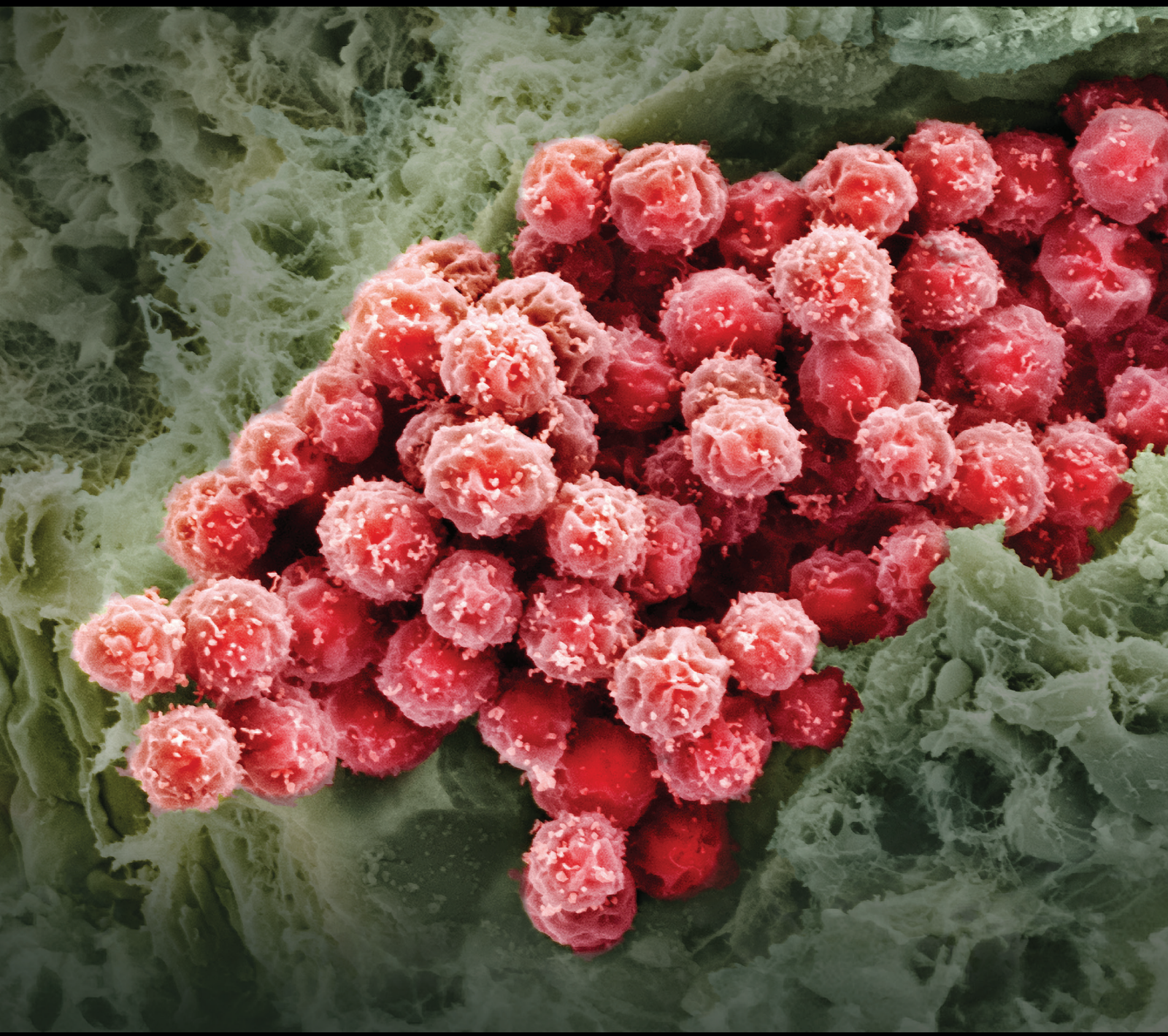
Stem Cells International

Stem Cells, Inflammation, and Fibrosis

Guest Editors: Vladislav Volarevic, Majlinda Lako, and Miodrag Stojkovic



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Editorial Board

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Editorial

Stem Cells, Inflammation, and Fibrosis

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Most organs are susceptible to fibrotic diseases and currently fibroproliferative diseases are believed to be responsible for around 45% of deaths in developed countries. Although considerable efforts are being devoted to the search for antifibrotic treatments, there are currently few effective therapies for fibrotic diseases that do not result in severe secondary effects.

Last decades' preclinical and clinical trials have led to application of stem cells as a novel therapeutic agent for the treatment of fibroproliferative diseases.

In this special issue of, several research groups contribute with original research articles as well as review articles that provide novel insights about the possible role of stem cells in modulation of inflammation and fibrosis.

Extensive research has been done on hypertrophic scar and keloid formation that has resulted in the plethora of treatment and prevention methods practiced today. In this issue, B. F. Seo and S.-N. Jung extensively review this body of research examining the mechanism and potential of stem cell therapy in the treatment of excessive scars.

Due to their immunomodulatory ability and capacity for self-renewal and differentiation into tissues of mesodermal origin, mesenchymal stem cells (MSCs) have been proposed as possible new therapeutic agents for the treatment of immune-mediated diseases. MSCs produce cytokines, chemokines, and growth factors that robustly regulate cell behavior in a paracrine fashion during the remodeling process. Due to their immunomodulatory effects and their ability to act on profibrotic factors such as oxidative stress, hypoxia, and the transforming growth factor-β1 (TGF-β1) pathway, MSC has already been highlighted in preclinical and clinical studies of inflammatory diseases. Accordingly, MSCs as well as their secretomes have been investigated as a novel therapeutic approach for the treatment of inflammatory and fibrotic diseases in several papers published in this issue.

Currently, the most effective therapy for advanced cirrhosis is liver transplantation, but its use is limited because of organ donor shortage, financial considerations, and the requirement for lifelong immunosuppression. An alternative approach such as stem cell transplantation has been suggested as an effective alternate therapy for liver cirrhosis. It is well known that both rodent and human stem cells, in the presence of growth factors, cytokines, chemical compounds, hepatocytes, or nonparenchymal liver cells, are able to differentiate into hepatic progenitor cells (HPC) or hepatocytes *in vitro*, but it is still controversial whether stem cell transplantation can completely regenerate injured liver *in vivo*. Here, A. T. Yang and colleagues report that TGF-β1 displays time-dependent dual effect on interplay between HPC and hepatic stellate cells *in vitro* and in animal model of liver fibrosis. Differential stimulation of HPC by TGF-β1 for 12 h versus 48 h produces opposing anti- and profibrotic effects indicating the importance of microenvironment and particularly TGF-β1 for HPC and hepatic stellate cells crosstalk in the pathogenesis of liver fibrosis.

In line with these findings, L. Zhang and colleagues also highlight the importance of microenvironment and signaling pathways for immunomodulatory capabilities of

MSC. They show that Delta-1 overexpression is able to significantly change immunomodulation of MSCs to immune cells, showing potent inhibition of mature T cell proliferation and a slight delay in mature B cell growth, thus providing a potentially novel approach for MSC-mediated modulation of lymphocyte lineage differentiation and development.

Recently published data suggest that MSCs are not constitutively immunosuppressive; they require a "licensing" step provided by molecules of acute phase inflammation, like IFN-γ and TNF-α. MSCs are immunosuppressive only when exposed to sufficiently high levels of these proinflammatory cytokines while, in the presence of low levels of TNF-α and IFN-γ, MSCs may adopt a proinflammatory phenotype (MSC1) and enhance inflammation by secreting chemokines that recruit inflammatory cells (particularly Th1, Th17 cells, monocytes, and neutrophils) to the sites of inflammation. The switch toward proinflammatory (MSC1) phenotype or anti-inflammatory (MSC2) phenotype may also depend on MSC stimulation through toll-like receptors (TLRs) expressed on their surface. Polarization to proinflammatory (MSC1) phenotype, important for early injury responses, can be influenced by lipopolysaccharide- (LPS-) dependent activation of TLR4, while double stranded RNA- (dsRNA-) dependent activation of TLR3 may induce the polarization into anti-inflammatory type (MSC2). The balance between these opposing pathways may serve to promote host defense on one hand and at the same time create a loop that prevents excessive tissue damage and promotes fibrosis. Galectin 3 (Gal-3) is protein known to affect proliferation, differentiation, activation, migration, and polarization of immune cells, playing an important role in inflammation and fibrosis. Here, B. S. Markovic and colleagues indicate that Davanat, the newly synthesized inhibitor of Gal-3, could be used for improvement of MSC-mediated polarization of macrophages towards immunosuppressive M2 phenotype. They show that pharmacological inhibition of Gal-3 in MSC enhances their capacity to promote alternative activation of peritoneal macrophages *in vitro* and *in vivo*, in an experimental model of colon inflammation.

Soft dental tissues represent an easily accessible source of stem cells that can be used for autogenic or allogenic cell therapy. Dental stem cells are MSCs capable of self-renewal, multilineage differentiation, and immunomodulation. In this issue, S. Yildirim and colleagues compared proliferation rate, differentiation potential, gene expression, and immunomodulatory effects of dental stem cells isolated from human exfoliated deciduous teeth (SHEDs), dental pulp stem cells (DPSCs), and dental follicle stem cells (DFSCs) on peripheral blood mononuclear cells providing new and important information for cell therapy and regenerative dentistry.

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Research Article

Pharmacological Inhibition of Gal-3 in Mesenchymal Stem Cells Enhances Their Capacity to Promote Alternative Activation of Macrophages in Dextran Sulphate Sodium-Induced Colitis

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Transplantation of mesenchymal stem cells (MSCs) reduces the severity of dextran sulphate sodium- (DSS-) induced colitis. MSCs are able to secrete Galectin-3 (Gal-3), a protein known to affect proliferation, adhesion, and migration of immune cells. We investigate whether newly synthetized inhibitor of Gal-3 (*Davanat*) will affect production of Gal-3 in MSCs and enhance their potential to attenuate DSS-induced colitis. Pharmacological inhibition of Gal-3 in MSCs enhances their capacity to promote alternative activation of peritoneal macrophages *in vitro* and *in vivo*. Injection of MSCs cultured in the presence of Davanat increased concentration of IL-10 in sera of DSS-treated animals and markedly enhanced presence of alternatively activated and IL-10 producing macrophages in the colons of DSS-treated mice. Pharmacological inhibition of Gal-3 in MSCs significantly attenuates concentration of Gal-3 in sera of DSS-treated animals, indicating that MSCs produce Gal-3 in this disease. In conclusion, our findings indicate that Davanat could be used for improvement of MSC-mediated polarization towards immunosuppressive M2 phenotype of macrophages.

1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are the two major forms of inflammatory bowel disease (IBD) and are characterized by an abnormal cell influx to the intestinal tissues and massive release of proinflammatory mediators [1]. One of the most common IBD-related animal models is the *Dextran Sulphate Sodium-* (*DSS-*) induced colitis, originally reported by Okayasu et al. [2]. The clinical features of the DSS-induced colitis are similar to human colitis and include weight loss, loose stool/diarrhea, and occult and gross rectal bleeding. DSS has a toxic effect on epithelial cells, resulting in invasion of intestinal bacteria into subepithelial tissue. Dendritic cells (DCs) and macrophages capture bacteria that have passed through DSS-injured colonic epithelium, and through activation of Toll-like receptors (TLRs), release proinflammatory cytokines (TNF-α, IL-12) and chemokines (macrophage inflammatory protein- (MIP-) 1α, monocyte chemotactic protein- (MCP-) 1, and keratinocyte-derived chemokine (CXCL1/KC), CCL11) which induce migration of inflammatory cells in the colon [3, 4].

Mesenchymal stem cells (MSCs) are adult, multipotent cells that can be found in almost all postnatal tissues [5, 6]. MSCs can alter immune response and regulate the proliferation, activation, and effector function of T lymphocytes, professional antigen presenting cells (DCs, macrophages, B lymphocytes), and NK cells, through cell-to-cell contact or through the production of soluble factors [7]. Due to their immunomodulatory properties, much interest has been focused in MSC-based therapy of inflammatory disorders, including IBD [8–10].

Recently, transplantation of MSCs has been found to reduce the severity of DSS-induced colitis [11–14]. Liu et al. [15] found that MSCs significantly alleviated the DSS-induced colitis, and the major sources for TGF-β1 were macrophages that were recruited by MSCs. Specific ablation of macrophages completely abolished the anti-inflammatory effects of MSCs [15].

Sioud et al. [16] showed that MSCs were able to secrete Galectin-3 (Gal-3), a protein known to affect proliferation, adhesion, and migration of immune cells. Because Gal-3 is widely expressed in immune cells (neutrophils, eosinophils, basophils, mast cells, DCs, monocytes, and macrophages, as well as in NK cells) [17], which are involved in pathogenesis of DSS-induced colitis, we investigated the role of Gal-3 produced by MSCs in this experimental model.

2. Materials and Methods

2.1. Cells. Murine MSCs isolated from bone marrow of C57BL/6 mice were purchased from Gibco (catalog number S10502-01). The cells were cultured in *Dulbecco's Modified Eagle Medium* (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin G, and 100 μg/mL streptomycin (Sigma-Aldrich Chemical, Munich, Germany), at 37°C in a 5% CO_2 incubator. MSCs in passage 6 were used throughout these experiments.

2.2. Animals. We used 6–8-week-old male wild type (WT) C57BL/6 mice for induction of DSS colitis. Male, 6–8-week-old, Gal-3$^{-/-}$ C57BL/6 mice (provided by Dr. Daniel Hsu, University of California, Sacramento, CA) were used in the coculture experiments. Targeted disruption of mouse Gal-3 gene was performed in C57BL/6 embryonic stem cells and mice homozygous for disrupted gene were obtained [18]. Mice were maintained in animal breeding facilities of Faculty of Medical Sciences, University of Kragujevac, Serbia. All animals received human care and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia. Mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle and were administered standard laboratory chow and water *ad libitum*.

2.3. Experimental Design. Experimental animals were divided into 4 groups: (1) wild type (WT); (2) WT + DSS (Dextran Sulphate Sodium); (3) WT + DSS + MSCs (mesenchymal stem cells); and (4) WT + DSS + MSCs + Davanat (Gal-3 inhibitor). Each group had 10 animals. To complete this study, 80 WT and 10 Gal-3$^{-/-}$ C57BL/6 animals were used.

Table 1: Criteria for scoring the Disease Activity Index of IBD (DAI)*.

Score	Weight loss	Stool consistency	Visible blood in feces
0	No weight loss	Normal	No bleeding
1	1–5%		
2	6–10%	Loose	Slight bleeding
3	11–15%		
4	>15%	Diarrhea	Gross bleeding

*DAI value is calculated as the sum of scores of weight loss, stool consistency, and blood in feces.

2.4. Induction of Acute Colitis. Colitis was induced in C57BL/6 with 3% w/v DSS (molecular weight 40 kDa; TdB Consultancy, Uppsala, Sweden) dissolved in drinking water given *ad libitum* (days 1–7) as previously described [2]. Control mice were given DSS-free water.

2.5. Administration of MSCs. On day 0, 12 h after DSS administration, mice were injected intraperitoneally (ip) with 0.5×10^6 MSCs diluted in 200 mL PBS or a vehicle control (PBS alone) [19].

2.6. Pharmacological Inhibition of Gal-3 in MSCs. In order to inhibit production of Gal-3 in MSCs, MSCs were treated with inhibitor of Gal-3 (Davanat, 15 μg/mL, kindly provided by Professor Klyosov and Professor Traber from Galectin Therapeutics Inc., Newton, MA) and MSCs + Davanat were administered on day 0 (the 1st day of DSS administration), intraperitoneally, according to previously published protocol [20].

2.7. Assessment of the Severity of Colitis. *Disease Activity Index* (DAI) was used to assess the clinical signs of colitis (Table 1). Body weight was measured daily and compared with the body weight measured on day 0 (the 1st day of DSS administration). The obtained results were presented as ±% body weight loss. The analysis of stool consistency and Hemoccult (Beckman Coulter) fecal occult blood test were performed daily [21].

2.8. Histology. For histological analysis, colons were removed from euthanized mice, rinsed with phosphate buffer solution (PBS), and cut longitudinally before being rolled into "Swiss roll," as previously described [22]. Swiss-rolled colons were fixed in formalin and embedded in paraffin and 5 μm sections were stained with hematoxylin and eosin (H&E) and examined in a blinded manner by pathologist. Sections were analyzed for damage of epithelium including damage of crypts, submucosal edema, hemorrhage, and infiltration of immune cells. The histology scores for each mouse were

Table 2: Histological scoring*.

Score	Infiltration	Damage of epithelium
0	No infiltration	Normal morphology
1	Infiltration around crypt basis	Loss of goblet cells
2	Infiltration reaching the lamina muscularis mucosae	Loss of goblet cells in large areas
3	Extensive infiltration reaching the lamina muscularis mucosae associated with mucosa thickening and oedema	Loss of crypts
4	Infiltration of the lamina submucosa	Loss of crypts in large areas

*Scores were calculated by adding the score for two parameters, giving a maximum score of 8.

calculated as the sum of "infiltration" and "damage of epithelium" subscores, as previously described (Table 2) [23].

2.9. Measurements of Cytokines in Serum. We used the commercial ELISA sets (R&D Systems, Minneapolis, MN) to measure the concentration of selected cytokines (Gal-3, TNF-α, IL-1β, IL-10, and TGF-β) according to the manufacturer's instructions [24]. Briefly, blood sample was collected from abdominal aorta during euthanasia procedure. Serum was separated by centrifugation and stored at −80°C. Gal-3, TNF-α, IL-1β, IL-10, and TGF-β serum levels were measured by enzyme-linked immunosorbent assay (ELISA).

2.10. Isolation of Immune Cells from Lamina Propria and Flow Cytometry Analysis. Isolation of immune cells from *lamina propria* was conducted as previously described [25]. Briefly, each colon was dissected away from cecum. The colons were cut in 3 cm long pieces and then cut longitudinally, so that 3 × 3 cm flaps of colonic tissue were made. The flaps were placed in a 50 mL conical tube and washed 3 to 5 times with 30 mL cold HBSS, calcium- and magnesium-free. After decanting the supernatant, the pieces were incubated in 20 mL HBSS/EDTA for 30 min in a 37°C water bath. Each tube was shaken regularly during the incubation to ensure that epithelial cells are disrupted from the mucosa. The pieces were sediment and supernatants were decanted. The remaining EDTA were washed out with 40 mL HBSS, calcium- and magnesium-free. The fragments of colonic tissue were placed in a 10 cm petri dish and cut into smaller pieces with a razor blade or scalpel. The pieces were aspirated with a pipette, transferred to a new 50 mL conical tube, and filled to 20 mL with DMEM supplemented with 10% fetal bovine serum (FBS). Then, 1 mL of 4000 Mandl units (3×10^6 Wünsch units)/mL collagenase D and 200 μL of 1 mg/mL DNase were added to the tube and incubated for 1 h in a 37°C water bath. The supernatants were filtered through a 100 μm nylon cell strainer into a clean 50 mL conical tube. A cold HBSS, calcium- and magnesium-free, was added to 50 mL. Cells were pelleted by centrifuging for 10 min at 450 ×g, at 4°C. The pellet was disrupted and cells were resuspended in 50 mL HBSS, calcium- and magnesium-free, and filtered through a 40 μm nylon cell strainer into a clean 50 mL conical tube. Cells were again pelleted by centrifuging for 10 min at 450 ×g, 4°C. The pellet was disrupted and cells were resuspended in 20 mL of 30% Percoll. Then, the cell suspension was carefully layered over 25 mL of 70% Percoll in a 50 mL conical tube and centrifuged for 20 min at 1100 ×g, room temperature, with as low an acceleration rate as possible and with the brake off. Clumping of cells was prevented by the addition of 1 mM EDTA to the solution. Epithelial cells float on the 30% Percoll layer, while immune cells were found between the 30% and 70% layer. Debris and dead cells were pelleted at the bottom of the conical tube.

Flow cytometry followed routine procedures by using 1×10^6 cells per sample and were incubated with antimouse F4/80, antimouse CD206, antimouse FcεRI, antimouse CD117, antimouse CD11c, antimouse CD11b, antimouse CD80, antimouse NK1.1, and antimouse CD3 conjugated with fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ), phycoerythrin (PE; BD Biosciences), peridinin chlorophyll protein (PerCP; BD Biosciences), or allophycocyanin (APC; BD Biosciences). For the intracellular staining, cells were previously stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 h at 37°C. Following extracellular staining, cells were fixed, permeabilized, and stained for TNF-α, IL-10, IL-12, and IL-1β by using conjugated antimouse antibodies. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by using the Flowing software analysis program.

2.11. Isolation and In Vitro Coculture of Peritoneal Macrophages. Macrophages were isolated from peritoneal cavity of untreated, healthy Gal-$3^{-/-}$ mice. Mice were injected with 5 mL of PBS ip and, after shaking, peritoneal lavage was performed. Macrophages were collected from the peritoneal cavity of mice under sterile conditions and cultured in complete DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. Isolated macrophages were plated at a density of 10^6 cells/well and cocultured with MSCs and MSCs + Davanat cells for 24 hours [26]. The levels of IL-10 and TGF-β were determined in cell culture supernatants by ELISA sets, according to manufacturer's recommendations. Phenotype of macrophages was determined by flow cytometry, as described above.

2.12. Statistics. Data were expressed as the mean ± SEM for each group. We tested for normality using Shapiro-Wilk's test and for homogeneity of variances using Levine's test. A paired samples t-test was used to compare two matched groups. Independent samples Student's t-test was otherwise used to compare two groups with Gaussian distribution. Fisher's exact test was used to assess survival differences

between groups. Statistical analyses were performed using SPSS 19.0 for Windows software (SPSS, Inc., Chicago, IL). All reported P values were 2-sided and $P < 0.05$ was considered statistically significant and highly significantly different when $P < 0.01$.

3. Results

3.1. Pharmacological Inhibition of Gal-3 in MSCs Significantly Attenuates Concentration of Gal-3 in Sera of DSS-Treated Mice. The concentration of Gal-3 in sera of DSS-treated mice that received MSCs correlates with pharmacological inhibition of this molecule in MSCs. As it is shown in Figure 1(a), pharmacological inhibition of Gal-3 in MSCs significantly attenuates concentration of Gal-3 in sera of DSS-treated animals, indicating that MSCs produce Gal-3 in this disease ($P < 0.05$; Student's t-test).

Inhibition of Gal-3 in MSCs did not alter their potential to prevent the development of DSS-induced colitis, according to survival rate (Figure 1(b)) ($P < 0.05$; Fisher's exact test), clinical parameters (Figure 1(c)), and colon length (Figure 1(d)) ($P < 0.05$; Student's t-test). All DSS-treated WT mice developed severe colitis with similar clinical symptoms: diarrhea, rectal bleeding, and weight loss. The presence of blood in the feces was detected one to two days after the start of DSS treatment, whereas gross bleeding and diarrhea were initially observed from day 4. Significant body weight loss (>5%) became prominent after four days of DSS treatment. Compared to DSS-only-treated animals, DSS-treated mice that received MSCs + Davanat or MSCs did not develop diarrhea, gross rectal bleeding, and significant body weight loss till the end of the experiment. These observations were also confirmed by the histological analysis (Figure 1(e)) ($P < 0.01$; Student's t-test). The DSS-treated group clearly exhibited a severe mucosal inflammatory cell infiltrate and disruption of crypt architecture (epithelial ulcerations and loss of goblet cells), whereas DSS-induced lesions were prevented in both MSCs + Davanat- and MSCs-only-treated animals (Figure 1(e), lower panels).

3.2. Pharmacological Inhibition of Gal-3 in MSCs Results in Increased Concentration of IL-10 in Sera of DSS-Treated Animals. In effort to investigate the effect of Gal-3 on immunomodulatory characteristics of MSCs in DSS-induced colitis, we analyzed concentration of cytokines in sera. There were significantly lower levels of inflammatory TNF-α and IL-1β and significantly higher levels of anti-inflammatory IL-10 and TGF-β in sera of DSS-treated mice that received MSCs + Davanat or MSCs only when compared to DSS-only-treated animals (Figure 2) ($P < 0.01$; Student's t-test). Importantly, the concentration of IL-10 was significantly higher (Figure 2(c)) ($P < 0.05$; Student's t-test) in sera of MSCs + Davanat-treated mice when compared to concentration of this cytokine in MSCs-only-treated mice with DSS-induced colitis.

3.3. Pharmacological Inhibition of Gal-3 in MSCs Results in Markedly Enhanced Presence of Alternatively Activated and IL-10 Producing Macrophages in the Colons of DSS-Treated Mice. Injection of MSCs resulted in significantly lower number of F4/80+ macrophages, FcεRI+CD117+ mast cells, inflammatory CD11c+CD11b+DCs, and CD3+NK1.1+NKT cells in colons of DSS-treated mice (Figure 3). Among all these cells, pharmacological inhibition of Gal-3 in MSCs affected only phenotype and cytokine production of macrophages (Figures 3(b) and 3(c)) in colons of DSS-treated animals. Although there was not any difference in the percentage of IL-12 and IL-1β producing F4/80+ colonic macrophages (Figures 3(d) and 3(e)) between MSCs and MSCs + Davanat groups, pharmacological inhibition of Gal-3 in MSCs results in markedly enhanced presence of alternatively activated and IL-10 producing macrophages in the colons of DSS-treated mice. The percentage of F4/80+CD206+ alternatively activated macrophages as well as macrophages that produced IL-10 was significantly higher in mice that received MSCs in which production of Gal-3 was inhibited (Figures 3(b) and 3(c)) ($P < 0.05$; Student's t-test) when compared with other experimental groups.

3.4. Pharmacological Inhibition of Gal-3 in MSCs Enhances Their Capacity to Promote Alternative Activation of Peritoneal Macrophages. In order to elucidate the role of Gal-3 produced by MSCs for alternative activation of macrophages, Gal-3$^{-/-}$ macrophages were cocultured with MSCs or MSCs + Davanat cells. Pharmacological inhibition of Gal-3 in MSCs enhances their capacity to promote alternative activation of peritoneal macrophages. There was significantly higher percentage of F4/80+CD206+ alternatively activated macrophages in population of peritoneal macrophages cocultured with MSCs + Davanat cells (Figure 4(a)) ($P < 0.01$; Student's t-test), while there was no significant difference in the percentage of inflammatory F4/80+CD11b+ macrophages between experimental groups (Figure 4(a)). In line with these findings, there was a significantly higher level of IL-10 in supernatants of macrophages cocultured with MSCs + Davanat cells (Figure 4(b)) ($P < 0.05$; Student's t-test).

4. Discussion

The immunoregulatory activity of MSCs offers a novel strategy in the design of therapeutic protocols aimed at suppressing pathologic immune responses responsible for the development of IBD. In this context, it has been shown that MSCs can exert their inhibitory effect not only on cells of adaptive immune response, but also on cells of the innate immunity, including DC, NK cells, and macrophages [7].

Macrophages have been identified as one of the most important cells for the induction of acute human colitis and DSS-induced colitis [27, 28]. Uptake of DSS by macrophages activates the Nlrp3 inflammasome resulting in increased production of inflammatory cytokines IL-1β and IL-18. In addition, production of nitric oxide (NO) and expression

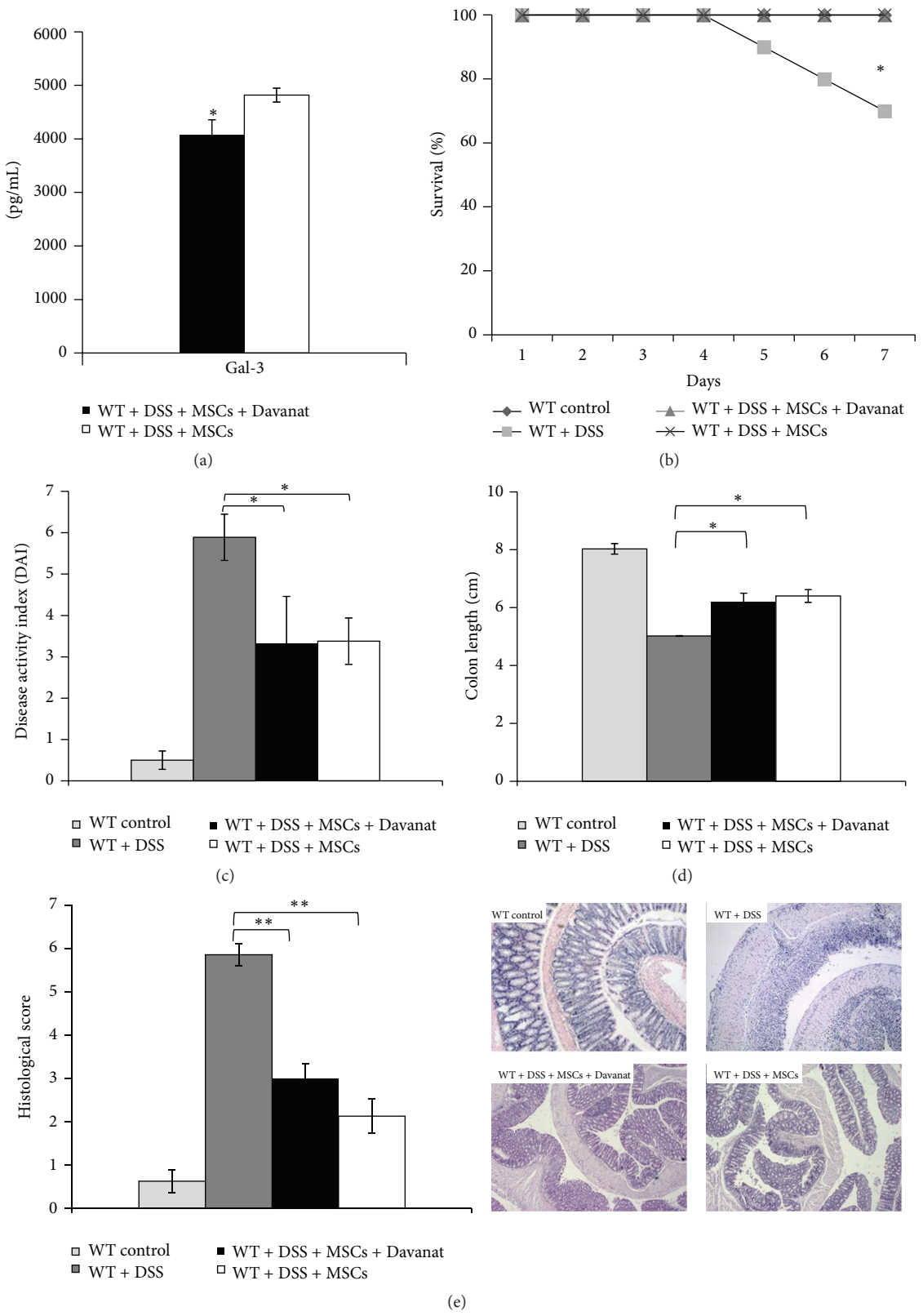

Figure 1: MSCs attenuate DSS-induced colitis. Water with 3% DSS was given to mice for 7 days; regular drinking water was fed to control mice. The concentration of Gal-3 in sera of MSCs groups (a). Survival rate of mice with colitis (b). Disease Activity Index (DAI) scored at day 7 using the following parameters: weight loss, stool consistency, and rectal bleeding (c). After DSS treatment length of the entire colon was measured (d). Histological examination was performed with hematoxylin and eosin staining (e). H&E staining images of representative colon tissues are shown at the same magnifications (100x) (e). Data presented as means ± SEM; $n = 10$ mice per experimental groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

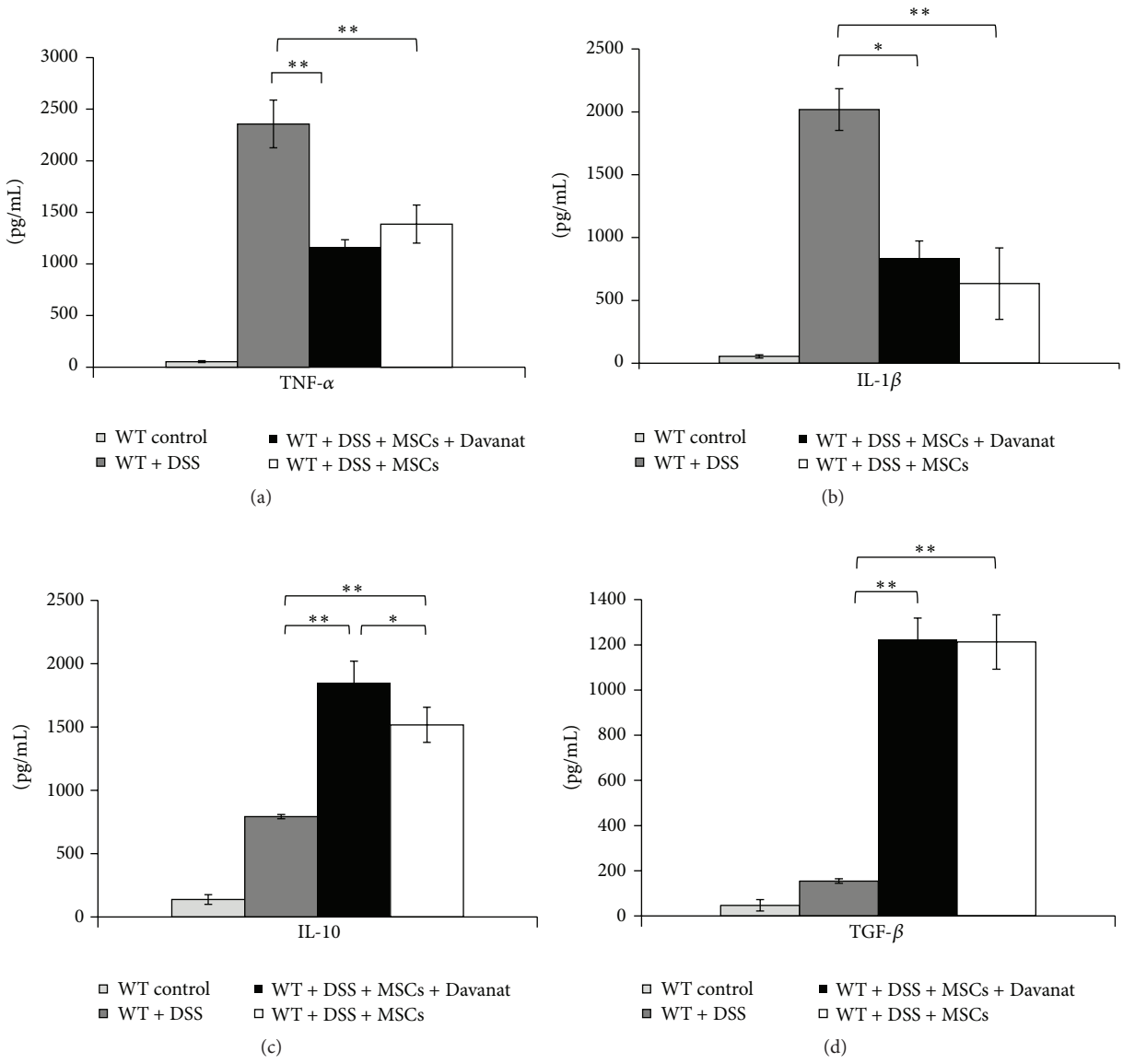

Figure 2: Pharmacological inhibition of Gal-3 in MSCs results in increased concentration of IL-10 in sera of DSS-treated animals. Levels of proinflammatory cytokines in the sera are shown: (a) TNF-α and (b) IL-1β. Levels of anti-inflammatory cytokines in the sera are shown: (c) IL-10 and (d) TGF-β. Values are mean ± SEM ($n = 10$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$.

of inducible nitric oxide synthase (iNOS) in macrophages exacerbate DSS-induced colitis [29].

Recently, Liu and coworkers suggest that MSCs transplantation may recruit macrophages to produce anti-inflammatory cytokines, which attenuate colitis [15]. In line with these findings, we found that MSCs significantly ameliorated the clinical and histopathological severity of DSS-induced colitis (Figure 1) that correlated with increased serum levels of IL-10 (Figure 2) and increased percentage of F4/80+CD206+ alternatively activated macrophages in colon (Figure 3).

A limited number of studies have been performed to investigate a potential of MSCs to educate macrophages to adapt an anti-inflammatory/immune-suppressive phenotype: to express higher levels of CD206 (marker for alternatively activated macrophages) and to increase production of anti-inflammatory cytokine IL-10. Kim and Hematti [30] were first to report that human bone marrow-derived MSC could promote the generation of alternatively activated macrophages.

In another study, Cutler and coworkers showed that monocytes isolated from peripheral blood displayed increased expression of CD206, lower levels of surface HLA-DR, and reduced capability of stimulating alloreactive T-cell response, after coculturing with umbilical cord-derived MSC [31]. Zhang et al. [32] showed that human gingiva-derived

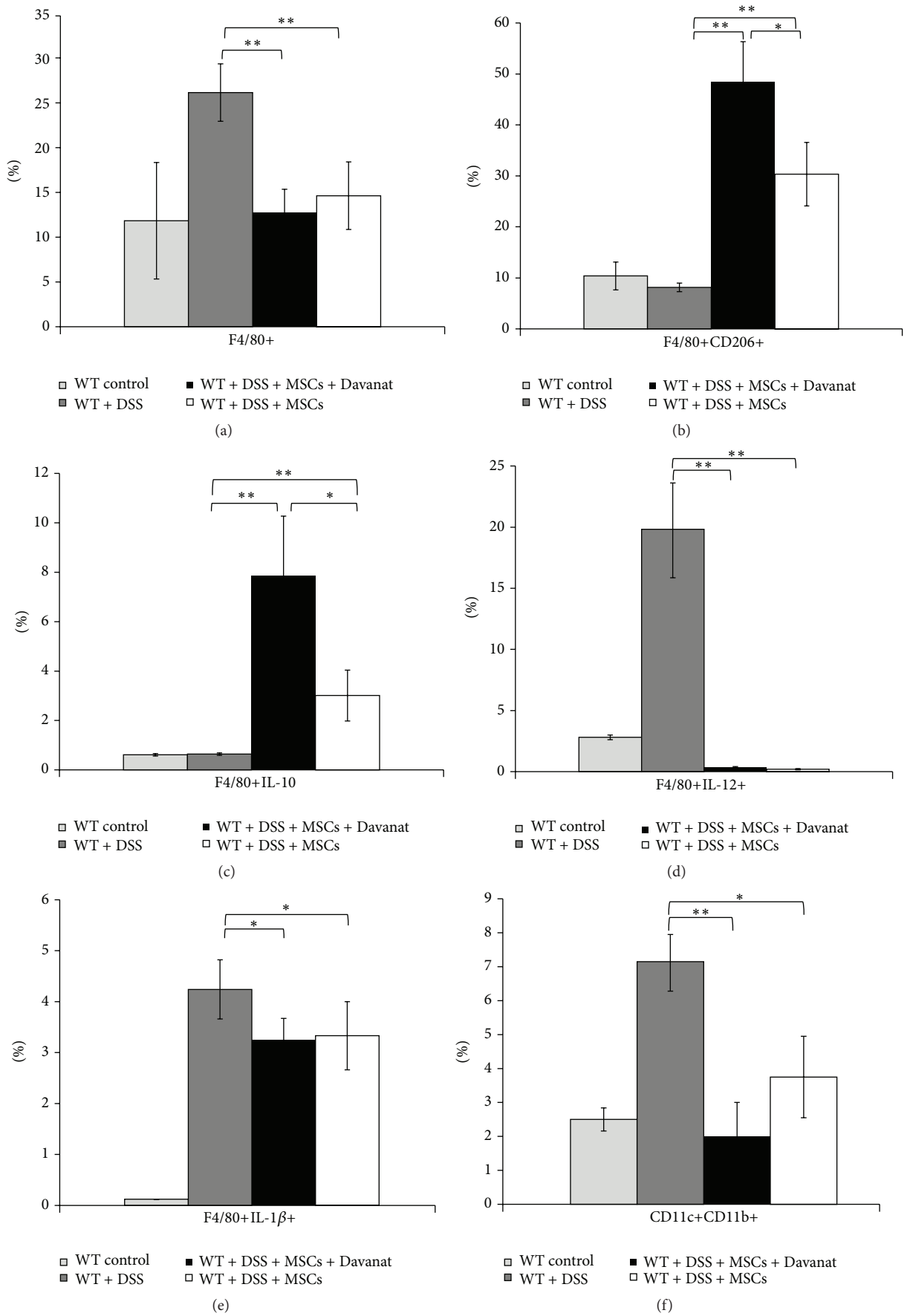

Figure 3: Continued.

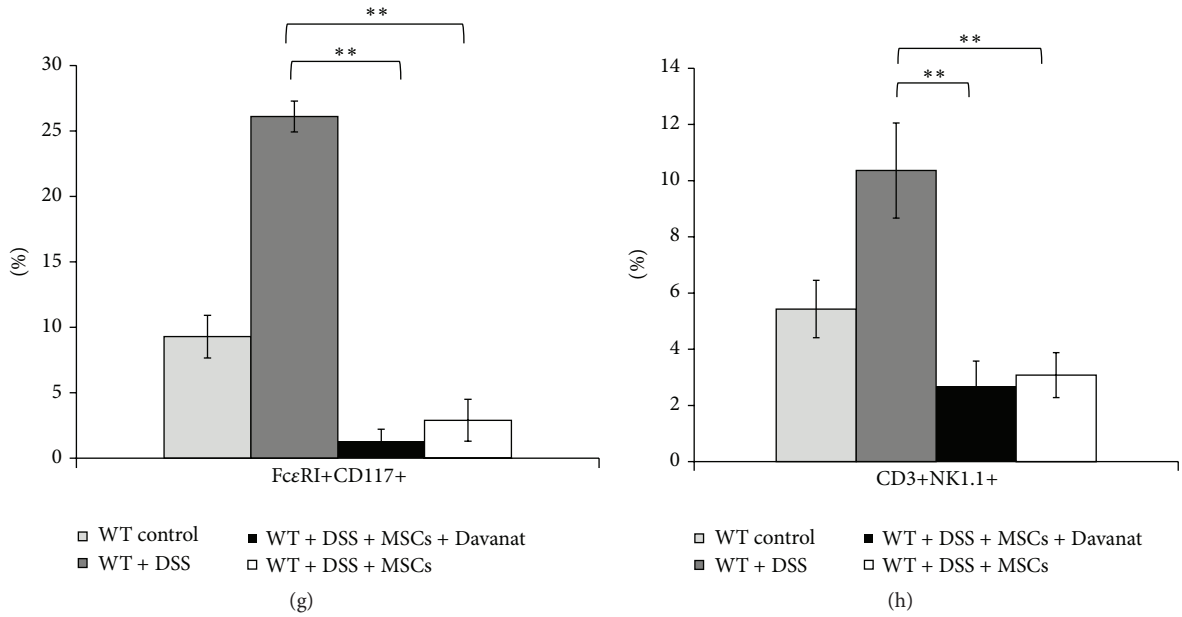

Figure 3: Pharmacological inhibition of Gal-3 in MSCs favored alternative activation of macrophages in the colon tissues of DSS-treated mice. The percentage of F4/80+ macrophages in colon tissue (a). Significant decrease in percentage of F4/80+CD206+ and F4/80+ IL-10+ macrophages in MSCs-treated mice (white bars), when compared to MSCs + Davanat-treated mice (black bars), after 7 days on DSS treatment (b and c). The percentage of IL-12- and IL-1β-producing F4/80+ macrophages (d and e). The percentages of inflammatory dendritic cells (f) and FcεRI+CD117+ mast cells (g) as well as CD3+NK1.1+NKT cells (h) are shown. Values are mean ± SEM ($n = 10$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$.

MSCs could induce polarization of M2 macrophages *in vitro* and confirmed this phenomenon *in vivo*. They reported that repeatedly infused human gingiva-derived MSCs could home to the wound site in close proximity with host macrophages and promote their polarization towards M2 phenotype [32].

Concerning the mechanisms underlying the M2 polarizing effect exerted by MSCs on macrophages, an essential role of different soluble factors has been demonstrated. By the use of specific neutralizing antibodies, Zhang and colleagues [32] showed an involvement of IL-6 and granulocyte-macrophage-CSF while Cutler et al. [31] reported the importance of PGE2 for the induction of M2 phenotype by MSC.

Herewith, we showed that Davanat-mediated pharmacological inhibition of Gal-3 in MSCs resulted in enhanced presence of F4/80+CD206+ alternatively activated and IL-10 producing macrophages in colon of DSS-treated animals (Figure 3) and increased serum levels of IL-10 (Figure 2) indicating the importance of Gal-3 produced by MSCs, for macrophage polarization towards M2 phenotype. In addition, we showed that pharmacological inhibition of Gal-3 in MSCs enhances capacity of MSCs to promote M2 polarization of macrophages and IL-10 production *in vitro* (Figure 4).

It is well known that Gal-3 plays important role in macrophage polarization and function [33–36]. In animal model of type 1 diabetes, macrophages of Gal-3 deficient mice produce less TNF-alpha and nitric oxide (NO) and are less effective in intracellular and extracellular killing compared with WT mice [37]. By using an animal model of immune mediated acute hepatitis [36], we showed that both genetic deletion and TD139-induced pharmacological inhibition of Gal-3 resulted in an increased number of IL-10-producing alternatively activated, M2-polarized macrophages in the livers.

Traber and coworkers demonstrated that Davanat significantly reduced expression of Gal-3 in portal and septal macrophages resulting in attenuated fibrosis in thioacetamide-induced liver disease [38]. Also, pharmacological inhibitor of Gal-3 managed to ameliorate hepatocellular damage, inflammation, and fibrosis in a mouse model of nonalcoholic fatty liver disease [39] and these effects are associated with a reduction of Gal-3 expression on liver macrophages.

5. Conclusion

In conclusion, Davanat-induced inhibition of Gal-3 did not significantly affect potential of MSCs to attenuate colitis but managed to enhance production of anti-inflammatory cytokine IL-10 in colonic macrophages and to promote their polarization towards immunosuppressive M2 phenotype. Our findings indicate that Gal-3 target drugs could be used for improvement of MSCs-mediated suppression of macrophages.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

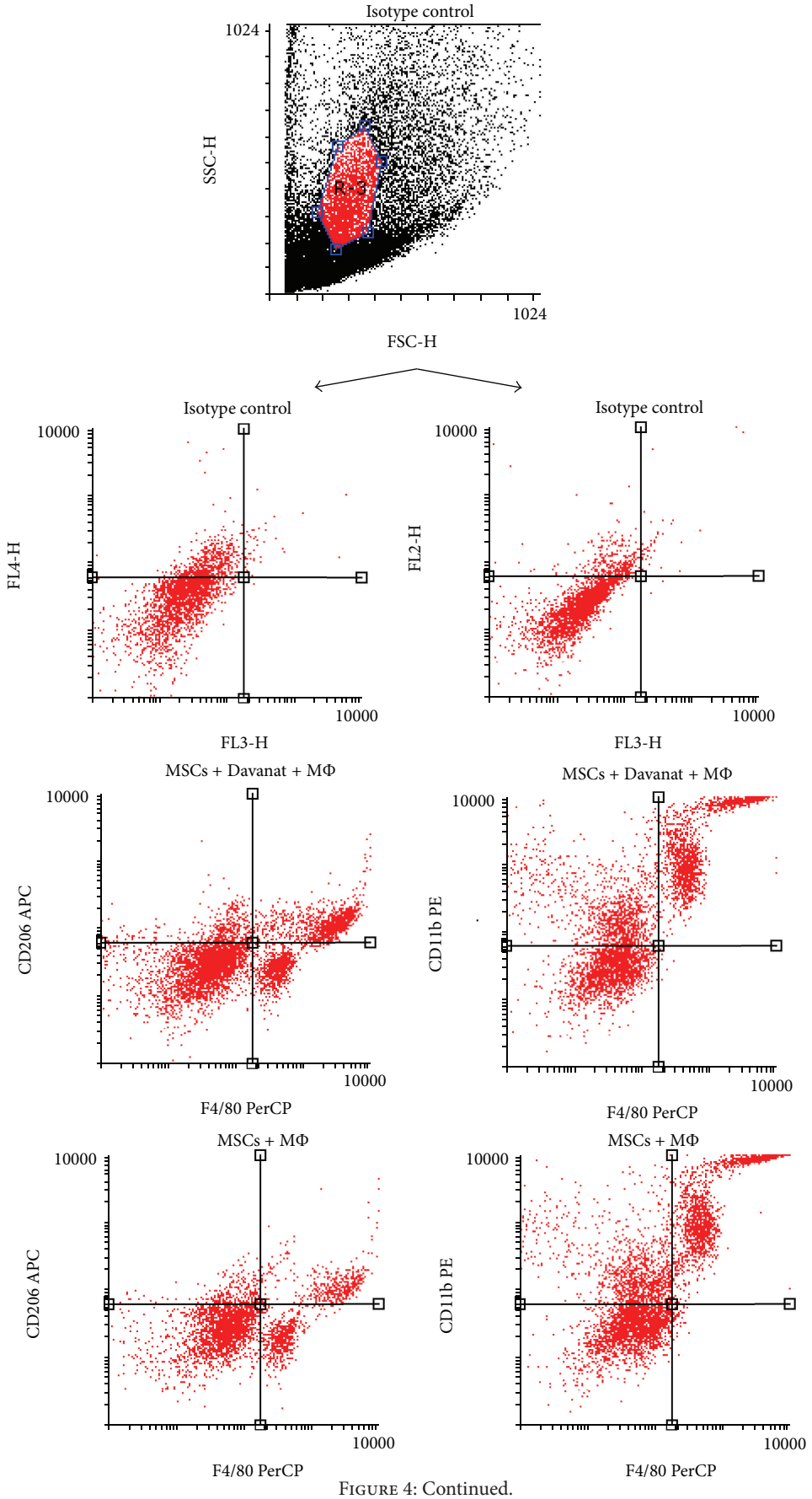

Figure 4: Continued.

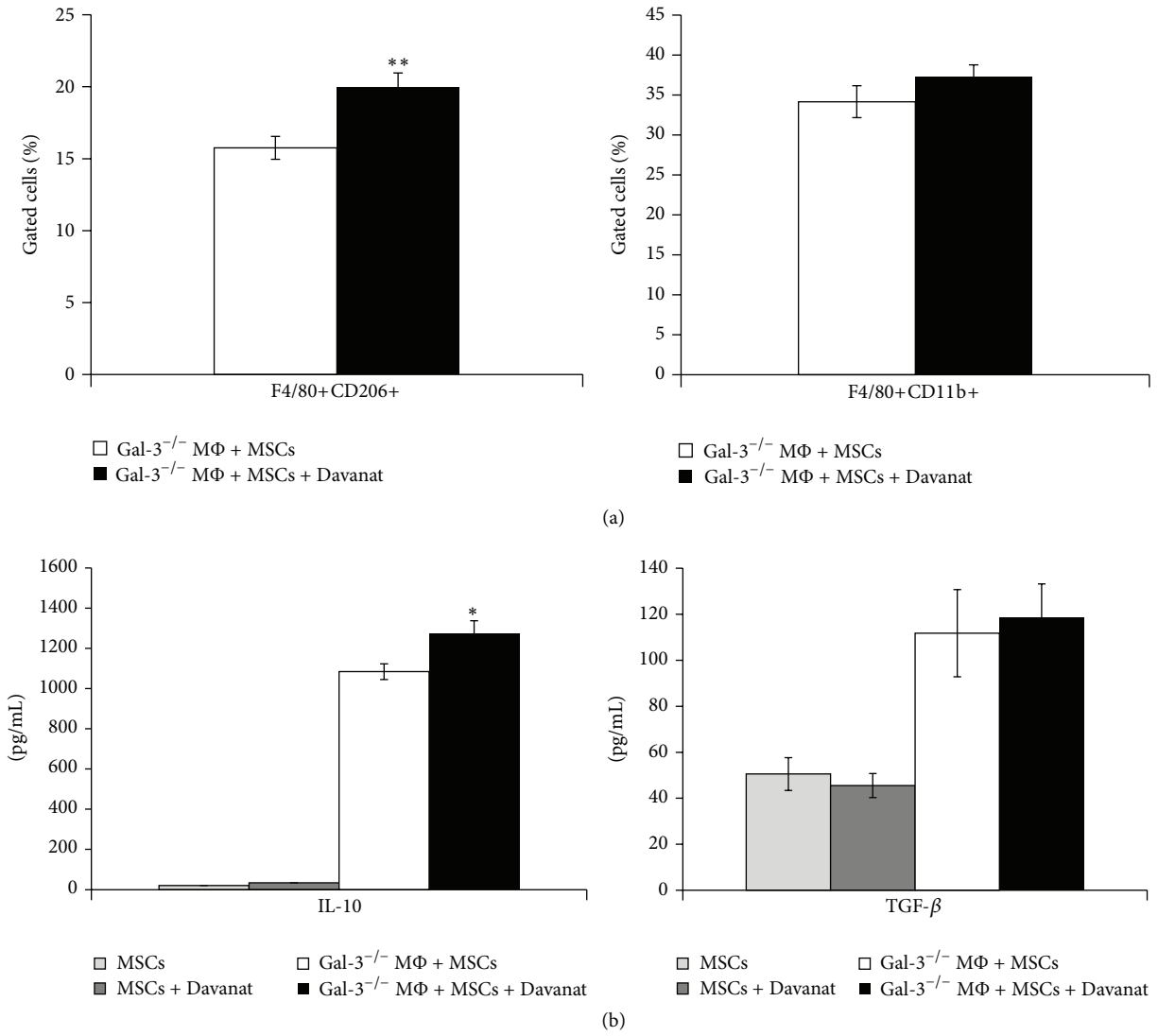

Figure 4: Pharmacological inhibition of Gal-3 in MSCs enhances their capacity to promote alternative activation of peritoneal macrophages. The significant increase in percentage of F4/80+CD206+ macrophages in MSCs + Davanat-treated Gal-$3^{-/-}$ macrophages (black bars), when compared to only MSCs-treated Gal-$3^{-/-}$ macrophages (white bars) (a). The percentage of F4/80+CD11b+ macrophages is shown (a). Representative flow cytometry dot plots are shown. The level of IL-10 and TGF-β in supernatants (b). Values are mean ± SEM ($n = 10$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$.

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Review Article

The Immunomodulatory Effects of Mesenchymal Stem Cells in Prevention or Treatment of Excessive Scars

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Excessive scars, including keloids and hypertrophic scars, result from aberrations in the process of physiologic wound healing. An exaggerated inflammatory process is one of the main pathophysiological contributors. Scars may cause pain, and pruritis, limit joint mobility, and cause a range of cosmetic deformities that affect the patient's quality of life. Extensive research has been done on hypertrophic scar and keloid formation that has resulted in the plethora of treatment and prevention methods practiced today. Mesenchymal stem cells, among their multifunctional roles, are known regulators of inflammation and have been receiving attention as a major candidate for cell therapy to treat or prevent excessive scars. This paper extensively reviews the body of research examining the mechanism and potential of stem cell therapy in the treatment of excessive scars.

1. Introduction

Excessive scarring, first described in the Smith papyrus about 1700 BC, is a persisting phenomenon that provides a spectrum of morbidities on the inflicted [1]. Specific to humans, they may occur after any type of injury including burns, lacerations, abrasions, piercings, surgical incisions, or injections. Hypertrophic scars or keloids are scars that present with an overabundance of dermal collagen, rising above skin level. Such lesions not only are cosmetically unattractive, but may also limit joint function and cause uncomfortable symptoms such as pain and pruritis. The resulting psychological burden affects the patient's quality of life and escalates health care costs [2].

Although the definitive process underlying such scar formation is yet to be elucidated, the upregulated, exaggerated inflammatory response has been found to be a critical step in achieving excessive scars [3–5]. Normal physiologic wound healing in human adults undergoes three overlapping phases: inflammation, proliferation, and remodeling [6]. Immediately after injury, platelet degranulation and activation of complement and coagulation cascades result in formation of a fibrin clot at the site of injury. This structure provides hemostasis and functions as the seat of wound chemotaxis. This temporary extracellular matrix (ECM) stimulates the recruitment of inflammatory cells (neutrophils, macrophages, epithelial cells, mast cells, endothelial cells, and fibroblasts), which in turn produce proinflammatory mediators including macrophage inflammatory protein-1alpha (MIP-1α), monocyte chemotactic protein-1 (MCP-1), RANTES, interleukin-1beta (IL-1β), and interleukin-6 [7, 8]. Inflammatory cells also deliver a wide range of growth factors, transforming growth factor-beta 1 (TGF-β1), transforming growth factor-alpha (TGF-α), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) [9, 10]. Proliferation begins within 48 hours to 10 days after injury, characterized by replacement of the temporary fibrin scaffold with a vascularized ECM synthesized by recruited fibroblasts. The construction of this granulation tissue, composed of procollagen, elastin, proteoglycans, and hyaluronic acid, provides the framework for vascular ingrowth and migration and proliferation of keratinocytes [11, 12]. During this stage, myofibroblasts, modified fibroblasts containing

actin filaments, mediate wound contraction to bring wound margins together [4]. With wound closure comes the remodeling phase, beginning around 14 to 21 days after injury. ECM is reorganized and degraded, during which a variety of proteolytic enzymes including matrix metalloproteinases (MMPs), and their inhibitors (TIMPs for tissue inhibitor of MMPs) play a major role. The proportion of type I collagen to type III collagen increases [3]. Maturation of the scar results in a decrease in cellularity and vascularity of the tissue [6]. The number of myofibroblasts is dramatically reduced through the process of apoptosis [13].

It is evident that the transformation of a fibrin clot into mature scar tissue requires a delicate balance between ECM deposition and degradation. Physiological wound healing requires timely resolution of the inflammatory response, and when this process is disrupted abnormalities in scarring will occur. There is substantial evidence in the literature that increased inflammation is a prerequisite for scarring [9, 14]. Fetal mammalian wound healing is scarless, incorporating fewer inflammatory cells and fewer inflammatory mediators, with a shorter inflammatory phase [15]. Oral mucosal wound healing results in reduced scar formation compared to skin, and studies have found that mucosa ECM components resemble that of fetal skin and have reduced inflammatory cell infiltration and proinflammatory mediators [16, 17].

Mesenchymal stem cells (MSCs) are characterized by their regenerative capacity and have been recognized as a legitimate player accelerating the wound healing process [18–21]. MSCs are able to be home to sites of injury, transdifferentiate into epidermal or dermal lineages, and have immunomodulatory, antifibrotic, and angiogenic abilities they exert by secreting an enormous array of paracrine growth factors or cell-to-cell contact [22–25]. Administration of MSCs regulates excessive inflammation, demonstrated by their therapeutic abilities in experimental models of inflammation related fibrotic diseases: lung injury, spinal cord injury, myocardial infarction, corneal injury, renal fibrosis, or liver cirrhosis [24, 26–31]. The ability and mechanisms with which MSCs attenuate the inflammatory process during cutaneous wound healing are an emerging focus of interest, which we will review while overviewing the current tide of research.

2. Overview of Current Research

There is a multitude of research that has been and is being performed on the effects of stem cells on the wound healing process. Most experimental designs are composed of stem cells of different origins applied in differing doses via (1) systemic injection, (2) local injection (at the wound site), or (3) seeded on a tissue engineered scaffold at various time points.

2.1. Preparation of Stem Cells. The most common type of stem cell used in wound healing and scar research is MSC. Although there have been some studies that applied murine embryonic stem cells (ESCs) to burn wound surfaces, ethical and legislative issues limit the expansion of further investigation [32–34]. MSCs, obtained from human bone marrow aspirate in early studies, are now harvested from various tissues throughout the body [35]. Adipose derived stem cells may be obtained during excisional surgery or liposuction [36]. The marrow space of long bones, periosteum, synovial fluid, nasal septum, gingiva, periodontal ligament, palatine tonsil, parathyroid gland, and fallopian tube also withhold MSCs [37–44]. While adult MSCs are most commonly utilized in research, limitations such as donor morbidity and limitation in proliferative capacity have led researchers to search for an alternative source. Extra-embryonic MSCs harvested after birth from prenatal tissue such as the placenta, umbilical cord, umbilical cord blood/Wharton's Jelly, or the dental pulp of the primary tooth are now major sources of MSCs [45–49].

MSCs in all experimental studies should meet the International Society for Cellular Therapy (ISCT) minimum criteria for MSCs: plastic-adherent when maintained in standard culture conditions, expression of CD105, CD73, and CD90 while lacking expression of CD45, CD23, CD14, or CD11b, CD79alpha or CD19 and HLA-DR surface molecules, differentiation to osteoblasts, adipocytes, and chondroblasts in vitro [50].

2.2. Wound Models. It is well known that there is a lack of a universal model of abnormal wound healing [51]. The porcine burn model comes closest in resemblance of human scar tissue; however, most studies were performed on previously established wound models of smaller animals [52]. An excisional or incisional wound is created, or a cytotoxic agent is injected into the dorsal skin of mice, rats, rabbits, or, rarely, pigs [36, 53–59]. Because loose skinned animals, including mice or rats, display rapid wound healing mediated by wound contracture, some studies used splints to resist against this process [58, 60, 61]. Fu and Li and Yun et al. used a burn wound produced by a heated brass bar pressed on the backs of minipigs or male Yorkshire pigs, respectively [62, 63].

2.3. Delivery of MSCs. While most earlier studies delivered MSCs to the site of injury via systemic intravenous or local subcutaneous or dermal injection, recent studies have been focusing on a more effective method of transport. Tissue engineering approaches combine stem cells with biomaterial scaffolds or matrices attempt to minimize unprogrammed cell death and/or migration from the wound. The incorporation of scaffolds is supported by researchers who argue that recapitulation of the stem cell microenvironment is necessary to enhance their potential [64, 65]. Acellular dermal matrices carrying adipose-derived stem cells have been effective in targeted cell delivery [66, 67]. Another group has found that poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), a natural polymer that has previously shown influence on reepithelialization, synergistically acts to downregulate the inflammation process in murine skin wound models [68]. Lam et al. found increased cell survival and proliferation and reduced scarring when they applied a patch harvested from

porcine small intestine submucosa (SIS) seeded with adipose-derived stromal cells onto murine skin excision wounds [69]. Fat grafting or cell-assisted lipotransfer into or beneath scar has been suggested as a natural method of delivering autogenous adipose derived stem cells but requires further evidence [70, 71].

The advantages in using tissue engineering include the possibility of designing a 3-dimensional structure tailored to the wound and the potential of attaching biomaterials that are synergistic with the cells.

2.4. Clinical Studies. The transition of MSC application from bench to bedside has yet many barriers to overcome. These include issues of safety, efficacy, and cost-effectiveness, among others. More clinical studies are warranted before development of a cell treatment. Most of the research on MSCs and scars involves animal models and is focused on how the cells exert their immunomodulatory abilities during the early stages of the wound healing process. There have been few reports on the use of stem cells in human scars. In 2014, Hemphill et al. found that injection of a heterogeneous mixture of 2 million human amniotic stem cells and amniotic membrane matrix directly into an intractably painful postsurgical scar resulted in a significant decrease in pain and visible decrease in scar tissue [72]. The application of fat grafts into neuropathic scars or hypertrophic scars shows pain alleviation or an increased scar quality. Fat grafts are a source of MSCs and adipose derived stem cells which are thought to be involved in this process [73].

3. Immunomodulatory Effects of MSCs That Downregulate Excessive Scarring

3.1. MSCs Are Capable of Homing the Site of Injury. MSCs that are injected systemically travel through the circulatory system to ultimately concentrate at the site of tissue damage [24, 74]. MSCs express chemotaxis toward a variety of wound healing cytokines in vitro such as PDGF, TNF-α, insulin-like growth factor-1, and IL-8, which explains their preferential migration toward wounds [75, 76].

3.2. MSCs Modulate Inflammatory Cells. MSCs produce a vast spectrum of paracrine factors. The main paracrine factors involved in immunomodulation are TGF-β, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), IL-10, IL-6, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and human leukocyte antigen G (HLA-G) [77–79]. Each of these factors is known to regulate different target immune cells. Other than such soluble factors, MSCs secrete extracellular vesicles (EVs), lipid bilayers that contain and transport the cytoplasmic components of the MSCs [25]. EV is an inclusive term that has recently been suggested to encompass both exosomes and microvesicles [80]. Several studies have reported the immunological potential of MSC EVs in vitro, and the ability of these EVs to attenuate an activated immune system in vivo [81, 82]. Along with cell-to-cell (MSC) contact, the MSC secretome including EVs and soluble factors modulates the inflammatory response.

3.2.1. Natural Killer (NK) Cells. MSCs are capable of inhibiting proliferation and function of NK cells, mediated by IDO, PGE2, and TGF-β1 [78, 79, 83]. Numerous studies have shown that MSCs only partially inhibit the proliferation of activated NK cells and are susceptible to lysis by activated cells [77]. HLA-G5 inhibits NK cell mediated cytolysis and decreases interferon-gamma (IFN-γ) secretion [84].

3.2.2. Dendritic Cells (DCs). Dendritic cells are antigen presenting cells that differentiate from monocytes or CD34+ hematopoietic stem cells until contact with antigens, after which they are activated into mature cells. MSCs are known to impair this differentiation process via PGE2 secretion [83].

3.2.3. Neutrophils. Neutrophils arrive at the wound through chemotaxis, traversing postcapillary venules to degrade pathogens with the granules within phagolysosomes, and then undergo apoptosis. IL-10 secreted by MSCs inhibit neutrophil invasion into the wound. MSCs secrete TNF-stimulated gene/protein-6 (TSG-6), which interacts with protein ligands to inhibit rolling and transendothelial migration of neutrophils. Dyer et al. have found that TSG-6 interacts with the glycosaminoglycan binding site of CXCL8 (IL-8), a chemokine produced by macrophages and transported to the surface of the endothelium, impairing neutrophil adhesion and migration [85].

3.2.4. Macrophages. Macrophages, early responders that arrive at the injury site hours later than neutrophils, are phagocytes that cleanse the wound of matrix and cell debris. They may be polarized depending on environment and may be typically classified into two main groups: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages generally withhold antimicrobial characteristics and promote a Th1 type response while M2 macrophages promote Th2 type responses. The M2 macrophage can be classified into M2a, M2b, and M2c macrophages that are defined by specific patterns of cytokine production. In general, M2 macrophages secrete less proinflammatory cytokines, have high production of anti-inflammatory cytokines such as IL-10, and induce resolution of the inflammatory phase. This is in the exception of M2b macrophages, which maintain high levels of inflammatory cytokines [86]. Many studies have demonstrated the ability of autologous or allogeneic MSCs to polarize macrophages toward an M2 phenotype in vitro mediated by paracrine mechanisms, enhancing expression of M2 associated macrophage genes [87]. Kim and Hematti have suggested a separate definition for MSC-educated macrophages that secrete high IL-10 and IL-6 and low IL-12 and TNF-α, to call them M2m, different from other subcategories. They propose the possibility of collecting monocytes through leukapheresis and coculturing these mononuclear cells with allogeneic MSCs to provide MSC-educated macrophages prepared for repair of wounds [87].

3.2.5. B Cells. B lymphocytes are manufacturers of antibodies in response to antigens. MSCs may block B cell proliferation

in the G0/G1 phase of the cell cycle without eliciting apoptosis [88]. Krampera et al. found that inhibition of proliferation was seen only in the presence of IFN-γ, which is probably mediated by MSC production of IDO. IDO is the first and rate-limiting enzyme of the essential amino acid tryptophan catabolism to kynurenine pathway, causing depletion and therefore halting growth. IFN-γ has IDO inducing effects [89]. The differentiation of B cells is also inhibited in the presence of B cells [88].

3.2.6. T Cells. Inhibitory effects of T cell proliferation by MSCs are mediated by both cell-to-cell contact and soluble factors. TGF-β1 and HGF work together to suppress T cell proliferation [90]. MSCs secrete PGE2 which prevents differentiation of CD4+ T cells into Th17 cells. MSCs also release IDO and enhance secretion of IL-10, which also inhibit cell proliferation [78]. NO has also been implicated as a mediator that downregulates T cell proliferation by phosphorylation of signal transducer and activator of transcription-5 (STAT5). STAT5 is a transcription factor required in activation and proliferation of T cells [91].

3.3. MSCs Downregulate Fibrosis. The major downregulators of fibrosis produced by MSCs are PGE2, IL-10, NO, HGF, and adrenomedullin. When cocultured with T cells, MSCs have increased expression of PGE2. PGE2 is produced from arachidonic acid with the aid of enzymes cyclooxygenase-1 or cyclooxygenase-2 [79]. PGE2, already described to inhibit or reduce proliferation or function of NK cells, DCs, T cells, and Treg cells, also induce T cells and macrophages to express higher levels of IL-10 [92, 93]. IL-10 is a major anti-inflammatory cytokine that inhibits neutrophil infiltration into the wound. Neutrophils are one of the first-responders to the inflammatory reaction, capable of ingesting microorganisms, releasing granules filled with enzymes and antimicrobial proteins, and constructing neutrophil extracellular traps that trap and kill microbes extracellularly. During phagocytosis, there is a burst of oxygen consumption, and much of the extra oxygen consumed is converted to highly reactive oxygen species (ROS) ("respiratory burst") [94]. Therefore IL-10 works to prevent further damage from neutrophil release of ROS. ROS are cytotoxic and have antimicrobial effects but also damage normal tissue and intensify collagen deposition by causing membrane lipid oxidation and induction of TGF-β [95]. NO are known to scavenge ROS, resulting in reactive nitrogen species which are less toxic.

HGF is a growth factor secreted by MSCs that modulate fibroblasts, the central player in fibrosis. Myofibroblasts, rich in alpha smooth muscle actin (SMA-α), are responsible for wound contraction and secretion of ECM and undergo apoptosis after wound maturation. The continued presence and activation of myofibroblasts is seen during excessive scarring. HGF downregulates fibroblast expression of TGF-β1, which drives myofibroblast differentiation, and collagens types I and III [96]. HGF upregulates fibroblast expression of MMPs, therefore enhancing degradation of the ECM. HGF also acts on keratinocytes, upregulating expression of VEGF-A, and is shown to induce angiogenesis without vascular inflammation [97, 98].

3.4. MSCs Are Able to Differentiate and Transdifferentiate into Dermal or Epidermal Cell Types. MSCs are characterized by their ability to differentiate and transdifferentiate into cells of different lineages. Capability to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro is included in the criterion of MSCs. However, when cocultured in vitro with keratinocytes, MSCs show transdifferentiation to keratinocytes [99, 100]. These results suggest that MSCs themselves may participate in regeneration of wound tissue.

3.5. MSCs Promote Angiogenesis. MSCs are recognized as powerful producers of bFGF and VEGF-A, growth factors that promote proliferation, migration, and differentiation of endothelial cells. Angiogenesis with stable vessels aids the normal progression of wound healing [101]. A summary of the immunomodulatory effects of MSCs can be seen in Figure 1.

4. Proinflammatory Capabilities of MSCs

Although the immunomodulatory functions of MSCs have been extensively investigated, there are also reports of proinflammatory capacities of these stem cells. This paradoxical ability has been noted under stimulation of certain infectious molecules. MSCs can be polarized into two uniform but distinct populations, MSC1 and MSC2 [102]. MSC1 is known to express proinflammatory factors while MSC2 expresses immunosuppressive factors. Toll-like receptors (TLRs), a family of transmembrane, immune regulatory pattern recognition receptors, play an important role in MSC-mediated immune responses. Thirteen analogs have been identified, and TLRs 1 through 6 are expressed at higher levels in human and murine MSCs. TLR3 and TLR4 agonists have been found to decrease the ability of MSCs in suppressing T cell proliferation [103]. Blockage of the TLR4 pathway is reported to lead to decrease in B cell activating factor, BAFF, a vital survival factor of B lymphocytes [104].

More clarification is required in the investigation of such proinflammatory pathways. Whether these findings exert an influence at the clinical level remains to be elucidated.

5. Conclusion

While there is an escalating volume of preclinical studies on the effect of MSCs on excessive scarring, more long term clinical studies are required to enable translation of this potential into treatment modalities. Issues such as cost effective methods of stem cell harvest, quantification, and delivery must also be addressed. Most research is focused on the prophylactic application of MSCs, applied during the wound healing process before scar maturation. The lack of a universal hypertrophic scar or keloid model may be partially responsible. However, many of patients tend to seek management for scars long after the remodeling phase of

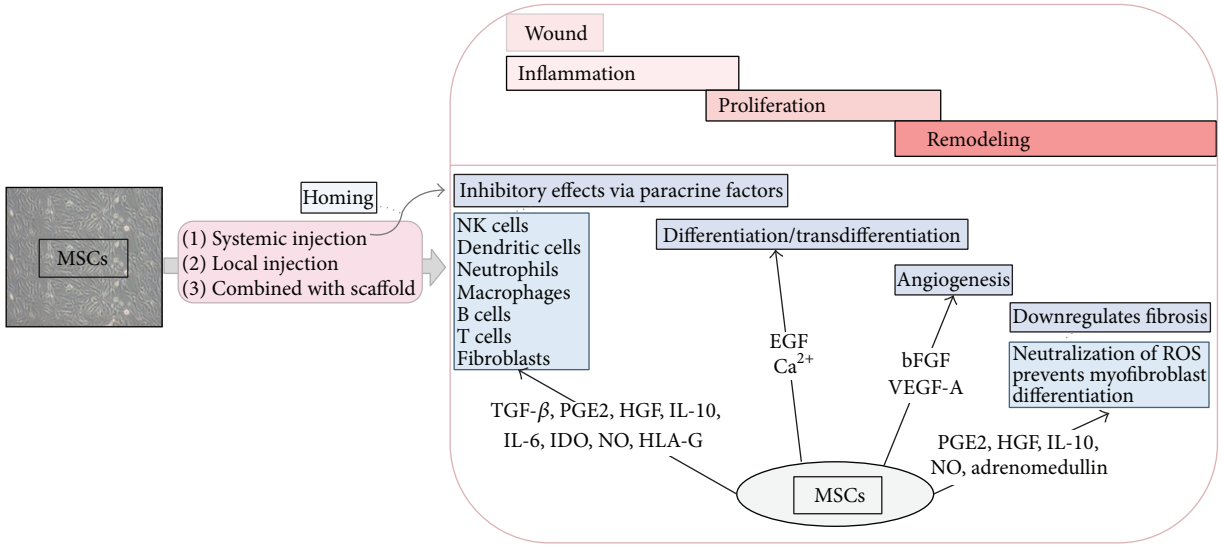

Figure 1: A summary of the immunomodulatory effects of MSCs that downregulate excessive scarring. MSCs are able to home the wound, where the stages of wound healing (inflammation, proliferation, and remodeling) are in progress. MSCs have been found to attenuate the activity of inflammatory cells, differentiate or transdifferentiate into epidermal cell lineages, escalate angiogenesis, and decrease fibrosis.

wound healing has finalized. Research on the effect of MSCs as a treatment modality of excessive scars may provide a promising solution for many other affected patients. Finally, although MSCs withhold enormous capacities for stem cell therapy in scar management, further insight into polarization and proinflammatory capacities is required before safe application in clinical treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

TGF-β1 Induces the Dual Regulation of Hepatic Progenitor Cells with Both Anti- and Proliver Fibrosis

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Transforming growth factor-beta 1 (TGF-β1) plays a central role in hepatic progenitor cells- (HPCs-) mediated liver repair and fibrosis. However, different effects of TGF-β1 on progenitor cells have not been described. In this study, both *in vitro* (HPCs cocultured with hepatic stellate cells (HSCs) in transwells) and *in vivo* (CCl_4-injured liver fibrosis rat) systems were used to evaluate the impacts. We found that HPCs pretreated with TGF-β1 for 12 hours inhibited the activation of HSCs, while sensitization for 48 hours increased the activation of HSCs. Consistent with these *in vitro* results, the *in vivo* fibrosis rat model showed the same time-dependent dual effect of TGF-β1. Regression of liver fibrosis as well as normalization of serum aminotransferase and albumin levels was detected in the rats transplanted with HPCs pretreated with TGF-β1 for 12 hours. In contrast, severe liver fibrosis and elevated collagen-1 levels were detected in the rats transplanted with HPCs pretreated with TGF-β1 for 48 hours. Furthermore, the TGF-β1-pretreated HPCs were shown to deactivate HSCs *via* enhancing SERPINE1 expression. Inhibition of SERPINE1 reversed the deactivation response in a dose-dependent manner.

1. Introduction

Hepatic progenitor cells (HPCs) are a group of small epithelial cells that reside in the smallest ducts of the biliary tree in the liver [1]. With advances of research technology, we have come to understand and appreciate the active participation of HPCs in both acute and chronic liver diseases. Depending on the disease process and the concurrent changes in the microenvironment, HPCs are pluripotent under different stimuli and can be classically differentiated into hepatocytes or cholangiocytes, or even myofibroblast cells or cancer cells [2–5].

Upon acute liver injury, when hepatocyte division is severely impaired or blocked, HPCs are activated and proliferate, and the expanding HPCs will begin to infiltrate along the liver plate towards the central vein; consequently, they differentiate themselves into either hepatocytes or cholangiocytes to restore the hepatic parenchyma and liver function [6]. Recent studies have shown the important role of HPCs in injury repair and fibrosis in both experimental models and patients with chronic liver disease [7, 8]. The HPC response correlates with the extent of hepatocellular injury and liver fibrosis [9]. In addition, inhibition of hepatic stellate cells (HSCs) activation or iloprost administration reduces HPCs activation increases of hepatocyte differentiation [10]. HSCs are resident perisinusoidal cells distributed throughout the liver, with a remarkable range of functions in normal and injured liver. During liver injury, various inflammatory and fibrogenic pathways contribute to the activation of HSCs, which increased fibrogenesis and altered matrix degradation; therefore, HSCs can be important therapeutic targets [11].

However, there has been ongoing debate whether the effects of the HPC response in liver fibrosis are antifibrotic or fibrogenic. In the CCl_4-induced rat model of hepatic failure with a two-thirds hepatectomy, HPCs effectively participated in repairing the damaged liver as shown by our previous

study [12]. Our recent data also have shown that transplanted HPCs ameliorate CCl_4-induced liver cirrhosis. However, a pluripotent differentiation of HPCs was observed. For instance, the expansion of the HPC compartment, which is known as ductular reactions, can clearly lead to transient amplification of the heterogeneous cell population, which is capable of differentiating into liver parenchymal and myofibroblast cells. Ductular reactions are often accompanied with HPCs activation as well as excessive deposition of extracellular matrix (ECM) around the portal areas, suggesting a direct correlation between ductular reactions and periportal fibrosis [5, 13].

Transforming growth factor-beta 1 (TGF-β1), a multifunctional cytokine, exerts its biological effects on tissue and organ development, cellular proliferation, differentiation, survival, and apoptosis [14]. In the liver, TGF-β1 is hypothesized to serve as the important link among liver regeneration, chronic injury, cirrhosis, and hepatocellular carcinoma. TGF-β1 is considered the most potent hepatic profibrogenic cytokine predominantly produced by activated mesenchymal cells upon chronic liver damage [15]. It is reasonable to suggest a regulatory axis from TGF-β1 to HPCs and then to HSCs in hepatic fibrosis. Their interrelationship can be dissected via partially mimicking the pathological microenvironment in fibrosis with HPCs exposed to a high concentration of TGF-β1.

To investigate the interrelationship among TGF-β1, HPCs, and HSCs in the imitated microenvironment, we utilized both *in vitro* and *in vivo* systems. In the *in vitro* study, HPCs pretreated with or without TGF-β1 were indirectly cocultured with HSCs. In the *in vivo* study, HPCs pretreated with or without TGF-β1 were transplanted into spleen with CCl_4-induced fibrosis. Furthermore, the mechanisms involved in this regulatory axis from TGF-β1 to HPCs and then to HSCs in hepatic fibrosis were studied using an epithelial-to-mesenchymal transition- (EMT-) related polymerase chain reaction (PCR) array.

2. Materials and Methods

2.1. Coculture of HPCs with HSCs. The rat hepatic progenitor cell line (WB-F344) was obtained from Academy of Military Medical Sciences. The rat hepatic stellate cell line (T6) was kindly provided by Dr. Friedman. Both WB-F344 cells and HSCs-T6 were plated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) (complete medium).

An indirect coculture system was assembled using transwell culture plates (0.4 μm pore size, 6-well Millicell; Millipore, Switzerland). This system allows cells to maintain contact through shared culture medium without mixing the two cell lines; 1×10^4 HSCs were plated on the lower chamber, and 4×10^4 HPCs were plated in the upper insert. The coculture system was maintained in an incubator supplied with 5% CO_2. For the monoculture groups, 1×10^4 HSCs were cultured in a separate dish and treated with the same medium as for the coculture system.

For the TGF-β1 treatment, HPCs were cultured with 10 ng/mL TGF-β1 in 5% FBS-DMEM medium for 6, 12, or 48 h. SERPINE1 inhibitor, 1H-indole-3-acetic acid, and α-oxo-1-(phenylmethyl)-5-[4-(trifluoromethoxy)phenyl] (PAI-039, Axon, USA, Cell Signaling Technology, Danvers, MA, USA) were added to the medium at a concentration of 10 μM simultaneously with TGF-β1 to confirm its blocking and reversing HPC influence on HSCs.

2.2. Quantitative Real-Time PCR (qPCR) Analysis of SERPINE1 Expression. Total cellular mRNA was extracted from harvested cells with a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was reversely transcribed using the Reverse Transcription System (Promega, Beijing, China). The following primers were used: forward, 5′-TCTCCAGGGGCCCTCTGAGGT-3′, reverse, 5′-TGCCCCTCTCCGCCATCACC-3′ (SERPINE1); forward, 5′-CCTGCCAAGTATGATGACATCAAGA-3′, reverse, 5′-GTAGCCCAGGATGCCCTTTAGT-3′ (GAPDH). SYBR Green-based qPCR was carried out on an instrument (Applied Biosystems, USA) for 2 min at 5°C before incubation for 10 min at 95°C to inactivate the reverse transcriptase, which otherwise interferes with the DNA polymerase. Forty cycles at 95°C for 15 s followed by 60°C for 60 s were performed. The mRNA expression of the target gene was normalized to GAPDH. The relative amounts were expressed as the means ± standard deviation (SD) from three independent experiments.

2.3. Western Blotting of Cell Differentiation-Related and Fibrogenic Markers. Cells were washed and lysed with buffer (250 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P40, 5 mM EDTA, 50 mM NaF, and 1 mM Na_3VO_4) supplemented with a protease inhibitor mixture (Roche Applied Science, USA). After boiling for 10 min, the lysates were separated in 12% sodium dodecyl sulfate-polyacrylamide gels. The blots were blocked using 5% nonfat dry milk in tris-buffered saline containing Tween 20 for 2 h at room temperature followed by incubation with the primary antibodies against albumin (ALB, diluted 1 : 500, Abcam, Cambridge, UK), α-fetoprotein (AFP, diluted 1 : 1000, R&D Systems, USA), α-smooth muscle actin (SMA, diluted 1 : 1000, R&D Systems, USA), TIMP-1 (diluted 1 : 1000, R&D Systems, USA), and PCNA (diluted 1 : 1000, Abcam, Cambridge, UK) at 4°C overnight. After washing, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (ZSGB Bio, China) for 1 h at room temperature, and reactivity was detected by the Enhanced Chemiluminescence Kit (Thermo Fisher Scientific Inc., USA). After signal detection, the membranes were incubated with an anti-β-actin antibody (diluted 1 : 2000, Sigma, US) as a loading control. The western blot detection was repeated three times.

2.4. Gene Expression by the EMT PCR Array. Differential expression of EMT genes was analyzed using the Rat EMT PCR Array (PARN-090ZC, Qiagen, Hilden, Germany). RNA was extracted according to standard protocols and converted to first strand cDNA using the RT2 First Strand Kit. The template was added to an instrument-specific, ready-to-use RT2 SYBR Green qPCR Master Mix. The resulting

mixture was added to the wells (25 μL/well) of the PCR array plate containing the predispensed gene-specific primer sets (25 μL for the 96-well plates), and PCR was performed. The threshold cycle (Ct) values for all the genes on each PCR array were calculated using the instrument-specific software, and the fold changes in gene expression for pairwise comparison were calculated using the $\Delta\Delta$Ct method.

2.5. HPCs Transplantation. All procedures involving laboratory animals were in accordance with guidelines for The Care and Use of Laboratory Animals issued by the Animal Care and Use Committee of the Friendship Hospital, Capital Medical University. Liver fibrosis was induced in male rats by twice-a-week intraperitoneal injections of 0.2 mL/100 g bodyweight of CCl_4 mixed with olive oil (2 : 3) for 2 weeks. Two days after completion of the CCl_4 treatment, 5×10^6 HPCs pretreated with TGF-β1 for 12 h (TGF-β1 pre-HPC (12 h) group, $n = 6$), with TGF-β1 for 48 h (TGF-β1 pre-HPC (48 h) group, $n = 6$), or without TGF-β1 (HPC group, $n = 6$) were diluted in 500 μL of phosphate-buffered saline (PBS) and then transplanted slowly into the rat spleen using a 23-gauge needle [16]. Rats treated with CCl_4 for 2 weeks and transplanted with 500 μL of PBS were used as the control (PBS group, $n = 4$). Following transplantation, no additional CCl_4 was administered to any of the four groups. All animals were sacrificed at 4 weeks after cell transplantation, and blood and liver samples were collected upon euthanization.

2.6. Examination of Liver Injury. Serum aminotransferase (Jiancheng Institute of Biotechnology, Nanjing, China) levels and albumin (Abcam, Cambridge, UK) were determined as biochemical evidence of liver injury. All blood samples were collected at the end of the experiment.

2.7. Histological Examination. Liver samples were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned. Hematoxylin and Eosin and Sirius Red staining were performed. Each sample was independently assessed and scored by two pathologists blinded to the study protocol, according to a fibrosis score system recently published by Cong et al. [17]. The severity of fibrosis was categorized into seven stages (0–6), where 0 indicates no fibrosis and 6 indicates cirrhosis.

2.8. Determination of Serum Levels of Collagen-1. Serum levels of collagen-1 were determined using an enzyme-linked immunosorbent assay system (Blue Gene, China) according to the instructions supplied by the manufacturer.

2.9. Immunohistochemical Staining of α-Smooth Muscle Actin. Immunohistochemical analysis was performed using rabbit anti-α-SMA (diluted 1 : 100, Abcam, Cambridge, UK). The detailed method has been published previously [17]. Five fields in each section were randomly selected to calculate the ratio of positive expression area.

2.10. Immunofluorescence Staining of SERPINE1. HPCs were incubated in media containing different concentrations of PAI-039 in DMSO for 48 h, fixed with 100% methanol for 5 min at −20°C, and blocked with PBS containing 10% goat serum, 0.3 M glycine, 1% bovine serum albumin, and 0.1% Tween for 2 h at room temperature. The HPCs were then incubated with rabbit anti-SERPINE1 (diluted 1 : 100, Abcam, Cambridge, UK) at 4°C overnight. After washing three times in PBS, the primary antibodies were reacted with the corresponding Alexa Fluor 488-conjugated anti-IgG (diluted 1 : 500, Abcam, Cambridge, UK) at 37°C for 30 min. Sections were examined under an Olympus CX41 fluorescence microscope (Olympus, Japan).

2.11. Statistical Analysis. All data are expressed as the mean ± SD from three independent experiments. Differences between mean values of multiple groups were analyzed using the nonparametric analysis of variance test (SPSS Inc., Chicago, IL, USA). Comparison between two groups was made using Student's t-test. $P < 0.05$ was considered to be significant.

3. Results

3.1. TGF-β1-Induced HPCs Differentiated into Mesenchymal-Like Cells. The exposure of HPCs to TGF-β1 for 0, 6, 12, 24, 36, and 48 h gradually altered the cell morphology from a typical polygonal shape and cobblestone monolayer appearance to elongated, spindle-shaped cells. As shown in Figure 1(a), we found that HPCs treated with TGF-β1 began to present phenotypic changes after 12 h, and remarkable phenotypic changes occurred after 48 h.

Furthermore, we observed the altered expression of differentiation markers in HPCs treated with TGF-β1. As shown in Figure 1(b), α-SMA was induced as early as 12 h, while the expression of other markers of progenitor cells, including ALB and AFP, was inhibited 24 h after TGF-β1 treatment, suggesting that although α-SMA was increased at 12 h after TGF-β1 induction, the HPCs still possessed the phenotype of stem cells. Since TGF-β1 treatment for 12 h induced a high level of α-SMA expression compared to treatment for 6 or 48 h, we chose TGF-β1 treatment for 12 h for all of the subsequent experiments.

3.2. HPCs Pretreated with TGF-β1 for 12 h Inhibited HSCs Growth. To mimic the *in vivo* microenvironment of fibrosis, we cocultured HSCs and progenitors pretreated with TGF-β1 for different periods of time (Figure 2(a)). We observed the effects of HPC exposure to TGF-β1 on the activation of HSCs.

The morphological changes of HSCs were only observed after they were cocultured with progenitors pretreated with TGF-β1 for 12 h (Figure 2(b)). In this group, the HSCs lost their typical spindle shape and became round or oval with round nuclei. The number of cells did not increase. No morphological changes in the other groups were observed; they still carried a typical HSC appearance, including spindle-shaped, star-shaped, or irregular cell bodies with oval or elongated nuclei.

3.3. Preexposure of HPCs to TGF-β1 for Different Periods of Time Caused Different Activations of HSCs. Next, we

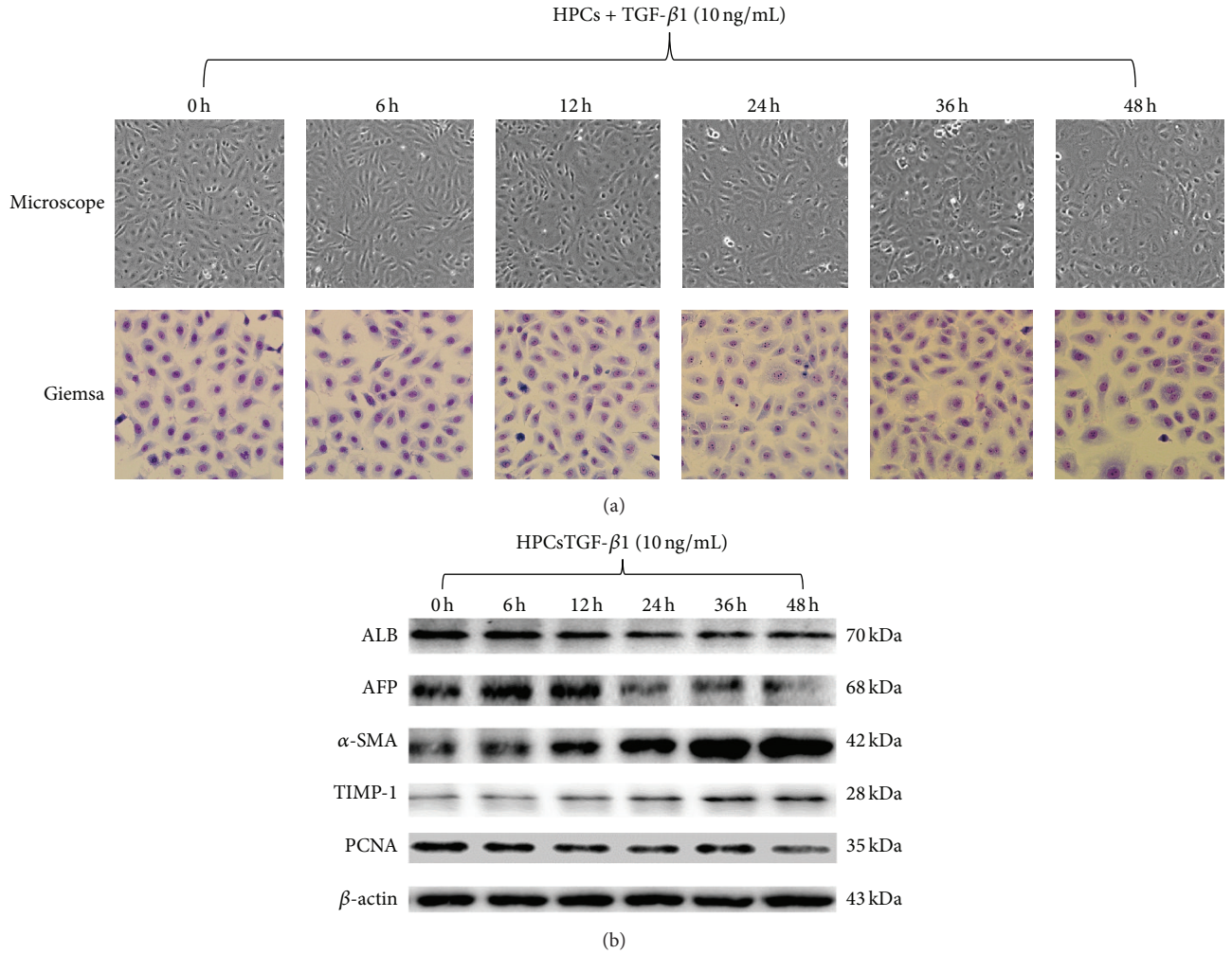


Figure 1: Transforming growth factor-beta 1 (TGF-β1) induced a mesenchymal morphology in HPCs. HPCs were cultured in the presence (10 ng/mL) or absence of TGF-β1 for 48 h. The phenotypic changes (transition toward a myofibroblast-like phenotype) were evaluated by phase-contrast microscopy and Giemsa staining (a). Changes in differentiation markers were evaluated by western blot (b).

analyzed the expression of fibrogenic genes in HSCs that were cocultured with HPCs pretreated with or without TGF-β1 for 6, 12, or 48 h. As shown in Figures 3(a)-3(b), HSCs cocultured with HPCs pretreated with TGF-β1 for 6 h showed no significant difference in α-SMA or TIMP-1 expression compared with no TGF-β1 treatment of HPCs; the HSCs cocultured with pretreated HPCs stimulated with TGF-β1 for 12 h had significantly lower α-SMA and TIMP-1 expression levels compared with untreated HPCs. However, HPCs pretreated with TGF-β1 for longer than 12 h had significantly increased α-SMA and TIMP1 expression levels, compared with untreated HPCs. These results demonstrated that preexposure of HPCs to TGF-β1 for different periods of time caused differential activation of HSCs.

Animals injected with HPCs exposed to TGF-β1 for 12 h had reduced progression of liver fibrosis and improved liver function.

We further evaluated the effects of HPCs exposed to TGF-β1 on liver fibrosis by an *in vivo* study. The rat model was injected with the HPCs that were exposed to TGF-β1 for 12 or 48 h. These time periods were selected based on the results of the *in vitro* study.

We stained liver sections with H&E and Sirius Red (Figure 4(a)). Semiquantitative grades of liver fibrosis in each group are shown in Table 1. The fibrosis scores demonstrated that the animals with either transplanted untreated HPCs or HPCs pretreated with TGF-β1 for 12 h had a reduced amount of fibrosis, compared with the spontaneous regression of fibrosis in the PBS group ($P < 0.05$). Importantly, compared with the HPC transplantation groups, the rats transplanted with HPCs pretreated with TGF-β1 for 12 h showed an additional significant reduction in liver fibrosis. The rats transplanted with HPCs pretreated with TGF-β1 for 48 h had a significantly increased fibrosis ($P < 0.05$, Table 1).

Because HSCs are important for liver fibrogenesis, we further examined whether the HPCs had an effect on HSCs activation. The rats transplanted with untreated HPCs or HPCs pretreated with TGF-β1 for 12 h showed significant suppression of HSC activation, as indicated by the decreased expression of α-SMA and collagen I (Figures 5(a)–5(c)),

Table 1: Quantitative evaluation of liver fibrosis.

Group	Number of rats	Liver fibrosis stage							Average stage
		S0	S1	S2	S3	S4	S5	S6	
PBS	4	0	0	0	1	2	1	0	4.0 ± 0.4
HPCs	6	0	0	3	3	0	0	0	$2.7 \pm 0.2^{*}$
HPCs pretreated with TGF-β1 for 12 h	6	0	4	2	0	0	0	0	$1.7 \pm 0.2^{*\#}$
HPCs pretreated with TGF-β1 for 48 h	6	0	0	0	0	1	3	2	$5.2 \pm 0.3^{*\#\$}$

The average scores are expressed as mean ± standard deviation (SD) in arbitrary units. $*$ indicates $P < 0.05$, compared to the group of PBS group. # indicates $P < 0.05$, compared to the group of HPCs group. $ indicates $P < 0.05$, compared to the group of HPCs pretreated with TGF-β1 for 12 h.

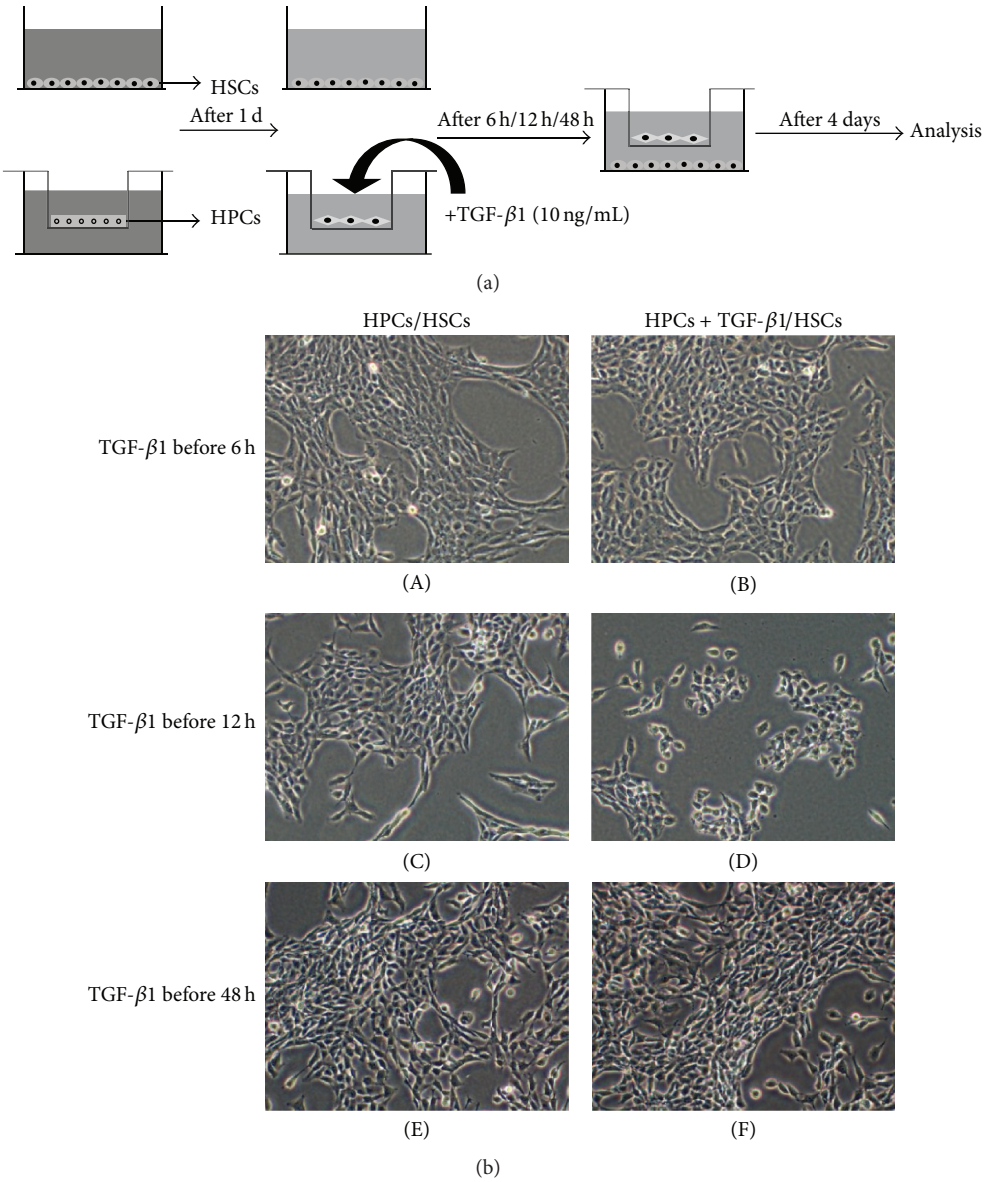


Figure 2: Influence of preexposure of HPCs to TGF-β1 for different periods of time on the morphology of HPCs. Schematic of the experimental procedure (a). After exposure to TGF-β1 for 12 h, the HPCs were cocultured with HSCs. The HSCs lost their typical spindle shape, while the other groups did not experience any morphological changes (b).

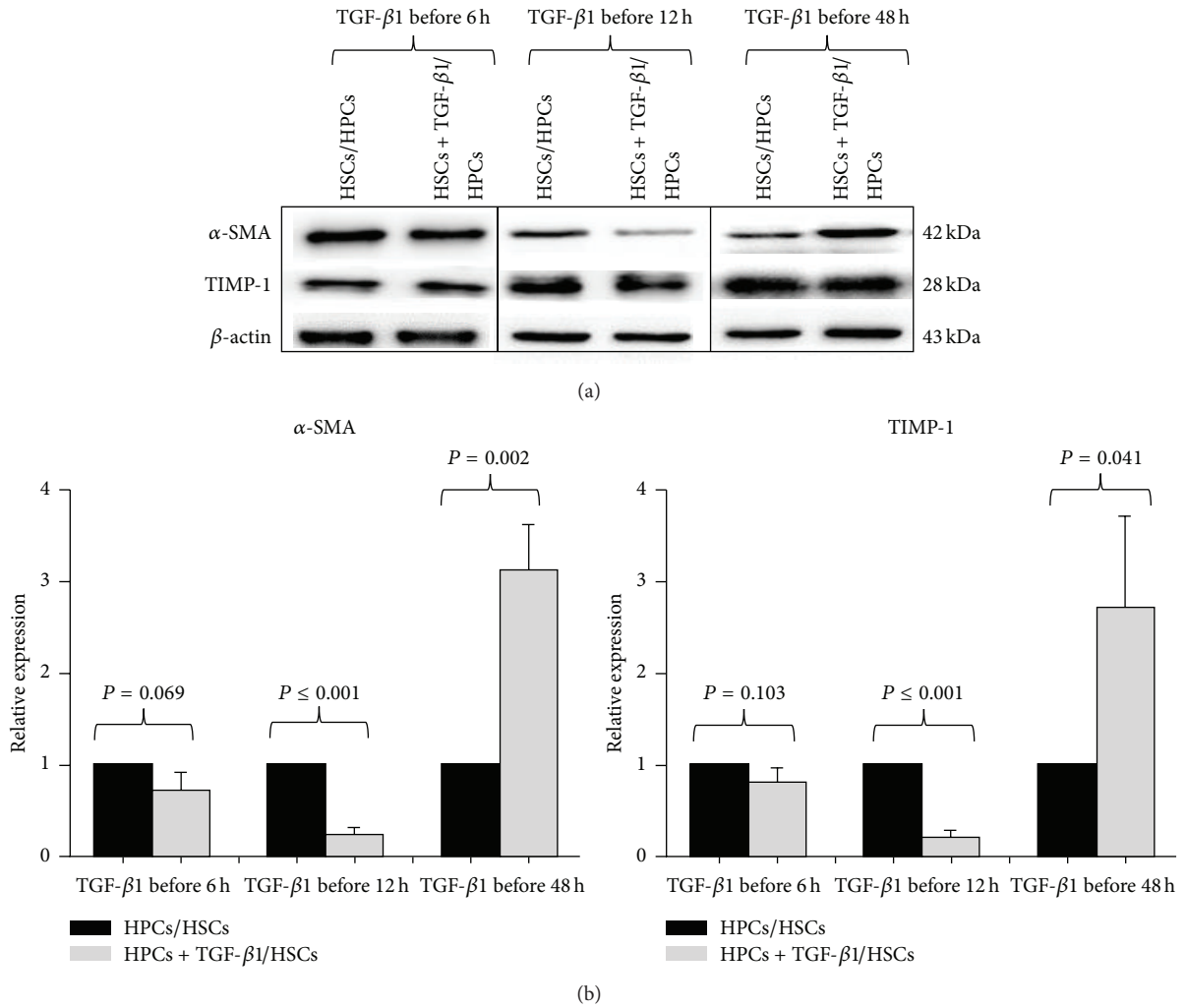


Figure 3: Influence of preexposure of HPCs to TGF-β1 for different periods of time on the activation of HPCs. The expression levels of α-SMA and TIMP-1 were measured by western blot (a). Quantification of the expression levels after normalization is shown in the lower panel (b). All data are expressed as the mean ± SD from three independent experiments.

suggesting that injection of HPCs exposed to TGF-β1 for an appropriate time is a novel therapeutic strategy to attenuate liver fibrosis.

In addition, we compared serum ALT and ALB levels across the four experimental groups. The serum ALT levels were significantly lower in the rats transplanted with the HPCs pretreated with TGF-β1 for 12 h than in the PBS injection group, and they tended to be even lower in the untreated progenitors-transplanted rats (Figure 5(d)), while the ALT levels in the rats transplanted with the progenitors pretreated with TGF-β1 for 48 h were the highest among the four groups. The serum ALB levels were significantly elevated in the untreated progenitors-transplanted rats compared with the control CCl_4-induced mice. As expected, the ALB level was further elevated in the group transplanted with HPCs pretreated with TGF-β1 for 12 h, compared with that in both the PBS- and untreated progenitors-transplanted groups. The rats transplanted with progenitors pretreated with TGF-β1 for 48 h showed the lowest levels among the four groups (Figure 5(e)).

3.4. SERPINE1 May Mediate the Effects of HPCs Pretreated with TGF-β1. We examined the gene expression profiles using an EMT PCR array and compared the relative expression levels of EMT genes in the HPCs exposed to TGF-β1 for 12 and 48 h. The layout of the EMT genes of the PCR array is shown in Figure 6(a). In addition, Figure 6(b) depicts the heat map showing the fold changes in the expression levels between the TGF-β1-treated progenitors and the control group. There were many red- and green-colored genes, which signaled both upregulated and downregulated gene expression by the TGF-β1-treated progenitors.

The genes with a 5-fold change in the expression level are listed in Table 1. Of 84 EMT-focused genes in this array, the expression of SERPINE1 and ITGA5 was different by 4.5-fold between untreated HPCs and HPCs treated with TGF-β1

Table 2: Variation in the ECM related gene expression between HPCs control and TGF-β1-treated HPCs in RT^2 profiler PCR array.

Group	Position	Gene bank	Symbol	Description	Upregulation or downregulation
HPC + TGF-β1 (12 h)/HPC	E7	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	12.8
	C6	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	−4.7
HPC + TGF-β1 (48 h)/HPC	A10	NM_000089	COL1A2	Collagen, type I, alpha 2	40.0
	D5	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	13.4
	G8	NM_004626	WNT11	Wingless-type MMTV integration site family, member 11	6.0
	E7	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	−5.1

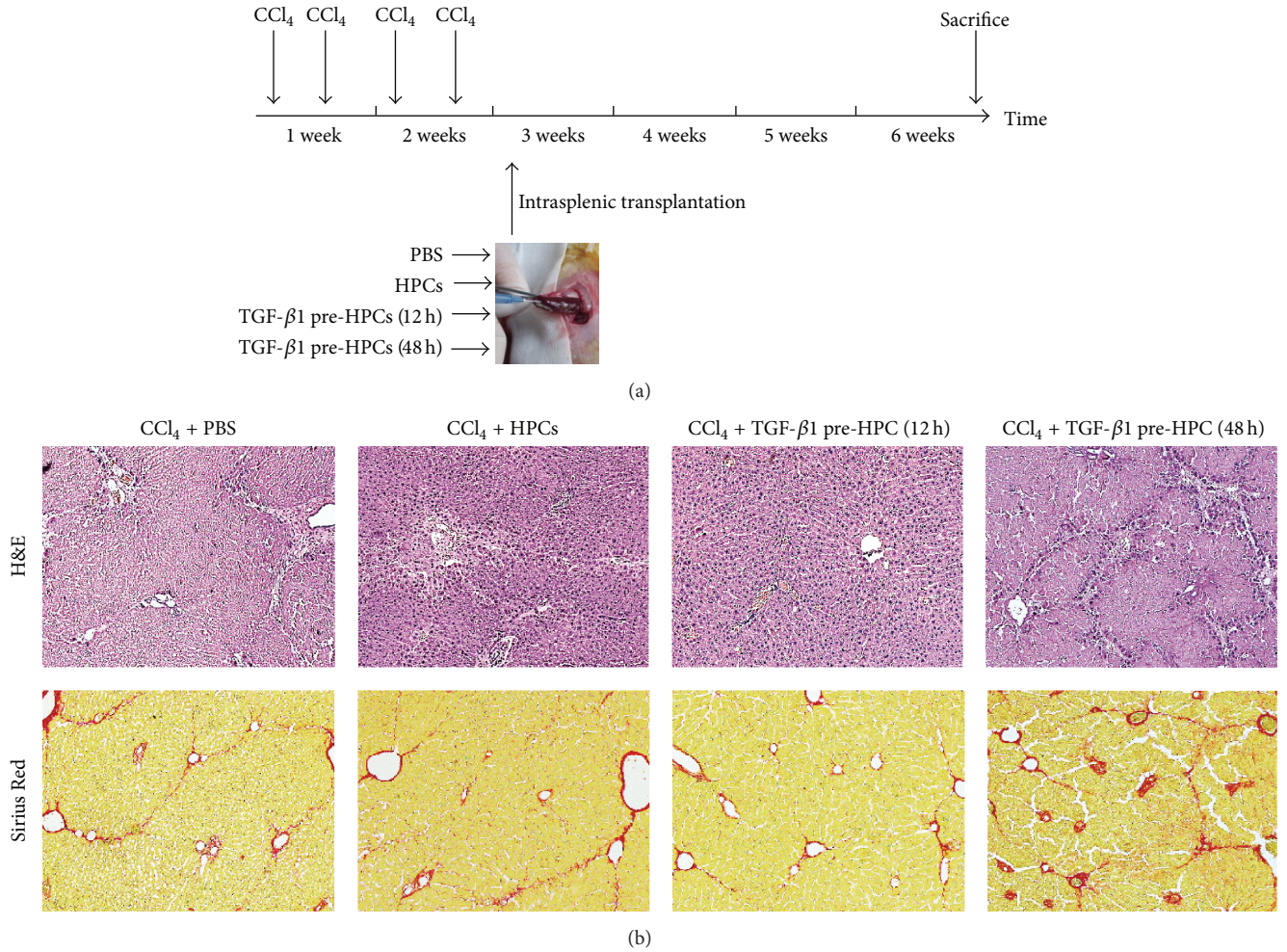


Figure 4: Therapeutic effects of transplanted HPCs, HPCs pretreated with TGF-β1 for 12 h, and HPCs pretreated with TGF-β1 for 48 h on recovery in the rat model with CCl_4-induced injury. Schematic description of the experiment (a). Hepatic collagen deposition was determined by H&E and Sirius Red staining (b).

for 12 h; a 5-fold difference in expression was detected in four genes between HPCs treated with TGF-β1 for 12 h and HPCs treated with TGF-β1 for 48 h. Collagen-1A2, matrix metalloproteinase-9, and WNT11 were upregulated, and only SERPINE1 appeared to be downregulated in the HPCs treated with TGF-β1 for 48 h (Table 2).

Real-time PCR was used to further verify the changed levels of SERPINE1 detected in the PCR array. The expression of SERPINE1 was upregulated in the progenitors treated with TGF-β1 for 12 h, and it was downregulated in the HPCs treated with TGF-β1 for 48 h, which further confirmed the previous results in the PCR array (Figure 6(c)).

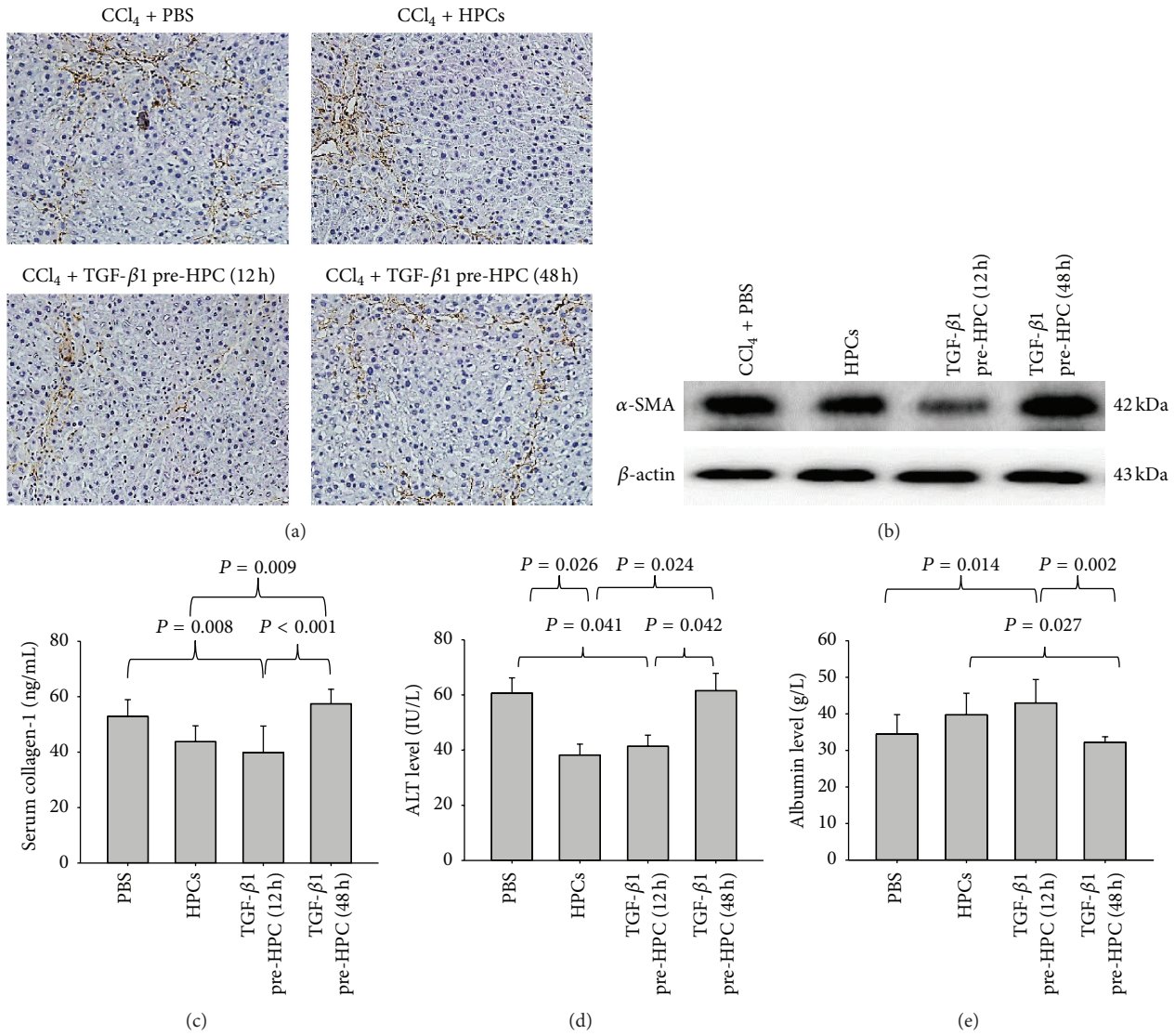


Figure 5: Fibrogenic markers of transplanted HPCs, HPCs pretreated with TGF-β1 for 12 h, and HPCs pretreated with TGF-β1 for 48 h on recovery in the rat model with CCl_4-induced injury. The expression of α-SMA in liver tissues was detected by immunohistochemistry (a) and western blot (b). The expression levels of collagen-1 in liver tissues were measured by an enzyme-linked immunosorbent assay (c). ALT (d) and ALB (e) in the blood samples collected at the end of the experiment were analyzed. All data are expressed as the mean ± SD from three independent experiments.

3.5. Inhibition of SERPINE1 in HPCs Prevented the Response of HSCs to HPCs. To further confirm the role of SERPINE1 in mediating the response of HSCs to progenitors, we used a chemical inhibitor (PAI-039) that blocks SERPINE1 expression to determine whether the response of HSCs to progenitors could be interrupted.

We first determined the optimal concentration of SERPINE1 by a cell counting kit-8 assay. Treatment with PAI-039 at ≥15 μM for 24 or 48 h reduced cell viability significantly (data not shown). Thus, we selected 1, 5, and 10 μM PAI-039 to test inhibition of SERPINE1 expression. As shown in Figures 7(a)-7(b), 5 and 10 μM PAI-039 remarkably inhibited SERPINE1 expression in a dose-dependent manner ($P < 0.05$).

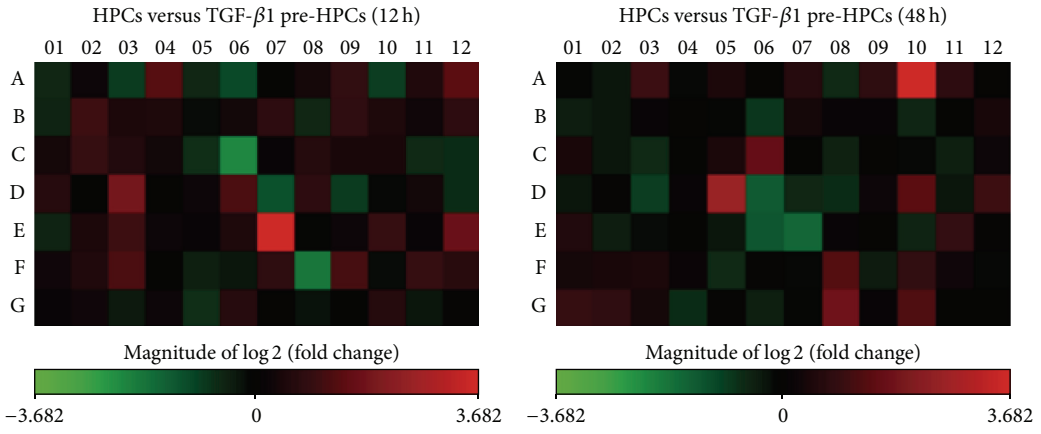
Finally, we examined whether inhibition of SERPINE1 affected the response of HSCs to progenitors. As shown in Figures 7(c)–7(e), PAI-039 efficiently blocked the response of HSCs to HPCs treated with TGF-β1 for 12 h. These results demonstrated that HPCs inhibited HSC activation largely *via* a SERPINE1-dependent mechanism.

4. Discussion

Little is known regarding the effect of HPCs on HSC activation and fibrosis under pathological conditions. TGF-β1 is a potent profibrogenic cytokine that is produced by activated mesenchymal cells upon liver injury. We assumed that the increased production of TGF-β1 in response to liver

	1	2	3	4	5	6	7	8	9	10	11	12
A	AHNAK	AKT1	BMP1	BMP7	CALD1	CAMK2N1	CAV2	CDH1	CDH2	COL1A2	COL3A1	COL5A2
B	CTNNB1	DSC2	DSP	EGFR	ERBB3	ESR1	F11R	FGFBP1	FN1	FOXC2	FZD7	GNG11
C	GSC	GSK3B	IGFBP4	IL1RN	ILK	ITGA5	ITGAV	ITGB1	JAG1	KRT14	KRT19	KRT7
D	MAP1B	MITF	MMP2	MMP3	MMP9	MSN	MST1R	NODAL	NOTCH1	NUDT13	OCLN	PDGFRB
E	PLEK2	PPPDE2	PTK2	PTP4A1	RAC1	RGS2	SERPINE1	SIP1	SMAD2	SNAI1	SNAI2	SNAI3
F	SOX10	SPARC	SPP1	STAT3	STEAP1	TCF3	TCF4	TFPI2	TGFB1	TGFB2	TGFB3	TIMP1
G	TMEFF1	TMEM132A	TSPAN13	TWIST1	VCAN	VIM	VPS13A	WNT11	WNT5A	WNT5B	ZEB1	ZEB2
H	B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

(a)



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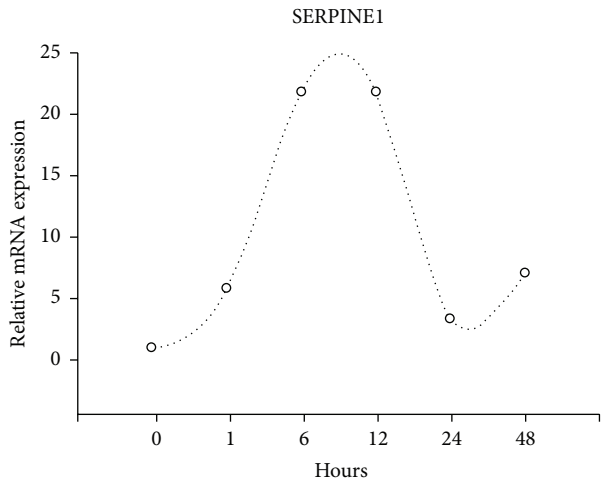

(c)

Figure 6: SERPINE1 is required for HPC-mediated amelioration of liver injury induced by an injection of CCl_4. The layout of the genes included in the EMT Pathway Finder PCR Array (a). The heat map of the variations in the expression levels of 84 genes between control HPCs and HPCs treated with TGF-β1 for 12 h or 48 h is shown as a fold increase or decrease (b). Validation of SERPINE1 expression by real-time PCR analysis (c). All data are expressed as the mean ± SD from three independent experiments.

injury would regulate the functions of HPCs, thus impacting the activity of HSCs in the repair of the damaged liver parenchyma. We aimed to investigate the potential TGF-β1–HPCs–HSCs regulatory axis. For this purpose, we cocultured HSCs with HPCs pretreated with TGF-β1 to mimic the pathological conditions with the assembled regulatory axis *in vitro*. In addition, to investigate the impacts of the TGF-β1-stimulated HPCs on liver fibrosis *in vivo*, HPCs pretreated with TGF-β1 were transplanted into rats with CCl_4-induced liver fibrosis. Furthermore, the potential role of SERPINE1

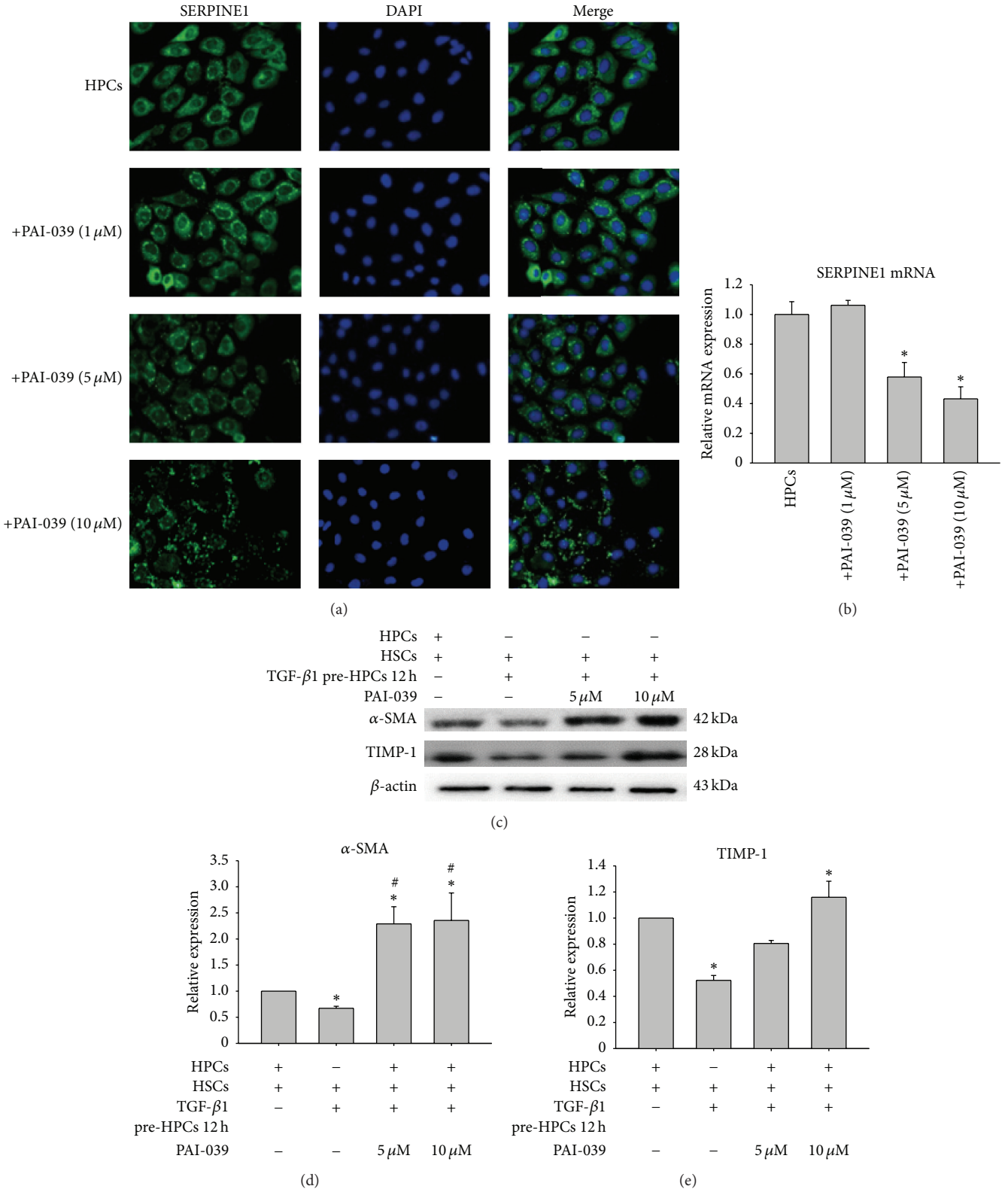

Figure 7: Influence of the SERPINE1 inhibitor on HSC activation after HSCs were cocultured with HPCs pretreated with TGF-β1 for 12 h. After incubation with various concentrations of the SERPINE1 inhibitor PAI-039 (0–10 μM) for 48 h, SERPINE1 expression was inhibited significantly as shown by immunofluorescence (a) and real-time PCR (b) analyses. Suppression of SERPINE1 expression prevented the response of HSCs to HPCs. Data are expressed as the mean ± SD from three independent experiments.

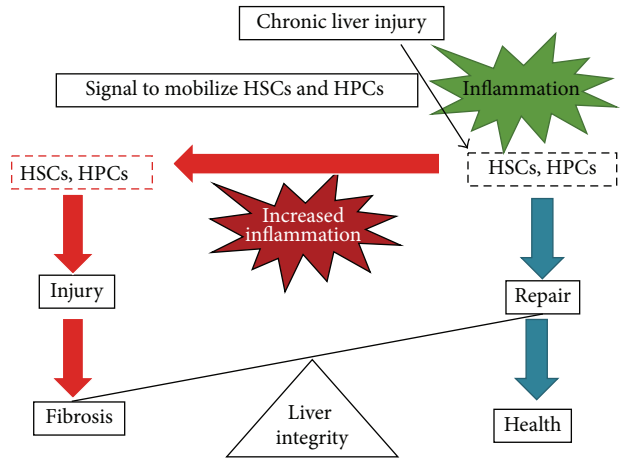

Figure 8: The schematic representation of the balance of HSCs and HPCs between injury and repair.

in mediating the effects of TGF-β1-pretreated HPCs on HSC activation was analyzed. We found that HPCs may function differently, depending on the time that the HPCs were sensitized with TGF-β1, and that HPCs may activate HSCs via reducing SERPINE1 expression.

The current study highlighted interesting but complicated interactions between TGF-β1, HPCs, and HSCs. It appears that TGF-β1 exerted profound impacts on HPCs, and the opposite impacts of HPCs on HSC activation and liver fibrosis were primarily determined by the time of TGF-β1 exposure, as shown in both coculture and transplantation studies. Our results demonstrated that a short-term exposure (12 h) of HPCs to TGF-β1 led to a reduction of the HSC number with a concomitant decrease in the activation of HSCs; more importantly, it prevented the progression of fibrosis and improved liver function. Meanwhile, a relatively longer exposure (48 h) led to increased activation of HSCs and worsened fibrosis in the liver. These findings suggested that the destination and ultimate function of pluripotent HPCs depend on the exposure to or regulation by TGF-β1, and it seems that the exposure time played a defining role in mediating pro- or antifibrogenesis. Our results not only confirmed different impacts on repairing the damaged liver tissue exerted by HPCs but importantly provided the evidence necessary to link the function of HPCs and TGF-β1 regulation.

We found that TGF-β1 could be a double-edged sword in the fibrotic process. It is known that TGF-β1 is a multifunctional cytokine whose function depends on which target cell it binds. It can regulate development, differentiation, regeneration, fibrogenesis, tumorigenesis, and metastasis [18, 19]. What is different from previous findings is that TGF-β1 may even exert two opposite regulations of the same HPCs in the progression course of chronic liver disease. TGF-β1 is widely regarded as a profibrogenic agent in chronic liver injury, and it stimulates myofibroblasts to produce cytokines and ECM. An overwhelming scar-forming wound-healing reaction can lead to distortion of hepatic architecture [18–20]. But a shorter TGF-β1 stimulation of HPCs may preferentially destine the differentiation of HPCs into hepatocytes that can restore the hepatic structure of the repaired tissue. Thus, TGF-β1 has beneficial effects. We also considered that TGF-β1 signaling proteins play a role in both maintaining the undifferentiated state of cells and initiating differentiation [21]. Nagy et al. have shown that treatment of rat liver epithelial cells with TGF-β induced the expression of ALB [22]. Similarly, our previous study demonstrated that connective tissue growth factor (CTGF), a downstream mediator of TGF-β1, can induce HPC differentiation into hepatocytes [23]. Further studies found that inhibition of the TGFβ–CTGF signaling axis by iloprost (an inhibitor of CTGF) resulted in a significant reduction of progenitor cell proliferation [10]. During hepatocyte regeneration and proliferation, TGF-β1 has an important tissue-mass-limiting cytostatic effect and controls inflammation by generating regulatory T cells [24]. Considering that TGF-β1 may exert antifibrotic activity, a simple inhibition of TGF-β1 may not be a wise approach to slow and reverse fibrosis.

The surrounding microenvironment is an important determinant to HPC behavior. HPCs are not only involved in tissue repair by differentiation into hepatocytes but also involved in fibrogenesis. As shown in Figure 8, when liver injury occurs, the environment at the injury site is filled with inflammatory chemo/cytokines that are released by infiltrated cells to promote the recruitment of stem or progenitor cells to the site of injury [25, 26]. If a repair is mainly achieved with hepatocytes, the original architecture of the liver will be restored. However, when liver injury is prolonged, the number of progenitor cells is decreased or the cells are not functional so that the repair fails. In this case, the balance is tipped toward a prolonged injury. Cell therapy should be designed to provide the appropriate cells within an adequate time frame and at a sufficient dose to restore the repair potential and capacity [27]. Wu et al. [28] have found that WB-F344 cells exposed to low doses of TGF-β1 for 18 weeks acquired tumorigenicity. Taken together, the surrounding microenvironment is the key element to control progenitor cell behavior and the balance between progenitor cell activation, proliferation, and differentiation. Improving the microenvironment should be taken into consideration

when progenitor cells are considered as a therapeutic option in chronic liver diseases.

Since preexposure of HPCs to TGF-β1 for 12 or 48 h elicited completely different effects on HSC activation, we found that elevated SERPINE1 levels may mediate HPC inhibition of HSC activation. The plasminogen activator and plasmin proteolytic cascades have an important role in stem cell-mediated regeneration, as most regenerative responses are associated with changes in the ECM [29]. von Montfort et al. have found that plasminogen activator inhibitor 1 plays a protective role in CCl_4-induced hepatic fibrosis in mice [30]. Thus, modulation of SERPINE1 expression may have a therapeutic impact on reversing the fibrotic process.

In summary, differential stimulation of HPCs by TGF-β1 for 12 h *versus* 48 h produced opposing anti- and pro-liver fibrotic effects. Our results further suggest that the antifibrotic function was possibly mediated through the upregulated expression of SERPINE1 in HPCs.

Conflict of Interests

No conflict of interests is declared by the authors.

Authors' Contribution

Ai-Ting Yang and Hong You participated in conception and design of research; Ai-Ting Yang, Dou-Dou Hu, Ya-Meng Sun, and Wen-Shan Zhao performed experiments; Ai-Ting Yang, Ping Wang, Min Cong, Tian-Hui Liu, Dong Zhang, and Hong You analyzed data; Ai-Ting Yang, Ji-Dong Jia, and Hong You interpreted results of experiments; Ai-Ting Yang and Hong You prepared the figures and drafted the paper; Ai-Ting Yang and Hong You edited and revised the paper. All authors approved the final version of the paper.

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Research Article

The Comparison of the Immunologic Properties of Stem Cells Isolated from Human Exfoliated Deciduous Teeth, Dental Pulp, and Dental Follicles

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Aim. To compare the effects of various mesenchymal stem cells, those isolated from human exfoliated deciduous teeth (SHEDs), dental pulp stem cells (DPSCs), and dental follicle stem cells (DFSCs), on human peripheral blood mononuclear cells (PBMCs). *Method*. Mesenchymal stem cells were isolated from three sources in the orofacial region. Characterization and PCR analyses were performed. Lymphocytes were isolated from healthy peripheral venous blood. Lymphocytes were cocultured with stem cells in the presence and absence of IFN-γ and stimulated with anti-CD2, anti-CD3, and anti-CD28 for 3 days. Then, lymphocyte proliferation, the number of $CD4^+FoxP3^+$ T regulatory cells, and the levels of Fas/Fas ligand, IL-4, IL-10, and IFN-γ in the culture supernatant were measured. *Results*. The DFSCs exhibited an enhanced differentiation capacity and an increased number of $CD4^+FoxP3^+$ T lymphocytes and suppressed the proliferation and apoptosis of PBMCs compared with SHEDs and DPSCs. The addition of IFN-γ augmented the proliferation of DFSCs. Furthermore, the DFSCs suppressed IL-4 and IFN-γ cytokine levels and enhanced IL-10 levels compared with the other cell sources. *Conclusion*. These results suggest that IFN-γ stimulates DFSCs by inducing an immunomodulatory effect on the PBMCs of healthy donors while suppressing apoptosis and proliferation and increasing the number of $CD4^+FoxP3^+$ cells.

1. Introduction

Stem cells possess self-renewal capacity and are able to differentiate into various types of cells in the body. Hence, they induce the repair and regeneration of tissues and organs, making stem cells an ideal source for cell therapy and regenerative medicine. Stem cells are primarily divided into two groups: embryonic stem cells and adult stem cells. Embryonic stem cells have pluripotent characteristics and can differentiate into most embryonic layers [1]. Due to ethical issues that surround the use of embryonic stem cells, most recent studies have focused on adult-derived stem cells.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells. MSC populations have been isolated from various sources, such as cord blood [2, 3], Wharton's jelly [4], the placenta [5, 6], bone marrow [7], teeth [8], and adipose tissue [9, 10].

A promising source of MSCs is dental tissue, which is easily accessible and can be isolated from many sources of the orofacial region, such as stem cells isolated from human exfoliated deciduous teeth (SHEDs) [8], dental pulp stem cells (DPSCs) [11], dental follicle stem cells (DFSCs) [12], and periodontal ligament stem cells (PDLSCs) [13]. These MSCs (SHED, DPSCs, and DFSCs) are able to differentiate into osteoblasts, adipocytes, and chondrocytes under suitable conditions. These cells also express MSC-specific markers, such as CD29, CD73, CD90, CD105, and CD166, and are negative for hematopoietic markers, including CD14, CD45, CD34, CD25, and CD28 [8, 11–13].

The immune system is a major defense mechanism that provides protection against foreign substances and produces a variety of cells and molecules that can recognize and eliminate the vast variety of possible foreign materials. The first protective barrier against microorganisms is the natural

(innate) immune response [14]. Regulatory T (Treg) cells play an important role in controlling immune responses to allergens, autoantigen tumor antigens, and infectious agents. These cells express the transcription factor fork head box P3 (FoxP3) [15, 16].

Recent studies on MSCs have reported their inhibitory effect on lymphocyte proliferation [17, 18]. Later, adaptive immune responses develop for specific pathogens. MSCs have immunosuppressive and immunomodulatory effects that are promising for the treatment of autoimmune diseases. MSCs suppress mitogen-stimulated memory and naive T cell responses. The suppressive effect of MSCs increases T cell viability and decreases the related cell apoptosis [19].

Most studies on T cells have reported mesenchymal stem-immunosuppressive cell interactions. T cells are the primary cellular effectors of the adaptive immune system and play a fundamental role in cell-mediated immunity. A number of reports have shown that the anti-inflammatory and immunomodulatory effects of MSCs are associated with the inhibition of T cell proliferation and cytokine production by effector T cell subsets.

MSCs affect the various types of T-helper cell subtypes (Th1, Th2, and Th17 cells) and Treg cells via various mechanisms. Human MSCs downregulate interferon-gamma expression via T-helper type 1, upregulate interleukin-4 expression via T-helper type 2 cells, increase the ratio of Treg cells, and cause shift from a proinflammatory environment towards an anti-inflammatory environment. The mechanisms that mediate the inhibitory effects of MSCs are not yet clearly defined [20]. Cell-cell contact and soluble factors are believed to induce suppressive effects. SHED cells were found to have a significant effect on Th17 cell inhibition compared with bone marrow mesenchymal stem cells (BMMSCs) [21].

In this study, we investigated the immunomodulatory effects of SHEDs, DPSCs, and DFSCs on the peripheral blood mononuclear cells (PBMCs) of healthy donors. Thus, coculturing of SHEDs, DPSCs, and DFSCs with PBMCs that were isolated from venous blood samples affected the immune system cells, indicating that stem cells play a primary role in the mechanisms of the immune system.

2. Materials and Methods

2.1. Isolation of Stem Cells. Dental pulp (DP), dental follicle (DF), and SHED cells were collected from the Marmara University Faculty of Dentistry Oral and Maxillofacial Surgery. The legitimate delegate of all patients provided informed consent according to the guidelines of the Ethics Committee of the Marmara University Medical Faculty in Istanbul, Turkey (09.2014.0015/70737436-050.06.04). Teeth were obtained from 3 children aged from 8 to 15 years and 3 adult donors aged 20–25 years undergoing third-molar extraction, and healthy human third-molar follicles were collected from the tooth buds of 3 healthy teeth aged 20–25 years. These pulps and follicles were transported in Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, NY 14072, USA) containing 1% penicillin/streptomycin (Gibco, USA). All laboratory work was performed in a laboratory in the Department of Pediatric Allergy-Immunology, Marmara University Research Hospital.

Dental pulps and follicles were isolated under sterile conditions. Pulps and follicles were enzymatically treated with 3 mg/mL collagenase type I (Gibco, USA) for 45 minutes at 37°C to completely digest pulps and follicles tissue. Then, 3 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin was added to digest the pulp and follicle tissue followed by centrifugation at 1200 rpm for 5 minutes. Cells pellets were obtained, and the supernatant was aspirated. DPSCs, SHEDs, and DFSCs were cultivated in T-25 flasks in a 5% CO_2 atmosphere under 37°C in culture medium composed of DMEM, 10% FBS, and 1% penicillin/streptomycin. The stem cells were washed with DPBS and provided with fresh culture medium. The culture medium was changed every 3 to 4 days until the cells reached confluence. The cells were detached with 0.25% trypsin-EDTA (Gibco, USA) when they reached 70–80% confluence. Adherent cells cultured for 3 passages were characterized and analyzed for specific surface markers. The cellular analyses and differentiation were performed using flow cytometry.

2.2. Flow Cytometry Analysis. To analyze the cell surface antigen expressions, the cells from the third passage were used. SHED, DFSCs, and DPSCs were incubated with antibodies for human CD73 phycoerythrin (PE), CD90 PE, CD146 fluorescein isothiocyanate (FITC), CD29 allophycocyanin (APC), CD105 PE, CD45 FITC, CD34 PE, CD14 PE, CD25 APC, and CD28 PE (BD Biosciences, San Diego, CA, USA) at room temperature in the dark. Control antibodies were phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated and allophycocyanin-conjugated mouse IgG1 and mouse IgG_2 (BD Biosciences, San Diego, CA, USA). The flow cytometry results were analyzed using BD FACS Calibur.

2.3. Differentiation of Stem Cells. To induce osteogenic (MesenCult, Stemcell Technologies, North America), adipogenic, and chondrogenic differentiation, a human MSC functional identification kit (Gibco, Grand Island, USA) was used. For differentiation, the cells were plated in 6-well plates (5×10^4 cell/well), and the differentiation medium was prepared according to the manufacturer's instructions and changed 3 times per week. After 14 days, the adipocytes and chondrocytes were stained with Oil Red O and Alcian blue, respectively, and after 28 days, the osteocytes were stained with Alizarin red.

2.4. Real-Time PCR Analysis. Total RNA was isolated from 10×10^6 SHEDs, DPSCs, and DFSCs at passage 3 using a high pure RNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. One microgram of total RNA was converted to cDNA using a transcriptor first strand cDNA synthesis kit (Roche, Mannheim, Germany). Equal amounts of cDNA were used for the real-time amplification of the target genes according to the manufacturer's recommendations using a LightCycler 480 Real-Time PCR

System (Roche Diagnostic, Mannheim, Germany). The gene expression of specific markers for MSCs, including ALPL, RUNX2, NANOG, NESTIN, NOTCH, and DSPP, was quantified relative to the housekeeping gene GAPDH. The RT-PCR conditions were as follows: preincubation for 10 minutes at 95°C for 1 cycle; amplification for 10 seconds at 95°C, 60°C for 30 seconds, and 72°C for 1 second for 45 cycles; and cooling for 10 seconds at 40°C for 1 cycle. The reaction mixture lacking cDNA was used as a negative control in each run. The real-time PCR results were analyzed using LightCycler software (version 2).

2.5. Lymphocyte Isolation. Peripheral blood was obtained from 10 healthy donors aged from 20 to 25 years and was added to heparin tubes. The legitimate delegate of all patients provided informed consent according to guidelines of the Ethical Committee of the Marmara University Medical Faculty in Istanbul, Turkey. PBMCs were obtained via Ficoll-Paque (GE Healthcare Bio-Sciences) density gradient from heparinized peripheral blood samples, as previously described [22]. The cells were cultured in RPMI (Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. PBMCs were stimulated with 5 μL of anti-CD2 (0.5 μg/mL, eBioscience, San Diego, CA)/anti-CD3 (0.5 μg/mL, Life Span Biosciences, USA)/anti-CD28 (0.5 μg/mL, Millipore, California) (CDmix) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h.

2.6. Coculture of Human PBMCs with SHEDs, DPSCs, and DFSCs. SHEDs, DPSCs, and DFSCs (5×10^4/well in a 48-well plate) were plated 48 h prior to the addition of ten times number of lymphocytes in the culture medium. SHEDs, DPSCs, DFSCs, and lymphocytes (1 : 10) were cocultured for 3 days. The cultures were stimulated using 5 μL of the CDmix and stimulated with and without 5 μL of IFN-γ (5 μg/mL, Millipore, CA, USA). Then, lymphocyte proliferation (carboxyfluorescein succinimidyl ester, CFSE), apoptosis (Fas/Fas ligand), $CD4^+FoxP3^+$ Treg cell expression and cytokine expression were analyzed via flow cytometry.

2.7. CFSE Assays for Lymphocyte Proliferation. After 3 days of coculturing, the cell proliferation behavior of the lymphocytes was quantified using carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Grand Island, USA). The cells were labeled with CFSE, and 10 μm CFSE dye was used to stain the lymphocytes after coculturing. The lymphocytes were stimulated in vitro with and without SHEDs, DPSCs, and DFSCs and were tested for CFSE dilution via flow cytometry.

2.8. Detection of Apoptosis of the Lymphocytes by Fas/Fas Ligand Labeling. After 3 days of coculturing, the apoptotic rate of the lymphocytes was quantified using a Fas/Fas ligand kit (BD Biosciences, USA), according to the manufacturer's instructions. The kit included CD95-FITC, CD178-biotin, IgG1 κ isotype control-biotin, and PE streptavidin.

2.9. $CD4^+FoxP3^+$ Treg Cell Assessment. After 3 days of coculturing, the Treg lymphocyte cells were quantified using a Human FoxP3 Buffer Set (BD Biosciences, USA). We determined the percentage of Treg ($CD4^+FoxP3^+$) markers that had developed from the lymphocytes. The cultures were assessed via flow cytometry using Human FoxP3 Buffer Kit according to the manufacturer's instructions. The kit included Buffer A, and anti-human CD4 and anti-human FoxP3 PE (BD Biosciences, USA).

2.10. Analysis of the Cytokine Expression Profiles. After 3 days of coculture, the supernatant percentages of IL-10, IL-4, and IFN-γ were measured. The cytokines were analyzed via flow cytometry using a BD cytometric bead array (CBA) human Th1/Th2/Th17 Kit (BD Biosciences, USA) according to the manufacturer's instructions.

2.11. Statistical Analyses. The differences between groups were analyzed via a one-way ANOVA test using SPSS v20 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 software. Graphs were generated using GraphPad Prism. P values less than 0.05 were considered significant.

3. Results

3.1. Isolation, Characterization, Differentiation, and Real-Time PCR Analysis of SHEDs, DPSCs, and DFSCs. SHEDs, DPSCs, and DFSCs attached sparsely to the culture flasks and exhibited a fibroblast-like and spindle-shaped morphology during the early days of incubation. The SHEDs began to proliferate in approximately 3 days and gradually formed small colonies (Figure 1(a)). The SHEDs reached 70% confluency in the primary culture 7 days after being plated in their first passage (P1). Most of the SHEDs exhibited fibroblast-like morphology in the later passages (P1, P2, and P3; Figures 1(b)–1(d)). The DPSCs began to proliferate in approximately 4-5 days and gradually formed small colonies (Figure 1(e)). The DPSCs reached 70% confluency in the primary culture 9 days after being plated in their first passage (P1). Most of the DPSCs exhibited a fibroblast-like morphology in the later passages (P1, P2, and P3; Figures 1(f)–1(h)). The DFSCs began to proliferate in approximately 2 days and gradually formed small colonies (Figure 1(a)). The DFSCs reached 70% confluency in the primary culture 5-6 days after being plated in their first passages (P1). Most of the DFSCs exhibited fibroblast-like morphology in the later passages (P1, P2, and P3; Figures 1(j)–1(l)). Then, immunophenotyping and differentiation of the three cell passages were observed.

The SHEDs, DPSCs, and DFSCs were analyzed via flow cytometry. These cells exhibited positive staining for CD29, CD73, CD90, CD105, and CD146 but were negative for CD14, CD25, CD28, CD34, and CD45 (Figures 2(a)–2(c)).

The SHEDs, DPSCs, and DFSCs differentiated into osteocytes, adipocytes, and chondrocytes. First, the osteogenic differentiation capability was investigated in vitro during a twenty-eight-day culture period in osteogenic induction medium. The SHEDs, DPSCs, and DFSCs were stained with Alizarin red, and the cells formed calcified bone nodule structures (Figures 3(a), 3(d), and 3(g)). Next, the in vitro adipogenic differentiation capability was assessed by culturing

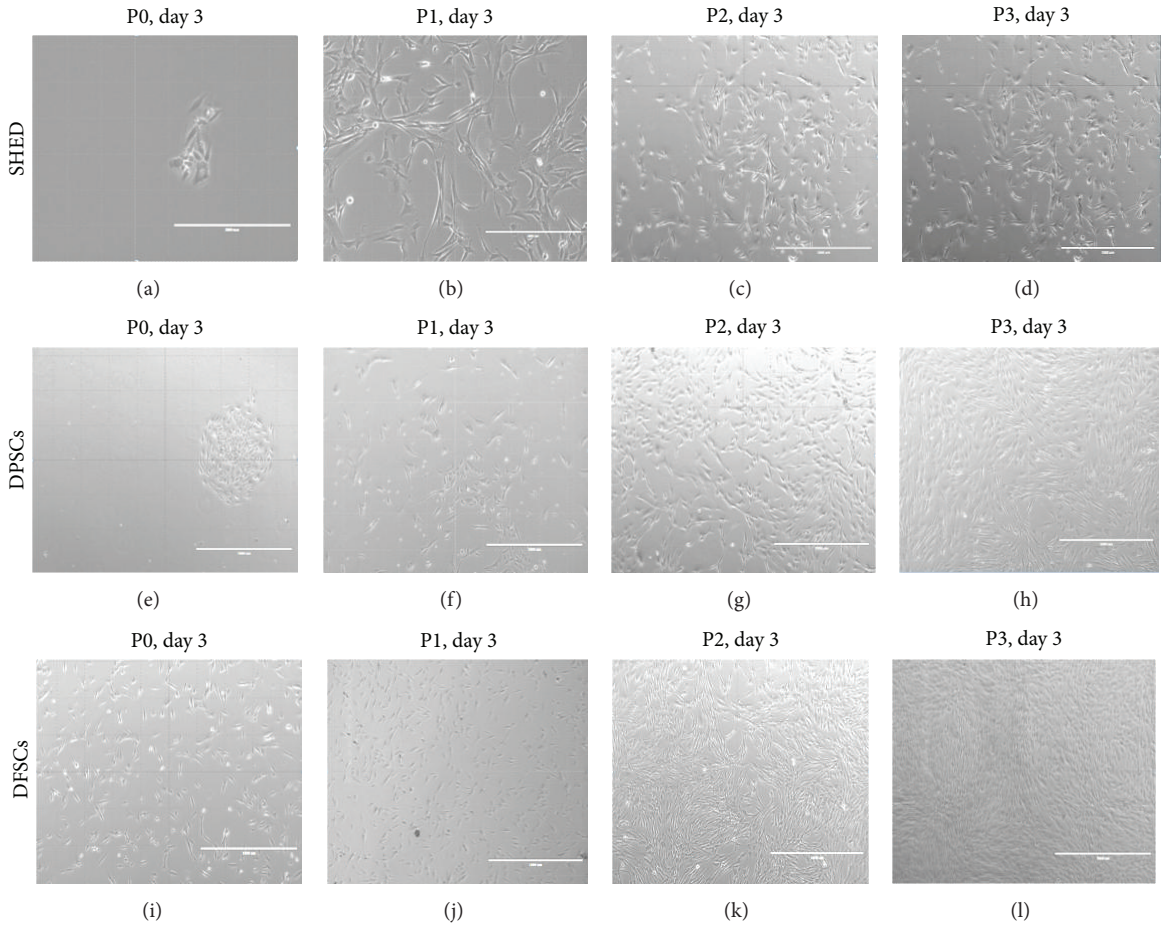

Figure 1: Morphological appearance of SHED, DPSCs, and DFSCs. Morphology of SHED, (a) P0: 3rd day, (b) P1: 3rd day, (c) P2: 3rd day, and (d) P3: 3rd day. Original magnifications: (a and b) ×20; (c and d) ×10. Morphology of DPSCs, (e) P0: 3rd day, (f) P1: 3rd day, (g) P2: 3rd day, and (h) P3: 3rd day. Original magnifications: (e, f, g, and h) ×10. Morphology of DFSCs, (i) P0: 3rd day, (j) P1: 3rd day, (k) P2: 3rd day, and (l) P3: 3rd day. Original magnifications: (i) ×20; (j, k, and l) ×10.

the cells in adipogenic induction medium and staining with Oil Red O. Intracellular lipid droplets were observed in these cells (Figures 3(b), 3(e), and 3(h)). Finally, the chondrogenic differentiation capability was investigated in vitro during a fourteen-day culture period in chondrogenic induction medium, and cell differentiation into chondrocytes was confirmed with Alcian blue staining. Intracellular proteoglycans were observed in these cells (Figures 3(c), 3(f), and 3(i)).

We analyzed the gene expression of specific markers in SHEDs, DPSCs, and DFSCs, including alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), NANOG, NESTIN, NOTCH, and dentin sialophosphoprotein (DSPP) relative to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The SHEDs, DPSCs, and DFSCs expressed ALPL, RUNX2, NANOG, NESTIN, NOTCH, and DSPP genes. The DFSCs expressed higher levels of all genes compared with SHEDs and DPSCs (Figures 4(a)–4(c)).

3.2. The DFSCs Suppressed Lymphocyte Proliferation Better Than the SHED Cells and DPSCs. Lymphocyte proliferation was quantified via flow cytometry. In the CFSE labeling assay, lymphocyte proliferation was suppressed at day 3. The proliferation of lymphocytes stimulated with the CDmix was significantly increased compared with unstimulated lymphocytes ($P < 0.001$). The proliferation of the lymphocytes stimulated with the CDmix in the presence and absence of IFN-γ was suppressed when the lymphocytes were cocultured with SHED cells, although the result was not significant ($P > 0.05$). The proliferation of the lymphocytes stimulated with the CDmix was suppressed when the lymphocytes were cocultured with DPSCs, although the result was not significant ($P > 0.05$). However, the proliferation of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$). The proliferation of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.05$) compared with the proliferation of the lymphocytes in the absence of IFN-γ. The proliferation of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with DFSCs ($P < 0.01$). In addition, the proliferation of the lymphocytes stimulated with the CDmix

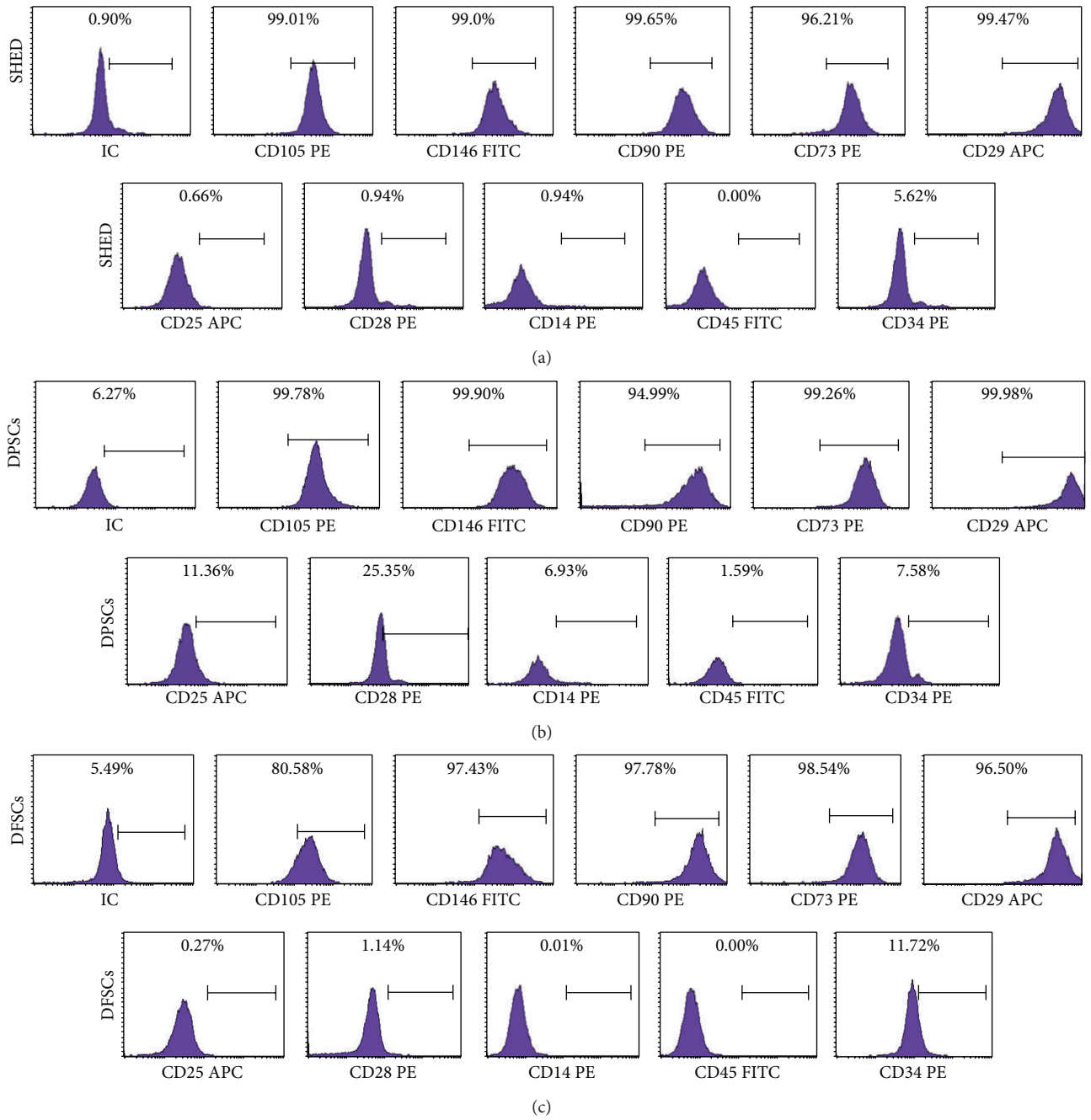

Figure 2: Representative flow cytometry analysis of cell surface markers in SHED, DPSCs, and DFSCs. Representative flow cytometry analysis of cell surface markers (a) on SHED in P3, (b) on DPSCs in P3, and (c) on DFSCs in P3.

in the presence of IFN-γ was significantly suppressed ($P < 0.001$), and the proliferation of the lymphocytes was significantly suppressed ($P < 0.05$) when stimulated with the CDmix in the presence of IFN-γ compared with in the absence of IFN-γ (Figures 5(a) and 5(b)).

3.3. The DFSCs Suppressed Apoptotic Effects Better Than SHED Cells and DPSCs. The Fas/Fas ligand rates of the lymphocytes were quantified via flow cytometry. The inhibitory effect of SHEDs, DPSCs, and DFSCs on the Fas (CD95) rate of the lymphocytes was significant. The Fas (CD95) rate of the lymphocytes stimulated with the CDmix was significantly increased ($P < 0.01$) compared with unstimulated lymphocytes. The Fas (CD95) rate of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with SHED cells ($P < 0.05$). In addition, the Fas (CD95) rate of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$). The Fas (CD95) rate of the lymphocytes stimulated with the CDmix was significantly

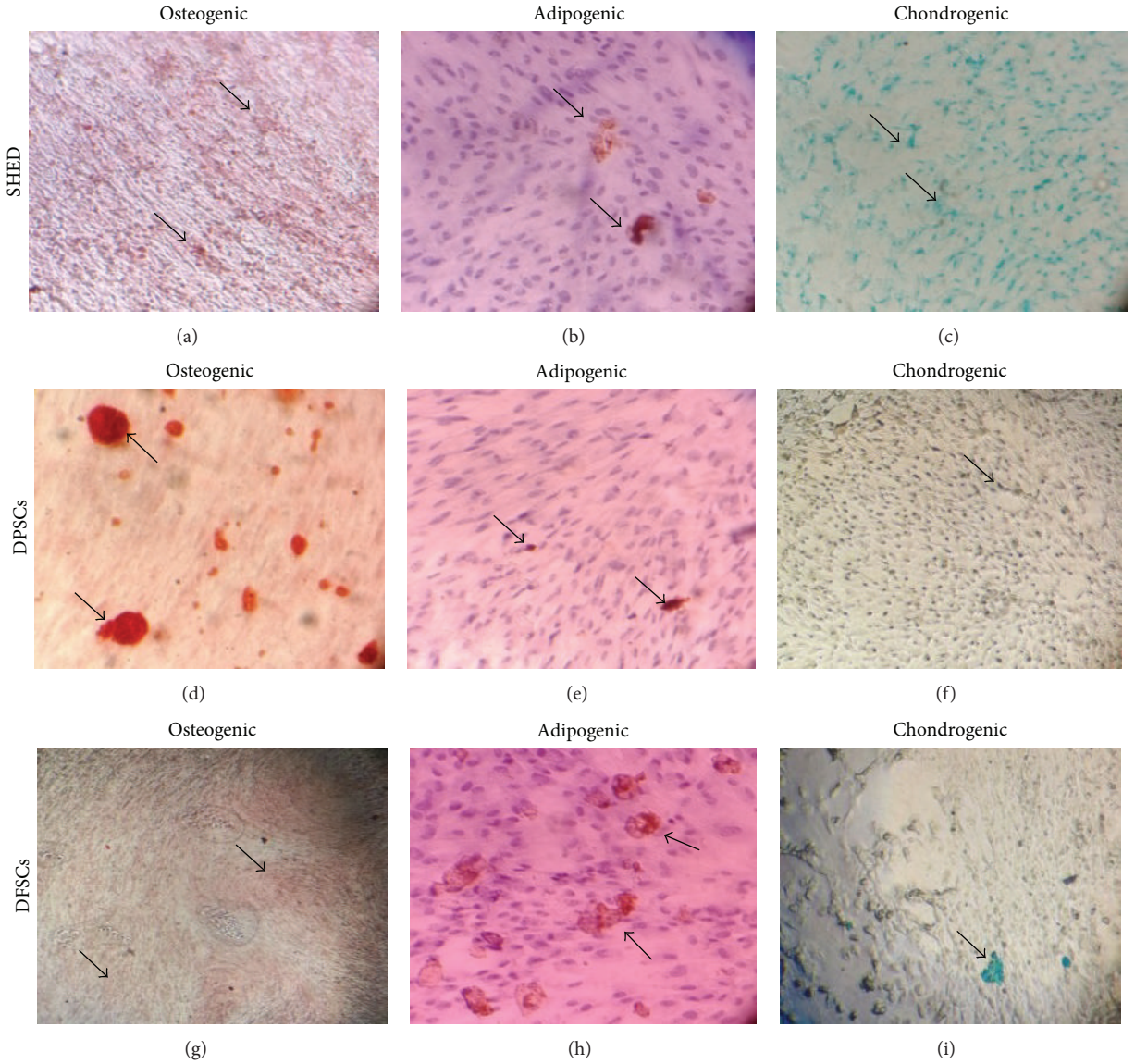

Figure 3: Differentiation analysis in SHED, DPSCs, and DFSCs. (a, d, g) Alizarin red staining of osteogenic induced SHED, DPSCs, and DFSCs. (b, e, h) Oil Red staining of adipogenic induced SHED, DPSCs, and DFSCs. (c, f, i) Alcian blue staining of chondrogenic induced SHED, DPSCs, and DFSCs.

suppressed when the lymphocytes were cocultured with the DPSCs ($P < 0.01$). In addition, the Fas (CD95) rate of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$). The Fas (CD95) rate of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with the DFSCs ($P < 0.05$). Finally, the Fas (CD95) rate of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$; Figures 6(a) and 6(b)).

The inhibitory effect of SHEDs, DPSCs, and DFSCs on the Fas ligand (CD178) rate of the lymphocytes was significant. The Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix was significantly increased ($P < 0.001$) compared with the unstimulated lymphocytes. The Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with SHED cells ($P < 0.05$). In addition, the Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.001$). The Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with the DPSCs ($P < 0.01$). In addition, the Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$). The Fas ligand (CD178) rate of the lymphocytes stimulated with the CDmix was significantly suppressed when the lymphocytes were cocultured with the DFSCs ($P < 0.05$). In addition, the Fas ligand (CD178) rate of the lymphocytes stimulated

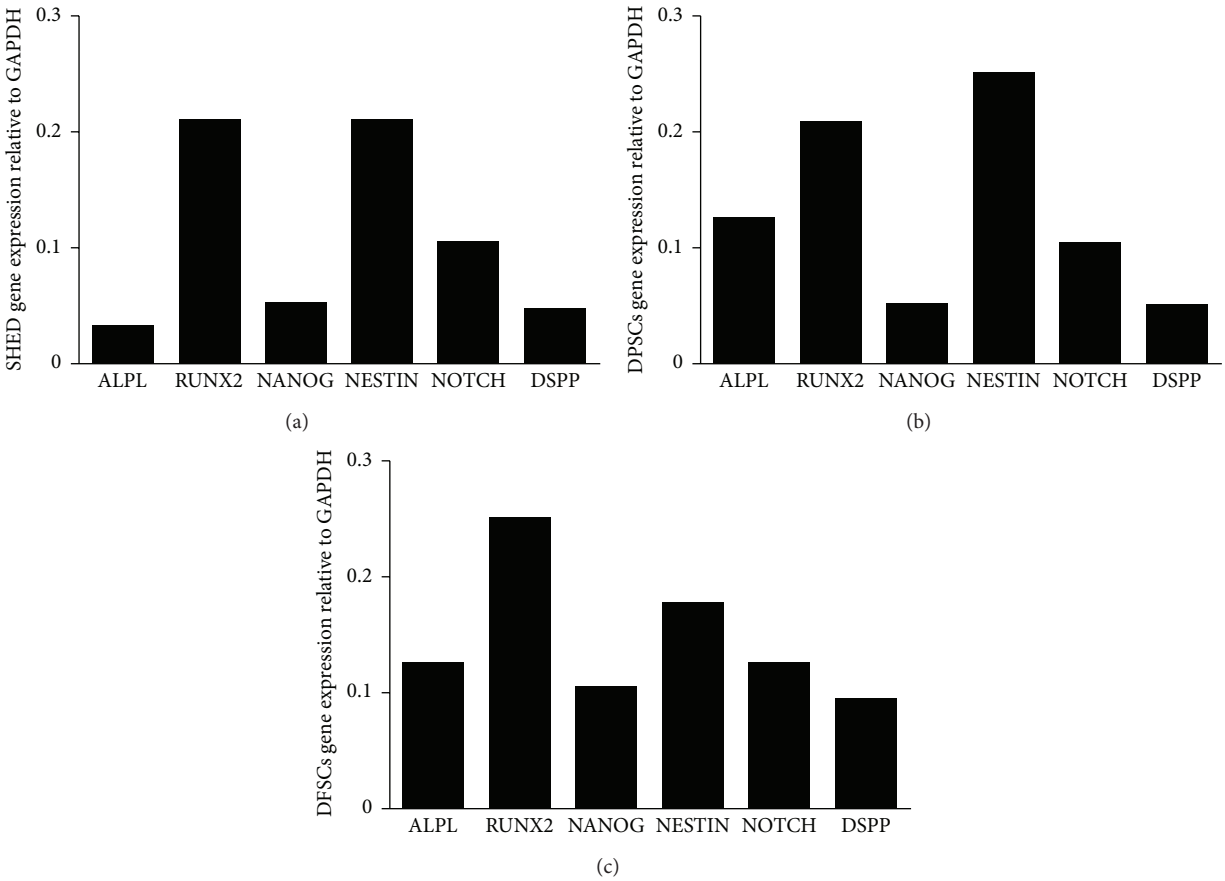

Figure 4: Gene expression of specific markers for SHED (a), DPSCs (b), and DFSCs (c), including ALPL (alkaline phosphatase), RUNX2 (runt-related transcription factor 2), NANOG, NESTIN, NOTCH, and DSPP (dentin sialophosphoprotein) according to housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was performed.

with the CDmix in the presence of IFN-γ was significantly suppressed ($P < 0.01$; Figures 7(a) and 7(b)).

3.4. Effects of SHEDs, DPSCs, and DFSCs on $CD4^+FoxP3^+$ Treg Cell Expansion of Lymphocytes. We studied the effects of SHEDs, DPSCs, and DFSCs on the Treg frequency. $CD4^+FoxP3^+$ Treg cells were significantly induced by the stimulated lymphocytes with the CDmix compared with unstimulated lymphocytes ($P < 0.0001$). The $CD4^+FoxP3^+$ Treg cells were significantly induced when stimulated with the CDmix in the presence of IFN-γ lymphocytes cocultured with SHED cells ($P < 0.05$). $CD4^+FoxP3^+$ Treg cells were significantly induced when stimulated with CDmix in presence of IFN-γ lymphocytes cocultured with DPSCs ($P < 0.01$). $CD4^+FoxP3^+$ Treg cells were significantly induced when lymphocytes stimulated with the CDmix were cocultured with DFSCs ($P < 0.05$). In addition, the $CD4^+FoxP3^+$ Treg cells were significantly induced when stimulated with the CDmix in the presence of IFN-γ lymphocytes when cocultured with DFSCs ($P < 0.05$; Figures 8(a) and 8(b)).

3.5. Effects of SHEDs, DPSCs, and DFSCs on IL-10, IL-4, and IFN-γ Cytokine Expression by Lymphocytes. The expression levels of IL-10, IL-4, and IFN-γ were determined via flow cytometry. IL-10 was significantly induced when lymphocytes stimulated with the CDmix were cocultured with the DFSCs ($P < 0.01$) and SHEDs ($P < 0.05$). In addition, IL-10 was significantly induced when lymphocytes stimulated with the CDmix were cocultured with DPSCs ($P < 0.05$) and with the CDmix in the presence of IFN-γ when the lymphocytes were cocultured with DPSCs ($P < 0.01$; Figure 9(a)).

IL-4 was significantly inhibited when lymphocytes stimulated with the CDmix were cocultured with DFSCs, DPSCs, and SHEDs ($P < 0.05$). In addition, IL-4 was significantly inhibited when stimulated with the CDmix in the presence of IFN-γ when the lymphocytes were cocultured with DFSCs, DPSCs, and SHEDs ($P < 0.05$; Figure 9(b)).

IFN-γ was significantly inhibited when lymphocytes stimulated with the CDmix were cocultured with SHEDs ($P < 0.05$). IFN-γ was significantly inhibited when stimulated with the CDmix in the presence IFN-γ when the lymphocytes were cocultured with DFSCs and DPSCs ($P < 0.05$; Figure 9(c)).

4. Discussion

In this study, the immunological impact of SHEDs, DPSCs, and DFSCs was evaluated in vitro. MSCs were first described

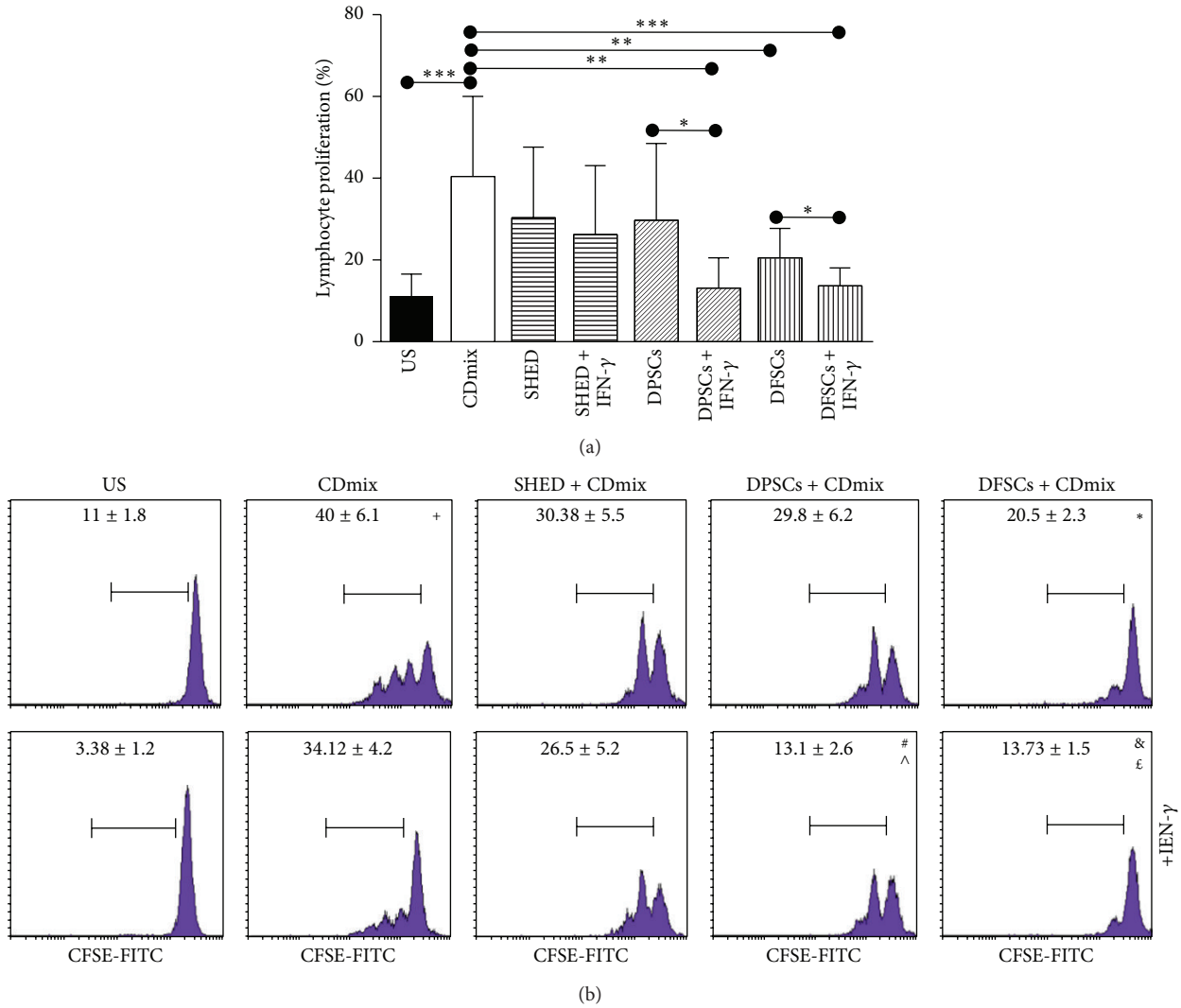

Figure 5: Inhibitory effect of SHED, DPSCs, and DFSCs on the proliferation of lymphocytes as detected by CFSE. (a) Inhibitory effect of SHED, DPSCs, and DFSCs on the proliferation of lymphocytes displayed statistically. (b) Inhibitory effect of SHED, DPSCs, and DFSCs on the proliferation of lymphocytes displayed by flow cytometry. $^{+}P < 0.001$, compared with US group. $^{*}P < 0.01$, compared with CDmix group. $^{\#}P < 0.05$, compared with DPSCs + CDmix group. $^{\&}P < 0.05$, compared with DFSCs + CDmix group. $^{\wedge}P < 0.01$, compared with CDmix group. $^{£}P < 0.001$, compared with CDmix group.

by Friedenstein in 1968 [7], and the minimal criteria for defining MSCs were developed by the International Society for Cellular Therapy. Accordingly, MSCs must be adherent to plastic surfaces in standard culture conditions and must express CD105, CD73, and CD90, whereas the expression of CD45, CD34, CD14, and CD11b must be absent, and MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [23].

MSCs can be isolated from various postnatal regions and tissues, such as cord blood [2, 3], Wharton's jelly [4], the placenta [5, 6], bone marrow [7], teeth [8], and adipose tissue [9, 10]. Dental tissues are promising tissue as a source of MSCs. MSCs in dental tissues have the potential to differentiate into other tissues. Dental tissue can provide dental mesenchymal stem cells, which include SHEDs, DPSCs, DFSCs, apical papilla mesenchymal stem cells (APSCs), and PDLSCs [24, 25]. Dental tissue MSCs represent a source that is easily accessible, and they have the potential to differentiate into other tissue cell lines and can be used to treat several diseases. We have isolated and used three types of dental tissue MSCs: SHEDs, DPSC, and DFSC. Furthermore, we investigated the effects of these MSCs on immune system cells. These cells can also be extracted with minimal invasiveness, unlike other cell types, and are therefore readily accessible.

Miura et al. isolated MSCs from human deciduous teeth; from each deciduous tooth, they obtained 15–20 cells. The authors showed that these cells were adherent to plastic surfaces and had characteristics of stromal cells. When the authors compared these cells to bone marrow stromal cells, they found that human deciduous teeth had a greater potential to proliferate and a higher multiplying potential than the bone marrow stromal cells. In addition, deciduous teeth

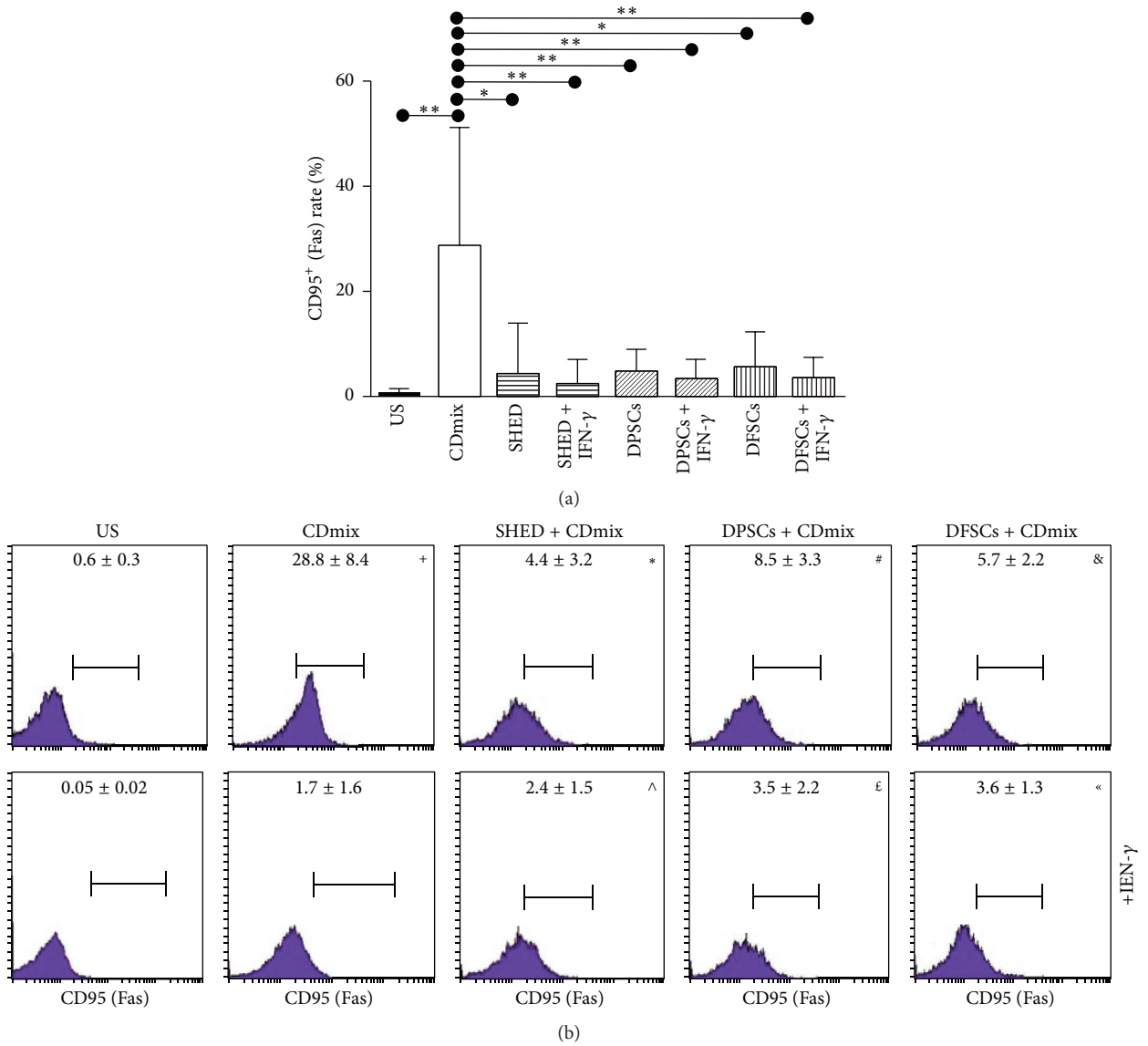

Figure 6: Inhibitory effect of SHED, DPSCs, and DFSCs on the apoptosis of lymphocytes as detected by Fas/FasLigand kit. (a) Inhibitory effect of SHED, DPSCs, and DFSCs on Fas (CD95) rate of lymphocytes displayed statistically. (b) Inhibitory effect of SHED, DPSCs, and DFSCs on Fas (CD95) rate of lymphocytes displayed by flow cytometry. $^{+}P < 0.01$, compared with US group. $^{*}P < 0.05$, compared with CDmix group. $^{\#}P < 0.01$, compared with CDmix group. $^{\&}P < 0.05$, compared with CDmix group. $^{\wedge}P < 0.01$, compared with CDmix group. $^{£}P < 0.01$, compared with CDmix group. $^{«}P < 0.01$, compared with CDmix group.

stem cells expressed STRO-1 and CD146 surface markers [8]. Suchánek et al. isolated SHEDs and showed that these stem cells expressed high levels of CD44, CD73, CD90, CD117, CD166, and HLA I, medium levels of CD29 and CD105, and low levels of CD45, CD63, and CD71 cell surface markers. Additionally, these cells were negative for CD18, CD31, CD34, CD49d, CD49e, CD106, CD133, CD184, CD197, CD146, and HLA II cell surface markers [1]. In our study, we isolated stem cells from human deciduous teeth according to the isolation protocol. These cells were adherent to plastic surfaces and exhibited a high proliferation potential. After isolation, we characterized the cells and showed that they expressed the CD146 surface marker and other stem cell markers, including CD73, CD90, CD105, and CD29, and that they were negative for CD14, CD45, CD34, CD25, and CD28 markers.

Tarle et al. compared the proliferation and differentiation potential and gene expressions of SHEDs and PDLSCs. In their study, they examined the osteogenic, adipogenic, and chondrogenic differentiation of SHEDs and PDLSCs and stained the differentiated cells with Alizarin red, Oil Red O, and von Kossa, respectively, to demonstrate the differentiation process [13]. In our study, we utilized osteogenic, adipogenic, and chondrogenic differentiation protocols and stained the cells with Alizarin red, Oil Red O, and Alcian blue, respectively, showing that SHEDs can differentiate into these three cell lines (osteogenic, adipogenic, and chondrogenic).

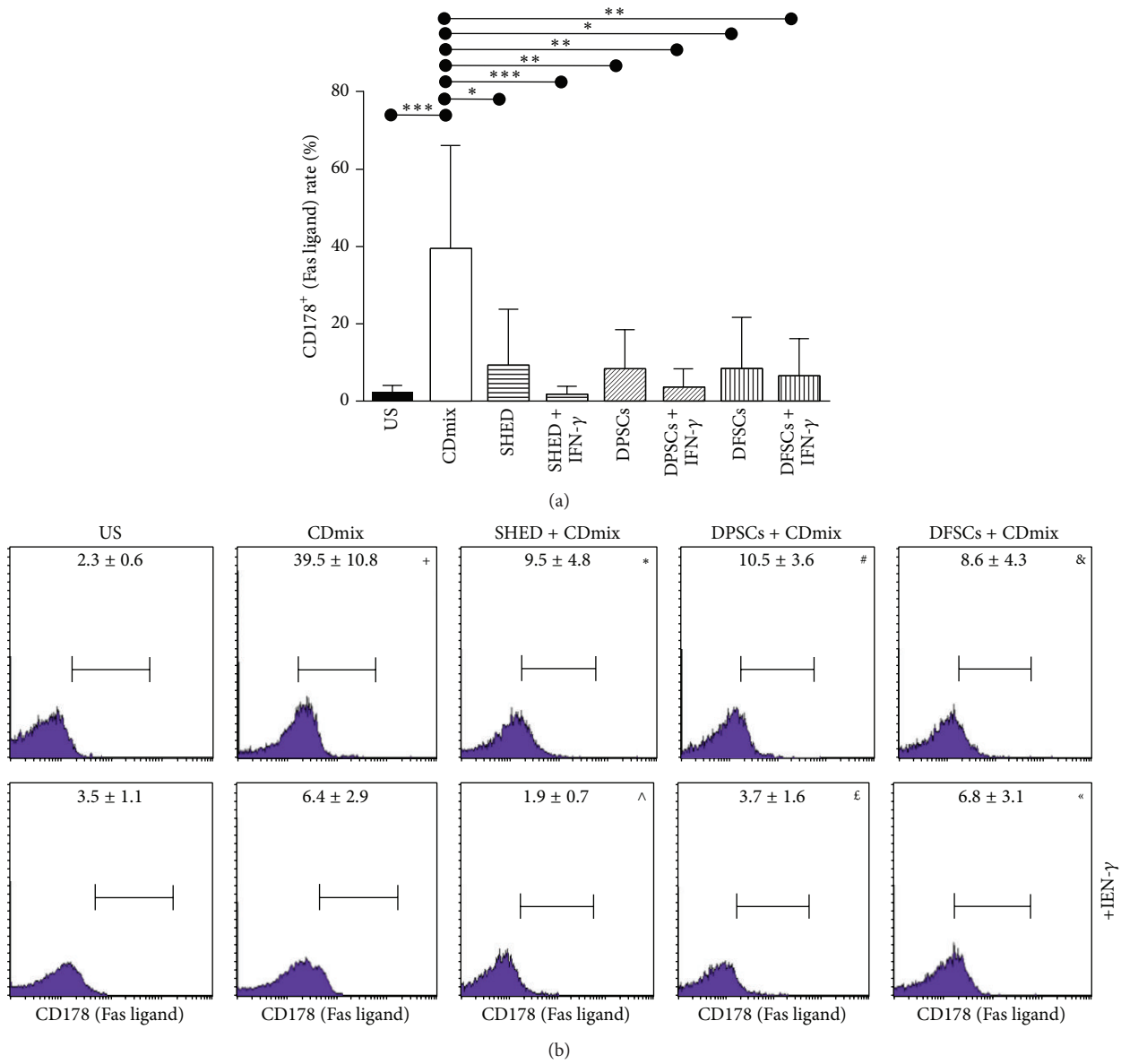

Figure 7: Inhibitory effect of SHED, DPSCs, and DFSCs on the apoptosis of lymphocytes as detected by Fas/FasLigand kit. (a) Inhibitory effect of SHED, DPSCs, and DFSCs on Fas ligand (CD178) rate of lymphocytes displayed statistically. (b) Inhibitory effect of SHED, DPSCs, and DFSCs on Fas ligand (CD178) rate of lymphocytes displayed by flow cytometry. $^{+}P < 0.001$, compared with US group. $^{*}P < 0.05$, compared with CDmix group. $^{\#}P < 0.01$, compared with CDmix group. $^{\&}P < 0.05$, compared with CDmix group. $^{\wedge}P < 0.001$, compared with CDmix group. $^{£}P < 0.01$, compared with CDmix group. $^{«}P < 0.01$, compared with CDmix group.

When osteogenic differentiation was performed, we demonstrated osteoblasts in the cell line. In adipogenic cultures, oil drops were observed, and, in the chondrogenic culture, cartilage and proteoglycans were observed after the staining protocol.

Gronthos et al. isolated MSCs from human dental pulp and found that dental pulp stem cells were inside the mineralized matrix and exhibited fibrosed and blood-veined tissue similar to pulp complexes. Isolated stem cells exhibited morphology of fibroblast-like cell colonies. These cells were compared with bone marrow stem cells and did not express hematopoietic lineage cell surface markers, including CD14, CD34, and CD45 [11]. Another study published in 2010 reported that isolated DPSCs exhibited fibroblast-like colonies and were negative for the CD11b, CD34, CD31, CD33, CD49b, and CD45 cell surface markers and positive for the CD44, CD73, and CD90 cell surface markers [26]. Doğan et al. showed that DPSCs were negative for CD34, CD45, and CD133 and positive for CD29, CD73, CD90, CD105, and CD166 [27]. We isolated dental pulp stem cells and showed that they were confluent on the 9th day in plastic culture flasks. The cells exhibited morphology of

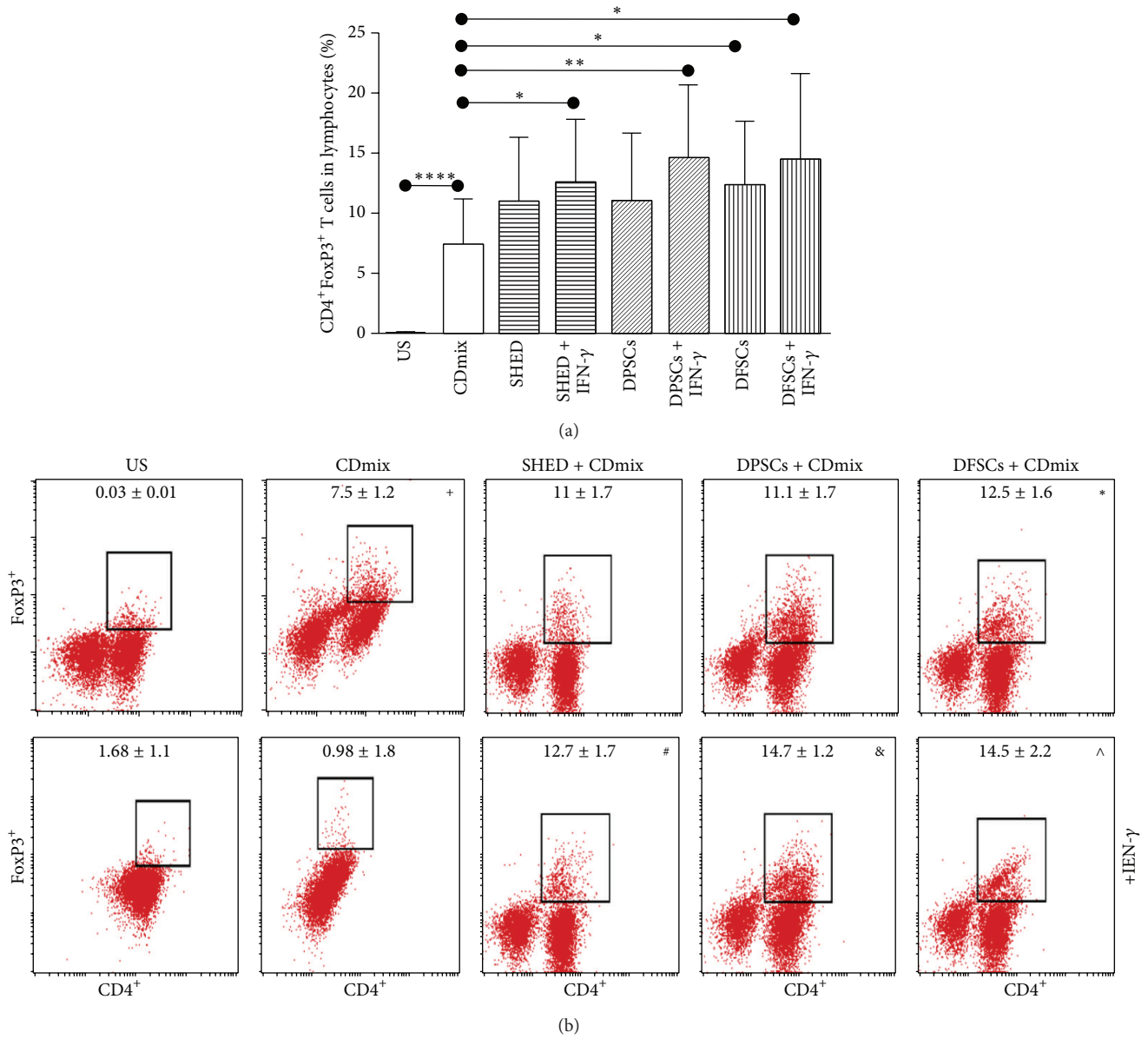

FIGURE 8: We therefore studied the effect of SHED, DPSCs, and DFSCs on Treg frequency. (a) Induced effects of SHED, DPSCs, and DFSCs on $CD4^+FoxP3^+$ Treg cells were displayed statistically. (b) Induced effects of SHED, DPSCs, and DFSCs on $CD4^+FoxP3^+$ Treg cells were displayed by flow cytometry. $^+P < 0.0001$, compared with US group. $^*P < 0.05$, compared with CDmix group. $^\#P < 0.05$, compared with CDmix group. $^\&P < 0.01$, compared with CDmix group. $^\wedge P < 0.05$, compared with CDmix group.

fibroblast-like colonies and were positive for CD73, CD90, CD105, CD29, and CD146 and negative for the hematopoietic markers CD14, CD45, CD34, CD25, and CD28.

Doğan et al. isolated DPSCs and performed immunocytochemical analyses and RT-PCR and induced differentiation to osteogenic, adipogenic, and chondrogenic cell lines. The differentiated cells were stained with von Kossa, Oil Red O, and Alcian blue, respectively [27]. Eslaminejad et al. isolated dental pulp stem cells and performed flow cytometry and RT-PCR, assessed the multiplying ratio, and performed odontogenic, chondrogenic, adipogenic, and osteogenic differentiation studies [26]. We isolated DPSCs and performed differentiation studies for osteogenic, adipogenic, and chondrogenic cell lines. After differentiation, we stained the cells with Alizarin red, Oil Red O, and Alcian blue, respectively, to show the differentiation potency. After staining, we examined the cells using a microscope, which revealed osteoblast nodules as osteogenic, oil drops as adipogenic, and proteoglycans as chondrogenic regarding the differentiation processes.

Dental follicle is a surrounding dental tissue that covers the dent. Dental follicles, cementum, periodontal ligaments, and alveolar bone marrow are mesenchymal tissues that together form the dent [28]. Dental follicle is a tissue that is easily accessible. Dental follicles are removed during surgical processes due to orthodontic diseases. Additionally, it is easy to isolate stem cells from dental follicles. Handa and

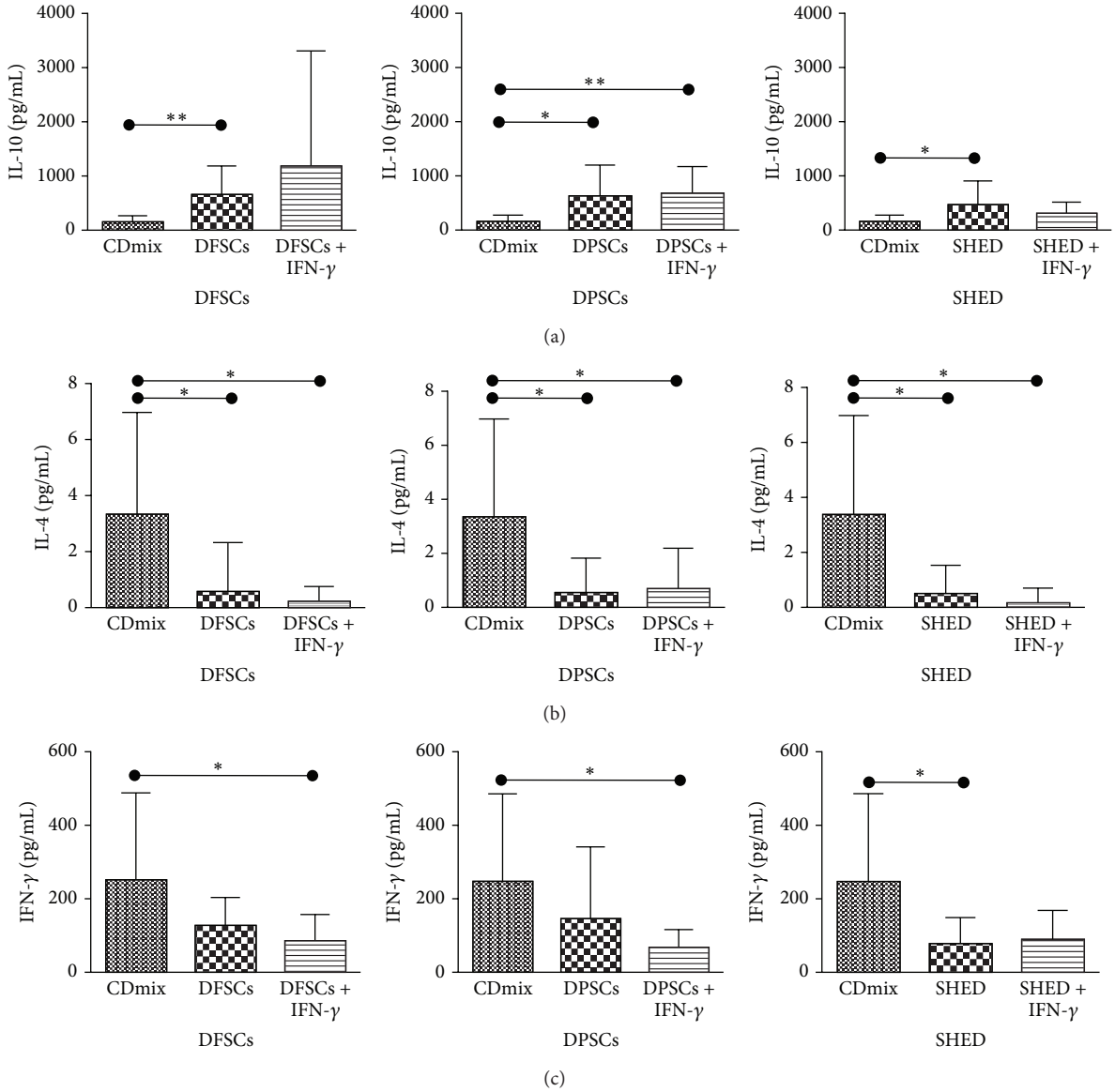

Figure 9: The immunoregulatory effects of SHED, DPSCs, and DFSCs on the expression of IL-10, IL-4, and IFN-γ cytokines in lymphocytes. (a) Induced effects of SHED, DPSCs, and DFSCs on the expression of IL-10 cytokines in lymphocytes. (b) Inhibited effects of SHED, DPSCs, and DFSCs on the expression of IL-4 cytokines in lymphocytes. (c) Inhibited effects of SHED, DPSCs, and DFSCs on the expression of IFN-γ cytokines in lymphocytes ($^{*}P < 0.05$, $^{**}P < 0.01$).

colleagues showed for the first time that DFSCs form a cementum-like matrix when the cells are differentiated in vitro. However, DFSCs form fibroblast-like colonies and are adherent to plastic surfaces [29]. Yokoi et al. showed that DFSCs are able to form periodontal ligaments [28]. Recent studies have also shown that DFSCs have a high proliferation capacity and can be differentiated toward osteogenic, adipogenic, and chondrogenic cell lines [30]. In our study, we isolated stem cells from dental follicles and achieved confluence on plastic surfaces on the 5th day.

Mori et al. isolated DFSCs and performed immunophenotype analyses. These cells expressed the surface markers CD73, CD146, CD90, CD44, CD105, and HLA I and were negative for CD45. Additionally, these cell lines showed a higher proliferation rate compared with bone marrow MSCs [12]. In contrast to the study by Mori et al., we showed that DFSCs do not express hematopoietic stem cell markers (CD14, CD45, CD34, CD25, and CD28). Additionally, DFSCs express CD73, CD90, CD105, CD29, and CD146, which are MSC markers.

Mori et al. showed that DFSCs could differentiate into osteogenic cell lines. We additionally showed that these cells have the potential to differentiate into osteogenic, adipogenic, and chondrogenic cell lines. After the differentiation process, we stained the cell lines with Alizarin red, Oil Red O, and Alcian blue to show the osteogenic, adipogenic, and

chondrogenic differentiation processes, respectively. After staining, we examined the cell lines using a microscope and observed that, in the osteogenic cell lines, osteoblasts were stained orange-red indicating osteoblast nodules, oil drops were stained red indicating adipogenic cell lines, and proteoglycans were stained blue indicating chondrogenic cell lines.

We compared three types of MSCs obtained from different dental sources. In addition, we compared the proliferation rate, differentiation potential, and gene expressions from RT-PCR among the DPSCs, SHEDs, and PDLSCs. Comparing the isolation steps, the colony-forming units, and the proliferation potential, the proliferation potential was higher in the DFSCs (DFSC > SHED > DPSC). Additionally, flow cytometry analyses were performed, revealing that the DPSCs expressed higher amounts of MSC markers compared with the SHED cells and DFSCs. The SHEDs expressed lower amounts of hematopoietic stem cell markers compared with DPSCs and DFSCs (SHED > DPSC > DFSC). As a result, DFSCs may be more effective due to their proliferation potential, colony-forming ability, and differentiation potential compared to other cell lines, in particular compared with SHEDs and DPSCs.

Miura and colleagues showed that SHEDs express the ALP gene as a marker of stromal and vascular system cell lines [8]. The ALP gene is a specific marker for nondifferentiated pluripotent stem cells. In addition, induced pluripotent stem cells express ALP [31]. It has also been shown that DFSCs express ALP when cultured in a hypoxic environment [32]. We showed that SHEDs, DPSCs, and DFSCs express the ALP gene. DFSCs expressed higher levels of the ALP gene compared with the SHEDs and DPSCs. In addition, we showed that SHEDs, DPSCs, and DFSCs expressed DSPP, RUNX2, NOTCH, and NESTIN genes. The DFSCs expressed higher levels of DSPP, RUNX2, and NOTCH genes. Compared with SHEDs and DPSCs, the DFSCs expressed higher levels of NANOG, a pluripotent stem cell marker.

Maintaining the continuity of biologic functions is the first target of cell-based therapy for the treatment injured tissues and organs. For this purpose, stem cells are the most important material used in cell-based therapies [33]. Stem cells from various sources (e.g., embryonic stem cells and bone marrow MSCs) have been used in experimental models both in vitro and in vivo. However, ethical considerations impede the use of embryonic stem cells, and the teratoma potential of these cell lines prevents their use in clinical trials [34]. Therefore, stem cell isolation studies now focus on MSCs derived from other tissues, such as muscle, cartilage, dental pulp, adipose tissue, neural tissue, and bone marrow [35].

MSC therapy is a promising biological therapy for the treatment of several diseases. Due to the simplicity of isolating MSCs, their rapid proliferation in culture conditions, and their promising differentiation properties compared to other cell lines, it has been suggested that most MSCs can be used in studies [36, 37]. In addition, MSCs are potential regulators of the immune system, and their teratoma risk is very low [37]. In this study, we examined the effect of MSCs isolated from deciduous teeth, dental pulp, and dental follicle on immune system cells. The results reported here are important for the use of these cell lines for treatment of several immune system diseases.

MSCs are highly promising due to their immunosuppressive and immunomodulatory effects on autoimmune diseases [38, 39]. There are limited experimental studies in this area. MSCs are used to treat diseases such as multiple sclerosis (MS) and amyotrophic lateral sclerosis and are thought to be effective in slowing or inducing regression of these diseases [40]. The immunogenicity of MSCs is very low, and they induce immunosuppressive effects, which represents the primary reason why the researchers have focused on MSCs in clinical applications. These cells do not express HLA-DR or costimulatory factors (CD80, CD86). MSCs stimulate Treg cells and prevent T cell activation, thus inhibiting B cell activation. MSCs present their immunosuppressive effects by suppressing T cell proliferation. In this study, we examined the immunosuppressive effects of SHEDs, DPSCs, and DFSCs by culturing each cell type with immune system cells from peripheral blood mononuclear cells.

MSCs suppress the response of naïve and memory T cells stimulated with mitogens. Demircan and colleagues showed that dental pulp stem cells induce a suppressive effect on T cell proliferation [41]. First, we examined the effects of SHEDs, DPSCs, and DFSCs on lymphocyte proliferation. All groups of MSCs induced suppressive effects on lymphocytes. Additionally, the cultures were separated into two groups: cultures with or without IFN-γ stimulation. The DFSCs were the most effective in suppressing lymphocytes in cultures with or without IFN-γ. Thus, stem cells exhibit inhibitory effects on T lymphocyte proliferation, in agreement with other studies. For the first time, we found that these cells exhibit the highest immunosuppressive effect in the presence of IFN-γ. In addition, the immunosuppressive efficiency of the SHEDs, DPSCs, and DFSCs was compared.

Some previous reports have shown that, after coculture, MSCs suppressed T lymphocytes, but the survival of T lymphocytes increased, and apoptosis decreased [42]. We also showed the suppression of lymphocyte apoptosis. Additionally, we performed a Fas/Fas ligand analysis to study apoptosis. We evaluated the three types of dental MSCs and compared their cocultures with lymphocytes. The results showed that all of the dental MSCs suppressed T lymphocyte apoptosis. In the cocultures with IFN-γ stimulation and the DFSCs cultured without IFN-γ, the suppression of T lymphocyte apoptosis was significantly decreased. Additionally, when compared to other dental MSCs, cocultures with SHEDs showed that expression of the Fas ligand (CD178) was significantly suppressed with the stimulation of IFN-γ.

Demircan and colleagues showed that dental stem cell and T cell proliferation cocultures increase the ratio of $CD4^+$ to $FoxP3^+$ T cells [41]. Flow cytometric analyses of the cocultures revealed that the numbers of $CD4^+FoxP3^+$ Treg cells increased compared with the lymphocyte cultures with and without MSCs. Additionally, IFN-γ stimulation increased the number of $CD4^+FoxP3^+$ Treg cells. The suppression effect of the DFSCs, SHEDs, and DPSCs was also compared. The DFSCs and DFSCs with IFN-γ simulation exhibited the highest increase in $CD4^+FoxP3^+$ Treg cells compared with the other dental MSCs.

Demircan and colleagues showed that dental stem cells and T cell proliferation cocultures increase the levels of IFN-γ cytokines [41]. Additionally, we analyzed the levels of IL-4 and IL-10 cytokines. After coculturing, the supernatants were collected, and a cytokine analysis was performed. The dental MSCs suppressed the expression of IL-4 and IFN-γ, whereas the expression of IL-10 was increased. IL-10 is secreted by macrophages and T lymphocytes and suppresses the expression of IL-12, IFN-γ, and TNF-α [43, 44].In our study, the MSCs increased the expression of IL-10 and suppressed IFN-γ.

5. Conclusions

In this study, we examined the immunologic effects of SHEDs, DPSCs, and DFSCs on lymphocytes of healthy donors in vitro. We observed that DFSCs were simply accessible and isolated and were able to differentiate into other cell types more efficiently compared with the other dental MSCs. The DFSCs, SHEDs, and DPSCs suppressed lymphocyte proliferation, increased Treg cells, decreased IL-4 and IFN-γ levels, and increased IL-10 levels. Additionally, DFSCs exhibited a higher immunomodulatory effect on immune system cells. Upon stimulation with IFN-γ, DFSCs exhibited immunomodulatory functions, suggesting that they can be used for the treatment of autoimmune, inflammatory, and allergic diseases.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgment

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Research Article

Delta-Like-1 Changes the Immunomodulatory Property of OP9 Cells

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As stromal cells and recently confirmed mesenchymal stem cells, OP9 cells support hematopoiesis stem cell (HSC) differentiation into the B lymphocyte lineage, yet Delta-like-1 (DL1) overexpressing OP9 (OP9DL1) cells promote the development of early T lymphocytes from HSC. However, the immunomodulatory capacity of OP9 or OP9DL1 on mature B and T cell proliferation has not been elucidated. Here, we show that OP9 and OP9DL1 have similar proliferation capacities and immunophenotypes except DL1 expression. Compared with OP9, OP9DL1 displayed more osteogenesis and less adipogenesis when cultured in the respective induction media. Both OP9 and OP9DL1 inhibited mature B and T cell proliferation. Furthermore, OP9 showed stronger inhibition on B cell proliferation and OP9DL1 exhibited stronger inhibition on T cell proliferation. With stimulation, both OP9 and OP9DL1 showed increased nitrate oxide (NO) production. The NO levels of OP9 were higher than that of OP9DL1 when stimulated with TNFα/IFNγ or LPS/IL4. Taken together, our study reveals a previously unrecognized role of OP9 and OP9DL1 in mature B and T cell proliferation. DL1 overexpression alone changed the properties of OP9 cells in addition to their role in early B cell development.

1. Introduction

The process of lymphocyte lineage differentiation and development from hematopoietic stem cells is highly influenced by soluble factors and cell contact-dependent signals within specific microenvironments, each of which supports the development of specific cell lineages. The bone marrow (BM) microenvironment supports B cell, but not T cell, lymphopoiesis [1], whereas the thymic environment is required for early T lymphocyte development [2]. *In vitro*, some BM-derived stromal cell lines have been applied to the formation of multiple hematopoietic cell lineages. One such cell line, OP9 stromal cells, has been found to support the development of multiple lineages, such as B cells, erythroid, and myeloid [3–5]; however, attempts to generate T cells from HSCs *in vitro* in the absence of the thymic microenvironment have been unsuccessful. The Notch signaling pathway is known to affect the developmental process of a variety of cell lineages [6–8]. When the OP9 cell line was retrovirally transduced to express the Notch ligand Delta-like-1 (namely, OP9DL1 cell line), it strongly promoted T cell lineage commitment and development and inhibited B cell lymphopoiesis *in vitro* [9].

Studies on the OP9 cell line demonstrated that OP9 are genuine mesenchymal stem cells (MSCs) with a multiple differentiation ability and immunomodulation capacity [10].

MSCs are multipotent stem cells capable of differentiating into multiple cell types, including osteoblasts and adipocytes, and can also regulate immune cell responses [11, 12]. Recently, a body of evidence [13–17] has indicated that MSCs produce a variety of cytokines such as nitric oxide (NO) and PGE_2 that display profound immunoregulatory properties by inhibiting the proliferation and function of several major types of immune cells, including natural killer cells, dendritic cells, and both T and B lymphocytes [18–20]. However, the underlying mechanisms of MSC immunomodulation have yet to be fully elucidated.

To date, studies on the effects of OP9 or OP9DL1 on T and B cells mainly focus on lineage commitment, differentiation, and function [7, 21, 22]. However, given the immunomodulatory properties of MSCs, the role of OP9 or OP9DL1 on mature T and B cell proliferation has not been investigated, which may be highly impactive considering the possible application of OP9 or OP9DL1 to T and B lymphocytes in *ex vivo* regeneration and expansion.

In this study, our results provide insight into the role of Delta-like-1 (DL1) in the properties of OP9. In addition to their different roles in promoting B or T cell development, OP9 and OP9DL1 show different capacities in inhibiting mature B or T cell proliferation.

2. Materials and Methods

2.1. Animals. C57BL/6 mice were purchased from the Laboratory Animal Center, Institute of Basic Medical Sciences, Beijing, China. Mice were maintained in a pathogen-free barrier facility, and all experiments were performed in accordance with the Institute of Basic Medical Sciences Guide for Laboratory Animals.

2.2. Cells. OP9 cells and Delta-like-1 overexpressing OP9 (OP9DL1) cell lines were gifts from Professor Bing Liu of Chinese PLA 307 Hospital [10] and were cultured in alpha minimum essential medium (α-MEM, Gibco) with 4 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 20% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. Bone marrow cells, flushed out from femurs and tibiae of 2~3-week-old mice, were filtered by 40 μm cell strainer and subsequently subjected to BD pharm lyse to remove red blood cells. $CD34^+$ cells were then selected from the bone marrow monocytes by $CD34^+$ MicroBead Kit (Miltenyi Biotec) and incubated together with OP9 and OPDL1 at ratio of 1 : 10 (OP9 or OP9DL1 : $CD34^+$ cells) in the α-MEM with 5 ng/mL Flt3L, 5 ng/mL IL-7, and 20% FBS. Peripheral T and B cells were isolated from murine spleens with CD3ε MicroBead Kit (Miltenyi Biotec) or B220 MicroBead Kit (Miltenyi Biotec), respectively. Next, the cells were activated with 1640 media containing 20% FBS in the presence of different stimulators (for T cells: PMA (50 ng/mL)/ion (1 μg/mL); for B cells: IL4 (25 ng/mL)/LPS (10 μg/mL)) for 24 h and then cultured alone or together with OP9 or OP9DL1 cells at different ratios for 36 h.

2.3. Cell Proliferation Assay. Cell proliferation was measured by BrdU incorporation and Ki67 assay. For BrdU incorporation, cells (1×10^5/well) were seeded in 6-well plate, 10 mM BrdU (BD) was added and incubated for 3 hours, and then the cells were collected and processed according to the protocol of BrdU flow kit (BD Pharmingen, San Diego, CA, USA). For Ki67 assay, cells (1×10^5/well) were seeded in 6-well plate, and 2 days later the cells were harvested and did according to the protocol of Ki67 Cell Proliferation Kit (Miltenyi Biotec Inc., Auburn, CA, USA). Data were collected on FACS Canto II (BD) and were analyzed with FlowJo software (TreeStar).

2.4. CFSE Staining. Peripheral $CD3^+$ T or $B220^+$ B cells were labeled with 5 μM carboxy fluorescein diacetate succinimidyl ester (CFSE, Invitrogen) for 7 min at 4°C. Labeling was terminated according to the manufacturer's protocol. After washing, cells were activated with stimulation factors as mentioned above for 24 h and then cultured with OP9 or OP9DL1. Cell division, as indicated by reduction of fluorescence intensity, was analyzed by flow cytometry.

2.5. Flow Cytometry. Antibodies anti-mouse CD29 (BD Pharmingen), CD31 (BioLegend), CD34 (BD Pharmingen), CD44 (BD Pharmingen), CD105 (BioLegend), CD45 (BD Pharmingen), Sca-l (BioLegend), DL1 (BD Pharmingen), B220 (BD Pharmingen), and CD3 (BD Pharmingen) were used for this study. The Ki67 kit was from Miltenyi Biotec Inc. (Auburn, CA, USA) and BrdU flow kit was from BD Pharmingen (San Diego, CA, USA). Data were collected on FACS Canto II (BD) and were analyzed with FlowJo software (TreeStar).

2.6. In Vitro Differentiation. For *in vitro* differentiation, cells were induced with osteogenic induction media containing 0.1 mM dexamethasone, 50 mM ascorbate-2 phosphate, and 10 mM glycerophosphate (Sigma). To induce adipogenic differentiation, cells were cultured in an adipogenic induction media containing 1 mM dexamethasone, 200 mM indomethacin, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 10 μg/mL insulin (Sigma). Alkaline phosphatase (ALP) assay and Oil Red O staining were performed as described previously.

2.7. Detection of NO. OP9 and OP9DL1 were stimulated with TNFα/INFγ, LPS/IL4, or PMA/ion for 6, 12, and 24 h, respectively. NO in culture supernatants was detected using a modified Griess reagent (Sigma-Aldrich). Briefly, all NO_3 was converted into NO_2 by nitrate reductase, and total NO_2 was detected by the Griess reaction.

2.8. Real-Time PCR. Total RNA was extracted with TRIZOL (Sigma) and reverse transcribed into cDNA with a reverse transcriptase kit (Takara). cDNA was used as a template in real-time PCR with SYBR Green reagent from TOYOBO (Shanghai, China) to determine specific

gene expression. Primer sequences were as follows: β-actin: CTTCCGCCTTAATACTTC (forward) and AAGCCTTCATACATCAAG (reverse); EBF1: ATGAAGAGGTTGGATTCTG (forward) and GCAGTTATTGTGTGATTC C (reverse); GATA3: CTGTCAGACCACCACCAC (forward) and CACACTCATTGATGTCAACC (reverse).

2.9. Statistical Analysis. Data are presented as mean ± SD. Statistical significance was assessed by unpaired two-tailed Student's t-test.

3. Results

3.1. The Immunophenotypes and the Proliferation Properties of OP9 and OP9DL1. It has been demonstrated that the OP9 cell line is genuine MSCs [10]. To examine whether OP9 cells overexpressing DL1 show a different immunophenotype than conventional OP9 cells, we analyzed surface markers indicated in Figure 1(a). The flow cytometric data showed that DL1 expression in OP9DL1 cells is significantly higher than that in OP9 cells. MSC surface molecules including CD29, CD44, and Sca-l were positive in both OP9 and OP9DL1 cells, while MSCs surface molecules CD105, hematopoietic lineage markers CD34 and CD45, and endothelial cell marker CD31 were almost absent in both OP9 and OP9DL1 cell lines. Next, we examined the proliferation ability of OP9 and OP9DL1 by Ki67 and BrdU labeling assays, respectively. The growth rate between OP9 and OP9DL1 did not differ significantly (Figures 1(b) and 1(c)).

3.2. Different Differentiation Capacities of OP9 and OP9DL1. Recently, there has been some debate on the effects of Notch receptor/ligand interaction on the differentiation of MSC into the osteocyte and adipocyte lineages [23, 24]. To investigate the effect of DL1 on OP9 differentiation, we examined the adipogenesis and osteogenesis of OP9 and OP9DL1 at different time intervals as indicated in Figures 2(a) and 2(b). Oil Red O staining revealed that the rate of adipocyte differentiation of OP9 cells was faster than that of OP9DL1 at each of the indicated time points (Figure 2(a)). Conversely, osteogenesis of OP9DL1 was more robust compared with that of OP9 cells, as determined by ALP staining (Figure 2(b)).

3.3. OP9 Supports the Development of BM CD34$^+$ Cells to B Cells, Whereas OP9DL1 Promotes the Differentiation of CD34$^+$ Cells to T Cells. To examine the ability of OP9 and OP9DL1 to support BM CD34$^+$ cell differentiation to B or T lymphoid lineages, coculture experiments of CD34$^+$ cells with OP9 or OP9DL1 were performed. CD34$^+$ cells were isolated from the femurs and tibiae of 2- or 3-week-old mice and were cultured with either OP9 or OP9DL1. The cells were collected and FACS analysis was performed at day 12 of coculture. As shown in Figures 3(a) and 3(b), there were B220$^+$ cells in CD34$^+$ cells with OP9 coculture and CD3$^+$ cells in CD34$^+$ cells with OP9DL1 coculture. Real-time PCR was used to determine the expression of EBF1 (B cell factor) or GATA3 (T cell factor). As expected, high EBF1 expression was found in the OP9 coculture group whereas high GATA3 expression was found in the OP9DL1 coculture group (Figures 3(c) and 3(d)). Similar to previous reports [5, 9], our results suggest that OP9 supports early B lymphocyte lineage development from CD34$^+$ while OP9DL1 promotes early T cell growth.

3.4. The Effect of OP9 and OP9DL1 on the Proliferation of Mature B Cells. The impact of OP9 or OP9DL1 on mature B cell proliferation has yet to be defined. Splenic B220$^+$ B cells were stained with CFSE and then incubated alone or together with OP9 or OP9DL1 at different ratios (OP9 or OP9DL1 versus B cells) in the presence of LPS plus IL4, respectively. As shown in Figure 4, both OP9 and OP9DL1 inhibit mature B cell proliferation as indicated by the reduction in CFSE intensity. Compared with OP9DL1, OP9 exhibited a much stronger immunosuppressive activity, and B cell proliferation was strikingly inhibited by OP9 at ratios as low as 1 : 80 (OP9 to B).

3.5. The Effect of OP9 and OP9DL1 on the Proliferation of Mature T Cells. As mentioned earlier, OP9DL1 promotes early T cell development, but its effect on mature T cell proliferation is unknown. To clarify the role of OP9DL1 on mature T cell growth, CD3$^+$ T cells stained with CFSE were cultured alone or together with OP9 or OP9DL1 cells at the indicated ratios (Figure 5). Mature T cell proliferation was directly assessed by CFSE labeling and monitoring of CFSE dilution. Unexpectedly, a marked reduction in T cell proliferation in the OP9DL1 cultures was observed compared with that in coculture with OP9 at each proportional hierarchy (Figure 5). Compared with the growth rate of T cells alone, OP9DL1 inhibits T cell proliferation more obviously with increasing ratios (OP9DL1 versus T cell), while OP9 only prohibits T cell growth at the highest ratio.

3.6. NO Production in OP9 and OP9DL1 Cells in the Presence of Different Stimulators. NO has been shown to be involved in the immunomodulation of MSCs to multiple immune cells [11]. To examine whether NO is related to the effect of OP9 and OP9DL1 on mature T or B cell proliferation, we assayed the NO level in culture supernatants from OP9 and OP9DL1 stimulated with TNFα/IFNγ, LPS/IL4, or PMA/ion for 6, 12, and 24 h, respectively (Figure 6). Our data showed that NO production was indeed increased in the culture media from each group of stimulation factors, regardless of stimulation duration of OP9 or OP9DL1, as compared with that of the no stimulation group. The NO levels of OP9 were higher than that of OP9DL1 when stimulated with TNFα/IFNγ or LPS/IL4 (Figure 6).

4. Discussion

It has been extensively demonstrated that OP9 promotes B lymphocyte lineage development and OP9DL1 contributes to the development of early T cells at the expense of B cell development [5, 9, 21, 25]. However, it was still unclear what role both OP9 and OP9DL1 cells have on mature T and B cell proliferation, respectively. Our findings demonstrated for the first time that OP9 exhibited strong immunosuppressive

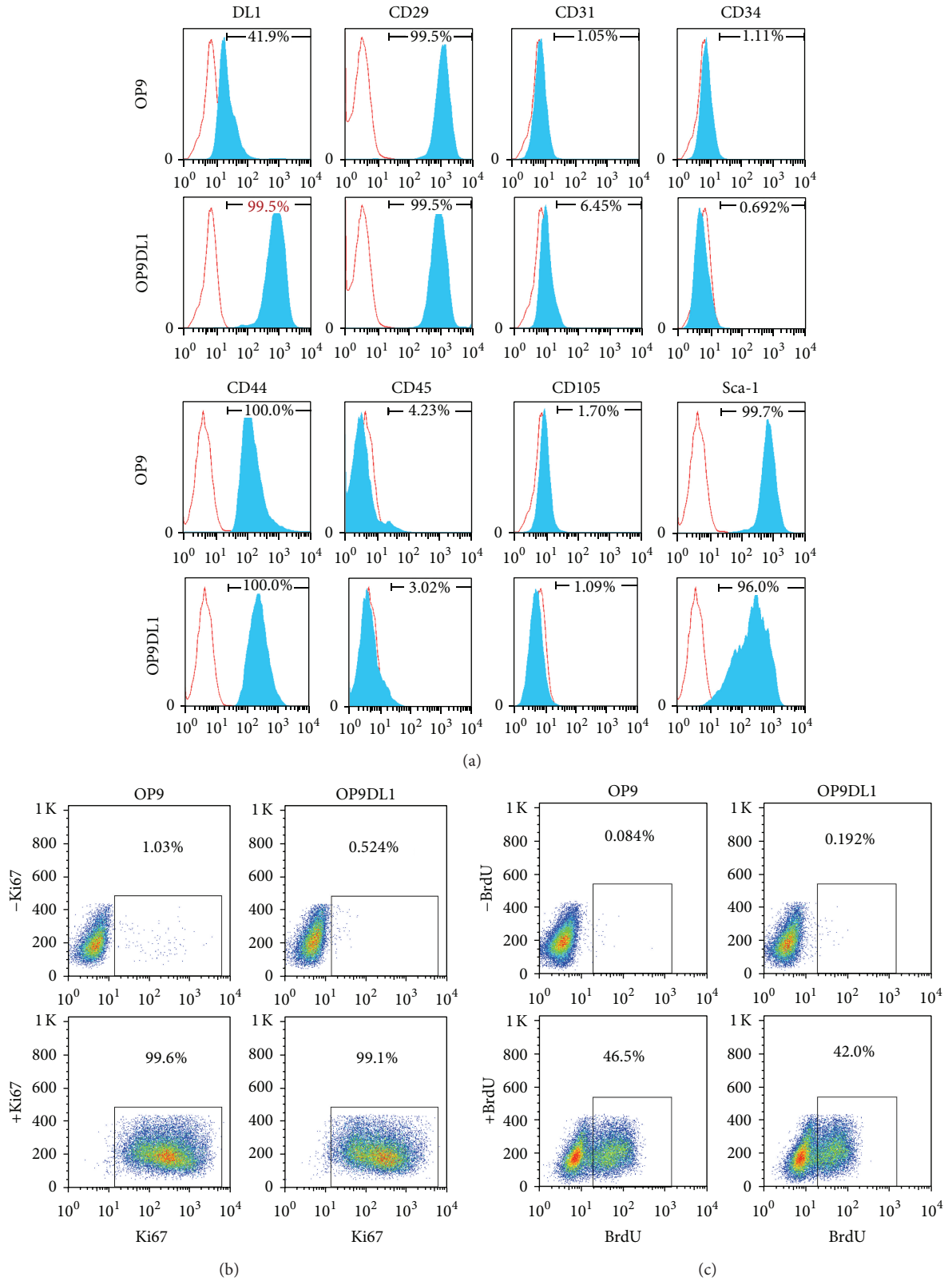

Figure 1: Comparisons between OP9 and OP9DL1 cells with regard to their immunophenotype and proliferation. (a) The indicated surface markers of OP9 or OP9DL1 cells were shown by FACS analysis, respectively. ((b)-(c)) The growth rate of OP9 and OP9DL1 cells was assayed by Ki67 or BrdU incorporation assays, respectively.

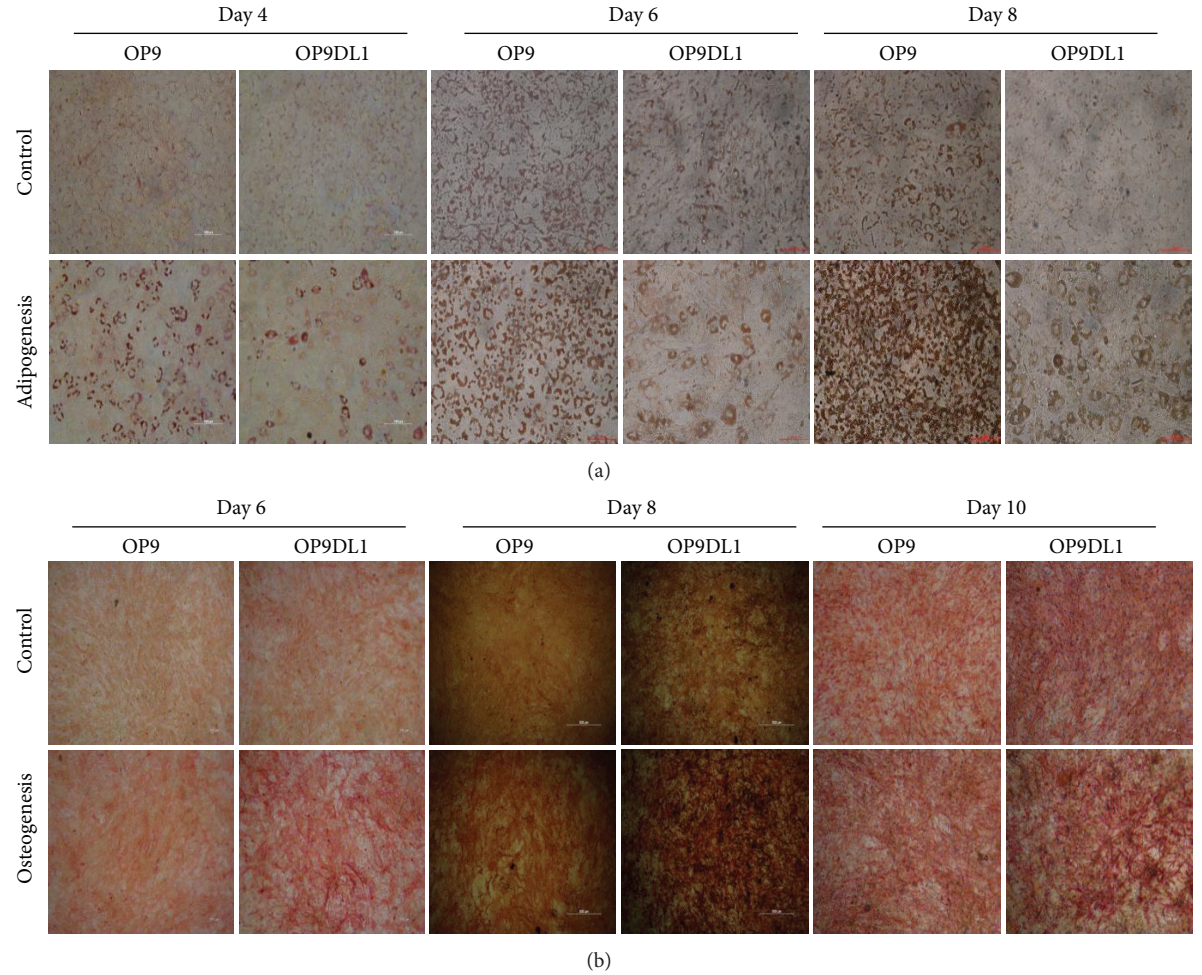

Figure 2: Differentiation ability of OP9 or OP9DL1 to adipocytes and osteoblasts *in vitro*. (a) Differentiation into adipocytes was shown by Oil Red O staining at the indicated time points, respectively. (b) The osteogenesis of OP9 and OP9DL1 was assayed by ALP staining at different times, respectively.

activity on mature B cell proliferation, while OP9DL1 showed enhanced inhibition capacity on mature T cell proliferation.

It is known that Notch1 engagement by Notch ligand DL1 can activate Notch1 signaling [7, 26, 27], and the Notch1 pathway regulates T and B lineage commitment and development [25, 28, 29]. It has been shown that inhibition of Notch signaling decreases CD4 or CD8 T cell proliferation [30] but has no effect on mature B cell growth [31]. To date, the effect of OP9 and OP9DL1 on mature T and B cell proliferation has not been clarified. Surprisingly, our data showed that OP9DL1 inhibits mature T cell proliferation and defers mature B cells growth (Figures 4 and 5), which is different from its role in early T and B cell development [9, 25]. In addition, in light of the contribution of OP9 to early B cell development, OP9 should also support mature B cells proliferation; however, we found that OP9 impeded the proliferation of mature B cells (Figure 4). These unforeseen results may be associated with the different responses of mature T/B cells to OP9 or OP9DL1 immunomodulation. In accordance with a previous report [10], our study shows that both OP9 and OP9DL1 have the same proliferation capacity and phenotypes similar to those of MSCs (Figure 1) and also differentiation capacities to adipocytes and osteocytes (Figure 2), suggesting that OP9 and OP9DL1 are both MSCs. Noticeably, our data show that OP9DL1 has stronger osteogenic and weaker adipogenic abilities than OP9, which help to clarify the controversy [23, 24] that Notch receptor/ligand interactions affect the differentiation ability of MSCs.

MSCs possess an immunomodulatory role on immune cells including T and B cells by direct cell-to-cell contact-dependent mechanisms [32, 33] and/or the production of soluble factors, such as indoleamine 2,3-deoxigenase [34], prostaglandin E2 [16–18], NO [11, 35, 36], TGFβ [37], hepatocyte growth factor [37], IL-10 [38], IFNγ [39], and TNFα [15]. It has been reported that DL1 activates Notch signaling [7] and Notch activation may be associated with increased NO production [40]. Our data showed that OP9DL1 did not generate more NO than OP9, and more NO is produced from the OP9 or OP9DL1 stimulation groups compared with that of the no simulation group. These results suggest that NO may be involved in regulating the growth of mature B and T cells during coculture with OP9 or OP9DL1. However, OP9DL1

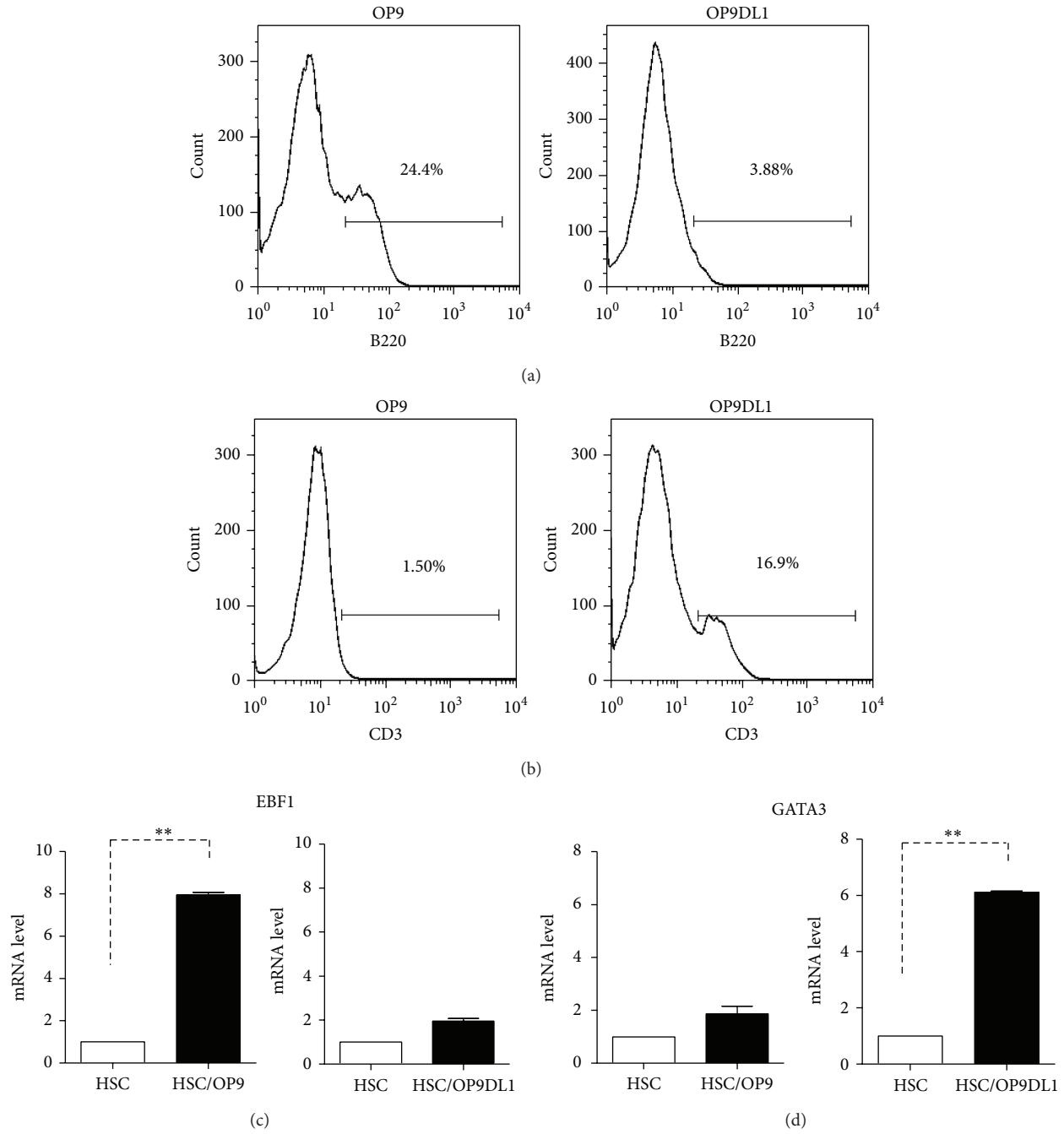

Figure 3: Effect of OP9 or OP9DL1 on differentiation of bone marrow $CD34^+$ cells. OP9 or OP9DL1 cells (5×10^4/well) were plated in 12-well plates with α-MEM medium plus 20% FBS 12 h prior to the addition of BM $CD34^+$ (5×10^5/well). The coculture was started with α-MEM medium plus 20% FBS containing a final concentration of 5 ng/mL each of IL-7 and Flt-3 ligand (Flt-3L). 12 days later, cells were collected for FACS analysis ((a) and (b)), and EBF1 or GATA3 gene expression was determined by real-time PCR after 5-day coculture, respectively ((c) and (d)). (Error bars present the SD of the mean values, $^{**}P < 0.01$.)

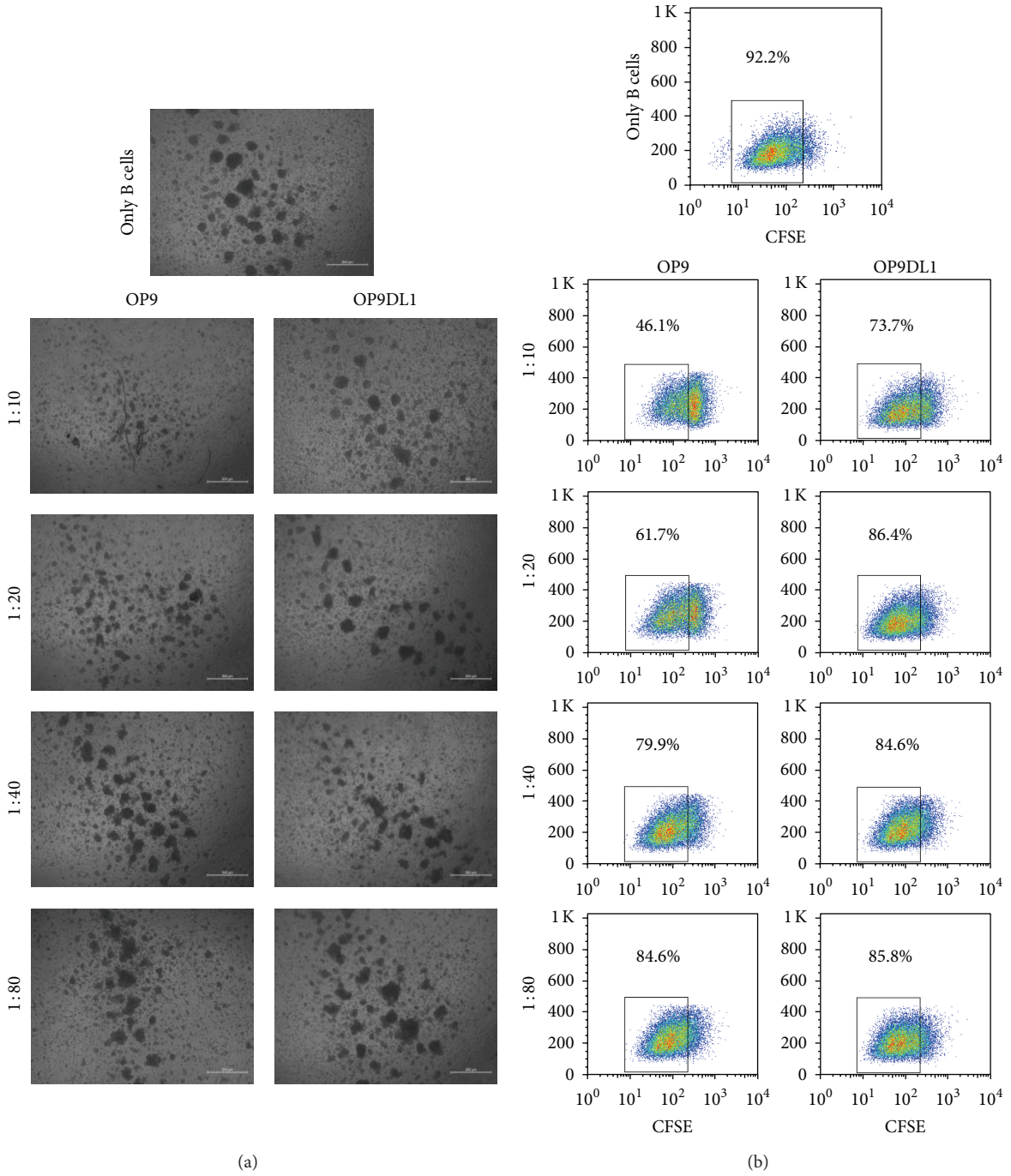

Figure 4: The effect of OP9 and OP9DL1 on proliferation of mature B cells. The B cells ($B220^+$) isolated from mice spleens were stained with CFSE, then exposed with 25 ng/mL IL4 plus 10 μg/mL LPS for 24 h, and subsequently cultured alone or together with OP9 or OP9DL1 cells at different rates for 36 h. At 36 h later, the morphology of B cells in each well was observed microscopically (a), then the B cells were analyzed by FACS, and proliferation was measured by the reduction in CFSE intensity. Cell growth rates are indicated by percentages in (b). Data are representative of three experiments.

inhibits mature T cells more and mature B cells less than OP9, which may indicate that DL1 contributes to the dominant immunomodulation role in a cell contact-dependent fashion.

Similar to previous studies, in our study, more B cells and less T cells are generated from $CD34^+$ cells in the OP9 coculture compared to the OP9-DL1 coculture group. Unlike

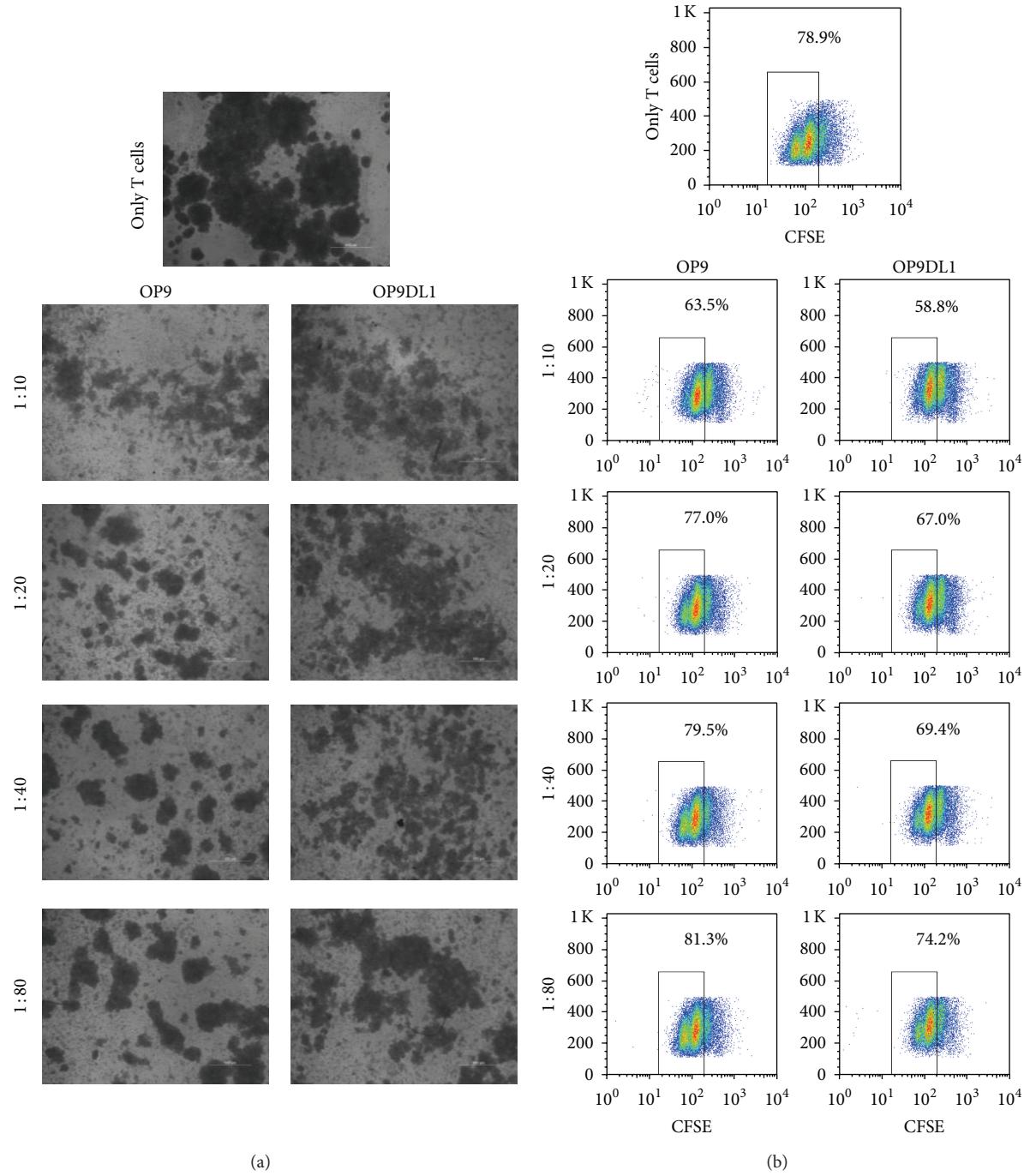

Figure 5: OP9 and OP9DL1 inhibit proliferation of mature T cells *in vitro*. $CD3^+$ T cells were isolated from murine spleens with CD3ε MicroBead Kits and labeled with CFSE. T cells were stimulated with PMA (50 ng/mL) plus ionomycin (1 μg/mL) for 24 h and then cultured alone or with OP9 or OP9DL1 at different ratios (OP9 or OP9DL1 cells to T cells). After 36 h, all of the cells were analyzed via microscope (a) and flow cytometry (b) for T cell proliferation as indicated by the reduction in CFSE intensity. Data are representative of three independent experiments.

mature T/B cells, the cell-cell contact may have a predominant role in early T/B development during coculture with OP9 or OP9DL1. Certainly, the underlying immunomodulatory mechanism of OP9 and OP9DL1 to mature T/B cells remains to be defined in further studies.

5. Conclusion

Our study elucidated that DL1 changes the immunomodulation of MSCs to immune cells, showing potent inhibition of mature T cell proliferation and a slight delay in mature B

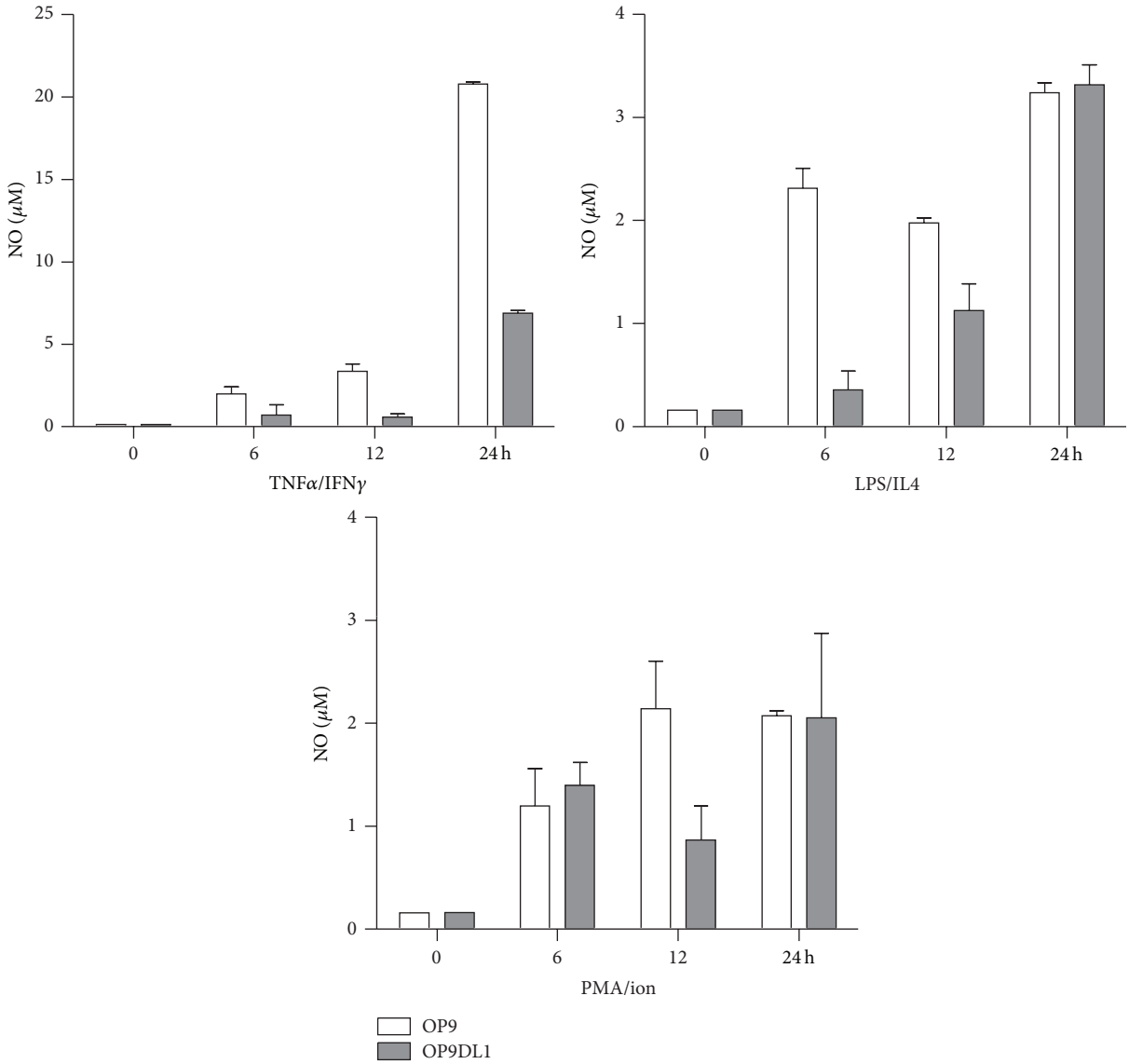

Figure 6: NO production in OP9 and OP9DL1 cells in the presence of different stimulators. OP9 and OP9DL1 were stimulated with TNFα/IFNγ (each 2 ng/mL), LPS (10 μg/mL)/IL4 (25 ng/mL), or PMA (50 ng/mL)/ion (1 μg/mL) for 24 h, respectively. Supernatants were collected at 6, 12, and 24 h and used for NO assay, respectively.

cell growth. These findings provide significant insight into the immunomodulation properties of MSCs, as well as large scale remodeling of mature T and B cells by using OP9 or OP9DL1 *ex vivo*.

Conflict of Interests

The authors declare no potential conflict of interests.

Authors' Contribution

Xiao-Xia Jiang and Nan-Zhu Fang conceived and designed the experiments. Lei Zhang, Rui-Jie Dang, Yan-Mei Yang, Dian-Chao Cui, Ping Li, Yan-Li Ni, and Tong Hao performed the experiments. Lei Zhang, Rui-Jie Dang, Yan-Mei Yang, Dian-Chao Cui, Ping Li, Yan-Li Ni, Tong Hao, Changyong Wang, Xiao-Xia Jiang, and Nan-Zhu Fang analyzed the data. Changyong Wang, Xiao-Xia Jiang, and Nan-Zhu Fang contributed reagents/materials/analysis tools. Lei Zhang, Rui-Jie Dang, Xiao-Xia Jiang, and Nan-Zhu Fang wrote the paper. Lei Zhang and Rui-Jie Dang contributed equally to this work.

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