

# CXCL12 is essential for migration of activated Langerhans cells from epidermis to dermis

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The initial step in Langerhans cell (LC) migration from the epidermis to the lymph node involves migration of maturing LC into the dermis. Here, we investigated the migration of LC out of the epidermis after exposure of the skin to contact allergens. *Ex vivo* intact human skin, epidermal sheets, and LC derived from the MUTZ-3 cell line (MUTZ-LC) were used to determine whether dermal fibroblasts play a role in mediating LC migration towards the dermis. Exposure of epidermal sheets or MUTZ-LC to allergens (nickel sulphate, 2,4-dinitrochlorobenzene, and cinnamaldehyde) or a cytokine maturation cocktail resulted in LC migration towards dermal fibroblasts. This was due to upregulation of CXCR4 on maturing LC and secretion of CXCL12/stromal derived factor-1 chemokine by fibroblasts. Neutralizing antibodies to either CXCL12 or CXCR4 completely blocked migration. Injection of CXCL12 neutralizing antibodies into intact human skin totally inhibited LC migration into the dermis. In contrast, neutralizing antibodies to CCL19/CCL21 did not inhibit migration into the dermis. We describe a novel and essential role of dermis-derived CXCL12 in initiating migration of maturing human LC to the dermis thus permitting their further journey to the draining lymph nodes.

**Key words:** Allergy · Chemotaxis · Cytokines · DC · Skin



See accompanying commentary by Villablanca and Rodrigo

## Introduction

Human skin is the external barrier to harmful environmental factors. Upon environmental assault (*e.g.* exposure to pathogens or allergens), Langerhans cells (LC) residing in the epidermis start to mature and migrate through the dermis [1, 2]. Pathogen- or allergen-exposed LC then migrate further into the afferent lymphatics towards the local lymph node where they are able to initiate a T-cell-mediated response [3]. LC migration is regulated by the sequential and differential expression of chemokines and

their receptors [4, 5]. Although migration to the lymph node is well documented, the chemokine ligands/receptors involved in the initial step of human LC migration out of the epidermis and into the dermis are as yet unknown.

Several studies describe an essential role of CCR7 in mediating migration of mature LC towards the lymphatics [6–10]. CCR7 is highly expressed on fully mature, but not on immature LC, and is the chemokine receptor for the ligands CCL19 (MIP-3 $\beta$ ) and CCL21 (6Ckine) [11–13]. Both of these chemokines are upregulated by lymphatic endothelial cells in response to allergen exposure [14]. Simultaneously, the chemokine receptor CCR6 on LC is downregulated upon maturation [15]. CCR6 is the receptor for CCL20 (MIP-3 $\alpha$ ), which is mainly produced by keratinocytes (KC) in clinically normal human skin. CCR6/CCL20 is thought to

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be of importance for epidermal homeostasis and homing of immature LC [16–19]. Taken together these reports show that loss of CCR6 and gain of CCR7 play important roles in the migration of mature LC out of the epidermis and into the lymphatic vessels. However, it is probable that other as yet unidentified signals trigger the initial step of migration of maturing LC out of the epidermis and into the dermis.

Dermal cells, such as fibroblasts, are prime candidates for the production of chemokines, which may facilitate the initial migration of LC out of the epidermis. This is supported by Kobayashi *et al.* [20] who suggested a possible role of soluble mediators secreted by dermal fibroblasts in LC migration. Fibroblasts are known to secrete many chemokines. Recently, it was shown that the constitutive chemokine CXCL12 is also secreted by fibroblasts and its secretion is enhanced upon exposure to stress factors, such as hypoxia, irradiation, and burns [21–25]. CXCL12 secretion is not restricted to fibroblasts though, since endothelial cells and DC are also located in the dermis and also secrete CXCL12 [23, 26, 27]. Low expression of CXCR4, the CXCL12 receptor, was found on the surface of freshly isolated immature LC. In contrast, mature LC have high CXCR4 expression on their cell surface [28–30]. The interaction between CXCL12 and CXCR4 has been shown to be involved in migration and trafficking of immature haematopoietic stem cells and mature DC [31–33]. Based on these data, we hypothesized a role of dermal-derived CXCL12 and its receptor CXCR4 in the initial step of LC migration out of the epidermis and into the dermis.

In this study we investigated the initial phase of LC migration out of the epidermis upon topical exposure of freshly excised skin and epidermal sheets to the contact allergens nickel sulphate ( $\text{NiSO}_4$ ), 2,4-dinitrochlorobenzene (DNCB), and cinnamaldehyde (CA). In addition to *ex vivo* skin-derived LC, MUTZ-3-derived LC were also used. MUTZ-3 is a human myeloid leukaemia cell line, which can differentiate in a TGF- $\beta$ -dependent manner into LC-like DC, expressing Langerin and bearing LC-specific Birbeck granules [34, 35]. MUTZ-3-derived LC acquire characteristic maturation markers, such as CD83 and CCR7 upon exposure to maturation inducing factors [36]. This LC cell line model was used in functional *in vitro* assays to support and confirm our *ex vivo* data. Furthermore, we used human dermal fibroblasts as a general source for dermal CXCL12. Our findings show that dermis-derived CXCL12 is a pivotal initial inducer of migration of LC out of the epidermis and into the dermis after exposure to maturation signals, such as contact allergens, cytokines, and prostaglandins.

## Results

### Allergen exposure induces LC migration out of the epidermis

To study the effect of allergen exposure on the migration of LC out of the epidermis, *ex vivo* skin was topically exposed to  $\text{NiSO}_4$

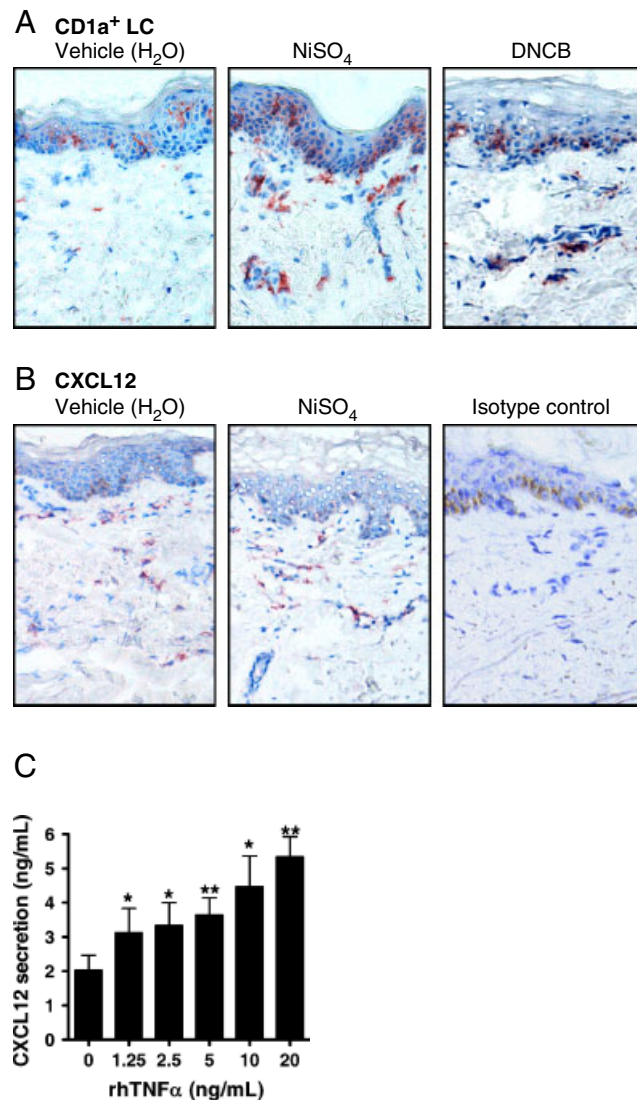


**Figure 1.** LC migrate upon exposure to  $\text{NiSO}_4$ . Staining of full thickness skin exposed to  $\text{H}_2\text{O}$  (vehicle),  $\text{NiSO}_4$ , or DNCB for 16 h (magnification  $200\times$ ): (A) CD1a immuno-staining; (B) CXCL12 immuno-staining. No staining was observed in (A) (data not shown) and (B) with the corresponding isotype (IgG1) control. Melanin (brown pigment) within the epidermis is visible and should not be confused with CXCL12 $^+$  (red) staining. Data are representative of five experiments performed in duplicate. (C) CXCL12 secretion (ng/mL) by fibroblasts upon exposure to serial dilutions of the pro-inflammatory cytokine rhTNF- $\alpha$ . Data represent the average of five experiments in duplicate  $\pm$  SEM. \* $p<0.05$  and \*\* $p<0.01$  were calculated using two-way ANOVA test followed by a Bonferroni post-test in comparison with the vehicle-exposed skin.

or DNCB. After 16 h more CD1a $^+$  LC could be detected in the dermis after  $\text{NiSO}_4$  or DNCB exposure, as compared with vehicle ( $\text{H}_2\text{O}$ )-exposed skin (Fig. 1A). In order to ascertain a possible physiological relevance of CXCL12 in initiating LC migration, the localization of CXCL12 was determined by immunohistochemistry in tissue sections obtained from skin biopsies. CXCL12 was found to be expressed in isolated, single cells within the dermis

(Fig. 1B) and its expression appeared slightly increased upon NiSO<sub>4</sub> exposure. The single cells were dispersed throughout the dermis in a similar manner to that observed for dermal fibroblasts (e.g. by Vimentin immunostaining, data not shown). Production of CXCL12 by dermal fibroblasts was confirmed *in vitro*, and was shown to be upregulated by the epidermal and contact allergen inducible cytokine TNF- $\alpha$  (Fig. 1C) [4, 5], but not by allergen (e.g. NiSO<sub>4</sub>) directly (data not shown).

### LC migration out of the epidermis upon allergen exposure is facilitated by fibroblast-derived CXCL12

To determine whether fibroblast-derived chemo-attractants (and more specifically CXCL12) were indeed involved in allergen-induced LC migration, epidermal sheets were isolated and topically exposed to a non-toxic concentration of NiSO<sub>4</sub> in a transwell system in the presence or absence of dermal fibroblasts, or in the presence of fibroblasts together with anti-CXCL12, in the lower chamber. Figure 2A shows that NiSO<sub>4</sub> exposure in the absence of fibroblasts results in retracted dendrites (*i.e.* rounder LC morphology, likely due to E-cadherin down-regulation [37]), allowing low-level migration of CD1a<sup>+</sup> LC from the epidermal sheets, as demonstrated by a decrease in LC density. When sheets were exposed to NiSO<sub>4</sub> and cultured in the presence of fibroblasts, this effect was greatly enhanced as evidenced by a massive migration of LC out of the epidermal sheets (Fig. 2A) and into the lower transwell chamber (Fig. 2B). CXCL12 neutralizing antibodies completely blocked this NiSO<sub>4</sub>-induced migration of LC out of epidermal sheets towards dermal fibroblasts in the lower transwell (Fig. 2A and B), although a retraction of dendritic protrusions was still apparent (Fig. 2A). Of note, the epidermal sheet isolation and culture procedure led to a basal LC activation level that also allowed for low but clearly detectable levels of migration of the LC from the sheets towards fibroblasts upon exposure to vehicle (*i.e.* H<sub>2</sub>O), see Fig. 2A and B. These data clearly demonstrate an important role of CXCL12 in the migration of LC towards dermal fibroblasts upon contact allergen exposure.

### Allergen-exposed LC migrates towards fibroblast-derived CXCL12 via CXCR4

In order to investigate the migrational behaviour of LC and the involvement of CXCL12 in more detail, we used the MUTZ-3 cell line which is capable of differentiating into CD1a<sup>+</sup> and Langerin<sup>+</sup> LC (Fig. 3A). As shown in Fig. 3A, immature MUTZ-LC express only low levels of the CXCL12 receptor CXCR4, whereas MUTZ-3-derived LC exposed to NiSO<sub>4</sub> or a cytokine maturation cocktail (CMC) undergo maturation, as shown by CD83 expression, and upregulate CXCR4. These results suggest that CXCR4 is upregulated rapidly upon maturation induction since 50% of the cells were already CXCR4<sup>+</sup> after 16 h of NiSO<sub>4</sub> exposure as compared with only 27% of the cells being CD83<sup>+</sup> (Fig. 3A). In contrast, no CCR7 upregulation was detectable at this time (Fig. 3A). Only

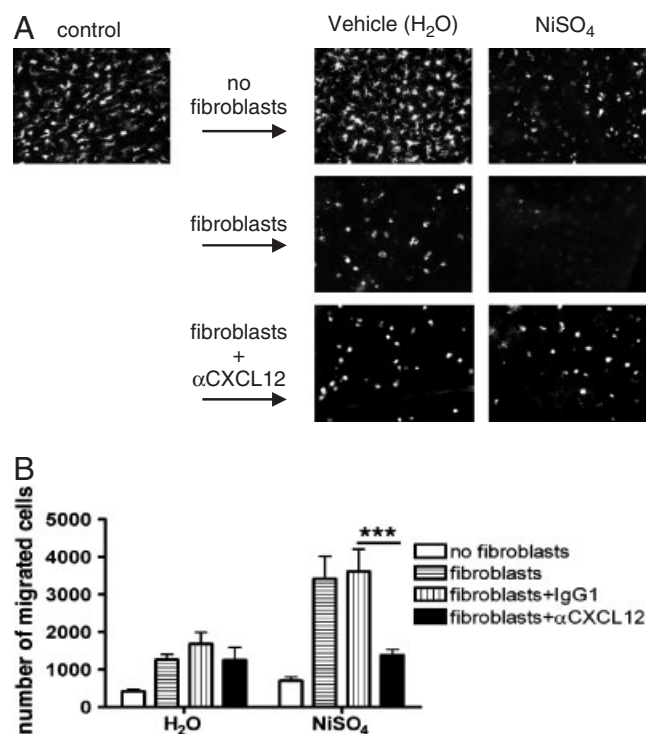


**Figure 2.** CXCL12 is secreted by fibroblasts and initiates LC migration out of the epidermis. (A) Staining of CD1a<sup>+</sup> cells in epidermal sheets in the upper transwell exposed to H<sub>2</sub>O (vehicle) and NiSO<sub>4</sub> for 16 h in the presence or absence of fibroblasts in the lower transwell chamber or fibroblasts in the presence of a neutralizing antibody against CXCL12 (magnification 100 $\times$ ). Data are representative of three experiments performed in duplicate. The control sample shows the epidermal sheet before treatment. (B) Number of CD1a<sup>+</sup> cells migrating from epidermal sheets exposed to H<sub>2</sub>O (vehicle) or NiSO<sub>4</sub> for 16 h in the presence or absence of fibroblasts or in the presence of fibroblasts plus either a control antibody IgG1 or a neutralizing antibody against CXCL12, as measured by flow cytometry. Data represent the average of four experiments in duplicate  $\pm$  SEM. Statistical differences \*\*\* $p$  < 0.001 were calculated using two-way ANOVA test followed by a Bonferroni post-test.

after 48 h of exposure to NiSO<sub>4</sub> or CMC, did LC express CCR7 (data not shown), indicating that in contrast to CXCR4, CCR7 is absent in immature LC and is upregulated in the later stages of LC maturation. The MUTZ-LC migrated towards fibroblasts upon NiSO<sub>4</sub> exposure in a comparable manner to *ex vivo* epidermal LC (Fig. 3B). Furthermore, MUTZ-LC exposed to other maturing agents, *e.g.* DNCB and CMC (*i.e.* a cocktail of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and PGE<sub>2</sub>), also migrated towards fibroblasts in a similar manner to MUTZ-LC exposed to NiSO<sub>4</sub> (Fig. 3B). Similarly, induction of LC migration towards CXCL12 was not confined to allergens, since both allergen- (NiSO<sub>4</sub> and DNCB) and CMC-exposed (*i.e.* maturing) MUTZ-LC showed enhanced migration towards recombinant human CXCL12 (rhCXCL12) (Fig. 3C). In contrast, immature LC did not migrate towards rhCXCL12. Fibroblast-mediated migration of mature LC was totally blocked by neutralizing antibodies to either CXCL12 (Fig. 3D) or its receptor CXCR4 (Fig. 3E), clearly demonstrating the dominance of the CXCR4/CXCL12 axis in this fibroblast-mediated migration of LC.

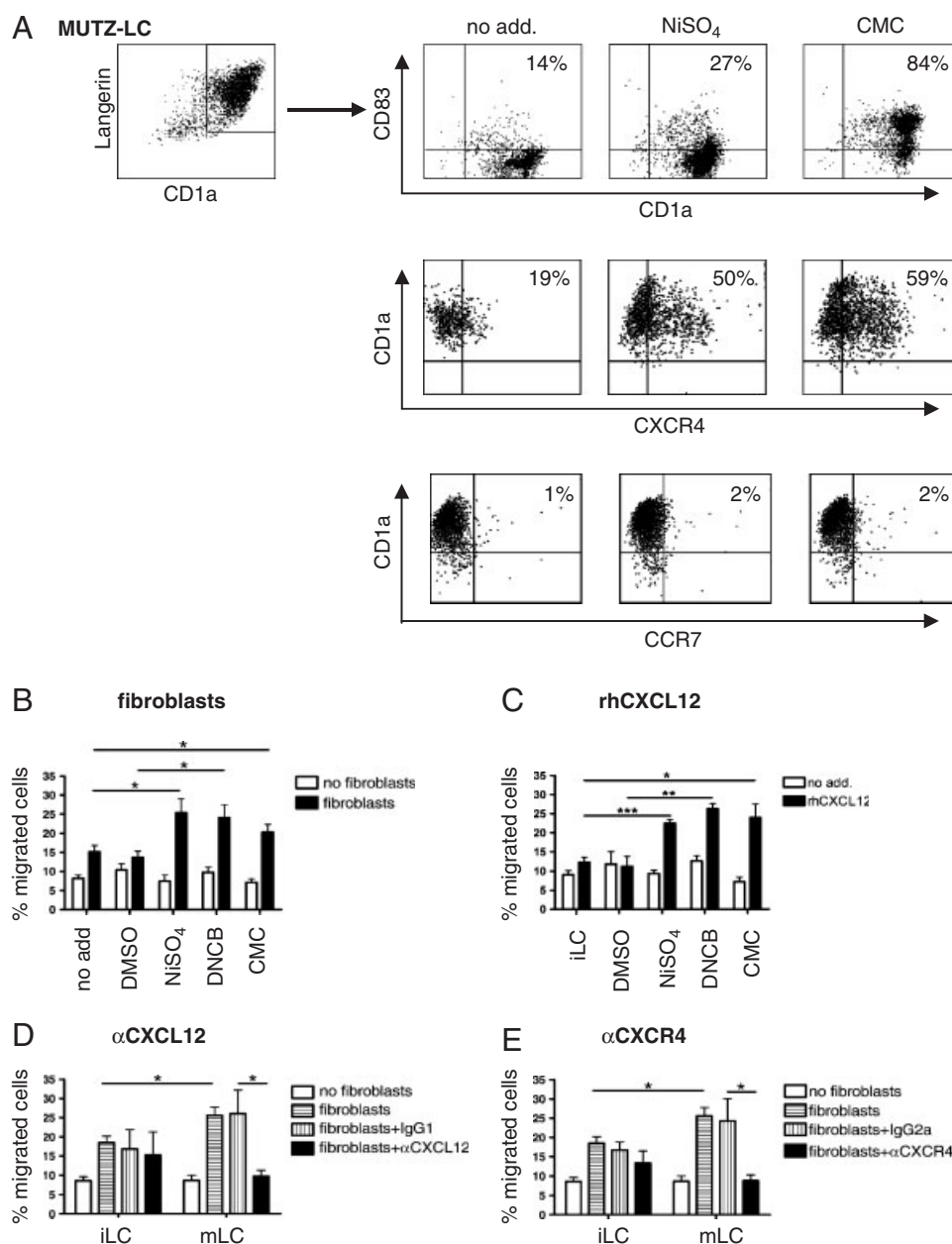


**Figure 3.** MUTZ-LC upregulate CXCR4 expression upon maturation and migrate towards fibroblasts in a CXCL12-dependent manner. (A) Phenotyping of MUTZ-LC (derived from the MUTZ-3 human myeloid leukaemia cell line). FACS dot plot and the employed gate for CD1a (x-axis) and Langerin (y-axis) double-positivity is shown. CD83, CXCR4, and CCR7 phenotyping of CD1a<sup>+</sup> MUTZ-LC either unexposed (no add) or exposed to NiSO<sub>4</sub> or CMC. Percentage of double positive cells is noted in the upper right corner of the plots. (B) Percentage of migrated CD1a<sup>+</sup> MUTZ-LC either unexposed (no add), or exposed to DMSO (vehicle control for DNCB), NiSO<sub>4</sub>, DNCB, or CMC in the presence or absence of fibroblasts. (C) Migration of LC towards rhCXCL12 is maturation-dependent. Immature LC (iLC) were exposed to allergens (NiSO<sub>4</sub> or DNCB) or CMC. DMSO was used as a vehicle control for the allergen DNCB. (D) Anti-CXCL12 blocks migration of mature LC (mLC) but not the basal low migration of iLC towards fibroblasts. (E) Anti-CXCR4 blocks migration of mature LC but not the basal low migration of immature LC towards fibroblasts. Data represent the average of four experiments in duplicate  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.005$  were calculated using two-way ANOVA test followed by a Bonferroni post-test.

### Allergen-induced LC migration is dependent on CXCL12 produced in the dermis of intact skin

To determine the relative contribution of CXCL12 to LC migration in intact human skin, *ex vivo* skin biopsies were injected with neutralizing antibodies against CXCL12 or against the CCR7

ligands CCL19/CCL21, prior to topical exposure with NiSO<sub>4</sub> (Fig. 4A and B), DNCB or CA (Fig. 4B). Only anti-CXCL12 and not anti-CCL19/21 efficiently blocked LC migration into the dermis upon exposure to any of these allergens. These results confirm the essential role of CXCL12 in mediating migration of maturing LC into the dermis.

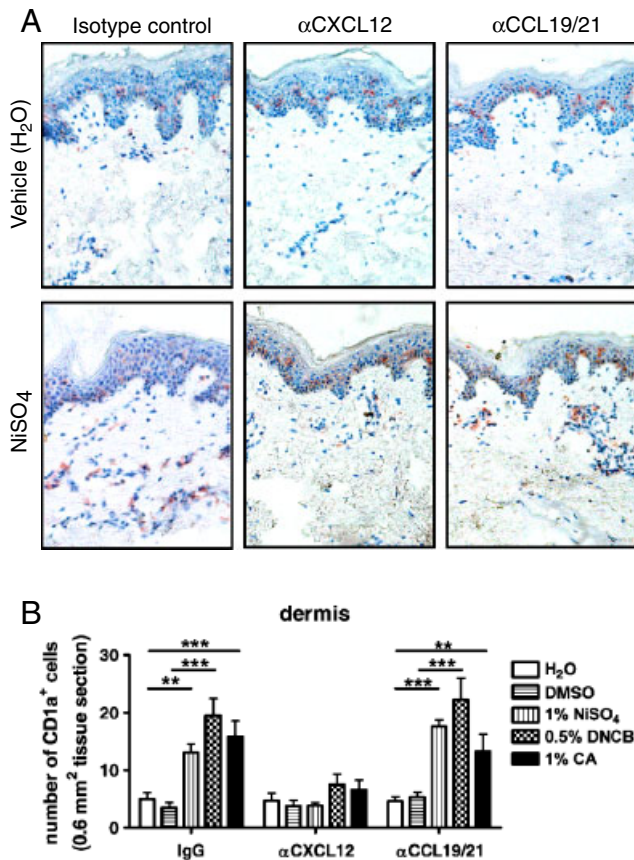


**Figure 4.** CXCL12 initiates migration of mature LC in *ex vivo* skin. (A) Staining of tissue sections obtained from full thickness skin biopsies (magnification 200 $\times$ ); shown are representative results for CD1a staining in the presence of intradermally injected isotype control antibody (IgG), neutralizing antibody to CXCL12, or a neutralizing antibody-cocktail to CCL19/CCL21 prior to topical exposure with H<sub>2</sub>O (vehicle) or NiSO<sub>4</sub> for 16 h. No immuno-staining was observed with the isotype control for CD1a. (B) The number of CD1a<sup>+</sup> cells in the dermis per 0.6 mm<sup>2</sup> tissue cross-section is shown. H<sub>2</sub>O was used as vehicle control for NiSO<sub>4</sub> and CA, whereas DMSO was used as vehicle control for DNCB. Data represent the average of three donors tested in quadruplicate  $\pm$  SEM for NiSO<sub>4</sub> and the average of two donors tested in quadruplicate  $\pm$  SEM for DNCB and CA. \*\**p* < 0.01 and \*\*\**p* < 0.001 were calculated using two-way ANOVA test followed by a Bonferroni post-test.

## Discussion

We have identified a novel and crucial role for dermis-derived CXCL12 in facilitating human LC migration out of the epidermis and into the dermis after exposure of the skin to allergens. These results highlight the role of CXCL12 in the initiation of an adaptive immune response upon antigenic contact through the skin. Initial migration of LC from the epidermis is a key event in eventual migration to the lymph node and in the generation of T-cell-mediated immunity. Dermis-derived CXCL12 is a key player as it specifically draws maturing LC into the dermis.

In our study, we have used fibroblasts as a general source of CXCL12. However, it should be noted that other dermis-derived cells also secrete CXCL12 [21–25] and therefore our findings may

not be restricted to just fibroblasts, but may involve dermis-derived stromal cells in general. We propose that dermis-derived cells play a role in creating a directional chemotactic gradient between the epidermis and the dermis, by secreting chemokines in response to epidermis-derived cytokines (e.g. TNF- $\alpha$ ).

The release of TNF- $\alpha$  by KC is induced during allergic contact dermatitis [19, 38–40]. Exposure to contact allergens results in secretion of IL-1 $\beta$  by LC which in turn stimulates epidermal KC to secrete TNF- $\alpha$ , and also IL-18 [41–43]. Strong evidence shows that these epidermis-derived cytokines are essential for the migration of LC out of the epidermis [41–43]. Furthermore, these cytokines have been reported to increase secretion of many chemokines by fibroblasts [44, 45] and this would explain the increased LC migration towards fibroblasts after allergen exposure. Indeed, here we show that one of these chemokines is CXCL12, which is induced by TNF- $\alpha$ . Furthermore, we show that CXCL12 is essential in mediating the migration of contact allergen-exposed CXCR4<sup>+</sup> LC from the epidermis into the dermis. CXCR4 acts upstream of CCR7-mediated LC migration since blocking antibodies to CXCL12 completely inhibited LC migration into the dermis of intact skin biopsies, whereas blocking antibodies to CCL19/CCL21 did not. This is in line with the absence of CCL21-secreting lymphatic endothelial cells directly underneath the basement membrane, making it unlikely that a CCL21 chemokine gradient would be sufficient to attract the LC out of the epidermis. Furthermore, our results indicate that LC gain *de novo* CCR7 expression only in the later stages of maturation, whereas CXCR4 expression is upregulated in the initial stages of maturation and emigration from the epidermis. During the migration of LC towards the lymphatics, the maturation of LC will be completed, resulting in CCR7 expression and migration of the mature LC into the paracortical T-cell areas of the draining lymph node [11].

We propose a novel two-step CXCL12-CCL19/21-dependent model by which LC are first drawn into the dermis by chemotaxis towards CXCL12 and subsequently further out towards lymphatic vessels and into the draining lymph nodes by chemotaxis towards both CXCL12 and CCL21, in order to present the hapten to T-cells, as suggested by several groups [1, 2] (Fig. 5). As CXCR4 is more rapidly upregulated during LC maturation as compared with CCR7, the advantage of our proposed two-step mechanism could be a rapid CXCR4-dependent migration of the LC, preceding the later CCR7-driven migration response. This would facilitate a more immediate LC response, which would otherwise be delayed by the relatively late onset of CCR7 expression.

Recently, there has been some degree of controversy with respect to the essential or non-essential role of LC in skin immune responses and the relative contribution of dermal DC. Our proposed two-step mechanism is in keeping with both models. In case of relatively low hapten concentrations with epidermal penetration only, engagement of LC and subsequent CXCR4 induction will facilitate rapid LC migration, while deeper, dermal penetration with higher hapten concentrations will directly activate and induce migration of dermal DC, the latter option making LC migration more or less redundant. Of note, the two-step migration model of LC would in theory also facilitate cross-talk

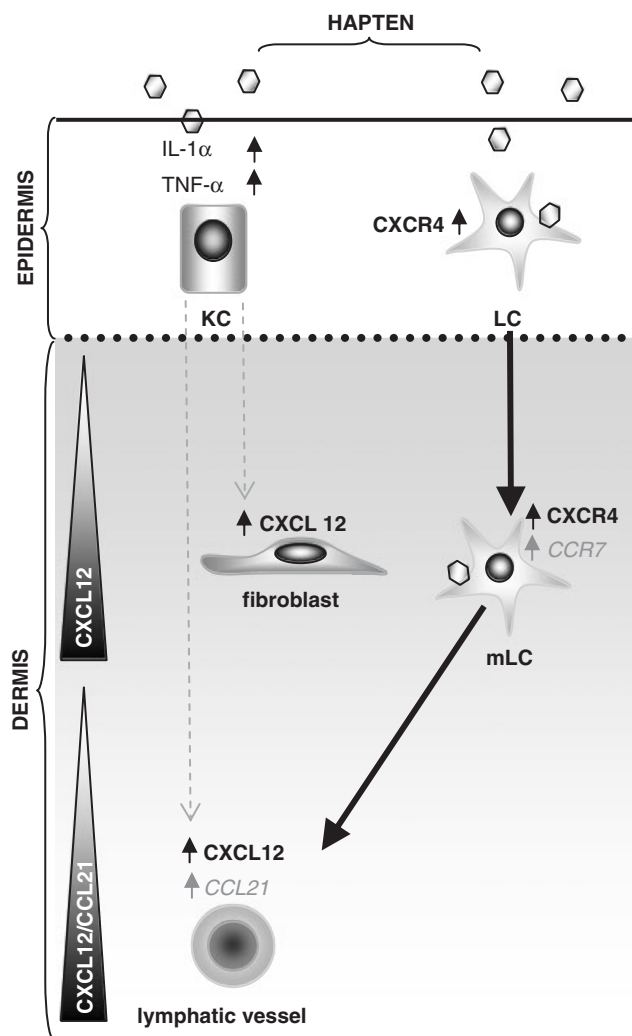


**Figure 5.** Schematic overview of the proposed two-step mechanism of LC migration. LC in the epidermis capture hapten and undergo subsequent activation accompanied by upregulated CXCR4 expression. Hapten-activated KC release fibroblast-activating cytokines (e.g. TNF- $\alpha$ ). Dermal fibroblasts in turn release higher levels of CXCL12, thus attracting hapten-activated CXCR4<sup>+</sup> LC into the dermis. Sequential expression of CCR7 subsequently mediates, in concert with CXCR4, further migration of the fully mature LC to the lymphatic vessels and draining lymph nodes where they then present hapten to naïve T cells. In this manuscript we show evidence for the existence of the essential role of CXCL12 in mediating LC migration from epidermis to dermis. CCL19/21-mediated migration from skin to lymph node has been shown by others [6–10]. Thick arrows indicate migration, whereas the dotted arrows indicate cytokine-mediated conditioning. Small vertical arrows indicate upregulation of the indicated molecules.

between LC and dermal DC in the dermis. Although transfer of herpes simplex virus antigens from LC to dermal DC has also been described [46], it has yet to be proven whether such a mechanism occurs with haptens and also whether haptens would retain their chemical reactivity during such a transfer. A non-essential role of LC in skin-immune responses has been proposed by two groups [47, 48]. These authors suggested that dermal DC in the absence

of LC were able to directly take up hapten. Such a situation could possibly occur *in vivo* in UV irradiated skin.

In accordance with our findings, a recent mouse study indicated an important role of the CXCL12/CXCR4 axis in the migration of cutaneous DC [49]. Cutaneous DC migration upon sensitization was not completely inhibited in CCR7 knock-out mice nor in mice that lack both the CCR7 ligands CCL19 and CCL21 [7, 8], Kabashima *et al.* [49] hypothesized a role for CXCL12/CXCR4. They showed that in mice, cutaneous DC migrated towards the lymph nodes in a CXCL12/CXCR4-dependent manner upon sensitization.

While our results describe in detail allergen-exposed LC migration, we also show that fibroblasts mediate migration of cytokine-matured LC. This implies a much broader role for fibroblasts and other dermis-derived stromal cells, and in particular CXCL12/CXCR4, in mediating LC migration. The CXCL12/CXCR4 axis may not only be restricted to allergen-induced LC migration, but may also be involved in cytokine- and/or pathogen-induced LC migration. Importantly, our results clearly show that the described role for CXCL12 cannot be overridden by the downstream chemokines CCL19/21, previously identified as crucial to lymph node homing. The identification of this central role of the CXCR4/CXCL12 axis in mediating mature LC migration may in future be exploited with respect to *in vitro* detection of contact sensitizers and novel vaccination strategies.

## Materials and methods

### Cell culture

Fibroblasts isolated from adult human abdominal skin were cultured in DMEM (ICN Biomedicals, Irvine, CA, USA) containing 1% UltrosorG (BioSeptra S.A., Cergy-Saint-Christophe, France), 1% penicillin-streptomycin (Invitrogen, Paisly, UK) at 37°C, 5% CO<sub>2</sub>.

The MUTZ-3 progenitor cell line was maintained as previously described [34]. MUTZ-3 were differentiated into LC by culturing at  $2 \times 10^5$  cell/mL in minimal essential media- $\alpha$  (MEM- $\alpha$ ; Gibco, Grand Island, NY, USA) supplemented with 20% v/v heat inactivated fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 1% penicillin-streptomycin, 2 mM L-glutamine (Invitrogen), 50  $\mu$ M 2-mercaptoethanol (Merck, Whitehouse Station, NY, USA), 100 ng/mL recombinant human Granulocyte Macrophage-Colony stimulating factor (Biosource International, Camarillo, CA, USA), 10 ng/mL TGF- $\beta$  (Biovision, Mountain View, CA, USA) and 2.5 ng/mL TNF- $\alpha$  (Strathmann Biotec, Hamburg, Germany). Maturation was induced by CMC, containing 100 ng/mL interleukin (IL)-6, 50 ng/mL TNF- $\alpha$ , 25 ng/mL IL-1 $\beta$  (Strathmann Biotec), and 1  $\mu$ g/mL PGE<sub>2</sub> (Sigma Chemical Co., St. Louis, MO, USA) for 24 h. Cells were cultured at 37°C, 5% CO<sub>2</sub>, 95% humidity.

The MUTZ-LC were exposed to 300  $\mu$ M nickel sulphate (NiSO<sub>4</sub>)(aq) (Merck), or 12  $\mu$ M DNCB (Sigma, and applied at less

than 0.1% v/v) dissolved in DMSO (Riedel-de Haën, Seelze, Germany) for 24 h. These chemical concentrations were determined from dose–response studies and were the concentrations where at least 75% of the cells were viable as determined by propidium iodide staining.

### LC migration from *ex vivo* skin

Healthy human abdominal skin was obtained after informed consent from patients undergoing dermal-lipectomy following hospital guidelines, and used directly after surgery. All fat was carefully removed. Pieces of skin of approximately 4 cm<sup>2</sup> were cut and placed on transwell inserts. Finn Chamber filter paper discs of 11 mm diameter (Epitest, Oy, Finland) impregnated with vehicle (H<sub>2</sub>O), 1% NiSO<sub>4</sub>, 0.5% DNCB dissolved in DMSO (applied at less than 0.1% v/v), or 1% CA (Sigma) were applied topically to the stratum corneum, as previously described [19]. Dose–response studies were performed for NiSO<sub>4</sub> (1, 5, and 10%), DNCB (0.25, 0.5, and 1%), and CA (1, 2, and 4%) and the lowest non-toxic concentration was chosen where migration in the presence of allergen was observed. Excised skin was incubated overnight in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker Europe, Verviers, Belgium) containing 10% heat inactivated fetal calf serum, 1% penicillin-streptomycin, and 50 µM DTT (Merck). Excised skin was harvested after 16 h for immunohistochemical analysis. At these chemical concentrations, no visible detrimental changes in tissue histology were observed by light microscopy and migrated LC were more than 85% viable as shown by propidium iodide incorporation studies. Therefore, the chemical concentrations used were considered non-cytotoxic.

For blocking assays, 20 µL of 10 µg/mL goat anti-human CXCL12 (AF-310-NA), or a neutralization cocktail of 20 µL of 12.5 µg/mL goat anti-human CCL19 (AF361) and goat anti-human 5 µg/mL CCL21 (AF366), was intradermally injected (all were polyclonal antibodies purchased from R&D Systems, Minneapolis, MN, USA). The Neutralization Doses<sub>50</sub> for these antibodies were determined at 1.5–6 µg/mL for CXCL12 in the presence of 2 ng/mL of rhCXCL12 $\alpha$ , 2–10 µg/mL for CCL19 in the presence of 0.05 µg/mL of rhCCL19, and 1–4 µg/mL for CCL21 in the presence of 50 ng/mL of rhCCL21, according to the manufacturer's data sheets. Isotype control to assess non-specific binding was 12.5 µg/mL goat IgG (R&D Systems). The number of migrated cells was quantified with the aid of Nis Elements AR version 2.10 software (Nikon Instruments Europe B.V., Amstelveen, The Netherlands).

### Epidermal sheet isolation

Split thickness healthy human abdominal skin was obtained with the aid of a dermatome (AESCLAP®, Braun, Tuttlingen, Germany) and washed in PBS (Braun, Melsungen, Germany). The epidermal sheet was then separated from the dermis by incubating in dispase (grade II, Roche, Basel, Switzerland) for

20–30 min at 37°C. Epidermal sheets were then washed in PBS before use in migration studies.

### Immunohistochemical and fluorescence staining

From the frozen skin explants, vertical 0.5 µm cryostat sections were cut and air-dried at room temperature on SuperFrost® gold slides (Menzel GmbH & Co. KG, Braunschweig, Germany). The sections were fixed in acetone (VWR, Amsterdam, The Netherlands) for 10 min and then incubated for 30 min with primary monoclonal antibodies directed against CD1a (OKT6; 1:500, Ortho Diagnostics Systems, Rochester, NY, USA) or CXCL12 (79018; 1 µg/mL, R&D Systems). After washing in PBS for 10 min, sections were incubated for another 30 min with goat anti-mouse conjugated to HRP (DakoCytomation, Glostrup, Denmark). After washing with PBS, the slides were incubated for 10 min with 3-amino-9-ethyl-carbazol (ICN Biomedicals) in the dark. Slides were counterstained with haematoxylin. The sections were washed three times in water, and embedded in Aquatex® (Merck).

The density of LC in epidermal sheets was determined by incubating the sheets overnight with PE labelled anti-CD1a (BD Pharmingen, San Diego, CA, USA). Subsequently, the epidermal sheets were washed three times in PBS and then examined using a fluorescence microscope (Nikon Eclipse 80i), G-2a Ex510-560, DM575, BA590.

### Transwell migration assay

#### Epidermal sheets

Human fibroblasts ( $5 \times 10^4$  cells/well) were allowed to adhere overnight in the lower chamber of a 6 transwell culture plate (Corning, Schiphol-rijk, The Netherlands). Epidermal sheets were placed on the transwell inserts (3 µm pore size). Subsequently, Finn Chamber filter paper discs 11 mm diameter (Epitest) impregnated with vehicle (H<sub>2</sub>O) or 1% NiSO<sub>4</sub> were applied topically to the stratum corneum. DMEM containing 1% UltrosorG, penicillin, and streptomycin was placed in the lower chamber only. The transwell migration assay was then carried out at 37°C, 5% CO<sub>2</sub>, in a humidified atmosphere for 16 h. The number of cells migrating out of the epidermal sheet into the lower chamber was analysed by flow cytometry.

#### MUTZ-LC

Human fibroblasts ( $1 \times 10^4$  cell/well) were allowed to adhere overnight in the lower chamber of a 24 transwell culture plate (Corning). Either immature or mature MUTZ-LC ( $5 \times 10^4$  cells/well) were placed in the transwell insert (5 µm pore size). When indicated, fibroblasts were incubated with a monoclonal antibody against human/mouse CXCL12 (R&D Systems, clone number 79014) for 30 min before combination with MUTZ-3 LC. When indicated, MUTZ-3 LC were placed in the upper transwell chamber together with a monoclonal antibody against human

CXCR4 (R&D Systems, clone number 44708). Migrating MUTZ-LC entering the lower chamber were analysed by flow cytometry after 16 h at 37°C, 5% CO<sub>2</sub>, in a humidified atmosphere in DMEM supplied with 1% UltrosorG, penicillin, and streptomycin. When indicated, rhCXCL12 (R&D Systems) was placed in the lower well in an optimum concentration of 0.1 ng/mL, as determined by dose–response studies (data not shown).

## ELISA

CXCL12 levels in culture supernatants of fibroblasts exposed for 4 h to serial dilutions of rhTNF- $\alpha$  (Strathmann Biotech) were determined by ELISA. A DuoSet CXCL12 development system (R&D Systems) was used essentially as described by the suppliers.

## Flow cytometry

The number and phenotype of migrating cells were determined by flow cytometry.

## Phenotyping

Cell staining was performed using, mouse anti-human CD83-PE (IgG2b, Immunotech, Marseille, France), mouse anti-human CD1a-FITC/PE (IgG1, BD Pharmingen), mouse anti-human CD207 (Langerin)-PE (IgG1, Immunotech), mouse anti-human CXCR4-FITC (IgG2a, R&D Systems), mouse anti-human CCR7-FITC (IgM, BD Pharmingen). Isotype controls to assess non-specific binding were mouse IgG1-PE, IgM-FITC, and IgG1-FITC (BD Pharmingen), mouse IgG1-PE (Immunotech), mouse IgG2b-PE (Immunotech), mouse IgG2a-FITC (R&D Systems). Cells were incubated with antibodies for 30 min, washed in PBS containing 0.1% BSA and 0.1% sodium azide and resuspended in the same buffer for FACS analysis.

## Quantifying

To quantify the number of migrated cells, an excess amount of Flow-Count Fluorospheres (Beckman Coulter, Fullerton, CA, USA) was added and the migrated cells were stained with mouse anti-human CD1a-FITC and mouse anti-human CD83-PE before analysis with an FACScan flow cytometer (Beckton Dickinson, San Jose, CA, USA). The data were subsequently analysed using CellQuestPro software.

## Statistical analysis

Percentages of migrated MUTZ-LC and absolute numbers of migrated LC were compared between conditions using the two-way ANOVA test followed by a Bonferroni post-test. Differences were considered significant when  $p < 0.05$ .

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**Abbreviations:** CA: cinnamaldehyde · CMC: cytokine maturation cocktail · DNCB: 2,4-dinitrochlorobenzene · KC: keratinocyte · LC: Langerhans cell

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