Macrophages in Diabetic Wounds Healing

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Macrophages play a prominent role in wound healing. In the early stages, they promote inflammation and remove pathogens, wound debris, and cells that have apoptosed. Later in the repair process, they dampen inflammation and secrete factors that regulate the proliferation, differentiation, and migration of keratinocytes, fibroblasts, and endothelial cells, leading to neovascularisation and wound closure. The macrophages that coordinate this repair process are complex: they originate from different sources and have distinct phenotypes with diverse functions that act at various times in the repair process. Macrophages in individuals with diabetes are altered, displaying hyperresponsiveness to inflammatory stimulants and increased secretion of pro-inflammatory cytokines. They also have a reduced ability to phagocytose pathogens and efferocytose cells that have undergone apoptosis. This leads to a reduced capacity to remove pathogens and, as efferocytosis is a trigger for their phenotypic switch, it reduces the number of M2 reparative macrophages in the wound. This can lead to diabetic foot ulcers (DFUs) forming and contributes to their increased risk of not healing and becoming infected, and potentially, amputation.

Keywords: macrophage ; inflammation ; diabetic foot ulcer ; chronic wound ; efferocytosis ; phenotype ; infection

1. Introduction

Non-healing ulcers are the most common cause of amputation and it is estimated that, worldwide, a leg is amputated due to diabetes every 30 seconds [1][2][3]. Approximately 84% of lower leg amputations will be in patients that have acquired a DFU prior to amputation [4][5].

Macrophages are one of the key cells that regulate the wound repair process [6][7]. Wounds without macrophages have delayed re-epithelialisation, impaired angiogenesis, reduced collagen deposition, and reduced cell proliferation [8]. They are not a homogenous population of cells and several different combinations of phenotypes with distinct functions present at different times in the repair process [6][9][10]. Macrophage function is altered in people with diabetes such that they have a reduced capacity to clear an infection and their function in the later stages of repair is altered, leading to a delay in the repair process [6][11][12][13][14][15].

2. Repair of an Acute Wound

Wound healing is complex and involves a series of coordinated and overlapping stages that need to come together for skin integrity to be restored [16][17]. These stages involve a range of distinct but often interlinked processes that include coagulation, inflammation, migration, proliferation, regeneration, and remodelling of the extra cellular matrix (ECM) [16][17]. These must all occur in a specific sequence, at a specific time and for a precise amount of time for an efficient and timely repair process. Delays in these steps can have detrimental outcomes, such as increased scar formation or the formation of non-healing wounds, and delays in closing the wound can lead to an increase in the risk of infection.

Wound healing begins first with haemostasis, followed by an inflammatory phase, a proliferative phase that leads to re-epithelization, and a remodelling phase, during which the scar matures [16][17]. While there is overlap in the phases, each phase is necessary for the next to be completed.

In the first stage of the repair process, haemostasis stems the flow of blood from the damaged tissue. This takes place in seconds to minutes and is achieved by vasoconstriction and the formation of a clot [18]. In the inflammatory phase, a number of immune cells, including tissue resident macrophages, and neutrophils and monocytes recruited to the site of injury from the blood, work with numerous cell types in and surrounding the injured skin to orchestrate the repair process [19][20].

The macrophages' role in the inflammatory phase is to clear pathogens and cell debris and to regulate, either directly or indirectly, the next stage in the repair process, the proliferative stage, through their ability to secrete cytokines and growth

factors that stimulate keratinocytes, fibroblasts, and endothelial cells to proliferate, differentiate, and migrate [15][21]. This culminates in a new extracellular matrix (ECM), which allows cells to migrate over and re-epithelialise the wound and for new blood vessels to form and populate the wound. During the remodelling phase, macrophages secrete enzymes that remodel and alter the structure of the ECM and the wound [15][21].

3. The Inflammatory Phase of Wound Healing and Macrophages

Studies in mice show that wound monocyte/macrophage numbers dramatically increase in an acute wound and then remain high from day 2 until around day 5, although this timing is wound-size dependent, with larger wounds taking longer [22]. The numbers then begin to decrease as re-epithelialisation occurs, falling to relatively low levels by day 7 and then returning to steady-state levels by day 14 [22]. Over this day 2–7 period, these cells have many different functions and so are responsible for a wide range of effects: they contribute to the initiation of inflammation but also resolve inflammation; they remove any pathogens that may have entered the wound; they clear up the neutrophils containing dead and partially digested microbes which have apoptosed and the remaining cell and ECM debris; they orchestrate the repair process through their secretion of cytokines and other factors, such as TGF- β1 and VEGF, that promote angiogenesis and attract fibroblasts that secrete extracellular matrix components necessary to rebuild the tissue; and they secrete enzymes that play a role in remodelling the ECM [7][16].

Source and Plasticity of Wound Associated Macrophages

The monocytes/macrophages observed in wounds are derived from a number of sources. Initially, they consist of the tissue resident macrophages located in the skin prior to injury, of which there are two kinds; in the epidermal layer, these are predominantly Langerhans cells; in the dermal layer, they are mainly dermal macrophages [9].

Next on scene is the early wave of monocytes, which enter through microhaemorrhages caused by blood vessels damaged during the injury itself [22]. Factors released from platelets and in the wound environment trigger their differentiation into macrophages [23]. In the wound, these cells would also be exposed to alarmins, which include danger-associated molecular patterns (DAMPs) such as HMGB-1 or ATP released from cells after tissue damage, that activate these cells to become M1 macrophages [24]. Once activated, they can contribute to the initial proinflammatory phase and help recruit bone marrow-derived monocytes from the circulation 24 h later in a process that involves the chemokine receptor present on macrophages, CX3CR1 [22]. To accommodate the recruitment of a significant number of monocyte cells from the blood, there is an increase in myeloid lineage committed multipotent progenitors and monocytes in bone marrow that results in a 70% increase in monocytes in circulation on day 2, with their levels returning to steady-state levels after around day 4 [22][25]. These monocytes are classified as either classical/pro-inflammatory monocytes that are $CD14^{+}CD16^{-}$ capable of differentiating into pro-inflammatory M1 macrophages or anti-inflammatory monocytes that are $CD14^{low}/CD16^{+}$ that give rise to mostly M2 macrophages [19]. In mouse models of wound repair, circulating monocytes can also be divided into two groups: $CX3CR1^{low}CCR2^{+}$ $Ly6C^{+}$ and $CX3CR1^{high}CCR2^{-}Ly6C^{-}$ [7]. The first group produces pro-inflammatory cytokines and is the first to enter a wound, with the second entering later [7]. Factors in the local wound environment stimulate these bone marrow-derived monocytes to differentiate into macrophages and the precise combination of these factors is what appears to dictate macrophage phenotype, although the original phenotype of the bone marrow monocytes may also dictate the macrophage phenotype. In addition, some immune cells can proliferate in the wound [26]. It is the inflammatory monocytes/macrophages derived from the circulating monocytes, but not the mature wound macrophages, that are able to proliferate in the wound and, at the mid-stages of healing, these cells constitute around 25% of macrophage population.

4. Macrophage Dysregulation and the Repair Process

One common factor in all ulcers, both diabetic and non-diabetic, along with the inability to heal, is the dysregulation of the inflammatory phase of wound healing [15]. In diabetes, this is compounded by the fact that high levels of glucose seen in diabetes alters cells of the immune system, including macrophages, one of the key orchestrators of the repair process and defence against infection [13][27][28][29]. This dysregulation potentially contributes further to non-healing wounds and to the increased risk of infections. In individuals with diabetes, M1 macrophages drive the elevated and prolonged non-resolving inflammatory phase seen in DFUs [30]. Approximately 80% of cells at the chronic wound margin are pro-inflammatory M1 macrophages and there are compelling data from mouse and human studies to suggest that the shift to M2-like phenotypes may not proceed as expected, despite this shift being necessary for the repair process to progress [29][30].

Macrophage Function Is Altered in People with Diabetes

What is happening to the macrophage to cause this dysregulation of the repair process in DFUs? A number of factors contribute to the altered macrophage phenotypes, such as infection in the wound, high glucose, and advanced glycosylation end products (AGEs). Sustained exposure to high glucose levels in vitro produces macrophages that have reduced phagocytic activity, and therefore a reduced ability to clear an infection, reduced nitric oxide production, and cells that secrete more proinflammatory cytokines when stimulated [31][32]. Similar experiments with macrophages sourced from mice and individuals with diabetes show these macrophages are hyperresponsive to inflammatory stimulants and therefore secrete more pro-inflammatory cytokines, have difficulties switching to the more reparative M2-like phenotypes and, when looked at within the wound setting, prolong the inflammatory phase (**Figure 1**) [11][27][29][33][34].

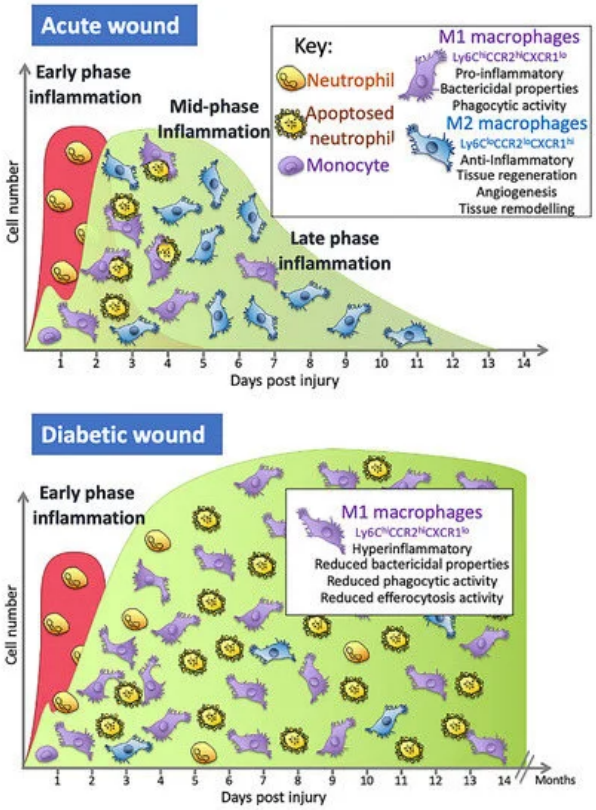

Figure 1. During the early stages of inflammation in an acute wound, the wound is predominantly populated with M1 macrophages that are pro-inflammatory. A switch in the predominant phenotype is seen later in the inflammatory phase, where the majority of macrophages are M2. In wounds of people with diabetes, macrophage numbers are altered and secrete more pro-inflammatory cytokines, have reduced phagocytic and efferocytosis abilities and are less likely to switch to the M2 phenotype in the mid- to late-inflammation phase that is as seen in normal acute wounds.

This switch in macrophage phenotype is essential for a timely repair process. Increasing the levels of M2 macrophages in wounds leads to an increase in cells secreting the anti-inflammatory cytokines that dampen inflammation and an increase in the growth factors necessary for proliferation, migration, and the repair process [16]. The importance of this switch has been seen in many studies indicating that, depending on the phenotype and the timing of their actions, macrophages may have a detrimental or positive effect, either damaging tissue or aiding the repair process. Studies using diphtheria toxin-mediated macrophage depletion models in mice show that M2 macrophages ($CD206^+/CD301b^+$) are critical for activation of reparative processes during the mid-stages of wound healing [35]. Blocking the switch to M2 macrophages in wounds using the inhibitor GW2580, which blocks the CSF-1 signalling cascade that drives macrophage differentiation, proliferation, and survival, results in persistent inflammation, less collagen, and increased M1 macrophages in the wound [36], while transplanting day 5 wound M2 ($CD206^+/CD301b^+$) macrophages to day 3 wounds in mice leads to significantly increased proliferation and fibroblast repopulation [36]. Collectively, these and other studies show that this M1 to M2 switch is important to a timely repair process and that the impaired switching to M2 phenotypes seen in diabetic wounds is associated with poor angiogenesis, decreased collagen deposition, and reduced wound closure (**Figure 1**) [34].

Hyperglycaemia leads to an increase in advanced glycation end products (AGEs), which are proteins or lipids glycated due to their exposure to sugars [32]. The diabetic wound environment accumulates both AGEs and macrophages that express high numbers of the receptor for AGEs (RAGE). In vitro, the addition of AGEs to M1 macrophages reduces their ability to phagocytose. The phagocytosis of apoptosed cells (efferocytosis) is a key process that activates the switch to M2 macrophages.

5. Concluding Remarks

The dysregulation of macrophage functions in diabetic individuals, such as the reduced ability to phagocytose pathogens and apoptosed cells, leads to a reduced ability to switch to the more reparative M2 phenotype. This results in an increase in the number macrophages being recruited to the wound, and their activation to become M1 macrophages. With few M2 macrophages present this leads to a delay in wound closure and in many cases further damage to the tissue. Further compounding this is the reduced ability to clear an infection, increasing the risk of amputation as the ultimate treatment to DFUs.

There are many questions left to answer before we fully understand the repair process, such as what are the exact phenotypes needed in DFUs to promote effective wound healing and can we alter macrophages to adopt those phenotypes? As our understanding of what is happening to macrophages in wounds increases, so will the opportunities to design and test new potential therapies that might complement existing treatments in future.

It is unclear exactly what control of wound inflammation might look like. It could be by the use of biomaterial dressings that might modulate the immune system or through the modulation of the skin's own host defence peptides to clear an infection and alter the inflammatory phase [37][38][39][40][41]. It might be as simple as reducing the number of monocytes/macrophages entering the wound by repurposing of drugs, for example the use of anti-integrin antibodies to reduce the macrophage load [42]. Alternatively, it might be altering the phenotype of the macrophage, reducing the factors such as TNF in the wound with an anti-TNF antibody to reduce inflammation, or the design of new therapies to dampen wound inflammation [43]. This would be in addition to wound dressings, potential debridement, offloading, and control of factors such as hyperglycaemia, and preventing and treating any infection that might occur in the wound.

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