Human umbilical cord blood mesenchymal stem cells effect on expression of leukaemic inhibitory factor and interleukin-10 in acute myeloid leukaemia

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Background
Haematological malignancies represent ~7% of all malignant diseases. Acute myeloid leukaemia (AML) is an aggressive and fatal disease. AML treatment basically remained unimproved in the last 20 years; it depends upon induction of cytotoxic chemotherapy. An average of less than 30% of AML patients survive for the long term. Mesenchymal stem cells (MSCs) are currently being investigated for an ever-expanding number of clinical indications based on their tissue-regenerative, immunomodulatory and anti-inflammatory effects. The leukaemic inhibitory factor gene (*LIF*) induces the differentiation of AML cells and inhibits their growth, while the interleukin-10 (IL-10) might be an efficient inhibitor of tumour metastasis.
Aim
The present work aimed to detect the effect of human umbilical cord blood-derived (HUCB-MSCs) on the expression of the *LIF* gene and on IL-10 in AML patients.
Materials and methods
The MSCs were separated from HUCB, and co-cultured with samples collected from peripheral blood of AML-insulted adults before chemotherapy. The expression of *LIF* gene and the IL-10 level were measured using the real-time PCR and enzyme-linked immunosorbent assay techniques, respectively, before and after the co-culture aiming to evaluate the immunomodulatory and anti-inflammatory effects of the MSCs.
Statistical analysis
The results were considered statistically significant if a '*P* value' was found to be less than 0.05 based on one-way analysis of variance analysis and paired *t*-test.
Results
The present study revealed that the group of AML cells co-cultured with HUCB-MSCs showed a significant increase in the expression level of *LIF* gene compared with the untreated group. The group of AML cells co-cultured with MSCs showed a significant decrease in the IL-10 concentration compared to that of the untreated group.
Conclusion
Our data demonstrated that co-culture of AML with MSCs represents a simple approach to inhibit leukaemic cells *in vitro*.

Keywords:
acute myeloid leukaemia, interleukin-10, leukaemia inhibitory factor, mesenchymal stem cells

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Introduction

Acute myeloid leukaemia (AML) is the most frequent haematological malignancy in adults, with an estimated worldwide annual incidence of 3–4 cases per 100 000 people. Despite intensive research on prognostic markers and new therapies, AML is still a disease with a highly variable prognosis among patients, and a high-mortality rate. Less than 50% of adult AML patients have a 5-year overall survival rate, and in the elderly, only 20% of AML patients survive for 2 years [1]. The AML is an aggressive and fatal disease caused by an increased proliferation and a block to differentiation capacity of the myeloid blasts [2].

The mesenchymal stem cells (MSCs) are undeveloped cells capable of proliferation, self-renewal, conversion to differentiated cells and regeneration of tissues. The stem cells of adult origin have been used clinically for more than 50 years in the treatment of haematological neoplasms such as leukaemia [3]. The stem cells have been used in replenishing the blood and the immune system damaged by the cancer cells or during treatment of cancer by chemotherapy or radiotherapy [4]. Thus,

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the stem cells may be potential candidates for immunotherapeutic approaches in AML patients. The MSCs seem to have a relevant role in AML as they were found having unique immunomodulatory properties [5], prevent spontaneous and induced apoptosis and may attenuate chemotherapy-induced cell death [6]. It was found that co-cultivation of a leukaemic cell line with the murine stroma cell line 'MS-5' can block apoptosis [6].

Leukaemia inhibitory factor (LIF) protein is a pluripotent cytokine with pleiotropic activities [7]. The *LIF* gene has been characterized by its ability to induce the differentiation of the murine myeloid leukaemia cell line (M1) and suppress its growth [8]. The *LIF* gene also maintains the pluripotency of embryonic stem cells, while it induces the differentiation of several myeloid leukaemia cells and inhibits their growth [9]. Generally, the *LIF* gene plays an important and complex role in cancer; this role depends upon the types of the cancer [10].

The cytokines have been reported to be important regulators of AML blast proliferation, but the responses to cytokines are variable [11]. Interleukin-10 (IL-10) is a polypeptide produced by the Th2 subset of T-helper lymphocytes, B lymphocytes, macrophages and monocytes in response to immunological challenge [12]. The IL-10 has been detected in the leukaemic cells of most AML cases; it suppresses the immune reactions [13]. The IL-10 is also an efficient inhibitor of tumour metastasis in-vivo at doses that do not have a direct effect on the normal cells [14].

Therefore, the present study aimed to investigate the effect of the MSCs on the expression of *LIF* gene, and the IL-10 level in AML; the elucidation of that effect may provide an important insight into the role of MSCs as a regenerative medical tool for AML.

Materials and methods

Materials

High-glucose Dulbecco's modified Eagle's medium (DMEM) [15], low-glucose-DMEM, PBS, foetal bovine serum, trypsin/EDTA 0.25%, penicillin solution (10 000 U/ml), streptomycin solution (10 000 U/ml) and l-glutamine 200 mmol/l (100×) were purchased from Gibco/BRL, Invitrogen, Applied Biosystems (Carlsbad, California, USA). IL-10 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Orgenium Laboratories, AviBion (Vantaa, Finland). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD33, CD34, CD41 and CD44, phycoerythrin (PE)-conjugated monoclonal antibodies against CD13, CD34 and CD105 were purchased from MiltnyiBiotec (Bergisch, Gladbach, Germany). SV total RNA isolation system was purchased from Promega (Madison, Wisconsin, USA). RNeasy extraction kit was purchased from Qiagen Gmbh (Hilden, Germany).

Groups

The present study included four groups of cultured cells as the following:

Group 1: control group: six peripheral blood (PB) samples derived from healthy humans; group 2: AML: 10 PB samples derived from 10 adult humans diagnosed with AML, group 3: MSCs: 10 human umbilical cord blood samples (HUCB) and group 4: MSCs co-cultured with AML cells: 10 co-cultured samples. The study was started after the approval of the Local Ethical Committee of the Faculty of Medicine, Cairo University. Informed consent was obtained from each patient before enrolment in the study.

Methods

The *LIF* gene expression and IL-10 level were measured using real-time PCR (qPCR) and ELISA techniques, respectively, before and after the co-culture. The cultured cells were detected using the specific surface markers by Navios flow cytometer (Beckman Coulter Life Sciences, Atlanta, Georgia, USA) at the Egyptian National Cancer Institute in Cairo.

Mesenchymal stem cells isolation, culture, and expansion

The MSCs were isolated from HUCB as was described previously [16,17]. HUCB was diluted with PBS in 1 : 2 ratio. Buffy coat was isolated by density-gradient centrifugation at 1800*g* for 30 min at room temperature and re-suspended in complete culture medium (5 ml of DMEM, 10% foetal bovine serum, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l l-glutamine). The cells were incubated at 37°C in 5% humidified CO_2. The MSCs were attached to the polystyrene surface of the culture flask and were further purified by passages. Nonadherent cells were removed by replacing the medium after 2 days [18]. The medium was replaced every other day. When the cells were grown up to 70–80% confluency, they were passaged to the next passage using trypsin/EDTA 0.25%.

Mesenchymal stem cells identification

The cultured MSCs were identified by their attachment to the polystyrene surface of the culture flask and also by their fibroblast-like morphology.

Moreover, MSCs were identified by flow cytometry analysis. In this technique, the cultured MSCs were harvested by treatment with 0.25% trypsin/EDTA and then were incubated for 1 h at 4°C with combinations of the following conjugated monoclonal antibodies: CD45 FITC, CD90 FITC, CD34 PE and CD105 PE. The control tubes were incubated with FITC-conjugated and PE-conjugated antibodies against human immunoglobulin G. The cells were stained with propidium iodide (1 μg/ml) before flow cytometry alanysis [19]. The cells were washed with PBS three times at room temperature, and then the labelled cells were detected immediately – after having been stained – using Navios flow cytometer (Beckman Coulter Life Sciences) according to the manufacturer's protocol. Thus, the fluorescence detectors identified the labelled cells based on their phenotypic markers using Navios software.

Isolation of leukaemic cells

PB samples were collected from 10 patients recently insulted with AML. The PB samples were diluted with an equal volume of 2% dextran solution and incubated at room temperature for 45 min. The supernatant was collected and centrifuged at 1.600*g* for 10 min. The pellet was re-suspended in 4 ml of PBS with 0.1% EDTA. The mixture was transferred into a 20-ml tube containing Ficoll-Paque, GE Healthcare, Chicago, Illinois, United States (ready-to-use reagent for in-vitro isolation of mononuclear cells) with a 2 : 1 ratio, respectively, and centrifuged at 2200*g* for 20 min. The buffy coat was thawed in human cell complete culture medium as was described previously ('Mesenchymal stem cell, isolation, culture, and expansion' section).

Identification of leukaemic cells

The blood cells from leukaemic samples were characterized by their adhesiveness to the polystyrene surface of the culture flask and their fusiform shape. Furthermore, leukaemic cells were identified by flow cytometry analysis. The leukaemic cells were suspended in PBS and incubated with combinations of the following conjugated monoclonal antibodies: CD33 FITC, CD41 FITC, CD13 PE and CD34 PE [20]. The labelled cells after having been stained were detected immediately by the fluorescence detectors of Navios flow cytometer (Beckman Coulter Life Sciences) according to the manufacturer's protocol as was described previously ('Mesenchymal stem cells identification' section).

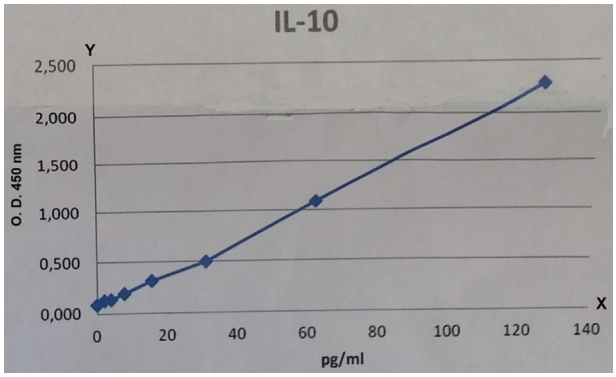
Co-culturing leukaemic cells with mesenchymal stem cells

Leukaemic cells were incubated at 37°C in 5% humidified CO_2. When large colonies developed (80–90% confluence), the cultures were washed twice with PBS and the cells were treated with 0.25% trypsin in 1 mmol/l EDTA for 5 min at 37°C. After centrifugation (at 2400*g* for 20 min), 2.5×10^5 cells were co-cultured with an equal number of MSCs and re-suspended in serum-supplemented complete culture and incubated in 50-cm^2 culture flasks [21].

Measurement of interleukin-10 concentration

24 h before harvesting the confluent adherent layers, the culture medium was collected in order to measure IL-10 concentration by human IL-10 ELISA kit according to the manufacturer's protocol. The level of IL-10 in cell culture supernatant was measured using 'Orgenium Laboratories IL-10 ELISA kit'. In this technique, 50 μl of the sample (culture medium) and 50 μl of each diluted standard (starting from 125 pg/ml) were added into appropriate wells. 50 μl of biotinylated antibody were added to all the wells containing the standards and samples (total reaction volume is 100 μl). 100 μl of prepared horseradish peroxidase-streptavidin solution were added to each well and incubated for 30 min at room temperature. 50 μl of tetramethylbenzidine – a soluble chromogenic substrate that yields a blue colour under the effect of horseradish peroxidase – were added to each well, and incubated in the dark for 20 min at room temperature. 25 μl of stop solution were added to each well, and the plate was read at 450 nm within 15 min according to the manufacturer's protocol [22]. The intensity of the produced blue colour gave an indication of the amount of IL-10 concentration in the cultured mediums. The results of the unknown samples were calculated against the standard. The standard curve is generated by plotting the average optical density (450 nm) – obtained for each of the standard concentrations – on the vertical axis (*y*) versus the corresponding IL-10 concentration (pg/ml) on the horizontal axis (*x*) (Fig. 1).

Figure 1



Human IL-10 standard curve. IL, interleukin.

Measurement of LIF gene expression

RNA extraction and cDNA synthesis: Total RNA was extracted from the cultured cells using RNeasy extraction kit (Qiagen Gmbh) according to the manufacturer's protocol. The amount of RNA was measured spectrophotometrically. The RNA integrity was tested on the Nanodrop. All samples had an optical density 260/280-nm ratio more than 1.8, indicating high purity. The extracted RNA was reverse transcribed into cDNA using cDNA reverse transcription kit (Stratagene, USA) according to the manufacturer's protocol.

qPCR: qPCR was performed using SYBR Green Universal Master Mix (2×) (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. The name and sequences of primers (purchased from Stratagene) for both *LIF* (target gene) and *β-actin* (control gene) are listed in Table 1. qPCR reactions were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the fluorescent TaqMan methodology. The thermal cycling conditions comprised of 10 min at 95°C, followed by 40 cycles (95°C denaturation for 15 s, 60°C annealing for 30 s and 72°C extension for 30 s) and finally incubation at 72°C for 10 min.

LIF gene expression analysis: The $2^{-\Delta\Delta C_t}$ method was used to quantify the *LIF* gene relative expression. This frequently used method presents expression levels in the number of folds, as compared to the expression level of a calibrator which is usually the biological control sample. The relative quantification value of *LIF* gene – normalized to the endogenous control ß-*actin* gene (housekeeping) and relative to a calibrator – is expressed as $2^{-\Delta\Delta C_t}$ (fold difference), where $\Delta C_t = C_t$ (*LIF* gene)$-C_t$ (ß-*actin*) and $\Delta\Delta C_t = \Delta C_t$ of sample of *LIF* gene $-\Delta C_t$ of calibrator of ß-*actin* gene.

Statistical analysis

The data were expressed as mean±SD. Statistical analysis was performed with statistical package for the social sciences (SPSS version 10; SPSS Inc., Delaware, Chicago, Illinois, USA). The results were considered statistically significant if a '*P* value' was found to be less than 0.05 based on one-way analysis of variance analysis and paired *t*-test.

Results

The identification of mesenchymal stem cells and acute myeloid leukaemia

The present study recorded the fibroblast-like cultured MSCs, and their adherence capabilities to the polystyrene surface in the culture and their differentiation capabilities into connective tissue (Fig. 2a and b); the current study also showed AML before and after co-culture with MSCs (Fig. 2c and d).

The current study also showed that the human MSCs are negative for both CD34 and CD45, but positive for both CD90 and CD105 (Fig. 3a and b), while AML cells are negative for both CD34 and CD41, but positive for both CD13 and CD33 as was proven by the immunophenotyping (Fig. 3c and d).

LIF gene expression

The results obtained using the $2^{-\Delta\Delta C_t}$ method showed that the mean±SD of the relative expression of *LIF* gene increased significantly from 1.00±0.00 in the control group (group 1) to 1.911±0.219 in the AML group (group 2) ($P<0.05$). Following MSCs co-culture (group 4), the mean±SD of the relative expression of the *LIF* gene increased significantly reaching 4.247±0.415 compared to the AML group (group 2) (Table 2 and Fig. 4).

Interleukin-10 concentration

The IL-10 concentration in cell culture supernatant increased significantly from 1.13±0.17 in the control group (group 1) to 4.28±0.22 in the AML group (group 2). Following MSCs co-culture, the mean ±SD of IL-10 concentration decreased significantly reaching 1.33±0.15 (Table 3 and Fig. 5).

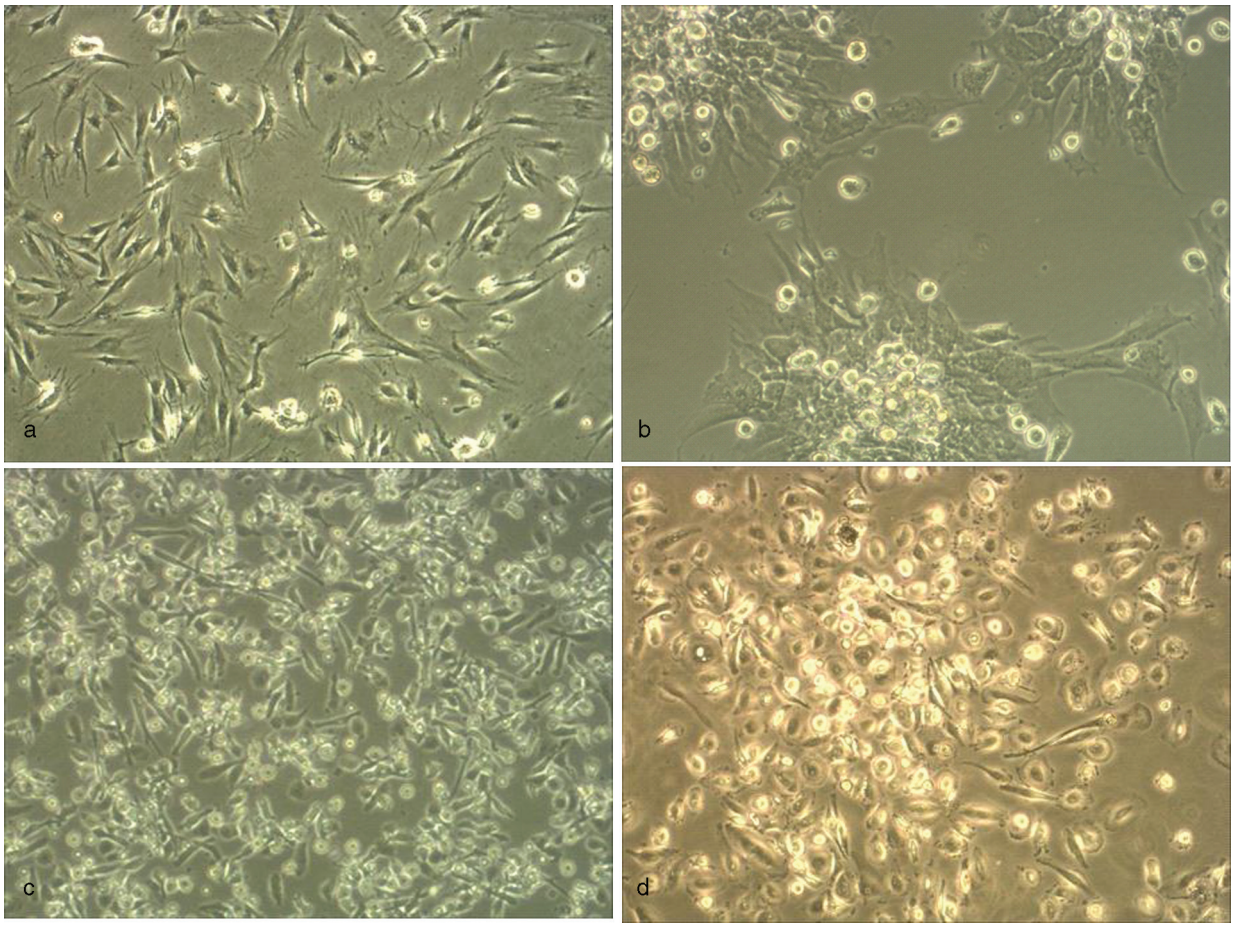
Discussion

In agreement with the previously reported data [23], the present study recorded the adherence capabilities of the fibroblast-like cultured MSCs to the polystyrene surface in the culture and their differentiation capabilities into connective tissue. The current study showed that the human MSCs are negative for both CD34 and CD45, but positive for both CD90 and CD105, as were proven by immunophenotyping. This is in accordance with the data [24,25] which reported that the MSCs

Table 1 List of human gene-specific primers

Genes	Forward primer (5′→3′)	Reverse primer (5′→3′)	cDNA fragment size (bp)
LIF	GTCAACTGGCTCAACTCAACG	TACGCGACCATCCGATACAGC	185
ß-*actin*	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG	133

Figure 2



(a) MSCs and AML cultures. Primary culture of MSCs on day 7; colony-forming unit-fibroblast (CFU-F) with rapidly proliferating MSCs. 200×. (b) MSCs and AML cultures. Primary culture of MSCs on day 5, the differentiation capabilities of MSCs into connective tissue. 400×. (c) MSCs and AML cultures. Primary culture of the leukaemic blast cells on day 7, the size of the colony is very large with a relatively equal distribution and elongated cells. 100×. (d) MSCs and AML cultures. Co-culture of MSCs with AML on day 5; it has been observed that the total number of viable leukaemic cells was diminished after co-culture with MSCs; while MSCs tend to make CFU-F. 200×. AML, acute myeloid leukaemia; MSC, mesenchymal stem cell.

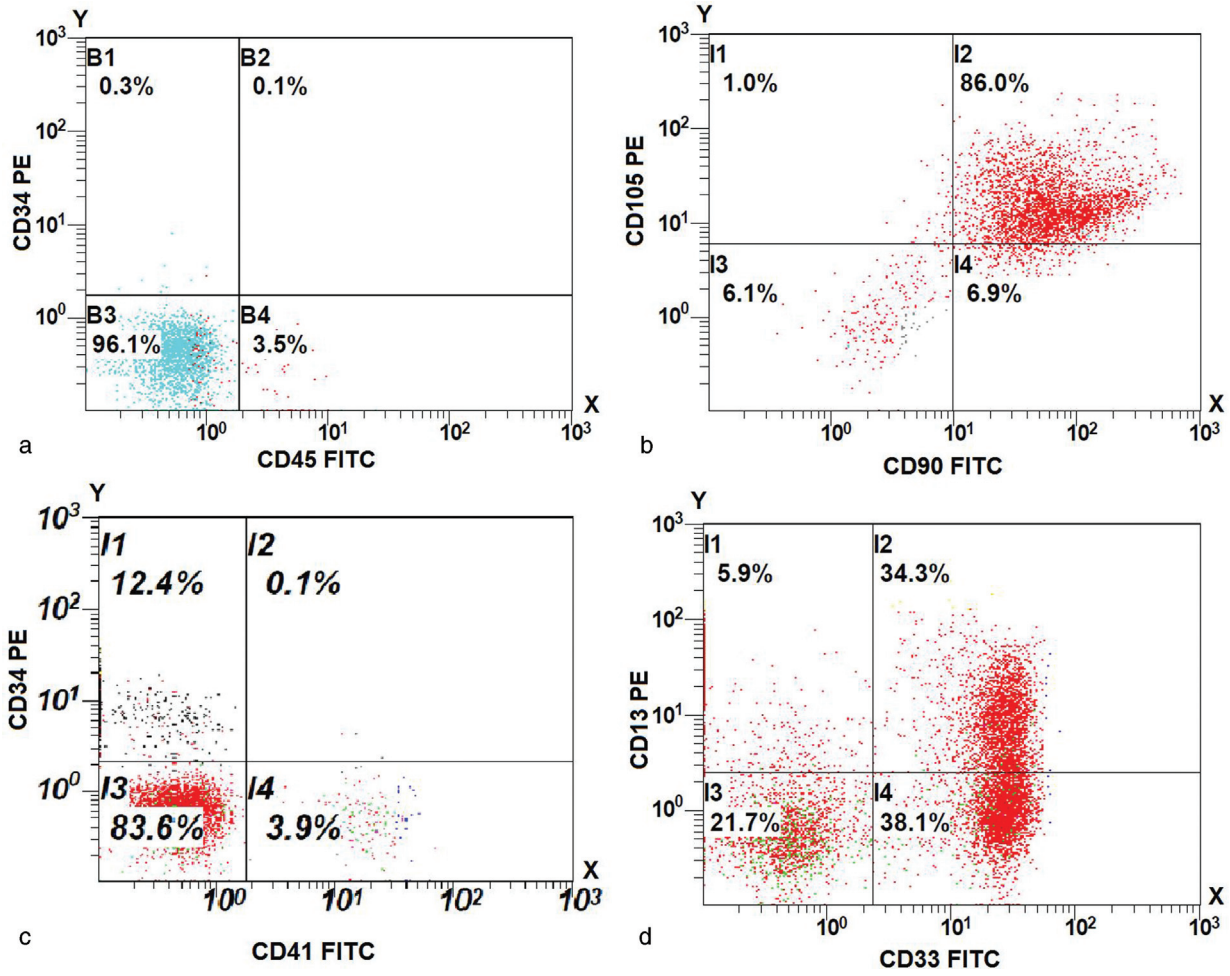
express CD105, CD90 and CD73, and that the MSCs lack the expression of CD45, CD34 and CD14. The current study also showed that AML cells were CD34-negative as was proven by flow cytometry, and this coincides with some researchers who showed that CD34 expression in AML is highly variable; they classified their patients into three groups based on the extent of CD34 expression [26].

In the present study, the AML group showed a significant increase in the relative expression of the *LIF* gene after co-culture with HUCB-MSCs. As regards IL-10 concentrations in the present study, there was a significant decrease in the IL-10 concentration in the cultured media of the AML group that was co-cultured with MSCs compared with that of the control group.

The high relative expression of the *LIF* gene in the MSC samples is in accordance with the results previously reported by Williams *et al.* [27] who reported that the *LIF* gene maintains the pluripotency of the embryonic stem cells, while it induces the differentiation of several myeloid leukaemia cells and inhibits their growth. Additionally, the present study confirmed previous findings that the *LIF* gene maintains the stem state of MSCs and other stem cells [9] through the finding of the high relative expression of the *LIF* gene in the MSCs.

The present study recorded a high relative expression of the *LIF* gene in the AML samples; this is in agreement with the data previously reported by Kim *et al.* [28] who documented that adherent layers derived from patients with chronic myeloid leukaemia, AML, myeloid dysplastic syndrome or hairy cell leukaemia secrete significantly higher levels of the LIF protein into the conditioned media, as compared to adherent layers derived from normal controls.

Figure 3



(a) Flow cytometric analysis of the cultured cells. Fluorescein isothiocyanate (FITC)-conjugated antibodies are on the *x* axis, while phycoerythrin (PE)-conjugated antibodies are on the *y* axis. (a) MSCs were CD34-negative and CD45-negative. (b) MSCs were CD90-positive and CD105-positive. (c) AML cells were CD34-negative and CD41-negative. (d) AML cells were CD13-positive and CD33-positive. AML, acute myeloid leukaemia; MSC, mesenchymal stem cell.

Table 2 Relative expression of the *LIF* gene in the four studied groups

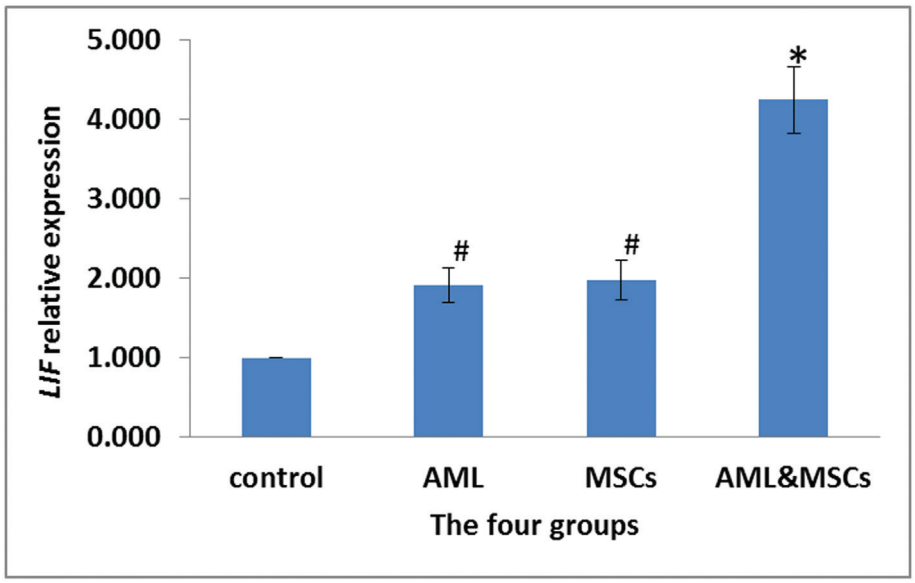
Group	Group 1 (control) (*n*=6)	Group 2 (AML) (*n*=10)	Group 3 (MSCs) (*n*=10)	Group 4 (AML and MSCs) (*n*=10)
LIF gene expression (mean ±SD)	1.00±00	1.911±0.219†	1.974±0.251†	4.247±0.415*

Relative *LIF* gene expression in the four studied groups. Data are mean±SD, *n*=number of samples per group. AML, acute myeloid leukaemia; LIF, leukaemia inhibitory factor; MSC, mesenchymal stem cell. *$P<0.05$, significantly different from the AML group. †$P<0.05$, significantly different from the control healthy group.

The present observations also revealed that the co-cultivation of MSCs with AML cells tends to upregulate the *LIF* gene expression. Moreover, the present study showed that there is a statistically significant difference between *LIF* expression in AML samples before and after co-culture with MSCs. Following the co-culture of AML cells with MSCs; the relative expression of the *LIF* gene increased significantly when compared to the AML group.

The present finding can be explained in the light of previously reported data that suggested that MSCs constitutively expressed mRNA for *LIF* among other cytokines such as 'IL-6', 'IL-11', 'macrophage colony-stimulating factor' (CSF) and 'stem cell factor' [29]; and then the *LIF* exhibits growth inhibitory effects on primary AML cells, most likely due to prolongation of the generation time of leukaemic stem cells. Furthermore, the *LIF* suppresses clonogenicity of several numbers of leukaemic cell lines such as 'HL-

Figure 4



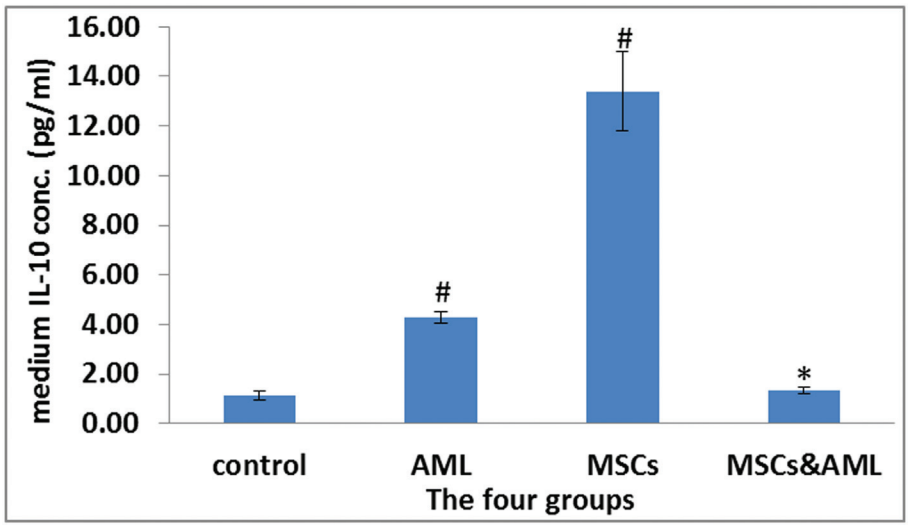
LIF gene relative expression in the four studied groups. Data are mean±SD, *significantly different from the AML group at $P<0.05$. #Significantly different from the control healthy group at $P<0.05$. AML, acute myeloid leukaemia; LIF, leukaemia inhibitory factor.

Table 3 Interleukin-10 concentration in the four studied groups

Group	Group 1 (control) (*n*=6)	Group 2 (AML) (*n*=10)	Group 3 (MSCs) (*n*=10)	Group 4 (AML and MSCs) (*n*=10)
IL-10 (pg/ml)	1.13	4.28†	13.4†	1.33*

IL-10 concentration in the four studied groups. Data are mean±SD, *n*: number of samples per group. AML, acute myeloid leukaemia; IL, interleukin; MSC, mesenchymal stem cell. *$P<0.005$, significantly different from the AML group. †$P<0.05$, significantly different from the control healthy group.

Figure 5



Concentration of IL-10 in the culture media of the four studied groups. Data are mean±SD, *significantly different from the AML group at $P<0.005$. #Significantly different from the control healthy group at $P<0.05$. AML, acute myeloid leukaemia; IL, interleukin.

60' and 'U937' cells in synergy with granulocyte-macrophage CSF and granulocyte-CSF [30].

The high relative expression of the *LIF* gene in AML samples that were co-cultured with MSCs makes MSCs a promising candidate for AML cell–based therapy; this agrees with previously reported results [31,32].The present study revealed that MSCs had a high-level of IL-10 in the cultured media. The present finding can be explained in the light of previously

reported data that suggested that MSC interactions with macrophages can change macrophages proinflammatory activity into anti-inflammatory via release of prostaglandin E2 from MSCs, which, in turn, binds to prostaglandin E2 receptor 2 and prostaglandin E2 receptor 4 on macrophages to induce high-level IL-10 production [33].

Moreover, the present study also showed that there was a statistically significant difference between IL-10 concentration in the AML medium before and after co-culturing with MSCs. The IL-10 concentration increased significantly in the AML group when compared to the control group; while it decreased significantly under the effect of MSCs co-culture. Our findings agree with the results of earlier studies [34,35], affirming that the MSCs have immunomodulatory properties with AML cells.

The high IL-10 level in MSCs cultured medium makes MSCs a perfect candidate for AML cell–based therapy; this is in agreement with the previously reported results [13]. These authors pointed out that IL-10 is an efficient inhibitor of tumour metastasis at doses that do not have a direct effect on the normal cells.

We perceive that co-culture of leukaemic blasts with MSCs might represent a simple approach to inhibit leukaemia cells *in vitro*. Our hypothesis can be supported by previous research which hypothesized that the MSCs represent the optimal candidate for cell–based therapy because they can be easily obtained from the bone marrow or the umbilical cord, and produce different cytokines that reduce apoptosis [36,37]. This co-culture system permits exploration of the protective effects of the MSCs against leukaemic cells, and serves as an improved method to explore therapeutic approaches targeting the leukaemic cells.

Conclusion

The present study showed a statistically significant increase in the *LIF* gene relative expression in AML cells after co-culture with MSCs when compared with AML cells; and a statistically significant decrease in IL-10 concentrations. Co-culture of AML with MSCs might represent a simple approach to inhibit leukaemia cells *in vitro*. This effect may be through the immunomodulatory and anti-inflamatory effects of MSCs. The MSCs represent the optimal candidate for cell therapy because they can be easily obtained from bone marrow or HUCB and expanded on a large scale before autotransplantation, raising no ethical problems.

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Nil.

Conflicts of interest

Conflicting interest: The authors declare that they have no competing interests.

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