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Survival and immunomodulation of stem cells from human extracted deciduous teeth expanded in pooled human and foetal bovine sera

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ABSTRACT

The immunomodulatory properties of mesenchymal stem cells (MSCs) from autologous and allogeneic sources are useful in stimulating tissue regeneration and repair. To obtain a high number of MSCs for transplantation requires extensive *in vitro* expansion with culture media supplements that can cause xeno-contamination of cells potentially compromising function and clinical outcomes. In this study stem cells from human extracted deciduous teeth (SHED) were cultured in Knockout™ DMEM supplemented with either pooled human serum (pHS) or foetal bovine serum (FBS) to compare their suitability in maintaining immunomodulatory properties of cells during *in vitro* expansion. No significant difference in cell survival of SHED grown in pHS (pHS-SHED) or FBS (FBS-SHED) was observed when co-cultured with complement, monocytes or lymphocytes. However, significant changes in the expression of sixteen paracrine factors involved in immunomodulation were observed in the supernatants of FBS-SHED co-cultures with monocytes or lymphocytes compared to that in pHS-SHEDs after both 24 and 120 h of incubation. Further analysis of changing protein levels of paracrine factors in co-cultures using biological pathway analysis software predicted upregulation of functions associated with immunogenicity in FBS-SHED and lymphocyte co-cultures compared to pHS-SHED co-cultures. Pathway analysis also predicted significant stimulation of HMGB1 and TREM1 signalling pathways in FBS-SHED co-cultures indicating activation of immune cells and inflammation. Though FBS supplementation does not impact survival of SHED, our combinatorial biological pathway analysis supports the idea that *in vitro* expansion of SHEDs in pHS provides optimal conditions to minimise xeno-contamination and inflammation and maintain their immunomodulatory properties.

1. Introduction

Mesenchymal stem cells (MSCs) are able to inhibit the activation of T-lymphocytes and natural killer cells, proliferation of B-lymphocytes, differentiation of monocytes, and maturation of dendritic cells (DCs) [1,2]. MSCs have been reported to express CD46, CD55, and CD59 that makes them resistant to complement-mediated cytotoxicity as well [3]. The immunomodulatory properties of MSCs from both autologous and allogeneic sources make them a popular source of stem cells for use in regenerative therapies [4,5]. Immunomodulatory properties of MSCs are also regulated by the presence of paracrine factors in the microenvironment. Induced immunosuppressive properties of MSCs have been seen in the presence of IL-6, IL-10, IFN-γ, and TNF-α [6–10]. Growth factors such as HGF and PDGF-BB also induce the immunosuppressive properties of MSCs [6,11]. Numerous clinical trials have reported that MSCs are safe for therapeutic use, however, lack of long-term clinical benefits has been considered a major drawback [12,13]. In many cases MSCs must be expanded *in vitro* prior to transplantation, often in foetal bovine serum (FBS) supplemented media [3,14], which contains N-glycolylneuraminic acid (Neu5Gc) monosaccharides, a xenoantigen found predominantly in non-human mammals that can modify MSC properties. Despite having immunosuppressive properties, hyper-immunogenicity to xeno-contaminated MSCs regardless of their source (autologous or allogeneic) has been reported in both *in vivo* and *in vitro* studies [3,15].

To address the issue of xeno-contamination, chemically defined xeno-free medium has been developed that needs to be supplemented with several recombinant human proteins [16,17]. However, these recombinant proteins, especially the growth factors and cytokines have the potential to prime the cells towards a particular lineage that could

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reduce the regenerative potential of MSCs [18–20]. Human serum, plasma, and platelet lysate have been proposed as possible replacements for FBS and recombinant protein supplementation [21–23] to reduce xeno-contamination during *in vitro* expansion of MSCs [3]. In recent years, the potential of pooled allogeneic plasma serum from adult AB-blood universal donors and pooled cord blood serum in maintaining the functional properties of MSCs has been reported [24–27]. Production of pooled human plasma or serum is confined to universal donors AB blood group which narrows down the number of donors. Due to donor scarcity, large scale production of cord blood serum is not practical as well. Moreover, this process destroys multipotent stem cells in the cord blood that could be a potential source of stem cells for regenerative therapy [28,29]. Platelet lysate, another potential substitute of FBS needs the addition of heparin to prevent gelatinisation of medium [30]. Pharmaceutical grade heparin can be produced either from animal tissues or synthetically. Hence, the source of heparin needs to be strictly labelled in the media [30]. Its working concentration also needs to be optimised as it has an effect on the viability and differentiation potential of MSCs [31].

Collection of blood irrespective of donors' blood group creates a larger donor pool compared to when the collection of blood is confined to a particular group. In this study we used pooled human serum (pHS) to expand stem cells from human extracted deciduous teeth (SHED) and tested their immunomodulatory properties in the presence of complement, monocytes, or lymphocytes. Furthermore, expression of paracrine factors in the microenvironment of SHED co-cultured with monocytes or lymphocytes has also been studied. Understanding and preserving the immunomodulatory properties of SHED during culture expansion is of interest due to their easy accessibility, limited risk to the donor, and that their regenerative potential is considered to be competitive when compared to other MSC populations [32,33]. In this study, we have attempted to explore the potential of pHS in maintaining the immunomodulatory properties of SHED, while FBS was used as a control. We report that SHED expanded in FBS have lower proliferative capacity compared to pHS. Furthermore, expression of the analysed paracrine factors in the microenvironment of SHED expanded in FBS (FBS-SHED) and lymphocyte co-cultures could be inflammatory compared to that in the microenvironment of SHED expanded in pHS (pHS-SHED) and lymphocyte co-cultures.

2. Materials and methods

2.1. Ethics approval

Extracted deciduous teeth and blood collection procedures for the current research were approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DFRD1301/0012 [L] and DFCO1107/0066 [L]). Written consent was obtained from the blood donors and guardians who agreed to donate their children's extracted teeth.

2.2. Preparation of pooled human serum (pHS)

pHS was prepared according to the procedure described before [34]. In brief, venous blood was collected from six healthy male donors aged between 21 and 35 years. Exclusion criteria are: smoking, alcohol consumption, drug and/narcotics addiction, diagnosis of any inflammatory diseases either chronic or at least in the last four weeks, major surgical treatment in the last year, and immunotherapy. 20 ml of blood from each donor (n = 6) was transferred into a 50 ml sterile centrifuge tube (Falcon®, Corning, NY, USA) containing no anticoagulant and allowed to stand at room temperature for an hour to facilitate coagulation. The coagulated blood was centrifuged at 400*g* for 15 min followed by a second round of centrifugation at 1800*g* for 15 min, after which the serum supernatant was transferred into 15 ml sterile centrifuge tubes (Falcon®, Corning). Six heat treated sera (57 ± 2 °C for 30 min) were combined to constitute the complement inactivated pHS.

2.3. Isolation, characterisation, and expansion of stem cells from extracted deciduous teeth (SHED)

2.3.1. Isolation of SHED

Sound intact extracted deciduous molars were collected from children (n = 3 biological replicates; ages 5–9 years) who were undergoing a planned serial extraction for management of occlusion at the Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya. SHED were isolated according to the established procedure described before [35,36]. Growth of stem cells from dental pulp tissue was observed under an inverted microscope (Primo Vert, Carl Zeiss, Jena, Germany) on day 3 to day 5 post-incubation and the first subculture of SHED was carried out on day 14.

2.3.2. In vitro maintenance of SHED

Until passage 3 SHED (n = 3 biological replicates) were maintained in 10% FBS (Gibco®, Thermo Fisher Scientific, Lot No. 10270) supplemented Knockout™ DMEM (Gibco®,Thermo Fisher Scientific, NY, USA). Subsequent cultures (from passage 4–7) were maintained in Knockout™ DMEM supplemented with either 10% FBS or 10% pHS. In order to minimise xeno-contamination, animal derived component free TrypLE™ express (Gibco®, Thermo Fisher Scientific) was used as cell dissociation reagent.

2.3.3. SHED as MSCs

Plastic adhering capability was confirmed by viewing the SHED containing culture flask under inverted microscope (Primo Vert, Carl Zeiss). To measure the specific surface antigen expression on SHED, cells were stained with human MSC phenotyping kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and MACSQuant® Analyzer 10 Flow cytometer (Miltenyi Biotec) was used to analyse data as described before [34]. In brief, nearly 70% confluent SHED were dissociated using TrypLE™ express (Gibco) and total number of cells were counted using trypan blue (Gibco). After that cells were aliquoted (1×10^6 cells each) into seven pre-labelled (i.e. 1, 2, PerCP, PE, APC, FITC and blank) microcentrifuge tubes, centrifuged at 300*g* for 10 min and supernatants were aspirated carefully. 500 µl of buffer (2% FBS containing DPBS) was added into the tube labelled 'blank', cells were resuspended and kept in ice until further use. Cells in the other microcentrifuge tubes were resuspended into 100 µl buffer each, followed by addition of 10 µl of the MSC phenotyping cocktail (anti-human: CD14-PerCP, CD20-PerCP, CD34-PerCP, CD45-PerCP, CD73-APC, CD90-FITC, CD105-PE), 10 µl of the isotype control cocktail (anti-human: mouse IgG1-FITC, mouse IgG1-PE, mouse IgG1-APC, mouse IgG1-PerCP, mouse IgG2a-PerCP), 10 µl of anti-human CD73-Biotin, 10 µl of anti-human CD105-PE, 10 µl of anti-human CD73-APC, and 10 µl of anti-human CD90-FITC into the tubes labelled 1, 2, PerCP, PE, APC, and FITC respectively. Cells were mixed gently and incubated in the dark at 4 °C for 10 min. The cells were then washed, and cell pellets in the tubes except labelled PerCP were resuspended into 500 µl of buffer and kept in an ice filled container. Cell pellet into the tube labelled PerCP was resuspended into 10 µl of anti-Biotin-PerCP followed by incubation in the dark at 4 °C for 10 min. The cells were washed again and resuspended in 500 µl of buffer. Tubes labelled 1 and 2 were used to determine the expression of specific surface antigen on SHED, while others were used to compensate the instrument.

StemPro® Adipogenesis, StemPro® Osteogenesis, and StemPro® Chondrogenesis Differentiation kits (Gibco®, Thermo Fisher Scientific) were used to assess the trilineage differentiation potential of SHED. After reaching 70% confluency, SHED were fed with corresponding differentiation (adipogenic, chondrogenic and osteogenic) media. Cells were fed with fresh differentiation medium on every third day. On day 14, adipogenic and chondrogenic differentiation were confirmed by

staining the cells with Oil Red O (Sigma-Aldrich, Steinheim, Germany) and Safranin O (Sigma-Aldrich) solution respectively. Osteogenic differentiation was confirmed on day 21 by staining the cells with Alizarin Red S (Sigma-Aldrich) solution. For all differentiation assays, SHED cultured in complete proliferation medium were used as the negative control.

2.4. Preparation of autologous serum and separation of immune cells

2.4.1. Preparation of human serum and isolation of peripheral blood mononuclear cells (PBMCs)

Venous blood was collected from healthy male donors (n = 3 biological replicates) aged 21–35 years following the same exclusion criteria mentioned above. Blood was collected from each donor in two steps: (i) 20 ml without any anticoagulant for individual serum preparation, and (ii) 30 ml in vacutainer containing sodium heparin (GmbH, Hamburg, Germany) for peripheral blood mononuclear cells (PBMCs) isolation.

Individual human serum was prepared according to the procedure mentioned above with the following modifications. After the final round of centrifugation, serum supernatant was aliquoted into two sterile 15 ml centrifuge tubes. One aliquot was heat treated at 57 ± 2 °C for 30 min to inactivate complement, while the other was left untreated to retain complement activity. Non-heat-treated sera (n = 3 biological replicates) were used as autologous human serum (AuHS) to their PBMCs, monocytes, and lymphocytes.

PBMCs were isolated according to the procedure described before [37]. Briefly, sodium heparinised blood was diluted with equal volume of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Gibco®, Thermo Fisher Scientific), and carefully added on Ficoll-Paque Plus (GE Healthcare, Upsala, Sweden) at a 4:3 ratio followed by centrifugation at 400*g* for 30 min with brake off. Buffy coat containing PBMCs was collected and washed twice with DPBS by centrifuging at 200*g* for 10 min. After discarding the supernatant, the pellet was mixed with Knockout™ DMEM. Cell number and viability were counted using trypan blue (Gibco®, Thermo Fisher Scientific) dye exclusion method.

2.4.2. Separation of monocytes and lymphocytes from PBMCs

PBMCs (n = 3 biological replicates) were seeded in Knockout™ DMEM supplemented with 10% (v/v) AuHS, 2 mM Glutamax (Gibco®, Thermo Fisher Scientific), and 50 units/ml Penicillin-Streptomycin (Gibco®, Thermo Fisher Scientific) at a density of 5.5×10^5 cells/ml and incubated in a 5% CO_2 humidified incubator at 37 °C. Following incubation, non-adherent lymphocytes were collected very carefully into 15 ml centrifuge tubes. Adherent monocytes (n = 3 biological replicates) were washed twice gently using DPBS to minimise lymphocyte contamination and fed with AuHS supplemented Knockout™ DMEM for further use.

Lymphocytes (n = 3 biological replicates) were centrifuged at 200*g* for 10 min. Supernatants were discarded and the pellets were re-suspended in AuHS supplemented Knockout™ DMEM for further experiments.

2.5. Effect of complement on SHED expanded in pHS and FBS

SHED (n = 3 biological replicates) maintained in FBS and pHS supplemented medium (FBS-SHED and pHS-SHED respectively) were seeded (passage 7) at a density of 100,000, 50,000, 25,000, 12,500 and 6255 cells/well in 96-well plates containing Knockout™ DMEM medium supplemented with 40% freshly prepared non-heat treated human serum (n = 3 biological replicates) to determine the cytotoxic effect of complement at different cell densities. Complement inactivated (55 ± 2 °C for 30 min) human sera (n = 3 biological replicates) from the same donors were used to supplement media as controls. After 12 h of incubation at 37 °C in 95% humidified air and 5% CO_2, the effect of complement on the survival of SHED was analysed using PrestoBlue® cell viability reagent (Invitrogen™, Thermo Fisher Scientific). Viability of SHED at 24 h of incubation was also analysed. Following incubation all the media were discarded and the wells were washed twice with DPBS. Knockout™ DMEM medium along with 10% PrestoBlue® reagent (v/v) was added to each well and the plates were further incubated for 2 h. Absorbance was measured at 570 nm with reference wavelength set at 600 nm by microplate reader (Infinite 200 PRO, Tecan, Switzerland). The absorbance values were converted to the corrected absorbance of PrestoBlue® reagent.

2.6. Survival of SHED expanded in pHS and FBS in the presence of immune cells

At passage 5, confluent FBS-SHED (n = 3 biological replicates) and pHS-SHED (n = 3 biological replicates) were dissociated and co-cultured with either monocytes (n = 3 biological replicates) or lymphocytes (n = 3 biological replicates) in 24-well plates at 1:10 and 1:50 ratio respectively. The culture media was supplemented with freshly prepared 10% untreated human serum of the corresponding monocytes or lymphocytes. Cultures of monocytes, lymphocytes, and SHED were maintained individually in the same media as control. After 24 and 120 h of incubation, supernatants were collected and centrifuged at 200*g* for 10 min. Following centrifugation supernatants were aliquoted into two equal parts and stored at −80 °C for cytotoxicity assay and immunoassay.

2.6.1. Cytotoxicity assay

Lactate dehydrogenase (LDH) assay kit (Sigma-Aldrich) was used to analyse the cytotoxic effect of monocytes and lymphocytes on SHED. LDH is an enzyme that is found in all living cells and is involved in the catalyses of the conversion of lactate into pyruvate to generate NADH. LDH is released during cell or tissue damage, thus it is considered as a marker for cellular injury or cytotoxicity. LDH activity is presented as milliunit/ml (nmole/min/ml). One unit of LDH activity is the amount of enzyme that generates 1 μmol of NADH per minute at 37° C. Absorbance at 450 nm is used to measure the presence of NADH in the samples.

2.6.2. Immunoassay

Supernatants collected from the co-cultures after 24 and 120 h of incubation were used to measure the amount of selected paracrine factors (fibroblast growth factor, 2FGF-2; granulocyte colony stimulating factor, G-CSF; granulocyte-macrophage colony stimulating factor, GM-CSF; macrophage colony stimulating factor, M-CSF; hepatocyte growth factor, HGF; interleukin 4, IL-4; interleukin 6, IL-6; interleukin 10, IL-10; interleukin 12, IL-12; interferon γ, INF-γ; leukaemia inhibitory factor, LIF; platelet derived growth factor BB, PDGF-BB; stem cell factor, SCF; stromal cell-derived factor 1a, SDF-1a; tumor necrosis factor α TNF-α; and vascular endothelial growth factor A, VEGF-A) in each culture by using Luminex-based ProcartaPlex human cytokine/chemokine 16plex immunoassay kit from e-Bioscience (affymetrix, e-Bioscience, Vienna, Austria). Paracrine factors were selected based on their involvement in cell survival and regulation of immune functions (Supplementary Table S1)

2.6.3. Molecular network analysis

Up or down regulation of paracrine factors in the supernatants of FBS-SHED and monocyte or lymphocyte co-cultures was calculated by comparing the concentration of each factor with the concentration of factors in supernatants from corresponding co-cultures of pHS-SHED and monocyte or lymphocyte respectively, and the values were presented as mean fold changes. The corresponding Entrez Gene IDs of the 16 paracrine factors along with the mean fold changes in concentration were imported into the core analysis tool of Ingenuity Pathway Analysis (IPA) (Ingenuity systems; www.ingenuity.com) to identify their roles in

regulating biological functions and pathways. Activation and inhibition of biological functions and signalling pathways were predicted by the 'z-score' calculated by the software on the basis of the expression pattern of downstream transcriptional regulators. The software also predicted activation and inhibition of the cascade of upstream transcriptional regulators that help to explain the causes behind the changes in the expression of analysed paracrine factors. Positive and negative z-score indicates activation or inhibition respectively. Z-score greater than +1.96 or smaller than −1.96 were considered significant ($p < 0.05$).

2.7. Data analysis

Data were analysed using independent sample *t*-test (SPSS version 22) and the significance level was set at $p < 0.05$.

3. Results

3.1. Classification of SHED as MSC-like cells

Plastic adherence and morphologically homogenous monolayers of spindle-shaped SHED at different passages were observed under inverted microscope (Primo Vert, Carl Zeiss) (Fig. S1 A, B). Immunophenotyping using flow cytometry showed that 95% of SHED expressed MSC positive markers (CD73, CD90, CD105) and 3% of SHED expressed MSC negative markers (CD14, CD20, CD34, CD45) (Fig. S1C). Adipogenic, chondrogenic, and osteogenic differentiation of SHED were observed following induced differentiation (Fig. S1 D–F), while no evidence of differentiation was observed in control cultures.

3.2. Complement and immune cells have no effect on FBS-SHED and pHS-SHED survival

FBS-SHED and pHS-SHED showed no significant difference in survival as measured by cell viability assays after 12 h of incubation in the presence or absence of complement (Fig. 1A, B). However, proliferation of pHS-SHED, using cell viability as an indirect measure of cell proliferation, was significantly higher ($p = 0.000$) compared to that of FBS-SHED 24 h post-incubation (Fig. 1C). Cytotoxic activity of monocytes or lymphocytes on FBS-SHED and pHS-SHED measured by LDH after 24 and 120 h of incubation showed no significant difference (Fig. 1D).

3.3. Paracrine factors in the co-cultures of FBS-SHED or pHS-SHED with monocytes

We next analysed the presence of sixteen paracrine factors (2FGF-2, G-CSF, GM-CSF, M-CSF, HGF, IL-4, IL-6, IL-10, IL-12, INF-γ, LIF, PDGF-BB, SCF, SDF-1a, TNF-α and VEGF-A) known to influence immunomodulatory properties of cells in the supernatants of FBS-SHED or pHS-SHED and monocyte co-cultures using Luminex-based immunoassay kit. In co-cultures of FBS-SHED and monocytes, expression of FGF-2, M-CSF, INF-γ a, IL-4 and SCF were not detected 24 h post-incubation. While in the pHS-SHED and monocyte co-cultures, expression of TNF-α and the five latter paracrine factors were also not detected. Among the detected paracrine factors, expression of PDGF-BB was significantly ($p < 0.05$) higher in FBS-SHED and monocyte co-cultures compared to that of pHS-SHED and monocyte co-cultures. Meanwhile, the expression of VEFG-A was significantly ($p < 0.05$) higher in pHS-SHED and monocyte co-cultures (Fig. 2).

At 120 h post-incubation, in both co-cultures (FBS-SHED or pHS-SHED with monocytes) only M-CSF was not expressed (Fig. 2). Expression of FGF-2 ($p < 0.05$), HGF ($p < 0.01$), PDGF-BB ($p < 0.01$), and SDF-1a ($p < 0.01$) were observed to be significantly higher in FBS-SHED and monocyte co-cultures compared to pHS-SHED and monocyte co-cultures. Meanwhile, the expression of IL-6 ($p < 0.05$) was significantly higher in pHS-SHED and monocyte co-cultures (Fig. 2).

3.4. Paracrine factors in the co-cultures of FBS-SHED or pHS-SHED with lymphocytes

Expression of paracrine factors that modulate the immunogenic properties of lymphocytes were also analysed in the supernatants of FBS-SHED or pHS-SHED and lymphocyte co-cultures using Luminex-based immunoassay kit. After 24 h of incubation, all sixteen selected paracrine factors were identified in co-cultures of FBS-SHED and lymphocytes. With the exception of FGF-2 and M-CSF, the remaining 14 paracrine factors were also detected in pHS-SHED and lymphocyte co-cultures. Among the expressed paracrine factors, PDGF-BB ($p < 0.01$) and SDF-1a ($p < 0.05$) were significantly higher in FBS-SHED and lymphocyte co-cultures compared to pHS-SHED and lymphocyte co-cultures (Fig. 3).

Expression of all sixteen selected paracrine factors were detected 120 h post-incubation in both co-cultures (FBS-SHED or pHS-SHED with lymphocytes). Expression of HGF and SDF-1a were significantly ($p < 0.01$) higher in FBS-SHED and lymphocyte co-culture compared to pHS-SHED and lymphocyte co-cultures. Meanwhile, significantly higher expression of VEGF-A ($p < 0.05$) was observed in pHS-SHED and lymphocyte co-cultures (Fig. 3).

Changes in expression of paracrine factors in FBS-SHED co-cultures either with monocytes or lymphocytes compared to pHS-SHED co-cultures either with monocytes or lymphocytes, measured in mean fold change at both 24 and 120 h post-incubation, are shown in Table 1.

3.5. Paracrine factors in the FBS-SHED and lymphocyte co-cultures at 24 h predicted to be more inflammatory

Entrez Gene IDs of the sixteen paracrine factors and their mean fold change in FBS-SHED co-cultures compared to pHS-SHED co-cultures (Table 1) were imported into biological pathway analysis software to understand which biological functions were affected by the serum supplementation. Biological pathways related to proliferation, viability, apoptosis, differentiation, migration, and inflammatory response were noted in particular (Table 2).

Pathway analysis predicted that after 24 h of FBS-SHED and monocyte co-culture, the prevailing microenvironment would cause significantly decreased proliferation ($p < 0.05$; $z = -2.449$) and differentiation ($p < 0.05$; $z = -2.203$) of hematopoietic progenitor cells, proliferation of myeloid cells ($p < 0.05$; $z = -2.209$), and increased apoptosis of myeloid cells ($p < 0.05$; $z = 2.364$) compared to the microenvironment of corresponding pHS-SHED co-cultures. After 120 h of incubation, a significant increase in the binding of cells ($p < 0.05$; $z = 2.330$), migration of hematopoietic progenitor cells ($p < 0.05$; $z = 2.192$), and migration of lymphatic system cells ($p < 0.05$; $z = 2.214$) were predicted in the microenvironment of FBS-SHED and monocyte co-cultures compared to pHS-SHED and monocyte co-culture (Table 2).

After 120 h of FBS-SHED and lymphocyte co-cultures, the culture microenvironment was predicted to significantly increase binding of leucocytes ($p < 0.05$; $z = 2.222$) and migration of hematopoietic progenitor cells ($p < 0.05$; $z = 2.389$) compared to the corresponding pHS-SHED and lymphocyte co-cultures. After 24 h of incubation, the microenvironment of the FBS-SHED and lymphocyte co-cultures was predicted to significantly increase proliferation of mononuclear leucocytes ($p < 0.05$; $z = 2.773$), cell viability of leucocytes ($p < 0.05$; $z = 3.088$), differentiation of leukocytes ($p < 0.05$; $z = 2.704$), activation of mononuclear leukocytes ($p < 0.05$; $z = 2.394$), adhesion of immune cells ($p < 0.05$; $z = 2.695$), immune response of antigen presenting cells ($p < 0.05$; $z = 2.186$), immune response of phagocytes ($p < 0.05$; $z = 2.166$), inflammatory response ($p < 0.05$; $z = 2.234$), stimulation of leukocytes ($p < 0.05$; $z = 2.602$), leukocytes migration ($p < 0.05$; $z = 3.136$) and migration of lymphatic

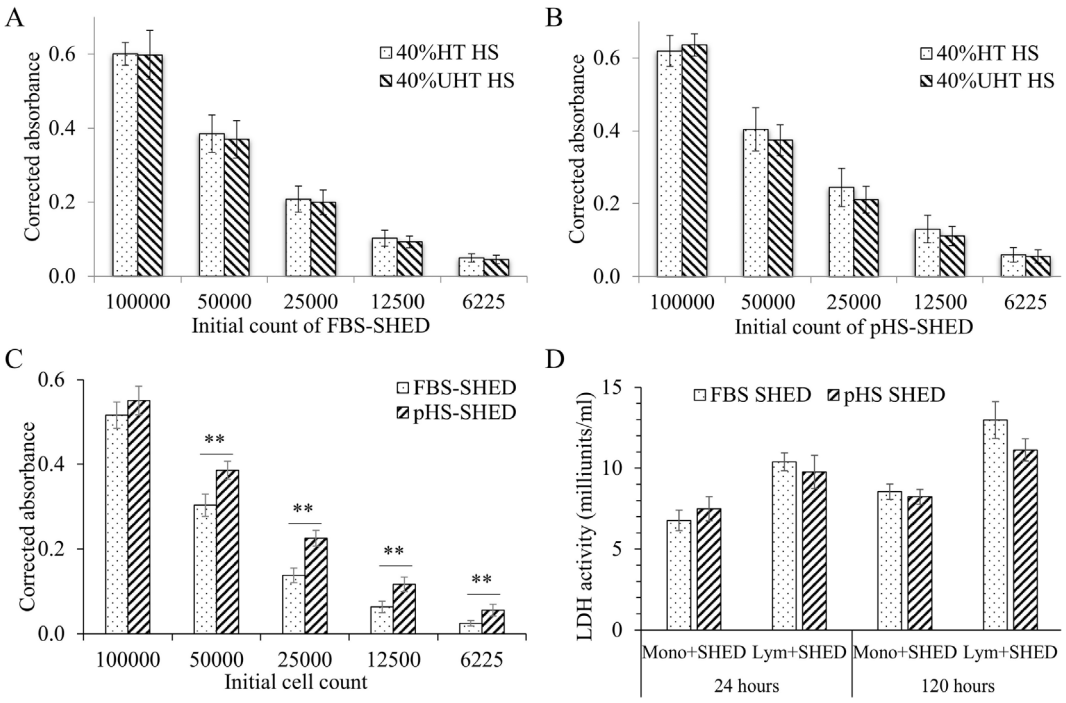

Fig. 1. Survival of SHED in the presence of complement, monocytes or lymphocytes. (A) Viability of SHED cultured in FBS (FBS-SHED, n = 3 biological replicates) in the presence or absence of complement (n = 3 biological replicates) after 12 h of incubation. (B) Viability of SHED cultured in pHS (pHS-SHED, n = 3 biological replicates) in the presence or absence of complement (n = 3 biological replicates) after 12 h of incubation. (C) Viability of pHS-SHED was significantly higher than that of FBS-SHED after 24 h of incubation when the cells were seeded at a density of ≤50,000 cells/well in a 96-well plate. (D) FBS-SHED (n = 3 biological replicates) and pHS-SHED (n = 3 biological replicates) showed no significant difference in their survival in the presence of monocytes (n = 3 biological replicates) or lymphocytes (n = 3 biological replicates) after 24 and 120 h of incubation. (*, $p < 0.05$; **, $p < 0.01$; HT, heat treated; UHT, un-heat treated; HS, individual human serum).

system cells ($p < 0.05$; $z = 2.396$) compared to that of pHS-SHED and lymphocyte co-cultures (Table 2).

Furthermore, after 24 h of incubation, activation of high mobility group box 1 protein (HMGB1) signalling (z score = 2.646), triggering receptor expressed on myeloid cells 1 (TREM1) signalling (z score = 2.000), and DCs maturation (z score = 1.342) in the FBS-SHED co-cultures compared to pHS-SHED were predicted (Fig. 4).

3.6. Predicted changes in the expression of upstream regulators

On the basis of the expression of analysed paracrine factors in the supernatants of different co-cultures, pathway analysis predicted changes in upstream regulators in cells of co-cultures. The results showed the predicted activation of eight upstream regulators (IL1B, IL15, IL18, NFkB, TLR2, TLR3, TLR4 and TLR6) and inhibition of three upstream regulators (CD28, HLA-DQ and IL37) within the cells of FBS-SHED co-culture with lymphocytes after 24 h of incubation compared to the cells of pHS-SHED co-culture with lymphocytes (Fig. 5).

4. Discussion

In order to yield a sufficient number of cells for regenerative therapies, MSCs are usually expanded in FBS-supplemented media. A number of studies have reported the presence of Neu5Gc, a sialic acid-based xenoantigen in FBS [3,14] that can contaminate MSCs and trigger complement-dependent cytotoxicity (CDC) and antibody dependant cell-mediated cytotoxicity (ADCC) [3,38,39]. Sialic acid enters cells through fluid-phase pinocytosis where it is converted to a monosaccharide in lysosomes and can be transported to the golgi to be added to glycoconjugates that are classified as xeno-autoantigens. Most humans produce anti-Neu5Gc antibodies and therefore MSCs cultured in FBS-containing medium can raise an unwanted immune response. However, cell survival and immunosuppressive properties of MSCs regardless of the media supplement used during culture expansion have been reported in several studies [2,40,41].

Several studies reported the effect of media supplements on the proliferation potential of MSCs, while no significant effect on the differentiation potential and phenotypic marker expression were observed [42–45]. Furthermore, subsequent culturing of adipose-derived MSCs in heat-inactivated normal human pooled serum supplemented medium for a week showed reduction of xeno-contamination from 80.01% to 2.08% that was caused due to isolation and expansion in FBS supplemented medium [3]. Successful isolation of MSCs using pooled cord blood serum or pHS has been reported earlier [46,47]. However, isolation of dental pulp derived stem cells (DPSC) was not successful when pHS concentration in the media was below 20% (v/v) [48]. Moreover, volume of primary pulp tissue collected from the deciduous tooth is not adequate to attempt isolation in multiple media. Hence in this study, the primary isolation, expansion, and characterisation were conducted in FBS supplemented media, and subsequent studies on the survival and immunomodulatory properties of SHED were conducted using pHS, FBS, and individual human serum.

According to the International Society for Cellular Therapy (ISCT), MSCs should have adherence to plastic, specific surface antigen expression, and trilineage (adipogenic, chondrogenic and osteogenic) differentiation potential [49]. Plastic adhering capability, trilineage differentiation potential, and specific surface antigen expression pattern confirmed SHED as MSCs (Fig. S1).

In our study, FBS-SHED and pHS-SHED did not show any significant difference in their survival in the presence or absence of complement, monocytes or lymphocytes, and these observations concur with previously published observations [2,3,25,40,41]. As shown by Komoda et al. (2010), the latter effect could be attributed to the expression of complement regulatory proteins such as CD46, CD55, and CD59 on

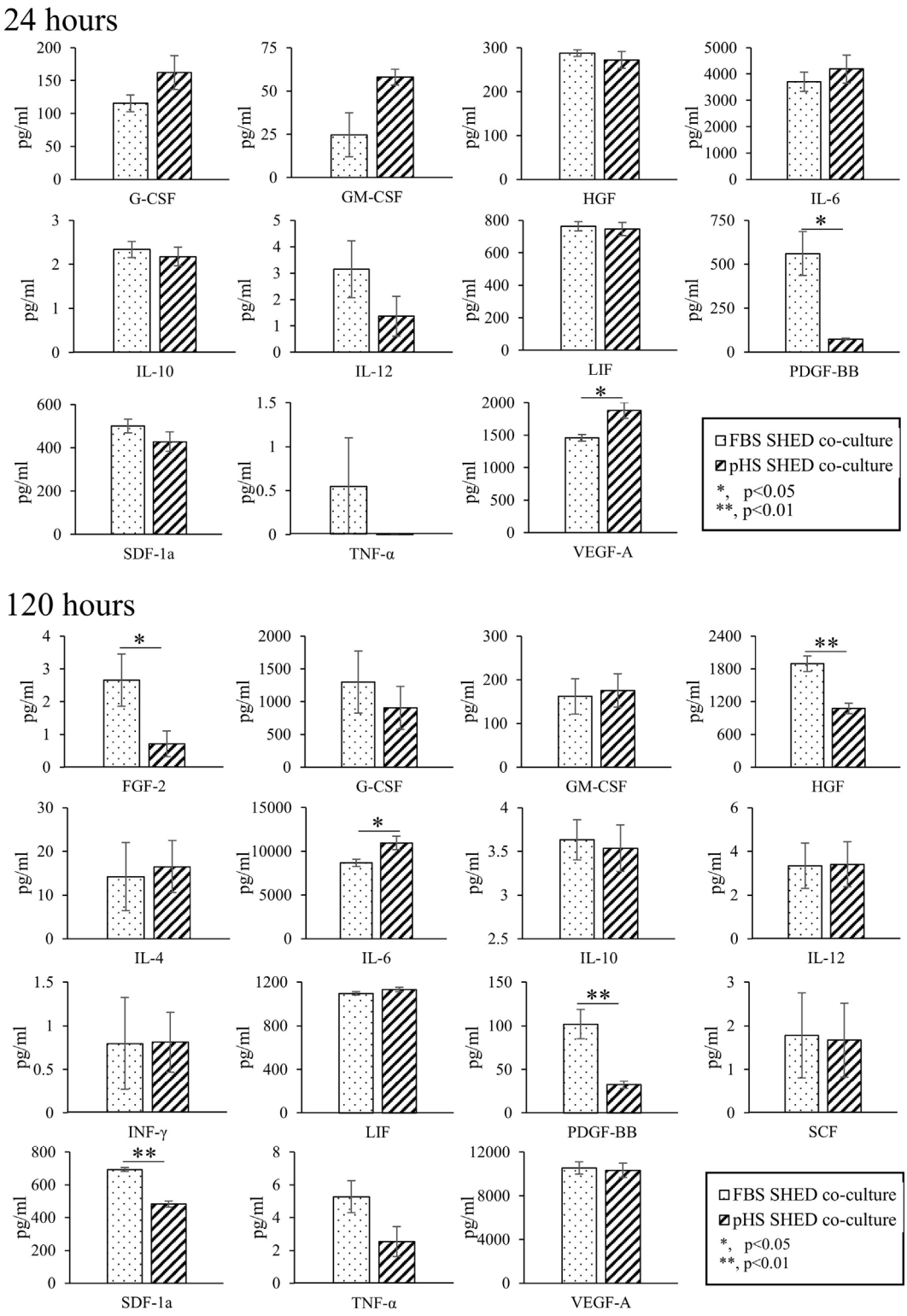

Fig. 2. Comparative expression of paracrine factors in the SHED and monocyte co-cultures. PDGF-BB was significantly higher in FBS-SHED (n = 3 biological replicates) and monocyte (n = 3 biological replicates) co-cultures compared to pHS-SHED (n = 3 biological replicates) and monocyte (n = 3 biological replicates) co-cultures at 24 h of incubation. Meanwhile, VEFG-A was significantly higher in pHS-SHED and monocyte co-cultures compared to FBS-SHED and monocyte co-cultures. At 120 h, FGF-2, HGF, PDGF-BB and SDF-1a were significantly higher in FBS-SHED (n = 3 biological replicates) and monocyte (n = 3 biological replicates) co-cultures compared to pHS-SHED (n = 3 biological replicates) and monocyte (n = 3 biological replicates) co-cultures. Whereas, the expression of IL-6 was significantly higher in pHS-SHED and monocyte co-cultures compared to FBS-SHED and monocyte co-cultures.

MSCs, making them resistant to CDC [3]. Secretion of complement inhibiting factor H from MSCs has also been reported and this could be another reason for survival of SHED cultured in FBS and pHS supplemented media in the presence of complement [50]. However, at 24 h post-incubation in non-heat treated human serum, significantly ($p = 0.000$) higher viability of pHS-SHED compared to FBS-SHED

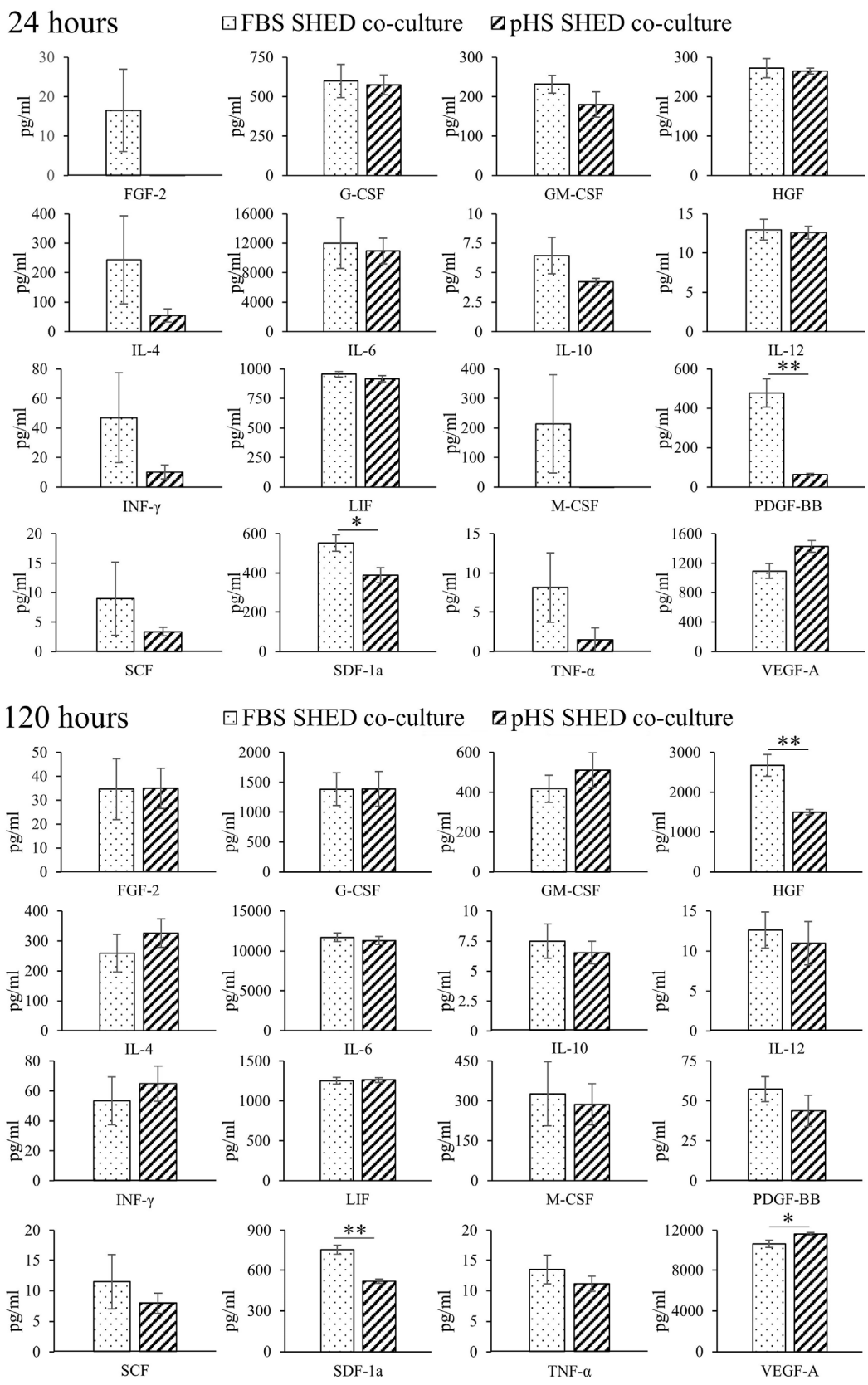

Fig. 3. Comparative expression of paracrine factors in the co-cultures of SHED with lymphocytes. At 24 h, PDGF-BB and SDF-1a were significantly higher in FBS-SHED (n = 3 biological replicates) and lymphocyte (n = 3 biological replicates) co-cultures compared to pHS-SHED (n = 3 biological replicates) and lymphocyte (n = 3 biological replicates) co-cultures. At 120 h post incubation expression of HGF and SDF-1a were significantly higher in FBS-SHED (n = 3 biological replicates) and lymphocyte (n = 3 biological replicates) co-cultures compared to pHS-SHED (n = 3 biological replicates) and lymphocyte (n = 3 biological replicates) co-cultures. Meanwhile, the expression of VEGF-A was significantly higher in pHS-SHED and lymphocyte co-cultures compared to FBS-SHED and lymphocyte co-cultures (* = $p < 0.05$, ** = $p < 0.01$).

(Fig. 1C) indirectly suggests that in the absence of differences in cell death between culture conditions, culture with pHS stimulates the proliferation of SHED.

Cell-mediated cytotoxicity studies also showed no significant difference between the survival of FBS-SHED and pHS-SHED in the presence of either monocytes or lymphocytes. It has reported that MSCs have the potential to prevent initial differentiation of monocytes to DCs by inhibiting the expression of DC marker CD83 and co-stimulatory molecule CD86 even in the presence of GM-CSF, IL-4, and lipopolysaccharides [51]. Reduction in the expression of CD83 on the matured

Table 1
Entrez Gene IDs of the 16 selected paracrine factors and their relative fold change in expression in the FBS-SHED co-cultures compared to that in the corresponding pHS-SHED co-cultures (Positive and negative values denote up- and down-regulation in the expression of paracrine factors respectively; the value 'zero, .00′ denotes that the particular paracrine factors were not detected in either FBS-SHED or pHS-SHED co-cultures; the value 'infinity, ∞' indicates that the particular paracrine factor was detected in either FBS-SHED or pHS-SHED co-cultures of corresponding immune cells).

Cytokines	Gene ID	24 h		120 h	
		$Cocul^{mono}$	$Cocul^{lym}$	$Cocul^{mono}$	$Cocul^{lym}$
FGF-2	NM_002006	0.00	∞	3.73	−1.01
HGF	NM_001010932	1.06	1.03	1.76	1.78
LIF	NM_002309	1.02	1.04	−1.03	−1.01
PDGF-BB	NM_033016	7.60	7.62	3.16	1.31
SCF	NM_000899	−2.00	2.66	1.06	1.45
SDF-1a	NM_199168	1.17	1.42	1.44	1.45
VEGF-A	NM_001171628	−1.29	−1.31	1.02	−1.09
G-CSF	NM_000759	−1.40	1.04	1.43	1.00
GM-CSF	NM_000758	−2.34	1.28	−1.08	−1.23
M-CSF	NM_000757	0.00	∞	0.00	1.14
INF-γ	NM_000619.2	0.00	4.64	−1.02	−1.22
TNF-α	HQ201306.2	∞	5.45	2.08	1.21
IL-4	NM_000589.3	0.00	4.47	−1.16	−1.26
IL-6	NM_000600	−1.13	1.10	−1.26	1.04
IL-10	AY029171.1	1.07	1.53	1.03	1.15
IL-12	NM_000882	2.31	1.03	−1.02	1.15

$Cocul^{mono}$, co-culture with monocytes; $Cocul^{lym}$, co-culture with lymphocytes

DCs in the presence of MSCs has also been reported by the same group [51]. Furthermore, expression of inhibitory molecules such as B7-H1, B7-H4, and HLA-G on MSCs has also been reported [52–54]. These properties help MSCs to survive in allogeneic conditions by inhibiting the proliferation and function of cytotoxic T cells, NK cells and B cells, and preventing differentiation of monocytes into antigen-presenting DCs [5,51]. Cell surface associated expression of inhibitory molecules such as B7-H1, B7-H4, and HLA-G [52–54] might also be the major factor in the survival of FBS-SHED and pHS-SHED in the presence of immune cells.

The presence of paracrine factors in the microenvironment plays an important role in the survival of MSCs and their role in tissue repair. Among different paracrine factors, IL-6 has been found to have suppressive effects on the generation of DCs from monocytes [51]. Inversely, TNF-α facilitates the generation of DCs [51]. Furthermore, maturation and function of DCs is regulated by secretion of IL-12 from them [51]. In this study, higher expression of IL-6 and lower expression of TNF-α and IL-12 in both pHS-SHED and FBS-SHED co-cultures with monocytes also support their survival. However, in the pHS-SHED co-culture with monocytes, the expression of IL-6 at 120 h was significantly ($p < 0.05$) higher compared to FBS-SHED co-culture, whereas the expression of TNF-α at 24 h was not detected in it (Fig. 2). It is noteworthy to mention that moderately higher expression of IL-12 in the FBS-SHED co-culture with monocyte could also be linked to the moderately lower expression of IL-6 and higher expression of TNF-α in it compared to pHS-SHED co-culture at 24 h post-incubation. IL-12 is a very important paracrine factor that plays vital role in linking innate and adaptive immunity, determining the fate of CD4 T cells and activating CD8 T cells [55,56]. In this study SHED expanded in FBS or pHS media were co-cultured with either monocytes or lymphocytes. Further studies on the secretome from the co-culture with PBMC could help to come to a proper conclusion on the effect of pHS in supporting the survival of MSCs including SHED over FBS following transplantation.

On the other hand, significantly higher expression of HGF ($p < 0.05$) that induces expression of IL-10 from monocytes in the presence of MSCs, enhancing their immunosuppressive properties [6], was detected in FBS-SHED and monocyte co-culture compared to pHS-SHED co-cultures at 120 h post-incubation. Significantly higher expression of PDGF-BB that has the potential to stimulate survival of MSCs by activating protein kinase B/akt [11] was also detected in the FBS-SHED and monocyte co-cultures at both 24 and 120 h post-incubation (Fig. 2).

Studies have shown that IFN-γ and TNF-α stimulate MSCs to become immunosuppressive towards the cells of the adaptive immune system [7–9]. IFN-γ in the presence of TNF-α or IL-1α induces the expression of intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) that help MSCs to display immunosuppressive properties [41]. IFN-γ-induced immunosuppressive function of MSCs with the concomitant presence of TNF-α, IL-1α, or IL-1β has also been reported [40]. IFN-γ induces the expression of indoleamine 2,3-dioxygenase in MSCs [57] that is known to inhibit NK cell and T-cell proliferation [58,59]. In our study, the presence of IFN-γ and TNF-α were detected in the co-cultures (FBS-SHED or pHS-SHED with lymphocytes) at both 24 and 120 h post-incubation (Table 1). Presence of these paracrine factors in the co-cultures' microenvironment is predicted to help both FBS-SHED and pHS-SHED cultured cell populations survive in the presence of lymphocytes.

Expression of paracrine factors in the co-cultures of SHED may help to explain their survival in the presence of innate and adaptive immune responses. To understand the role of the selected paracrine factors on the culture microenvironment we conducted combinatorial biological pathway analysis. After 24 h of incubation, pathway analysis predicted significant increases in inflammation-related biological functions in the microenvironment of the FBS-SHED and lymphocyte co-cultures compared to pHS-SHED and lymphocyte co-cultures (Table 2). This observation was supported by the predicted significant activation of HMGB1 signalling pathway in the FBS-SHED and lymphocyte co-cultures after 24 h of incubation (Fig. 4). Several studies have reported that HMGB1 promotes inflammation, tissue destruction, and the activation of immunity [60–62]. However, predicted higher expression of TREM1 (Fig. 4) and its up-stream regulators such as TLR2, TLR3, TLR4, and TLR6 (Fig. 5) in the FBS-SHED and lymphocyte co-cultures 24 h post-incubation remains elusive. TREM1 is usually expressed on monocytes and neutrophils and is recognised as an inducer of inflammation by itself or by inducing the expression of toll-like receptors (TLRs) [63]. Notably, in this study lymphocytes were isolated using depletion technique which may contain some progenitor cells. Hence, software analysis predicted activation of TREM1 pathway and its up-stream regulator TLRs in the FBS-SHED and lymphocyte co-cultures 24 h post-incubation (Fig. 5), possibly due to the presence of monocytes and neutrophils which differentiated from the progenitor cells. Further studies are needed to address this issue.

5. Conclusion

The effect of complement and cell-mediated cytotoxicity assays showed that SHED maintain their immunomodulatory properties when cultured in FBS and pHS. This finding further supports previously published results on the immunomodulatory properties of MSCs expanded in FBS supplemented media *in vitro*. However, the immunoassay of sixteen paracrine factors and software prediction of biological pathways indicate that pHS is a better supplement for expansion of SHED than FBS on the basis of reduced inflammation.

Conflicts of interest

The authors confirm that there are no conflicts of interest related to this study.

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Table 2
Predicted activation or inhibition of biological functions in FBS-SHED co-cultures compared to corresponding pHS-SHED co-cultures. Ingenuity Pathway Analysis (Ingenuity systems; www.ingenuity.com) predicted activation or inhibition of biological functions maintained by the 16 analysed paracrine factors in FBS-SHED (n = 3 biological replicates) co-cultured with monocytes (n = 3 biological replicates) or lymphocytes (n = 3 biological replicates) compared to pHS-SHED (n = 3 biological replicates) co-cultured with monocytes (n = 3 biological replicates) or lymphocytes (n = 3 biological replicates) respectively after 24 and 120 h of incubation.

Biological Functions (p value)	Cytokines analysed																Increase (↑) Decrease(↓)			
	FGF-2	HGF	LIF	PDGF-BB	SCF	SDF-1A	VEGF-A	G-CSF	GM-CSF	M-CSF	INF-γ	TNF-α	IL-4	IL-6	IL-10	IL-12	24 hrs		120 hrs	
																	$Cocul^M$	$Cocul^L$	$Cocul^M$	$Cocul^L$
Proliferation of hematopoietic progenitor cells (4.94E−15)	–	–	–	–	√	√	–	√	√	–	–	√	–	√	√	–	↓	–	–	–
Proliferation of mononuclear leukocytes (1.08E−15)	–	–	–	–	√	√	–	√	√	√	√	√	√	√	√	–	–	↑	–	–
Proliferation of myeloid cells (4.32E−12)	–	–	–	–	√	–	–	√	√	–	–	√	–	–	√	–	↓	–	–	–
Apoptosis of myeloid cells (3.64E−12)	–	–	–	–	√	–	–	√	√	–	–	√	–	√	√	–	↑	–	–	–
Cell viability of leukocytes (2.10E−22)	–	√	–	–	√	√	–	√	√	√	√	√	√	√	√	–	–	↑	–	–
Differentiation of hematopoietic progenitor cells (7.04E−14)	–	–	–	–	√	√	–	√	√	–	–	√	–	√	√	–	↓	–	–	–
Differentiation of leukocytes (1.84E−19)	–	√	√	–	√	–	–	√	√	√	√	√	√	√	√	–	–	↑	–	–
Activation of mononuclear leukocytes (7.53E−12)	–	–	–	–	–	–	–	–	√	√	√	√	√	√	√	–	–	↑	–	–
Adhesion of immune cells (2.50E−19)	–	√	–	–	√	√	√	–	√	√	√	√	√	√	√	–	–	↑	–	–
Binding of cells (5.33E−13)	√	√	–	–	√	√	√	–	–	–	–	√	√	√	√	–	–	–	↑	–
Binding of leukocytes (1.06E−10)	√	–	–	–	√	√	–	–	–	–	–	√	√	–	√	–	–	–	–	↑
Immune response of antigen presenting cells (4.73E−11)	–	√	–	–	–	–	–	–	√	–	√	√	√	–	–	–	–	↑	–	–
Immune response of phagocytes (6.28E−12)	–	√	–	–	–	–	–	√	√	–	√	√	√	–	–	–	–	↑	–	–
Inflammatory response (1.87E−14)	–	–	–	–	√	√	–	–	√	√	√	√	√	√	√	–	–	↑	–	–
Stimulation of leukocytes (2.70E−15)	–	–	–	–	–	–	–	√	√	–	√	√	√	√	√	–	–	↑	–	–
Leukocyte migration (1.99E−18)	–	√	–	–	√	√	–	√	√	√	√	√	√	√	√	–	–	↑	–	–
Migration of hematopoietic progenitor cells (2.13E−14)	–	√	–	–	√	√	–	–	–	–	√	√	√	–	–	–	–	–	↑	↑
Migration of lymphatic system cells (7.63E−14)	√	√	–	–	–	√	–	√	–	–	–	√	√	√	√	–	–	↑	↑	–

Increase (↑) and decrease (↓) were measured by the positive and negative z-scores. z-score > +1.96 or < −1.96 were considered significant. [√, involved; –, uninvolved/unchanged; $Cocul^M$, co-culture with monocytes; $Cocul^L$, co-culture with lymphocytes]

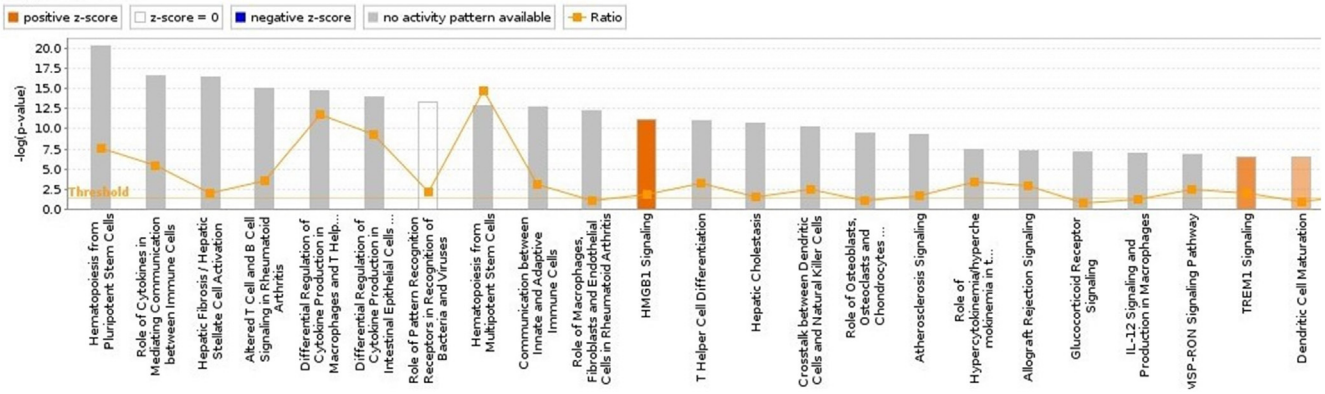

Fig. 4. Predicted activation and inhibition of signalling pathways in the FBS-SHED co-culture with lymphocytes 24 h post-incubation. Activation and inhibition were predicted by Ingenuity Pathway Analysis (Ingenuity systems; www.ingenuity.com).

Upstream Regulator	z-score				Name of the analysed paracrine factors controlled by the upstream regulator
	24 hours		120 hours		
	$Cocul^{Mono}$	$Cocul^{Lym}$	$Cocul^{Mono}$	$Cocul^{Lym}$	
CD28	-	-2.129	-0.569	0.010	GM-CSF, IFN-γ, TNF-α, IL-4, IL-10
HLA-DQ	-	-2.000	0.000	-1.000	GM-CSF, TNF- α, IL-6, IL-10
IL1B	-	2.003	0.950	-0.171	FGF-2, G-CSF, GM-CSF, IFN-γ, TNF-α, VEGF-A, IL-6, IL-10
IL15	-	1.984	-	0.004	GM-CSF, IFN-γ, TNF-α, IL-10
IL18	0.709	2.399	0.709	0.796	GM-CSF, IFN-γ, TNF-α, SDF-1a, IL-4, IL-6, IL-10
IL37	0.763	-2.170	0.025	-0.468	G-CSF, GM-CSF, IFN-γ, TNF-α, IL-6
NFkB	0.647	2.150	0.647	1.347	GM-CSF, IFN-γ, TNF-α, SDF-1a, IL-6, IL-10
TLR2	-	2.219	-0.840	-0.192	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6, IL-10
TLR3	-0.131	2.179	-0.131	0.665	GM-CSF, IFN-γ, TNF-α, IL-6, IL-10
TLR4	0.507	2.521	-0.323	0.955	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6, IL-10, IL-12A
TLR6	-	2.236	-1.000	-0.447	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6
$Cocul^{Mono}$, co-culture with monocytes/macrophages; $Cocul^{Lym}$, co-culture with lymphocytes, -, no activity pattern found (Z-score values greater than +1.96 or smaller than -1.96 considered significant, p<0.05)					

Fig. 5. Predicted changes in the expression of upstream regulators. On the basis of the changes in the expression of analysed cytokines in FBS-SHED (n = 3 biological replicates) co-cultures with monocytes (n = 3 biological replicates) or lymphocytes (n = 3 biological replicates) compared to that in the corresponding pHS-SHED (n = 3 biological replicates) co-cultures Ingenuity Pathway Analysis (IPA; www.ingenuity.com) predicted the activation or inhibition of upstream regulators. IPA also identified the analysed paracrine factors that could be controlled by the upstream regulators.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.04.018.

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