Altered macrophage phenotypes impair wound healing

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ABSTRACT
One of the defining features of chronic wounds is their high levels of inflammation. Patients present with high levels of inflammation, around 80%, of cells at the wound margin being macrophages and with wound fluid laden with pro-inflammatory cytokines. The latter is in part responsible for preventing wound closure, along with the low levels of growth factors. In healthy acute wounds, two types of macrophages can be found: pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages, with more M1 cells present early post-injury and M2 appearing later to regulate repair and wound closure. This pro-repair M2 phenotype, that secretes a range of mediators including growth factors that regulate re-vascularisation and closure of a wound, is lacking in chronic wounds, leading to excessive inflammation, enhanced degradation within the wound, reduced matrix deposition and lack of closure. This review will discuss the alterations seen in chronic wound macrophages, how this might affect the repair process and some potential reasons for their dysregulation.

Keywords: Inflammation, macrophage, M1 and M2, wound.

INTRODUCTION
Most wounds heal or at least close relatively quickly, but for some people wounds can take much longer to close, with wounds that do not heal in a timely manner (4–6 weeks) being defined as chronic. These wounds typically occur on the lower limbs of mainly ageing patients, many of whom have an underlying chronic disease, such as diabetes or chronic venous insufficiency, that can contribute to wound chronicity. Impaired healing can last from months to years and have an enormous impact on patient quality of life. In addition to being painful, wounds are often malodorous, can result in loss of mobility and social isolation, leading to depression in some cases. Treatments can be unreliable with their outcomes often unpredictable. Worldwide, complications from these chronic wounds lead to one major amputation every 30 seconds, which amounts to over 2500 limbs lost a day, with one in four amputees requiring contralateral amputation and/or re-amputation. For diabetic foot ulcer patient amputees the mortality rate is high, at 39–80% five years post-amputation. Compare this to the 10% mortality rate for breast cancer patients at five years (data from 2009–2013) post-diagnosis then this figure is alarming. The financial impact of chronic wounds is also substantial, with wound management accounting for around 3% of the total health care expenditure in developed countries. It is estimated that $2.85 billion per annum is spent on chronic wounds in Australia alone and with the increasing age and population with diabetes, the number of patients with chronic wounds is expected to rise dramatically. While in-roads have been made into understanding the repair process, there is still much to understand about why these wounds occur, how they differ from an acute wound and how they might be altered to improve wound healing. Improved understanding will aid in the targeting of therapies to prevent or speed up healing of chronic wounds.

REPAIRING SKIN
Wound healing is a highly complex, multistage process that starts the instant an injury occurs. It involves skin cells such as keratinocytes, fibroblasts, endothelial cells, the recruitment of immune cells, the laying down of new extracellular matrix...
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As mentioned above, wound macrophages play a range of roles and regulate the function of numerous other cells in the wound. These functions need to be coordinated in a timely and sometimes sequential fashion. Macrophages do this by altering their phenotype through cues in the wound environment. Broadly speaking, macrophages have been classified as pro-inflammatory M1 or anti-inflammatory/repairative M2 macrophages (Table 1)\textsuperscript{11-13}. This classification is an oversimplified one as multiple M2 phenotypes exists, although not all exist in wounds. \textit{In vivo}, there is a continuum of phenotypes between M1 and M2 that evolve as wound healing progresses (Figure 1). M1 macrophages, found in the early stages post-wounding, are activated by stimulants in the local wound environment; this can include interferon-\(\gamma\) (IFN-\(\gamma\)), tumour necrosis factor (TNF) and damage-associated pattern molecules (DAMPs). The latter are biomolecules released by damaged ECM and injured cells that act as endogenous danger signals to tell the immune system that an injury has occurred. These M1 macrophages are prolific producers of pro-inflammatory cytokines, such as TNF and interleukin-6 (IL-6), and other mediators that regulate the early stages of wound healing (Table 1). They are also proficient phagocytes and engulf spent neutrophils. This phagocytosis step, known as efferocytosis, along with environmental factors (IL-4 or IL-13) can push M1 macrophages into an M2 anti-inflammatory/repairative phenotype. M2 macrophages can be separated \textit{in vitro} into four phenotypes, M2a-d, based on their stimulation, surface markers and the cytokines, chemokines, growth factors and other mediators they produce and, in most cases, secrete (Table 1). M2 macrophages produce anti-inflammatory cytokines that act to switch off inflammation in the wound. They also act as a master regulator of repair by producing various growth factors, such as vascular endothelial growth factor (VEGF) that regulates formation of new blood vessels, and transforming growth factor-\(\beta\) (TGF-\(\beta\)), which plays a key role in regulating proliferation, migration, differentiation, and ECM production during the proliferation and maturation phases of repair.

MACROPHAGES ARE DYSREGULATED IN CHRONIC WOUNDS

In an acute wound at the start of the inflammatory phase, approximately 85\% of macrophages have an M1 pro-
inflammatory phenotype but by five days post-injury only 15% of the macrophages found in the wound are M1 macrophages\textsuperscript{14,15}. In chronic wounds this shift in phenotypes does not occur and approximately 80% of the cells found at the chronic wound margin are macrophages with an M1 pro-inflammatory phenotype\textsuperscript{16-19} (Figure 2). As a result, patients present with high levels of pro-inflammatory cytokines, such as TNF and IL-6, as well as iNOS in their wound fluid\textsuperscript{17,20,21}. These high levels of TNF and other pro-inflammatory mediators leads to the recruitment of more immune cells and it disrupts both the levels and delicate balance between matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs)\textsuperscript{22}. In an acute wound these MMPs play a role in the remodelling of the ECM and their activity is finely tuned by their inhibitors, the TIMPs. However, when these MMPs have free reign, as seen in chronic wounds, either by their overexpression and/or a reduction in their inhibitors, their excess proteolytic activity leads to the destruction of the ECM scaffold that cells require to repopulate and close the wound\textsuperscript{22}. This impaired wound healing has been studied in mice and the addition of recombinant TNF into mouse wounds delays the repair process and leads to senescence of fibroblasts\textsuperscript{17}. A number of therapeutic antibodies that inhibit TNF exist including Infliximab and Adalimumab. When used topically (Infliximab) or subcutaneously (Adalimumab) in chronic venous ulcers (CVU) of patients whose wounds failed to respond to any previous conventional treatment it improved wound closure\textsuperscript{23,24}. Although these studies were limited in that they had no placebo-treated patients, their results mimicked results seen in mouse studies where inhibiting TNF improved impaired wound healing in diabetic mice compared to control mice\textsuperscript{17}.

The detrimental effects of TNF are compounded by the lack of M2 macrophages. These cells are key drivers of the proliferative stage, and so chronic wound fluid contains much lower levels of growth factors, such as TGF\textsubscript{β}1, VEGF and insulin-like growth factor-1 (IGF1), than would be expected\textsuperscript{19,25}. In an acute wound, the number of cells with an M2 phenotype normally increases as the wound matures. Using M2 macrophages transplanted from the later (day 5) proliferative stages to the earlier inflammatory stage (day 3) it has been shown that these M2 cells increase fibroblast proliferation and vascular regeneration\textsuperscript{15}.

One of the big questions is then why are chronic wounds populated by an excess of M1 macrophages with very few M2 macrophages in the wound? Studies using the cFMS kinase inhibitor GW2580 to block the M1 to M2 switching of phenotypes in acute wounds show that failure to switch to M2 lengthens the inflammatory stage, suggesting defects in the switch might contribute to the protracted inflammation
In vitro M2 macrophages can be formed by stimulating macrophages with certain cytokines, such as IL-4, IL-10 or IL-13 (Table 1). However, in an acute wound where macrophages switch from M1 to M2 quite readily, little extracellular IL-4 and IL-13 has been found. In fact, the addition of M2a or M2c macrophages, formed in vitro by stimulation with IL-4 or IL-10 respectively, to wounds had little effect on wound closure in mice, suggesting that IL-4, IL-10 and IL-13 may not play a role in driving this switch. One other way to drive the M1 to M2 switch in macrophages is by their phagocytosing dead neutrophils that have apoptosed. In vitro, cells formed this way are of an M2b macrophage phenotype that produces high levels of IL-10 and TGF-β. Wounds are typically populated with spent neutrophils in the early stages of repair and their clearance by macrophages could drive the switch from M1 to M2. Therefore, the macrophage phagocytic ability, or lack of, could ultimately alter whether a wound will remain in a pro-inflammatory state or heal in a timely manner.

Unlike macrophages from acute wounds, cells taken from patients with diabetes with chronic wounds show these macrophages have a defect in their ability to phagocytose apoptosed neutrophils (Figure 2). This deficit is a result of hyperglycaemia and its associated advanced glycated end products (AGEs), which alter the macrophages ability to phagocytose prior to them entering the wound. These macrophages are also activated prior to entering the wound rather than by factors in the wound. The inability to phagocytose increases the number of apoptotic cells in these wounds and inhibits the M1 to M2 switch in phenotype, generating more inflammation. What then might be responsible for the defective M1 to M2 switch in other types of chronic wounds? Chronic venous disease patients present with venous hypertension in lower limb deep and superficial veins, which results in changes to the endothelium and macrophage activation. Consequently, as seen with patients with diabetes, macrophages in these patients are altered prior to entering the wound and there is low-level chronic inflammation prior to the wound forming. Alterations are due to the chronic inflammation and to their phagocytosing red blood cells that have leaked into tissue and increasing their intracellular iron levels. Iron-loaded macrophages taken from the wound margin of iron-dextran-treated mouse models show that these cells are predominantly of a pro-inflammatory M1 phenotype with an intermediate anti-inflammatory M2 phenotype (TNF-α, IL-12β, IL-6, INOS, IL-4, IL-10, CD204 and CD206), which alter the macrophages ability to phagocytose prior to them entering the wound. These macrophages are also activated prior to entering the wound rather than by factors in the wound. This inability to phagocytose increases the number of apoptotic cells in these wounds and inhibits the M1 to M2 switch in phenotype, generating more inflammation. What then might be responsible for the defective M1 to M2 switch in other types of chronic wounds? Chronic venous disease patients present with venous hypertension in lower limb deep and superficial veins, which results in changes to the endothelium and macrophage activation. Consequently, as seen with patients with diabetes, macrophages in these patients are altered prior to entering the wound and there is low-level chronic inflammation prior to the wound forming. Alterations are due to the chronic inflammation and to their phagocytosing red blood cells that have leaked into tissue and increasing their intracellular iron levels. Iron-loaded macrophages taken from the wound margin of iron-dextran-treated mouse models show that these cells are predominantly of a pro-inflammatory M1 phenotype with an intermediate anti-inflammatory M2 phenotype (TNF-α, IL-12β, IL-6, INOS, IL-4, IL-10, CD204 and CD206), which alter the macrophages ability to phagocytose prior to them entering the wound. These macrophages are also activated prior to entering the wound rather than by factors in the wound. This inability to phagocytose increases the number of apoptotic cells in these wounds and inhibits the M1 to M2 switch in phenotype, generating more inflammation. What then might be responsible for the defective M1 to M2 switch in other types of chronic wounds? Chronic venous disease patients present with venous hypertension in lower limb deep and superficial veins, which results in changes to the endothelium and macrophage activation. Consequently, as seen with patients with diabetes, macrophages in these patients are altered prior to entering the wound and there is low-level chronic inflammation prior to the wound forming. Alterations are due to the chronic inflammation and to their phagocytosing red blood cells that have leaked into tissue and increasing their intracellular iron levels. Iron-loaded macrophages taken from the wound margin of iron-dextran-treated mouse models show that these cells are predominantly of a pro-inflammatory M1 phenotype with an intermediate anti-inflammatory M2 phenotype (TNF-α, IL-12β, IL-6, INOS, IL-4, IL-10, CD204 and CD206), which alter the macrophages ability to phagocytose prior to them entering the wound.
SUMMARY
Chronic wounds, regardless of the aetiology, are characterised by an increased and protracted inflammatory stage, with high levels of M1 macrophages that are unable to switch to the M2 phenotype. This switch in an acute wound dampens inflammation and progresses the proliferative stage of repair but is missing from chronic wounds. For diabetic and chronic venous ulcers, immune cells are pre-primed prior to entering the wound by the low levels of chronic inflammation seen in these patients and factors such as AGEs and iron that alter macrophage phenotype. This pre-priming alters their ability to form M2 macrophages. In diabetes this is due to an inability to phagocytose spent neutrophils, in chronic venous ulcers the cause remains to be determined but may also be an inability to phagocytose. Future research will determine whether this is the case and how these cells might be altered to progress wound healing.

REFERENCES


