IL-17 and IL-22 Promote Keratinocyte Stemness in the Germinative Compartment in Psoriasis

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IL-17 and IL-22 promote keratinocyte stemness in the germinative compartment in psoriasis

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**Abbreviations**

aldehyde dehydrogenase, ALDH
brilliant violet, BV
caffeic acid phentenyl ester, CAPE
cytokeratin 10, K10
epidermal growth factor, EGF
EpiLife Defined Growth Supplement, EDGS
interleukin, IL
keratinocytes, KCs
KC serum-free medium, KFSM
keratinocyte stem cells, KSCs
matrix metalloproteinase 9, MMP9
neonatal human epidermal keratinocytes, HEKn
nuclear factor κB, NF-κB
quantitative real time PCR, qPCR
tumor necrosis factor alpha, TNF-α
Abstract

Psoriasis is an inflammatory skin disorder characterized by the hyperproliferation of basal epidermal cells. It is regarded as T-cell mediated, but the role of keratinocytes (KCs) in the disease pathogenesis has reemerged with genetic studies identifying KC-associated genes. We applied flow cytometry on KCs from lesional and non-lesional epidermis to characterize the phenotype in the germinative compartment in psoriasis and observed an overall increase in the stemness markers CD29 (2.4 fold), CD44 (2.9 fold), CD49f (2.8 fold) and p63 (1.4 fold). We found a reduced percentage of cells positive for the early differentiation marker, cytokeratin 10 (K10), and a greater fraction of CD29+ and involucrin+ cells in the psoriasis KCs than in non-lesional KCs. The upregulation of stemness markers was more pronounced in the K10+ cells. Furthermore, the psoriasis cells were smaller, indicating increased proliferation. Treatment with IL-17 and IL-22 induced a similar expression pattern of an upregulation of p63, CD44 and CD29 in normal KCs and increased the colony-forming efficiency and long-term proliferative capacity, reflecting increased stem cell-like characteristics in the KC population. These data suggest that IL-17 and IL-22 link the inflammatory response to the immature differentiation and epithelial regeneration by acting directly on KCs to promote cell stemness.
**Introduction**
Psoriasis is widely regarded as an inflammatory T-cell-mediated disorder, where the hyperproliferation and disturbed maturation of the keratinocytes (KCs) are believed to be driven by Th1- and Th17-associated cytokines and chemokines (Harden et al., 2015, Lowes et al., 2014). Genetic data support the role of a dysregulated immune system by showing a strong association with HLA-C, IL12B and IL23R. Th17 cells and their downstream effector cytokines, interleukin (IL)-17 and IL-22, are central to pathogenesis, which is supported by the effectiveness of treatments targeting related pathways (Harden et al., 2015, Tsoi et al., 2015). Although recent genetic advances suggest that both immune and epidermal components contribute to disease susceptibility, the role of KCs in the formation of the psoriatic lesion has received less attention.

The marked increase in proliferation and the disturbed differentiation of KCs in psoriasis (Hawkes et al., 2017) led to psoriasis long being regarded as a KC-driven disease (Lowes et al., 2014). The psoriatic epidermis manifests an incompletely formed granular layer and a stratum corneum where the KCs are not fully differentiated and still retain their nuclei (Raychaudhuri et al., 2014). It also demonstrates an increased growth fraction of epidermal KCs and an approximately 30-fold increase in the production of cells compared with the normal epidermis (Weinstein et al., 1985). The mechanism of this enhanced proliferation is not fully understood and no reduction in cell cycle time has been observed in plaques (Castelijns et al., 2000, van Ruissen et al., 1996). Epidermal KCs also display disturbed tissue homeostasis and dysregulated apoptosis, which may contribute to the thickened epidermal (Eding and Enerback, 2017). Epidermal homeostasis is maintained through the activation of stem cells in the basal layer and the subsequent proliferation of early progenitor cells, often referred to as transient amplifying cells (Ghadially, 2012). In psoriasis, the phenotype of the proliferating epidermal KC subpopulation is still poorly defined. Here, using the best available markers of differentiation and stemness, we describe an overall more immature
phenotype of psoriatic KCs. Furthermore, we found that IL-17 and IL-22 alter the expression of stem cell markers in KCs to a pattern that is reminiscent of that observed in psoriatic KCs.
Results

Flow cytometric analysis of the germinative compartment reveals an increased expression of stem cell markers in the psoriatic epidermis

To characterize the phenotype in the germinative compartment in psoriasis, KCs from psoriatic lesional and non-lesional skin were isolated and analyzed with flow cytometry. Trypsin was used to dissociate the epidermal sheets, which enriched the immature and early differentiating cells of the epidermis as trypsin does not dissociate terminally differentiated cells. To exclude doublet and cell debris, a gate was placed based on scatter properties. Within this population, cells were further gated on the lack of expression of CD45, a cell surface marker that is found on cells of hematopoietic origin. The derived KCs were analyzed based on their marker expression, evaluating the MFI in non-bimodal populations and further subgating the bimodal populations where positive and negative cells could be clearly distinguished. An illustrative flowchart as well as the strategy for singlet gating, scatter gating and CD45- gating can be found in Figure 1a and 1b. The autofluorescence of unstained cells is shown in Figure S1. The gates for the bimodal populations can be seen in Figure 2. As expected, CD45 revealed a higher percentage of leukocytes in the lesional psoriatic skin than in the control skin (2.3% ±0.4 vs. control 0.7% ± 0.1; p<0.05, data not shown). In the KC population that resulted from gating the CD45- cells, we found a higher expression of all the investigated stem cell markers, i.e. CD29, CD44, CD49f and p63, in psoriatic KCs than in the KCs from non-lesional skin, with a 2.4-fold upregulation of CD29, a 2.9-fold upregulation of CD44, a 2.8-fold upregulation of CD49f and a 1.4-fold upregulation of p63 (Figure 1c). We also found a tendency towards an upregulation of the proliferation-associated marker CD71 in the psoriatic KCs (p=0.08). The KCs derived from lesional psoriatic skin were markedly smaller (Figure 1d) than their non-lesional counterparts, indicative of an increase in proliferative capacity (Barrandon and Green, 1985).
The markers K10, CD29 and involucrin displayed a bimodal expression pattern with one positive and one negative cell population, which were altered in the KCs derived from psoriatic lesions. The most notable difference was the markedly smaller fraction of K10+ cells in the lesional cell fractions compared with the non-lesional ones (51.3% ±5.5 vs. control 86.8% ±1.8, p<0.001; Figure 2). Furthermore, the cells derived from psoriatic lesions contained a larger fraction of CD29+ cells than the non-lesional cells (29.4% ±5.5 vs. control 9.1% ±1.2, p<0.05; Figure 2). The KCs from lesional psoriatic skin also contained a slightly larger proportion of involucrin+ cells than the corresponding non-lesional cells (71.3% ±13.0 vs. control 63.8% ±13.1, p<0.05; Figure 2).

As we observed a notable difference in the fraction of K10- and K10+ cells between psoriatic and non-psoriatic KCs, we performed further gating on this marker (Figure S2). We found that psoriasis-derived K10+ cells displayed a more prominent upregulation of the stem cell markers than the corresponding cells from non-lesional skin. The psoriatic K10+ population demonstrated a 1.5-fold upregulation of the expression of CD29, a 3.0-fold upregulation of CD44, a 2.2-fold upregulation of CD49f and a 1.6-fold upregulation of p63 (Figure S2a, S2b) compared to the non-lesional cells. The K10- population demonstrated an upregulation of only CD44 and involucrin (Figure S2a, S2c). The elevated levels of these markers suggest that, despite being in early differentiation, the psoriatic K10+ cells still retain an immature and proliferative phenotype that is not seen in their non-lesional skin counterparts. A detailed description of the specific marker expression in the K10+/- and CD29+/- populations is presented in Figures S2 and S3, respectively. The cell distribution obtained by the combined gating of K10 and CD29 is shown in Table 1a, demonstrating the altered frequency (% cells) of the populations.

CD44, which is an established stem cell marker in mammary epithelia (Al-Hajj et al., 2003, Wright et al., 2008), displayed an overall increase in the psoriasis cell population (Figure 1c).
This upregulation was pronounced even when cells were divided into K10+/− and CD29+/− populations (Figure S2, S3). The altered expression of CD44 remained pronounced when keratinocytes were dual-gated for K10 and CD29, displaying an increase in the K10+CD29+, K10+CD29− and K10-CD29+ populations (Table 1b).

We confirmed the flow cytometric findings using immunofluorescence staining on tissue sections of psoriatic skin and control skin using antibodies directed against CD44, K10, p63, CD29 and involucrin (Figure S4). We also performed co-staining for the combination of K10 and CD44 as well as K10 and p63 to further visualize the localization of the markers (Figure S5). We were able to confirm the differential expression of these markers in psoriasis compared to controls and describe their spatial distribution.

CD44 was found to be present in all layers of the epidermis. In the psoriasis skin, the expression was particularly localized to the basal layers, with a gradually lower expression towards the skin surface, confirming an increased level of CD44 expression in the psoriatic epidermis, as well as a pronounced expression in the K10− cell layers. K10 expression was visible in all layers except the innermost. Psoriasis skin revealed a homogenous expression in the outer layer, but more cells at the innermost layer lacked K10 expression. P63 was faintly expressed in the basal layers in the control skin, while it in psoriasis formed a gradient towards the skin surface with the strongest expression in the basal layer and no expression in the outmost layers. As expected and previously described, CD29 stained the basal layers both in psoriatic and control skin but was found in additional cell layers in the psoriatic epidermis, reflecting the increased percentage of CD29+ cells that we observed with flow cytometry.

While CD29 is also found in pre-adipocyte cells (Festa et al., 2011), the expression we observed was primarily localized to the epidermis. Involucrin was in psoriasis found in the outermost layer, as well as scattered through the epidermis, and displayed a stronger expression in psoriatic skin than in control skin.
**A psoriatic microenvironment of IL-17, IL-22 and TNF-α increases the expression level of stem cell markers and alters the phenotypic behavior of KCs**

As the psoriatic KCs displayed an overall immaturity, we hypothesized that stimuli present in the psoriatic microenvironment would be a contributing cause. Because of the strong Th17 component in psoriasis (Hawkes et al., 2017) and as it has been suggested that IL-22 increases proliferation and delays differentiation (Dudakov et al., 2015) we hypothesized that IL-17 and IL-22 would be key mediators in altering the KC phenotype in psoriasis. Indeed, treatment of cultured neonatal human epidermal KCs (HEKn) with a combination of IL-17, IL-22 and TNF-α gave rise to an expression pattern similar to that observed in the psoriatic KCs, with an increase in CD44, p63 and CD29 and a decrease in K10. These changes were observed at both mRNA (Figure 3a) and protein (Figure 3b) levels. This was a synergistic effect, as TNF-α alone did not affect the levels of CD44 and p63 (Figure S6).

One characteristic feature of KC stem cells (KSCs) is a long-term proliferative potential (Li et al., 1998). Treatment of biopsy-derived KCs with IL-22 and IL-17 gave rise to a 3.9 and 6.3 times larger cell number, respectively, after 46 days of culture than untreated cells (Figure 4a). When an equal number of cells were seeded in each passage, the proliferation increased for each passage, suggesting an increased proliferative rate as a function of long-term exposure to IL-17 and IL-22 (Figure 4b). Furthermore, cell sensitivity to the cytokines appeared to increase with time (Figure S7). There was no indication of increased differentiation or apoptosis in the untreated cells, as they increased exponentially (Figure 4c). These findings suggest that IL-17 and IL-22 increase long-term proliferation in KCs. Like the reduced size observed in the lesion-derived psoriatic KCs, the size of the KCs decreased after treatment with IL-17 and IL-22 (Figure S8). We also found a large number of condensed nuclei in the treated cells, which is indicative of active cell proliferation (Bazile et al., 2010).
Furthermore, the colony-forming capacity (clonogenicity) is another indicator of stem cell-like characteristics in KCs (Doucet and Owens, 2015, Moestrup et al., 2017). The number of colonies is indicative of the growth potential of the cells, while the size of the colonies reflects their stage in terminal differentiation (Barrandon and Green, 1985). We found that HEKn treated with IL-17 or IL-22 demonstrated a greater colony-forming capacity than untreated cells (Figure 4d), with an increase in both the number of colonies and the size of the colonies. Based on the flow cytometric analysis of psoriatic KCs, we anticipated that lesion-derived psoriatic KCs seeded at colony density would exhibit a colony-formation pattern comparable to that of undifferentiated KCs. Indeed, when compared with normal KCs seeded at colony density for 10 days, psoriatic KCs formed a strikingly larger number of colonies, including colonies of larger size (Figure 4e).

Next, we explored the molecular mechanisms by which IL-17 and IL-22 promote KC stemness. The principal mediators of IL-17 and IL-22 signaling are nuclear factor (NF)-κB and STAT3 respectively, whereas the MAPK pathway has been implicated in a tissue context-dependent manner (Amatya et al., 2017, Dudakov et al., 2015). We found that the selective MEK1 & MEK2 inhibitor U0126 (Duncia et al., 1998) and the NF-κB inhibitor caffeic acid phenethyl ester (CAPE), prevented the upregulation of CD44 (Figure 5a) and p63 mRNA (Figure 5b) in response to IL-17 and IL-22. Interestingly, the inhibition of the small GTPase, RAC1, a regulator of the ERK pathway and a recently emerging mediator of psoriasis pathogenesis (Winge et al., 2016) reduced the CD44 and p63 mRNA expression (Figure 5a, 5b). The effects on the protein level were verified by flow cytometry and are presented in Figure 5c. These findings suggest that IL-17 and IL-22 induce CD44 and p63 through the RAC1/MEK/ERK/NF-κB pathway.
CD44 has previously been shown to increase RAC1 upon binding to the CD44 ligands hyaluronan and streptococcal polysaccharide (Bourguignon, 2014). Since RAC1 has been implicated in psoriasis pathogenesis, we used siRNA to silence CD44 and analyzed the expression of S100A7, S100A8, S100A9 and β-defensin 2, all of which are psoriasis-associated antimicrobial peptides regulated by IL-17 and IL-22. Interestingly, the basal level of S100A7, S100A8 and S100A9 were strongly suppressed in CD44 siRNA transfected cells compared to cells transfected with negative control siRNA. In response to stimulation by IL-17/TNF-α or IL-22/TNF-α, we detected a diminished upregulation of the S100 proteins that reached statistical significance for S100A7 in response to IL-17/TNF-α (Figure S9).
**Discussion**

KSCs are located in the basal layer and display limited, slow division and the potential to self-renew (Watt and Jensen, 2009). The search for a molecular signature for stem cells in the epidermis began in the 1990s, when Jones and Watt demonstrated that CD29 expression on the surface of epidermal KCs correlated with a high proliferative potential in vitro (Jones and Watt, 1993). Although progress has been made in defining molecular markers for hair follicle epidermal stem cells residing in the bulge (Blanpain and Fuchs, 2006) stem cell markers for human interfollicular epidermis are less well defined (Ghadially, 2012, Kretzschmar and Watt, 2014). Here, we used the putative stem cell markers, CD29, CD44, CD49f and p63 (Ghadially, 2012, Moestrup et al., 2017, Pincelli and Marconi, 2010) to characterize the differentiation state of psoriatic KCs in the germinative compartment. We also included the early differentiation, proliferation-associated CD71 and the early differentiation marker, K10, as well as the established differentiation marker, involucrin, which has been suggested to be aberrantly expressed in psoriasis (Eckert et al., 1993, Ishida-Yamamoto and Iizuka, 1995).

With these markers of stemness and differentiation, we were able to identify higher levels of all the investigated stem cell markers in the psoriasis-derived KC fraction than in the fraction from normal skin. In addition, the psoriasis cell fraction contained a much larger population of K10- cells, as well as a larger proportion of CD29+ cells. The increased number of K10-cells is in line with findings indicating that several rows of suprabasal cells are negative for K10 transcripts or protein (Bernerd et al., 1992).

A high KC expression of CD29 correlates with a high proliferative potential in vitro (Jones and Watt, 1993). Upon KC commitment to terminal differentiation, CD29 is downregulated (Levy et al., 2000). Previous attempts to identify the proliferating population in psoriasis have focused on sorting on the high CD29 expression (Jones et al., 2007) which identified a cell population with increased mRNA levels of CD49f and decreased levels of K10 mRNA.
(Franssen et al., 2005). Similarly, in 1993, Bata-Csorgo et al. suggested that the proliferative population in the psoriatic epidermis is CD29+, with CD29+K10- cells as the primary hyperproliferative population in the psoriatic skin (Bata-Csorgo et al., 1993).

We observed a larger proportion of both K10-CD29+ cells and K10-CD29- cells in the psoriasis fraction than in the non-lesional fraction, as described in Table 1a. This contrasts with the findings of Bata-Csorgo et al. who report the same relative proportion of K1/K10-cells in the normal and psoriatic CD29+ fraction (both 65.4% of CD29+ cells). The reason for this discrepancy is not clear, but it may relate to differences in protocols. The large fraction of K10-CD29- cells indicates that many of the K10- cells in psoriasis may not be highly proliferative. This is supported by the elevated expression of involucrin that we observed in the K10- population. Instead, we found that the increase in stem cell markers was pronounced, especially in the K10+ fraction, which clearly suggests that K10+ cells are abnormal in psoriasis.

The most pronounced increase in the investigated stem cell markers was the upregulated CD44 expression in psoriasis-derived cells, which was persistent in all subpopulations, regardless of whether they were subdivided on K10 or CD29 expression. CD44 is firmly established as a stem cell marker in mammary epithelial cells (Charafe-Jauffret et al., 2009), which have the same ectodermal origin as epidermal KCs. In breast cancer stem cells, the knockdown of CD44 induces cell differentiation (Pham et al., 2011). Furthermore, a study by Szabo et al. has proposed a CD44+ aldehyde dehydrogenase (ALDH)+ epidermal KC population that is enriched in epidermal stem cells, with the CD44+ALDH+ cells displaying an ability for self-renewal, holoclone formation and multipotency (Szabo et al., 2013). The inflammatory environment in the psoriatic plaque makes CD44 of even more interest, as this receptor can act as a co-receptor of inflammatory mediators like endothelial growth factor (EGF) and serve as an activator of matrix metalloproteinase (MMP)9 (Misra et al., 2015).
We hypothesized that the mechanism for driving the immature, stem cell-like features of psoriasis KCs is derived from the microenvironment in the psoriatic plaque. Both IL-17 and IL-22 are key cytokines in psoriasis. IL-22 contributes to many of the main features of psoriasis, such as acanthosis, loss of the granular layer and hyperkeratosis, while also promoting KC differentiation, inducing antimicrobial peptide production and delaying differentiation (Boniface et al., 2005, Wolk et al., 2009a, Wolk et al., 2009b). It has been suggested that IL-17 inhibits proliferation in a 3D model of normal human skin (Donetti et al., 2014) but does not affect terminal differentiation (Donetti et al., 2014, Wolk et al., 2009a). However, in the imiquimod-based mouse model of psoriasis, signaling through the IL-17 pathway reduces K10 levels, promotes KC hyperproliferation and attenuates KC differentiation (Ha et al., 2014). Furthermore, IL-17 increases wound healing kinetics in mice (MacLeod et al., 2013).

The combination of IL-22, IL-17 and TNF-α with IL-1α and oncostatin M reduces the expression of the KC differentiation factors, K10, K1, desmoglein-1, desmocollin-1 and loricrin (Rabeony et al., 2014).

We found that IL-17 and IL-22, in combination with TNF-α, increased the expression of the investigated stem cell markers in normal KCs to a pattern similar to that observed in the entire psoriasis fraction. The increase in stem cell marker expression was accompanied by cellular characteristics typical of early progenitor cells, with an increased long-term proliferation ability and colony-forming efficiency. Large, abundant colonies were also seen in cultured treated KCs. Previous studies have demonstrated that the cells that give rise to the largest colonies form holoclones, which express CD29 and p63 (Jones and Watt, 1993, Pellegrini et al., 2001). These cells also express high levels of CD49f and low levels of CD71 and display many stem cell features, including quiescence and long-term growth capacity (Webb and Kaur, 2006, Webb et al., 2004).
The capacity of IL-17 and IL-22 to promote stemness, in a TNF-α microenvironment, was abolished upon treatment with inhibitors of the RAC1/MEK/ERK/NF-κB pathway. This pathway is of particular interest in psoriasis, since the expression of a constitutively active form of RAC1 in mice induces psoriasiform skin lesions with a transcriptional overlap with human psoriasis (Winge et al., 2016). Interestingly, RAC1 is also activated in response to the binding of streptococcal polysaccharide to the KC cell surface CD44 (Cywes and Wessels, 2001) as well as the binding to its ligand hyaluron (Bourguignon, 2014). We demonstrated that CD44 mediates the expression of the IL-17/IL-22 downstream targets S100A7, S100A8 and S100A9, which is in line with these previous reports and suggest a role for CD44 in the regulation of these key psoriasis mediators. This suggests that RAC1 is involved both in CD44 regulation and mediating downstream effects of IL-17 and IL-22.

In conclusion, we describe an overall more immature phenotype of psoriatic KCs. This immaturity is likely to be caused by the actions of the psoriatic key cytokines, IL-17 and IL-22, as these cytokines gave rise to a similar phenotype, induced proliferation, promoted a proliferation-like morphology and enhanced the colony-forming capacity of normal KCs. The data suggest that IL-17 and IL-22 in the psoriatic microenvironment may act on KCs to promote proliferation and keep the KCs in an immature state.
Materials and Methods

Isolation of human epidermal KCs

Skin punch biopsies (4 mm) were obtained from psoriasis patients at the local dermatology clinic. All the participating patients had been examined by a dermatologist. The patients were not receiving any systemic treatment. The psoriasis skin punch biopsies were obtained from an active, untreated, psoriatic lesion and control skin was obtained from a non-lesional area from the same patient to minimize confounding variation. All the participants had given their written informed consent. The lesional and the non-lesional biopsies from each patient were simultaneously obtained, processed, stained and analyzed, rendering a completely matched comparison. KC isolation was performed by placing the punch biopsy in 3.8% sterile ammonium thiocyanate (flow cytometry) for 30 minutes at room temperature or in dispase over night at 4°C (culture). Following incubation, the epidermis was removed and placed in trypsin with mechanical disruption for 30 minutes at 37°C.

Staining and flow cytometry

The KC suspension was filtered through a 70 µm mesh and fixed and permeabilized using the transcription factor buffer set (BD Biosciences, San Jose, CA). Fixation took place over night at 4-8°C and antibody staining was performed on ice for 50 minutes. To avoid excessive spectral overlap and minimize the risk of compensation artifacts, cells were stained with either of two separate panels (Table S1). The following antibodies were used for analysis with flow cytometry: anti-CD29-brilliant violet (BV)510, anti-CD44-PE.Cy7, anti-CD45-FITC, anti-CD49f-PerCP.Cy5.5, anti-CD71-BV421 (BD Biosciences), anti-cytokeratin 10 (K10)-APC and anti-involucrin-PE (Bio-Techne, Abingdon, UK). Anti-p63 (Bio-Techne) was conjugated using the Lynx rapid RPE conjugation kit (AbD Serotech, Kidlington, UK). Analysis was performed on a Gallios flow cytometer (Beckman Coulter, Bromma, Sweden). Between 2,000
and 10,000 events were collected. Data were analyzed using Kaluza analysis software (Beckman Coulter).

**Cells for culture and culture conditions**

The culture conditions for the KCs are described in the supplement. Treatment with IL-17 (10 ng/mL) or IL-22 (20 ng/mL) alone, or in combination with TNF-α (10 ng/mL) was performed for 48h (qPCR) or 72h (immunocytochemistry). For inhibitor experiments, the cells were pretreated for one hour with CAPE (20 µM), Stattic (2 µM), SB203580 (20 µM) and U0126 (20 µM), all from Abcam, Cambridge, UK, or the RAC1 inhibitor (50 µM; Calbiochem), before the addition of cytokines or vehicle DMSO. To downregulate CD44 expression, HEKn were transfected with 50 nM CD44 siRNA (Qiagen) 8h before addition of IL-17 and IL-22 in combination with TNF-α for 48h. AllStars Negative control siRNA (Qiagen) served as control. Transfection was performed using FlexiTube premix siRNA.

**RNA extraction, cDNA synthesis and qPCR**

Cells were lysed in RLT buffer (Qiagen, Hilden, Germany) and RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Vilnius, Lithuania). qPCR was performed using SYBR green for the expression analyses of CD44, p63, CD29 and K10 and using predesigned TaqMan gene expression assays for the analyses of S100A7, S100A8 and S100A9 (Applied Biosystems, Foster City, CA), on a real-time 7500 HT system (Applied Biosystems). The primer sequences are listed in Table S2. Expression data were normalized to RPLP0 using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

**Immunofluorescence**
HEKn and skin punch biopsies from healthy controls and lesional psoriasis skin were fixed, paraffin-embedded and stained for CD44, p63, K10, CD29 and Involucrin using a standard protocol. A detailed description of the staining process can be found in the supplement. Alexa Fluor 488 and 555 were used as secondary antibodies. Negative controls were obtained by omitting the primary antibody and displayed no staining. Fluorescence intensity was analyzed with Image J software (version 1.51j, NIH, USA).

**Colony-forming efficiency assay and long-term proliferation**

Colony-forming efficiency and proliferation assays were performed on KCs isolated from skin biopsies and HEKn. We cultured the cells without a feeder layer, as described by Doucet et al. (Doucet and Owens, 2015). Briefly, second-passage KCs were seeded and treated with IL-17 (5 ng/mL) or IL-22 (10 ng/mL). After 10 days, colonies were fixed and stained with 0.05% crystal violet. The cells in the proliferation assay were cultured for 46 days and counted upon passaging. A detailed description of the culture process can be found in the supplement.

**Statistics**

Differences between groups were analyzed using Student’s t-test or the Mann Whitney U-test. A p-value of less than or equal to 0.05 was considered significant. The data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed in GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA).
Conflict of interest

The authors have no conflicts of interest.

Acknowledgement

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References


## Tables

### Table 1a. K10 and CD29 subpopulations

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Non-lesional (% cells)</th>
<th>Psoriasis (% cells)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>K10+CD29-</td>
<td>83.0 ± 1.7</td>
<td>43.2 ± 4.1</td>
<td>p&lt;0.0001</td>
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<tr>
<td>K10-CD29+</td>
<td>5.3 ± 0.8</td>
<td>21.3 ± 5.0</td>
<td>0.02</td>
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<tr>
<td>K10-CD29-</td>
<td>7.9 ± 2.0</td>
<td>27.4 ± 4.6</td>
<td>0.007</td>
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<tr>
<td>K10+CD29+</td>
<td>3.8 ± 0.9</td>
<td>8.1 ± 2.3</td>
<td>n.s.</td>
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</tbody>
</table>

### Table 1b. Marker expression within K10 and CD29 subpopulations

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Non-lesional (MFI)</th>
<th>Psoriasis (MFI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K10+CD29-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>6.24±1.9</td>
<td>15.6±2.3</td>
<td>p&lt;0.01</td>
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<td>CD49f</td>
<td>8.83±1.3</td>
<td>16.4±2.6</td>
<td>n.s.</td>
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<tr>
<td>CD71</td>
<td>0.75±0.0</td>
<td>3.21±1.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>p63</td>
<td>5.13±1.0</td>
<td>7.20±0.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>involucrin</td>
<td>102±23.8</td>
<td>104±14.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>K10-CD29+</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>2.8±0.3</td>
<td>13.9±2.4</td>
<td>p&lt;0.05</td>
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<tr>
<td>CD49f</td>
<td>27.5±3.7</td>
<td>50.3±9.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD71</td>
<td>1.02±0.1</td>
<td>2.82±0.7</td>
<td>p=0.05</td>
</tr>
<tr>
<td>p63</td>
<td>7.18±1.3</td>
<td>7.19±1.0</td>
<td>n.s.</td>
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mean ± S.E.M
n.s., not significant
Figure Legends

Figure 1. KCs from psoriatic lesions display an immature phenotype and signs of proliferation

Events collected on a Beckman Coulter Gallios flow cytometer were gated to exclude doublets and cell debris. A schematic of the gating strategy is shown in panel (a), and panel (b) displays the gating to obtain the keratinocyte (KC) population, starting with the singlet gate to exclude doublets (top image), followed by the FS/SS gate (middle image) and the CD45 gate where CD45+ cells were removed from the analysis by placing a KC gate on the CD45- cells (bottom image). KCs derived from psoriatic lesions and non-lesional skin were stained for the expression of CD29, CD44, CD49f, p63 and CD71. Panel (c) shows the fluorescence overlay between the lesional (grey histograms) and non-lesional cells (transparent histograms), as well as the median fluorescence intensity (MFI) levels. The x-axis in the histograms displays the fluorescence levels and the y-axis displays the count. Brackets in the histograms show the fluorescence at which cells were considered positive for each marker. Panel (d) shows the median FSC values as a measurement of cell size; n=4-7, *p≤0.05, **p<0.01.

Figure 2. Keratinocyte sub-populations in psoriatic epidermis

The panels show the representative dot plots demonstrating the proportion of K10+-/-, CD29+-/- and involucrin +/- populations in KCs derived from psoriatic lesions (lesional) and non-lesional skin (non-lesional). The dot plots display the fluorescence on the x-axis and the linear side scatter area (SS) on the y-axis. The graphs show the percentage of K10+, CD29+ and involucrin+ cells; n=4-7, *p≤0.05, ***p<0.001.
Figure 3. Treatment with IL-17, IL-22 and TNF-α produces an in vivo-like phenotype
Cultured neonatal human epidermal KCs (HEKn) were treated with IL-17 (10 ng/mL), IL-22 (20 ng/mL) or a combination of either IL-17 or IL-22 with TNF-α (10 ng/mL) for 48h (qPCR) or 72h (immunocytochemistry). Panel (a) shows the mRNA expression of CD44, p63, CD29 and K10 in HEKn cultured with IL-17, IL-22, IL-22+TNF-α, IL-17+TNF-α or IL-17+IL-22+TNF-α. Panel (b) shows the protein expression in HEKn cultured with IL-17+IL-22+TNF-α; n=3-4, *p≤0.05. The scale bar for the immunocytochemistry images is shown in the first panel and represents 50 µm.

Figure 4. IL-17 and IL-22 promote proliferation and increase colony-forming efficiency in normal KCs
Early-passage KCs from normal skin biopsies were seeded and continually passaged upon 80% confluence. Every three days, the medium was changed to fresh medium containing IL-17 (5 ng/mL) or IL-22 (10 ng/mL). The total cell yield was assessed after 46 days of culture (a). Panel (b) shows the proliferation at each passage. Panel (c) shows the total yield of cells after 46 days of cell culture in absolute numbers; n=3-5, *p≤0.05, **p<0.01, ***p<0.001. (d) Cultured HEKn were seeded at colony density and were treated with IL-17 (5 ng/mL) or IL-22 (10 ng/mL) for 10 days and subsequently stained with crystal violet (n=6). Panel (e) shows the colony formation of psoriatic KCs obtained from two psoriasis patients (psoriasis 1 and 2) and one non-psoriasis control (normal).

Figure 5. NF-κB and MAPK mediate IL-17- and IL-22-dependent effects on KC stem cell markers
Cultured HEKn cells were pretreated for one hour with the inhibitors U0126 (20 µM, MEK), CAPE (20 µM, NF-κB), SB203580 (20 µM, p38 MAPK), Stattic (2 µM, STAT3) or Rac1 inhibitor (50 µM), before treatment with IL-17 (10 ng/mL) or IL-22 (20 ng/mL) in combination with TNF-α (10 ng/mL) for 48h. The mRNA expression of CD44 (a) and p63 (b) was related to untreated controls. The reduced protein expression of CD44 following inhibitor treatment was confirmed using flow cytometry (c); n=3-4, *p<0.05, **p<0.01.
Supplementary Material

Cells for culture and culture conditions

KCs isolated from skin biopsies were cultured in KC serum-free medium (KFSM) with L-glutamine supplemented with 25 µg/mL bovine pituitary extract and 1 ng/mL EGF (Gibco, Gaithersburg, MD). HEKn (Cascade Biologics, Life Technologies, Carlsbad, CA) were cultured in complete EpiLife medium supplemented with 1% EpiLife defined growth supplement (EDGS), CaCl₂ (0.06 µM; all from Cascade Biologics, Paisley, UK). All media contained 1% amphotericin B (Gibco) and 1% penicillin/streptomycin (Lonza, Verviers, Belgium).

Immunofluorescence

HEKn were fixed in 4% formaldehyde and permeabilized with 0.1% saponin, followed by incubation with primary antibody overnight at 4°C and subsequent incubation with secondary Alexa Fluor 488 conjugated antibody (Molecular Probes, Eugene, OR, USA). Skin punch biopsies were obtained from healthy controls and lesional psoriasis skin and were fixed and paraffin-embedded. The sections were deparaffinized in Histolab-clear (Histolab Products, Gothenburg, Sweden) and rehydrated in ethanol. Heat-induced antigen retrieval was performed in citrate (pH = 6) antigen retrieval buffer (DAKO, Glostrup, Denmark). 5% bovine serum albumin was used as blocking agent. Incubation with primary antibody was carried out overnight at 4°C followed by 1h incubation with Alexa Fluor 555 conjugated secondary antibody. DAPI nuclear counterstain was performed.

The following primary antibodies were used; CD44 (BD Biosciences), p63 (Abcam), K10 (Epitomics, Burlingame, CA), CD29 (Novus Biologicals, Littleton, CO) and Involucrin (Novus Biologicals) and secondary Alexa Fluor 488 and Alexa Fluor 555 conjugated antibodies (Molecular Probes, Eugene, OR, USA).
Colony-forming efficiency assay and long-term proliferation

Second passage KCs were seeded at 500 KCs/well in 12 well plates for the colony formation assay and 10,000 KCs/well in 6 well plates for the proliferation assay. The day after seeding the cells were treated with IL-17 (5 ng/mL) or IL-22 (10 ng/mL). Every three days, the medium was changed and fresh cytokines added. After 10 days, the colonies were fixed in 4% formaldehyde and stained with 0.05% crystal violet. The cells in the proliferation assay were continually passaged when 80% confluency was reached. At each passage the number of cells in each well was determined, and the cells were replated at 10,000 cells per well irrespective of cell yield. The cumulative number of cells derived since the first plating of 10,000 cells was also determined at each passage. The total cell yield after 46 days of culture was calculated under the assumption that all the cells from the previous passages had been replated.
Supplement figure legends

**Figure S1.** Histograms demonstrating the autofluorescence of unstained KCs in each fluorochrome channel.

**Figure S2.** A detailed description of the K10+/− population. Panel (a) displays the dot plots with the gating strategy to determine the positive and negative population. Panel (b) displays the median fluorescence intensity of each respective marker and the percentage cells positive for each marker in the K10+ population, and panel (c) displays the median fluorescence intensity (MFI) of each respective marker and the percentage cells positive for each marker in the K10− population; n=4-7, *p≤0.05, **p<0.01.

**Figure S3.** A detailed description of the CD29+/− population. Panel (a) displays the dot plots with the gating strategy to determine the positive and negative population. Panel (b) displays the median fluorescence intensity (MFI) of each respective marker and the percentage cells positive for each marker in the CD29+ population, and panel (c) displays the median fluorescence intensity of each respective marker and the percentage cells positive for each marker in the CD29− population; n=4-7, *p≤0.05.

**Figure S4.** Immunofluorescence describing the localization of CD44, p63, K10, CD29 and involucrin in psoriasis and control skin. Markers are stained red, bottom pictures include DAPI counterstaining of cell nuclei (blue). Photos are representative of three independent experiments. The scale bars for the immunofluorescence photos are shown in the first panel set and represent 50 µm.

**Figure S5.** Double immunofluorescence staining of (a) CD44 (green) and K10 (red) or (b) p63 (green) and K10 (red) of control and psoriasis skin. Photos are representative of three experiments. The scale bars are shown in the rightmost panels and represent 50 µm.
**Figure S6.** mRNA expression analysis of CD44 and p63 in HEKn treated with (gray) or without (white) TNF-α (10 ng/mL). n=3; ns, non-significant.

**Figure S7.** For details on experimental procedure, see figure 4. Comparison of the sensitivity of normal KCs to cytokine treatment after passage 1 and passage 5; **p≤0.01.

**Figure S8.** Representative images showing cell and nuclear size of cultured neonatal human epidermal KCs (HEKn) after treatment with IL-17 (5 ng/mL) or IL-22 (10 ng/mL). The scale bar is 500 µm.

**Figure S9.** qPCR analyses of the mRNA expression of CD44, S100A7, S100A8 and S100A9 in CD44 siRNA-transfected HEKn treated with IL-22+TNF-α or IL-17+TNF-α. Gene expression was compared to untreated negative control siRNA-transfected cells. n=4, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001
Singlet gate

FS/SS gate

Keratinocyte (KC) gate

MFI in non-bimodal KC populations (Fig. 1c)

% positive KCs in bimodal populations (Fig. 2)

Gate on K10+ or K10- population

Gate on CD29+ or K29- population

MFI in entire K10+ or K10- population

% positive cells in K10+ or K10- population (Fig. S2)

MFI in entire CD29+ or CD29- population

% positive cells in CD29+ or CD29- population (Fig. S3)

MFI/SC-A

Non-lesional

Lesional

**

0.08

**
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Table S3. Marker expression in KC subpopulations

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n.s., not significant