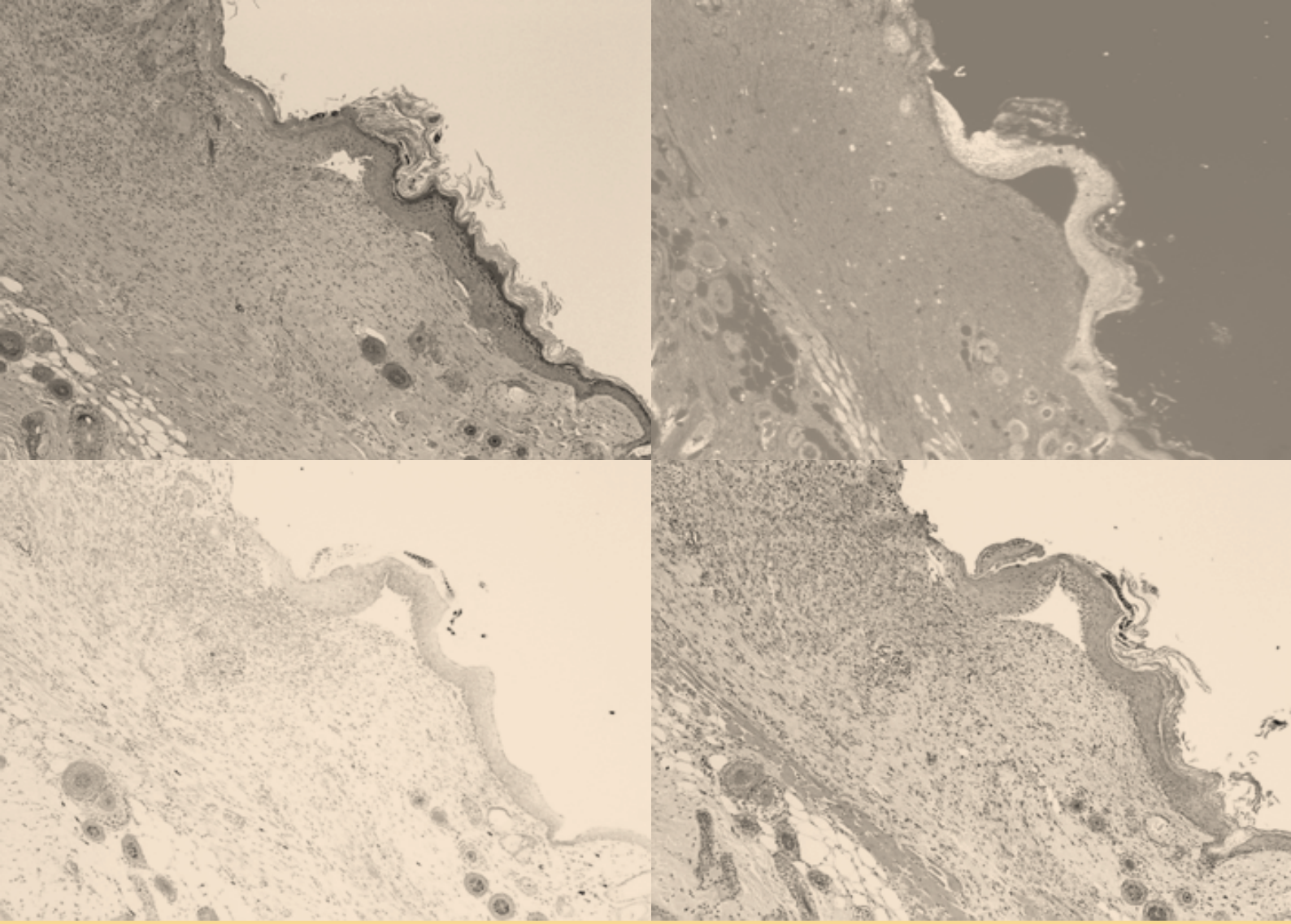

Ana Luísa Alves Tellechea

CELLULAR AND MOLECULAR MECHANISMS OF IMPAIRED WOUND HEALING IN DIABETES: UNDERSTANDING THE ROLE OF SUBSTANCE P AND MAST CELLS

Doctoral Thesis in Pharmacy, specialization in Cellular and Molecular Biology, supervised by Professor Aristidis Veves and by Doctor Eugénia Carvalho, co-supervised by Professor Teresa Cruz Rosete and presented to the Faculty of Pharmacy of the University of Coimbra.

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Cover: Panel of modified histological and immunohistochemical images of a mouse skin specimen showing part of the wound and peri-wound area: (1) Hematoxylin & Eosin (H&E) staining; (2) co-staining for CD68 and tumor necrosis factor-alpha (TNF- α); (3) Toluidine Blue staining; (4) CD31 staining. Images were modified to match the colors of the thesis cover according to the visual identity rules of the University of Coimbra.

Ana Luísa Alves Tellechea

Cellular and Molecular Mechanisms of Impaired Wound Healing in Diabetes: Understanding the role of Substance P and Mast Cells

Doctoral Thesis in Pharmacy, specialization in Cellular and Molecular Biology, supervised by Professor Aristidis Veves and by Doctor Eugénia Carvalho, co-supervised by Professor Teresa Cruz Rosete and presented to the Faculty of Pharmacy of the University of Coimbra.

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Ana Luísa Alves Tellechea

Mecanismos Celulares e Moleculares da Cicatrização Deficitária de Feridas na Diabetes: Compreender o papel da Substância P e dos Mastócitos

Tese de Doutoramento em Farmácia, na especialidade de Biologia Celular e Molecular, orientada pelo Professor Doutor Aristidis Veves e pela Doutora Eugénia Carvalho, co-orientada pela Professora Doutora Teresa Cruz Rosete e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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This PhD project was performed at the Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School, Boston, MA, USA and at the Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Coimbra, Portugal, under the supervision of Professor Aristidis Veves (BIDMC, Harvard Medical School) and Doctor Eugénia Carvalho (CNC, University of Coimbra) and co-supervision of Professor Teresa Cruz Rosete (CNC and Faculty of Pharmacy, University of Coimbra), supported by a doctoral fellowship from the Portuguese Foundation for Science and Technology (SFRH/BD/48624/2008).

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“And once the storm is over, you won’t remember how you made it through, how you managed to survive. You won’t even be sure whether the storm is really over. But one thing is certain, when you come out of the storm, you won’t be the same person who walked in. That’s what this storm is all about.”

Haruki Murakami

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Table of Contents

List of abbreviations

Ag	Antigen(s)
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
b-FGF /FGF-2	Basic fibroblast growth factor/Fibroblast growth factor-2
BM	Bone marrow
CCL	Chemokine (C-C motif) ligand
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CRF/CRH	Corticotropin-releasing factor/hormone
CSF-1	Colony-stimulating factor 1
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAB	Diaminobenzidine
DEJ	Dermo-epidermal junction
DFU	Diabetic foot ulcer(s)
DM	*Diabetes Mellitus*
DMN	*Diabetes Mellitus* neuropathy
DNA	Deoxyribonucleic acid
DSCG	Disodium cromoglycate (cromolyn)
EC	Endothelial cell(s)
ECM	Extracellular matrix
EGF	Endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell(s)

FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor
FDA	Food and Drug Administration
FFPE	Formalin fixed paraffin embedded
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hours
H&E	Hematoxylin and eosin
HbA1c	Hemoglobin A1c
HbO_2	Oxygen saturation of hemoglobin
hfp	High power field
HG	High glucose
HIF-1α	Hypoxia-inducible transcription factor-1 alpha
HLA	Human Leukocyte Antigen
HUVEC	Human umbilical cord vein endothelial cell(s)
i.m.	Intramuscularly
i.p.	Intraperitoneally
i.v.	Intravenously
IACUC	Institutional Animal Care and Use Committee
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-gamma
Ig	Immunoglobulin(s)
IHC	Immunohistochemistry
IL	Interleukin(s)
iNOS	Inducible nitric oxide synthase
IRB	Institutional Review Board
KC	Interleukin-8 related protein in rodents
KitIg	Kit ligand
LFA	Leukocyte-function-associated antigen

LIRB4	Leukocyte immunoglobulin-like receptor B4
LPS	Lipopolysaccharide
LT	Leukotriene(s)
MC	Mast cell(s)
MCP-1	Monocyte chemoattractant protein 1
MHSI	Medical Hyperspectral Imaging
min	Minutes
MIP	Macrophage inflammatory protein
mKitL	Membrane-bound kit ligand
MMP	Metalloproteinase(s)
MNC	Mononuclear cells
MS	Malphighian stratum
MSC	Mesenchymal stem cell(s)
NEP	Neutral endopeptidase
NG	Normal glucose
NGF	Nerve growth factor
NI	Neuro-ischemia
NK	Neurokinin
NK1R	Neurokinin 1 receptor
NKA	Neurokinin A
NKR	Neurokinin receptor(s)
NO	Nitric oxide
NO	Nitric oxide synthase
NPY	Neuropeptide Y
NS	Non significant
NT	Neurotensin
OCT	Optimal cutting temperature compound
OEC	Outgrowth endothelial cell(s)

PAD	Peripheral arterial disease
PAF	Platelet activating factor
Par-1	Protease-activated receptor-1
PB	Peripheral blood
PBS	Phosphate buffer solution
PDGF	Platelet-derived growth factor
PG	Prostaglandin(s)
PMN	Polymorphonuclear neutrophil(s)
PNS	Peripheral nervous system
PRP	Platelet rich plasma
PVD	Peripheral vascular disease
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rh	Human recombinant
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
SDF-1α	Stromal cell-derived factor 1 alpha
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SGPG	Serglycin proteoglycan
sKitL	Soluble kit ligand
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
SP	Substance P
ssDNA	Single stranded deoxyribonucleic acid
STZ	Streptozotocin

TAC1	Tachykinin 1
TBP	Tata binding protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.1% Tween-20
TCC	Total contact casting
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptors
TNF-α	Tumor Necrosis Factor alpha
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell(s)
WT	Wild type
α-MSH	Alpha-melanocyte stimulating hormone
α-SMA	Alpha-smooth muscle actin
β-hex	Beta-hexosaminidase

List of related publications

The work included in this thesis is based on the following **publications**:

1. **Tellechea A.***, Leal E.C.*, Veves A., Carvalho E. Inflammatory and angiogenic abnormalities in diabetic wound healing: the role of neuropeptides and therapeutic perspectives. *The Open Circulation & Vascular Journal* (*TOCVJ*) 2010, *3,* 43-55.*equally contributing authors.

2. **Tellechea A.**, Kafanas A., Leal E.C., Tecilazich F., Kuchibhotla S., Auster M.E., Kontoes I., Paolino J., Carvalho E., Pradhan Nabzdyk L., Veves A.. Increased skin inflammation and blood vessel density in human and experimental diabetes. *International Journal of Lower Extremity Wounds* (*IJLEW*) 2013, 12(1):4-11.

3. Leal E.C.*, Carvalho E.*, **Tellechea A.***, Kafanas A., Tecilazich F., Kearney C., Kuchibhotla S., Auster M.E., Kokkotou E., Mooney D.J., LoGerfo F.W., Pradhan-Nabzdyk L., Veves A. Substance P promotes Wound Healing in Diabetes. (*Submitted for publication to Diabetes* - DB14-0882) ***equally contributing authors**.

4. **Tellechea A.**, Leal E.C., Kafanas A., Auster M.E., Kuchibhotla, S., Ostrovsky Y., Tecilazich F., Zabolotny J.M., Carvalho E., Weng Z., Petra A., Pradhan-Nabzdyk L., Theoharides T.C., Veves A. Mast Cells are involved in Impaired Diabetic Wound Healing. (*In preparation*)

5. **Tellechea A.**, Silva E., Kuchibhotla, S., Anum S., Auster M.E., Pradhan-Nabzdyk L., Shih W., Mooney D.J., Veves A. New Biomaterials for Diabetic Wound Healing. (*In preparation*)

Abstract

Diabetes mellitus (DM) is one of the most predominant chronic diseases worldwide, representing a serious problem of public health. One of the most debilitating and costly complications of DM is the development of chronic, non-healing diabetic foot ulcers (DFU). Despite the efforts to develop new treatments for DFU, the currently available therapies are scarce and their efficacy limited. Therefore, understanding the complex multifactorial pathways that lead to DM-impaired wound healing is crucial in order to identify novel therapies for DFU.

Diabetic peripheral neuropathy (DPN), peripheral vascular disease (PVD), reduced resistance to infection, and biochemical abnormalities are major risk factors for DFU development. In addition, DM-related systemic inflammation and DPN-associated lack of neuropeptides have recently been suggested as important contributors to DFU failure to heal. However, there has been very little clinically-driven basic research into the mechanisms underlying the pathophysiology of DFU. The main objectives of this thesis are to identify the cellular and molecular mechanisms through which chronic inflammation and neuropeptide deficiency lead to wound healing failure in DM, and to develop new strategies that can reverse them, thus restoring the healing process.

To evaluate whether the chronic pro-inflammatory environment observed systemically in DM was present at the skin level, skin biopsies from diabetic (DM) patients and several DM animal models, together with their respective non-diabetic (non-DM) controls, were assessed for inflammation. The results showed not only increased inflammation, but also increased blood vessel density - confirmed by a specific marker - in the skin biopsies from all DM groups. These findings clearly indicate a pro-inflammatory state in DM skin.

Neuropeptides secreted by peripheral small nerve fibers participate in wound healing. The neuropeptide Substance P (SP) has shown to promote healing in non-DM and DM corneal

injuries and in non-DM cutaneous wounds. However, little is known regarding its effect and mechanisms of action in DM skin wounds. Therefore, the effect of local SP treatment, as well as the impact of SP- or SP receptor-deficiency were studied. The main findings were that SP improves DM wound healing mainly by promoting the acute inflammatory response at the early stages of healing and resolving inflammation at the later stages, thereby enabling progression to the proliferative phase. Conversely, SP deficiency was found to be associated with the chronic inflammation and healing failure that occur in DM. Additionally, SP levels were reduced in the skin of both DM human subjects and mice, whereas the local expression of the enzyme that digests SP - neutral endopeptidase (NEP) - was elevated. This work suggests that developing strategies to protect and locally deliver SP to the wound could be highly beneficial for DFU treatment.

Skin mast cells (MC) are located in close proximity to nerve endings and can be stimulated by neuropeptides, namely SP. Moreover, MC have been implicated in all phases of wound repair. However, there is scarce information about their function in DM skin and participation in DM-abnornal healing. To investigate this subject, MC were evaluated in the skin of DM patients and mouse models. DM skin showed increased pre-wounding MC degranulation that was associated with local and systemic inflammation. In addition, post-wounding MC degranulation was observed in non-DM mice but not in DM, while MC stabilization reversed this effect and ameliorated healing in DM mice. Moreover, MC deficient mice, with or without DM, showed abnormal wound healing. These results indicate that the presence of intact MC in unwounded skin and post-wounding degranulation are required for optimal healing, while pre-wounding MC degranulation has similar effects to MC absence. Taken together, these findings suggest MC stabilization as a potential target for novel DFU therapies.

Finally, new biomaterials that offer targeted and controlled delivery of multiple effectors involved in wound repair, namely neuropeptides and endothelial cell (EC) precursors,

were tested in experimental models of DM wound healing with promising results. This indicates that such tested bioproducts may prove useful for DFU treatment.

In summary, the results presented in this thesis clarify the mechanisms of DM-impaired wound healing and identify possible novel therapeutic strategies for DFU with high translational potential. Future studies will be required to validate these results in other pre-clinical models and to optimize treatments.

Resumo

Diabetes mellitus (DM) é uma das doenças crónicas mais predominantes no Mundo, representando um grave problema de saúde pública. Uma das complicações mais debilitantes e dispendiosas da DM consiste no desenvolvimento de úlceras crónicas, não cicatrizantes, do pé diabético (DFU). Apesar dos esforços para desenvolver novos tratamentos, as terapias actualmente disponíveis são escassas e de eficácia limitada. Assim, compreender as vias complexas e multifactoriais conducentes à cicatrização deficitária de feridas diabéticas (DM) é crucial para identificar novas estratégias terapêuticas para DFU.

A neuropatia diabética periférica (DPN), doença vascular periférica (PVD), reduzida resistência a infecção e anomalias bioquímicas são os principais factores de risco para o desenvolvimento de DFU. Além disso, a inflamação sistémica relacionada com DM e a deficiência em neuropeptídeos associada à DPN, foram recentemente propostos como factores importantes para o insucesso na cicatrização. No entanto, a investigação translacional dos mecanismos subjacentes à fisiopatologia da DFU é escassa. Os principais objectivos desta tese são identificar os mecanismos celulares e moleculares através dos quais a inflamação crónica e a deficiência em neuropeptídeos comprometem a cicatrização na DM, e desenvolver novas estratégias que os consigam reverter e restaurar o processo de cicatrização.

Para avaliar se o ambiente pro-inflamatório crónico observado sistemicamente na DM está presente ao nível da pele, analisou-se a inflamação em biópsias de pele de pacientes diabéticos (DM) e vários modelos animais DM, e dos seus respectivos controlos não diabéticos (non-DM). Observou-se não só um aumento da inflamação, como também da densidade de vasos sanguíneos, confirmada por marcação específica, nas biópsias de todos os grupos DM. Estes resultados indicam claramente um estado pro-inflamatório na pele DM.

Neuropeptídeos segregados pelas pequenas fibras nervosas periféricas participam na cicatrização de feridas. O neuropeptídeo Substância P (SP) revelou promover a cicatrização de feridas em córneas non-DM e DM, e em feridas cutâneas non-DM. No entanto, os seus efeitos e mecanismos de acção em feridas da pele DM continuam por esclarecer. Assim, o efeito do tratamento local com SP, bem como o impacto da deficiência em SP ou no seu receptor foram estudados. Os principais resultados revelaram que a SP melhora a cicatrização de feridas DM ao promover a resposta inflamatória nas fases iniciais da cicatrização e resolver a inflamação nas fases mais avançadas, permitindo a progressão para a etapa proliferativa. Contrariamente, a deficiência em SP demonstrou estar relacionada com a inflamação crónica e o insucesso na cicatrização que ocorrem na DM. Além disso, os níveis de SP estavam reduzidos na pele de pacientes e ratinhos DM, enquanto que a expressão local da enzima que degrada a SP, *neutral endopeptidase* (NEP), estava aumentada. Estes resultados sugerem que o desenvolvimento de estratégias de protecção e entrega local de SP às feridas poderá ser benéfico para o tratamento de DFU.

Os mastócitos (MC) da pele estão localizados perto de terminações nervosas e podem ser estimulados por neuropeptídeos, nomeadamente SP. Além disso, estão envolvidos em todas as fases da reparação de feridas. No entanto, existe pouca informação sobre a função dos MC na pele DM e sobre a sua participação na cicatrização anormal na DM. Para investigar este assunto, avaliaram-se os MC na pele de pacientes e ratinhos DM. A pele DM apresentou maior desgranulação dos MC associada a inflamação sistémica e local. Observou-se desgranulação dos MC pós-ferida apenas nos ratinhos não-DM, enquanto que a estabilização dos MC reverteu este efeito e melhorou a cicatrização nos DM. Além disso, ratinhos com défice em MC, com ou sem DM, revelaram cicatrização anómala. Estes resultados indicam que a presença de MC intactos na pele e a sua desgranulação pós-ferida são cruciais para uma cicatrização óptima, enquanto que a desgranulação dos MC pré-ferida apresenta efeitos semelhantes à ausência de MC. Estes

resultados sugerem a estabilização dos MC como um potencial candidato a novas terapias para DFU.

Por fim, novos biomateriais que oferecem uma entrega direccionada e controlada de múltiplos efectores envolvidos na cicatrização de feridas, nomeadamente neuropeptídeos e precursores de células endoteliais (EC), foram testados em modelos experimentais de cicatrização de feridas DM com resultados promissores, enfatizando o potencial terapêutico destes bioprodutos no tratamento de DFU.

Resumindo, os resultados apresentados nesta tese esclarecem os mecanismos celulares e moleculares envolvidos na cicatrização deficitária de feridas DM e sugerem novas estratégias terapêuticas para DFU com elevado potencial translacional. Estudos futuros serão necessários para validar estes resultados noutros modelos pré-clínicos e optimizar as terapias.

Chapter I

General Introduction

1. Chapter I - General Introduction

1.1. Anatomy and physiology of the skin

The skin is the largest organ of the body, accounting for approximately 16% of the total body weight with a surface area of 1.8m^2 in an average adult[1]. It provides an efficient barrier that prevents physical, chemical, and microbiological assaults from the external environment, as well as the loss of water, solutes and proteins from the internal milieu[2-5]. Body thermoregulation, vitamin D synthesis, immune surveillance and recognition, repair, and communication are other functions of the skin[4, 6]. Anatomically, the skin comprehends three anatomic compartments: the epidermis, of ectodermal origin, the dermis and the hypodermis or subcutis, both of mesodermal anlage (**Figure 1.1.**).

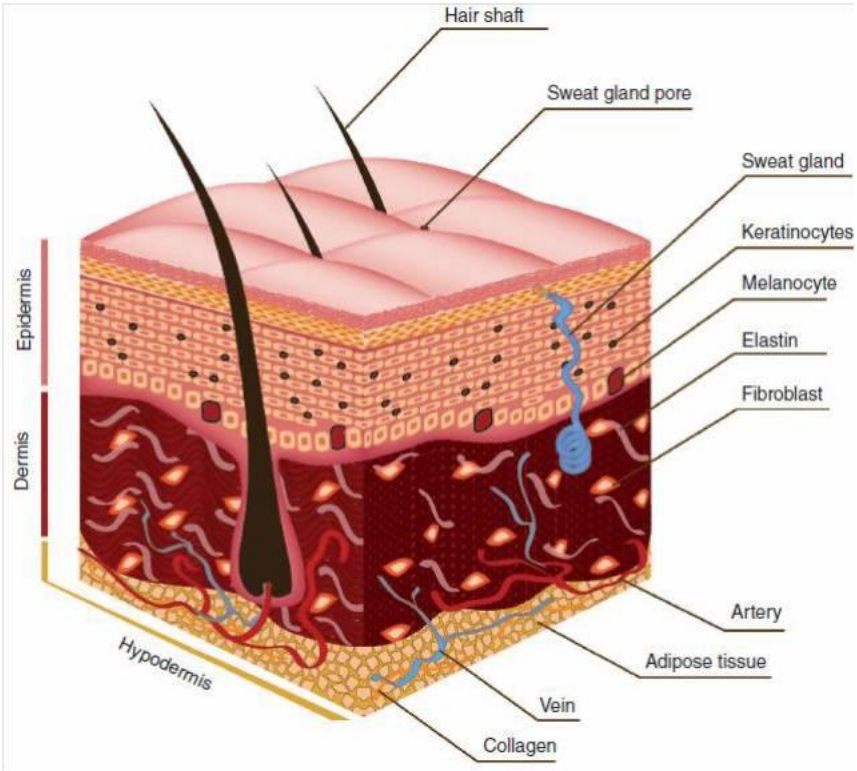


Figure 1.1. Anatomy of the skin. Diagram showing the skin layers, its appendages and the main cellular constituents. Obtained from Pereira *et al.,2013* [6] with permission of Future Medicine Ltd.

Blood and lymph vessels, as well as nervous tracts traverse the subcutis and dermis, which also contains the epithelial skin adnexae: pilosebaceous and sweat glands. Regional and functional variations occur along with differences in the relative thickness of the skin layers, blood and nervous supply, as well as type and density of the epithelial adnexae.

1.1.1. Epidermis

The **epidermis** is the outermost layer of the skin. It is composed by (i) a superficial stratum corneum (SC), where the physical skin barrier is primarily localized, made of highly resistant, keratin-rich, anucleated but metabolically active, tightly bound flat cells, the corneocytes and (ii) a deeper malpihghian stratum (MS) made of nucleated keratinocytes organized in a multilayered fashion (brick wall). Keratinocytes comprise the major cell type of the epidermis (>80%). They originate from stem cells, at the basal layer and as they move from the basement membrane to the surface they differentiate into the robust corneocytes, a process known as cornification or keratinisation. Approximately two weeks are needed for a basal keratinocyte to fully differentiate into a corneocyte (cornification) and about the same time is required for a newly formed corneocyte to detach from the outer SC (desquamation)[3, 6]. All these processes must be coordinated and highly regulated to enable a protective and fully functional role of the keratinocyte/corneocyte. Keratinocytes synthesize keratin, a structural protein that provides rigidity and permeability to the epidermis. They also subserve antigen presentation, anti-oxidative and detoxifying functions, as well as producing antimicrobial peptides, mucin, and a myriad of cytokines. Melanocytes (responsible for skin coloration), Langerhans cells (involved in skin immunosurveillance and skin contact sensitization) and Merkel cells, which associate with nerve terminals and act as mechanoreceptors (involved in touch sensation), are also present in the MS, but in smaller numbers[7]. The epidermis is avascular, depending on dermal blood vessels for oxygenation, provision of nutrients and removal of metabolic waste products.

1.1.2. Dermo-epidermal junction (DEJ)

The **dermo-epidermal junction** (DEJ) is a complex structure composed of the plasma membrane of keratinocytes and the basement membrane. The most obvious function of DEJ is to bind the epidermis tightly to the dermis. It provides support to the epidermis, determines cell polarity, and serves as a selective barrier for cellular and molecular traffic between layers. It is thought that the basement membrane influences epidermal differentiation and maintains the proliferative state of the basal layer. It is a crucial structure for wound healing, in that it provides a scaffold for keratinocyte adhesion and migration[8].

1.1.3. Dermis

The **dermis** is 10 to 40 times thicker than the epidermis. It contains different resident cells, namely the fibroblasts, which synthesize the dermal extracellular matrix (ECM), which is composed of both the robust collagen and elastic fibers, and also of non-fibrous glycoproteins[3, 9]. The former are responsible for the tension resistance and elastic properties of the skin respectively. The non-fibrous proteins of the ECM constitute a gel where the fibrous dermal proteins are immersed and assembled in complex networks interacting with cells, cytokines and growth factors. The multiplicity of these interactions determines their numerous and variable functional roles[10]. Additional dermal resident cells include dermal dendrocytes, mast cells (MC), macrophages, endothelial cells (EC) and vascular smooth muscle cells (VSMC)[6]. In contrast to the epidermis, the dermis is highly vascularised and innervated and contains folliculosebaceous and sudoriparous (eccrine and apocrine) adnexae. The blood vascular and eccrine sweat glands have crucial thermoregulatory functions, and the sebaceous glands contribute to the skin barrier function as well as to vitamin D synthesis.

1.1.4 Hypodermis

The **hypodermis** or **subcutis** varies in thickness depending on the body region. It is mainly constituted by adipose tissue, acting as an insulator and conserving energy[3, 6]. The lobules of fat cells are separated by fibrous septa made up of large blood vessels and collagen. Some authors have refused to include this layer as part of the skin referring to it as subcutaneous tissue, subcutaneous adipose tissue or subcutaneous fat. In response to this controversy, Driskell and colleagues have recently attempted to redefine the nomenclature for the cells and adipose tissue underlying the reticular dermis as **intradermal adipocytes** and **dermal white adipose tissue** respectively[11]. The resident intradermal adipocyte precursor cells enclose mature hair follicles and have been implicated in hair cycle regulation[12, 13]. In addition, it has been shown that these cells stimulate fibroblast migration and dermal regeneration during wound healing[14]. Further investigation is needed to better understand the role of intradermal adipocytes and dermal white adipose tissue in cutaneous wound healing.

1.2. Physiology of normal wound healing

Wound healing is an evolutionary conserved phenomenon, and is an innate response that aims at restoring tissue integrity. This complex and dynamic process involves several coordinated and overlapping phases, including coagulation-inflammation, proliferation and maturation[15] (**Figure 1.2.**). In normal skin, the epidermis and dermis function in a balanced manner to provide a protective barrier against the external environment. Immediately after a skin break is produced, blood components are released into the wound site and, after 2-3 hours, a fibrin clot is formed. This provides provisional repair and activates a cascade of events that culminate with wound closure. The aggregated platelets (thrombocytes) release cytokines and growth factors that recruit inflammatory cells to the area of injury. **Inflammation** can last from hours to days. Neutrophils arrive first to remove contaminating bacteria[16], and are followed by monocytes, which then differentiate into macrophages. Macrophages play an important role in enhancing the inflammatory response and tissue

debridement as they phagocyte bacteria and damaged tissue, and release proteases. Other cell types respond to the initial inflammatory signals and migrate to the wound site, including fibroblasts, epithelial cells, endothelial cells (EC), as well as circulating and local progenitor cells. Upon arrival, these cells start to proliferate and, through **neovascularization** and extracellular matrix (ECM) deposition, form a contractile granulation tissue that brings the wound margins closer. At the same time, a fresh surface epithelium covers the wound area. At the end of the proliferative phase, which usually takes 1-2 weeks, fibroblasts differentiate into myofibroblasts to promote contraction and eventual closure of the wound. The fibrin matrix and granulation tissue progressively differentiate and give place to collagen and scar tissue, approaching the structural and functional characteristics of the mature dermis, finally repairing the damaged barrier. While wound closure is the visible sign of complete healing; however, tissue regeneration continues through a dynamic and balanced process of collagen synthesis, reorganization, crosslinking and degradation. In fact, the remodeling step can last up to 18 months and, as this phase progresses, the tensile strength of the scar increases[17].

In summary, wound healing requires the orderly initiation and arrest of many complex biological phenomena including cell proliferation, cell migration, cell differentiation and ECM deposition[16]. These processes involve direct cell–cell and cell–matrix interactions, as well as the indirect crosstalk between different cell types by soluble mediators. Therefore, not only a correct balance of signaling molecules (growth factors, cytokines and chemokines), but also an effective and controlled cellular response from participating cells are essential for proper tissue repair[18-20].

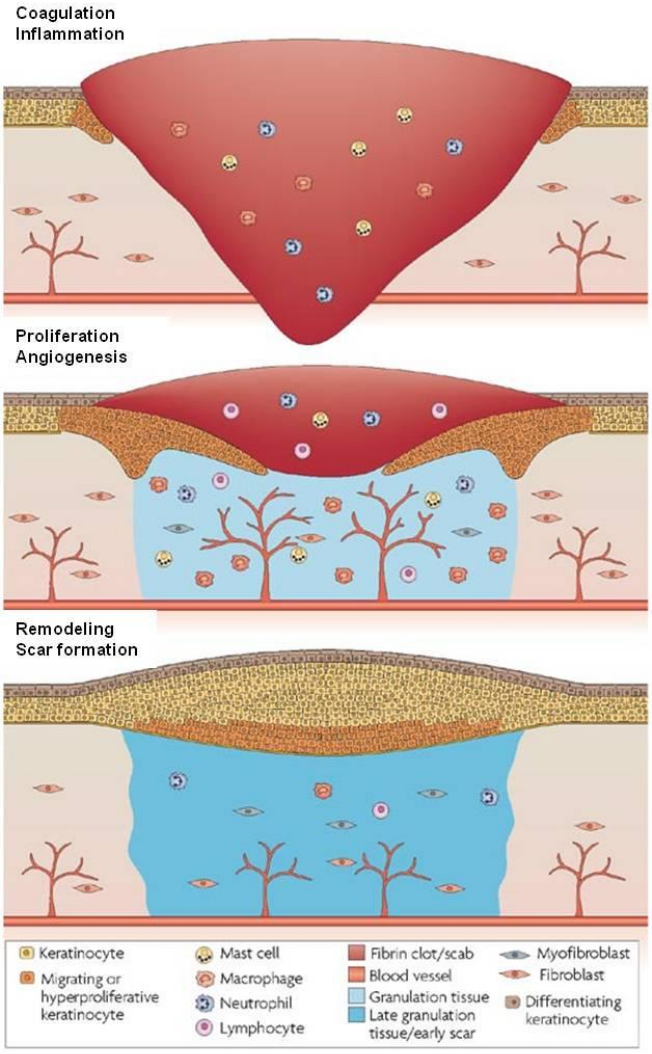


Figure 1.3. Normal wound healing process. Images representing the wound healing process, focusing on the main skin structures and the cell types involved in each phase. Adapted from *Schäfer & Werner, 2008*[21] with permission of Nature Publishing Group.

1.2.1. Inflammation in wound healing

Inflammation is essential to wound healing. However, to achieve successful healing, the inflammatory response must be tightly controlled in time, space and magnitude.

In physiologic conditions, skin injury causes a rapid onset of acute inflammation, which is mostly regulated by two principal effectors that are recruited from the circulation - polymorphonuclear neutrophils (PMN) and monocyte-derived macrophages. Neutrophils appear earlier than monocytes, arriving at the wound site within minutes, and become the

predominant cells during the first days, reaching their peak at day 2 post-injury. Therefore, the initial inflammatory phase is characterized by a massive infiltration of PMN. As this phase progresses, the number of PMN declines and is overcome by macrophages. PMN and monocytes from dermal blood vessels are actively attracted to the wound site by chemotatic signals, including cytokines and growth factors secreted by resident cells, such as interleukin 1-beta (IL-1 β), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), complement factor 5a, fibrin by-products, platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and even foreign epitopes, such as lipopolysaccharides and formyl-methionyl peptides derived from invading microorganisms[22-25]. These signals stimulate the secretion of adhesion molecules, including endothelial selectins (P- and E-selectins) and ICAM-1 and -2, crucial for neutrophil diapedesis[25]. The initial inflammatory trigger also leads to the rapid activation of resident skin immune cells - mast cells (MC), T cells, and Langerhans cells[26-28]. MC play an important role in leukocyte recruitment, as they release other potent chemoattractants, such as tumor necrosis factor-alpha (TNF-α), histamine, proteases, leukotrienes, and pro-inflammatory cytokines[26]. PMN destroy the invading microorganisms through several strategies, including bursts of reactive oxygen species (ROS)[29]. Studies suggest that wound neutrophils participate not only in the inflammatory response, but in many other aspects of repair, such as resolution of the fibrin clot and provisional ECM, angiogenesis, and re-epithelialization[30]. Once they have completed their tasks, neutrophils undergo apoptosis or are engulfed and degraded by macrophages, which are present at the wound site within 2 days after injury.

As monocytes migrate to the wounded area, they mature into macrophages that bind to specific proteins of the ECM and phagocyte microorganisms, foreign particles and cell debris, controlling and fighting infection. Monocyte/macrophage homing to the site of injury is regulated by different chemotactic agents, including growth factors, pro-inflammatory cytokines, and chemokines such as monocyte chemo-attractant protein-1/Chemokine (C-C motif) ligand 2 (MCP-1/CCL2), macrophage inflammatory protein-1 (MIP-1) and

RANTES/Chemokine (C-C motif) ligand 5 (CCL5)[18, 31-33]. Important sources of these molecules include platelets entrapped in the fibrin clot, as well as keratinocytes from the wound margins, fibroblasts, and leukocytes. Both forms of macrophages, “classically activated” (M1, pro-inflammatory) and “alternatively activated” (M2, anti-inflammatory), are present in the early stages of inflammation, but M2 macrophages predominate later in the repair process[34, 35]. Like neutrophils, the role of macrophages in wound healing is not limited to their immunological functions. Besides acting as antigen-presenting cells and phagocytic agents, macrophages also release a cocktail of growth factors, cytokines and reactive oxygen and nitrogen species at the wound site, which further promotes cell proliferation and the synthesis of ECM molecules by resident skin cells[34, 35].

Among the substances secreted by PMN and/or macrophages are TNF-α, IL-1, IL-6, PDGF, TGF-α and TGF-β, epidermal growth factor (EGF), basic-fibroblast growth factor (b-FGF/FGF2), vascular endothelial growth factor (VEGF), and nitric oxide (NO). These molecules contribute to the activation, proliferation and migration of fibroblasts and EC from the adjacent dermis, inducing the granulation tissue formation[16, 36, 37]. Therefore, this inflammatory cell influx is crucial in the transition between the process of inflammation and the actual repair phase. One of the major roles of the inflammatory cytokines released by these cells is to regulate angiogenesis, which they accomplish in concert with signals from other cells present at the wound site and in circulation.

1.2.2. Neovascularization in wound healing

Neovascularization generates the mature circulatory system, one of the first and major systems to create and maintain metabolic homeostasis through the supply of oxygen and nutrients, as well as the removal of waste products[38, 39]. In normal mature tissues the vessels are in general quiescent as cells produce low levels of pro-angiogenic molecules and high levels of angiogenesis inhibitors. In the presence of a skin injury, platelets, inflammatory cells, fibroblasts and injured EC secrete pro-angiogenic factors that trigger the formation of new blood vessels within the granulation tissue. During wound healing, neovascularization

takes place to replace the damaged capillaries and re-establish the supply of oxygen and nutrients to the wound. Neovascularization of the wound's granulation tissue occurs as a result of angiogenesis and/or vasculogenesis[38] (**Figure 1.4**).

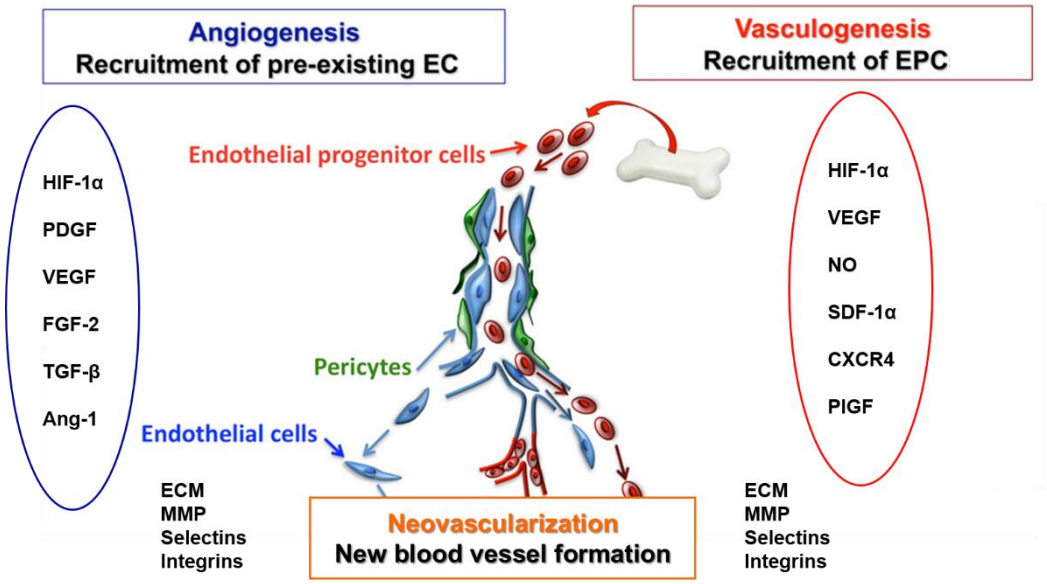


Figure 1.4. Angiogenesis and vasculogenesis. Image representing angiogenesis and vasculogenesis, two different processes of new blood vessel formation during wound healing, as well as the main cells and factors involved in each phenomenon. Adapted from *Castillo-Melendez et al, 2013*[40] with permission of Frontiers Science Production.

1.2.2.1. Angiogenesis in wound healing

Angiogenesis is the process by which new capillaries are developed from pre-existing blood vessels, after destabilization of the endothelial tubular structure. During wound healing, resident EC of the wound's adjacent mature vascular network, in response to pro-angiogenic signals, proliferate, migrate, and remodel into new capillaries that grow within the wound substrate and form a cord-like structure[38, 41-44]. Several molecules, including growth factors, ECM proteins, matrix metalloproteinases (MMP), integrins and cytokines, interplay co-ordinately in highly complex scenarios to replace the damaged vessels and re-establish the vasculature, restoring the supply of oxygen and nutrients to the wound site.

In the wound area, the local low oxygen tension stimulates hypoxia-inducible transcription factor-1 alpha (HIF-1α), which in turn increases the production of angiogenic growth factors such as VEGF[45, 46] and the expression of the chemokine receptor CXCR4 [47]. Meanwhile, activated platelets release PDGF[48], VEGF[49], FGF-2[50], TGF-β[50] and angiopoietin-1 (Ang-1)[51]. Furthermore, other cells present at the wound area, such as fibroblasts, inflammatory cells, keratinocytes and EC secrete growth factors and cytokines. The result is a potent chemotactic signal that activates EC to migrate to the ECM, proliferate and form a new immature vascular structure[38, 52-54]. Invasion of EC depends on vascular permeability, peri-endothelial support loss and disruption of the basal membrane[55]. Proteolytic enzymes, including serine proteases, urokinase plasminogen activator, and MMP, are released by EC to digest the basement membrane and ECM components[56]. MMP digest specifically the ECM components of the provisional matrix and stimulate cell proliferation and migration, either directly through activation of EC or indirectly via interaction with adhesion molecules and integrins[56]. As EC enter the wound tissue, they must continue to proliferate, arrange three-dimensionally as channels and produce a basal lamina[57]. Fibroblasts produce ECM proteins that act as a scaffold support for the new vascular network, through which EC can migrate, and serve as a reservoir and modulator for growth factors such as FGF-2, VEGF and PDGF[44, 58]. Finally, PDGF recruits VSMC and pericytes to provide stabilization and maturation of the new vasculature[59-61].

1.2.2.2. Vasculogenesis in wound healing

Vasculogenesis is the *de novo* formation of blood vessels from the differentiation of bone marrow-derived precursor cells[38]. During wound healing, bone marrow-derived endothelial progenitor cells (BM-EPC) are mobilized to the peripheral circulation and subsequently to the site of injury, where they differentiate into mature EC and give rise to a new vascular network[62-64]. BM-EPC respond primarily to local tissue ischemia by angiogenic chemokine gradients that promote their recruitment and subsequent homing to the wound site[63, 65, 66]. Upon the hypoxic signal, VEGF is released and upregulates the production of NO in the

bone marrow (BM) through the activation (phosphorylation) of the stromal BM nitric oxide synthase (NOS). In this process, the endothelial isoform of nitric oxide synthase (eNOS) plays a central role[67, 68]. Elevated NO levels stimulate EPC production from the BM, proliferation and mobilization to the bloodstream, whereas the chemokine stromal cell-derived factor-1α or chemokine (C-X-C motif) ligand 12 (SDF-1α or CXCL12)/chemokine (C-X-C motif) receptor 4 (CXCR4) axis promotes the homing of these cells to the injured area. In addition to VEGF, placental growth factor (PlGF) also exerts its vasculogenic effects through VEGF receptor 1 (VEGFR1) with subsequent metalloproteinase-9 (MMP-9) secretion. MMP-9 mediates the conversion of membrane-bound kit ligand (mKitL) to soluble kit ligand (sKitL), which contributes to EPC proliferation, recruitment and maturation[69]. Furthermore, adhesion molecules, such as selectins and β-integrins, as well as activated platelets promote adhesion and differentiation of EPC[70]. VSMC may also sequester circulating EPC through chemokine secretion[71]. Once recruited and firmly adhered to the site of injury, EPC contribute to the maintenance of the endothelial monolayer but also exert paracrine effects by producing pro-angiogenic growth factors and cytokines, which in turn promote mobilization of adjacent mature EC[72].

1.3. Pathophysiology of wound healing in Diabetes *Mellitus* (DM)

1.3.1. DM and its complications

Diabetes *Mellitus* (DM) is a serious problem of public health, and one of the most prevalent chronic diseases worldwide. In 1992, Zimmet first used the term “epidemic of diabetes”, highlighting the alarming rate at which human suffering and economic burden were increasing as a consequence of the disease[73]. Recent studies estimate that approximately 285 million worldwide suffer from DM and its prevalence is expected to rise to 439 million by 2030[74-76]. In 2013, it was estimated that DM affects 24.4 million in the USA[77] and 56.3 million in Europe[77], with Portugal accounting for approximately 1 million (12.9% of its population)[78]. Aging and poor lifestyle habits of the population are considered to be the main reasons for these escalating numbers[75].

DM is a clinical condition characterized by chronic hyperglycemia and by imbalances in carbohydrate, lipid, and protein metabolism. This metabolic disorder is caused by an inability to efficiently mobilize glucose from the bloodstream into the cells, due to abnormalities in insulin secretion, insulin sensitivity, or both[79, 80].

There are two major forms of diabetes – Type 1 DM, accounting for approximately 5% of the total cases, and Type 2 DM, accounting for almost 95% of the diabetic population[81]. A third type is gestational DM, which only occurs during pregnancy. Genetic abnormalities affecting β-cell function and insulin action, pancreatic diseases, certain pharmacological or chemical agents, and other conditions can also cause other types of diabetes.

Type 1 DM is characterized by an autoimmune process that selectively destroys pancreatic β-cells, leading to absolute insulin deficiency and, consequently, to the total dependence on exogenous insulin to sustain life[80, 82]. In the past, Type 1 DM was also referred to as juvenile onset diabetes, because it was more likely to occur during childhood, with a peak incidence between the ages of 8 and 14. It is now well recognized that there is also an increased risk for Type 1 DM onset in adult life, between 60 and 80 years old[83, 84].

Type 2 DM is characterized by resistance to insulin action which can be combined with a relative insulin deficiency. Type 2 DM was previously referred to as adult onset diabetes, due to its likelihood of occurring later in life[85]. However, the prevalence of T2DM in the younger population is on the rise likely due to the growing incidence of obesity in younger people, including children[85]. There are several different medications available for type 2 DM patients that may improve insulin sensitivity, increase the amount of available insulin by stimulating its endogenous production, or provide exogenous insulin. Non-pharmacological approaches, such as an adequate diet and regular physical exercise can also help prevent, delay or control type 2 DM[86, 87].

The chronic hyperglycemia present in DM is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

As a result, DM complications include stroke, coronary heart disease, and peripheral vascular/arterial disease (PVD/PAD) - **macrovascular complications** -, as well as retinopathy, nephropathy, neuropathy - **microvascular complications**. In fact, DM patients are at 2- to 4-fold higher risk of death from cardiovascular disease[81]. Moreover, DM is the major cause of blindness in adults over the age of 65, and of preventable blindness in adults from 20 to 74 years old[81]. DM is also the leading cause of chronic kidney disease and renal failure[81].

Neuropathy is another microvascular complication of DM that accounts for considerable morbidity and mortality. There are several neuropathic syndromes associated with DM. However, the most common manifestation is chronic distal symmetrical polyneuropathy, also referred to as diabetic peripheral neuropathy (DPN). DPN has been identified by the Toronto Diabetic Neuropathy Consensus Panel as "a symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycemia exposure and cardiovascular risk covariantes."[88] It involves abnormalities in the activity of the nerves and thus may affect autonomic, motor and sensory functions. Therefore, it is a major risk factor for the development of **diabetic foot ulcerations** (**DFU**). DPN affects approximately 50% of the entire diabetic population, irrespective of Type 1 or Type 2 DM, and its prevalence almost doubles in patients with DM when compared to non-DM subjects[89-91].

1.3.2. Diabetic Foot Ulceration (DFU)

One of the most severe and debilitating complications of DM is the development of chronic, non-healing foot ulcers. **Diabetic foot ulceration** (**DFU**) is estimated to occur in 15%-20% of diabetic patients, often requires prolonged hospitalizations for its management, severely impairs quality of life and is the major cause of non-traumatic amputations in the western world[92, 93]. In addition, it consumes 25–50% of the total cost of DM care[94].

The pathophysiologic relationship between DM and impaired healing is complex and multifactorial. In contrast to acute wounds, which progress through the phases of wound healing linearly and orderly in healthy individuals, chronic wounds in diabetic patients become stalled in different phases and progression does not occur in synchrony due to DM-associated neuropathy, vascular disease, impaired immune function and biochemical abnormalities[15, 16, 95] (**Figure 1.5.**).

According to different reports, the prevalence of **peripheral arterial disease** (**PAD**) in patients with DFU varies from 10% to 60%[96-99]. Additionally, it has been shown that the severity of PAD can be associated with higher rates of lower extremity amputation, morbidity and mortality[100].

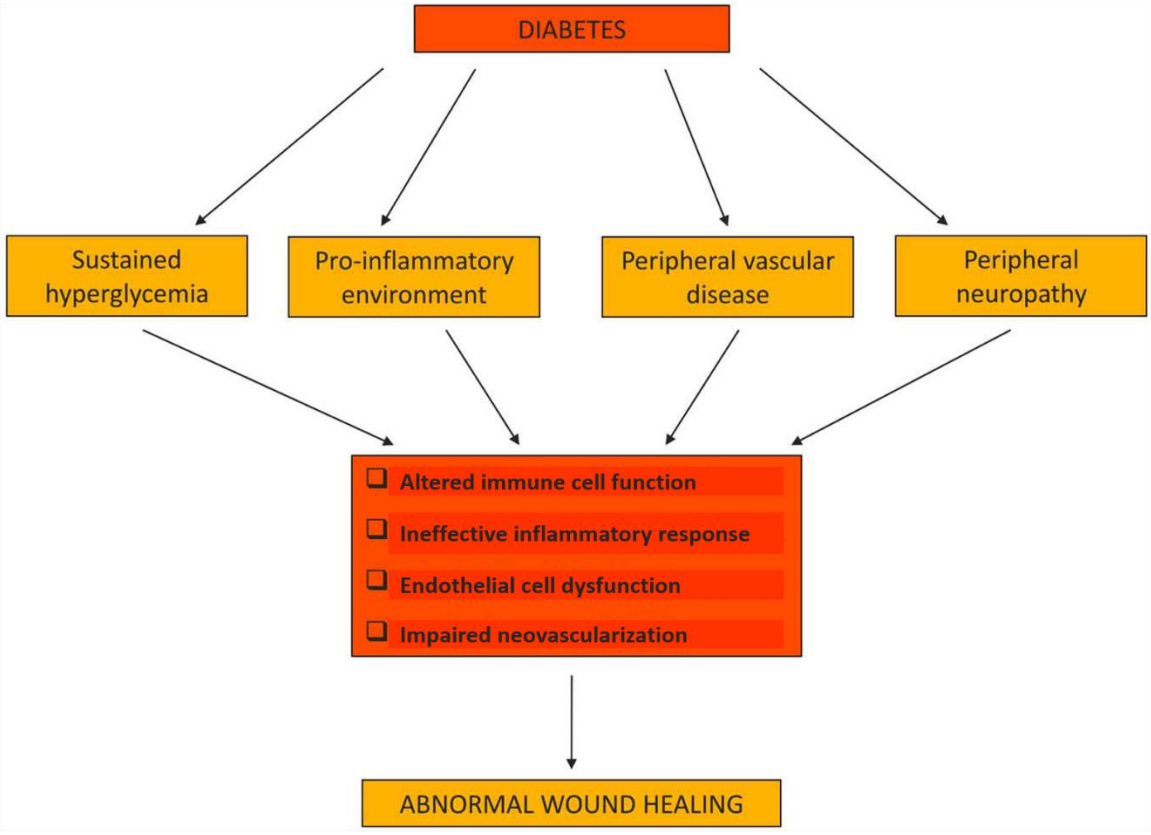


Figure 1.5. Overview of DM-impaired wound healing. Obtained from *Tellechea et al, 2010[101].*

Diabetic peripheral neuropathy (**DPN**) is characterized by a progressive loss of nerve fibers that eventually leads to loss of pain perception. It has been estimated that up to 85%

of DFU are associated with DPN[102], making it one of the most important contributing factors to ulcer development and failure to heal. The mechanisms through which DPN triggers the pathogenic pathway for ulceration are complex. The reduction of protective sensitivity, including sensitivity to pain and heat, leads to a diminished perception of pain stimuli. In addition, DPN affects the motor system causing a progressive weakening of the intrinsic muscle components[103]. Furthermore, the autonomous component of neuropathy leads to anhydrosis resulting in dry skin with lowered barrier function, and increases the arterio-venous shunting, leading to altered skin and bone perfusion[104].

Two other important factors contribute to wound healing impairment in DM, namely the functional changes in microcirculation and abnormalities in cellular activity, as well as the expression of the various growth factors and cytokines that are involved in tissue repair.

Additional risk factors for the development of DFU include long duration of DM, prior history of ulceration, and prior amputation.

1.3.3. Inflammation in diabetic wound healing

A DM setting, either of type 1 or type 2, is characterized by sustained hyperglycemia and chronic elevation of pro-inflammatory mediators. This environment induces and perpetuates the inflammatory responses, leading to a chronic inflammatory state. However, this condition is considered a low-grade inflammation, since the hyperglycemic background causes impaired cellular defense mechanisms that fail to mount an acute response.

Studies have shown that the prolonged inflammatory phase in DM wound healing delays the formation of mature granulation tissue and reduces the wound tensile strength[22]. In addition, analysis of the fluid of DM wounds from both animal models and human patients has shown insulin-degrading activity, which has in turn been correlated with the levels of hemoglobin A1c (HbA1c)[105]. This indicates a straight relationship between sustained hyperglycemia and the wound proteolytic environment. Moreover, the expression and activity of some MMP, such as MMP-2, -8, -9, -14 and -26 was found to be increased in DM

chronic wounds compared to normal acute wound healing, whereas the tissue inhibitors of MMP - TIMP-1 and TIMP-2 - were found to be reduced[106-109]. This MMP/TIMP imbalance suggests that the increased proteolytic activity in DM wounds contributes to poor formation of new connective tissue, further impairing the healing process.

Furthermore, macrophage efferocytosis is impaired in DM wounds, resulting in increased apoptoptic burden and imbalanced inflammatory status with higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory mediators[110, 111]. Also, in DM, neutrophils show reduced chemotatic and phagocytic activities, rendering the wounds more prone to infection[112, 113]. In fact, DM patients have over 50% higher risk of wound infection and are also more likely to develop biofilms compared to non-DM subjects[114-117]. Naturally, infection and biofilm formation further hinders the healing process.

As a result, and in contrast to normal wound healing, where inflammation occurs in a sequential, regulated and self-resolving manner, the immune and inflammatory responses in diabetic wounds appear prolonged and non-effective. It is currently believed that aberrant expression and activity of growth factors and cytokines is the main reason why DFU fail to progress to the proliferative phase and are stalled in a chronic inflammatory state. Further understanding of this process could help identify and develop new therapeutic strategies.

1.3.4. Neovascularization in diabetic wound healing

As a result of complex and multifactorial pathophysiological processes, both angiogenesis and vasculogenesis appear dysfunctional in DM-associated impaired wound healing (summarized in **Table 1.**).

1.3.4.1. Angiogenesis in diabetic wound healing

Dysfunctional angiogenesis is involved in several DM complications, including retinopathy, nephropathy, neuropathy, and abnormal wound healing[15, 118-121]. However, the pathogenesis of DM angiogenic complications remains largely unknown[122-124].

Topical administration of D-glucose (22 mM) to wounds of non-DM rats was shown to inhibit the normal angiogenic process[125], suggesting a direct role for hyperglycemia in DM-impaired neovascularization. In addition, a number of growth factors essential for wound healing, including the pro-angiogenic molecules bFGF/FGF-2 and PDGF, were found to be reduced in experimental DM wounds[126-128]. Similarly, VEGF, a key player in vascular growth, has been shown to be deficient in both experimental and human diabetic wounds[129], and topical application of VEGF has been proven to accelerate healing in a diabetic mouse model[130]. Fibroblast abundance and function are reduced in DM wounds, showing a diminished proliferative capacity and decreased production of angiogenic factors and ECM components[38, 131, 132]. Moreover, the increased expression of MMP and reduced expression of tissue inhibitor of metalloproteinase (TIMP)-1 and -2 in DFU, also contributes to abnormal angiogenesis and failure to heal[106, 108, 109, 133, 134]. Finally, the abnormal hypoxic environment in DM wounds further impairs angiogenesis[135, 136].

1.3.4.2. Vasculogenesis in diabetic wound healing

Increasing evidence suggests that tissue repair mechanisms are compromised in DM as a result of an impaired BM-EPC response[39, 137-140].

The number and function – proliferation, adhesion and incorporation into the vascular structure - of circulating EPC have been shown to be reduced in DM[39, 137, 138]. In addition, EPC recruitment from the circulation and homing to the site of injury were reported to be greatly impaired[141, 142]. These changes are likely to be involved in the pathogenesis of vascular disease in DM, and contribute to poor healing through abnormal vasculogenesis.[137]

EPC are mobilized by eNOS activation in the BM and eNOS activity was shown to be decreased in DM mice[65], thus preventing EPC from reaching the wound site in significant numbers. In addition, the recruitment of circulating EPC to the site of injury is dependent on the ischemia-induced upregulation of SDF-1α/CXCL12, which was found to be reduced in

epithelial cells derived from wounds of streptozocin (STZ)-induced DM mice[65]. More importantly, the local injection of recombinant SDF-1α/CXCL12 protein significantly enhanced EPC recruitment to the wound site and improved healing in DM mice. Despite these promising data from animal models, there is still limited information regarding human subjects.

	Normal wound healing	Diabetic wound healing
ANGIOGENESIS	Pro-angiogenic molecules are secreted by platelets, inflammatory cells, fibroblasts and keratinocytes	Growth factors including VEGF, FGF-2 and PDGF, are reduced in diabetic wounds, possibly due to fibroblast dysfunction
	Local ECs lose their interaction with neighboring ECs	Resident ECs of the diabetic wound may fail to support new vessel formation
	MMPs digest the ECM components of the provisional matrix and stimulate cell activation, proliferation and migration	The balance between ECM acumulation and remodelling is disturbed due to an increased MMP/TIMP ratio
	Local cells continue to produce pro-angiogenic growth factors and cytokines	The proteolytic environment of the diabetic wound compromises cellular proliferation and angiogenesis
	ECs migrate, proliferate, grow and mature into new vessels	Disruption of new vessel formation and abnormal hypoxic conditions prevent angiogenesis
VASCULOGENESIS	EPCs are produced in the BM and recruited to the peripheral circulation through hypoxic and chemotactic signals	EPC proliferation is impaired in diabetes
	Increased VEGF and PlGF levels activate VEGFR-1 and stimulate MMP-9 secretion	Growth factors expression (including VEGF) is decreased due to hyperglycemia
	MMP-9 stimulates the secretion of soluble kit-ligand (sKitL) from the membrane bound mKitL, inducing EPC mobilization to circulation	Reduced number of EPCs compromises vasculogenesis. EPC recruitment is also impaired in DM.
	Circulating EPCs differentiate into late EPCs and gain specific EC markers	EPCs are dysfunctional in diabetes and blood supply is reduced
	Late EPCs arrive to the wound, adhere and further differentiate into mature ECs and secrete more pro-angiogenic factors	The mechanisms of EPC homing to the site of injury, including SDF-1$^{\alpha}$/CXCR4 axis, are compromised

Table 1. Major differences in neovascularization between normal and diabetic wounds. Adapted from *Stojanic et al., 2012*[143] with permission of Springer.

A recent study by Tecilazich and colleagues has shown decreased EPC numbers in DM patients with complications, with or without DFU[144], suggesting that DPN affects EPC abundance. The same study reported increased serum levels and skin expression of SDF-1α/CXCL12 in DFU patients but found no association between these baseline levels and complete healing or reduction in ulcer size[144]. However, complete wound closure was associated with a parallel reduction in circulating EPC, suggesting enhanced homing during the healing process. In contrast, DM mice and rabbits that have impaired wound healing

showed increased EPC after wounding when compared to non-DM animals, suggesting impaired trafficking of the progenitor cells[144].

Growth factors, such as VEGF and GM-CSF, can act not only as pro-angiogenic but also as pro-vasculogenic factors, by inducing the release of EPC from the BM. However, the nonspecific effects in other cells, such as white cells and platelets, or the leaky-capillary effect, especially in the retina, raise concerns in using these factors to treat DM patients with chronic non-healing wounds[145-148].

1.4. Neuropeptides

Increasing evidence suggests an important regulatory role of peripheral nerves and cutaneous neuro-immunology in normal wound healing[149]. The peripheral nervous system (PNS), acting through neuropeptides, not only relays sensory information to the central nervous system (CNS) but also participates in the inflammatory, proliferative, and reparative processes that occur after injury. Cutaneous nerve fibers and inflammatory cells release neuromediators, including cytokines and neuropeptides. Neuropeptides mediate their actions by binding to specific receptors found on various cells in the skin, including immune cells, Langerhans cells, EC, MC, fibroblasts and keratinocytes[149-151]. Several neuropeptides expressed in the skin are known to be involved in wound healing, including substance P (SP), neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), corticotropin-releasing factor/hormone (CRF/CRH), alpha-melanocyte stimulating hormone (α-MSH) and neurotensin (NT)[149].

1.4.1. Substance P (SP)

One neuropeptide that has been known to play an essential role in wound healing is **Substance P** (**SP**). This 11-amino acid peptide is a member of a family of structurally related peptides called tachykinins. SP is present in many areas of the central and peripheral nervous systems. In the periphery, it is located especially in areas of immunologic importance, such as the skin, gastrointestinal tract, and respiratory tract[152]. SP is

synthesized in the dorsal root ganglia, from which it migrates centrally to the dorsal horn of the spinal cord and peripherally to nerve terminals of sensory neurons[153]. Tachykinins exert their actions by activating 3 primary types of neurokinin (NK) receptors: NK1R, NK2R, and NK3R, with NK1R being the predominant and with highest affinity. All 3 receptors are members of the superfamily of receptors coupled to G-regulatory proteins. Receptor stimulation leads to the activation of phospholipase C and thus to the generation of inositol triphosphate and diacylglycerol, as well as to the release of Ca^{2+} from internal stores[154-156].

1.4.1.1. SP in wound healing

SP-positive nerve fibers supply the dermis and epidermis as well as innervate dermal blood vessels and hair follicles. Several exogenous and endogenous factors can stimulate the release of SP from peripheral nerves into the skin, including physical stimuli (skin break, heat, ultraviolet radiation, scratching), allergens, and inflammatory mediators (bradykinin, prostaglandins, proteases, cytokines)[157].

During a skin insult, SP is released by the sensory nerve fibers and activates several target cells, including endothelial cells (EC), mast cells (MC), fibroblasts and keratinocytes via NK receptors[150]. SP causes vasodilation through direct action on VSMC and enhanced production of NO by the endothelium[158, 159]. In addition, SP can increase vascular permeability and protein extravasation, enhancing leukocyte infiltration to the site of injury[152]. SP also acts as a strong chemoattractant for immune cells, which can stimulate the production of several cytokines from MC, macrophages and neutrophils.

MC are closely associated with SP-positive sensory nerves in many tissues, including the skin. There is a bidirectional communication between MC and primary sensory neurons, as neuropeptides stimulate MC and MC mediators regulate neuropeptide release. SP induces TNF-α[160] and VEGF[161] release from MC, thus participating in inflammation, immunity, and angiogenesis, all of which are important to wound healing. In addition to cytokines, SP stimulates the release of MMP and ROS from neutrophils, and activates their phagocytic

function. Human Langerhans cells also express SP[162], which in turn regulates T-cell proliferation[163] and other dermal cells, such as keratinocytes. In addition, SP modulates leukocyte adhesion to microvascular EC in the skin[164] by upregulating cell adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, P-selectin, and leukocyte-function-associated antigen (LFA)-1 in EC and lymphocytes[165-168]. This will lead to proliferation of EC and angiogenesis in the wound area. The effects of SP in the skin after injury are summarized in **Figure 1.6.**.

SP can also exert a trophic function in wound healing. This neuropeptide was shown to mediate healing, after UV-induced damage of the skin, by stimulation of angiogenesis or epidermal cell proliferation[169]. Moreover, SP induced angiogenesis in an *in vitro* model using human umbilical cord vein EC (HUVEC) cultured on a basement membrane (Matrigel) substrate and it stimulated EC differentiation into capillary-like structures in a dose-dependent manner[164]. Furthermore, capillary and fibroblast proliferation were enhanced by SP treatment after operative repair of the Achilles tendon in rats. SP was also shown to induce proliferation of human dermal fibroblasts through a NK1R-dependent mechanism[170].

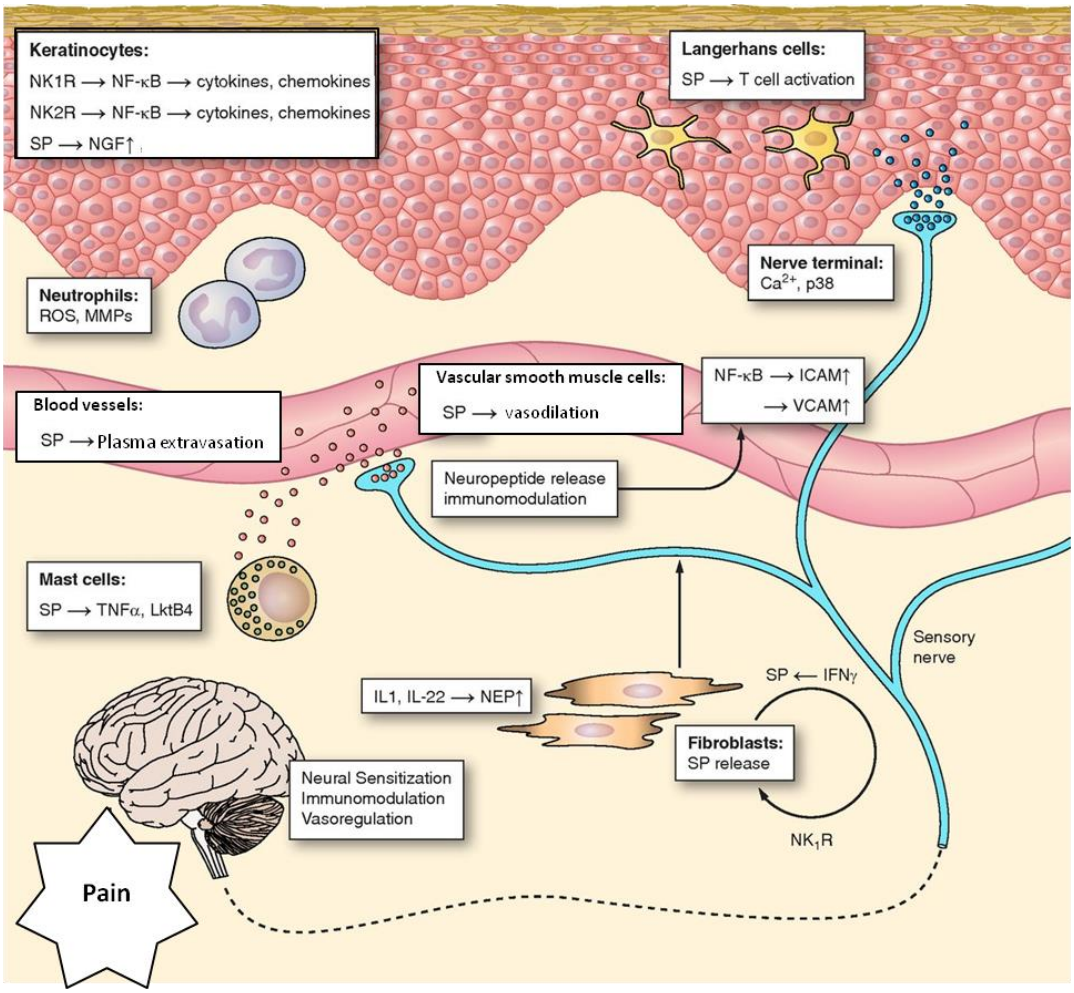


Figure 1.6. SP functions in wounded skin. Image representing SP release after skin injury and its main functions, with focus on inflammation. Adapted from *Steinhoff et al., 2014*[157] with permission of The American Physiological Society.

In a corneal epithelial wound healing model using DM rats, the combination of an SP-derived molecule (FGLM-NH2) with insulin-like growth factor 1 proved to have beneficial effects[171]. Due to the avascular nature of the cornea, these findings suggest that SP may have an effect not only on vascular cells but also epithelial cells. In a recent study, SP was topically administered to excisional wounds of non-DM rats and resulted in increased wound contraction and wound size reduction[172]. Local application of SP accelerated all phases of the healing process according to histological findings. At the molecular level, SP treatment increased TNF-α and reduced IL-10 levels during the first days after wounding, suggesting a pro-inflammatory effect during the acute inflammatory response. On the other hand, SP

increased VEGF and TGF-β1 expression during the later stages of healing, suggesting a role in angiogenesis and tissue regeneration. Gibran and collaborators[173] have demonstrated that exogenous SP improves wound healing kinetics in a db/db DM mouse model and have also shown fewer nerves in the epidermis of both DM patients and db/db mice.

SP serum levels are significantly decreased in type 1 DM subjects and further reduced in subjects with DPN when compared with controls[174]. Similarly, morphological and immunohistochemical analysis in DM subjects have shown a depletion of SP in the central and peripheral nervous system[175, 176], including the skin[177, 178]. In addition, SP gene expression and protein levels were shown to be reduced in experimental type 1 DM using a rabbit ear wound healing model[179, 180].These results suggest that insufficient nerve-derived mediators, including SP, contribute to the impaired response to injury.

The biological action of SP is regulated by a cell surface metallopeptidase - **neutral endopeptidase** (**NEP**) - which degrades SP. The skin of patients with DPN was found to have elevated NEP activity that may contribute to the deficient neuroinflammatory signaling and abnormal healing[181]. Furthermore, the skin from DM db/db mice showed increased NEP activity, whereas NEP inhibition was found to accelerate wound closure[182] .

In summary, it is evident that SP plays an important role in the inflammatory and angiogenic phases of wound healing. Therefore, it is anticipated that insufficient levels of SP, which occur in DPN, would significantly compromise wound repair. However, there is still little information regarding the effects of SP in DM cutaneous wounds, which are characterized by chronic inflammation, neuroischemia (NI), and overexpression of MMP, which degrade ECM proteins and growth factors involved in tissue repair.

1.5. Mast cells (MC)

1.5.1. MC development and maturation

Mast cells (**MC**) are immune cells that originate from hematopoietic pluripotent stem cells in the bone marrow[183-186]. Committed MC-progenitors are released into the bloodstream and subsequently home to virtually every organ in the body. MC differentiate and mature under the influence of tissue-specific growth factors and cytokines, resulting in distinct phenotypes in different tissues[184, 185, 187]. Therefore, it is not surprising that *in vivo*-differentiated MC are heterogeneous and able to exert different functions according to the tissue microenvironment. In fact, several polarized subsets have been identified in rodents and humans[188-190].

One of the most important endogenous factors involved in the development of MC is **stem cell factor** (**SCF**)/**Kit ligand** (**Kitlg**). It binds to the tyrosine kinase receptor **c-Kit** on the cell surface, activating a cascade of events that culminate with the production of proteins essential to the viability, development, proliferation and function of MC[191, 192 193, 194]. Consequently, Kitlg-defective $Kit^{Sl}/Kit^{Sl\text{-}d}$, as well as Kit-defective $Kit^{W}/Kit^{W\text{-}v}$ ($Kit^{W/W\text{-}v}$) and $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ ($Kit^{W\text{-}sh/W\text{-}sh}$ or *sash*) mice constitutively have profoundly reduced numbers of MC in their tissues[194-197].

Other important regulators of MC development, survival, expansion, maturation, and function are IL-1β, IL-3, IL-4, IL-6, IL-9, IL-10, IL-33, nerve growth factor (NGF), **SP**, transforming growth factor-beta (TGF-β), glucocorticoids, and interferons[198].

Mature MC are more abundant in tissues interfacing with the external environment, including the skin, the respiratory and gastrointestinal tracts, as well as in proximity with blood vessels[199, 200].

A particular feature of mature MC, especially those which reside in the skin and other connective tissues, is that their cytoplasm is filled with numerous granules, where preformed

mediators are stored[201]. MC were first identified by Nobel Laureate Paul Ehrlich in 1878, who speculated that the purpose of these cells was to feed the surrounding tissue with their dense granules, and therefore named them mast cells (from the Greek word for breast).

MC activation leads to the exocytosis of several mediators. In the early phase, activated MC rapidly release **preformed mediators** through degranulation and increase their expression of various **lipid mediators**. In the late phase, the activated cells secrete newly synthesized **cytokines**, **chemokines** and **growth factors**[129,130] (**Figure 1.7.**)[198, 201].

1.5.2. MC mediators and activation triggers

MC granules contain various **preformed mediators**, including **biogenic amines** - mostly histamine and serotonin; **enzymes** - beta-hexosaminidase (β-hex), tryptase and chymase; **proteoglycans** - serglycin proteoglycan (SGPG), heparin, chondroitin sulfate and hyaluronic acid; as well as the preformed cytokine **tumor necrosis factor-alpha** (**TNF-α**)[202, 203]. Upon stimulation, MC release these preformed mediators through rapid **degranulation** (5-30 minutes)[204].

During MC activation, **lipid mediators**, such as leukotriene B4 (LTB4), leukotriene C4 (LTC4), prostaglandin D2 (PGD2) and platelet activating factor (PAF) are synthesized from arachidonic acid, made available through the action of phospholipases[201].

MC activation also induces ***de novo*** **synthesis** and **delayed secretion** (12-24 hours later) of various **cytokines** and **chemokines**[202, 205], including interferon-α (IFN-α), IL-1β, IL-3, IL-4, IL-5, IL-6, IL-13, TNF, IL-8 (or CXCL8) and chemokine (C-C motif) ligand 2 (CCL2, or MCP-1), as well as **growth factors** such as SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF), NGF and VEGF[198, 202]. Some of these biological mediators can be exocytosed to the extracellular environment through **selective release**, a process that occurs independently from degranulation[206].

MC are the effector cells in immunoglobulin E (IgE)-mediated allergic responses[207-210]. Once exposed to allergic antigens (Ag), B cells produce IgE that binds to its high affinity Fcε receptor I (FcεRI) on the cell surface of MC, a process called priming[211-214]. Re-exposure to the same antigen, or other macromolecules, leads to aggregation and cross-linking of FcεRI receptors by the Ag-IgE complexes, resulting in massive degranulation and rapid release of preformed mediators. The **degranulation** process involves mobilization of cytoplasmic vesicles along microtubules towards the plasma membrane, followed by the calcium-dependent assembly of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) active complexes, leading to membrane fusion and granule exocytosis[204, 215].

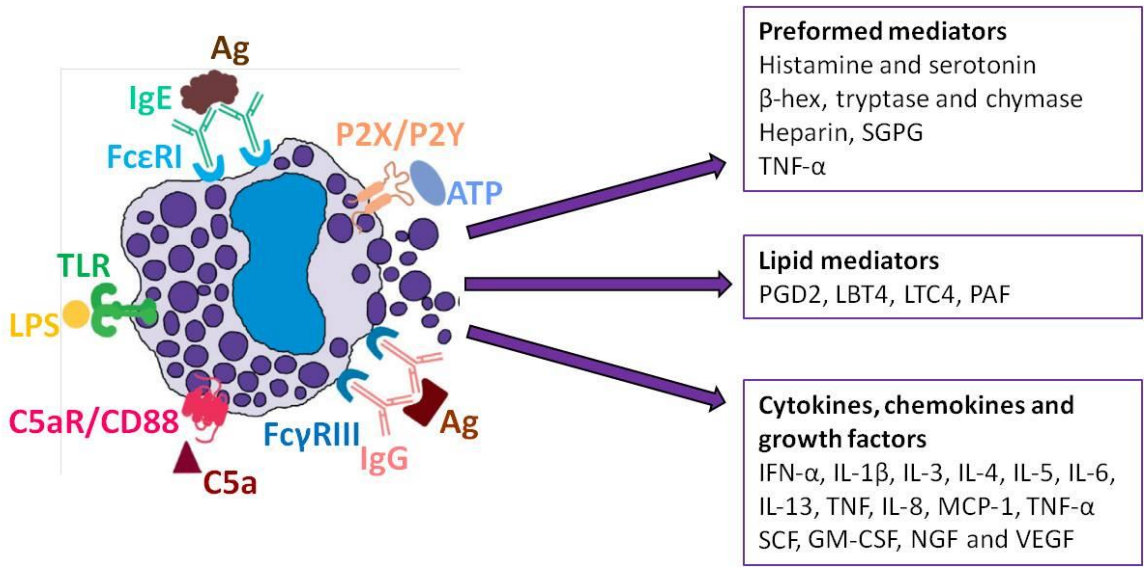


Figure 1.7. Exocytosed mediators from activated MC. Diagram representing MC activation showing different triggers and some of the most important MC mediators. Adapted from *Douaiher et al, 2014*[198] with permission of Elsevier.

MC can also be activated by numerous non-allergic triggers, through IgE-independent mechanisms. Studies have shown MC degranulation induced by thrombin via protease-activated receptor-1 (Par-1)[216, 217], by Ag-IgG complexes via FcγRIIa or FcγRIIIa[218], by ATP via P2X, P2Y, and adenosine receptors[219-221], by complement-derived anaphylatoxins via the C3a and C5a receptors[222, 223] (**Fig. 1.4.**). In addition, some chemical/pharmacological

agents such as compound 48/80[224] and opioids[225] cause histamine release from MC granules.

Moreover, MC can initiate inflammatory and immune responses without causing massive degranulation and anaphylactic shock, a process named **"selective"** or **"differential" release**[206]. For example, MC can release specific mediators such as serotonin or eicosanoids without histamine[206]. Lipopolysaccharide (LPS) can activate Toll-like receptors (TLR) on the cell surface of mouse and human MC, leading to *de novo* synthesis and release of TNF-α and several other cytokines, without causing degranulation[226, 227]. IL-1 stimulates human MC to selectively release IL-6 through small vesicles (40–80 nm) unrelated to the secretory granules (800–1000 nm)[228]. Also, corticotropin releasing hormone/factor (CRH/CRF) causes VEGF exocytosis from human cultured MC without degranulation.[229]

Furthermore, skin MC are located in proximity to sensory nerve endings and can be stimulated by **neuropeptides[230]** such as neurotensin (NT)[231], NGF[232] and **SP**[233]. Unlike the IgE-mediated process, peptide- and drug (such as SP and compound 48/80)- induced MC activation occurs through different G-protein coupled receptors[234, 235], and also via receptor-independent mechanisms by direct or indirect activation of G proteins[236 237, 238].

MC function is also regulated by inhibitory signals via CD200[239], leukocyte immunoglobulin-like receptor B4 (LIRB4)[240, 241] FcγRIIb[242], and IRp60/CD300a[243].

In summary, MC are not only heterogeneous, but also dynamic cells, as their phenotype can change depending on the context of both the stimulatory and inhibitory factors present in the tissue at a given moment. Therefore, understanding MC biology in the complex environment of a wound is a complex and challenging quest.

1.5.3. MC in wound healing

MC have long been recognized as effectors of IgE-dependent allergic reactions, but there is an emerging body of evidence for their participation in numerous other physiological and pathophysiological responses, particularly in innate and acquired immunity[244-248], auto-immunity[249, 250], and inflammation[200, 251]. Interestingly, MC have been implicated in all phases of wound healing[252-257].

Mature MC are abundant in healthy skin. They are present in both dermis and hypodermis, strategically located in the vicinity of nerves, hair follicles, and blood vessels[258]. Skin MC activation occurs early after tissue injury but the exact mechanisms are not fully understood[257, 259]. Pathogens, LPS and other pathogen products, and cytokines[200, 260] are likely to be involved, as well as pain signals, such as SP[261], and mechanical changes[255].

After a skin break, activated MC induce vascular permeability by rapidly releasing histamine, leukotrienes and VEGF[262-264]. This allows accumulation of plasma-derived fibrinogen into the site of injury, which subsequently is converted to fibrin[262]. MC participate in the fibrin clot formation, but also prevent excessive clotting, as they secrete tryptase-heparin complexes that degrade the excess fibrinogen[265]. Moreover, several studies have shown that MC activation promotes neutrophil recruitment and accumulation at the wound site[26, 266-268]. MC also contribute to inflammation by releasing chemokines, pro-inflammatory cytokines, histamine and others mediators that activate tissue-resident macrophages[269] and keratinocytes[270].

Additionally, MC promote the proliferative phase of wound healing by stimulating proliferation and migration of several skin cell types, namely fibroblasts, EC, and keratinocytes. In fact, activated MC release several factors that induce fibroblast proliferation, such as bFGF/FGF-2[271]. The close proximity with EC suggests that MC participate in angiogenesis. In fact, MC-derived factors such as VEGF, PDGF and heparin stimulate EC migration and vascular growth[272-275]. In addition, MC proteases actively

participate in ECM remodeling by degrading ECM proteins. MC have also been implicated in keratinocyte stimulation and proliferation, through secretion of several factors including not only keratinocyte growth factor and epidermal growth factor but also histamine, IL-1α, IL-1β, IL-6, and tryptase[266, 276, 277].

Furthermore, several experimental findings indicate that MC participate in the maturation/remodeling phase of healing. Besides stimulating fibroblast proliferation and migration, MC have been implicated in the differentiation of fibroblasts to myofibroblasts by increasing alpha-smooth muscle actin (α-SMA) expression and stimulating contraction[278-280]. In addition, recent studies reported that MC and fibroblasts can form gap junctions that have been shown to induce fibroblast proliferation, migration and contraction[281-284]. Even stronger evidence suggests that MC are involved in scar formation[285-287]. Numerous studies have shown increased MC abundance and activation in fibrotic or hypertrophic scars and keloids, both in animal models and in human subjects. Conversely, fetal healing, which occurs without scaring, is characterized by low numbers of MC that do not degranulate[256].

Despite the evidence that MC play an important role in wound healing, participating in all phases of repair, many questions remain to be answered. For example, the importance of MC in combating infection during the wound healing process has not been studied. Similarly, MC have been shown to have immunomodulatory proprieties, by secreting both pro- and anti-inflammatory mediators. However, it has not been examined whether MC can help resolve the acute inflammatory phase of wound healing. Furthermore, it is still not understood how MC are activated after injury and whether this heterogeneous and dynamic cell type adopts different phenotypes during the different stages of healing.

1.6. Strategies to improve diabetes-impaired wound healing

Recent reports estimated that DFU treatment accounts for 20-25% of the total cost of DM care[94, 288]. More importantly, DFU are the leading cause of non-traumatic amputations, preceding 85% of lower extremity amputations[289]. Despite the severity of the problem and

all the available diagnostic and therapeutic tools, DFU remains an unmet clinical need. Current and future DFU therapies are summarized in **Figure 1.8.**.

1.6.1. Current therapies for DFU

1.6.1.1. Wound care and general DFU management

Current management of DFU relies mainly on standard wound care which involves frequent debridement of non-viable tissue, followed by application of dressings that help maintain a clean and moist wound environment. In addition, pressure off loading is essential, since DFU typically develop in the plantar region, which is a high pressure area. While treatment of infection is often needed to control and minimize the wound bioburden, revascularization is performed to restore adequate blood flow. Naturally, general measures to control the disease and other risk factors are of great importance in preventing the development and/or recurrence of DFU.

1.6.1.1.1. Debridment and wound dressings

Debridement consists in removing from a wound bed all non-viable tissue and foreign elements that inhibit healing – namely hyperkeratotic epidermis (callus), necrotic dermal tissue, foreign debris, and bacterial components. Debridement should be performed to the level of tissue bleeding, with precision and care in order to avoid removing significant amounts of viable healthy tissue. In this way it enables better visual assessment of the wound base, alleviates the local pressure caused by the callus, and stimulates growth factor secretion, by "introducing" an acute wound in a chronic wound environment[290, 291]. In addition, it allows for the wound to better respond to topical treatments. There are several debridement methods, including surgery, wet-to-dry dressings, biosurgery (i.e., maggots therapy), enzyme preparations, polysaccharide beads or paste dextranomer polysaccharide, and hydrogels[292]. Sharp debridement using a scalpel or tissue nipper remains the most commonly used technique[272].

Development of appropriate **wound dressings** for DFU remains an important challenge. An ideal wound dressing should be able to form a protective barrier against secondary infections, provide a clean and moist wound environment, drain wound exudates, accelerate angiogenesis, and promote tissue regeneration. Dressing composition ranges between natural, modified or synthetic polymers, and can be used as single components or as mixtures or combinations. Formulations include films, foams, hydrocolloids and hydrogels. Interestingly, dressings can also be used as delivery systems to release factors and/or therapeutic agents that promote healing such as active principles, growth factors, peptides, cells and/or other bioactive substances[293]. Currently there is no evidence to recommend one type of wound dressing as being more effective than others. Selection is based on both dressing cost and the properties offered by each dressing type[294].

1.6.1.1.2. Pressure off-loading

Pressure reduction is essential in plantar DFU due to its location. Several techniques are used to redistribute the load on the plantar surface of the foot, including total contact casting (TCC) and therapeutic footwear, such as surgical shoes or half shoes and felted foam dressings. TCC seems to have the best outcomes in terms of wound healing rate[295]. However, because TCC may cause secondary skin lesions and does not allow daily assessment of the wound, its use in DFU management is limited[291].

1.6.1.1.3. Management of infection

Not all DFU patients need antibiotic therapy, but all DFU should be frequently assessed for the presence of wound infection. If positive, the severity of the infection should be evaluated after drainage of purulent collections and debridement of callus and necrotic tissue. Broad-spectrum antimicrobial therapy should be started immediately after debridement when bacterial infection is confirmed. Therapy may then be changed according to the culture results. Most patients with mild or moderate infections can be treated with oral antibiotics; in contrast, patients in poor clinical conditions who present systemic inflammation should

be evaluated for necrotizing infections, gangrene, or deep abscesses, which often require urgent surgical intervention. In some cases, early surgical intervention is the best option as it may reduce the risk of lower extremity amputation[296].

1.6.1.1.4. Revascularization

As mentioned earlier in this chapter, PAD is common in DM patients and it is a strong predictor of DFU development and failure to heal[297]. Therefore, it is extremely important to control for PAD in DFU management. First, lifestyle changes (e.g.: cessation of smoking, low fat diet, weight loss) should be implemented, followed by pharmacological agents (antiplatelet therapy, anticoagulants and low-density cholesterol-lowering drugs) and finally, if needed surgical procedures (angioplasty, endarterectomy, grafting or bypass). Importantly, revascularization must be performed only if needed and only after resolution of infection[291].

1.6.1.1.5. General measures

The same general measures that are important for DM control are crucial for DFU prevention and management, and should be the first priority in DM care. These include good glycemic control, blood pressure normalization, dyslipidemia management, appropriate diet, and smoking cessation.

1.6.1.2. Adjunctive treatment options

Adjunctive treatments for DFU have been used for many years and new therapies are emerging. These therapeutic strategies include growth factors, extracellular matrix proteins, bioengineered skin substitutes, hyperbaric oxygen therapy, negative pressure wound therapy, and electrical stimulation.

1.6.1.2.1. Growth factors

Growth factors play a key role in several cellular phenomena during the repair process, including cell proliferation, cell migration, neovascularization, and synthesis of ECM

molecules[298]. In addition, many of the important growth factors for proper wound healing have been found to be reduced in DM wounds[15, 299]. Thus, over the past two decades, topical formulations of exogenous growth factors have been used to enhance wound healing in DM[300].

Becaplermin or human recombinant PDGF (rhPDGF-BB) is the only growth factor based product that has earned US Food and Drug Administration (FDA) approval for use in DFU[301]. It is marketed in a gel form as Regranex Gel and has been reported to improve healing rate[302]. However, the clinical use of becaplermin remains limited. Cost seems to be a major factor, along with the variability in patient responsiveness[303-305]. Of note, FDA added a black box warning to the safety labelling of this product, as patients who use 3 or more tubes of becaplermin present increased risk of cancer mortality [291].

Other exogenous growth factor therapies such bFGF, recombinant human EGF (rh-EGF), recombinant human VEGF (rh-VEGF), NGF, and growth factors isolated at high concentrations from platelet rich plasma (PRP) have been used to stimulate wound healing[130, 298, 300, 306, 307]. However, there is not sufficient clinical evidence of positive outcomes for their use in DFU[308].

Since normal acute healing involves the complex interaction of several growth factors that work synergistically, it is more likely that the combination of multiple factors will have a beneficial effect in wound healing rather than a single exogenous growth factor application. In addition, it may be advantageous to combine growth factor therapies with approaches to retain growth factors in the wound site since the wound environment is highly proteolytic. In fact, some studies have already attempted different strategies to increase the half-life of growth factors in the wound with promising results[309-311].

1.6.1.2.2. Bio-engineered skin substitutes

3-dimensional bio-engineered equivalents of the dermis and/or epidermis have been used clinically in wound healing for three decades. Scaffolds for DFU treatment can be

categorized as acellular or cellular, according to the absence or presence respectively of live cells.

Acellular scaffolds can be prepared by decellularization of human or animal tissues, or by assembly of biological and synthetic polymers. They contain matrix components and growth factors that help the repair process. For example, the product Oasis Wound Matrix is obtained from decellularized porcine-derived small intestinal submucosa and contains several important dermal elements such as collagen, hyaluronic acid, proteoglycans, fibronectin, and growth factors[312]. By excluding living cells, these scaffolds have reduced cost, lowered regulatory requirements, and prolonged shelf life compared to cellular scaffolds.

In the cellular scaffolds category, two products have been approved by the FDA for DFU: Apligraf and Dermagraft. Apligraf is a bi-layered product composed of a bovine-derived collagen matrix seeded with allogenic dermal fibroblasts and epidermal keratinocytes derived from human neonatal foreskin, and a stratum corneum[313]. Dermagraft is an allogeneic, human neonatal-derived dermal matrix, composed of fibroblasts cultured on a collagen gel reinforced with a polyglactin mesh[314]. Both products have been shown to accelerate wound closure[315, 316] and the proposed mechanism is active secretion of cytokines, growth factors, and ECM components during the healing process. In theory, cellular scaffolds should perform better than both acellular scaffolds and growth factor-based therapies. The viable cells not only retain the residual matrix, but also secrete growth factors and ECM components in the physiologically appropriate proportion and sequence to enhance wound healing. However, studies comparing the effectiveness of the different products are scarce.

1.6.1.2.3. Hyperbaric oxygen therapy

In DM-associated microvascular disease, the distance between capillaries is increased and their function is impaired. Oxygen needs to diffuse longer distances, and therefore higher

PO2 is required at the edge of the capillaries[317]. It has been reported that oxygen therapy increases tissue PO2, stimulates angiogenesis, promotes collagen synthesis and helps control tissue infection[318, 319]. Despite these promising effects, there is little evidence on the clinical efficacy of hyperbaric oxygen therapy. In fact, a recent study on a large population of DM patients reported that hyperbaric oxygen therapy failed both to improve the healing prognosis and to reduce the likelihood of amputation[320].

1.6.1.2.4. Negative pressure wound therapy

Negative pressure wound therapy involves the application of local sub-atmospheric pressure to a wound[**321**]. It accelerates healing and promotes wound closure[**322**]. Several mechanisms have been proposed to explain the effectiveness of negative pressure including drainage of the excess third-space fluid[**323, 324**], reduction of bacterial load, mechanical effects on the wound bed, delivery of oxygen and nutrients, increased cellular proliferation[325], or increased granulation tissue and vasculogenesis via EPC mobilisation[**326**]. However, a retrospective review of 25 patients reported an increased bioburden in wounds that received negative pressure therapy[**321**]. The recommendations of an international expert panel on the use of negative pressure wound therapy were positive for non-ischemic DFU[**327**]. Nevertheless, more evidence is needed to validate the use of negative pressure wound therapy for DFU.

1.6.1.2.5. Electrical shock wave therapy

The application of shock waves to the wound seems to have bacteriostatic and bactericidal effects, as well as proliferative effects as suggested by increased neutrophil and macrophage migration, and increased fibroblast and endothelial cell growth in culture[328]. In addition, electrical shock wave therapy is believed to stimulate the early expression of pro-angiogenic factors. As such, it results in new blood vessel formation, increasing blood supply to the wound and promoting healing[329]. However, evidence on the clinical efficacy of electrical shock wave therapy for DFU is still weak[308].

1.6.2. Future therapeutic perspectives for DFU

Despite the currently available treatments, 25% of DFU patients will have to undergo amputation. Next-generation DFU therapies are currently being developed in preclinical and clinical settings.

1.6.2.1. Stem cell therapies

Stem cell-based therapies introduce adult stem cells into the wound to promote healing through active release of trophic factors. Stem cells have been tested in both animal and human studies with promising results. Local application of allogeneic MSCs has been used in animal models of wound healing with favourable results[330]. Intramuscular (i.m.) injections of autologous peripheral blood mononuclear cells (PB-MNC), bone marrow mesenchymal stem cells (BM-MSC) or bone marrow-derived mononuclear cells (BM-MNC) into the lower limb of patients with DM and critical limb ischemia increased blood flow and resulted in complete wound closure, with no major adverse effects[331, 332]. Local application of autologous BM cells to a DM patient with chronic venous and neuro-ischemic wounds increased tissue vascularisation and improved wound healing with no systemic adverse events[333], while local application of BM-MSC using a fibrin polymer spray system in patients with chronic ulcers had positive outcomes[334]. Despite all these studies describing beneficial effects of MSC topical delivery to DM wounds, completed human trials have not yet been reported.

1.6.2.2. Gene therapies

In face of the barriers to growth factor based therapies - cost of purified recombinant production, low bioavailability in the wound environment, and toxicity associated with repetitive doses – the delivery of genes encoding for growth factors has been proposed as a potential treatment for DFU. Topical application of a collagen gel containing a replication-defective adenovirus encoding for PDGF resulted in complete ulcer healing in DM patients with neuropathic ulcers, while no serious safety concerns were observed [335]. Others studies

have shown that local delivery of VEGF using adenovirus vectors ameliorates wound healing in experimental DM[336, 337]. Although it has been tested in human subjects and animal models with favourable results, the use of gene therapy in DM wound healing has not received clinical approval yet.

1.6.2.3. Other potential therapies

Novel therapeutic approaches for DFU should be directed towards correcting the factors that lead to DM-associated impaired wound healing. Based on the current knowledge, neuropathy and associated neuropeptide deficiency, chronic inflammation and impaired neovascularisation seem to be major contributors to the abnormal healing that occurs in DM. Therefore, **cell delivery**, **neuropeptide treatment**, **pro-inflammatory cytokine inhibition or anti-inflammatory cytokine supplementation**, effective **delivery of multiple growth factors** and/or **protease inhibition** all seem good candidates. In fact, these approaches have already been tested in preliminary studies with positive outcomes. In addition to stem cells, other cells including EC and fibroblasts have been used in animal models to improve healing of chronic wounds. For example, a study by Pedroso et al[338] showed that the co-transplantation of $CD34^+$ cells with $CD34^+$-derived EC enhanced healing in a chronic wound DM mouse model, by attenuating the exacerbated inflammatory reaction and increasing the neovascularization of the wound. Others have reported positive outcomes in healing and scarring by topical application of dermal fibroblasts into the wounds of mice and rabbits[339, 340].

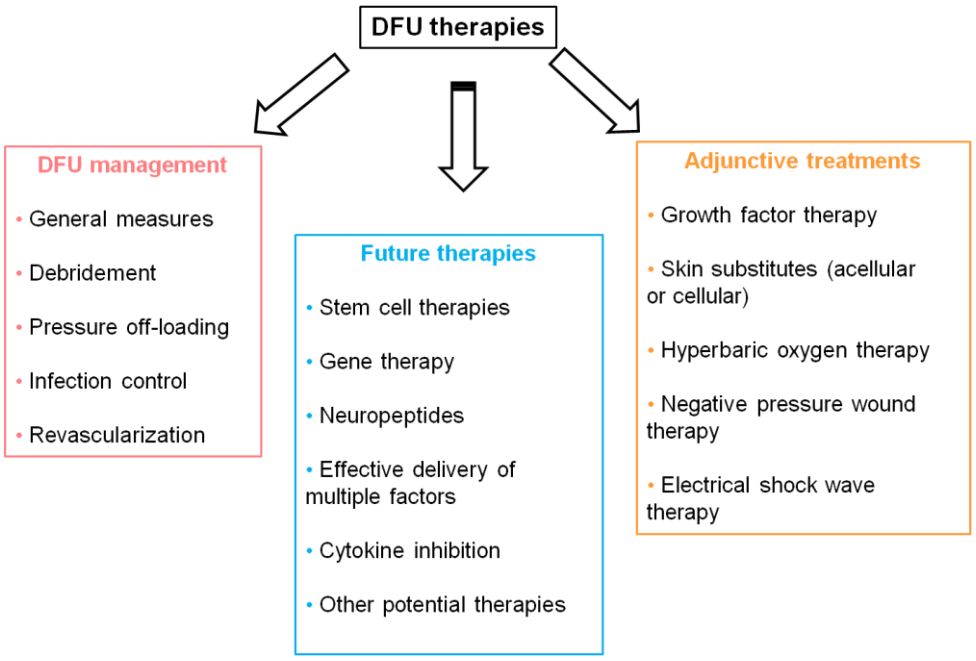


Figure 1.8. DFU therapies. Diagram summarizing the current and future therapies for DFU.

As mentioned earlier in this chapter, several studies have emphasized the role of neuropeptides in wound healing. In addition to SP, neurotensin has been proposed as a good candidate for DFU treatment. Moura and colleagues have recently shown that neurotensin regulates the macrophage response to injury *in vitro* [341] and improves wound healing in a DM mouse model by controlling the exacerbated inflammatory reaction[342, 343].

The combination of growth factors, such as epidermal growth factor and erythropoietin[344] or VEGF and FGF4[345], has demonstrated beneficial effects in experimental models of DM wound healing through synergistic mechanisms.

Inhibition of the IL-1β pathway in DM mice has been shown to promote the transition from a pro-inflammatory to an anti-inflammatory or reparative macrophage phenotype that stimulates the release of growth factors, and therefore ameliorates wound healing[346]. Conversely, IL-10 overexpression promoted healing in mouse models[347, 348].

Recently, a group has developed a selective MMP-9 inhibitor and shown that it accelerates wound closure and re-epithelialization in a db/db mouse mode[349].

Despite the costs, all these new approaches are promising, especially if used in combination, which adds to the total costs. However, as many promising therapies have failed in the past, efficacy must first be fully recognized before establishing the minimum requirements for economics.

1.7. Main objectives

DM is a serious problem of public health that has reached epidemic proportions. One of the major complications of DM is development of chronic non-healing DFU, as they severely impair the quality of life of patients and represent a heavy burden to the healthcare system. Despite its impact in public health and general economy, there is currently no satisfactory therapy for DFU. Therefore, it is urgent to better understand the mechanistic pathways involved in DM healing impairment in order to identify new therapeutic strategies with high potential for DFU treatment.

There has been limited clinically driven basic research into the cellular and molecular mechanisms of the pathophysiology of DM-associated impaired wound healing and this research strategy may be very useful to the field.

The **main hypotheses** underlying the present work are:

1) in DM there is not only systemic but also **local skin inflammation** that hinder the progression from the inflammatory to the proliferative phase of wound healing, resulting in **reduced angiogenesis, granulation** and **re-epithelilization**, culminating to deficient tissue repair;

2) diabetic neuropathy and associated **local SP deficiency** is a major factor contributing to impaired wound healing in DM;

3) lack of SP in association with the DM-induced pro-inflammatory state lead to **MC dysfunction** resulting in aberrant secretion of cytokines and angiogenic factors that further impair wound healing;

4) an ideal product for DFU treatment should be composed of state of the art **biomaterials** that can provide a localized, targeted and sustained presentation of appropriately **validated effectors and cells** that are well-adapted to the wound environment.

Therefore, the **main aims** of this thesis are:

1) to evaluate **inflammation** and **blood vessel density** in **DM skin**:

1.1) to assess skin inflammation in both human and experimental animal models of DM and compare it to their respective non-DM controls;

1.2) to assess skin blood vessel density in both human and experimental animal models of DM and compare it to their respective non-DM controls.

2) to investigate the **role of SP** in **DM-impaired wound healing**:

2.1) to evaluate the effect of local SP treatment in non-DM and DM animal models of wound healing;

2.2) to evaluate the effect of SP-deficiency in non-DM and DM mouse models of wound healing;

2.3) to evaluate the effect of SP receptor NK1R-deficiency in non-DM and DM mouse models of wound healing;

2.4) to study SP and NK1R skin expression in both human and mouse models of DM and neuropathy.

3) to investigate the **role of MC** in **DM-impaired wound healing**:

3.1) to assess the abundance and activation state of MC in both human and mouse models of DM and neuropathy;

3.2) to evaluate the effects of MC stabilization in non-DM and DM mouse models of wound healing;

3.3) to evaluate the effects of MC-deficiency in non-DM and DM mouse models of wound healing;

3.4) to evaluate the effects of SP local treatment in MC-deficient mice;

3.5) to study the effect of high glucose on MC degranulation and mediator release in MC cultures.

4) to test the potential of new **biomaterials** as delivery systems for **DM wound therapies**:

4.1) to evaluate the ability of recently developed injectable alginate gels to offer controlled release of neuropeptides;

4.2) to evaluate the ability of the alginate gels to deliver endothelial precursor cell to DM mouse wounds;

4.3.) to evaluate the ability of the alginate gels to serve as a multi-therapy delivery system to DM mouse wounds;

4.4.) to test the safety and efficacy of new DNA-based gels in mouse models of non-DM and DM wound healing.

Chapter II

Increased Skin Inflammation and Blood Vessel Density in Human and Experimental Diabetes

2. Chapter II - Increased Skin Inflammation and Blood Vessel Density in Human and Experimental Diabetes

2.1. Abstract

Systemic inflammation is associated with impaired wound healing in Diabetes *Mellitus* (DM) patients. Using immunohistochemistry techniques, we investigated changes in skin inflammation and skin blood vessels in human and experimental diabetes. Comparing to the non-DM human subjects, the total number of inflammatory cells per biopsy and the number of inflammatory cells around blood vessels, a strong indication of inflammation, were higher in DM subjects irrespective of their risk for developing diabetic foot ulcer. Inflammatory cell infiltration was robustly increased in all DM animal models compared with their non-DM controls. The number and density of blood vessels and CD31 positive proliferating endothelial cells around preexisting skin vessels was also higher in the DM patients. However, there were no differences in the skin blood flow between the non-DM and DM subjects. The number of skin blood vessels was also increased in the DM animals; however, these differences were less obvious than the ones observed for inflammatory cells. We conclude that skin inflammation and skin blood vessel density is increased in DM human subjects and in rodent and rabbit models of DM.

2.2. Introduction

Foot ulcers and impaired wound healing are common problems in DM as they can affect 15-20% of all patients during their lifespan resulting in impaired quality of life and extremely high rates of low extremity amputations[350, 351]. As the DM pandemic continues unabated and DM patients live longer, it should be expected that the incidence of diabetic foot ulcers (DFU) would increase over the next few decades. Neuropathy, peripheral arterial disease and reduced resistance to infection have been identified as major risk factors for the development of DFU[352, 353].

Recent studies by our group have shown that systemic inflammation is associated with impaired wound healing in diabetic (DM) patients[354]. In addition, we have shown that skin biopsies from both the forearm and dorsum of the foot of DM patients have increased inflammatory cell infiltration when comparing with non-diabetic (non-DM) control subjects. However, there is little information regarding the blood vessel density in the skin of DM patients. Furthermore, it is not known whether the skin changes observed in DM patients are also present in various experimental animal models of DM commonly used in wound healing studies. Given the lack of satisfactory animal models, the possible similarities and differences between human and experimental DM may prove very helpful in designing mechanistic studies in the future.

In the present study we used immunohistochemistry techniques to compare the inflammatory cell infiltration and blood vessel density changes between non-DM and DM rabbits, rats and mice. We also compared the animal study results with the results obtained from the forearm and dorsal foot skin biopsies of non-DM and DM human subjects.

2.3. Materials and Methods

2.3.1. Human subjects

Diabetic (DM) patients and healthy control (non-DM) subjects were recruited and enrolled in the study. The diabetic subjects were divided to those at low risk for developing foot ulceration and those at high risk according to their neuropathic status[355]. The exclusion criteria were: presence of foot ulceration at the time of recruitment; clinically present peripheral arterial disease (PAD); end stage renal disease (patients on renal dialysis or kidney transplantation); any other serious chronic disease that can affect wound healing. The cohort was well characterized in terms of gender, age, duration of DM, and clinical condition. All subjects attended the Joslin-Beth Israel Deaconess Foot Center and the General Clinical Research Center where they had a full physical examination and all tests described below. All DM patients received education about foot care and were regularly

seen by their podiatrist as required. The protocol was approved by the Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center. All participants gave written informed consent.

2.3.1.1. Forearm and foot skin samples

For the **forearm skin biopsies**, a 2 mm skin punch biopsy was taken from the volar aspect of the forearm. For the **foot skin samples**, discarded skin specimens were obtained from subjects who underwent foot surgery for various reasons.

2.3.1.2. Histology and immunohistochemistry (IHC)

Hematoxylin and Eosin (H&E) and CD31 immunohistochemistry staining was performed in all biopsies. Biopsies were embedded in optimal cutting temperature compound (OCT) and frozen in liquid nitrogen immediately after collection, stored at – 80°C and were analyzed by a pathologist with expertise in dermatopathology (AK). 5 µm sections were cut and fixed in acetone immediately before staining. To study skin inflammation, sections were stained with H&E according to standard protocols for cryosections. The number of inflammatory round cells was counted in each biopsy. In our previous study round cells were confirmed as being inflammatory cells by staining for CD45RO, a marker of lymphocytes and Factor XIIIa, a marker of dermal dendrocytes[354]. To evaluate skin blood vessel density, immunohistochemistry for CD31 was performed using a mouse monoclonal antibody (JC70A, Abcam, Cambridge, MA, USA). CD31 is commonly used as a marker for endothelial cells. In this study, immunohistochemistry for CD31 was performed with the purpose of identifying both proliferating endothelial cells and skin blood vessels. Endothelial cells were characterized as proliferating when they were observed as single, CD31 positive cells that were located in the proximity of pre-existing blood vessels but did not have a lumen[356]. The number of blood vessels was counted and expressed per square millimeter (mm^2).

2.3.1.3. Skin blood flow

Skin blood flow was monitored using a Laser Doppler Perfusion Imager (Lisca PIM 2.0, Lisca Development AB, Linkoping, Sweden) as previously described[357]. In brief, a 2x2 cm skin area was scanned after all subjects were acclimatized in a room with controlled temperature for 30 minutes. The skin area was the same that was used to perform skin biopsies after skin blood flow measurements were completed.

2.3.2. Animal studies

All animal studies were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols.

2.3.2.1. Rabbit model

New Zealand White male rabbits weighing 3.0 to 3.2 kg were obtained from Millbrook Farms (Amherst, MA). Six rabbits were made diabetic by injecting two doses of 50 mg/kg of alloxan monohydrate via the marginal ear vein, 48 hours apart. Seven rabbits in the non-diabetic (non-DM) group received vehicle alone (saline). Rabbits with fasting blood glucose over 250 mg/dl were considered diabetic (DM). Ten days after alloxan administration, rabbits were anesthetized by intramuscular injection of ketamine (25 mg/kg i.m.) and xylazine (3 mg/kg i.m.) and skin biopsies from the rabbit ear were obtained using a 6 mm punch biopsy.

2.3.2.2. Rat model

Eight-week old male Sprauge-Dawley rats were obtained from Charles River Laboratories. Ten rats were made diabetic by administering intraperitoneally a single dose of streptozotocin (STZ) (60mg/Kg, i.p.) in citrate buffer (0.1M). The non-diabetic (non-DM) group (9 rats) received equal volume of vehicle alone. Rats with fasting blood glucose over 250 mg/dl were considered diabetic (DM). Six to eight weeks after STZ treatment, rats were anesthetized using ketamine (35 mg/kg i.m.) and xylazine (2.5 mg/kg i.m.) and 6 mm full thickness skin punch biopsies were obtained from the shaved dorsum of the rats.

2.3.2.3. Mouse models

C57BL6/J and WBB6F1/J male mice were obtained from Jackson Laboratories. Nine 8-weeks-old mice were made diabetic by administering 50 mg/kg STZ (i.p daily for 5 consecutive days) in citrate buffer (0.1M). In the non-diabetic (non-DM) group, 9 mice were treated with vehicle alone. Fasting blood glucose was monitored a week after the last injection and mice with blood glucose over 250 mg/dl were considered diabetic (DM). Six to eight weeks after STZ treatment, mice were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and 6 mm full thickness skin punch biopsies were obtained from the shaved dorsum of the mice.

2.3.2.4. Histology and IHC

Skin samples were fixed in 10% formalin immediately after collection and embedded in paraffin blocks (FFPE). 6 μm FFPE sections were cut and stained with H&E according to standard techniques for paraffin sections. Analysis was performed by an observer (AT) who was unaware of the group each biopsy belonged under the supervision of the pathologist (AK). Round inflammatory cells and blood vessels were counted in four different high power fields (hfp, 400x magnification) per section. In order to avoid the risk of misclassifying other cell types as inflammatory, which in most cases are transversely cut fibroblasts/fibrocytes that may appear round, we mainly counted round cells with a diameter of 6-12 microns in the proximity of blood vessels where inflammatory cells are first observed during the inflammatory process. We also took into consideration additional subtle morphological details and we excluded cells with characteristics that are compatible with fibroblasts/fibrocytes (diameter 12-15 microns, bland looking nucleus). Immunohistochemistry (IHC) for CD31 was performed using a purified rat monoclonal antibody (BD 550274, BD Biosciences, San Jose, CA, USA) for mouse, a rabbit polyclonal antibody (250590, ABBiotech San Diego, CA) for rat and a rat monoclonal antibody (AB 56299, Abcam, Cambridge, MA, USA) for rabbit specimens. Briefly, sections were deparaffinized, hydrated and subjected to proteinase K treatment for antigen retrieval.

Overnight incubation with the primary antibody at 4°C was followed by biotinylated secondary antibody (Vector laboratories, Burlingame, CA,USA) and avidin-biotin-peroxidase complex by using the Vectastain elite ABC rat kit and diaminobenzidine (DAB) as chromogen development (Vector laboratories, Burlingame, CA, USA). The number of CD31 positive endothelial cells and/or the number of blood vessels were counted in four different high power fields (hpf, 400x magnification) per section. Results are expressed as average number/hpf.

2.3.3. Statistical analysis

Data analysis was performed using Minitab (Minitab, State College, PA). Analysis of variance (ANOVA) and the t-test were employed for the comparisons among the groups for normally distributed data. Non-parametrical data were analyzed through Kruskal-Wallis analysis of variance. The Pearson test was used to for the calculation of the correlation coefficient.

2.4. Results

2.4.1. Human forearm skin biopsies

We examined the forearm skin biopsies from 12 healthy non-DM subjects (age 60 ± 6 years, 4 males). DM subjects were divided into two groups, 10 DM subjects with neuropathy not severe enough to be at risk of developing foot ulceration (DM low-risk) (age 56 ± 9 years, 4 males, 3 Type 1 DM, DM duration 12 ± 13 years), and 50 neuropathic DM subjects at risk of foot ulceration (DM high-risk) (age 56 ± 8 years, 37 males, 21 Type 1 DM, diabetes duration 22 ± 14 years). DM duration was 32 ± 13 years in Type 1 DM and 13 ± 9 in type 2 DM patients ($p<0.001$) but there were no differences in diabetes duration between DM low-risk and DM high-risk. There were no other major differences in the clinical characteristics among these three groups (**Table 2**).

	Non-DM (N=12)	DM low-risk (N=10)	DM high-risk (N=50)	p value
Age (yrs)	60 ± 6	56 ± 9	56 ± 8	NS
Males (%)	33	40	74 *	* <0.05 vs non-DM and low-risk
DM duration (yrs)	NA	12 ± 13	22 ± 14	NS
DM type (1/2)	NA	3/7	21/29	NS
BMI	29.2 ± 8.0	35.2 ± 5.3	33.6 ± 6.9	NS

Table 2. Clinical characteristics of the three studied groups. Data represents mean ± SD.

2.4.1.1. Hematoxylin and Eosin (H&E) analysis

Comparing to the non-DM subjects, the total number of inflammatory cells per biopsy was higher in both DM groups (low-risk and high-risk) (Non-DM: 83 ± 44, DM low-risk: 143 ± 51, DM high-risk: 110 ± 39, $p<0.01$) (**Figure 2.1. A**). Similar results were observed for the number of inflammatory cells around vessels, a strong indication of inflammation (non-DM: 8 ± 7, DM low-risk: 17 ± 9, DM high-risk: 14 ± 11, $p<0.05$) (**Figure 2.1. B**). Furthermore, there were no differences between Type 1 and Type 2 DM subjects in both the total number of inflammatory cells in the dermis (Type 1 DM: 118 ± 47 vs. Type 2 DM: 112 ± 40, p=NS) and the number of inflammatory cells around vessels (Type 1 DM: 12 ± 9 vs. Type 2 DM: 15 ± 11, p=NS).

The number of skin vessels also trended to be higher in the two diabetic groups when compared to the non-DM subjects but failed to reach statistical significance (non-DM: 5.9 ± 2.9, DM low-risk: 7.2 ± 2.2, DM high-risk: 6.8 ± 2.2, p=NS).

2.4.1.2. CD31 staining analysis

The number of CD31 positive proliferating endothelial cells around pre-existing skin vessels was higher in both low-risk and high-risk DM subjects (non-DM: 0 ± 0, DM low-risk: 0.5 ± 0.5, DM high-risk: 0.5± 0.7, $p<0.05$) (**Figure 2.1. C**). The number of skin blood vessels per mm^2 was marginally higher in the two DM groups (non-DM: 21 ± 9, DM low-risk: 23 ± 7, DM high risk: 28± 12, p=0.057) (**Figure 2.1. D**). However, when the two DM groups were

merged in one group, the number of vessels per mm^2 was higher in the DM group when compared to the non-DM subjects (DM: 27 ± 11 vs. non-DM: 21 ± 9, $p<0.05$) (**Figure 2.1. E**). There were no differences between Type 1 and Type 2 DM subjects in both proliferating cells (Type 1 DM: 0.5 ± 0.6 vs. Type 2 DM: 0.5 ± 0.7, p=NS) and blood vessels (Type 1 DM: 26 ± 12 vs. Type 2 DM: 28 ± 11, p=NS). A strong correlation was observed between the number of inflammatory cells around the vessels and the number of CD31 positive proliferating cells around pre-existing skin vessels ($r=0.44$, $p <0.0001$) and the number of skin blood vessels per mm^2 ($r=0.24$, $p <0.05$).

2.4.1.3. Skin blood flow

There were no differences in the skin blood flow between the DM and non-DM subjects (non-DM: 1.01 ± 0.23, DM low-risk: 1.27 ± 0.41, DM high risk: 1.05 ± 0.37, arbitrary units, p=NS) (**Figure 2.1. F**). No correlation was observed between the number of vessels and skin blood flow.

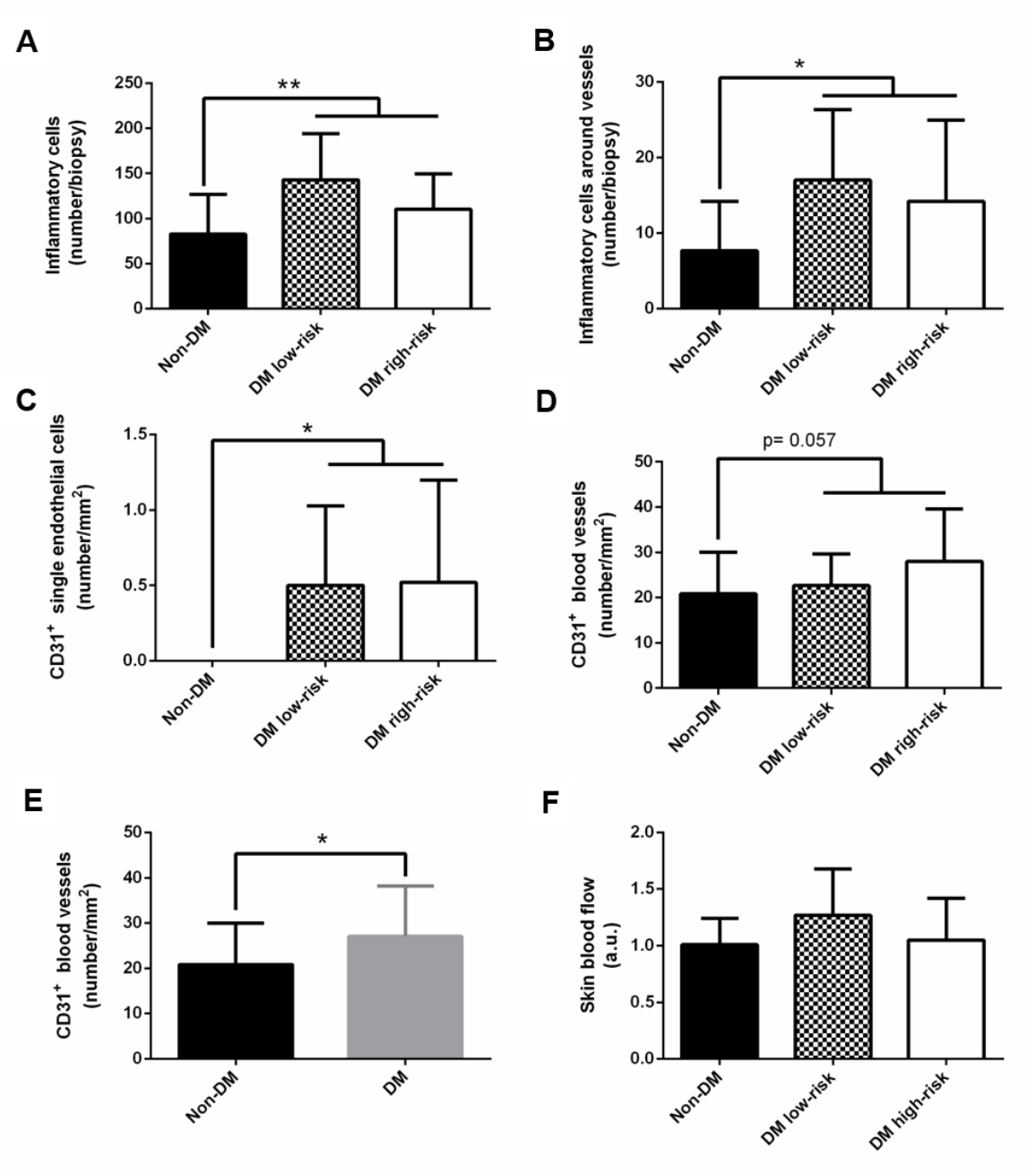


Figure 2.1. Skin inflammation, blood vessel density, and blood flood in forearm skin of non-DM, DM low-risk, and DM high-risk subjects. Number of **(A)** total inflammatory cells, **(B)** inflammatory cells around blood vessels, **(C)** single CD31+ proliferating endothelial cells and **(d)** CD31+ blood vessels in non-DM, DM low-risk and DM high-risk groups. **(E)** Number of CD31+ blood vessels in non-DM and DM (low-risk + high-risk) groups. **(F)** Skin blood flow in non-DM, DM low-risk and DM high-risk. Data represents mean ± SD. * $p < 0.05$, ** $p < 0.01$.

2.4.2. Human foot skin biopsies

We have also investigated discarded skin specimens from the foot that were obtained during foot surgery from 7 non-DM healthy subjects (age 55±18, 3 males) and 5 DM subjects (age 54±16, 3 males). The results were similar to the ones observed in the forearm biopsies although they failed to reach statistical significance, most likely due to the small number of participants. Thus, H&E staining showed that DM patients trended to have higher number of skin blood vessels per biopsy when compared to the non-DM (45 ± 57 vs. 27 ± 20, p=NS) while the number of CD45RO expressing cells around blood vessels, a marker of lymphocytes, also trended to be elevated in the DM patients (88 ± 26 vs. 77 ± 34, p=NS).

2.4.3. Rabbits

2.4.3.1. H&E analysis

The number of inflammatory cells per visual field from ear skin biopsies was higher in the DM rabbits when compared to the non-DM ones (2.2 ± 1.2 vs. 0.8 ± 0.2, $p<0.05$) (**Figure 2.2. A, B, and C**). The number of blood vessels per visual field, when assessed by morphological analysis, was also higher in the DM rabbits (5.5 ± 2.3 vs. 2.6 ± 0.8, $p<0.01$) (**Figure 2.2. A, B and D**). When non-DM and DM animals were considered as one group, a strong correlation was observed between the number of inflammatory cells and skin vessels, $r=0.83$, $p<0.0001$.

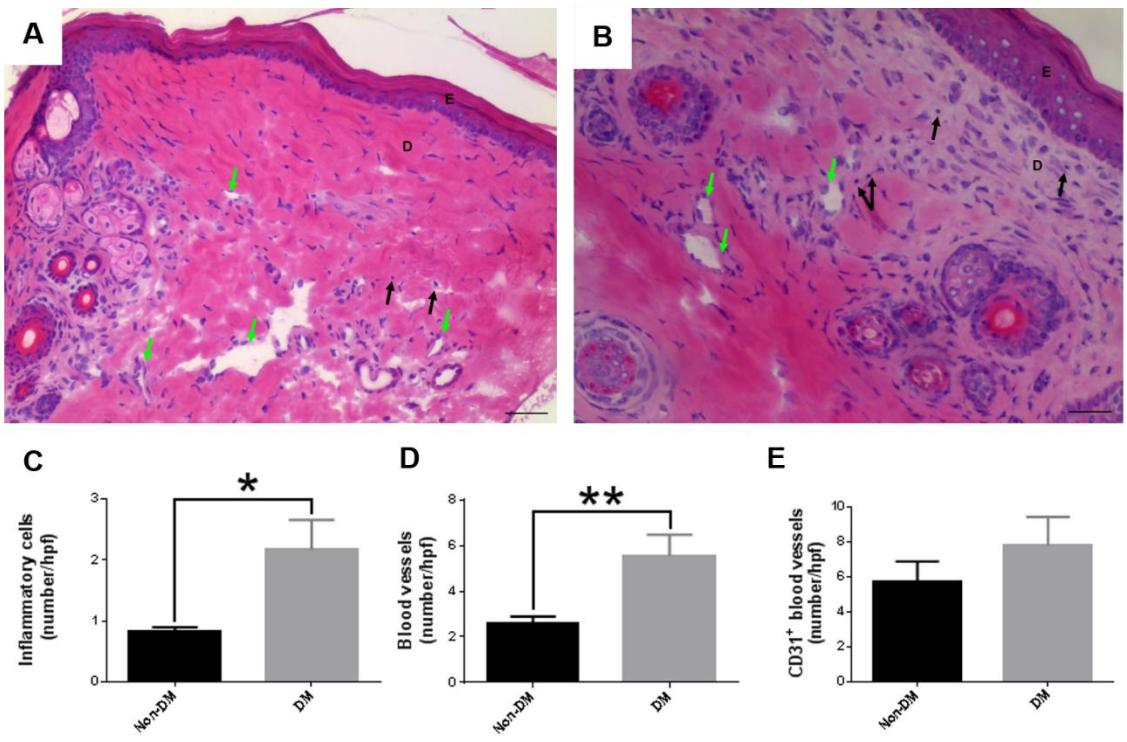


Figure 2.2. Skin inflammation and blood vessel density in ear skin biopsies from non-DM and DM rabbits. Representative images of H&E staining in **(A)** non-DM and **(B)** DM rabbit ear skin. E: epidermis, D: dermis; black arrows: round inflammatory cells; green arrows: blood vessels; scale bar: 50 µm. Number of **(C)** inflammatory cells and **(D)** blood vessels assessed by H&E staining. **(E)** Number blood vessels assessed by CD31 staining. Data represents mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

2.4.3.2. CD31 staining analysis

The number of skin blood vessels, when assessed by CD31 staining, trended to be higher in the DM rabbits compared to non-DM rabbits, although no statistical difference was observed, probably due to small number of tested animals (7.8 ± 4.0 vs. 5.7 ± 3.1, p=NS) (**Figure 2.2. E**).

2.4.4. Rats

2.4.4.1. H&E Analysis

Inflammation, assessed as the number of inflammatory round cells, was higher in the skin of DM rats when compared to the non-DM rats (3.5 ± 0.8 vs. 1.0 ± 0.3, p <0.0001) **(Figure 2.3. A, B, and C).** The number of skin blood vessels was also higher in the DM rats compared to the non-DM rats (3.5 ± 0.9 vs. 2.7 ± 0.6, p<0.05) (**Figure 2.3. A, B, and D**).

2.4.4.2. CD31 Staining Analysis

The number of skin blood vessels identified by CD31 positive staining was increased in the DM rats compared to the respective non-DM controls (5.3 ± 1.3 vs 3.8 ± 0.7, p<0.01) (**Figure 2.3. E**).

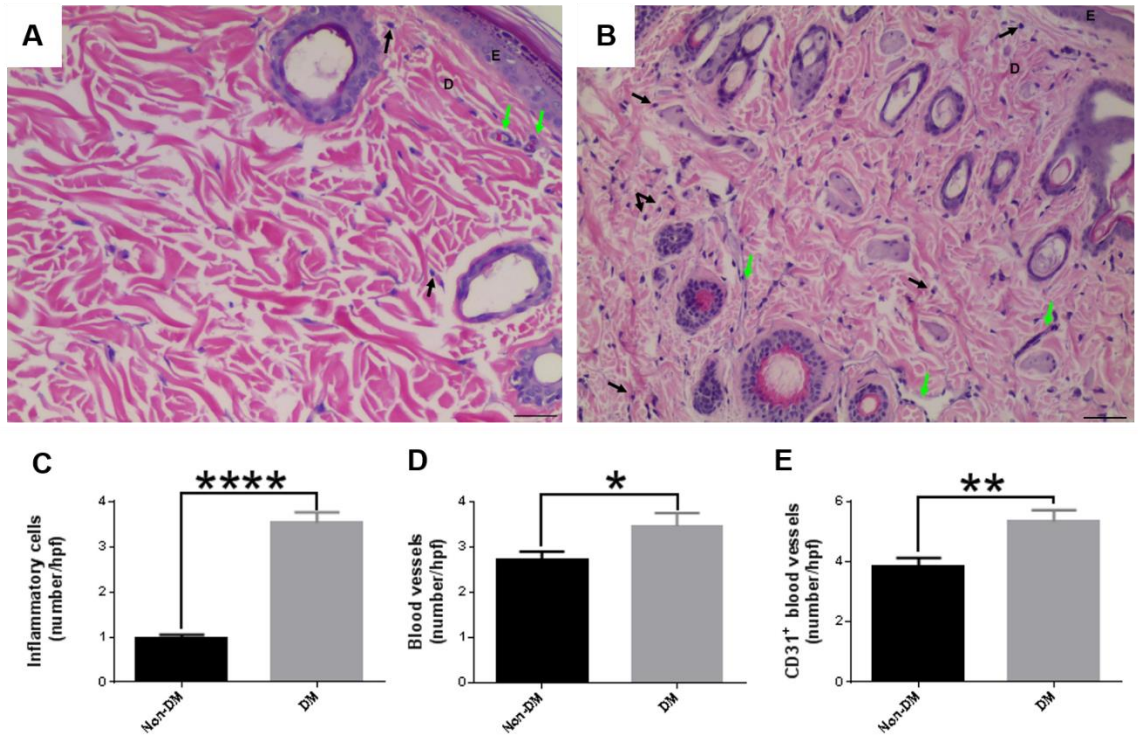


Figure 2.3. Skin inflammation and blood vessel density in dorsal skin biopsies from non-DM and DM rats. Representative images of H&E staining in **(A)** non-DM and **(B)** DM rat dorsal skin. E: epidermis, D: dermis; black arrows: round inflammatory cells; green arrows: blood vessels; scale bar: 50 µm. Number of **(C)** inflammatory cells and **(D)** blood vessels assessed by H&E staining. **(E)**

Number blood vessels assessed by CD31 staining. Data represents mean ± SEM. * p < 0.05, ** p < 0.01, **** p <0.0001.

2.4.5. Mice

2.4.5.1. H&E Analysis

DM C57BL6 mice had higher inflammatory cells per visual field than their respective non-DM C57BL6 controls (3.8 ± 1.4 vs 1.3 ± 0.6, p <0.001) (**Figure 2.4. A, B, and C**). The number of blood vessels, identified by morphological analysis, was also higher in the DM mice from the same strain (5.3 ± 1.4 vs. 3.2 ± 1.1, p<0.01) (**Figure 2.4. A, B and C**). A strong correlation was observed between the number of inflammatory cells and vessels when non-DM and DM animals were grouped together, r=0.79, p<0.0001.

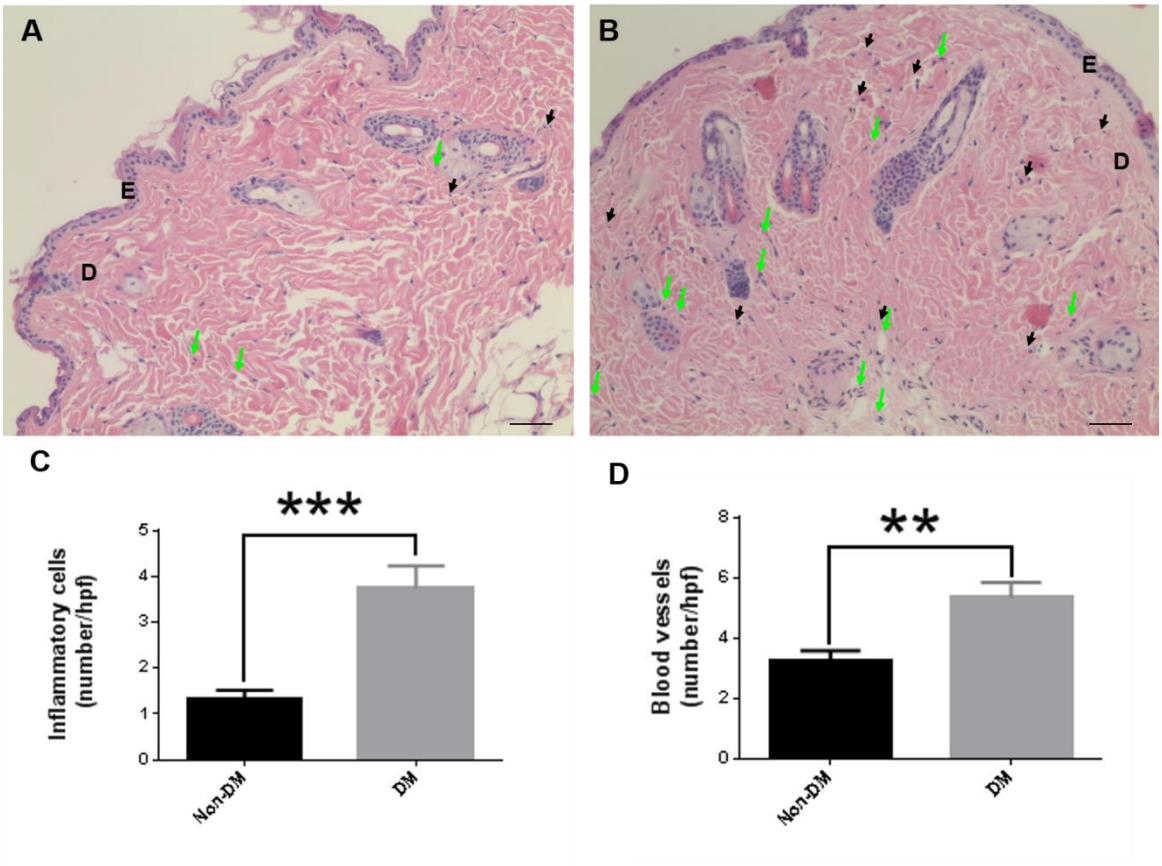


Figure 2.4. Skin inflammation and blood vessel density in dorsal skin biopsies from non-DM and DM C57BL6 mice. Representative images of H&E staining in **(A)** non-DM and **(B)** DM C57BL6 mouse dorsal skin. E: epidermis, D: dermis; black arrows: round inflammatory cells; green arrows:

blood vessels; scale bar: 50 µm. Number of **(C)** inflammatory cells and **(D)** blood vessels assessed by H&E staining. Data represents mean ± SEM. ** p < 0.01, *** p <0.001.

Compared to non-DM WBB6F1, DM WBB6F1 mice also had higher number of inflammatory cells (12.1 ± 1.4 vs. 8.6 ± 1.7, p<0.001) (**Figure 2.5. A, B, and C**) and higher number of skin blood vessels (3.8 ± 1.6 vs. 1.4 ± 0.8, p<0.001) (**Figure 2.5. A, B, and D**). In addition, a correlation existed between these two parameters (p=0.54, p<0.05).

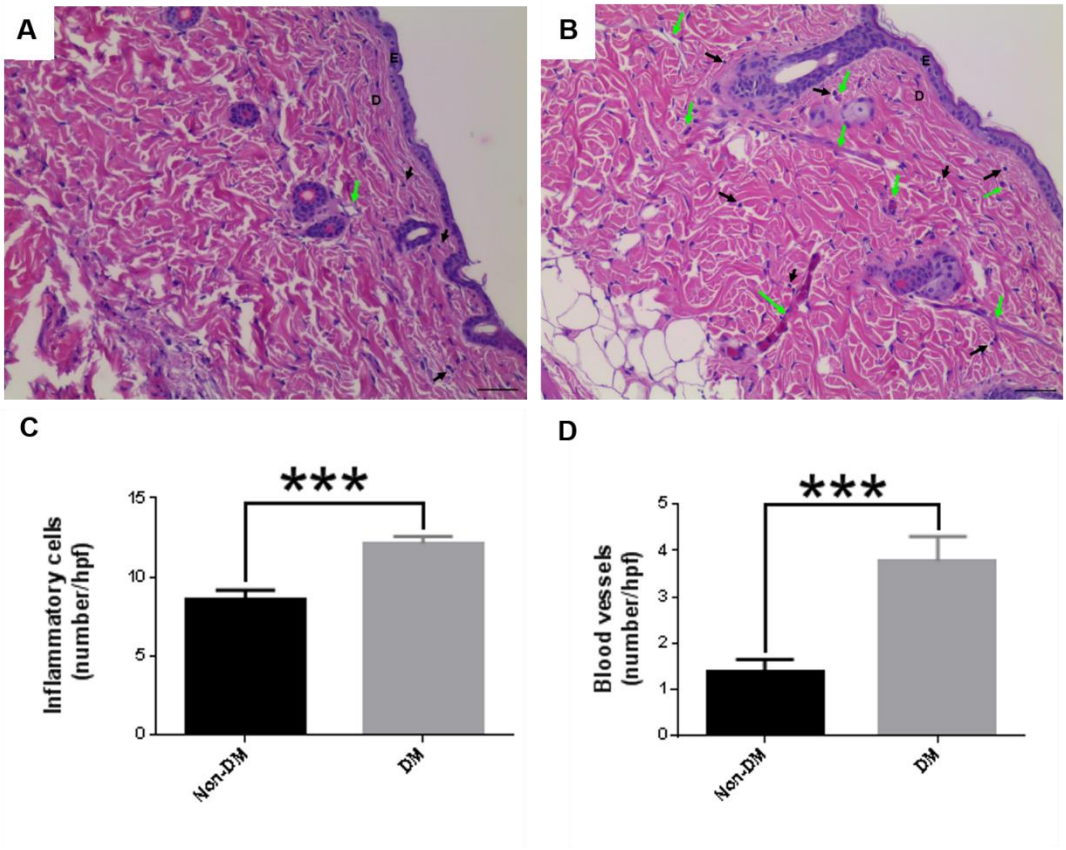


Figure 2.5. Skin inflammation and blood vessel density in dorsal skin biopsies from non-DM and DM WBB6F1 mice. Representative images of H&E staining in **(A)** non-DM and **(B)** DM WBB6F1 mouse dorsal skin. E: epidermis, D: dermis; black arrows: round inflammatory cells; green arrows: blood vessels; scale bar: 50 µm. Number of **(C)** inflammatory cells and **(D)** blood vessels assessed by H&E staining. Data represents mean ± SEM. *** p <0.001.

Finally, we compared differences between the two mice strains. Within the non-DM mice, WBB6F1 mice had higher number of inflammatory cells (8.6 ± 1.7 vs. 1.3 ± 0.6, p <0.0001) compared to C57BL6 mice, while similar differences existed between the DM mice (12.1 ± 1.4 vs. 3.2 ± 1.1, p<0.0001). However, the number of skin blood vessels was lower in both the non-DM WBB6F1 mice when compared to the non-DM C57BL6 mice (1.4 ± 0.8 vs. 3.2 ± 1.1, p<0.001) and the DM WBB6F1 compared to the DM C57BL6 mice (3.8 ± 1.6 vs. 5.3 ± 1.4, p=0.05).

2.4.5.2. CD31 staining analysis

We confirmed the blood vessel results obtained by H&E staining analysis, and extended them by identifying the number of single endothelial cells with CD31 staining (**Figure 2.6. A and B**). The number of CD31 positive skin blood vessels was increased in the DM group when compared to the non-DM group (3.7 ± 0.8 vs 1.8 ± 0.5, p<0.01) (**Figure 2.6. C**). The number of CD31 positive endothelial cells was also higher in the skin of the DM WBB6F1 mice when compared to their respective non-DM mice (6.8 ± 1.5 vs 3.8 ± 1.0, p<0.01) (**Figure 2.6. D**).

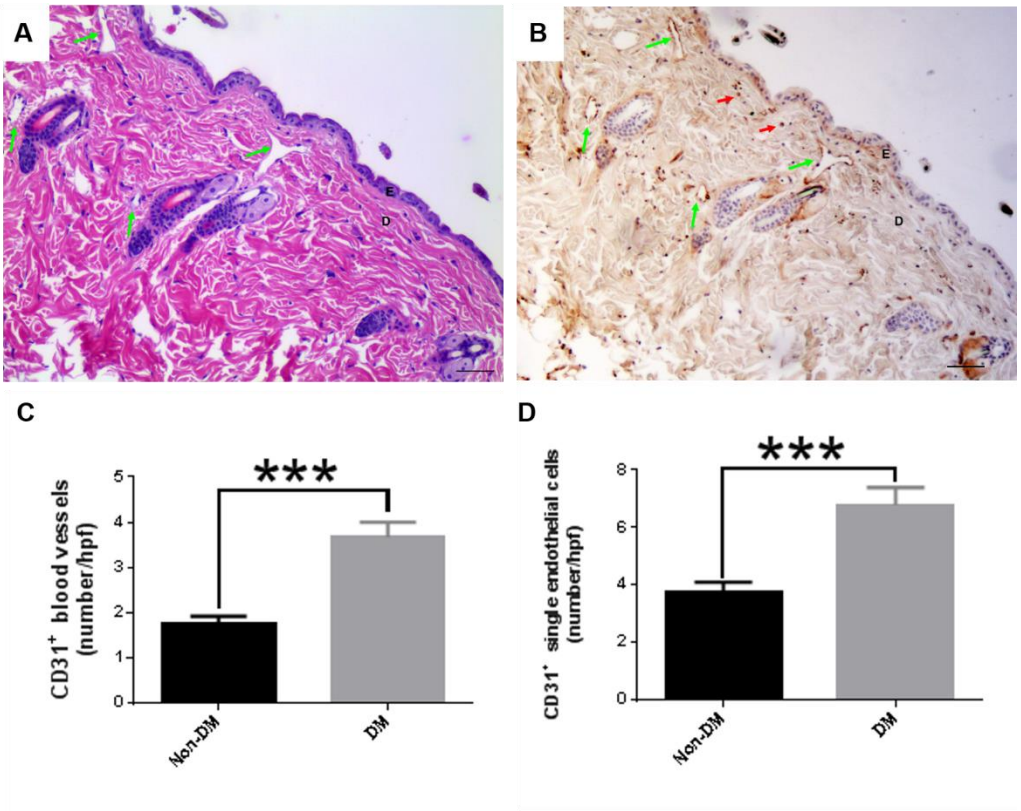


Figure 2.6. Skin blood vessel density and endothelial cell number in dorsal skin biopsies from non-DM and DM WBB6F1 mice. Representative images of H&E staining in **(A)** and **(B)** DM CD31 staining in the same mouse skin biospsy. E: epidermis, D: dermis; black arrows: round inflammatory cells; green arrows: blood vessels; red arrows: single CD31 positive cells; scale bar: 50 µm. Number of **(C)** blood vessels and **(D)** single endothelial cells assessed by CD31 staining. Data represents mean ± SEM. *** p <0.001.

2.5. Discussion

The main finding of the present study is that there is increased skin inflammation in both human and animal models of DM. This is, to the best of our knowledge, the first study to report increased inflammation at the skin level in both human and experimental DM. In addition, we have observed an increase in the number of skin blood vessels in various animal models of experimental DM that is similar to the one observed in DM patients. Finally, strong correlation was observed between the blood vessel density and inflammatory cell infiltration in both human and animal models of DM.

DM, especially type 2, and obesity are associated with increased systemic inflammation, as depicted by the elevated circulating inflammatory cytokines[358, 359]. In addition, inflammation in the adipose tissue has been proposed as one of the main factors that lead to the development of insulin resistance and type 2 DM[360, 361]. However, there is limited information regarding inflammation at the skin level. A previous study from our group was one of the first to describe increased number of inflammatory cells in the dermis, especially around hair follicles and blood vessels constituting a specific sign of inflammation, in DM[354]. In the present paper, we have expanded these observations and have shown that these changes are present in both non-neuropathic and neuropathic patients. Of interest, the present findings are in contrast with previous studies from our group that have indicated that systemic inflammation, assessed by the measurement of serum inflammatory cytokines, is present only in diabetic patients with neuropathy but is absent in diabetic patients without neuropathy[362]. These results indicate that in DM, skin inflammation probably manifests before the development of systemic inflammation and need further investigation.

An increase was also noticed in the endothelial cells proliferating around pre-existing skin blood vessels and the density of skin blood vessels in the DM patients while there were no changes in the skin blood flow. These results suggest that although there is increased endothelial cell proliferation and new vessel formation, this does not result in an increase of functional vessels that can augment skin blood flow. To the best of our knowledge, there are no previous reports regarding changes in skin blood vessels in DM. Previous studies have reported either reduced density of functional capillaries in subjects with metabolic syndrome[363, 364] or no changes in subjects with impaired glucose tolerance and type 2 DM[365]. Furthermore, our previous studies have shown impaired endothelial and smooth muscle function in the microcirculation resulting in impaired vasodilation in subjects with pre-diabetes, and in DM subjects with or without complications[357, 366]. These data indicate that although blood vessel density is increased, the functional capacity of these vessels,

especially under conditions of stress, remains limited and more studies are required to further explore this finding.

The possible mechanisms that are involved in the observed increased skin blood vessel density are not well understood. Inflammation is known to promote angiogenesis and may play a major role in the observed results[367, 368]. Previous studies in obese subjects have also shown an increase in serum Vascular Endothelial Growth Factor (VEGF) and have led to the hypothesis that adipocytes produce angiogenic factors that stimulate neovascularization which in turn plays an important role in allowing fat mass expansion[369-371]. Further studies will be required to investigate whether similar mechanisms can also affect angiogenesis at the skin level.

No differences were observed in both skin inflammation and skin vessel changes between Type 1 and 2 DM subjects. As expected, the duration of DM was longer in the type 1 patients although it was considerably long in both groups. Nonetheless, these results indicate that skin changes are similar in both types of DM of long duration.

Increased inflammatory cell infiltration and increased blood vessel density were also observed in the skin of various experimental animal models of DM that are typically used in wound healing studies. We opted to study all these animal models because there is no single model that satisfactorily represents the human condition representing skin and wound healing changes. Current consensus is that findings should be confirmed in more than one animal model. The rabbit ear model has the advantages that similarly to human wounds, rabbit wounds heal mainly by re-epithelialization, and allows the introduction of ischemia and neuropathy by ligating the central and the rostral ear artery and the central and rostral nerves respectively[179, 372]. On the other hand, rodents heal mainly by contraction but are easier to manipulate and also allow the study of various genetically engineered models. Despite the above differences, similar results were observed for both skin inflammation and skin blood vessel density in these various models. The consistency of these findings in

different species provides strong validation of the observed results. This can enable the conduction of future mechanistic studies to further understand the underlying mechanisms leading to the observed results in more than one animal model.

The number of inflammatory cells was clearly increased in all studied DM models, indicating that skin inflammation is prominent in these diabetic models. On the other hand, the changes in the skin vessel density, although heading in the same direction did not achieve statistical significance in all models. Thus, no statistical significance was reached regarding differences in blood vessel number between non-DM and DM human skin biopsies when assessed by H&E analysis despite significant differences in CD31 analysis. No differences were observed in the CD31 analysis in the rabbit ear, despite the existence of such difference when H&E analysis was employed. The main reason for these findings was that the skin vessel changes were less prominent than the inflammatory cell changes and larger numbers of human and large animals would be required to reach statistical significance in all measurements. Despite these limitations, the specificity of CD31 analysis and the confirmation it provided that that the structures observed by H&E analysis are in fact blood vessels, clearly indicate an increase in the skin blood vessel numbers in DM humans, rats and mice and a similar, but less prominent, increase in DM rabbits. Given the logistic limitations such as the number of animals that can be studied, mainly due to costs and availability of animal facilities, our results suggest that mice may be the preferred animal model to study DM-related changes in the skin vessels.

The study has its limitations. Only type 1 DM animal models were studied. However, there are no major differences in the wound healing processes between type 1 and 2 DM animal models and the models presented in this study are the commonly used models of diabetic wound healing. Also, no differences were observed regarding inflammation or vascular density at the skin level between type 1 and type 2 DM subjects. Therefore, we believe that the choice of animal models should not influence the observed results. Moreover, no statistical differences were reached in the human foot skin specimens. The main reason

for this was the small subject numbers. It should be noted that due to obvious risks, no biopsies were obtained from the feet of diabetic subjects. Instead, the foot skin samples from diabetic subjects came from discarded tissues during various operations. However, the fact that the observed results from the discarded skin of the foot were similar to those from the skin biopsies of the forearm, clearly indicate that changes at both sites in DM subjects are similar and supports the hypothesis of a generalized effect of DM. In addition to the above, the inflammatory cells were identified by H&E staining, a commonly used technique that lacks specificity. However, our previous studies using CD45RO staining have clearly indicated that our analysis specifically evaluates inflammatory cells in the human biopsies [354]. Furthermore, specific precautions, described in the methods section, were taken while analyzing the animal biopsies to exclude other types of cells, mainly transversely cut fibroblasts and/or fibrocytes that appear round, despite the fact that they are ovoid or spindle shaped. We therefore strongly believe that the observed cells were inflammatory and that there were no biases that could have affected our results.

In summary, our results indicate that there is increased number of skin inflammatory cells and blood vessels in human DM and also in rodent and the rabbit ear models of experimental DM.

Chapter III

Substance P promotes Wound Healing in Diabetes

3. Chapter III - Substance P promotes Wound Healing in Diabetes

3.1. Abstract

Diabetic foot ulceration (DFU) is a major complication of Diabetes *Mellitus* (DM). Substance P (SP) is known to be involved in wound healing but its effect in DM cutaneous wounds remains unclear. We examined the effect of SP topical treatment in skin wounds from DM mice and rabbits. We also studied the impact of deficiency in SP or its receptor on wound healing, using the tachykinin 1 *knock out* (TAC1KO) and neurokinin 1 *knock out* (NK1RKO) mouse models, respectively. SP improved wound healing in the DM neuro-ischemic rabbit ear model and in all mouse models except the NK1RKO. Wild type diabetic (WT DM), NK1RKO, and TAC1KO mice had impaired wound healing, and presented low grade inflammation before and during all stages of wound healing, failing to mount a robust acute inflammatory response to injury. Their skin M1/M2 macrophage ratio was elevated before wounding and failed to increase during the early stages of healing. SP treatment was able to reverse these conditions in all models except the NK1RKO. We also evaluated SP levels in DM and control human subjects. DM subjects had reduced serum levels and reduced skin gene expression of SP. In addition, DM skin presented increased expression of the enzyme neutral endopeptidase (NEP) that degrades SP. Taken together, these results indicate that SP promotes healing of DM wounds and reverses the chronic pro-inflammatory state in DM skin, suggesting that SP could be a potential therapy for DFU.

3.2. Introduction

Diabetic foot ulceration (DFU) is one of the most serious and debilitating complications of DM[373]. Diabetic peripheral neuropathy (DPN) and vascular disease are considered the main risk factors for DFU development, while chronic inflammation, increased MMP-9 skin expression, and aberrant growth factor signaling are major contributors to DFU failure to heal[354].

DPN is associated not only with loss of pain sensitivity, but also with lack of neuropeptides, and there is growing interest in the role of various neuropeptides, such as substance P (SP) and neuropeptide Y (NPY), in DM wound healing[149, 374]. Neuropeptides secreted by the small nerve fibers, both sensory and autonomic, play an important role during the inflammatory and proliferative phases of wound healing. Most of these molecules exert their actions by binding to specific receptors that are found in various cells in the skin, including endothelial cells (EC), mast cells (MC), fibroblasts and keratinocytes[150]. Previous studies in our unit have shown reduced neuropeptide expression in the skin of DM rabbits, which was accompanied by a chronic pro-inflammatory state, indicated by an elevated M1/M2 macrophage ratio, as well as an increased pro-inflammatory cytokine expression before wounding, and resulted in impaired wound healing[179, 375].

Substance P (SP), one of the main neuropeptides, is an 11-amino acid peptide member of the tachykinin peptide family[376] that is expressed in several areas of the central and peripheral nervous system, including peripheral nerves and skin[152]. In response to skin injury, SP is released by C-nociceptive fibers into the epidermis and papillary dermis. It exerts its effects mainly via its high affinity neurokinin 1 receptor (NK1R) and is degraded by the enzyme neutral endopeptidase (NEP)[377]. SP causes vasodilation and increased vascular permeability[378], enhances the delivery and accumulation of leukocytes[152], induces IL-8 and IL-6 secretion[379-381] and is mitogenic towards smooth muscle cells, fibroblasts and EC[382-384].

SP has been shown to promote wound healing in non-DM and DM corneal wounds that mainly involve epithelial cells[171, 385], and in non-DM cutaneous wounds [172, 386]. However, there is little information regarding its effect in DM skin wounds that are characterized by chronic inflammation, neuroischemia and increased expression of MMP that degrade proteins and growth factors involved in tissue repair[354]. Furthermore, the mechanisms of action of SP in wound healing remain unclear.

In this study, we investigated the effect of SP in DM and non-DM mouse, and DM neuro-ischemic rabbit models of cutaneous wound healing. We also investigated the translational potential of these findings by evaluating the SP serum levels, as well as the SP and NEP skin expression in DM patients and non-DM control subjects.

3.3. Materials and methods

3.3.1. Animal studies

All animal studies were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols.

3.3.1.1. Mouse models

We used 16- to 18-week-old male C57BL/6J wild type (WT), $NK1R^{-/-}$ (originated at Dr. Gerard's Laboratory, Children's Hospital, Boston) and $Tac1^{-/-}$, B6.Cg-*Tac1*<tm1Bbm>/J (Jackson Labs, Bar Harbor, Maine) mice. A subset of mice from each genotype received 50 mg/kg of streptozotocin (STZ, i.p daily for 5 consecutive days) in citrate buffer (0.1M) to induce DM. In the non-DM groups, mice were treated with vehicle alone. Fasting blood glucose was monitored a week after the last injection and mice with blood glucose levels over 250 mg/dl were considered DM. Body weight and blood glucose levels were monitored frequently and NPH insulin (0.1–0.2 units, as needed) was administered to DM mice to avoid significant weight loss.

3.3.1.2. Wound creation, monitoring and treatment

Eight weeks after STZ or vehicle only treatment, mice were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and two circular 6 mm full thickness were created on the shaved dorsum of the mice using a punch biopsy tool. Baseline (Day-0) skin biopsies were collected for histological and molecular analysis. Wound closure kinetics was monitored daily over a 10-day period by measuring the wound size using acetate tracing followed by analysis with Image J software (NIH). Data was presented as percentage of

original wound size (Day-0) over the study period. One of the wounds in each mouse received daily topical application of SP in saline (32µg/5µl/wound) while the other was treated with saline alone. At the end of the study, 3 (Day-3) or 10 (Day-10) days after wound creation, mice were euthanized and blood and wound tissue were collected for further analysis.

3.3.1.3. Medical Hyperspectral Imaging (MHSI)

Tissue oxygen saturation of hemoglobin (HbO_2) from mouse unwounded (Day-0) or peri-wound (Day-3 or Day-10) skin was monitored with a non-invasive Medical Hyperspectral Imaging System (MHSI, HyperMed Inc, Berlington, MA, USA)[387]. Imaging was performed on the shaved dorsum of the anesthetized mice, either before wound creation (Day-0) or prior to sacrifice (Day-3 or Day-10).

3.3.1.4. Tissue Collection

At Day-0, the circular 6 mm diameter skin sections were cut and collected. At the end of the study, Day-3 or Day-10 post-wounding, mice were euthanized and 1 cm X 1 cm skin sections that included the wound margins were cut. Each skin sample was further divided into different sections for morphologic and molecular analysis. For morphologic analysis and immunohistochemistry, tissue was fixed in 10% formalin and subsequently embedded in paraffin (FFPE). For immunofluorescence, skin was embedded in optimal cutting temperature media (OCT) and gradually frozen in dry ice, then stored at -80°C. For gene expression or protein analysis, tissue was snap frozen in liquid nitrogen and stored at -80°C.

3.3.1.5. Histological analysis

FFPE sections (5 µm) underwent routine histological processing with hematoxylin and eosin (H&E) for morphologic analysis. Epidermis regeneration was scored as: 0- absent, 1- incomplete regeneration, 2- complete regeneration, 3- complete maturation. The granulation tissue size was measured to assess granulation tissue formation.

3.3.1.6. Immunohistochemistry (IHC)

FFPE sections (5 µm) were stained with the following antibodies: rabbit anti-SP (Santa Cruz Biotechnology, St Cruz, CA), rabbit anti-NK1R (Santa Cruz Biotechnology, St Cruz, CA), rat anti-CD31 (BD Biosciences, San Jose, CA), and rabbit anti-NEP (Millipore, Billerica, MA). Samples were analyzed by a blinded observer and positive staining was quantified using two random high-power fields (hpf, 400x magnification). OCT frozen skin sections (5 µm) were co-stained for detection of M1 macrophages, with CD68 (AbCam, Cambridge, MA) and TNF-α (Serotec, Oxford, United Kingdom), or M2 macrophages, with CD68 and CD206 (Santa Cruz Biotechnology, St Cruz, CA). Each sample was counterstained with DAPI and visualized using a fluorescent microscope. A blinded observer counted the triple positive cells in two random hpf.

3.3.1.7. Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Standard quantitative real-time RT-PCR (qRT-PCR) was performed to evaluate and compare levels of specific RNA transcripts between RNA samples from non-DM and DM C57BL6/J WT, NK1RKO and TAC1KO mouse skin biopsies at baseline (unwounded skin, Day-0) and at the end point of the studies, 3 or 10 days post-wounding (wound samples, Day-3 or Day-10). Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was prepared from 1 µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) prior to thermal cycling using a Stratagene MX3000P qRT-PCR machine (Stratagene, La Jolla, CA). Gene amplification reactions were performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix Reagent (Stratagene, La Jolla, CA) in duplicate for each cDNA sample using 5 µL cDNA per reaction. Primer sequences (**Table 3**) were obtained from Integrated DNA Technologies (Coralville, IA). Thermal cycling was performed under the following conditions: segment 1: 3 min at 95°C; segment 2 (40 cycles): 10 sec at 95°C, 22 sec at 60°C; segment 3: 1min at 95°C, 30sec at 55°C, 30sec at 95°C. Gene expression was determined by the $^{\Delta\Delta}$CT method of relative quantification obtained as 2^{-}

$^{\Delta\Delta}$CT, and normalized to the TATA box binding protein (TBP) levels. The data is presented as the fold change over the gene expression in the skin from WT non-DM mice at baseline (Day-0).

Primer	Mouse primer sequences	
	Forward primer (5'-3')	Reverse primer (5'-3')
SP	TGGACATGGCCAGATCTCTCACAA	GCATCGCGCTTCTTTCATAAGCCA
NEP	CAGCCTCAGCCGAAACTACA	GCAAAAGCCGCTTCCACATA
NK1R	GCCAGAACATCCCAACAGG	GGCGAAGGTACACACAACCA
VEGFA	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT
VEGFR2	AAACACTCACCATTCCCAGG	CGCAAAGAGACACAT TGAGG
SDF1-alpha	GAGAGCCACATCGCCAGAG	TTTCGGGTCAATGCACACTTG
CXCR4	AGCATGACGGACAAGTACC	GATGATATGGACAGCCTTACAC
PDGF	ATTAGAGGTGCAGTGTGCGTGTGA	AGGGCACATGAGGAAGAAGACACA
FGF2	ACCGGTCACGGAAATACTCCAGTT	AACAGTATGGCCTTCTGTCCAGGT
EGF	ACGGCACAGTTTGTCTTCAATGGC	TGTTGGCTATCCAAATCGCCTTGC
IL-6	TGGCTAAGGACCAAGACCATCCAA	AACGCACTAGGTTTGCCGAGTAGA
TNF-α	TTCCGAATTCAGTGGAGCCTCGAA	TGCACCTCAGGGAAGAATCTGGAA
KC	ATTAGGGTGAGGACATGTGTGGGA	AATGTCCAAGGGAAGCGTCAACAC
MCP-1	ACTGCATCTGCCCTAAGGTCTTCA	AGAAGTGCTTGAGGTGGTTGTGGA
MMP-9	TCCAACTCACTCACTGTGGTTGCT	AGACTGCCAGGAAGACACTTGGTT
TBP	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG
Primer	Human primer sequences	
	Forward primer (5'-3')	Reverse primer (5'-3')
SP	TGTGTCTCAGGGCTGAAATG	TATGGAACCACAAACCGTGA
NEP	GATGACAATGGCAGAAACTT	CTTGAAATTGCCTGGACTGT
NK1R	TTGGCCCACAAGAGAATGAGGACA	AGTACCACTCGTTGTGGACAGCAT
TBP	TTCCACTCACAGACTCTC	ACAATCCCAGAACTCTCC

Table 3. Primer sequences used for q-RT-PCR analysis.

3.3.1.8. Western blot analysis

Mouse baseline skin (Day-0) or wounded skin samples from days 3 (Day-3) and 10 (Day-10) post-wounding were homogenized in T-PER tissue protein extraction reagent (Thermo scientific, Rockford, IL) supplemented with 1 mM dithiothreitol (Sigma, St. Louis, MO), phosphatase and protease inhibitor cocktails (Boston BioProducts, Ashland, MA). Lysates were kept at 4°C for 15 min and then centrifuged at 13,000 *g* for 15 min. The supernatant protein content was quantified using the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Proteins were denatured by adding Laemmli's SDS sample buffer (Boston BioProducts, Ashland, MA) and heating the samples for 5 min at 95°C. Equal amounts of protein (40 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% polyacrylamide gels (BioRad, Hercules, CA). The resultant gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes by a semi-dry electroblotting system (BioRad, Hercules, CA). The membranes were then blocked for 1 hour (h) at room temperature (RT), in tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. Incubation with the primary antibodies - rabbit anti-MMP9 (Cell Signaling Technology, Danvers, MA), rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit NEP (Millipore, Billerica, MA), rabbit anti-NK1R (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-beta actin (Sigma, St. Louis, MO) - was performed overnight at 4°C. The membranes were washed in TBS-T, and incubated for 1 hour at RT with the appropriate anti-fluorescein alkaline phosphatase conjugate (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA). Protein signals were detected using the ECF substrate kit (GE HealthCare Life Sciences, Pittsburgh, PA), according to the manufacturer's instructions. Bands were visualized using a VersaDoc imaging system (Model 3000, BioRad, Hercules, CA) and the band density was evaluated with the Quantity One software (Version 4.5.2, BioRad, Hercules, CA). For quantification, the band density for each protein of interest was normalized to the loading control, beta-actin. The data is presented as the

percent change over the protein levels in the skin from WT non-DM mice at Day-0 (% of WT non-DM baseline).

3.3.1.9. Multiplex analysis

Immediately after collection, blood was centrifuged at 14000 rpm, at 4°C for 20 minutes. Serum was then collected and stored at -80°C until analysis. Serum protein levels of inflammatory cytokines and biochemical markers of endothelial function were measured using a Luminex 200 apparatus (Luminex, Austin, TX) and Millipore multiplex immunoassay panels (Millipore, Chicago, IL).

3.3.2. Diabetic rabbit ear model

New Zealand White male rabbits weighing 3.0 to 3.2 kg (Millbrook Farms, Amherst, MA) received alloxan monohydrate (75mg/kg i.v.) to become DM. Rabbits with fasting blood glucose over 250 mg/dl were considered diabetic. 30 days after alloxan treatment, a neuro-ischemic ear wound was created[179, 180]. Wounds were either untreated, treated with alginate gel alone (90µl/wound) or with alginate gel encapsulating SP (32µg/90µl/wound).

3.3.3. Human subjects

DM patients and healthy control subjects were recruited and enrolled in the study. The exclusion criteria were: presence of foot ulceration at the time of recruitment; clinically present peripheral arterial disease (PAD); end stage renal disease (patients on renal dialysis or kidney transplantation); any other serious chronic disease that can affect wound healing. The cohort was well characterized in terms of gender, age, duration of DM, and clinical condition. All subjects attended the Joslin-Beth Israel Deaconess Foot Center and the General Clinical Research Center where they had a full physical examination and all tests described below. All DM patients received education about foot care and were regularly seen by their podiatrist as required. Protocols were approved by the Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center. All participants gave written informed consent.

3.3.3.1. Serum samples

Serum from healthy control subjects and DM patients was analyzed for the measurement of SP using a Luminex 200 apparatus (Luminex, Austin, TX) and Millipore multiplex immunoassay panels (Millipore, Chicago, IL).

3.3.3.2. Forearm and foot skin samples

For the **forearm skin samples**, a 2 mm skin punch biopsy was taken from the volar aspect of the forearm. Forearm skin biopsies were used for SP, NK1R, and NEP immunohistochemistry. For the **foot skin samples**, discarded skin specimens were obtained from subjects who underwent foot surgery for various reasons. Foot skin specimens were used for western blot and real time q-RT-PCR.

3.3.4. Statistical Analysis

All statistical analysis was performed using Minitab (Minitab, State College, PA). The statistical power analysis was based on preliminary data or data from other studies in our unit. Analysis of variance (ANOVA) and the t-test were employed for the comparisons among the groups for normally distributed data. Non-parametrical data were analyzed through Mann-Whitney and Kruskal-Wallis tests.

3.4. Results

3.4.1. Animal studies

3.4.1.1. SP treatment improves wound healing

WT DM mice had delayed wound healing when compared with WT non-DM from Day-5 to Day-10 post-wounding (**Figure 3.1. A and B**). Topical treatment with SP accelerated wound closure from Day-8 to Day-10 post-wounding in both WT non-DM and DM mice (**Figure 3.1. B**). In addition, SP treatment accelerated healing in the rabbit DM neuro-ischemic wound model, which mimics neuropathy and ischemia that are commonly present in human DFU

(**Figure 3.1. C and D**). These results suggest that pharmacologic levels of SP improve wound healing in DM.

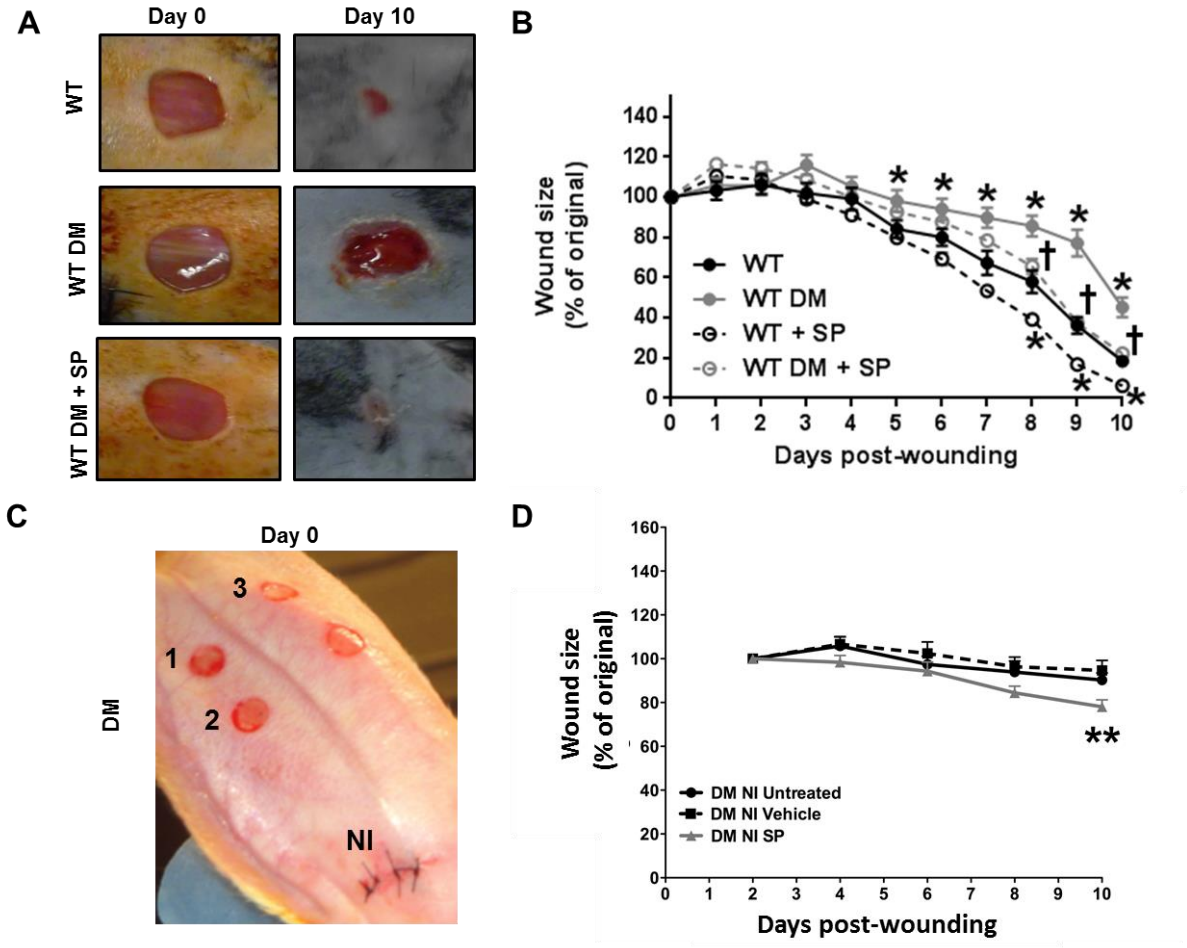


Figure 3.1. Substance P (SP) topical treatment accelerates wound healing. (A) Representative images of the wounds at baseline (Day-0) and (Day-10) of wild-type non-diabetic (WT), WT diabetic (WT DM), and WT diabetic SP treated wounds (WT DM + SP). **(B)** WT DM showed delayed healing compared to WT non-DM. SP accelerated wound closure in both WT and WT DM mice. Data represent mean ± SEM.*p<0.05, compared to WT. †p<0.05, compared to DM. **(C)** Neuro-ischemic wound healing rabbit ear model. Day-0 wounds 1: untreated; 2: vehicle-treated; 3: SP-treated; NI: neuro-ischemia. (D) SP improved wound healing in a DM NI rabbit wound healing model. Data represent mean ± SEM. **p<0.01 compared to untreated and vehicle-treated wounds.

3.4.1.2. The absence of SP or its receptor affects wound healing

To further investigate the role of SP in wound healing, we used two genetically modified mouse models: (i) mice deficient in the neurokinin 1 receptor (NK1RKO), the receptor of SP that is most abundant and has highest affinity, and (ii) mice deficient in the tachykinin 1 (TAC1) gene that encodes for SP and other tachykinins (TAC1KO). Both non-DM and DM NK1RKO and TAC1KO mice showed impaired wound healing when compared with respective WT controls (**Figure 3.2. A and B**).

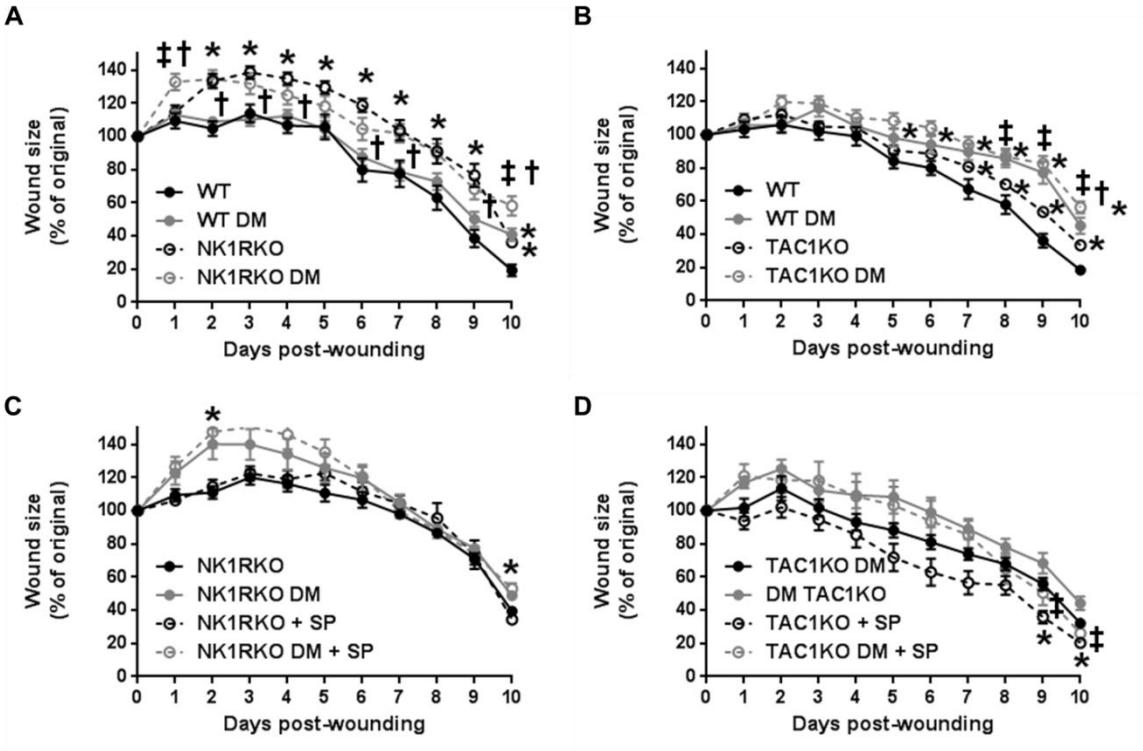


Figure 3.2. NK1R- or SP-deficiency impair wound healing. The effect of SP is mediated by NK1R. (A) Non-DM and DM NK1RKO mice showed delayed wound closure. *p<0.05 compared to WT non-DM; †p<0.05 compared to WT DM; ‡p<0.05 compared to NK1RKO. **(B)** Non-DM and DM TAC1KO mice showed delayed wound closure. *p<0.05 compared to WT; †p<0.05 compared to DM; ‡p<0.05 compared to TAC1KO. **(C)** SP treatment did not affect healing kinetics in NK1RKO mice. *p<0.05 compared to NK1RKO. **(D)** SP treatment improved wound healing in non-DM and DM TAC1KO mice. Data represent mean ± SEM.*p<0.05 compared to TAC1KO; ‡p<0.05 compared to TAC1KO DM.

3.4.1.3. NK1R mediates the SP effect in wound healing

To evaluate whether the SP effect in wound healing was mediated mainly by NK1R rather than by NK2R or NK3R, SP was topically applied to the wounds of NK1RKO mice. SP treatment did not affect wound closure kinetics in NK1RKO mice (**Figure 3.2. C**). SP accelerated wound closure in both non-DM and DM TAC1KO mice (**Figure 3.2. D**), suggesting that SP is the main tachykinin expressed by the TAC1 gene involved in the wound healing process.

3.4.1.4. SP effect on tissue granulation

We evaluated granulation tissue formation by histological analysis at Day-10 post-wounding. Granulation tissue was significantly reduced in WT DM and in both DM and non-DM NK1RKO and TAC1KO mice (**Figure 3.3**.). These results suggest that the lack of SP or its receptor significantly affect dermal repair.

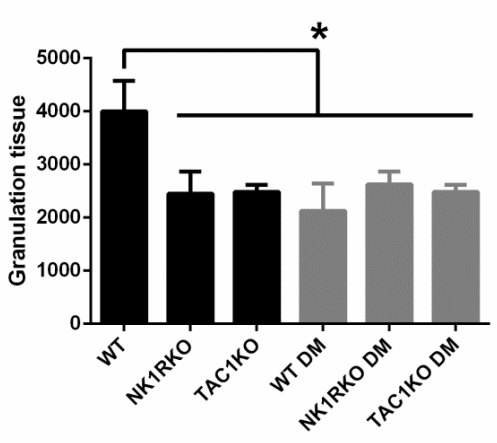


Figure 3.3. Granulation tissue formation is reduced in WT DM, TAC1KO and NK1RKO wounds. Granulation tissue was reduced in Day-10 wounds from WT DM and both non-DM and DM TAC1KO and NK1RKO mice when compared to the WT non-DM controls. Data represent mean ± SEM.*p<0.05.

3.4.1.5. Skin gene expression of SP is reduced in diabetes

At baseline (Day-0), SP gene expression was reduced in the skin of WT DM compared to WT non-DM mice. When compared to baseline, SP skin gene expression was increased in WT non-DM mice at both Day-3 and Day-10 post-wounding. A similar but not as pronounced response to injury was observed in the WT DM mice, resulting in a significant reduction at Day-3, and a marginal reduction at Day-10 when compared to WT non-DM at the same time points (**Figure 3.4. A**). Local SP treatment of WT non-DM wounds reduced SP gene expression at Day-10. In addition, SP skin gene expression was increased in NK1RKO DM mice compared to WT DM mice both at Day-0 and Day-10 (**Figure 3.4. B**). Interestingly, NK1R baseline skin gene expression was increased in TAC1KO DM mice (**Figure 3.4. C**). These results indicate that SP skin gene expression is reduced in DM mice before and during all phases of wound healing, while there is a compensatory overexpression of SP or its receptor in mice that lack NK1R or SP, respectively.

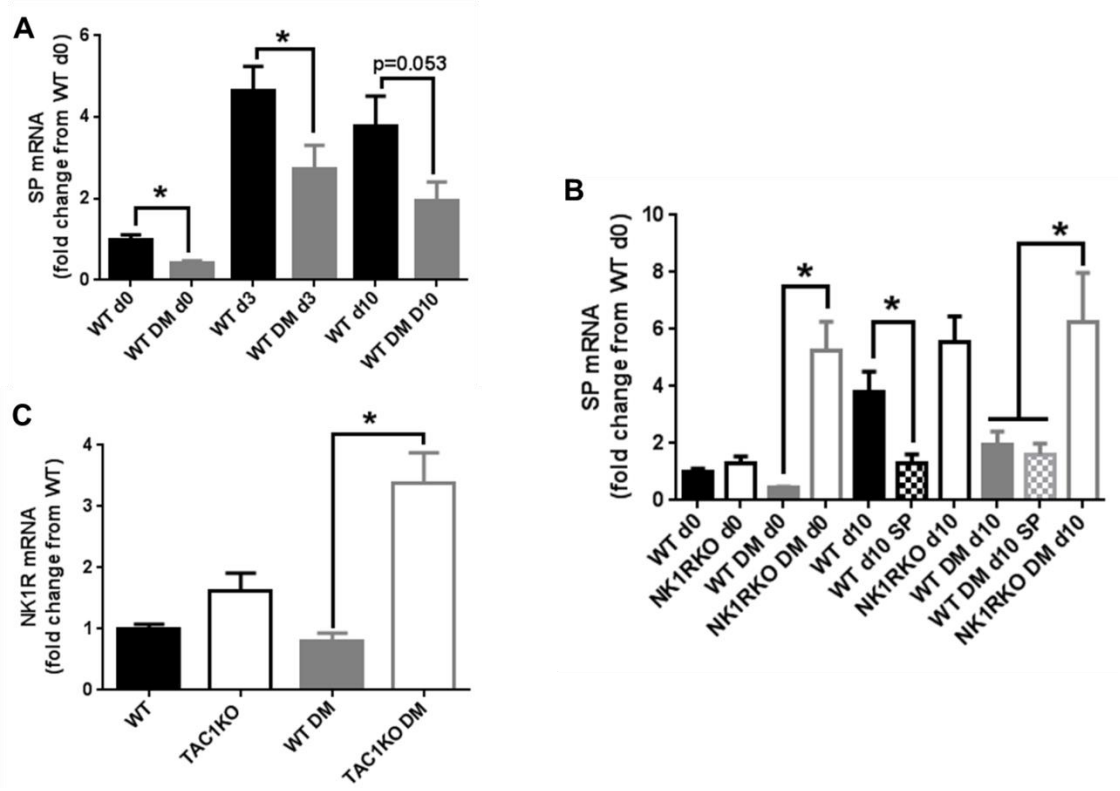


Figure 3.4. SP skin gene expression is reduced in DM. (A) SP skin gene expression was decreased in WT DM mice at baseline and after wounding. **(B)** SP skin gene expression was

increased in NK1RKO DM mice at Day-0 and Day-10. Local treatment with SP reduced SP expression in WT wounds. **(C)** Skin NK1R gene expression was increased in TAC1KO DM mice at baseline and trended to be increased in non-DM TAC1KO when compared to their respective WT controls. Data represent mean ± SEM. *p<0.05, †p<0.05 compared to WT non-DM Day-0.

3.4.1.6. Skin gene and protein expression of NEP is increased in WT DM and NK1RKO mice but not in TAC1KO mice

At baseline, skin gene expression of NEP, the enzyme that breaks down SP, was increased in WT DM mice and in both non-DM and DM NK1RKO and TAC1KO mice (**Figure 3.5. A**). In addition, baseline skin protein expression was increased in WT DM and in non-DM and DM NK1RKO mice (**Figure 3.5. B-D**). In WT non-DM wounds, NEP levels peaked at Day-3 but returned to pre-wounding levels by Day-10. In contrast, in WT DM mice, NEP levels remained elevated (**Figure 3.5. E and F**), indicating that this is an additional factor that contributes to reduced SP bioavailability.

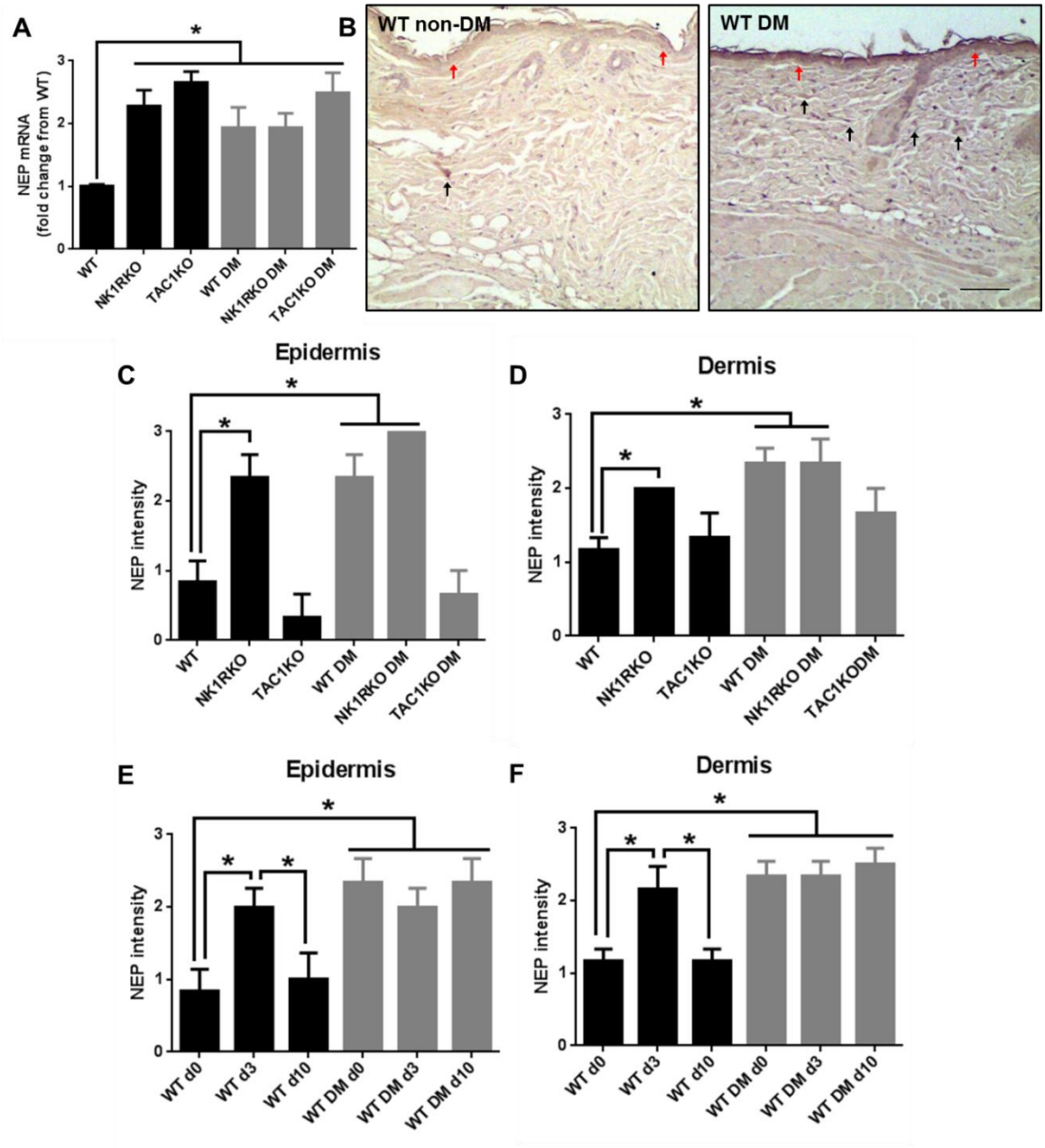


Figure 3.5. NEP skin expression is increased in WT DM, NK1RKO and TAC1KO mice. **(A)** NEP skin gene expression was elevated at baseline in WT DM and both NK1KO and TAC1KO mice. **(B)** Representative images of NEP staining in baseline (Day-0) skin from WT and WT DM mice. Arrows point to NEP^{+} positive cells in epidermis (red arrows) and dermis (black arrows). Scale bar: 100 µm. NEP expression was increased in both epidermis **(C)** and dermis **(D)** of WT DM and NK1RKO mice at baseline. In WT non-DM mice, epidermal **(E)** and dermal **(F)** NEP expression increased at Day-3 but returned to baseline levels by Day-0, whereas in DM mice it remained elevated throughout the healing process. Data represent the mean ± SEM. *p<0.05.

3.4.1.7. SP promotes the acute inflammatory response to skin injury

Skin gene expression of IL-6 and KC (mouse homolog for human IL-8) was increased at baseline in WT DM mice compared to WT non-DM. In addition, whereas in WT non-DM mice it peaked at Day-3 and lowered at Day-10, in WT DM mice it was persistently elevated. SP treatment further increased IL-6 and KC gene expression at Day-3 and reduced it at Day-10 in both WT non-DM and DM mice (**Figure 3.6. A and C**). Moreover, IL-6 and KC skin gene expression was elevated in both non-DM and DM NK1RKO and TAC1KO mice at baseline when compared to their WT controls (**Figure 3.6. B and D**). Similarly, skin gene expression of TNF-α was elevated in Day-10 wounds from WT DM mice and SP treatment reduced it (**Figure 3.7. A**), whereas it was increased in baseline skin of NK1RKO and TAC1KO mice. (**Figure 3.7. B**)

Serum IL-6 levels were increased in DM NK1RKO and DM TAC1KO mice at baseline (**Figure 3.6. E**). At Day-3, an increase of IL-6 circulating levels from baseline was observed in all groups. Of interest, at Day-10, serum IL-6 remained elevated in WT DM and NK1RKO DM mice, while it returned to baseline levels in all the remaining conditions. Similarly, higher serum levels of KC were observed in WT diabetic and/or transgenic mice (**Figure 3.6. F**).

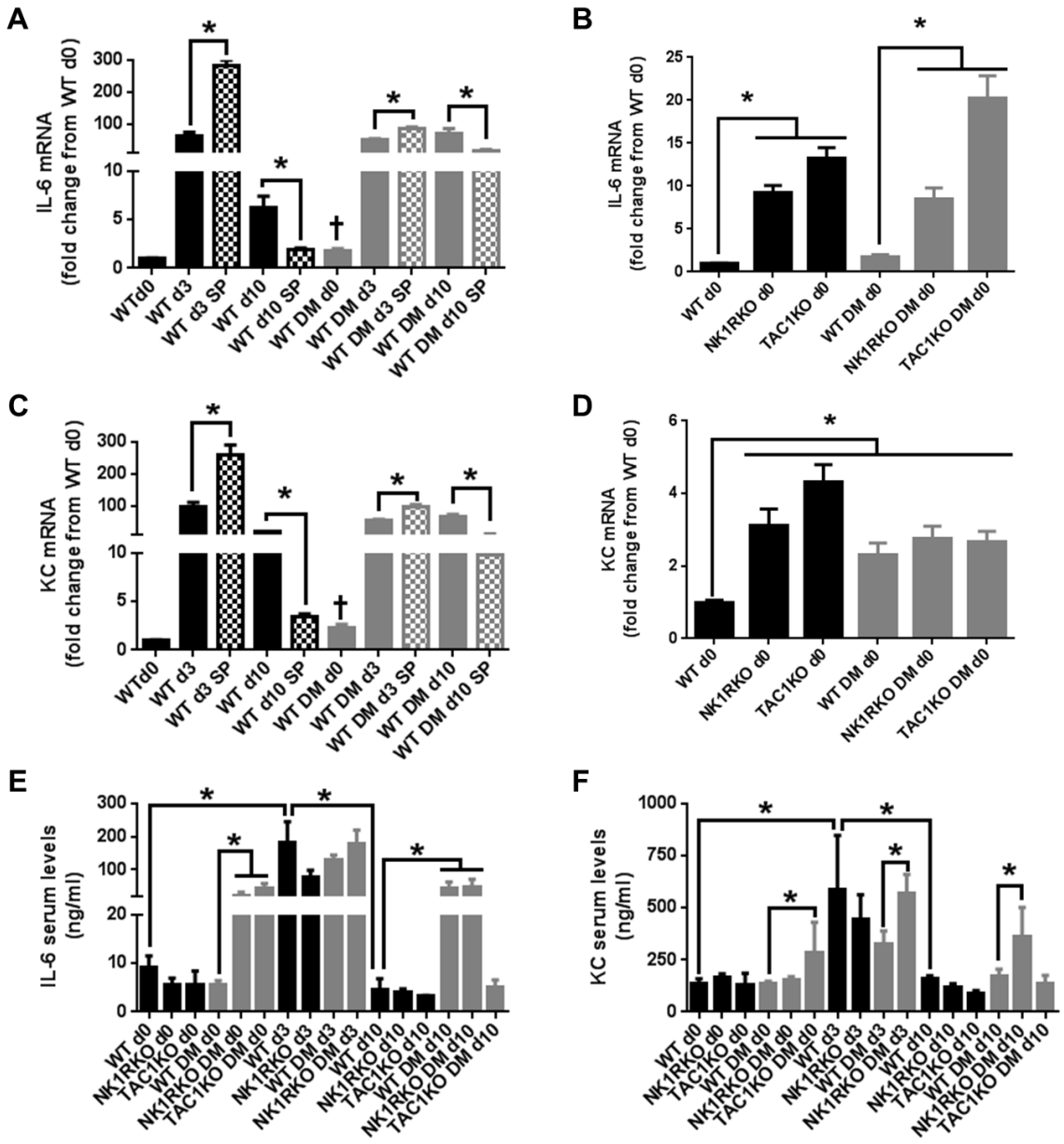


Figure 3.6. Substance P promotes the acute inflammatory phase of wound healing. WT DM mice showed an increase in IL-6 **(A)** and KC **(C)** skin gene expression at baseline. At Day-3, IL-6 and KC skin gene expression increased from baseline and SP further induced this increase. At Day-10, cytokine gene expression decreased from Day-3 in WT non-DM mice but not in WT DM mice. SP treatment reduced cytokine expression at Day-10. NK1RKO and TAC1KO mice had elevated IL-6 **(B)** and KC **(D)** skin gene expression at baseline. Serum levels of IL-6 **(E)** and KC **(F)** peaked at Day-3 and returned to baseline at Day-10 in WT non-DM mice, but not in WT-DM or NK1RKO mice. Data represent mean ± SEM. *p<0.05; †p<0.05 compared to WT non-DM Day-0.

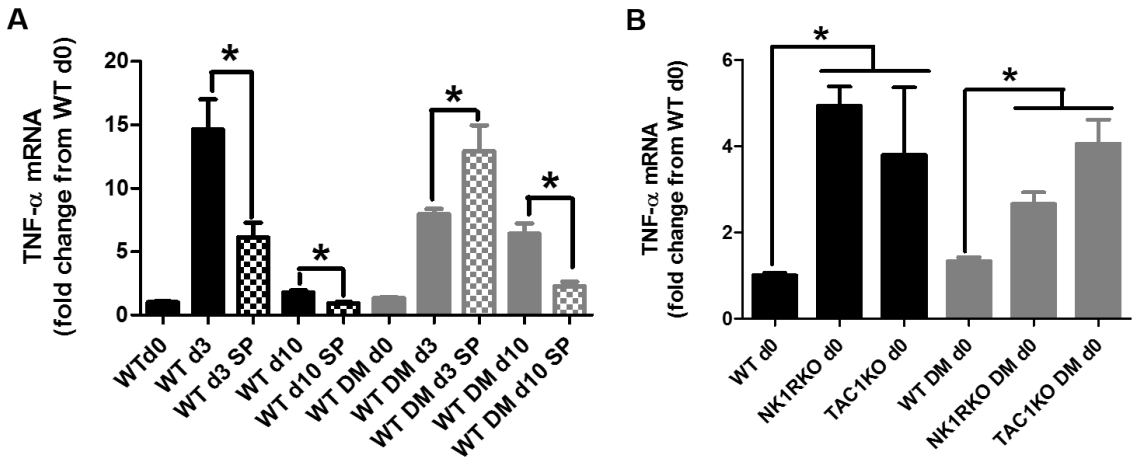


Figure 3.7. TNF-α gene expression is reduced during the acute inflammatory phase and elevated during the later stages of healing in DM wounds. SP reverses this effect while SP- or NK1R-deficiency shows increased TNF-α baseline skin expression. (A) TNF-α skin gene expression was reduced at Day-3 and elevated at Day-10 in DM wounds compared to non-DM. SP treatment reversed this effect resulting in TNF-α expression similar to non-DM levels. **(B)** Non-DM and DM NK1RKO and TAC1KO mice showed increased TNF-α expression at baseline. Data represent mean ± SEM *p<0.05

The above results indicate that DM and/or lack of SP or its receptor are associated with low grade inflammation before wounding, inability to mount a robust acute inflammatory response in the early stages of wound healing and persistence of low grade inflammation at the later phases of healing. In contrast, SP treatment promotes an early acute inflammatory response, which enables progression to the proliferative phase of healing.

3.4.1.8. SP modulates macrophage activation phenotype during wound healing

We evaluated M1 and M2 macrophage polarization in wound healing progression (**Figure 3.8. A**) and calculated the M1/M2 ratio that best reflects the inflammatory state of the tissue. In WT non-DM skin, the M1/M2 ratio increased at Day-3 and returned to baseline levels by Day-10. In contrast, in WT DM skin, the M1/M2 ratio was increased at baseline and did not significantly change during wound healing. At Day-10, SP treatment reduced the DM-

induced elevated M1/M2 ratio (**Figure 3.8. B**). Similarly to WT DM mice, non-DM and DM NK1RKO and TAC1KO mice showed a persistently elevated M1/M2 ratio (**Figure 3.8. C**).

The skin gene expression of monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes to areas of inflammation where they differentiate to macrophages, was increased at Day-3 in both WT non-DM and DM mice when compared to Day-0. At Day-10, MCP-1 skin gene expression decreased from Day-3 in both groups, but was increased in WT DM mice compared to non-DM (**Figure 3.8. D**). SP treatment further increased MCP-1 expression at Day-3 and reduced it at Day-10 in both WT non-DM and DM wounds. TAC1KO non-DM and DM mouse skin showed higher MCP-1 gene expression at Day-0 (**Figure 3.8. E**).

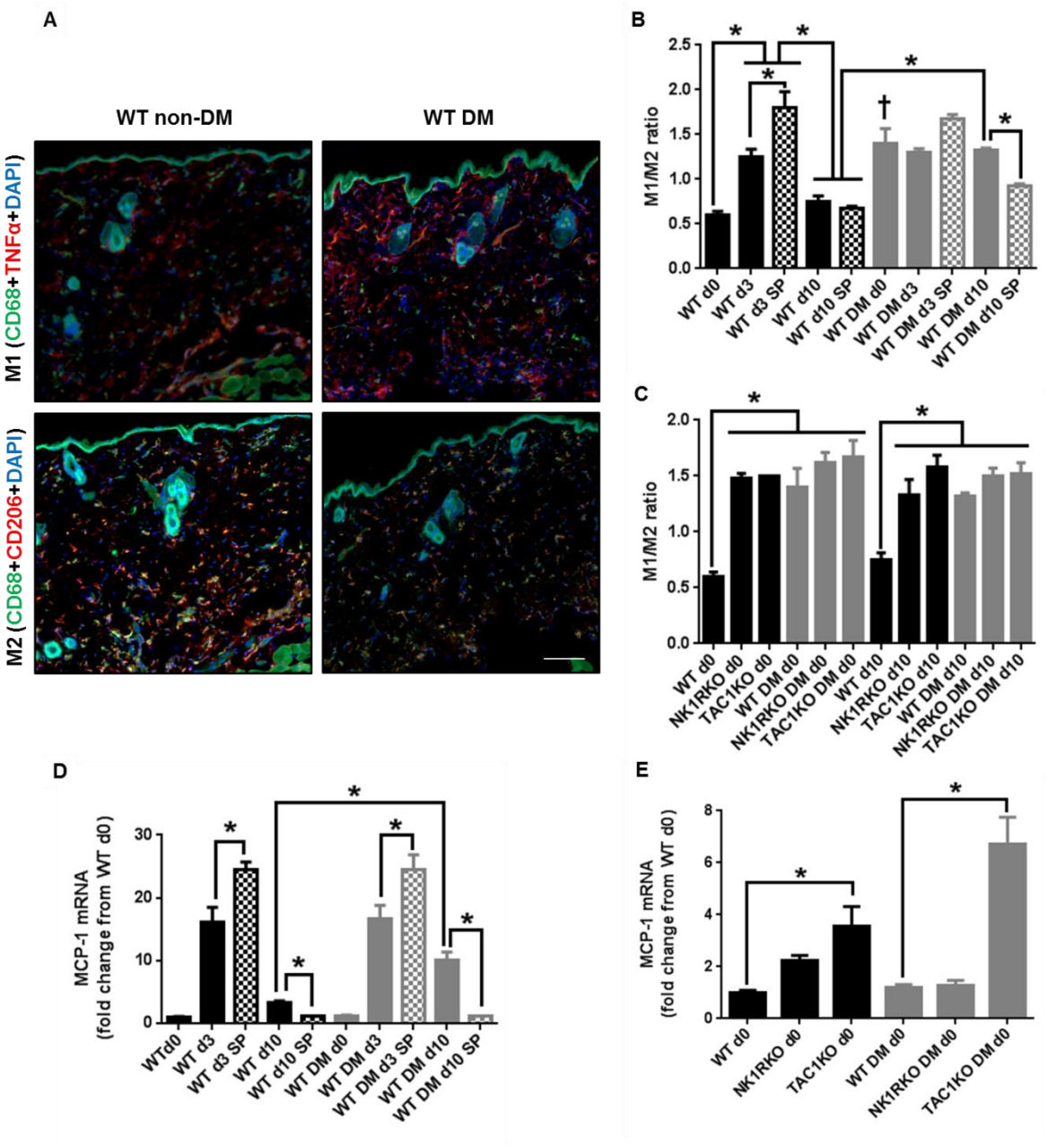


Figure 3.8. SP modulates skin macrophage phenotype during healing. (A) Representative images of M1 and M2 macrophages in WT non-DM and DM mouse skin. Scale bar: 100 µm. M1 (upper panel) and M2 (lower panel) are denoted by the yellow-orange stain resulting from triple positive stain with CD68, TNFα and DAPI (for M1) or CD68, CD206 (for M2) and DAPI. **(B)** M1/M2 ratio was higher in DM at Day-0. In non-DM, M1/M2 peaked at Day-3 and returned to baseline levels at Day-10, while it was persistently elevated in DM. SP increased M1/M2 at Day-3 and reduced it at Day-10. **(C)** M1/M2 was increased in non-DM and DM NK1RKO and TAC1KO at Day-0 and Day-10. **(D)** MCP-1 skin gene expression peaked at Day-3 and returned to pre-wounding levels at Day-10 in WT non-DM, but not in DM mice. In both WT non-DM and DM mice, SP treatment further increased

MCP-1 at Day-3 and reduced it at Day-10. **(E)** MCP-1 gene expression was increased at baseline in TAC1KO non-DM and DM mice. Data represent mean ± SEM. *p<0.05; †p<0.05 compared to WT non-DM Day-0.

The above results indicate that, similarly to DM, SP- or NK1R-deficiency result in pro-inflammatory activation of skin macrophages before wounding. In addition, SP plays an important role in regulating macrophage function during healing: it recruits macrophages to the wound during the early stages of healing, and shifts macrophage activation to the regenerative M2 phenotype during the later stages.

3.4.1.9. MMP-9 skin expression is increased in WT DM and in SP or NK1R deficient mice while SP reduces MMP-9 wound expression

At Day-0, MMP-9 skin gene expression was increased in the WT DM mice (**Figure 3.9. A**). At Day-3, WT non-DM and DM mice showed increased MMP-9 skin gene expression compared to baseline; however, this increase was more pronounced in DM wounds and SP treatment reduced it. MMP-9 baseline skin gene expression was elevated in non-DM NK1RKO and TAC1KO mice when compared to WT (**Figure 3.9. B**). Similar results were found regarding MMP-9 skin protein levels. Thus, MMP-9 protein levels were increased in WT DM mice at Day-0 when compared to WT non-DM. An increase from baseline was observed at Day-3 in both WT non-DM and WT DM mice (**Figure 3.9. C**). At Day-10, MMP-9 was higher in WT DM compared to WT non-DM wounds, while SP treatment further reduced it in the WT non-DM. NK1RKO wounds showed a decrease in MMP-9 levels at Day-10 (**Figure 3.9. D**), whereas DM TAC1KO wounds showed an increase at Day-0 and Day-10 (**Figure 3.9. E**), when compared to WT controls. These results suggest that similarly to DM, lack of SP leads to chronically elevated skin MMP-9 expression, while SP treatment reduces wound MMP-9 expression.

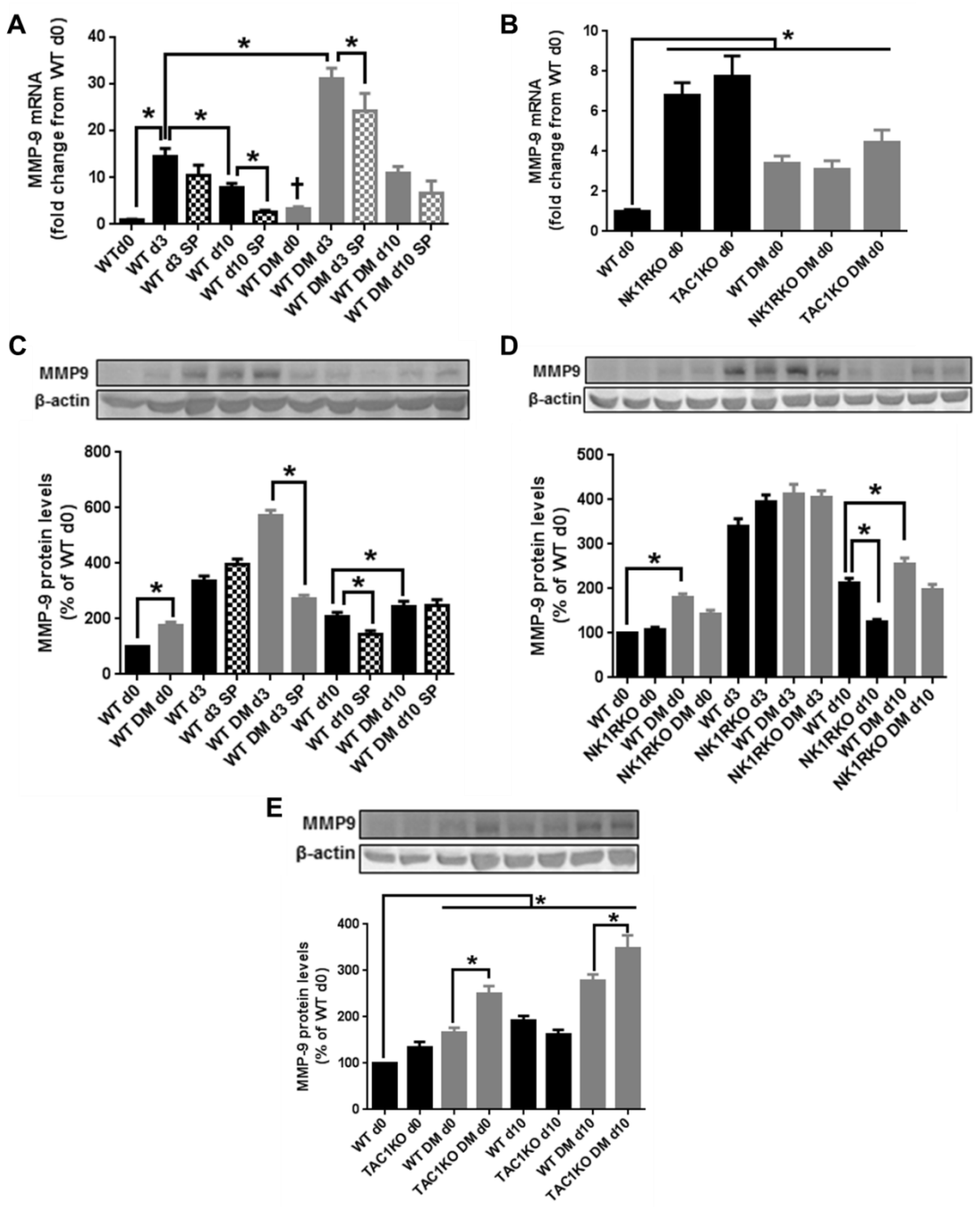


Figure 3.9. Diabetes, SP deficiency, and NK1R deficiency increase MMP-9 skin expression, while SP treatment reduces it. (A, C) DM increased skin MMP9 gene **(A)** and protein **(C)** expression at baseline and caused an exaggerated increase at Day-3, which was reduced by SP treatment. **(B)** NK1RKO and TAC1KO mice showed increased MMP-9 gene expression at Day-0. **(D)** NK1RKO mice showed decreased MMP9 protein levels in skin at Day-10. **(E)** TAC1KO DM mice showed increased MMP-9 protein levels at Day-0 and Day-10. Data represent mean ± SEM. *p<0.05. †p<0.05 compared to WT non-DM Day-0.

3.4.1.10. SP does not play a major role in wound neovascularization

We evaluated the effect of SP on the number of CD31 positive endothelial cells and blood vessels in the skin of our various mouse models (**Figure 3.10. A**).

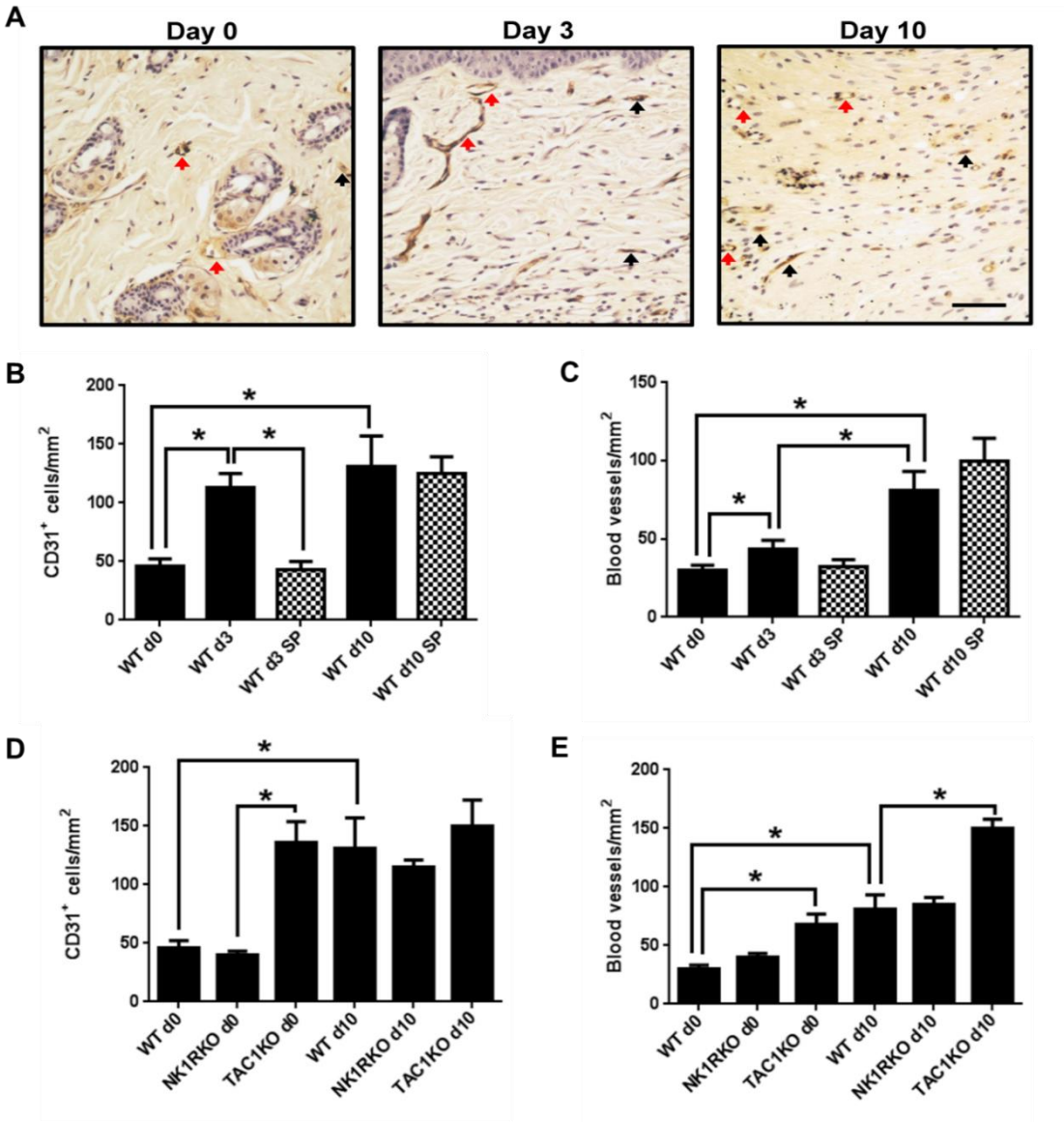


Figure 3.10. SP-deficiency increases skin blood vessel density. (A) Representative images of CD31 staining in WT mouse skin at Days 0, 3 and 10. Red arrows: blood vessels; black arrows: single CD31 positive cells. Scale bar: 50 µm. **(B, C)** The number of $CD31^+$ cells and vessels increased post-wounding in WT mice. **(D)** TAC1KO mice showed increased $CD31^+$ cells at baseline. **(E)** TAC1KO skin had increased blood vessel density at Day-0 and Day-10. Data represent mean ± SEM. *$p<0.05$.

As expected, the number of blood vessels and single endothelial cells increased post-wounding in WT mice (**Figure 3.10. B, C**). No major differences were observed between the study groups except that TAC1KO mice showed increased number of $CD31^+$ cells and increased number of blood vessels at Day-0 (**Figure 3.10. D, E**). These results suggest that similarly to DM (see Chapter II), SP-deficiency increases the baseline skin blood vessel density.

At Day-0, VEGF skin gene expression was reduced in WT DM mice but was increased in both non-DM and DM NK1RKO and TAC1KO mice. At Day-3, SP treatment did not affect VEGF gene expression. However, at Day-10, SP reduced VEGF expression in both WT non-DM and DM wounds (**Figure 3.11. A**). Similar results were observed for the expression of VEGF receptor 2 (VEGFR2), SDF1-α and its receptor CXCR4 (**Figure 3.12.**).

VEGF protein levels were reduced in WT DM mice at Day-0, while they were increased in non-DM NK1RKO mice at Day-0, and in DM NK1RKO mice at Day-0, Day-3 and Day-10. Non-DM TAC1KO mice had reduced VEGF protein levels, while DM TAC1KO mice had increased expression at both Day-0 and Day-10 (**Figure 3.11. B**).

These results indicate that VEGF skin expression is reduced in DM and increased in SP- or NK1R-deficiency, while SP treatment reduces it without affecting wound neovascularization.

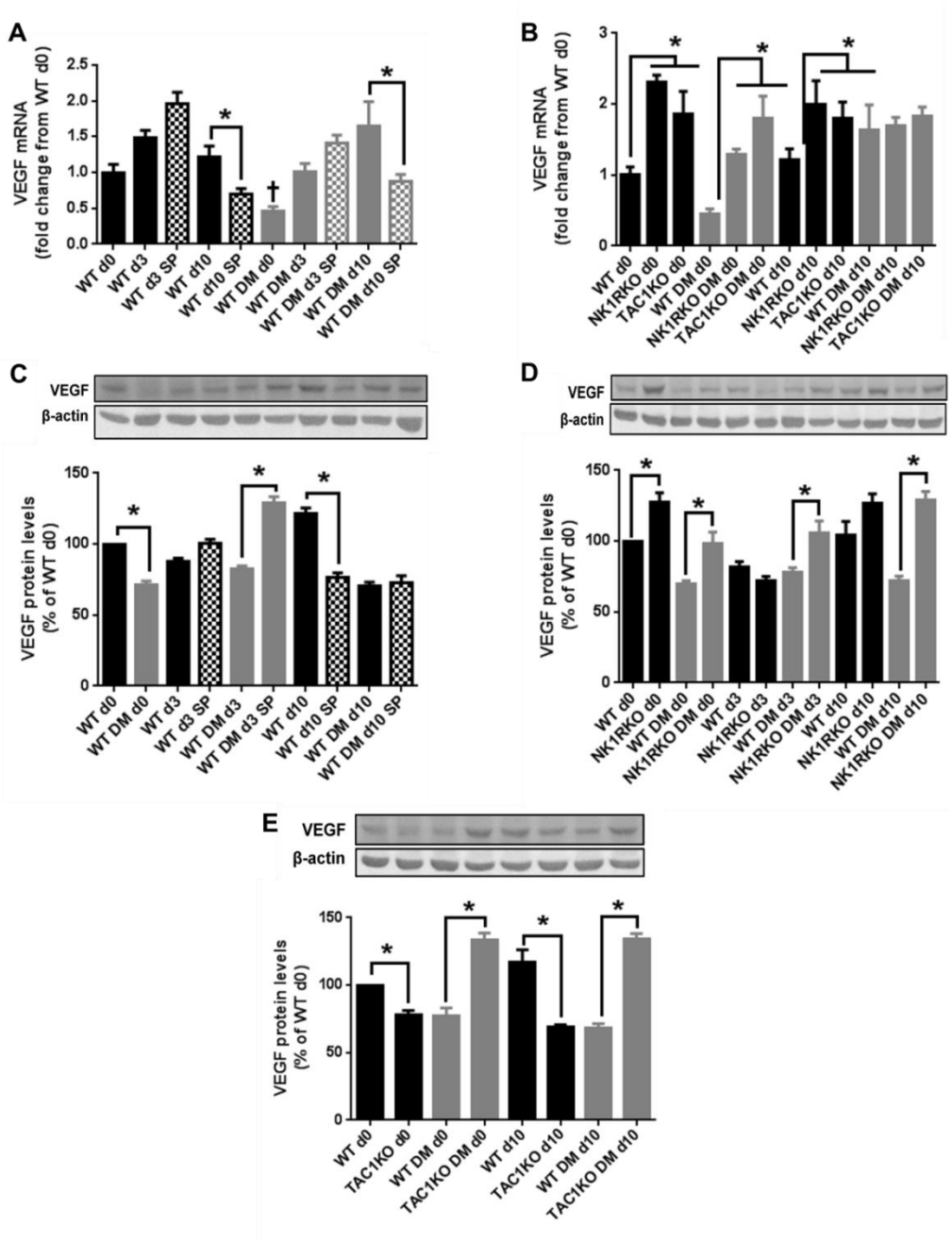


Figure 3.11. VEGF skin expression is altered in DM and SP- or NK1R-deficiency. (A) VEGF skin gene expression was decreased in WT DM at Day-0. **(B)** VEGF gene expression was increased in TAC1KO and NK1RKO at Day-0 and Day-10. **(C)** VEGF skin protein expression was decreased at baseline in WT DM. SP treatment increased VEGF levels at Day-3 in DM wounds and decreased it at Day-10 in non-DM wounds. **(D)** NK1RKO mice had higher skin VEGF protein expression. **(E)** VEGF protein was reduced in non-DM TAC1KO but increased in DM TAC1KO. Data represent mean ± SEM. *p<0.05. †p<0.05 compared to WT non-DM Day-0.

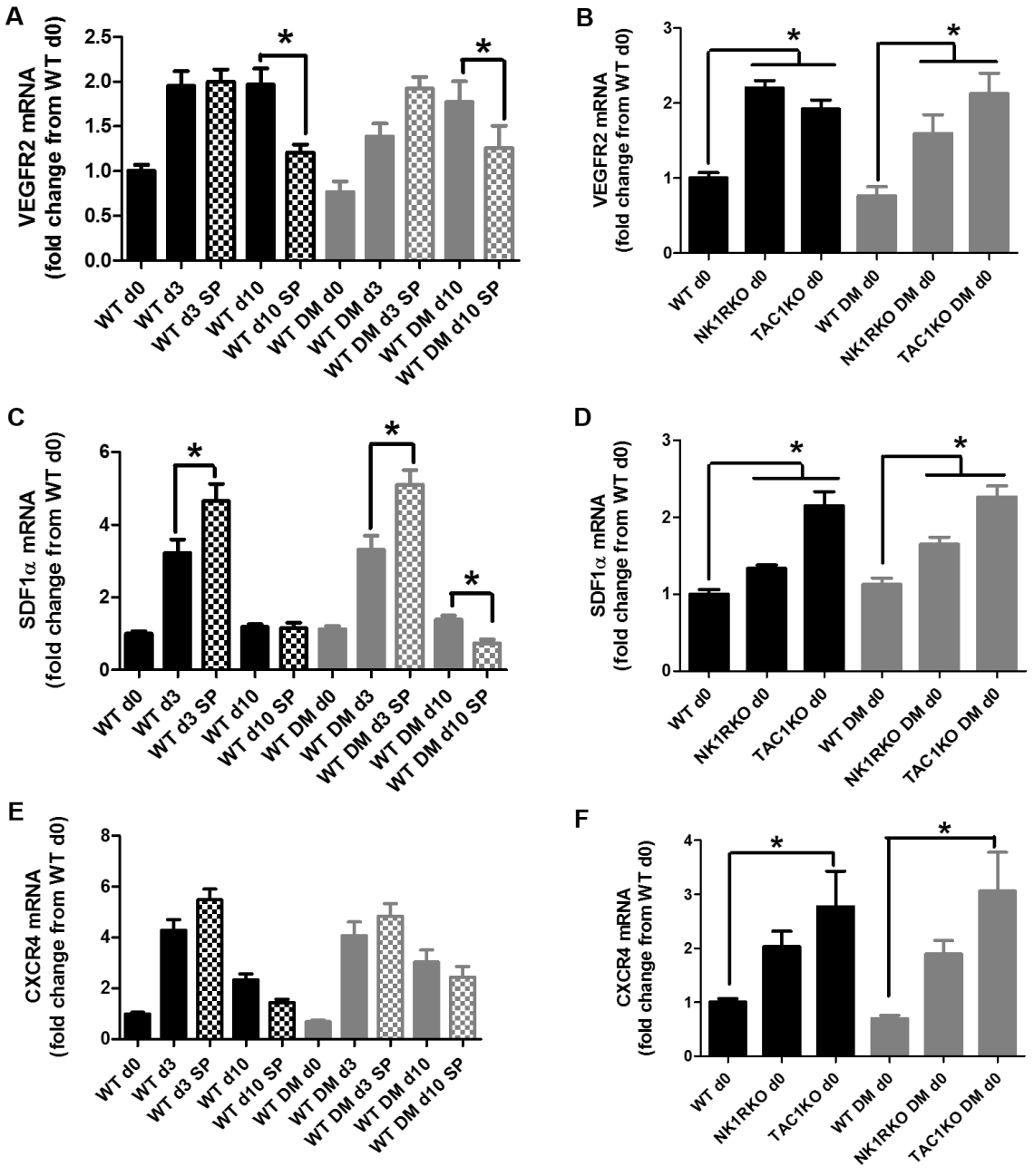


Figure 3.12. SP treatment reduces skin expression of pro-angiogenic and pro-vasculogenic factors at the later stages of healing while SP- or NK1R-deficiency increases it at baseline. Skin gene expression of VEGFR2 **(A)**, SDF1-α **(C)**, and CXCR4 **(E)** increased post-wounding in both WT non-DM and WT DM mice. SP treatment reduced VEGFR2 **(A)** and SDF1-α **(C)** expression at day-10 but increased SDF1-α expression **(C)** at Day-3. NK1RKO and/or TAC1KO mice, with or without DM, showed increased expression of VEGFR2 **(B)**, SDF1-α **(D)**, and CXCR4 **(F)** at baseline. Data represent mean ± SEM. *p<0.05.

3.4.1.11. SP- or NK1R-deficiency affect skin expression of pro-angiogenic growth factors; however, SP local treatment does not have a significant effect

SP treatment did not affect wound gene expression of PDGF, FGF2, or EGF (**Figure 3.13. A, C, E**). However, NK1RKO and TAC1KO mice showed increased growth factor expression at baseline and through wound healing progression (**Figure 3.13. B, D, F**).

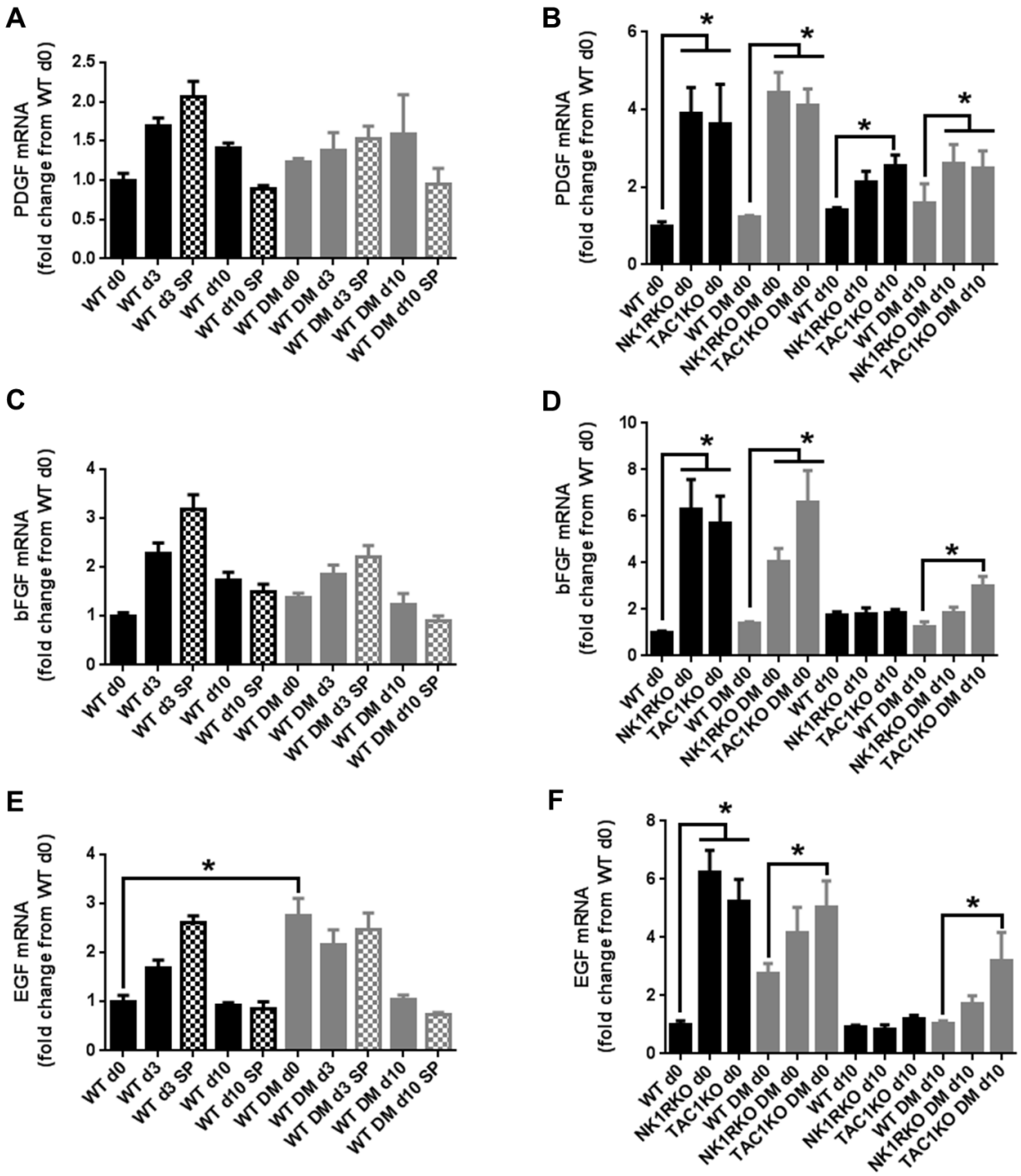


Figure 3.13. SP- or NK1R-deficiency alter growth factor skin gene expression, while SP treatment does not affect it. SP treatment did not change wound expression of PDGF **(A)**, FGF2

(C), and EGF **(E)**. However, NK1RKO and TAC1KO mice had higher pre-wounding skin gene expression of all the factors above **(B, D, F)**.

These results are in agreement with the increased growth factor expression we have previously observed in DM neuropathic (DMN) patients with non-healing DFU[354] and suggest a resistance to their action rather than lack of their expression.

3.4.1.12. DM and SP- or NK1R-deficiency affect skin oxygenation

In order to evaluate skin oxygenation, we measured hemoglobin oxygen saturation (HbO_2) levels, either in unwounded skin (Day-0) or in the peri-wound skin (Day-3 or Day-10) of the studied mouse models. No major differences were observed between groups at Day-0 or Day-3. However, at Day-10, WT DM mice as well as non-DM and DM NK1R and TAC1KO mice had reduced HbO_2 saturation when compared to WT non-DM mice (**Figure 3.14.**). These results suggest that DM and the lack of SP or its receptor lead to peri-wound hypoxia, which may contribute to the impaired healing.

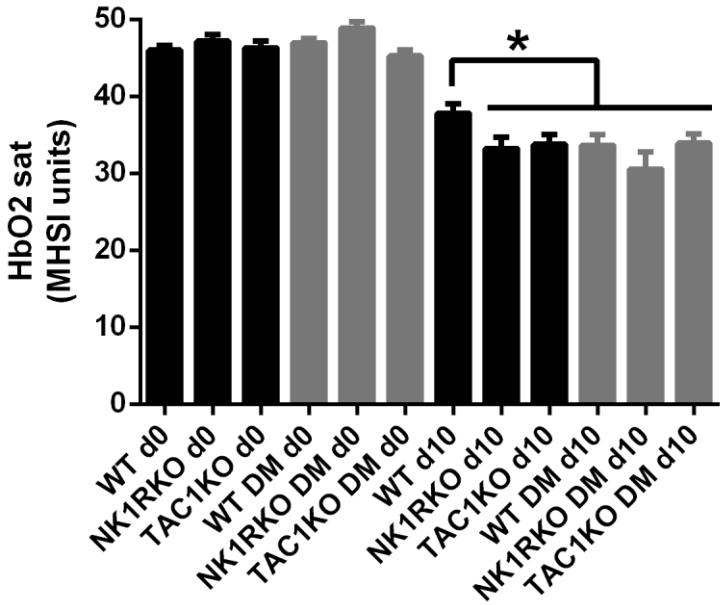


Figure 3.14. DM and SP- or NK1R-deficiency lead to periwound hypoxia. No differences were observed in skin hemoglobin oxygen (HbO_2) saturation at Day-0. At Day-10, WT DM mice and both

non-DM and DM NK1R and TAC1KO mice showed reduced HbO_2 saturation. Data represent mean ± SEM. *p<0.05.

3.4.2. Human studies

3.4.2.1. SP serum levels are reduced in diabetic patients but SP expression is increased in inflammatory skin cells

SP serum levels and skin gene expression were reduced in DM patients (**Figure 3.15. A, B**) when compared to non-DM control subjects. There was no difference in the number of epidermal cells expressing SP between DM and non-DM subjects. However, DM skin expressed SP in a larger number of endothelial, dendritic and inflammatory cells (**Figure 3.15. C**).

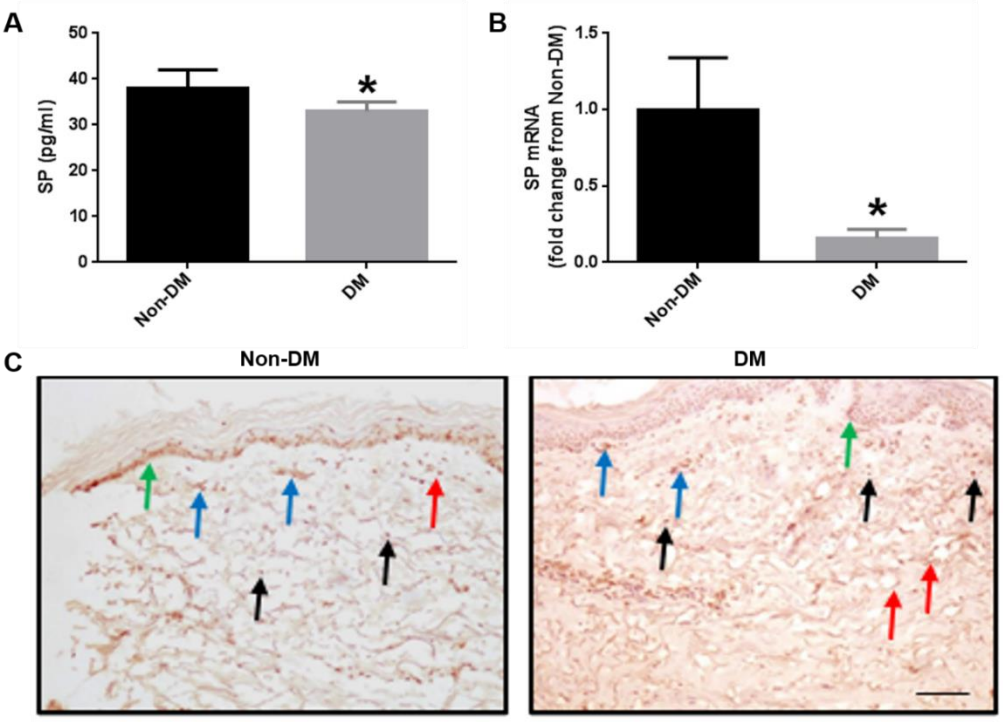


Figure 3.15. Substance P levels are altered in DM patients. Serum levels **(A)** and skin gene expression **(B)** of SP were reduced in DM compared to non-DM subjects. **(C)** Representative images of SP staining in forearm skin biopsies. SP was expressed in larger number of endothelial and inflammatory cells in forearm skin biopsies from DM. Arrows: black-inflammatory cells; blue-

fibroblasts, stromal, and dendritic cells; red-endothelial cells; green-epithelial cells. Scale bar: 100 µm. Data represent mean ± SEM. *p<0.05.

These results are in agreement with previous studies that have shown SP production by endothelial cells[388] and inflammatory cells, including macrophages, lymphocytes and dendritic cells[389-391]. In accordance with other reports[389-392], NK1R expression was also increased in endothelial cells, dendritic cells and inflammatory cells of DM skin (**Figure 3.16. A**). However, total NK1R protein expression was reduced in the skin of DM patients (**Figure 3.16. B**).

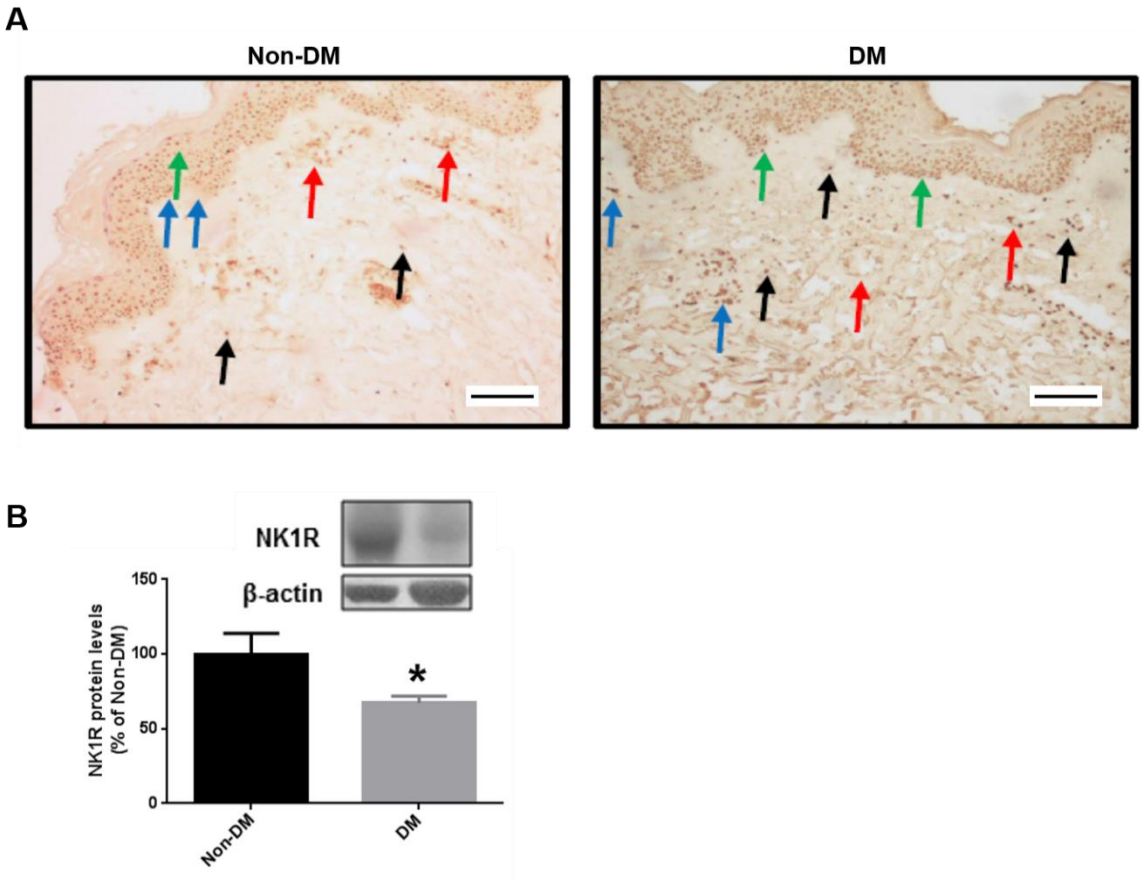


Figure 3.16. NK1R is expressed in several skin cells and its skin gene expression is increased in DM. (A) NK1R was expressed in larger number of endothelial cells, dendritic and inflammatory cells in foot skin specimens of DM patients. Arrows: black-inflammatory cells; blue-fibroblasts, stromal, and dendritic cells; red-endothelial cells; green-epithelial cells. Scale bar: 100 µm. **(B)** NK1R

protein levels, assessed by western blot, were significantly decreased in DM foot skin when compared to non-DM. Data represent mean ± SEM. *p<0.05.

3.4.2.2. NEP expression is increased in the skin of DM patients

NEP expression was increased in the epidermis and dermis of both forearm (**Figure 3.17.**) and foot (**Figure 3.18.**) skin specimens from DM patients. NEP gene and protein expression was also increased in foot skin of DM patients compared to non-DM subjects (**Figure 3.19.**).

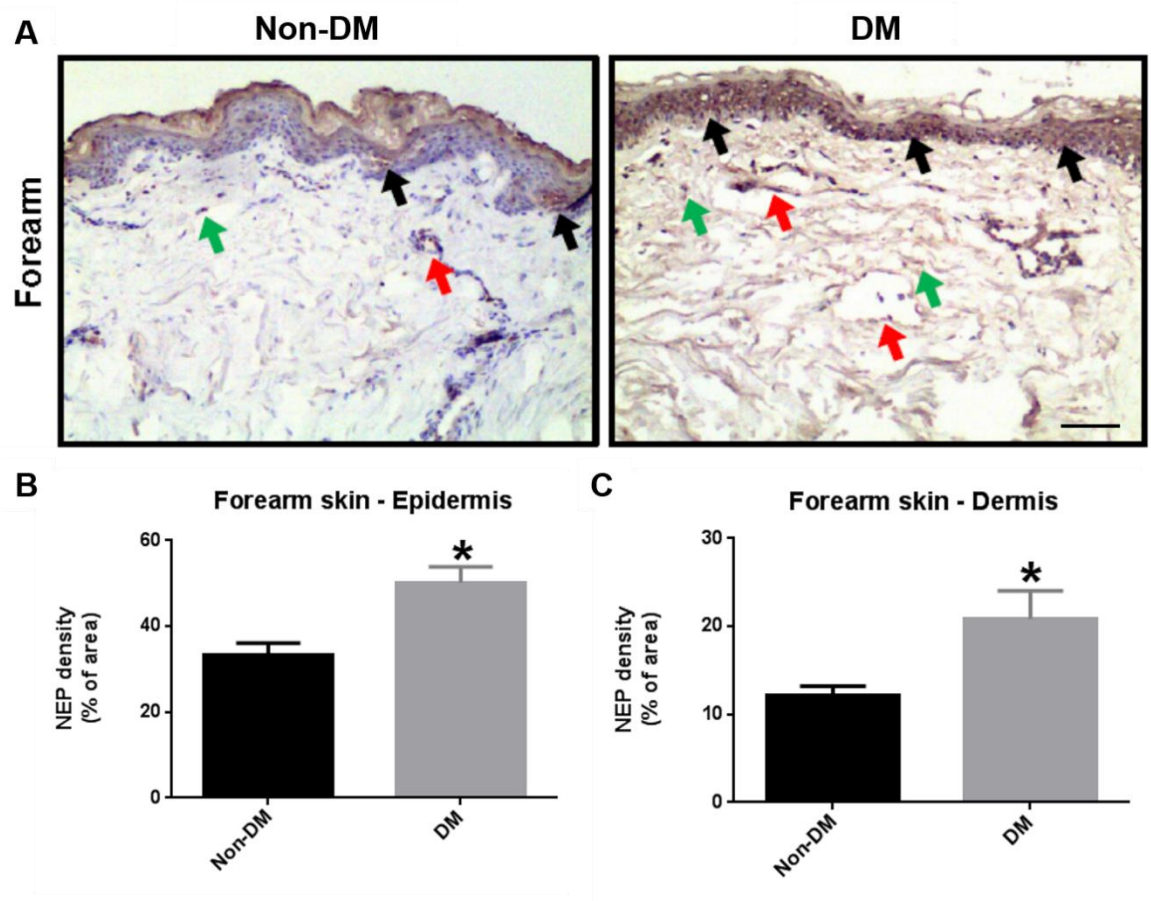


Figure 3.17. NEP staining is increased in the forearm skin of DM patients. (A) Representative images of NEP staining in forearm skin biopsies from non-DM and DM subjects. NEP staining is membranar and cytoplasmic and stains the epidermis (black arrows), stromal cells (green arrows) and endothelial cells (red arrows). Scale bar: 100 µm. NEP intensity was elevated in the epidermis

(B) and dermis **(C)** of forearm skin biopsies from DM patients. Data represent the mean ± SEM. *p<0.05 compared to non-DM.

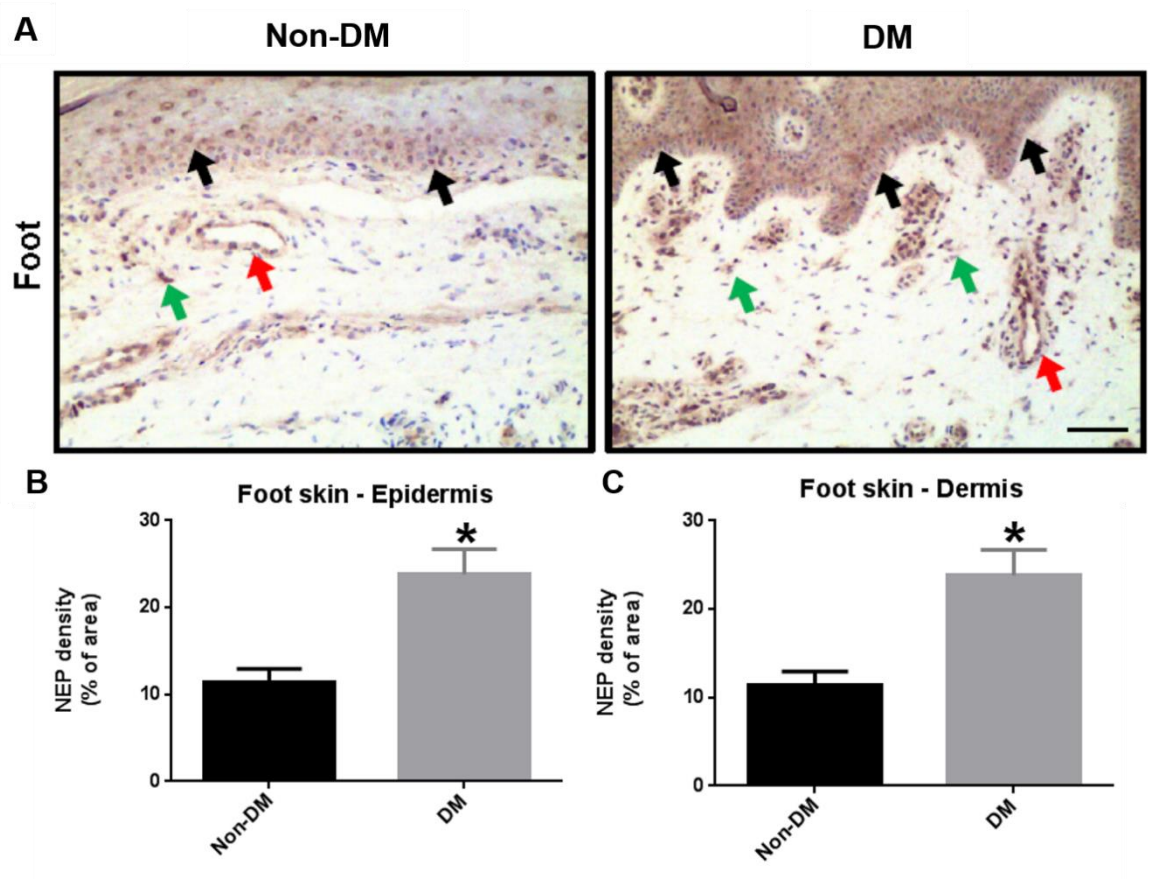


Figure 3.18. NEP staining is increased in the foot skin of DM patients. (A) Representative images of NEP staining in foot skin specimens from non-DM and DM subjects. NEP positive staining was observed in epidermal (black arrows), stromal (green arrows) and endothelial cells (red arrows). Scale bar: 100 µm. NEP intensity was higher in the epidermis **(B)** and dermis **(C)** of foot skin specimens from DM patients. Data represent the mean ± SEM. *p<0.05 compared to non-DM.

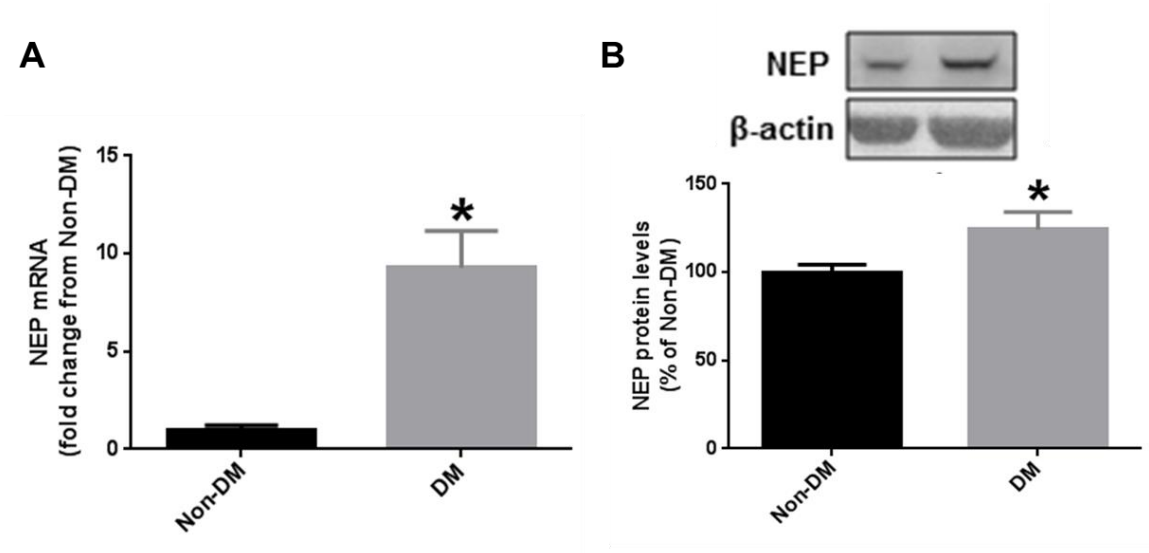


Figure 3.19. NEP skin gene expression and protein levels are increased in DM. NEP mRNA **(A)** and protein levels were increased in the foot skin of DM patients. Data represent mean ± SEM. *p<0.05 compared to non-DM.

3.5. Discussion

Normal wound healing is divided into three phases with limited overlap: coagulation-inflammation, proliferation, and remodeling[15, 16, 393]. In DFU, this linear progression from one phase to the next does not occur, resulting in persistence of a chronic pro-inflammatory state that is present in the majority of the wound area[15, 17, 394]. Previous studies have shown that SP can improve acute skin wound healing in non-DM[386], and corneal wound healing in both non-DM and DM animal models[171]. However, its effect on DM cutaneous wound healing, where an abnormal interplay of multiple cell types and a chronic pro-inflammatory environment co-exist, has not been investigated.

Our results indicate that SP ameliorates wound healing mainly by acting during the inflammatory phase: during the early stages of healing (Day-3), SP treatment increased the local and systemic expression of IL-6, KC (mouse equivalent of human IL-8) and other pro-inflammatory cytokines that are known to play a pivotal role in this phase [395], whereas it

reduced their expression at the later stages (Day-10), indicating resolution of inflammation and progression to the proliferative phase. On the other hand, lack of SP or of its receptor (as depicted in TAC1KO and NK1RKO mice, respectively), showed similar effects to DM, namely: increased basal cytokine expression, inability to mount a robust acute inflammatory response at Day-3, and persistence of a low grade pro-inflammatory state by Day-10. This is the first study, to the best of our knowledge, to demonstrate that lack of SP is associated with the chronic pro-inflammatory state and the failure to mount an adequate acute inflammatory response to skin injury that occurs in DM.

We also present evidence that SP regulates macrophage activation during the various phases of wound healing. During the inflammatory phase M1 activated macrophages initiate an acute inflammatory response, while during the proliferative phase M2 macrophages promote angiogenesis and granulation tissue formation[396-398]. In non-DM mice, the M1/M2 ratio peaked at Day-3 and returned to baseline levels at Day-10. In contrast, and in parallel with the pro-inflammatory cytokines profile, the baseline skin M1/M2 ratio was increased in DM mice, as well as in NK1RKO and TAC1KO mice. SP treatment further increased the M1/M2 ratio in non-DM mice at Day-3 and reduced it in DM mice at Day-10. Similarly, SP increased wound MCP-1 skin expression at Day-3 and reduced it at Day-10. MCP-1 is implicated in macrophage recruitment to tissues, and it was found to be overexpressed in the adipose tissue of obese subjects[397, 399]. In addition, a persistent M1 polarization, which is associated with a chronic pro-inflammatory state, has also been reported in chronic venous ulcers[400].

SP treatment or SP-deficiency had an impact in the skin expression of MMP-9, an extracellular protein which is involved in the breakdown of ECM proteins and growth factors[15]. Timely controlled expression and degradation of metalloproteinases (MMP) promotes healing in acute wounds, and MMP-9 overexpression has been reported in DM and other chronic wounds[134, 401-404]. In addition to confirming chronically increased

expression of MMP-9 in DM skin, our data suggest that SP plays an important role in this pathway.

No major changes associated with SP- or NK1R-deficiency were observed in neovascularization during wound healing. According to clinical studies[65, 144, 354], this is likely achieved by an overexpression of VEGF and its receptors VEGFR1 and VEGFR2, as well SDF1-α and CXCR4. SP treatment reduced the expression of most of these pro-angiogenic factors in both DM and non-DM wounds. Since MMP-9, which is known to be involved in the breakdown of these proteins, was found to be increased in the skin of SP- and NK1R-deficient mice, the observed increase in the pro-angiogenic factors is probably a compensatory response. Taking together, these results suggest that the main role of SP in wound healing is to promote its progression to the proliferative phase, with minimal effect once this progression has been achieved.

An additional important finding is that at baseline, TAC1KO and NK1RKO mice mimicked the chronic pro-inflammatory state observed in WT DM mice, including increased IL-6, KC, TNFα, MMP-9, and M1/M2 ratio. These findings suggest that the neuropathy-associated lack of SP may be a major factor contributing to the chronic pro-inflammatory state in DM.

As there is limited information regarding the role of SP in DFU, we investigated human skin and serum specimens from non-DM and DM subjects. Our results indicate reduced SP serum levels and reduced SP skin gene expression in DM. Furthermore, the expression of NEP was increased in the skin of DM patients. These results suggest that reduced SP and increased NEP expression are the two main mechanisms for SP deficiency at the skin level that contributes to the development and failure to heal of DFU.

There is currently no satisfactory experimental model of DM wound healing and the consensus it that more than one model should be studied in order to obtain reliable results. We confirmed our mouse findings in the neuro-ischemic DM rabbit ear model that heals

with minimal contraction, is characterized by chronic inflammation and increased M1/M2 ratio before healing[180], and is probably the closest animal model to the human neuro-ischemic DFU[149, 179, 180]. Since SP had a similar effect in both mouse and rabbit models, we believe that they satisfactorily represent the human condition and that the results support the progression to phase I/II clinical trials.

The study has its limitations. We studied only Type 1 DM animal models. However, there are no differences in DFU pathophysiology and natural history between human Type 1 and 2 DM, while the chosen models are commonly used in wound healing studies. In addition, the streptozotocin (STZ)-induced DM model allowed us to study the effect of DM in genetically modified mice. Furthermore, we did not splint the mouse wounds to influence possible healing by contraction. The main reason for this is that previous studies have alerted to potential confounding effects of splinting, namely that the modulation of extracellular and intracellular tension may inadvertently affect wound healing[405, 406]. In addition, in our studies the wound size of WT DM and transgenic mice tended to increase in the first three days, indicating reduced contraction. Furthermore, the confirmation of the mouse findings in the rabbit ear model, which heals mainly by re-epithelialization with minimal contraction, clearly indicates that the lack of splinting was not a major factor for the observed results.

Our findings suggest novel mechanisms related to impaired wound healing in DM and can potentially lead to the development of new DFU treatments. Thus, systemic inflammation and reduced SP skin bioavailability result in a chronic skin pro-inflammatory state that results in the inability to mount an acute inflammatory response, leading to abnormal healing and eventual development of a chronic wound. NEP inhibitors have been proposed as possible therapeutic agents but their use is hampered because it results in hydrolysis of a large number of peptides and has been associated with serious side effects such as angioedema[182, 407]. However, local treatment with SP or SP analogues has the potential not

only to promote DFU healing but also to reverse the chronic pro-inflammatory state present in DM skin without major adverse effects expected.

Chapter IV

Mast Cells are involved in

Impaired Diabetic Wound Healing

4. Chapter IV - Mast Cells are involved in Impaired Diabetic Wound Healing

4.1. Abstract

Diabetes *Mellitus* (DM)-associated impaired wound healing significantly increases hospitalization and impairs quality of life of patients. However, diabetic foot ulceration (DFU) remains an unmet clinical challenge. Mast cells (MC) interact with peripheral sensory nerves and participate in wound healing, but there is little information on their number, activation state and function in DM skin. Here we showed that MC degranulation is increased in unwounded forearm skin of DM patients and dorsal skin of DM mouse models. Post-wounding MC degranulation increased in non-DM mice but not in DM. MC stabilization was able to rescue DM-associated wound healing impairment in mice and also shifted the macrophages to the anti-inflammatory M2 phenotype. MC were a major source of VEGF in unwounded skin, but VEGF was reduced in DM. Both non-DM and DM mice deficient in MC had impaired wound healing compared to their respective wild-type (WT) controls. In addition, topical treatment with the MC trigger Substance P (SP) did not affect wound healing in MC deficient mice, but improved it in controls. In conclusion, the presence of non-degranulated MC in unwounded skin is required for proper wound healing and MC stabilizers may be a novel treatment for DFU.

4.2. Introduction

Foot ulceration, and associated impaired wound healing in diabetic patients (DM), is a major problem that significantly impairs their quality of life, leading to prolonged hospitalization and often resulting in amputations[350, 351]. DM wounds occur almost exclusively in the presence of diabetic peripheral neuropathy (DPN) and there is considerable evidence that several neuropeptides, including Substance P (SP), are involved in wound healing[15]. In

addition, a recent prospective study has shown that increased inflammation and aberrant circulating levels of growth factors predispose neuropathic DM to wound healing failure[354].

Mast cells (MC) participate in all phases of wound healing[267, 408]. More specifically, MC degranulation leads to vasodilation and secretion of various growth factors and cytokines that are involved in the repair process[409, 410]. In addition, previous studies have reported that MC are mandatory for the inflammatory phase of wound healing as they recruit neutrophils to the site of injury[26, 266-268] and secrete cytokines that activate tissue-resident macrophages[269]. Moreover, MC stimulate fibroblast proliferation through the production of interleukin-4 (IL-4) and bFGF[271], promote wound tissue granulation, cell migration and proliferation, angiogenesis, and collagen maturation[255, 411, 412] during the proliferative phase. Finally, they have been implicated in wound contraction[278-280] and scar formation[284-287, 413] during the remodeling phase.

Despite the aforementioned evidences that MC are involved in wound repair, the role of MC in DM-impaired wound healing has not yet been investigated to the best of our knowledge. In fact, there is currently very little information on the number and state of activation of skin MC in DM. In humans, MC are localized around blood vessels and in fat depots where they influence development of local inflammation and adipocytokine release[206, 414]. MC contribute to the development of diet-induced obesity and DM by producing IL-6 and interferon-gamma (IFN-γ)[415]. They have also been implicated in the development of severe local insulin-induced lipo-atrophy[416]. On the other hand, MC interact with peripheral sensory nerves and can be stimulated by SP[233] and other neuropeptides[230]. In addition to degranulation, SP can stimulate selective VEGF release from MC without degranulation[417].

In this study, we investigated possible changes in MC abundance and degranulation in the skin of DM patients and DM mice. We also examined the role of MC in mouse models of non-DM and DM wound healing. In addition, we investigated a possible interaction between

SP and MC in wound healing. Finally, we evaluated the effects of high glucose in MC degranulation and mediator release from human MC *in vitro*.

4.3. Materials and methods

4.3.1. Human studies

All subjects gave informed consent and the protocol was approved by the Institutional Review Board (IRB) of the Beth Israel Deaconess Medical Center.

4.3.1.1. Forearm skin biopsies

2 mm forearm skin punch biopsies were obtained from 10 non-DM and 56 DM subjects who participated in a prospective study in our unit. Details about these two groups and the biopsy procedures have been described elsewhere[354]. Peripheral diabetic neuropathy was present in 48 (86%) DM patients while the nerve axon reflex, an index of subclinical peripheral neuropathy, was reduced at the forearm level of the DM group. Forearm skin biopsies were used to evaluate total, degranulated, and non-degranulated MC by toluidine blue staining (0.1%), and to evaluate HLA-DR$^+$/CD68$^+$ (M1) and CD206$^+$/CD68$^+$ (M2) macrophages by fluorescent immunohistochemistry.

4.3.1.2. Foot skin specimens

Foot skin discarded specimens that were collected during foot surgeries from healthy non-DM subjects (n=5) and DM patients (n=6) were used to evaluate M1 and M2 macrophages by fluorescent immunohistochemistry, and to evaluate gene expression of TNF-α, IL-1β and IL-10 by q-RT-PCR.

4.3.1.3. Foot skin specimens

From the same population above, IL-6 and TNF-α serum levels were measured by using a Luminex 200 apparatus (Luminex Corporation Austin, TX) and Millipore multiplex immunoassay panels (Millipore Corporation, Chicago, IL). IL-33 was measured in serum samples from age and sex-matched 39 healthy control subjects (non-DM), 39 diabetic

patients without complications (DM), 36 diabetic patients with neuropathy (DMN) and 43 diabetic patients with an active foot ulceration using commercially available IL-33 ELISA assay kit (R&D Systems, Minneapolis, MN).

4.3.2. Animal studies

All animal studies were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols.

4.3.2.1. Mouse models

C57BL6/J mice and genetically MC-deficient WBB6F1/J-Kit^{W}/Kit^{W-v} (Kit^{W}/Kit^{W-v}) mice together with their congenic normal WBB6F1/J-$Kit^{+/+}$ wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were made diabetic (DM) by administering streptozotocin (STZ, 50 mg/kg i.p daily for 5 consecutive days) in citrate buffer (0.1M). In the non-DM groups, mice were treated with vehicle alone. Fasting blood glucose was monitored a week after the last injection and mice with blood glucose over 250 mg/dl were considered DM. To evaluate the effect of MC stabilization on wound healing, C57BL/6J WT non-DM and STZ-DM mice were treated with disodium cromoglycate (DSCG, 50 mg/kg i.p. daily for 10 consecutive days) prior to wound creation. To use a different mouse model of MC deficiency, sash Kit^{W-sh}/Kit^{W-sh} mice were generated in our laboratory from breeder mice obtained from Jackson Laboratories. B6.Cg-Kit^{W-sh}/HNihrJaeBsmJ females were crossed with C57BL/6J males. The heterozygotes from the first generation were then intercrossed and the homozygotes (Kit^{W-sh}/Kit^{W-sh} and Kit^{+}/Kit^{+}) mice from the second generation were used in our study. Sash mutants were genotyped based on mouse coat appearance. Non-DM age-matched male Kit^{W-sh}/Kit^{W-sh} mice and their respective WT B6.Cg controls from the colony were used in wound healing studies.

4.3.2.2. Wound creation, monitoring and treatment

Eight weeks after STZ or vehicle only treatment, mice were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and two circular 6 mm full thickness were created on the shaved dorsum of the mice using a punch biopsy tool. Baseline (Day-0) skin biopsies were collected for histological and molecular analysis. Wound closure kinetics was monitored daily over a 10-day period by measuring the wound size using acetate tracing followed by analysis with Image J software (NIH). Data was presented as percentage of original wound size (Day-0) over the study period. For the SP studies, one of the wounds in each mouse received daily topical application of SP in saline (32µg/5µl/wound) while the other was treated with saline only (5µl/wound). At the end of the study, ten days after wound creation, (Day-10), mice were euthanized and blood and wound tissue were collected for further analysis.

4.3.2.3. Medical Hyperspectral Imaging (MHSI)

Tissue oxygen saturation of hemoglobin (HbO_2) from unwounded (Day-0) or peri-wound (Day-10) mouse skin was monitored with a non-invasive Medical Hyperspectral Imaging System (MHSI, HyperMed Inc, Berlington, MA, USA). Imaging was performed on the shaved dorsum of the anesthetized mice, either before wound creation (Day-0) or prior to sacrifice (Day-10).

4.3.2.4. Tissue Collection

At Day-0, the circular 6 mm diameter skin sections were cut and collected. At the end of the study, Day-10 post-wounding, mice were euthanized and 1 cm X 1 cm skin sections that included the wound margins were cut. Each skin sample was further divided into different sections for morphologic and molecular analysis. For morphologic analysis and immunohistochemistry, tissue was fixed in 10% formalin and subsequently embedded in paraffin (FFPE). For immunofluorescence, skin was embedded in optimal cutting

temperature media (OCT) and gradually frozen in dry ice, then stored at -80°C. For gene expression or protein analysis, tissue was snap frozen in liquid nitrogen and stored at -80°C.

4.3.2.5. Histological analysis

FFPE sections (5 µm) underwent routine histological processing with hematoxylin and eosin (H&E) for morphologic analysis. Epidermis regeneration was scored as: 0- absent regeneration, 1- incomplete regeneration, 2- complete regeneration, 3- complete maturation.

To study the number and activation state of MC, FFPE sections (5 µm) were deparaffinized and stained metachromatically with 0.1% toluidine blue, pH 2 (cytoplasmic granules appear purple on a blue background). The number of total, degranulated and non-degranulated MC was determined by a blinded observer. MC degranulation was determined as extensive (>50% of granules exhibiting fusion, alterations in staining, and extrusion from cell), moderate (10-50% of granules altered as mentioned above) or absent.

4.3.2.6. Immunohistochemistry (IHC)

FFPE sections (5 µm) underwent routine histological processing with hematoxylin and eosin (H&E) for morphologic analysis. To evaluate the number of endothelial cells, CD31 staining was performed on FFPE sections (5 µm) using a purified rat monoclonal antibody (BD 550274, BD Biosciences, San Jose, CA) as described previously[418] (Chapter II). OCT frozen skin sections (5 µm) were co-stained with CD68 (AbCam, Cambridge, MA) and TNF-α (Serotec, Oxford, United Kingdom) for detection of M1 macrophages, or with CD68 and CD206 (Santa Cruz Biotechnology, St Cruz, CA) for detection of M2 macrophages. Each sample was counterstained with DAPI and visualized using a fluorescent microscope. A blinded observer counted the triple positive cells in two random high power fields (hpf, 400x magnification).

4.3.2.7. Western blot analysis

Day-0 and Day-10 mouse skin samples were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Rockford, IL) supplemented with 1 mM dithiothreitol (Sigma, St. Louis, MO), phosphatase and protease inhibitor cocktails (Boston BioProducts, Ashland, MA). Lysates were kept at 4°C for 15 min, centrifuged at 13,000 *g* for 15 min. The supernatant protein content was quantified using the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). After denaturation, equal amounts of protein (20 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% polyacrylamide gels (BioRad, Hercules, CA) and transferred onto PVDF membranes (Bio-Rad, Hercules, CA) by a semi-dry electroblotting system (BioRad, Hercules, CA). Blots were blocked, incubated with the primary antibodies anti-VEGF (Abcam, Cambridge, MA) and anti-MMP-9 (Millipore, Billerica, MA), and subsequently with HRP-conjugated secondary antibodies (SIGMA, St. Louis, MO), and finally detected using LumiGlo chemiluminescent substrate kit (KPL, Gaithersburg, MD). For quantification, band densitometry was normalized to the loading control, beta-actin (SIGMA, St. Louis, MO), using the ImageJ software (NIH). The data is presented as the fold change over the protein levels in the Day-0 skin from WT non-DM mice (fold change from WT non-DM baseline).

4.3.2.8. Multiplex analysis

Immediately after collection, blood was centrifuged at 14000 rpm, at 4°C for 20 minutes. Serum was then collected and stored at -80°C until analysis. Serum protein levels of inflammatory cytokines and biochemical markers of endothelial function were measured using a Luminex 200 apparatus (Luminex, Austin, TX) and Millipore multiplex immunoassay panels (Millipore, Chicago, IL).

4.3.3. Cell culture studies

4.3.3.1. Human mast cell (MC) culture

LAD2 mast cells (MC) were cultured in StemPro-34 SFM Medium 1X (5.5mM glucose) (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml recombinant human stem cell factor (rhSCF, from Biovitrum, Sweden) and 1% U/ml penicillin/streptomycin, in an incubator with 5% CO_2 and air environment at 37 °C. Mannitol powder (molecular weight 182.17 g/mol) and glucose solution (molecular weight 180.16 g/mol) were dissolved in MiliQ Water to create solutions of 1.1M. StemPro-34 SFM Medium was supplemented with 24.5 mM glucose solution for a final concentration of 30 mM to prepare high glucose (HG) media. StemPro Medium was supplemented with 24.5 mM mannitol, as an osmotic control for a final concentration of 30 mM to prepare normal glucose media (NG). All media were prepared as fresh solutions and the appropriate dilutions were made. Media were supplemented with the sugars immediately before addition to cells. Cells were maintained in HG or NG for 2 weeks before the studies were performed.

4.3.3.2. MC degranulation assay

To evaluate MC degranulation, beta-hexosaminidase (β-hex) release was assayed using a fluorometric assay. Briefly, LAD2 cells ($0.5x10^5$/tube) were stimulated for 30 min with SP (1μM), supernatant fluids were collected and cell pellets were lysed with 1% Triton X-100. Supernatants and cell lysates were incubated in reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1.5 h and then 0.2 M glycine was added to stop the reaction. Absorbance was measured at 405 nm in a plate reader, and the results were expressed as the percentage of β-hex released over the total (n=4 per condition).

4.3.3.3. MC mediator release

For VEGF release, LAD2 cells were triggered with SP (1 μM) for 24 hr. VEGF was measured in the supernatant fluids using commercially available VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). For IL-8 release, LAD2 cells were

triggered with SP (1 μM) for 24 hr. IL-8 was measured in the supernatant fluids using commercially available IL-8 ELISA assay kit (R&D Systems, Minneapolis, MN). For TNF-α release, LAD2 cells were triggered with SP (1 μM) for 24 hr. TNF-α was measured in the supernatant fluids using commercially available TNF ELISA assay kit (R&D Systems, Minneapolis, MN). For MCP-1 release, LAD2 cells were triggered with SP (1 μM) for 4H. MCP-1 was measured in the supernatant fluids using commercially available MCP-1 ELISA assay kit (R&D Systems, Minneapolis, MN).

4.3.4. Statistical analysis

All statistical analysis was performed using Minitab (Minitab, State College, PA). The statistical power analysis was based on preliminary data or data from other studies in our unit. For parametrically distributed date, differences among experimental groups were analyzed using student's t-test or one-way ANOVA followed by Fisher's post hoc test. For non-parametrical data, Mann-Whitney and Kruskal-Wallis tests were used. The Pearson correlation coefficient was calculated. Statistical significance was defined for a p value < 0.05.

4.4. Results

4.4.1. Human studies

4.4.1.1. The number of total and degranulated dermal MC is increased in DM patients and correlates with inflammation

Forearm skin biopsies from DM patients presented increased number of total and degranulated MC, and reduced number of non-degranulated cells, compared to healthy control (non-DM) subjects (**Figure 4.1. A-D**). In addition, MC degranulation was mostly observed in the proximity of round inflammatory cells (**Figure 4.1. A**), suggesting that dermal MC activation is associated with skin inflammation. Moreover, MC degranulation positively correlated with the dermis inflammatory cell number (**Figure 4.1. E**), and with the circulating levels of the inflammatory markers IL-6 and TNF-α (**Figures 4.1. F, G**).

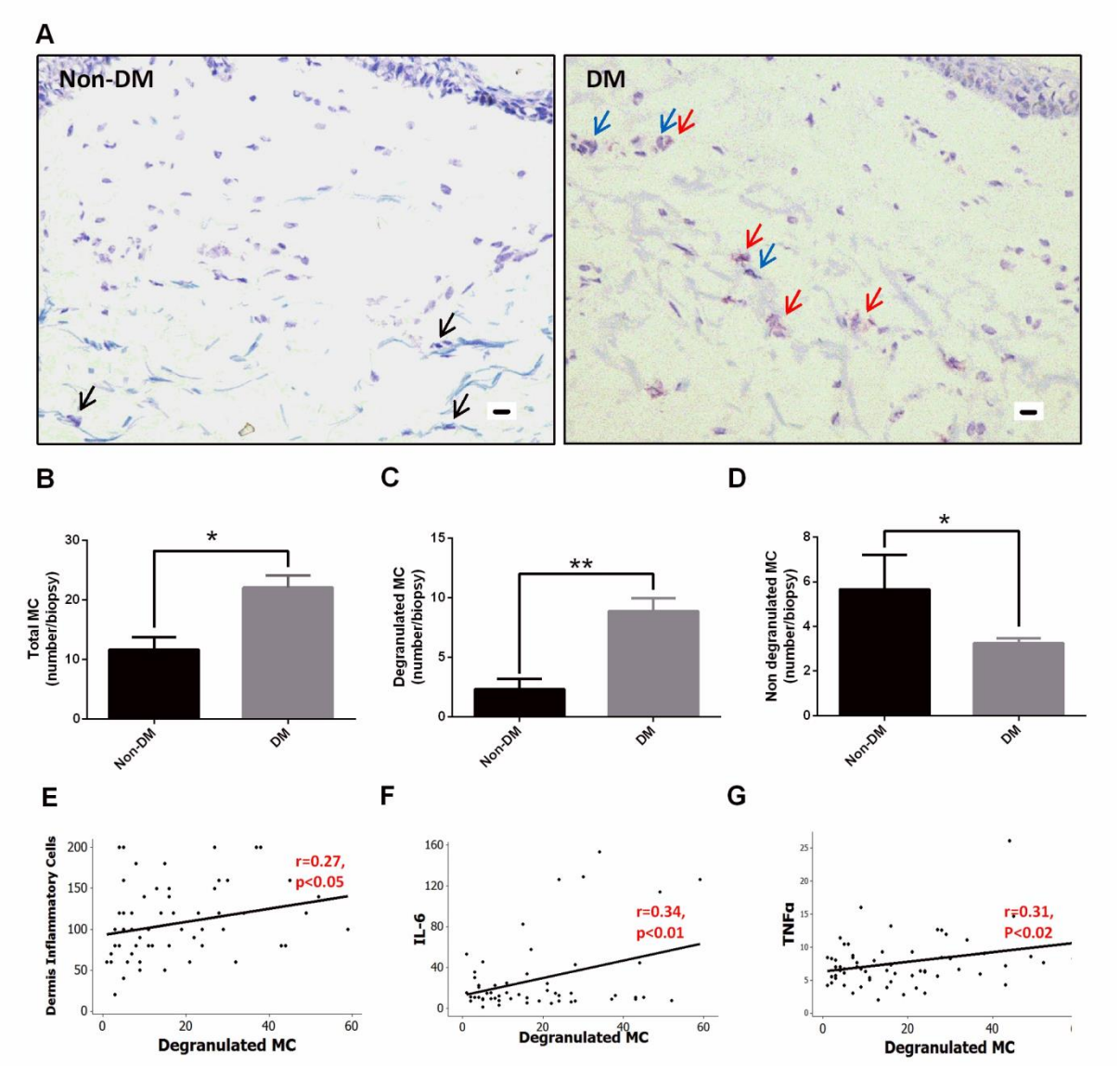


Figure 4.1. The number of degranulated skin MC is increased in DM patients and is associated with inflammation. **(A)** Representative images of non-degranulated (black arrows) and degranulated (red arrows) MC in forearm human skin biopsies from non-DM and DM subjects (scale bar: 10 µm). Degranulated MC were in proximity with inflammatory cells (blue arrows). The numbers of total **(B)** and degranulated **(C)** MC were increased in DM, whereas the number of non-degranulated MC **(D)** was reduced. Data represent mean ± SEM. *p<0.05, **p<0.01. **(E, F)** A significant correlation was observed between degranulated MC and the dermis round inflammatory cells **(E)**, the serum IL-6 **(F)** and TNFα **(G)**.

4.4.1.2. IL-33 levels are increased in the serum of DFU patients

We evaluated the circulating levels of IL-33 in serum samples from i) control healthy subjects (non-DM), diabetic patients without complications (DM), diabetic patients with neuropathy (DMN), and DFU patients. IL-33 was higher in the DFU patients when compared to the non-DM controls (**Figure 2**).

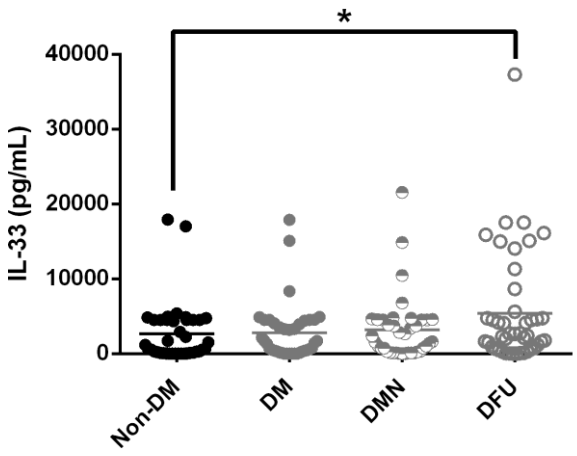


Figure 4.2. IL-33 is increased in the serum of patients with DFU. We evaluated the levels of IL-33 in the serum of i) healthy control subjects (non-DM, n=39), ii) diabetic patients without complications (DM, n=39), iii) diabetic patients with neuropathy (DMN, n=36), and iv) diabetic patients with an active foot ulcer (DFU, n=43). IL-33 was higher in the serum of DFU patients when compared to the healthy non-DM controls. Data represent individual values and mean of each group. * $p<0.05$.

4.4.1.3. Macrophage activation is polarized towards M1 in the skin of DM patients

We evaluated the number of M1 and M2 macrophages in the skin of DM and non-DM subjects without lesions (**Figure 4.3. A-C**). The number of M1 macrophages was increased in the forearm skin of DM when compared to non-DM subjects, while no differences were observed in M2 macrophages (**Figure 4.3. B**). Furthermore, at the foot skin level, the M1/M2 macrophage ratio was increased in DM (**Figure 4.3. C**).

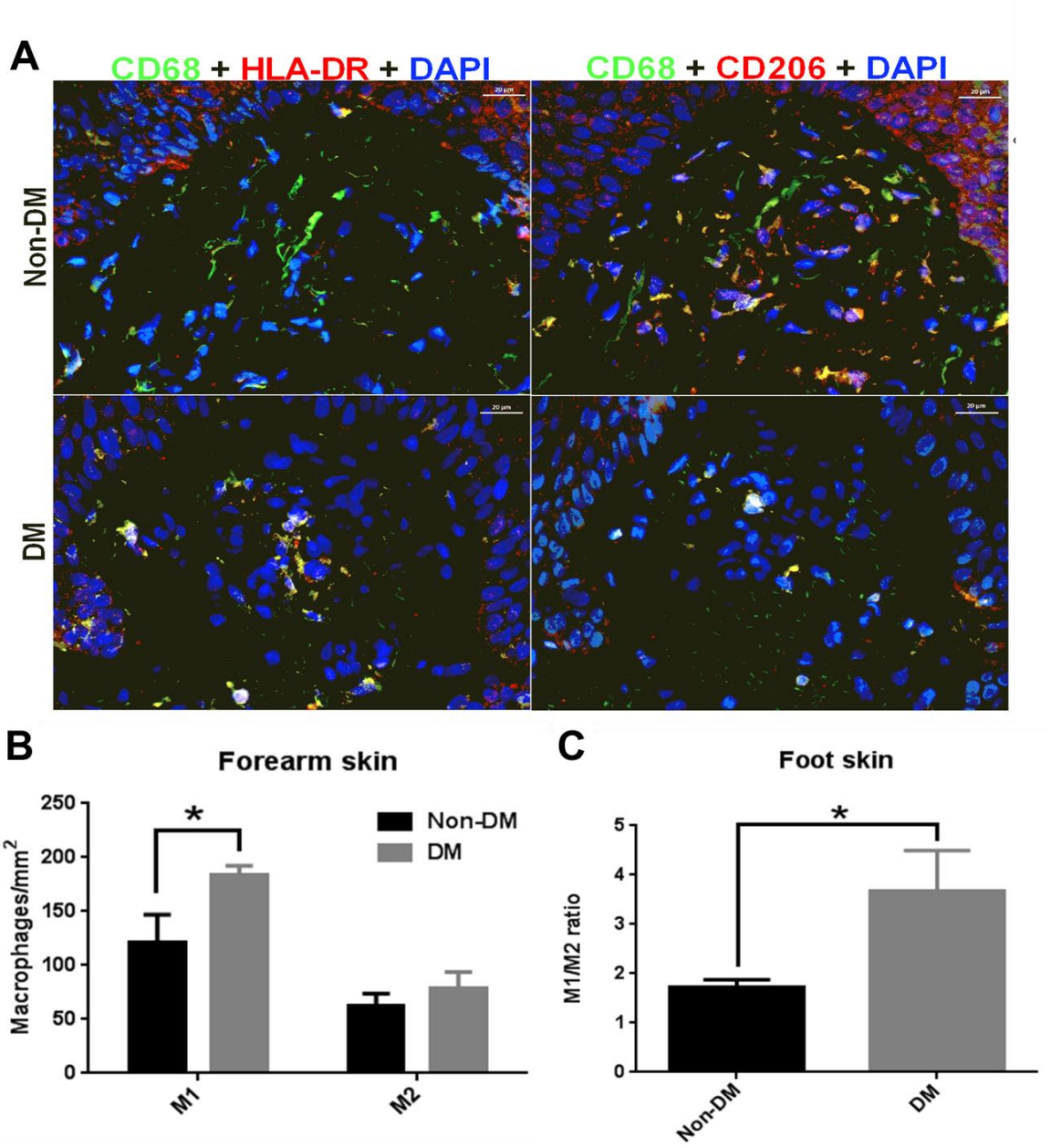


Figure 4.3. Macrophage activation is polarized towards the M1 activation in the skin of DM patients. (A) Representative images of M1 and M2 macrophages in foot skin specimens from non-DM and DM subjects. Scale bar: 20 μm. **(B)** The number of M1 macrophages was increased in forearm skin biopsies of DM. **(C)** M1/M2 ratio was higher in the skin of DM subjects at the foot level. Data represent mean ± SEM. *p<0.05.

Gene expression of the M1-associated pro-inflammatory cytokines TNF-α (**Figure 4.4. A**) and IL-1β (**Figure 4.3. B**) was elevated in the foot skin of DM patients, whereas the M2-associated anti-inflammatory cytokine IL-10 was reduced (**Figure 4.3. C**).

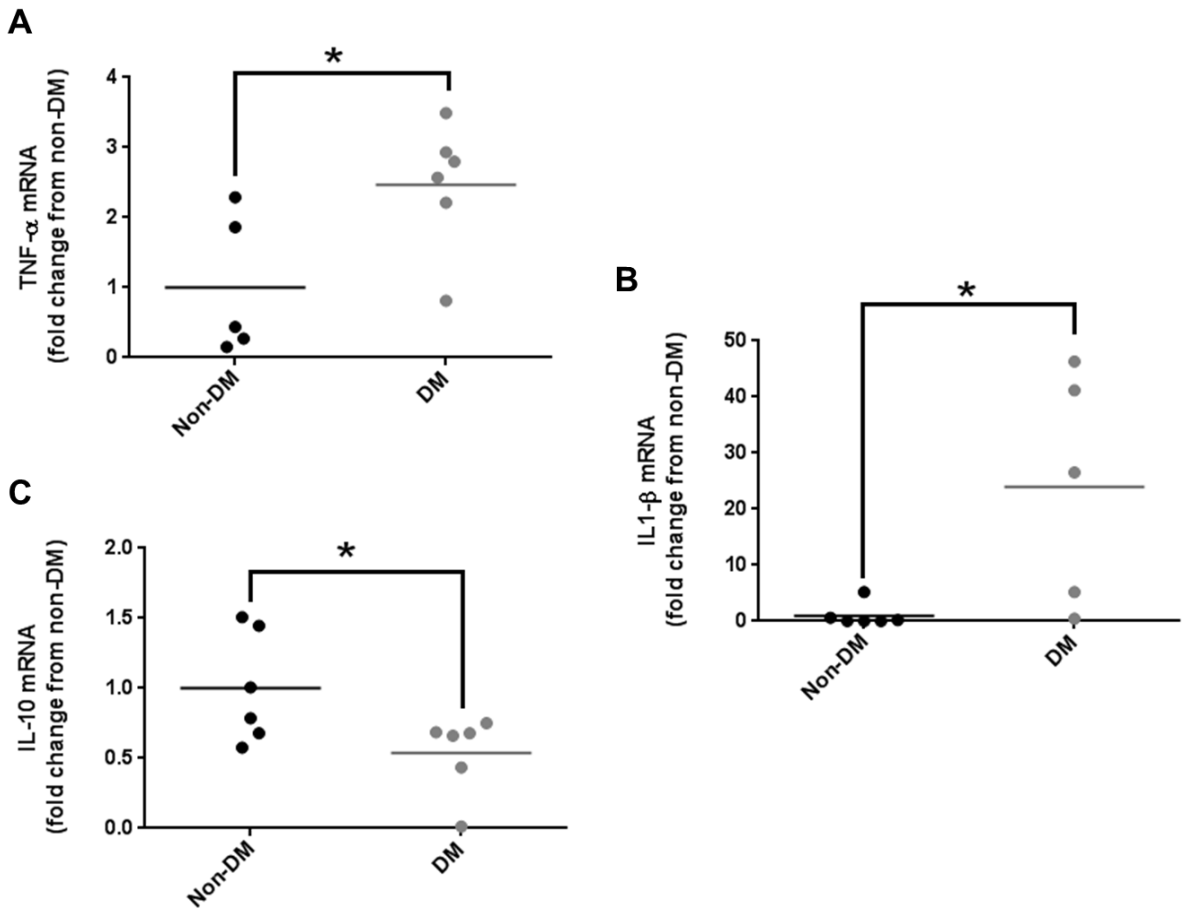


Figure 4.4. Skin gene expression of M1- and M2-associated cytokines is altered in DM. Foot skin gene expression of **(A)** TNF-α and **(B)** IL-β was increased in DM subjects, whereas **(C)** IL-10 was reduced, when compared to non-DM. Data represent Data represent mean. * $p<0.05$.

4.4.2. Mouse studies

4.4.2.1. The number of degranulated MC is increased in the skin of DM mice

We examined MC in C57BL/6J WT non-DM and DM mouse skin biopsies before wounding (Day-0) and at 10 days post-wounding (Day-10) (**Figure 4.5. A-D**). No differences were observed in terms of total MC counts between non-DM and DM mice (**Figure 4.5. B**). However, and similarly to the findings in human skin (**Figure 4.1. A**), DM mice showed extensive degranulation of MC (**Figure 4.5. A-C**). We also employed a 10-day daily i.p. injection of the MC stabilizer disodium cromoglycate (DSCG) as previously described[415], before wound creation, to evaluate its effect on MC degranulation in DM. DSCG treatment

increased the total MC counts in DM mice (**Figure 4.5. B**) and reduced the degranulated MC in DM mice to a level similar to non-DM controls (**Figure 4.5. C**). Compared to Day-0 skin, MC degranulation increased in the Day-10 wounds of non-DM mice, but no further degranulation was observed from Day-0 to Day-10 post-wounding in the DM mice. DSCG treatment was able to effectively reverse this effect (**Figure 4.5. D**). In addition, we evaluated skin MC degranulation in genetically modified mice lacking tachykinin 1 (TAC1) gene, which encodes for SP and other tachykinins. Similarly to WT DM, both non-DM and DM TAC1 *knock out* (TAC1KO) mice had increased skin MC degranulation at baseline, when compared to WT (**Figure 4.5. E**). DM and SP-deficiency did not have an additive effect in skin MC degranulation, as there were no differences between non-DM and DM TAC1KO mice.

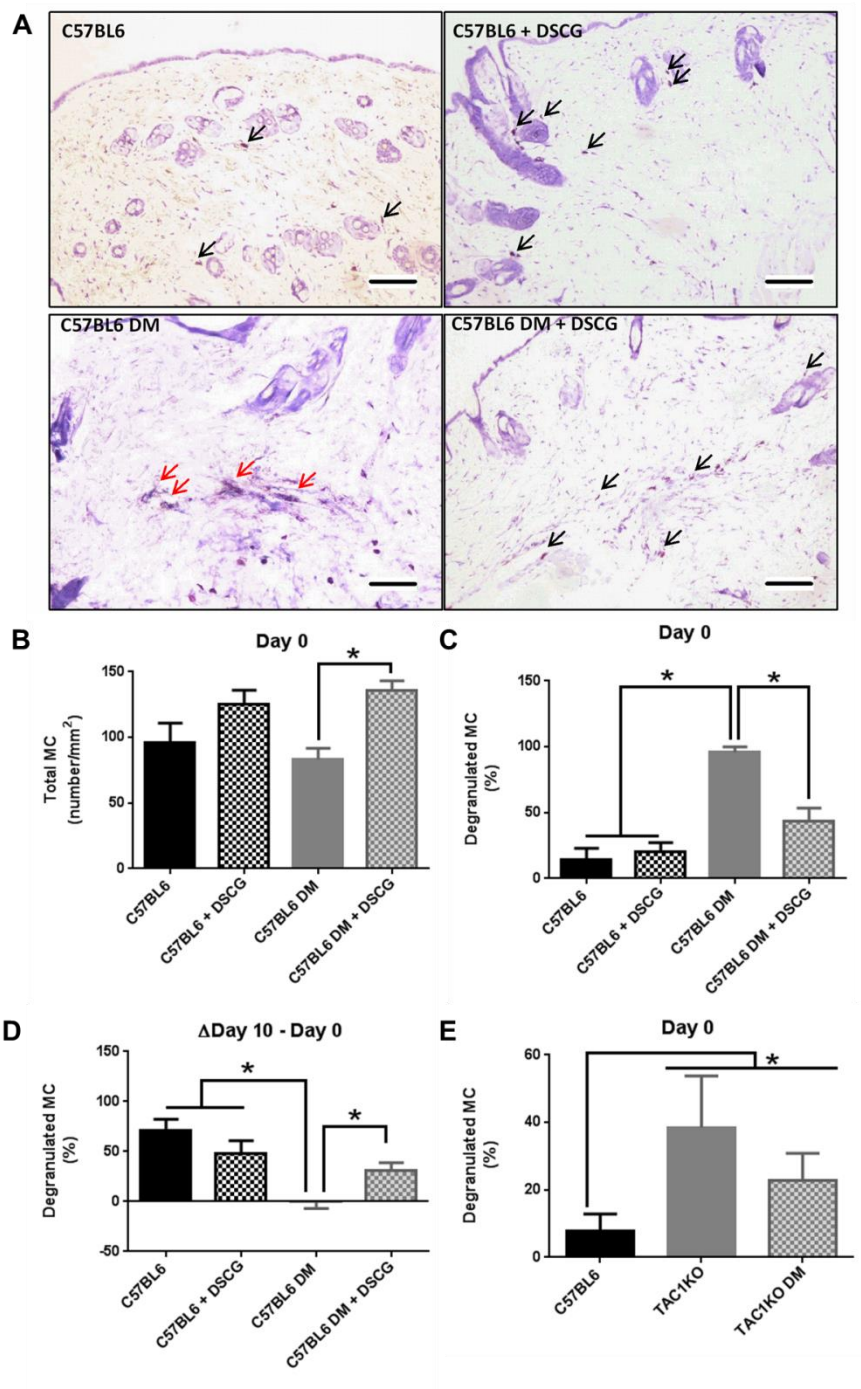


Figure 4.5. The number of degranulated MC is increased in unwounded skin of DM mice. (A) Representative images of non-degranulated (black arrows) and degranulated (red arrows) MC in Day-0 skin biopsies from C57BL6 non-DM and DM mice, non-treated and DSCG-treated. Scale bar: 100µm. **(B)** DSCG increased total MC numbers in DM mice. **(C)** MC degranulation was increased in DM and DSCG reduced it. **(D)** At Day-10, MC degranulation increased from baseline in all groups except DM non-treated. **(E)** Non-DM and DM TAC1KO mice presented increased skin MC degranulation when compared to C57BL6 WT mice. Data represent mean ± SEM. *p<0.05.

4.4.2.2. Pharmacological stabilization of MC improves wound healing in DM mice

We evaluated the effect of MC pharmacological stabilization, induced by DSCG treatment, on wound healing progress in C57BL6/J WT non-DM and DM mice. As expected, and in agreement with our previous results (Chapter III), DM mice showed delayed wound closure when compared to non-DM mice. DSCG treatment was able to accelerate wound closure in the DM mice at the later stages of healing (from Day-6 to Day-10 post-wounding), achieving similar kinetics as the non-DM mice (**Figure 4.6. A, B**). In contrast, DSCG had no significant effect in non-DM mice, in which minimal pre-wounding degranulation was observed.

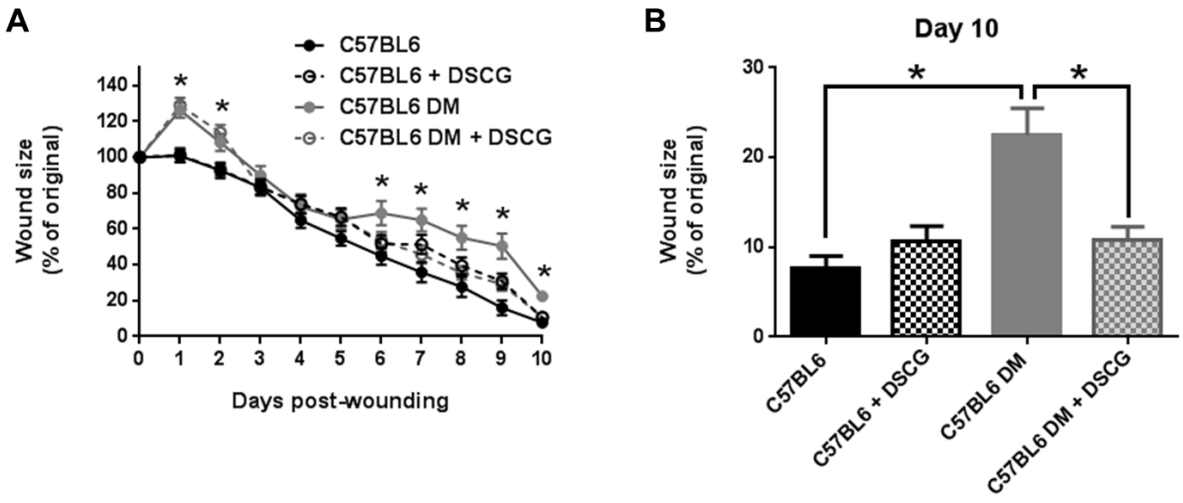


Figure 4.6. MC stabilization accelerates wound closure in DM mice. (A) Wound healing progress over a 10-day period in WT C57BL6 non-DM and DM mice, non-treated and DSCG-treated. **(B)** Wound size at Day-10 post-wounding in C57BL6 WT non-DM and DM mice, non-treated and DSCG-treated. Data represent mean ± SEM. *p<0.05, compared to C57BL6 WT non-DM.

4.4.2.3. Wound healing is impaired in MC deficient mice

We evaluated wound healing progress in MC deficient *Kit*^{W}/*Kit*$^{W-v}$ mice and their WBB6F1/J WT controls, in both non-DM and DM settings. The healing rate was reduced in non-DM *Kit*^{W}/*Kit*$^{W-v}$ mice, from Day-5 until Day-10 post-wounding (**Figure 4.7 A, B**). DM *Kit*^{W}/*Kit*$^{W-v}$ mice also showed delayed wound closure, from Day-8 to Day-10 post-wounding (**Figure**

4.7. C, D). In order to exclude the possibility that the observed healing impairment was related to this particular transgenic model, we also evaluated wound healing in a second MC deficient mouse model - the $Kit^{W\text{-}sh/}Kit^{W\text{-}sh}$ - that possesses an inversion mutation upstream of the c-kit promoter region and its phenotype, and, in contrast with the $Kit^{W}/Kit^{W\text{-}v}$ strain, has normal levels of other differentiated hematopoietic and lymphoid cells[196, 419]. $Kit^{W\text{-}sh/}Kit^{W\text{-}sh}$ mice showed an even more pronounced healing impairment when compared to their respective B6.Cg WT controls from the colony, observed from early stages (Day-1) until the end of the study, Day-10 (**Figure 4.7. E, F**).

4.4.2.4. Topical treatment with Substance P (SP) accelerates wound healing in both WT non-DM and DM mice, but not in MC deficient mice

Previous studies in our unit have shown that local treatment with SP improves wound healing in non-DM and DM C57Bl6/J WT mice (Chapter III)[420]. Here, we investigated the effect of SP treatment in non-DM and DM WBB6F1/J WT and MC deficient mice. In agreement with our previous findings, topical SP treatment accelerated wound closure in both WBB6F1/J WT non-DM and WBB6F1/J WT DM mice (**Figure 4.7. A-D**). However, no differences were observed between the non-treated and SP-treated wounds of $Kit^{W}/Kit^{W\text{-}v}$ mice in the absence or presence of DM (**Figure 4.7. A-D**).

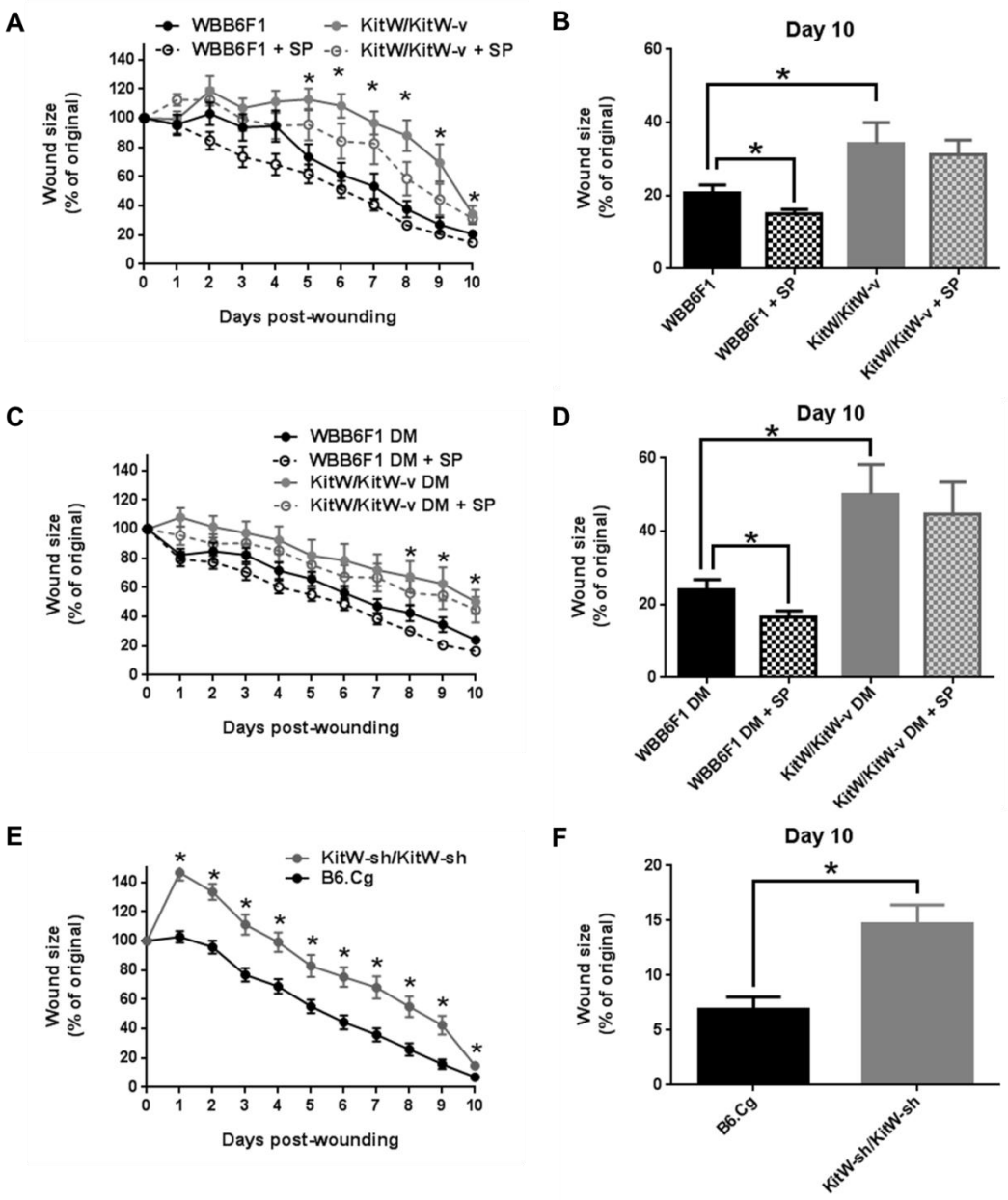


Figure 4.7. Functional MC are required for proper wound healing. Wound healing progress over a 10-day period in the following models: **(A, B)** Kit^{W}/Kit^{W-v} and WBB6F1 mice, non-treated and SP-treated; **(C, D)** DM Kit^{W}/Kit^{W-v} and DM WBB6F1 WT mice, non-treated and SP-treated; **(E, F)** Kit^{W-sh}/Kit^{W-sh} and B6.Cg WTmice. **(A-D)** Wound healing was delayed in Kit^{W}/Kit^{W-v} mice without or with DM, when compared to their respective non-DM or DM WBB6F1 WT controls. SP improved healing at Day-10 post-wounding in both WBB6F1 WT non-DM and DM mice, but failed to have an effect in the Kit^{W}/Kit^{W-v} mice. **(E-H)** Wound healing was delayed in MC deficient Kit^{W-sh}/Kit^{W-sh} mice when compared to their respective B6.Cg WT controls. Results represent mean ± SEM of 10-15 mice. * $p<0.05$ compared to the respective WT control.

4.4.2.5. Wound re-epithelialization and angiogenesis are improved in DM DSCG-treated mice and impaired in KitW/KitW-v mice

Wound re-epithelialization at Day-10 post-injury was reduced in DM mice when compared to their respective non-DM C57BL6/J controls (**Figure 4.8. A, B**). More importantly, re-epithelialization was increased in DM DSCG-treated mice when compared to DM non-treated mice (**Figure 4.8. A, B**). We also performed the same histological analysis in the Day-10 wounds of Kit^{W}/Kit^{W-v} mice, in the absence or presence of DM, together with their respective WBB6F1/J WT controls. As with the C57BL6/J strain, wound re-epithelialization was reduced in WBB6F1/J WT DM when compared to their non-DM controls. In addition, non-DM and DM Kit^{W}/Kit^{W-v} mice had lower re-epithelialization than their respective non-DM and DM WT controls. Finally, DM Kit^{W}/Kit^{W-v} wounds had more incomplete re-epithelialization than non-DM Kit^{W}/Kit^{W-v} wounds (**Figure 4.8. C**). DSCG treatment increased wound angiogenesis, assessed by the number of CD31 positive cells, in C57BL6/J WT DM mice (**Figure 4.8. D**), while it had no effect on wound granulation (**Figure 4.8. E**).

4.4.2.6. Skin hemoglobin oxygen saturation is reduced in WT DM mice and in MC deficient mice

Skin oxygen saturation of hemoglobin (HbO_2), measured by medical hyperspectral imaging (MHSI), was reduced in the peri-wound area of C57BL6/J STZ-DM mice without MC stabilization compared to both DM DSCG-treated mice and non-DM mice at Day-10 (**Figure 4.9. A**). HbO_2 was also reduced in the peri-wound skin of both non-DM and STZ-DM Kit^{W}/Kit^{W-v} mice when compared to their respective WT controls at Day-10 (**Figure 4.9. B, C**). Moreover, Kit^{W-sh}/Kit^{W-sh} mice showed lower levels of skin HbO_2 at both Day-0, and Day-10 (**Figure 4.9. D**).

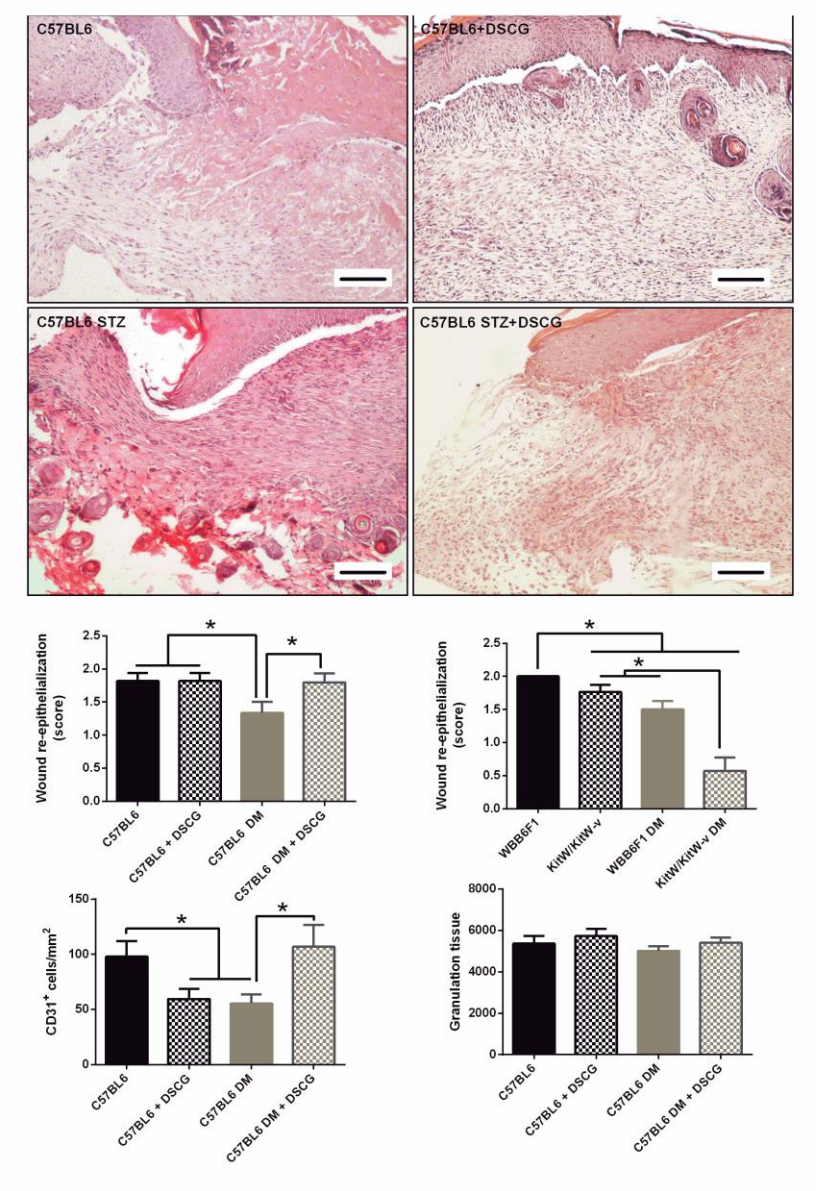


Figure 4.8. Re-epithelialization is reduced in DM and Kit^{W}/Kit^{W-v} wounds. DSCG improves re-epithelialization and angiogenesis in DM wounds. (A) Representative H&E images of Day-10 wounds from C57BL6 non-DM and DM mice, non-treated and DSCG-treated. Scale bar: 100 µm. **(B)** Re-epithelialization was reduced in C57BL6 DM wounds when compared to both non-DM and DM DSCG-treated. **(C)** Re-epithelialization was reduced in WBB6F1 DM when compared to non-DM. Non-DM and DM Kit^{W}/Kit^{W-v} wounds had lower re-epithelialization than their respective WBB6F1 controls. DM Kit^{W}/Kit^{W-v} wounds had more incomplete re-epithelialization than non-DM Kit^{W}/Kit^{W-v}. **(D)** CD31 positive cells were reduced in DM mice compared to non-DM but DSCG treatment reversed this effect. **(E)** No significant differences were observed in granulation tissue. Data represent mean ± SEM. * $p<0.05$.

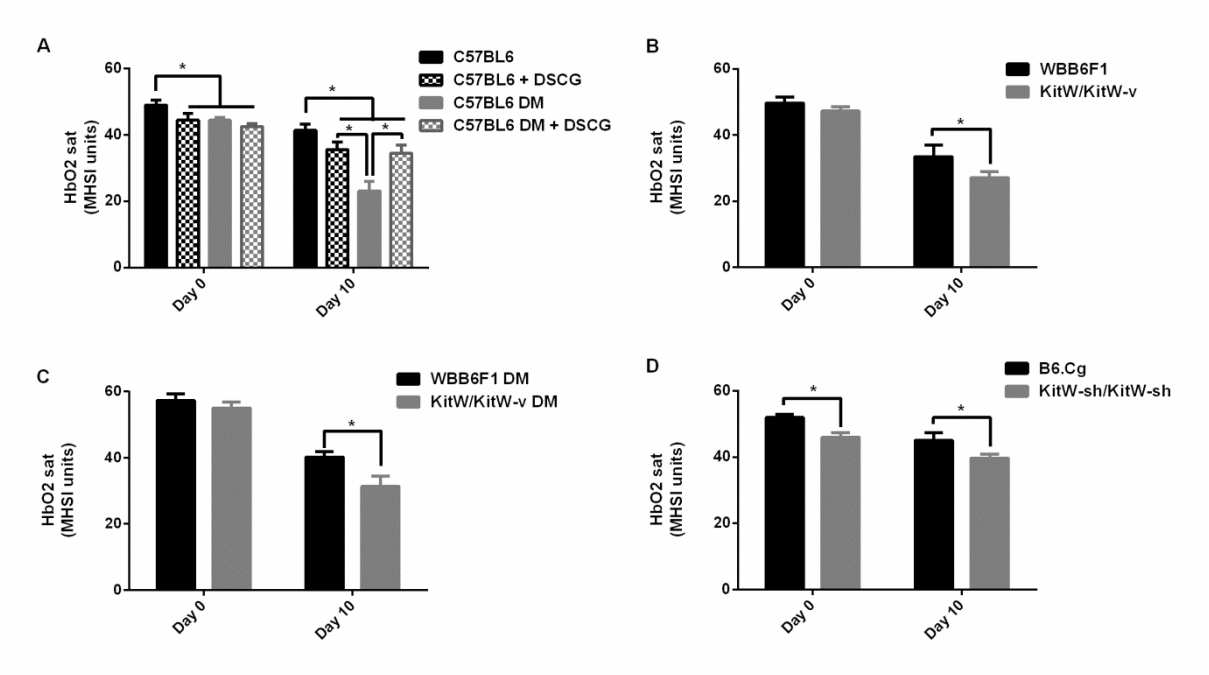


Figure 4.9. Skin oxygen saturation of hemoglobin is reduced in the peri-wound area of STZ-DM mice and MC deficient mice. **(A)** Oxygen saturation of hemoglobin (HbO_2) was reduced in the peri-wound area (Day-10) of C57BL6 DM mice compared to DM DSCG-treated mice and non-DM mice. **(B, C)** HbO_2 was reduced in the peri-wound skin (Day-10) of both non-DM **(B)** and DM **(C)** KitW/*Kit*$^{W-v}$ mice when compared to their respective WBB6F1 controls. **(D)** *Kit*$^{W-sh}$/*Kit*$^{W-sh}$ mice showed lower levels of skin HbO_2 at both Day-0 and Day-10 compared to their B6.Cg controls. Data represent mean ± SEM. * $p<0.05$.

4.4.2.7. M1/M2 macrophage ratio is increased in DM mouse wounds and pharmacological stabilization of MC is able restore it to normal levels

We evaluated the number M1 and M2 macrophages in both unwounded (Day-0) and peri-wound (Day-10) skin of C57BL6/J WT non-DM and DM mice, non-treated and treated with DSCG. The peri-wound skin of DM mice showed a higher dermal M1/M2 macrophage ratio at Day-10. MC stabilization by DSCG treatment in DM mice drastically reduced the M1/M2 ratio, restoring it to similar levels as the non-DM mice (**Figure 4.10. A, C**).

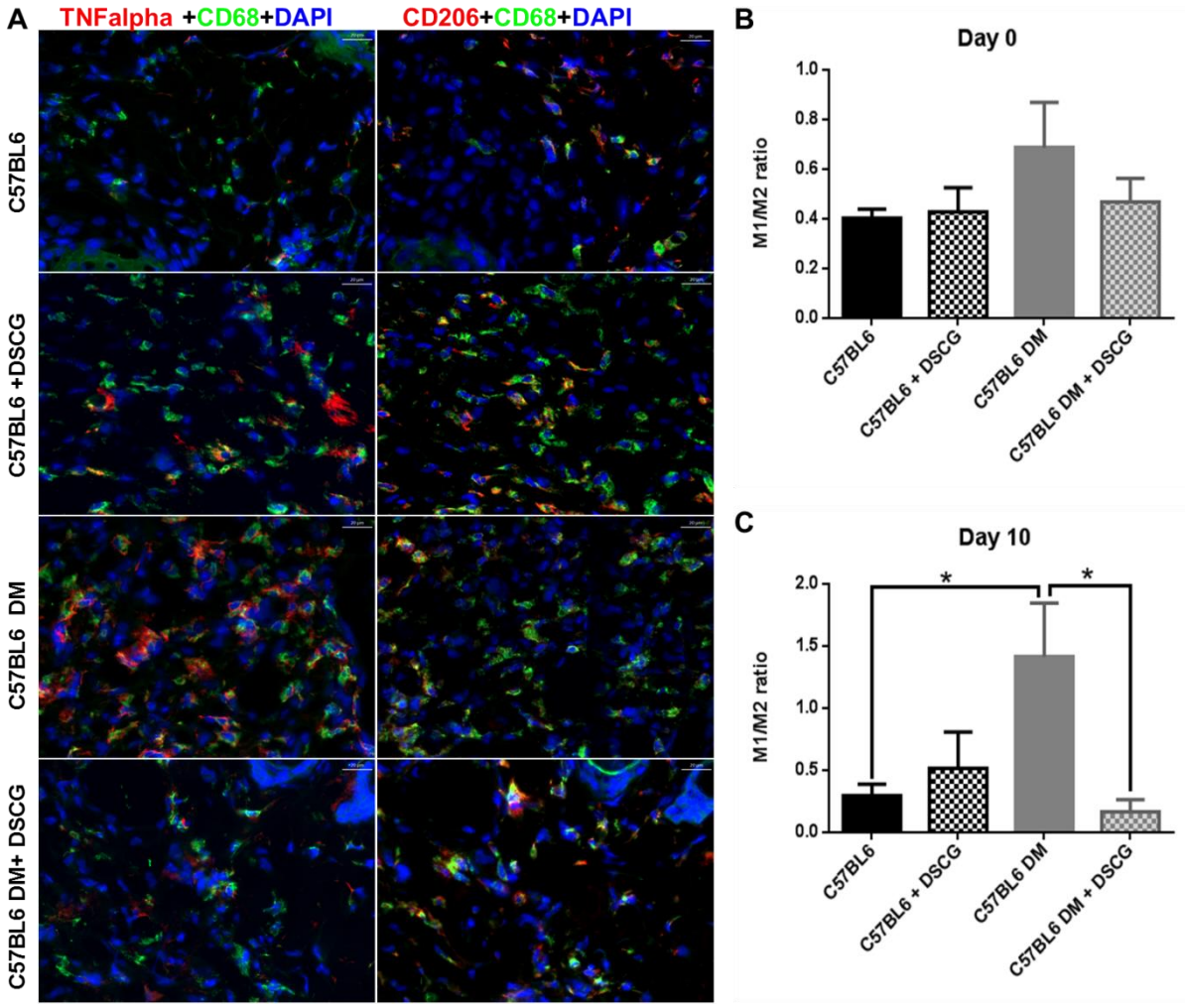


Figure 4.10. M1/M2 macrophage ratio is increased in the peri-wound skin of C57BL6 DM mice and DSCG treatment restores it to normal levels. (A) Representative images of M1 and M2 macrophages from peri-wound (Day-10) skin of C57BL6 non-DM and DM mice, non-treated and DSCG-treated (scale bar: 20µm). **(B)** There was a trend for the M1/M2 ratio to be increased in the skin of DM mice at Day-0, but failed to reach statistical significance. **(C)** At Day-10, DM mice had higher dermal M1/M2 ratio and DSCG treatment reduced it to levels similar to non-DM. Data represent mean ± SEM. * $p<0.05$.

We also evaluated the M1/M2 macrophage ratio in Day-0 and Day-10 skin specimens from non-DM and DM MC deficient $Kit^{W}/Kit^{W\text{-}v}$ mice, together with their respective WBB6F1 WT controls (**Figure 4.11. A**). The M1/M2 ratio was markedly increased in the peri-wound skin at Day-10 of WBB6F1/J DM and of $Kit^{W}/Kit^{W\text{-}v}$ DM mice, whereas it was marginally increased in the wounds of non-DM $Kit^{W}/Kit^{W\text{-}v}$ mice ($p=0.055$) (**Figure 4.11. C**). The same trend was

observed in unwounded skin (Day-0), although not as pronounced and not significant, as in Day-10 (**Figure 4.10 B** and **Figure 4.11 B**).

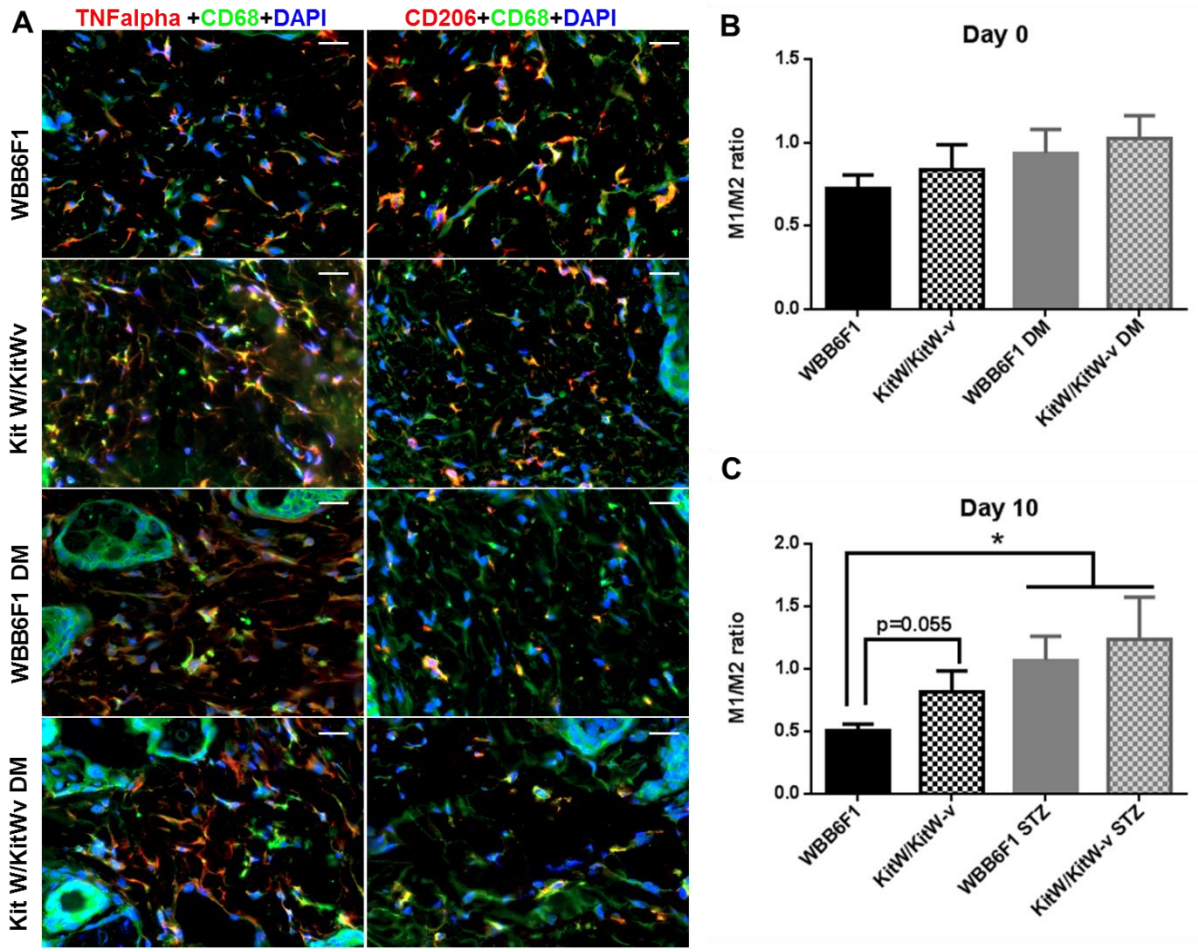


Figure 4.11. M1/M2 macrophage ratio is increased in the peri-wound skin of WBB6F1 DM mice and $Kit^{W}/Kit^{W\text{-}v}$ DM mice. (A) Representative images of M1 and M2 macrophages from peri-wound (Day-10) skin of non-DM and DM $Kit^{W}/Kit^{W\text{-}v}$ mice and their respective non-DM and DM WBB6F1 WT controls (scale bar: 20µm). **(B)** No significant differences were observed in the non-wounded (Day-0) skin M1/M2 ratio between WBB6F1 and $Kit^{W}/Kit^{W\text{-}v}$ mice. **(C)** Both DM WBB6F1 and DM $Kit^{W}/Kit^{W\text{-}v}$ mice had higher M1/M2 ratio in the peri-wound (Day-10) skin compared to non-DM WBB6F1. Non-DM $Kit^{W}/Kit^{W\text{-}v}$ mice had a marginally increased M1/M2 ratio compared to WBB6F1 (p=0.55). Data represent mean ± SEM. * $p<0.05$.

4.4.2.8. Skin levels of VEGF and MMP-9 are altered in WT DM and in MC deficient mice

VEGF protein expression was reduced in unwounded (Day-0) skin in both STZ-DM mice non-treated and DSCG-treated, and in MC deficient *Kit^{W}/Kit^{W-v}* mice when compared to their respective controls (**Figure 4.12. A-C**). In addition, VEGF was reduced in DM wounds at Day-10 (**Figures 4.12. A, C**). DSCG-treated DM mice showed a similar increase post-wounding as non-DM mice (**Figure 4.12. C**).

MMP-9 protein expression was increased in Day-0 skin and in Day-10 wounds of both C57BL6/J and WBB6F1/J WT DM mice, compared to their respective non-DM controls (**Figure 4.13. A, C**). DSCG treatment reduced MMP-9 protein expression in Day-10 wounds from C57BL6/J DM mice (**Figure 4.13. A**). MMP-9 was also elevated in Day-10 wounds from *Kit^{W}/Kit^{W-v}* mice (**Figure 4.13. B**). Topical wound treatment with SP reduced MMP-9 expression at Day-10 in C57BL6/J WT mice but failed to have an effect on the *Kit^{W}/Kit^{W-v}* mice (**Figure 4.13. B**).

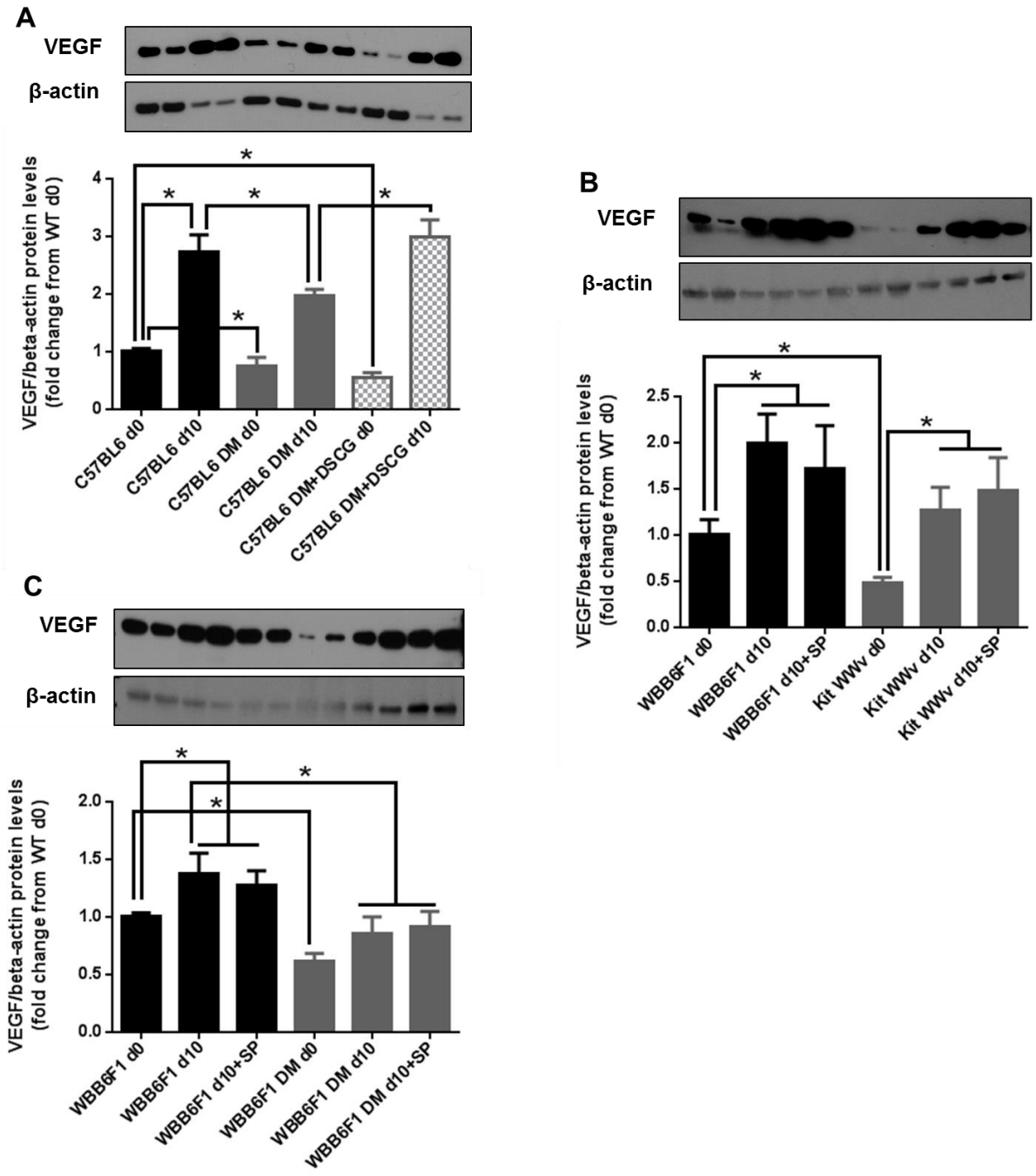


Figure 4.12. VEGF protein levels are reduced in the skin of STZ-DM, as well as in Kit^{W}/Kit^{W-v} mice. We evaluated the skin protein levels of VEGF at Day-0 and 10 from: **(A)** non-DM and DM C57BL6 mice, non-treated and DSCG-treated; **(B)** Kit^{W}/Kit^{W-v} mice and their WBB6F1 controls, non-treated and SP-treated; **(C)** non-DM and DM WBB6F1/J mice, non-treated and SP-treated. **(A)** VEGF was reduced in Day-0 skin from C57BL6 DM mice. At Day-10, VEGF was reduced in DM but DSCG treatment restored it. **(B)** Kit^{W}/Kit^{W-v} mice showed reduced VEGF at Day-0. **(C)** Similar results were observed in WT WBB6F1 mice. Data represent mean ± SEM. * $p<0.05$.

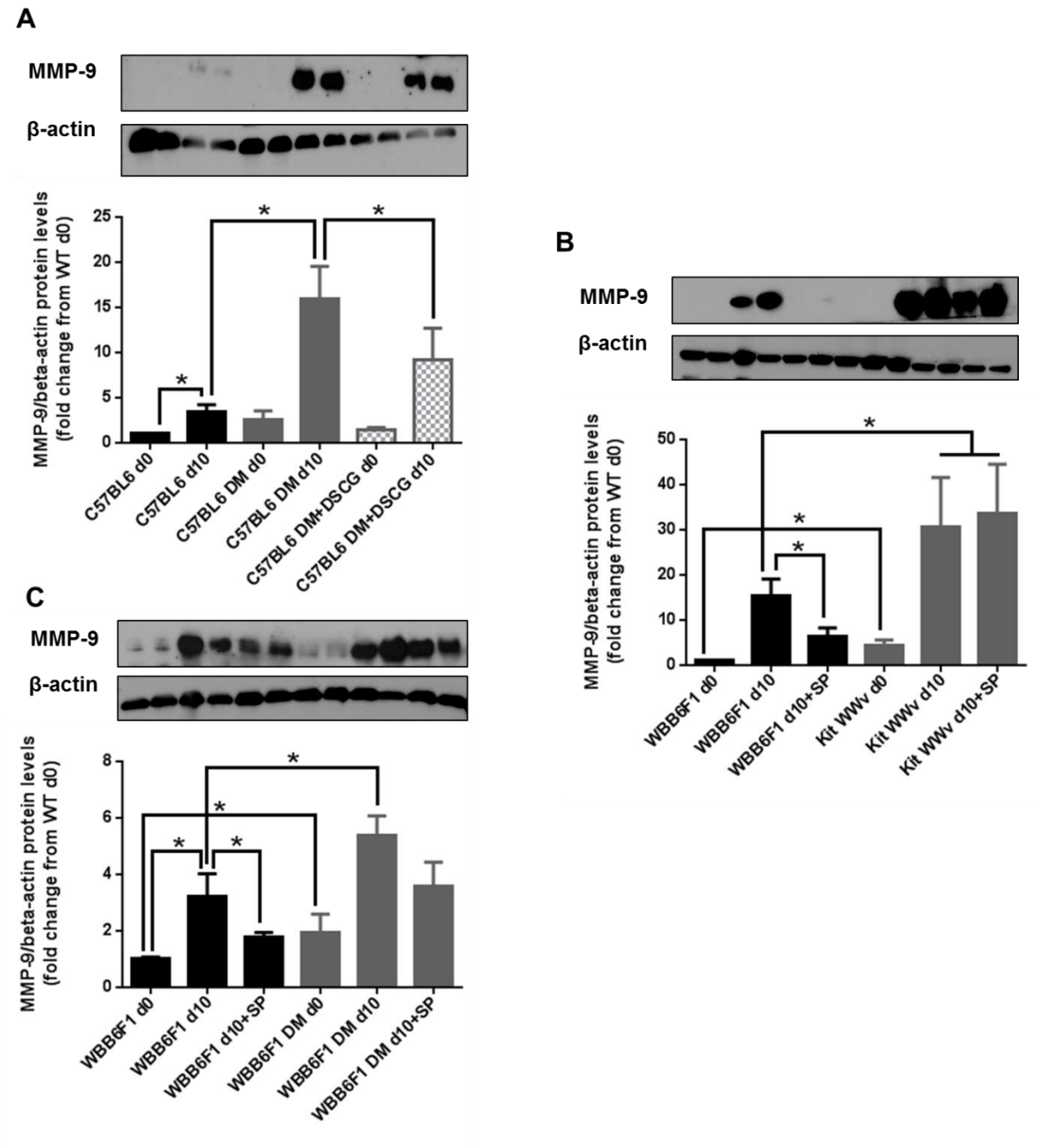


Figure 4.12. MMP-9 protein levels are increased in the skin of WT DM and Kit^{W}/Kit^{W-v} mice. We evaluated the skin protein levels of MMP-9 at Day-0 and Day-10 from: **(A)** non-DM and DM C57BL6 mice, non-treated and DSCG-treated; **(B)** Kit^{W}/Kit^{W-v} mice and their WBB6F1 controls, non-treated and SP-treated; **(C)** non-DM and DM WBB6F1/J mice, non-treated and SP-treated. **(A)** MMP-9 was increased in DM mouse wounds at Day-10 and DSCG treatment reduced it. **(B)** MMP-9 was elevated in the Day-10 wounds of Kit^{W}/Kit^{W-v} mice. **(B)** SP treatment reduced MMP-9 in the Day-10 wounds of WBB6F1 non-DM mice, but did not have any effect in the Kit^{W}/Kit^{W-v} wounds. **(C)** Similar results were observed in WBB6F1 WT mice. Data represent mean ± SEM. * $p<0.05$.

4.4.2.9. Circulating levels of cytokines are consistent with chronic inflammation in DM

Serum levels of the pro-inflammatory cytokines KC (mouse homolog for human IL-8) and IL-12 (p40), increased post-wounding (Day-10) in non-DM mice. However, such increase failed to occur in either WBB6F1/J DM mice or in non-DM and DM *Kit*^{W}/*Kit*$^{W-v}$ mice (**Figure 4.13. A, B**). A similar profile was observed for the circulating levels of TNF-α and IFN-γ, although not as profound or significant as for KC and IL-12 (**Figure 4.13. C, D**). In addition, *Kit*^{W}/*Kit*$^{W-v}$ mice trended to have lower serum levels of VEGF at baseline (**Figure 4.13. E**).

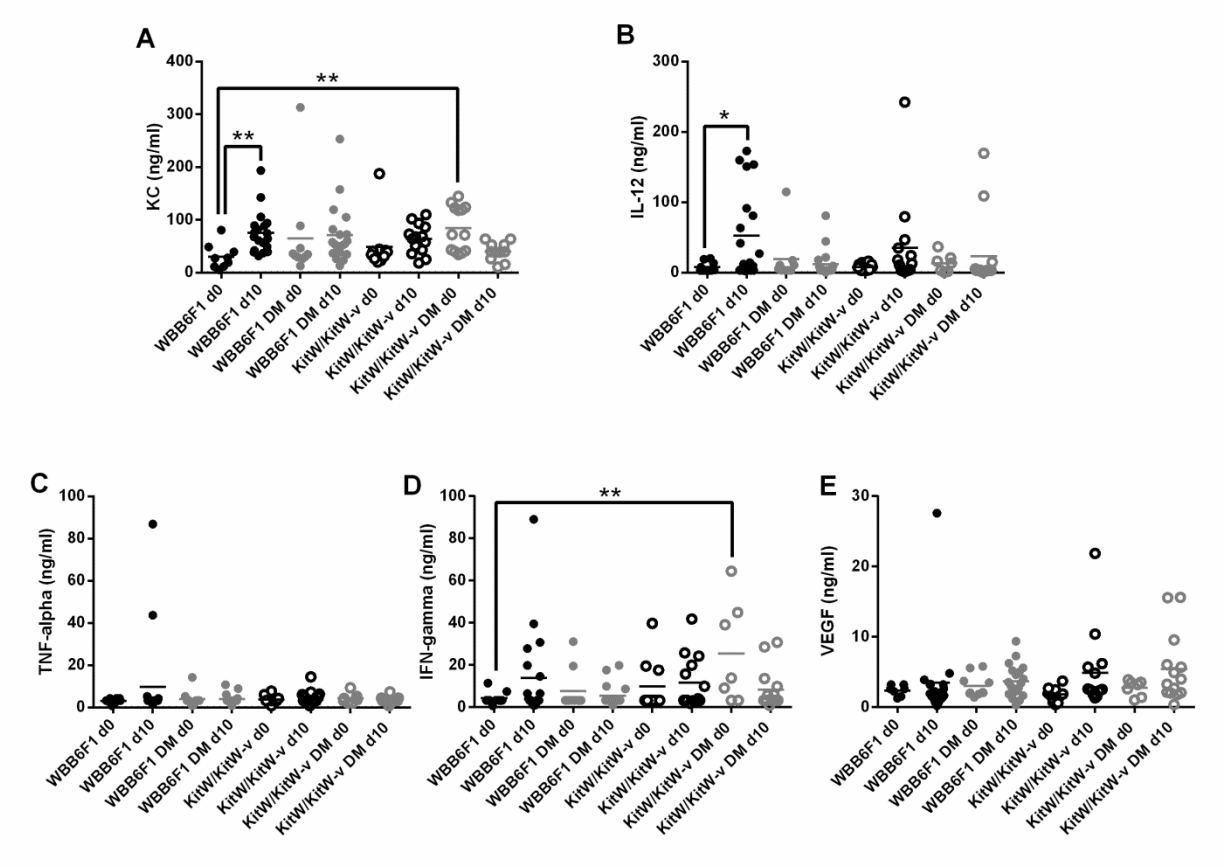


Figure 4.13. Circulating levels of some inflammatory cytokines are altered in DM. Serum levels of **(A)** KC (mouse homolog for human IL-8) and **(B)** IL-12 increased post-wounding (Day-10) in WBB6F1 non-DM mice but not in DM WBB6F1 or *Kit*^{W}/*Kit*$^{W-v}$ mice. Day-0 levels of KC were elevated in STZ-DM *Kit*^{W}/*Kit*$^{W-v}$ mice. **(C-D)** A similar profile was observed for TNF-α and IFN-γ. (E) VEGF trended to be reduced at Day-0 in *Kit*^{W}/*Kit*$^{W-v}$ mice. Data represent mean.* $p<0.05$

4.4.3. Cell culture studies

4.4.3.1. High glucose does not increase in vitro MC degranulation

We evaluated MC degranulation in human LAD2 cells cultured for two weeks in normal glucose (NG) or high glucose (HG), in the absence or presence of the MC trigger SP. SP treatment induced degranulation in both NG and HG cultured MC, but no differences were observed between NG and HG or NG+SP and HG+SP cultured MC (**Figure 4.14. A**).

4.4.3.2. High glucose reduces VEGF release from MC

We evaluated VEGF release in human LAD2 cells in NG or HG, in the absence or presence of the MC trigger SP. VEGF release was reduced in LAD2 cells cultured in HG compared to NG (**Figure 4.14. B**), suggesting that hyperglycemia *per se* affects MC secretion of VEGF. SP did not have any effect on VEGF release from MC either in NG or HG.

4.4.3.2. High glucose increases the release of IL-8 from MC in the presence of SP

We evaluated the release of several pro-inflammatory MC mediators from human LAD2 cells in NG or HG, in the absence or presence of the MC trigger SP. SP increased the release of IL-8, TNF α, and MCP-1 in both NG and HG cultured MC. IL-8 release was increased in MC cultured in HG+SP when compared to NG+SP (**Figure 4.14. C**). However, no differences were observed in terms of TNF α or MCP-1 release between NG and HG in the absence or presence of SP (**Figure 4.15. A, B**).

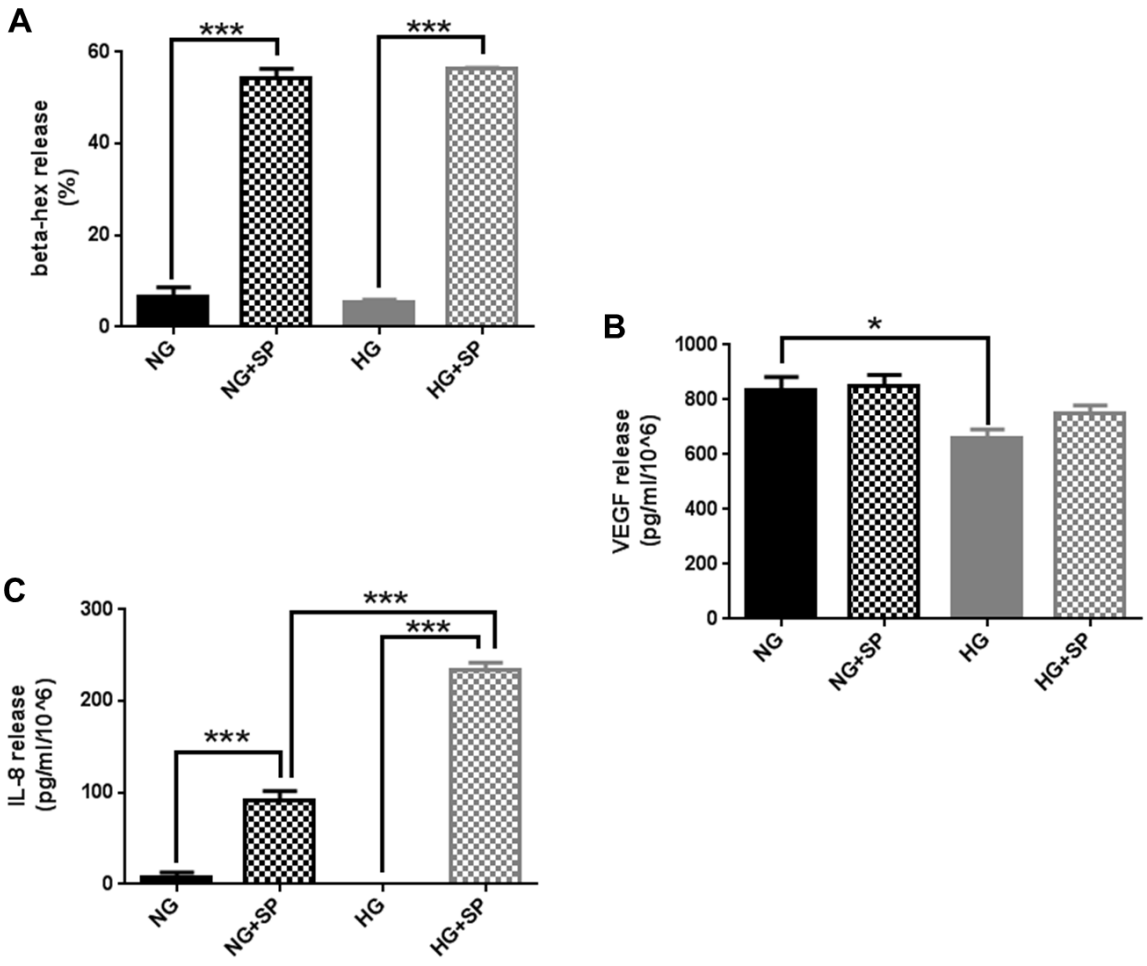


Figure 4.14. High glucose does not affect in vitro MC degranulation, but influences VEGF and IL-8 release. (A) SP increased beta-hexosaminidase (beta-hex) release from LAD2 cells cultured in NG or HG. HG did not have any effect on beta-hex release. **(B)** HG reduced VEGF release from LAD2 cells. SP did not affect VEGF release from LAD2 cells, either in NG or HG. **(C)** SP increased IL-8 release from LAD2 cells cultured either in NG or HG. No differences were observed between NG and HG alone. However, IL-8 release from LAD2 cells in HG and triggered with SP was 61% higher than LG+SP. Data represent mean ± SEM. * $p<0.05$, *** $p<0.001$.

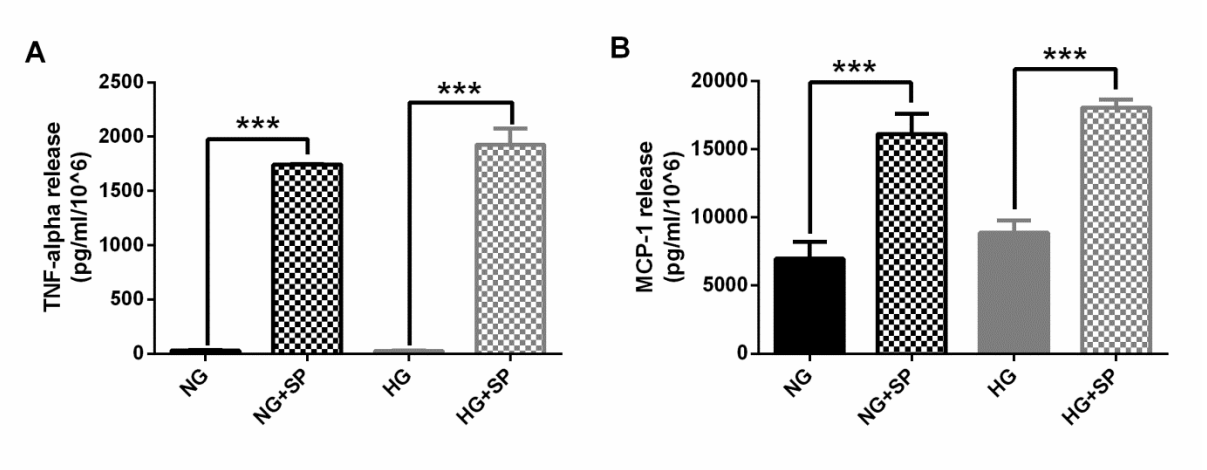


Figure 4.15. High glucose does not affect in vitro MC release of TNF-alpha or MCP-1. SP increased the release of TNF-alpha **(A)** and MCP-1 **(B)** in both LG and HG cultured MC. No differences were observed in terms of TNF-alpha **(A)** or MCP-1 **(B)** release between NG and HG or between NG+SP and NG+SP. Results represent mean. *** $p<0.001$.

4.5. Discussion

The main findings of the present study are that both human and experimental DM are associated with skin MC degranulation that contributes to chronic inflammation and impaired wound healing. MC stabilization in DM mice improves wound healing while MC deficiency, in the presence or absence of DM, causes impairs wound healing. Furthermore, MC exert their effects by promoting M2 polarization while SP improves wound healing, at least partly, through its effects on MC.

MC progenitors are released by the bone marrow and are attracted by circulating chemokines to the dermis and hypodermis. Once in the skin, MC maturation is greatly affected by the presence of cytokines in the local microenvironment, including IL-6, IL-33 and TNF-α, and is characterized by considerable heterogeneity [421]. Mature skin MC are activated by several factors, including cytokines IL-3 and IL-33, and exocytose their granules that contain numerous proteases such as tryptase, histamine, serotonin and heparin[422, 423].

Our study, to the best of our knowledge, is the first to report increased MC degranulation in both human and experimental DM skin. In addition, we show that human degranulated MC were in close proximity with the dermis inflammatory cells and correlated with serum levels IL-6 and TNF-α. Finally, we show that serum IL-33, which promotes MC degranulation during wound healing, was increased in DM patients with foot ulceration. Previous findings published by our group have also shown that DM leads to a chronic inflammatory state not only systemically, but also locally at the skin level[354, 418]. The results presented in Chapters II and III also corroborate the persistence of a pro-inflammatory environment in DM skin. Therefore, our data indicate that the local and systemic inflammation that is present in DM is probably the main factor responsible for the MC activation.

MC have been shown to play a role in all phases of wound healing[198, 421]. Thus, during the inflammatory phase, MC regulate the accumulation of neutrophils and macrophages in the wound area[26, 266-269], during the proliferative phase promote the proliferation of fibroblasts[279], angiogenesis[424] and re-epithelialization[281-284], and during the remodeling phase augment scar formation[285-287, 413]. In the present study, we report for the first time that MC stabilization improved wound healing in DM mice in which we documented increased MC degranulation before wounding but had no effect on non-DM mice, which showed no pre-wounding MC degranulation. In agreement with previous studies, we demonstrated that non-DM mice have considerable skin MC degranulation after injury[268, 269]. However, we also showed that this post-wounding degranulation is absent in DM mice and believe that these findings have not been reported previously. Furthermore, we showed that MC deficient mice, with or without DM, had impaired wound healing when compared to their respective MC sufficient controls. These results indicate that the presence of intact MC in unwounded skin and a timely post-wounding degranulation are required for optimal wound healing, while pre-wounding MC degranulation has similar effects to MC absence.

Macrophage phenotype can be broadly characterized as “classically activated” pro-inflammatory (M1) or “alternatively activated” immunomodulatory (M2) despite the

existence of numerous phenotypes that cover the full spectrum between these two[425, 426]. M1 activation is required during the acute inflammatory phase of wound healing but is also present in chronic wounds that are characterized by chronic inflammation[400], while M2 activation during the proliferation phase promotes angiogenesis and collagen production[427]. Our results show that in unwounded human forearm and foot skin of DM patients macrophages are shifted towards M1 activation. Furthermore, we show increased skin expression of the cytokines TNF-α and IL-1β that promote M1 activation and are known to contribute to impaired wound healing and development of chronic wounds when persistently elevated[346, 354], while the expression of IL-10 that promotes M2 activation and skin repair was reduced[428]. These results strongly support the hypothesis that the chronic inflammatory state that is present in DM before ulcer development plays a major role in the failure to heal the DFU [354].

In our mouse model, MC stabilization restored the elevated macrophage M1/M2 ratio post-wounding in DM mice to levels similar to non-DM mice, while a smaller, non-significant improvement was observed pre-wounding. In addition, similar results to DM mice were observed in MC deficient mice. These results strongly indicate that M2 polarization may be one of the major factors responsible for the observed improvement in wound healing by MC stabilization in DM, as a reduced M1/M2 ratio at Day-10 post-wounding suggests a progression from the inflammatory to the proliferative phase.

Substance P (SP) has been shown by us to improve wound healing in both non-DM and DM mice and its skin expression is reduced in both human and experimental DM (Chapter III)[420]. Here we show that SP exerts its beneficial effects in wound healing only in the presence of MC, as no changes were observed between SP-treated and untreated wounds in MC deficient mice. Furthermore, genetically modified mice lacking the TAC1 gene that encodes SP and other tachykinins had increased pre-wounding MC degranulation. However, MC degranulation was not different between non-DM and DM TAC1KO mice suggesting that although DM and SP-deficiency independently cause MC degranulation in

unwounded skin, they do not have an additive or synergistic effect. MC are located close to dermal nerve fibers that secrete SP and they express the neurokinin 1 receptor (NK1R)[408], through which SP mainly affects wound healing (Chapter III)[420]. These findings suggest that SP prevents MC degranulation in the unwounded skin and it mediates its beneficial effects in wound healing, at least partially, through MC function.

MC stabilization prevented the post-wounding VEGF reduction and reduced the elevated MMP-9 levels that were noticed in the DM mice, while MC-deficiency had a similar effect to DM in the various mouse models tested. Similar results were also observed in the wound re-epithelization and angiogenesis, and in the peri-wound hemoglobin oxygen saturation. In addition, DM or MC deficient mice failed to increase post-wounding the KC and IL-12 cytokines, despite rather high pre-wounding levels. VEGF, MMP-9, KC and IL-12 play an important role in wound healing and our results are in agreement with the current understanding that DM is characterized by chronic inflammatory state and failure to mount an acute inflammatory response to injury[180, 354]. Furthermore, our data indicate that MC absence or pre-wounding degranulation plays a role in all the above aberrations while MC stabilization in DM reverses those abnormalities and affects multiple wound healing parameters that include wound size reduction, re-epithelialization and angiogenesis.

While most studies have reported a MC role in wound healing [196, 255, 266, 267, 279, 410, 413, 424, 429], other investigators have not noticed any effect of MC deficiency in wound healing [430-432]. Although the reasons for this discrepancy are not clear, it should be emphasized that the above studies were based on various types MC deficient mice and did not include DM mice nor employed MC stabilization in MC sufficient mice with increased degranulation. Nonetheless, a possible reason why the models of MC deficiency in some of these studies[430, 431] may have normal wound healing is that they do not lack the full range of cells that are currently considered as MC. In addition, the study of splinted wounds may be another reason for the observed changes[432] as other studies that employed splinting have shown robust deficiencies in the most important parameters of wound healing, including cell

proliferation, angiogenesis, granulation and collagen maturation despite a lack of difference in wound closure[255]. Given the concerns that splinting may alter the wound healing phenotype[405], we opted to avoid it in the present studies.

In order to further elucidate the role of MC in DM wound healing, we performed additional cell culture studies. The observed results are complimentary to the *in vivo* data as they indicate that although hyperglycemia did not affect mast cell degranulation, it nonetheless reduced VEGF release, one of the main factors that affect angiogenesis and wound healing, and increased IL-8 release, one of the most prominent inflammatory cytokines that are involved in impaired wound healing[433]. These results are compatible with previous studies that have shown that MC have considerable heterogeneity and plasticity and under various conditions, such as IL-33 stimulation, can undergo partial degranulation and/or selectively release various effectors[434, 435].

The study has its limitations. We did not investigate changes during the remodeling phase where MC have been extensively implicated in extensive scarring[413]. However, scarring is not a problem in DFU as there is neither keloid formation nor functional problems related to extensive scarring. In addition, we have focused mainly on the mouse model. The main reason for this is the availability of genetically modified MC deficient mice. Nonetheless, the fact that we involved more than one type of mice and the similarity between the human and mouse findings regarding the MC function in DM makes us confident about the translational value of the observed results.

In summary, the main finding of the present study is that MC stabilization can ameliorate DM-impaired wound healing. Therefore, the use of cromolyn-based products, or other agents that prevent MC degranulation such as flavonoids[436, 437], or even the development of novel MC stabilizers that can be topically applied in the wound area, such as small molecule orai/CRAC channel blockers that can inhibit MC degranulation[438] and T-cell activation [439] has the potential to be an effective novel therapeutic approach for DFU.

Chapter V

New Biomaterials for Diabetic Wound Healing

5. Chapter V - New Biomaterials for Diabetic Wound Healing

5.1. Abstract

Diabetic foot ulcers (DFU) represent a severe health problem and a major unmet clinical challenge. Despite the numerous attempts to develop therapies for DFU, very few have been approved and their efficacy is limited. Current consensus is that a multi-therapy approach is more likely to be beneficial for Diabetes *Mellitus* (DM) wound care than a single treatment. In addition, strategies to increase the bioavailability of the therapeutic agents in the wound environment are believed to be advantageous. In this study, we propose to use novel biomaterials for the treatment of DM wounds. We show that these new materials – alginate- and deoxyribonucleic acid (DNA)- based gels – are biocompatible and can be directly injected into the wound margins without major adverse effects in our mouse model of DM wound healing. In addition, they allow the incorporation of multiple bioactive factors and provide sustained release of effector cells – outgrowth endothelial cells (OEC) – and molecules – neuropeptides – that have been previously validated in experimental models of DM wound healing and/or hindlimb ischemia. We also demonstrate that the combination of OEC and the neuropeptide substance P (SP) in the gels has a better wound healing outcome than delivery of OEC alone. In addition, we observed that sub-therapeutic doses of the growth factor VEGF are required for the transplanted cells to exert their beneficial effects in skin repair. In summary, alginate and DNA scaffolds could serve as potential delivery systems for the next-generation DFU therapies.

5.2. Introduction

Foot problems represent one of the major DM complications as they are the leading cause of non-traumatic lower extremity amputations[350] and carry a considerable socio-economic burden[440]. Given the alarming rise of the DM prevalence in the general population, it can only be expected that the number and cost of diabetic foot ulcers (DFU) will intensify in the future.

Despite the severity of the problem, no new products for DFU treatment have been licensed recently. The only three commercially available products - Becaplermin, Apligraf, and Dermagraft - were developed in the 1990s[291, 441] and are characterized by moderate efficacy[314, 315, 442].

Studies from our research groups have identified possible mechanistic interventions that can improve the management of DFU. Namely, we have demonstrated a persistent inflammatory state in DM that is present at baseline (Chapters II, III and IV)[180, 418, 443-445] and throughout the impaired wound healing process (Chapters III and IV)[180, 420, 445]. In addition, we have found reduced levels of the neuropeptide Substance P (SP) in human and experimental DM (Chapter III)[179, 180, 420, 446]. Moreover, we have shown that both SP (Chapter III)[420, 446] and neurotensin (NT)[342, 343] modulate inflammation and improve wound healing in DM mouse models. Furthermore, we have reported reduced endothelial progenitor cell (EPC) numbers in DM patients with complications[144], while studies from collaborators have shown beneficial effects of endothelial cell precursor transplantation in mouse models of DM wounds[338] and hindlimb ischemia[447].

A major problem of drug delivery without proper control is that it frequently requires large doses of the bioactive agent to achieve a therapeutic effect. The use of such doses, beyond the waste and additional costs, can lead to increased toxicity and/or undesirable adverse effects. This is particularly true for topical administration of neuropeptides, since they are rapidly inactivated in the protease-rich wound environment[448]. In addition, it is now clear that the ability of transplanted cells to orchestrate regeneration is highly dependent on their interaction with host cells, and the gene expression profile of the transplanted cells is crucial for their therapeutic effect[449-451]. Current systems hold the transplanted cells in one location, limiting their interactions, and provide little control over gene expression. To overcome these issues, next generation delivery systems should protect bioactive agents in the wound environment while allowing interaction with the host cells. Moreover, these systems should

provide sustained release of the bioactive molecules and cells, and adequately modulate the fate, location and phenotype of transplanted cells.

Biomaterials based on alginate, a polysaccharide widely used in dentistry and as an excipient in the pharmaceutical industry[452] can be used as a multi-therapy delivery system for wound healing. Biodegradable, injectable alginate gels have already been developed[453, 454], and demonstrated to protect cells following transplantation, promoting proliferation of the cells inside the material, and releasing the cells into the wound site over time[447, 455, 456].

Biomaterials based on DNA are also new, innovative and very interesting candidates as the construction of DNA composite materials offers much greater control and versatility than has been available previously. Scaffolds built solely from synthetic polymers typically do not display the biological motifs that guide normal cellular behavior in the ECM (e.g. adhesion, matrix synthesis and degradation, motility), while scaffolds built solely from biological materials lack control over mechanical properties and spatiotemporal organization. DNA building blocks which are 'programmable' structures offer a far finer level of spatial and mechanical control compared to other self-assembling biomolecules currently used for organ and tissue engineering applications, such as ECM self-assembly of collagen fibrils and gels.

In this study, we tested the effect of alginate gels encapsulating (i) neuropeptides (SP and NT), (iii) human umbilical cord-derived outgrowth endothelial cells (OEC), that have a similar behavior to EPC[447], (iii) or a combination of OEC and SP on DM wound healing. We also evaluated the potential use of DNA hydrogels as delivery systems for wound therapy.

5.3. Materials and methods

5.3.1. Biomaterials

5.3.1.1. Alginate gels

Alginate gels were prepared as previously described[447, 457]. Release kinetics of VEGF[454, 457], as well as the ability of OEC to migrate outward from the macroporous alginate scaffolds had already been tested elsewhere[447] with positive results for prolonged periods of time (over 2 weeks).

Release kinetics for SP and NT from the alginate gels were performed by measuring the cumulative release of SP or NT into the media using commercially available ELISA kits for SP (R&D Systems, Minneapolis, MN) and NT (Bachem, Torrance, CA), respectively.

5.3.1.2. Deoxyribonucleic acid (DNA) gels

Deoxyribonucleic acid (DNA) hydrogels were prepared in the lab of Dr. Shih. Two Y-scaffold DNA gels were designed based on an adaptation of the model by Xing et al[458]. Each Y-scaffold was assembled from three single-stranded DNA (ssDNA) strands. Each strand of one Y-scaffold was 46 nucleotides long with the following components: 16 complementary sequences and 14 nucleotide ‘sticky ends’. The hybridization of ‘sticky ends’ of the first Y-scaffold to the second Y-scaffold resulted in the formation of a hydrogel. Each Y-Scaffold is pre-annealed at 3mM and stoichiometrically combined in a 1:1 ratio.

5.3.2. Mouse model

Wild-type (WT) C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were made diabetic (DM) by administering 50 mg/kg STZ (i.p daily for 5 consecutive days) in citrate buffer (0.1M). In the non-DM groups, mice were treated with vehicle alone. Fasting blood glucose was monitored a week after the last injection and mice with blood glucose over 250 mg/dl were considered DM.

5.3.2.1. Wound creation, monitoring and treatment

Eight weeks after STZ or vehicle treatment, mice were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and two circular 6 mm full thickness wounds were created on the shaved dorsum of the mice using a punch biopsy tool. Wound closure kinetics was monitored daily over a 10-day period by measuring the wound size using acetate tracing followed by analysis with Image J software (NIH). Data was presented as percentage of original wound size (Day-0) over the study period.

On Day-0, immediately after wound creation, the gels were injected into the tissue surrounding the wounds. For the studies using alginate gels, only DM mouse models were used. Treatments were as follows: 1a) alginate gel only (60µl/wound), 1b) alginate gel encapsulating a combination of SP (32µg/60µl/wound) and NT (50µg/60µl/wound); 2a) alginate gel encapsulating a sub-therapeutic dose of bioactive VEGF (3µg/60µl/wound), 2b) alginate gel encapsulating VEGF (3µg/60µl/wound) and OEC ($1x10^6$OEC/60µl/wound), 2c) alginate gel encapsulating VEGF (3µg/60µl/wound), OEC ($1x10^6$OEC/60µl/wound) and SP (32µg/60µl/wound); 3a) alginate gel only (60µl/wound), 3b) alginate gel encapsulating OEC ($1x10^6$OEC/60µl/wound). For the studies using DNA-based gels, both DM and non-DM mice were used. The study groups were divided as follows: 4a) non-DM untreated wounds, 4b) non-DM DNA gel treated wounds (60µl/wound), 4c) DM untreated wounds, 4d) DM DNA gel treated wounds (60µl/wound).

5.3.2.2. Histological analysis and immunohistochemistry (IHC)

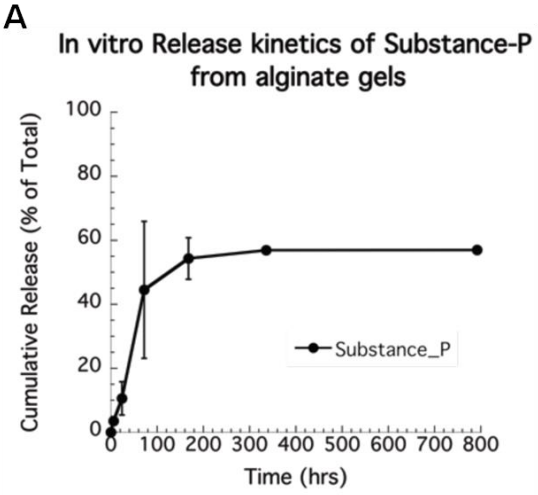
At the end of the study, Day-10 post-wounding, mice were euthanized and 1 cm X 1 cm skin sections that included the wound margins were cut. For morphologic analysis and immunohistochemistry, tissue was fixed in 10% formalin and subsequently embedded in paraffin (FFPE). For morphologic analysis and evaluation of the inflammatory cell infiltrate, FFPE sections (5 µm) underwent routine histological processing with hematoxylin and eosin (H&E).

5.4. Results

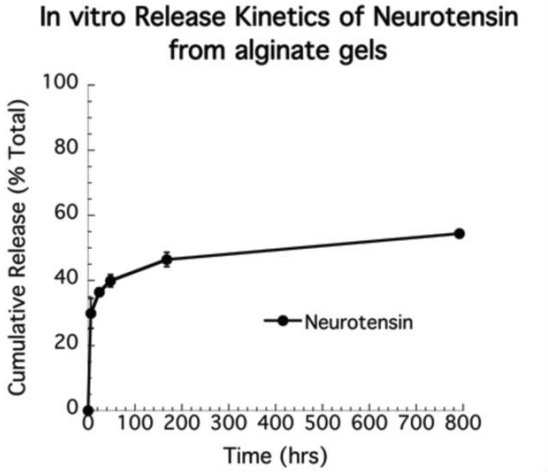
5.4.1. Alginate gels offer continuous release of Substance P (SP) and Neurotensin (NT) for more than 10 days

We evaluated SP (**Figure 5.1. A**) and NT (**Figure 5.1. B**) release kinetics from the alginate hydrogels at several time points up to one month. Both neuropeptides were cumulatively released from the gels during the time period tested. At one week, the closest time point to our *in vivo* wound healing studies, the cumulative release of SP and NT was 54.3% and 46.4%, respectively (**Figure 5.1. C**).

A



B



C

	6 hrs	12hrs	24hrs	48 hr	3 day	1 wk	2 wk	1 month
Neurotensin	29.9%	_	36.4%	39.9%	_	46.4%	_	54.4%
Substance_P	3.5%	10.6%	_	_	44.5%	54.3%	56.9%	57.0%

Figure 5.1. Alginate gels offer sustained release of SP and NT for more than 10 days. *In vitro* release kinetic profiles of SP **(A)** and NT **(B)** from alginate hydrogels measured for 800 hours. Data represent mean ± SEM. **(C)** Table showing the mean cumulative release (as percentage of total) of NT and SP at the studied time points.

5.4.2. Alginate gels releasing SP and NT improve wound healing in DM mice

A single injection of alginate gel with SP and NT into the wound margins reduced the wound size in DM mice at Day-10 post-wounding (**Figure 5.2. A**). In fact, there was 54% wound size reduction at Day-10 in the wounds that received the neuropeptide combination compared to the wounds that received vehicle only (**Figure 5.2. B**).

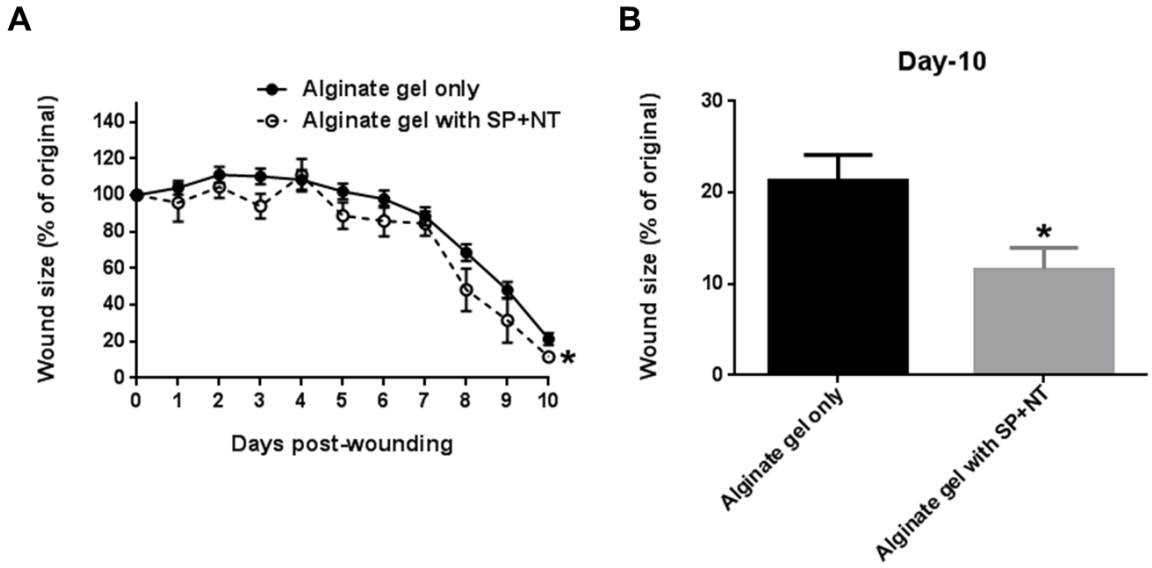


Figure 5.2. Topical treatment with alginate gel encapsulating NT and NT reduces wound size in DM mice. (A) Wound healing progress of DM wounds treated with alginate gel only or with alginate gel with SP+NT over 10 days. **(B)** Day-10 wound size of DM wounds treated with alginate gel only or with alginate gel with SP+NT. Data represent mean ± SEM. * $p < 0.05$, compared to alginate gel only.

5.4.3. Alginate gel deployment of outgrowth endothelial cells (OEC) accelerates wound healing in DM mice. The combination of cell therapy and SP treatment acts additively, if not synergistically

We evaluated the effect of (i) alginate gel with VEGF, (ii) alginate gel with VEGF+OEC, and (iii) alginate gel with VEGF+OEC+SP in our mouse model of DM wound healing. Healing was accelerated in the wounds that received alginate gel with VEGF+OEC compared to the ones that received alginate gel with VEGF (**Figure 5.3. A, B**). In addition, healing was

improved in the wounds that received alginate gel with VEGF+OEC+SP compared to both wounds that received alginate gel with VEGF only or with VEGF+OEC (**Figure 5.3. A, B**). These results indicate that the combination of OEC and SP has an additive, if not synergistic effect in DM wound healing, suggesting that multi-therapy approach may be more beneficial than single treatments.

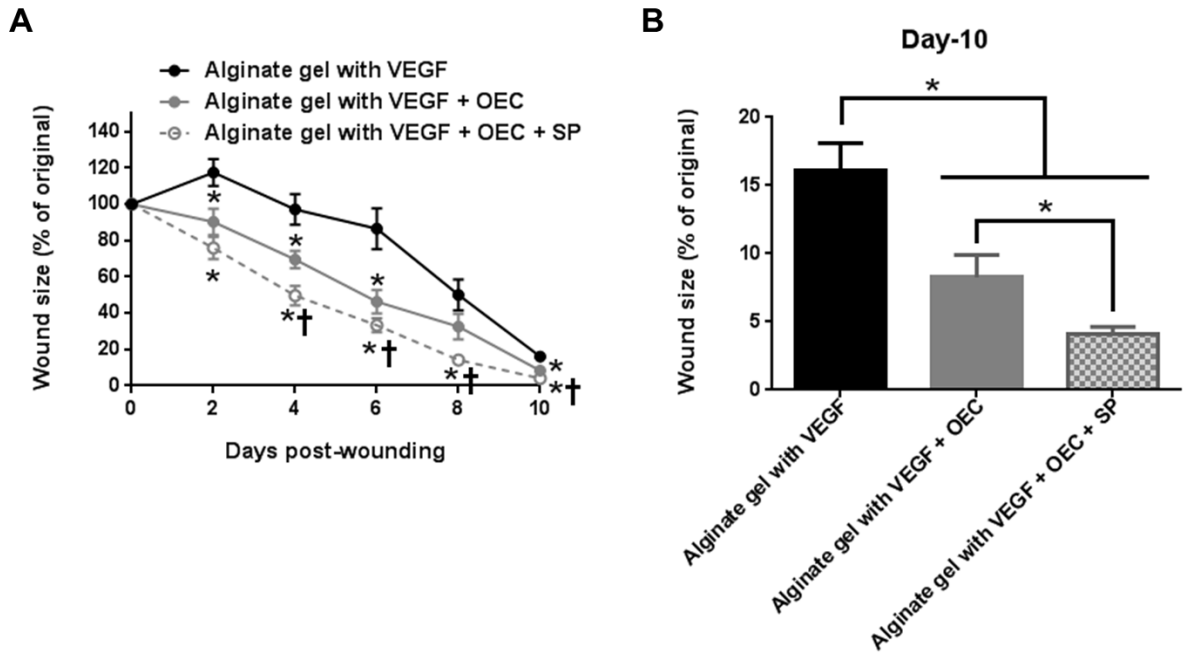


Figure 5.3. Topical treatment with alginate gels encapsulating VEGF and OEC improves wound healing in DM mice. The combination of VEGF and OEC with SP in the alginate gels further improved healing. (A) Wound healing progress of DM wounds treated with (i) alginate gel with VEGF, (ii) alginate gel with VEGF+OEC, or (iii) alginate gel with VEGF+OEC+SP over 10 days. **(B)** Day-10 wound size of DM wounds treated with (i) alginate gel with VEGF, (ii) alginate gel with VEGF+OEC, or (iii) alginate gel with VEGF+OEC+SP. Data represent mean ± SEM. * $p < 0.05$, compared to alginate gel with VEGF. †$p<0.05$, compared to alginate gel with VEGF+OEC.

5.4.4. Sub-therapeutic doses of VEGF are needed to assure OEC function in vivo

We evaluated the effect of (i) alginate gel only (without VEGF) and (ii) alginate gel with OEC (without VEGF) in our model of DM wound healing. No differences were observed in terms of healing kinetics over the studied 10-day period between the wounds that received

different treatment (**Figure 5.4 A, B**). Taken together, these results suggest that sub-therapeutic doses (3 µg) of VEGF have to be incorporated in the alginate gel encapsulating the OEC so they can exert their beneficial effect in wound healing.

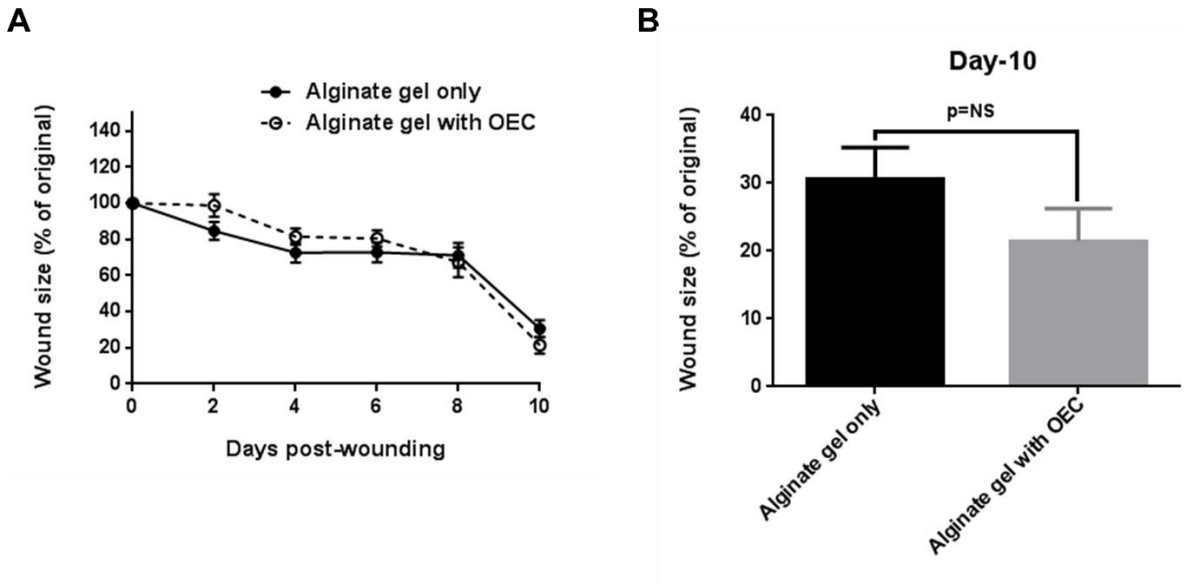


Figure 5.3. Topical treatment with alginate gels encapsulating OEC without VEGF does not affect wound healing in DM mice. (A) Wound healing progress of DM wounds treated with (i) alginate gel only (without VEGF) and (ii) alginate gel with OEC (without VEGF). **(B)** Day-10 wound size of DM wounds treated with (i) alginate gel only (without VEGF) and (ii) alginate gel with OEC (without VEGF). Data represent mean ± SEM.

5.4.5. DNA-based hydrogels are suitable material for drug and/or cell delivery in our mouse model of wound healing

We evaluated the effect of DNA gels in wound healing using both non-DM and DM mice. As expected, and according to our previous studies, DM mice showed impaired healing when compared to non-DM (**Figure 5.5. A, B**). Injection of DNA gel in the tissue surrounding the wounds trended to reduce wound size in both non-DM and DM mice, but failed to reach statistical significance (**Figure 5.5. A, B**).

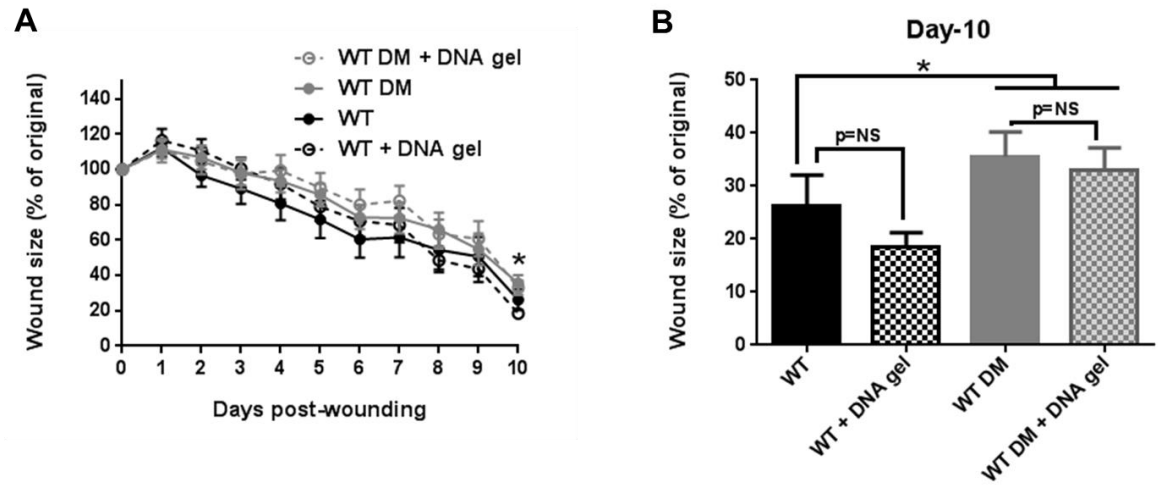


Figure 5.5. Topical treatment with DNA gels did not significantly affect wound healing in DM mice. (A) Wound healing progress of non-DM and DM wounds, untreated or treated with DNA gels **(B)** Day-10 wound size of non-DM and DM wounds, untreated or treated with DNA gels. Data represent mean ± SEM. * $p < 0.05$, compared to WT.

There were no signs of immune reaction to the hydrogel in the mouse wounds. In addition, histological analysis showed no difference between untreated and DNA gel treated wounds in terms of extent and intensity of inflammatory cell infiltrate (**Figure 5.6. A-C**), indicating that the DNA-based material did not induce an immune or inflammatory reaction. These preliminary findings suggest that DNA-based gels are a suitable material to serve as scaffold for the release of specific cells, neuropeptides, growth factors or other molecules to the wound area as a therapeutic approach.

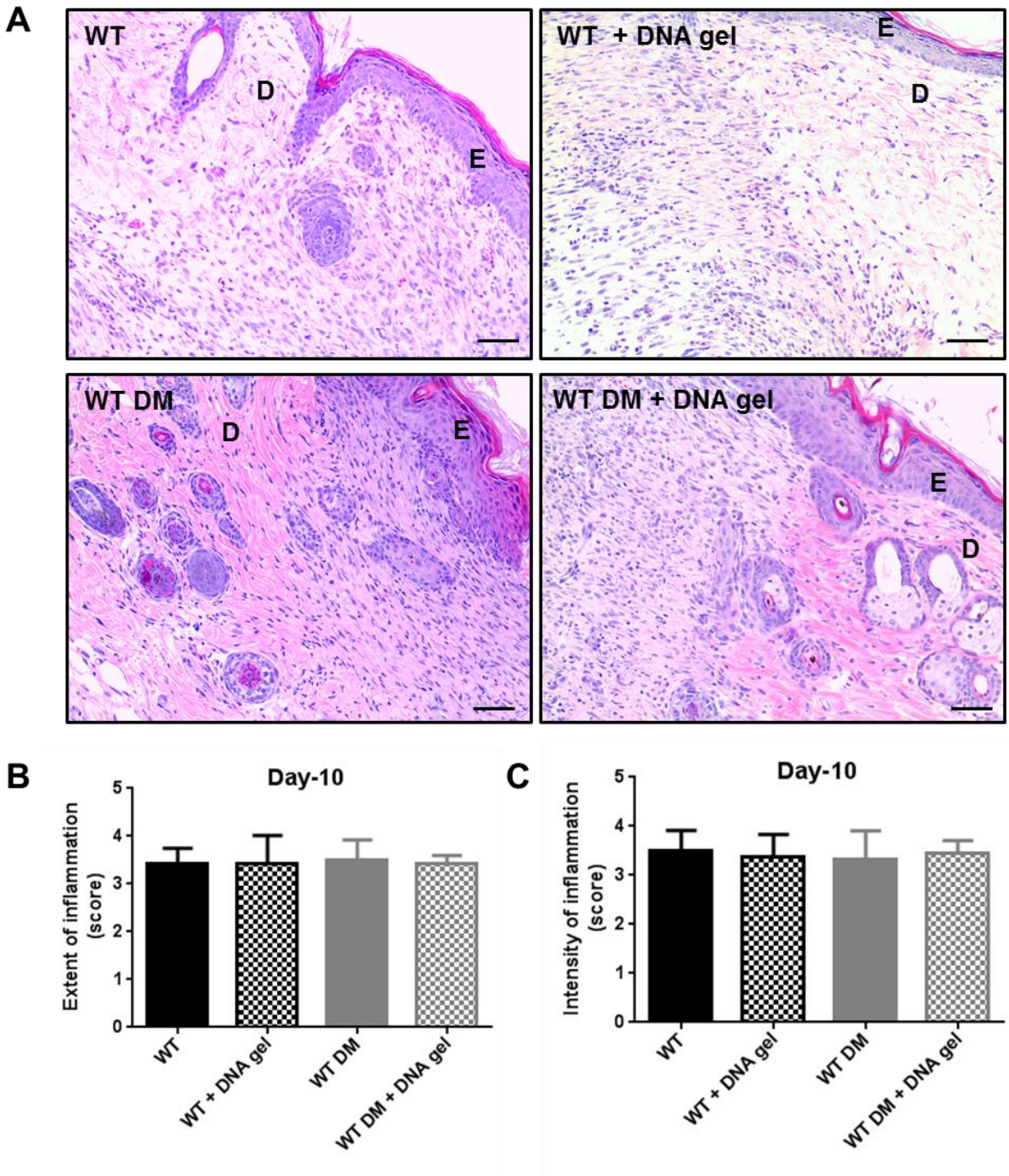


Figure 5.6. DNA gels did not increase wound inflammation in both non-DM and DM mouse models. (A) Representative H&E images of the peri-wound skin of (i) WT non-DM untreated, (ii) WT non-DM DNA gel treated, (iii) WT DM untreated, and (iv) WT DM DNA gel treated wounds. E – epidermis; D – dermis. Scale bar: 100 µm. No differences were observed in terms of extent **(B)** and intensity **(C)** of inflammatory cell infiltrate between the different groups. Data represent mean ± SEM.

5.5. Discussion

Wound healing is a complex physiological process that is severely impaired in DM. The current consensus is that there are multiple mechanisms involved in DM-associated impaired wound healing, including neuropathy and associated neuropeptide-deficiency, chronic inflammation, reduced intracellular signaling of growth factors, and impaired neovascularization. Therefore, the development of new products that can affect all the above mechanisms would be highly beneficial for DFU. In addition, it is well recognized that the cells and molecules that participate in wound repair interact with and influence each other, and that many effectors act synergistically to promote healing. Moreover, the hyperglycemic and proteolytic environments that characterize the DM wound impair the viability and function of cells, and reduce the bioavailabiltity of active molecules. Hence, novel delivery systems providing protection and controlled release of multiple factors are far more promising than a single bolus administration for DFU treatment.

In this study we tested new biomaterials as potential delivery systems for DM wound therapeutics. We used injectable alginate hydrogels to incorporate and release a combination of neuropeptides – substance P (SP) and neurotensin (NT) that have shown to improve DM-impaired healing when individually applied to the wounds[342, 343, 446]. We also used the same macroporous alginate polymer scaffolds to encapsulate and deliver to the wounds endothelial precursor cells, either in single therapy, or in combination with SP. Finally, we applied a new DNA nanoparticle system into both non-DM and DM mouse wounds to evaluate its potential adverse or beneficial effects in wound healing.

Our main finding is that the combination of multiple effectors, namely endothelial precursor cells and neuropeptides, is more advantageous than single treatments for DM-wound healing. In addition, we show that outgrowth endothelial cells (OEC) require the presence of minimal doses of VEGF to have a beneficial effect in wound healing when administrated via alginate hydrogels. This is in accordance with a previous study from our collaborators that reports that the inclusion of low doses of VEGF increased cell migration from the gels[447].

Since we incorporated in the gel sub-therapeutic doses of VEGF – 3 µg instead of the 10 µg or 20 µg commonly used for wound healing studies[130, 459] – and there was a significant improvement in the wounds treated with endothelial precursor cells + VEGF when compared to the wounds that received VEGF only, we are confident that the beneficial effect on wound healing is mediated by the cells and not by the low dose of VEGF. We also show that the alginate gels provide sustained release of the neuropeptides that have been previously validated for DM wound healing in our mouse model[342, 343, 446] and that peri-wound injection of the alginate hydrogels improves healing. Finally, we demonstrate safety and biocompatibility of DNA-based hydrogels, which are able to incorporate active molecules[460, 461], in our mouse model of wound healing, suggesting that this new biomaterial could serve as a promising delivery system for wound therapy.

Taken together, our findings indicate that the new biomaterials tested prove to be safe and efficacious for wound healing in our mouse models and suggest that they could serve as novel delivery systems for wound therapeutics. Although these data are only preliminary, we believe that they are promising and could originate novel bioproducts for DFU treatment. Further investigation is needed to optimize the combination of biomaterials and effectors, evaluate the safety and efficacy of the products in experimental models of DM wound healing, and, in case of positive outcomes, explore their translational capabilities.

Chapter VI

Concluding remarks

6. Chapter VI - Concluding Remarks

Diabetic foot ulceration (DFU) is a serious and debilitating clinical complication of Diabetes *Mellitus* (DM) that is associated with high morbidity and mortality[98, 462-464]. In addition, DFU contributes substantially to the toll DM takes on the health care system[94, 465-467]. As DM has been recognized as an 'epidemic' for more than two decades[73], and its prevalence worldwide continues to rise[75], it is expected that the impact of DFU in health and economy will strengthen. Despite the severity of the problem, DFU remains an unmet clinical challenge. Therefore, understanding better the mechanisms that contribute to wound healing impairment in DM is of major importance to the design of new therapies.

Diabetic peripheral neuropathy (DPN), peripheral vascular disease (PVD) and reduced resistance to infection have been identified as major risk factors for the development of DFU[352, 353], while systemic chronic inflammation[354], impaired neovascularization[15], increased local levels of MMP-9[109], and abnormal skin expression of growth factors[354] are important contributors to DFU failure to heal.

Results reported in Chapter II of this thesis demonstrate that, in addition to the systemic inflammation that occurs in DM, there is local chronic inflammation at the skin level. Moreover, there is increased blood vessel density and increased numbers of proliferating endothelial cells (EC) in the skin of DM subjects, but this does not translate to enhanced blood flow. This evidence suggests that despite the increased EC proliferation and blood vessel formation in DM skin, the resulting blood vessels are not functional. Furthermore, the changes in inflammation and blood vessel density observed in human DM skin were also present in several animal models of alloxan- or streptozotocin (STZ)-induced DM, suggesting that these are valid experimental models to study skin changes in DM. Accordingly, the findings in Chapters III, IV and V show impaired cutaneous wound healing in STZ-DM mouse models when compared to their non-DM controls.

It has long been recognized that DPN is associated with a higher risk for DFU development and failure to heal. Initially it was thought that DPN contributed to DFU mainly by causing pain insensitivity, thereby leading to continuous trauma to the injured foot[468]. However, more recent studies have suggested that the DPN-induced neuropetide-deficiency has a role in DM-impaired healing[149, 173]. One of the most studied neuropeptides, Substance P (SP), has been shown to promote wound healing in non-DM and DM corneal wounds that mainly involve epithelial cells[171, 385], and in non-DM cutaneous wounds[172, 386, 469]. However, there is little information regarding the effects of SP in DM skin wounds, and the exact mechanisms of SP actions in wound healing are still not fully understood.

As reported in Chapter III, SP ameliorates non-DM and DM cutaneous wound healing mainly by promoting the early acute inflammatory response and enabling the progression to the proliferative phase of healing. This seems to be achieved through regulation of: (i) skin expression of pro-inflammatory cytokines that are involved in wound repair, such as IL-6 and IL-8, (ii) dermal macrophage activation phenotype, and (iii) skin expression of MMP-9, during wound healing progression. In addition, SP-deficiency was found to be associated with the chronic pro-inflammatory state and the failure to mount an adequate acute inflammatory response to injury that occurs in DM. Moreover, reduced SP circulating levels and skin gene expression were observed in human DM, whereas skin expression of neutral endopeptidase (NEP), which degrades SP, was increased in DM patients. These findings indicate that reduced SP and increased NEP expression are major contributors for SP-deficiency in DM skin and suggest that the development of systems that offer protection and controlled delivery of SP could serve as potential new therapeutic products for DFU.

SP is also known to be a trigger for mast cells (MC)[160, 161, 233], while MC have been recognized to interact with nerve endings[27] and participate in wound repair[252-256, 267, 408]. However, there is currently very little information on the role of MC in DM-impaired wound healing.

Chapter IV describes increased skin MC degranulation in both human and experimental DM. Moreover, in non-DM mice, skin MC degranulation occurred in response to tissue injury, whereas in DM mice no further degranulation was observed post-wounding. This chronic MC degranulation present in DM skin was found to be associated with increased inflammation and impaired healing. Conversely, MC stabilization in DM mice improved wound healing and reduced the exacerbated local inflammatory reaction at the later stages of healing by promoting M2 macrophage polarization and reducing MMP-9 levels at the wound site. Interestingly, both non-DM and DM mice that lack MC showed markedly impaired wound healing. Also, in Chapter IV, the results obtained in Chapter III were extended and confirmed in a different mouse strain. Thus, SP accelerated wound healing in both non-DM and DM wild-type (WT) mice, but failed to have an effect in the MC-deficient mouse models. Furthermore, tachykinin 1 *knock out* (TAC1KO) mice that lack SP and other tachykinins, had increased pre-wounding skin MC degranulation. These results suggest that SP prevents MC pre-wounding degranulation and exerts its beneficial effects in wound healing, at least partially, through MC. Taken together, the findings presented in Chapter IV indicate that the presence of non-degranulated MC in unwounded skin and a timely post-wounding degranulation are required for optimal wound healing, while DM-induced chronic MC degranulation has similar effects to MC absence, and therefore suggest that the use of MC stabilizers may prove useful in the development of new treatments for DFU.

The complex nature of the wound healing process and the diversity of cellular and molecular mechanisms that lead to DFU pathophysiology have hampered the success of several products tested for DFU treatment. Therefore, the development of new products that can combine effector cells and/or bioactive molecules to act synergistically in the multiple pathways that are altered in DM is likely to be more beneficial than single therapy in DFU management.

The results reported in Chapter V show that macroporous alginate polymer scaffolds allow encapsulation and controlled delivery of cells and/or molecules that have previously shown

to promote healing in DM mouse models. In addition, both the alginate scaffolds and a DNA-based gel proved to be biocompatible and allowed direct injection into the peri-wound skin, without significant adverse effects. Moreover, the combination of endothelial cell (EC) precursors with SP in the alginate gels offered better healing outcomes than single treatment with EC progenitors, suggesting that these effectors have an additive or even synergistic effect in wound healing. In summary, although preliminary, the findings presented in Chapter IV suggest that the use of these new biomaterials to deliver previously validated effectors is a promising strategy to improve DM wound healing.

In summary, the results presented in this thesis indicate that DM-induced systemic inflammation, in combination with neuropathy-associated SP deficiency lead to a chronic pro-inflammatory environment at the skin level, which is characterized by increased baseline (pre-wounding) skin (i) inflammation, (ii) blood vessel density, and (iii) MC degranulation. This in turn causes an inability to mount an acute inflammatory response in the early stages of healing and a failure to resolve inflammation at the later stages, compromising the progression to the proliferative and remodeling phases, and culminating in the development of a chronic, non-healing wound (**Figure 6**). Interestingly, the results presented in this thesis show that there is and increased rather than reduced number of immune and inflammatory cells, as well as increased rather than reduced EC and blood vessel density in DM skin, suggesting that the problem resides in the function and not in the abundance of cells. These findings not only provide new mechanistic insights into the pathophysiology of DFU but also open avenues for novel therapeutic approaches for DFU. In fact, the use of new biomaterials that offer controlled release of cells and bioactive molecules as potential candidates for next-generation DFU therapies has also been discussed in this thesis (Chapter V).

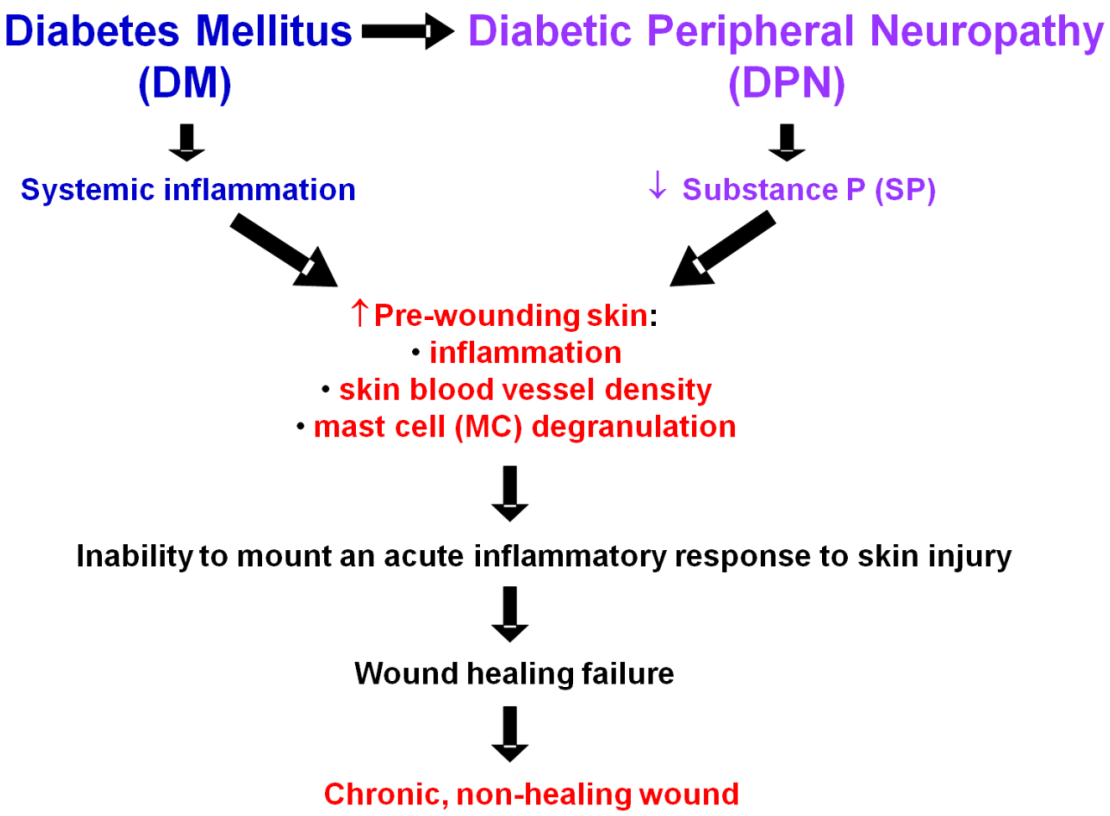


Figure 6. Proposed mechanisms for DM-impaired wound healing. DM-induced pro-inflammatory environment, in combination with DPN-associated SP deficiency lead to chronic inflammation at the skin level, as depicted by increased baseline skin inflammation, blood vessel density and MC degranulation. This chronic inflammation hampers the acute inflammatory response to skin injury, culminating in healing failure and development of a chronic wound.

In future studies, it would be important to identify the most effective combination of the previously validated effectors to promote DM wound healing, and to develop and test the final product. In addition, the development of biomaterial formulations as a bandage- or dressing-type gel for topical application to the wound would have great advantages over the injectable products. Moreover, it would be interesting to explore the effects of SP analogues and of other molecules that are capable of preventing MC degranulation. For instance, SP analogues could be modified to be more resistant to the action of NEP, while other MC stabilizers, such as plant-derived flavonoids[436, 437], or new orai/CRAC channel blockers[438]

may prove more efficient that cromolyn in inhibiting MC degranulation and improving DM wound healing.

Chapter VII

References

7. Chapter VII - References

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