Mesenchymal Stem Cells Regulate *IL8* and *TGFA* Expression in a Novel Leucocytes Depleted Platelet-Rich Plasma-Skin Equivalent in a Preliminary *in vitro* Study of Chronic Wound Healing

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Keywords: Skin equivalent, Wound healing, Inflammation, Mesenchymal stem cell, Cell therapy

Posted Date: September 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-73514/v1>

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Abstract

Background: Chronic leg ulcerations are associated with Haemoglobin disorders, Type 2 Diabetes Mellitus, and long-term venous insufficiency. Mesenchymal stem cells (MSCs) ability to modulate the inflammatory response represents the fundamental requisite for their applicability as a treatment of chronic wounds.

Methods: This study aimed to develop a novel bioactive platelet-rich plasma (PRP)-leukocytes-depleted scaffold to reproduce typical clinical wound of patients with poor chronic skin perfusion and low leucocytes infiltration. After scratching the wound model to mimic injury three conditions were compared; an untreated condition, a condition treated with recombinant TNF to mimic an inflammatory state and a condition treated with TNF and also with MSCs to evaluate how the latter's immunomodulatory properties affect the therapeutic outcomes in an inflammatory state. Gene expression of *IL8* and *TGFA* were analysed in biological triplicates of the three conditions. Statistical analysis was done through a paired student t-test and a $p < 0.05$ was considered significant.

Results: We set up a skin model that consisted of a leukocyte-depleted, platelet-rich plasma scaffold, with embedded fibroblasts as dermal equivalent and seeded keratinocytes on it as multi-layered epidermidis. *IL8* expression increased upon scratching ($p=0.014$) and continued to increase up to day 1 ($p=0.048$). *IL8* expression decreased upon administration of TNF ($p=0.005$) but then increased again. *IL8* expression decreased in the untreated condition after day 1 as the natural healing process took place and was lower than in treated conditions in day 8 ($p=0.048$). *TGFA* expression decreased upon scratching ($p=0.006$) and increased again in day 1, more so in the untreated than in the treated conditions ($p=0.02$). *TGFA* expression decreased again in day 4 in the study group before increasing sharply ($p=0.027$) in day 8 to reach pre-scratch levels.

Conclusion: This study found that a leukocyte-depleted PRP-based skin equivalent can be useful in the study of treatments of chronic wounds. This study also indicates that MSCs appear to modulate the expression of *IL8* by switching from an immunosuppressive phenotype to a pro-inflammatory phenotype. These results indicate that the administration of MSCs could offer a potential therapeutic approach for the treatment of leg ulcers in patients with poor skin perfusion.

Background

Leg ulcers are common, and their incidence rises with age, leading to a negative impact on the quality of life and considerable cost for the health service [1]. Haemolytic disorders such as Sickle Cell Disease and β -thalassaemia are common in Malta and other Mediterranean countries due to a number of β globin gene mutations [2]. Haemoglobin disorders are associated with chronic cutaneous wounds due to peripheral hypoxia [3, 4], lower bioavailability of nitric oxide (NO), iron overload, and an impaired endothelial function [5]. Chronic leg ulceration has also been seen in Type 2 Diabetes Mellitus (T2DM)

and in long-term venous insufficiency where poor perfusion and altered metabolism set up a chronic inflammation that impairs repair and wound closure [6, 7].

Although many therapies have been explored for leg ulcers, none has been completely satisfactory. In our clinic, we obtained complete rapid closure of a longstanding indolent ulcer in the ankle of a β -thalassaemia patient with a homozygous β globin mutation using a mixture of Platelet-Derived Wound Healing Factor (PDWHF) prepared from a pooled platelet concentrate of a matching blood group. The application of the PDWHF resulted in a remarkable change in ulcer appearance which was completely cured after 141 days of treatment. The locally applied PDWHF appears to have stimulated the granulation tissue formation and accelerated the reepithelization.

Due to their ability to differentiate into various cell types and their immunomodulatory properties [8–10] mesenchymal stem cells (MSCs) are thought to have therapeutic potential in the healing of various types of skin defects including leg ulcers, trauma, burn wounds, and scar excision [11] due to their involvement in every stage of the wound healing process [12]. The resolution of inflammation is essential to successful wound healing, and chronic inflammation can lead to poor healing outcomes. Studies have shown that MSCs possess both anti-inflammatory and pro-inflammatory properties, promoting inflammation when the immune system is under-activated and exerting immunosuppressive effects when the immune system is over-activated to avoid damage secondary to inflammation [12, 13]. MSCs stimulated by pro-inflammatory cytokines, including tumor necrosis factor (TNF) and interferon-gamma (IFN- γ), modulate immunosuppression by secreting high levels of soluble factors, including indoleamine 2,3-dioxygenase (IDO), prostaglandin E₂ (PGE₂), nitric oxide (NO), hepatocyte growth factor (HGF), haem oxygenase (HO), cyclooxygenase-2 (COX-2), interleukin-4 (IL-4) and interleukin-1 (IL-1) receptor antagonist preserving T-cell activation. In addition, the MSCs exhibit a pro-inflammatory phenotype by secreting pro-inflammatory chemokines, including metalloproteinase-1 (MIP-1), interleukin 8 (IL-8) and interleukin 10 (IL-10) which enhance the T-cell response [14–16].

To study wound healing a skin substitute scaffold can be used. This is a three-dimensional organotypic culture [17] typically used for skin biology studies and testing of topically applied products. The epithelial cells and the fibroblasts of the skin equivalent secrete cytokines, chemokines, and growth factors that favour skin regeneration and provide a protective layer over the wound [18, 19].

Our objectives were a) to generate a novel bioactive platelet-rich plasma (PRP)-leukocytes-depleted scaffold to develop an *in vitro* model of the typical clinical wound of patients with poor chronic skin perfusion and low leucocytes infiltration and b) to determine whether MSCs affect cytokine expression in this wound model. An air-liquid interface model was used to reproduce a full-thickness skin consisting of a co-culture of epithelial cells and fibroblasts, seeded into a PRP-leukocytes-depleted scaffold.

The PRP leukocytes-depleted scaffold was stimulated with calcium chloride (CaCl₂) which primes and degranulates platelets, hence providing the support structure [20] and being a source of growth factors involved in haemostasis, wound healing and tissue regeneration in various injured tissues [21]. The PRP-

leukocytes-depleted scaffold aimed to avoid the excessive cytokine expression from white blood cells that may delay or impede the wound healing process. Although the inflammatory response is fundamental for wound healing, a subset of inflammatory cells can result in delayed healing [22]. Furthermore, leukocyte-depleted PRP is more predictable, as growth factor release and fibrin scaffold integrity are conserved when exposed to inflammatory conditions [23].

TNF is a cytokine with pleiotropic effects upon cell growth, inflammation, and immune responsiveness. Whereas the local effects of TNF are usually beneficial to the host, when generated at higher concentrations within chronic inflammatory lesions, the proinflammatory effects of TNF often become deleterious and systemic [24]. Recombinant TNF (rTNF) was therefore administered to mimic an inflammatory condition.

Expression of the genes encoding IL-8 and transforming growth factor- α (TGFA) involved in the inflammatory phase [25] and the proliferative phase [26] of wound healing was analysed to evaluate how the leucocyte depleted PRP-based skin equivalent, the rTNF administration, and the MSC infusion affect the final therapeutic outcomes in an inflammatory state.

Materials And Methods

Skin tissue biopsies discarded after medical interventions were obtained to create a skin equivalent model through a co-culture of fibroblasts and epithelial cells. Pooled Buffy coats were used to isolate peripheral blood-derived MSCs.

Isolation and cell culture of primary epithelial cells and fibroblasts

The skin biopsies were washed in Dulbecco's phosphate-buffered saline (PBS) and then suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (50 $\mu\text{g}/\text{mL}$ gentamycin, 0.25 $\mu\text{g}/\text{mL}$ amphotericin B, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin) as well as the antimycotic amphotericin B (Sigma-Aldrich, Milan, Italy). This was followed by overnight digestion at 4 °C with 1 mg/mL Collagenase/Dispase (COLLDISP-RO Roche, UK). The following day, they were incubated at 37 °C for 1 h with 0.25% trypsin-EDTA solution (Sigma-Aldrich) to separate the epidermis from the dermis.

The dermis and epidermis were cut into small pieces of approximately 1 mm² each and incubated separately at 37 °C for 2 h with 0.25% Trypsin-EDTA solution and mixing every 10 min. The enzymatic action was stopped by adding complete medium supplemented with 10% fetal bovine serum (FBS). The digested tissues were centrifuged at 100 $\times$ g for 10 minutes at room temperature to obtain cell pellets. The epithelial cells derived from the epidermis were plated in a 6-well plate and cultured with Stemline Keratinocyte medium II supplemented with 5 $\mu\text{g}/\text{mL}$ hydrocortisone and 10 ng/mL human recombinant keratinocyte growth factor (KGF) (Sigma-Aldrich). The dermal cells were plated in a 6-well plate and

cultured with DMEM/F12 (1:1 mix) supplemented with 50 µg/mL L-ascorbic acid and 5 ng/mL human recombinant fibroblast growth factor (FGF-basic) (Sigma-Aldrich). Both complete culture media also contained 10% FBS, 100 µg/mL insulin, 50 µg/mL gentamycin, 0.25 µg/mL amphotericin B, 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). Both cell types were cultured at 37 °C in a humidified atmosphere and 5% CO₂.

Flow Cytometry analysis of primary Epithelial Cells

Immunofluorimetric characterization of the epithelial stem cells was performed using monoclonal antibodies (moAbs) against Fluorescein Isothiocyanate (FITC) CD34 (Hematopoietic Progenitor Cell Antigen-1, Miltenyi Biotec, Surrey, UK), Phycoerythrin (PE) CD133/2 (Prominin-1, Miltenyi Biotec), PE-CD326 (epithelial cell adhesion molecule, clone 187 eBioscience, Waltham, USA) Alexa Fluor 488 CD29 (Integrin beta-1, clone TS2/16, Bio Legend, UK) Phycoerythrin-Cy5 (PE-Cy5) CD44 (CD 44 molecule, clone IM7, eBioscience) and Allophycocyanin (APC) CD90 (Thy-1, clone 5E10, Bio Legend) surface antigens. PE-IgG1 FITC-IgG1, PE-Cy5 and APC-IgG1 were used as corresponding isotype controls. Flow cytometry assay was done on adherent and 70% confluent epithelial cells. Cells were incubated at 37°C for 10 min with 0.05% Trypsin-EDTA and the enzymatic action was stopped by adding 10% FBS in PBS. Cells were washed with PBS, and 200 µL of cell suspension was stained with 5 µL of each moAb for 20 min in the dark at room temperature. The cells were analysed with the FACS Aria II (FACS Diva version 6.1.2, Becton Dickinson, USA) and raw data analysis was performed using FCS Express 7, De Novo software. Initial forward scatter (FSC) and side scatter (SSC) distribution parameters of the cell populations were applied to exclude cell debris.

Isolation and culture of peripheral blood-derived MSCs

MSCs were isolated from a buffy coat in an EDTA anti-coagulating bag using a gradient density media separation on Histopaque-1077 (Sigma-Aldrich) at 500 x g for 25 min at 20 °C. Isolated mononuclear cells were subsequently washed with PBS and centrifuged at 100 x g for 10 min at room temperature. The cell suspension containing the MSCs was resuspended in 12 mL of fresh cell culture medium (MesenCult Proliferation Kit, Stem Cell Technologies, UK) supplemented with 50 µg/mL gentamycin, 0.25 µg/mL amphotericin B, 100 units/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich) and plated in a 75 cm² flask. MSCs were cultured at 37 °C in a humidified atmosphere and 5% CO₂. After 48 hours, the culture medium was changed to remove the non-adherent cells. The medium was changed every three to four days until confluence was reached.

Flow cytometry analysis of the MSCs

Cytofluorimetric characterization and cell sorting of MSCs was done with moAbs against the surface antigens FITC- CD105 (Endoglin, clone 43A3, Bio Legend), Alexa Fluor 488 CD29 (Integrin beta-1, clone TS2/16, Bio Legend), PE-Cy5 CD44 (CD 44 molecule, clone IM7, eBioscience), PE-Cyanine7 (PE/Cy7) CD73 (Ecto-5'-nucleotidase, clone TY/11.8, Bio Legend), PE-CD34 (clone 561, Bio Legend) and Peridinin Chlorophyll Protein Complex (PercP) CD45 (Leukocyte common antigen, clone HI30, Bio Legend). FITC-IgG1, PE-Cy5-IgG1, PE-Cy7-IgG1, PE-IgG1 and PerCP-IgG1 were used as corresponding isotype controls.

Adherent and confluent at 70% at first passage MSCs were treated with 0.05% Trypsin-EDTA for 10 minutes at 37 °C and the enzymatic action was stopped by adding 10% FBS in PBS. Cells were washed with PBS, and 200 µL of cell suspension was stained with 5 µL of each moAb for 20 min at room temperature in the dark. The cell suspension was analysed with FACS Aria II (FACS Diva version 6.1.2, Becton Dickinson), and raw data analysis was performed using FCS Express 7, De Novo software. Initial forward scatter (FSC) and side scatter (SSC) distribution parameters of the cell populations were applied to exclude cell debris.

Differentiation of the MSCs into Osteocytes, Chondrocytes, and Adipocytes

The StemPro™ Supplement kits (Gibco™, Thermo Fisher Scientific, Waltham, USA) were used to differentiate the MSCs into three lineages: osteocytes, chondrocytes, and adipocytes. The culture media were supplemented with 50 µg/mL gentamycin, 0.25 µg/mL amphotericin B, 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). Differentiations were carried out according to the manufacturer's protocol. MSC differentiation into the three lineages was assayed using standard protocols by Von Kossa staining for osteocytes, Toluidine Blue for chondrocytes, and Oil Red O staining for adipocytes. Counterstaining was done with a 5% aluminum-sulphate solution for osteocytes and with Mayer's Haematoxylin Solution for adipocytes.

Cell count and viability

Cell count and viability of the three cellular components involved in the study were performed before the assembly of the experimental model and administering the MSCs to the wound model. The assay was performed with Countess TM II Automated Cell Counter using a 0.4% Trypan Blue solution (Thermo Fisher Scientific) as per the manufacturer's protocol. The analyses were performed in triplicate.

Preparation of the leukocyte-depleted PRP-based skin equivalent

Apheresis donor platelet concentrates were transferred into 50 mL centrifuge tubes and centrifuged for 20 min at 300 x g at 4°C. The platelet pellet was resuspended in 5 mL residual plasma to form the PRP. 1 mL of PRP was mixed with 1 mL 20 mM calcium chloride (CaCl₂) solution and 6×10^4 cells/cm² fibroblasts. The mixed solution was poured into a 24 mm trans-well with a 0.4 µm pore polyester membrane insert and a cell growth area of 4.67 cm² (Corning Trans-well, Sigma-Aldrich) and left at 37°C in a CO₂ incubator for 24 h. The CaCl₂ worked as activator for the formation of autologous thrombin and allowed the PRP-based derma equivalent to solidify.

The epithelial cells were seeded on the PRP-based derma equivalent with a density of 6×10^4 cells/cm² to establish the prototype skin equivalent. This skin model was cultured with an air-liquid interface system allowing the development of a multi-layered skin equivalent. Once fully developed, the skin equivalents were collected and fixed for 24 h at room temperature in 10% neutral buffered formaldehyde for

sectioning and processing using a short histology protocol, with an overnight incubation step in 60% ethanol. The tissue-equivalent sections were embedded in wax blocks, cut at 5 µm, and transferred to slides and stained with Mayer's Haematoxylin-Eosin solutions (Sigma-Aldrich).

The *in vitro* experimental model of wound healing

In order to create an *in vitro* experimental model of wound healing, a scratch injury was inflicted. Administration of human rTNF (Bachem AG, Switzerland) was used to mimic an inflammatory condition. The infusion of peripheral blood-derived MSCs was done 24 h after the administration of the rTNF to evaluate how their immunomodulatory properties affect the final therapeutic outcomes in an inflammatory state. The three different conditions of wound healing created in the study were:

Control: a scratch injury was inflicted but no rTNF or MSC was subsequently administered.

Treated: A wound was inflicted and 30 ng/ml of rTNF was administered 3 h after wound infliction. The purpose of this condition was to reproduce an inflammatory condition.

Study: A wound was inflicted, 30 ng/ml of rTNF was administered 3 h after wound infliction, and 6×10^4 cells/cm² MSCs were infused 24 h after the administration of rTNF. The purpose of this condition was to study whether the presence of MSCs alters the inflammatory response.

Skin equivalent models from all conditions were collected at day 0 (3 h after wound infliction) and then at 1, 2, 4- and 8-day intervals after the wound was inflicted. The *in vitro* model was repeated in biological triplicate.

RNA extraction and qPCR

RNA was extracted from 5×10^5 adherent MSCs, 5×10^5 adherent fibroblasts, and the leukocyte-depleted PRP-based skin equivalents using the Pure Link® RNA Mini Kit (Thermo Fisher Scientific). In brief, leukocyte-depleted PRP-based skin equivalents were harvested quickly and were immediately digested and homogenised with 45 µl of Digestion Buffer and 5 µl of Proteinase K using a homogenizer (Speed Mill PLUS Analytic Jena AG, Germany). The cycles of homogenisation were: 30 sec high speed, 1 min pause, 30 sec high speed. RNA was then extracted from the homogenised tissue according to the manufacturer's protocol.

RNA quality control and measurement of the amount of total RNA were performed using a Nanodrop 2000 Instrument (Thermo Fisher Scientific). Complementary DNA (cDNA) was produced from 50 ng of RNA from each sample using the Revert Aid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qPCR was performed using Rotor-Gene Q Series Software 2.1.0. (Qiagen, Valencia, California, USA). One microliter of the neat cDNA was amplified in a final volume of 20 µL with 5x HOT FIREPol Eva Green qPCR Super mix (Solis BioDyne, Tartu, Estonia) and primers for *IL8*, *TGFA*, *CD34*, *CD45*, *CD90*, *CD73*, *CD105*, *CD44*, *CD29*, and *ACTB* (Table 1). Thermal cycling proceeded for 12 min at 95 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72°C for 30 s. All real-time PCR reactions were performed in

triplicate. The relative expressions of mRNAs were calculated using the comparative Ct method ($2^{(-\Delta\Delta Ct)}$) and normalized against the endogenous reference gene *ACTB* (encoding β -Actin), and the data are reported as the mRNA fold change. The expression levels of the skin equivalent models before scratching were used as calibrators.

Table 1
Primer sequences and amplicon sizes for each gene analysed in the study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fragment size
<i>CD34</i>	TGAAGCCTAGCCTGTCAC	ATAAGACCTCCAGCTGTGCG	180 bp
<i>CD44</i>	CCAATGCCTTTGATGGACCA	GAATCAGATGGACACTCACA	314 bp
<i>CD45</i>	GTGTTTCATCAGTACAGACG	GCTGTCATTTCAACCACAAC	191 bp
<i>CD73</i>	ATGGTGTGGAAGGACTGATC	CATCGCTCAGAAAGTGAGG	310 bp
<i>CD90</i>	TGCTCTTTGGCACTGTGG	CTGCTCCTGCTCTCCCTCT	248 bp
<i>CD29</i>	GGATTCTCCAGAAGGTGGTTTCG	GGAGATGGGAAACTTGGTGGCA	121 bp
<i>CD105</i>	GGGGTCAACACCACAGAG	CACATCCTGAGGGTCCTG	261 bp
<i>IL8</i>	GAGAGTGATTGAGAGTGGACCAC	AAACTGGGTGCAGAGGGTTGTG	90 bp
<i>TGFA</i>	GGTCCGAAAACACTGTGAGTGG	AAGAGCCCAGAGGAGGAGTTTG	108 bp
<i>ACTB</i>	AGTCCTAGCTACTCCGGAGGC	CGGCTATTCTCGCAGCTCAC	113 bp

Statistical analysis

The data are presented as mean $\pm$ standard deviation (SD). The statistical significance was determined using the Wilcoxon signed-rank test, a nonparametric alternative to paired t-test, or a paired student t-test. $P < 0.05$ was considered statistically significant.

Results

Successful isolation and culture of primary epithelial cells, fibroblasts, and skin equivalent.

The epithelial cells displayed a short and wide spindle-shape and epithelial morphology like a "pavement stone" (Fig. 1A). The cultures reached confluence and were ready to passage within two weeks. The growth of fibroblasts from the dermal tissue was observed by day 10 after tissue plating. The dermal tissue was removed from the plate 15 days after plating, where the proliferating fibroblasts initiated the primary culture reaching confluence at day 20 after plating (Fig. 1B)

The skin equivalent model was assembled through a co-culture of epithelial cells and fibroblasts-seeded into the *PRP-leucocyte depleted scaffold* and cultured in an *air-liquid interface system* allowing the development of a multi-layered skin equivalent (Fig. 1C).

Microscopical observation of the scaffold confirmed the presence of confluent and multi-layered epithelial cells (Fig. 1D), and the presence of proliferating spindle-shaped fibroblasts (Fig. 1E) on the PRP scaffold. Histological examination of the skin equivalent showed the presence of different epithelial cells organized in a typical skin tissue. The nuclei of the epithelial cells and the fibroblasts were stained in violet (Fig. 1F).

Characterization of primary culture of epithelial cells and fibroblasts.

The presence of epithelial stem cells and proliferating epithelial cells in the skin equivalent allows for the development of a multi-layered epithelium. In order to identify the presence of epithelial stem cells and committed epithelial progenitors on the primary epithelial cell culture we evaluated the expression of well-established markers such as the epithelial stem cell markers CD34 (CD34 molecule) [27] and the CD133 (Prominin-1) [28], CD90 [29] and the committed progenitor epithelial cells marker CD326 (epithelial cell adhesion molecule) [30].

Epithelial cells were also identified through the presence of specific lineage markers such as CD29 and CD44.

PE-IgG1 FITC-IgG1, PE-Cy5 and APC-IgG1 were used as negative controls to identify and quantify the percentage of positivity of the stained population for each marker. 45.67% of the cultured epithelial cells were positive for CD29 (Fig. 2A), 29.53% were positive for CD44 (Fig. 2B), 12.72% were positive for CD90 (Fig. 2C), and 9.73% were positive for CD34 (Fig. 2D), 6.62% were positive for CD326 (Fig. 2E) and 2.52% were positive for CD133 (Fig. 2F).

Gene expression analysis of the primary fibroblasts culture showed high expression of *CD105* (mean $\pm$ SD: 25.4 ± 0.07), and *CD90* (28.5 ± 0.11), while a lower expression of *CD73* (2.68 ± 0.32 SD) was detected (Fig. 2G). Data also showed the absence of expression of the genes encoding the hemopoietic markers *CD45* (-2.62 ± 0.40) and *CD34* (-3.69 ± 0.40). Analysis of the products on 1.5% agarose gel confirmed the presence of the qPCR products (Fig. 2G).

Characterization of peripheral blood-derived MSCs

FITC-IgG1, PE-Cy5-IgG1, PE-Cy7-IgG1, PE-IgG1 and PerCP-IgG1 conjugated isotypes were used as negative controls to identify and quantify the percentage of positivity of the stained population for each marker. Flow cytometric analysis performed on peripheral blood-derived MSCs at the first passage showed the

presence of two separate populations, p1 (Fig. 3A) and p2 (Fig. 4A) with different immunophenotypic patterns.

Population P1 (Fig. 3A) was found positive for CD29 (Fig. 3B), CD44 (Fig. 3C), CD73 (Fig. 3D) and CD105 (Fig. 3E). It was also found negative for the hemopoietic markers CD34 (Fig. 3F) and CD45 (Fig. 3B-F).

Population P2 (Fig. 4A) was found highly positive for CD44 (Fig. 4B) and slightly positive for CD29 (Fig. 4C), CD73 (Fig. 4D), and (CD105 Fig. 4F). It was also found negative for the hemopoietic markers CD34 (Fig. 4E) and CD45 (Fig. 4B-F).

Isolation, culture and gene expression of MSCs

Peripheral blood isolated mesenchymal stem cells showed their properties to form colonies (Fig. 5A), to adhere (Fig. 5B and Fig. 5C) and to have a spindle-shape fibroblastic picture (Fig. 5B and Fig. 5C)

The ability of the MSCs to differentiate into the three lineages was analysed by histological staining and microscopic observation. Adipocytic differentiation was reached at day 14 after stimulation, as determined by the presence of characteristic lipid vacuoles in the cytoplasm. The Oil Red O stained the fat in the lipid vacuoles while the nuclei were counterstained with Mayer's Haematoxylin solution. Osteogenic differentiation was achieved at day 21 after stimulation and was confirmed by the presence of dark-coloured calcium deposits stained with Von Kossa solution. Chondrogenic differentiation was noted at day 21 after stimulation as determined by the presence of proteoglycans stained with Toluidine Blue solution.

Gene expression analysis confirmed that the expression of genes for MSC-specific markers such as CD44 (mean $\pm$ SD: 15.27 ± 0.25), CD73 (14.12 ± 1.00), and CD90 (11.68 ± 2.02) was high, and low for CD105 (5.65 ± 0.88) and CD29 (4.93 ± 1.13) (Fig. 5D). Data also showed the absence of expression of the genes encoding the hemopoietic markers CD45 (-1.38 ± 0.25) and CD34 (-2.25 ± 0.40). Analysis of the qPCR products on an 1.5% agarose gel confirmed the presence of the products (Fig. 5D).

Viability of the various cell types used for the wound model

Cell viability tests were performed in triplicate on the three cellular cell types: fibroblasts, epithelial cells, and MSCs. Viability (mean $\pm$ SD) was $85.89 \pm 0.51\%$ for fibroblasts, $85.67\% \pm 0.58\%$ for epithelial cells and $85 \pm 3.2\%$ for MSCs.

Effects of scratch injury, rTNF, and MSC on gene expression in skin equivalent model

We investigated the response of the skin equivalent cells in our experimental, leukocyte-depleted PRP-based skin equivalent model of a chronic wound associated with low skin perfusion. For this purpose, we performed real-time qPCR analysis to evaluate the level of expression of genes involved in the inflammatory (*IL8*) (Fig. 6A) and proliferative (*TGFA*) (Fig. 6B) phases at various time points in response to the scratch injury, rTNF administration, and MSC infusion.

In these experiments, we monitored a control condition which received only the scratch and was used to verify the inflammation and proliferation of an untreated wound, while the treated condition and the study condition were administered a scratch injury and rTNF, or a scratch injury, rTNF, and MSC respectively (Fig. 6A,B).

As shown in Fig.xx, the expression of *IL8* increased markedly within 3 h of the scratch injury, whereas the expression of *TGFA* decreased.

A decrease in the expression of *IL8* was noted in day 1 in the condition treated with rTNF (30 ng/ml) when compared with the untreated control condition (Fig. 6A).

The expression of *IL8* decreased steadily in the control condition from day 2 onwards but increased slightly in the treated condition. The expression of *IL8* decreased significantly in the study condition in day 4 and almost reached the levels which were present prior to the injury but then increased again in day 8 (Fig. 6A).

The expression of *TGFA* increased in the first two days and decreased in day 4 in all groups. A significant decrease was noted in the study group between day 4 and day 8 (Fig. 6B).

Discussion

Skin equivalents represent the first examples of three-dimensional organotypic cultures which are often used for in vitro research study of normal and abnormal skin biology [17, 31].

In this study, we developed a new wound model on which to observe the immune response during the wound healing process through the expression of *IL8* and *TGFA*. The control condition was not treated, the treated condition was given an additional inflammatory stimulus through the administration of 30 ng/mL rTNF which regulates genes that code for inflammatory mediators [32], while the study condition was administered with 30 ng/mL rTNF and 6×10^4 cells/cm² MSCs. This model is shown in Fig. 7.

Studies on monolayer cultures of keratinocytes submerged into culture media do not resemble the true nature of the physiological process of wound healing [33]. Multi-layered differentiated models are comparable to native skin and produce excellent results when analysing epithelial attachment, proliferation, differentiation, and dermal remodelling [34]. Such models support further expertise of skin biology and skin diseases, without the complexity of the intrinsic interactions found in native skin [34, 35].

One example of a skin model cultured in an air-liquid interface system is the In Vitro Reconstructed Human Epidermis [36], which does not contain fibroblasts. On the other hand, de-epidermidised dermis and collagen framework, which includes only the fibroblasts, basement membrane, and extracellular matrix (ECM) components and which are the most commonly used dermal equivalents [34] is not

sufficient to reproduce a skin model. It was shown that the inclusion of fibroblasts enhances the production of extracellular matrix proteins, generating a more normal epidermal architecture [34]. Therefore, the interaction between epidermal and dermal components is needed for adequate wound healing. In another study, a skin equivalent was developed by implanting keratinocytes onto the upper surface of a collagen scaffold, occupied with fibroblasts and culture at the air-liquid interface [37].

It is known that platelet-rich plasma contain more than 300 biologically active molecules containing growth factors and pro-inflammatory and immune-modulating cytokines that can activate the platelets themselves, perpetuating the inflammatory cycle [27].

Moreover, the platelets trapped into the fibrin matrix release the growth factors slowly over a duration of seven days, in contrast with the use of exogenous thrombin, where almost all growth factors are discharged during the first hour [38].

Several studies reported positive results on the application of PRP in stimulating the wound healing process [39] and increasing keratinocyte migration [35]. Previous attempts to use platelet-derived products were tested in skin tissue engineering. One study described the use of platelet lysate, combined with chitosan and 107 hyaluronic acid dressing [40]. At the same time, others used platelet lysate in conjunction with a collagen/gelatin scaffold [41] or a collagen type I gel which was mixed with PRP [42]. These studies all showed promising outcomes, supporting the approach of the use of platelet-derived products in wound healing.

Our novel skin model consisted of a leukocyte-depleted, platelet-rich plasma scaffold, with embedded fibroblasts, as dermal equivalent and seeded keratinocytes on it as multi-layered epidermis (Fig. 7). Calcium chloride was used as an activator to initiate the formation of autologous thrombin from prothrombin, forming a fibrin clot that provided a surface for keratinocyte seeding and enabled the skin cells to mature into stratum corneum and basal, spinous and granular layers. The lack of leukocytes allowed for the mimicking of typical chronic wounds of patients with poor skin perfusion and low leukocyte infiltration. The leukocyte-depletion allowed for the evaluation of the immunomodulatory properties of the infused MSCs, which modulate the *IL8* and *TGFA* secretion.

We subsequently used our new wound model to analyse cytokine gene expression under three conditions: control, treated, and study conditions.

In physiological wound process, the ECM components, such as fibronectin, glycosaminoglycans and collagens, regulate the dynamic and interactive process of wound healing. [43] The platelets are early modulators of the healing process [44] and the blood clot formed upon platelets activation provides a provisional “scaffolding” containing fibrin molecule and plasma fibronectin. This occurs during the first 24 hours after the injury and enables formation of a temporary matrix in the wound bed. [45] Therefore, our PRP-based scaffold as dermal equivalent reassembles the physiological scaffolding formed during the hemostatic phase and required for the normal wound process.

The initial wave of inflammatory phase is characterized by *IL8* production by platelet α -granules and skin resident cells to reduce blood loss and fill the tissue gap with a blood clot rich in platelets, macrophages, leukocytes and mast cells producing/secreting cytokines and growth factors [46–49].

The inflammatory response occurs within hours of the occurrence of the damage as a localized or systemic protective response. It is activated by molecules expressed by pathogens or associated with tissue injury and are recognized by Toll-like receptors (TLRs) present on skin resident cells [50]. TLRs activation in response to injury and inflammation is responsible for the upregulation of *IL8* [51–53].

A significant upregulation of IL8 expression was noted three hours after the scratch injury when compared to the levels exhibited just before, thereby confirming the success of our scaffold in mimicking the wound. On the other and, the scratch injury exhibited a down regulatory effect on the expression of *TGFA*.

In addition to the induction of inflammation by chemokines, other molecules such as TNF promote the inflammatory response following wounding.

It has been shown that the prolonged stimulation of their TLR receptors causes downregulation of TLR2 and TLR4, most likely as a self-regulatory mechanism to prevent overactive skewing of the immune response [54]. In our model we noted a significant down regulation of *IL8* following the administration of rTNF which appears to indicate the delayed the activation of the inflammatory response. The progression of *IL8* in the treated group occurred in delay (i.e., at a later time point) when compared with the control group.

TLR ligation triggers the release of inflammatory mediators initiating innate immune responses mainly through the activation of macrophages, neutrophils, leucocytes, and stromal cells including MSCs, thus creating an inflammatory environment [55–56].

Neutrophils and monocytes/macrophages represent the key cells of the inflammatory phase [57] as their simultaneously release of large number of cytokines and growth factors are crucial to initiate the next phase of the healing process [58]. Neutrophils appear in the wound area a few minutes after the injury [59] and are replaced after two or three days by monocytes that undergo a transformation into macrophages [60]. Macrophages are cells of great importance for the healing process [61] as they participate in phagocytosis and are also the main source of cytokines and growth factors stimulating the proliferation of fibroblasts and collagen biosynthesis [62–63].

It was noted that a decreased influx of neutrophils in the first 4 days after the infliction of a wound has a negative impact on healing outcomes. [64]

It is well known that macrophages switch phenotypes from an M1 pro-inflammatory phenotype to an M2 pro-repair phenotype leading to the reduction of inflammatory markers and the promotion of the proliferation phase. [65]. Moreover, macrophages secrete PDGF, TGF- α , and bFGF, which modulate the epithelialization, collagen accumulation, and angiogenesis. [66]. During the proliferative phase there is an

increase in migration and proliferation of fibroblasts and endothelial cells as well as keratinocytes, which secrete bFGF, EGF, VEGF, bFGF, and PDGF, TGF- α and KGF. TGF- α mRNAs were isolated in both wound macrophages [67] epidermal keratinocytes at the wound edge. [68]. Based on its expression level, *TGFA* can be considered as a biomarker of the early phase of re-epithelialization. [69]

The results obtained with our model indicate that all three conditions studied were in an inflammatory state throughout the study as shown by the lower expression of *TGFA* when compared with *IL8*.

The absence of leucocytes, which promote the resolution of the inflammation by releasing numerous potent cytokines, probably led to a delay of the proliferative phase.

In experimental models, the stimulation of MSCs with the pro-inflammatory cytokine TNF upregulates expression of a subset of TLRs, thus increasing the sensitivity of MSCs to the inflammatory milieu [70]. We postulate that the MSC infusion could modulate the expression of *IL8* and that the decrease of *IL8* expression in the study condition at day 2 and especially at day 4 could indicate that the presence of MSCs inhibited the inflammatory response in contrast with an increase in the treated condition.

TLR4 receptor activation triggers the MSC1 population which exhibits a pro-inflammatory profile while activation of the TLR3 receptor activates the MSC anti-inflammatory phenotype MSC2 [14, 15, 71]. MSCs are known to display an anti-inflammatory phenotype in an inflammatory environment as characterized by increased mRNA expression of *IL6* [72].

Our data suggest the possibility that the MSCs modulate the inflammatory response, switching from an immunosuppressive phenotype to a pro-inflammatory phenotype and regulating the *IL8* expression. Presumably, this switch in our model occurred between day 4 and day 8, showing a substantial increase of mRNA expression in the study condition in day 8 when compared with the treated condition.

TGFA was down regulated throughout all time points in all the three conditions in our study. Interestingly, the changes in expression of *TGFA* had a similar pattern of to the changes in the expression of *IL8* between days 2 and 8 in the study condition. We hypothesize that the modulation of *IL8* could affect the expression level of *TGFA*.

Keratinocytes and fibroblasts could have both contributed equally to synthesize *IL8*.

We also suggest that the presence of a pro-inflammatory cytokine (TNF) stimulates MSCs to exert their immunomodulatory properties secreting directly *IL8* or/and having a paracrine effect on *IL8* and *TGFA* production by acting on the resident skin cells.

Further investigation is necessary to address the specific cellular source of *IL8* and *TGFA* production and the cellular target of the MSCs paracrine action and hence to evaluate the clinical relevance of the infusion of MSCs in a scenario of a low blood supply in the site of the wound.

Conclusion

A leukocyte-depleted, platelet-rich plasma scaffold was developed which allowed for the mimicking of typical chronic wounds of patients with poor skin perfusion and low leukocyte infiltration. The wound model indicated accurately how the resident skin cells produce *IL8* and deregulate *TGFA*, in response to damage in a physiological and inflammatory state. The change in the expression level of *IL8* confirms that an inflammatory condition was created. Notwithstanding the absence of leukocytes, MSCs modulated the *IL8* expression in response to damage in an inflammatory environment by acting directly on skin resident cells. Therefore, MSCs could have therapeutic potential for the treatment of ulcers in patients with poor skin perfusion.

List Of Abbreviation

APC	Allophycocyanin
bFGF	Basic Fibroblast Growth Factor
CaCl₂	Calcium Chloride
CD	Cluster of differentiation
cDNA	Copy DNA
COX-2	Cyclooxygenase-2
Ct	Cycle threshold
DAMP	Danger-Associated Molecular Pattern
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate.
FL	Fluorescence
FSC	Forward Scatter
FGF- basic	Fibroblast growth factor
HGF	Hepatocyte Growth Factor
HO	haem oxygenase
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
KGF	Keratinocyte growth factor
MMP	Matrix metalloproteinases
MoAbs	Monoclonal Antibodies
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cells
NO	Nitric oxide

PBS	Phosphate-buffered saline
PCR	Polymerase Chain reaction
PDWHF	Platelet-Derived Wound Healing Factor
PDGF	Platelet Derived Growth Factor
PerCP	Peridinin Chlorophyll Protein Complex
PGE2	Prostaglandin E2
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5
PE/Cy7	PE-Cyanine7
PRP	Platelet Rich Plasma
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rTNF	Recombinant TNF
SSC	Side Scatter
SD	Standard Deviation
T2DM	Type 2 Diabetes Mellitus
TGF-β	Transforming growth factor beta
TGF-α	Transforming growth factor alpha
TIMPs	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptors
TNF	Tissue Necrosis Factor
VEGF	Vascular endothelial growth factor
Δ Ct	Delta Ct
$\Delta\Delta$ Ct	Delta Delta Ct

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are grateful to Dr Kevin Schembri Cardiothoracic Specialist at Mater Dei Hospital for providing the skin tissue biopsies. The authors are grateful to Dr Alex Aquilina, Director of the National Blood Transfusion Centre for providing the pooled buffy coat bags and the apheresis-pooled, leukocyte-depleted platelet products.

Ethics approval and consent to participate

This research was approved by The University of Malta Research Ethics Committee with protocol reference number 56120L7. Apheresis-pooled, leukocyte-depleted platelet products were obtained from the National Blood Transfusion Centre.

Consent for publication

Not applicable

Funding

This research was supported by a fellowship granted by the Foundation for Medical Service (FMS), Malta Enterprise, and research funds from the Faculty of Medicine and Surgery, University of Malta. Funding reference number MDSRA01-01.

Author Contributions

ES: Conceptualized the project; isolated, cultured and characterized primary cells; assembled the skin model; performed the in vitro model of wound healing; acquired and analyzed data and wrote the first draft of the manuscript. **GGa:** reviewed and edited the manuscript **GGr:** isolated and cultured the primary cells, assembled the skin model; performed the in vitro model of wound healing; **SST:** performed the histological staining of the skin equivalent **AF:** Conceptualized the project; reviewed, edited and approved the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or is available upon request.

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Figures

Figure 1

Morphology of the primary epithelial cells, fibroblasts, and skin equivalent A) Seeded epithelial cells showing their characteristic "pavement stone" morphology; B) Fibroblasts reaching confluence 15 days after the plating of the dermal cells; C) Typical air-liquid interface system composed by a PRP-leucocyte depleted scaffold in a 24 mm trans-well. The skin model was assembled through a co-culture of epithelial cells and fibroblasts-seeded in the PRP-leucocyte depleted scaffold; D) Confluent epithelial cells growing in multilayers on the surface of the scaffold; E) Confluent fibroblasts embedded into the scaffold; F) Transversal section of the skin equivalent showing the presence of different epithelial cells organized into

the different layers of the skin. Nuclei of the epithelial cells are stained in violet. The image also shows the presence of the fibroblasts (nuclei stained in violet) embedded in the PRP-leucocyte depleted scaffold stained in pink reassembling the extracellular matrix of the skin. Scale bar = 100 μ m.

Figure 2

Flow cytometry to identify the epithelial cells, and mRNA analysis to verify the fibroblasts identity A-F) Flow cytometry scatter plots of the adherent epithelial cells side scatter (SSC)/ Fluorescence dot plots of each marker; epithelial cells were found positive for CD29 (A) and CD44 (B) lineage markers and also for CD90 (C), CD34 (D), CD326 (E), and CD133 (F) stemness markers; G) mRNA expression of genes involved in fibroblast characterization was determined by qPCR. Transcript levels were normalized to the ACTB reference gene using the 2- Δ Ct method. The data are presented as mean $\pm$ standard deviation (SD). The graph bar shows expression level of the genes CD90, CD73, CD105, CD45, and CD34 of cultured fibroblasts. Gene expression was confirmed by 1.5% agarose gels.

Figure 3

Flow cytometry analysis to verify the MSCs identity of Population P1 Flow cytometry to determine the positivity of MSCs. Dot plots analysis shows that population P1 (A) was positive for the specific lineage markers CD29 (B), CD44 (C), CD73 (D) and CD105 (E) and negative for the hemopoietic markers CD34 (F) and CD45 (B-F).

Figure 4

Flow cytometry analysis to verify the MSCs identity of Population P2 Flow cytometry to determine the positivity of MSCs. Dot plots analysis shows that population P2 (A) was highly positive for the specific lineage marker CD44 (B) and slight positive for CD29 (C), CD73 (D) and CD105 (F). It was negative for the hemopoietic markers CD34 (E) and CD45 (B-F).

Figure 5

Isolation culture and gene expression of MSCs Isolated MSC showed their characteristics to form colonies (A), to adhere (B) and having a spindle-shape fibroblastic picture (B and C). Photos taken at day 6 of the first passage (A), at day 15 of the first passage (B) and at the second passage (C). Scale bare 100 μm qPCR analysis of native MSCs mRNA expression of characteristic MSC genes was done during the second passage (D). Transcript levels were normalized to the ACTB reference gene using the 2-ΔCt method. The data are presented as mean± SD. The graph bar showing the mRNA expression level of CD90, CD73, CD105, CD44, CD29, CD45, and CD34 of cultured MSCs. Expression was high for CD44, CD73, CD90 and low for CD105 and CD29. Gene expression was confirmed by analysis of the products on 1.5% agarose gels.

Figure 6

IL8 and TGFA Gene Expression Bar graphs showing the log fold change expression levels of the genes IL8 (A) and TGFA (B) of the control, treated, and study conditions for the five time points at which they were measured. The mRNA expression was determined by qPCR. Relative transcript levels were normalized to the ACTB reference gene. The expression levels of the skin models before scratching were used as a calibrators. Data presented as log fold change using the $2^{-\Delta\Delta C_t}$ method. P values were

worked out through a paired student t-test. $p < 0.05$ was considered as statistically significant and is shown in the figure where applicable (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 7

Experimental wound model and the changes in the expression of the genes Schematic representation of the effects of scratch assay, rTNF administration, and MSC infusion on gene expression in our skin equivalent model. IL8 and TGFA expression levels were evaluated at different time points. Down arrows in red represent downregulation while up arrows in green represent upregulation. Grey sideways arrows indicate no change in expression levels.