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Improved viability and activity of neutrophils differentiated from HL-60 cells by co-culture with adipose tissue-derived mesenchymal stem cells

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ABSTRACT

Neutropenia is a principal complication of cancer treatment. We investigated the supportive effect of adipose tissue-derived mesenchymal stem cells (AD-MSCs) on the viability and function of neutrophils. Neutrophils were derived from HL-60 cells by dimethylformamide stimulation and cultured with or without AD-MSCs under serum-starved conditions to evaluate neutrophil survival, proliferation, and function. Serum starvation resulted in the apoptosis of neutrophils and decreased cell survival. The co-culture of neutrophils and AD-MSCs resulted in cell survival and inhibited neutrophil apoptosis under serum-starved conditions. The survival rate of neutrophils was prolonged up to 72 h, and the expression levels of interferon (IFN)-α, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor, and transforming growth factor (TGF)-β in AD-MSCs were increased after co-culture with neutrophils. AD-MSCs promoted the viability of neutrophils by inhibiting apoptosis as well as enhancing respiratory burst, which could potentially be mediated by the increased expression of IFN-α, G-CSF, and TGF-β. Thus, we conclude that the use of AD-MSCs may be a promising cell-based therapy for increasing immunity by accelerating neutrophil function.

1. Introduction

Neutropenia is a condition characterized by a decreased number of neutrophils in the bloodstream and represents a leading cause of death in cancer patients with immunity problems. Neutropenia is also a principal complication of various treatments for a number of malignancies [1,2]. Neutrophils play an important role in the acute inflammatory response, with a primary role in the clearance of extracellular pathogens. Neutrophils are also involved in the activation, regulation, and effector functions of innate and adaptive immune cells. Therefore, neutrophils have a crucial role in the pathogenesis of a broad range of diseases, including infections caused by intracellular pathogens, autoimmunity, chronic inflammation, and leukemia [3]. Infection is also a principal complication of treatments for a number of malignancies in the clinic, and neutropenia is often a causative factor for such infections. Thus, effective strategies are necessary to sustain the levels of functional neutrophils.

Using white blood cell (WBC) differential analysis, absolute neutrophil count was determined by calculating the product of the number of WBCs and the fraction of neutrophils among the WBCs. An absolute neutrophil count of fewer than 1500 per microliter is generally diagnosed as neutropenia [4]. Neutropenia can arise due to decreased neutrophil production, destruction of neutrophils after production, or the pooling of neutrophils.

Medications that induce bone marrow or neutrophil damage, such as cancer chemotherapy, are a primary cause of neutropenia. Neutropenia treatment protocols are developed based on underlying cause, severity, and presence of associated infections or symptoms [1]. Some commonly used treatment methods are as follows: (1) the administration of WBC growth factors, such as recombinant granulocyte colony-stimulating factor (rG-CSF) in severe neutropenia; (2) granulocyte transfusions; (3) corticosteroid therapy or immune globulin injection for some cases of immune-mediated neutropenia; and (4) antibiotics and/or antifungal medications to treat infections [4].

Administration of rG-CSF is commonly used to treat neutropenia because it can promote neutrophil activation, proliferation, and differentiation [5]. However, rG-CSF is not appropriate for long-term clinical use due to its instability in *in vivo* systems, resulting in its chronic administration to achieve optimal therapeutic effects [5,6]. To overcome this limitation, developments of new therapeutic strategies are strongly recommended.

Recently, allogeneic hematopoietic stem cell (HSC) transplantation (HSCT) has been suggested as the only curative treatment

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in patients with severe congenital neutropenia compared with G-CSF [7]. While HSCT is considered the only curative option for these patients, further study is necessary to determine the precise role of HSCT, because transplantation outcomes after malignant transformation are not well documented [8]. Allograft transplantation using HSCT may not supply sufficient amounts of HSCs, and HSC collection is often inconvenient for these patients.

Mesenchymal stem cells (MSCs) from adipose tissue represent a promising substitute for HSCs [9]. MSCs can be derived from various tissues, including brain, cord blood, adipose tissue, and bone marrow (BM) [9,10]. These stem cell sources have numerous limitations, including the invasive procedures required to obtain the cells and a lack of an adequate number of cells for clinical use [10]. Although BM is a good source of MSCs (BM-MSCs), obtaining a sufficient volume by BM harvesting requires an invasive procedure. In contrast, adipose tissue-derived MSCs (AD-MSCs) are advantageous for clinical use because they are relatively easy to obtain by liposuction under topical anesthesia. MSCs can also differentiate into various cell types and possess immunosuppressive abilities; they are considered a promising cell source for clinical research in tissue regeneration, graft-versus-host disease, and autoimmune diseases [11–13]. Several recent studies reported that BM-MSCs promoted the viability of neutrophils [3,14,15]. Therefore, it is necessary to evaluate the therapeutic efficacy of AD-MSCs toward neutrophils to overcome the limitations of BM-MSCs.

We investigated the therapeutic potential of AD-MSCs on the function and survival of neutrophils differentiated from HL-60 cells using dimethylformamide (DMF) treatment under serum-starved conditions.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute Medium (RPMI 1640), penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Welgene (Korea). DMF, phorbol 12-myristate 13-acetate (PMA), dimethylsulfoxide (DMSO), Alizarin Red S, and Oil red O were purchased from Sigma (St. Louis, MO). PE-mouse anti-human CD11b, PE-mouse anti-human CD14, PE-mouse anti-human CD35, and fluorescein isothiocyanate (FITC)-mouse anti-human CD71 antibodies were purchased from Biolegend (San Diego, CA). PE-mouse IgG1 and FITC-mouse IgG1 were purchased from Becton Dickinson (Franklin Lakes, NJ). TRIzol, and adipogenic and osteogenic differentiation media were purchased from Invitrogen (Carlsbad, CA). The reverse transcription kit and master mix for real-time polymerase chain reaction (RT-PCR) were purchased from Promega (Madison, WI) and KAPA Biosystems (Boston, MA), respectively. PE-mouseIgG1, FITC-mouse IgG1 IgG2a, and FITC Annexin V Apoptosis detection kits were purchased from BD Pharmingen (San Diego, CA).

2.2. Cell culture and neutrophil-like differentiation

HL-60 cells were purchased from the ATCC (Manassas, Virginia), and AD-MSCs were provided by RNLBio (Seoul, Korea). Cells were cultured in RPMI 1640 and DMEM supplemented with 10% FBS, respectively, at 37 °C in a humidified atmosphere of 5% CO_2.

HL-60 cells were differentiated into neutrophils by treatment with 0.8% DMF. Differentiated HL-60 cells (D-HL-60) were assessed on days 4–7 to determine phenotypic alterations using flow cytometric analysis with a FACS Calibur system (BD, San Diego, CA). D-HL-60 cells were stained with surface antigens, PE-CD11b, PE-CD35, and PE-CD71. Non-specific staining was assessed with either IgG1 or FITC-IgG1 as an isotype control.

2.3. Determination of surface antigen on AD-MSCs

Cell surface antigen profiling of AD-MSCs was performed by flow cytometry. The antibodies for flow cytometry were as follows: hematopoietic cell markers, PE-CD14, PE-CD34, and PE-CD45; and primitive cell markers, PE-CD73, PE-CD90, and PE-CD105.

2.4. Mesodermal differentiation of AD-MSCs

For adipogenic and osteogenic differentiation, cells were seeded at a density of 1×10^5 cells per well in a 12-well plate and cultured with adipogenic and osteogenic media, respectively for 3 weeks. Media were changed every 3–4 days. After 3 weeks of culture, AD-MSCs were stained with Oil red O and a 2% Alizarin Red S solution, respectively. The lipid droplets and mineralized spots were visualized by phase contrast microscopy.

2.5. Measurement of respiratory burst by chemiluminescence assay

D-HL-60 cells (1×10^5 cells) were seeded and co-cultured with AD-MSCs (5×10^4 cells/well) in a 96-well plate. Respiratory activity was assessed by measuring oxidative burst by chemiluminescence assay after stimulation with PMA. Luminol was used as a substrate. The chemiluminescence response was measured using an automated luminometer (Multi-Biolumat, Berthold, Germany).

2.6. Determination of D-HL-60 viability

To assess the effects of AD-MSCs on the viability of D-HL-60 cells, we seeded AD-MSCs at a density of 5×10^4 cells/well in a 96-well plate. D-HL-60 cells (1×10^5 cells) were the added onto the AD-MSCs pre-seeded in each well. Cells were maintained with varying concentrations of FBS (0%, 2%, 4%, 6%, 8%, and 10%) for 24 h, and the viability of D-HL-60 cells was determined by an MTT [3-(4,5-dimethylthizol-2-yl)2,5-diphenyl tetrazolium bromide] assay. Briefly, D-HL-60 cells were transferred into a new 96-well plate, 20 μl MTT solution (5 mg/ml in PBS) was added to each well and the cells were incubated for an additional 4 h. The supernatant was removed, and DMSO (200 μl/well) was added to each well. The cells were incubated at room temperature for 15 min on a microplate shaker to create the formazan precipitate. Dissolved formazan was measured by absorbance at 570 nm using an enzyme-linked immunosorbent assay plate reader.

2.7. Apoptosis assay

D-HL-60 cells were cultured at varying concentrations of FBS in either the presence or absence of AD-MSCs, as described above. The cells were washed with PBS and resuspended in 500 μl isotonic binding buffer [0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$]. Annexin V and propidium iodide (PI) were stained according to the manufacturer's instructions (BD Pharmingen), and the cells were analyzed by flow cytometry.

2.8. Quantitative RT-PCR

AD-MSCs co-cultured with or without D-HL-60 cells at two different FBS concentrations (2% or 10%) were collected to compare expression levels of interferon (IFN)-α, IFN-β, G-CSF, granulocyte–macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)-β by quantitative RT-PCR. The detailed RT-PCR procedure and the primer pairs for IFN-α, IFN-β, G-CSF, GM-CSF, and TGF-β are listed in the Supplementary information.

2.9. Statistical analysis

All experiments were conducted as three independent sets, followed by statistical analyses using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All values were expressed as the mean ± standard error of the mean (SEM). Statistical significances between the groups were determined by Student's *t* test ($^*p < 0.05$).

3. Results

3.1. Evaluation of DMF-induced neutrophil differentiation of HL-60 cells

To assess DMF-induced granulocytic differentiation of HL-60 cells, we analyzed the expression levels of several surface antigens. On differentiation day 5 (D5), levels of granulocytic differentiation markers CD11b and CD35 were increased during DMF-induced neutrophil differentiation to 65 ± 0.7% and 75 ± 1.4%, respectively. The expression of CD11b was decreased to 26 ± 2.1% on D7 (Fig. 1A). In contrast, the expression of CD71, a transferring receptor that plays an essential role in cell proliferation, significantly decreased in a time-dependent manner (Fig. 1A). Based on these results, we chose D5 as an optimum differentiation period and used this time-point for further study. Changes in the expression patterns of various surface antigens are shown in Fig. 1B.

3.2. Enhanced viability of D-HL-60 cells by AD-MSCs

AD-MSCs showed a typical MSC surface phenotype. Cultured MSCs were negative for the non-hematopoietic cell-specific

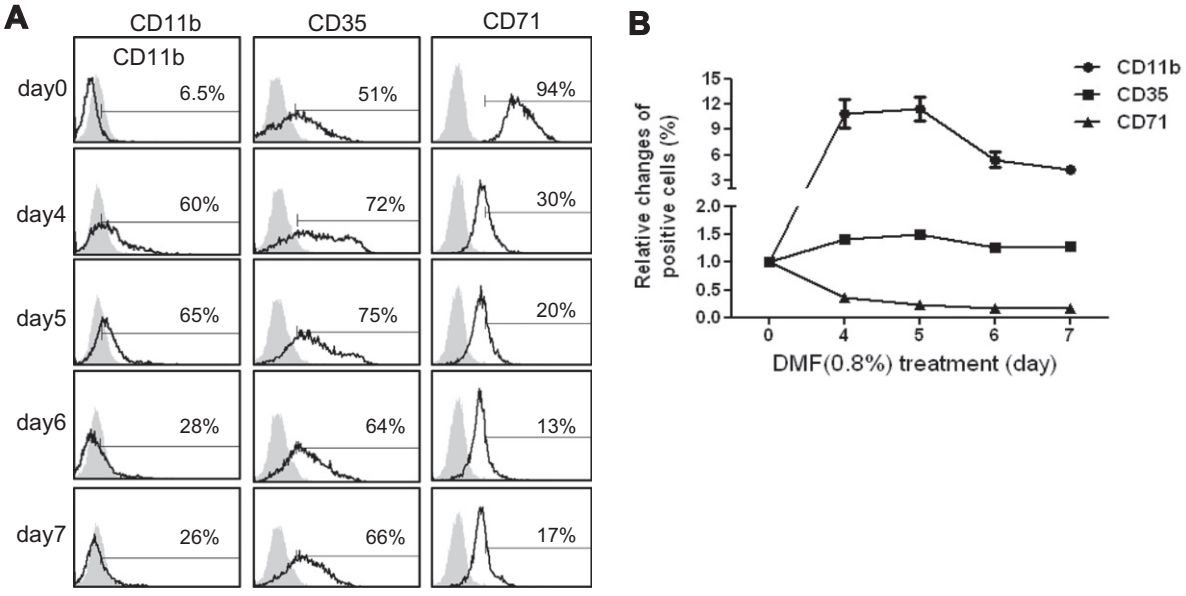

Fig. 1. Differentiation of HL-60 cells into neutrophils. HL-60 cells were treated with 0.8% DMF to induce differentiation into granulocytes. Levels of neutrophil markers, CD11b, CD35, and CD71, were measured by flow cytometry (A). Relative changes of positive cells compared to the initial time (day 0) are presented (B).

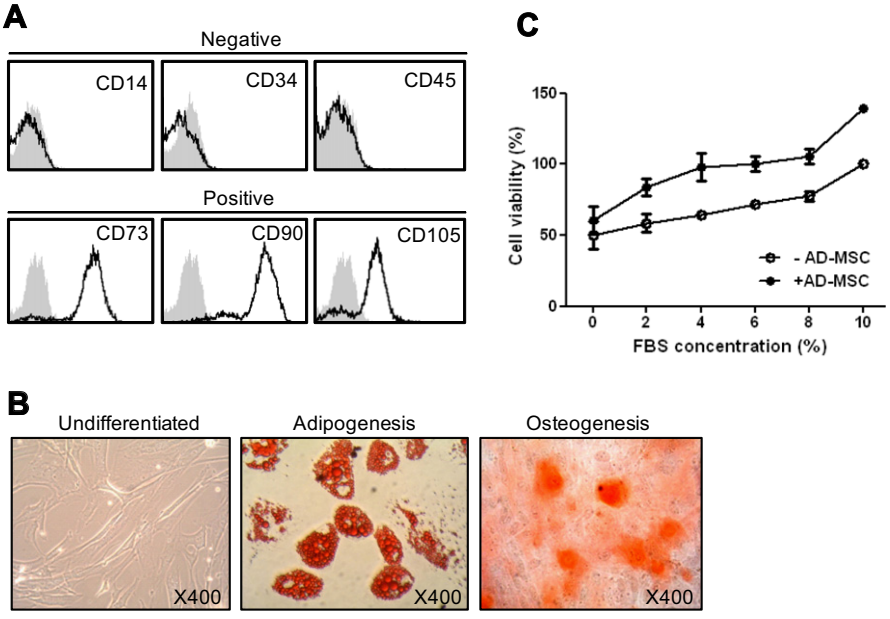

Fig. 2. Characteristics of AD-MSCs and viabilities of D-HL-60 cells co-cultured with AD-MSCs. AD-MSCs showed a typical MSC surface phenotype by flow cytometry (A). Mesodermal differentiation of AD-MSCs, adipogenesis, and osteogenesis were investigated by Oil red O and Alizarin Red S staining, respectively (B). Cell viability of D-HL-60 cells was measured (C). Significance differences between D-HL-60 cells with and without AD-MSCs were determined by Student's *t* tests at each FBS concentration ($^*p < 0.05$).

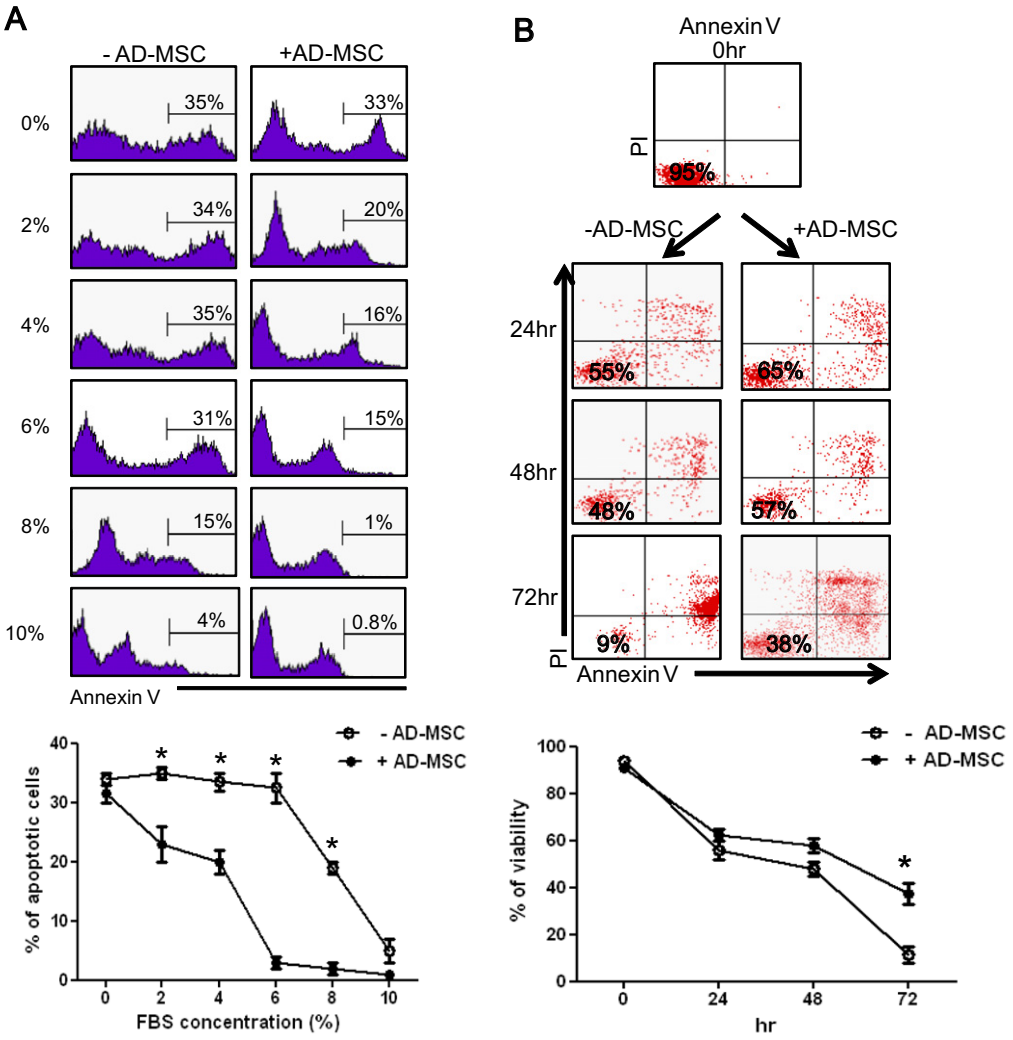

Fig. 3. Inhibition of apoptosis and prolonged viability of D-HL-60 cells by co-culture with AD-MSCs. D-HL-60 cell apoptosis (A) and cell viability (B) with or without AD-MSCs were evaluated by flow cytometry under varying concentrations of FBS (A) and incubation time (B). Statistically significant differences in D-HL-60 viability with and without AD-MSCs were determined by Student's *t* tests at each FBS concentration (*$p < 0.05$).

markers CD14, CD34, and CD45 but were positive for the MSC markers CD73, CD95, and CD105 (Fig. 2A).

To confirm the characteristics of AD-MSCs as effective MSCs, we evaluated their adipogenic- and osteogenic differentiation properties. Morphological changes and formation of cytoplasmic lipid droplets were noticeable after 3 weeks of adipogenic induction, as visualized by Oil red O staining. Upon osteogenic induction, the morphology of the AD-MSCs changed from spindle-shaped to flattened and spreading. Calcified deposits were detected by Alizarin Red S staining (Fig. 2B).

To assess the effect of AD-MSCs on neutrophil survival, we cultured neutrophils with or without AD-MSCs under varying concentrations of FBS for 24 h (Fig. 2C). AD-MSCs significantly enhanced the viability of D-HL-60 cells at 24 h ($p < 0.05$, between groups with and without AD-MSC). In contrast, AD-MSCs did not have any beneficial effects on cell viability under serum-free conditions (0%). Thus, synergistic effects of AD-MSCs on cell viability can be achieved with FBS concentrations between 2% and 10%.

3.3. Inhibition of apoptosis of D-HL-60 cells by AD-MSCs

To confirm whether the increased viability of D-HL-60 cells in the presence of AD-MSCs was due to anti-apoptotic activity, flow cytometry analysis was performed using Annexin V and PI to compare the degree of apoptosis under varying FBS concentrations. AD-MSCs significantly decreased the apoptosis of D-HL-60 cells at 24 h in the presence of 2–8% FBS (Fig. 3A) and in the presence of 2% FBS at 72 h compared to D-HL-60 cells cultured alone (Fig. 3B).

3.4. Reactive oxygen species generation in D-HL 60 cells and gene expression relevant to survival and growth

We examined the activation of respiratory burst using a luminol-enhanced chemiluminescence assay.

D-HL-60 cells generated a respiratory burst upon PMA stimulation in a cell population-dependent manner (Fig. 4A). Reactive oxygen species (ROS) generation by D-HL-60 cells was significantly increased by co-culture with AD-MSCs, and was significantly accelerated at high concentration of FBS (10%) compared to serum-starved conditions ($p < 0.05$; Fig. 4B).

We compared gene expression levels of IFN-α, IFN-β, G-CSF, GM-CSF, and TGF-β by RT-PCR relevant to neutrophil survival and growth. AD-MSCs were cultured with or without D-HL-60 cells at either 2% or 10% FBS. AD-MSCs highly expressed IFN-α and TGF-β in the presence of D-HL-60 cells at both FBS concentrations ($p < 0.05$, Fig. 4C). AD-MSCs showed significant increases in G-CSF, a classical neutrophil stimulating factor, when co-cultured with D-HL-60 cells ($p < 0.05$). Co-culture of D-HL-60 cells with

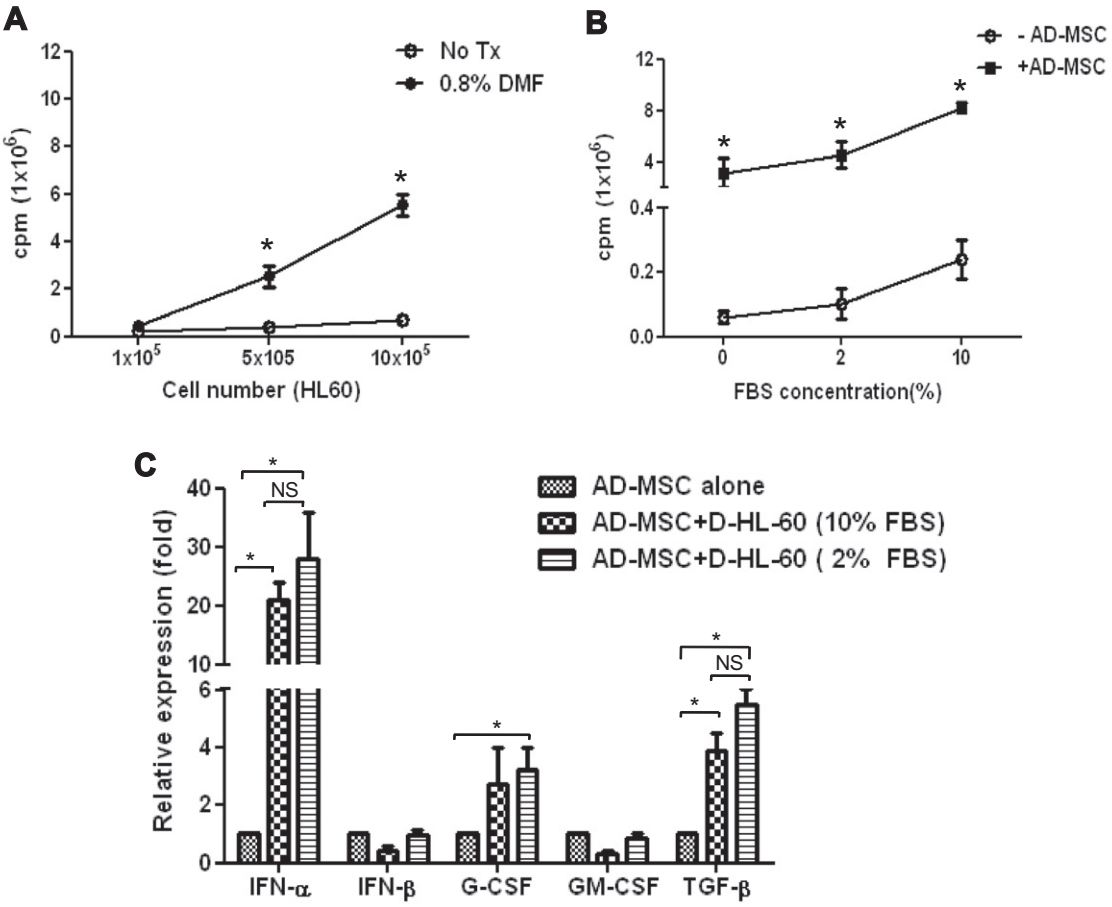

Fig. 4. AD-MSCs enhanced the respiratory burst of D-HL-60 cells and triggered the expression of neutrophil cell survival and growth factor mRNAs. D-HL-60 cells generated a respiratory burst upon PMA stimulation in a D-HL-60 cell population-dependent (A) and FBS concentration-dependent manner (B). The levels of IFN-α, G-CSF, and TGF-β mRNA in AD-MSCs co-cultured with D-HL-60 cells were determined by RT-PCR. Data are expressed as the mean ± SEM. Significant differences between D-HL-60 cells with or without AD-MSCs were determined by Student's *t* test (*$p < 0.05$). NS: not statistically significant.

AD-MSCs showed no significant differences between the 2% and 10% FBS conditions.

4. Discussion

Immune cells such as neutrophils and macrophages are required to infiltrate and operate efficiently within infected environments [16]. The lack of functional neutrophils in neutropenia decreases immunogenicity and can cause lethal infection in cancer patients undergoing chemotherapy. Therefore, effective strategies to recover or sustain functional neutrophils are needed. As a representative molecule, G-CSF has been widely used to treat neutropenia because of its ability to promote neutrophil activation, proliferation, and differentiation. However, the therapeutic effects of G-CSF are limited by its short-term persistence, and therefore require repetitive treatment.

We adopted AD-MSCs as a supportive cell therapy system by increasing the survival and proliferation of neutrophils. In this study, we showed that AD-MSCs protected and enhanced the survival of DMF-treated HL-60 cells by reducing their rate of apoptosis and promoting respiratory burst. The AD-MSCs used in this study exhibited a typical MSC surface phenotype, as measured by flow cytometry. AD-MSCs were confirmed by their negative expression of non-hematopoietic cell-specific markers (i.e., CD14, CD34, and CD45) and the positive expression of MSC-specific markers (i.e., CD73, CD95, and CD105; Fig. 2A). AD-MSCs also showed specific multi-lineage differentiation capacities for adipogenesis and osteogenesis (Fig. 2B).

Similar to our results, Cassatella et al. [14] showed that BM-MSCs activated by either polyinosinic:polycytidylic acid (poly I:C) or lipopolysaccharide enhanced the survival of neutrophils up to 72 h. Brandau et al. [15]. also showed that the incubation of MSC supernatants for 24 h reduced the rate of apoptosis in lipopolysaccharide-stimulated neutrophils by the secretion of interleukin-8/macrophage inhibitory factor. However, it is currently unclear whether these effects are due to MSCs alone or ligand-induced inflammatory conditions. In this regard, we attempted to understand the effects of MSCs alone on neutrophils using a co-culture system of AD-MSCs with either DMF-induced or non-induced HL-60 cells.

Human promyelocytic leukemia HL-60 cells can be induced to mature into terminally differentiated cells using many chemotherapeutic agents and are a well-established model for the study of acute promyelocytic leukemia [17]. These cells can differentiate into granulocyte-like cells upon exposure to a variety of agents such as DMSO, retinoic acid, or DMF. We evaluated the effects of direct AD-MSC exposure on D-HL-60 cells at various FBS concentrations. Serum or serum fractions are an indispensable supplement for classical cell culture media. Neutrophils generally have a short life span, thus prolongation of the neutrophil life span is critical for their effectiveness against pathogens [18]. In some pathological situations, shortened neutrophil survival (i.e., neutropenia) due to apoptosis may increase susceptibility to severe and recurrent infections [19].

To explore the effects of AD-MSCs on neutrophil viability, decreased FBS supplementation was applied to D-HL-60 cell

cultures to induce apoptosis. We used varying concentrations of FBS (0%, 2%, 4%, 6%, 8%, and 10%) with 10% FBS representing the standard concentration used in cell culture systems. We showed that the viability and proliferation of D-HL-60 cells increased in the presence of AD-MSCs even in the presence of 2% FBS (Fig. 2 and 3A). We further investigated enhanced D-HL-60 survival using an apoptosis assays. Apoptosis is programmed cell death invoked by cells in response to internal and externals insults. Low FBS concentrations potently induce apoptosis in neutrophils due to either the lack of essential components or sustainable survival signals. Although the AD-MSC-mediated inhibition of apoptosis is not statistically significant in 0% serum, a noticeable reduction in apoptosis was observed. The pattern of apoptosis reduction observed at 2–8% FBS concentrations reflects the capability of AD-MSCs to inhibit neutrophil cell death (Fig. 3A). Because circulating neutrophils have a shortened life span, it is particularly important to determine whether MSCs are able to prolong D-HL-60 cell life span. AD-MSCs were capable of limiting cell death in neutrophils under serum-deprived conditions for up to 72 h (Fig. 3B).

Because MSCs regulate apoptosis and mitosis under conditions of ischemia/reperfusion in a paracrine manner [20,21], we suggest that co-culture with AD-MSCs might accelerate cell proliferation and survival of D-HL-60 cells. Identifying the major secreted molecules from AD-MSCs that might affect D-HL-60 is still needed. Human BM-MSCs are beneficial in genetic disorders and hematological diseases [22,23]. Human BM-MSC treatment reduced mortality in an acute kidney injury mouse model by inducing protection against acute kidney injury-related peritubular capillary changes [23]. In this regard, AD-MSCs can be further considered as an efficient cell support system to aid in neutrophil survival and function.

Neutrophils play pivotal roles in immunity by generating ROS, important determinants of functional neutrophils, via nicotinamide adenine dinucleotide phosphate-oxidase during respiratory bursts [24,25]. The phagocyte respiratory burst, which generates superoxide, is one of the most important mechanisms for destruction of invading microbial pathogens. Polymorphonuclear respiratory burst is also a critical host defense mechanism [25]. During the differentiation of HL-60 cells, superoxide generation can be induced by specific stimuli, such as PMA. Respiratory burst activity of D-HL-60 cells after PMA treatment was profoundly enhanced in the presence of AD-MSCs (Fig. 4A and B). These results suggested that AD-MSCs support neutrophil activity and prolong survival. We next analyzed the effects of AD-MSCs on the expression of representative factors involved in neutrophil growth and survival, including IFN-α, IFN-β, G-CSF, and TGF-β, by quantitative RT-PCR. Expression levels of IFN-α, G-CSF, and TGF-β were increased in the presence of D-HL-60 cells (Fig. 4C). In summary, we observed that neutrophils starved by serum deprivation were rescued from apoptosis in the presence of AD-MSCs. Moreover, neutrophil function, as measured by respiratory burst, was enhanced by the addition of AD-MSCs.

Taken together, results of the present study verified the potential role of AD-MSCs in the support of neutrophil survival and function after infection, thereby overcoming the weakened immunity caused by a lack of neutrophils. Further development of an *in vivo* model to assess the effects of AD-MSCs on the protection of neutrophils in harsh circumstances is currently needed. In this regard, we suggest that AD-MSCs may be an ideal cell population for future clinical cell therapies and may possess promising potential for the treatment of neutropenia.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.049.

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