 20 min in the dark. After centrifugation of the mixture, the cells were washed three times with PBS, resuspended, and analyzed using flow cytometry.

2.3. Cell Culture and Processing. N9 cells were cultured in DMEM complete medium containing 10% FBS and 1% penicillin-streptomycin. The cells were subcultured when they reached 80-90% confluence. N9 cells and MSC were cultured in an indirect coculture system using Transwell filters at a cell ratio of 1 : 1. In this study, MSC were inoculated into the upper chamber, and N9 cells were inoculated into the lower chamber (Figure 1(d)). N9 cells were treated with 1 μg/mL LPS for 24 hours to induce inflammation. MSC were treated with 10 μM TIIA for 48 hours to prepare TIIA-MSC. In this study, we used MSC modified with TIIA, not a mixture of TIIA and MSC. Therefore, TIIA was discarded after TIIA treatment. The TREM2 gene of MSC was silenced by TREM2 siRNA by treating the cells with siRNA for 48 hours. The siRNA was discarded after the treatment. The details of the experimental conditions

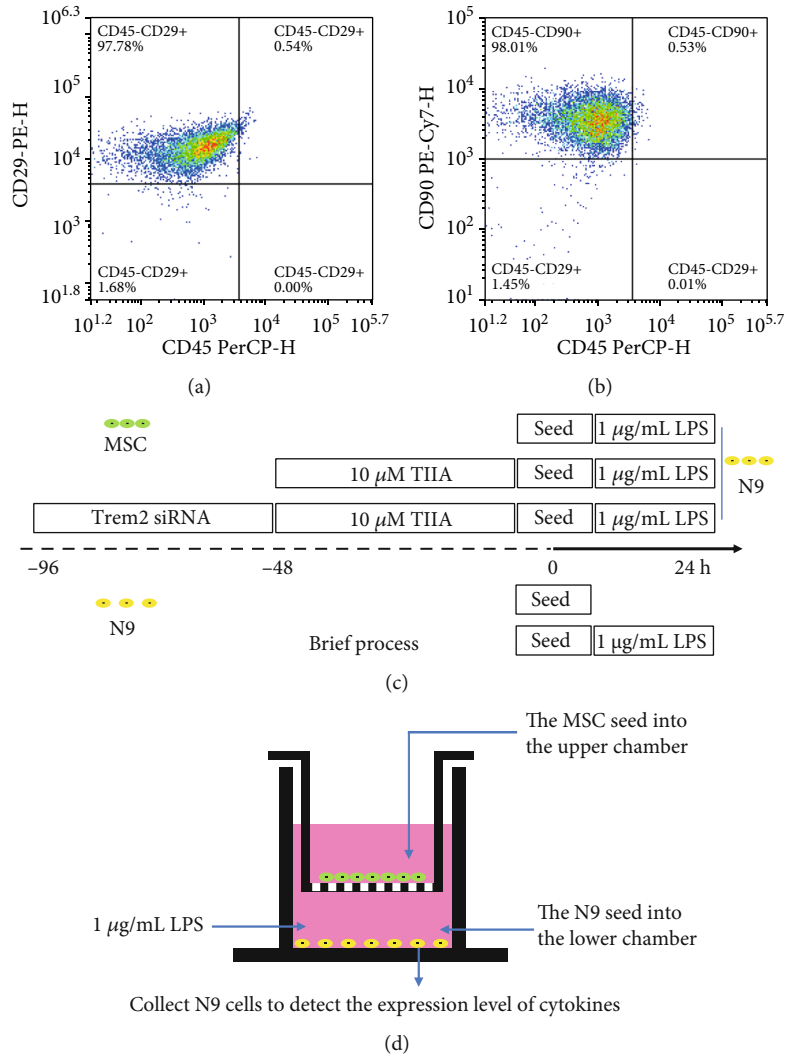

Figure 1: The identification of MSC by flow cytometry and the schematic representation of the study design. (a, b) Flow cytometry was used to detect the surface antigens of MSC: CD29 (97.78%), CD90 (98.01%), and CD45 (0.54%). (c) Brief descriptions of the experimental procedures and experimental conditions. (d) Transwell system used in the present study.

and specific procedures used in this study are shown in Figure 1(c).

2.4. Cell Transfection. When the MSC reached 80-90% confluence, the cell culture medium was replaced with 1 mL of serum-free medium. To prepare transfection reagents, 125 μL Opti-MEM was added to 2 sterilized EP tubes. Five microliter Lipofectamine 3000 was added to one of the tubes, 12.5 μL siRNA (the dry siRNA powder was dissolved in DEPC water; 125 μL/1 OD) was added to another, and the tubes were mixed and incubated at room temperature for 5 min. Next, the content of the above two EP tubes was combined and incubated at room temperature for 15 min. Afterward, the mixture was applied to the MSC dropwise. Six hours after transfection, 1 mL of complete medium with a serum content of 20% was added to the six-well plate. Finally, the transfection efficiency was determined by RT-qPCR and Western blotting at 48 hours after transfection [16].

2.5. RT-qPCR. Forty-eight hours after transfection, the total RNA from cultured cell samples was extracted using TRIzol lysis buffer through a series of rinse, elution, and centrifugation steps. The concentration and purity of RNA (OD260/OD280) were measured with a UV-Vis spectrophotometer. cDNA was synthesized by reverse transcription using a HiFi-Script cDNA first-strand synthesis kit. A fluorescent PCR machine was used to perform fluorescent quantitative PCR. The composition of the reaction mixture was as follows: RNase Free dH_2O 9.5 μL, cDNA 1 μL, upstream primer 1 μL, downstream primer 1 μL, and 2 × SYBR Green PCR Master Mix 12.5 μL. The reaction steps were as follows: predenaturation at 95°C 10 min; followed by 40 cycles of denaturation at 95°C for 10 s; annealing at 58°C for 30 s; extension at 72°C for 30 s. The primer sequences were as follows: TREM2 forward primer 5′-CATGTACTTATGACGCCTTGAA-3′, reverse primer 5′-TCTGCGATGACT GTGCTCC-3′; GAPDH forward primer: 5′-TCAACGGCACAGTCAAGG-

3′, reverse primer 5′-TGAGCCCTTCCACGATG-3′. GAPDH was used as an internal control, and the relative expression of TREM2 was calculated according to the $2^{-\triangle\triangle Ct}$ method.

2.6. Western Blotting. N9 cells were incubated with lysis buffer for 30 min at 4°C, and insoluble material was removed by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was carefully collected to obtain the total protein. The protein concentration was determined using the BCA kit. Equal amounts of protein were separated by sodium dodecylbenzene sulfonate gel electrophoresis (SDS-PAGE) for 2 hours and transferred to PVDF membranes with a constant current of 300 mA for 80 min. The blots were blocked with fat-free milk solution in TBST for 1 hour at room temperature. The blots were washed with TBST three times and incubated with the primary antibody overnight at 4°C. The blots were washed with TBST three times for 5 min each time followed by incubation with the corresponding secondary antibody solution for 2 hours at room temperature. Then, PVDF membranes were washed with TBST three times and visualized using ECL solution. The gray value of each antibody band was analyzed with Quantity One software version 4.6.1 software.

2.7. Statistical Analyses. All data were statistically analyzed with SPSS 22.0 and expressed as the mean ± SEM from at least three independent experiments. Significant differences between groups were analyzed by one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of MSC. To identify whether the isolated and cultured cells were MSC, we used flow cytometry to detect MSC markers. The flow cytometry results showed that the cells expressed CD29 (97.78%) and CD90 (98.01%) and almost no expression of the hematopoietic cell marker CD45 (0.54%) (Figure 1). These results are consistent with the standard surface markers of mouse MSC.

3.2. Transfection Efficiency Test. In this study, three TREM2 interference sequences (siRNA-1, siRNA-2, and siRNA-3) were designed and transfected into MSC. After 48 h, the transfection efficiency was measured by RT-qPCR and Western blotting (Figure 2). Compared with siRNA-NC, TREM2 interference (siRNA-1, siRNA-2, and siRNA-3) significantly reduced the mRNA expression level of TREM2 (Figure 2(a)); in addition, Western blotting results showed that, compared with siRNA-NC, siRNA-1 and siRNA-2 downregulated the levels of TREM2 protein, and the effect of siRNA-1 was more pronounced than that of siRNA-2 (Figure 2(b)). The results showed that transfection of TREM2 siRNA was successful, and because siRNA-1 showed the best downregulation, it was used for subsequent experiments.

3.3. Changes in the Levels of Inflammatory Factors. Western blotting was used to determine the expression levels of the inflammatory factors IL-1β, IL-6, and TNF-α, and the results are shown in Figure 3. Compared with the control group, LPS significantly increased the protein levels of IL-1β, IL-6, and TNF-α, indicating that LPS stimulated the inflammatory response of N9 cells. Compared with the LPS-treated cells, both MSC and TIIA-MSC downregulated the expression levels of IL-1β, IL-6, and TNF-α, but TIIA-MSC showed a more pronounced downregulatory effect on inflammatory factors. Compared with MSC, TIIA-MSC significantly reduced IL-6. After the TREM2 gene was silenced using TREM2 siRNA in MSC, the ability of TIIA-MSC to reduce inflammatory factors in N9 cells was suppressed. Compared with TIIA-MSC, TREM2 siRNA significantly suppressed the reduction of IL-6 and TNF-α levels, but this effect seemed to be less obvious in the regulation of IL-1β expression.

4. Discussion

TREM2 is a type of immunoglobulin-like receptor that is highly expressed on microglia. Large-scale gene sequencing in the population has shown that a genetic mutation of TREM2 is closely associated with AD [17]. In AD, TREM2 can initiate microglial responses by maintaining cell energy and biosynthesis [18], which participates in the response of microglia to Aβ plaque deposition [19]. Interestingly, a large amount of its extracellular soluble fragment (sTREM2) is present in the cerebrospinal fluid of AD patients and is closely related to the tau protein level, which is one of the characteristic features in the pathology of AD. sTREM2 can increase the survival rate of microglia in the brain by regulating phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), which can induce inflammation and enhance the viability of microglia [20]. *In vitro*, upregulation of TREM2 expression reduced the number of apoptotic neurons and increased the expression of some anti-inflammatory factors. Conversely, downregulation of TREM2 expression increased the number of apoptotic neurons and promoted the expression of proinflammatory factors [21]. *In vivo*, intracerebroventricular injection of TREM2 overexpression lentivirus can promote the transformation of microglia to the M2 type and reduce neuronal apoptosis [22].

Interestingly, TREM2 is also expressed in MSC, and the activation of TREM2 can inhibit the immune activation of MSC and promote their differentiation [23]. .TREM2-modified MSC have better neuroprotective effects [9]. This study found that compared with MSC, TIIA-MSC significantly reduced the expression levels of IL-6, and the downregulatory effect was more significant. At the same time, silencing the *TREM2* gene in MSC greatly suppressed the ability of TIIA-MSC to reduce inflammatory factors in N9 cells. Compared with TIIA-MSC, TREM2 siRNA significantly attenuated the decline in IL-6 and TNF-α, but this effect seemed to be less obvious for IL-1β. We suspect that the effect of TIIA-MSC on IL-1β was not apparent because the affected part of IL-1β is secreted into the medium, and our assay did not detect this part of IL-1β. Through the present data, it can be preliminarily surmised that the TREM2 signaling pathway is the key to the neuroinflammatory regulation of TIIA-MSC.

MSC can mediate and regulate the balance of the immune response through paracrine mechanisms and the interaction between MSC and immune cells. Bone marrow-derived MSC exosomes play an immunomodulatory role in various autoimmune-related diseases [24] and can reduce tissue

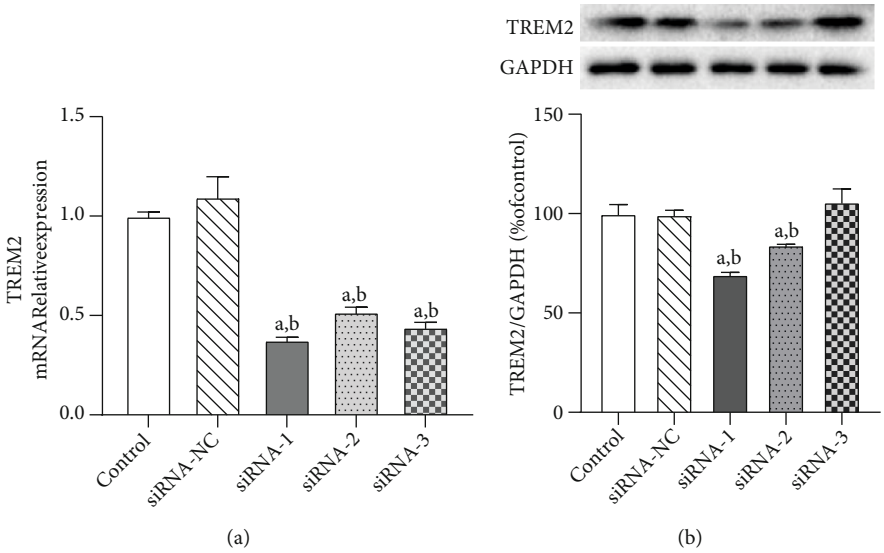

Figure 2: RT-qPCR and Western blotting analyses of TREM2 siRNA transfection efficiency. (a) Relative expression levels of TREM2 mRNA. (b) Expression levels of TREM2 protein. $^{a}P < 0.05$ vs. control, $^{b}P < 0.05$ vs. siRNA-NC, mean ± SEM, $n = 3$.

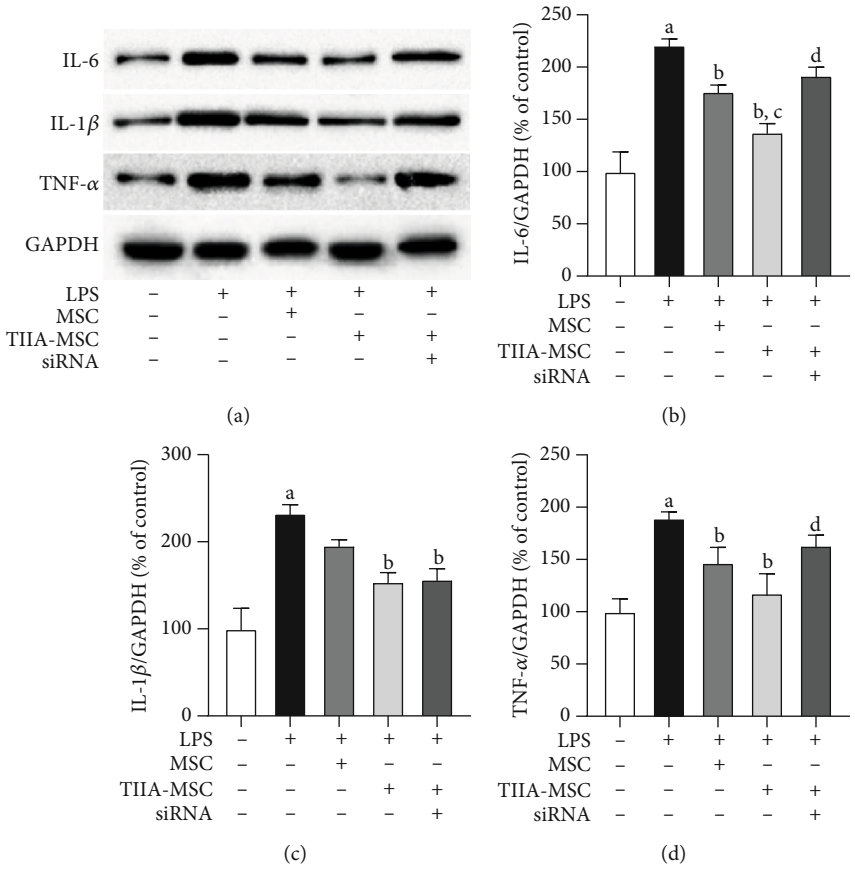

Figure 3: Inflammatory factor (IL-1β, IL-6, and TNF-α) protein expression. (a) Representative Western blotting results for IL-1β, IL-6, TNF-α, and GAPDH. (b) Relative protein expression of IL-6. (c) Relative protein expression of IL-1β. (d) Relative protein expression of TNF-α. $^{a}P < 0.05$ vs. control, $^{b}P < 0.05$ vs. LPS, $^{c}P < 0.05$ vs. LPS + MSC, $^{d}P < 0.05$ vs. LPS + TIIA − MSC, mean ± SEM, $n = 3$.

damage and promote tissue repair [25]. Therefore, we speculate that the enhanced inhibitory activity of TIIA-MSC may be related to the regulation of the TREM2 signaling pathway to affect the secretion of exosomes. In addition, the role of MSC in regulating neuroinflammation may be due to their directed differentiation into microglia with specific phenotypes [26]. Therefore, another possible reason for the function of TIIA-MSC is that they promote the expression of TREM2 in MSC and further differentiate into microglia with high TREM2 expression, thereby negatively regulating neuroinflammation and exerting a protective effect. However, this study also has certain limitations. For example, we did not test microglial proliferation, anti-inflammatory cytokines, or chemokines, such as CCL2, CXCL1, and IL-10. These will be assessed in our future studies.

5. Conclusions

The superior efficiency of TIIA-MSC in inhibiting the inflammatory response of N9 cells induced by LPS is related to the regulation of the TREM2 signaling pathway. This not only provides support for the application of TIIA to pretreat MSC but also provides new ideas for applying MSC for the treatment of neuroinflammation-related AD.

Data Availability

The authors declare that all the data supporting the findings in this study are available from the corresponding author through email on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Nanqu Huang and Juan Huang contributed equally to this work.

Acknowledgments

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Research Article

Curcumin and Mesenchymal Stem Cells Ameliorate Ankle, Testis, and Ovary Deleterious Histological Changes in Arthritic Rats *via* Suppression of Oxidative Stress and Inflammation

Rania H. Ahmed,[1] Sanaa R. Galaly,[1] Nadia Moustafa,[1] Rasha Rashad Ahmed,[1] Tarek M. Ali,[2] Basem H. Elesawy,[3] Osama M. Ahmed,[4] and Manal Abdul-Hamid[1]

[1]*Cell Biology, Histology and Genetics Division, Department of Zoology, Faculty of Science, Beni-Suef University, P.O. Box 62521, Beni-Suef, Egypt*
[2]*Department of Physiology, College of Medicine, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia*
[3]*Department of Pathology, College of Medicine, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia*
[4]*Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, P.O. Box 62521, Beni-Suef, Egypt*

Correspondence should be addressed to Manal Abdul-Hamid; medo_bio@yahoo.com

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Rheumatoid arthritis (RA) is a chronic inflammatory condition, an autoimmune disease that affects the joints, and a multifactorial disease that results from interactions between environmental, genetic, and personal and lifestyle factors. This study was designed to assess the effects of curcumin, bone marrow-derived mesenchymal stem cells (BM-MSCs), and their coadministration on complete Freund's adjuvant- (CFA-) induced arthritis in male and female albino rats. Parameters including swelling of the joint, blood indices of pro-/antioxidant status, cytokines and histopathological examination of joints, and testis and ovary were investigated. RA was induced by a single dose of subcutaneous injection of 0.1 mL CFA into a footpad of the right hind leg of rats. Arthritic rats were treated with curcumin (100 mg/kg b.wt./day) by oral gavage for 21 days and/or treated with three weekly intravenous injections of BM-MSCs (1×10^6 cells/rat/week) in phosphate-buffered saline (PBS). The treatment with curcumin and BM-MSCs singly or together significantly ($P < 0.05$) improved the bioindicators of oxidative stress and nonenzymatic and enzymatic antioxidants in sera of female rats more than in those of males. Curcumin and BM-MSCs significantly ($P < 0.05$) improved the elevated TNF-α level and the lowered IL-10 level in the arthritic rats. Furthermore, joint, testis, and ovary histological changes were remarkably amended as a result of treatment with curcumin and BM-MSCs. Thus, it can be concluded that both curcumin and BM-MSCs could have antiarthritic efficacies as well as protective effects to the testes and ovaries which may be mediated *via* their anti-inflammatory and immunomodulatory potentials as well as oxidative stress modulatory effects.

1. Introduction

Rheumatoid arthritis (RA) is the most severe destructive inflammatory arthritis. It is a chronic autoimmune condition through which nonsuppurative proliferative synovitis contributes to destruction of the articular cartilage and bone resulting in multiple joint inflammation. RA is more common among women than among men [1, 2]. The severity of the disease ranges from person to person, with joint damage varying from mild pain and irritation to severe inflammation. RA also affects joint pairs (two hands, two feet) and can affect small joints in wrists and hands. Many joints such as knees, elbows, shoulders, feet, and ankles can be also affected over time and deformity occurs. In addition, other organs such as the skin, eyes, and lungs can be affected, and neuropathy, anemia, fatigue, and heart disease may occur [3]. Although

the etiology of RA is unclear, disease susceptibility is associated with inheritance of certain allelic types of major histocompatibility complex (MHC) class II genes [4].

The mechanism of the joint degeneration effects in rheumatoid arthritis involves direct cell damage by cytotoxic CD^{8+} T-cells or other lytic cells. On other hand, the damaging effects of cytokines are triggered by CD^{4+} T-cells which know their antigenic targets, or by non-T-cells which release inflammatory mediators like tumor necrosis factor-α (TNF-α) and interleukin- (IL-) 1β [5]. In addition, Ahmed [6] suggested that the cytokine imbalance of CD^{8+} and CD^{4+} Th1/Th2 with a predominance of Th1 cytokines has pathogenic importance. TNF-α, a proinflammatory Th1 cytokine, serves a key role in the pathophysiological processes of RA [7, 8]. It is mainly released from activated inflammatory cells including macrophages, T-lymphocytes, and natural killer cells [9]. It contributes to the stimulation of other inflammatory cytokines, including interleukin- (IL-) 1, 6, 8, and 17 [7, 10]. TNF-α and other proinflammatory cytokines potentially amplify differentiation and activation of osteoclasts which in turn induce synovial hyperplasia, angiogenesis, cartilage erosion, and bone damage [11–13]. On the other hand, Th2 cytokines including IL-4 and IL-10 have anti-inflammatory effects, and their increases results in improving inflammation and arthritis [14, 15].

Reactive oxygen species (ROS) often participate in the pathogenesis of different diseases, including RA. ROS also play a central role both upstream and downstream of the TNF-α and nuclear factor-kappa B (NF-κB) pathways, which are at the center of the inflammatory response. RA-related inflammation is associated with altered signaling pathways, resulting in elevated levels of inflammatory cytokine markers, lipid peroxides, and free radicals. The natural protection mechanism involves antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as nonenzymatic antioxidant and reduced glutathione (GSH). The defect in such protective mechanism contributes to toxic oxidative free-radical accumulation and consequent degenerative changes [14, 16]. Due to the adverse effects and toxicity arising from the use of antiarthritic drugs, more focus is placed in discovering safer, more efficient, natural product-based, alternative medicines with antioxidant activities [17–19].

Curcumin or diferuloylmethane is a polyphenolic yellow pigment derived from turmeric (*Curcuma longa*) and has been reported to exhibit numerous activities including antioxidant and anti-inflammatory properties [20, 21]. Curcumin is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide, 1% carboxymethyl cellulose, and acetone [20, 22]. The fact that curcumin in solution exists primarily in its enolic form has an important role in the radical-scavenging ability of curcumin [20]. Many chronic disorders, including inflammatory arthritis, intestinal disease, chronic anterior uveitis, pancreatitis, and malignancies may benefit from curcumin [20]. Curcumin has also been shown to decrease many proinflammatory cytokines and their release mediators such as nitric oxide synthase (NOS), interleukin-8 (IL-8), interleukin-1 (IL-1), and TNF-α [21, 22].

The mesenchymal stem cell (MSC) population mainly resides in the bone marrow but may be present in other tissues (e.g., fat) and are capable of multilineage differentiation and self-renewal [23]. Under appropriate stimulation, MSCs can differentiate into 3 mesenchymal lineages: chondrocytes, adipocytes, and osteoblasts [23]. MSCs can also be induced experimentally to differentiate into neural and myogenic cells [24]. Multiple publications have confirmed that adherent cells (MSCs) isolated from various tissues meet the minimal criteria corresponding to the basic MSC phenotype, such as the expressions of CD73, CD90, and CD105 [25]. However, MSCs derived from different tissues can also express mesenchymal, hematopoietic, and endothelial tissue developmental markers [26], and they also produce molecules which directly involve immune response regulation, like programmed death ligand 1 (PDL-1) and PDL-2 inhibitory molecules, the costimulatory molecule CD28, and different cytokine arrays [27]. Therefore, MSCs can control immune response to these molecules. *In vivo*, MSC immunoregulatory function has also been observed; treatment with MSCs in humans enhanced the outcome of allogeneic transplantation through reducing graft-versus-host disease (GVHD) and facilitating hematopoietic engraftment [28]. MSCs have been widely used in animal models to prevent the autoimmunity recurrence in lupus-pronounced mice [29], to promote improvement of experimental autoimmune encephalomyelitis [30], and to enhance amelioration of CFA-induced arthritis in rats [14, 15]. Due to the success of MSC therapy in the treatment of some autoimmune disorders in animal models [30] and humans [28], the current research is aimed at examining the potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) either singly or in combination with curcumin in the therapy of RA in male and female Wistar rats.

2. Materials and Methods

2.1. Chemicals. Complete Freund's Adjuvant (CFA) (10 mL; each 1 mL of CFA contains 1 mg of *Mycobacterium tuberculosis*, heat-killed and dried, 0.85 mL paraffin oil, and 0.15 mL mannide monooleate) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Curcumin was obtained from Hedel–De Han AG, Germany. DMEM (Dulbecco's modified Eagle's medium), trypsin/EDTA, penicillin-streptomycin solution, and fetal bovine serum (FBS) were obtained from Lonza, Belgium. Sodium hydrogen carbonate was obtained from LOBA Chemie, India. Culture flasks and culture consumables were obtained from Greiner Bio-One (Germany). Other reagents and all chemicals used were of analytical quality and high purity.

2.2. Preparation of Complete Culture Medium. To prepare the complete culture medium, 10 mL FBS and 1 mL penicillin-streptomycin solution were added to 89 mL DMEM for each preparation according to Sun et al. [31] and Ahmed et al. [32].

2.3. BM-MSC Isolation and Culture. The isolation and culture method of BM-MSCs used in this study is based on

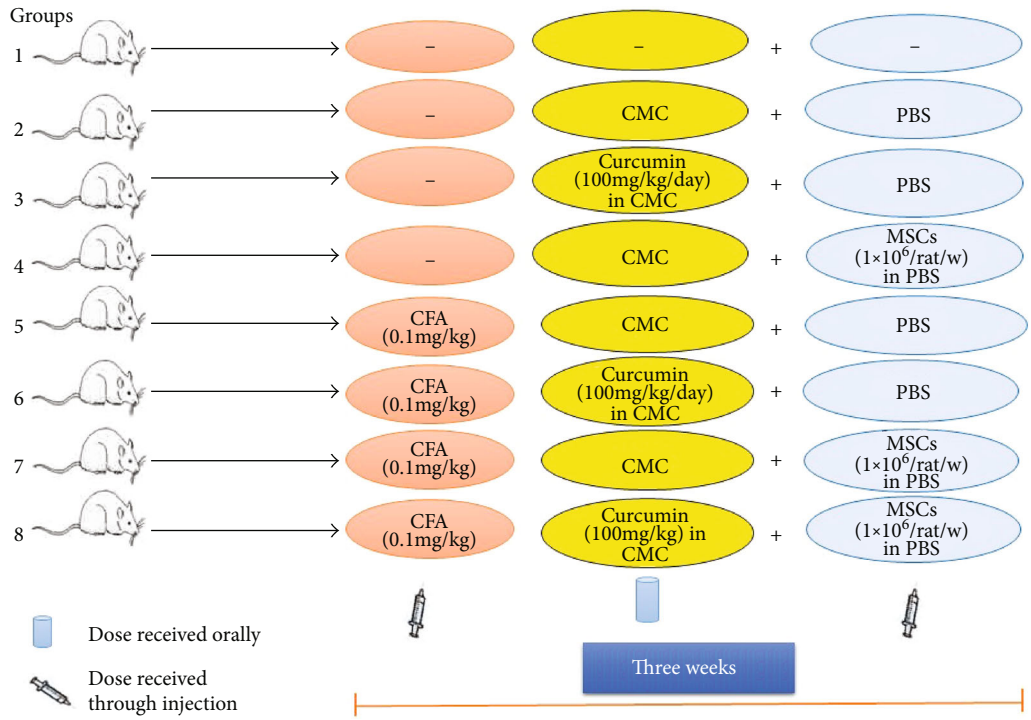


Figure 1: Experimental design and animal grouping.

the procedure of Ahmed et al. [32] and Chaudhary and Rath [33]. Also, the viability of cultured cells are tested by staining with trypan blue (0.4%), and it was found to be 96-98%.

2.4. Experimental Animals. The used experimental animals in this study were randomly bred 48 adult males and 48 females of laboratory albino rats of Wistar strain weighing 110-150 g. The animals were delivered from the Experimental Animals Helwan Station, Egyptian Organization for Biological Products and Vaccines (VACSERA), Helwan, Cairo, Egypt. The animals were held in plastic cages that have wired covers and kept in normal laboratory conditions during the course of the experiment. The animals were not treated with antibiotics, insecticides, or vitamins and were fed a standard commercial diet (ATMID Company, Giza, Egypt) and tap water *ad libitum*. All experimental procedures were performed in accordance with recommendations, instructions, and guidelines stated by the Ethics Committee for Care and Use of Animals, Faculty of Science, Beni-Suef University, Egypt (Ethical Approval Number: BSU/FS/2018/7).

2.5. Experimental Design. Experimental animals (Figure 1) were organized into 16 groups (6 animals for each), eight groups including male rats and the other eight groups including female rats as follows:

(1) Group 1: normal group that did not receive any treatment or vehicle.

(2) Group 2 (control group): rats within this group received the equivalent volumes of 1% CMC (5 mL/kg b.wt./day) as vehicle 1 by oral gavage daily and PBS (as vehicle 2) in the lateral tail vein weekly for three weeks. The equivalent volume of phosphate-buffered solution was given.

(3) Group 3: curcumin control group. Rats were daily supplemented with curcumin by oral gavage. Curcumin was dissolved in 1% CMC (carboxymethyl cellulose) at 2% concentration and was administrated orally (100 mg/kg b.wt./day) [34]. This group was also weekly given the equivalent volume of PBS.

(4) Group 4 (mesenchymal stem cells (MSCs) control group): in this group, the rats weekly received injection of MSCs (1×10^6/rat) in PBS. This group was daily given the equivalent volume of 1% CMC by oral gavage for 21 days.

(5) Group 5 (arthritic control group): rats were subcutaneously injected with CFA (0.1 mL (0.1 mg)/kg b.wt. single dose) into a foot pad of the right hind leg [35] to induce RA. This group was also given the equivalent volumes of 1% CMC by daily oral administration and PBS by weekly intravenous injection.

(6) Group 6 (arthritic group treated with curcumin): rats were injected with CFA like in group 5 and orally treated with curcumin like in group 3. This group was also weekly given the equivalent volume of PBS by intravenous injection.

(7) Group 7 (arthritic group treated with BM-MSCs): in this group, rats were injected with CFA like group 5 in parallel to injection of BM-MSCs in the tail vein like group 4. This group was daily given the equivalent volume of 1% CMC by oral gavage for 21 days.

Table 1: Effect of gender, group and their interactions on paw circumference in rats (one-way and two-way ANOVA).

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	17.055	15	1.137	46.748	$P < 0.001$
	Within groups	1.946	80	0.024		
	Total	19	95			
Two-way ANOVA	Gender	0.196	1	0.196	8.067	$P < 0.001$
	Treatment	15.529	7	2.218	91.211	$P < 0.001$
	Gender-treatment	1.330	7	0.190	7.812	$P < 0.001$
	Error	1.946	80	0.024		
	Total	110.925				

$P < 0.001$ is significant effect at $\alpha = 0.001$.

Table 2: Effect of tested materials (MSCs and curcumin) on circumference of paw region (cm) of arthritic group.

Parameter	Right hind leg paw circumference at (cm)	
Group	Male rats	Female rats
Normal (water)	1.925 ± 0.017^{a}	1.996 ± 0.016^{a}
CMC/PBS	1.943 ± 0.018^{a}	1.995 ± 0.020^{a}
MSCs	1.978 ± 0.028^{a}	2.028 ± 0.060^{a}
Curcumin	$1{,}986 \pm 0.027^{a}$	1.998 ± 0.017^{a}
Arthritic group (CFA)	3.856 ± 0.231^{b}	9.500 ± 0.215^{c}
Arthritic group treated with curcumin	2.023 ± 0.059^{a} 880.87	2.060 ± 0.097^{a} 1425.00
Arthritic group treated with MSCs	2.031 ± 0.084^{a} −72.87	2.018 ± 0.070^{a}
Arthritic group treated with MSCs and curcumin	2.255 ± 0.192^{a}	2.025 ± 0.022^{a}

Data are expressed as mean ± SE. The number of animals in each group is six. Values with the same superscript letter are similar (nonsignificant, $P > 0.05$), whereas others are significant (significant, $P < 0.05$).

(8) Group 8 (arthritic group treated with both MSCs and curcumin): rats were injected with CFA (like group 5) in parallel to oral administration of curcumin like groups 3 and 6 and injection of BM-MSCs in the tail vein like groups 4 and 7.

2.6. Tissues Sampling. The ankle circumference of the right hind leg of each rat was measured at the end of the experiment, and rats were sacrificed under mild anesthesia in each group. The ankle circumference was measured by wrapping a cotton thread around the ankle, and the length of the wrapped thread was measured by ruler. By centrifugation of blood at 3000 rpm for 15 minutes, sera were separated and the clear and nonhemolyzed supernatant sera were rapidly removed and kept at -20°C while being used in biochemical analysis. For histopathological analysis, paw and hind ankle, testes, and ovaries were removed and then fixed in neutral-buffered formalin (10%).

2.7. Paw Edema Level. The circumference of the right hind paw above the tarsal pad was determined by using a piece of cotton thread and wrapping it around the paw just above the tarsal pad as an indicator of the swelling rate and paw edema in different groups. The circumference was measured using a meter ruler [18, 36]. The measurements were taken on the 21th day of adjuvant induction.

2.8. Oxidative Stress Markers. In serum, the thiobarbituric acid-reactive substances (TBARS) were measured according to Preuss et al. [37] to determine lipid peroxidation (LPO). Glutathione reduced form (GSH) level was measured colorimetrically using the Ellman reagent as protein-free sulfhydryl content [38]. In addition, glutathione-S-transferase (GST) activity was calculated according to Habig et al. [39], and glutathione peroxidase (GPx) activity was determined by using the method of Kar and Mishra [40] in serum. Finally, superoxide dismutase (SOD) activity was detected according to the colorimetric method of Nishikimi et al. [41].

2.9. Detection of Serum TNF-α and IL-10 Levels. TNF-α levels in the serum of normal and experimental groups were measured using ELISA kits which were purchased from R&A Systems, USA, according to the manufacturer's instructions [42]. The level of IL-10 was determined using specific ELISA kits purchased from R&A Systems, USA, in the serum of control and experimental groups. According to the manufacturer's instructions, the concentrations of

Table 3: One-way and two-way analysis for serum TNF-α (pg/mL) level in normal, arthritic, and arthritic-treated rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	19177.342	16	1198.584	43.907	$P < 0.001$
	Within groups	2156.54	79	27.298		
	Total	21333.890	95			
Two-way ANOVA	Gender	395.444	1	395.444	14.415	$P < 0.001$
	Treatment	17417.571	7	2488.224	90.702	$P < 0.001$
	Gender-treatment	1326.246	7	189.464	6.906	$P < 0.001$
	Error	2149.629	80	27.433		
	Total	470842.89				

$P < 0.001$indicates significant effect at $\alpha = 0.001$.

Table 4: Effect of MSCs and curcumin on serum TNF-α (pg/mL) level in arthritic rats.

Parameter Group Group	TNF-α (pg/mL serum)	
	Male rats	Female rats
Normal	55.59 ± 1.48^{a}	63.19 ± 5.06^{a}
CMC/PBS	58.25 ± 0.88^{a}	61.57 ± 0.91^{a}
MSCs	64.84 ± 0.76^{a}	56.47 ± 1.56
Curcumin	66.58 ± 1.20^{a}	56.31 ± 3.14^{a}
Arthritic group (CFA)	96.43 ± 1.31^{b}	103.50 ± 3.37^{c}
Arthritic group treated with curcumin	84.26 ± 1.85^{d}	64.70 ± 1.73^{a}
Arthritic group treated with MSCs	74.05 ± 1.29^{d}-	72.16 ± 1.23^{d}
Arthritic group treated with MSCs and curcumin	56.62 ± 1.57^{a}	61.40 ± 2.44^{a}

Data are expressed as mean ± SE. The number of animals in each group is six. Values with the same superscript letter are similar (nonsignificant, $P > 0.05$), whereas others are significant (significant, $P < 0.05$).

Table 5: One-way and two-way analysis for serum IL-10 (pg/mL) level in normal, arthritic and arthritic-treated rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	15720.113	16	982.507	8.832	$P < 0.001$
	Within groups	8788.131	79	111.242		
	Total	24508.244	95			
Two-way ANOVA	Gender	477.265	1	477.265	4.341	$P < 0.05$
	Treatment	11886.638	7	1698.091	15.444	$P < 0.001$
	Gender-treatment	3348.098	7	478.300	4.350	$P < 0.001$
	Error	8796.243	80	109.953		
	Total	897550.02				

$P < 0.001$indicates significant effect at $\alpha = 0.001$, and $P < 0.05$ indicates significant effect at $\alpha = 0.05$.

IL-10 were measured by using a spectrophotometer at 450 nm.

2.10. Histological Preparations

2.10.1. Paraffin Section Preparation. Hind ankle region and paw tissue samples from male and female rats, testes, and ovaries were fixed for 24 h in 10% neutral-buffered formalin (pH 6.8). Tissue samples were embedded in paraffin wax after dehydration, sectioned at 4 to 5 μm, and finally stained with hematoxylin and eosin for histopathological analysis [43].

2.11. Statistical Analysis. Two-way analysis of variance (ANOVA) [44] accompanied by one-way ANOVA using the SPSS/PC program (version 20.0; SPSS, Chicago, IL) and post hoc LSD test was used to statistically analyze biochemical variable measurements ($P < 0.05$ was considered to be significant). Two-way ANOVA was applied to assess

Table 6: Effect of MSCs and curcumin on serum IL-10 (pg/mL) level in arthritic rats.

Parameter	IL-10 (pg/mL)	
Group	Male rats	Female rats
Normal	104.96 ± 1.6^{a}	103.44 ± 4.42^{a}
CMC/PBS	104.33 ± 2.06^{a}	102.16 ± 3.58^{a}
MSCs	103.08 ± 3.39^{a}	104.70 ± 7.18^{a}
Curcumin	105.82 ± 4.53^{a}	105.38 ± 3.34^{a}
Arthritic group (CFA)	79.83 ± 2.17^{b}	53.26 ± 1.00^{c}
Arthritic group treated with curcumin	91.40 ± 6.32^{a}	88.51 ± 4.62^{a}
Arthritic group treated with MSCs	85.37 ± 6.38^{a}	89.22 ± 4.94^{a}
Arthritic group treated with MSCs and curcumin	96.25 ± 2.21^{a}	97.53 ± 5.01^{a}

Data are expressed as mean ± SE. The number of animals in each group is six. Values with the same superscript letter are similar (nonsignificant, $P > 0.05$), whereas others are significant (significant, $P < 0.05$).

Table 7: One-way and two-way analysis to test the effect of gender, treatment, and their interactions on MDA content in rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	344.312	15	22.954	18.389	$P < 0.001$
	Within groups	99.858	80	1.248		
	Total	444.169	95			
Two-way ANOVA	Gender	48.920	1	48.920	38.403	$P < 0.001$
	Treatment	285.120	7	40.740	31.981	$P < 0.001$
	Gender-treatment	8.158	7	1.165	0.915	$P > 0.05$
	Error	101.910	80	1.274		
	Total	2027.001				

$P < 0.001$indicates significant effect at $\alpha = 0.001$, while $P > 0.05$ indicates insignificant.

Table 8: One-way and two-way analysis to test the effect of gender, treatment, and their interactions on GSH content in rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	260.032	15	17.335	10.822	$P < 0.001$
	Within groups	128.145	80	1.602		
	Total	388.168	95			
Two-way ANOVA	Gender	6.531	1	6.531	4.079	$P < 0.05$
	Treatment	251.022	7	35.860	22.397	$P < 0.001$
	Gender-treatment	2.526	7	0.361	0.225	$P > 0.05$
	Error	128.089	80	1.601		
	Total	4975.856				

$P < 0.001$indicates significant effect at $\alpha = 0.001$, and $P < 0.05$ indicates significant effect at $\alpha = 0.05$, while $P > 0.05$ is insignificant.

the effects of gender, treatment, and gender/treatment interaction.

3. Results

3.1. Effect on Paw Edema. The right hind leg circumference, at the paw region in CFA-injected animals at day 21 of CFA injection and as a result of curcumin and MSC treatments, is shown in Tables 1 and 2.

The CFA-induced arthritic male and female rats exhibited a significant increase in the hind paw edema at day 21 as compared with the normal group. The arthritic effect in female rats is more severe than in male rats. The groups of the arthritic male and female rats treated with curcumin, MSCs, and their combination showed a significant amelioration of the elevated values of paw edema as compared to the arthritic animals and the values returned to nearly normal (Table 2).

Table 9: One-way and two-way analysis to test the effect of gender, treatment, and their interactions on GST content in rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	1096.462	15	73.097	14.362	$P < 0.001$
	Within groups	407.168	80	5.090		
	Total	1503.630	95			
Two-way ANOVA	Gender	21.556	1	21.556	4.232	$P < 0.05$
	Treatment	994.629	7	142.090	27.897	$P < 0.001$
	Gender-treatment	79.974	7	11.425	2.243	$P < 0.05$
	Error	407.472	80	5.093		
	Total	29070.773				

P<0.001 indicates significant effect at $\alpha = 0.001$, and $P < 0.05$ indicates significant effect at $\alpha = 0.05$.

Table 10: One-way and two-way analysis to test the effect of gender, treatment, and their interactions on GPx activity in rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	381.014	15	25.401	14.032	$P < 0.001$
	Within groups	144.912	80	1.811		
	Total	525.925	95			
Two-way ANOVA	Gender	10.140	1	10.140	5.437	$P < 0.05$
	Treatment	336.466	7	48.067	25.775	$P < 0.001$
	Gender-treatment	30.130	7	4.304	2.308	$P < 0.05$
	Error	149.189	80	1.865		
	Total	6250.769				

$P < 0.05$ indicates significant effect at $\alpha = 0.05$.

Table 11: One-way and two-way analysis to test the effect of gender, treatment, and their interactions on SOD activity in rats.

Source of variation		Sum of squares	D.F.	Mean squares	F ratio	P value
One-way ANOVA	General effect					
	In between groups	212.628	15	14.175	10.941	$P < 0.001$
	Within groups	103.647	80	1.296		
	Total	316.275	95			
Two-way ANOVA	Gender	5.956	1	5.956	4.595	$P < 0.05$
	Treatment	203.057	7	29.008	22.347	$P < 0.001$
	Gender-treatment	3.406	7	0.487	0.375	$P > 0.05$
	Error	103.847	80	1.298		
	Total	5047.615				

$P < 0.001$indicates significant effect at $\alpha = 0.001$, and $P < 0.05$ indicates significant effect at $\alpha = 0.05$, while $P > 0.05$ is insignificant.

Regarding one-way ANOVA, the general effect was very highly significant between groups ($P < 0.001$) (Table 1) throughout the experiment.

Concerning two-way ANOVA, it was noticed that the effect of treatment, gender, and treatment-gender interaction was significant at $P < 0.001$ (Table 1).

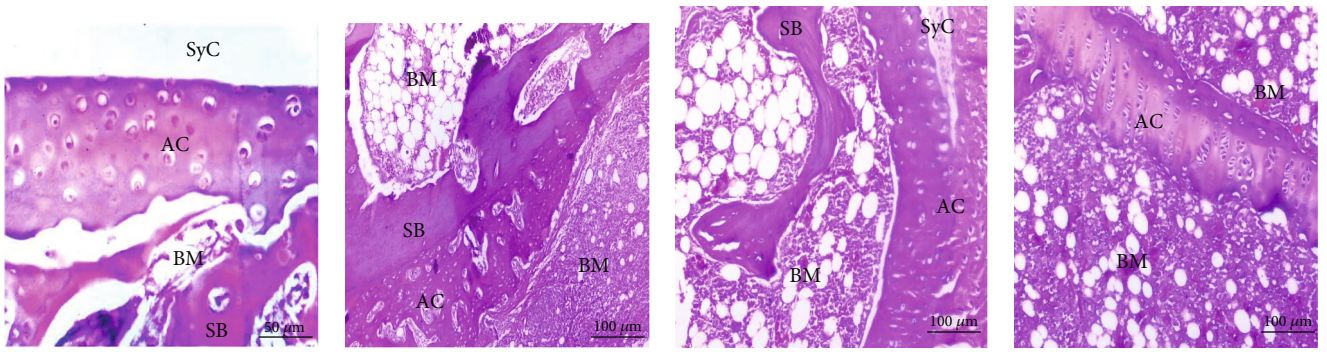
3.2. Effect on TNF-α and IL-10 Serum Inflammatory Cytokine Levels. The data showing the pattern of changes in serum TNF-α and IL-10 levels are represented in Tables 3, 4, 5, and 6.

A significant elevation in serum TNF-α level was noticed in CFA-induced arthritic rats when it was compared with normal rats; the arthritic effect is more severe in female than in male rats. CFA-injected rats that were treated with curcumin and/or MSCs exhibited a marked decrease in the elevated serum TNF-α level in comparison with arthritic control rats and when compared with either the curcumin- or MSC-treated arthritic groups (Table 4). Regarding one-way ANOVA, the general effect between groups on serum TNF-α level was highly significant ($P < 0.001$) (Table 3) throughout the experiment. Concerning two-way ANOVA,

Table 12: Protective effect of MSCs and curcumin against CFA-induced changes in LPO; GSH concentration; and GST, GPx, and SOD activities in serum of all experimental groups.

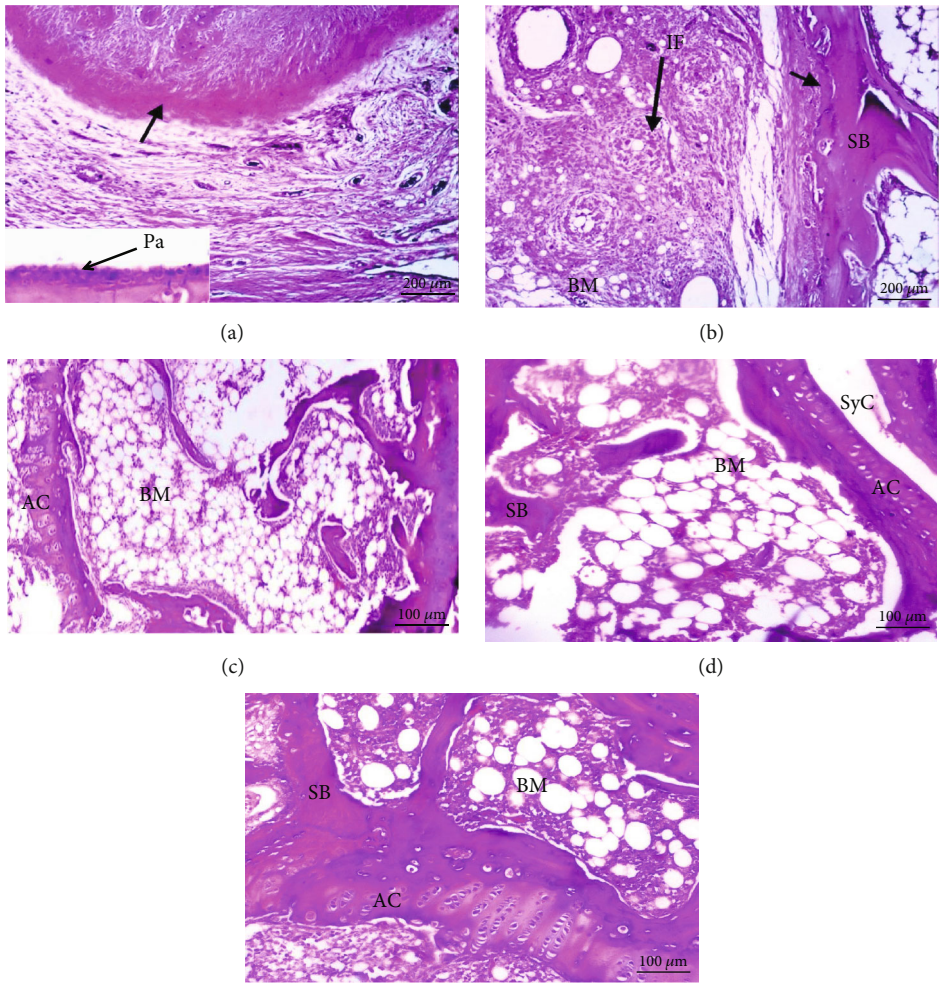
Parameter		Group							
		Normal	CMC/PBS	Curcumin	MSCs	Arthritic group	Arthritic group treated with curcumin	Arthritic group treated with MSCs	Arthritic group treated with MSCs and curcumin
LPO (nmol/mL)	Male rats	2.625 ± 0.461^{a}	2.817 ± 0.399^{a}	2.632 ± 0.451^{a}	2.847 ± 0.313^{a}	7.530 ± 1.99^{b}	2.613 ± 0.351^{a}	3.23 ± 2.07^{a}	2.612 ± 0.397^{a}
	Female rats	4.701 ± 0.440^{a}	3.827 ± 0.460^{a}	3.858 ± 0.504^{a}	3.572 ± 0.398^{a}	9.500 ± 1.002^{c}	3.953 ± 0.517^{d}	5.475 ± 0.404^{a}	3.582 ± 0.530^{a}
GSH (nmol/mL)	Male rats	8.106 ± 0.428^{a}	7.468 ± 0.393^{a}	8.148 ± 0.590^{a}	7.858 ± 0.426^{a}	3.299 ± 0.233^{b}	7.425 ± 0.433^{a}	7.336 ± 0.360^{a}	7.753 ± 0.81^{a}
	Female rats	7.375 ± 0.726^{a}	7.221 ± 0.447^{a}	7.566 ± 0.659^{a}	7.381 ± 0.509^{a}	2.088 ± 0.072^{b}	6.978 ± 0.555^{a}	6.900 ± 0.561^{a}	7.222 ± 0.549^{a}
GST (U/mL)	Male rats	18.541 ± 0.400^{a}	18.496 ± 0.842^{a}	18.053 ± 0.419^{a}	18.453 ± 0.532^{a}	5.690 ± 0.506^{b}	17.755 ± 1.21^{d}	16.811 ± 2.31^{d}	17.970 ± 0.948^{a}
	Female rats	18 ± 0.968^{a}	18.034 ± 0.584^{a}	18.203 ± 0.863^{a}	18.880 ± 0.540^{a}	11.268 ± 0.573^{c}	17.853 ± 0.809^{d}	18.78 ± 0.830^{d}	18.39 ± 0.590^{a}
GPx (U/mL)	Male rats	8.541 ± 0.400^{a}	8.498 ± 0.415^{a}	8.566 ± 0.761^{a}	9.363 ± 0.352^{a}	1.901 ± 0.227^{b}	7.583 ± 0.668^{d}	7.798 ± 0.589^{d}	8.925 ± 0.938^{a}
	Female rats	8.725 ± 0.465^{a}	9.083 ± 0.550^{a}	8.780 ± 0.436^{a}	7.988 ± 0.837^{a}	3.771 ± 0.360^{c}	8.058 ± 0.281^{d}	8.030 ± 0.451^{d}	8.672 ± 0.436^{a}
SOD (U/mL)	Male rats	7.001 ± 0.342^{a}	7.446 ± 0.383^{a}	7.251 ± 0.563^{a}	8.156 ± 0.313^{a}	3 ± 0.085^{b}	7.330 ± 0.403^{a}	7.253 ± 0.518^{a}	6.728 ± 0.696^{a}
	Female rats	7.915 ± 0.405^{a}	8.058 ± 0.537^{a}	7.568 ± 0.383^{a}	7.855 ± 0.746^{a}	3.431 ± 0.371^{c}	7.638 ± 0.461^{a}	7.716 ± 0.465^{a}	7.925 ± 0.405^{a}

Data are expressed as mean ± SE. The number of animals in each group is six. In the same column, values with the same superscript letter are similar (nonsignificant, $P > 0.05$), whereas others are significant (significant, $P < 0.05$).



(a) (b) (c) (d)

FIGURE 2: Photomicrographs of sections in the ankles of male rats from control groups: (a) water, (b) CMC, (c) curcumin, (d) stem cells showing normal articulating cartilage (AC), synovial cavity (SyC), sponge bone (SB), and bone marrow (BM) (H&E).



(a) (b) (c) (d) (e)

FIGURE 3: Photomicrographs of sections in the ankles of male rats. (a) From the RA group showing necrosis of articular cartilage (↗). The insert shows pannus formation (↙). (b) From the RA group showing necrosis of articular cartilage (↓) and inflammatory cell infiltration (IF). (c–e) From arthritic-treated groups with curcumin, MSCs, and a combination of curcumin and MSCs, respectively, showing a nearly normal structure of articulating cartilage (AC), synovial cavity (SyC), sponge bone (SB), and bone marrow (BM).

it was revealed that the effects of treatment, gender, and treatment-gender interaction were very highly significant at $P < 0.001$ (Table 3).

A significant decrease in serum IL-10 level was shown in CFA-induced arthritic rats when compared with normal rats after 21 days; the decrease is more pronounced in female

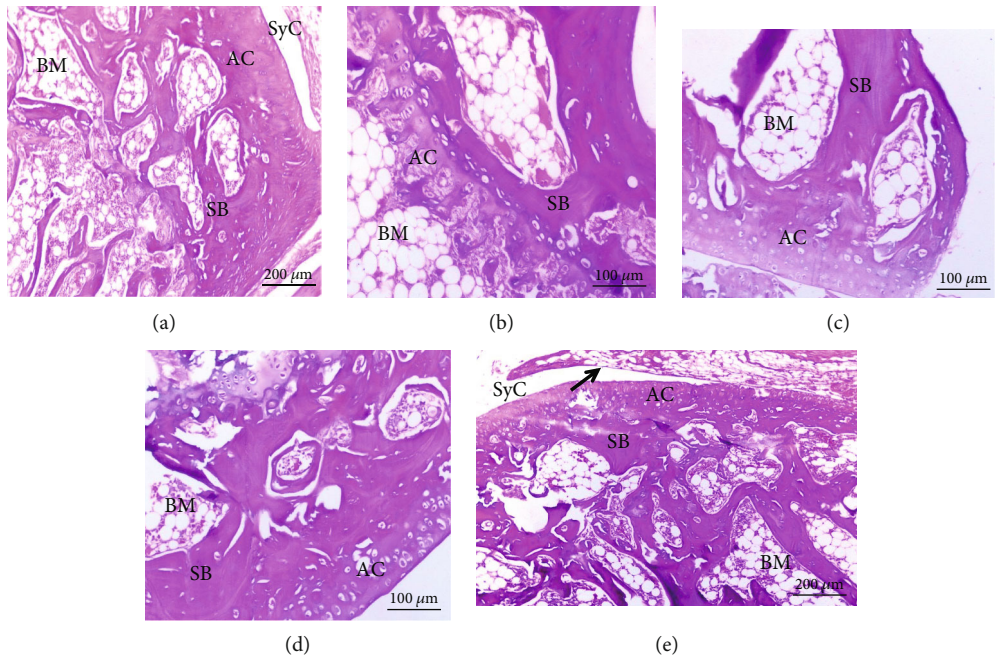


Figure 4: Photomicrographs of sections in the ankles of female rats from control groups: (a, b) water; (c) CMC; (d) curcumin; (e) stem cells showing normal articulating cartilage (AC), synovial cavity (SyC), synovial membrane (↗), sponge bone (SB), and bone marrow (BM).

than in male rats. The treatment of CFA-injected rats with MSCs and/or curcumin produced a significant increase in IL-10 level after 21 days in comparison to arthritic control rats; the combinatory effects were the most potent (Table 6). Regarding one-way ANOVA, the general effect between groups on serum IL-10 level was very highly significant ($P < 0.001$) (Table 5) throughout the experiment. Concerning two-way ANOVA of normal-arthritis effect, it was revealed that the effects of treatment and treatment-gender interaction were very highly significant ($P < 0.001$), while the effect of gender is only significant ($P < 0.05$) (Table 5).

3.3. Oxidative Stress Markers. The data showing the effects on LPO, GSH content, and antioxidant enzymes in serum are represented in Tables 7, 8, 9, 10, 11, and 12.

Table 12 shows changes in LPO and antioxidant parameters for all groups. MDA level, as an indicator of LPO, exhibited a significant increase ($P < 0.05$) in male and female rats in comparison with the normal group. CFA injection resulted in MDA level increase in female rats more than in male rats. On the other hand, the treatment with curcumin, MSCs, and a mixture of both induced a potential reduction of elevated MDA in both male and female rats.

Concerning the two-way ANOVA. The MDA level of arthritic rats treated with curcumin, MSCs, and combination effects, it was noticed that the effects of treatment and gender were very highly significant ($P < 0.001$), while the effects of gender-treatment interaction were nonsignificant ($P > 0.05$) (Table 7).

The level of the nonenzymatic antioxidant, GSH, and the activities of antioxidant enzymes including GST, GPx, and SOD showed a significant depletion in CFA-induced arthritic rats. On the other hand, the treatment with curcumin, MSCs, and their combination induced a significant improvement in GSH, GST, GPx, and SOD activities in both male and female rats; the effects of curcumin, MSCs, and their combination were more or less similar.

Concerning the one-way ANOVA, CFA caused significant effects in GSH levels (Table 8) along with GST (Table 9), GPx (Table 10), and SOD (Table 11) activities ($P < 0.001$) in both male and female rats compared to the normal group.

Concerning the two-way ANOVA, in the case of GSH content, it was noticed that the effects of treatment were very highly significant ($P < 0.001$), and the effects of gender were significant ($P < 0.05$), while the effects of gender-treatment interaction were nonsignificant ($P > 0.05$) (Table 8). In the case of GST and GPx activities, it was noticed that the effects of gender and gender-treatment interaction were significant ($P < 0.05$), and the effects of treatment were very highly significant ($P < 0.001$) (Table 9). Finally, in the case of SOD, it was revealed that the effects of gender were significant ($P < 0.05$), and the effects of treatment were significant ($P < 0.001$), while the effects of treatment-gender interaction were nonsignificant ($P > 0.05$) (Table 10).

3.4. Histopathological Results. The histological alterations of the articular ankle joint in various groups of male and female rats are depicted in Figures 2–5. In normal rats, the bone surfaces in the synovium are covered by an articular cartilage that lacks a perichondrium. The heads of the two articulated bones are enclosed and joined by an articular capsule consisting of two parts, the outer and inner parts. The outer part is a sheath of fibrous tissue (fibrous capsule) that extends well beyond each bone's articular cartilage. The inner part is called a synovial membrane and lines the fibrous capsule and is reflected in the bone that covers right up to the articular cartilage. Therefore, the joint cavity between two

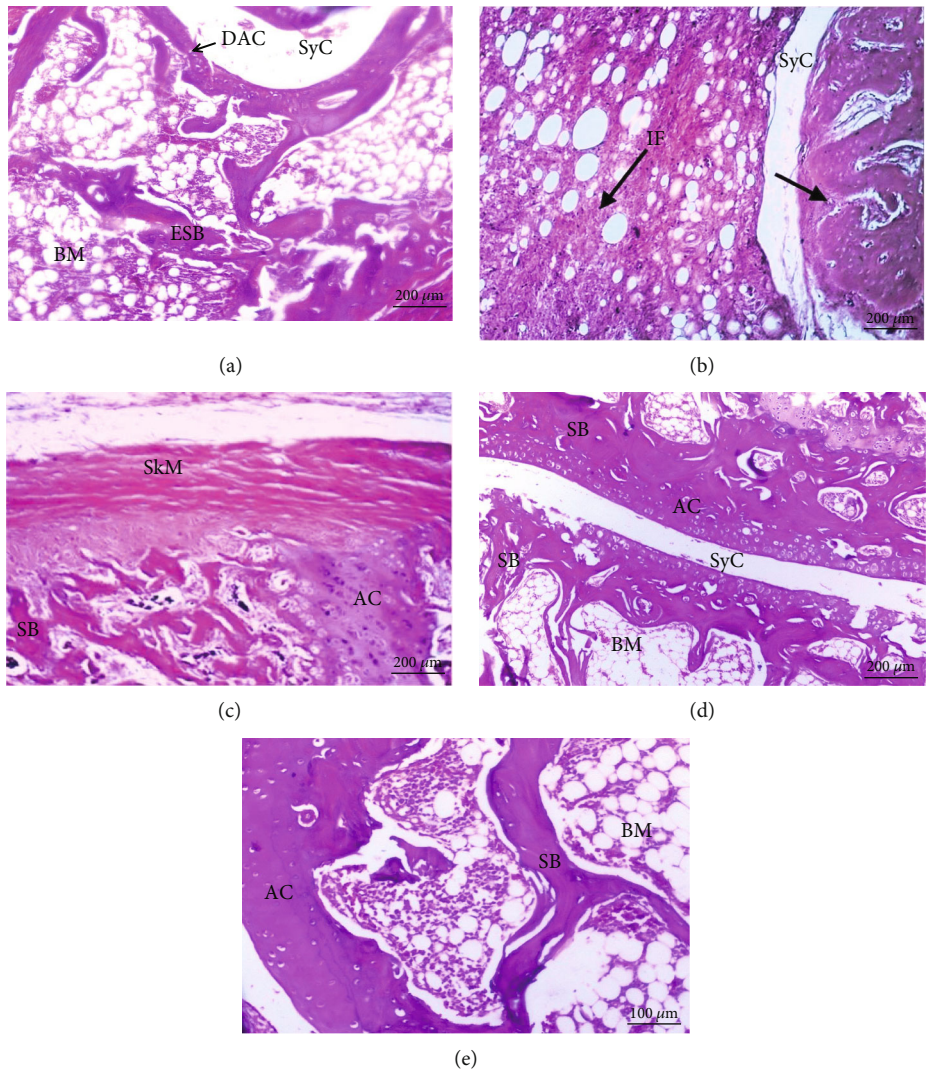


Figure 5: Photomicrographs of sections in an ankle of a female rat (a) from the RA group showing damaged articulating cartilage (DAC) and eroded sponge bone (ESB); (b) from the RA group showing necrosis of articular cartilage (↘) and massive inflammatory cell infiltration (IF); (c–e) from arthritic-treated groups with curcumin, MSCs, and a mixture of curcumin and MSCs, respectively, showing a nearly normal structure of articulating cartilage (AC), synovial cavity (SyC), skeletal muscle (SkM), sponge bone (SB), and bone marrow (BM).

articulated bones is lined everywhere with either articular cartilage or synovial membrane. The synovial membrane is a thin sheath of fibrous connective tissue, with a dense network of blood and lymph capillaries. Ankle joint sections of male rats (Figures 2(a)–2(d)) and female rats (Figures 4(a)–4(e)) from normal, CMC, and combined curcumin and MSC groups, respectively, showed the normal histological structure of an ankle with normal articulating cartilage, synovial cavity, sponge bone, and bone marrow.

CFA-administered arthritis male rats showed necrosis of cartilage with inflammatory cell infiltration in ankle joint sections, degeneration of cartilage, and pannus formation (Figures 3(a) and 3(b)).

CFA-administered arthritis female rat ankle joint sections showed severe necrosis of cartilage with massive inflammatory cell infiltration, severe degeneration of cartilage, and eroded spongy bone (Figures 5(a) and 5(b)). This indicated that the arthritic effect was more severe in female rats than in male rats.

CFA-administered male rats (Figures 4(c)–4(e)) and female rats (Figures 5(c)–5(e)) treated with curcumin, MSCs, and a mixture of curcumin and mesenchymal stem cells showed a nearly normal section structure of articulating cartilage, synovial cavity, sponge bone, and bone marrow nearly similar to the normal control groups.

The ovary of control normal rats (Figures 6(a)–6(d)) from normal, CMC, curcumin, and MSCs, respectively, showed a normal morphology. The ovary consists of two distinct regions: an outer cortex that contains numerous follicles at various stages of maturation and an inner central medulla, which did not appear in these histological sections. The surface of the ovary is covered with germinal epithelium. It contains corpus luteum and different primordial follicles including primary follicles and secondary follicles.

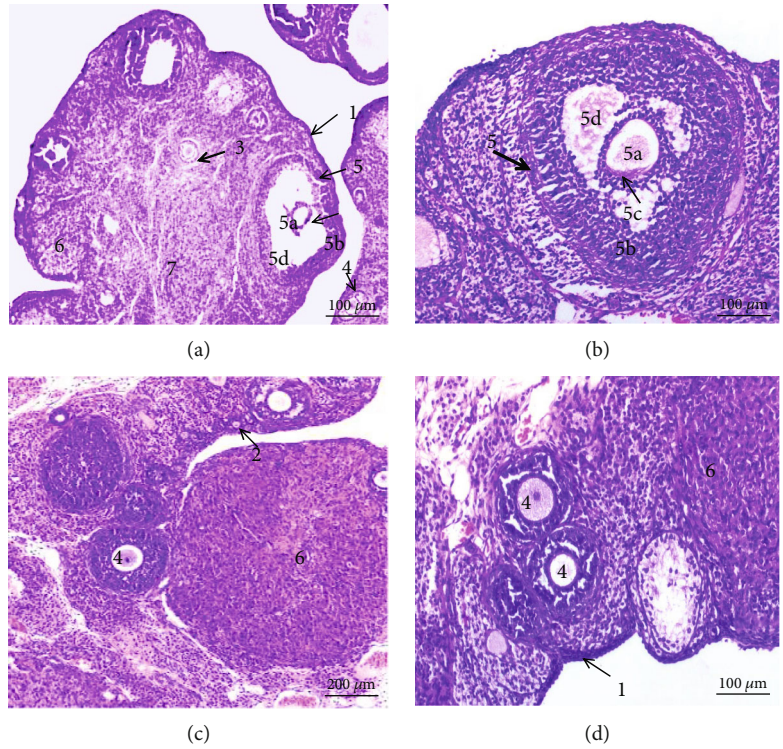


Figure 6: Photomicrographs of control rat ovarian sections: (a) water; (b) CMC; (c) curcumin; (d) stem cells showing normal histological structure. (1) Germinal epithelium; (2) primordial follicles; (3) primary follicles; (4) secondary follicles; (5) mature (antral or Graafian) follicle including (5a) oocyte (immature ovum), (5b) granulosa cells (stratified cuboidal epithelium), (5c) zona pellucida, and (5d) antrum; (6) corpus luteum; (7) medulla (H&E).

The secondary follicle contains an oocyte surrounded by two or more layers supporting granulosa cells and a follicular antrum filled with liquor follicle, and the follicle is surrounded by theca interna. Mature Graafian follicles are seen beneath the epithelium. Graafian follicles consist of an enlarged oocyte that floats freely within liquor folliculi surrounded by clear zona pellucida, corona radiata, well-defined zona granulosa, and compact theca folliculi.

The histopathological examination of arthritic ovarian sections revealed multiple luteal structures in ovarium medulla, stromal hyperemia, and infiltration of mononuclear cells (Figures 7(a) and 7(b)).

Sections of arthritic rats treated with curcumin (Figure 7(c)), MSCs (Figure 7(d)), and a combination of curcumin and mesenchymal stem cells (Figure 7(e)) revealed nearly normal structure.

Testes of normal rats (Figures 8(a) and 8(b)), CMC (Figure 8(c)), curcumin (Figure 8(d)), and stem cells (Figure 8(e)) revealed a normal seminiferous tubule morphology. Every tubule has epithelial cells including Sertoli cells and germ cells that demonstrated the complete spermatogenesis process (Figure 8(b)). Sertoli cells were usually located in the seminiferous tubule toward the basement membrane. Spermatogonia stood on seminiferous tubule basal lamina. Primary spermatocytes were immediately above them, identified by their large nuclei having coarse chromatin clumps and copious cytoplasm. Due to the rapid division processes, secondary spermatocytes in these sections were not seen. Therefore, there were small, rounded spermatids with rounded nuclei above the primary spermatocytes that proceeded in a long metamorphosis to become recognizable spermatozoa (Figure 8(b)).

Testicular tissue sections obtained from CFA-treated rats displayed several histopathological changes as showed in Figures 9(a)–9(c). Atrophy and focal necrosis in germinal cells, spermatogenic arrest, and congestion were noticeably observed (Figure 9(a)). Pyknotic nuclei, interstitial edema, and damaged seminiferous epithelium and germ cells are also seen (Figure 9(b)). The seminiferous tubules showed irregular variable size and congestion in intercellular space (Figure 9(c)). Testes treated with curcumin, MSCs, and mixture of MSCs plus curcumin (Figures 9(d)–9(f)), respectively, revealed apparent normal seminiferous tubules. Spermatogenic layers were well organized, the tubules had restored their regular shape, and sperms in most of the tubules were observed.

4. Discussion

Currently, stem cell therapy has been declared as one of the most important and promising treatments for the near future. This kind of therapy could improve or even reverse some degenerative diseases and have potential applications in replacement and regenerative medicines and RA. Also, using plant constituents in RA treatment has attracted many researchers due to the side effects of conventional drugs.

RA, one of the most common chronic inflammatory autoimmune diseases, is distinguished by systemic

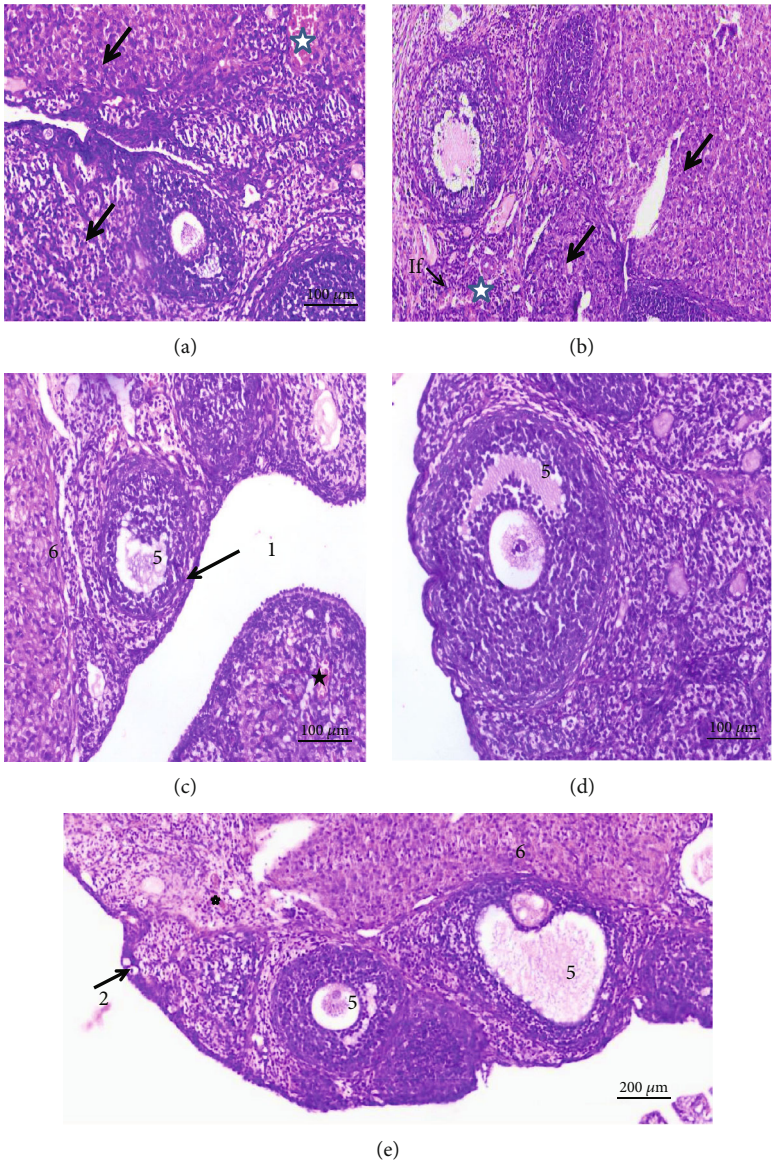


Figure 7: Photomicrographs of rat ovarian sections (a, b) from the RA group showing numerous luteal structures (arrow) in ovarium medulla and hyperemia in the stroma (star); (c–e) from arthritic-treated groups with curcumin, stem cells, and a mixture of curcumin and stem cells, respectively, showing nearly normal histological structure except for a few hyperemic areas in the stroma (star) and mononuclear cell infiltration (IF). (1) Germinal epithelium, (2) primordial follicles, (5) mature (antral or Graafian) follicle, and (6) corpus luteum (H&E).

inflammation, permanent synovitis, edema, and production of autoantibodies [45]. Because of their multipotent differentiation abilities, cell therapy using MSCs is the most common new technique in tissue repair and regeneration [14, 15, 32, 46]. Additionally, MSCs have therapeutic potential to joint and bone diseases through the secretion of a number of immune modulating substances and cell-to-cell interactions leading to antiapoptotic, antifibrotic, immunosuppressive, and proangiogenic properties [47]. Curcumin, a polyphenolic yellow pigment derived from *Curcuma longa* Linn, is a member of the compound family curcuminoid. Curcumin, derived from diferuloylmethane, is an important antioxidant which has been used as herbal therapy and as a dietary factor in many Eastern countries. Curcumin has also been shown to inhibit many proinflammatory cytokines and mediators such as IL-1, IL-8, and nitric oxide synthase [48]. Consequently, curcumin's beneficial effects on inflammatory disorders are due to the suppression of immune functions of T-cells, specially Th1, which plays a key role in the pathogenesis of chronic inflammatory disorders such as arthritis (Figure 10) [14, 15, 18, 49].

In the present study, due to treatment with MSCs and curcumin, the increased right hind leg ankle joint circumference of male and female arthritic rats was significantly reduced. This decrease in the joint circumference of the ankle represents the swelling rate decrease that can be due to edema reduction, inflammatory process attenuation, and synovial tissue hyperplasia reduction as demonstrated by

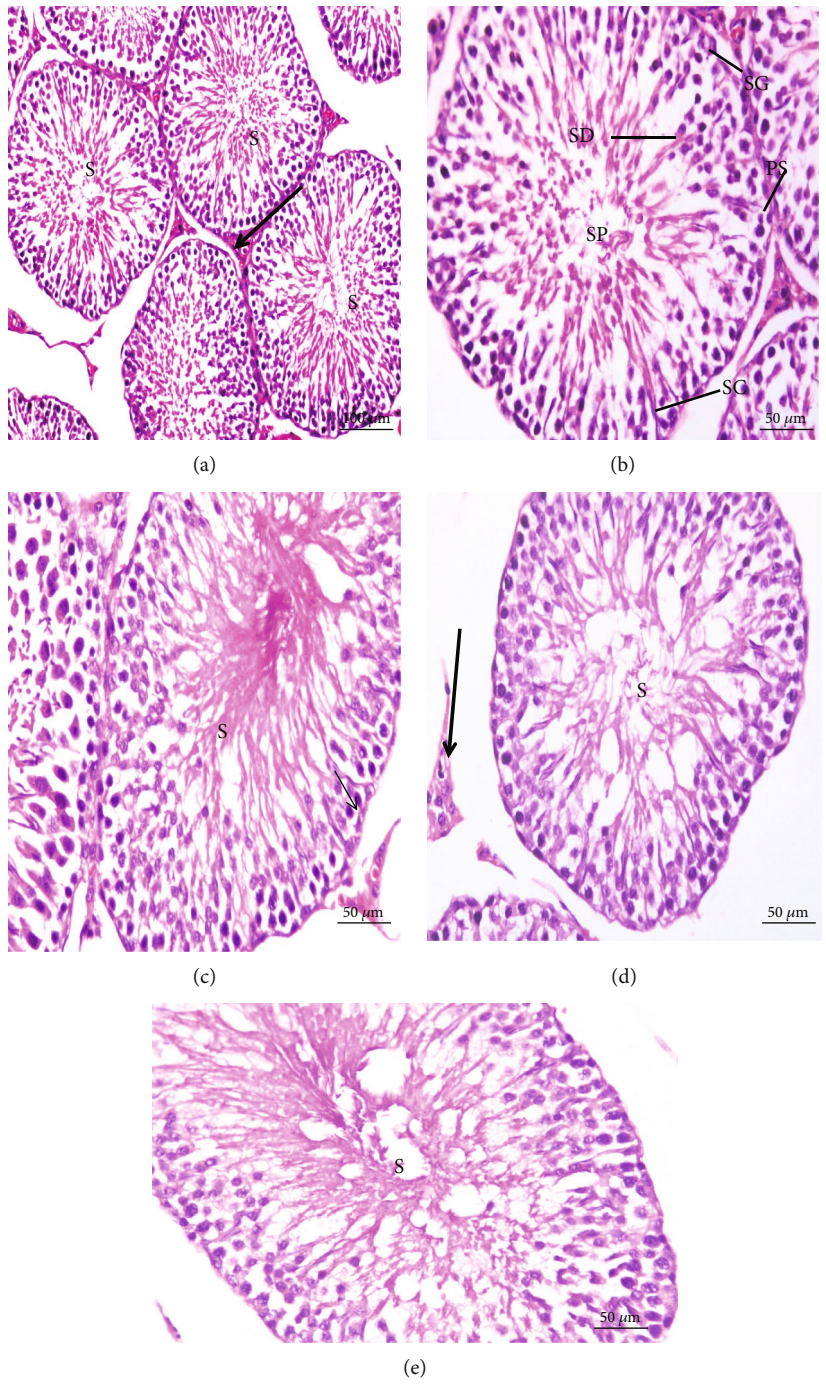


Figure 8: Photomicrographs of sections in testis of control rat groups: (a) water; (b) magnified cross section of (a); (c) CMC; (d) curcumin; (e) stem cells showing normal histological structure seminiferous tubules (S), interstitial space containing Leydig cells (arrow), spermatogonia (SG) resting upon the basal lamina of the seminiferous tubules that are oval in shape, primary spermatocytes (PS), recognized by their large nuclei, spermatids (SD) with rounded nuclei, Sertoli cells (SC) with nuclei located basally, and sperms in the lumen of the tubules (SP) (H&E).

the histological results in the current study and stated by previous publications [22, 50].

Serum concentrations of TNF-α and IL-10 were determined in the current study to elucidate their potential anti-inflammatory roles in the mechanisms of action of curcumin and MSCs. The TNF-α serum proinflammatory cytokine was significantly elevated in arthritic rats, and the effect was more deteriorated in female than in male arthritic rats. The IL-10 serum level of anti-inflammatory cytokine was depleted in arthritic rats and also was more deteriorated in female than in male arthritic rats. Therefore, changes of these cytokines ensure that Th1 cytokines are dominant over Th2 cytokines (Figure 10). Many previous authors supported this evidence [22, 51].

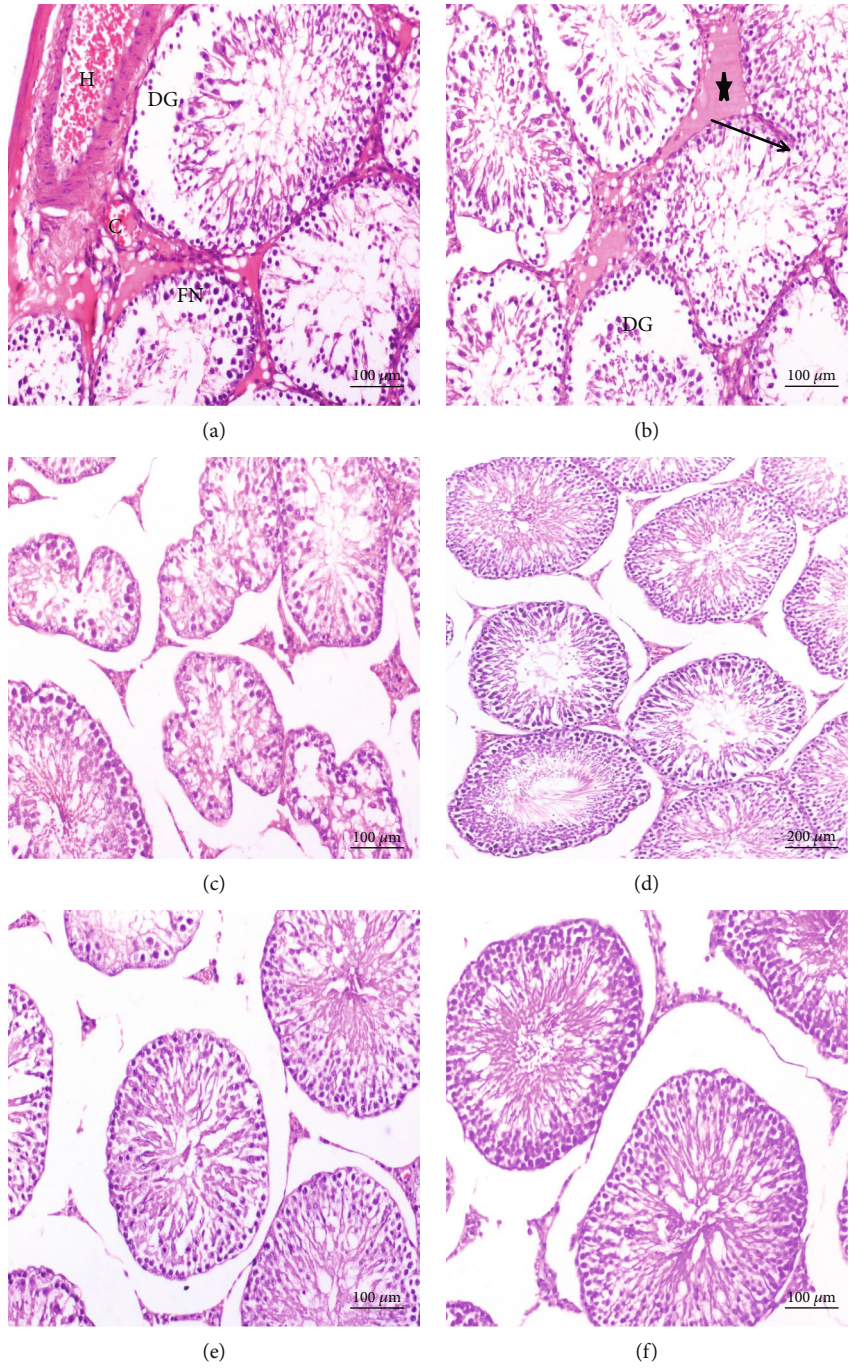


Figure 9: Photomicrographs of rat testis sections. (a) From the RA group showing capillary congestion (C), hemorrhage (H), and focal necrosis in germinal cells (FN). (b) From the RA group showing pyknotic nuclei (thin arrows) of primary spermatocytes (PS), interstitial edema (star), and degeneration of germ cells (DG). (c) From the RA group showing irregular variable-sized seminiferous tubules and atrophy in seminiferous tubules, with most tubules showing a marked decrease in the number of spermatogenic cells; consequently, the lumen of the tubules appeared with few or no sperms observed. (d–f) From arthritic-treated groups with curcumin, stem cells, and a mixture of stem cells and curcumin, respectively, showing a nearly normal histological structure of the seminiferous tubules (H&E).

In the present study, numerous histopathological changes in bone, ovarian, and testicular tissues were noticed in arthritic rats. The ankle joint of CFA-administered arthritic rats exhibited deleterious histological changes including necrosis, eroded articulating cartilage, and pannus formation [52]. These histopathological alterations may be attributed to the increase in the oxidative stress; antioxidant defense system suppression; elevation in the proinflammatory and inflammatory cytokines represented by increased IL-1β, IL-6, and COX-1 mRNA expression; and depletion of anti-inflammatory cytokines represented by decreased IL-4 mRNA expression. The improvement of ankle joint histological architecture as a result of treatment of arthritic rats with MSCs and curcumin may be due to their ability to

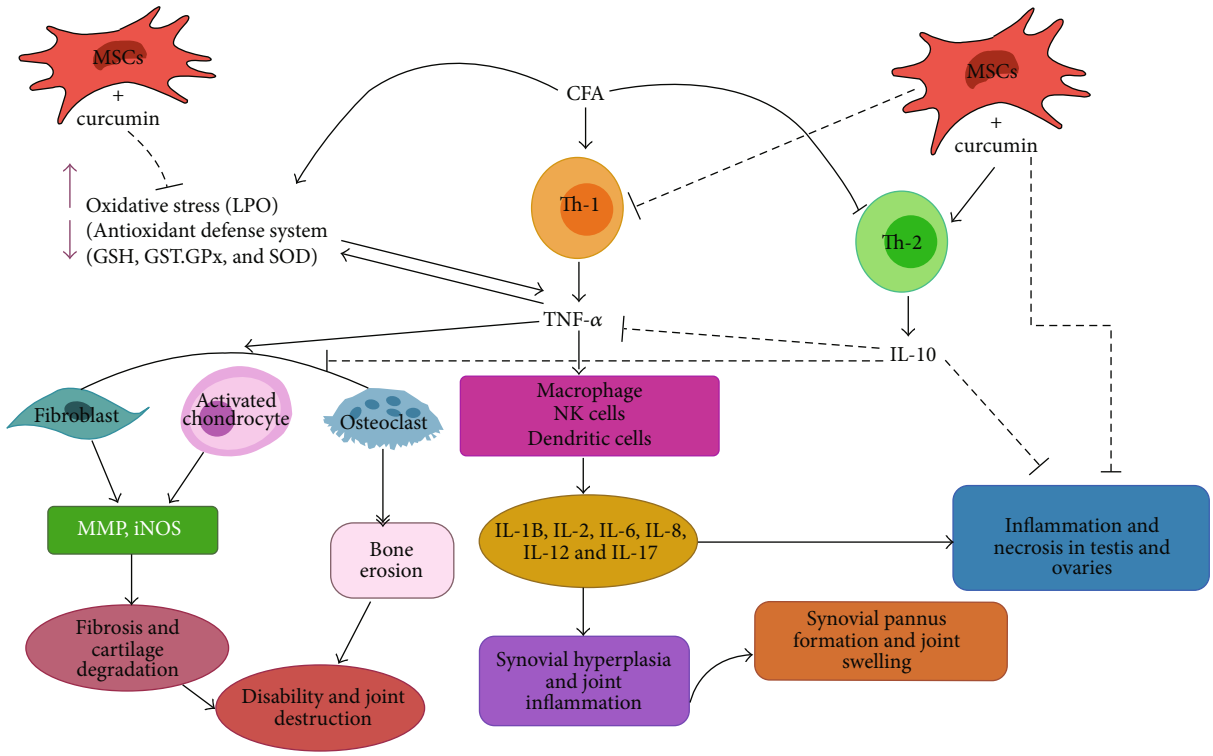


Figure 10: Schematic figure showing the mode of actions of CFA to induce arthritis *via* increasing LPO and TNF-α (Th1 cytokine) and decreasing IL-10 (Th2), thereby resulting in synovial hyperplasia and joint, testis, and ovary necrosis, and inflammation. The treatment with curcumin and/or MSCs can counteract these actions *via* enhancing the antioxidant defense system and anti-inflammatory mechanisms. $\rightarrow$: activation; $\perp$: inhibition.

scavenge lipid peroxides and free radicals, enhance the antioxidant defense system, and suppress inflammatory status. MSCs are able to inhibit osteoclast-mediated bone resorption, resulting in bone degradation through induction of T regs and reduction in the development of inflammatory cytokines that aid osteoclastogenesis. It has been demonstrated that osteoclastogenesis is inhibited by MSCs through production of osteoprotegerin or using interactions with osteoclast precursors via CD200/CD200 receptor interactions [53]. Garimella et al. [54] suggested that MSC injection into the collagen-induced arthritis (CIA) mice prevented bone loss *via* decreasing bone marrow osteoclast precursors but the mechanisms remain unclear.

Antioxidants are compounds which can delay, inhibit, or avoid oxidation of compounds, capture free radicals, and reduce oxidative stress. The body has an effective mechanism for preventing and neutralizing the free-radical-caused damage. This is accomplished by a group of endogenous antioxidant enzymes like SOD and CAT and the nonenzymatic antioxidant, GSH. Oxidative stress leads to cellular function deregulation that leads to different pathological conditions when the balance between ROS production and antioxidant defense is lost [55]. In rheumatoid arthritis, oxygen free radicals are implicated as tissue damage mediators. The involvement of free radicals is well studied in various inflammatory conditions, such as synovitis and rheumatoid arthritis. In the present study, the results revealed significant lipid peroxidation increase and decrease in antioxidant enzymes as well as GSH in male and female arthritic rats. Polyphenols have the ability of protecting cells from oxidative stress. However, polyphenol compounds may have antioxidant/prooxidant properties, depending on the source and concentration of free radicals [56]. The combination of curcumin and MSCs revealed a significant decrease of LPO when compared with the arthritic group. Significant normalization of the levels of antioxidant enzymes (GST, GPx, and SOD) and GSH promoted the potent antiarthritic curcumin activity with MSC combination. Arthritic rats treated with curcumin revealed a significant increase in GSH, GST, GPx, and SOD levels when compared to arthritic control. In this study, it was determined that administration of MSCs plus curcumin in arthritic rats significantly attenuated the changes in LPO, GPx, SOD, GST, and GSH. LPO was significantly reduced in arthritic rats treated with curcumin and MSCs as compared to the arthritic control, where all values approximately returned to the normal level.

It was shown in this study that the ovarian tissue of the arthritic female rats had multiple luteal structures in ovarium medulla, stromal hyperemia, and infiltration of mononuclear cells. According to Kim and Boone [57], at the penultimate stage of follicular development in the ovary, FasL is present in granulosa cells and may be the signal that causes apoptosis of granulosa cell during atresia.

In the present study, testicular changes include necrosis in germinal cells, pyknotic nuclei, interstitial edema, atrophy, vacuolation, and blood vessel congestion may be due to an increase of free radicals and elevation of inflammatory cytokines. *In vitro* studies on seminiferous tubule cultures revealed that IFN-γ and TNF-α caused germ cell apoptosis *via* the Fas-FasL system [58–60]. In the same regard, Rival et al. [61] also reported that IL-6 induced germ cell apoptosis.

The controls on the immune cells and inflammatory cytokines that are involved in RA are due to MSCs. The activation and proliferation of B-cells and T-lymphocytes are inhibited by MSCs *via* cytokine secretion (paracrine effect) and also a cell-cell direct contact effect [62, 63]; thus, they have a protective effect against ovarian and testicular tissue damage induced by RA.

Curcumin can ameliorate the destructive damage of testis and ovary tissues because of the ability to scavenge lipid peroxides and free radicals, enhance the antioxidant defense system, and suppress inflammatory status, which are elevated due to CFA that induce arthritis.

5. Conclusion

In conclusion, the present study shows that CFA induced oxidative stress and ankle, ovarian, and testicular damage not. The administration of curcumin and BM-MSCs singly or in combination provides potential protective activities against oxidative stress changes and articular inflammatory cell infiltration and ameliorates the histopathological effects of CFA male and female rats; the combinatory effects are more potent in both male and female arthritic rats. Consequently, we advise using the combination of mesenchymal stem cells and curcumin due to their antioxidant and anti-inflammatory properties and their ameliorating role in histopathological changes. However, clinical studies are required to assess the efficacy and safety of this combination before approval of its application for treatment in human beings.

Abbreviations

CFA: Complete Freund's adjuvant
BM-MSCs: Bone marrow-derived mesenchymal stem cells
RA: Rheumatoid arthritis
PBS: Phosphate-buffered saline
TNF-α: Tumor necrosis factor-α
IL: Interleukin
MHC: Major histocompatibility complex
Th: T helper
ROS: Reactive oxygen species
NF-κB: Nuclear factor-kappa B
CAT: Catalase
SOD: Superoxide dismutase
GPx: Glutathione peroxidase
GSH: Reduced glutathione
NOS: Nitric oxide synthase
HSCs: Hematopoietic stem cells
PDL: Programmed death ligand
GVHD: Graft-versus-host disease
FBS: Fetal bovine serum
DMEM: Dulbecco's modified Eagle's medium
g: Gram
CMC: Carboxymethyl cellulose
rpm: Rounds per minute
TBARS: Thiobarbituric acid-reactive substances
LPO: Lipid peroxidation
GST: Glutathione-S-transferase
ANOVA: Analysis of variance
H: Hour
mRNA: Messenger ribonucleic acid
CIA: Collagen-induced arthritis
IFN-γ: Interferon-γ.

Data Availability

The raw data supporting the conclusions of this article will be made available by the corresponding author upon reasonable request.

Ethical Approval

All experimental procedures were performed in accordance with recommendations, instructions, and guidelines stated by the Ethics Committee for Care and Use of Animals, Faculty of Science, Beni-Suef University, Egypt (Ethical Approval Number: BSU/FS/2018/7).

Consent

Not applicable.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

RRA, SRG, NM, and MA conceived and designed the experiments. RHA performed the experiments and analyzed the data. TMA, BHE, and OMA provided the experimental technical support and assisted in completing the study at different stages. RHA drafted the manuscript. RRA, SRG, NM, and MA finalized the paper. All authors are in agreement with the contents of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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Review Article

An Overview of the Safety, Efficiency, and Signal Pathways of Stem Cell Therapy for Systemic Lupus Erythematosus

Qian Yang,[1] Yiping Liu,[1] Guangyong Chen,[1] Wancong Zhang,[2,3] Shijie Tang,[2,3] and Tianbiao Zhou[1]

[1]*Department of Nephrology, Second Affiliated Hospital of Shantou University Medical College, Shantou 515041, China*
[2]*Department of Plastic Surgery and Burn Center, The Second Affiliated Hospital of Shantou University Medical College, Shantou 515041, China*
[3]*Plastic Surgery Institute of Shantou University Medical College, China*

Correspondence should be addressed to Shijie Tang; sjtang3@stu.edu.cn and Tianbiao Zhou; zhoutb@aliyun.com

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Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organs and tissues. Mesenchymal stem cells (MSCs) are considered a good source for autoimmune disease and hematological disease therapy. This review will summarize the efficacy, safety, and mechanisms of MSC therapy for SLE. MSC therapy can reduce anti-dsDNA, antinuclear antigen (ANA), proteinuria, and serum creatinine in SLE patients. In animal models of SLE, MSC therapy also indicates that it could reduce anti-dsDNA, ANA, proteinuria, and serum creatinine and ameliorate renal pathology. There are no serious adverse events, treatment-related mortality, or tumor-related events in SLE patients after stem cell treatment. MSCs can inhibit inflammatory factors, such as MCP-1 and HMGB-1, and inhibit inflammation-related signaling pathways, such as the NF-κB, JAK/STAT, and Akt/GSK3β signaling pathways, to alleviate the lesions in SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by overexpression of antibodies and activation of an inflammatory response that commonly affects the skin, joints, kidneys, and central nervous system and can lead to disability and death [1–3]. SLE is one of the top 20 leading causes of death in females between 5 and 64 years of age [3]. SLE is four times more prevalent in black women than in white women, and patients of African descent tend to develop disease earlier and have higher mortality [3]. Conventional therapy for SLE involves steroid and/or immunosuppressive drug treatment. However, conventional therapy is only effective in mild SLE patients, and the drugs have significant side effects over long-term use, such as infection, gastrointestinal syndrome, herpes zoster, or varicella [4–7]. Due to the high relapse and low survival rate of SLE, novel treatments have been investigated in recent years and some progress has been made.

Mesenchymal stem cell (MSC) transplantation is regarded as a novel treatment for SLE, and clinical research and animal experiments have been conducted with encouraging outcomes. MSCs can be derived from abundant sources, such as marrow, human umbilical cord, and adipose tissue [8]. MSCs have the potential to regulate the immune system and control inflammation. MSC treatment can alleviate the lesions of the glomerulus and renal interstitium and improve renal function with few adverse effects [9]. MSCs can modulate the inflammatory microenvironment and influence abnormal activation of signal pathways in SLE patients and lupus mice. They also modulate T lymphocyte proliferation and differentiation [10–14]. Thus, MSC therapy is a potential treatment for SLE patients. This review pays attention to the efficacy and safety of MSC therapy for SLE,

based on both preclinical and clinical studies, and further discusses the immunomodulatory function which involves lymphocytes, cytokines, and signaling pathways.

1.1. The Efficacy of Mesenchymal Stem Cell Therapy for SLE: A Preclinical Model. The efficacy of MSC therapy in animal experiments was evaluated in recent years (Table 1). Liu et al. [15] found that human placenta-derived MSC treatment could improve the renal function and decrease the proteinuria levels of MRL/lpr mice. In addition, anti-double-stranded DNA (anti-dsDNA) antibody levels decreased in the MSC group compared to a control group treated with normal saline. The immune complex immunoglobulin G (IgG) deposited in glomeruli was observed to be significantly less in the MSC group when compared with the control group. Thus, the renal destruction was alleviated and the glomerular swelling and lymphocyte infiltration were slightly less than before MSC treatment. The deposition of electron dense material in the glomeruli was also reduced. Ruan et al. [16] gave weekly intravenous (IV) injections of human umbilical cord mesenchymal stem cells (H-UC-MSCs) for 4 weeks to B6.Fas mice and showed that the levels of antinuclear, anti-histone, and anti-dsDNA antibodies in the MSC treatment group decreased compared with the B6.Fas mouse model group. Renal function improved as the 24-hour urine protein was lower in the MSC treatment group than the lupus control group. As for renal pathology, the proliferation of glomerular mesangial cells and tubulointerstitial fibrosis were reduced following MSC treatment, as well as lowering immune complex deposition in the glomeruli compared to the lupus group. MSC transplantation ameliorated mouse lupus by decreasing the 24 h proteinuria and serum anti-dsDNA antibody [11, 16, 17]. Ma et al. discovered that H-UC-MSC treatment improved renal function, decreased proteinuria and plasma levels of Cr by inhibiting C5 activation, which is involved in the complement system, and can induce an inflammatory response in lupus nephritis [18]. Researchers have shown that MSC therapy diminishes the deposition of immune complexes and complement components (IgG, IgA, and C3) in lupus glomeruli [18]. As a result, inflammatory infiltration and mesangial hyperplasia of the glomeruli decrease after MSC treatment in lupus mice [10, 18]. Zhang et al. [19] found decreased lymphocyte infiltration, mesangial cell proliferation, and interstitial fibrosis in glomeruli following treatment with adipose-derived mesenchymal stem cells (ADMSC). ADMSC treatment could also decrease the proteinuria, serum anti-dsDNA antibody, and serum Cr. Zhang et al. showed that after MSC treatment, the level of the anti-dsDNA antibody exhibited a declining trend compared with other groups [20]. Mononuclear cell infiltration and the deposition of IgG in glomeruli and tubules also decreased. Sun et al. infused allogenic bone marrow mesenchymal stem cells (BMMSCs) into MRL/lpr mice at the early stage and the mature stage [21]. The serum level of the anti-dsDNA antibody, immunoglobulin, serum albumin, and ANA was reduced at both these stages. In addition, they found that C3 and IgG deposition decreased in the kidney after MSC treatment. Similar to the above studies, Ji et al. showed that BMMSC transplantation can decrease the levels of anti-dsDNA, IgG, IgM, ANA, and proteinuria [22]. However, they showed that at high dose, MSCs had the effect on immunosuppression and production of proteinuria, whereas the low dose had no effect. Jang et al. [13] found that infusion of BMMSCs during the preclinical phase could delay the time when the anti-dsDNA antibody increased and lupus nephritis progressed. As for the mice that had already developed nephritis, infusion of MSCs could reduce the level of the anti-dsDNA antibody, the deposition of immune complexes and complement factors (C3), and the infiltration of CD4+ T cells, as well as B220+ B cells, in the kidneys [13]. Zhou et al. found that the levels of anti-dsDNA antibodies, serum Cr, and 24 h proteinuria decreased following MSC treatment [23]. BMMSCs ameliorated late-stage renal pathology by reducing the expression of vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and fibronectin (FN) in the kidney [23]. Thus, MSCs can regulate cytokines to delay the development of renal fibrosis and decrease the level of proteinuria.

1.2. Clinical Efficacy and Safety of Stem Cell Therapy for SLE

1.2.1. Clinical Efficacy of Stem Cell Therapy for SLE. MSCs have been tentatively applied for SLE treatment in recent years. Various clinical research studies have shown MSC therapy to have encouraging potential for this specific immune disease. Barbado et al. intravenously injected three SLE patients with MSCs (1.5 million MSCs/kg) and found that the level of proteinuria decreased below 0.6 g/24 h at 1 month after MSC treatment when compared with baseline [24]. Serum creatinine (Cr) levels of two of the patients also decreased while the serum Cr of the third patient was normal throughout the entire study. Leng et al. assessed the efficacy of MSC treatment in a 10-year follow-up study that enrolled 24 Chinese patients with severe SLE [25]. All patients were treated with the combination of high-dose immunosuppressive therapy with autologous peripheral blood stem cell transplantation. More than half of the patients completed the 10-year follow-up, and their median proteinuria level decreased from 4.00 g/24 h before treatment to 0.00 g/24 h at 5 years after transplantation, which was maintained until the end of the 10-year follow-up. Liang et al. evaluated serum indices and renal function of 15 refractory SLE patients with MSC treatment (bone marrow mesenchymal stem cells (BMMSCs)) [26]. The follow-up study demonstrated that sera from 11 of 15 patients had anti-dsDNA antibody levels that decreased below baseline at both 1 and 3 months after transplantation. The 24 h proteinuria of nearly all patients except one decreased significantly at 1 week from 2538.0 ± 382.3 to 1430.7 ± 306.3 mg and further decreased continually at the 1-, 3-, 6-, and 12-month follow-ups. Another short-term clinical study also showed recovery of renal function of SLE patients with both 24 h urine protein and serum Cr decreasing at the 12- to 18-month follow-up [21]. One case reported a refractory SLE patient suffering from several complications, such as arthritis, skin vasculitis, and high titers of anti-dsDNA antibody, who was treated with high-dose immune suppression medication and stem cell transplantation. The serum Cr and proteinuria were decreased at

Table 1: Efficacy of MSC therapy in animal experiments.

Author, year	Stem cell type	Animal model	Groups	Handing methods	Treatment effect
Liu et al. [15]	pMSCs	MRL/lpr mice, BALB/C mice	The control group ($n = 10$) The vehicle group ($n = 10$) The LEF group ($n = 10$) The MSC group ($n = 10$)	(pMSCs) 1×10^6 cells via the tail vein	Proteinuria ↓, anti-dsDNA ↓, IgG deposition ↓, renal injury ↓
Ruan et al. [16]	H-UC-MSCs	B6.Fas mice	Normal control (C57BL/6 mice) group ($n = 10$), model (B6.Fas mice) group ($n = 10$), low-dose treatment (B6.Fas mice) group ($n = 10$), medium-dose treatment (B6.Fas mice) group ($n = 10$), high-dose treatment (B6.Fas mice) groups ($n = 10$)	High: 2×10^6 cells via the tail vein Medium: 1×10^6 cells via the tail vein Low: 0.5×10^6	Anti-nuclear ↓, anti-histone ↓, anti-dsDNA antibody ↓, proliferative mesangial cells ↓, tubulointerstitial fibrosis ↓, 24 h proteinuria ↓
Ma et al. [18]	UC-MSCs	B6.lpr mice	MSC group ($n = 8$) C5aRA, Merck group ($n = 8$) Control group ($n = 8$)	MSCs (1×10^6) via the tail vein	Proteinuria ↓, plasma levels of creatinine ↓, mesangial matrix ↓, inflammatory cell infiltration ↓, IgG ↓, IgA ↓, C3 ↓
Zhang et al. [19]	ADSCs	MRL/lpr mice	Control group ($n = 5$) ADSC group ($n = 5$) CTX group ($n = 5$)	ADSCs (1×10^6) via the tail vein	Proteinuria ↓, serum anti-dsDNA antibody ↓, serum creatinine ↓, renal pathology ↓
Zhang et al. [20]	H-UC-MSCs	B6.lpr mice	MSC group FLS group Phosphate-buffered saline (PBS) treatment group	UC-MSCs (1×10^6) via the tail vein	Anti-dsDNA antibody ↓, IgG ↓, mononuclear cell infiltration ↓
Sun et al. [21]	BMMSCs	MRL/lpr mice	BMMSC group ($n = 12$) Control group ($n = 12$) CTX group ($n = 12$)	BMMSCs (0.1×10^6 cells per 10 g body weight) via the tail vein	Anti-dsDNA antibody ↓, immune globulin ↓, serum albumin ↓, ANA ↓, C3 ↓, IgG ↓
Ji et al. [22]	BMMSCs	MRL/lpr mice	Low-dose MSC group ($n = 10$) High-dose MSC group ($n = 10$) PBS group ($n = 10$) Control group ($n = 10$)	BMMSCs (0.05×10^6 cells per 10 g body) MSC (0.2×10^6 cells per 10 g body)	Anti-dsDNA antibody ↓, IgG ↓, IgM ↓, ANA ↓, proteinuria ↓
Jang et al. [13]	BMMSCs	NZB/W mice	BMMSC group ($n = 10$-11) PBS group ($n = 10$-11)	BMMSCs (1×10^6) via retro-orbital injection of the venous sinus	Anti-dsDNA antibody ↓, immune complexes and complement factors ↓, CD4+ T cells ↓, B220+B cells ↓
Zhou et al. [23]	BMMSCs	MRL/lpr mice	Control group ($n = 5$) CTX-treated group ($n = 4$) MSC group ($n = 4$) MSC+CTX group ($n = 4$)	BMMSCs (1×10^6) via the tail vein	Anti-dsDNA antibody ↓, serum creatinine ↓, 24 h proteinuria ↓

follow-up 9 months posttransplantation, and the anti-dsDNA titer dropped to undetectable levels [27]. Some other case reports also revealed that refractory SLE patients who had been treated with conventional immunosuppressants and glucocorticoids with no response, and their condition steadily declined. After programing stem cell transplantation therapy, their anti-dsDNA and serum anti-nuclear antibodies decreased [28–31]. The results also indicated that Cr and Cr clearance became normal as well [32].

1.2.2. The Safety of Stem Cell Therapy in SLE. The safety of stem cell therapy is important and is an essential precondition for clinical application. The safety of MSC clinical applications is still the most concerning issue. In the past years, MSCs were used for the treatment of liver diseases [33], diabetes mellitus [34], and idiopathic Parkinson's disease [35], with no significant adverse effects reported in most clinical trials. Stem cell therapy for SLE has less adverse effects compared with conventional treatment (steroid and/or and immunosuppressive drugs). According to the data from some follow-up studies and case reports, there were no serious adverse events [21, 36–38], treatment-related mortality [36], or tumor-related events [36] after stem cell treatment of SLE patients [39]. In a long-term follow-up study that

enrolled 81 refractory SLE patients, 11 infection events occurred in patients with MSC treatment [40]. The immediate adverse events after MSC infusion were mild and included dizziness and a warm sensation that disappeared in a short time. Delayed adverse events were mainly bacterial and viral infections during follow-up and most resolved. However, as advanced and refractory SLE patients suffered from multiple system dysfunction, it is doubtful that the delayed infection events were caused by MSC treatment [37]. An observational study demonstrated that mortality of severe SLE patients after transplantation mainly included original disease, transplant-related complications, and infections [41]. The transplant-related mortality was mainly caused by the transplant centers' lack of experience and the severity of disease [41]. As for child-bearing SLE patients, MSC therapy reduced the activity of lupus and risk of pregnancy complications. MSC therapy also improved the fetal outcome when compared with traditional treatment [42].

1.3. Effect of MSCs on Inflammatory Factors and Potential Signaling Pathways of MSCs in SLE. MSC therapy was considered an appropriate treatment for autoimmune diseases like SLE, because MSCs exhibit immunosuppressive potential only in an inflammatory environment [43]. MSCs promote anti-inflammatory actions and inhibit proinflammatory progression in SLE and regulate the imbalance of the inflammatory response [44]. Monocyte chemoattractant protein-1 (MCP-1) and high-mobility group box chromosomal protein 1 (HMGB-1) are proinflammatory cytokines that are correlated with renal pathogenesis in SLE [45]. MSCs can reduce the overexpression of MCP-1 and HMGB-1 mRNA and decrease the serum and urine level of these two proinflammatory cytokines to delay the kidney damage [45].

Furthermore, Choi et al. [46] found that MSC transplantation increased serum levels of interleukin-4 (IL-4) and interleukin-10 (IL-10) when compared with the control group. The study revealed that the anti-inflammatory action and the regulation of the T cell subset ratio by MSCs relied on hypoxia-inducible factor 1α (HIF-1α) and mammalian target of rapamycin (mTOR) [43]. mTOR and HIF-1α play a critical role in regulating cell growth and proliferation (e.g., Th17 cells), which are related to the abnormal development of T cells in lupus mice and SLE patients [43]. The Th17 cells promote an inflammatory response by secreting interleukin-17 (IL-17) [12, 47]. MSCs modulate the proportion of Th17 cells by cell-cell contact [47]. IL-17 is a proinflammatory agent that infiltrates glomeruli and causes the destruction of renal tissue in part [43]. IL-17 can also promote various cells to secrete inflammatory cytokines [12]. It has been suggested that ADMSC treatment inhibits the mTOR pathway and the expression of HIF-1α, as well as the expansion of Th17 cells. Thus, the secretion of IL-17 by Th17 cells is decreased in the lupus kidney, which reduces the progression of lupus [43]. Moreover, MSCs also downregulate Th17 cells and IL-17 to suppress the inflammatory response by secreting several cytokines, including IL-10, prostaglandin E2 (PGE2), and TGF-β [12]. TFN-γ, which exists in the internal environment of lupus mice, upregulates the secretion of these cytokines. These cytokines play an important role in reducing Th17 cell differentiation and proliferation directly or indirectly [12]. In contrast, interleukin-23 (IL-23) plays a crucial role in Th17 cell differentiation and function [47]. ADMSC downregulates the expression of IL-23 mRNA level, thereby reducing the level of IL-23 [47]. MSC therapy promotes the apoptosis of abnormal T cells in lupus and regulates the number of Th1 and Th2 cells [11]. Imbalance of Th1 and Th2 cells has been observed in SLE patients and mouse models of lupus [44]. Th1 cells are proinflammatory cells that produce interferon-γ (IFN-γ) and IL-2, whereas Th2 cells are anti-inflammatory cells that produce IL-4, IL-5, IL-6, and IL-10 [23, 44]. MSCs decrease Th1 proinflammatory cytokines and increase Th2 anti-inflammatory cytokines [44]. They also reduce other inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-12, which play a role in the proinflammatory response and inhibition of Th2 cells [44]. T follicular helper (Tfh) cells are a subtype of CD4+ T helper cells that contribute to the pathogenesis and symptoms of SLE [20]. The increased proportion of Tfh cells was observed in lupus mice and SLE patients [13, 20]. The overactivation of Tfh cells in SLE results in the overexpression of C-X-C chemokine receptor 4, programmed cell death-1, and interleukin-21. These molecules influence the differentiation of B cells [20]. Studies show that MSCs downregulate the percentage of Tfh cells [19] through inhibiting the Tfh cell proliferation and differentiation of naïve T cells [20] and decreasing their circulating precursors [13]. The inhibition of Tfh cells is mediated by iNOS. The activation of iNOS involves the nuclear factor kappa-B (NF-κB), STAT, and Akt signaling pathways via direct cell-to-cell contract of MSCs and CD4+ T cells [20]. Tfh cells have the capacity to develop long-lived plasma cells. Long-lived plasma cells exist in an inflammatory environment and cause prolonged destruction of organs, which makes SLE symptoms difficult to ease. Thus, the reduction of Tfh cells can lead to a reduction in long-lived plasma cells [13].

The abnormal activation of the complement system and deposition of immune complexes also promote the progression of lupus nephritis. C3 and C5 are important molecules of the complement system [18]. Ma et al. demonstrated that MSCs inhibit the activation of C5 by secreting factor H (FH) [18]. The IFN-α, produced by SLE cells, promotes FH secretion by MSCs. FH suppresses the activation of C5 by the cleavage of C3 and competitive combination with activators of the classical pathway. Tolerogenic regulatory T cells (Tregs), a special subtype of CD4+ T cells, which can suppress immune responses and proinflammatory cytokine production, are reduced in SLE. Many studies have demonstrated that MSCs can upregulate Treg cells to modulate excessive autoimmunity [16, 21, 46, 48, 49]. Furthermore, MSCs increase the Treg cells of SLE patients via secreting soluble human leukocyte antigen-G (sHLA-G), a nonclassical HLA class I molecule that is involved in immunosuppression (Figure 1) [48].

Stem cell therapy for SLE is associated with signaling pathways such as the NF-κB [15], STAT [39], and Akt/GSK3β signaling pathways [22], which are related to the synthesis of downstream inflammatory mediators [15]

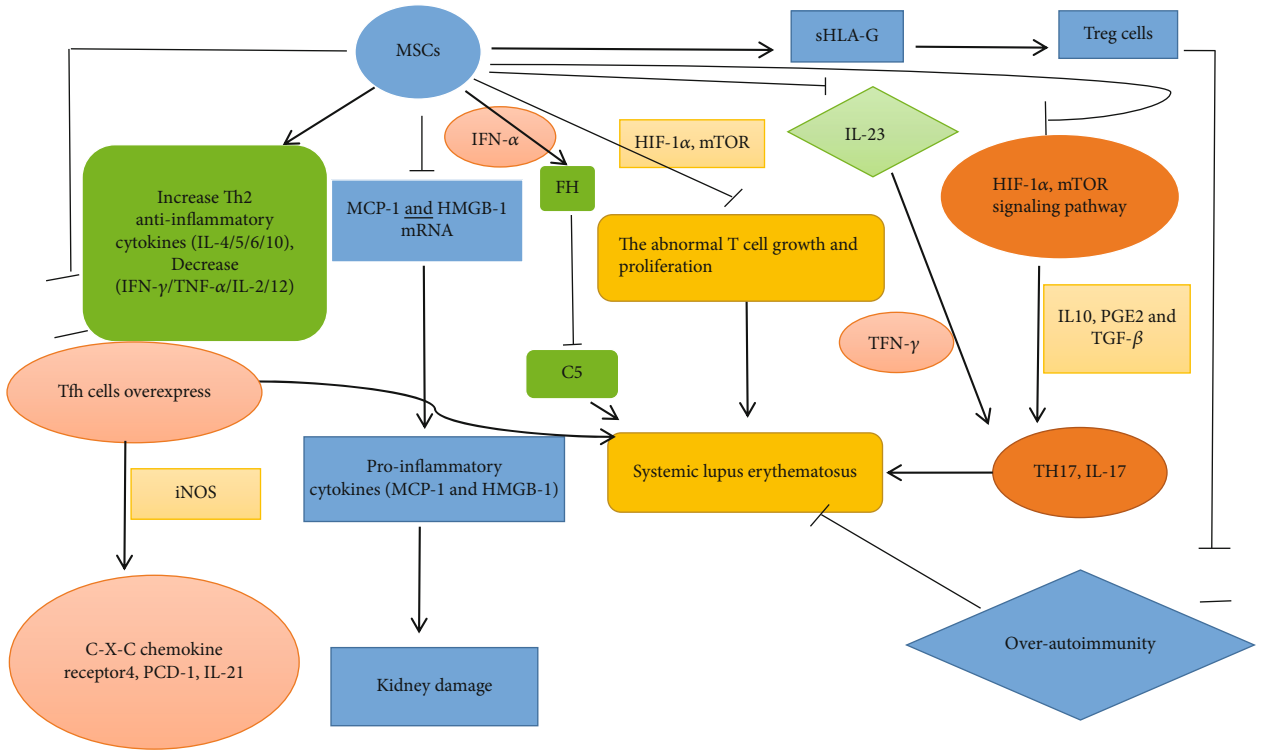

Figure 1: Effect of MSCs on cytokine production in systemic lupus erythematosus.

and the regulation of T cells, including Th1, Th2, Treg, and Tfh cells [20, 22, 50].

NF-κB is a transcription factor for many inflammatory cytokines. NF-κB can efficiently induce the expression of inflammatory cytokines (IL-1, IL-6, and TNF-α), adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1); intercellular cell adhesion molecule-1 (ICAM-1)), chemokines, and inflammatory enzymes (iNOS, COX-2). It has been demonstrated that the inflammatory microenvironment of SLE patients with lupus nephritis increases NF-κB expression in the glomerular endothelial and mesangial cells, and overexpression of NF-κB could induce expression of inflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes in turn, causing a vicious cycle [15]. MRL/lpr mice were treated with human placenta-derived mesenchymal stem cells (pMSCs) to reduce NF-κB mRNA and the protein level of phospho-NF-κB p65 and their protein synthesis and to further downregulate NF-κB signaling pathway activation. Thus, the expression of downstream TNF-α, plasminogen activator inhibitor-1 (PAI-1), and ICAM-1 decreases in MRL/lpr mouse kidneys [15]. PAI-1 is a proinflammatory cytokine that is related to a hypercoagulable state of blood and causes glomerular microthrombi. Therefore, MSC treatment could suppress abnormal NF-κB signal pathway activation to reduce the inflammatory microenvironment and ameliorate lupus symptoms.

CD1c+ DCs are a subset of dendritic cells that can suppress the proliferation and differentiation of T cells and regulate T regulatory cells and Th2 cells in an IL-10-dependent manner in various organs such as the liver. Research shows that specific CD1c+ DCs might play an important role in ameliorating immune dysfunction of SLE patients [39]. The number of CD1c+ DCs is negatively correlated with the activity and severity. UC-MSC transplantation therapy can upregulate CD1c+ DC numbers through FLT3L (Fms-related tyrosine kinase 3-ligand), a regulator that binds to FLT3 to stimulate DC proliferation. Lupus CD8+ T cells produce IFN-γ, and this cytokine enhances the expression of FLT3L, in MSCs, in a manner that is mediated by the JAK/-STAT signaling pathway. AG490 inhibits the JAK/STAT signaling pathway, causing the number of CD1c+ DCs as well as the expression of FLT3L to decrease [39].

STAT3 is a protein that is encoded by a gene on chromosome 17 and contributes to Tfh cell differentiation. It is demonstrated that BMMSCs can inhibit the gene expression and phosphorylation of STAT3 to prevent naïve T cells from differentiating to Tfh cells [50]. Recent research has also found that abnormal proliferation and activation of autoreactive T cells play an important role in the development of SLE [20, 22, 43].

High-dose MSC transplantation can suppress the activation of lupus T cells through the Akt/GSK3β signaling pathway to further modulate the immune disorder and ameliorate SLE abnormalities [22]. PI3K is the major upstream molecule that activates Akt to influence diverse downstream targets such as GSK3β, mTOR, and p2, which can regulate the cell cycle [22]. In MRL/lpr mice, activation of the PI3K/Akt/GSK3β signaling axis is prevented and lupus T cells accumulate in G0/G1 while the number of S phase T cells decreases. This suggests that MSC treatment influences lupus T cells by increasing the activity of $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ and decreasing the activity of CDK2, which is a cyclin-dependent kinase associated with the cell cycle

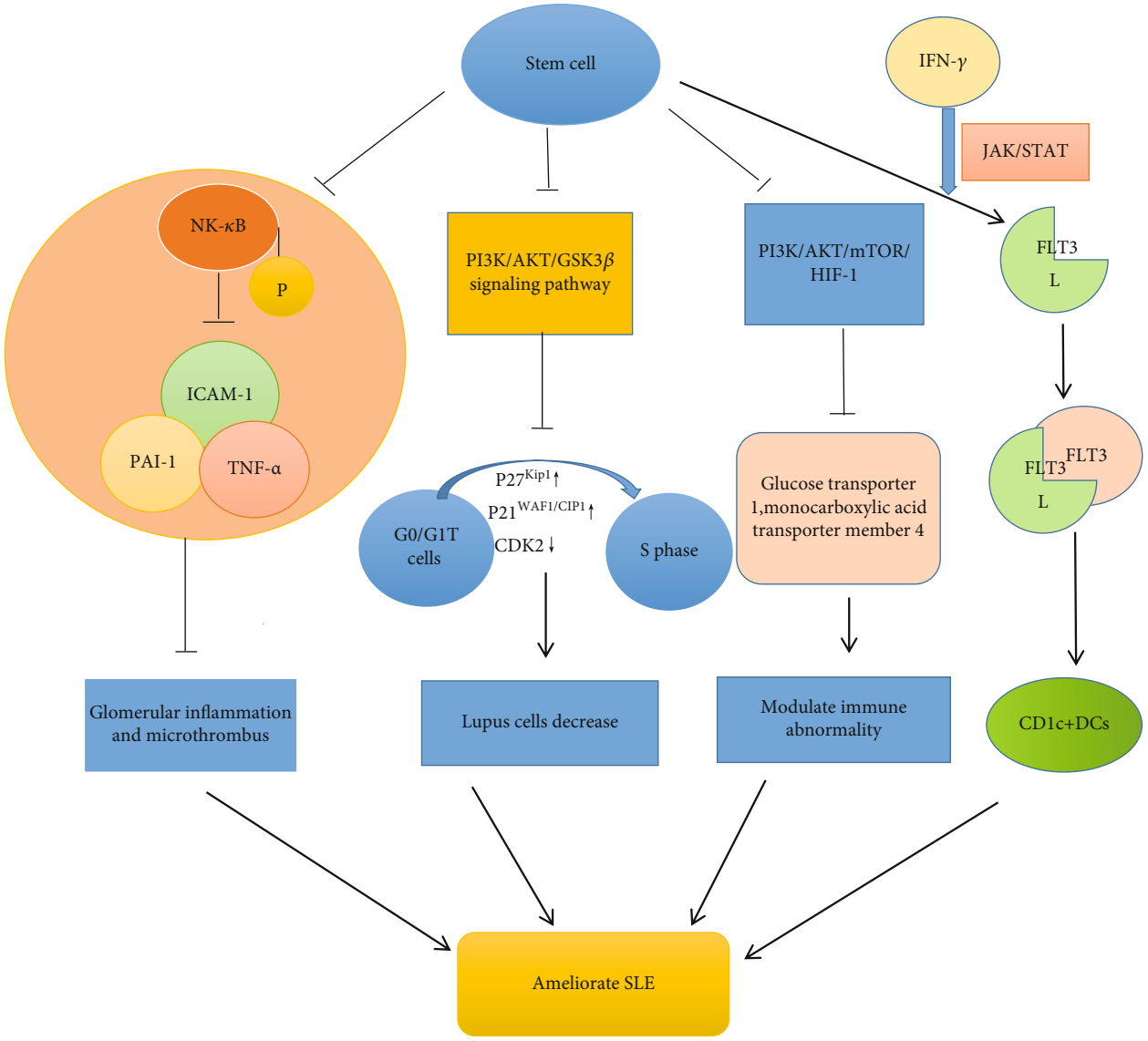

Figure 2: Stem cell therapy influences abnormal activation of signal pathways in systemic lupus erythematosus.

progression through G1. This study reveals that high-dose MSC treatment has a negative effect on cell growth of lupus T lymphocytes and could inhibit G1/S transition of abnormal T cells via inhibition of the PI3K/Akt/GSK3β signaling pathway in the abnormal T cells [22]. The mTOR is a downstream target of Akt, and it increases the HIF-1α protein level by promoting HIF-1α mRNA transcription. HIF-1α plays an important role in Th17 cell differentiation and IL-17 expression. It acts on its target cytokines as well as monocarboxylic acid transporter member 4 and glucose transporter 1 and various glycolytic enzymes to regulate aerobic glycolysis in Th17 cells. The suppression of Akt indirectly decreases the expression of mTOR and HIF-1α, thereby reducing Th17 cells and IL-17 in glomeruli and tubules, supporting the model that MSC treatment suppresses the activation of the Akt/mTOR/HIF-1α/Th17 pathway to modulate immune abnormality in SLE (Figure 2) [43].

2. Conclusions

Stem cell transplantation treatment has been studied as an alternative therapy for autoimmune disease in recent years. Traditional therapy has little effect on refractory SLE patients and has accumulating drug toxicity, as well as causing drug complications. Stem cell therapy decreases the level of serum autoantibodies (anti-dsDNA, ANA), proteinuria, and Cr and improves lupus renal pathology and reduces the inflammatory response in SLE patients, as well as animal models. There are no serious adverse events, treatment-related mortality, or tumor-related events in SLE patients after stem cell treatment. MSCs can inhibit inflammatory factors, such as MCP-1 and HMGB-1, and inhibit activation of the NF-κB, JAK/STAT, and Akt/GSK3β signaling pathways to alleviate the lesions in SLE. In addition to the studies above, the different effects of different types of stem cells need to be studied in the future.

Data Availability

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

QY looked up a lot of pertinent papers and wrote the manuscript. YL, GC, and WZ reviewed and checked the article. ST and TZ modified and polished the article and reviewed the article. The authors have read and approved the final manuscript. Qian Yang and Yiping Liu should be regarded as joint first authors.

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