Autocrine Regulation of Re-Epithelialization After Wounding by Chemokine Receptors CCR1, CCR10, CXCR1, CXCR2, and CXCR3

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This study identifies chemokine receptors involved in an autocrine regulation of re-epithelialization after skin tissue damage. We determined which receptors, from a panel of 13, are expressed in healthy human epidermis and which monospecific chemokine ligands, secreted by keratinocytes, were able to stimulate migration and proliferation. A reconstructed epidermis cryo(freeze)-wound model was used to assess chemokine secretion after wounding and the effect of pertussis toxin (chemokine receptor blocker) on re-epithelialization and differentiation. Chemokine receptors CCR1, CCR3, CCR4, CCR6, CCR10, CXCR1, CXCR2, CXCR3, and CXCR4 were expressed in epidermis. No expression of CCR2, CCR5, CCR7, and CCR8 was observed by either immunostaining or flow cytometry. Five chemokine receptors (CCR1, CCR10, CXCR1, CXCR2, and CXCR3) were identified, the corresponding monospecific ligands (CCL14, CCL27, CXCL8, CXCL1, CXCL10, respectively) of which were not only able to stimulate keratinocyte migration and/or proliferation but were also secreted by keratinocytes after introducing cryo-wounds into epidermal equivalents. Blocking of receptor–ligand interactions with pertussis toxin delayed re-epithelialization, but did not influence differentiation (as assessed by formation of basal layer, spinous layer, granular layer, and stratum corneum) after cryo-wounding. Taken together, these results confirm that an autocrine positive-feedback loop of epithelialization exists in order to stimulate wound closure after skin injury.

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INTRODUCTION

Chemokines constitute a family of structurally related chemotactic cytokines. Many chemokines are constitutively expressed and show increased secretion upon cutaneous damage. Chemokines were first described to direct migration of inflammatory cells (neutrophils, macrophages, monocytes, lymphocytes) into the wound bed (Gillitzer and Goebeler, 2001; Yoshie *et al.*, 2001). However, the presence of chemokine receptors on skin residential cells (e.g., keratinocytes, fibroblasts, endothelial cells) indicates that chemokines may also contribute to regulation of epithelialization, granulation tissue formation, angiogenesis, and tissue remodeling.

Our previous study focused on the role of chemokines in dermal repair and in particular on mesenchymal stem cells (Kroeze *et al.*, 2009). This study focuses on epidermal regeneration. Re-epithelialization occurs from a viable epidermal progenitor cell pool that resides in the basal layer of the epidermis and in dermal appendages such as hair follicles (Clayton *et al.*, 2007; Gurtner *et al.*, 2008). Re-epithelialization involves keratinocyte migration and proliferation, followed by differentiation in order to regenerate the epidermis during wound closure. Keratinocyte migration begins 3–6 hours after wounding and proceeds with proliferation and differentiation (Martin, 1997). Several hours after the onset of migration, keratinocyte proliferation is increased distal from the migrating edge (Martin, 1997; Jacinto *et al.*, 2001). As wound-healing processes are triggered very early after wounding and before infiltrating cells enter the wound area, it is possible that keratinocytes initiate re-epithelialization in an autocrine manner.

Chemokine receptor–ligand interactions have been described to be involved in re-epithelialization. Steude *et al.* (2002) reported in an *in vitro* skin model that CXCL1 and CXCL8 induce keratinocyte migration by binding to the receptor CXCR2 (Rennekampf *et al.*, 1997, 2000). In addition, several other ligand–receptor interactions (CXCL12/CXCR4, CCL17/CCR4, and CCL27/CCR10) have been described to be involved in migration and proliferation of keratinocytes (Florin *et al.*, 2005; Fujimoto *et al.*, 2008). *In vivo* results using CXCL11−/− and CXCR3−/− mice demonstrated a delayed re-epithelialization after wounding,

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which suggested an autocrine regulation of re-epithelialization (Yates *et al.*, 2008, 2009). Whereas all of these studies clearly indicate a role for chemokines in re-epithelialization, until now no distinction has been made between autocrine and paracrine regulation. As most studies generally associate chemokine expression with inflammatory cell recruitment, these studies often suggest paracrine regulation of re-epithelialization. We suggest that an early autocrine regulation would be favorable as it would result in an immediate response to tissue damage before infiltrating cells are able to induce a chemokine cascade and amplified wound-healing response. Paracrine regulation of re-epithelialization would be expected to result in a delayed response as the cells would have to respond to a chemokine gradient originating from another cell type, e.g., infiltrating cell or fibroblast. By using a reconstructed epidermis wound-healing model, we were able to determine whether autocrine chemokine regulation is involved in re-epithelialization and identify putative chemokines involved.

In this study, we describe chemokine receptor expression and tissue location in native healthy human epidermis. We describe which monospecific binding chemokines for these receptors are secreted by keratinocytes and whether these same chemokines can stimulate keratinocyte migration, proliferation, and differentiation. An *in vitro* reconstructed epidermal equivalent wound-healing model was used to determine which chemokines were secreted by keratinocytes and whether or not the chemokine receptor blocker pertussis toxin was able to inhibit re-epithelialization. We show that pertussis toxin is able to inhibit re-epithelialization by preventing chemokines secreted by epidermal keratinocytes from binding to their receptors. This study identifies chemokine receptors involved in an autocrine regulation of re-epithelialization after skin tissue damage.

RESULTS

Chemokine receptor expression on epidermal cells

If autocrine chemokine regulation initiating re-epithelialization occurs, it would be expected that chemokine receptors would already be present on the surface of cells within normal healthy epidermis in order for these cells to immediately respond to tissue damage without delay. Therefore, we first determined the tissue location (immunohistochemical staining) and cell-surface expression (flow cytometry) of chemokine receptors in human epidermis (Figure 1). Chemokine receptors CCR1, CCR3, CCR4, CCR6, CCR10, CXCR1, CXCR2, CXCR3, and CXCR4 were expressed in the epidermis (Figure 1, Table 1). Immunohistochemical staining occurred throughout the epidermis for CCR1, CCR4, CXCR1, CXCR2, and CXCR4, in line with flow cytometry, which showed a similar homogenous expression for these receptors. CCR10 was also expressed throughout the epidermis, but flow cytometry identified a heterogenous intensity of expression of CCR10. Chemokine receptors CCR3, CCR6, and CXCR3 showed a differential expression within the epidermis; CCR3 and CCR6 were expressed predominantly in suprabasal epidermal layers, whereas CXCR3 was expressed in basal and lower spinous layers. The heterogenous expression of these receptors was confirmed by flow cytometry. No expression of CCR2, CCR5, CCR7, and CCR8 was observed by either immunostaining or flow cytometry.

Chemokine receptor expression was also studied in primary cultured keratinocytes, which had been incorporated into a three-dimensional reconstructed epidermis *in vitro* (Figure 1). These epidermal equivalents demonstrated similar results with regard to chemokine receptor tissue location and cell-surface expression to that observed in normal human epidermis. Therefore, the epidermal equivalent closely resembles *in vivo* human skin (epidermal tissue architecture as well as chemokine receptor expression) and ascertains the use of this model for further experiments.

Chemokine-mediated keratinocyte migration

Re-epithelialization after wounding involves both keratinocyte migration and proliferation. To determine whether the chemokine receptors identified on the cell surface of epidermal keratinocytes could be involved in epithelialization, we next determined the effect of monospecific chemokines (ligands that bind to only one receptor; CCL14/CCR1; CCL20/CCR6; CCL22/CCR4; CCL24/CCR3; CCL27/CCR10; CXCL1/CXCR2; CXCL10/CXCR3; CXCL12/CXCR4) on keratinocyte migration in a chemotaxis transwell assay. For CXCR1, no monospecific chemokine has been identified, and therefore CXCL8 was used, which also binds to CXCR2.

Three different types of response were observed: (i) Chemotaxis—a strong dose-dependent increase in migration of keratinocytes toward CCL14, CCL22, CCL27, CXCL1, and CXCL10 was observed (Figure 2a). When an equal concentration of each of these chemokines (125 ng ml^{-1}) was placed in the upper and lower well, no increase in keratinocyte migration occurred compared with the medium control, indicating that chemotaxis, rather than chemokinesis, was involved; (ii) Chemokinesis—a strong dose-dependent increase in migration of keratinocytes was also observed toward CXCL8 (Figure 2b). However, the mode of action was chemokinesis rather than chemotaxis, as equal concentrations of CXCL8 (125 ng ml^{-1}) in the upper and lower compartments still induced a greater migratory response compared with control; (iii) No effect on migration—CCL20, CCL24, and CXCL12 were not able to increase cell migration compared with the medium control (Figure 2c).

Chemokine-mediated keratinocyte proliferation

Having identified the chemokine ligand–receptor pairs involved in initiating keratinocyte migration, we next determined which chemokine ligand receptor pairs could stimulate keratinocyte proliferation.

A 2-fold increase in proliferation compared with unsupplemented cultures was observed after exposure of keratinocytes to CCL24, CXCL1, CXCL8, and CXCL12. Characteristic peaks in the dose–response data indicated that an optimal working concentration existed for each chemokine (Figure 3a). In contrast, CCL14, CCL20, CCL22, CCL27, and CXCL10 were not able to increase cell proliferation (Figure 3b).

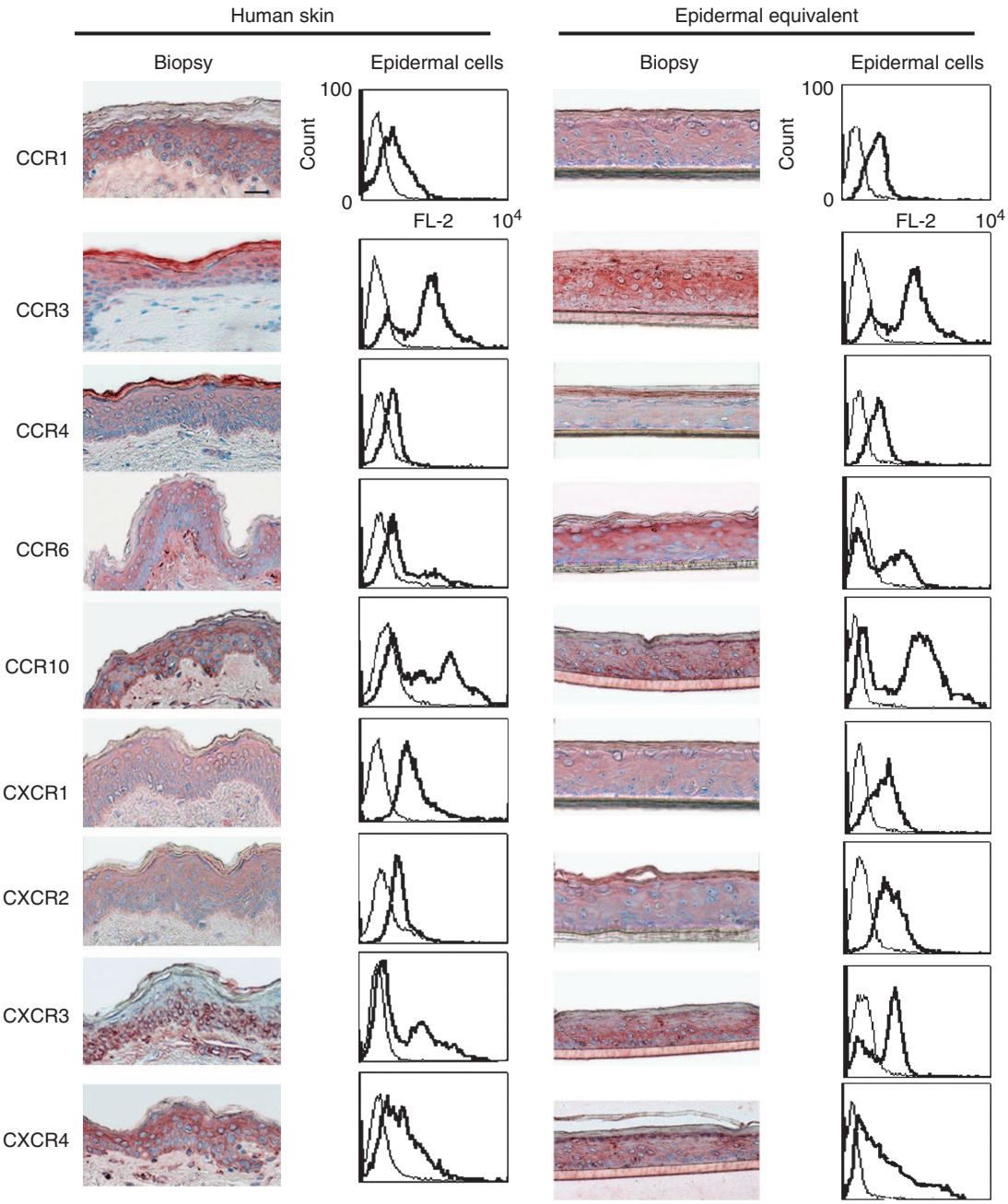

Figure 1. Chemokine receptor expression on human epidermal keratinocytes. Immunohistochemical staining was used to locate chemokine receptors in the epidermis of human skin and epidermal equivalents. Flow cytometry was used to determine chemokine receptor expression on keratinocyte cell surface. Tissue sections or keratinocytes isolated from fresh skin tissue or epidermal equivalents were stained with mAbs against the indicated phycoerythrin-labeled chemokine receptors (thick line in histogram). Each histogram plot contains a phycoerythrin-labeled isotype-matched control (thin line). Data shown are from one individual donor (skin biopsy/epidermal equivalent and keratinocyte isolation matched) and are representative experiments from three donors performed in duplicate. Bar = 250 μm.

Taken together, our results on monospecific binding chemokines can be subdivided into four groups depending on how they mediate keratinocyte migration and proliferation: (i) CXCL1/CXCR2, CXCL8/CXCR1 and CXCL2, and CXCL12/CXCR4 interactions increase both keratinocyte migration and proliferation; (ii) CCL14/CCR1, CCL22/CCR4, CCL27/CCR10, and CXCL10/CXCR3 interactions increase only cell migration; (iii) CCL24/CCR3 interaction increases only proliferation; and (iv) CCL20/CCR6 has no effect on migration or proliferation (Table 1). Note that additional nonspecific chemokines that are able to bind to multiple chemokine receptors are also summarized in Table 1. However, because of their nonspecific binding nature, these chemokines were not used to investigate specific receptor function.

Table 1. Summary of chemokine receptor expression on epidermal keratinocytes and the effect of receptor-specific chemokines on chemotaxis and proliferation

Chemokine receptor	Present on keratinocyte	Epidermal location	Receptor-specific ligand[1]	Chemotaxis	Proliferation	Other chemokine ligands and their receptors[2]	
CCR1	Yes	All epidermal layers	CCL14	Yes	No	CCL3	CCR1, 5
						CCL5	CCR1, 3, 4
						CCL7	CCR1, 3
						CCL8	CCR1, 2, 3, 5
						CCL13	CCR1, 2, 3
						CCL15	CCR1, 3
						CCL16	CCR1, 2, 5
						CCL23	CCR1
CCR3	Yes	SB	CCL24	No	Yes	CCL5	CCR1, 3, 4
						CCL7	CCR1, 2, 3
						CCL8	CCR1, 2, 5
						CCL11	CCR3
						CCL13	CCR1, 2, 3
						CCL15	CCR1, 3
						CCL24	CCR3
						CCL26	CCR3
						CCL28	CCR10
CCR4	Yes	All epidermal layers	CCL22	Yes	No	CCL5	CCR1, 3, 4, 5
						CCL17	CCR4
CCR6	Yes	SS^U, SG	CCL20	No	No	None	
CCR10	Yes	All epidermal layers	CCL27	Yes	No	CCL28	CCR3, 10
CXCR1	Yes	All epidermal layers	CXCL8[3]	Yes[4]	Yes	CXCL6	CXCR1, 2
CXCR2	Yes	All epidermal layers	CXCL1	Yes	Yes	CXCL2	CXCR2
						CXCL3	CXCR2
						CXCL5	CXCR2
						CXCL8	CXCR1, 2
CXCR3	Yes	BL, SS^L	CXCL10	Yes	No	CXCL4	CXCR3
						CXCL9	CXCR3
						CXCL11	CXCR3
CXCR4	Yes	All epidermal layers	CXCL12	Yes	Yes	None	
CCR2	No						
CCR5	No						
CCR7	No						
CCR8	No						

Abbreviations: BL, basal layer; SB, suprabasal layer; SG, stratum granulosum/granular layer; SS^L, lower stratum spinosum/spinous layer; SS^U, upper stratum spinosum/spinous layer.
[1]Monospecific binding chemokines investigated in this study.
[2]Data derived from Zlotnik *et al.* (2006).
[3]Also a ligand for CXCR2.
[4]Chemokinesis instead of chemotaxis.

Chemokines secreted by keratinocytes in response to wounding
Once we had identified the key chemokine receptors responsible for initiating proliferation and migration, it was next important to determine whether the monospecific ligands for these receptors were indeed secreted by keratinocytes after epidermal wounding. To investigate this, we used our previously described *in vitro* epidermal wound-healing model that consists of reconstructed epidermis (keratinocytes) grown on human acellular donor dermis (Breetveld *et al.*, 2006). Because of the air-exposed culture method, complete epidermal differentiation occurs, resulting in a basal layer, spinous layer, granular layer, and stratum corneum similar to native healthy skin. In this model, a full-thickness standardized wound was introduced by cryofreezing a defined region of the epidermis. Chemokine secretion before and after wounding was analyzed. Figure 4 shows that the epidermal equivalent secretes basal levels of CCL14, CCL20, CCL27, CXCL1, CXCL8, and CXCL10. Increased secretion of CCL14, CCL27, and CXCL10 (and increased trend for CCL20, CXCL1, and CXCL8) was observed 24 hours after wounding. It is noteworthy that these chemokines were also secreted by excised healthy human skin, confirming the relevance of the *in vitro* model (Table 2). Chemokines CCL22, CCL24, and CXCL12 were not secreted by epidermal

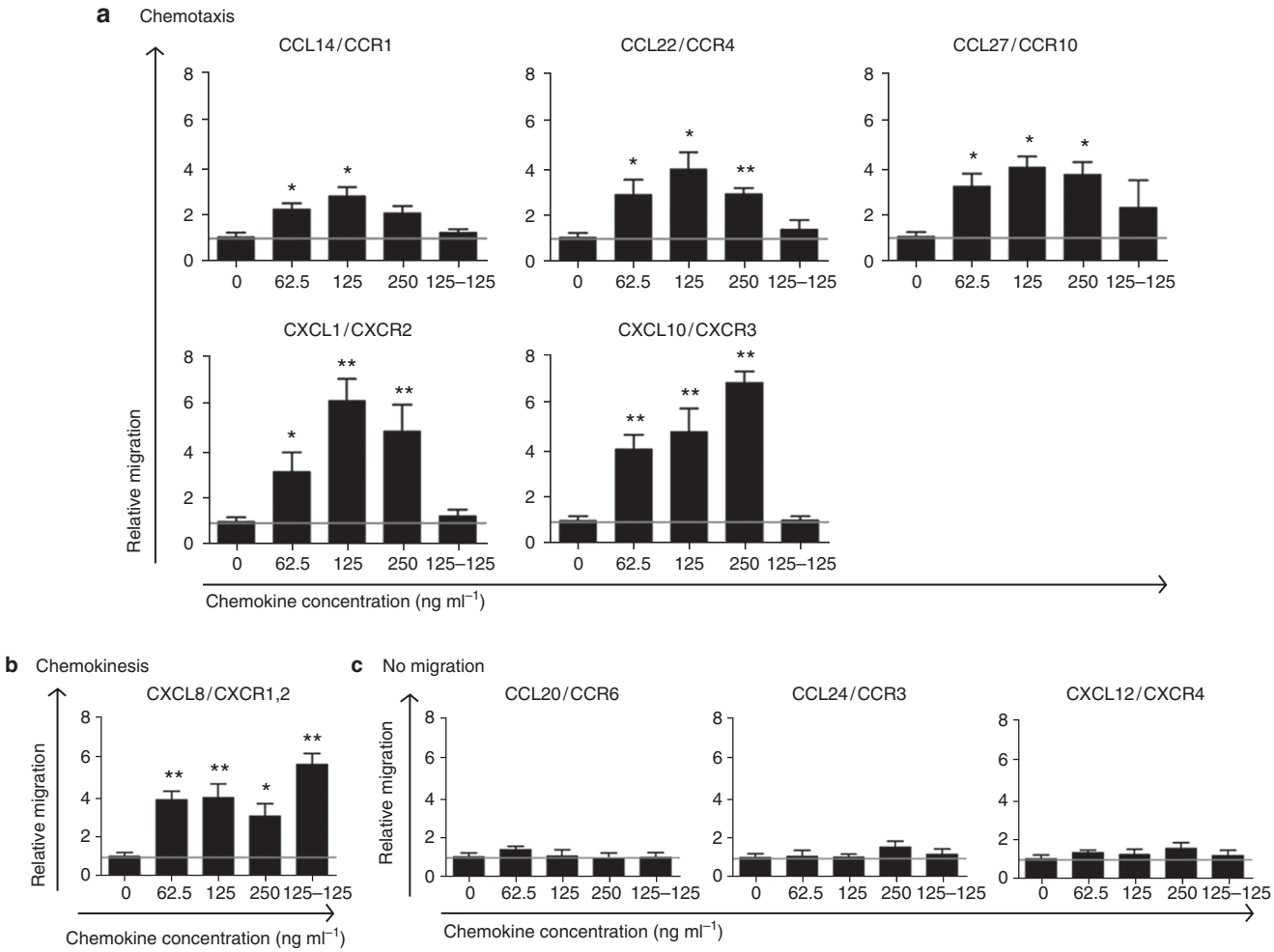

Figure 2. Migration of keratinocytes upon chemokine exposure. Cultured keratinocytes were seeded into the upper chamber of a chemotaxis transwell with chemokines in the lower well. As a control, an equal concentration of chemokine (125 ng ml^{-1}) in the upper and lower well distinguished chemotaxis from chemokinesis (125–125). Keratinocyte migration from the upper to the lower transwell surface is expressed relative to unsupplemented cultures. Three groups of chemokines could be distinguished based upon their effect on keratinocyte migration: (**a**) inducing chemotaxis, (**b**) inducing chemokinesis, and (**c**) no effect on keratinocyte migration. Statistical significant differences between supplemented and unsupplemented keratinocytes were calculated using one-way analysis of variance test, followed by Dunnett's test. Differences were considered significant when $*P<0.05$ or $**P<0.01$.

equivalents either before or after wounding, and CCL24 and CXCL12 were also not secreted at detectable levels by excised skin (15 pg ml^{-1} is the detection limit of ELISA). However, CCL22 was detectable in excised skin.

It is noteworthy that chemokine receptor expression remained unaltered after wounding as observed by immunohistochemical analysis of the migrating epithelial tongue and flow cytometric analysis of keratinocytes incorporated into a scratch-wound-healing assay (see Kroeze *et al.*, 2009 for method; data not shown).

Autocrine regulation of re-epithelialization after wounding by chemokines

As keratinocytes secrete a number of chemokine ligands (CCL14, CCL20, CCL27, CXCL1, CXCL8, and CXCL10) that are able to bind to a single receptor present on their cell surface, it is most probable that an autocrine regulation of re-epithelialization occurs. To confirm this, the degree of re-epithelialization in the wound-healing model was determined after wounding in the presence or absence of a chemokine receptor blocker (pertussis toxin). In this model, the regenerating epidermis forms underneath the dead epidermal tissue (Breetveld *et al.*, 2006). Re-epithelialization occurs from the wound margins. A representative photograph of a wound margin is shown in Figure 5a. Incubation with pertussis toxin showed a dose-dependent inhibition of re-epithelialization (Figure 5). A 50% decrease in re-epithelialization of the dermal matrix was observed after supplementing culture medium with 800 ng ml^{-1} pertussis toxin. This finding confirms that chemokines secreted by keratinocytes do indeed provide an immediate autocrine feedback loop to initiate wound closure upon tissue injury.

Although re-epithelialization was clearly inhibited by pertussis toxin, epidermal differentiation was not affected (Figure 5a). Although the outgrowth of the epidermal tongue was less, the epidermis which did regenerate showed an

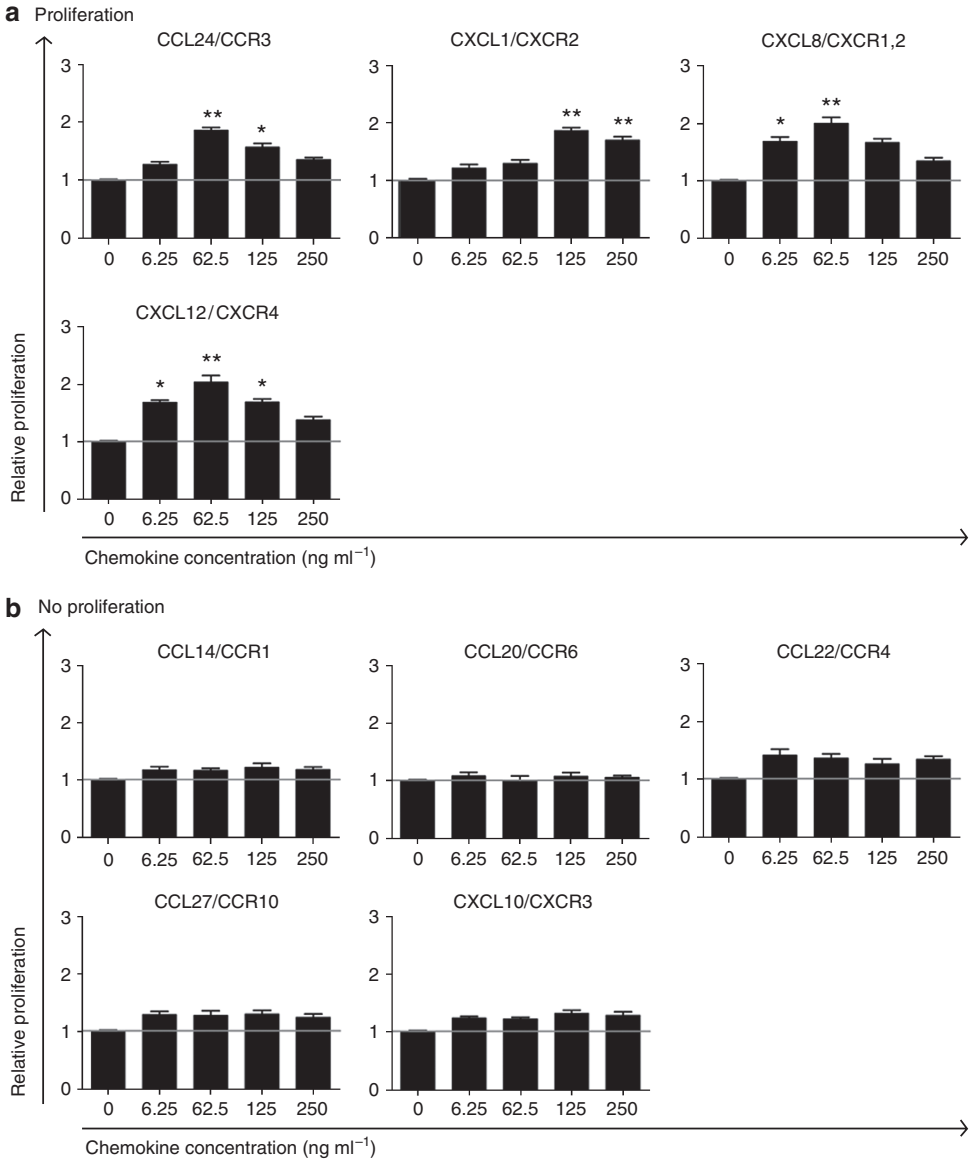

Figure 3. Proliferation of keratinocytes upon chemokine exposure. Proliferation was assessed using a lactate dehydrogenase assay. Cultured keratinocytes were cultured in the presence of chemokines. Keratinocyte proliferation is expressed relative to unsupplemented keratinocytes. Two groups of chemokines could be distinguished based upon their ability to stimulate keratinocyte proliferation. Statistically significant differences between supplemented and unsupplemented keratinocytes were calculated using one-way analysis of variance test, followed by Dunnett's test. Differences were considered significant when $*P<0.05$ or $**P<0.01$.

equal number of cell layers consisting of a newly formed basal layer, spinous layer, granular layer, and stratum corneum similar to control cultures (without pertussis toxin). Therefore, chemokines did not regulate differentiation in an autocrine manner.

DISCUSSION

Here we show that an autocrine regulation of re-epithelialization exists, which has a direct effect on stimulating wound closure. Keratinocytes secrete chemokines, which in turn are able to bind to receptors already present on their cell surface. This autocrine loop initiates migration and/or proliferation, but does not influence epidermal differentiation.

In this study, we have identified five chemokine receptors (CCR1, CCR10, CXCR1, CXCR2, and CXCR3) that are expressed on the surface of keratinocytes. Their corresponding ligands (which notably can only bind monospecifically to these receptors) are not only able to stimulate keratinocyte migration and/or proliferation, but are also secreted by keratinocytes. In addition, the chemokine receptor blocker, pertussis toxin, was able to partially block re-epithelialization in a fully defined (serum free) *in vitro* epidermal wound-healing model consisting only of keratinocytes. Chemokine receptors were already expressed in normal unwounded epidermis, allowing for immediate re-epithelialization after wounding, and were not further upregulated after wounding.

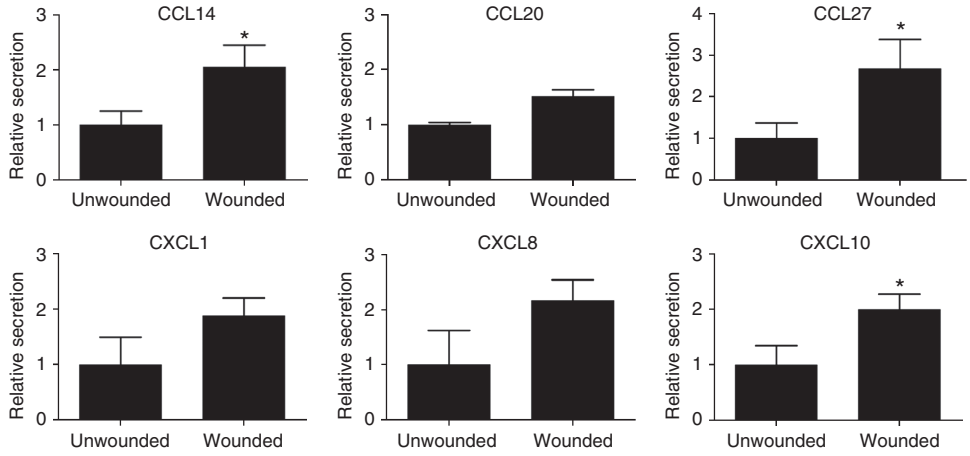

Figure 4. Increased chemokine production by keratinocytes 24 hours after wounding. Culture supernatants were collected after 24 hours from unwounded and cryo (freeze)-wounded epidermal equivalent cultures. Chemokine secretion was measured by using ELISA. Each bar represents the mean ± SEM of three independent experiments each conducted in duplicate. Statistical significant differences between unwounded and wounded epidermal models were calculated using one-way analysis of variance test, followed by Dunnett's test. Differences were considered significant when $*P<0.05$.

Table 2. Chemokine ligand secretion by excised adult skin and epidermal equivalents

Chemokine ligand	Excised skin	Epidermal equivalent
CCL14	112 ± 16.7 pg ml^{-1}	64.9 ± 15.9 pg ml^{-1}
CCL20	3.21 ± 0.58 pg ml^{-1}	10.6 ± 1.42 pg ml^{-1}
CCL27	43.3 ± 16.1 pg ml^{-1}	197 ± 149 pg ml^{-1}
CXCL1	24.9 ± 7.09 ng ml^{-1}	620 ± 305 pg ml^{-1}
CXCL8	105 ± 13.5 ng ml^{-1}	349 ± 205 pg ml^{-1}
CXCL10	787 ± 349 pg ml^{-1}	203 ± 70.5 pg ml^{-1}
CCL22	139 ± 18.1 pg ml^{-1}	ND
CCL24	ND	ND
CXCL12	ND	ND

Abbreviation: ND, not detectable.
Chemokine amounts were measured by ELISA of culture supernatants from 4-cm^2 excised skin or epidermal equivalents collected over 24 h. Data represent three different donors each in duplicate (n=3 ± SEM). Detection limit of ELISA=15 pg ml^{-1}.

Taken together, these results confirm that an autocrine regulation of epithelialization exists in order to stimulate wound closure after skin injury.

In addition to autocrine regulation, our results also identify chemokine receptors that may be associated with paracrine regulation. Three of the nine chemokines in our study, namely CCL22, CCL24, and CXCL12, were not secreted by keratinocytes in the wound-healing model. However, their corresponding receptors (CCR4, CCR3, and CXCR4) were present on epidermal keratinocytes *in vivo*, and their ligands (CCL22, CCL24, and CXCL12) were able to induce migration and/or proliferation *in vitro*. In line with these results, chemokine CCL24 has been reported to be secreted by dermal fibroblasts and is known to induce keratinocyte proliferation (Dulkys *et al.*, 2001; Petering *et al.*, 2001). For CXCL12, we and others have previously shown that dermal fibroblasts secrete this ligand, which in turn stimulates keratinocyte proliferation via its only receptor CXCR4 (Florin *et al.*, 2005; Ouwehand *et al.*, 2008). With regard to CCR4, Katou *et al.* (2001) reported that its ligand CCL22 is secreted by macrophages and epidermal Langerhans cells. In addition to CCL22, CCR4 has two other ligands (CCL5 and CCL17; not monospecific). Both of these ligands are produced by keratinocytes and function as mitogens for keratinocytes, suggesting that CCR4 may be involved in the autocrine regulation of keratinocyte migration but via CCL5 and CCL17 rather than via CCL22 (Li *et al.*, 1996; Tsuda *et al.*, 2003; Fujimoto *et al.*, 2008). As we were only able to detect secretion of CCL22 from excised skin, it is possible that the ligands CCL24 and CXCL12 were secreted at very low amounts, were directly taken up by adjacent cells, or were bound to the dermis, rather than being released into the culture supernatant.

We identified one chemokine receptor (CCR6) that was present on the surface of differentiated keratinocytes in the upper layers of the epidermis and the ligand (CCL20) of which did not influence keratinocyte migration or proliferation in any way, even though its secretion was increased upon epidermal damage. This finding is in line with others who have identified CCL20 as a chemokine with antimicrobial properties, which is secreted from differentiated keratinocytes (Tohyama *et al.*, 2001; Pernet *et al.*, 2003). It is therefore possible that CCL20/CCR6 is regulated in an autocrine manner with its role being to control pathogen infection after wounding rather than wound closure.

Whereas reports by others have emphasized the important role of chemokines in wound healing, these reports did not distinguish between autocrine and paracrine regulation of re-epithelialization and only focused on one or two ligand–receptor interactions. For example, reports describe upregulation of CXCR2 (receptor for CXCL1 and CXCL8) early after wounding in undifferentiated keratinocytes

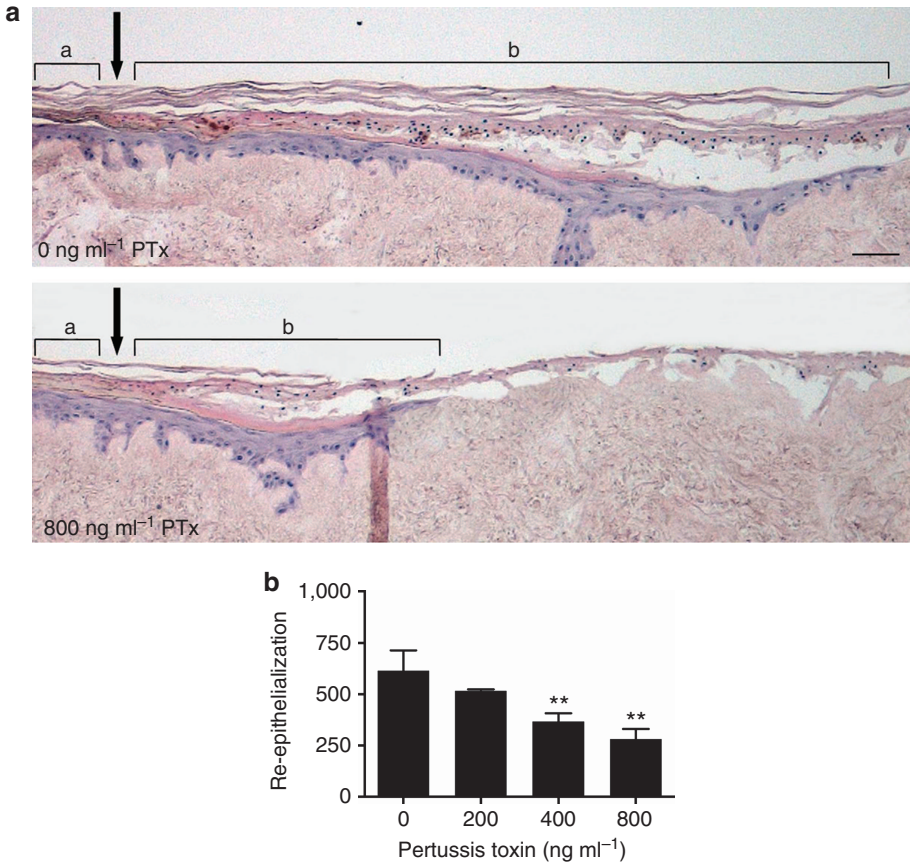

Figure 5. Pertussis toxin inhibits re-epithelialization in an epidermal wound model. Cryo(freeze)-wounds were introduced into epidermal equivalents and the medium was supplemented with 0, 200, 400, and 800 ng ml^{-1} pertussis toxin (PTx). Cultures were harvested 12 days later. (**a**) A representative photograph showing histology of re-epithelialization in the epidermal equivalent cultured in unsupplemented and supplemented (800 ng ml^{-1} PTx) medium. a, Unwounded epidermis; b, newly formed epidermis; arrow, wound margin; bar = 250 μm. (**b**) Re-epithelialization was measured as the distance the newly formed epidermis migrated into the wound bed. Each bar represents the mean ± SEM of three independent experiments conducted in duplicate. Statistical significant differences between supplemented and unsupplemented epidermal equivalents were calculated using one-way analysis of variance test, followed by Dunnett's test. Differences were considered significant when $^{**}P<0.01$.

(Nanney *et al.*, 1995), and mice lacking CXCR2 show impaired re-epithelialization after wounding (Devalaraja *et al.*, 2000). In addition, expression of CXCR3 (receptor for CXCL10) on keratinocytes (Satish *et al.*, 2003) and a delayed re-epithelialization in mice lacking CXCR3 and CXCL11 has been reported (Yates *et al.*, 2008, 2009). Low *et al.* (2001) described delayed wound healing in MCP1 knockout mice. Fujimoto *et al.* (2008) described CCR10 expression on epidermal keratinocytes and increased migration to its recombinant ligand CCL27, and Petering *et al.* (2001) described proliferation of human keratinocytes after stimulation with CCL24, which may be inhibited by anti-CCR3. Our study clearly distinguishes the autocrine from the paracrine mechanisms involved in wound healing, and this information will enable us and others to identify target molecules and cells for developing new wound-healing strategies in the future.

Importantly, in our study, we show that multiple chemokine receptors are involved in the autocrine regulation of re-epithelialization. Therefore, mutation or blocking of a single receptor would enable a bypass mechanism via the other receptors to take place, thus ensuring that wound closure still occurs even if a single gene should malfunction. Indeed, studies using knockout mouse show delayed wound healing but not the absence of wound healing (Low *et al.*, 2001; Yates *et al.*, 2008, 2009).

In conclusion, we show that autocrine regulation of re-epithelialization occurs via chemokine receptors and their ligands. Such a response may facilitate early triggering of wound closure. This early autocrine response is most probably combined with paracrine responses from infiltrating cells and neighboring skin cells in order to amplify the wound-healing cascade.

MATERIALS AND METHODS

Isolation and culture of human keratinocytes and epidermal equivalents

Human adult skin was obtained from healthy donors (with written informed consent) undergoing abdominal dermolipectomy and was used directly after surgery. The VU University medical center approved the experiments described in this paper. The study was conducted according to Declaration of Helsinki Principles.

Epidermal cells were isolated from human skin essentially as described earlier (Waaijman *et al.*, 2010). In brief, epidermis was

isolated from human skin by overnight incubation at 4 °C in dispase (Roche, Mannheim, Germany). Thereafter, a cell suspension was made by incubation in trypsin (Gibco, Invitrogen, Paisley, UK) for 15 minutes at 37 °C. Freshly isolated keratinocytes were used for flow cytometry analysis or further cultured. Subconfluent, first-passage keratinocytes cultured in keratinocyte medium (DMEM/Ham's F12 (3:1), 1% UltroserG, 1% penicillin–streptomycin, 1 μmol l^{-1} hydrocortisone, 1 μmol l^{-1} isoproterenol, 0.1 μmol l^{-1} insulin and 2 ng ml^{-1} keratinocyte growth factor) were used for experiments to assess keratinocyte migration and proliferation and to construct the *in vitro* wound-healing model.

To construct epidermal equivalents for the wound-healing model and receptor analysis, keratinocytes were seeded onto acellular de-epidermized dermis or onto 24-mm diameter transwells (pore size of 0.4 μm; Corning, Corning, NY), respectively (Breetveld *et al.*, 2006), and cultured submerged for 1 week in keratinocyte medium. After 1 week, cultures were lifted to the air–liquid interface and cultured for a further week in DMEM/Ham's F12 (3:1), 0.2% UltroserG, 1% penicillin–streptomycin, 1 μmol l^{-1} hydrocortisone, 1 μmol l^{-1} isoproterenol, 0.1 μmol l^{-1} insulin, 1.0×10^{-5} mol l^{-1} L-carnitine, and 1.0×10^{-5} mol l^{-1} L-serine supplemented with 2 ng ml^{-1} keratinocyte growth factor and 50 μg ml^{-1} ascorbic acid. Thereafter, UltroserG and keratinocyte growth factor were omitted from the culture medium for 2 days before introducing the cryo-wound and for the duration of the experiment. The medium was refreshed twice a week. Unless otherwise stated, all culture additives were obtained from Sigma (St Louis, MO).

Immunohistochemical analysis

Human skin biopsies and epidermal equivalents were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde, and processed for conventional paraffin embedding. Sections (5 μm) were cut, deparaffinized, and rehydrated in preparation for immunohistochemical analysis of chemokine receptors. Immunohistochemical procedures were performed as described previously (Kroeze *et al.*, 2009). Photographs were taken with a computer-assisted microscope (Nikon, Dusseldorf, Germany). Antibodies and corresponding isotypes used were obtained from (BD Biosciences, Franklin Lakes, NJ) generated in mice, and reactive with human unless stated otherwise: CCR1 (53504, IgG2b), CCR2 (48607, IgG2b), CCR3 (5E8, IgG2b), CCR4 (1G1, IgG1), CCR5 (2D7, IgG2a), CCR6 (11A9, IgG1), rat anti-human CCR7 (3D12, IgG2a), rat anti-human CCR8 (191704, IgG2b, R&D Systems, Minneapolis, MN), rat anti-human CCR10 (314305, IgG2a, R&D Systems), CXCR1 (5A12, IgG1), CXCR2 (CDw128b, IgG2b), CXCR3 (1C6/cxcr3, IgG1), and CXCR4 (12G5, IgG2a, R&D Systems). Staining for each receptor was performed using skin or epidermal equivalents derived from the same three different donors and in duplicate.

Flow cytometric analysis

Epidermal cells isolated from human epidermis or epidermal equivalents were examined for cell-surface expression of chemokine receptors. Cells were incubated for 30 minutes with antibodies, washed in PBS supplemented with 0.1% BSA and 0.1% sodium azide, and then resuspended in the same buffer for FACS analysis. Cells were measured on a FACScan and analyzed with Cell Quest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Phycoerythrin-labeled antibodies and corresponding isotypes were the same as those used for immunohistochemistry (see above). Staining for each receptor was performed using skin or epidermal equivalents derived from three different donors in duplicate.

Chemotaxis assay

Chemotactic migration of second-passage keratinocytes to chemokine ligands was assessed by the Boyden well chamber technique as described previously for dermal- and adipose-derived stromal cells (Kroeze *et al.*, 2009). Before starting the experiment, the transwell inserts were coated with collagen IV, and keratinocytes were incubated overnight in serum-free medium (DMEM/Ham's F12 (3:1), 1% penicillin–streptomycin, 1 μmol l^{-1} hydrocortisone, 1 μmol l^{-1} isoproterenol, and 0.1 μmol l^{-1} insulin). The cell suspension (200 μl; 5×10^5 per ml in serum-free medium) was loaded into the upper well of the chamber and allowed to attach for 4 hours. After 4 hours, different concentrations of chemokine were placed in the lower well and migration was allowed for 24 hours. The number of migrated cells was determined by counting the number of nuclei in a 40-fold magnification area with a computer-assisted fluorescence microscope (Nikon, Dusseldorf, Germany). Migration after exposure to chemokine is given relative to control. Experiments were conducted using three different donors, each in duplicate.

Proliferation assay

Proliferation of second-passage keratinocytes in response to chemokine ligands was assessed by a modified lactate dehydrogenase assay. Lactate dehydrogenase released into the media after lysis of the cells is representative of the total number of cells. Second-passage keratinocytes were cultured in a collagen IV-coated 48-well plate in DMEM/Ham's F12 (3:1), 0.1% UltroserG 1% penicillin–streptomycin, 1 μmol l^{-1} hydrocortisone, 1 μmol l^{-1} isoproterenol, and 0.1 μmol l^{-1} insulin. Subconfluent cell monolayers (75%) were exposed to different concentrations of chemokine (0–500 ng ml^{-1} medium) for 24 hours. After incubation, cells were washed with phospate-buffered saline and lysed with 0.1% triton in PBS for 30 minutes at 4 °C. Lactate dehydrogenase assay mixture was added to the lysate for 30 minutes at room temperature and then absorbance was measured at a wavelength of 492 nm. Proliferation after exposure to chemokines is given relative to control. Experiments were conducted using three different donors, each in duplicate.

Wound-healing model and chemokine receptor blocking

Full-thickness wounds were made in epidermal equivalents after 1 week of air-exposed culture as described previously (Breetveld *et al.*, 2006). Wounds were defined as extreme cryo-wounds (freeze-wounds; 1 mm wide and 2 cm long) resulting in cell death of that entire region of the epidermis while leaving the rest of the culture intact and viable. One wound was introduced into each culture. After wounding, epidermal equivalents were further cultured air-exposed. Directly after wounding, culture medium was supplemented with the chemokine receptor inhibitor (pertussis toxin; 0, 200, 400, and 800 ng ml^{-1}) or left unsupplemented.

After 12 days, wound closure (re-epithelialization and differentiation of newly formed epidermis) was analyzed on hematoxylin/eosin-stained paraffin sections (5 μm) with the aid of a Nikon microscope and Osteomeasure software (Osteometrics, Atlanta, GA). Re-epithelialization was measured as the distance that the newly formed epidermis had migrated into the wound bed.

Re-epithelialization after exposure to pertussis toxin-supplemented medium was compared with control unsupplemented medium and is given relative to control. As re-epithelialization occurred from both wound margins, duplicate readings were obtained for each wound and averaged. Differentiation was assessed by histology with respect to the presence or absence of a basal layer, stratum spinosum, stratum granulosum, and stratum corneum. Experiments were conducted using epidermal equivalents constructed from three different pools of five skin donors in duplicate.

ELISA

Freshly excised adult skin (4 cm^2) and epidermal equivalents with and without full-thickness cryo-wounds (see below) were placed in transwells (2.4 cm diameter, 3 μm pore size) with 1.5 ml culture medium in the lower compartment for 24 hours (Breetveld *et al.*, 2006). Culture supernatants were collected and stored at −20 °C for further analysis. For chemokine quantification in culture supernatants, ELISA reagents were used in accordance with the manufacturer's specifications. CCL14, CCL20, CCL22, CCL27, CXCL1, CXCL10, and CXCL12 were measured by commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System (Minneapolis, MN). For CXCL8/IL-8, a Pelipair reagent set (CLB, Amsterdam, The Netherlands) was used. For CCL24, a Quantikine Immunoassay (R&D System) was used.

Statistics

All experiments were conducted in duplicate using skin cells derived from three different donor pools, each pool derived from three different donors. When intact freshly excised skin was used, three different donors were used with an intra-experiment/donor duplicate. All data are presented as mean ± SEM. Differences were evaluated by one-way analysis of variance *post hoc* Dunnet's, using computer program GraphPad Prism (GraphPad Software, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Breetveld M, Richters CD, Rustemeyer T *et al.* (2006) Comparison of wound closure after burn and cold injury in human skin equivalents. *J Invest Dermatol* 126:1918–21

Clayton E, Doupé DP, Klein AM *et al.* (2007) A single type of progenitor cell maintains normal epidermis. *Nature* 446:185–9

Devalaraja RM, Nanney LB, Qian Q *et al.* (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol* 115:234–44

Dulkys Y, Schramm G, Kimmig D *et al.* (2001) Detection of mRNA for eotaxin-2 and eotaxin-3 in human dermal fibroblasts and their distinct activation profile on human eosinophils. *J Invest Dermatol* 116:498–505

Florin L, Maas-Szabowskin N, Werner S *et al.* (2005) Increased keratinocyte proliferation by JUN-dependent expression of PTN and SDF-1 in fibroblasts. *J Cell Sci* 118:1981–9

Fujimoto S, Uratsuji H, Saeki H *et al.* (2008) CCR4 and CCR10 are expressed on epidermal keratinocytes and are involved in cutaneous immune reaction. *Cytokine* 44:172–8

Gillitzer R, Goebeler M (2001) Chemokines in cutaneous wound healing. *J Leukoc Biol* 69:513–21

Gurtner GC, Werner S, Barrandon Y *et al.* (2008) Wound repair and regeneration. *Nature* 453:314–21

Jacinto A, Martinez-Arias A, Martin P (2001) Mechanisms of epithelial fusion and repair. *Nat Cell Biol* 3:E117–23

Katou F, Ohtani H, Nakayama T *et al.* (2001) Macrophage-derived chemokine (MDC/CCL22) and CCR4 are involved in the formation of T lymphocyte-dendritic cell clusters in human inflamed skin and secondary lymphoid tissue. *Am J Pathol* 158:1263–70

Kroeze KL, Jurgens WJ, Doulabi BZ *et al.* (2009) Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. *J Invest Dermatol* 129:1569–81

Li J, Ireland GW, Farthing P *et al.* (1996) Epidermal and oral keratinocyte are induced to produce RANTES and IL-8 by cytokines stimulation. *J Invest Dermatol* 106:661–6

Low QE, Drugea IA, Duffner LA *et al.* (2001) Wound healing in MIP-1alpha(−/−) and MCP-1(−/−) mice. *Am J Pathol* 159:457–63

Martin P (1997) Wound healing—aiming for perfect skin regeneration. *Science* 276:75–81

Nanney B, Mueller SG, Bueno R *et al.* (1995) Distribution of melanoma growth stimulatory activity or growth related gene and the interleukin-8-receptor B in human wound repair. *Am J Pathol* 147: 1248–60

Ouwehand K, Santegoets SJ, Bruynzeel DP *et al.* (2008) CXCL12 is essential for migration of activated Langerhans cells from epidermis to dermis. *Eur J Immunol* 38:3050–9

Pernet I, Reymermier C, Guezennec A *et al.* (2003) Calcium triggers beta-defensin (hBD-2 and hBD-3) and chemokine macrophage inflammatory protein-3 alpha (MIP-3alpha/CCL20) expression in monolayers of activated human keratinocytes. *Exp Dermatol* 12:755–60

Petering H, Kluthe C, Dulkys Y *et al.* (2001) Characterization of the CC chemokine receptor 3 on human keratinocytes. *J Invest Dermatol* 116:549–55

Rennekampf HO, Hansbrough JF, Kiessig V *et al.* (1997) Role of melanoma growth stimulatory activity (MGSA/Gro) on keratinocyte function in wound healing. *Arch Dermatol Res* 289:204–12

Rennekampf HO, Hansbrough JF, Kiessig V *et al.* (2000) Bioactive interleukin-8 is expressed in wound and enhances wound healing. *J Surg Res* 93:41–54

Satish L, Yager D, Wells A (2003) *J Invest Dermatol* 120:1110–7

Steude J, Kulke R, Christophers E (2002) Interleukin-1-stimulated secretion of interleukin-8 and growth-related oncogene-alpha demonstrates greatly enhanced keratinocyte growth in human raft cultured epidermis. *J Invest Dermatol* 119:1254–60

Tohyama M, Shirakara Y, Yamasaki K *et al.* (2001) Differentiated keratinocytes are responsible for TNF-alpha regulated production of macrophage inflammatory protein 3alpha/CCL20, a potent chemokine for Langerhans cells. *J Dermatol Sci* 27:130–9

Tsuda T, Tohyama M, Yamasaki K *et al.* (2003) Lack of evidence for TARC/CCL17 production by normal human keratinocytes *in vitro*. *J Dermatol Sci* 31:37–42

Waaijman T, Breetveld M, Ulrich M *et al.* (2010) Use of a collagen/elastin matrix as transport carrier system to transfer proliferating epidermal cells to human dermis *in vitro*. *Cell Transplant* 19:1339–48

Yates CC, Whaley D, Hooda S *et al.* (2009) Delayed reepithelialisation and basement membrane regeneration after wounding in mice lacking CXCR3. *Wound Rep Reg* 17:34–41

Yates CC, Whaley D, Y-Chen A *et al.* (2008) ELR-Negative CXC chemokine CXCL11 (IP-9/I-TAC) facilitates dermal and epidermal maturation during wound repair. *Am J Pathol* 173:643–52

Yoshie O, Imai T, Nomiyama H (2001) Chemokines in immunity. *Adv Immunol* 78:57–110

Zlotnik A, Yoshie O, Nomiyama H (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* 7:243