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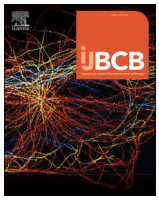

Enhancement of wound healing in diabetic mice by topical use of a peptide-ionic liquid conjugate

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ABSTRACT

Diabetic foot ulcers (DFU) are one of the most devastating complications of diabetes, with high impact on patient's quality of life. In worst scenarios, DFU can lead to severe amputation or even death. DFUs are an easy target for microbial pathogens and their effective healing is hampered by the galloping increase of microbial resistance to antibiotics, including from the most prevalent pathogens in DFU, *e.g. Staphylococcus aureus*. As such, available antibiotics show poor efficacy in the treatment of DFU, leading to a chronic condition that is exacerbated by poor healing rates due to the persistent inflammation, poor oxygenation and low angiogenesis, leading to high risk of ischemia, among other conditions that typically affect patients with diabetes. Our group has recently designed new peptide-based strategies towards the topical treatment of DFU, whereby peptide-ionic liquid conjugates emerged as highly promising agents. One of the best such conjugates, C_{16}-Im-PP4, results from coupling an imidazolium-based ionic liquid with intrinsic antimicrobial activity to the *N*-terminus of a collagen boosting peptide used in cosmetics, the pentapeptide-4. C_{16}-Im-PP4 showed excellent *in vitro* properties, namely, wide-spectrum antimicrobial action and collagen-boosting effect on human dermal fibroblasts, prompting the *in vivo* study here reported. The peptide-ionic liquid conjugate was applied topically on wounds of mice with diabetes. The results show multitargeted actions, at a dose of 1 μg/wound including: i) anti-inflammatory; ii) antioxidant; iii) pro-collagenic; vi) pro-angiogenic; v) antimicrobial; and vi) improved wound maturation effects. Altogether, these results identify this technology as a novel topical treatment for DFU.

1. Introduction

According to the International Diabetes Federation (IDF), in 2021 half a billion people were living with diabetes, and many more people with undiagnosed diabetes. Therefore, diabetes is one of the most rapidly expanding global health threats of the 21st century. The IDF estimates that, by 2045, there will be 783 million people with diabetes in the 20–79 years range (International Diabetes Federation, 2024). For a longer and better quality of life, patients with diabetes should control their glucose levels daily and adopt healthy lifestyles to delay the onset and severity of the relevant complications arising from diabetes. The principal complications to prevent are nephropathy, retinopathy, neuropathy, peripheral artery disease and foot ulceration, while paying attention to the emergence of early symptoms of these conditions. It has

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been reported that about 15 % of the patients with diabetes develop diabetic foot ulcers (DFU), which are one of the most devastating complications of diabetes, that can lead to severe amputation or even death (Akkus and Sert, 2022). The standard of care for DFU typically involves off-loading, vascular assessment, surgical debridement of the open wound, and control of infection (Everett and Mathioudakis, 2018). The risk of infection is much higher in DFU than in a "regular" acute wound, as diabetes-driven biochemical and physiological imbalance hamper the normal healing process, which gets stalled in the inflammation phase. Healing progression towards the proliferation and remodeling phases is therefore blocked, and the wound remains open while offering an ideal environment for microbial pathogens to thrive. In other words, a diabetic foot infection (DFI) emerges. DFI are further aggravated by the increasing prevalence of multidrug resistant (MDR) strains that are not effectively depleted by current antibiotics (Senneville et al., 2023). Indeed, in a large prospective observational study with 299 patients with DFI, healing was achieved in only 46 % of the cases after 12 months of antibiotic treatment, whereas reinfection occurred in 10 % of the apparently resolved cases. DFI presents a polymicrobial profile, where *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* are amongst the most prevalent species (Zhang et al., 2023).

In the past decade, antimicrobial peptides (AMPs) have re-gained momentum as valuable substitutes for antibiotics. AMPs have been used in the management of DFI: for instance, the glycopeptide vancomycin is employed in the treatment of moderate to severe methicillin-resistant *S. aureus* (MRSA) DFI with, for instance, recent antibiotic use or prolonged hospitalization (Senneville et al., 2023). AMPs are therefore an important asset for the management of infections, including of open wounds. These AMPs typically exhibit potent bactericidal action against Gram-positive and Gram-negative bacteria, including MDR strains, and often also against fungi. Moreover, many AMPs also show immunomodulatory, pro-angiogenic, proliferative, regenerative effects, while being less prone to inducing microbial resistance due to their membranolytic action on pathogen cells (Ji et al., 2024). As such, AMPs are quite promising towards effective management of DFU/DFI, as this demands for multi-action therapeutic agents capable of promoting not only antimicrobial prevention/treatment, but also other effects that can jointly contribute to overcome the non-healing nature of these complex chronic non-healing wounds. This underpins the efforts that are being made towards the design of a growing panoply of bioactive peptides, as well as on their production through simpler and greener processes (Da Silva et al., 2021; Teixeira et al., 2023). According to Caiyun Fu and co-workers, more than 80 therapeutic peptides have already reached the market, despite their well-known liabilities like, e.g., poor *in vivo* stability, as well as solubility and aggregation issues (Wang et al., 2022). This reflects the considerable interest and room for improvement that exist regarding the conversion of bioactive peptides into clinically relevant agents, either by using advanced delivery systems (Deshayes et al., 2022) or through rational of suitable derivatives and/or peptidomimetics (Mohammad et al., 2015).

Our group has been dedicated to the design of new peptide-based strategies towards the development of a new active pharmaceutical ingredient (API) for the topical treatment of DFU. By aiming at a new API that could concomitantly exert antimicrobial action and skin regeneration effects, we have taken pentapeptide-4 (PP4), a collagen-boosting peptide (CBP) used in high-end anti-aging cosmetics, as our core skin-regenerating structure, and modified it through conjugation with antimicrobial moieties, including AMPs and ionic liquids (ILs) (Gomes et al., 2019, 2020, 2021). The wounds of diabetic patients have a decrease in collagen deposition, and this led to a loose extracellular matrix in the wound bed, which difficult several cellular functions such as migration and proliferation important in wound healing (Huang and Kyriakides, 2020). The pro-collagen effect of the peptide will contribute for a better formation of the granulation tissue with increased collagen deposition and promote a better healing. This has paved the way towards the advancement of peptide-ionic liquid conjugates (PILC) as new leads for the topical treatment of complex non-healing wounds like DFUs. PILC, which are easily produced by conjugating an antimicrobial IL to the *N*-terminus of the peptide through "click chemistry", showed the desired dual-action *in vitro*, namely, wide-spectrum microbicidal activity, including against MDR strains of both Gram-positive and Gram-negative bacteria, and against three different species of *Candida* fungi (*C. albicans*, *C. glabrata* and *C. parapsilosis*), alongside significant collagen-boosting activity on human dermal fibroblasts. As a result, we have advanced for a pilot *in vivo* assessment with PILC, C_{16}-Im-PP4 (Fig. 1A) for its wound healing effects on diabetic mice (Gomes et al., 2022). The promising results thus far obtained are herein reported and discussed.

2. Materials and methods

2.1. Chemical synthesis of C_{16}-Im-PP4

The PILC of interest, C_{16}-Im-PP4, resulted from the *N*-terminal modification of peptide PP4 with a 1-hexadecylimidazolium-based IL, as described elsewhere (Gomes et al., 2022). The target PILC was obtained as a fluffy white solid, and its purity and chemical structure were confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization-ion trap mass spectrometry (ESI-IT MS), respectively. The results agreed with previously reported data (Gomes et al., 2022). Accurate quantitation of this PILC was performed by microvolume spectrometry at 205 nm, using a Thermo Scientific™ NanoDrop™ One system and with method 31 that assumes an extinction coefficient ε_{205} of 31 $mL.mg^{-1}.cm^{-1}$ (Loughrey et al., 2024). The stock solution (2 mg/mL) for *in vivo* experiments, was prepared by dissolving the solid PILC in saline 0.9 % NaCl solution, which was then further diluted to the two working solutions (100 μg/mL and 500 μg/mL).

2.2. Animals

Eight-week-old male C57BL/6 J mice (Charles River Laboratories, France) were housed in certified local facilities, at normal room temperature for rodents (21 ± 3°C) under a 12-hour light/dark cycle, with free access to water and food.

2.3. In vivo diabetic wound healing model

Diabetes was induced by intraperitoneal injections of 50 mg/kg streptozotocin (STZ) for five consecutive days, as used in previous work (Leal et al., 2015). After one week, mice with blood glucose levels above 250 mg/dL were considered diabetic. The animals were kept diabetic for 6 weeks prior to the wound healing experiment and the weight and blood glucose levels were measured before randomly assign the animals for the different treatment groups (Table SM1). Analgesia was given before wounding (0.05 mg/kg buprenorphine, subcutaneous) and every 6–8 h up to 24 h after wounding (0.10 mg/kg buprenorphine, subcutaneous). The animals were anesthetized with 5 % isoflurane, and maintained with 2.5 %, combined with oxygen (0.5 L/min). Then, after the dorsal hair was removed, skin was sterilized with Betadine, and two full-thickness wounds were made with a 6 mm diameter biopsy punch tool. The mice were randomly divided into three treatment groups: C_{16}-Im-PP4 – 1 μg (n = 4), C_{16}-Im-PP4 – 5 μg (n = 4), or control – saline 0.9 % NaCl solution (n = 3, control). The treatments were applied topically to each wound two times daily during the 10-day course of the treatment period. The wound size was monitored and measured by acetate tracing and quantified with Fiji software (NIH Image, USA). On day 10 after wounding, mice were anesthetized with ketamine/xylazine (100/10 mg/kg, intraperitoneally) and euthanized by cervical dislocation. The wounded skin was harvested and cryopreserved in optimal cutting temperature gel (OCT, VWR) at −80 °C, or fixed in 4 %

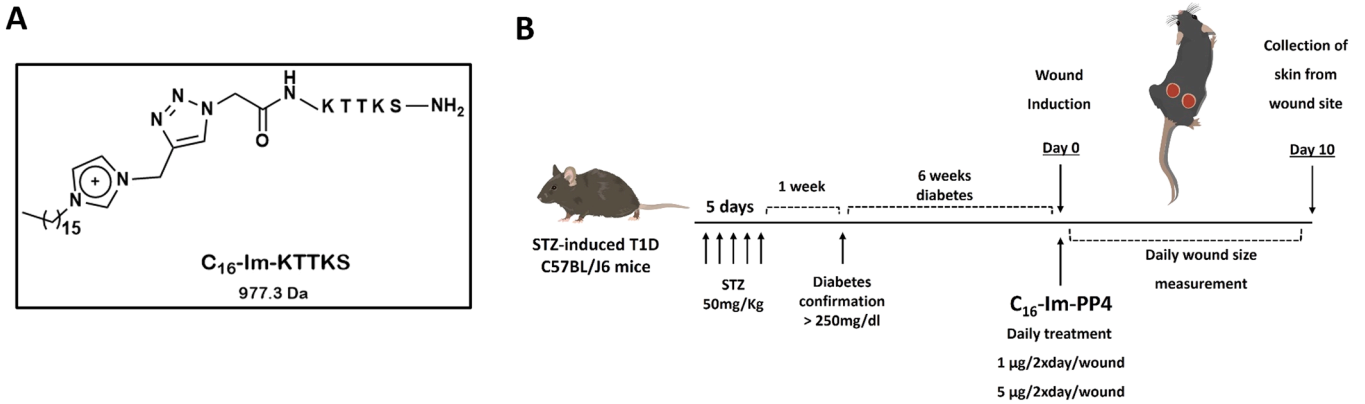

Fig. 1. (**A**) Structure of C_{16}-Im-PP4, the PILC selected for study herein reported (KTTKS is the one-letter amino acid sequence of pentapeptide-4, the collagen-boosting peptide used as building block of the PILCs developed by us). (**B**) The experimental design and timeline of diabetes and wound induction on C57BL/6 J mice.

paraformaldehyde (PFA; Sigma) in phosphate buffered saline (PBS) at 4 °C, for further analyses. A schematic view of the *in vivo* model used is given in Fig. 1B.

2.4. Histological analysis

After fixation (4 % PFA) at 4°C for 48 h, the wounded skin was included in paraffin. Paraffin-embedded skin Section (5 μm) were stained with Hematoxylin & Eosin (H&E, Sigma, Germany), and Masson-Goldner's Trichrome kit (MT, Carl Roth, Germany), according to manufacturers' protocol, to evaluate the wound structure and collagen deposition, respectively. The wound image sections, obtained with combinations of images (100x magnification), were acquired using a Zeiss AxioImager Z2 upright widefield microscope. Collagen deposition was determined as the percentage of collagen in the wound bed relative to control wound samples, the image analysis process was performed using the image analyzed with Fiji software (NIH, USA).

A histology scoring system was used to evaluate the effect of the PILC treatment in wound healing progression by using H&E and MT-stained skin sections. This system was based on a previously validated histological scoring method for murine skin wounds (van de Vyver et al., 2021). In brief, this scoring system for murine cutaneous wounds evaluates key parameters in each phase of healing to establish an overall histology score, ranging from 0 (open/unhealed wound) to 12 (completely healed wound). Those parameters include re-epithelization (none - 0; partially - 1; total −2), epithelial thickness (hypoplasia - 0; hypertrophy - 1; normal 95–2), keratinization (No – 0; Yes – 2), granulation tissue (thin GT– 0; thick GT– 1; presence of intact dermis formation – 2), remodeling (None – 0; Partial - 1, presence of either: collagen deposition or dermal white adipose tissue – 1; Complete - 2, presence of: dermal white adipose tissue, skin Appendages, panniculus carnosus regeneration), and scar (hypoplasia – 0; hypertrophied – 1; normal – 2).

2.4.1. Immunohistochemistry

The presence of M1- and M2-like macrophages (CD68 +TNF-α and CD68 +CD206 respectively), T lymphocytes (CD3), inflammatory markers (IL-6 and MCP-1), and angiogenesis (CD31, endothelial cell marker) present in the wounded skin samples was assessed by immunohistochemistry.

Skin cryosections (10 μm thickness) were fixed in ice-cold acetone for 10 minutes, and permeabilized at RT with PBS with 1 % tween (PBS-T) and 0.2 % Triton X-100 for 30 minutes. Subsequently, the samples were blocked with 50 μl of 10 % goat serum for 30 minutes at RT. Then, samples were placed in a humidified chamber and incubated overnight at 4 °C with the primary antibodies: rabbit anti-CD68 (1:100, Abcam, UK) and rat anti-TNF-α (1:200, AbD Serotec, Portugal) for M1-like macrophages; rabbit anti-CD68 (1:100, Abcam, UK) and rat anti-CD206 (1:200, Santa Cruz, Santa Cruz, USA) for M2-like macrophages; rat anti-CD31 (1:200, PECAM-1, Merck Millipore, Germany) for endothelial cells; rabbit anti-CD3 (1:100, Abcam, UK) for T-cells; rabbit anti-IL-6 (1:100, Abcam, UK) for IL-6; rabbit anti-MCP-1 (1:100, Abcam, UK) for MCP-1. The samples were then incubated at RT for 1 h, with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000) for nuclei staining and the secondary antibody, anti-rat (1:500, conjugated to Alexa Fluor 568, Invitrogen) and anti-rabbit (1:500, Alexa Fluor 468 conjugated, Invitrogen). The 3–4 random images at the wound site were obtained using the Carl Zeiss LMS 710 confocal microscope, with 400x magnification, and acquired using the Zen Blue software. The number of cells, area or fluorescent staining was analyzed using Fiji software (NIH Image, USA).

2.4.2. Dihydroethidium assay (DHE)

The dihydroethidium (DHE) assay was performed to detect and measure the production of reactive oxygen species (ROS) in skin cryosections. Skin cryosections (10 μm) were incubated with 10 μM DHE (Invitrogen, USA) in a humidified dark chamber at 37°C for 30 min, followed by fixation with 4 % PFA in PBS, for 5 min at RT in the dark, and counterstaining with DAPI. The images were acquired with a confocal microscope (Zeiss LSM 710) with 200 × magnification (5 random imagens at the wound site for each sample). The images were acquired using the Zen Blue software and analyzed with Fiji software (NIH, USA).

2.4.3. Wound microbiota load

For the assessment of microbial load at the wound site, the microbiota was collected using a sterile gauze humidified with 20 μl of saline 0.9 % on days 0, 3, 7, and 10 of the experiment. At day 0, i.e., prior to wounding, only one gauze was used and placed over the non-wounded skin for 1 min; whereas at days 3, 7 and 10 post-wounding, each mouse had two wounds with the same treatment, and one gauze was used and placed over each wound for 1 min. Each gauze was then placed on the center of a plate with Trytpic Soy Agar (TSA), and then the gauze dressings were discarded. All the plates were kept in an incubator at 37 °C for 10 days. On day 10, the overall microbial load was determined through the observation of the number of colony-forming units (CFUs).

2.5. Statistical analysis

The statistical analysis was performed using GraphPad Prism (GraphPad Prism Software version 9.3.1, USA). of the specified number of independent experiments. Statistical analysis between groups were evaluated using a student's *t*-test and the results are presented as mean ± SD. *p* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Chemical synthesis

The target PILC was successfully obtained in high purity (<95 %) and in adequate amounts to perform the *in vivo* experiments. Additional details are given in the Supplementary Material section (Figs. SM1 and SM2).

3.2. In vivo studies

3.2.1. Improved wound closure and histological analyses

The weight and glycemia of the animals were similar between the mice groups (see Table SM1). The PILC C_{16}-Im-PP4, was tested *in vitro* for its toxicity at 1 and 5 μg of peptide, the maximum concentration used in the experiments herein performed. As expected from our previous studies, (Gomes et al., 2022) this PILC showed no toxicity against HaCaT keratinocytes at this concentration (see Figure SM3). This experiment was followed by a scratch assay using HaCaT cells, which showed that – under the *in vitro* conditions used – the PILC did not improve cell migration at 24 h or 48 h of incubation, either in high or in low glucose levels (Figures SM4 and SM5). However, when tested in the wounds of diabetic mice, it had a clear positive impact on wound closure, with statistically significant differences between the control and the treated groups observed as early as day 2 (Fig. 2A). However, no significant differences between the two doses (1 μg *vs* 5 μg) tested were observed, that could be due to the limited number of animals used in the assay. At the end of the *in vivo* experiment (day 10), the wounded skin was collected to perform histological analyses. Hematoxylin-eosin (H&E) and Masson's Trichrome staining indicate that the wounds treated with C_{16}-Im-PP4 present higher collagen deposition (Fig. 2C and D) that is in agreement with previous *in vitro* data regarding collagen-inducing effects on human dermal fibroblasts, (Gomes et al., 2022) this PILC was able to enhance collagen deposition at the wound site in the *in vivo* diabetic animal model. Using the Histology Scoring System proposed by van de Vyver and co-workers, (van de Vyver et al., 2021) it was possible to conclude that the PILC tested C_{16}-Im-PP4 improved wound maturation compared to the untreated tissue, as depicted in Fig. 2E. No statistically significant differences could be observed between the two doses tested, which agrees with observations on wound closure (Section 3.2.1).

3.2.2. Anti-inflammatory effects

The recruitment of inflammatory cells such as macrophages is an important step in the healing and tissue repair process, but the abnormal prolongation of a pro-inflammatory stage leads to stall wound healing progression and a state of chronicity that impedes wound closure and a functional healthy skin. In other words, a switch from a pro- to an anti-inflammatory state is needed for healing to advance properly. Using the skin tissue collected from wounds on day 10, M1- (pro-inflammatory) and M2-like (anti-inflammatory) macrophages were identified and quantified. As shown in Fig. 3 A and B, treatment with the C_{16}-Im-PP4 led to a significant decrease in M1-like macrophages per field, compared with the control tissue, with no statistical difference observed between both C_{16}-Im-PP4 doses used. Conversely, the same treatment led to an increase in macrophages of the M2-like phenotype, despite not being statistically significant (Fig. 3 C and D) possibly due to the limited number of animals in the pilot study. However, according with these findings, treatment with the C_{16}-Im-PP4 led to a significant decrease in the M1/M2 ratio, denoting a clear and statistically significant difference in the inflammatory response between treated and control groups (Fig. 3E). Overall, the anti-inflammatory action of PILC C_{16}-Im-PP4 is evident, while similar effects were obtained for both doses tested.

To confirm these observations, we further analyzed the expression of pro-inflammatory cytokines, namely interleukin (IL-6) and monocyte chemoattractant protein-1 (MCP-1 or CCl2). A statistically significant decrease in the expression of both cytokines was observed upon treatment with C_{16}-Im-PP4, corroborating the anti-inflammatory action of this PILC (Fig. 4 A,B,D,E). Additionally, the number of CD3-positive cells was determined as an indicator of improved healing, since T cells are recruited during the inflammatory phase and their decrease is an indicator of healing progression into the proliferation and remodeling phases (Short et al., 2021). As shown in Fig. 4 C and E, the number of CD3-positive cells was decreased at day 10 for treated versus control groups.

3.2.3. Antioxidant and pro-angiogenic effects

High levels of ROS and poor angiogenesis are amongst the hallmarks of diabetes, which highly contribute to the non-healing profile of diabetic wounds. As such, we have further evaluated how both ROS levels and angiogenesis could be affected by topical treatment with C_{16}-Im-PP4. To determine the effect of this PILC on the generation of ROS, we have used a standard assay that uses the fluorescent probe DHE for detection of the ROS in the skin tissue (E et al., 2021). Fig. 5 A and B shows that treatment with the test PILC causes a statistically significant decrease in the amount of ROS in the tissue samples at day 10 post-wounding, as compared to the control group (C_{16}-Im-PP4, 1 μg: 56.9 ± 6.3 % of control; C_{16}-Im-PP4, 5 μg: 54.7 ± 6.9 % of control). To assess the effect of C16-Im-PP4 on angiogenesis, we performed an immunohistochemical analysis using CD31 to detect the presence of endothelial cells in histological tissue sections. As shown in Fig. 5 C and D, the test PILC increased the area of $CD31^+$ cells per field, therefore improving angiogenesis and, consequently, oxygenation at the wound site for an improved wound healing.

3.2.4. Antimicrobial action

The major complication on DFU, in addition to, but also, in relation to its slow/non-healing nature, is microbial infection leading to a DFI. This can result from (i) colonization by external pathogens, (ii) imbalance or phenotype change in commensal microbiota, or (iii) both combined (Wang et al., 2023). In this study, a non-infected diabetic wound healing mouse model was used. Yet, evaluation of antimicrobial action is equally relevant in this case, as it serves to predict the ability of the treatment to prevent undesired microbial colonization of the wound. As such, we have made a qualitative assessment of the effect of the test PILC on the overall wound microbiota load, with results shown on Fig. 6. At day 0, immediately before wound induction, only a few colony forming units (CFUs) were detected in all test groups. Yet, a marked increase in CFUs could be observed at days 3 and 7 in the control (non-treated) group, whereas much fewer CFUs were detected in the treated groups. Interestingly, there is an apparent dose-effect dependence, although further studies are needed to confirm this observation.

4. Discussion

Herein, we advance C_{16}-Im-PP4 a new compound resulting from the conjugation of an antimicrobial ionic liquid with a collagen-boosting peptide, as a promising candidate for the topical treatment of DFU. We have previously found C_{16}-Im-PP4, as well as related PILCs, to have promising properties *in vitro*, such as collagen-inducing effects on human dermal fibroblasts, and antimicrobial action against Gram-positive and Gram-negative bacteria, including MDR bacterial clinical isolates, as well as against three different species of *Candida* fungi (Gomes et al., 2022). We have now also confirmed the effectiveness of C_{16}-Im-PP4 on a first pilot *in vivo* study on a well validated diabetic wound healing model using C57BL/6 J mice with STZ-induced diabetes (Sanapalli et al., 2021).

The pilot study allowed us to confirm the ability of the test PILC C_{16}-Im-PP4 to increase the wound healing rate, which did not occur in the *in vitro* scratch assays, where the compound did not promote migration of keratinocytes, at either high or low glucose levels. This lack of correlation is not surprising, given the rather complex multifactorial nature of

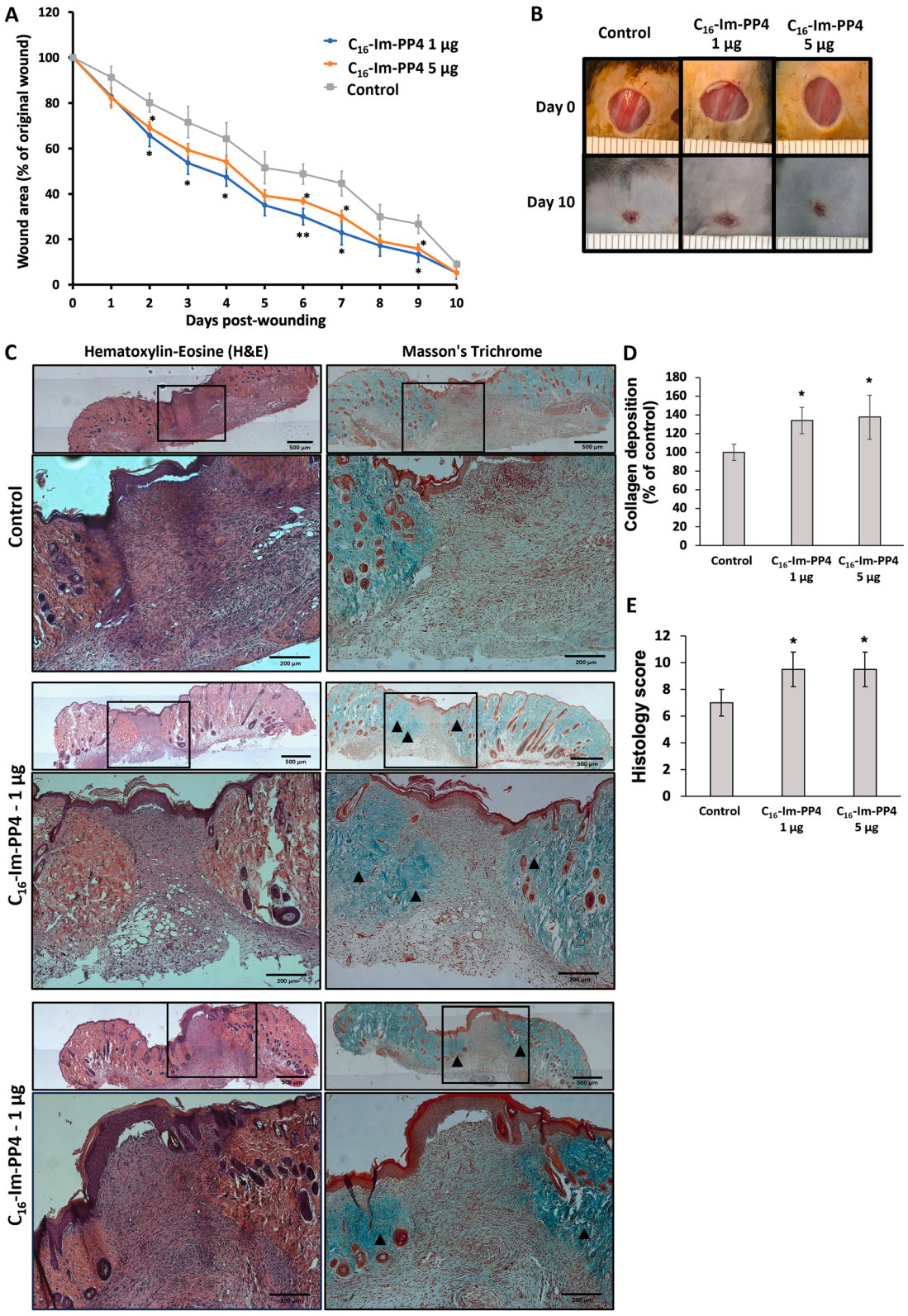

Fig. 2. *In vivo* effect of PILC in diabetic wound healing. (A) Effect of the C_{16}-Im-PP4 treatment on wound closure in a diabetic mouse model. Statistical analysis was performed using a student's *t*-test. Results are presented as means ± SD; $*p < 0.05$ * $*p < 0.01$ compared to control. (B) Representative images of wound healing progression in the diabetic mouse model on days 0 and 10 post-injury. (C) Representative images of H&E (left) and Masson's Trichrome (right) stainings at day 10 after wounding. Scale bar in full skin - 500 µm; Scale bar in wounds – 200 µm. Triangles indicate increased collagen deposition in wound. (D) Collagen deposition. (E) Histology score. Data are expressed as mean ± SD of 4 animals (each condition of the C_{16}-Im-PP4 treatment) and mean ± SD of 3 animals (control). The student *t*-test was used for the represented comparisons. $*p < 0.05$.

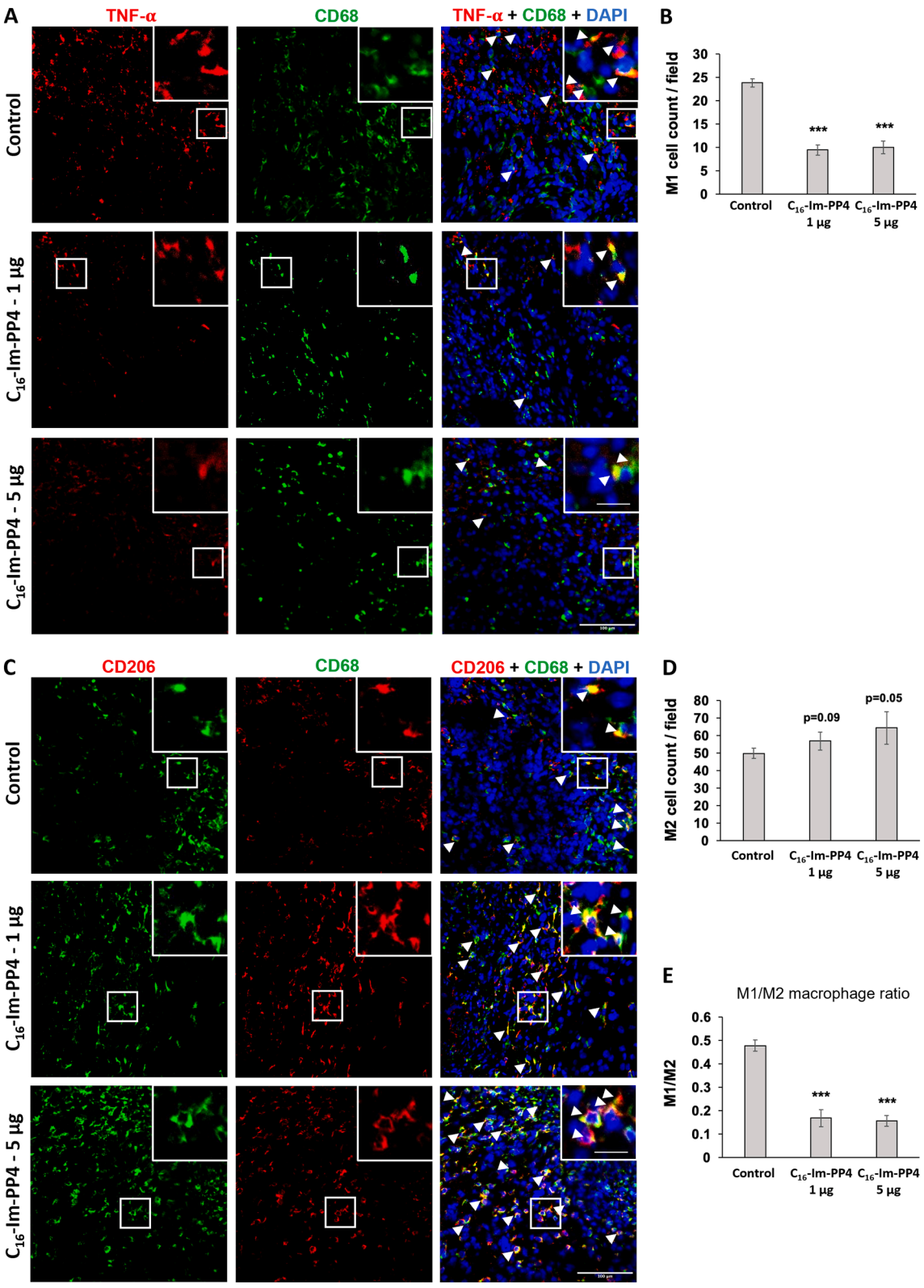

Fig. 3. Effect of C_{16}-Im-PP4 treatment on the expression of M1- and M2-like macrophages in the wounds of diabetic mice at day 10 post-injury. (A) Representative images of M1-like macrophages. This phenotype was determined by the expression of CD68 (green) and TNF-α (red). Cell nuclei were stained with DAPI (blue). Arrows indicate M1-like macrophages. Scale bar - 100 μm. (B) Number of M1-like macrophages per field at the wound site. Statistical analysis was conducted using a student's *t*-test. (C) Representative images of M2-like macrophages. This phenotype was determined by the expression of CD68 (green) and CD206 (red). Cell nuclei were stained with DAPI (blue). Arrows indicate M2-like macrophages. Scale bar – 100 μm. (D) Number of M2-like macrophages per field at the wound site. (E) M1/M2 macrophage ratio. Statistical analysis was conducted using a student's *t*-test. The top right square (scale bar – 20 μm) in each image is a magnification of the small square in the corresponding image. Three fields were measured per sample. The results are presented as means ± SD. * $^{**}p < 0.001$.

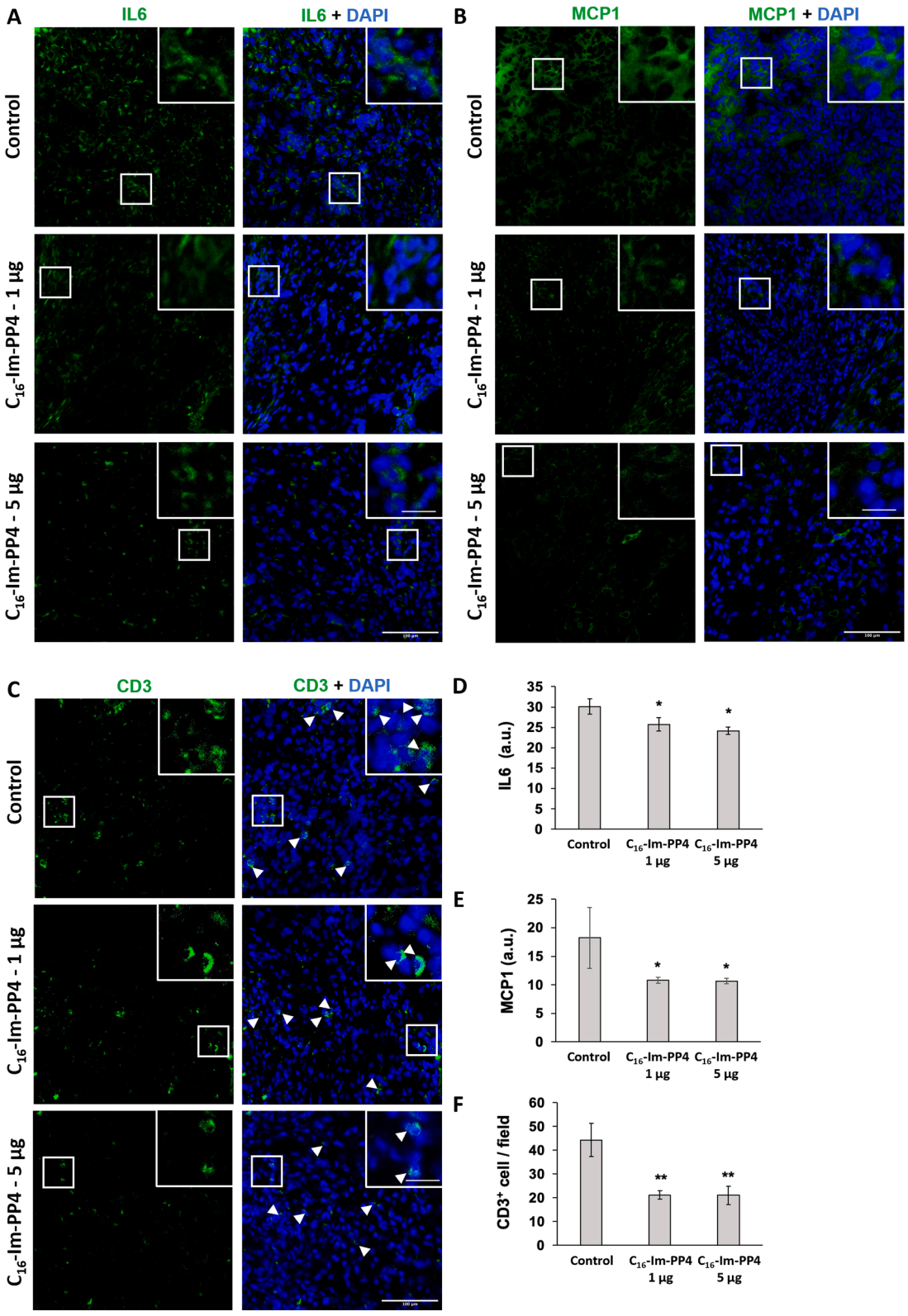

Fig. 4. Effect of the C_{16}-Im-PP4 treatment on the levels of pro-inflammatory cytokines, IL-6 and MCP-1, and on the numbers of $CD3^+$ cells in the wounded tissue of diabetic mice at day 10 post-injury. (A) Representative images of IL-6 (green). (B) Representative images of MCP-1 (green). (C) Representative images of $CD3^+$ cells (green). Cell nuclei were stained with DAPI (blue). Arrows point to $CD3^+$ cells. Scale bar – 100 μm. (D) Percentage of IL-6 expression levels relative to the control group. (E) Percentage of MCP-1 expression levels relative to the control group. (F) Number of $CD3^+$ cells per field. The top right square (scale bar – 20 μm) in each image is a magnification of the small square in the corresponding image. Four fields were measured per sample. Statistical analysis was performed using a student's *t*-test. Results are presented as means ± SD. *$p < 0.05$, * *$p < 0.01$. * *$p < 0.01$. Arrows point to $CD3^+$ cells.

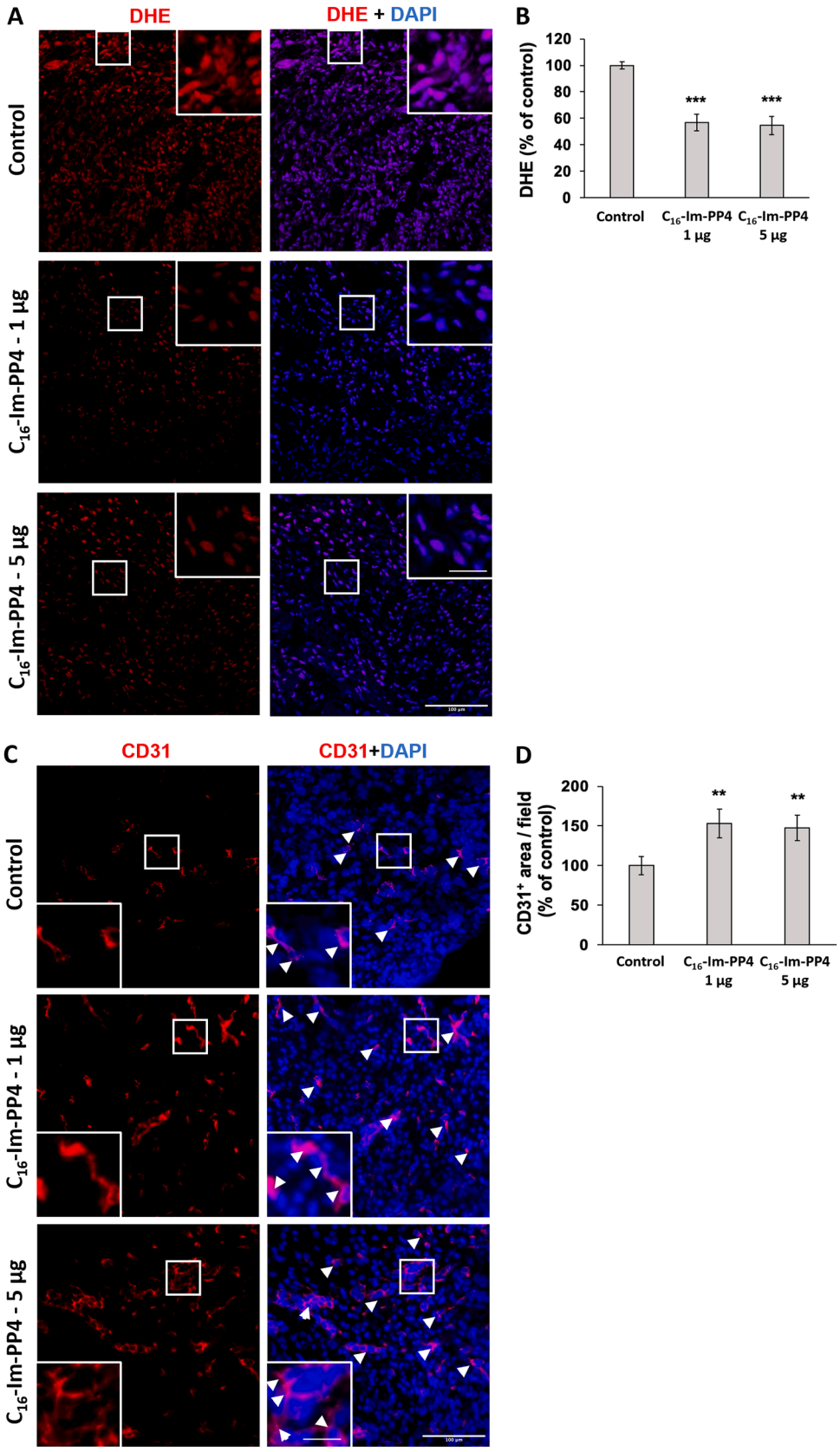

Fig. 5. The effect of C16-Im-PP4 treatment on the levels of ROS and angiogenesis in wounds of diabetic mice at day 10 post-injury. (A) Representative images of DHE assay (red). (B) Percentage of ROS expression levels relative to the control group, five fields were measured per sample (C_{16}-Im-PP4, 1 µg: 56.9 ± 6.3 % of control; C_{16}-Im-PP4, 5 µg: 54.7 ± 6.9 % of control). (C) Representative images of endothelial cells stained with CD31 (red). Cell nuclei were stained with DAPI (blue). Arrows point to endothelial cells. Scale bar −100 µm. (D) Area of CD31 + cells per field at the wound site, four fields were measured per sample. The bottom left square

(scale bar – 20 μm) in each image is a magnification of the small square in the corresponding image. Statistical analysis was performed using a student's *t*-test. Results are expressed as means ± SD. * *p < 0.01, * **p < 0.001.

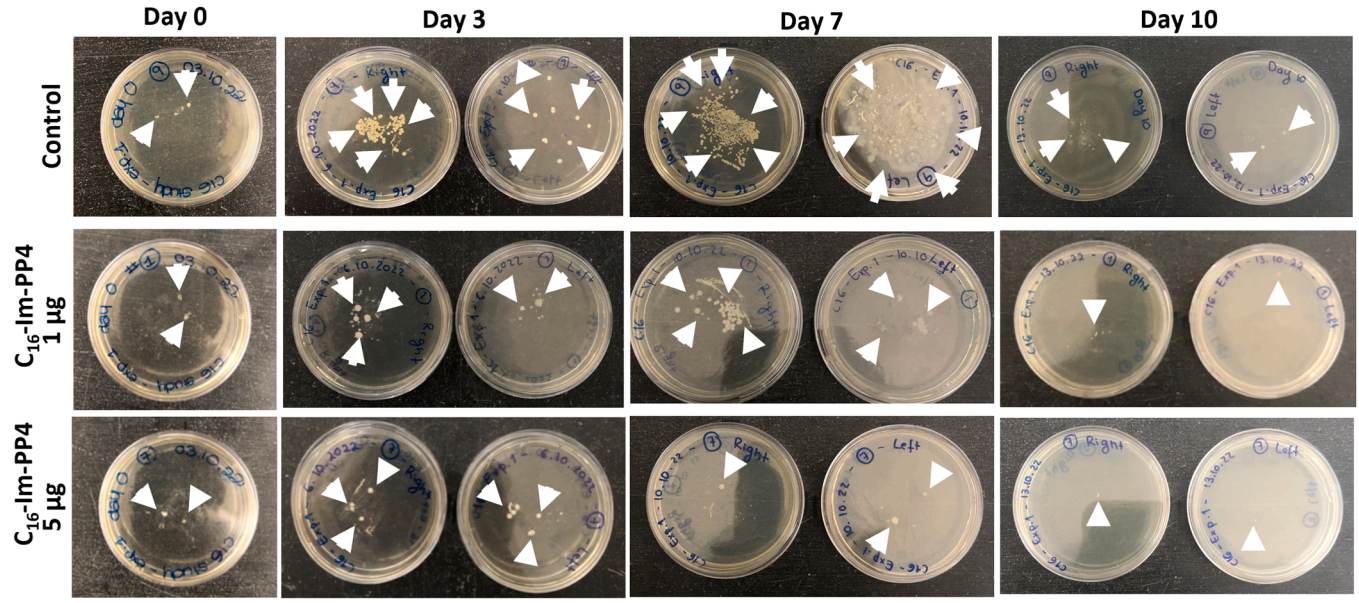

Fig. 6. Effect of C_{16}-Im-PP4 (1 and 5 μg) on the overall wound microbial load in skin of diabetic mice. Representative images of the microbial load at day 0 and days 3, 7, and 10 post-wounding. At day 0, the images represent the microbiota collected from non-wounded skin. At days 3, 7, and 10 post-wounding, the two images represent both wounds induced in the mouse dorsum that received the same treatment. The images from days 0, 3, 7 and 10 are from the same mouse of each condition. White arrows point to colony-forming units.

healing, and highlights the need to complement simple scratch assays with other studies prior to making any assumptions on the healing ability of any test compound. Remarkably, the compound herein evaluated was able to promote several effects all of which are relevant for healing: improved wound maturation markers, including the presence of partially intact dermis in granulation tissue with skin appendages; decreased inflammatory cell infiltration; increased collagen deposition; decreased pro-inflammatory markers; increased angiogenesis; and decreased levels of ROS. All these correlates with the enhanced wound healing rate observed and were similar at both doses tested; in other words, C_{16}-Im-PP4 is effective in exerting its multiple pro-healing effects at the lowest dose tested (1 μg/wound).

A relevant finding of this work was the robust anti-inflammatory action of PILC C_{16}-Im-PP4. The healing process in DFU is typically stalled at the inflammation phase, which keeps pro-inflammatory cytokines as well as the M1/M2 balance of macrophage phenotypes at very high levels (Lin et al., 2022). These prevent healing to evolve normally until the wound is properly resolved. T-lymphocytes also play an important role in the inflammatory stage. These cells are recruited to the wound and kept at high numbers while healing does not proceed into the subsequent stages; T-lymphocytes may remain at the wound site after the inflammation stage is over and healing progresses, however, at low levels (Wang et al., 2019). Based on this, and on the fact that, at day 10, the PILC-induced a decrease in $CD3^+$ cell numbers, reaching levels that were clearly lower for the treated *versus* the control groups. Thus, treated wounds reached a more advanced maturation stage compared to the non-treated ones.

Another trait of DFU is the high production of ROS that is induced by the hyperglycemic environment at the wound (Martins-Green and Saeed, 2020; Deng et al., 2021). In turn, the imbalance in the oxidative/antioxidant rate due to high levels of ROS, may contribute to chronic inflammation, triggering a vicious cycle that hampers healing. As reported, C_{16}-Im-PP4 causes a decrease in ROS in the wound skin tissue, thus, helping to promote wound healing. This is in accordance with the decrease in inflammatory cell numbers that are high contributors to ROS production in wounds. Interestingly, while a few cosmetic peptides are known to possess intrinsic antioxidant activity, peptide PP4 from which the test conjugate derives is used in high-end cosmetics not as an antioxidant but rather as an anti-aging agent able to stimulate collagen synthesis (Ngoc et al., 2023). This leads to the question of whether the antioxidant action exerted by the PILC could be due to its imidazolium moiety. A previous report from Ivanova and co-workers supports this hypothesis, as imidazolium-derived phenolic compounds were found to display improved antioxidant activity compared to the unconjugated phenols (Gerasimova et al., 2021).

Peripheral arterial disease (PAD) is one of the major harmful consequences of diabetes, encompassing low blood perfusion and consequently low oxygen supply to tissues and consequently to the wound bed. As such, there is a high risk of severe lower limb ischemia in diabetes, which can cause gangrene in the DFU, ultimately leading to amputation of the affected part of the foot or leg (Cai et al., 2022). One way to counteract this problem, while helping to accelerate healing, is to promote neovascularization, which is also impaired in patients with diabetes. As previously reported by Toshikazu Kondo and co-workers, angiogenesis is also decreased in STZ-induced diabetic mice (Ishida et al., 2019). Therefore, this is a suitable model to evaluate the pro-angiogenic potential of test compounds. In our work, higher area of $CD31^+$ cells could be observed in wound tissues collected from the treated mice as compared to those from the control group. This highlights the ability of C_{16}-Im-PP4 to induce neovascularization and it is further indicative of healing progression from the inflammatory to the proliferative stage, where formation of granulation tissue and new blood vessels takes place. Additional *in vitro* studies using endothelial or vascular smooth muscle cells could help to understand the potential mechanism behind the observed pro-angiogenic effect.

Altogether, the above findings demonstrate the strong potential of PILC like C_{16}-Im-PP4 to efficiently promote healing of DFU and of other complex wounds. Yet, one should keep in mind that DFUs are also at a

very high risk of infection, due both to their non-healing nature that impedes wound closure, and to other diabetes-related imbalances, including PAD and hyperglycemia. These diabetic conditions jointly create a suitable environment for microbial pathogens to thrive or even for commensal skin microbiota, e.g., *Staphylococcus aureus*, to switch its phenotypes exacerbating the virulence and resistance to the treatments (Radzieta et al., 2021). Bearing this in mind, we investigated whether C_{16}-Im-PP4 could reduce the "natural" (i.e., non-induced) bacterial colonization of the skin wounds inflicted on diabetic mice. As described in the results section, the microbial load was clearly reduced in the treated as compared to the control groups. This agrees with our previous findings on the potent action of PILC against a wide spectrum of microbial pathogens, including fungi and MDR clinical isolates of Gram-positive and Gram-negative bacteria (Gomes et al., 2022). Moreover, these results are in accordance with germ-free mice experiments which provide valuable insights into the role of commensal microbiota in wound healing. These studies have shown that germ-free mice exhibit enhanced angiogenesis, reduced inflammatory cell accumulation, and increased in M2-like macrophages which contributes to accelerated wound closure rates and decreased wound scaring compared to conventional mice. Taken together, these findings suggest that a reduction in the overall commensal microbiota is beneficial for tissue repair (Canesso et al., 2014).

The ongoing investigation is now focused on a better assessment of the antimicrobial action of C_{16}-Im-PP4 in diabetic wounds, not only to evaluate and establish a dose-response correlation, but also – and more importantly – to determine how C_{16}-Im-PP4 and related PILC can alter wound microbiota, i.e., change the relative proportions of different microbial species hopefully favoring non-pathogenic ones. Indeed, as highlighted by Zielińska *et al.*, the microbiota composition in DFU could be a key factor towards healing (Zielińska et al., 2023). Based on *in vitro* data, we anticipate that the PILC will be effective in promoting microbicidal action and proper healing in infected diabetic ulcers. If this assumption is confirmed, a new approach for DFI management that avoids use of topical antibiotics will emerge, in line with the IWGD-F/IDSA guidelines. PILC, including C_{16}-Im-PP4, are peptide-based constructs that, by virtue of exerting microbicidal action through microbial membrane disruption, are unlikely to induce development of antimicrobial resistance, while suitable for topical use.

5. Conclusion

The work herein described paves the way towards advancement of a new type of API, consisting of peptide-ionic liquid conjugates (PILCs), for development of a topical agent for the treatment of DFU. Herein, one such PILC was tested in non-infected wounds of diabetic mice, in a pilot study, that shown an overall improvement of healing rate, faster wound maturation, decreased levels of inflammation and oxidation markers, and increased neovascularization. It further showed the ability to decrease the microbial load in the wound bed. Jointly, these features contribute to overcome the chronicity that typically characterizes diabetic non-healing wounds, by promoting the normal progression of the healing process from the inflammatory to the proliferation and subsequent remodeling stages. This is vital for a proper resolution of the wound and restoration of the skin.

In short, results now reported unveil C_{16}-Im-PP4 and related PILCs as valuable tools for topical treatment of DFU with no symptoms of infection, or of infected DFU after biofilm removal by debridement. Indeed, debridement opens a window of opportunity for application of topical products that promote healing, by improving wound maturation and keeping microbiota under control, therefore avoiding reinfection. Ongoing work will expectedly contribute to the consolidation of PILC as a new chemotype for topical treatment not only of DFU, but also of DFI.

Ethics approval

All the experimental protocols involving animals were approved by the animal research ethics committee of the Center for Neuroscience and Cell Biology and the Faculty of Medicine of the University of Coimbra (ORBEA_213_2019/28082019) and by the national (Directorate-General for Food and Veterinary of the Portuguese Ministry of Agriculture) research ethical committee. Also, the animal protocols were in accordance with the European Directive 2010/63/EU and the Portuguese Decree-law (113/2013) for the use of animals for scientific purposes.

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CRediT authorship contribution statement

Ferraz Ricardo: Writing – review & editing, Conceptualization. **Calheiros Daniela:** Writing – review & editing, Conceptualization. **Gonçalves Teresa:** Writing – review & editing. **Gomes Ana:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **C. Leal Ermelindo:** Writing – review & editing, Investigation. **Da Silva Jessica:** Writing – review & editing, Investigation. **Teixeira Inês:** Investigation. **Carvalho Eugénia:** Writing – review & editing. **Gomes Paula:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

To Whom It May Concern,

The authors declare a conflict of interest. An international patent application protecting peptide-ionic liquid conjugate, whose *in vivo* activity is disclosed in this manuscript, for the prevention and/or treatment of skin disorders, has been filed by the following authors: Ana Gomes, Ricardo Ferraz, Paula Gomes (PCT/IB2023/056516).

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Data availability

Data will be made available on request.

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