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Comparative Immunomodulatory Properties of Adipose-Derived Mesenchymal Stem Cells Conditioned Media From BALB/c, C57BL/6, and DBA Mouse Strains

Seyed Mahmoud Hashemi,[1] Zuhair Mohammad Hassan,[1*] Ali Akbar Pourfathollah,[1] Sara Soudi,[1,2] Abbas Shafiee,[2] and Masoud Soleimani[3**]

[1]*Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[2]*Stem Cell Biology Department, Stem Cell Technology Research Center, Tehran, Iran*
[3]*Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

ABSTRACT

Adipose tissue-derived mesenchymal stem cells (AD-MSCs) have been shown to be capable of differentiating into multiple cell type and exert immunomodulatory effects. Since the selection of ideal stem cell is apparently crucial for the outcome of experimental stem cell therapies, therefore, in this study we compared AD-MSCs conditioned media (CM) from BALB/c, C57BL/6, and DBA mouse strains. No significant difference was found in the morphology, cell surface markers, in vitro differentiation and proliferation potentials of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice. The immunological assays showed some variation among the strains in the cytokines, nitric oxide (NO), and indoleamine 2,3-dioxygenase (IDO) production and immunomodulatory effects on splenocytes functions. Our results indicated a suppression of splenocytes proliferation in the presence of AD-MSC CM from the three inbred mouse strains. However, BALB/c CM exerted a higher suppression of splenocytes proliferation. AD-MSCs isolated from C57BL/6 and BALB/c mice produced higher levels of TGF-β than those from DBA mice. Furthermore, IL-17 and IDO production was higher in AD-MSCs isolated from BALB/c mice. Our results indicated an increased production of TGF-β, IL-4, IL-10, NO, and IDO by splenocytes in response to CM from BALB/c AD-MSCs. In conclusion, our results showed that the immunomodulatory properties of mouse AD-MSCs is strain-dependent and this variation should be considered during selection of appropriate stem cell source for in vivo experiments and stem cell therapy strategies. J. Cell. Biochem. 114: 955–965, 2013.

KEY WORDS: IMMUNOMODULATORY PROPERTIES; ADIPOSE-DERIVED MESENCHYMAL STEM CELLS; CONDITIONED MEDIA; MOUSE STRAINS

Mesenchymal stem cells (MSCs) are mesoderm-derived non-hematopoietic stromal cells that are capable of differentiating in vitro and in vivo to mesenchymal lineages, including osteoblasts, chondrocytes, and adipocytes. In addition, MSCs have been shown to be capable of differentiating into multiple cell type such as hepatocytes, myocytes, neurons, and cardiomyocytes [reviewed in Uccelli et al., 2008; Mohseny and Hogendoorn, 2011]. MSCs have been isolated from various tissues other than the bone marrow, including adipose tissue and connective tissue of most organs. Previous studies have shown that MSCs have been isolated and cultured from human, rodents, and many other species. While, these studies suggest that there are some variations in the phenotypes and functional capacities of MSCs isolated from different tissues or strains [English et al., 2010]. Over the last decade, there has been increasing evidence about the in vitro and in vivo immunomodulatory properties of MSCs [reviewed in Uccelli et al., 2008]. Several recent reports have demonstrated that MSCs have immunomodulatory or immunosuppressive effects on many immune cells, including dendritic cells (DCs) [Ivanova-Todorova et al., 2009], T lymphocytes [Rasmusson et al., 2005; Sato et al., 2007; Lu et al., 2009; Yang et al., 2009; Svobodova et al., 2011], and B-lymphocytes [Che et al., 2012]. In vitro studies have demonstrated that both cell–cell contact [Sheng et al., 2008] and soluble factors [Sato et al., 2007; Lu et al., 2009] contribute to the

*Correspondence to: Zuhair Mohammad Hassan, Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Ale-Ahmad Avenue, P.O. Box 14115-331, Tehran, Iran. E-mail: hasan_zm@modares.ac.ir
**Correspondence to: Masoud Soleimani, Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Ale-Ahmad Avenue, P.O. Box 14115-331, Tehran, Iran. E-mail: soleim_m@modares.ac.ir
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immunomodulatory function of MSCs. The mechanisms of immune modulation by MSCs depend on the secretion of anti-inflammatory factors, such as interleukin-10 (IL-10) [Yang et al., 2009], transforming growth factor-β (TGF-β) [Nemeth et al., 2010], indoleamine 2,3-dioxygenase (IDO) [DelaRosa et al., 2009], nitric oxide [Sato et al., 2007], prostaglandin-E2 (PGE-2) [Yanez et al., 2010], and human leucocyte antigen-G (HLA-G) [Selmani et al., 2009]. It has also been reported that, MSCs can induce regulatory T cells (T-reg) generation [Ge et al., 2010; Svobodova et al., 2011]. Furthermore, MSCs induce cell division arrest and apoptosis of T lymphocytes [Jarvinen et al., 2008; Yang et al., 2009]. Several recent reports have demonstrated the phenotypic and functional characterization of adipose-derived MSCs (AD-MSCs) [Uccelli et al., 2008; Gimble et al., 2011]. AD-MSCs are becoming an alternative source of bone marrow derived (BM-MSCs) because adipose tissues are abundant and accessible. In addition, the immunomodulatory properties of AD-MSCs have been demonstrated both in vivo and in vitro [Puissant et al., 2005]. Since mouse is suitable experimental models of immunological diseases, the selection of ideal murine MSCs is apparently crucial for the outcome of in vivo and in vitro experiments with MSCs. There are no reports comparing the immunomodulatory properties of AD-MSCs in different mouse strain. Therefore, in this study we have compared immunomodulatory properties of AD-MSCs derived from BALB/c, C57BL/6, and DBA mouse strains.

MATERIALS AND METHODS

ANIMALS

Male BALB/c, C57BL/6, and DBA mice (6–7 weeks old) were purchased from the Pasteur Institute, Tehran, Iran. The animals were housed in specific pathogen-free conditions and maintained under standard laboratory conditions. All mice were allowed to acclimatize for 1 week with a maximum of five mice per cage. Mice were allowed free access to feed and water, all experiments were carried out according to the Tarbiat Modares university guidelines for animal care.

MESENCHYMAL STEM CELL ISOLATION AND CULTURE

The isolation of MSCs from adipose tissue was carried out on minced samples obtained from abdominal fat tissue. After washing in the phosphate-buffered saline (PBS), extracellular matrix was digested with 0.075% type I collagenase (37°C for 30 min) and centrifuged at 500*g* for 5 min, and the pellet was then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin, and streptomycin (all from Invitrogen). After 72 h, nonadherent cells were removed. When adherent cells were confluent, they were trypsinized, harvested, and expanded. All the experiments were performed using AD-MSCs at passages 3.

PREPARATION OF AD-MSCS CONDITIONED MEDIA

In order to prepare AD-MSCs conditioned media (CM), 1.0×10^6 AD-MSCs were plated in 75 cm^2 tissue culture flask. When the AD-MSCs confluency was near 90%, the cells were cultured in serum free DMEM medium for 48 h. The supernatant was filtered through a 0.22 μm membrane and stored at −80°C. To assess the suppression of the splenocytes proliferation, and cytokine, IDO and NO production by splenocytes, the splenocytes were cultured in prepared CM mixed with 50% fresh splenocyte culture media and the plates were incubated for 3 days at 37°C at 5% CO_2 in a humid atmosphere. The suppression of the splenocytes proliferation in the absence and presence of 5 μg/ml PHA was determined. Serum free DMEM was used as control.

SURFACE MARKER ANALYSIS

The expression of surface markers was evaluated using monoclonal antibodies against mouse CD 73, CD105, CD44, CD29, Sca-1, CD90, VEGFR, CD31, CD11b, CD45, and CD34 (all from eBioscience). The AD-MSCs at passage 3 were treated with 0.25% trypsin-EDTA, harvested, and washed. Then incubated with the specific antibodies or isotype control antibodies in 100 μl of 3% bovine serum albumin (Sigma) in PBS for 1 h at 4°C. The cells were then fixed with 1% paraformaldehyde (Sigma) and analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA) and Cyflogic software (CyFlo Ltd.).

MULTILINEAGE DIFFERENTIATION

The AD-MSCs at passage 3 were analyzed for their ability to differentiate into osteoblasts and adipocytes. To induce osteogenic differentiation, the cells were treated with 10 mM beta-glycerol-phosphate (Merck), 50 μg/ml ascorbic acid biphosphate (Sigma), and 100 nM dexamethasone (Sigma) for 3 weeks. Osteogenic differentiation was assessed with Alizarin Red staining. Adipocyte differentiation was achieved in the presence of 250 nM dexamethasone (Sigma) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) 5 μM insulin (Sigma), 100 μM indomethacin (Sigma), for 3 weeks. Oil Red O staining was used to determine the accumulation of oil droplets in the cytoplasm.

COLONY FORMING UNITS-FIBROBLAST (CFU-F) AND POPULATION DOUBLING TIME ASSAY

Colony forming units-fibroblast (CFU-F) assay was used to evaluate the self-renewal potential of AD-MSC. The cells were resuspended in the culture medium to a concentration of 10 viable cells/ml. Ten-milliliter of this cell suspension was plated in a 10 cm Petri dish. The medium was changed 2 times/week. On the Day 14, cultures were fixed and stained with Giemsa and colonies 2 mm or larger were counted. Three replicate plates were used for the experiments to obtain a mean value. Moreover, the population doublings were determined and calculated.

SPLENOCYTES PROLIFERATION ASSAY

The spleen was removed from the animal and placed in cold RPMI-1640 media. Splenocytes were extracted using a 5 ml syringe with a 23 G needle. RBC was lysed with ammonium chloride solution and cells were washed twice. Cell suspensions were washed in cold RPMI-1640 media, counted in 0.2% trypan blue, and dispensed at a density of 5×10^5 cells/well in 96-well plates. As splenocyte culture media, RPMI-1640 supplemented with 10% heat inactivated FBS, 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine, and 10 mM HEPES was used. To assess the suppression of the

splenocytes proliferation, the splenocytes were cultured in prepared CM mixed with 50% fresh splenocyte culture media and the plates were incubated for 3 days at 37°C at 5% CO_2 in a humid atmosphere. The suppression of the splenocytes proliferation in the absence and presence of 5 μg/ml PHA was determined. Serum free DMEM was used as control.

In addition, AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice at passage 3 were added to the cultures with a ratio of AD-MSCs to splenocytes of 1:1, 0.2:1, 0.1:1, and 0:1 and cell proliferation was determined. The percentage growth inhibition was calculated using the formula:

$$\%\,\text{Splenocytes proliferation} = \frac{\text{OD(Splenocytes} + \text{MSCs)} - \text{OD(MSCs)}}{\text{OD(Splenocytes} + \text{Media)}} \times 100$$

Cell proliferation was determined by MTT [3,(4,5-dimethylthiazal-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The cells, cultured in a 96-well plate, were incubated for 4 h in the presence of MTT (5 mg/ml; Sigma) followed by addition of 0.1 ml dimethyl sulfoxide (DMSO). The formazan crystals were dissolved and the absorbance was read at 570 nm by ELISA reader.

IN VITRO CYTOKINE PRODUCTION

TGF-β, IFN-γ, IL-4, IL-17, and IL-10 in cell culture supernatants from AD-MSC and cultured splenocytes in the presence of AD-MSCs CM from C57BL/6, BALB/c, and DBA mice were tested using the ELISA kit (Bender MedSystems). Tests were performed according to the manufacturer's instructions. Splenocytes at a concentration of 1×10^6 cells/well were cultured in the 24-well plates in the presence of AD-MSC CM, 5 μg/ml PHA or complete media, in the total volume of 1 ml. RPMI-1640 supplemented with 10% heat inactivated FBS, 100 μg/ml streptomycin, 100 U/ml penicillin was used. The plates were incubated for 48 h at 37°C in a humidified 5% CO_2 atmosphere. Supernatants were collected after centrifugation. In order to examine cytokine production by splenocytes (in the presence of CM), AD-MCSs CM were used as blank to subtract background absorbance of the cytokines produced by AD-MCSs.

DETERMINATION OF IDO ACTIVITY

We evaluated the biological activity of IDO by measuring the level of kynurenine in culture supernatants from AD-MSC and cultured splenocytes in the presence of AD-MSC CM [Mahanonda et al., 2007]. A 100 μl quantity of culture supernatants was mixed with 50 μl of 30% trichloroacetic acid, vortexed, and centrifuged at 8,000*g* for 5 min. Then, a 75 μl of the supernatant was added to an equal volume of Ehrlich reagent (100 mg *p*-dimethylbenzaldehyde in 5 ml glacial acetic acid) in a 96-well microtiter plate, and the absorbance was read at 490 nm. AD-MCSs CM were used as blank to subtract background absorbance of IDO produced by AD-MCSs.

NITRIC OXIDE PRODUCTION

We measured NO production in culture supernatants from AD-MSC as well as cultured splenocytes in the presence of AD-MSC CM by the Griess reaction. Briefly, nitrite was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 ml samples of the medium. The optical density at 550 nm (OD 550) was measured with a microplate reader. The concentrations were calculated by comparison with OD 550 of the standard solutions of sodium nitrite (MERCK) prepared in the culture medium. AD-MCSs CM were used as blank to subtract background absorbance of NO produced by AD-MCSs.

STATISTICAL ANALYSIS

All data are expressed as the mean ± SD of at least three experiments. Statistical analysis was done using the one-way analysis of variance (ANOVA) to compare results. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

CHARACTERIZATION OF AD-MSCS

Expression of specific cell surface markers by AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice at passage 3 were examined by flow cytometry. AD-MSCs isolated from three different mouse strains were negative for CD11b, CD34, CD45, and CD31 expression but positive for CD44, CD73, CD90, CD105, Sca-1, CD29, and VEGFR expression (Fig. 1). No significant difference was found in the percentage of specific cell surface markers by AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice except for expression of CD73 that was significantly ($P < 0.05$) lower in the BALB/c cells than in other strains. Moreover, C57BL/6, BALB/c, and DBA AD-MSCs exhibit fibroblastic morphology (Fig. 2A–C) and were able to differentiate toward osteogenic and adipogenic lineages. Alizarin red staining indicated mineralization of the extracellular matrix of differentiated cells (Fig. 2D–F). Additionally, Oil Red O staining demonstrated accumulation of lipid droplets in AD-MSCs (Fig. 2G–I). There was no difference in differentiation between the inbred strains.

COLONY FORMING UNITS–FIBROBLAST (CFU-F) AND POPULATION DOUBLING TIME ASSAY

The colony forming capacity of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mouse at passage 3 was examined by comparing CFU-F assay. The number of CFU-F formed per 100 C57BL/6 AD-MSCs was higher although, the difference was not statistically significant (Fig. 2J). No statistically significant differences were found between AD-MSCs isolated from different mouse strains in their abilities to generate CFU-F (Fig. 2J). In addition, the results indicated that after three passages the doubling times of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice ranged between 65 and 85 h and do not vary between the different strains (Fig. 2K).

IN VITRO IMMUNOSUPPRESSIVE EFFECTS OF MSCS ON SPLENOCYTES PROLIFERATION

To investigate the ability of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice to suppress splenocytes proliferation, AD-MSC CM (Fig. 3A,B) and different doses of MSCs (Fig. 3C) were added to the culture. The results indicated a significant ($P < 0.05$) suppression of splenocytes proliferation in the presence of AD-MSC CM in comparison with control media. In addition, AD-MSCs isolated from BALB/c mice showed a significant ($P < 0.05$) suppression in comparison with other strains. Furthermore, the percentage of

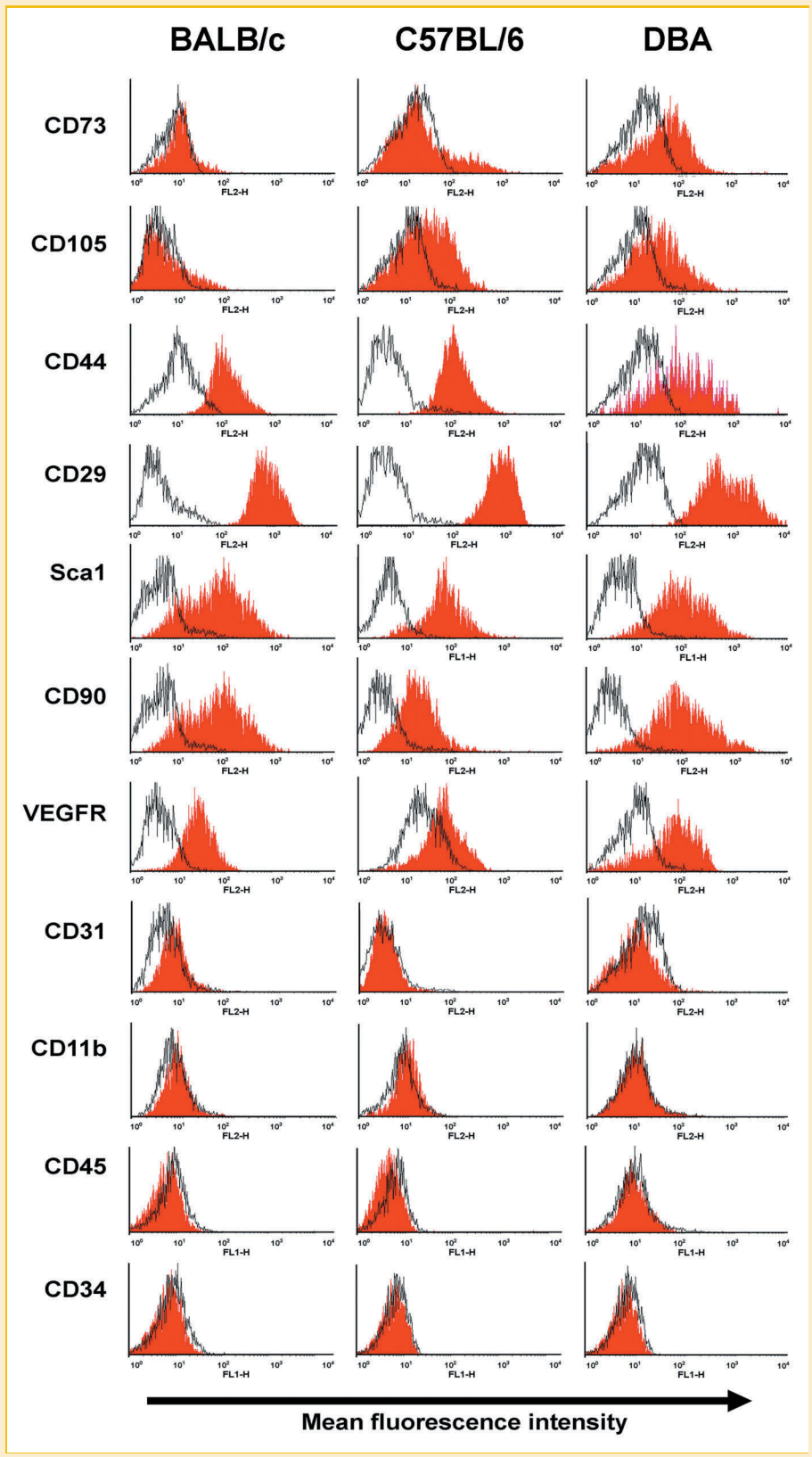

Fig. 1. Representative flow cytometric analysis of surface expression of phenotypic markers in mouse AD-MSCs isolated from different mouse strain. Isotype controls are presented as white histograms and analyzed markers as red histograms. The figure shows one representative results from four independent experiments.

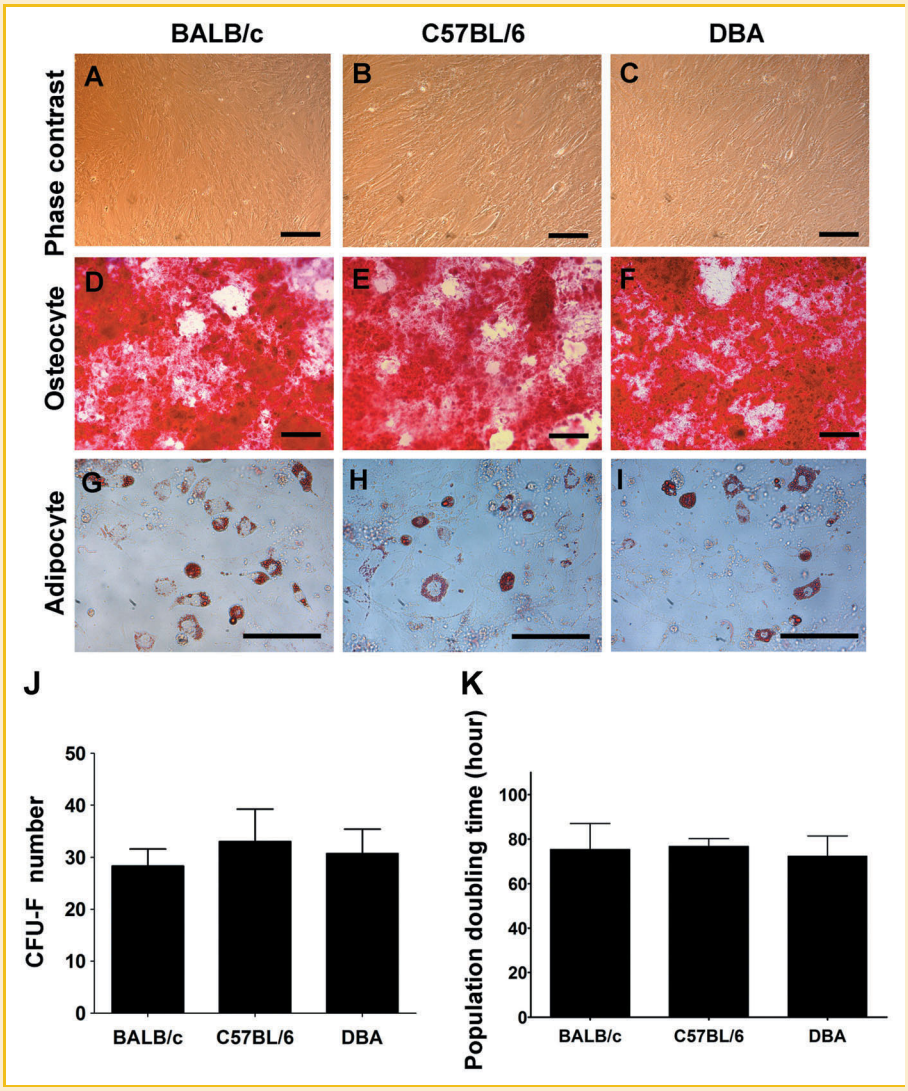

Fig. 2. Phase contrast microscopy of AD-MSCs isolated from C57BL/6, BALB/c and DBA mice at passage 3 (A–C). Differentiation into osteocytes after induction culture was assessed by Alizarin Red S staining for calcium mineralization (D–F). Adipogenic differentiation was determined by Oil Red O staining for lipid vacuoles (G–I). Control cultures in normal growth medium were also stained and were negative (data not shown). The figure shows one representative results from four independent experiments. Scale bars represent 100 μm. Colony forming units-fibroblast (CFU-F) (J) and population doubling time assay (K) of AD-MSCs isolated from C57BL/6, BALB/c and DBA mice at passage 3. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ significantly different from other group.

splenocytes proliferation was significantly lower in the presence of PHA compared to splenocytes cultured without PHA. To assess the involvement of cell–cell contact, AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice at passage 3 were added to splenocytes cultures with a ratio of AD-MSCs to splenocytes of 1:1, 0.2:1, 0.1:1, and 0:1 and cell proliferation was determined (Fig. 3C). The cells significantly ($P < 0.05$) inhibited splenocytes proliferation in a dose-dependent manner with 80% inhibition at the highest dilution tested (1:1). AD-MSCs isolated from BALB/c mice showed a significant ($P < 0.05$) suppression at 1:1 ratio in comparison with other strains and there was no significant differences in other ratios between three strains.

CYTOKINE PRODUCTION BY AD-MSCS

Analysis of TGF-β, IFN-γ, IL-4, IL-17, and IL-10 production demonstrated that, AD-MSCs consistently secreted TGF-β and IL-17 in culture medium, but no, IL-4, IL-10, and IFN-γ (Fig. 4A–E). AD-MSCs isolated from C57BL/6 and BALB/c mice produced significantly ($P < 0.05$) higher levels of TGF-β than those from DBA mice. Furthermore, IL-17 production was significantly ($P < 0.05$) higher in AD-MSCs isolated from BALB/c mice as compared with those from C57BL/6 and DBA mice.

IDO AND NO PRODUCTION BY AD-MSCS

Nitric oxide and IDO are important mediators of immunomodulation by MSCs. Analysis of NO production demonstrated that, AD-MSCs consistently secreted NO after three passages and there was no significant difference in the release of NO between the three strains (Fig. 5A). In this study, we observed that AD-MSCs isolated from BALB/c mice produced significantly ($P < 0.05$) higher levels of IDO in comparison with AD-MSCs isolated from C57BL/6 and DBA mice

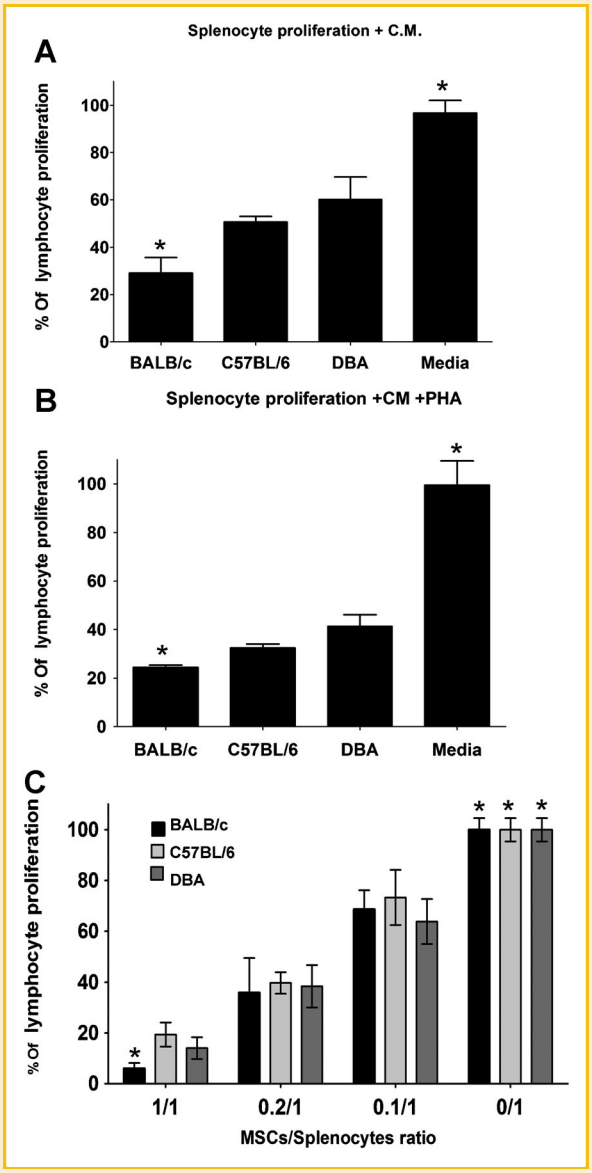

Fig. 3. The inhibitory effect of AD-MSCs conditioned media and AD-MSCs isolated from C57BL/6, BALB/c and DBA mice at passage 3 on splenocytes proliferation. Splenocytes were incubated for 3 days with AD-MSCs conditioned media in the absence (A) and presence (B) of PHA as a stimulator. Splenocytes were cultured alone as control or in combination with different concentrations of AD-MSCs in the presence of PHA (C). Results are expressed as the percent of splenocytes proliferation obtained in the absence of AD-MSCs conditioned media and AD-MSCs. Serum free DMEM was used as control (media). Data are expressed as the mean ± SD of three independent experiments. *$P < 0.05$ significantly different from other group.

(Fig. 5B). However, there was no significant difference in the release of IDO between the C57BL/6 and DBA strains.

EFFECTS OF AD-MSCS CONDITIONED MEDIA ON CYTOKINE PRODUCTION BY SPLENOCYTES

We next analyzed the capacity of AD-MSCs CM from C57BL/6, BALB/c, and DBA mice to modulate TGF-β, IFN-γ, IL-4, IL-17, and IL-10 production by splenocytes. Figure 7A–E shows that the culture of splenocytes with AD-MSCs CM from C57BL/6, BALB/c, and DBA mice resulted in increased TGF-β, IL-4, and IL-10 production and decreased IFN-γ and IL-17 production by unstimulated splenocytes. The results showed that AD-MSCs CM from BALB/c mice significantly ($P < 0.05$) decreased IFN-γ and IL-17 production and significantly, ($P < 0.05$) increased IL-4, and IL-10 production compared to AD-MSCs CM from C57BL/6 and DBA mice. In addition, after culture of splenocytes with PHA and AD-MSCs CM from three inbred strains of mice increased TGF-β, IL-4, and IL-10 production and decreased IFN-γ and IL-17 production were seen in comparison with culture medium (Fig. 6F–J). Moreover, the result indicated that AD-MSCs CM from BALB/c significantly ($P < 0.05$) decreased IL-17 production and significantly ($P < 0.05$) increased IL-4, and IL-10 production compared to the C57BL/6 and DBA CM (Fig. 6G,I,J). However, there was no significant difference in the release of TGF-β between the different strains (Fig. 6F).

EFFECTS OF AD-MSCS CONDITIONED MEDIA ON IDO AND NO PRODUCTION BY SPLENOCYTES

To examine the effect of AD-MSCs CM on the IDO and NO production by splenocytes, we measured the production of IDO and NO by splenocytes in the presence and absence of PHA (Fig. 7). As shown in Figure 7A, NO production by stimulated and unstimulated splenocytes significantly ($P < 0.05$) increased after culturing with AD-MSCs CM from C57BL/6, BALB/c, and DBA mice in comparison with culture media only. The results indicated that AD-MSCs CM from BALB/c significantly ($P < 0.05$) increased NO production compared to AD-MSCs CM from C57BL/6 and DBA mice in the in the presence and absence of PHA (Fig. 7B). Moreover, IDO production by stimulated and unstimulated splenocytes significantly ($P < 0.05$) increased after culturing with CM from three inbred mice in comparison with culture media and there was no significant differences between three inbred strains of mice (Fig. 7C,D).

DISCUSSION

In the current study, AD-MSCs were isolated from three inbred strains of mice and evaluated to determine their phenotype and capacity to differentiate into adipocyte and osteocyte. We also characterized the immunomodulatory effects of AD-MSCs CM from C57BL/6, BALB/c, and DBA mice. The isolated AD-MSCs from three inbred strains of mice presented similar morphology and cell surface markers. In addition, no significant difference was found in the in vitro differentiation and proliferation potentials of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice. On the other hand, the immunological assays showed some variation among the strains in the cytokines, NO, and IDO production. It is well known that MSCs exert broad immunomodulatory effects [reviewed in Nauta and Fibbe, 2007; Uccelli et al., 2008]. Therefore, we hypothesized that AD-MSCs isolated from inbred mouse strains with different T helper immune backgrounds are different in their immunomodulatory functions. Since mouse is one of the most useful experimental models of immunological diseases, the selection of an ideal strain of mice to develop experimental models of human diseases and stem cell therapy studies appear to be a crucial factor.

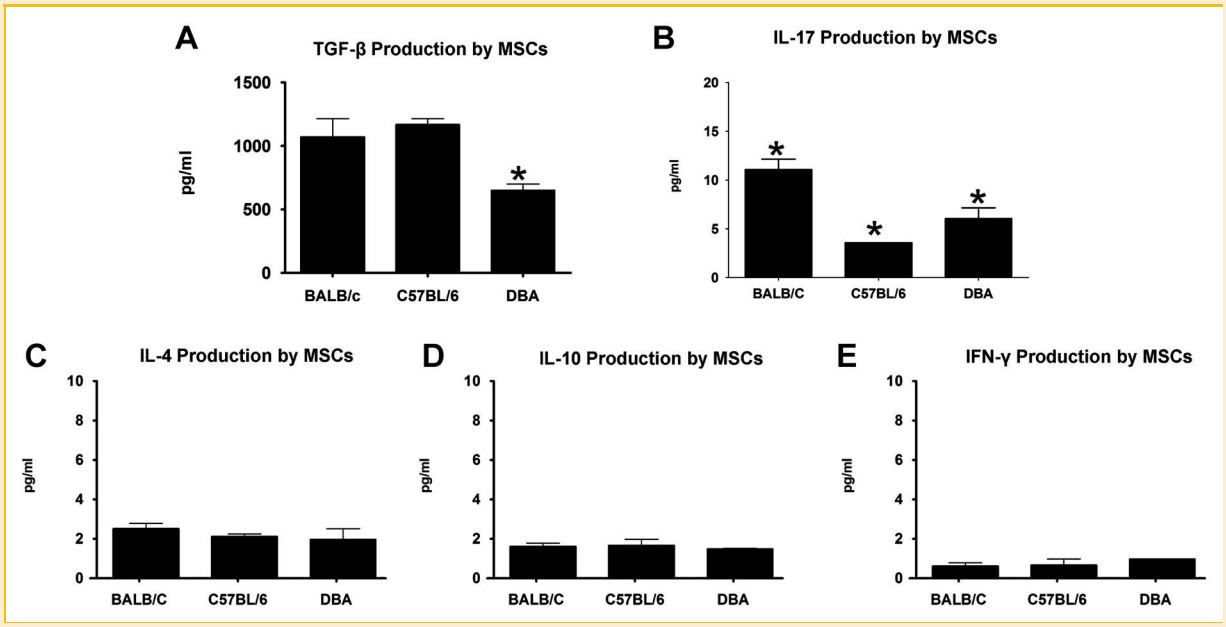

Fig. 4. TGF-β (A), IL-17 (B), IL-17 (C), IL-14 (D), IL-10 (E), and IFN-γ production by AD-MSCs isolated from C57BL/6, BALB/c and DBA mice after three passages. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ significantly different from other group.

Some strains of mice are Th1-biased (C57Bl/6, DBA/2, AKR, and CBA), while the others are Th2-biased strains (BALB/c, BP2, and A/J) [De Vooght et al., 2010]. In this study splenocytes from (C57BL/6 × BALB/c) F1 mice were employed because they would not respond to parental antigens [Mills et al., 2000] and to minimize the effects of genetic background differences in splenocyte immune function. In addition, a number of previous studies have compared phenotype and functional characteristics of MSCs obtained from different human or mouse tissues [Keyser et al., 2007; Yoo et al., 2009; Lee et al., 2012]. Moreover, there are recent comparative studies in which BM-MSCs from different strains were analyzed [Peister et al., 2004; Li et al., 2008; Barzilay et al., 2009]. However, to our knowledge, this is the first comparative study of phenotype, differentiation, and immunomodulatory effects of AD-MSCs from different mouse strains.

Immunophenotyping of AD-MSCs isolated from three different mouse strains at passage 3 showed that expression of surface markers were compatible with those previously reported for mouse AD-MSCs [Yamamoto et al., 2007]. However, it has been reported that BM-MSCs from different strains also differed in their expression of the cell-surface markers [Phinney et al., 1999; Peister et al., 2004; Li et al., 2008]. In addition, no statistically significant differences were found between CFU-Fs frequency of AD-MSCs isolated from different strains. Phinney et al. [1999] reported that the number of CFU-Fs derived from the BM-MSCs of five different inbred mouse strains varies dramatically and the BM-MSCs of BALB/c mice yield the highest number of CFU-Fs. In another study, it has been reported that BM-MSCs from C57BL/6 and BALB/c expanded more rapidly than the other, and the cells from the different strains had different media requirements [Peister et al., 2004]. In a previous study [Sung et al., 2008], the number of CFU-Fs of the AD-MSCs from the C57BL/6 strain was significantly higher than that of the C3H and BALB/c strains [Sung et al., 2008].

It is well known that MSCs can inhibit T lymphocytes proliferative responses to stimulation by allogeneic cells or by mitogens [Rasmusson et al., 2005; Sato et al., 2007; Lu et al., 2009; Yang et al., 2009; Sioud et al., 2011; Svobodova et al., 2011]. In this report, we compared this inhibitory effect in three inbred strains of mouse, and our results indicated a significant ($P < 0.05$) suppression of splenocytes proliferation in the presence of AD-MSC CM in all three inbred mouse strains. However, the splenocyte proliferation was suppressed more significantly ($P < 0.05$) by CM from BALB/c CM than by the other two strains (Fig. 3A,B). Furthermore, the involvement of cell–cell contact was assessed by co-culture of splenocytes and AD-MSCs with a ratio of AD-MSCs to splenocytes of 1:1, 0.2:1, 0.1:1, and 0:1. The cells inhibited splenocytes proliferation in a dose-dependent manner with 80% inhibition at the highest dilution tested (1:1). In agreement with these results, other groups have demonstrated that mouse and human MSCs isolated from different tissues have an inhibitory effect on T lymphocyte proliferation triggered by allogeneic cells or mitogens such as PHA or IL-2 [Sudres et al., 2006; Jarvinen et al., 2008; Yang et al., 2009; Cutler et al., 2010]. In a recent study, the culture supernatant of BM-MSCs of both BALB/c and C57BL/6 effectively inhibited the proliferation of T lymphocyte and they reported that T cell contact with MSCs is not necessary to inhibit T cell proliferation [Yang et al., 2009]. Several studies have revealed that these inhibitory effects of MSCs are mediated by both cell–cell contact and soluble factors such as PGE2, IDO, NO, and TGF-β [Yang et al., 2009; Shi et al., 2011]. The production of NO by MSCs has also been reported as a potential mechanism of inhibition of lymphocyte proliferation [Sato et al., 2007; Shi et al., 2011]. IDO production by MSCs is another suggested mechanism that induces the depletion of tryptophan, which is an essential amino acid for lymphocyte proliferation [Meisel et al., 2004; Uccelli et al., 2008]. Therefore, in

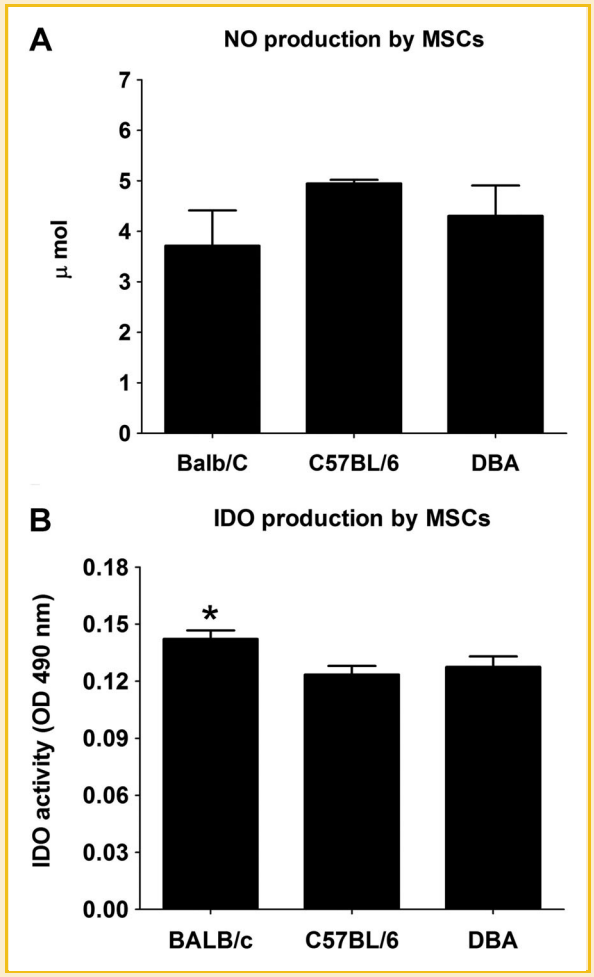

Fig. 5. Nitric Oxide (NO) (A) and indoleamine 2,3-dioxygenase (IDO) (B) production by AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice after 3 passage. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ significantly different from other group.

this study, we analyzed the NO, IDO, and cytokine production by AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice. Our findings show that AD-MSCs isolated from BALB/c mice produced significantly ($P < 0.05$) higher levels of IDO in comparison with AD-MSCs isolated from C57BL/6 and DBA mice (Fig. 5B). AD-MSCs isolated from C57BL/6 and BALB/c mice produced significantly ($P < 0.05$) higher levels of TGF-β than those from DBA mice. Furthermore, IL-17 production was significantly ($P < 0.05$) higher in AD-MSCs isolated from BALB/c mice as compared with those from C57BL/6 and DBA mice. Our results confirm the observation that TGF-β, HGF, IDO, PGE-2, and NO mediate T lymphocyte suppression by MSCs [Sato et al., 2007; DelaRosa et al., 2009; Nemeth et al., 2010; Yanez et al., 2010]. Specifically, neutralizing antibodies against TGF-β, HGF, an inhibitor of PGE-2, or an inhibitor of IDO production reverses the inhibition of T-cell proliferation by MSCs [Meisel et al., 2004; Sato et al., 2007]. Moreover, MSCs from iNOS−/− mice had reduced the abilities to suppress T-cell proliferation [Sato et al., 2007]. It has been reported that the cytokine profile of AD-MSCs is similar to that reported for BM-MSCs and MSCs isolated from different tissues [Puissant et al., 2005; Kilroy et al., 2007; Kang et al., 2008]. However, there are some discrepancies between the results obtained by different studies that reflect organ- or species-specific differences in the immunomodulatory effects of MSCs. Moreover, other factors such as HLA-G, hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), and PGE-2 are associated with the immunomodulatory effects of MSCs [Nasef et al., 2008; Uccelli et al., 2008]. Although in the present study, we have not compared these soluble factors in the three inbred strains.

We next analyzed the capacity of AD-MSCs CM from C57BL/6, BALB/c, and DBA mice to modulate TGF-β, IFN-γ, IL-4, IL-17, IL-10, IDO, and NO production by splenocytes. Our in vitro results indicated an increased production of TGF-β, IL-4, IL-10, NO, and IDO; and decreased production IFN-γ and IL-17 by unstimulated splenocytes in response to AD-MSCs CM. In addition, after culture of splenocytes with PHA and AD-MSCs CM from three inbred strains of mice increased TGF-β, IL-4, IL-10, IDO, and NO production and decreased IFN-γ and IL-17 production were seen in comparison with culture medium (Fig. 6F–J and Fig. 7B,D). Many reports indicated that three types of CD4+ helper T lymphocytes (Th), Th1, Th2, and Th17, were implicated in the pathogenesis of different autoimmune diseases and characterized by their distinct cytokine profiles [reviewed in Steinman 2007]. IL-2, IFN-γ, and TNF-α are Th1 cytokines and IL-4, IL-5, IL-6, IL-10, and IL-13 are Th2 cytokines. Regulatory T cells and Th3 cells produce TGF-β and IL10 and another novel T helper subtype (Th17) produce IL17. In recent years, it has been clarified that MSCs have in vitro and in vivo potential to suppress immune response through a shift in the Th1/Th17 to Th2 cell balance, which indicates a shift from a pro-inflammatory (IFN-γ-producing) state to an anti-inflammatory (IL-4-producing) state [Uccelli et al., 2008; Li et al., 2010]. Recent in vivo studies have indicated that transplantation of MSCs derived from bone marrow and adipose tissue polarize host immune response towards a Th2 cytokine profile by down-regulating Th1 cytokines and/or up-regulating of Th2 cytokines [Batten et al., 2006; Constantin et al., 2009; Lu et al., 2009; Bouffi et al., 2010; Darlington et al., 2010; Choi et al., 2011]. Moreover, our result was confirmed by the in vivo and in vitro observations that MSCs inhibit Th17 differentiation and IL-17 production [Constantin et al., 2009; Rafei et al., 2009; Duffy et al., 2011; Tatara et al., 2011].

Our results suggest that AD-MSCs CM from BALB/c mice is more potent than other strains in polarization of the immune response towards a Th2 cytokine production profile and suppression of Th1 and Th17 profiles. The differences in the immunological properties of AD-MSCs isolated from three inbred mouse strains could be related to the difference in the genetic and immunological backgrounds that have been reported to be Th1 in C57BL/6 and DBA mice and Th2 BALB/c mice. Th2 strains are completely resistant to the development of some autoimmune disease, whereas other Th1 strains are susceptible. The differences among MSCs isolated from different strains may explain conflicting data in the literature concerning the immunomodulatory effects of MSCs isolated from various species, strains, and tissues.

In conclusion, our results showed that the immunomodulatory properties of mouse adipose tissue-derived mesenchymal stem cells

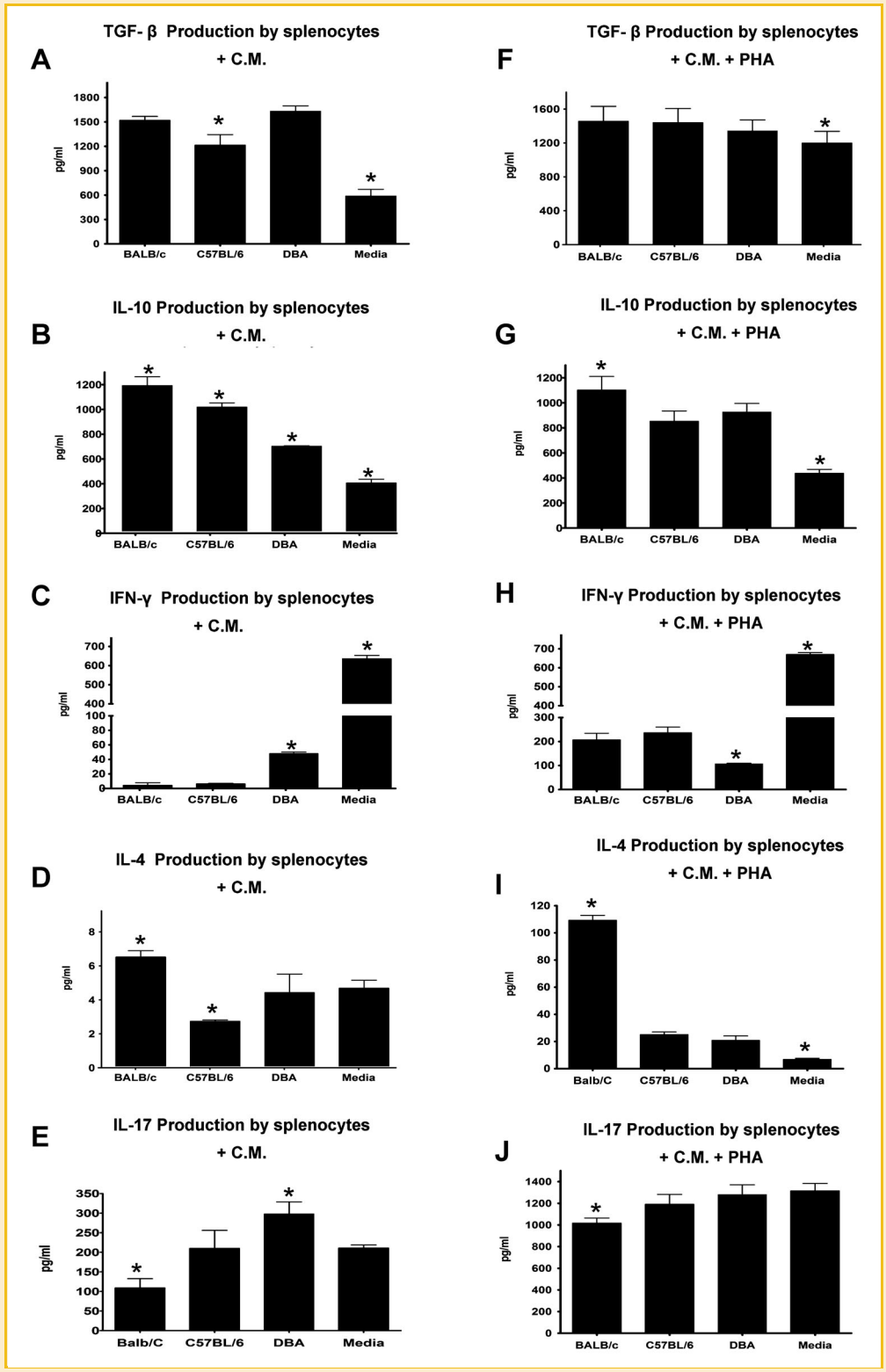

Fig. 6. The effect of the conditioned media of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice at passage 3 on splenocytes cytokine production. Splenocytes were incubated for 3 days with AD-MSCs conditioned media in the absence (A–E) and presence (F–J) of PHA as stimulator. Serum free DMEM was used as control (media). Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ significantly different from other group.

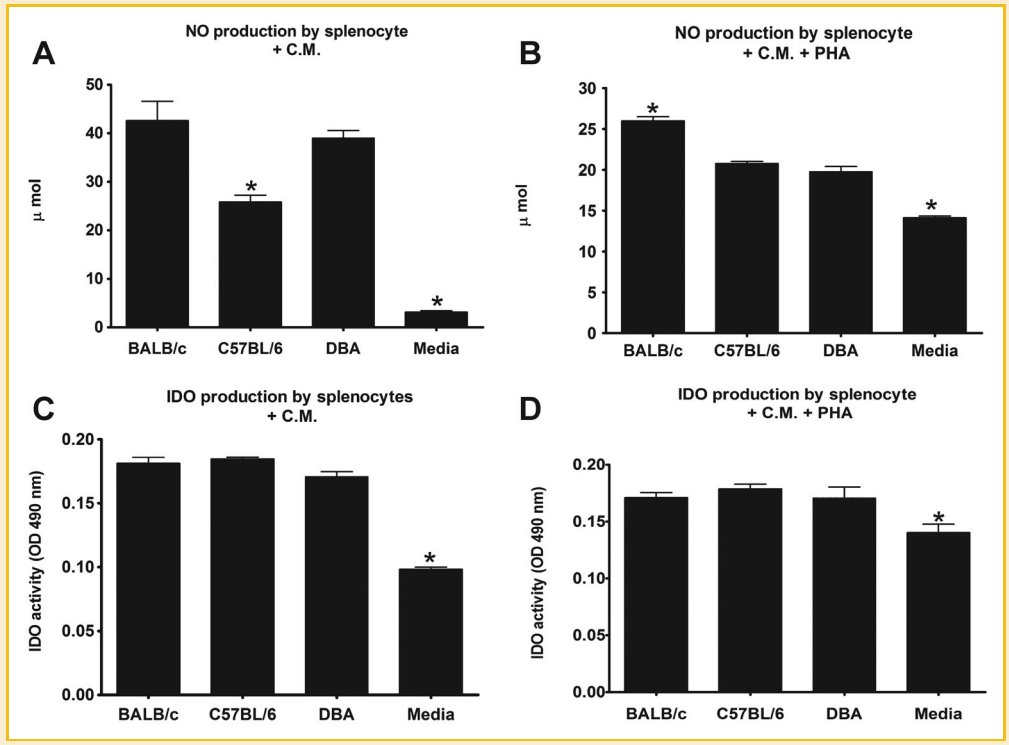

Fig. 7. The effect of the conditioned media of AD-MSCs isolated from C57BL/6, BALB/c, and DBA mice at passage 3 on splenocytes Nitric Oxide (NO) and indoleamine 2,3-dioxygenase (IDO) production. Splenocytes were incubated for 3 days with AD-MSCs conditioned media in the absence (A,C) and presence (B,E) of PHA as a stimulator. Serum free DMEM was used as control (media). Data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$ significantly different from other group.

is strain-dependent and this variation should be considered during selection of appropriate stem cell source for in vivo experiments and stem cell therapy strategies.

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