

## Theme Issue Article

# Neutrophil function in the healing wound: adding insult to injury?

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### Summary

Cells of the innate immune system, including neutrophils and macrophages, are a highly visible component of normal wound healing in adult mammals. The role of inflammatory cells in the healing wound has been widely investigated, and evidence for both positive and negative influences exists. Several recent

investigations support the emerging paradigm that robust inflammation is detrimental to wound closure. This developing information suggests that the functional role of inflammatory cells in wound healing must be reevaluated.

### Keywords

Wound healing, neutrophils, macrophages, inflammation

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## The role of neutrophilic granulocytes in healing

The first nucleated cell to infiltrate the wound bed after the integrity of the skin has been disrupted is the neutrophilic granulocyte or neutrophil. This innate immune cell mediates the first line of defense and marks the start of the inflammatory response of wound healing. The main function of neutrophils at the wound site is to decontaminate the open wound by the destruction of invading microbes. In the circumstance of injury to barrier surfaces, such as the skin, a very rapid and effective response by neutrophils minimizes the chance for infectious complications. Macrophages, which are considered essential to healing (1), are not abundant at the wound site until 24 to 48 hrs after neutrophils, and cells of the adaptive immune response not until 7 days post injury. Hence, the neutrophil provides a singularly rapid, albeit relatively nonspecific, immune response to injury. After neutrophils complete their task, tissue repair and restoration commences. In skin wounds, epidermal keratinocytes near the wound edge become mobilized to migrate and

proliferate over the denuded wound surface. These processes lead to a restoration of the epithelial surface and the barrier function of the skin.

Despite numerous reports of neutrophil persistence in situations of impaired healing (2-4), the effect of neutrophils on normal wound healing remains incompletely understood. Indeed, neutrophils have previously been regarded as a cell type with minor influence on healing of uninfected wounds, a concept that is largely derived from an investigation of the effects of neutrophil depletion on wound healing in a study that was performed by Simpson and Ross (5). In this study, no discernable differences in fibrin content or distribution were observed in wounds of neutropenic and control guinea pigs. Furthermore, no differences in percent wound volume occupied by macrophages, or in macrophage phagocytosis was seen. Using a similar approach of neutrophil depletion to study the effects of neutropenia on wound healing in a murine model, we obtained results consistent with but also extending the study conducted by Simpson and Ross (5). In our murine study, we also did not find significant differences in collagen deposition, wound disruption

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strength, or macrophage infiltration in wounds of neutropenic mice (6). Apart from these parameters of dermal healing, our study extended the findings of Simpson and Ross by investigating epithelial healing in the absence of neutrophils. Somewhat surprisingly, we found that neutrophil-depleted mice displayed significantly accelerated wound re-epithelialization. In genetically diabetic mice, an example of delayed healing associated with an aberrant and excessive neutrophilic response, wound closure was accelerated by 50% following systemic neutrophil-depletion (6). Taken together, these results suggest that neutrophils delay normal wound closure and may contribute to the healing impairment in diabetic individuals. Our results also suggest that epidermal and dermal healing are not necessarily linked, since epithelialization, but not the regeneration of tensile strength in the dermis, was accelerated in the absence of neutrophils. Overall, the data strongly suggests a direct negative effect of neutrophils on keratinocyte functions essential to wound closure.

Neutrophils are known to produce a variety of growth factors that could promote the revascularization and repair of injured tissue, such as IL-8 (7) and VEGF (8). However, the majority of studies support a negative role for neutrophils in normal tissue repair (9, 10). Neutrophils produce many bioactive substances that can accelerate tissue damage. For instance, within the wound, neutrophils secrete proteases that can convert wound cytokines to an active or inactive form and can induce substantial tissue damage. The neutrophil proteases elastase and PR-3 are both capable of cleaving elastin and a variety of extracellular matrix proteins, including fibronectin, laminin, vitronectin, and collagen IV. Since the extracellular matrix serves as a supporting scaffold, and influences keratinocyte functions such as migration and proliferation, modification of the ECM by neutrophils could have important consequences for epithelial repair. Indeed, the incubation of skin with neutrophil elastase has been shown to cause separation of the dermal and epidermal layers (11). Interestingly, the addition of neutrophils to a monolayer of keratinocytes leads to a protease-dependent keratinocyte detachment (12).

While neutrophils may impair healing, macrophages are generally believed to play a positive role, and augmentation of macrophages has been shown to accelerate wound healing (13). One interesting possibility is that an enhancement in macrophage function, favorable to healing, in tandem with the depletion of neutrophils, accounts for the accelerated re-epithelialization observed in neutrophil-depleted mice.

## Predictions

Our *in vivo* and *in vitro* studies strongly support the emerging hypothesis that neutrophils influence the healing process in a negative manner. This hypothesis leads to two very simple predictions. First, less neutrophils would be present in wounds that heal very well, and second, more neutrophils would be found in

wounds that heal very poorly. In the following two paragraphs, we review the evidence that both of these predictions are in fact correct.

## Neutrophils in wounds that heal very well

In contrast to adult skin, in which injured skin is repaired resulting in a fibrous scar, fetal dermal healing is characterized by scarless regeneration (14-16). During this remarkable process of regeneration, the normal dermal architecture is completely restored not unlike the ability of certain amphibians to regenerate complete limbs (17, 18). For reasons that remain incompletely understood, fetal wounds contain very little if any inflammatory response (16, 19, 20). Since scarless repair in fetuses does not appear to be a function of the fetal environment (21, 22), the absence of inflammation, especially the absence of neutrophils, may be at least in part responsible for the rapid and flawless repair of fetal wounds (23). In support of this concept, scar formation appears to accelerate when inflammation is provoked in fetal wounds (24-26). Recent studies in the PU1.1 knockout mouse, a mutant strain with an incomplete immune system, also support the concept that inflammatory cells may negatively influence repair (27). In these mutant mice, little inflammation occurs at the wound site, and the repair appears to be scar-free, similar to the embryo.

A final example of a tissue that heals with minimal inflammation, rapidly, and often with minimal scar formation, is the oral mucosa. To examine differences in the inflammatory response of oral and dermal murine healing, we studied cellular infiltration and cytokine production in equivalent size excisional wounds of the tongue and skin. Consistent with the view that neutrophils influence the healing process in a negative manner, we found significantly lower numbers of neutrophils in rapidly healing oral wounds. In addition, oral wounds contain fewer macrophages and T-cells as well as reduced levels of the cytokines IL-6 and KC (28).

## Neutrophils in wounds that heal very poorly

The concept that neutrophils influence healing in a negative manner predicts that wounds with abundant and persistent neutrophil infiltration would heal very poorly. In fact, in chronic wounds, a protracted inflammatory response is manifest in terms of the continued presence of inflammatory leukocytes, most notably neutrophils (29-31). The continued production of pro-inflammatory cytokines by neutrophils attracts and activates additional inflammatory cells, further resulting in the release of bioactive substances such as proteases causing extensive tissue damage. The factors that lead to the unresolved inflammatory response in chronic wounds include recurrent

physical trauma, ischemia reperfusion injury, diabetes, and contamination of wounds. Several other circumstances of delayed healing also exhibit enhanced inflammation. The wounds of diabetic individuals, which heal slowly and with poor dermal quality, demonstrate robust inflammation (32). Advancing age is also correlated with abnormal inflammatory activity and delayed healing of both acute and chronic wounds (33, 34).

The mechanisms by which neutrophils might impair healing are certainly many. Activated neutrophils release a battery of microbicidal substances that are essential in combating invading pathogens. However, some of these factors, such as reactive oxygen species, cationic peptides, eicosanoids, and proteases delay the healing process by harming cells and by directly damaging the newly formed structures of the healing wound. Neutrophil elastase, for instance, can degrade virtually every component of the extracellular matrix, as well as proteins as diverse as clotting factors, complement, immunoglobulins, and cytokines (35).

Neutrophil mediated tissue damage has been documented in a variety of pathologic situations besides aberrant wound healing, with one such example being ischemia reperfusion injury (36). The reperfusion of ischemic tissue is characterized by a massive infiltration of neutrophils that results in damaging of the vasculature and surrounding tissue. The inhibition of neutrophil infiltration alleviates the reperfusion injury (36). Other examples of neutrophil-induced tissue damage include certain pulmonary diseases (37-39), as well as autoimmune diseases, such as Wegener's granulomatosis (40, 41). The molecular mechanism of endothelial damage in patients suffering from Wegener's granulomatosis, characterized by systemic vasculitis, is particularly interesting. The binding of anti-neutrophil cytoplasmic antibodies (ANCA) to surface bound serine protease, Proteinase 3 (PR3), leads to release of the molecule. PR3 then binds to a specific surface receptor on endothelial cells, which triggers cellular activation, detachment, apoptosis, and cytolysis. Thus, some of the cell and tissue injury caused by neutrophils can have a rather sophisticated underlying molecular mechanism.

### **Some possible benefits of neutrophil – epithelial cell interactions in wounds**

The current evidence suggests that neutrophils slow the keratinocyte proliferation and migration necessary for epithelial repair, most probably by accelerating keratinocyte differentiation. This neutrophil-induced acceleration of keratinocyte differentiation would generally have a negative effect on wound closure, and little benefit of neutrophils in wounds beyond decontamination seems readily apparent. Yet the ability of neutrophils to induce keratinocyte differentiation might provide an important safeguard against malignant transformation in the hyperprolifera-

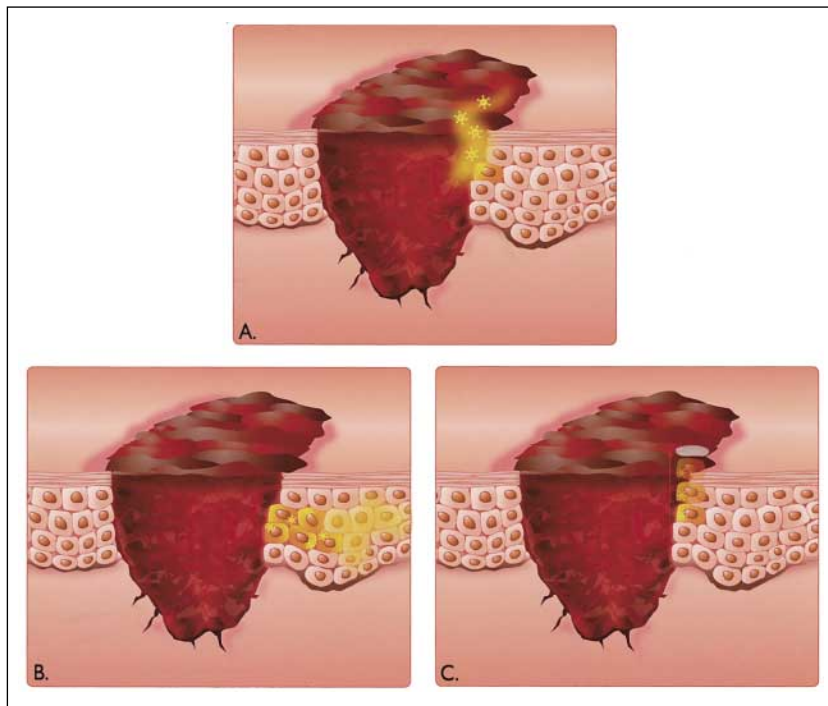
tive epithelium of the wound. Within the context of wound healing, the brief and limited infiltration of neutrophils might impede malignant transformation by moderating the robust hyperproliferation and response to growth factors. Given that hyperproliferation can predispose to malignancy, such a temporary neutrophil counterbalance might be beneficial (42). In the case of prolonged inflammation, a circumstance well known to promote tumor development, any counterbalancing influence of neutrophils would be overcome by the effect of repeated oxidative stress on genetic stability (43, 44).

In addition to protection against tumor formation, the acceleration of keratinocyte differentiation in wounds may constitute a mechanism of local immune defense. Neutrophils are very effective in eliminating extracellular microorganisms such as bacteria. But once pathogens access the intracellular compartments of a cell, neutrophils are largely ineffective in ridding the pathogen, and cells of the adaptive immune system are needed. Unfortunately, the adaptive response against a newly encountered microorganism takes approximately seven days to become fully activated. During this lag, the intracellular pathogen could potentially spread and infect an enormous number of cells (Fig. 1A, B). Perhaps the neutrophilic response is a simple and yet well-adapted form of limiting the survival of potentially infected cells. In accelerating keratinocyte differentiation, infected cells will quickly be lost due to the natural differentiation program that culminates in shedding of cornified cells (Fig. 1A, C). Importantly, in contrast to removing cells via the induction of apoptosis or necrosis, the removal of keratinocytes by accelerated differentiation allows for the maintenance of the skin's barrier function, preventing further infection with microorganisms.

### **Why would acute inflammation, a process unfavorable to wound closure, evolve as a component of the healing process?**

In order to speculate on the answer to this question, one must consider two possible reasons for why mammals, including man heal today the way they do. First, it may be the most advantageous way to heal. A second possibility is that the process was the most advantageous way to heal in times when evolutionary pressure demanded selection of the fittest.

If the way we heal today was (and is still) the most advantageous way to heal, then some advantage to the delay in wound closure must be assumed. This seems counter-intuitive, as rapid wound closure seems to be the single most important process in the prevention of wound infection and possible death. Yet there seem to be several instances in which fastest closure is not necessarily the best solution. When a wound is heavily contaminated, clearance of the infection is needed prior to wound closure. Delayed closure could improve the likelihood that neutrophils



**Figure 1: Possible benefits of neutrophil-epithelial cell interactions in wounds.** (A) Open wounds provide easy access for pathogens, including intracellular pathogens, such as viruses (stars). (B) During the initial inflammatory phase, cells of the innate immune response are effective at removal of extracellular pathogens. However, because few cells of the adaptive immune response are present in the early wound, keratinocytes are extremely susceptible to infection by intracellular microbes at this stage. (C) The accelerated differentiation of keratinocytes, possibly induced by neutrophils, would reduce the threat of viral spread via an increased rate of shedding of infected cells. This temporary halt in wound closure would be beneficial in reducing viral spread.

can effectively combat infections for several reasons. First, neutrophils have an enormous oxygen requirement in order to produce reactive oxygen intermediates, which help combat infections. The wound bed becomes hypoxic shortly after closure. Neutrophils could be compromised in their generation of oxygen intermediates during this time if the wound was rapidly closed, diminishing their effectiveness against bacterial infections. In addition, the maintenance of an open wound by neutrophils would allow the sloughing of necrotic debris, ultimately facilitating healing. In fact, surgeons often choose to artificially keep wounds open until the infection is cleared. Simply put, optimal neutrophil function and pathogen clearance may require delayed closure. Therefore, a delay in closure remains the best way to heal.

A second possible reason why a delay in wound closure, mediated by neutrophils, persists, is that this scheme provided a selective advantage in times when evolutionary pressure demanded selection of the fittest. To understand the way we heal today, one must consider what type of injuries had to be healed in primitive man. Typical injuries were probably irregularly shaped tear wounds due to rocks and other sharp objects in the surroundings, blunt trauma inflicted by other humans, and bite wounds from wild animals. The feature that all of these wounds share is that they were heavily contaminated. Since neither antibiotics, surgical intervention nor clean water was available, the healing strategy was selected to ensure survival in most cases. The neutrophilic response insured wound decontamination and enhanced the chance of survival, a benefit bought for the price of delayed wound closure.

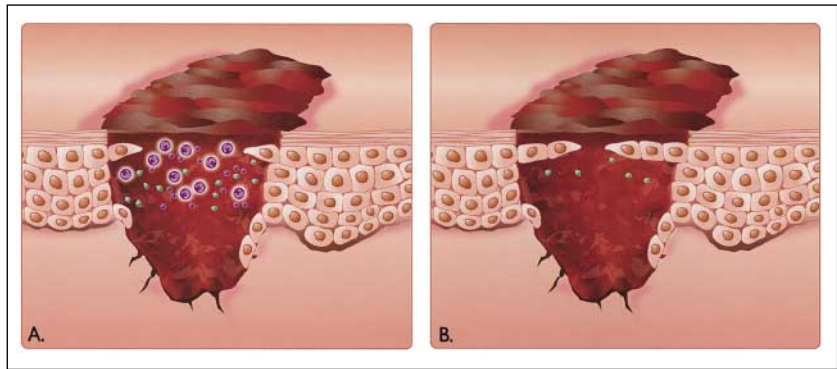
## Is inflammation the chicken or the egg?

Most living organisms have the ability to heal. However, not all organisms have a sophisticated immune response activated concomitantly with injury to protect from infection. During the course of evolution, cells participating in the primary repair process and cells of the immune system had to adapt to work in parallel and in concert. Perhaps comparable to the adaptation of mitochondrial precursors to phylogenetically old eukaryotes, both cell types adapted to working in concert. Furthermore, one system influences the other, and control over the entire process (of healing) is carried by both alike. Despite the tempting assumption that the immune system came after the process of healing, one could argue that healing in and by itself is the oldest form of an immune system. In fact, most immunologists consider the skin an important part of the innate immune system. Assuming this premise is accurate, it is then not surprising that cells of the immune system, namely neutrophils, and keratinocytes communicate with each other in a direct manner.

## Do neutrophils affect the function of other cells? A reconsideration of macrophage function in healing

Macrophages have been quoted as “essential”, “indispensable”, and “most important to a successful outcome of wound heal-

**Figure 2: The effect of neutrophils on wound closure.** (A) Neutrophils, observed here in early wounds, may cause a delay in wound closure by the secretion of substances that accelerate keratinocyte differentiation. This delay would cause wounds to remain open while microbial decontamination occurs. (B) Following decontamination of the wound, neutrophil content would diminish, possibly by the phagocytosis by arriving macrophages. The elimination of neutrophils would allow keratinocytes to proliferate and migrate freely to close the wound.



ing”, as these cells have been shown to produce growth factors that stimulate angiogenesis and fibrogenesis (1, 13). From a phylogenetic perspective, it is hard to understand how an immune cell can be indispensable to healing of a sterile wound, and indeed recent studies question the concept that macrophages are needed for optimal healing (27). An alternative explanation of the importance of macrophages in wounds, other than mediating proliferative processes, seems to be needed. One possibility is that macrophages are critical to the removal of apoptotic neutrophils. Interestingly, Meszaros et al. have demonstrated that macrophages, which arrive in the wound bed just after the neutrophils and just prior to neutrophil disappearance, are capable of inducing neutrophil apoptosis *in vitro* (45). Furthermore, neutrophil-derived fragments have been found in phagosomes inside of macrophages (46). Perhaps the most important function of macrophages to wound healing is to accelerate the regression of the inflammatory response via the elimination of neutrophils.

## Immune and non-immune cell interaction

Immune cells are rarely considered by specialists of disciplines other than immunology, such as physiologists, endocrinologists, neurologists. Clearly, the immune system is not an entity that functions in isolation. There are multiple points of interaction between immune- and non-immune cells. For instance, several studies determined that the nervous system influences and is influenced by the immune system (47-49). Studies by other investigators and ourselves on the immune component of healing once again emphasize the importance of immune and non-immune cell interaction. The understanding of one complete system, such as the nervous or immune system, is a great task.

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However, it will be dwarfed by understanding the interactions between systems. This underlines once again that we are far from understanding the complex biologic process of wound repair in its entirety.

## Neutrophils and wounds: a look to the future

The study of the role of the neutrophil in wounds, initiated in the 1970s, remains an area of new discovery and surprises. Neutrophils are rapidly recruited to sites of injury, yet once a wound is sufficiently decontaminated, neutrophils are rapidly lost. With the loss of neutrophils, a negative regulator of re-epithelialization is removed, allowing the completion of wound closure (Fig. 2). If a wound is heavily contaminated, neutrophils receive survival signals. The prolonged neutrophilic infiltrate stalls re-epithelialization until neutrophils perform their tasks, and considerable additional tissue damage results from this physiologic response to infection. The response of the neutrophil to injury can be seen as both a strength and weakness of the healing process.

As our studies and those of others suggest, in the healing of a clean wound in a healthy individual, the negative effects of neutrophils probably outweigh the positive effects. In clean surgical wounds, neutrophils are undesirable because they delay wound closure and cause additional tissue damage. Whether this fact can be exploited to enhance healing, such as through pharmacological inhibition of neutrophil infiltration, remains open to study. Undoubtedly, a careful balance must be achieved in order to facilitate repair without risking infection.

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