Chapter 10 Summary with options for chemokine therapies of burn and chronic wounds

SUMMARY WITH OPTIONS FOR CHEMOKINE THERAPIES OF BURN AND CHRONIC SKIN WOUNDS

This thesis focuses on the evaluation of growth factors, cytokines and chemokines present in the wound environment of burn, chronic and surgical wounds. The first chapters describe a differential expression of these mediators in wound exudates isolated from the 3 different wound types. Our results indicate that development of a universal therapy for these wounds is not an option. Since surgical wounds often heal by first intention we were mainly interested in burn and chronic wounds, which provide difficulties in closing. Our results in conjunction with clinical observations indicate that in chronic wounds granulation tissue formation needs to be stimulated whereas in burn wounds excess granulation tissue formation and inflammation rather should be prevented since this frequently leads to excessive scar formation. In this last chapter, we focus on chemokines which may stimulate processes like granulation tissue formation, inflammation and scar formation. Consequently, a stimulatory cocktail of these chemokines would be favoured for chronic wounds while inhibition of these chemokines, e.g. by means of an inhibitory cocktail, would be indicated for burn wounds. Finally, we emphasize the potential role of skin substitutes in facilitating closure of different wounds.

This final chapter first discusses current therapies for burn and chronic wounds. Then, we use the knowledge obtained in **chapters 2 - 9** to propose potential new therapies for burn and chronic wounds.

CURRENT THERAPIES FOR BURN WOUNDS

Currently the "gold standard" treatment for full thickness burn wounds is transplantation with an autologous, meshed split-thickness graft. This is an adequate procedure to close the injury. However, it is very problematic in severely burned patients due to limited availability of donor sites with healthy skin. Moreover, severe scarring of the donor sites may occur. The burn wounds heal with an irregular mesh pattern and often hypertrophic scarring. These scars are defined as raised, but staying within the confines of the original lesions (1). Hypertrophic scarring causes a cosmetic problem but also impairs skin function by reduced scar elasticity and scar contractures. Contractures place patients at risk for additional medical problems including interference with skin and graft healing. Functionally, contractures of the lower extremities interfere with mobility, seating, and ambulation. Contractures of the upper extremities may affect activities of daily living, such as grooming, dressing, eating, and bathing, as well as fine motor tasks (42).

Several reports describe that a lack of dermal tissue in the wound is one of the main factors responsible for scarring (1-3). This is due to the function of the dermis, which provides

tensile strength and elasticity of the skin. Dermal tissue is synthesized by fibroblasts which have extensive crosstalk with other cell types in the dermis e.g endothelial and infiltrating cells, as well as keratinocytes in the epidermis. The presence of the dermis is essential for promoting tissue regeneration (4). Therefore, application of an epidermal substitute alone on full-thickness wounds is insufficient for healing and may cause hypertrophic scarring and skin fragility (5,6). Indeed, several studies have shown that the use of dermal substitutes in combination with a thin split-thickness skin graft provides a beneficial effect on burn scar outcome (7-10). However, some disadvantages are associated with the clinical use of dermal substitutes. For example, the dermal substitute Integra shows slow vascularisation, which may facilitate wound infection leading to loss of the graft. Furthermore, this dermal substitute is applied in a two-step grafting procedure making it very costly (10). In 2000 Van Zuijlen et al. reported the successful application of another dermal substitute, Matriderm®, in combination with a split skin graft (SSG) in acute burn wounds in a one-step procedure (11). A one-step operation procedure reduces the risk of infection and treatment costs. Still, however, no difference in scar elasticity was found in burn wounds treated with Matriderm® and split-skin grafts (SSG) compared to SGG alone at 12 months and 12 years follow-up (8, 12). But, a significantly improved scar elasticity was found when topical negative pressure therapy was added to the therapy with Matriderm® and SGG (43). It was hypothesized that topical negative pressure therapy increases vascularization of the dermal substitute thereby improving graft take and scar quality of burn wounds. Since such combination therapies are costly and logistically very demanding wider clinical introduction may be slow.

Below we describe two hypothetical therapies for facilitating wound closure and improving scar quality of burn wounds, based on the results described in this thesis. The first focuses on applying skin substitutes and the second focuses on influencing the environment of the burn wound.

TOWARDS NEW THERAPIES FOR BURN WOUNDS

Application of a skin substitute (SS) and neutralizing TNF-α (Figure 1)

In severely burned patients a combined therapy of dermal substitute and SSG can improve scar quality as described above. However, this therapy still requires autograft tissue. When donor skin sites are a limiting factor, tissue engineered skin substitutes, containing both dermis and epidermis, would then ideally be used. Tissue engineered skin substitutes contain living cells and are thought to function not only by directly closing the wound but also by continuously secreting chemokines, angiogenic and growth factors which stimulate wound healing (13,14). **Chapter 5** and previous research from our laboratory showed that when a full thickness autologous skin substitute (cultured from autologous keratinocytes

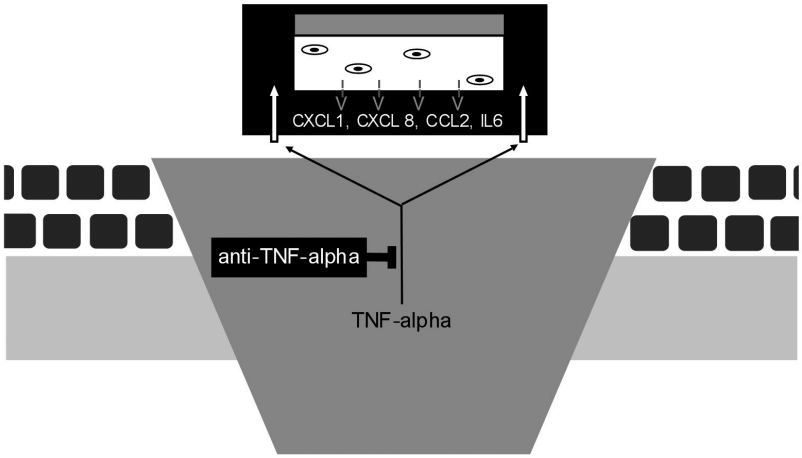

Figure 1: Schematic cross section of a burn wound.
Depicted are the application of the SS and the presence of TNF-α in the wound bed of burn wounds. TNF-α increases secretion of wound healing mediators from the SS. This increase is inhibited by neutralization of TNF-α.

and fibroblasts) is applied to a *chronic wound* it stimulates wound closure (15). However, when the same skin substitute was applied to a *surgical excision wound*, hyper-granulation was observed together with excessive secretion of wound exudate and delayed wound healing. Still, the final scar quality 1 year later was acceptable and similar to standard autograft techniques. This result prompted an *in vitro* study in which we found that wound exudates isolated from both acute (surgical excision) wounds and chronic leg ulcers induced strong secretion of wound healing mediators (CCL2/ MCP1, IL6, CXCL1/Gro-α, CXCL8/ IL8) from the SS into the wound bed. These wound healing mediators are known to stimulate the formation of granulation tissue and initiate wound healing explaining the beneficial clinical effects (16, 17). Indeed, the surgical excision wound (similar to a burn wound) has a good tendency to heal. It is often the extent of the trauma and size of the wound which prevents wound closure. But, our results show that the wound bed of surgical excision wounds becomes over-activated by the interaction of wound exudate and skin substitute. Due to similarities between burn- and excision wounds in their tendency to heal a negative effect on wound healing can also be expected when a SS is applied to burn wounds. Our results (**Chapter 5**) show that this adverse effect on wound healing may be a result of TNF-α present in burn wound exudates over-stimulating the skin substitute, since *in vitro* neutralizing of TNF-α in wound exudate reduced the secretion of wound healing mediators known to be up-regulated by TNF-α. Therefore, a potential combined therapy of a skin substitute with topical anti-TNF-α

is proposed that may provide a novel means to heal surgical and burn wounds since it would be expected to inhibit the excess granulation tissue formation shown in chapter 5 whilst at the same time directly closing the wound.

Since rapid closure of burn wound (reduces risk of infection and improves functional outcome) an autologous skin substitute may appear less suitable for burn wounds regarding the 2-3 wk culture process of an autologous SS. Therefore, a temporary wound cover (eg. human allograft) which temporarily closes the wound and initiates healing, may be an option to bridge the time needed for culturing an autologous SS.

Literature shows that TNF-α antagonists (e.g. Etanercept and Infliximab) are now being extensively evaluated in the setting of chronic wound healing. Of note, preliminary clinical studies and case reports provide evidence of the clinical potential of these compounds in deep ulcers on the leg caused by pyoderma gangrenosum, an immune system dysfunction (45). Also, among autoimmune skin disorders psoriasis has been shown to improve under anti-TNF-therapies (46). In these studies the anti-TNF-α is administered systemically. We hypothesize that a topical application might be preferred since it should cause less side effects.

Inhibitory cocktail (Figure 2)

Chapter 4 shows that burn wounds exhibit excessive secretion of inflammatory mediators (such as CCL2, CCL5, CCL18, CCL27 and CXCL12) compared to surgical excision wounds. Inflammation is known to play a crucial role in regulating scar tissue formation in adult wounds, with excessive inflammation leading to adverse wound healing (40,41). Inhibition of inflammatory chemokines by using neutralizing antibodies may decrease inflammation, which could be beneficial for burn wound healing. Of course, when developing an 'inhibitory cocktail' for burn wounds, with neutralizing antibodies for inflammatory chemokines, care must be taken not to inhibit closure. Therefore, histatin may be a good candidate to include in such a cocktail as it was demonstrated in chapter 9 that this saliva-derived peptide stimulates re-epithelialization. We suggest that an inhibitory cocktail contacting anti-CCL5, anti-CCL27 and histatin would be in favor of burn wound healing. More detail on the potential working action of this proposed cocktail is described below.

Neutralization of CCL5

CCL5/ RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted). CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites (19). Neutralization of CCL5 may partly reduce inflammation which is in favor of optimal wound closure. Beside regulating inflammation, CCL5 influences more processes affecting wound healing. These will now be discussed.

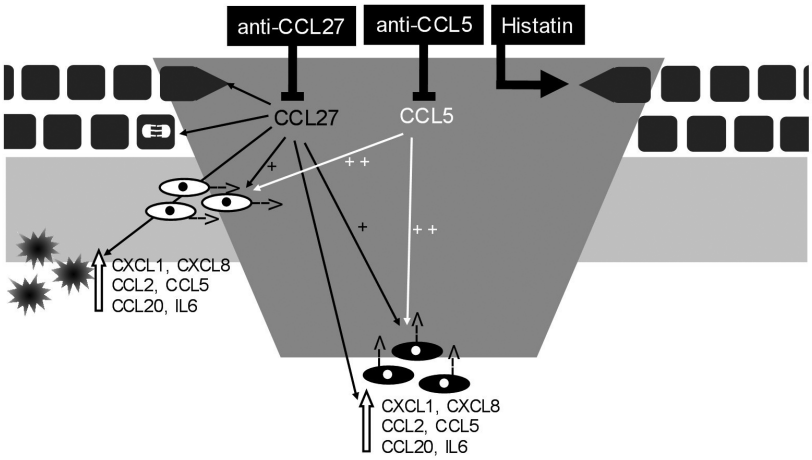

Figure 2: Schematic cross section of a burn wound.

CCL5 and CCL27 are present in the wound bed of burn wounds where they regulate several wound healing processes. These wound healing processes are inhibited by neutralization of CCL5 and CCL27. Histatin stimulates keratinocyte migration.

It is nature's evolution to promote whatever steps are necessary to immediately close an injury. For healing deep third degree burn wounds, in which the adipose tissue is exposed, this fast healing may be partly achieved by homing of adipose derived mesenchymal stem cells into the wound bed. These adipose mesenchymal stem cells have been previously described as having potent myofibroblast characteristics (high α-smooth muscle actin expression and high contractile properties) and therefore are likely to be involved not only in rapid healing but also in adverse scar formation ((18); unpublished results from our lab). In chapter 6 we show that CCL5 is also

very potent in inducing adipose- and dermal mesenchymal stem cells to migrate upon injury. This indicates that CCL5/ RANTES may be involved in hypertrophic scar formation in burn wounds by causing attraction of scar forming adipose mesenchymal stem cells into the wound bed. This conclusion was further substantiated in **chapter 3** showing expression of CCL5 already one hour after injury and in **chapter 4** demonstrating increased CCL5 expression in 3rd degree burns compared to surgical- and chronic wounds.

These results on CCL5 suggest that neutralization of CCL5 might facilitate potential therapies for deep burn wounds aimed at facilitating closure and improving scar quality. Immediate administration of e.g. neutralizing antibodies onto 3rd degree burn wounds would be expected to reduce the migration of scar forming adipose derived mesenchymal stem cells into the wound bed and reduce inflammation.

Neutralization of CCL27

Chemokines are often described as redundant since there are few unique receptor-ligands pairs; most receptors are shared by more than one ligand and vice versa. The problem with developing neutralizing therapies is therefore that an antibody that blocks the action of one chemokine or one chemokine receptor, may have little effect in the clinic, since another chemokine (receptor) will simply takes its place. CCL27 is an exception in this regard. CCL27 interacts solely with the CCR10 receptor (20). Intriguingly, CCL27 is secreted by keratinocytes which also express CCR10 suggesting an autocrine role in wound healing (21,22). **Chapter 8** shows that CCL27 is not only a chemoattractant for immunologically active effector T-cells, but also for dermal mesenchymal stem cells and scar forming adipose mesenchymal stem cells, though less potent as CCL5. In addition to stimulating homing of adipose stem cells to the wound bed, CCL27 induces secretion of granulation stimulating and inflammatory factors from adipose stem cells and monocytes as described in **chapter 8**. From **chapter 4** we know that CCL27 expression is increased in burn wounds as compared to surgical and chronic wounds and **chapter 3** shows that it is secreted already one hour after wounding. These results suggest that excessive secretion of CCL27 may play a role in scar formation. Neutralization of CCL27 in burn wounds extending into the adipose tissue, therefore, would reduce migration of scar forming adipose stem cells into the wound bed and decrease secretion of granulation stimulating factors by these cells and monocytes thereby improving scar quality. Additionally, anti-CCL27 would reduce the inflammatory response in burn wounds by lowering the local accumulation of T-cells and thus the T-cell mediated inflammation and reducing the secretion of inflammatory chemokines by ASCs and monocytes thereby facilitating wound closure.

Application of histatin

It has been an historical observation that oral wounds heal substantially faster and with less scarring and inflammation than cutaneous wounds (23). Histatins present in saliva are possibly involved in this superior oral wound healing (24). **Chapter 9** shows that indeed histatin could stimulate re-epithelialization in an *in vitro* human skin model. These results suggest that topical application of histatin would offer a potential therapy aimed at improving re-epithelialization in both burn and chronic wounds. However, it should be noted that thusfar little is known with regards to whether histatin might positively or negatively influence scar formation. This is currently under investigation. **Chapter 9** also shows that histatin exerts its effect by signaling through a G-protein coupled receptor coupled to G*ai*, as is prototypical for stimulation of chemokine receptors (25). Interestingly, CCL28 (a mucosa-associated chemokine, with strong expression in other tissues associated with mucosal epithelial surfaces and a ligand for CCR10 and CCR3 (20)) is also secreted in saliva and the C-terminus of human

CCL28 has a significant sequence similarity to histatins (26). This is not the case for other chemokines including CCL27. CCL28 is also unique in showing antimicrobial activity, similar to histatins (26). Whether histatin may act on wound healing also via chemokine receptor CCR3 is currently unknown.

CURRENT THERAPIES FOR CHRONIC WOUNDS

Chronic wounds represent a major health burden and drain on resources. Standard care of chronic wounds includes pressure relief, debridement and compression bandages (27-29). Occlusive dressings are also regularly used for chronic wound care which promote a moist environment to assist healing by promoting re-epithelialisation (30,31). Additionally dressings prevent further trauma, minimize infection risk and optimize wound environment.

Gold standard for treatment of venous ulcers is compression therapy. A more multidisciplinary approach should be employed because of the multifaceted nature of diabetic foot ulcers and pressure ulcers, but elevation of the affected foot and pressure relief are essential components of the treatment and are initiated at first presentation (44). Despite this many chronic ulcers are therapy resistant and need more advanced therapies. One example is the use of negative wound pressure to stimulate healing. Vacuum Assisted Closure (V.A.C.) therapy is believed to stimulate chronic wound healing by reduction of exudates, increase in perfusion, removal of infectious materials and formation of granulation tissue (32). For the patient a treatment with VAC therapy is associated with a reduced mobility because of the need to remain connected to a mechanical device. Additionally a randomized controlled trial on VAC therapy in patients with chronic wounds showed that VAC therapy is associated with higher costs compared to the convential dressings, due to the costs of the device and its disposable materials.

Recent advances in chronic wound healing have led to topical application of growth factors to chronic wounds (33,34). The function of growth factors is to attract various cell types into the wound, stimulate cellular proliferation, and promote angiogenesis. Clinical results from topical application of growth factors to chronic wounds have not been as rewarding as first hoped. This is not surprising considering the complexity of the wound healing process. The only currently available product proven to be efficacious in randomized, double-blind studies is PDGF (becaplermin, Regranex®). Although results were modest it demonstrated to reduce healing time and improve the incidence of complete wound healing in stage III and IV diabetic ulcers (33, 35). Regranex, therefore, is currently indicated for use together with other wound care measures to help granulation (healing) of full-thickness, neuropathic, chronic, diabetic ulcers (36). Regranex® is used on neuropathic ulcers up to 5 cm^2 in size since these ulcers are caused by a nerve problem, and not by a problem with the blood supply to the area affected. Other cytokines/growth factors currently under study *in vitro* are TGF-β, EGF,

FGF and IGF-1. The diversity of growth factors playing a role in wound healing has stimulated studies with preparations rich in growth factors. Anitua et al. describe the use of a small volume of plasma enriched in platelets releasing multiple growth factors in accelerating healing of chronic ulcers (37). Another continuous source of growth factors, but also of cytokines and chemokines, are autologous skin grafts and bioengineered skin equivalents. Use of autograft skin tissue is not favored since it requires relatively large amounts of donor skin and induces wounds with a clinical risk for impaired healing. Application of only a dermal matrix can be successful in small wounds since epithelialisation follows from the wound margins. Two-step methods have been described in which donor dermis is first applied and allowed to attach followed by application of cultured keratinocytes for diabetic foot ulcers (38). This method, although potentially promising, is hindered by logistics around timing of dermal take and culture of keratinocytes. Ideally, a full thickness skin substitute (SS) is required to close deep wounds in a one-step protocol. Apligraf®, an allogeneic full thickness SS, has been FDA-approved to promote the healing of ulcers that have failed standard wound care (39). Apligraf® is indicated for partial and full-thickness venous ulcers and full-thickness neuropathic diabetic foot ulcers of greater than 1 month duration which have not adequately respond to conventional ulcer therapy (39). Appligraf® is an allogeneic product thus implies rejection and a potential risk of disease transmission from allogeneic keratinocytes and fibroblasts. Additionally, often multiple applications are required to increase the success rate in healing chronic wounds. Ideally, a full-thickness SS should form a permanent skin tissue that will not be rejected by the immune system of the patient. Below we describe two potential therapies for facilitating wound closure of chronic ulcers. The first focuses on an autologous skin substitute and the second focuses on influencing the environment of the chronic wound.

TOWARDS NEW THERAPIES FOR CHRONIC WOUNDS

Application of a full-thickness skin substitute (SS) (Figure 3)

In contrast to burn wounds where our results clearly indicate neutralization of inflammatory chemokines would be beneficial, chronic wounds need activation (**chapter 4**). Previous results from our laboratory indicate that a tissue engineered, autologous full thickness skin substitute is suitable for healing therapy resistant leg ulcers (15). However, the means by which this SS stimulated the inert wound bed to heal was unknown. Here we showed that exposure of a full thickness SS to wound exudates isolated from chronic leg ulcers resulted in a strongly amplified increase in secretion of wound healing mediators from the SS (**chapter 2** and **5**). The proposed mechanism apparently resides in the cross-stimulatory interaction between the wound exudate and SS. **Chapter 4** and **5** demonstrate that IL-1α in the wound exudate of chronic ulcers induces an increase in secretion of potent granulation tissue

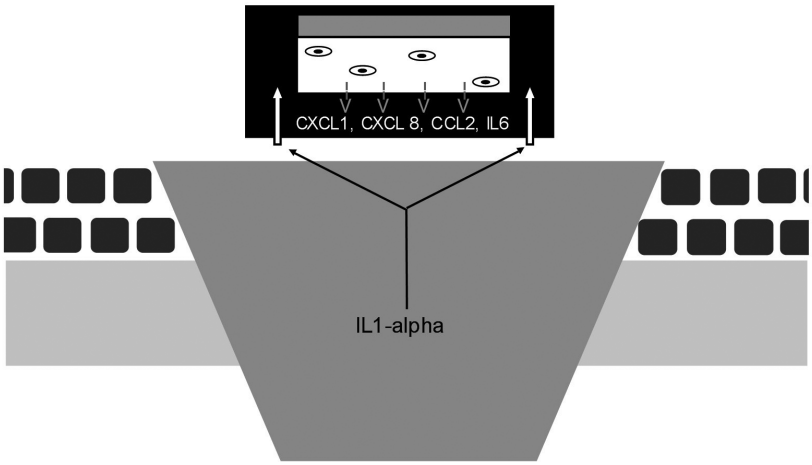

Figure 3: Schematic cross section of a chronic wound.
Depicted are the application of the SS and the presence of IL-1α in the wound bed of chronic wounds. IL-1α increases secretion of wound healing mediators from the SS.

stimulating factors from the SS (CCL2/ MCP-1, IL-6, CXCL1/Gro-α, CXCL8/ IL-8). This increased secretion of wound healing mediators in conjunction with the healthy keratinocytes and fibroblasts within the SS which are applied onto the wound bed trigger the previously unresponsive, inert wound bed to heal. Application of a SS to the wound bed revitalizes the inert wound bed of chronic wounds and therefore we propose this as the most optimal wound healing strategy for chronic wounds.

Stimulatory cocktail (Figure 4)

Our results suggest that application of recombinant chemokines, cytokines and growth factors may overcome the unresponsiveness of cells in the chronic wound bed and restore the balance between active and inactive (protease degraded) inflammatory mediators (**chapter 4**). Skin substitutes form a living slow-release system continuously providing active factors thus revitalizing the inert wound bed. Of note, SS represent an advanced therapy modality and not an off-the-shelf product. As a logistically more simple alternative, a stimulatory cocktail (crème or ointment) might also provide an attractive option for less severe chronic ulcers. Results described in **chapter 6** suggest that application of recombinant human CCL5 and CCL27 can mobilize multi-potent dermal and adipose mesenchymal stem cells facilitating wound healing and closure. Indeed, **chapter 8** indicates that application of recombinant

CCL27 to the wound bed of chronic wounds will result in keratinocyte proliferation and mobilization towards wound closure whilst at the same time stimulating infiltrating monocytes and deep subcutaneous ASCs to secrete chemokines related to the inflammatory phase of wound healing and granulation tissue formation. Interestingly, both of these 2 chemokines were poorly expressed in chronic wound exudates when compared to other chemokines eg. IL-8 (**chapter 2**). It must be noted that we showed that chemokines present in the wound exudates of chronic wounds are active and that chronic wound healing may result from the unresponsiveness of the cells in the wound. Thus still, for closure of chronic wounds supplementation with healthy fibroblasts, such as present in the SS, may be necessary.

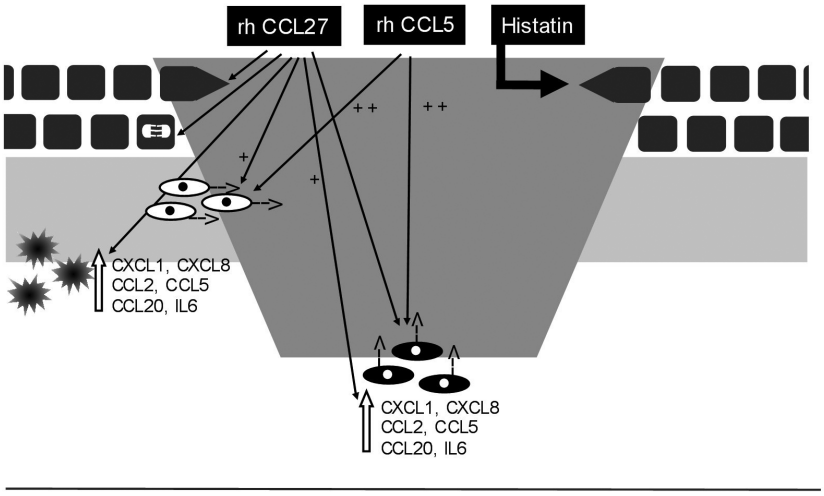

Figure 4: Schematic cross section of a chronic wound.
Application of rhCCL5 and rhCCL27 on the chronic wound bed will stimulate wound healing processes. Histatin stimulates keratinocyte migration.

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