The role of inflammation in cutaneous repair

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ABSTRACT

Inflammation is a fundamental component of the normal adult wound healing response occurring even in the absence of infection. It performs many beneficial roles such as the clearing of damaged cells and extracellular matrix (ECM), the removal of pathogens that might otherwise multiply and spread, and the secretion of mediators that regulate other aspects of wound healing such as proliferation, re-epithelialisation and wound remodelling. Yet, excess and/or prolonged inflammation is detrimental to wound healing and leads to increased fibrosis and scarring, which can be disfiguring and, in cases such as contractures, can lead to disability. Furthermore, excessive inflammation is a major contributing factor to the persistence of chronic non-healing wounds, which are “stuck” in the inflammatory phase of healing and fail to re-epithelialise. Current research suggest that the type of immune cells, their timing and the level of inflammation in a wound could have a dramatic effect on whether a wound heals in a timely fashion and the final quality of the repaired tissue. Studies suggest that altering the level of inflammation might be beneficial in terms of reducing scarring and improving the rate of healing in chronic wounds. This review looks at the role of the major immune cells in normal and impaired wound healing and strategies that might be used to reduce inflammation in wounds.

Keywords: Inflammation, macrophages, scar formation, chronic wounds, and regeneration.

CUTANEOUS TISSUE REPAIR

The skin, as the biggest organ of the human body, performs a number of essential functions such as temperature regulation and acting as a barrier to harmful pathogens. Preservation of skin integrity and its timely restoration through the wound healing process is necessary to support these functions. Cutaneous wound healing involves a complex and tightly regulated series of molecular events facilitated by an array of cell types, some resident and others recruited to the site of injury, including fibroblasts, keratinocytes, neutrophils and macrophages1-3. The process can be separated into four overlapping and interdependent phases: haemostasis, inflammation, proliferation and maturation1. These phases arise regardless of the type of injury, whether it is a scald, contact burn or a cut.

The repair process begins immediately upon injury, when haemostasis and coagulation are initiated1. During this stage, clotting factors and platelets collaborate to plug the wound and stop bleeding. Exposed collagen in combination with clotting factors from the injured skin induces platelets to form aggregates and secrete pro-inflammatory factors. Together with the aggregated platelets, cross-linked fibrin and fibronectin form a plug inhibiting blood loss and serving as a provisional wound support until collagen production is commenced. Additional blood loss is prevented through the temporary constriction of blood vessels initiated by the release of local pro-inflammatory factors. Platelets and other inflammatory cells release chemokines, cytokines and growth factors inducing vasodilation. This, in turn, leads to the recruitment of additional platelets and phagocytes to the site of injury, marking the beginning of the inflammatory phase (Figure 1). In a non-infected acute wound this inflammatory phase
typically lasts for approximately 5–7 days\(^1\). During this time the proliferation phase commences, covering days 3–10 post injury, whereby wound re-epithelialisation occurs, the vascular network is restored and granulation tissue is formed. Fibroblasts synthesise collagen III and fibronectin, which are organised into loose bundles that act as a framework for angiogenesis to occur, and also aid wound contraction\(^2\). New blood vessels sprout from existing endothelial cells migrating towards the wound guided by chemotaxis\(^3\). Proliferation and migration of keratinocytes promotes re-epithelialisation\(^4\). Together with granulocytes and macrophages, keratinocytes aid in the formation of the granulation tissue and initiation of remodelling. Finally, the wound maturates and extracellular matrix (ECM) protein remodelling continues up to a year after wounding. The process of wound healing ultimately ends with the formation of a scar where collagen III is substituted with collagen I, which is stronger and organised in parallel bundles differing from the basket-weave collagen in uninjured skin. The new collagen fibres are rearranged along tension lines, inducing wound contraction and decreasing the wound surface. The formation of the granulation tissue is terminated through apoptosis, resulting in avascular and acellular tissue\(^6\). This maturation process can take up to one year or longer.

**Figure 1: Cutaneous tissue repair.** During coagulation platelets that spill from damaged blood vessels plug the vascular defect and form a fibrin-rich clot that acts as a scaffold for infiltrating cells and contains chemoattractants to recruit neutrophils and macrophages to the wound site. Neutrophils and macrophages remove tissue debris and microbes and release of reactive oxygen species. Macrophages are activated and secrete large amounts of pro-inflammatory cytokines. Apoptosed neutrophils are phagocytosed by macrophages, which allows macrophages to adapt their phenotype and produce anti-inflammatory cytokines. Macrophages also produce pro-fibrotic growth factors (TGF\(\beta\)) as well as pro-angiogenic factors (VEGF, PDGF). Activated fibroblasts proliferate and migrate into the wound bed to form granulation tissue. Fibroblasts within this granulation tissue transform into specialist contractile myofibroblasts that produce large amounts of loosely organised collagen. Keratinocytes initiate the epidermal repair by migrating from the wound edges over newly laid ECM to fill the wound. MMPs regulate the remodelling of the connective tissue into parallel collagen bundles, ultimately resulting in scar formation.

**THE INFLAMMATORY PHASE OF WOUND HEALING**

**Immune cells are key regulators of the wound environment**

Inflammation in the wound occurs even in the absence of infection and is brought about predominantly by resident mast cells and mast cell precursors recruited from the circulation, along with neutrophils, monocytes and T cells that enter tissue from the blood after injury\(6,7\). Within hours of injury, resident mast cells degranulate, releasing an array of cytokines, amines and enzymes including histamine, which causes the local blood vessels to dilate and become porous. This, in combination with their secreted chemoattractants, supports the extravasation of inflammatory cells into the wounded area. New mast cells are recruited to the area and they persist in high numbers during the healing process. The exact role(s) mast cells play has been controversial, with partially contradictory results for roles in re-epithelialisation, angiogenesis, immune cell infiltration and fibrosis. Although it must be said, there are a few potential issues with the models used to generate these data. A more recent wound study has used transgenic mice with mast cells containing a Cre-inducible diphtheria toxin receptor where mast cells can be specifically depleted using diphtheria toxin\(^8\). This study found no significant differences in the kinetics of re-epithelialisation, the formation of vascularised granulation tissue, or scar formation when compared to control mice\(^4\). This suggests that at least in terms of wound closure and scar formation mast cells appear not to have a significant role.

Neutrophils and macrophages enter the wound relatively early in the wound healing process although with slightly different timings\(3,9\). The bulk of neutrophil recruitment occurs over the initial 6–12 hours of the healing process, with numbers in the wound peaking around one day post injury\(3,10\). Once neutrophils have entered the wound they secrete proteinases and antimicrobial products that kill microbes and remove pathogens from the site of injury in a process known as phagocytosis. In the wound they are also responsible for clearing up cell debris, again by phagocytosis. Through the secretion of cytokines and chemokines, neutrophils are also able to entice other immune cells to the wound and regulate resident fibroblasts and keratinocytes. Their recruitment ceases around day 2 when numbers decline dramatically due to apoptosis (a form of programmed cell death)\(^10\). Spent neutrophil fragments are then phagocytosed by macrophages, which helps to resolve inflammation during the wound healing process\(^11\).

Prior to injury, skin contains only a few resident macrophages and due to their low numbers they play only a minor role in inflammation during skin repair\(^12,14\). Thus, the majority of wound-associated macrophages are predominantly derived from circulating monocytes. Monocyte entry into the wound occurs in two waves\(^13\). Initially a small pool of monocytes enters the wound early post-injury through leaky vasculature as a result of the initial injury\(^9\). This leakage is transient and ceases as the vessels are plugged\(^19\). Upon injury, the production of their precursor cells, the myeloid progenitor cell, in the bone marrow increases, leading to a dramatic rise in circulating monocytes\(^12,15\). These monocytes must then actively cross the endothelium to
enter the wound and differentiate in situ into wound-associated macrophages. They continue to enter the wound in large numbers over then next 48 hours, and their numbers remain stable until 4 to 5 days post-injury when they begin to decrease, reaching steady-state levels by day 14\(^8\)\(^{12}\).

**The macrophage M1/M2 paradigm during cutaneous repair**

Once monocytes have infiltrated the wound, signals from the local wound environment trigger them to differentiate into macrophages\(^{16,17}\). Macrophages are classically activated through factors such as IFN-γ or through their pattern recognition receptors. These receptors activate when they detect pathogens or certain proteins released from broken cells or damaged ECM\(^{17,18}\). The differentiation process creates cells with an increased capacity to phagocytose (ingest and kill pathogens and clean up debris), and secrete enzymes such as matrix metalloproteinases (MMPs) that aid in the remodelling of the wound, pro-inflammatory cytokines such as TNF that regulate other cells, and growth factors such as TGFβ. Once activated, these macrophages, known as M1 macrophages, become predominantly pro-inflammatory in the wound environment\(^{16,17}\). M1 macrophages have a strong microbicidal activity through increased levels of reactive oxygen species, such as superoxide anions, oxygen and nitrogen radicals that help tackle and prevent infection. M1 macrophages are also voracious phagocytes removing pathogens, dying cells and debris that might act as stimuli to increase inflammation. They also produce large volumes of pro-inflammatory cytokines (for example, TNFα, IL-6, IL-23) to regulate other cells (Figure 3).

Macrophages are highly dynamic cells and can alter their functional phenotype as the environmental cues they receive change, leading to adjustments in their primary functions\(^9\). After phagocytosis of dead (apoptosed) neutrophils, or in response to stimuli such as IL-10 and IL-4, wound-associated macrophages alter and mature into M2 type macrophages (Figure 3)\(^{18}\). They become more reparative and begin releasing growth factors, such as TGFβ, VEGF and PDGFβ, that recruit endothelial cells (pro-angiogenic) and fibroblasts; they promote myofibroblast differentiation and secrete ECM components\(^{17}\). M2 macrophages also begin secreting anti-inflammatory cytokines such as IL-10 that act to switch off inflammation. The phagocytosis of apoptotic neutrophils is a major trigger in the switch from M1 to the reparative anti-inflammatory M2 type\(^{46}\). This is a simplistic view of what happens to these cells and it is more likely that between switching from M1 to the M2 there is a continuous spectrum of phenotypes.

Through the early stages of inflammation (day 1 post-wounding) the majority of macrophages express high levels of the cell surface markers Ly6c, as well as CCR2, and resemble an M1 phenotype producing large amounts of the pro-inflammatory cytokines TNFα, IL-6 and IL-1 and enzymes such as MMP-9 but little TGFβ\(^{12,20}\). By day 7 the majority of wound-associated macrophages express low levels of Ly6c and secrete little to no pro-inflammatory cytokines but produce more TGFβ and IL-10 as well as expressing the mannose receptor (CD206), which is more representative of the M2 phenotype\(^{12,20}\).

**Inflammatory cells are necessary to promote wound healing**

Neutrophils and macrophages are necessary to promote aspects of the wound healing process. They play a role in clearing the wound of pathogens, cell debris and damaged ECM. Macrophages also promote the advancement of healing through secretion of growth factors, chemokines and cytokines that activate the proliferation phase. Therefore, some inflammation is beneficial, and necessary for the timely repair of injured skin. While neutrophils are not absolutely necessary for wounds to re-epithelialise, studies in mice suggest that their absence might prolong closure rates and reduce neovascularisation\(^{21}\). It has also been suggested that failure to recruit neutrophils deprives macrophages from phagocytosing apoptotic neutrophils, a step that is important in altering the phenotype of the

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**Figure 2:** Inflammation in wound healing. The inflammatory phase of tissue repair under different wound healing conditions is shown.
macrophage to become more reparative in the wound\textsuperscript{24}. Studies on macrophages will be discussed below.

**EXCESSIVE INFLAMMATION AND INCREASED NUMBERS OF MACROPHAGES COMPROMISE WOUND HEALING OUTCOMES**

Inflammation and macrophages regulate scar formation

Evolutionarily wise the immediate objective of the repair process is to restore tissue integrity and homeostasis. However, this hasty repair process (fibrosis) comes at a price and the new tissue is not an exact replica of the uninjured tissue\textsuperscript{25}. The level of scar formation and its location then determines functionality of the skin. Healed skin is typically acellular and contains bundles of collagen fibres aligned in one direction rather than the normal organised lattice/basket weave structures seen in normal healthy and uninjured skin. Collagen helps provide tensile strength to the healed skin, although not quite reaching the strength of that found in uninjured skin. It is now becoming increasingly clear that the degree of inflammation and macrophages in the wound can dictate the amount of fibrosis and scar formation\textsuperscript{26}.

The kinetics of neutrophil and macrophage recruitment and egress after tissue damage described earlier is representative of an acute wound. Alterations in this process can have a profound impact on healing outcomes (Figure 2). While studies specifically targeting neutrophils show their depletion has no effect on fibrosis, the same cannot be said for macrophages. It is clear that macrophage numbers in the wound can dictate the level of scar formation, with reduced numbers being linked to less fibrosis. In foetal wounds, when monocytes have yet to develop, wounds heal scar-free\textsuperscript{2,26,27}. This improved healing process perseveres until the monocyte lineage develops (3rd trimester)\textsuperscript{28}. Although the lack of macrophages is not the only difference between foetal and adult wounds, these studies suggest that macrophages may potentially play a role in fibrosis. Also areas of the body that are characterised by low levels of macrophages and reduced inflammation, such as the oral mucosa, heal faster and with less scarring\textsuperscript{29}. These findings, together with other studies, have led to the suggestion that having high macrophage numbers in a wound to prevent infection might come at the price of fibrotic repair. Although it is not quite as simple as this, since macrophages play other important roles, there is now increasing evidence from more sophisticated functional mouse studies that macrophages regulate scar formation. Studies in mice where macrophages have been depleted in wounds show reduced scarring. Earlier studies using mice lacking the PU.1 transcription factor (Sfp1-/-), which are deficient in macrophages, neutrophils, B cells, mast cells and eosinophils, and thus have low levels of inflammation, show improved healing rates with much less fibrosis than control mice\textsuperscript{26}. These studies suggested inflammation regulated through these cells plays a role in fibrosis and scarring and together with other studies have led to a number of investigations into the exact role of these cells in wound healing.

More recently targeted studies to specifically deplete macrophages have helped reveal a role for these cells in scar formation\textsuperscript{1,2,22}. Administration of diphtheria toxin to transgenic mice containing diphtheria toxin-sensitive macrophages prior to wounding and during the healing process ablates all macrophages\textsuperscript{22}. This leads to a delay in re-epithelialisation, perhaps due to the reduced cell proliferation seen in these mice, and impaired angiogenesis\textsuperscript{22}. Importantly, there is also reduced collagen deposition in these wounds. These results suggest that macrophages promote wound closure and fibrosis. To further dissect their role, macrophages have been depleted over specific time frames during healing\textsuperscript{21}. Loss of macrophages from the inflammatory stage (days 0–5) of healing resulted in minimised scarring, again supporting the idea that macrophages regulate scar formation\textsuperscript{21}. These mice showed a reduced rate of epithelialisation during the inflammatory stage\textsuperscript{22}. However, once the diphtheria toxin injection was stopped at day 4, allowing the mice to produce new macrophages, wound closure was rapid compared to control animals and wounds were similarly healed by day 14 except they had greatly reduced scar formation\textsuperscript{21}. Remarkably, the removal of macrophages over days 4–9 led to severe haemorrhage in the wound, a lack of maturation and wound closure did not occur. Depletion of macrophages over the latter stages (days 9–14) of repair had no effect on the outcome\textsuperscript{21}. These results together suggest that if we could reduce early inflammation (days 0–5) there is the potential to reduce scar formation.

**High levels of inflammation and macrophages contribute to the chronicity of wounds**

In addition to regulating scar formation, excessive numbers of neutrophils and macrophages increase inflammation and impair the healing process, contributing to the chronicity of wounds (Figure 2)\textsuperscript{31,34}. Excess pro-inflammatory mediators and enzymes produced by macrophages and neutrophils negatively impact the wound environment. For example, high levels of matrix metalloproteinases, such as MMP9 secreted by these cells, causes tissue damage through excessive degradation of the ECM that would normally provide a scaffold for new cells to migrate into the wound\textsuperscript{24}. Another contributor to chronicity occurs through the secretion of pro-inflammatory cytokines, such as TNF, which exacerbates inflammation. While neutrophil numbers in the wound are high, it has been shown that 80\% of wound margin cells are pro-inflammatory macrophages. Increased numbers of macrophages and sustained inflammation are observed in human chronic wounds, suggesting that a reduction in macrophage numbers might be beneficial in their treatment. Studies undertaken in impaired wound healing models of obese (ob/ob) and diabetic (db/db) mice show macrophage numbers are elevated and that they persist at sites of injury for much longer than in control mice\textsuperscript{26,37}. Attractively, wound closure in diabetic mice can be improved by attenuating wound inflammation. Similarly dampening inflammation through antibody-based macrophage depletion is able to restore disturbed tissue regeneration in healing-impaired, obese mice\textsuperscript{36}.

Interestingly, in mouse models with impaired healing, such as the obese (Ob/Ob) and defective TLR-signalling (MyD88-/-) mice, the increased levels of macrophages seen in the wound are predominantly of the pro-inflammatory M1 phenotype with much less of the reparative non-inflammatory M2 macrophages\textsuperscript{1,2,22,38}. While this work has been done in mice, human venous leg ulcers also have
increased numbers of M1 macrophages that contribute to chronic inflammation, tissue breakdown and impaired wound healing\(^9\). These findings suggest that perhaps the normal environmental stimulus for switching phenotypes might not be present or that the increased levels of inflammation are damaging the wound environment perpetuating inflammation. For example, high levels of enzymes found in chronic wounds, such as the MMPs, secreted from neutrophils and macrophages lead to the degradation of new ECM proteins. These proteins would normally provide scaffolding for new cells to enter and fill the wound. Additionally, the damaged ECM components might act as a stimulus to the pattern-recognition receptors keeping macrophages in the M1 state\(^\text{35}\).

Pro-inflammatory macrophages have also been seen to concomitantly express both M1 and M2 markers, suggesting that there is a glitch in the switch from pro-inflammatory M1 to the anti-inflammatory M2 phenotype, leading to the persistent and excessive inflammation seen in impaired healing\(^\text{38,40}\). These results support the idea that the different macrophage subpopulations are derived from an appropriate (in normal wound healing) or defective (in impaired healing) in situ response to the wound environment rather than being recruited from circulating monocytes\(^\text{47}\).

STRATEGIES TO DAMPEN INFLAMMATION

There are a number of potential strategies to specifically dampen inflammation in wounds. These include preventing immune cells entering a wound and being activated to secrete pro-inflammatory cytokines; inhibiting the pro-inflammatory cytokines that regulate and, in the case of chronic wounds, perpetuate chronicity; removal of factors that lead to the release of products that activate immune cells, such as those released when cells or ECM is damaged in chronic wounds.

ALTERING RECRUITMENT OF IMMUNE CELLS

In diseases, such as psoriasis, where infiltration of immune cells is a determining factor in disease progression, preventing the immune cells that regulate inflammation exiting the blood and entering the skin has been used as a successful strategy to reduce inflammation and symptoms. To exit the blood, immune cells must first adhere to the endothelial cell wall, where they roll along the wall until they arrest, migrate to the cell junctions and cross the endothelium to enter the wounded tissue\(^\text{41,42}\). The initial contact of monocytes to vascular endothelium occurs via cell adhesion molecules (CAMs) such as the ICAMs and the selectins\(^\text{41}\). Integrins on the immune cell surface act as the cognate ligands for these adhesion molecules, allowing them to attach to the endothelial cell wall\(^\text{41}\). Studies in mice deficient in both P- and E-selectins, which are typically found on the endothelium, show markedly reduced neutrophil and macrophage recruitment and significantly impaired closure of wounds, with reduced epithelial migration and granulation tissue formation\(^\text{4\text{1,42}}\). These results are unsurprising given the more recent conditional depletion of macrophages studies, which showed that loss of macrophages during the mid-phase of healing (days 4–9) led to severe haemorrhage in the wound, a lack of maturation and loss of wound closure. A number of other studies have been undertaken using antibodies to integrin molecules on immune cells in rabbit models of burn injuries where integrins have been targeted early in the process. For example, using a rabbit model of thermal injury, it has been shown that in the first 24 hours the level of TNF can be reduced after treatment with anti-integrin alpha L antibodies. Other burn wound studies show that damage in the zone of stasis (the tissue surrounding the necrotic burn area) can be reduced when targeting ICAM-1 and integrin β2\(^\text{43,44}\). While these studies have shown the treatment was beneficial in the first few days, they did not look at the longer term effects such as healing time and scar formation. However, a clinical trial using a monoclonal antibody against ICAM-1 (Enlimomab), administered within 6 hours of injury to burns patients showed that there was a significant increase (threefold) in the number of patients that healed within 21 days\(^\text{46}\). They found no significant differences in scar formation, but the number of patients that attended the follow-up scar assessment procedure was greatly reduced\(^\text{46}\). How this treatment would affect acute wounds is unknown and whether targeting immune cell extravasation at a slightly later time point (for example, day 2) might be more beneficial in reducing scar formation is not yet known, but one might imagine that if you time administration correctly you might be able to reduce scar formation.

Pro-inflammatory cytokines as therapeutic targets

Due to the negative influence of exacerbated inflammation on wound healing, depletion of pro-inflammatory cytokines secreted by the immune cells once they have entered the wound has been considered to be a promising therapeutic target to accelerate repair and improve wound-healing outcomes. A reduction in their levels in many diseases where inflammation plays a role in the pathological process has been found to be beneficial. For example, clinical application of an antibody that targets TNF (Infliximab) is used to dampen inflammation in rheumatoid arthritis. Topical application of anti-TNF antibodies to mice that have impaired healing has been shown to reduce immune cell recruitment, alter immune cell activation and phenotype, and accelerate the wound repair process\(^\text{47}\). Application of antibodies against other pro-inflammatory cytokines (IL-1β and IL-17) has also been tested in mice wound models\(^\text{1,2,22}\). These studies show improved skin repair in normal and impaired healing mice models compared to controls suggesting that targeting pro-inflammatory cytokine may be beneficial during the repair process. Moreover, inhibition of the IL-1β pathway using a neutralising antibody in diabetic mice wounds induces a switch from pro-inflammatory M1 to healing-associated M2 macrophage phenotypes\(^\text{22}\). These wounds generally have increased levels of wound growth factors, and improved healing, which also confirms that macrophage maturation benefits wound healing\(^\text{22}\). This strategy has yet to be tested in patients.

Reducing molecules in the wound that activate immune cells

The elevated levels of enzyme activity in chronic wounds contribute to increased tissue destruction that includes the release of intracellular proteins and fragments of ECM components that are capable of binding to macrophages and neutrophils and activating them\(^\text{48}\). This activation then leads to the secretion of proinflammatory cytokines that increase the level of inflammation. Activated macrophages and neutrophils are also responsible for secreting many of these enzymes

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and so more damage leads to more inflammation, which, in turn, leads to more damage and a vicious cycle begins, perpetuating chronicity in the wound.

MMPs are one such family of enzymes whose levels are significantly increased in chronic wounds (MMPs1, 2, 8, 9) along with decreased levels of their inhibitors (TIMPs1, and 2). The imbalance in MMPs and TIMPs could be responsible for uncontrolled ECM turnover, inflammation, dysregulated cell growth and migration in wound tissue. Given the significance of MMPs in inflammation-associated diseases such as chronic wounds, these proteases have been and still are considered promising therapeutic targets. However, most of the MMP inhibitors are broad-spectrum and since there are also other MMPs in the wound that have beneficial physiological functions are not suitable for use in wounds. The design of specific inhibitors has been unsuccessful to date as MMPs share very similar structures around and within their active sites. Protease-absorbent dressings have been used to ‘mop up’ protease in chronic wounds, although whether they are beneficial in healing is unclear. For example, one study using an oxidised, regenerated cellulose/collagen matrix dressing showed reduced levels of MMP2 in venous leg ulcers but found no effect on the overall healing rate.

**CONCLUSION**

Future research will determine whether targeting macrophage migration, activation or its secretory products in the early stages of tissue repair might reduce inflammation and lower scar formation. Data from mice studies suggest that dampening inflammation has the potential to reduce scar formation and that the same strategies might also be beneficial to chronic wound healing.

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