Human amniotic fluid stem cells (hAFSCs) may be useful for regenerative medicine because of their ability to differentiate into all three germ layers and to modulate immune response with different types of secretion molecules. This last issue has not been completely elucidated. The aim of this study was to investigate the secretome profile of the hAFSC, focusing on the role of hepatocyte growth factor (HGF) in immunoregulation through short and long cocultures with human peripheral blood mononuclear cells. We found that HGF produced by hAFSCs exerts a cytoprotective role, inducing an increase in caspase-dependent apoptosis in human immune cells. This study provides evidence supporting the hypothesis that amniotic fluid is an ideal source of stem cells for expansion and banking properties for therapeutic use. hAFSCs not only are less immunogenic but also can secrete immunoregulatory factors that may be useful in autoimmune diseases or allogenic implants.

**INTRODUCTION**

Mesenchymal stem cells (MSCs) show immunomodulatory activity and secrete a wide spectrum of cytokines and chemokines that suppress inflammatory responses, that block mixed lymphocyte reactions and other immune reactions, and that have proven therapeutic effects against conditions such as graft-versus-host disease [1]. Immunoregulation by bone marrow MSCs (BM-MSCs) is thought to result from both direct interactions between the stromal and immune cells [2–4] and the actions of anti-inflammatory soluble factors released by the stromal cells [5, 6]. However, the relatively limited proliferation of BM-MSCs under standard conditions, suitable for manufacture of a clinical product, presents a potential drawback for their medical application [7]. Tissue engineering and cell therapy will be enhanced by improved cell sources.

Human amniotic fluid stem cells (hAFSCs) are broadly multipotent, can be expanded extensively in culture, are not tumorigenic, and can be readily cryopreserved for cell banking. hAFSCs resemble MSCs in many respects including surface marker expression and differentiation potential. The population of multipotent stem cells from amniotic fluid is obtained by immunoselection for c-Kit (CD117) [8], the cell surface receptor for stem cell factor, and is characterized by a high capacity for self-renewal and the ability to differentiate toward lineages representative of all three germ layers including glial cells, neurons, hepatocytes, osteocytes, chondrocytes, and adipocytes [8–11].

Both hAFSCs and BM-MSCs express surface markers CD29, CD44, CD73, CD90, and CD105, but hAFSCs also express the more primitive stem cell markers SSEA4 [8], Oct4, Nanog, and Sox2 [12]. The two cell types also have a similar immune antigen surface profiles with positive major histocompatibility complex (MHC) class I expression but little to no MHC class II expression [13]. Moreover, amniotic fluid cells may have an immunoprivileged status because fetal cells must possess mechanisms to avoid destruction by the maternal immune system during development [14].

In vivo experiments with hAFSCs have not had successful results. hAFSCs xenotransplanted in a rat model of myocardial infarction, with or without cyclosporine treatment, or in intact heart from immunocompetent or immunodeficient
animals were acutely rejected [15]. These authors have continued to investigate the immune regulatory potential of hAFSCs [16] because the prior experiment was cross-species. It may be possible to use amniotic fluid cells in an allogenic fashion, in addition to a potential autologous use for congenital defects. The authors found that exposure to an inflammatory milieu leads to activation of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), which becomes the central immunosuppressive enzyme affecting T-cell proliferation in several stem cell types, including hAFSCs [17], even if some other molecules may be involved [16, 18]. In fact, c-Kit-positive cells from amniotic fluid were described as exerting an immunomodulatory effect through cytokines separate from IDO secretion [18]. Moorefield et al. demonstrated that AFSCs secrete multiple factors in common with MSCs that are known to be involved in immune regulation, including growth-related oncogene and monocyte chemotactic protein (MCP) family members and interleukin-6 (IL-6). AFSCs, activated by exposure to peripheral blood mononuclear cells (PBMCs), released several additional cytokines compared with BM-MSCs [1].

Among secreted cytoprotective factors, hepatocyte growth factor (HGF) and matrix metalloproteinases 2 and 9 were identified [1, 19, 20]. HGF is a cytokine with angiogenic potential and is likely released in the blood, suggesting its endocrine role in targeting the injury site. Endogenous HGF is required for self-repair of injured liver, kidneys, lungs, and other organs. In addition, HGF exerts protective effects on epithelial and nonepithelial organs (including heart and brain) through antiapoptotic and anti-inflammatory signals. In line with other soluble factors associated with regenerative processes, HGF-1 possesses immunomodulatory activity [21]. Treatment of dendritic cells with HGF-1 reduces the ability to induce generation of inflammatory Th1 cells [22]. Furthermore, studies have shown that in vivo administration of HGF-1 protects against autoimmune diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, through stimulation of regulatory T cells producing immune-suppressive cytokines [23, 24]. MSC production of HGF-1 has been shown to be critical in several in vivo therapeutic activities of MSCs in models of autoimmunity [21].

We investigated the role of HGF, a soluble factor with both regenerative and immune modulatory activities that is secreted by hAFSCs, in the regulation of immune response. This study demonstrated that hAFSCs may counteract allogenic rejection by promoting cell survival, also through HGF secretion.

**Materials and Methods**

**Adult Human Tissue Isolation and Cell Culture**

Amniocentesis samples taken during the second trimester of pregnancy (6 back-up flasks obtained from different donors) were provided by the Laboratorio di Genetica, Ospedale Santa Maria Nuova (Reggio Emilia, Italy). All samples were collected with the informed consent of the donors (aged ≥35 years) according to Italian law and ethics committee guidelines.

The hAFSCs were isolated, as described previously [8]. Human amniocenteses cultures were harvested by trypsinization and subjected to c-Kit immunoselection by MACS technology (Milteny Biotech, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). The hAFSCs were subcultured routinely at 1:3 dilution and were not allowed to expand beyond 70% of confluence. The hAFSCs were grown in culture medium (Minimum Essential Medium, α modification, supplemented with 20% fetal bovine serum [FBS], 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin; EuroClone, Milan, Italy, http://www.euroclongroup.it) [25]. Osteogenic differentiation was obtained after 2 weeks of culture in a classic osteogenic medium, as described by Maraldi et al. [25, 26].

Neurogenic differentiation occurred after 3 weeks in medium containing retinoic acid [9].

**Assessment of Cytokines/Chemokines Levels by Antibody Microarrays**

The levels of cytokines IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-13, interferon-β (IFN-β), tumor necrosis factor-α (TNF-α) and chemokines IL-8 and MCP-1 were assessed in conditioned media obtained after 5 days of culture of hAFSCs (preactivated or not) with the Quantibody Human Inflammation Array 1 (Ray Biotech, Norcross, GA, http://www.raybiotech.com). In detail, the kit provides microarray slides, each spotted with 16 copies of a cytokine/chemokine antibody array. The slide comes with a 16-well removable gasket that allows for the process of 16 samples in 1 slide. Every array consists of 6 × 8 matrices in quadruplicate for each antibody and 2 positive controls.

Prior to processing, the samples were diluted 1:10 in the appropriate diluent buffer provided in the kit, and the assays were conducted according to a sandwich enzyme-linked immunosorbent assay (ELISA) protocol, according to the manufacturer’s instructions. The fluorescent signal was read with a ScanArray GX scanner (PerkinElmer, Waltham, MA, http://www.perkinelmer.com) and quantified with the ScanArray Express software (PerkinElmer). The quantification of each cytokine or chemokine was achieved by interpolating the fluorescent signals to the standard curve, generated by processing some arrays with specific standards containing predetermined cytokines concentrations (picograms per milliliter) [27].

**Immunofluorescence and Confocal Microscopy**

For immunofluorescence analysis, samples were processed as described previously [28]. Confocal imaging was performed by a Nikon A1 confocal laser scanning microscope (Nikon, Tokyo, Japan, http://www.nikon.com), as described previously [29]. Primary antibody against HGF (rabbit anti-HGF H-170, catalog number sc-13087) was purchased from Santa Cruz Biotechnology (Dallas TX, http://www.scbt.com). The primary antibody was diluted 1:50 and incubated for 1 hour at room temperature.

The confocal serial sections were processed with ImageJ software (NIH, Bethesda, MD, http://imagej.nih.gov/ij/) to obtain three-dimensional projections, as described previously [30]. The image rendering was performed with Adobe Photoshop software (Adobe Systems, San Jose, CA, http://www.adobe.com).

**Mononuclear Cell Separation**

Human PBMCs were separated from peripheral blood of healthy donors by gradient centrifugation (Ficoll-Hypaque, Lymphoprep; Axis-Shield, Dundee, U.K., http://www.axis-shield.com) at room temperature [31].

The concentration of isolated PBMCs was adjusted to 2 × 10^6 cells per milliliter in RPMI 1640 (EuroClone) including 10% FBS. Twenty hours later, PBMCs were washed and used for the experiments described below.
Coculture Experiments
The hAFSCs were seeded for all experiments in multiwell plates (6-well culture plates) when confluence was reached (approximately 300,000 cells per well) and were pre-exposed or not to PBMCs for 24 hours prior to coculture with fresh isolated PBMCs.

Cocultures in contact were obtained by adding 2 ml of PBMC suspension (2 × 10⁶ cells per milliliter) to hAFSC culture plates. For Transwell culture, PBMCs were seeded onto polycarbonate membrane cell culture inserts (Corning, Corning, NY, http://www.corning.com). For conditioned medium (CM) experiments, PBMCs were suspended in RPMI obtained from hAFSC culture (i.e., CM) for up to 5 days. In some experiments, 1 μg/ml of anti-HGF neutralizing antibody (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com) was added to the coculture. In general, cocultured cells and controls were maintained for 5 days.

Western Blotting
Whole-cell lysates from hAFSCs and PBMCs were processed as described previously [32]. Some sample were pretreated for 30 minutes with inhibitors of the Akt pathway [33]: a reversible inhibitor of PI3K LY294002 (25 μM, Calbiochem no. 440202; EMD Millipore, Billerica, MA, http://www.emdmillipore.com), an allosteric Akt inhibitor MK-2206 (0.1 μM, no. 51078; Selleck Chemicals, Houston, TX, http://www.selleckchem.com), and a dual ATP-competitive PI3K and mammalian target of rapamycin inhibitor BEZ235 (0.1 μM, no. 10565; Cayman Chemical, Ann Arbor, MI, https://www.caymanchem.com).

Primary antibodies were used against phospho-c-MET, phospho-Akt, phospho-ERK (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com); PARP, HGF, β-actin (Santa Cruz Biotechnology); and cleaved caspase 3 and 9 (Sigma-Aldrich).

HGF Immunoassay
Concentration of HGF in the conditioned media of cultured hAFSCs was measured by using a sandwich ELISA, according to the manufacturer’s instructions (Boster Biological Technology, Pleasanton, CA, http://www.bosterbio.com) [34]. Conditioned media were centrifuged before testing. Samples were run in duplicate. A standard curve was constructed using known concentrations of recombinant human HGF (0–8,000 pg/ml).

Evaluation of Apoptosis in Lymphocytes by Flow Cytometry
Apoptosis was quantitatively measured using propidium iodide. The staining process was performed according to the manufacturer’s instructions. Data were obtained using flow cytometric analysis with fluorescence-activated cell sorting within 1 hour.

Apoptosis Assay
PBMCs (10⁶ cells) were removed from coculture and washed with phosphate-buffered saline, and 50 μl of this suspension (0.5 × 10⁶ cells) was allowed to adhere to the slide for 10 minutes at room temperature. After 3 washes with 50 μl of binding buffer (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM CaCl₂), the specimen was incubated for 15 minutes with 50 μl of double staining solution (binding buffer containing 0.25 μl of annexin V-Cy3 and 0.25 μl of CFDA; Sigma-Aldrich). Finally, the specimen was washed 5 times with 50 μl of binding buffer, mounted with 15 μl of binding buffer, and visualized under fluorescence microscopy.

Statistical Analysis
In vitro experiments were performed in triplicate. For quantitative comparisons, values were reported as mean ± SD based on triplicate analysis for each sample. To test the significance of observed differences among the study groups, analysis of variance with post hoc Bonferroni correction or Student’s t test were applied. A p value <.05 was considered statistically significant.

RESULTS
The hAFSCs were isolated from a heterogeneous cell population of second-trimester amniotic fluid. After the selection of c-Kit-positive cells, the culture maintained markers typical not only of the mesenchymal profile. In Table 1 we present a summary of the characterization of hAFSCs obtained in our laboratory. These data were previously published in part [9, 35] and were obtained with different techniques, such as Western blot and immunofluorescence. As already reported by other authors, we confirmed that hAFSCs exhibit positivity for stromal mesenchymal markers, such as CD73, CD90, and CD105 [8, 18, 35, 36] and Str-1 and CD271 [9].

Moreover, hAFSCs selected for c-Kit and maintained in culture for several passages expressed proteins typical of more primitive stem cells features, such as SSEA4, Oct4, TRA-1-81, FOXO1, Sox2 [35], and Nanog [18]. Consequently, the differentiation potential observed for hAFSCs was consistent with the expression of some pluripotency-associated markers. We previously reported successful differentiation in osteoblasts [25, 26]; in adipocytes, myocytes, and pancreatic cells [34]; and in glial and neuronal cells [9].

Regarding the immunoregulatory potential of second-trimester hAFSCs, it has been noted that human leukocyte antigen A (HLA-A), HLA-B, and HLA-C are expressed, unlike HLA-DR [1, 36]. Moreover, the production in the secretum of some immune-modulating molecules has been proved for hAFSCs [1, 18, 36].

In order to shed a light on this issue, we used a microarray designed to test the presence in the secretum of several cytokines and chemokines: IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, MCP-1, IFN-γ, and TNF-α. Table 2 shows only the highest values obtained from the analysis of HGF in standard growth conditions or pre-exposed to PBMCs (AFSC active). This analysis confirmed the presence of IL-6 in hAFSC secretem [1], mostly in activated hAFSC medium. In this last condition, we also observed a detectable value of IL-8. The presence of MCP-1 is still evident in unactivated hAFSCs, but pre-exposure to PBMCs causes a large increase (10 times).

Immune modulation can be exerted by other soluble factors, IDO [18] and HGF [37]. HGF has been shown to exert regenerative activity outside the liver, including stimulation of angiogenesis. In line with other soluble factors associated with regenerative processes, HGF possesses immune modulatory activity. Consequently, we focused our attention on the role of HGF in the immunosuppressive effects of hAFSCs. HGF expression in hAFSCs was evaluated in different culture conditions. Figure 1A shows the Western blot analysis of total lysates of hAFSCs, even after 2 weeks of culture in osteogenic differentiation medium or after 3 weeks in neurogenic medium. In all of these conditions, HGF was detectable at comparable
intensities, indicating that this property was maintained during differentiation.

Results obtained by Western blot were confirmed by immunofluorescence. Undifferentiated hAFSCs strongly expressed HGF within the cytoplasm in a spot-like distribution (Fig. 1B). HGF production by hAFSCs seeded at confluence was measured in culture within the cytoplasm in a spot-like distribution (Fig. 1B). HGF-proteins. Undifferentiated hAFSCs strongly expressed HGF indicating that this property was maintained during differentiation.

Table 2. Summary of the characterization of hAFSCs isolated in our laboratory

| Multipotent mesenchymal stem cell markers | Technique | Reference |
|------------------------------------------|-----------|-----------|
| CD73                                    | WB        | [35]      |
| CD90                                    | WB        | [35]      |
| CD105                                   | WB        | [35]      |
| CD271                                   | IF        | [9]       |
| Stro1                                    | WB        | Data not shown |

Pluripotency-associated markers

| markers                  | Technique | Reference |
|--------------------------|-----------|-----------|
| Oct4                     | WB, IF    | [9, 35]   |
| Nanog                    | WB        | Data not shown |
| SSEA4                    | WB        | [35]      |
| Sox2                     | IF        | [35]      |
| TRA-1-81                 | WB        | Data not shown |
| c-Kit                    | WB, IF    | [9]       |
| FOXO1                    | IF        | [35]      |

Abbreviations: hAFSCs, human amniotic fluid stem cells; IF, immunofluorescence; WB, Western blot.

Table 2. Inflammation microarray of conditioned media

| Culture         | IL-6     | IL-8     | MCP-1    |
|-----------------|----------|----------|----------|
| hAFSCs          | 16.6 ± 0.6| 13.0 ± 0.4| 147.7 ± 16.0|
| hAFSCs active   | 718.1 ± 11.0   | 544.9 ± 38.0   | 1,242.2 ± 81.0   |

The table shows only the highest values (pg/ml) obtained from the analysis of conditioned media from 5 days of human AFSC cultures previously activated (AFSC active) or not (AFSCs) for 24 hours with peripheral blood mononuclear cells in the Transwell condition. *p < .0001 compared with AFSC values.

Abbreviation: hAFSCs, human amniotic fluid stem cells.

In order to better investigate the effect of interaction between hAFSCs and PBMCs, coculture experiments were performed for longer times. Samples of immune cells exposed to different kinds of coculture: (a) in CM, culture medium was derived after 5 days of hAFSCs in RPMI serum free; (b) in the Transwell condition, hAFSCs were in coculture with PBMCs but physically separated by a membrane; (c) in coculture of hAFSCs and PBMCs, PBMCs and hAFSCs were physically in contact.

PBMCs were then collected and analyzed by fluorescence-activated cell sorting (FACS), Western blot, and confocal microscopy.

Figure 3A shows representative images of PBMCs alone or exposed to CM, Transwell, and hAFSC-PBMC coculture with hAFSCs analyzed by Western blot for apoptotic markers. The cleaved form of PARP and caspase 3 significantly increased in the presence of hAFSC secretum in all the three coculture conditions.

Annexin V analysis was performed on PBMCs cultured alone (control) and PBMCs cocultured with normal hAFSC culture or activated hAFSCs to confirm apoptotic cell death in immune cells. Figure 3B shows representative images of PBMCs cultured alone or exposed to hAFSCs or activated hAFSCs, labeled by annexin V. The presence of cells stained with red rings increased in both exposure conditions with respect to the PBMCs cultured alone, indicating the onset of an apoptotic process, mostly in cells exposed to activated hAFSC conditioned medium. In order to quantify apoptosis occurring in PBMCs, FACS analysis was performed after staining with propidium iodide (Fig. 3C). The percentage of apoptotic cells in the presence or absence of CM obtained from normal hAFSC culture or activated hAFSCs is shown; in both conditions, the cells in the sub-G1 phase reach ~10% and 20%, respectively. These values are significantly different from the control and between each other.

The conditioned medium obtained after 5 days was chosen to test the efficacy of anti-HGF neutralizing antibody in reducing the apoptotic process. In fact, the conditioned medium is cell-free; therefore, it is possible to perform preincubation for 1 hour with the antibody in a shaking condition. Apoptosis was quantitatively assessed by cyttofluorimetric analysis (Fig. 3D). The percentage of apoptotic PBMCs obtained from CM of hAFSCs in the presence of anti-HGF significantly decreased, although it did not reach the percentage of leukocytes cultured alone. Furthermore, no significant differences in the sub-G1 level were observed in PBMCs with the addition of anti-HGF neutralizing antibody in comparison with PBMCs cultured alone.

This effect has been confirmed by Western blot analysis. The expression of PARP and active caspase 9 and 3 was assessed in...
whole leukocyte lysates cultured alone and cocultured with hAFSCs active in the Transwell condition (Fig. 3E). The increase of these markers confirms the presence of apoptotic death.

**DISCUSSION**

MSCs can give rise to differentiated cells of the mesodermal lineage including bone, fat, cartilage, tendon, and muscle [41–43]. In addition, their ability to evade immunosurveillance after cell transplantation and to suppress immune response has made BM-MSCs a particularly attractive candidate for clinical use [44, 45]. In particular, it was observed that BM-MSCs could suppress lymphocyte proliferation and activation in response to the allogenic activation or chemical stimulation in vitro or in vivo [43, 46, 47].

Among stem cell sources, the amniotic fluid contains multiple cell types derived mainly from exfoliating surfaces of the developing fetus [48]. These include cells from the fetal skin, respiratory system, and urinary and gastrointestinal tracts, along with populations of MSCs [49]. Immunoselection for c-Kit (CD117), the cell surface receptor for stem cell factor, designated AFSCs with no signs of malignant transformation, chromosomal abnormalities, or loss of differentiation potential [8].

In this study, we confirmed that hAFSCs cultured in our experimental conditions were positive for the mesenchymal stem cell markers CD73, CD90, CD105, CD271, and Stro-1 but also for typical markers of embryonic stem cells, such as SSEA4, Oct4, Nanog, FOXO1, Sox2, and TRA-1-81.

CD271, also known as low affinity nerve growth factor receptor (LNGFR) or p75NTR, belongs to the low-affinity neurotrophin receptor and tumor necrosis factor receptor superfamily [50]. This cell surface marker potentially defines an MSC precursor subpopulation with immunosuppressive and lymphohematopoietic engraftment-promoting properties [51]. The expression of this marker in most amniotic fluid stem cell populations should suggest immune-regulation potential.

Differentiation plasticity can be justified by the expression of several pluripotent stem cell markers. SSEA4, an early embryonic glycolipid antigen, is commonly used as a marker for undifferentiated pluripotent human embryonic stem cells and cleavage to blastocyst-stage embryos but also identifies the adult mesenchymal stem cell population.

The transcription factor Oct4 has essential functions for the maintenance of pluripotent embryonic and germ cells of mammals [52], Sox2, a member of the Sox81 transcription factor family, is an important transcriptional regulator in pluripotent stem cells. Together with Oct4 and Nanog, they cooperatively control gene expression in pluripotent stem cells and maintain their pluripotency. The FOXO transcription factors have an essential role in maintaining stem cell identity [53]. Finally, all human pluripotent stem cells express TRA-1-81 antigen.
AFSCs are able to suppress inflammatory responses in vitro, and soluble factors are an essential for communication between lymphocytes and AFSCs. Considering their extensive self-renewal capacity, possibility for banking, and absence of tumorigenicity, AFSCs can be evaluated as a superior source of stable, well-characterized, “off the shelf” immunomodulatory cells for a variety of immunotherapies and have the ability to avoid allogenic rejection [1].

It has been proven that AFSCs modulate lymphocyte proliferation in a different manner according to gestational age. Second-trimester AFSCs showed low expression of HLA class I molecules and absence of HLA II, and their features were associated with lower sensitivity to natural killer (NK) cell-mediated lysis. Second-trimester AFSCs did not efficiently inhibit T and NK cell proliferation but could suppress B-cell proliferation, which was not affected by the first- and third-trimester AFSCs [36]. In contrast, the mechanism of hAFSC immunoregulation by soluble factors has been elucidated only in part. Under growth conditions, amniotic fluid stem cells and BM-MSCs release relatively low levels of very few cytokines, but on activation by PBMCs, high levels of cytokines are released [1]. In fact, the microarray shown in Table 2 demonstrated the activating effect of pre-exposure to PBMCs on the secretion of immunomodulatory factors.

Based on these observations, we decided to evaluate the immunosuppressive actions of hAFSCs directed toward the whole peripheral blood leukocyte population, focusing on the secreted factor HGF. HGF is a potent immunomodulatory factor that inhibits dendritic cell function along with differentiation of IL-10-producing regulatory T cells, decrease in IL-17-producing T cells, and downregulation of surface markers of T-cell activation [23]. Apoptotic effects of HGF and c-Met are not yet fully understood [39] but have been observed in several cell lines such as ovarian carcinoma cells, breast carcinoma cells, mouse sarcoma cells, and mouse hepatocarcinoma cells [54–57]. Yang et al. [58] found that hepatic stellate cells send an apoptotic message to T cells and, at the same time, inhibit antigen-specific T-cell activity. Bottai et al. [37] demonstrated the secretion of HGF by hAFSCs isolated from third trimester amniotic fluid and focused on its endocrine role in targeting the injury site in an animal model without evaluating its possible immunoregulating capacity.

Initially we demonstrated that HGF is released by hAFSCs in the medium and after different types and durations of differentiation protocols, suggesting that the potential immunomodulatory property could be maintained in vivo during differentiation and after engraftment. HGF production is time dependent and enhanced by hAFSC preactivation with PBMCs, a condition reflecting the realistic situation occurring after the in vivo implant.

We next examined the effect of short time exposure to hAFSC secretum on PBMCs compared with pure HGF. Figure 2 shows that 24 hours with hAFSC CM activates the typical HGF pathway of c-MET/Akt in PBMCs and induces exogenous HGF; however, this phenomenon is not linked to the obvious survival stimulus, as demonstrated by the unexpected effect of Akt inhibitors.

PBMC apoptotic portion was increased by the presence of hAFSCs in all coculture conditions via a caspase-dependent process. The apoptotic effect was augmented by increased HGF production after hAFSC activation. By neutralizing the HGF activity, the immunosuppressive action of the hAFSCs was partially abolished. These results suggest that hAFSCs possess immunomodulatory properties involving HGF production.

CONCLUSION

Our findings have provided new information about the immune modulatory properties of hAFSCs. It is clear that immunomodulation
is not a peculiar feature of MSC-like cells but actually a general property of stem cells that may be induced or enhanced by inflammatory stimuli. Understanding these mechanisms may help identify novel therapeutic strategies and recognize the most effective stem cell class for interfering with damage-mediated inflammation and inducing tissue regeneration and organ repair [16]. Consequently, further in vivo studies will clarify whether these considerations for MSCs may be applied to c-Kit-positive stem cell populations such as AFSCs.

We conclude that AFSCs can be considered as a new therapeutic tool for many pathologies, taking into account the differentiation potential in vitro toward cell lineages belonging to the three germ layers and the different ways to regulate immune responses.

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**Figure 3.** Apoptosis of immune cells exposed to human AFSC (hAFSC) coculture. (A): Western blot analysis of lysates of peripheral blood mononuclear cells (PBMCs) exposed for 3 days to hAFSCs by conditioned medium, Transwell condition, or direct contact. Then immunoblot directed to cleaved PARP and caspase 3 was performed. Presented data are representative of three independent experiments, and the gray density values are shown in the graph. ****, \( p < .0001 \); ***, \( p < .001 \). (B): Representative immunofluorescence images of annexin V (red) and CFDA (green) labeling of PBMCs exposed for 3 days to media (CM) obtained from 5 days of hAFSCs preactivated (AFSC active) or not (AFSC). (C): Representative fluorescence-activated cell sorting (FACS) analysis of propidium iodide (PI) fluorescence of lymphocytes exposed for 3 days to media (CM) obtained from 5 days of hAFSCs preactivated (AFSC active) or not (AFSC) for 24 hours. *, \( p < .05 \); **, \( p < .01 \) vs. control; ***, \( p < .001 \) vs. AFSC. (D): Representative FACS analysis of PI fluorescence of lymphocytes cultured alone, after exposure to CM of activated hAFSCs, and in presence of anti-HGF neutralizing antibodies. Histograms represent the means of three different experiments \( \pm \) SD. ***, \( p < .001 \) vs. control; ***, \( p < .0001 \); **, \( p < .01 \) vs. CM. Abbreviations: AFSC, amniotic fluid stem cells; a-HGF, anit-hepatocyte growth factor; Casp3, caspase 3; cl, cleaved; CM, conditioned medium; CO, direct contact; Ctrl, control; TW, Transwell.
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AUTHOR CONTRIBUTIONS
T.M.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; F.B., M.G., and M.Z.: collection of assembly of data; A.D.P.: manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
The authors indicated no potential conflicts of interest.

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