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Effects of peripheral blood mononuclear cells, alone or in combination with acellular dermal matrices, in diabetic patients with non healing chronic wounds

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SUMMARY:

1. INTRODUCTION

Diabetes mellitus (DM) has a significant global impact, costing more than 760 billion dollars annually, approximately 10% of adult health care costs. Currently, 500 million people have diabetes mellitus, and this number is predicted to rise significantly in the coming years. In fact, diabetes is projected to affect 783.2 million people globally by 2045 (representing 7.8% of the global population) [1]. In 2019 alone, complications due to diabetes caused over 4 million deaths. These data clearly show that diabetes and its associated conditions are serious global health issues [2]. Diabetes patients frequently have poorly healing wounds, persistent infections and inflammation, and slower epithelial regeneration.
Approximately 15% of people with T2DM experience ulcers on the lower extremities, known as diabetic foot ulcers (DFUs), with an alarming recurrence rate (40% within one year and 65% within five years) and no effective methods exist to predict its incidence [3,4]. Moreover, DFUs precede 84% of all lower limb amputations related to diabetes. This underscores the urgent need to better understand the underlying pathological mechanisms that lead to ulcer formation and impaired wound repair in diabetic patients.

Wound healing is a natural physiological reaction to tissue damage. It depends on complex interactions among different cell types and a network of soluble signaling molecules, including cytokines, chemokines, growth factors, and metabolites. The healing process progresses through four coordinated and partly overlapping phases: haemostasis, inflammation, proliferation (re-epithelialization), and remodelling (scar maturation). Macrophages and fibroblasts are among the many cell types involved in wound repair [5].

Macrophages, a type of immune cell, are the first to eliminate debris and pathogens from an injury site. Their released cytokines and growth factors recruit and activate fibroblasts, which are crucial for tissue healing and scar formation. Fibroblasts produce extracellular matrix components, which are essential for structural support and wound closure. Macrophages change from pro-inflammatory (M1) to healing-promoting (M2) phenotypes based on the wound environment [5]. M2 macrophages produce anti-inflammatory cytokines and growth factors, which drive fibroblasts for collagen deposition, angiogenesis, and wound contraction. Macrophage dysfunction is linked to poor wound healing in diabetes [6]. During the inflammatory phase of wound healing, macrophages become proinflammatory/M1 and secrete

cytokines like IL1β, IL6, IL10, IL12, IFNɣ, and TNFα [5]. These cytokines promote inflammation and the clearance of dead tissue and cell debris. During the proliferative phase of wound healing, anti-inflammatory/M2 macrophages dominate and promote cell proliferation, repair, and tissue remodelling [6]. Diabetes is thought to cause aberrant macrophages due to epigenetic alterations and decreased interaction with other cell types, including neutrophils, fibroblasts, endothelial cells, and lymphocytes [7-13].

Notably, chronic hyperglycemia in diabetes contributes to multiple systemic complications and drives several local disturbances within the wound microenvironment. These include chronic inflammation, pathological angiogenesis, oxidative stress caused by hypoxia, neuropathy, the formation of advanced glycation end-products, and impaired neuropeptide signaling [4].

In this work, we examine the role of peripheral blood mononuclear cells, alone or in combination with acellular dermal matrices, as therapeutic strategies for diabetic non-healing chronic wounds.

2. THE HEALING PROCESS

Wound healing is a complex and precise biological process that involves various cell types and mediators. The process consists of four stages: haemostasis (immediately after damage), inflammation (1-4 days), proliferation (4-21 days), and remodeling (21 days to 2 years) [14,15]. Diabetes patients have slower wound healing than healthy people. Diabetics suffer with wound healing due to the delayed start and control of inflammation. Macrophages cause and resolve wound inflammation.

The conventional macrophage polarization concept identifies two distinct phenotypes: "classically" activated M1 macrophages generated by lipopolysaccharide or interferon-γ, and "alternatively" activated M2 macrophages triggered by IL-13 or IL-4 [14]. M1 macrophages have pro-inflammatory, anti-infection, and anti-tumor immunological activities, which can cause inflammation and autoimmune disorders. M2 macrophages have anti-inflammatory and tissue repair activities, but may promote tumor growth. Macrophage activation is thought to occur on a continuous spectrum, with M1 and M2 being the two extremes. Macrophage polarization is dynamically reversible, allowing M1 macrophages to convert to M2 macrophages in response to changing microenvironmental conditions [15].

Macrophages play a crucial role in wound healing, especially in the inflammatory and proliferative phases. During wound inflammation, pro-inflammatory chemicals attract macrophages to the afflicted area, where they change into the M1 type and consume necrotic tissue and apoptotic neutrophils. In the proliferative phase, damaged macrophages primarily polarize towards the M2 type, which promotes healing through inflammation suppression, angiogenesis, and re-epithelialization. M1 macrophages' phagocytic activity is crucial for the phenotypic change of damaged macrophages [14,15].

Macrophages activated in high-glucose settings are more likely to become senescent, complicating the M1-to-M2 transition. Proper balance of M1 and M2 macrophage polarization is crucial for clearing necrotic tissue, resolving inflammation, and repairing wounds. To speed up wound healing, medicines or immunosuppressants can polarize macrophages towards a pro-repair subtype or recruit subpopulations beforehand.

2.1 Haemostasis

Immediately following tissue injury, mast cell degranulation improves capillary permeability and promotes vasodilation, which enhances bleeding and allows immune cells to move through the wound site.

Meanwhile, the coagulation cascade begins, resulting in the development of a provisional scab composed of temporary matrix components [16]. Simultaneously, activated keratinocytes, fibroblasts, and platelets release various soluble signaling molecules, including: (a) growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF); (b) chemokines like IL-8 (CXCL8) and CXCL2; (c) danger-associated molecular patterns (DAMPs) including histones, genomic DNA, adenosine 5′-triphosphate (ATP), and high mobility group box 1 (HMGB1); and (d) cytokines such as thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 [14,15,17,18]. All of these inflammatory mediators work as danger signals that recruit circulating immune cells, initiate local inflammatory responses, and subsequently stimulate the proliferative activity of resident tissue cells.

2.2 Inflammation

A strong inflammatory response, initiated during the haemostasis phase, begins to clear the wound of debris, damaged tissue, and microorganisms. This inflammatory stage is marked by: (a) the recruitment of immune cells such as neutrophils, monocytes/macrophages, mast cells, and T cells; (b) the accumulation of inflammatory mediators including cytokines, chemokines, and lipid-derived signals; and (c) the secretion of extracellular matrix–degrading enzymes, such as matrix metalloproteinases (MMPs) and collagenases, which collectively contribute to swelling, heat, and pain [19].

It is well-established that an appropriate and carefully regulated inflammatory response is essential for starting the proliferative and remodelling phases of tissue repair. [20,21]. On the other hand, the uncontrolled or excessively prolonged inflammation, characteristic of diabetic wounds, disrupts these subsequent phases and is strongly implicated in the development of ulceration [22,23].

Neutrophils play a central role in this process. Although absent in intact skin, their recruitment to injured tissue is orchestrated by resident T cells, mast cells, and macrophages [24].

Neutrophils provide key proteolytic enzymes (such as elastase, cathepsin G, and urokinase-type plasminogen activator) that facilitate re-epithelialization [25–27], along with reactive oxygen and nitrogen species, cytokines (IL-1β, TNF, IL-6, IL-12p40, TGF-β), and chemokines (CCL2, CCL3, CCL5, CXCL1, CXCL2) [28]. Elevated neutrophil numbers—and an increased neutrophil-to-lymphocyte ratio—are recognized biomarkers of impaired healing in individuals with T2DM [29].

Furthermore, T2DM promotes increased neutrophil extracellular trap formation (NETosis), a mechanism associated with delayed repair; blocking NETosis has been demonstrated to accelerate wound closure [22,30]. Persistent neutrophil activation and NETosis further amplify inflammation through the release of mitochondrial DNA and histone H4 [31], rather than progressing toward normal resolution via macrophage-mediated clearance of apoptotic neutrophils [21]. This clearance normally promotes self-limiting inflammation and shifts eicosanoid production from pro-inflammatory to pro-resolving mediators [32–34]. In diabetic wounds, however, dysregulation of neutrophil recruitment and activity delays inflammation [22,31,35].

Interestingly, many of these inflammatory abnormalities appear to be caused by epigenetic reprogramming of innate immune cells prior to their entry into the wound, rather than by excessive local glucose concentrations. This reprogramming is caused by systemic T2DM-associated factors such as chronic hyperglycemia, which shifts progenitor cells toward pro-inflammatory activity [36]. This transition is further supported by broad systemic inflammation in diabetes individuals and animal models [37]. Nonetheless, the specific epigenetic processes behind neutrophil and monocyte/macrophage dysfunction in diabetes are not fully characterized and remain incompletely understood.

Chemokines produced by mast cells, keratinocytes, and fibroblasts, such as MCP-1, control monocyte migration to injured skin using CCR2 and CCR5 [38]. Monocytes and macrophages are crucial regulators of inflammation, proliferation, and remodelling due to their different functional capacities. [39]. In healthy wounds, macrophages transition between pro-inflammatory M1 and pro-repair M2 phenotypes. In contrast, macrophages in T2DM are strongly shifted into M1 state [36]. M1 macrophages exhibit high phagocytic activity and secrete abundant pro-inflammatory cytokines—including IL-1β, IL-1α, IL-6, TNF, and IL-12—which intensify inflammation, raise neutrophil recruitment, and sustain the low-grade

inflammatory environment typical of chronic DFUs [36,40]. As a consequence, the reduced number of M2 macrophages and the increased M1:M2 ratio result in reduced production of growth factors (PDGF, FGF, VEGF) and anti-inflammatory cytokines (IL-10, TGF-β, TGF-α), all of which are crucial for initiating proliferation and resolving inflammation [23,41].

Skin-resident T cells are likewise essential for modulating local inflammation during healing.

In summary, the extended inflammatory phase characteristic of diabetic wounds—and the resulting defects in wound closure and tissue remodeling—arises not only from elevated local pro-inflammatory signals but also from deficiencies in anti-inflammatory mediators produced by regulatory cell types such as M2 macrophages. Despite growing insight into these processes, the mechanisms governing their dysregulation require further investigation.

2.3. Proliferation

After the inflammatory stage subsides, the proliferative phase begins, marked by the development of granulation tissue. This tissue is composed of fibroblasts, immune cells, and newly formed capillaries, which enable epithelial cells to migrate across the wound surface during re-epithelialization [36]. As described earlier, fibroblasts, keratinocytes, mast cells, and M2 macrophages apply strong regenerative effects by secreting cytokines (e.g., IL-10, IL-35), growth factors (e.g., TGF-β, TGF-α, FGF, EGF), chemotactic molecules that recruit stem cells (e.g., CXCL8, SDF-1), and by coordinating extracellular matrix (ECM) remodeling through MMPs and TIMPs [23,33,39–42,43–45].

Chronic and slow-healing wounds—including DFUs—show reduced proliferation of skin-resident cells as well as impaired activation of stem cells. While this is partly driven by the prolonged inflammatory phase, additional factors associated with T2DM, such as protein glycation, impaired angiogenesis, and resulting oxidative stress, extend the proliferative phase unnecessarily, sometimes preventing wound closure entirely [36]. Alarmingly, T2DM patients often exhibit dysfunctional and reduced populations of circulating stem cells, including endothelial progenitors [46,47], which stands in contrast to normal wound healing, where healthy neovascularization is a defining component of the proliferative phase [19]. The diminished vessel-forming capacity of endothelial progenitors, combined with a lack of stem cells, significantly compromises re-epithelialization in diabetic wounds [48,49]. In response, stem cell–based interventions—particularly those involving mesenchymal stem cells

(MSCs)—have emerged as promising therapeutic options for chronic wounds such as DFUs. These therapies and other novel treatment strategies will be addressed in the following sections.

Successful wound closure requires dermal reconstruction before basal keratinocytes can complete epithelial coverage [44]. This involves rebuilding the dermal collagen matrix, the structural scaffold that supports subsequent cell migration and organization. Fibroblasts and myofibroblasts are key drivers of this process [50], aided by macrophages, mast cells, and lymphocytes through VEGF- and TGF-β-dependent pathways [39–41]. Neovascularization is equally essential, supplying oxygen and nutrients to support tissue repair [51]. M2 macrophages further promote angiogenesis via direct interactions with endothelial cells and through paracrine signaling [52]. However, in diabetic wounds, monocyte polarization toward the M2 phenotype is impaired, while pro-inflammatory polarization is enhanced. Likewise, fibroblasts exposed to T2DM conditions show diminished activation, reduced collagen synthesis, and weakened paracrine signaling, including decreased TGF-β pathway activity [53].

The proliferative phase is arguably the most critical determinant of successful wound repair, as it relies on the coordinated progression of angiogenesis and epidermal resurfacing. When dysregulated, this phase becomes inefficient or stalls altogether, resulting in chronic, non-healing ulcers. Although the complex network of cells and signaling pathways makes this process difficult to fully understand, several key contributors to T2DM-associated wound impairment have been identified, including fibroblast dysfunction, altered macrophage polarization, dysregulated growth factor activity, and persistent inflammatory stimuli left over from the unresolved inflammatory phase.

2.4 Remodelling

During the fourth and final stage of wound repair—the remodeling phase—granulation tissue is reinforced through the accumulation and organization of extracellular matrix (ECM) proteins, finally forming scar tissue [50]. The major goal of this phase is to restore normal skin integrity by reducing cellular and vascular content and increasing collagen concentration. As in the proliferative phase, fibroblasts, myofibroblasts, and M2 macrophages are essential contributors to this stage of healing [44,50, 54].

Collagens make up approximately 85% of the dermis and undergo continuous restructuring during healing, determining the final characteristics of the resulting scar [55]. In normal wound repair, collagen III is gradually degraded and replaced with collagen I, a process regulated largely by TGF-β and FGF signaling pathways [56]. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) also regulate the turnover and deposition of ECM components, thereby guiding scar architecture [57,58]. Through these mechanisms, fibroblasts and macrophages remodel collagen fibers so they become thicker, denser, and more interwoven—improving tensile strength and overall scar quality in healthy wounds [59].

Throughout remodeling, the ECM remains highly dynamic, evolving toward a mature and organized structure. Its composition is crucial for skin regeneration, as provisional ECM proteins such as fibronectin and vitronectin interact with diverse cell populations and modulate their behaviour [44]. Notably, therapeutic delivery of ECM components from decellularized skin has been shown to enhance wound healing, making ECM-based biomaterials an appealing treatment strategy [60-64].

In contrast, wounds associated with T2DM show significant structural and functional abnormalities. Impaired angiogenesis leads to persistent hypoxia and oxidative stress within the wound microenvironment [36]. Recent studies show that oxidative stress in diabetic wounds promotes macrophage polarization toward the pro-inflammatory M1 phenotype [36]. Elevated M1 macrophage numbers sustain inflammation and alter expression patterns of MMPs and TIMPs, disrupting ECM remodeling throughout the late proliferative and remodeling phases [44,65]. Strategies aimed at shifting macrophages toward a pro-regenerative M2 phenotype—by enhancing angiogenesis or reducing oxidative stress—have shown promise in restoring more normal healing trajectories [36].

Fibroblast dysfunction is also a major contributor to impaired remodeling. In a 3D in vitro system, fibroblasts derived from DFUs produced ECM structures that were approximately half as thick as those generated by fibroblasts from healthy skin [66]. These thinner matrices contained disproportionately high levels of collagen I and fibronectin [66]. Notably, topical application of fibronectin has been shown to improve DFU wound healing by promoting angiogenesis and reducing inflammatory cytokine production, apoptosis, and oxidative stress [67]. Together, these findings suggest that enhancing or correcting fibroblast function—potentially through ECM-based treatments—may help compensate for the deficiencies observed in DFU fibroblasts.

3. DIABETIC WOUNDS

Physiological wound healing occurs in four regulated phases. The initial haemostasis phase involves vasoconstriction, platelet aggregation, and activation of coagulation pathways. This is followed by the inflammatory phase, during which immune cells infiltrate the wound and release inflammatory mediators; macrophages secrete matrix metalloproteinase-9 (MMP-9), while neutrophils generate neutrophil extracellular traps (NETs). In the proliferative phase, inflammation decreases and skin cells, as well as keratinocytes producing epidermal growth factor (EGF), proliferate and move to the wound site. Finally, the remodeling phase is characterized by extracellular matrix deposition, tissue reorganization, and neovascularization mediated by fibroblasts releasing fibroblast growth factors (FGFs) and endothelial cells producing vascular endothelial growth factor (VEGF).

In diabetic wounds, factors as ischemia, hypoxia, and a hyperglycemic microenvironment disrupt these coordinated healing stages, leading to delayed or failed wound repair and increased the risk of complications. Although the mechanisms underlying DFU development are multifactorial and complex, current treatment options remain limited in their ability to promote effective healing. Consequently, there is a critical need for novel therapeutic strategies that are both cost-effective and capable of restoring normal wound healing. A more comprehensive understanding of the molecular and regulatory mechanisms governing DFU repair is therefore essential and warrants further investigation.

3.1. Hyperglycemia

In patients with diabetes mellitus, chronic hyperglycemia plays a central role in delayed wound closure and the development of diabetic foot ulcers (DFUs) through mechanisms that include atherosclerosis, dysfunction of skin-resident cells, and peripheral neuropathy. Elevated glucose levels promote atherosclerotic changes that limit the delivery of oxygen and nutrients to wounded tissue, thereby impairing repair processes [68].

Hyperglycemia also induces endothelial cell dysfunction, which compromises pressure-induced vasodilation—a protective mechanism essential for skin integrity and wound healing [69]. Beyond vascular effects, excessive glucose disrupts keratinocyte and fibroblast protein synthesis, migration, and proliferation, all of which are required for effective re-epithelialization [70-72]. In DFUs, the expression of key keratinocyte-associated proteins

involved in differentiation and adhesion, including cytoskeletal keratins (K2, K6, K10) and laminin-5 β3 chain precursor (LM-3A32), is altered. Reduced LM-3A32 expression interferes with epithelial cell survival and attachment to the basement membrane, further hindering re-epithelialization [73-78].

Oxidative stress represents another major mechanism of hyperglycemia-induced impairment. Reduced activity of antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase, leads to excessive free radical accumulation. Prolonged hyperglycemia also increases the formation of advanced glycation end products (AGEs) and activates pathways including the polyol, hexosamine, protein kinase C, and AGE signaling cascades, all of which elevate reactive oxygen species (ROS) levels. While ROS are necessary during early wound healing, sustained overproduction damages peripheral nerves and vasculature, resulting in sensory, motor, and autonomic neuropathies that significantly increase DFU risk. Collectively, these hyperglycemia-driven changes make the skin more vulnerable to injury and infection and substantially delay wound repair [73,79-83].

3.2. Neuropathy

Diabetic neuropathy—whether sensory, motor, or autonomic—contributes both to DFU formation and impaired wound healing. Diabetic foot neuropathy is strongly associated with patient age, duration of disease, and the degree of glycemic control. Among the complications of diabetes, damage to the peripheral and autonomic nervous systems is particularly prevalent. Hyperglycemia, elevated levels of advanced glycation end products (AGEs), excessive acylcarnitine accumulation, and oxidized low-density lipoproteins (LDLs) disrupt motor and sensory function by damaging neuronal structure and activity. There are two main ways that AGEs cause neuropathy: they directly affect protein function and, when they bind to AGE receptors (RAGEs), they change intracellular signaling and gene expression. Reactive oxygen species (ROS) and inflammatory mediators are produced more frequently as a result of this interaction, which seriously impairs the release and transport of neurotransmitters. Acetyl-CoA is converted to acylcarnitine when cellular transport systems are overloaded by excess metabolic substrates. Cellular stress, axonal degeneration, and mitochondrial dysfunction in Schwann cells and dorsal root ganglion (DRG) neurons are all brought on by this process, which eventually results in irreversible nerve damage. The expression of important molecules involved in neural plasticity, such as neuromodulin, β-tubulin, heat shock proteins, and poly

(ADP-ribose) polymerase in the DRG, is altered by hyperglycemia, according to experimental studies conducted in rodent models [84-86].

Experimental studies in rodent models have demonstrated that hyperglycemia alters the expression of key molecules involved in neural plasticity, including neuromodulin, β-tubulin, heat shock proteins, and poly (ADP-ribose) polymerase in the DRG. Additional abnormalities, such as altered spliceosome function, changes in motor neuron protein expression, and upregulation of GW bodies involved in mRNA processing, have been identified as critical contributors to diabetic neuropathy.

Moreover, LDLs in diabetic patients are readily oxidized by ROS and subsequently bind to oxidized LDL receptors and RAGE, triggering signaling cascades involving caspase-3 and RNA-mediated pathways. These processes amplify inflammatory responses and ROS accumulation, leading to progressive and irreversible nerve tissue injury. Dysregulation of polyol and inositol metabolism, degradation of Na^+/K^+-ATPase activity, neurovascular insufficiency, impaired neurotrophic support, defective axonal transport, and nonenzymatic glycation of neuronal proteins have also been reported in diabetic nerves. Collectively, these pathological alterations disrupt protein processing, increase oxidative stress, impair mitochondrial function, and ultimately result in the loss of peripheral nerve function.

Autonomic neuropathy reduces sweat gland activity, leading to dry, fissured skin that is prone to itching and infection. Motor neuropathy alters foot biomechanics, increasing plantar pressure and causing localized ischemia and tissue breakdown. Dysfunction of the Achilles tendon reflex is often an early indicator. Sensory loss reduces pain perception, allowing injuries to progress unnoticed. Overall, neuropathic skin displays reduced nerve density and diminished regenerative capacity [87-88].

The combined effects of sensory and motor neuropathy lead to abnormal plantar pressure distribution and altered gait. Over time, repeated pressure induces hyperkeratosis, subcutaneous hematoma formation, and eventual skin breakdown. These lesions, exacerbated by neuropathy and increased mechanical load, often progress into chronic, non-healing ulcers.

3.3. Microvascular Complications

Peripheral arterial disease (PAD) is common in patients with DFUs and is strongly associated with poor healing outcomes and increased amputation risk. Studies report PAD prevalence rates exceeding 40% among DFU patients. Revascularization strategies have demonstrated

significant benefits in eligible individuals by improving perfusion, reducing amputation rates, and lowering mortality.

Among these patients, atherosclerosis is the predominant cause of peripheral vascular pathology. In diabetes, disturbances in carbohydrate, lipid, and protein metabolism, together with elevated reactive oxygen species (ROS) levels and activation of inflammatory mediators, result in endothelial dysfunction. [89-90]

Endothelial injury, abnormal smooth muscle cell function, chronic inflammation, a hypercoagulable state, and platelet dysfunction collectively promote the development of atherosclerosis. Hyperglycemia, insulin resistance, elevated free fatty acids, and the accumulation of advanced glycation end products (AGEs) suppress endothelial nitric oxide synthase (eNOS) activity and enhance oxidative stress. Simultaneously, these metabolic abnormalities activate pro-inflammatory signaling pathways, including nuclear factor-κB (NF-κB) and activator protein-1. Under these inflammatory conditions, leukocytes adhere to the vascular endothelium, migrate into the arterial wall, ingest lipids, and differentiate into foam cells, thereby driving atherosclerotic plaque formation. As macrophages and low-density lipoproteins (LDLs) accumulate within the arterial intima to form foam cells, VSMCs migrate toward these lipid-rich regions, where they contribute to ECM synthesis and deposition, further accelerating plaque development. In addition, impaired endogenous platelet inhibition in diabetic patients facilitates platelet activation. Activated endothelial cells release excessive adhesion molecules and platelet agonists, which promote leukocyte recruitment and activation. This platelet–leukocyte interaction enhances plaque progression and instability, increasing the risk of plaque rupture, thrombosis, and vascular occlusion [89-90].

Due to impaired circulation, DFUs exist in a chronically hypoxic environment. Hypoxia alters gene expression across different skin cell populations, with endothelial cells and macrophages upregulating angiogenic and inflammatory pathways, while keratinocytes and fibroblasts primarily exhibit metabolic adaptations. Clinical studies demonstrate that lower tissue oxygenation correlates with worse healing outcomes. In fact hyperbaric oxygen therapy is widely used to counteract hypoxia in DFUs, although its precise molecular mechanisms remain under investigation [91-92].

Anaemia is frequently observed in patients with diabetes, particularly those with DFUs. While several studies associate anaemia severity with ulcer depth, infection, amputation risk, and mortality, other reports fail to demonstrate a significant prognostic relationship. Consequently,

the role of anaemia as a predictor of DFU healing remains controversial and warrants further study [93-98].

Notably, the distribution of atherosclerotic disease in the lower extremities differs between diabetic and nondiabetic individuals. In patients with diabetes, distal arteries—particularly the posterior and anterior tibial arteries—are most commonly affected, whereas the femoral and popliteal arteries are less frequently involved, and the iliac arteries are typically spared. As tibial or proximal arterial occlusion progresses, inadequate perfusion of the foot compromises skin integrity, leading to ischemic ulcers or gangrene. Consequently, peripheral vascular disease represents both the initiating cause of diabetic foot ulcers and the principal determinant of amputation and mortality. Atherosclerosis remains the central pathological process underlying peripheral arterial disease, and plaque rupture in the diabetic milieu frequently precipitates thrombosis, arterial occlusion, lower-limb ischemia, and ultimately DFU formation.

3.4. Barrier Disruption and Infection

Diabetic foot infection (DFI) represents a frequent and severe complication in individuals with diabetes. Reduced arterial perfusion to the lower limbs combined with multiple forms of neuropathy substantially increases susceptibility to infection.

3.4.1. Transepidermal Water Loss

The skin barrier depends on intact lipid layers, cell junctions, antimicrobial defences, and enzymatic activity to prevent dehydration and infection. Aging and diabetes share similar barrier defects, including reduced lipid content, impaired hydration, and increased AGEs. Although findings on transepidermal water loss (TEWL) in diabetes are inconsistent, altered sweating patterns and autonomic dysfunction may mask underlying barrier impairment. Reduced skin hydration has been associated with poor microcirculation and inferior healing outcomes, while hyperglycemia further disrupts tight junctions and epidermal structure, increasing susceptibility to infection [99-105].

3.4.2. Antimicrobial Peptides

Antimicrobial peptides (AMPs) are essential for maintaining microbial balance and preventing infection. In healthy skin, fibroblasts can differentiate into adipocytes that produce cathelicidin (LL-37), which supports both antimicrobial defence and wound repair. In DFUs,

cathelicidin production is markedly reduced, and although other AMPs may be upregulated, their levels appear insufficient to control microbial growth. Additionally, common antidiabetic medications can further suppress AMP expression. Novel therapeutic strategies employing AMP delivery via hydrogels, nanoparticles, and nano-polymers have shown promising antimicrobial and wound-healing effects in experimental models [106-112].

3.4.3. Bacterial Diversity

Advanced sequencing technologies have revealed significant microbial dysbiosis in diabetic skin and DFUs. Diabetic wounds commonly harbour Staphylococcus, Streptococcus, Pseudomonas, and Enterobacteriaceae species, with increased bacterial diversity observed in more severe infections.

In addition, fungal pathogens—most notably *Candida albicans*, *Candida parapsilosis*, and *Candida tropicalis*—are frequently identified. Among these, *C. parapsilosis* is the most prevalent yeast isolated from diabetic foot ulcer (DFU) tissue, likely due to its frequent colonization beneath the nails. This complex microbial environment presents significant therapeutic challenges, particularly in cases involving concurrent bacterial and fungal infections. Many of these pathogens secrete proteases that disrupt epidermal integrity, facilitating infection and tissue damage. Biofilm formation further limits antibiotic penetration, prompting interest in localized and sustained drug delivery systems [113-116].

3.4.4. pH and the Microbiome

Although intact diabetic skin generally exhibits a near-normal pH, DFU wounds are significantly more alkaline than acute wounds. Alkaline conditions enhance biofilm formation and alter bacterial antibiotic sensitivity, highlighting the need to consider wound pH when designing antimicrobial therapies [117-118].

3.5. Inflammation and Immune Dysfunction in Chronic Wounds

Persistent DFIs are closely associated with immune dysfunction, including impaired leukocyte activity and dysregulated macrophage-driven inflammation.

Unlike acute wounds, DFUs are characterized by persistent inflammation marked by excessive neutrophil and macrophage infiltration and prolonged secretion of proinflammatory cytokines. Chronic hypoxia, insufficient angiogenesis, and sustained ROS production further

exacerbate tissue damage. Reduced expression of connective tissue growth factors, TGF-β, and collagen impairs fibroblast function and delays wound closure [119-124].

A failure to transition from inflammation to proliferation has been linked to dysregulation of signaling pathways such as p38 MAPK and altered expression of regulatory microRNAs. Impaired angiogenesis has also been associated with reduced levels of the long noncoding RNA MALAT1, whereas restoring MALAT1 expression improves fibroblast activity and wound healing in diabetic models [125-128].

Immune dysfunction in diabetes includes impaired phagocytosis, leukocyte dysfunction, and sustained dominance of proinflammatory M1 macrophages. Abnormal T-cell accumulation and dysregulated cytokine profiles further compromise host defence. Hematologic biomarkers such as elevated platelet-to-lymphocyte and neutrophil-to-lymphocyte ratios have emerged as potential indicators of DFU severity and prognosis [129-142].

4. MONOCYTES

Macrophages and monocytes play a fundamental role in the body's defense systems, tissue repair, and cellular regulation. These cells are essential for healthy wound healing, collagen synthesis, blood vessel creation, and wound closure, according to research using mice devoid of monocytes and macrophages [143,144]. Healing may be delayered or reduced when macrophage phenotypes are out of balance and monocyte/macrophage regulation has been altered [144–148].

Macrophages' phenotypes change during wound healing and are categorized into two types: M1 or “classical,” macrophages, identified by $CD14^{+}CD16^{-}$ expression in humans and $Ly6C^{+}$/high in mice, which are pro-inflammatory, and “non-classical,” M2 macrophages, which regulate inflammation characterized by CD14^low/$^{-}CD16^{+}$ in humans and $Ly6C^{-}$/low in mice [149,150]. Both populations are actively recruited to sites of tissue injury.

Increased wound-associated monocytes/macrophages and a failure to transition from a pro-inflammatory to a pro-healing state have been related with impaired wound healing in diabetic individuals [151, 152]. Additionally, chronic inflammation in diabetic wounds has been linked to a reduction in phagocytic activity. [153,154].

Macrophages and monocytes are central, but they are not the only actors. recent studies confirmed that lymphocytes T, and specifically the subpopulation Regulatory T-cells (Treg), support in the tissue regeneration and healing, with an important role in the angiogenesis and tissue regeneration of diabetic wounds [155].

According to a so-called "immune-centric revolution" or "macrophage centred approach," the impact of the immune system on regenerative therapies has recently been known [156–158].

Olingly et al. [159] demonstrated that circulating non-classical monocytes are directly recruited into wounds, where they localize to a perivascular niche and differentiate into M2 macrophages that support tissue repair. In addition, local administration of the small molecule FTY720, which selectively attracts non-classical monocytes, was shown to enhance post-injury vascular remodeling, further supporting an angiogenic role for peripheral blood monocytes. Blood-derived non-classical monocytes therefore represent a major source of alternatively activated M2 macrophages and play a crucial role in regulating inflammation and promoting regeneration.

Pro-inflammatory monocytes ($CD14^{+}CD16^{-}$ in humans; $Ly6C^{+}$/high in mice) originate primarily from the bone marrow and spleen and increase in circulation following injury,

whereas under steady-state conditions they display limited endothelial adhesion [160]. These inflammatory monocytes have a short lifespan—approximately 20 hours in mice—and their numbers fluctuate depending on continuous recruitment from hematopoietic reservoirs, reaching a peak around 48 hours after injury. In contrast, anti-inflammatory monocytes persist longer in circulation, with a half-life exceeding two days in mice [160].

Anti-inflammatory monocytes adhere to the vascular endothelium through β2 integrins such as LFA-1 and the adhesion molecule L-selectin (CD62L). This interaction allows them to patrol the endothelium even under homeostatic conditions, enabling a rapid response to tissue damage and facilitating vascular repair when required. These observations suggest that, in addition to resident tissue macrophages, a population of functionally "resident" monocytes may also exist [160,161].

An alternative classification system further subdivides human monocytes into three subsets: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{\wedge}dimCD16^{++}$), and non-classical ($CD14^{++}CD16^{+}$) monocytes. In healthy individuals, classical monocytes account for approximately 85% of circulating monocytes, whereas intermediate and non-classical subsets represent about 5% and 10%, respectively [162].

Under inflammatory conditions, classical monocytes preferentially differentiate into M1 macrophages, while non-classical monocytes give rise to M2 macrophages that contribute to tissue repair [163,164].

Although classical inflammatory monocytes are recruited to wounds in greater numbers than non-classical monocytes, accumulating evidence indicates that inflammatory monocytes can transition toward an anti-inflammatory phenotype and subsequently differentiate into M2 macrophages [160,165]. This plasticity was demonstrated by Arnold and colleagues in a skeletal muscle injury model, where initially inflammatory, highly phagocytic monocytes converted into M2 macrophages that promoted myogenesis and muscle fiber growth [166].

Notably, recent clinical observations showed that implantation of autologous peripheral blood mononuclear cells—prepared using a point-of-care selective filtration device—into non-healing diabetic wounds induced macrophage polarization from M1 to M2, resulting in complete wound closure. Inflammatory monocytes expressing the $CD14^{++}CD16^{+}$ phenotype are markedly increased during aging and in chronic inflammatory diseases. Furthermore, circulating $CD16^{++}$ monocytes have been proposed as a potential biomarker for predicting diabetic foot ulcer outcomes, as higher percentages of these cells and elevated MMP-3 levels

were observed in patients with healed wounds compared to those with non-healing lesions [167, 168].

4.1 Definition and overview

The term "monocytes" actually refers to the mononuclear phagocytic system, which is a highly malleable and diverse cell population, that represent around 10% of all peripheral leukocytes.

Monocytes were initially observed in the second part of the 1800s. Ehrlich created a cytological method in 1880 and used nuclear morphology to characterize white blood cells (WBC). In this manner, he categorized polymorphonuclear cells with neutrophilic (neutrophils), acidophilic (eosinophils), or basophilic (basophils) granules [169] and mononuclear leukocytes, which are distinguished by a large kidney-shaped nucleus that he called "Übergangszelle" or "transitional cells" now known as monocytes. It appears that Artur Pappenheim introduced the term "monocytes" in 1910 [170]. The ability of these cells to migrate and differentiate into tissue macrophages in response to acute insults as inflammatory stimuli was then described in the first in vitro experiments [171].

4.2 Monocyte heterogeneity and subsets

Monocytes are a heterogeneous cell population showing differences in terms of morphology, phenotype and functions.

The classification of macrophages into M1 and M2 subtypes is a simplified representation of a more complex continuum of subtypes. Some scientists refer to this scenario as "the macrophage spectrum," where cells exhibit different levels of M1- or M2-like features [172]. Macrophages can change phenotypes based on their surroundings. To categorize the complex and dynamic macrophage population, various classifications and nomenclatures exist based on activation, release, and surface markers. The distinction between in vitro and in vivo phenotypes of macrophages remains unclear [173]. Wound macrophages can acquire distinct phenotypes based on parameters such as anatomical setting, location (center/edge), environment (moist/dry), and infection status [174].

4.2.1 Macrophages' Classification Based on Cell Surface Markers

Monocytes show distinct phenotypes due to the different expression, on the cell surface, of two markers: CD14, the lipopolysaccharide (LPS) receptor antigen, and CD16, the

immunoglobulin G Fcγ receptor. According to the nomenclature of the Nomenclature Committee for the International Union of Immunological Societies, monocytes are classified into three subpopulations: classical CD14++CD16-, intermediate CD14++CD16+ and non-classical CD14+CD16++ monocytes [175]. Each of these subgroups exhibits unique traits in the expression of chemokine receptors and cell adhesion molecules, leading to distinct phenotypes and activities. (**Figure 1**).

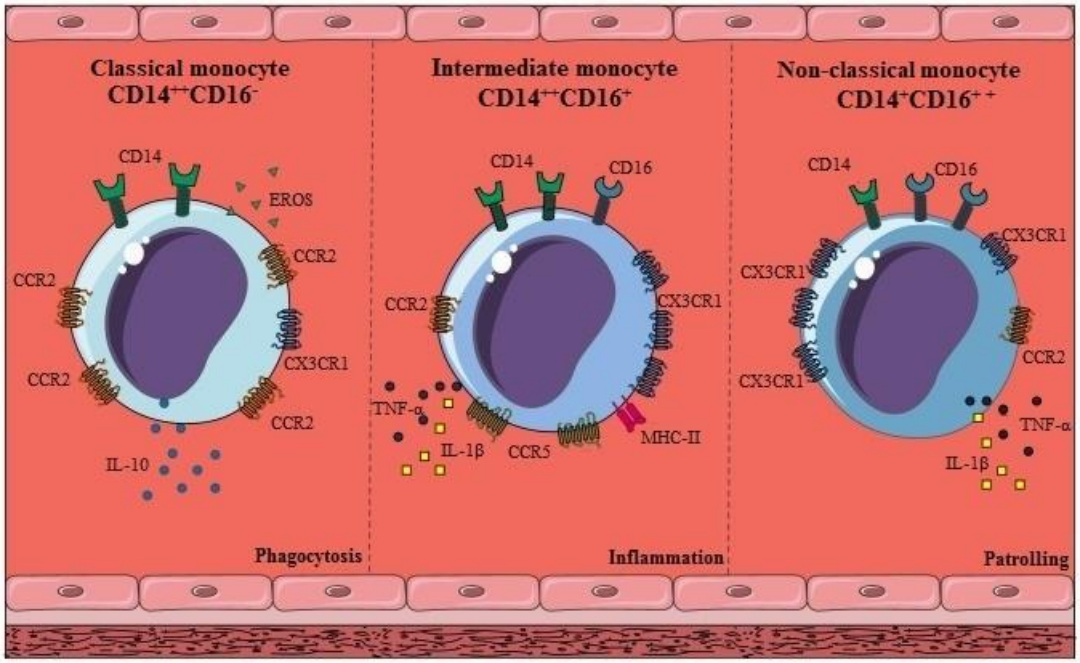


Figure 1. Human monocyte subsets.

Classical monocytes represent the largest subgroup: 80-90% of the total monocyte count. These cells display a high expression of the chemokine receptor (CCR) type 2, that is the receptor of MCP-1, of chemokine receptor (CXCR) 1,2 and 4 and high levels of the cell adhesion molecule L-Selectin (CD62L) while CX3CR1 (the fractalkine receptor) and CCR5 show lower levels. Following stimulation with LPS, this subset produces several chemokines and cytokines such as interleukin (IL)-10, IL-6, IL-8, IL-1β, TNF-α and CCL2 (chemokine ligand also known as MCP-1). The main function is linked to the immune response, specifically, due to the high expression of myeloperoxidase, several receptors such as, CD32, CD64, signal-regulatory protein α (SIRPα), and CD36 that highlights their role in the phagocytic process. These cells are considered as inflammatory mediators as they are rapidly recruited to inflamed tissue due to the recognition, by their receptor CCR2, of MCP-1 produced by tissue resident-macrophages. Furthermore, classical monocytes are rich in genes

related to angiogenesis and coagulation thus underlining their involvement in tissue repair and wound healing [176].

Non-classical monocytes (2-8% of total circulating monocytes) show phagocytic competences too; these are also ROS producers; however, their production of cytokines is greatly reduced. Concerning molecule expression on cell surface, non-classical monocytes display ICAM-1, opsonin receptors FcγRI and FcγRII, major histocompatibility complex (MHC) class II and CX3CR1 but low levels of CD11b and CD33 while they do not express CCR2 and CD62L at all. The main function is the patrolling of the blood circulation for the control and removal of damaged cells. This activity is due to the interaction between fractalkine (CX3CL1) expressed on endothelial cells, and its receptor CX3CR1 allowing the attraction and arresting of monocytes to the endothelium. Moreover, non-classical monocytes show a high expression of genes involved in the rearrangement of the cytoskeleton [176].

Intermediate monocytes (2-10% of total circulating monocytes) appear as an intermediate state between classical and non-classical monocytes, as these cells show the expression of a mixture of markers that characterize both classical and non-classical subsets. The expression of CCR1, CCR2 and CXCR2 characterizes the intermediate monocytes. Furthermore, CX3CR1 and CCR5 are present on the cell surface, as well as MHC class II that is more expressed in this group than the others are. Intermediate monocytes produce large quantities of ROS and pro-inflammatory cytokines such as TNF-α and IL-1β following stimulation with LPS; however, they are also the main producers of the anti-inflammatory IL-10 [177].

To summarize, the monocyte population is made up of three subsets with various phenotypes; however, whether these phenotypes are distinct or form a broad continuum is still unknown. To date, it is considered that the subset of monocytes exhibits dynamic changes rather than obvious distinctions in phenotype and function.

The observation that circulating monocytes change phenotypic from one group to another, followed by an increase in CD16 expression, lends support to this hypothesis. Monocyte subsets undergo dynamic alterations under diseases including severe systemic inflammation [178].

4.2.2 Macrophages Classification Based on Release

Macrophages involved in wound healing can be divided in vivo into three main subtypes according to the stage of repair and the mediators they secrete: pro-inflammatory, pro-wound healing, and pro-resolving macrophages.

Pro-inflammatory macrophages appear soon after tissue injury and contribute to the early inflammatory phase by releasing nitric oxide, reactive oxygen species, pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ, as well as matrix metalloproteinases MMP-2 and MMP-9, which degrade the extracellular matrix.

During the proliferative phase, pro-wound healing macrophages secrete high levels of growth factors including PDGF, IGF-1, VEGF, and TGF-β1. These mediators promote cell proliferation, formation of granulation tissue, and angiogenesis. To limit excessive matrix degradation and allow extracellular matrix reconstruction, these macrophages also produce TIMP-1, an inhibitor of metalloproteinases.

In the final phase of repair, pro-resolving macrophages reduce inflammation by releasing IL-10, arginase-1, and TGF-β1. They also secrete MMP-12 and MMP-13, which contribute to extracellular matrix remodeling and strengthening, helping restore tissue balance and minimize fibrosis. Pro-inflammatory and pro-resolving macrophages share some functional characteristics, as their activities partially overlap during tissue proliferation and remodeling.

Among M2 macrophage subtypes, M2a cells synthesize collagen precursors and growth factors that activate fibroblasts and produce high amounts of PDGF, supporting angiogenesis. M2b macrophages, identified by surface markers such as CD86, CD68, and MHCII, limit inflammation by secreting anti-inflammatory cytokines including IL-10, IL-6, IL-1β, and TNF, along with nitric oxide and various MMPs. In vitro, macrophages acquire the M2b phenotype after engulfing neutrophils.

M2c macrophages express CD206, MERTK, and CD163 and are induced by glucocorticoids, IL-10, and TGF-β. They produce high levels of IL-10, MMP-9, IL-1β, and TGF-β, while generating low amounts of IL-12. Often described as deactivated macrophages, M2c cells closely resemble pro-resolving macrophages and can arise from M1 macrophages through changes in gene expression.

M2 macrophages may polarize into M2a, M2b, or M2c phenotypes. Additionally, M2d macrophages, activated by IL-6 and adenosine, lack expression of CD206 and dectin-1 and secrete large quantities of VEGF, IL-10, and TGF-β, while suppressing TNF-α and IL-12 to further reduce inflammation.

4.2.3 Macrophages Classification Based on their origin

Macrophages can be categorized into two principal groups based on their origin: (a) tissue-resident macrophages (RTMs), known in the skin as dermal macrophages, which originate from embryonic progenitors in the yolk sac and are established before birth, maintaining themselves through self-renewal; and (b) circulating monocytes that are recruited to sites of injury and subsequently differentiate into macrophages. In skin wounds, macrophages arise from both resident dermal macrophages and infiltrating monocytes, with the latter contributing to a greater extent.

Resident dermal macrophages respond rapidly to tissue damage by recognizing damage-associated molecular patterns (DAMPs) or, in the presence of infection, pathogen-associated molecular patterns (PAMPs). This recognition triggers the release of reactive oxygen species, initiating a local pro-inflammatory response. Dermal macrophages also recruit neutrophils to combat infection. Their primary function is to preserve skin integrity, maintain tissue homeostasis, and support repair processes. These cells are characterized by the expression of CD64 and MERTK and by low or absent CCR2 expression. They exhibit strong phagocytic capacity but a relatively slow turnover. Following tissue remodeling, dermal macrophages self-renew and remove apoptotic cells during the resolution phase, thereby restoring homeostasis.

Lineage-tracing studies have shown that most tissue-resident macrophages originate prenatally from the yolk sac or foetal liver, whereas circulating monocytes are continuously generated through haematopoiesis. Advanced imaging approaches, including intravital microscopy and confocal multiplex imaging, have revealed that RTMs protect tissues from excessive inflammation through a so-called “cloaking” mechanism that limits neutrophil-induced damage. Disruption of this protective function results in uncontrolled inflammatory responses, excessive neutrophil accumulation, and collateral tissue injury, necessitating the recruitment of additional circulating monocytes to regulate inflammation. These findings identify RTMs as a previously unrecognized immune checkpoint that prevents chronic inflammatory damage.

Circulating monocytes enter injured tissues following activation of the inflammatory cascade, where they differentiate into macrophages. In vivo imaging has demonstrated that the first wave of monocytes infiltrates wounds concurrently with neutrophils, rather than at a later stage as previously assumed. While resident macrophages initiate rapid, short-lived local inflammatory responses, monocyte-derived macrophages are recruited from the bloodstream

over an extended period—approximately 24 hours in mice—after tissue injury. Their recruitment is driven by signals from damaged tissue, including both DAMPs and PAMPs.

A well-known PAMP is lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, which is recognized by toll-like receptor 4 and activates NF-κB signaling, leading to the expression of pro-inflammatory genes. DAMPs, such as extracellular DNA, RNA, and ATP released from dying cells, also attract immune cells to injury sites. In addition, chemokines and cytokines including IL-1, IL-6, TNF-α, and MCP-1 (CCL2) efficiently promote monocyte recruitment. MCP-1 is particularly important for inflammatory angiogenesis, as it drives the migration of circulating monocytes into ischemic or damaged tissues, and it has been shown to enhance healing in diabetic wounds by restoring effective macrophage responses.

Resident and monocyte-derived macrophages coexist within the same wound environment and may display different activation states. Recent studies have identified two distinct macrophage subsets in skin wounds with different origins and functions: CX3CR1^high macrophages, which derive from tissue-resident macrophages and predominantly exhibit an M2-like phenotype, and CX3CR1^low/negative macrophages, which originate from recruited monocytes and display both M1 and M2 activation profiles. Migratory monocytes populate peripheral tissues in substantial numbers and actively collaborate with resident macrophages to protect tissues. While RTMs are primarily involved in preventing the initiation of excessive inflammation, recruited monocytes play a key role in the subsequent resolution phase.

4.3 Monocyte development

Monocytes are derived from hematopoietic stem cells (HSC), which produce a diverse population of multipotent progenitors (MPP) that develop into two distinct precursors: common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). The former generates lymphoid cells such B, T, and natural killer cells, whilst the latter produces megakaryocyte and erythrocyte progenitors (MEP) or granulocyte and macrophage progenitors (GMP). Basophils, neutrophils, and eosinophils derive directly from GMP, as do monocyte-macrophage/dendritic cell progenitors (MDP). At this time, two separate lineages emerge: dendritic cells evolve from common dendritic cell precursors (CDP), whereas common monocyte progenitors (cMoP) produce monocytes [179]. (**Figure 2**)

The development of monocytes from cMoP involves an intermediate stage of pre-monocytes with high CXCR4 expression. After 24 hours, these cells develop into monocytes with low

CXCR4 but high CCR2 [180]. Monocytes in CCR2-deficient mice remain confined in the bone marrow, resulting in fewer circulating monocytes [181]. As a result, it is likely that CCR2 and its ligands CCL2 and CCL7, rather than CXCR4, regulate monocyte departure from the bone marrow into the bloodstream.

Although the bone marrow generates a large number of monocytes, the spleen also produces a small amount of MDP and cMoP [182]. The spleen is a tiny reserve of monocytes. During inflammation, IL-1β promotes emergency extramedullary monopoiesis [183].

Under homeostatic conditions, classical monocytes circulate for roughly a day before migrating to tissues to develop into tissue-resident macrophages. Monocyte-derived macrophages differ significantly from their circulating ancestors; they adapt to the local environment and acquire capabilities comparable to the macrophages they replace [184]. Monocytes, on the other hand, can move to tissue and preserve their traits and identity, forming a local reservoir [185].

An additional method is the shift of classical monocytes to the non-classical group via intermediate monocytes, which stay in the bloodstream for about 4 days [178]. Non-classical monocytes have a longer life span, lasting about 2 days in mice and up to 7 days in humans. This longer life span provides a consistent quantity of non-classical monocytes even under pathological settings, particularly after inflammatory stimuli that stimulate the differentiation of classical monocytes to macrophages, preventing the shift to non-classical monocytes. The amount of non-classical monocytes is thus heavily influenced by the organism's physiological condition; in example, exercise and aging can impact the balance of monocyte subsets [178].

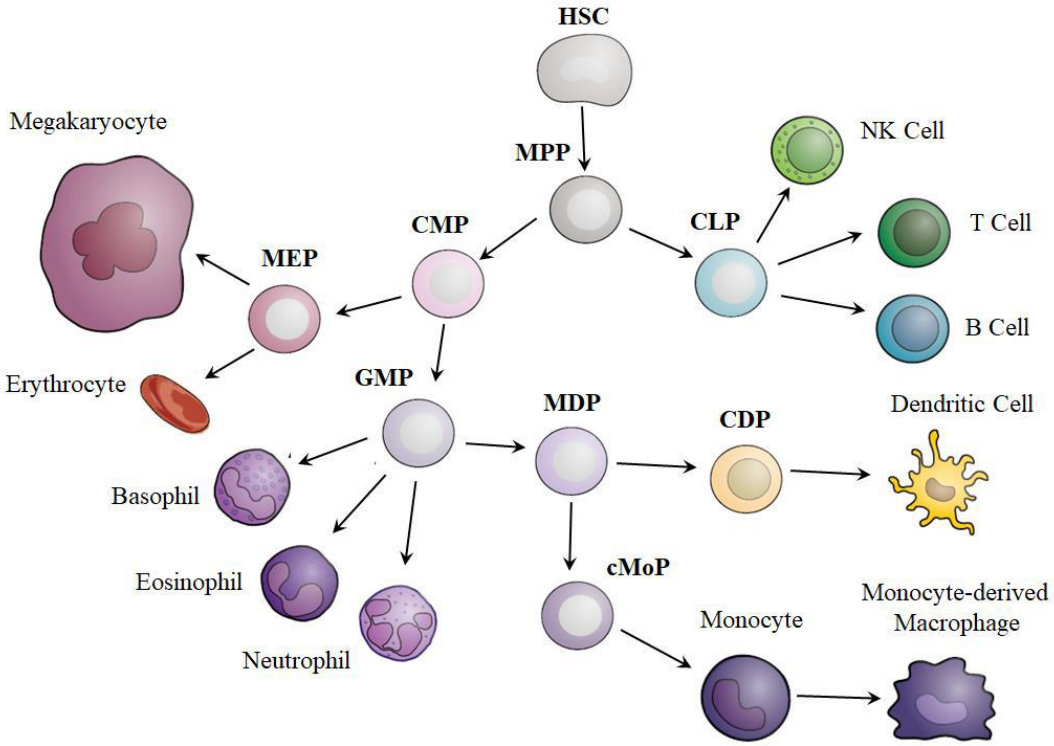


Figure 2. Hematopoietic development in the bone marrow.

CDP: Common Dendritic cells Precursors; **CLP**: Common Lymphocyte Precursors; **cMoP**: common Monocyte Precursors;**CMP**: Common Myeloid Precursors; **GMP**: Granulocyte Macrophage Precursors; **HSC**:Hematopoietic Stem Cells; **MDP**: Monocyte-Macrophage/Dendritic cell Precursors; **MEP**: Megakaryocyte/Erythrocyte Precursors; **MPP**:Multipotent Precursors;

4.4 Role of M1 and M2 Macrophages in Wound Healing

M1 macrophages show up at the site of injury right after the homeostatic stage and participate in pathogen phagocytosis by eliminating or destroying injured cells during the inflammatory stage. Additionally, M1 macrophages stimulate other immune cells by releasing chemokines (CXCL8-11) and pro-inflammatory cytokines (TNF-, IL-1_, and IL-6), which have a positive feedback effect on antigen-presenting cells (APCs) or inactivated monocytes [186]. M1 macrophages are characterized by increased activity of nitric oxide (NOS) and reactive oxygen species (ROS), as well as positive expression of co-stimulatory molecules (CD86 and CD80), toll-like receptor-2 (TLR-2), TLR-4, and MHC-II on the cell surface. [187].

As the healing process progresses to the proliferative/regenerative stage, the macrophage population shifts from M1 to M2 [187,188].

M2 macrophages regulate M1 macrophages and are divided into four subtypes (M2a, M2b, M2c, and M2d) based on their released cytokines and surface markers [186,189,190].

The M2a subtype has increased expression of CD206, FIZZ1, TGF-_, CCL17, and CCL22 in response to IL-4 and IL-13, which operate as anti-inflammatory and wound-healing factors [189,191].

M2c macrophages express CD206, CD163, and MERTK, produce high quantities of TGF-_, IL-10, MMP-9, and IL-1_, and can be stimulated by IL-10 and glucocorticoids [187,192]. M2a and M2c macrophages are pro-healing and pro-remodeling [191]. They promote fibroblast and keratinocyte migration and proliferation, and recruit endothelial stem cells for granulation tissue and neovascularization. Cytokines, chemokines, and growth factors play a crucial role in coordinating cell proliferation and remodeling during normal wound healing [193,194]. In diabetic wounds, cross-talk between cells was reduced [187].

M1 macrophages initiate sprouting angiogenesis by secreting proangiogenic cytokines and VEGF, while M2 macrophages support vessel regression and remodelling [195]. M2 macrophages produce MMP and VEGFs, which are necessary for remodeling [196]. Switching from M1 to M2 macrophages at the conclusion of the pro-inflammation stage to the proliferation/remodelling stage is crucial for optimal wound healing, allowing for enough quantities of M2 macrophages. (**Figure 3**)

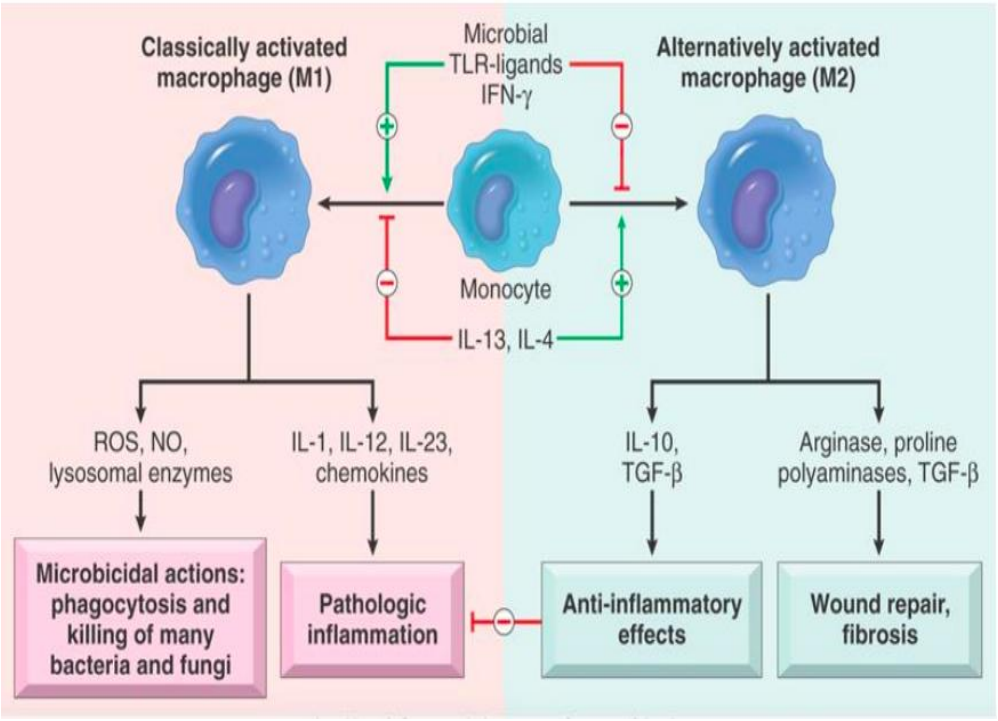


Figure 3. M1/M2 macrophages role.

4.5 Switch of M1 and M2 Macrophages at the Molecular Level

M1 and M2 macrophages can be recruited from peripheral locations or polarized locally from monocytes/resting macrophages [197]. Environmental alterations in cytokines, miRNAs, transcription factors, and exosomes might trigger the production of M2 and M2 subtype macrophages during wound healing [196-199]. Attenuating innate immune cytokines including IFN-, TNF-α, and IL-1_ can improve M1 polarization and survival by increasing the expression of M2-associated markers (CD206, CD36, or CD163) and cytokines (TGF-β, CCL2, CXCL8, and CCL3) [200,201].

Extracellular stimuli activate M2-polarizing signaling, shifting the molecular hub from an M1-associated profile (NF-_B-, IRF5-, STAT1-, and AKT2-dominant) to an M2-associated profile (IRF4-, STAT6-, PPAR--, and AKT1-dominant) [197]. IL-10, an anti-inflammatory cytokine involved in wound regeneration, has been shown to increase the amount of the IL-4R chain on the cell surface. This makes macrophages more sensitive to IL-4 and IL-13, leading to an M2-polarizing trend [202]. The IL-4/IL-13 signaling pathway activates IL-10R, IL-6R, and IL-4R via STAT3 [197, 202]. Antiinflammatory cytokines' feedback regulation alters the environment, promoting M2 polarization.

Pro-inflammatory M1 macrophages migrate to the injury site to eliminate germs, necrotic cells, and foreign material in the early stages of wound healing, immediately following hemostasis. As repair begins in acute wounds, the macrophage population gradually switches to the M2 phenotype, which supports anti-inflammatory responses and tissue regeneration. This phenotypic transformation, known as macrophage polarization, is commonly regarded as an important phase in wound healing [203-204].

M2 polarization promotes fibroblast, keratinocyte, and endothelial cell migration and proliferation, allowing for the regeneration of the dermis, epidermis, and blood vessels [205,206]. Diabetic wounds cause a disruption in coordinated cellular communication [207]. During this phase, both M1 and M2 macrophages help with neovascularization, first by driving vessel sprouting and then by supporting the formation of anastomoses between newly formed vessels [208,209].

In vivo time-lapse imaging has shown that macrophages play an active role in creating functional vascular connections. After arriving at the injury site, macrophages extend filopodia or lamellipodia to adhere to the endothelial ends of blood vessels, facilitating the repair of torn vasculature via direct adhesion and mechanical force. This macrophage-driven repair mechanism has been observed not only in the brain, but also in peripheral blood

arteries. Supporting these findings, macrophages have been shown to secrete significant quantities of VEGF-C, which stabilizes tip cell fusion and increases vascular network complexity [207-210].

Gurevich and colleagues found that after tissue injury in mice and zebrafish, macrophages can induce angiogenic sprouting, promote new vessel development, and direct future vascular remodeling. Using an in vitro human co-culture system, they also found that pro-inflammatory M1 macrophages are required to initiate early sprouting angiogenesis via localized distribution of pro-angiogenic cytokines and VEGF. However, a timely transition from the M1 to M2 phenotype is required for normal vessel maturation and regression in later phases [211].

During the final remodeling phase of healing, macrophages produce metalloproteinases, which destroy the temporary extracellular matrix and cause apoptosis. In chronic wounds, macrophages frequently remain in the pro-inflammatory M1 state without switching to the anti-inflammatory M2 phenotype, which is thought to hinder tissue recovery. As a result, controlling the transition from M1 to M2 macrophages may be a viable technique for promoting inflammation to proliferation during wound healing [212-215].

It remains unclear whether the M1-to-M2 transition occurs through differentiation of newly recruited circulating monocytes, polarization of resident macrophages at the injury site, or both. Evidence suggests that this switch is influenced by changes in the local microenvironment, including cytokine profiles, microRNAs, transcription factors, exosomes, and the regulation of inflammatory receptors. Efficient clearance of apoptotic cells by macrophages (efferocytosis) is also required to resolve inflammation, and emerging data indicate that microRNA-21 plays a role in enhancing this process. Recent studies further demonstrate that exosomes derived from M2 macrophages can directly reprogram M1 macrophages into the M2 phenotype with near-complete efficiency. These reprogrammed M2 macrophages produce metalloproteinases and VEGF and are essential for angiogenesis and re-epithelialization during the proliferative phase of wound healing [216-220].

4.6 Monocytes/Macrophages and Lymphocytes in Diabetic Wound Healing

Diabetes is a metabolic condition that causes low-grade systemic inflammation, which has a profound impact on the immune system with a delayed proliferation/remodelling phase characterized by a stalled non-healing state. Dysregulated metabolic pathways and immunological disorders have an impact on the entire wound healing process. An important issue is the imbalance of macrophage polarization. An RNA sequencing analysis of wound tissues from DFU patients and healthy people found a greater M1/M2 ratio in DFU tissues than in healthy skin.

Hypoxia and the development of advanced glycation end products impair M1 macrophages' phagocytic activity. In diabetic wounds, M1 macrophages release proinflammatory cytokines such IL-1β, IL-6, and tumor necrosis factor-α, attracting monocytes to the site of inflammation and driving their development into the M1 phenotype.

Chronic wounds accumulate M1 macrophages, which prevents them from transitioning to the M2 phenotype and proceeding from the inflammatory phase to the proliferative granulation phase.

As a result, persistent inflammation remains, causing poor wound healing. Current research focuses on treatments that promote wound healing by polarizing macrophages to the M2 phenotype.

In a normally healing wound (Figure 4), immediately after neutrophil infiltration, an initial wave of monocytes enters the injured tissue and differentiates into pro-inflammatory M1 macrophages. These cells secrete cytotoxic and inflammatory mediators, including IL-1β, TNF-α, IL-6, and reactive oxygen species, to eliminate pathogens, degrade necrotic tissue, and remove damaged cells. This early inflammatory phase is followed by a second wave of anti-inflammatory M2 macrophages, which instead support tissue repair by releasing TGF-β, IL-10, and other anti-inflammatory cytokines.

M2 macrophages primarily function as producers of growth factors and immunoregulatory mediators while retaining the capacity to clear apoptotic cells. In addition, they recruit endothelial progenitor cells and stimulate angiogenesis within the wound, thereby enabling granulation tissue formation and the development of new blood vessels [221].

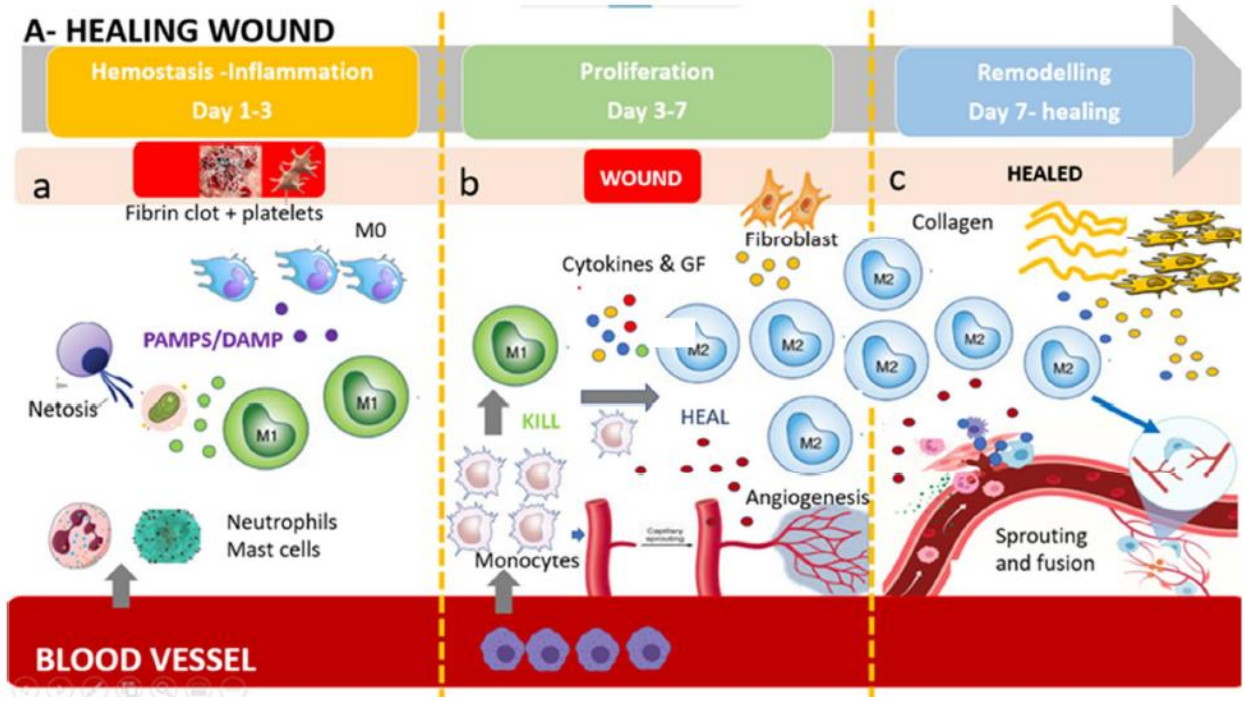


Figure 4. The healing wound:

(a) **Inflammatory phase:** Platelets form a fibrin clot at the wound site, releasing chemoattractants that recruit inflammatory cells, including neutrophils and mast cells. These cells secrete pro-inflammatory cytokines. Neutrophils perform NETosis (Neutrophil Extracellular Trap formation) to trap and destroy pathogens. Tissue-resident macrophages respond to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). Meanwhile, the first wave of monocytes infiltrates the wound and differentiates into M1 macrophages, which carry out phagocytosis.

(b) **Proliferative phase:** After inflammation resolves, the proliferative phase begins. Angiogenesis occurs via vessel sprouting. Infiltrating monocytes differentiate into M1 and M2 macrophages. M1 macrophages continue to maintain an inflammatory response by releasing cytokines and reactive oxygen species while clearing dead bacteria and apoptotic neutrophils. Over time, M1 macrophages polarize into M2 macrophages, which adopt a pro-regenerative phenotype. M2 macrophages release anti-inflammatory cytokines, growth factors, and proteases, which replace the provisional extracellular matrix with collagen, stimulate fibroblast proliferation, and promote new vessel formation. This coordinated activity leads to granulation tissue formation and keratinocyte coverage.

(c) **Remodeling phase:** Tissue remodeling is mediated by macrophages, fibroblasts, and myofibroblasts, which reorganize the provisional ECM into mature, functional tissue. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) play a central role, ensuring the tissue gains tensile strength and functionality. Angiogenesis nears completion, and macrophages contribute to vessel fusion, creating a fully connected and functional vascular network.

Source: Rehak, L.; Giurato, L.; Meloni, M.; Panunzi, A.; Manti, G.M.; Uccioli, L. The Immune-Centric Revolution in the Diabetic Foot: Monocytes and Lymphocytes Role in Wound Healing and Tissue Regeneration—A Narrative Review. J. Clin. Med. 2022, 11, 889.

In contrast, diabetic wounds (Figure 5) display profound structural and functional abnormalities, including impaired angiogenesis, which results in a hypoxic environment and elevated oxidative stress [36]. Healing of diabetic foot ulcers does not follow the normal sequential phases of repair but instead remains trapped in a chronic, non-healing state. This

condition is characterized by persistent, dysregulated inflammation, insufficient angiogenesis, impaired epithelial migration, reduced responsiveness to growth factors, and excessive fibrosis [222]. Thus, recent studies have emphasized M2-enrichment approaches, such as the administration of M2 macrophages directly or the elimination of factors that obstruct M2 recruitment or polarization, for dealing with DFUs.

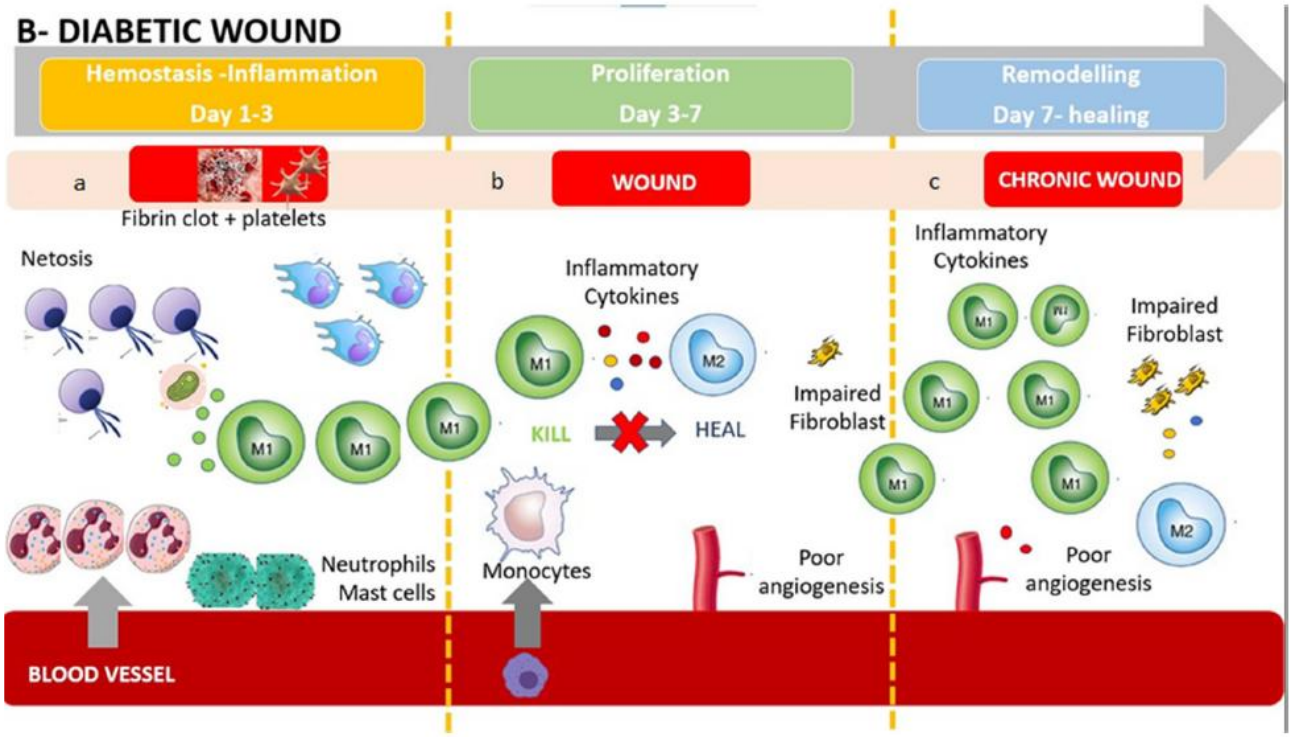


Figure 5. Diabetic wound:

(a) **Inflammatory phase in impaired wounds:** In poorly healing wounds, there is an excessive influx of neutrophils and mast cells, leading to an exaggerated inflammatory response. This causes collateral tissue damage and prolongs the inflammatory phase. The sustained release of inflammatory cytokines maintains M1 macrophage activation, which further amplifies inflammation.

(b) **Proliferative phase in impaired wounds:** Monocyte recruitment is limited due to arterial blockage and impaired microcirculation. Poor angiogenesis, combined with glycated proteins, reduces fibroblast activity. The resulting hypoxic environment increases oxidative stress, which promotes M1 macrophage polarization and further impairs fibroblast function. This leads to defective extracellular matrix (ECM) remodeling and a persistent inflammatory environment. Polarization toward the M2 pro-regenerative phenotype is minimal or absent, causing additional accumulation of M1 macrophages.

(c) **Remodeling phase in impaired wounds:** Resident wound cells remain dysfunctional and inflammatory. Collagen reorganization is incomplete, producing weak, non-functional tissue prone to re-injury and ulceration. Macrophages remain predominantly in the M1 phenotype, and the wound fails to heal.

Source: Rehak, L.; Giurato, L.; Meloni, M.; Panunzi, A.; Manti, G.M.; Uccioli, L. The Immune-Centric Revolution in the Diabetic Foot: Monocytes and Lymphocytes Role in Wound Healing and Tissue Regeneration—A Narrative Review. J. Clin. Med. 2022, 11, 889.

Macrophages play a key role in wound healing, and transitioning to anti-inflammatory M2 phenotypes is required for effective healing. Questions persist about monocyte recruitment and macrophage differentiation, specifically whether monocytes are predisposed to differentiate into one of two phenotypes, M1 or M2, or whether macrophages polarize from M1 to M2 (or vice versa) within the wound. Several techniques have been used to immune-modulate macrophages, causing them to polarize into M2 and/or M1 phenotypes (Figure 6).

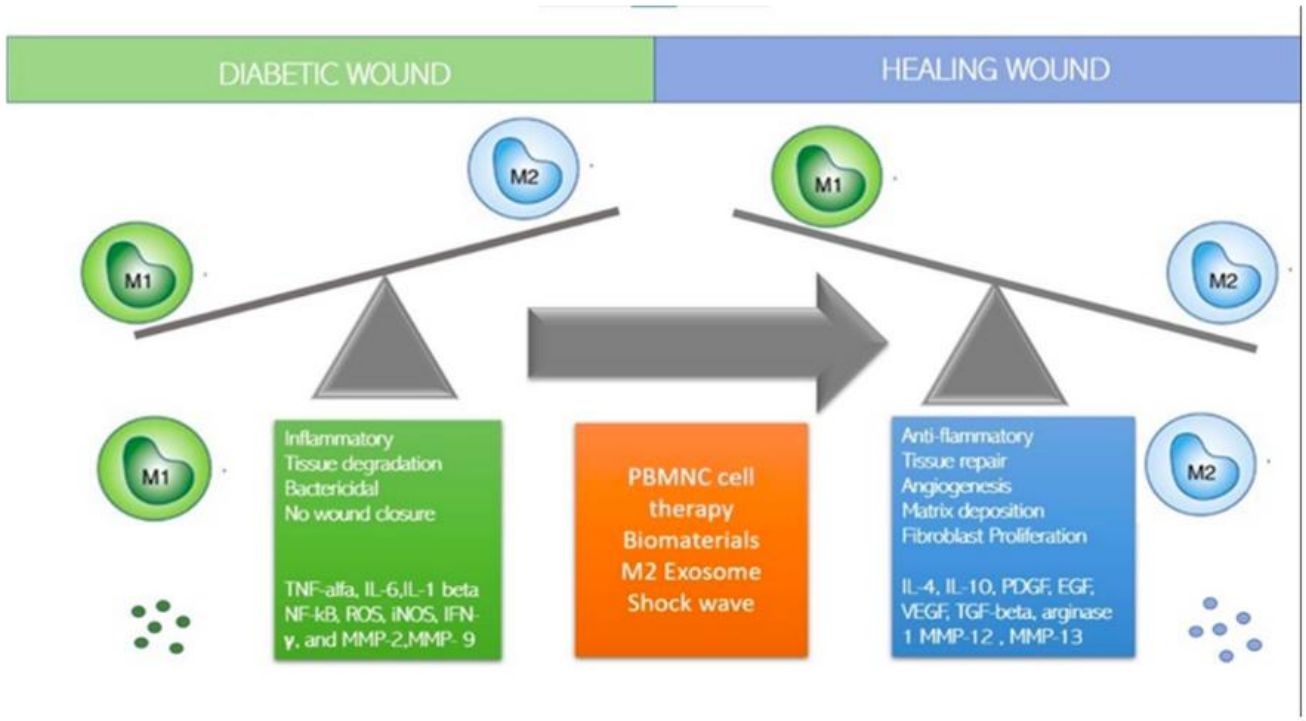


Figure 6. How to switch to M2: strategies.

Source: Rehak, L.; Giurato, L.; Meloni, M.; Panunzi, A.; Manti, G.M.; Uccioli, L. The Immune-Centric Revolution in the Diabetic Foot: Monocytes and Lymphocytes Role in Wound Healing and Tissue Regeneration—A Narrative Review. J. Clin. Med. 2022, 11, 889.

In the following sections, the distinct roles and altered behaviours of neutrophils, monocytes/macrophages, and lymphocytes in diabetic wounds will be discussed.

4.6.1 Neutrophils in diabetic wounds

A hallmark of impaired wound healing in diabetes is the elevated presence of neutrophils and an increased neutrophil-to-lymphocyte ratio. Neutrophils combat infection in part by forming neutrophil extracellular traps (NETs), which consist of decondensed chromatin decorated with

cytotoxic proteins. However, NETs can also damage healthy tissue. Neutrophils from type 1 and type 2 diabetic patients and mice are predisposed to excessive NET formation (NETosis), which contributes to delayed wound healing. Studies have shown that reducing NET formation accelerates wound closure. In normal wound healing, neutrophil apoptosis followed by phagocytosis by infiltrating monocytes and macrophages helps resolve inflammation. This process limits inflammatory cell infiltration and shifts eicosanoid production from pro-inflammatory to anti-inflammatory mediators. In diabetic wounds, however, persistent neutrophil activation and defective regulatory mechanisms prolong the inflammatory phase. Notably, the transcription factor FOXM1, essential for activating and recruiting inflammatory cells, is downregulated in diabetic patients [223-229].

4.6.2 Monocytes and macrophages in diabetic wounds

Dysregulation of the inflammatory phase in diabetic wounds is associated with epigenetic priming of innate immune cells toward a pro-inflammatory phenotype, likely driven by hyperglycemia [36]. This systemic inflammatory bias is observed in both diabetic patients and animal models [230]. In mice, impaired healing correlates with an altered immune cell composition, including increased neutrophils and reduced macrophages, monocyte-derived Langerhans cells, dendritic cells, and eosinophils [231]. Normally, infiltrating monocytes differentiate into inflammatory M1 and reparative M2 macrophages. In diabetic wounds, M1 polarization dominates, and the switch to M2 is severely impaired, resulting in fewer M2 macrophages and a higher M1:M2 ratio [36,54,59,152,214-216]. Consequently, the secretion of growth factors (PDGF, FGF, VEGF) and anti-inflammatory cytokines (IL-10, TGF-β) is reduced, impairing the proliferation phase and effective regulation of inflammation. Elevated M1 activity in diabetic conditions increases TNF-α levels and hinders keratinocyte migration, while high glucose environments further promote M1 polarization. Therapeutic approaches, such as negative pressure wound therapy, have been shown in mice to suppress macrophage inflammation and autophagy, promoting healing [232,233].

Macrophages are critical for neovascularization, as they produce VEGF and other angiogenic factors. Deficiencies in macrophage numbers contribute to impaired angiogenesis in diabetic wounds. Reduced VEGFR1 signaling and decreased M2 macrophage activity also limit blood vessel formation, which is essential for the proliferative phase, granulation tissue formation, and re-epithelialization. In addition, diabetic $CD34^+$ stem cells show impaired vessel reparative function, while injection of freshly isolated $CD14^+$ monocytes into ischemic limbs

of diabetic mice improves vascular growth and healing, highlighting the angiogenic potential of these cells [234-240].

4.6.3 Lymphocytes in diabetic wounds

Lymphocytes play a key role in both diabetic and non-diabetic wound healing. A lack of lymphocytes impairs healing, and when combined with diabetes, wound repair is further compromised, resulting in excessive M1 polarization, defective angiogenesis, and delayed closure. Type 2 diabetic tissues show increased $CD8^+$ T-cell infiltration. Systemic inflammation in diabetes may limit regulatory T-cell (Treg) migration and favour Th17 infiltration, which promotes neutrophilic recruitment and prolongs inflammation. Topical retinoic acid can accelerate diabetic wound healing by inducing T-cell plasticity and converting Th17 cells into Tregs, underscoring the importance of T cells in controlling inflammation. Reduced $CD4^+$ T-cell numbers correlate with lower vascular density, while Treg administration enhances angiogenesis via paracrine mechanisms. Decreased natural killer cells and elevated IFN-γ are associated with diabetic foot complications and may serve as predictive markers for infection [155,227,241-243].

4.6.4 Keratinocytes and fibroblasts in diabetic wounds

Keratinocyte secretion of the chemokine Ccl2 is regulated by Nrf2, a transcription factor essential for tissue regeneration. In diabetic wounds, Nrf2 activation is impaired, reducing Ccl2-mediated macrophage EGF production, which in turn limits keratinocyte proliferation and wound repair. Diabetic foot ulcers exhibit diminished proliferation of skin resident cells, stem cells, and progenitor cells, likely due to protein glycation, reduced angiogenesis, and oxidative stress, which prolong the proliferative phase. Fibroblasts in diabetic wounds show decreased extracellular matrix (ECM) production. Specifically, fibroblasts from diabetic foot ulcers produce ECM that is roughly half as thick as that of normal fibroblasts and display altered composition, with higher levels of collagen type I and fibronectin [66,153, 207].

5. DERMAL SUBSTITUTES

5.1 Overview

Temporary or permanent skin coverings used to heal wounds are known as skin substitutes. They are frequently used for acute wounds like burns or chronic wounds like diabetic or venous ulcers. They can be biologically, synthetically, or biosynthetically generated. The application of skin replacements can enhance both functional and cosmetic results in addition to wound healing rates. [244] Since the development of cultured epidermal autografts (CEAs) in the 1970s, Epicel (Vericel Corporation, Cambridge, MA), and the first dermal substitute, Integra (Integra Life Science Corporation, Plainsboro, NJ), in the early 1980s, bioengineered skin substitutes have been available for a number of decades [245]. The ideal skin substitute should be able to promote the tissue regeneration, minimally or non-antigenic, non-toxic, immunologically compatible, regenerative, protective, nonpathogenic, and long-lasting and, much like autologous skin transplants. Furthermore, it needs to defend against bacteria and be able to maintain an appropriate wound healing environment: moist, oxygenated, and sterile [246]. See table 1.

Property	Elaboration
Non-immunogenic	• Components do not induce tissue rejection.
Bio-compatible	• Infiltrating cells can effectively adhere to scaffold material. • Integrates readily into existing ECM; cells can deposit/extract ECM
Regenerative	• Does not inhibit or promotes angiogenic function • Minimizes sub-optimal granulation of tissue & scarification • Beneficially modulates regenerative cells such as macrophages & fibroblasts • Facilitates rapid epithelial cell coverage
Protective	• Provides coverage for underlying structures • Minimizes disruptive "floating" in the wound bed • Retains & maintains a moist environment, reducing oxidative stress
Non-pathogenic	• The low number of applications to minimize infection risk • Sterile preparation method & donor source do not confer disease
Durable	• The substitute does not degrade before regenerative action. • Complicating conditions (infection, T2DM-induced glycosylation, etc.) do not compromise scaffold flexibility & function

Table 1. Ideal proprerties of skin substitutes.

A 2020 report from the Agency for Healthcare Research and Quality identified 76 commercially available skin substitute products [247]. (see table 2).

Product	Manufacturer	Replaced region
Human-derived acellular dermal matrices		
AlloPatch	Musculoskeletal Transplant Foundation (dba MTF Biologics) Edison, NJ	Dermal
AlloPatch Pliable	Musculoskeletal Transplant Foundation (dba MTF Biologics) Edison, NJ	Dermal
Alloskin AC Acellular Dermal Matrix	AlloSource, Centennial, CO	Dermal
AlloSkin RT	AlloSource, Centennial, CO	Dermal
Coll-e-derm	Parametrics Medical, Leander, TX	Dermal
DermACELL Human Acellular Dermal Matrix and DermACELL AWM	LifeNet Health, Virginia Beach, VA	Dermal
Dermapure	Tissue Regenix Group, San Antonio, TX	Dermal
DermaSpan Acellular Dermal Matrix	Zimmer Biomet (manufactured by Biomet Orthopedics, Warsaw, IN)	Dermal
FlowerDerm	Flower Orthopedics, Horsham, PA	Dermal
GammaGraft	Promethean LifeSciences, Inc., Pittsburgh, PA	Dermal
GraftJacket RTM	Wright Medical Group N.V., Memphis, TN	Dermal
hMatrix ADM	Bacterin International, Inc., Belgrade, MT	Dermal
InteguPly	Aziyo Biologics, Silver Spring, MD	Dermal
Matrix HD Allograft	RTI Surgical, Alachua, FL	Dermal
Human amnion chorion membrane grafts		
AlloWrap	AlloSource, Centennial, CO	Dermal
AltiPlast	Aziyo Biologics, Silver Spring, MD	Dermal
AltiPly	Aziyo Biologics, Silver Spring, MD	Epidermal / Dermal
AmnioBand	Musculoskeletal Transplant Foundation (dba MTF Biologics), Edison, NJ	Dermal
Amnioexcel	Integra LifeSciences Corp. acquired Derma Sciences, Plainsboro, NJ	Dermal
AmnioFill Human Placental Tissue Allograft	MiMedx Group, Inc., Marietta, GA	Dermal
AmnioFix Amnion/Chorion Membrane Allograft	MiMedx Group, Inc., Marietta, GA	Dermal
Amniomatrix Human Amniotic Suspension Allograft	Integra LifeSciences Corp. acquired Derma Sciences, Plainsboro, NJ	Dermal
Artacent Wound	Tides Medical, Lafayette, LA	Dermal
BioDFactor Viable Tissue Matrix	Integra LifeSciences, originally BioD, LLC	Dermal
Biodfence	Integra LifeSciences, originally BioD	Dermal
Biovance Amniotic Membrane Allograft	Alliqua Biomedical, Langhorne, PA	Dermal
Cellesta Amniotic Membrane	Ventris Medical, Newport Beach, CA	Dermal
Cygnus Amnion Patch Allografts	Vivex Biomedical, Atlanta, GA	Dermal
Dermavest and Plurivest Human Placental Connective Tissue Matrix	Aedicell, Inc., Honeoye Falls, NY	Dermal
EpiCord	MiMedx, Marietta, GA	Dermal
Epifix	MiMedx, Marietta, GA	Dermal
FlowerAmnioPatch and FlowerAmnioFlo	Flower Orthopedics, Horsham, PA	Dermal
Genesis Amniotic Membrane	Genesis Biologics, Anaheim, CA	Dermal
Integra BioFix Amniotic Membrane Allograft	Integra LifeSciences Corp., Plainsboro, NJ	Dermal
Integra BioFix Flow Placental Tissue Matrix Allograft	Integra LifeSciences Corp., Plainsboro, NJ	Dermal

Table 2.

Product	Manufacturer	Replaced region
Interfyl Human Connective Tissue Matrix	Alliqua Biomedical, Langhorne, PA	Dermal
Neox Wound Allografts	Amniox Medical, Inc., Miami, FL	Dermal
NuShield	Organogenesis, Inc., Canton, MA	Dermal
PalinGen Membrane & Hydromembrane	Amnio Technology LLC, Phoenix, AZ	Dermal
Restorigin Amniotic Tissue Patches	Parametrics Medical, Leander, TX	Dermal
Revita	StimLabs, LLC, Roswell, GA	Dermal
WoundEx Membrane and WoundEx Flow	Skye Biologics, Inc., El Segundo, CA	Dermal
Xwrap Amniotic Membrane-Derived Allograft	Applied Biologics, Scottsdale, AZ	Dermal
Architect stabilized collagen matrix	Harbor MedTech, Inc., Irvine, CA	Dermal
Bio-ConneKt Wound Matrix	MLM Biologics, Inc., Alachua, FL	Dermal
CollaWound collagen sponge	Collamatrix Co., Ltd., Miaoli County, Taiwan	Dermal
Xenografts		
Cytal wound matrix	Acell, Inc., Columbia, MD	Dermal
Endoform dermal template	Hollister Wound Care, Libertyville, IL	Dermal
Excellagen	Taxus Cardium Pharmaceuticals Group, San Diego, CA	Dermal
EZ Derm	Mölnlycke Health Care, Norcross, GA	Dermal
Geistlich Derma-Gide	Geistlich Pharma North America Inc., Princeton, NJ	Dermal
Helicoll	EnColl Corp., Fremont, CA	Dermal
Integra Matrix Wound Dressing; originally Avagen wound dressing.	Integra LifeSciences Corp., Plainsboro, NJ	Dermal
Integra Flowable Wound Matrix	Integra LifeSciences Corp., Plainsboro, NJ	Dermal
MicroMatrix	Acell, Inc., Columbia, MD	Dermal
Miroderm	Miromatrix Medical, Inc., Eden Prairie, MN	Dermal
Ologen Collagen Matrix	Aeon Astron, Europe B.V.	Dermal
Kerecis Omega3 Wound (originally Merigen wound dressing)	Kerecis, Arlington, VA	Dermal
Oasis Wound Matrix	Smith & Nephew, Inc., Fort Worth, TX	Dermal
PriMatrix Dermal Repair Scaffold	Integra LifeSciences Corp., Plainsboro, NJ	Dermal
Puracol and Puracol Plus Collagen Wound Dressings	Medline Industries, Northfield, IL	Dermal
PuraPly Antimicrobial (PuraPly AM) Wound Matrix (formally called FortaDerm)	Organogenesis, Inc., Canton, MA	Dermal
Talymed	Marine Polymer Technologies, Inc., Burlington, MA	Dermal
TheraForm Standard/Sheet Absorbable Collagen Membrane	Sewon Cellontech Co., Seoul, Korea	Dermal
Synthetic grafts		
Hyalomatrix tissue reconstruction matrix	Anika Therapeutics, Bedford, MA	Dermal
Restrata	Acera Surgical, Inc., St. Louis, MO	Dermal
Combined natural and synthetic grafts		
Integra Bilayer Matrix Wound Dressing	Integra LifeSciences Corp., Plainsboro, NJ	Dermal
Integra Dermal Regeneration Template and Integra Omnigraft Regeneration Template	Integra LifeSciences Corp., Plainsboro, NJ	Dermal

Source: Adapted from Snyder D, Sullivan N, Margolis D, et al. Skin Substitutes for Treating Chronic Wounds [Internet]. Rockville (MD): Agency for Healthcare Research and Quality (US); Published February 2, 2020. Findings. Accessed April 10, 2021. https://www.ncbi.nlm.nih.gov/books/NBK554222/.

Table 2 (continued).

The most prevalent kind of product that is sold commercially is an acellular skin substitute. These goods come from animal tissues, human placental membranes, or donated human dermis [246]. They retain growth factors and cytokines to aid in healing, but they are decellularized to leave a scaffold for recipient cells to infiltrate. Acellular skin replacements do not elicit a robust immune response since they are immunologically inactive due to these processing techniques. Dermal substitutes made from biologic materials (allograft or xenograft) are the most often used commercially available acellular substitutes [246]. To reduce the host immune response and enhance absorption into the wound, human-derived acellular dermal matrices (ADM) are sterilized and decellularized to eliminate immunogenic cellular material, such as major histocompatibility complex (MHC) proteins [247-250].

5.2 Use of dermal substitutes in DFU

Because of their excellent safety profile and positive therapeutic results documented in the literature, fully acellular dermal substitutes are frequently utilized in the treatment of diabetic foot ulcers (DFUs).

For wound healing and remodeling, intact ECM components are essential [251]. Type I collagen makes up the majority of acellular dermal matrices (ADMs), with type III collagen coming in second. There has been a lot of attention due to the number of human fibroblasts and early studies showing their chemotactic attraction to collagens I, II, and III [252]. The release of wound and extracellular matrix (ECM)-affecting products, including growth factors, cytokines, and enzymes, is impacted by monocyte adherence to collagen types I and III [253-256].

Immunosuppressive effects in M2 macrophages have been associated with collagen I fiber-rich 3D environments [257], suggesting possible therapeutic benefits for DFU-resident macrophages. As with collagen type I deposition, an excess of type III collagen in hypertrophic wound scars does not produce enough immunomodulatory effects to restore normal wound healing [258].

Some proprietary ADMs have been shown to have a mechanistic influence, however the precise function of ADMs in DFUs is yet unknown. According to a study, the M1:M2 macrophage polarization ratio observed in DFUs can be restored to that of wounds that heal properly by utilizing a xenogenic ADM [259].

It has been demonstrated that ADMs enhance microvascular blood flow in DFUs [260]. These results demonstrate the significance of angiogenesis and the macrophage ratio in chronic

wounds, which makes ADMs an intriguing subject for further research. Beyond DFU, ADMs are frequently utilized for a variety of illnesses, such as burn treatment, plastic surgery, and uncommon skin conditions including epidermolysis bullosa [261-264]. By controlling important growth factors and cytokines, ADMs can enhance wound closure and resolution [265-267].

These materials should also successfully encourage polarization toward the anti-inflammatory M2 macrophage phenotype within the context of a macrophage-centered therapy approach.

Dermal replacements' tissue source, decellularization technique, and any ensuing alterations all have a significant impact on their clinical efficacy. The wound microenvironment following implantation can be greatly impacted by both the tissue's origin and the decellularization process [268]. Clinical results and immunological responses may be further changed by further treatments like cross-linking or the addition of bioactive chemicals. Fibroblasts are known to be drawn to collagen-based materials, and it has been demonstrated that monocyte adherence to collagen types I and III controls the release of growth factors, cytokines, and enzymes necessary for proper wound healing [269].

Macrophage behaviour is significantly influenced by surface shape and the presence of active components within scaffolds. Because of this, assessing dermal substitutes' immunomodulatory qualities is especially crucial for diabetic and chronic wounds. It has been demonstrated that some structural characteristics, such as scaffold shape, influence macrophage polarization. For example, fibrous collagen scaffolds with box-shaped pores and closely controlled fiber spacing (40–100 μm) enhance macrophage elongation and differentiation toward the M2 phenotype [270].

Macrophage reaction is also significantly influenced by scaffold pore size. Greater angiogenesis, more VEGF-positive cells, and fewer M1 macrophages have all been linked to larger holes (around 360 μm). Furthermore, immunological activity can be modulated by collagen-functionalizing substances like chondroitin sulfate. It has been demonstrated that higher chondroitin sulfate concentrations increase phagocytic activity, the generation of reactive oxygen species, and the release of pro- and anti-inflammatory mediators by macrophages and monocytes [272].

Different effects on macrophage polarization have been shown by comparative in vitro investigations assessing commercially available dermal replacements. Certain biomaterials, like OASIS® and INTEGRA®, have been shown to increase the expression of M1-associated genes while downregulating anti-inflammatory and pro-fibrotic M2a markers, indicating a

potentially adverse inflammatory profile for chronic wounds. While PriMatrix® produced a mixed response involving both pro- and anti-inflammatory markers, other materials, such AlloMend®, showed little effect on macrophage activation [269-274].

Further evidence that dermal substitutes had varying effects on macrophage recruitment and polarization over time came from in vivo investigations. Some matrices showed a dynamic shift toward M2 polarization at later stages of recovery, while others encouraged persistent macrophage infiltration or a protracted M1-dominant response.

Recent clinical and experimental evidence highlighted the capacity of particular dermal replacements to actively control inflammation in diabetic wounds. In particular, the dermal substitute Nevelia®, composed of a porous type I collagen matrix with a silicone layer, has been shown to significantly increase M2 macrophage polarization in diabetic foot ulcers.

Histological and immunofluorescence analyses showing increased macrophage activation and a shift toward reparative phenotypes corroborated clinical studies showing better healing rates in individuals treated with Nevelia® compared with standard treatment. These findings were consistent across both in vivo and in vitro models, demonstrating that Nevelia® enhances tissue repair by pushing macrophages toward an M2 pro-regenerative state [275-278]. These evidences are scheduled in the table 3.

	Primary Material Composition	Source and Other Components
Nevelia	Porous resorbable double layer matrix 2 mm thickness made of stabilized native collagen type I and a silicone sheet 200 mm thickness mechanically reinforced with a polyester fabric. The extraction procedure and the freeze-drying process allow the structuring of the collagen into a matrix with optimal hydrophilicity, pore structure and pore size (20–125 µm)	Bovine, Native collagen Type I. No glycosaminoglycan (GAG) added to improve cell attachment and proliferation. Glutaraldehyde Cross-linking
Integra	Bilayer system for skin replacement made of a porous matrix of fibers of cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) that is manufactured with a controlled porosity and defined degradation rate. The Integra pore size of 20 to 125 µm allows influx of cells.	Bovine Tendon Type I Collage Shark cartilage -derived chondroitin-6-sulphate (GAG). Glutaraldehyde Cross-linking
PriMatrix	Acellular dermal tissue matrix. comprising of both type I and type III collagen derived from fetal bovine dermis. This matrix is processed in a way to maintains the extracellular matrix in its native and undamaged state while removing all lipids, fats, cells, carbohydrates and non-collagenous proteins.	Fetal Bovine collagen type I and type III collagen. No cross-link
Oasis Wound Matrix	Lyophilized, decellularized porcine small intestine submucosa (SIS). Matrix is derived from a single layer of porcine small intestinal submucosa (SIS) technology. The technology provides an intact three-dimensional extracellular matrix which allows for host cell migration. The SIS is freeze-dried and sterilized with ethylne oxide gas in preparation for clinical use	Porcine small intestine submucosa (SIS). No cross-link

	Primary Material Composition	Source and Other Components
Allomend	Decellularized donated human dermal tissue, with significant removal of cellular debris (including DNA and RNA), proteins and antigens. The process does not require the use of detergents or enzymes, thereby mitigating the possibility of harmful residuals in the tissue. The decellularization process also inactivates microorganisms through cellular disruption. USA only, not available in Europe	Human dermal tissue No cross link
DermaMatrix	Cadaveric human allograft treated with a disinfectant solution that combines detergents with acidic and antiseptic reagents. USA only, not available in Europe	Human dermal tissue No cross link
Dermacell	Decellularized regenerative human tissue matrix allograft processed using proprietary technology that removes at least 97% of donor DNA without compromising the desired biomechanical structure or biochemical properties. USA only, not available in Europe	Human dermal tissue No cross link

Table 3.

Overall, these investigations confirm the role of scaffolds as active regulators of inflammation and tissue regeneration in diabetic wound healing and highlight the significance of scaffold design and immunomodulatory qualities in the effectiveness of dermal substitutes.

Dermal scaffolds are dermal tissue-derived or dermis-like matrices that can either integrate into the host ECM or be cleaved, allowing the healing wound to re-epithelialize and mature [279]. Wound substitutes that duplicate native dermis have been proven to promote wound healing, especially when paired with additional cellular or molecular components [280,281]. Compared to conventional animal models, in vitro models of human skin, such as 3D cell cultures, have made it simpler to assess skin substitutes [282]. Dermal scaffolds and other skin replacements might not completely integrate with the fibroblast-derived extracellular matrix components of the host. This can result in problems during extraction or inhibit desirable ECM/collagen deposition during wound healing remodeling. Dermal scaffolds may enhance treatment results, particularly when wound resolution is poor, according to recent research. [283,284]

5.3 Cell-Supplemented Dermal Matrices

ADMs have shown themselves to be highly adaptable, efficient, and capable doing much more on their own. Skin replacements' therapeutic potential can be boosted by employing stem and progenitor cells with proven healing capabilities, such as mesenchymal stem cells (MSCs) and fibroblasts [285-287]. When combined with ADMs, in vitro co-cultivation of MSCs with skin substitutes has been shown to enhance diabetic wound healing [280,288,289] and provide trophic factors for tissue regeneration [288].

Because of their ability to stimulate wound neovascularization through paracrine action and their anti-inflammatory characteristics, MSCs have a significant restorative effect [290].

The minimal immunogenicity of autologous and allogeneic MSCs is demonstrated by the lack of substantial differences in clinical outcomes [291]. This increases the likelihood of completely allogenic treatments without the chance of tissue rejection. Adipose-derived stem cells are safe and effective when used with ADMs, according to a recent comprehensive study [292]. Active clinical trials have demonstrated the safety and effectiveness of MSC-mediated treatment for DFUs.

Combining additive cellular and molecular components such growth factors, proteases, and MSCs with ADMs has been found to be a safe and effective treatment for DFUs, surpassing existing standards of care.

EXPERIMENTAL STUDY

Effects of topical monocyte infiltration, dermal substitutes application, and their combination on the healing of chronic non-healing diabetic foot ulcers: a case–control study.

6. ABSTRACT

Background: Diabetic foot ulcers (DFUs) represent a major complication of diabetes mellitus and are associated with impaired wound healing. Original regenerative and immunomodulatory therapies have been proposed to improve healing outcomes.
Objective: To evaluate the association between topical monocyte infiltration, dermal substitute application, and their combination with wound healing outcomes in patients with DFUs.
Methods: A retrospective case–control study was conducted including patients with DFUs treated at a specialized wound care center. Adult patients with Wagner grade 1–3 DFUs were included. Cases were patients who achieved favorable healing outcomes (complete healing within 16 weeks or ≥50% ulcer area reduction at 12 weeks), while controls showed unfavorable outcomes such as poor or absent healing. Patients were categorized according to treatment received: topical monocyte infiltration, dermal substitute application, combination therapy, or standard care. Multivariate logistic regression was performed to estimate odds ratios (ORs) for healing.
Results: A total of 32 patients were included (20 cases, 12 controls). Combination therapy was associated with significantly higher odds of favorable healing compared to standard care (OR 5.9231, 95% CI 0.6281-55.855; $p < 0.01$). Both monocyte infiltration and dermal substitutes alone were also associated with improved outcomes, though with lower effect sizes.
Conclusions: The combined use of topical monocyte infiltration and dermal substitutes may synergistically enhance the healing of diabetic foot ulcers. Prospective randomized studies are warranted to confirm these findings.
Keywords: diabetic foot ulcer; wound healing; monocytes; dermal substitute; regenerative therapy

7. INTRODUCTION

Diabetic foot ulcers (DFUs) are a common and severe complication of diabetes mellitus, affecting up to 25% of patients during their lifetime and they are a leading cause of hospitalization and non-traumatic lower limb amputation worldwide. Impaired angiogenesis, chronic inflammation, and immune dysfunction contribute to delayed wound healing and poor clinical outcomes such increased risk of infection and amputation.

Conventional management strategies—including debridement, off-loading, infection control, and advanced dressings—often fail to achieve satisfactory healing. Advanced wound care strategies have been developed to address these pathophysiological mechanisms. In recent years, regenerative approaches such as dermal substitutes and immunomodulatory therapies have gained increasing attention.

Monocytes play a critical role in wound healing by regulating inflammation, promoting angiogenesis, and orchestrating tissue regeneration through macrophage differentiation. Dermal substitutes, on the other hand, provide a temporary extracellular matrix scaffold that supports cell migration and neovascularization.

The potential synergistic effect of combining topical monocyte infiltration with dermal substitutes has not been extensively studied. Despite encouraging preclinical and early clinical evidence, limited data exist regarding the combined use of immunomodulatory and scaffold-based therapies in DFUs. This case–control study aims to assess the association between these therapeutic approaches and healing outcomes in patients with diabetic foot ulcers.

8. MATERIALS AND METHODS

Study Design and Reporting Standards

This retrospective case–control study reviewed clinical data from patients with diabetic foot ulcers (DFUs) treated at Plastic and Reconstructive Surgery Unit of Policlinico of Foggia, a tertiary care center, between January 2024 and December 2025. The study was designed, conducted, and reported in accordance with the Declaration of Helsinki and Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Due to the retrospective nature, informed consent was waived and data were anonymized prior to analysis.

Participants

Consecutive adult patients (≥18 years) with type 1 or type 2 diabetes mellitus and a diagnosis of DFU of at least 4 weeks' duration were screened for inclusion. Eligible wounds were below the ankle and classified as Wagner grade 1–3. Exclusion criteria included critical limb ischemia (ankle–brachial index <0.5 or toe pressure <30 mmHg) not amenable to revascularization, active osteomyelitis requiring surgical intervention at baseline, active systemic infection, immunosuppressive or corticosteroid therapy, active malignancy, absence of severe anaemia (Hb > 8 g/dL), absence of coagulation disorder/thrombocytopenia (PLT > 50,000/L) and pregnancy.

All patients had a minimum follow up of 12 weeks.

Data Sources and Measurements

Medical records were abstracted for demographic, clinical, laboratory, and ulcer-related data using a standardized case report form. Ulcer size was measured through digital planimetry, and area was expressed in square centimeters (cm^2). Ulcer depth, presence of exposed bone or tendon, and signs of infection were documented. Ulcers were graded according to the Wagner classification system. Peripheral arterial disease was assessed by ankle–brachial index and toe pressures. Laboratory data including HbA1c, complete blood count, and inflammatory markers were recorded within 4 weeks of baseline assessment.

Case and Control Definition

- **Cases:** Patients who achieved complete ulcer healing within 16 weeks or ≥50% reduction in ulcer area at 12 weeks.
- **Controls:** Patients with <50% reduction in ulcer area, ulcer worsening, infection requiring hospitalization, or minor amputation within follow-up.

Cases and controls were matched in a 1:1 ratio based on age (±5 years), sex, diabetes duration (±5 years), baseline ulcer area (±20%), and Wagner grade.

Treatment Protocols

Patients were categorized based on the received treatment:

- **Group A:** Topical monocyte infiltration
- **Group B:** Dermal substitute application
- **Group C:** Combination of topical monocyte infiltration and dermal substitute
- **Group D:** Standard care alone (control group)

A. Topical Monocyte Infiltration Protocol

Monocyte/macrophage-based interventions have been proposed to enhance wound healing by modulating inflammation and promoting tissue repair. Peripheral blood monocytes can be enriched and applied topically to increase reparative cellular presence at the wound site.

Topical monocyte infiltration was performed using autologous peripheral blood–derived monocytes. Peripheral blood samples were collected under sterile conditions and processed using standardized cell separation techniques. Monocytes were applied topically to the wound bed following thorough debridement. The procedure was repeated at 45-day intervals, for a total of 3 applications.

Cell Source and Preparation:

All patients underwent cell therapy with a concentrated solution of autologous PB-MNCs, produced by point of care HemaTrate ® Blood Filtration SystemCook Regentec – Indianapolis, Indiana–USA). Participants were treated by injecting a concentrate of MNCs obtained from 120 ml of peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated by by gravity filtration. One hundred and twenty ml of acid-citrate-dextrose (ACD)-anticoagulated peripheral blood was loaded in the upper blood bag and gravity filtration was allowed. The captured MNCs were harvested by sterile saline backflush and immediately implanted. (Figures 7,8).

All the procedures were performed in the operatory/surgery room with anaesthesiologic support (propofol and/or peripheral block).

Application Procedure:

- Following wound bed preparation (sharp debridement and cleaning), the monocyte suspension was applied directly to the wound surface and perilesional tissue.
- Multiple microinjections (e.g., 0.2–0.3 mL) were performed around the wound margins and base, at intervals of 1–2 cm and to a mean depth of 1.5–2 cm, using a 25–27 G needle to ensure even distribution, as described in similar mononuclear cell therapy studies. Figures 9,10.

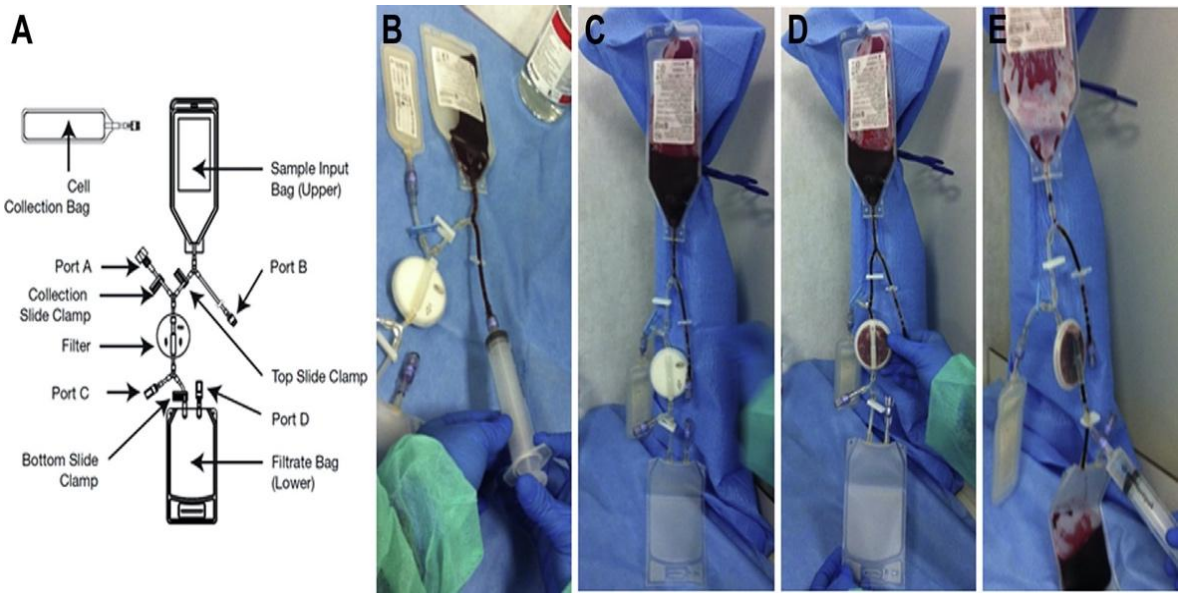


Figura 7.

Figure 8.

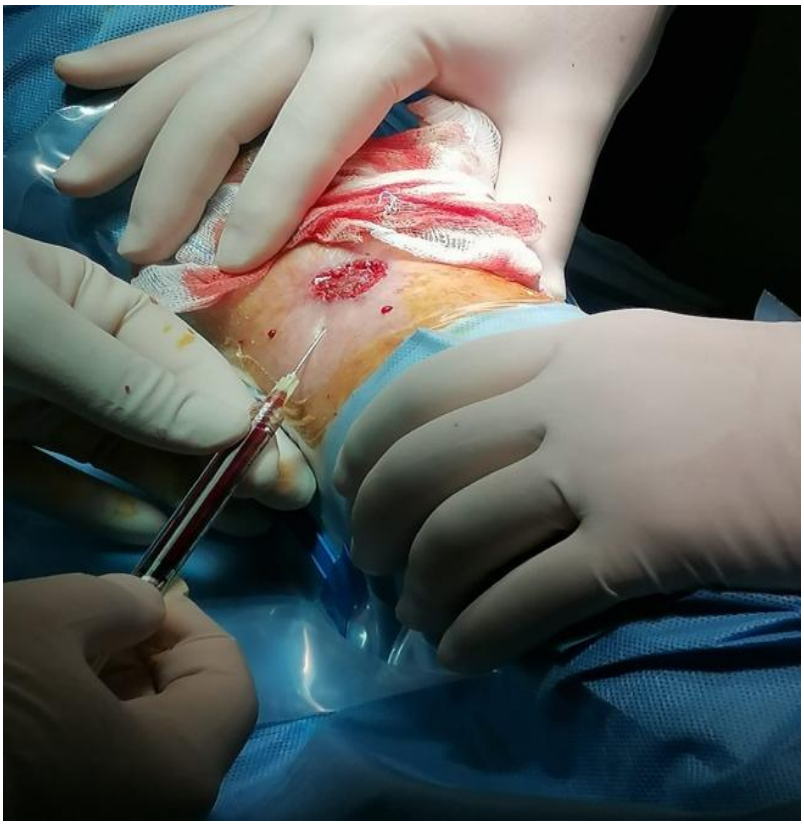


Figure 9.

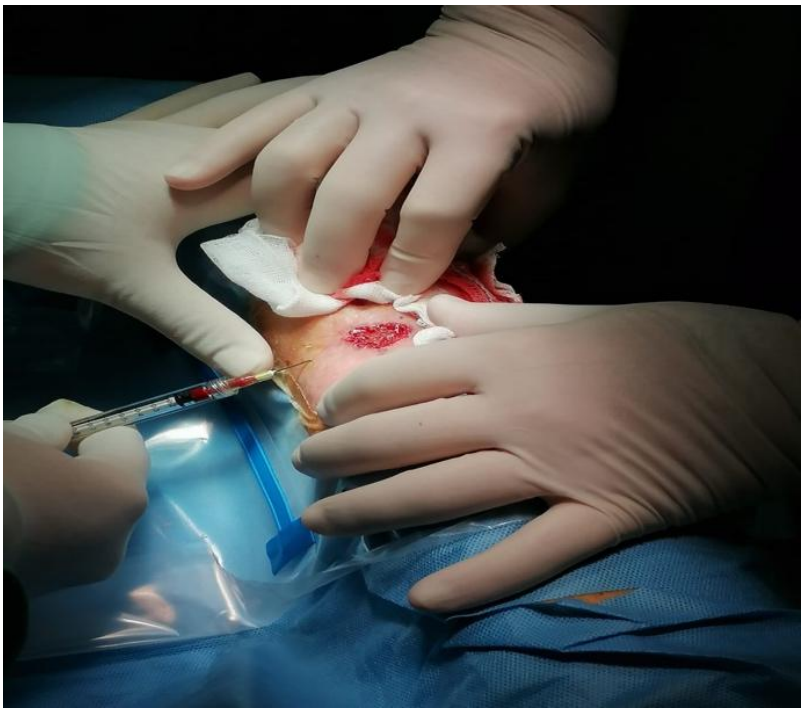


Figure 10.

B. Dermal Substitute Application

Dermal substitute products (e.g., acellular matrices or collagen-based scaffolds) were selected based on wound characteristics and availability. In particular we used in four cases Integra Dermal Regeneration Template® (LifeScience) and in the other four cases Pelnac® (Gunze). Following debridement and haemostasis, the fenestrating and meshing devices were placed to cover the wound bed and secured using standard fixation techniques and covered with non-adherent dressings. Dressings were applied per manufacturer and clinical protocol, with changes every **7** days or as clinically indicated.

C. Combination Therapy

Patients in the combination group received topical monocyte infiltration immediately prior to dermal substitute application (4 Integra® and 4 Pelnac®) to potentially enhance local immune modulation before matrix placement.

D. Standard Care

All patients received standard evidence-based wound care, including:

- Sharp debridement
- Moist wound dressings
- Pressure off-loading using removable or non-removable devices
- Infection management based on microbiological cultures
- Optimization of glycemic and vascular status

Follow-Up and Outcome Measures

Patients were followed weekly or biweekly in the outpatient clinic. Ulcer dimensions were measured at each visit. Healing status, adverse events, signs of infection, and need for additional interventions were recorded. Primary outcome was favourable healing (complete epithelialization or ≥50% area reduction). Secondary outcomes included time to healing (weeks), reduction of ulcer area (%), incidence of local infection, need for minor amputation, and ulcer recurrence within 6 months.

Bias and Confounding Control

Matching and multivariate adjustment were used to control for confounders including age, sex, duration of diabetes, baseline ulcer size, HbA1c, and peripheral arterial disease. Sensitivity analyses were conducted excluding patients with major comorbidities.

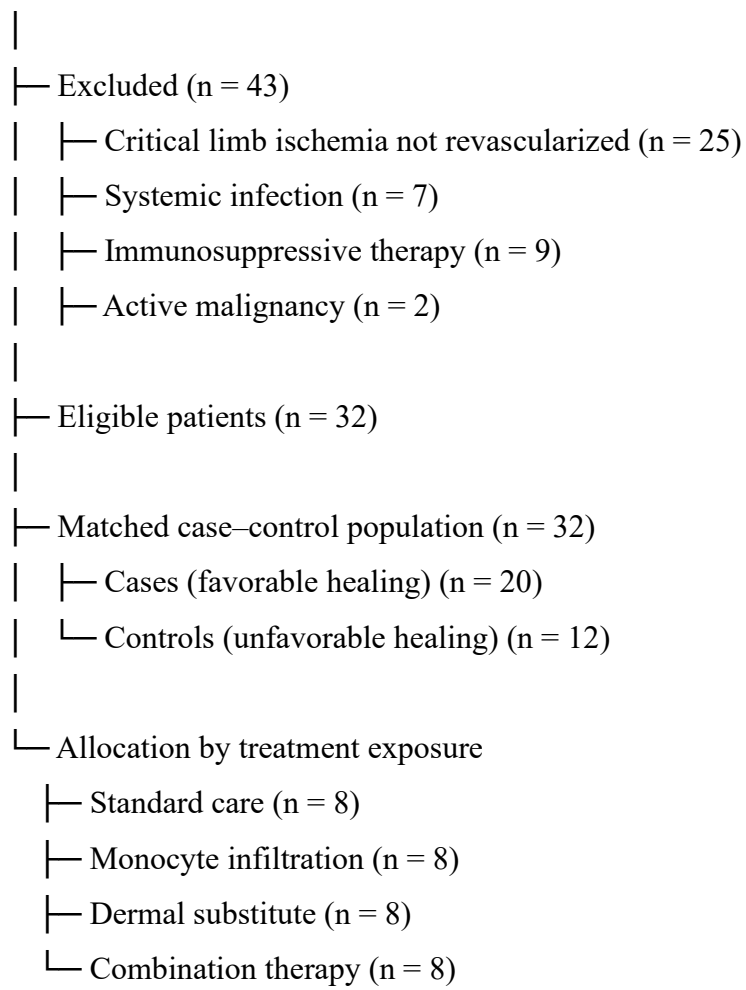
Statistical Methods

Analyses were performed using statistical software (SPSS version XX; IBM Corp., Armonk, NY). Continuous data were reported as mean ± SD or median (IQR), and compared using t-tests or Mann–Whitney U tests as appropriate. Categorical data were expressed as frequencies and percentages and were compared using chi-square or Fisher's exact tests. Multivariate logistic regression was used to calculate adjusted odds ratios (ORs) with 95% confidence intervals (CIs) to estimate the association between treatment and favorable healing outcomes. Covariates included age, BMI, HbA1c, smoking status, peripheral arterial disease, baseline ulcer area, and treatment modality. A two-sided p-value <0.05 was considered statistically significant.

9. RESULTS

Study Population

During the study period, a total of 75 patients with diabetic foot ulcers were screened for eligibility. After application of inclusion and exclusion criteria, 32 patients were included in the matched case–control analysis, comprising 20 cases with favorable healing outcomes and 12 controls with unfavorable outcomes. See the flow diagram.

Patients assessed for eligibility (n = 75)


Baseline demographic, metabolic, and ulcer-related characteristics were comparable between cases and controls, reflecting the effectiveness of the matching procedure. No statistically significant differences were observed in age, sex distribution, smoking, diabetes duration, baseline ulcer area, Wagner grade, HbA1c levels, or BMI **(Table 4).**

Table 4. Baseline characteristics of the study population

Characteristic	Cases (n=20)	Controls (n=12)	p-value
Age (years)	75.2 ± 6.3	77.5 ± 9.4	0.55
Male sex, n (%)	13 (65)	8 (66.67)	0.96
Smoking, n (%)	11 (55)	6 (50)	0.61
BMI	31.3± 2.9	32.1± 3.3	0.59
Diabetes duration (years)	16.7 ± 8.9	18.6 ± 8.5	0.29
HbA1c (%)	7.52 ± 0.71	7.63 ± 0.79	0.38
Wagner grade 2–3, n (%)	18 (90)	10 (83.3)	0.78
Baseline ulcer area (cm^2)	25.2 ± 6.3	31.3± 9.4	0.66

Distribution of Treatment Modalities

The distribution of treatment exposure differed between cases and controls (Table 5). Combination therapy (topical monocyte infiltration plus dermal substitute) was more frequently observed among cases, whereas standard care alone was more prevalent among controls.

Specifically:

- 35% of cases received combination therapy compared with 8.3% of controls,
- 30% of cases received monocyte infiltration alone compared with 16.7% of controls,
- 30% of cases received dermal substitutes alone compared with 16.7% of controls,
- 5% of cases received standard care only compared with 58.3% of controls.

Table 5. Treatment modalities among cases and controls

Treatment group	Cases n:20 (%)	Controls n:12 (%)
Combination therapy	7 (35)	1 (8.3)
Monocyte infiltration	6 (30)	2 (16.7)
Dermal substitute	6 (30)	2 (16.7)
Standard care	1 (5)	7 (58.3)

Primary Outcome: Favorable Ulcer Healing

The proportion of favorable healing differed significantly between treatment groups (Figure 11).

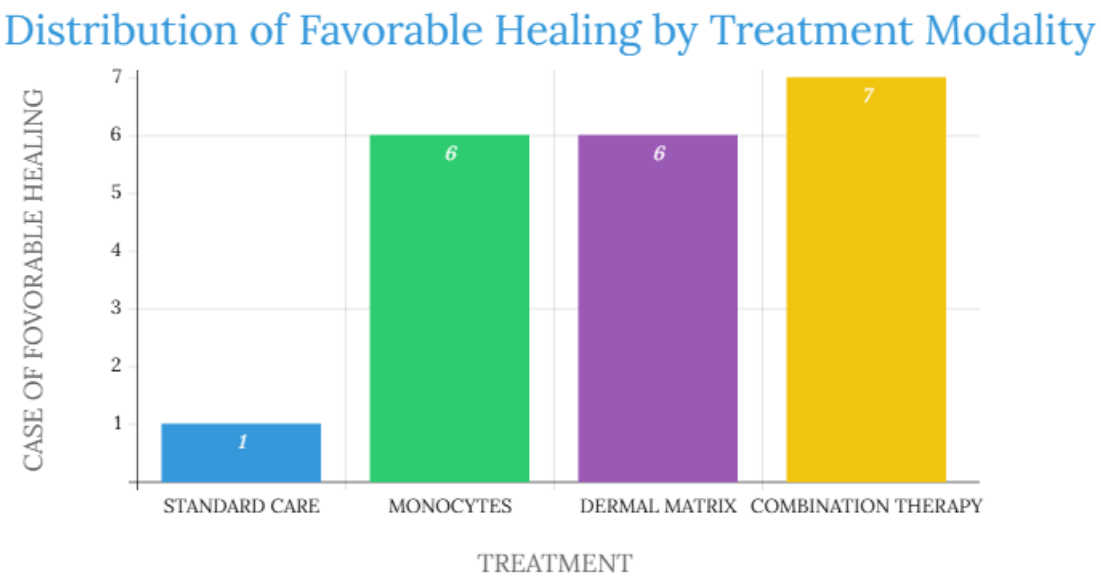


Figure 11. Distribution of Favorable Healing by Treatment Modality (Bar Plot)

Bar plot showing the percentage of patients with favorable healing outcomes according to treatment modality: standard care, topical monocyte infiltration, dermal substitute, and combination therapy.

It shows the proportion of patients achieving favorable healing outcomes across the four treatment groups. Patients receiving combination therapy exhibited the highest rate of favorable healing (87.5%), followed by monocyte infiltration alone (75%) and dermal substitute alone (75%). The lowest proportion of favorable healing was observed in the standard care group (12.5%). The difference between combination therapy and standard care was statistically significant ($p < 0.01$).

In univariate analysis, all advanced treatment modalities were associated with higher odds of favorable healing compared with standard care. The strongest association was observed for combination therapy. See table 6.

Table 6. Multivariate logistic regression analysis for favorable healing

Treatment	OR	95% CI	p-value
Monocyte infiltration	2.1429	0.3562-12.8903	0.4051
Dermal substitute	2.1429	0.3562-12.8903	0.4051
Combination therapy	5.9231	0.6281-55.855	< 0.01
Standard care	0.0376	0.0037-0.3803	0.0055

In multivariate logistic regression analysis adjusting for HbA1c, smoking status, peripheral arterial disease, body mass index, and baseline ulcer area, the associations remained statistically significant (Table 3).

Compared with standard care (OR 0.0376, 95% CI 0.0037-0.3803; p=0.0055):

- topical monocyte infiltration and dermal substitute application were associated with increased odds of favorable healing and showed a similar association (OR 2.1429, 95% CI 0.3562-12.8903; p = 0.4051),
- combination therapy demonstrated the highest likelihood of favorable healing (OR 5.9231, 95% CI 0.6281-55.855; p < 0.01).

When combination therapy was directly compared with monocyte infiltration or dermal substitute alone, a trend toward superior efficacy was observed, suggesting a potential synergistic effect. Odds ratios confirmed the superior association of combination therapy with healing (Figure 12).

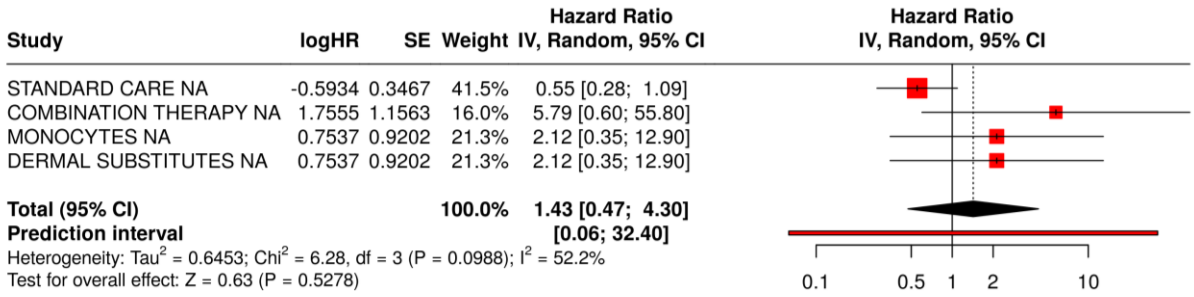


Figure 12. Association Between Treatment Modality and Favorable Healing (Forest Plot) derived from multivariate logistic regression analysis.

Forest plot showing adjusted odds ratios (ORs) and 95% confidence intervals for favorable ulcer healing according to treatment modality. Odds ratios were adjusted for age, HbA1c, smoking status, peripheral arterial disease, baseline ulcer area, and body mass index. Standard care was used as the reference group.

Secondary Outcomes

Combination therapy was also associated with shorter time to healing and lower rates of infection and minor amputation.

Time to Healing

Median time to complete healing was shorter in patients receiving combination therapy compared with other groups. Median healing time was:

- 16 weeks in the combination therapy group (range: 15-18 weeks),
- 23 weeks in the monocyte infiltration group (range: 22-25 weeks),
- 21 weeks in the dermal substitute group (range: 20-23 weeks),
- 39 weeks in the standard care group (range: 37-42 weeks).

The difference between combination therapy and standard care reached statistical significance ($p = 0.05$). Time-to-healing analysis demonstrated faster ulcer closure with combination therapy (Figure 3).

Ulcer Area Reduction

At 12 weeks, mean percentage reduction in ulcer area was significantly greater in patients receiving combination therapy compared with standard care (69% vs 7%, $p < 0.01$). Monocyte infiltration and dermal substitute therapy alone were also associated with greater area reduction, although with wider variability.

Infection and Amputation Rates

Local wound infection occurred less frequently in the combination therapy group compared with standard care (0% vs 12.5%). Similarly, the need for minor amputation was lowest among patients receiving combination therapy (0%) and highest in the standard care group (25%). Although the study was not powered primarily for these outcomes, the observed trends were clinically relevant.

Ulcer Recurrence

Among patients achieving complete healing, ulcer recurrence within 6 months occurred in 0% of the combination therapy group, compared with 16.7 % in the monocyte infiltration group, 0% in the dermal substitute group, and 0% in the standard care group.

Safety and Adverse Events

No serious adverse events related to topical monocyte infiltration were recorded. Mild transient local pain or erythema at the injection site was reported in 100% of applications and resolved spontaneously. No systemic adverse reactions were observed. Dermal substitute application was well tolerated, with no device-related complications requiring treatment discontinuation.

10. DISCUSSION

This case–control study suggests that topical monocyte infiltration and dermal substitute application are each associated with improved healing outcomes in patients with diabetic foot ulcers (DFUs), with the greatest benefit observed when the two approaches are combined. These findings support the hypothesis that simultaneously targeting immune dysregulation and extracellular matrix deficiency—two hallmarks of chronic diabetic wounds—may result in synergistic therapeutic effects.

The observed benefit may be explained by complementary mechanisms: monocytes modulate inflammation and promote angiogenesis, while dermal substitutes provide a structural scaffold that enhances cellular integration and tissue regeneration.

Biological Rationale and Mechanistic Considerations

Chronic DFUs are characterized by a prolonged inflammatory phase, impaired macrophage polarization, reduced angiogenic signaling, and dysfunctional extracellular matrix remodeling [293,294]. In diabetic wounds, macrophages often remain locked in a pro-inflammatory (M1-like) phenotype, leading to persistent tissue damage and delayed progression to the proliferative phase of healing [200].

Monocytes and monocyte-derived macrophages play a central role in coordinating wound repair through regulation of inflammation, angiogenesis, and fibroblast activity [212]. Topical monocyte infiltration may enhance the local pool of immune cells capable of differentiating into reparative macrophages, thereby restoring the balance between inflammation and tissue regeneration. Experimental and early clinical studies have demonstrated that monocyte/macrophage-based therapies can increase expression of pro-angiogenic and pro-fibrotic mediators such as VEGF, PDGF, and TGF-β, all of which are known to be deficient in diabetic wounds [295,296].

Dermal substitutes address a different but complementary aspect of impaired healing. By providing a temporary extracellular matrix scaffold, they facilitate cellular migration, neovascularization, and organized collagen deposition [297]. However, their effectiveness may be reduced in wounds with unresolved inflammation or insufficient cellular activity. The superior outcomes observed with combination therapy in this study suggest that immunomodulation by monocytes may optimize the wound microenvironment, enhancing dermal substitute integration and function.

Comparison with Existing Literature

Several randomized and observational studies have demonstrated that dermal substitutes improve healing rates and reduce time to closure in DFUs when compared with standard care alone [298–300]. Meta-analyses have further confirmed their role as effective adjuncts in advanced wound management, particularly in complex or non-healing ulcers [301].

Evidence supporting immune cell–based therapies in DFUs is more limited but growing. Preclinical studies and small clinical series have shown that modulation of macrophage activity can accelerate wound healing and improve angiogenesis in diabetic models [213,296]. Nevertheless, clinical data on combined regenerative strategies remain scarce.

To our knowledge, few studies have evaluated the concurrent use of immune-based therapies and dermal scaffolds in DFUs. Our findings are consistent with experimental data suggesting that scaffold-based therapies yield superior outcomes when paired with strategies that enhance cellular recruitment and immune regulation [302]. This study therefore contributes novel real-world evidence supporting a multimodal regenerative approach.

Clinical Implications

From a clinical standpoint, the results highlight the potential role of combination regenerative therapy in the management of complex DFUs, particularly in patients with large, chronic, or treatment-refractory ulcers. Current international guidelines emphasize the importance of individualized, multidisciplinary care for DFUs, and advanced therapies are increasingly recommended when standard measures fail [303].

Although cost and resource availability remain important considerations, improved healing outcomes may offset initial expenses by reducing infection rates, hospital admissions, and amputations—outcomes known to significantly impact patient quality of life and healthcare systems [4].

Study Limitations

Despite matching and multivariate adjustment, residual confounding cannot be excluded. Variability in treatment protocols, including differences in dermal substitute type and timing of monocyte infiltration, may also have influenced outcomes.

Furthermore, the absence of immunological or molecular biomarkers limits mechanistic interpretation. Long-term outcomes such as durability of healing and recurrence beyond six months were not fully assessed and warrant further investigation.

Future Research Directions

Prospective randomized controlled trials are needed to confirm these findings and define standardized protocols for combination therapy. Future studies should incorporate biomarkers of inflammation and immune activity to better understand mechanisms of action and identify patients most likely to benefit.

Cost-effectiveness analyses and stratified studies based on ulcer severity, ischemic status, and metabolic control will be essential to guide clinical implementation. Integration of combination regenerative strategies into multidisciplinary DFU care pathways represents a promising area for further research.

11.CONCLUSION

In conclusion, this study provides real-world evidence that combining topical monocyte infiltration with dermal substitutes may enhance healing outcomes in diabetic foot ulcers more effectively than standard care or either approach alone. The synergistic effect likely reflects complementary biological mechanisms: immunomodulation and angiogenesis driven by monocytes, and structural support provided by dermal scaffolds. By addressing both immune dysregulation and structural deficits, this multimodal strategy represents a promising direction in advanced wound care and aligns with the translational focus of contemporary regenerative wound care paradigms and management, and merits further investigation. These results support the rationale for prospective randomized controlled trials to confirm efficacy and define optimal treatment protocols.

12.REFERENCES

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