RESEARCH ARTICLE

The role of bone marrow mesenchymal stromal cell derivatives in skin wound healing in diabetic mice

Tomas de Mayo¹, Paulette Conget², Silvia Becerra-Bayona³, Claudia L. Sossa³,⁴, Virgilio Galvis³,⁵,⁶, Martha L. Arango-Rodríguez⁴*

¹ School of Medicine Clínica Alemana Universidad del Desarrollo, Lo Barnechea, Santiago, Chile, ² Center for Regenerative Medicine, School of Medicine Clínica Alemana Universidad del Desarrollo, Lo Barnechea, Santiago, Chile, ³ Universidad Autónoma de Bucaramanga (UNAB), Bucaramanga, Colombia, ⁴ Production Unity of Advanced Therapy, Fundación Oftalmológica de Santander, Clínica Carlos Ardila Lulle (FOSCAL Internacional), Bucaramanga, Colombia, ⁵ Centro Oftalmológico Virgilio Galvis, Bucaramanga, Colombia, ⁶ Fundación Oftalmológica de Santander FOSCAL, Bucaramanga, Colombia

* martha.arango@foscal.com.co

Abstract

Mesenchymal stromal cells (MSCs) have shown to be a promising tool in cell therapies to treat different conditions. Several pre-clinical and clinical studies have proved that the transplantation of MSCs improves wound healing. Here, we compare the beneficial effects of mouse bone marrow-derived allogeneic MSCs (allo-mBM-MSCs) and their acellular derivatives (allo-acd-mMSCs) on skin wound healing in Non-Obese Diabetic (NOD) mice. One dose of allo-mBM-MSCs (1×10⁶ cells) or one dose of allo-acd-mMSCs (1X) were intradermally injected around wounds in 8–10 week old female NOD mice. Wound healing was evaluated macroscopically (wound closure) every two days, and microscopically (reepithelialization, dermoepidermal junction, skin appendage regeneration, leukocyte infiltration, vascularization, granulation tissue formation, and density of collagen fibers in the dermis) after 16 days of MSC injection. In addition, we measured growth factors and specific proteins that were present in the allo-acd-mMSCs. Results showed significant differences in the wound healing kinetics of lesions that received allo-acd-mMSCs compared to lesions that received vehicle or allo-mBM-MSCs. In particular, mice treated with allo-acd-mMSCs reached significantly higher percentages of wound closure at day 4, 6 and 8, relative to the allo-mBM-MS Cs and vehicle groups (p < 0.05), while wound closure percentages could not be statistically distinguished between the allo-mBM-MS Cs and vehicle groups. Also, allo-acd-mMSCs had a greater influence in the skin would healing process. Specifically, they caused a less pronounced inflammatory severe response (p < 0.0001), more granulation tissue formation at an advanced stage (p < 0.0001), and higher density of collagen fibers (p < 0.05) compared to the other groups. Nevertheless, at day 16, both allo-mBM-MS Cs and allo-acd-mMSCs revealed a higher effect on the recovery of the quality skin (continuous epidermis; regular dermoepidermal junction and skin appendages) relative to untreated lesions (p < 0.0001), but not between them. On the other hand, ELISA analyses indicated that the allo-acd-mMSCs contained growth factors and proteins relevant to wound healing such as IGF-1, KGF, HGF, VEGF, ANG-2, MMP-1, CoL-1 and PGE2. Compared to allo-
acd-mMSCs, the administration of allo-mBM-MSCs is insufficient for wound healing in diabetic mice and delays the therapeutic effect, which maybe explained by the fact that trophic factors secreted by MSCs are critical for skin regeneration, and not the cells per se, suggesting that MSCs may require some time to secrete these factors after their administration.

Introduction

Ideal healing of a skin wound requires an integration of complex biological and molecular events of cell migration and proliferation, extracellular matrix deposition, angiogenesis, and remodeling [1]. Impairment of any of such processes would lead to wound chronicity. Despite having numerous causes, wound chronicity is primarily associated with diabetes, atherosclerosis, venous/pressure ulcers, vasculitis, and trauma [2]. Given the increasing prevalence of chronic wounds worldwide, and their marked outcomes on patient morbidity and mortality (let alone amputations), it is crucial to consider adequate and effective interventions to treat such debilitating wounds [3].

Multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), are an outstanding tool for cell therapy applications, not only because of their multipotent nature, but also due to their ability to home and engraft into damaged tissues [4, 5], release trophic factors [6], promote neovascularization [7], manage oxidative stress [8] and trigger an anti-inflammatory response [9]. MSCs are procured from live donors [10] and can be both efficiently expanded \textit{ex vivo} [11], and transplanted without previous conditioning of the patients as opposed to total bone marrow or hematopoietic stem cell transplantation [12].

Several pre-clinical and clinical studies have previously reported that autologous or allogeneic MSCs from different sources are safe and therapeutic in the treatment of chronic wounds [13], diabetic foot ulcers [14], pressure ulcers [15], burn injuries [16, 17], surgical wounds [18, 19], limb ischemia [20], and radiation burns [21]. These studies indicate that wounds treated with MSCs show a qualitative improvement in histological characteristics. Specifically, they report superior rete ridge architecture, multi-layered structure, improved dermal-epidermal junction and the formation of new skin appendage structures, such as hair follicles and sebaceous glands [22–26].

Even though the mechanisms by which MSCs ameliorate skin damage have been a subject of debate for years, two theories may currently explain MSC therapeutic effects: bioactive soluble factor production (growth factors, cytokines and specific proteins) or MSC differentiation into dermal and epidermal cells [27, 28]. Nevertheless, it is still controversial whether MSCs can contribute significantly to the regeneration of injured skin via tissue specific differentiation. For instance, Wu \textit{et al.} used an excisional wound model in normal and diabetic mice to prove that only a small percentage of the MSCs that migrate to injury sites in response to chemotactic signals become incorporated and survive within wounded tissue [29]. Likewise, other studies have revealed that transplanted MSCs do not need to be close to the damaged site to promote wound repair and functional recovery [30].

Considering these previous findings, we compare the beneficial effects of mouse bone marrow-derived allogenic MSCs (allo-mBM-MSCs) and their acellular derivatives (allo-acd-mMSCs) to cutaneous wound healing in a full thickness excisional wound model in diabetic mice [31]. Allo-mBM-MSCs or their allo-acd-mMSCs were injected into Non-Obese Diabetic (NOD) mice. Wound healing was assessed macroscopically every two days in terms of wound closure, and microscopically after 16 days of the injection (reepithelialization, dermoepidermal
junction, skin appendage regeneration, leukocyte infiltration, vascularization, granulation tissue formation, and density of collagen fibers in the dermis). The results revealed that although both treatments promoted tissue repair/regeneration, the allo-acd-mMSCs were the most effective tool for accelerating wound closure and the recovery of the damaged tissue. Based on ELISA analyses, the presence of relevant paracrine factors in allo-acd-mMSCs may play a significant role in the modulation of the wound healing process.

Materials and methods

Animals

C57BL/6 and NOD mice (Jackson Laboratory, Bar Harbor, ME) were used in the experiments. They were kept at constant temperature and humidity, with a 12:12 hour light-dark cycle and unrestricted access to standard diet and water. The animals were anesthetized with 1.1 mg/kg of 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich) or sevofluorane (Abbott, Japan) when required. All animal procedures were approved by the Ethics Committee of the School of Medicine Clínica Alemana—Universidad del Desarrollo School of Medicine (approval ID:2011–14).

Isolation and ex vivo expansion of allo-mBM-MSCs

Allo-mBM-MSCs were collected by flushing the femurs and tibias of 8-week-old C57BL/6 male mice with Alpha-MEM medium (Gibco, Auckland, NZ) followed by centrifugation. Cells were plated at a density of $1 \times 10^6$ nucleated cells/cm$^2$ and cultured in Alpha-MEM supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan Utha) and 80 μg/mL gentamicin (Sanderson Laboratory, Chile). Cultures were maintained at 37˚C in 5% CO2 and 95% air atmospheric condition. After 72 hours, non-adherent cells were removed and fresh medium was added to the cells.

Adherent cells were detached after 96 hours using 0.25% trypsin and 2.65 mM EDTA (Life Technologies, Grand Island, NY), then centrifugated and sub-cultured at 7,000 cells/cm$^2$. Allo-mBM-MSCs were injected after one subculture.

Allo-acd-mMSCs preparation

The allo-acd-mMSCs were produced using allo-mBM-MSC cultures at 80% confluence (passage 1) in 10 cm tissue culture dishes ($1 \times 10^6$ cells approximately). Allo-mBM-MSCs were pre-washed twice with serum-free Alpha-MEM medium, maintained using 3 mL/dish of this medium and incubated for 24 hours under normoxia condition (37˚C in a humidified atmosphere containing 95% air and 5% CO2). The medium was collected and centrifuged at 1500 rpm for 5 min. The supernatant was re-centrifuged at 3000 rpm for 3 min followed by collection of the second supernatant, named allo-acd-mMSCs to remove all cell debris. The pH was confirmed using an electric pH meter. Total allo-acd-mMSCs were collected, filtered, mixed, and aliquoted in 500 μL for storage at −20˚C.

Animal diabetic model: Induction and evaluation of diabetes

Diabetes was induced by a single intraperitoneal (i.p) injection of streptozotocin (STZ 200 mg/kg; CALBIOCHEM, San Diego, CA). For its activation, the STZ was prepared in citrate buffer solution (pH 4.5) immediately before its application. STZ was administered during the fasting state to pre-diabetic (8–10 week old) female NOD mice (body weight of 19–23 g). Hyperglycaemia usually occurred 2 days after STZ injection, and was verified using blood collected from the tail vein with a portable glucose meter (Accu-Check Go; Roche, Nutley, NJ, USA). Mice with blood sugar levels greater than or equal to 250 mg/dL after two consecutive
measurements were considered diabetic. Once hyperglycaemia was confirmed, diabetic mice were randomly assigned to receive one of the following treatment options: allo-mBM-MSCs, allo acd-mMSCs or vehicle (saline solution with 5% autologous plasma). During the study, glucose levels were measured every four days.

**Excisional skin wound model**

One week after administration of STZ, 8–10 week old female NOD mice were anesthetized with avertin and the hair was removed from the dorsal surface. Excisional biopsy wounds were made using a 6 mm punch on one side of the midline extending through the panniculus carnosus as previously described by Galiano et al. [31]. The number of mice used for each condition was: 25, 27 and 25 for the allo-mBM-MSC, the allo-acd-mMSC and the vehicle treatment, respectively.

**MSC and acellular derivative injection**

Allo-mBM-MSCs derived from 8-week-old C57BL/6 male mice (1x10^6 cells in 60 μL of vehicle) were injected intradermally in the periphery of the wound at four sites. Similarly, one dose (1X) of allo-acd-mMSCs (100 μL) or vehicle (60 μL) was intradermally injected around the wound. After the injections, tegaderm (3M, London, ON, Canada) was immediately placed over the wounds. Animals were housed individually during the experiment.

**Evaluation of the wound healing process**

Wound size was measured in duplicate using a digital caliper (Mitutoyo Sul Americano LTDA, Brazil) every two days after the allo-mBM-MSC or allo-acd-mMSC administration to compute the wound areas. Also, digital photographs of the wounds were taken (FUJIFILM-Finepix HS20 EXR). Time elased to wound closure was defined as the time in which the wound bed became completely reepithelialized and filled with new tissue. The percentage of wound closure was calculated using the formula [(area of original wound – area of actual wound)/(area of original wound)] x 100. All wounds were closed after 16 days and the mice were sacrificed by an overdose of anesthesia (ketamine 50 mg/kg – xylazine 5 mg/kg, Centrovet). Skin samples from the affected area and 2 mm of the surrounding skin were obtained using an 8 mm biopsy punch.

**Histological analyses**

Harvested skin samples from euthanized mice were fixed in 10% formalin, and embedded in paraffin. Four μm thick skin sections were cut using a Leica RM 2125 RTS microtome (Wetzlar, Germany) and stained with haematoxylin-eosin (Sigma-Aldrich) and Masson’s trichrome (Diagnostic Biosystems, Pleasanton, CA). Computational analysis was performed to quantitatively assess dermo-epidermal junction integrity. In brief, the border of the region with incomplete junction was manually selected using the open access software ImageJ, which calculated the traced area in pixels. Depending on this area, a score was assigned: 0: >2,801 pixels (corresponding to injunction); I: 1,201–2,800 pixels; II: 751–1,200 pixels; III: 401–750 pixels; and IV: 0.1–400 pixels (corresponding to complete junction) (S1 Fig).

A qualified pathologist standardized valid histological criteria in regular haematoxylin-eosin staining to estimate the degree of leukocyte infiltration (unquantifiable subjective parameters). For these purposes, an estimation of the area occupied by inflammatory cells (lymphocytes, plasma cells, eosinophils, macrophages, neutrophils, etc.) was determined in the superficial or mild/deep dermis. The following scale was used: absent (no apparent...
inflammatory response); mild (< 10% of the area covered by inflammatory cells); moderate (10 to 50% of the area covered by inflammatory cells) and severe (> 50% of the area covered by inflammatory cells).

In addition, a classification of granulation tissue stage (young or advanced) was assessed based on the degree of the fibroblastic response, vascular proliferation, correlation with the inflammatory response, and gross morphological evaluation of collagen fibers.

Moreover, appendage-like structures in each wound section were studied using the Masson’s trichrome staining. Also, morphometric computational analysis of dermal collagen was conducted with Masson’s trichrome staining to provide quantitative analysis of the density and the intensity of dermal collagen fibers according to the method described by Miot and Brianzi [32].

On the other hand, keratinocytes were stained with a monoclonal antibody against epidermal keratin subunits (Abcam Inc, Cambridge, MA) and visualized with a secondary antibody Alexa Fluor 488 (Abcam Inc, Cambridge, MA, USA). Similarly, vascularization was identified with polyclonal Von Willebrand Factor antibody (Dako, Denmark), followed by incubation with secondary antibody Alexa Fluor 488 (Cell Signaling, Massachusetts, USA). Nuclear staining was performed with DAPI (AppliChem, Germany). All slides were examined under a Leica DM2000 microscope (Wetzlar, Germany), and images were captured with a Leica DFC 295 camera (Wetzlar, Germany).

**ELISA assays**

The allo-acd-mMSCs samples were evaluated for their content of specific growth factors and proteins relevant to wound healing via ELISAs. Specifically, the following factors were measured per manufacturer’s instructions: collagen type 1 (CoL-1), keratinocyte growth factor (KGF), matrix metalloproteinase 1 (MPP-1), matrix metalloproteinase 3 (MPP-3), angiopoietin 1 (Ang-1), angiopoietin 2 (Ang-2), human insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE₂) and epidermal growth factor (EGF) (kits from R&D Systems, Abcam and MyBioSource).

**MSC Characterization**

**Colony forming unit (CFU) assay.** Allo-mBM-MSCs cells were suspended in alpha-MEM supplemented and plated at 17,000 nucleated cells/cm² density in triplicate. Non-adherent cells were removed with fresh medium, which was changed twice a week. Cells were stained with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO) in 10% methanol for 20 minutes on day 21. The colonies so formed were counted after four washes and the results were expressed as CFU/million nucleated cells plated (S2A Fig).

**Proliferation assay.** First passaged allo-mBM-MSCs were sub-cultured at 4,000 cells/cm²; the medium was changed every three days. The amount of cells was determined on days 0, 3, 6, 9 and 12 after staining with 0.5% crystal violet in 10% methanol for 20 minutes. Cell-incorporated crystal violet was solubilized after four washes by incubation with phosphate buffer in methanol (50:50) and spectrophotometrically quantified (570 nm absorbance) (S2B Fig).

**MSC differentiation assay.** Allo-mBM-MSCs were cultured either in osteogenic (0.1 μM dexamethasone, 10 mM β- glycerophosphate, 50 μg/ml ascorbic acid [33]) or adipogenic (1μM dexamethasone, 100 μg/ml 3-isobutyl- 1-methylxanthine (IBMX) medium [34]) medium for 21 days, respectively (S2C Fig).

**Immunophenotyping.** Although there is no current consensus with respect to murine MSCs markers (versus those for human MSCs), immunophenotyping was performed by flow cytometry analysis. In brief, cells were incubated with anti-CD45.2 clone 104 (APC-eFluor780
conjugated, eBioscience), anti-CD11b, clone M1/70 (PE-Cy-conjugated, eBioscience), anti-Sca-1, clone D7 (PE-conjugated, eBioscience), anti-CD90.2 clone 53–2.1 (PE-Cy7-conjugated, BD Pharmingen™) and anti-ASMA, clone 1A4 (FITC-conjugated, Sigma) (S2D–S2F Fig).

Statistical analysis

Stat Graph Prism 5.0 software was used for statistical analysis. Data are reported as the mean ± standard error of the mean. Comparison of experimental groups was performed using ANOVA followed by Dunn’s multiple test, $p < 0.05$. The association or independence of categorical variables were compared using Pearson’s chi–square test, a $p < 0.0001$ values was accepted as statistically significant.

Results

Wound closure kinetics in diabetic mice

In order to determine whether the paracrine effect of allo-acd-mMSCs was sufficient in the wound healing process, allo-acd-mMSCs and allo-mBM-MSCs were injected into excisional wounds in NOD mice. As shown in Fig 1A, wound closure started to be noticed after 4 days for the allo-acd-mMSCs treated mice, and became more evident after 8 days compared to the allo-mBM-MSCs and vehicle counterparts (4 vs 6 days, respectively). In addition, wound closure percentage was significantly higher in the allo-acd-mMSC treated mice, relative to the other two groups (Fig 1B). Specifically, mice treated with allo-acd-mMSCs reached significantly higher percentages of wound closure at days 4, 6 and 8, relative to the allo-mBM-MSCs and vehicle groups ($p < 0.05$), while wound closure percentages between the allo-mBM-MSCs and vehicle samples could not be statistically distinguished. Moreover, the data suggested that 50% of wound closure was reached approximately after 5 days for the allo-acd-mMSCs treated mice, whereas for the other two conditions the same extent of wound closure was achieved after approximately 7 days of treatment. Cumulatively, the results revealed that allo-acd-mMSCs were the most effective tool for wound closure.

Recovery of regenerated skin in diabetic mice

In order to determine the quality of the newly-formed skin, reconstitution of the dermo-epidermal junction, new skin appendage structure and reepithelialization at day 16 were evaluated. Significant differences in the complete reconstitution of the dermo-epidermal junction were identified in mice treated with allo-mBM-MSCs and allo-acd-mMSCs (score IV) compared to animals treated with vehicle ($p < 0.001$) (Fig 2A and 2B). Likewise, new skin appendage structure (hair follicles or sebaceous glands) were detected in the wounds treated with allo-acd-mMSCs and allo-mBM-MSCs compared to wounds treated with vehicle ($p < 0.001$) (Fig 2C and 2D).

Reepithelialization were observed between cutaneous lesions treated with the vehicle and the ones which received either allo-acd-mMSCs or allo-mBM-MSCs, the wounds showed the formation of normal layers of the epithelium as demonstrated by the expression of pancytokeratin, an epithelial tissue marker (S3A Fig).

Skin healing process in diabetic mice

Sixteen days after the start of the treatment, the influence of allo-acd-mMSCs on wound healing was evaluated by estimating: i) the magnitude of inflammatory infiltrates, ii) granulation tissue formation (including its stages), iii) the density of the collagen fibers in the cutaneous lesion and iv) vascularization. As shown in Fig 3A, more severe leucocyte infiltration was
found in the dermal connective tissue of the allo-mBM-MSC and vehicle treated groups compared to the group treated with allo-acd-mMSCs (p < 0.001).

Comparison of the extent of granulation tissue formation in the dermal connective tissue indicated higher granulation tissue in advanced stage in most of the animals treated with allo-acd-mMSCs relative to the animals treated with allo-mBM-MSCs (p < 0.001) (Fig 3B). Moreover, young-stage granulation tissue formation was characterized by distinct histopathological changes such as an acute inflammatory process with mild to moderate interstitial edema, the presence of collagen fiber deposits, and the fibroblastic response. On the contrary, granulation tissue formed at an advanced stage was identified by a scarce inflammatory infiltrate, less
Fig 2. Histological analysis of dermo-epidermal junction and appendage-like structure formation of skin wounds 16 days after treatment with allo-mBM-MSCs and allo-acd-mMSCs in NOD mice. (A) Wound histological representative images of haematoxylin-eosin staining showing dermo-epidermal junction, which are indicated by arrows. Scale bar = 100 μm. (B) Frequency of animals with the histological scores for dermo-epidermal junction. The score was assigned based on the junction level and was quantified using ImageJ (scores: IV: 0.1–400 pixels (complete junction), III: 401–750 pixels, II: 751–1,200 pixels, I: 1,201–2,800 pixels and 0: > 2,801 (incomplete junction). (C) Representative images of Masson’s trichrome staining illustrating appendage-like structure in the dermis indicated by arrows. Scale bar = 50 μm. (D) Frequency of animals with the score for appendage-like structure. (n = 25, 25 and 27 for vehicle, allo-mBM-MSCs and allo-acd-mMSCs groups, respectively). Analysis of Pearson’s chi-square test was used. * indicates a significant difference (p < 0.0001). Abbreviations: NOD, Non-Obese Diabetic; allo-mBM-MSCs, mouse bone marrow-derived allogeneic MSCs; allo-acd-mMSCs and mouse bone marrow acellular derivatives allogeneic MSCs.

https://doi.org/10.1371/journal.pone.0177533.g002
pronounced fibroblastic reaction, high presence of dense collagen fiber deposition and emerging hyalinization, which was sometimes admixed with hemosiderin-laden macrophages.

In the same way, a higher density and intensity of collagen fibers were found in the allo-acd-mMSCs-treated group (mean 242) compared to the allo-mBM-MSC (mean 204) and vehicle (mean 208) groups ($p < 0.05$, Fig 3C). These data suggested that a more intense remodeling process takes place in the allo-acd-mMSCs samples, compared to its allo-mBM-MSCs and

Fig 3. Histological analysis of leucocyte infiltration, granulation tissue and collagen fibers of skin wounds 16 days after treatment with allo-mBM-MSCs and allo-acd-mMSCs in NOD mice. Frequency of animals with the histological scores for: (A) leucocyte infiltration in the dermis, (B) granulation tissue formation stage and (C) quantitative analysis of the intensity of dermal collagen fibers. ($n = 25, 25$ and 27 for vehicle, allo-mBM-MSCs and allo-acd-mMSCs groups, respectively). Pearson’s chi-square test and variance (ANOVA) analysis were used, * indicates a significant difference $p < 0.0001$ and ** $p < 0.05$. Abbreviations: NOD, Non-Obese Diabetic; allo-mBM-MSCs, mouse bone marrow-derived allogeneic MSCs and allo-acd-mMSCs, mouse bone marrow acellular derivatives allogeneic MSCs.

https://doi.org/10.1371/journal.pone.0177533.g003
vehicle counterparts, in which the collagen fibers were less dense and organized. In addition, allo-acd-mMSCs showed greater vascular proliferation compared to allo-mBM-MSCs or vehicle treated wounds (S3B Fig).

Profile of paracrine factors found in allo-acd-mMSCs

Paracrine factors secreted by MSCs play a crucial role in wound healing [25, 35–38]. Therefore, the levels of growth factors and specific proteins relevant to wound healing were quantified to gain insight into the relationship between paracrine factor concentration and the observed response in terms of wound closure kinetics, characteristics of the regenerated skin and the healing process. As illustrated in Fig 4, the growth factors and proteins with the highest concentration in the allo-acd-mMSCs were CoL-1, KGF, MPP-1 and Ang-2. In contrast, IGF-1, HGF, PGE2 and VEGF were present in lower concentrations. Finally, EGF, Ang-1 and MPP-3 were not detected in the allo-acd-mMSCs.

Discussion

The present study was designed to examine the impact of allo-acd-mMSCs relative to allo-mBM-MSCs on diabetic wound healing toward the application of novel therapeutic approach for this condition. The results demonstrated that allo-acd-mMSCs possess more robust therapeutic properties than allo-mBM-MSCs per se for wound healing in diabetic mice. Particularly, the current work revealed that injection of allo-acd-mMSCs accelerated wound closure, resulting in improved healing, relative to allo-mBM-MSC injection. These results are consistent with previous studies that suggest that paracrine factors play a key role in MSC contribution to skin wound healing [22, 25, 35–41]. Nevertheless, our findings are in contrast to the results obtained by Wu et al., who demonstrated that the beneficial effect of MSCs on wound healing in nondiabetic and diabetic mice is primarily achieved through cell differentiation, proposing a direct contribution of MSCs to cutaneous regeneration [29]. Despite this fact, the literature evidences that the contribution of MSC differentiation to wound healing is limited because of poor engraftment and survival of MSCs at the injured site [38, 42].
Significant improvement regarding the functional and histological quality of healed skin, such as superior rete ridge architecture, multilayered structure and major dermoepidermal junction, was detected in a greater degree in the groups treated with allo-mBM-MSCs and allo-acd-mMSCs, relative to the control group (vehicle). These results are consistent with other studies that have demonstrated that wound reepithelialization is the main mechanism contributing to wound closure in the presence of BM-MSCs or BM-progenitors cells [43, 44]. In addition, allo-mBM-MSCs or their allo-acd-mMSCs stimulated the formation of new skin appendage structure, for instance, hair follicles or sebaceous glands, characteristics that are considered signs of skin regeneration. This potential benefit has also been reported by other authors who used BM-MSCs, human umbilical cord derived MSCs [45], or products derived of these cells such as exosomes [46] or conditioned medium [29].

Historically, MSCs have been considered hypoimmunogenic because they exhibit low expression levels of MHC class I markers, no expression of MHC class II markers, and no expression of costimulatory molecules, which allow them to avoid immunosurveillance [5, 47, 48]. In addition, it is known that MSCs have an immunoregulatory effect given by different putative mechanisms including immune cell interaction, production of soluble factors and promoting of T-reg generation [49]. However, in the present work, large severe leukocyte infiltration in wounds that received allo-mBM-MSCs was found, relative to the allo-acd-MSCs group, after 16 days of treatment. These results are in agreement with some studies that have previously reported concerns related to the immunogenicity of transplanted allogeneic MSCs [5, 48, 50]. MSCs have been correlated to not be intrinsically immunoprivileged, as assumed earlier [51]. Specifically, some reports indicated that allogenic MSCs can up-regulate the expression of MHC II and costimulatory molecules under certain conditions such as in the presence of inflammatory milieu through the IFN-γ stimulation [5]. MSCs can be lysed by activated NK cells by the interaction of NK receptors (NKp30, NHG2D and DNAM-1) with the MSCs ligands [5, 52], induce memory T cells [53] and trigger the formation of IgG antibodies after subcutaneous or intracardial injection, which results in the rejection of allogeneic MSC grafts [54]. On the other hand, other studies have shown that the presence of soluble factors derived of MSCs such as TGF-β, HGF and PGE2, are capable of suppressing T-cell responses in vitro [5]. In this context, the last two factors were found in allo-acd-MSCs, suggesting that the absence of severe leukocyte infiltration in wounds that received allo-acd-MSCs, could be due to soluble factors of this type.

The formation of new blood vessels (neovascularization) is considered one of the early essential processes in wound healing to sustain both the newly formed granulation tissue and the survival of keratinocytes and re-epithelialization [44, 55]. Based on macroscopic evaluation, the extent of granulation tissue formation in the bed of the wounds at day 4 was higher in the allo-mBM-MSCs and allo-acd-mMSCs groups compared to the vehicle (S4 Fig).

Likewise, from the histological assessments, the experimental group treated with allo-acd-mMSCs showed greater vascular proliferation and granulation tissue in advanced stage relative to the groups treated with allo-mBM-MSCs or vehicle at day 16. For the latter groups, the granulation tissue was in a young phase with abundant proliferation fibroblastic type. This observation is consistent with one of the reported therapeutic functions of MSCs in wound healing where the early induction of granulation tissue followed by neovascular network formation leads to a fast healing process [55].

Another aspect to take into account about the quality of healed skin is the restoration of its dermal mechanical properties, which largely depend on the specific arrangement of collagen fibers [40]. In this context, non-healing wounds are associated with atypical matrix deposition that may be related to the overproduction or insufficient presence of growth factors [56]. The results of the present work demonstrated that wounds receiving allo-mBM-MSCs or vehicle
had reduced density and organization of collagenous fibers at the dermal matrix compared to allo-acd-mMSC treated wounds. In particular, collagen fibers in the allo-mBM-MSC and vehicle treated wounds still appeared relatively thin and were parallel-oriented to the skin, which indicated improper wound maturation. On the contrary, allo-acd-mMSC treated wounds had thicker collagen fibers, distributed in a network-like manner mimicking normal skin.

Furthermore, the success of the wound healing process depends on a regulated secretion of growth factors, cytokines and chemokines that are involved in a complex integration of signals that coordinate cellular processes [57]. Non-healing wounds have been associated with the overproduction (acute wound) or insufficient presence (chronic wounds) of growth factors such as EGF, IGF-1, FGF-2, PDGF-BB, VEGF, Ang-1, SDF-1, KGF, MMP-9, or cytokines such as TGF-β, IL-1, IL-6, IL-8, and TNF-alpha [22, 56, 58]. These molecules could contribute towards wound repair and skin regeneration by suppressing inflammation, angiogenesis and stimulating skin stem cell proliferation and differentiation into new keratinocytes [23, 29, 55, 59–61]. We examined the presence of several of these growth factors in the allo-acd-mMSCs, and found a set of key bioactive molecules implicated in the wound healing process, namely, IGF-1, KGF, HGF, VEGF, Ang-2, Col-1, MMP-1 and PGE2. The pro-angiogenic growth factors IGF-1, Ang-2 had the highest concentration in the allo-acd-MSCs compared to VEGF. According to the literature, these factors have been reported to possibly enhance endothelial cell proliferation and neovascularization, a critical stage in tissue regeneration [62, 63]. In the same way, Col-1, which is found in the extracellular matrix, has been reported as crucial element in the restoration of the dermal mechanical properties and in a better remodeling of dermal fibrous architecture [40]. Our ELISA results showed high levels of this protein in the allo-acd-mMSCs.

Conclusions
Cumulatively, our study demonstrated that administration of allo-acd-mMSCs is more effective in wound healing and cutaneous regeneration than allo-mBM-MSCs or vehicle in diabetic mice. The results revealed that allo-acd-mMSCs accelerated the kinetics of wound closure, reduced severe leukocyte infiltration, increased granulation tissue formation, and remodelled the collagen deposition orientation. Also, our data suggested that the presence of important bioactive molecules in the allo-acd-mMSCs could have initiated the wound healing mechanism and facilitated the host response to tissue repair. Allo-acd-MSCs might serve as a novel therapeutic approach to treat chronic wounds caused by pathological conditions or other trauma.

Supporting information
S1 Fig. Histological scores of dermo-epidermal junction integrity. Representative images of haematoxylin-eosin staining of the dermo-epidermal junction integrity. Image J was used for estimating unbound area size. Scores: IV: 0.1–400 pixels (complete junction), III: 401–750 pixels, II: 751–1,200 pixels, I: 1,201–2,800 pixels and 0: > 2,801 (incomplete junction). Scale bar = 100 μm and applied to all images. (TIF)

S2 Fig. Characterization of isolated mBM-MSCs from C57BL/6 mice. (A) Bone marrow abundance was determined by CFU assay. Data are mean ± s.e.m (n = 6). (B) Proliferation kinetics was evaluated by crystal violet staining (570 nm absorbance) over a period of 12 days. Data are mean ± s.e.m (n = 4). (C) mBM-MSCs differentiate to mesodermal lineages in vitro. Passage 2 mBM-MSCs were used for potential differentiation. Adipogenic differentiation: cells
were cultured in adipogenic differentiation medium for 21 days. Oil Red O staining was performed to detect lipid accumulation. Osteogenic differentiation: cells were cultured in osteogenic differentiation medium for 21 days. Alizarin Red staining was performed to detect calcium accumulation. Scale bar 100 μm. (D) Flow cytometry analysis of mBM-MSC surface markers. mBM-MSCs expressed anti-CD90.2, anti-Sca-1, and anti-ASMA but not anti-CD45.2 and anti-CD11b. Representative images for 2 animals per group. (E) Statistical data of flow cytometry. Abbreviations: mBM-MSCs, mouse bone marrow-derived MSC and CFU, colony formation unit.

**S3 Fig. Effects of allo-mBM-MSCs and allo-acd-MSCs on reepithelialization and wound vascularity.** Representative images of cutaneous wound sections on day 16 in NOD mice treated with vehicle, allo-mBM-MSCs and allo-acd-mMSCs were immunostained with (A) an anti-pan-cytokeratin antibody (green) and (B) an anti-von Willebrand Factor (vWF) positive blood vessels (green), both primary antibodies were probed with Alexa Fluor488 secondary antibody and nuclei (blue) were counterstained with 4-6-diamidino-2-phenylindole (DAPI). Representative results of 4 animals per experimental groups. Scale bar 50 μm. Abbreviations: NOD, Non-Obese Diabetic; allo-mBM-MSCs, mouse bone marrow-derived allogeneic MSCs; allo-acd-mMSCs, mouse bone marrow acellular derivatives allogeneic MSCs; vWF, von Willebrand Factor; DAPI 4-6-diamidino-2-phenylindole.

**S4 Fig. Effects of allo-mBM-MSCs and allo-acd-MSCs on early granulation tissue formation on wound in NOD mice.** Invasion of granulation tissue is shown from the wound margins into the wound bed after 4 days of treatment. Allo-mBM-MSCs and allo-acd-mMSCs groups showed higher presence of tissue with the following features: wet, reddish, soft and granular in gross appearance compared to the vehicle group (only sparse granulation tissue was observed). Scale bar = 50 μm (applied to all images). Abbreviations: allo-mBM-MSCs, mouse bone marrow-derived allogeneic; acelullar derivatives, allo-acd-mMSCs.

**Acknowledgments**
The authors would like to thank Dr. Viviana Guiza-Arguello for English editing of the paper.

**Author Contributions**

**Conceptualization:** MLA-R.

**Data curation:** MLA-R TdM SB-B.

**Formal analysis:** MLA-R TdM SB-B.

**Funding acquisition:** MLA-R PC.

**Investigation:** MLA-R TdM SB-B.

**Methodology:** MLA-R PC.

**Project administration:** MLA-R.

**Resources:** MLA-R PC SB-B CLS VG.

**Software:** MLA-R TdM SB-B.
Supervision: MLA-R PC.
Validation: MLA-R TdM SB-B.
Visualization: MLA-R.
Writing – original draft: MLA-R.
Writing – review & editing: MLA-R TdM PC SB-B CLS VG.

References
1. Singer AJ, Clark RA. Cutaneous wound healing. The New England journal of medicine. 1999; 341 (10):738–46. https://doi.org/10.1056/NEJM199909023411006 PMID: 10471461.
2. Morton LM, Phillips TJ. Wound healing and treating wounds: Differential diagnosis and evaluation of chronic wounds. Journal of the American Academy of Dermatology. 2016; 74(4):589–605. https://doi.org/10.1016/j.jaad.2015.08.068 PMID: 26979352.
3. Fan K, Tang J, Escandon J, Kirnser RS. State of the art in topical wound-healing products. Plastic and reconstructive surgery. 2011; 127 Suppl 1:44S–59S. https://doi.org/10.1097/PRS.0b013e3181f6275 PMID: 21200273.
4. Ezquer M, Ezquer F, Ricca M, Allers C, Conget P. Intravenous administration of multipotent stromal cells prevents the onset of non-alcoholic steatohepatitis in obese mice with metabolic syndrome. Journal of hepatology. 2011; 55(5):1112–20. https://doi.org/10.1016/j.jhep.2011.02.020 PMID: 21356258.
5. Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. Diabetes. 2008; 57(7):1759–67. https://doi.org/10.2337/db08-0180 PMID: 18586907.
6. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. Journal of cellular biochemistry. 2006; 98(5):1076–84. https://doi.org/10.1002/jcb.20886 PMID: 16619257.
7. Ball SG, Shuttleworth CA, Kiely CM. Mesenchymal stem cells and neovascularization: role of platelet-derived growth factor receptors. Journal of cellular and molecular medicine. 2007; 11(5):1012–30. https://doi.org/10.1111/j.1582-4934.2007.00120.x PMID: 17979880.
8. Valle-Prieto A, Conget PA. Human mesenchymal stem cells efficiently manage oxidative stress. Stem cells and development. 2010; 19(12):1885–93. https://doi.org/10.1089/scd.2010.0093 PMID: 20380515.
9. Uccelli A, Prockop DJ. Why should mesenchymal stem cells (MSCs) cure autoimmune diseases? Curr Opin Immunol. 2010; 22(6):768–74. https://doi.org/10.1016/j.coi.2010.10.012 PMID: 21093239.
10. Hoogduijn MJ, Betjes MG, Baan CC. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? Current opinion in organ transplantation. 2014; 19(1):41–6. Epub 2013/11/28. https://doi.org/10.1097/MOT.0000000000000036 PMID: 24275893.
11. Minguell JJ, Ercies A, Conget P. Mesenchymal stem cells. Experimental biology and medicine. 2001; 226(6):507–20. Epub 2001/06/09. PMID: 11395921.
12. Rasmusson I. Immune modulation by mesenchymal stem cells. Experimental cell research. 2006; 312 (12):2169–79. https://doi.org/10.1016/j.yexcr.2006.03.019 PMID: 16631737.
13. Falanga V, Iwamoto S, Chartier M, Yuft T, Butmarc J, Kouttab N, et al. Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue engineering. 2007; 13(6):1299–312. https://doi.org/10.1089/tten.2006.0276 PMID: 17518741.
14. Voštassak, J, Danisovic L, Kubes M, Bakos D, Jarabeck L, Ulicna M, et al. Autologous biograft and mesenchymal stem cells in treatment of the diabetic foot. Neuroendocrinology letters. 2006; 27 Suppl 2:134–7. PMID: 17159798.
15. de la Garza-Rodea AS, Knaan-Shanzer S, van Bekkum DW. Pressure ulcers: description of a new model and use of mesenchymal stem cells for repair. Dermatology. 2011; 223(3):266–84. https://doi.org/10.1159/00034628 PMID: 22116308.
16. Gardien KL, Middelkoop E, Ulrich MM. Progress towards cell-based burn wound treatments. Regenerative medicine. 2014; 9(2):201–18. https://doi.org/10.2217/rme.13.97 PMID: 24750061.
17. Liu L, Yu Y, Hou Y, Chai J, Duan H, Chu W, et al. Human umbilical cord mesenchymal stem cells transplantation promotes cutaneous wound healing of severe burned rats. PLoS one. 2014; 9(2):e885348. https://doi.org/10.1371/journal.pone.00885348 PMID: 24586314.
18. Stoff A, Rivera AA, Sanjib Banerjee N, Moore ST, Michael Numnum T, Espinosa-de-Los-Monteros A, et al. Promotion of incisional wound repair by human mesenchymal stem cell transplantation.
19. Chen L, Tredget EE, Liu C, Wu Y. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. PloS one. 2009; 4(9):e7119. https://doi.org/10.1371/journal.pone.0007119 PMID: 19771171.

20. Bura A, Planat-Beard V, Bourin P, Silvestre JS, Gross F, Grolleau JL, et al. Phase I trial: the use of autologous cultured adipose-derived stroma/stem cells to treat patients with non-revascularizable critical limb ischemia. Cytotherapy. 2014; 16(2):245–57. https://doi.org/10.1016/j.jcyt.2013.11.011 PMID: 24438903.

21. Bey E, Prat M, Duhamel P, Benderitter M, Brachet M, Trompier F, et al. Emerging therapy for improving wound repair of severe radiation burns using local bone marrow-derived stem cell administrations. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2010; 18(1):50–8. https://doi.org/10.1111/j.1524-475X.2009.00562.x PMID: 20082681.

22. Kim SW, Zhang HZ, Guo L, Kim JM, Kim MH. Amniotic mesenchymal stem cells enhance wound healing in diabetic NOD/SCID mice through high angiogenic and engraftment capabilities. PloS one. 2012; 7(7):e41105. https://doi.org/10.1371/journal.pone.0041105 PMID: 22818301.

23. Kong P, Xie X, Li F, Liu Y, Lu Y. Placenta mesenchymal stem cell accelerates wound healing by enhancing angiogenesis in diabetic Goto-Kakizaki (GK) rats. Biochemical and biophysical research communications. 2013; 436(2):410–9. https://doi.org/10.1016/j.bbrc.2013.07.086 PMID: 23995518.

24. Kuo YR, Wang CT, Cheng JT, Wang FS, Chiang YC, Wang CJ. Bone marrow-derived mesenchymal stem cells enhanced diabetic wound healing through recruitment of tissue regeneration in a rat model of streptozotocin-induced diabetes. Plastic and reconstructive surgery. 2011; 128(4):872–80. https://doi.org/10.1097/PRS.0b013e3182174329 PMID: 21921763.

25. Shrestha C, Zhao L, Chen K, He H, Mo Z. Enhanced healing of diabetic wounds by subcutaneous administration of human umbilical cord derived stem cells and their conditioned media. International journal of endocrinology. 2013; 2013:922454. https://doi.org/10.1155/2013/922454 PMID: 24089612.

26. Bruna F, Contador D, Conget P, Erranz B, Sossa CL, Arango-Rodriguez ML. Regenerative Potential of Mesenchymal Stromal Cells: Age-Related Changes. Stem Cells Int. 2016; 2016:1461648. https://doi.org/10.1155/2016/1461648 PMID: 27247575.

27. Zhang S, Wu Q, van Velthoven MH, Chen L, Car J, Rudan I, et al. Smartphone versus pen-and-paper data collection of infant feeding practices in rural China. Journal of medical Internet research. 2012; 14(5):e119. Epub 2012/09/20. https://doi.org/10.2196/jmir.2183 PMID: 22989894.

28. Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. Journal of immunology. 2008; 180(4):2581–7. PMID: 18250469.

29. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem cells. 2007; 25(10):2648–59. https://doi.org/10.1634/stemcells.2007-0226 PMID: 17615284.

30. Basioyuny HS, Salama NM, Maadawi ZM, Farag EA. Effect of bone marrow derived mesenchymal stem cells on healing of induced full-thickness skin wounds in albino rat. International journal of stem cells. 2013; 6(1):12–25. PMID: 24298370.

31. Galliano RD, Michaels JT, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2004; 12(4):485–92. https://doi.org/10.1111/j.1067-1927.2004.12404.x PMID: 15260814.

32. Miot HA, Brianzoni M. Morphometric analysis of dermal collagen by color clusters segmentation. Anais brasileiros de dermatologia. 2010; 85(3):361–4. PMID: 20676470.

33. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. Journal of cellular biochemistry. 1997; 64(2):278–94. PMID: 9027588.

34. Nuttal ME, Patton AJ, Olivera DL, Nadeau DP, Gowen M. Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteopenic disorders. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. 1998; 13(3):371–82. https://doi.org/10.1359/jbrm.1998.13.3.371 PMID: 9525337.

35. Chen L, Xu Y, Zhao J, Zhang Z, Yang R, Xie J, et al. Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. PloS one. 2014; 9(4):e96161. https://doi.org/10.1371/journal.pone.0096161 PMID: 24781370.
36. Tamari M, Nishino Y, Yamamoto N, Ueda M. Acceleration of wound healing with stem cell-derived growth factors. The International journal of oral & maxillofacial implants. 2013; 28(6):e369–75. https://doi.org/10.11607/jomi.te17 PMID: 24278952.

37. Fong CY, Tam K, Cheyyatraivendran S, Gan SU, Gauthaman K, Armugam A, et al. Human Wharton’s jelly stem cells and its conditioned medium enhance healing of excisional and diabetic wounds. Journal of cellular biochemistry. 2014; 115(2):290–302. https://doi.org/10.1002/jcb.24661 PMID: 24038311.

38. Heo SC, Jeon ES, Lee IH, Kim HS, Kim MB, Kim JH. Tumor necrosis factor-alpha-activated human adipose tissue-derived mesenchymal stem cells accelerate cutaneous wound healing through paracrine mechanisms. The Journal of investigative dermatology. 2011; 131(7):1559–67. https://doi.org/10.1038/jid.2011.64 PMID: 21451545.

39. Jun EK, Zhang Q, Yoon BS, Moon JH, Lee G, Park G, et al. Hypoxic conditioned medium from human amniotic fluid-derived mesenchymal stem cells accelerates skin wound healing through TGF-beta/SMAD2 and PI3K/Akt pathways. International journal of molecular sciences. 2014; 15(1):605–28. https://doi.org/10.3390/ijms15010605 PMID: 24399894.

40. Mehanna RA, Nabil I, Attia N, Bary AA, Razek KA, Ahmed TA, et al. The Effect of Bone Marrow-Derived Mesenchymal Stem Cells and Their Conditioned Media Topically Delivered in Fibrin Glue on Chronic Wound Healing in Rats. BioMed research international. 2015; 2015:846062. https://doi.org/10.1155/2015/846062 PMID: 26236740.

41. Ma D, Kua JE, Lim WK, Lee ST, Chua AW. In vitro characterization of human hair follicle dermal sheath mesenchymal stromal cells and their potential in enhancing diabetic wound healing. Cytoterapy. 2015; 17(8):1036–51. https://doi.org/10.1016/j.jcyt.2015.04.001 PMID: 25998158.

42. Li TS, Takahashi M, Oshימה M, Qin SL, Kubo M, Muramatsu K, et al. Myocardial repair achieved by the intramyocardical implantation of adult cardiomyocytes in combination with bone marrow cells. Cell transplantation. 2008; 17(6):695–703. PMID: 18912527.

43. Nakamura Y, Ishikawa H, Kawai K, Tabata Y, Suzuki S. Enhanced wound healing by topical administration of mesenchymal stem cells transfected with stromal cell-derived factor-1. Biomaterials. 2013; 34(37):9393–400. https://doi.org/10.1016/j.biomaterials.2013.08.053 PMID: 24054847.

44. Fiorina P, Pietramaggiore G, Scherer SS, Jurewicz M, Mathews JC, Vergani A, et al. The mobilization and effect of endogenous bone marrow progenitor cells in diabetic wound healing. Cell Transplant. 2010; 19(11):1369–81. https://doi.org/10.3727/096368910X514288 PMID: 20977829.

45. Yu Y, Huang S, Ma K, Fu X, Han W, Sheng Z. Promising new potential for mesenchymal stem cells derived from human umbilical cord Wharton’s jelly: sweat gland cell-like differentiative capacity. Journal of tissue engineering and regenerative medicine. 2012; 6(8):545–54. https://doi.org/10.1002/term.468 PMID: 21916019.

46. Zhang J, Guan J, Niu X, Hu G, Guo S, Li Q, et al. Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. Journal of translational medicine. 2015; 13(1):49. https://doi.org/10.1186/s12967-015-0417 PMID: 25638205.

47. El Haddad N, Heathcote D, Moore R, Yang S, Azzi J, Mfarrej B, et al. Mesenchymal stem cells express serine protease inhibitor to evade the host immune response. Blood. 2011; 117(4):1176–83. https://doi.org/10.1182/blood-2010-06-287979 PMID: 21076046.

48. Schu S, Nosov M, O’Flynn L, Shaw G, Treacy O, Barry F, et al. Immunogenicity of allogeneic mesenchymal stem cells. Journal of cellular and molecular medicine. 2012; 16(9):2094–103. https://doi.org/10.1111/j.1582-4934.2011.01509.x PMID: 22151542.

49. Fiorina P, Jurewicz M, Augello A, Vergani A, Dada S, La Rosa S, et al. Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. J Immunol. 2009; 183(2):993–1004. https://doi.org/10.4049/jimmunol.0900803 PMID: 19561093.

50. Ellopoulos N, Stagg J, Lejeune L, Pomme L, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005; 106(13):4057–65. https://doi.org/10.1182/blood-2005-03-1004 PMID: 16118325.

51. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemsze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006; 108(6):2114–20. https://doi.org/10.1182/blood-2005-11-011650 PMID: 16690970.

52. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006; 107(4):1484–90. https://doi.org/10.1182/blood-2005-07-2775 PMID: 16239427.
53. Zangi L, Margalit R, Reich-Zeliger S, Bachar-Lustig E, Beilhack A, Negrin R, et al. Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. Stem Cells. 2009; 27(11):2865–74. https://doi.org/10.1002/stem.217 PMID: 19750539.

54. Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic in vitro, intracardiac injection elicits an immune response in vivo. Transplantation. 2007; 83(6):783–90. https://doi.org/10.1097/01.tp.0000258649.23081.a3 PMID: 17414713.

55. Hong SJ, Jia SX, Xie P, Xu W, Leung KP, Mustoe TA, et al. Topically delivered adipose derived stem cells show an activated-fibroblast phenotype and enhance granulation tissue formation in skin wounds. PloS one. 2013; 8(1):e55640. https://doi.org/10.1371/journal.pone.0055640 PMID: 23383253.

56. Badillo AT, Redden RA, Zhang L, Doolin EJ, Liechty KW. Treatment of diabetic wounds with fetal murine mesenchymal stromal cells enhances wound closure. Cell and tissue research. 2007; 329(2):301–11. https://doi.org/10.1007/s00441-007-0417-3 PMID: 17453245.

57. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2008; 16(5):585–601. https://doi.org/10.1111/j.1524-475X.2008.00410.x PMID: 19128254.

58. Reiber GE, Vileikyte L, Boyko EJ, del Aguila M, Smith DG, Lavery LA, et al. Causal pathways for incident lower-extremity ulcers in patients with diabetes from two settings. Diabetes care. 1999; 22(1):157–62. PMID: 10333919.

59. Rustad KC, Wong VW, Sorkin M, Glotzbach JP, Major MR, Rajadas J, et al. Enhancement of mesenchymal stem cell angiogenic capacity and stemness by a biomimetic hydrogel scaffold. Biomaterials. 2012; 33(1):80–90. https://doi.org/10.1016/j.biomaterials.2011.09.041 PMID: 21963148.

60. Khosrotehrani K. Mesenchymal stem cell therapy in skin: why and what for? Experimental dermatology. 2013; 22(5):307–10. https://doi.org/10.1111/exd.12141 PMID: 23614735.

61. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PloS one. 2008; 3(4):e1886. https://doi.org/10.1371/journal.pone.0001886 PMID: 18382669.

62. Aguilera V, Briceno L, Contreras H, Lamperti L, Sepulveda E, Diaz-Perez F, et al. Endothelium trans-differentiated from Wharton’s jelly mesenchymal cells promote tissue regeneration: potential role of soluble pro-angiogenic factors. PloS one. 2014; 9(11):e111025. https://doi.org/10.1371/journal.pone.0111025 PMID: 25412260.

63. Li M, Zhao Y, Hao H, Dai H, Han Q, Tong C, et al. Mesenchymal stem cell-conditioned medium improves the proliferation and migration of keratinocytes in a diabetes-like microenvironment. The international journal of lower extremity wounds. 2015; 14(1):73–86. https://doi.org/10.1177/1594734615569053 PMID: 25759411.