Mesenchymal Stem Cell Transplantation Inhibits Abnormal Activation of Akt/GSK3 β Signaling Pathway in T Cells from Systemic Lupus Erythematosus Mice

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Key Words

Systemic lupus erythematosus • Bone marrow mesenchymal stem cell • Transplantation • Cell cycle • Akt • GSK3 β

Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by activation and proliferation of autoreactive T cells and B cells. We examined changes in cell cycle progression of T cells from MRL/lpr mice with or without allogeneic bone marrow mesenchymal stem cells (BMMSCs) treatment and analyzed the expression of cell cycle associated proteins. In addition, the Akt/GSK3 β protein kinase cascade was studied. We demonstrated that high-dose MSCs transplantation effectively ameliorated disease activity in MRL/lpr mice. BMMSCs treatment inhibited G1/S transition of the abnormal lupus T lymphocytes. Moreover, it increased the expression of p21^{WAF1/CIP1} and p27^{Kip1} and decreased the expression of CDK2. Furthermore, high-dose MSCs inhibited abnormal activation of the Akt/GSK3 β signaling pathway of T cells from MRL/lpr mice. Our results suggest that high-dose BMMSCs transplantation successfully treated MRL/lpr lupus

mice by inhibiting abnormal activation of Akt/GSK3 β signaling pathway of T cells.

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune and potentially fatal disease characterized by activation and proliferation of autoreactive T cells and B cells with production of autoantibodies against nuclear and endogenous antigens [1-3]. The kidney is a major site of immune complex formation and/or deposition, and lupus nephritis is a major cause of morbidity and mortality, in both human SLE and murine models of lupus, such as MRL/lpr mice. In the development of SLE, T cells proliferation and the subsequent clonal expansion of T cells play a critical role. Therefore, inhibiting the activation and proliferation of T cells could offer a potential treatment for lupus.

Mesenchymal stem cells (MSCs) are multi-potent cells found in several adult tissues. They exhibit immunomodulatory and regulatory effects on T and B lymphocytes, dendritic cells, and natural killer cells [4-10]. It was reported that stimulated T cells in the presence of MSCs were arrested at the G1 phase [11, 12]. Recent

clinical and basic researches have highlighted the potential of using MSCs to treat SLE [13-15]. However, the molecular mechanisms in which MSCs suppress lupus T-cell activation and modulate the immune response are still unknown.

PI3K/Akt pathway has been found to be an important axis in regulating diverse cellular processes. PI3K is a major upstream molecule in this axis and another key component in this pathway is PTEN, which serves as a negative regulator that inhibits PI3K signaling. Akt activation can stimulate proliferation through multiple downstream targets impinging on cell-cycle regulation, such as GSK3 β , mTOR and p21 [16]. In some recent studies, the PI3K/Akt signaling pathway has been found to be upregulated in lupus T cells [17, 18] and B cells [19]. Indeed, we have also previously shown that abnormal activation of the Akt/GSK3 β signaling pathway increased the proliferation of lupus T lymphocytes [20].

In this study, we explored the effects of allogenic BMSCs in treating MRL/lpr mice *in vivo* and investigated changes in cell cycle progression and abnormalities in the PI3K/Akt signaling axis in T cells from MRL/lpr mice with or without allogenic BMSCs treatment.

Materials and Methods

Mice

C57BL/6 (B6) and MRL/lpr mice were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). All mice used for this study were bred and housed in a specific pathogen-free colony at Animal Research Center of Sun Yat-sen University, China. All animal experiments were performed in accordance to the National Institutes of Health Guidelines on Laboratory and were approved by the Animal Care Committee, Sun Yat-sen University.

MSCs isolation and ex vivo expansion

Mouse MSCs were isolated and cultured as described previously [21]. Six to eight week-old C57BL/6 mice were sacrificed. Bone marrow cells were obtained by flushing femurs and tibias with sterile PBS. Cells were cultured in DMEM (GIBCO) supplemented with 15% fetal bovine serum (GIBCO), 3.7 g/liter NaHCO₃, and a combination of 100 U/ml penicillin and 100 μ g/ml streptomycin and plated at a density of 25*10⁶ cells/ml. Non-adherent cells were removed after 3 hours by medium change. After an additional 8 h of culture, fresh medium was replaced and was repeated every 8 h for up to 72 h of initial culture. Then medium containing non-adherent cells was continued be replaced every 3-4 days of culture. When cell confluence was achieved, adherent cells were detached with

0.25% trypsin, 2.65 mM EDTA (GIBCO) for 2 min at room temperature, and cultured all lifted cells. After two subcultures, adherent cells were characterized.

In Vitro Differentiation Assay.

In vitro adipogenic and osteogenic induction of mouse MSCs were performed as previously described [22, 23]. MSCs were incubated with standard adipogenic or osteogenic differentiation media respectively. Lipid vacuole formation in the adipogenic cultures was determined by 0.3% Oil-red-O (Sigma). Osteogenic monolayer cultures were stained for matrix mineralization with Alizarin red (Sigma).

Allogenic Mouse MSCs Transplantation into MRL/lpr Mice

MRL/lpr mice aged 14 weeks were randomly divided into 3 groups to receive MSCs (n=10/group). MSCs were resuspended in 0.1mL PBS and were injected into MRL/lpr mice via tail vein under general anesthesia. The first group of MRL/lpr mice received low-dose MSCs (0.05*10⁶ cells per 10 g body), the second group received high-dose MSCs (0.2*10⁶ cells per 10 g body) and the third group received 0.1mL PBS (untreated). And age matched C57BL/6 mice were used. All mice were sacrificed one month later for further analysis.

Measurement of bioactive factors in blood serum and urine

Blood serum and urine were collected from mice. All of the samples were stored at -20°C until used and re-centrifuged before the application for ELISA. Anti-dsDNA IgG, anti-dsDNA IgM and ANA were measured using commercial available kits (Cusabio, USA). Urine was collected from individual mice housed in metabolic cages for 24 h. The 24h urine protein was measured at Sun Yat-sen memorial Hospital of Sun Yat-sen University.

Assessment of renal pathology

Kidneys were harvested from mice 30 days after treatment. Samples were fixed by overnight immersion in 10% formaldehyde and paraffin-embedded. Paraffin Sections were used for Hematoxylin and Eosin (H&E), Periodic Acid Schiff (PAS), Periodic Acid-silver Methenamine (PASM) and Masson trichrome staining.

T-cell isolation and culture

Spleens and lymph nodes were removed from mice. Splenocytes were depleted of red blood cells using lysis buffer (Sigma) and lymph nodes were crushed to obtain single-cell suspensions. Cell suspensions were subsequently treated with magnetic activated cell sorting (MACS) anti-CD90.2-conjugated microbeads, according to the instructions of the manufacturer (Miltenyi Biotech). Positively selected cells contained on average 95% T cells, as assessed by flow cytometric analysis with a CD3e monoclonal antibody (mAb). T cells were suspended in RPMI 1640 (GIBCO) plus 10% heat-inactivated FBS, penicillin, streptomycin, 50 mM 2-mercaptoethanol and activated for 48 hr in 0.5 μ g/ml CD3 antibody (2C11) for cell cycle analysis.

Fig. 1. Allogeneic BMMSCs transplantation reduced levels of autoantibodies. (A-D) ELISA quantified that levels of anti-dsDNA IgG (A), IgM (B), nuclear antibodies (ANA) (C) and urinary protein (D) (mean $\pm$ SD) were significantly reduced in the MSCs (0.2×10^6 cells per 10 g body) treated MRL/lpr mice ($n = 10$) when compared to un-treated MRL/lpr mice ($n = 10$) 20 and 30 days after treatment (* $p < 0.05$, ** $p < 0.01$, ns = not statistically significant v.s MRL/lpr).

Cell cycle analysis

Activated T cells were centrifuged at 1,200 rpm for 5 min. The resulting pellets were washed with 1 ml of ice-cold PBS and fixed with 70% ethanol at 4°C for 24 h. Fixed cells were washed twice with PBS and incubated with 0.5 µg/ml RNase A and 50 µg/ml propidium iodide for 30 min at room temperature in the dark. The percentage of cells in the G0/G1 phase, S phase, and G2/M phase were determined according to relative DNA content analyzed using a FACScan flow cytometer. (Becton Dickinson, USA).

Western blotting analysis

Purified T cells were lysed with RIPA buffer (50 mM Tris HCl pH 7.4; 150 mM NaCl; 0.1% SDS and 1% NP40) including protease and phosphatase inhibitors (Roche, Germany). Equal amounts of protein samples were separated by SDS-polyacrylamid gel electrophoresis and transferred onto a PVDF membrane (Millipore, USA). The filters were immunoblotted with specific primary antibodies, HRP-conjugated secondary antibodies, and visualized by enhanced chemiluminescence (Pierce, USA). Blots were re-probed using Restore Plus Western Blot stripping buffer (Pierce, USA). Antibodies against phospho-AKT^{Thr308}, phospho-AKT^{Ser473}, phospho-GSK3 β ^{Ser9}, p27^{Kip1}, AKT, and GSK3 β were purchased from Cell Signaling Technology (USA). The antibodies against Cyclin-dependent kinase-2 (CDK2) were from BD Transduction Laboratories (USA) and the antibodies against p21^{WAF1/CIP1} were from Santa Cruz Biotechnology (USA).

Statistical analysis

All data are expressed as the mean $\pm$ SD of, at least, triplicate determinations. Statistical difference between the values was examined by Student's t-test. Values for $p > 0.05$ (not significant, ns), $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) are indicated in the figures.

Results

Effects of allogeneic BMMSCs transplantation on levels of autoantibodies and renal function of MRL/lpr mice

SLE-like multisystemic autoimmune disorders appeared at age 12-13 weeks in MRL/lpr mice. Consistent with human findings, MRL/lpr mice showed a remarkable increase of anti-doublestrand DNA (dsDNA) IgG and IgM and antinuclear antibody (ANA) in the peripheral blood and an increased level of 24h urine protein. Similar to other studies[14, 15, 24, 25], allogeneic MSCs transplantation resulted in a significant reduction of anti-dsDNA IgG and IgM and ANA antibody in serum and a decrease of 24h urine protein in MSC 0.2 group(which received 0.2×10^6 cells per 10 g body) 20 and 30 days after injection, as compared with untreated MRL/lpr mice.

Fig. 2. Allogenic BMMSCs transplantation ameliorated renal abnormalities in MRL/lpr mice. PAS staining can detect glycogen, and it can also show the basal membrane and mesangial matrix. PASM stain can show glomerular basal membrane (GBM) more refined, as GBM is silver-positive in PASM stain. Glomerulus in Masson stain can show deep red of fibrinoid necrosis and dark blue of mesangial matrix. MRL/lpr mice showed renal disorders such as nephritis with glomerular basal membrane disorder and mesangium cell over-growth. Allogenic BMMSCs (0.2×10^6 cells per 10 g body) transplantation resulted in a reduced basal membrane disorder and mesangium cell over-growth in glomerular. (The first panels, H&E staining; the second panels, PAS staining; the third panels, PASM staining; the last panels, MASSON staining). Representative images of MSC 0.05 (0.05×10^6 cells per 10 g body), MSC 0.2 (0.2×10^6 cells per 10 g body) and untreated MRL/lpr group (n = 10).

Group	Cell cycle distribution (%)		
	G0/G1	G2/M	S
MSC 0.05	72.76±1.47	5.09±1.79	22.15±2.08
MSC 0.2	78.49±2.49**	4.62±0.97	16.89±2.00**
MRL/lpr	71.51±2.92	4.01±1.33	24.48±2.44
Control	82.15±1.45***	4.56±1.07	13.29±1.67***

Table 1. Distribution of activated T cells according to cell cycle phase. Data are presented as mean±SD (n=10) (** p<0.01 v.s MRL/lpr, *** p<0.001 v.s MRL/lpr).

However, there was no significant difference between untreated and low-dose MSCs (0.05×10^6 cells per 10 g body) treatment lupus mice (Fig. 1), suggesting low-dose MSCs treatment had no effect on autoantibody production and proteinuria. Histological analysis with H&E, PAS, PASM and Masson staining revealed that allogenic MSCs transplantation was able to ameliorated renal

abnormalities, such as nephritis with glomerular basal membrane disorder and messangial proliferation (Fig. 2). These experimental evidences indicated that allogenic MSCs transplantation was effective in improving renal function and in treating lupus nephritis in MRL/lpr mice after 20 days. However, only high infusing dose exhibited a therapeutic effect.

Cell cycle distribution of T lymphocytes from MRL/lpr mice with or without MSCs treatment

Abnormal activation and proliferation of lymphocytes are associated with the development of lupus, and we have previously reported that there were significant differences in the distribution of G0/G1 and S stages of activated T lymphocytes between SLE and control patients [20]. To explore the possible mechanisms by which allogeneic MSCs transplantation improves lupus phenotypes in MRL/lpr mice, we focused on T cells from lupus mice with or without transplantation in the present study. We examined the distribution of cell cycle of T lymphocytes between MRL/lpr mice and controls *in vitro* activation assay. As demonstrated in Table 1, the G0/G1 population of activated T cells from MRL/lpr mice was decreased while the population of S phase was strongly increased as compared with control group. High-dose MSCs transplantation induced T cells of lupus mice to accumulate at the G0/G1 cell cycle checkpoint. We showed a significant increase in the number of G0/G1 phase cells and a decrease in the number of S phase cells in MSCs 0.2 treatment group. However, we did not observe a marked difference in cell cycle dynamics between MSCs 0.05 treatment group and untreated MRL/lpr mice. The results showed that high-dose MSCs treatment had an inhibitory effect on G1/S transition of the abnormal lupus T lymphocytes.

Detection of p27^{Kip1}, p21^{WAF1/CIP1} and CDK2 levels in T cells from MRL/lpr mice

We next detected the molecular mechanisms associated the distribution of cell cycle in lupus T cells. Cell cycle progression is a complex process, which is tightly controlled by cyclins, CDKs and CDKIs, such as p27^{Kip1}, p21^{WAF1/CIP1} and CDK2. We showed by western blots that T cells from MRL/lpr lupus mice exhibited significantly lower levels of p27^{Kip1} and p21^{WAF1/CIP1}. In contrast, the expression of CDK2 was increased. We further analyzed the expression of these proteins among untreated and MSCs treatment groups. It showed that MSCs (0.2*10⁶ cells/10 g body) treatment resulted in an increase in the activity of p27^{Kip1} and p21^{WAF1/CIP1} and a decrease in the activity of CDK2. However, there was no significant difference between untreated and low-dose MSCs treatment lupus mice, which was in accordance with the distribution of cell cycle studies (Fig. 3).

Fig. 3. Expression of CDK2, p27^{Kip1} and p21^{WAF1/CIP1} of T cells were evaluated by western blot analysis. The mean± SD values of 5 individual samples (derived from independent mice) are plotted (* p<0.05, ** p<0.01 v.s MRL/lpr).

Fig. 4. Expression of pAkt^{Thr308}, pAkt^{Ser473}, Akt, pPTEN, PTEN, pGSK3 β ^{Ser9} and GSK3 β of T cells were evaluated by western blot analysis. The mean $\pm$ SD values of 5 individual samples (derived from independent mice) are plotted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ v.s MRL/lpr).

Changes in the phosphorylation levels of Akt and its downstream target GSK3 β in T cells from MRL/lpr mice with or without MSCs treatment

The PI3K/Akt/GSK3 β signaling pathway has been implicated in the regulation of cell growth of lupus lymphocytes. To further examine the possible signaling pathway involved in the effects of MSCs on lupus T cells, the phosphorylation levels of Akt and its downstream PTEN and GSK3 β were analyzed. Akt activation is controlled by phosphorylation at Ser473 and Thr308 and the activity of GSK3 β can be reduced by phosphorylation at Ser9. In this study, the phosphorylation levels of Akt and GSK3 β were increased in T cells of lupus mice compared with the control group. Whereas, the phosphorylation level of PTEN was decreased. High-dose

MSCs treatment suppressed the phosphorylation levels of Akt and more significant change was observed in pAkt^{Thr308}. In addition, the expression of pGSK3 β ^{Ser9} was also decreased. On the contrary, the phosphorylation level of PTEN was increased in MSCs treatment group (Fig. 4).

Discussion

In this study, we used MRL/lpr mice as a SLE mouse model to investigate the role of the BMMSCs in SLE disorder. We demonstrated that allogeneic BMMSCs transplantation blocked cell cycle progression of T cells from MRL/lpr lupus mice and the PI3K/AKT/GSK3 β signaling pathway may play an important role in this

MSCs-mediated inhibition of cell proliferation.

Our data suggested that high-dose BMMSCs transplantation reduced levels of several autoantibodies and improved renal function in MRL/lpr mice. This observation was consistent with previous studies [14, 15, 24, 25]. However, some recent studies showed treatment with MSCs *in vivo* had no effect on (NZB*NZW)F1 mice [26, 27]. Such discrepancies can be due to many factors, including differences in culture conditions, ratios of MSCs to target cells, different SLE mouse models, etc. We cultured BMMSCs according to the protocol [21] on the basis of the frequent medium change in primary culture and diminishing the trypsinization time to obtain a purified population of MSCs. In our study, MSCs showed a dose dependent inhibition that only high infusing dose ($0.2 \times 10^6/10g$) exhibited a therapeutic effect. MRL/lpr mice are homozygous for Fas mutation which cause a striking reduction in Fas mRNA expression and show lupus-like disease [28]. It was reasonable that in our lupus model, MSCs inhibited the abnormal proliferation of T cells and reduced autoantibody production. Unlike MRL/lpr mice, 40% of splenic antibody-secreting cells in (NZB*NZW)F1 mice were nondividing, long-lived plasma cells [29]. Thus, serum autoantibodies in this animal model of lupus could be maintained by these long-lived plasma cells and not affected by the presence of MSCs.

T lymphocytes play a pivotal role in the pathogenesis of SLE. Previous *in vitro* experiments demonstrated that T cells in the presence of MSCs remained in the G0/G1 phase of the cell cycle after stimulation [11, 12]. In this study, we focused on the action of MSCs on T lymphocytes. We investigated the cell cycle distribution of T cells from MRL/lpr mice with or without MSCs treatment. In agreement with these recent studies, our data showed that high-dose MSCs transplantation inhibited CD3-mediated activation and G0/G1 transition in abnormal lupus T cells. Cell cycle progression is a complex process, which is tightly controlled by cyclins, CDKs and CDKIs. It was reported that mice deficient in p21^{WAF1/Cip1} developed a fatal lupus-like syndrome [30]. Our findings also implied that MSCs target a signaling pathway involved not only in the up-regulation of CDK inhibitor p21^{WAF1/CIP1} and p27^{Kip1} but also in the down-

regulation of CDK2 expression. These findings suggested that high-dose MSCs transplantation influenced abnormal expression of the cell cycle regulatory proteins in T cells from MRL/lpr mice *in vivo* and thus blocked G0/G1 transition of abnormal lupus T cells.

The PI3K/Akt signaling pathway has been shown to play a key role in regulating cell proliferation [31]. Several recent studies described an association between this pathway and SLE [19, 32], and Akt axis might be a good therapeutic target in human SLE. Akt activation is controlled by phosphorylation at Ser473 and Thr308 and is phosphorylated on these two sites respectively [33]. The phosphorylation of Thr308 *in vivo* is not dependent on phosphorylation of Ser473 or vice versa [34]. Our results showed that the expression of Akt^{Thr308} and Akt^{Ser473} were increased in T cells from MRL/lpr mice. High-dose MSCs treatment suppressed the phosphorylation levels of Akt and more significant change was observed in pAkt^{Thr308}. GSK3 β was reported to play an important role in the regulation of cell proliferation and apoptosis. The activity of GSK3 β can be reduced by phosphorylation at Ser9. Akt phosphorylate and inhibit GSK3 β and therefore enhance the stability of the G1-to-S-phase cell cycle transition proteins [16, 35]. It was reported that GSK3 β inactivation determined activated T cell survival [36]. In this study, we found that the phosphorylation level of Ser9 GSK3 β was higher in T cells from MRL/lpr mice, and its expression was decreased in disease remission mice.

In conclusion, this study showed the immunosuppressive effects of MSCs directly on T cells from MRL/lpr mice *in vivo* and this immunosuppressive mechanism involved in the inhibition of abnormal activation of the Akt/GSK3 β protein kinase cascade in T cells of lupus.

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