Chapter 8  Keratinocyte, monocyte and endothelial cell-derived CCL27 promotes cell migration, proliferation and chemokine release in wound healing

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Keratinocyte, monocyte and endothelial cell-derived CCL27 promotes wound healing processes

ABSTRACT

Cutaneous wound healing is an interactive process involving skin residential cells, stem cells and infiltrating immune cells. The aim of this study was to determine the role of skin specific chemokine CTACK/CCL27 in wound healing and importantly to determine whether CCL27 could influence proliferation, migration or an inflammatory response from mesenchymal stem cells residing in the dermis (DSC) or subcutaneous adipose tissue (ASC). In addition to DSC and ASC, other skin residential cells (keratinocytes, human-microvascular-endothelial cells (hMVEC)) and infiltrating cells (granulocytes, monocytes) were studied. CCL27 was secreted into exudates after major burn injury in humans. Also, in a human excised skin burn wound model, CCL27 expression was increased in the outgrowing epidermis. CCL27 was not only secreted by keratinocytes, but also by hMVEC and monocytes after stimulation with factors related to skin trauma (VEGF and LPS respectively). All studied cells expressed the CCL27 receptor CCR10. Notably, CCL27 induced secretion of key mediators involved in angiogenesis, granulation tissue formation and inflammation (VEGF, CXCL1, CXCL8, IL-6, CCL2, CCL5, CCL20) from ASC and monocytes but not from DSC, granulocytes, keratinocytes or hMVEC. CCL27 stimulated migration and proliferation only of keratinocytes. The DSC and granulocytes neither secreted CCL27 nor responded significantly to CCL27 with regards to migration, proliferation or secretion of angiogenic/inflammatory mediators even though they expressed CCR10. Taken together these results indicate that ASC may contribute to skin wound healing by secreting angiogenic factors and that full-thickness wounds, which penetrate the adipose tissue, may respond more vigorously to CCL27 than superficial dermal wounds.
INTRODUCTION

Cutaneous wound healing is an interactive process involving skin residential cells, stem cells and infiltrating immune cells. Upon tissue damage, a cascade of cytokines and chemokines is released which initiates the first phase of wound healing, known as inflammation. Inflammatory cytokines are released by platelets and injured skin resident cells in order to attract stem cells, granulocytes, monocytes and lymphocytes into the wound bed (1-6). Infiltrating immune cells in turn release more cytokines and chemokines, target invading pathogens and initiate wound repair. The escalating secretion of growth factors and cytokines by these infiltrating cells further amplifies the inflammatory response and activates neighbouring skin residential cells and regenerative stem cells to proliferate, migrate and differentiate. The wound becomes re-epithelialized and granulation tissue is deposited (7-9). The newly formed collagen becomes reorganized and cross-linked in order to further repair and strengthen the dermis (1). As described above, many different cell types are involved in the wound healing process and extensive crosstalk takes place between these cells. Adipose derived mesenchymal stem cells (ASC) are likely to be involved in skin regeneration and wound healing due to their subcutaneous location as well as their ability to self-renew, their multi-lineage differentiation potential and their migration capacities (10,11). However, whereas much is known about ASC contribution to chondrogenic, osteogenic and adipogenic regeneration (12,13), surprisingly little is known about the ability of ASC to contribute to skin regeneration and wound healing. Furthermore, it is unknown how ASC respond during the inflammatory phase of skin trauma.

Previously we have shown that ASC and dermal derived stem cells (DSC), earlier referred to as fibroblasts, both display a mesenchymal stem cell phenotype (CD31-, CD34+, CD45-, CD54+, CD90+, CD105+, CD166+) and show similar multi-lineage differentiation potential. Also we have shown that both ASC and DSC migrate predominantly towards chemokine CCL5 (6) which is present in the wound fluid of cutaneous wounds (unpublished data). However, it is still unclear how the migrated DSC and ASC respond to epidermal derived chemokines once they enter the wound bed. In this study, we investigate the role of the skin specific chemokine CCL27 on wound healing and in particular whether this chemokine can influence ASC and DSC proliferation, migration and/ or inflammatory mediator release.

CCL27 expression has been reported to be up regulated in wounded skin of mice (14). It is described as a tissue-specific chemokine, since it has been reported to be selectively and constitutively expressed by keratinocytes in the basal layer of the epidermis (15). CCL27 was first described to play a role in skin homing of CLA+ CD4+ memory T cells expressing CCR10 during immune surveillance (16-18). It has also been reported in mice to attract CD34+ bone marrow-derived cells to the epidermis where they may trans-differentiate into keratinocytes and in doing so contribute to skin regeneration (14). The only known receptor for CCL27 is CCR10, a G protein-coupled seven-transmembrane domain receptor (15). The receptor
of CCL27 is expressed on many skin residential cells (keratinocytes, DSC, melanocytes and dermal endothelial cells) as well as on ASC and inflammatory cells (T-cells, Langerhans cells and plasma cells) involved in wound healing (6,19-21). This indicates that CCL27 may play a critical and multi-functional role in wound healing. However, the effect of CCL27 on the different cells involved in wound healing is largely unknown.

The aim of this study was to determine the role of CCL27 in wound healing and importantly to determine whether this skin specific chemokine could influence proliferation, migration or an inflammatory response from stem cells residing in the dermis (DSC) and sub-cutaneous adipose tissue (ASC). To meet our aim, CCL27 was studied in a human *in vitro* burn wound model (23), which resembles major skin trauma and gives the opportunity to investigate wound closure and factors regulating inflammation and granulation tissue formation in a physiologically relevant *in vitro* model. In order to assess the cell type specificity of the response to CCL27, ASC and DSC were compared with other cell types (keratinocytes, hMVEC, granulocytes and monocytes) involved in skin wound healing. CCL27 was found to be not only secreted by keratinocytes, but also by hMVEC and monocytes after stimulation with factors (e.g.: VEGF and LPS respectively) related to skin trauma. Notably, CCL27 acted as an early pro-inflammatory mediator by stimulating only ASC and monocytes, but not DSC, keratinocytes or hMVEC to release inflammatory mediators involved in the formation of granulation tissue and angiogenesis. This study indicates that ASC but not DSC contribute to the angiogenic/inflammatory response mediated by chemokine CCL27 after skin trauma.

**MATERIAL AND METHODS**

**Wound exudates**

*Burn wounds:* In 10 patients with 3rd degree burn wounds, eschar tissue was removed 11-21 days post burn. Eschar was removed to the depth where viable tissue was reached and wound exudates were collected from the viable tissue interface. Exudates were further handled as described below.

*Intracutaneous wound exudates:* In 12 patients undergoing elective surgery for breast reduction, incisions were made and drainage fluid was collected at 1 hour and 24 hours after wound closure.

One ml PBS containing protease inhibitor cocktail (1:100) was directly added to the exudates followed by gentle shaking at 4°C for 1 hour. After incubation samples were centrifuged and supernatant was stored at -80°C until further analysis. For the burn wound exudates the supernatant was normalized for total protein content using Bio-Rad Protein Assay (BioRad Laboratories, Hercules, California) essentially as described by supplier.
In vitro wound model

Human adult skin was obtained from healthy individuals undergoing abdominal dermolipectomy and was used directly after surgery. Skin (0.8 mm thick dermatomed, Acculan II, Braun, Tuttligen, Germany) was cut into pieces of 4 cm². For full thickness burn injury, a metal device attached to a Weller® soldering station (WSD 81 Cooper Tools, Besigheim, Germany), heated continuously at 128°C, was applied for 10 seconds to the epidermis. Unwounded skin was used as control. The (damaged) skin samples were placed dermis side down on a transwell (0.4 μm pore size; Costar Corning Incorporated, Corning, NY) and cultured at the air–liquid interface as earlier described (22). Culture medium was renewed every 24 hours. Cultures were harvested and culture supernatant were collected at different time intervals (day 0, 1, 2, and 7) for histological analysis, CCL27 and CCR10 immunostaining and CCL27 ELISA.

Cell isolation and culture

Dermal (DSC) and adipose (ASC) derived mesenchymal stem cells were isolated from human adult skin as previously described (6). Cells derived from the dermis and adipose tissue were cultured under identical conditions and used for experiments at passage 3. It should be noted that DSC were isolated and cultured using identical protocols to those previously described for dermal fibroblasts, indicating that cells commonly referred to as DSC and fibroblasts are the same cell population (23,24).

Epidermal keratinocytes were isolated from neonatal foreskin, essentially as described earlier (25). Keratinocytes were used for further experiments at passage 1.

Granulocytes were obtained from fresh human blood using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and further isolated as described by Ellerbroek et al. (26). Freshly isolated granulocytes were used for further experiments. The isolated granulocytes were CD15+ and CD45low+ as determined by flow cytometric analysis.

Monocytes were isolated from fresh human blood using Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and further isolated as previously described (27). Monocytes were used immediately for experiments and were CD14+, CD86+, HLA-DR+ and CD1a- as determined by flow cytometric analysis.

Human micro vascular endothelial cells (hMVEC) were isolated from neonatal foreskin as described previously (28). For all experiments cells were used between passage 6 and 10.

Exposures: When indicated cells were exposed to different concentrations of recombinant human (rh)-CCL27 (R&D Systems, Minneapolis, MN, U.S.A) (0, 62.5, 125, 250 and 500 ng/ml). When indicated cells were stimulated with culture medium containing 10 ng/ml TNF-α (R&D Systems, Minneapolis, MN), (keratinocytes, ASC and DSC), 10 nM N-Formylmethionyl-leucyl-phenylalanine (fMLP) (GenScript, Piscataway, NJ) (granulocytes), 100 ng/ml Lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO) (monocytes) or 10 ng/ml vascular endothelial
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growth factor (VEGF) (PrepoTech, Rocky Hill, NJ) (hMVEC). Culture supernatants were harvested (granulocytes after 4h, monocytes after 16h and keratinocytes, ASC, DSC and hMVEC after 24h) and stored at −20 °C for further analysis by ELISA. Viability of the cells after CCL27 exposure was determined as described below. Proliferation experiments were performed with keratinocytes, ASC, DSC and hMVEC. Prior to use, cells were cultured in the absence of growth factors for 24 hrs with the exception of hMVEC (in absence of endothelial cell growth factor (ECGF extracted from bovine hypothalamous Maclag et al. (29) cells did not survive). As described above cells were exposed to different concentrations of (rh)-CCL27 (0, 62.5, 125, 250 and 500 ng/ml) and stimulation media containing 5 ng/ml EGF (keratinocytes, ASC and DSC) or 10 ng/ml VEGF (hMVEC). Cultures were harvested after 3 days and the relative number of cells present was measured using the MTT (ASC, DSC and hMVEC) or LDH (keratinocytes) assay (see below).

**Cell proliferation**

ASC, DSC and hMVEC proliferation was determined with an MTT assay which measures mitochondrial activity which correlates to cell number (30). The assay was performed as described by the supplier (Sigma-Aldrich, St. Louis, MO). Keratinocyte proliferation was determined by quantifying intracellular lactate dehydrogenase (LDH) activity released into the culture medium from the cytosol upon total cell lysis with 0,1% triton in PBS (31). LDH activity was measured with a LDH detection kit (Roche Applied Science, Mannheim, Germany) in accordance to the manufacturer’s specifications. Propidium iodide staining (Invitrogen, Paisly, UK) was used to determine cell viability of granulocytes and monocytes (32).

**CCL27 immunostaining & CCR10 analysis**

Frozen cryostat tissue sections were stained for CCL27 as described earlier (17). Paraffin tissue sections were stained for CCR10 as described by Kroeze et al. (chapter 7 of this thesis). CCR10 expression was determined on the cell surface by flow cytometry as described earlier (6).

**Chemotaxis assay**

Chemotactic migration of cells towards CCL27 was assessed with the aid of a modified Boyden well chamber technique using a 24-transwell system with 3μm (granulocytes), 5μm (monocytes) or 8μm (keratinocytes. DSC, and ASC) poresize transwells (Costar Corning Incorporated, Corning, NY) essentially as described by Kroeze et al (6). For all cell types a positive chemoattractant was included in the assay: keratinocytes = 5 ng/ml EGF; granulocytes = 10 nM fMLP; monocytes = 125 ng/ml CCL2 (R&D Systems, Minneapolis, MN) and ASC = 250 ng/ml CCL5 (R&D Systems, Minneapolis, MN) and DSC = 250 ng/ml CCL5. Chambers were
incubated for 30 min (granulocytes), 16hrs (monocytes) or 24hrs (Keratinocytes, DSCs, ASCs and hMVECs) at 37°C in a humidified atmosphere.

The number of migrated granulocytes (CD15-FITC) and monocytes (HLA-DR-FITC) were quantified by FACScan flow cytometer as previously described (32).

The number of keratinocytes, DSCs, and ASCs were quantified as previously described (6). For DSCs and ASCs the number of nuclei present in 40 fold magnification on the underside of the transwell were counted. For keratinocytes total fluorescence intensity of the cells within 40 fold magnification was measured as described by Kroeze et al. (chapter 7 of this thesis).

**Enzyme-linked immunosorbent assay for cytokine and growth factor production**

All reagents were used in accordance to the manufacturer’s specifications. For CCL2, CCL5, CCL20, CCL27, CXCL1, VEGF and II-6 commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, MN) were used. For CXCL8 quantification, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used.

**Statistical analysis**

VU University medical center approved all the experiments described in this manuscript. The study was conducted according to Declaration of Helsinki 1975. Unless otherwise stated at least three independent experiments were performed, with each experiment having an intra experimental duplicate. Mann Whitney U test, paired t-test, or repeated measures ANOVA test followed by a Dunnett’s multiple comparison test were used to determine statistical significance as indicated in the figure legends. Differences were considered significant when \( p < 0.05 \).

**RESULTS**

**CCL27 and its receptor CCR10 are increased in skin wounds**

In order to determine whether CCL27 is secreted upon infliction of a human full thickness skin wound we first determined the amount of CCL27 present in intracutaneous drainage fluid isolated from surgical wounds and from exudates isolated from 3rd degree burn wound eschar. The former involves minimal tissue damage where the latter involves extensive tissue damage and tissue necrosis. CCL27 was detected in intracutaneous wound fluid collected at 1 hour (8953 ± 1433 pg/ml) and 24 hours (17973 ± 9043 pg/ml) after wounding. It was also detected in burn eschar exudates (198.5 ± 108.3 pg/total protein in mg). In line with
CCL27 secretion into wound exudates, CCL27 secretion was increased in culture supernatants collected during the first 24hrs after inflicting a full thickness burn in ex vivo healthy adult skin (23) compared to skin which had only undergone surgical excision (control) (Figure 1a and b). Levels of CCL27 remained slightly elevated for up to 7 days. In order to determine the

Figure 1: Increased CCL27 after major burn injury.
(a) Relative CCL27 secretion by unwounded excised skin (white bare) and burned skin (black bar). CCL27 secretion per 4 cm² excised skin per 24 hours per ml culture supernatant as determined by ELISA is shown for day 0-1 and day 6-7 after burning (mean ± SEM; n=3 in duplicate; *p < 0.05 by paired t-test). (b) Hematoxylin/eosin and (c) CCL27 staining and (d) CCR10 staining of re-epithelializing wound 2 days after burning. bar = 50μm
localization of CCL27 during wound healing, immunostaining was performed on tissue sections of the re-epithelializing burn wound (Figure 1c). CCL27 expression was clearly increased in the migrating front of the epidermis 2 days after injury. In agreement with Homey et al., CCL27 staining was also found in the dermis on endothelial cells of the superficial dermal plexus (arrows, figure 1c) (21). No increase above that of normal CCL27 basal layer expression was observed in the adjacent unwounded areas of the skin or in control skin, which had not been burned (Figure 1c, data not shown).

Having confirmed that CCL27 was up regulated upon infliction of major skin trauma, we next determined whether cells, in addition to keratinocytes, were able to produce CCL27. As expected keratinocytes showed a distinct basal secretion of 23 pg/ml CCL27 in contrast to ASC, DSC, hMVEC, granulocytes and monocytes where secretion was not detectable (Figure 2). Since after wounding, cells are stimulated by factors from the direct environment and by invading pathogens, we next stimulated each cell types with a relevant stimulation factor. Upon stimulation with TNF-α keratinocytes showed 15 fold increase in CCL27 secretion. Notably, monocytes and hMVECs were also able to secrete CCL27 upon stimulation with LPS and VEGF respectively (Figure 2). ASC, DSC and granulocytes did not secrete CCL27 upon stimulation with factors related to skin trauma, which are known to activate these cells. Taken together these results indicate that CCL27 has an immediate and sustained release after inflicting injury and during burn wound closure.

**Figure 2: Secretion of CCL27 by cells involved in wound healing.**
Basal secretion of CCL27 and stimulated secretion of CCL27 by cultured cells is determined by ELISA. Keratinocytes, hMVECs, monocytes, ASCs, DSCs and granulocytes were stimulated with 10 ng/ml TNF-α, 5 ng/ml VEGF, 100 ng/ml LPS, 10 ng/ml TNF-α, 10 ng/ml TNF-α and 10 nM FLMP respectively. Data are represented as the mean (± SEM) secretion of CCL27 in pg/ml corrected per hour and per 100.000 cells. (*p < 0.05, **p < 0.01 and ***p < 0.001 by paired t-test).
Next we determined which cells expressed the CCL27 receptor CCR10. CCR10 was expressed in all epidermal cell layers in human skin and was increased in the migrating front of the epidermis after injury (Figure 1d). Staining was also observed in single cells within the dermis and on blood vessels. Flow cytometry confirmed receptor expression on the cell surface of all cell types studied, with the highest expression being on keratinocytes (Table 1). ASC and DSC as well as monocytes, hMVEC and granulocytes showed a low but clear CCR10 expression.

### Table 1: CCR10 expression on cells involved in wound healing

<table>
<thead>
<tr>
<th>Cell type</th>
<th>% Cells expressing CCR10</th>
<th>MFI</th>
</tr>
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<tbody>
<tr>
<td>ASC</td>
<td>5.15 +/- 0.25</td>
<td>1.70 +/- 0.34</td>
</tr>
<tr>
<td>DSC</td>
<td>4.48 +/- 0.05</td>
<td>1.60 +/- 0.11</td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>38.00 +/- 7.00</td>
<td>30.62 +/- 8.24</td>
</tr>
<tr>
<td>Monocyte</td>
<td>11.85 +/- 1.80</td>
<td>2.74 +/- 0.42</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>6.10 +/- 1.01</td>
<td>3.84 +/- 1.46</td>
</tr>
<tr>
<td>hMVEC</td>
<td>3.65 +/- 0.54</td>
<td>1.34 +/- 0.26</td>
</tr>
</tbody>
</table>

1 The mean number of cells expressing CCR10 is shown as a percentage of total cell number +/- standard deviation (n ≥ 3). 2 MFI (Mean Fluorescence Index) is the ratio between the mean fluorescence intensity of CCR10 sample and its isotype control and is shown as a percentage of total cell number +/- standard deviations (n ≥ 3).

**CCL27 increases secretion of inflammatory and angiogenic mediators by ASC and monocytes but not DSC**

During wound healing, the inflammatory response is amplified by the release of a large number of chemokines from neighbouring and infiltrating cells. Since all cell types, including ASC and DSC, expressed CCR10 we next determined whether rh-CCL27 was able to stimulate secretion of typical mediators which influence angiogenesis, granulation tissue formation and inflammation (VEGF, CXCL1, CXCL8, IL-6, CCL2, CCL5 and CCL20) (Figure 3). Notably, rh-CCL27 stimulated ASC to increase secretion of VEGF, CXCL1, CXCL8 and IL-6, but not CCL2, CCL5 or CCL20. In contrast to ASC, no increased cytokine secretion was observed by DSC. For comparison, rh-CCL27 stimulated monocytes to increase secretion of all investigated cytokines, but not VEGF (Figure 3) whereas the other cell types (keratinocytes, granulocytes and hMVEC) did not increase mediator secretion in response to rh-CCL27 (data not shown).

**CCL27 stimulates keratinocyte migration and proliferation**

Since mobilization of cells into the wound bed is dependent on chemotaxis, we next determined which cells could migrate in a chemotactic transwell assay towards CCL27. Keratinocyte migration was increased 4 fold towards rh-CCL27 and a dose-dependent trend was observed for both ASC and DSC towards CCL27 (Figure 4). The response was chemotactic rather than chemokinetic since no significant increase in migration of these three cell types
Figure 3: Increased migration towards CCL27 by keratinocytes.

Cells were incorporated into a chemotaxic transwell assay in the presence of rh-CCL27 supplemented in the lower well. Placing an equal concentration of rh-CCL27 in upper and lower well blocks the migratory response indicating chemotaxis rather than chemokinesis. Keratinocyte migration is significantly increased by rh-CCL27. Monocytes, ASCs, DSCs and granulocytes showed no significant chemotaxis towards rh-CCL27. Data are represented as the mean (± SEM) relative migration to the underside of the transwell filter. (*p < 0.05 and **p < 0.01 by paired t-test)
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was observed when CCL27 was placed in both the upper and lower well. CCL27 had no effect on the migration of granulocytes, monocytes and hMVEC.

In order to determine the influence of CCL27 on cell proliferation, cells were incubated in the absence or presence of different concentrations rh-CCL27 (0, 62.5, 125, 250 and 500 ng/ml) for 24 hrs and 72 hrs. No difference in cell number for all cell types studied was observed after the 24 hr incubation period confirming that the increase in mediator secretion described above for ASC and monocytes was a direct effect of CCL27 exposure and not an indirect effect due to increased cell numbers. After 3 days exposure to rh-CCL27, only keratinocytes showed a slight but significant increase in proliferation compared to unexposed keratinocytes (1.3 fold by rh-CCL27 compared to 2 fold by EGF). None of the other cell types showed an increase in proliferation in response to rh-CCL27 (data not shown).

Figure 4: Rh-CCL27 stimulates monocytes and ASCs, but not keratinocytes, granulocytes, HMVECs and DSCs to secrete inflammatory chemokines.

The effect of rh-CCL27 on the secretion of chemokines involved in inflammation and granulation tissue formation was analyzed by ELISA. Data are represented as the mean (± SEM) production of chemokines in pg/ml corrected per hour and per 100,000 cells, (*p < 0.05, **p < 0.01 and ***p < 0.001 by repeated measures ANOVA test followed by a Dunnett’s multiple comparison test)
DISCUSSION

In this study, we have identified a putative role for CCL27 in wound healing. We provide evidence that CCL27 has pro-inflammatory properties and is capable of stimulating ASC but not DSC to secrete angiogenic factors upon skin trauma. Our study is to our knowledge the first to describe a tissue specific chemokine exerting a pro-inflammatory effect on stem cells located within the vicinity of the cutaneous tissue. We show that CCL27 is abundant in wound exudates. In addition to keratinocytes, also monocytes and hMVEC are able to secrete CCL27 upon stimulation. We show that CCL27 not only stimulates keratinocyte proliferation and migration, but remarkably stimulates ASC and monocytes to secrete cytokines and VEGF involved in inflammation and granulation tissue formation (Table 2).

We found that CCL27 is secreted after major burn injury in humans and is also present in intracutaneous wound fluid. This is in line with Inokuma et al. who showed that in mice CCL27 is up regulated in excision wounds compared with normal skin (14). It has been hypothesized that CCL27 secreted by keratinocytes may diffuse into the dermis where it binds to the surface of endothelial cells in the papillary dermis (21). However, secretion by other cell types in their study could not be excluded. In agreement, we observed in our full thickness burn wound model, an increase in CCL27 expression in the outgrowing epidermis and expression in microvascular structures within the dermis. Also the expression of CCR10, the receptor of CCL27, is increased in the migrating epidermis. Notably, we found that not only keratinocytes were able to secrete CCL27, but that monocytes and hMVEC, when stimulated with LPS and VEGF respectively, also secrete CCL27. LPS is a molecule present in the outer membrane of gram-negative bacteria, and VEGF is an important factor mediating angiogenesis. Both LPS and VEGF are present during wound healing (33,34). Keratinocytes, as expected, constitutively secreted CCL27 and this secretion could be further increased by stimulation with factors involved in skin wound healing, like TNF-α (15).

Notably, CCL27 triggered ASC but not DSC to secrete mediators (VEGF, CXCL1, CXCL8 and IL-6) which have also been reported to play a role in granulation tissue formation and angio-

Table 2 Summary of properties of CCL27/CCR10 interactions.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CCR10 expression</th>
<th>Constitutive</th>
<th>Inducible</th>
<th>Chemokine secretion</th>
<th>Migration</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DSC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Keratinocyte</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Monocyte</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>hMVEC</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>nd</td>
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</table>

+ = positive response; +/- = moderate response; - = negative response and nd = not done
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generation (1,7,8). The cause of this differential response is unknown since both ASC and DSC express CCR10 to similar extents on their cell surface. Our findings imply that full-thickness wounds, which penetrate the adipose tissue, may respond more vigorously to CCL27 than superficial dermal wounds. This is in line with the clinical observation that excess granulation tissue formation resulting in adverse scar formation (e.g.: hypertrophic scar) mainly occurs during healing of deep wounds which penetrate below the viable dermis (35), such as 3rd degree burns and suggests a putative role for CCL27 in scar formation. From an evolutionary point of view, the difference between ASC and DSC may be considered to be related to the function of the ASC which, in part, is to close life threatening deep wounds rapidly by strongly promoting the formation of granulation tissue and an inflammatory response. Our finding is also in line with others who showed that ASC showed greater release of angiogenic factors (VEGFa, HGF, Ang-1) than DSC (36).

Interestingly, we found that CCL27 stimulated secretion of angiogenic/ inflammatory factors VEGF, IL-6, CXCL1 and CXCL8 by ASC, but not secretion of potent inflammatory mediators CCL2, CCL5 and CCL20 (1,7,8,37). This result is in line with findings from others who reported that cytokine TNF-α increased secretion of IL-6 and CXCL8 from ASC (38,39) but did not increase transcription of CCL20 from ASC (38). Further reports describe that ASC exhibit strong angiogenic but also strong anti-inflammatory potential (36,38,40). The differential regulation of cytokine secretion, which we observed may possibly be related to this characteristic of ASC. Indeed, MSC in general are described to induce secretion of immunosuppressive cytokines which has led to their therapeutical testing in a range of autoimmune conditions (10,38,41).

In addition to ASC, monocytes were the only other cell type to respond to CCL27 by increasing cytokine secretion (but not VEGF secretion). This response was not restricted to cytokines which stimulate angiogenesis but also included the potent inflammatory mediators CCL2, CCL5 and CCL20. This indicates that CCL27 triggers infiltrating monocytes to escalate the inflammatory response upon skin trauma.

Our results show that CCL27 has potent pro-inflammatory properties and may explain the observed up-regulation described in a number of skin diseases such as allergic contact dermatitis, atopic dermatitis and psoriasis (15,42-45). It has been hypothesized that CCL27 may be responsible for the enhanced recruitment of lymphocytes resulting in chronic relapsing inflammatory skin (10,42,44) and the maintenance of memory T cells in skin after an allergic contact dermatitis reaction (17). Our finding that ASCs and monocytes respond to CCL27 by secreting inflammatory and angiogenic chemokines identifies a new function for CCL27 in promoting and maintaining skin inflammation.

Notably, the effect of CCL27 on cell function is cell type specific (see Table 2). The major functions of CCL27 were to stimulate keratinocyte migration/ proliferation and to induce mediator release from ASC and monocytes. All cell types expressed the CCR10 receptor and three of the cell types were able to secrete the ligand CCL27 (keratinocytes, monocytes and hMVEC). Since keratinocytes, hMVEC and monocytes are able to secrete CCL27 and also
express the receptor CCR10, it is possible that an autocrine feedback loop is involved. Interestingly, DSC, hMVEC and granulocytes did not respond significantly to CCL27 by any of the parameters studied even though these cell types expressed the receptor CCR10. The function of the CCR10 receptor on these cells therefore still remains unknown.

In conclusion, in a previous study we have shown that chemokine CCL5, present in wound exudates, is a potent chemoattractant for both ASC and DSC and therefore is able to draw these stem cells into the wound bed where tissue repair is required (6). This study shows that chemokine CCL27 is increased upon introduction of skin trauma and triggers specifically subcutaneous ASC and infiltrating monocytes to secrete chemokines involved in inflammation and granulation tissue formation, whilst at the same time stimulating keratinocytes to migrate in order to close the wound. Superficially located DSC and hMVEC as well as granulocytes do not respond to CCL27 with regards to chemokine release, proliferation or migration. Our results suggest that CCL27 may trigger ASC, but not DSC to produce granulation tissue forming factors upon severe full thickness skin trauma where adipose tissue is exposed. This in line the evolutionary point of view that it is important to rapidly close life threatening deep wounds.
REFERENCES


20. Gahrning LC. Osborne AV. Reed M. Rogers SW. Neuronal nicotinic alpha7 receptors modulate early neutrophil infiltration to sites of skin inflammation. *J Neuroinflammation* 2010;7:38.


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