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Human keratinocytes' response to injury upregulates CCL20 and other genes linking innate and adaptive immunity

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Abstract

In the early stages of wound healing, keratinocytes become “activated” and release inflammatory molecules such as interleukin-1 and interleukin-8 that are linked to innate immune responses and neutrophil recruitment. It is unclear, however, whether keratinocytes release molecules linked to adaptive immune responses, e.g. CCL20, in their early state of activation without signals from infiltrating T cells. This study aims to isolate the immediate alterations in protective and inflammatory gene expression that occur in epidermal keratinocytes, with a particular focus on molecules associated with cell-mediated immunity. We used dispase-separated epidermis, followed by intercellular disassociation by trypsinization, as a model for epidermal injury. We obtained a pure population of keratinocytes using flow cytometry. As a control for uninjured epidermis, we performed laser capture microdissection on normal human skin. Sorted keratinocytes had an early burst of upregulated gene expression, which included CCL20, IL-15, IL-23A, IFN- κ , and several antimicrobial peptides. Our results provide insight into the potential role of keratinocytes as contributors to cell-mediated inflammation, and expand knowledge about gene modulation that occurs during early wound healing. Our findings may be relevant to cutaneous diseases such as psoriasis, where micro-injury can trigger the formation of psoriatic plaques at the site of trauma.

Keywords

wound healing; keratinocytes; skin

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CONFLICTS OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Wound healing is a dynamic process that involves overlapping phases of inflammation, formation of granulation tissue, re-epithelialization, matrix formation, and tissue remodeling (Barrientos *et al.*, 2008). The role for keratinocytes (KCs) in the re-epithelialization process involves regenerative maturation, whereby KCs alter their programs of proliferation and differentiation in order to physically restore the disrupted epidermal barrier (Mansbridge and Knapp, 1987). The early inflammation process is largely attributed to fibroblasts, infiltrating macrophages, and neutrophils, which are recruited by the release of platelet degranulation products such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF- β) (Barrientos *et al.*, 2008; Singer and Clark, 1999).

The capacity for KCs to upregulate their expression of inflammatory products in response to injury has not been well defined. Current evidence largely links KCs to activation of pathways that direct innate immune responses, e.g., neutrophil recruitment into sites of injured epidermis (Marionnet *et al.*, 2003; Roupe *et al.*, 2010; Sextius *et al.*, 2010); however, more research is needed to define the isolated response of KCs to injury, including their potential to recruit and activate cells of adaptive immunity.

Prior *in vivo* wound healing studies have attempted to define the epidermal response to injury, however, it was unclear what role was played by dermally-derived cells such as fibroblasts and inflammatory cells (Cole *et al.*, 2001; Dickel *et al.*, 2010; Johnston *et al.*, 2010; Marionnet *et al.*, 2003; Nickoloff and Naidu, 1994; Roupe *et al.*, 2010; Sextius *et al.*, 2010). *In vitro* studies using cultured KCs and three dimensional skin equivalents have the advantage of a system populated solely by KCs (Dayem *et al.*, 2003; Koria *et al.*, 2003), but these cell models are intrinsically growth-activated and have a different gene expression profile from normal skin (Gazel *et al.*, 2003). In an effort to isolate the epidermal response to injury, Roupé et al. removed the dermis by dissection and used sterile NaCl to purge infiltrating inflammatory cells. They found that injury resulted in the upregulation of numerous antimicrobial peptides and cytokines of innate immunity, including IL-8. They also found that release of IL-8 in response to tissue injury resulted in prominent chemotactic activity of neutrophils. Although there was still the remaining potential for dermal cells, such as fibroblasts and immune cells, to be present in the analyzed tissue, their microarray study provides the best representation of the changes in gene transcription relating to innate immunity that occur several days after injury (Roupe *et al.*, 2010). However, the intrinsic short-term response of KCs to injury, as well as their potential to upregulate genes related to cell-mediated immunity, has not yet been defined by gene profiling.

In this study, we found trypsin-disassociated human KCs isolated from normal skin rapidly upregulated numerous antimicrobial peptides and inflammatory cytokines, including CCL20, IL-15, IL-23A, and IFN- κ . The generation of these products in response to cell disassociation provides insight into the potential role of the KC as a contributor to cell-mediated inflammation.

RESULTS

Isolation of a pure population of KCs by FACS and normal epidermis by laser capture microdissection (LCM)

We obtained biopsies of normal skin, isolated the epidermis with dispase, and used trypsin to produce cell disassociation. After a 16-hour culture, we used flow cytometry to obtain a pure population of KCs (Figure 1a). In order to detect potential T cell contamination, we also stained the epidermal cell suspensions for CD3 positivity and found minimal infiltrates (0.06%) (Figure S1).

We used epidermis isolated by laser capture microdissection (LCM) from normal skin as a model for KCs in steady state. This method allowed us to compare the sorted KCs with a model that most closely represented *in vivo* gene expression of normal epidermis (Espina *et al.*, 2006) and minimized dermal contamination. Additionally, we purchased normal adult human KCs (PromoCell) and grew them in long-term culture.

Following RNA extraction and amplification, we measured relative gene expression using HG U133 plus 2.0 chips for the sorted KCs, KCs isolated by LCM (KC LCM), and long-term cultured KCs (KC *in vitro*). Principal component analysis (Figure 1b) revealed that sorted KCs clustered with KC *in vitro*, and not KC LCM, providing further evidence that sorted KCs were not significantly contaminated by other cell types, including other resident epidermal cells. In addition, KC LCM samples segregated from their corresponding dermis and bulk samples, indicating the precision of the epidermal microdissection.

Sorted KCs rapidly upregulate genes related to innate and adaptive immunity compared to KCs in steady state

We compared gene expression between sorted KCs and KC LCM. With a criteria of absolute fold-change (FCH) > 4-fold and false discovery rate (FDR) < 0.01, we identified 5,413 probe sets encoding 3,198 unique differentially expressed genes (DEG). There were 1,721 unique, upregulated DEG and 1,477 unique, downregulated DEG. The full list is available in Table S1.

In response to cell disassociation, changes in mRNA abundance for many genes was impressive compared to KC LCM (Table 1). A heatmap with the top 100 DEG reflects the sharp contrast in gene expression between the two KC populations, and gene expression differences were seen consistently across individual samples (Figure 1c). Accordingly, 14 genes listed in Table 1 had >100-fold increases in mRNA and 5 had >1000-fold increases. CCL20, IL-15, and IL-23A were upregulated in sorted KCs, which have the potential to activate or recruit cells of the adaptive immune system. Genes related to innate immunity were also upregulated in sorted KCs, including members of the S100 family of proteins (S100A7, S100A8, S100A9) and other AMPs (RNASE7, NGAL, elafin). With the exception of RNASE7, these AMPs have also been identified in other wound healing studies (Marionnet *et al.*, 2003; Roupe *et al.*, 2010; Sorensen *et al.*, 2006). Upregulated cytokines/chemokines (IL-1 α , IL-8, TNF, IFN- κ , and CXCL1), with the exception of IFN- κ , are also consistent with other wound healing studies (Dickel *et al.*, 2010; Marionnet *et al.*, 2003; Nickoloff and Naidu, 1994; Roupe *et al.*, 2010). There were genes associated with

hyperproliferation as well, including ligands of the epidermal growth factor receptor (AREG, TGF- α , HB-EGF), known to be upregulated in activated KCs due to autocrine stimulation by preformed IL-1 (Cole *et al.*, 2001; Marionnet *et al.*, 2003; Nickoloff and Naidu, 1994). The matrix metalloproteinases (1, 3, and 9) that shed these growth factors from the surface of the KCs, and STAT3, which is a downstream effector of the EGFR signaling cascade (David *et al.*, 1996) were also upregulated.

The downregulated genes in the sorted KCs included Langerhans cell-specific (HLA-DQB2, HLA-DPA1, CD1A, CD207) and melanocyte-specific genes (PNPLA3), as these cells were present in the KC LCM samples. Of the 1477 unique downregulated genes, the above were among the 40 most highly downregulated genes, confirming that our experimental system was successful in the removal of Langerhans cells (LC) and melanocytes by FACS from the trypsinized epidermal single cell suspensions. One caveat to our analytic approach is that high expression of inflammatory (or other) genes by LCs or melanocytes, with low level expression in resting KCs, may have led to underestimation of gene regulation in sorted KCs.

Two-step QRT-PCR confirms DEGs in KC sorted vs. KC LCM

To confirm microarray data, we extracted RNA from sorted KCs and KC LCM and performed two-step quantitative reverse-transcription-PCR (qRT-PCR). All of the genes we chose (AREG, CCL20, CXCL2, S100A9, TNF, IL-8, IL-15, IL23A, IFNK) were significantly upregulated ($p < 0.05$) in KC sorted compared to KC LCM (Figure 2).

Sorted KCs have increased inflammatory gene expression but reduced markers of hyperproliferation compared to KCs in long term culture

Cultured KCs are well-recognized as having an altered phenotype that is similar to the proliferative activation and altered differentiation stages of wound healing (Gazel *et al.*, 2003). To compare proliferative and inflammatory gene expression of sorted KCs to cultured KCs, we isolated RNA from *in vitro* cultured KCs and analyzed global gene expression with HG U133 plus 2.0 chips. Overall levels of inflammatory products were much higher in sorted KCs compared to cultured primary KCs (KC *in vitro*), including CCL20 (390-fold), CXCR4 (277-fold), S100A7 (113-fold), IL-8 (55-fold), CXCL3 (47-fold), CCL27 (43-fold), IL-1F9 (39-fold), CXCL2 (36-fold), TNF (26-fold), IL-33 (24-fold), HB-EGF (20-fold), IL-20 (16-fold), TNFAIP3 (12-fold), and IL-15 (8-fold) (Table S2). In contrast, markers of KC hyperproliferation keratin 6A (KRT6A) and keratin 6B (KRT6B), (Mansbridge and Knapp, 1987; Stoler *et al.*, 1988; Weiss *et al.*, 1984), were more highly expressed in KC *in vitro* compared to sorted KCs (4-fold and 11-fold respectively). Overall, the range of cytokine and chemokine upregulation in the sorted KCs seemed to more closely resemble the acute KC inflammation phase rather than the proliferative/differentiation changes that occur as a longer range response to wound healing.

There is a high degree of overlap between genes upregulated in sorted KCs and cytokine-treated KCs in culture

As gene and transcriptomal analysis revealed the activation of both innate and adaptive immune pathways, we wanted to understand better the potential upstream effectors and

resultant signaling cascades present in the sorted KCs. Accordingly, we compared our list of upregulated genes in sorted KCs vs. KC LCM to those of cytokine-treated cultured KCs in previously published studies (Figure S2) (Nogales et al., 2008; Yano et al., 2008; Zaba et al., 2009). Interestingly, the greatest degree of overlap occurred with IFN- γ -treated KCs. This finding was unexpected because cells that synthesize IFN- γ are not present in our model system. There was also considerable overlap with KCs treated with IL-1, IL-17 and TNF whereby 44, 54, and 43 percent of the DEG in these cytokine-treated KCs overlapped with our list.

TNF regulates the expression of CCL20 and IL-23A but not IL-15 or IFN- κ in cultured KCs

Sorted KCs had increased expression of cytokines/chemokines that are known to activate cell-mediated immunity (CCL20, IL-15, IL-23A) and other novel inflammatory genes (IFN- κ) compared to KCs in steady state. To test the role of TNF in the regulation of these genes, we treated cultured KCs from three different primary cell lines with or without TNF for 24 hours. Etanercept was also added to some KCs to neutralize TNF. We found KCs treated with TNF upregulated their expression of CCL20 ($p = 0.0005$) and IL-23A ($p = 0.0038$) mRNA in compared to negative controls (Figure 3a). We demonstrated that TNF was responsible for this effect because there was no increase CCL20 or IL-23A mRNA following the neutralization of TNF with Etanercept. IL-15 and IFN- κ mRNA expression were not shown to be regulated by TNF (Figure S3).

CCL20 protein is decreased in disassociated epidermal cells treated with Etanercept

To test the whether endogenous TNF produced by KCs regulates CCL20 expression during cell disassociation, we cultured trypsinized epidermal cells with or without Etanercept. We obtained normal adult skin ($n=3$), used dispase to isolate the epidermis, and used trypsin to cause cell disassociation. Following trypsinization, we cultured the cells for 16 hours +/- Etanercept. For a negative control, we used epidermal cells whose mRNA was extracted immediately following trypsinization. We found that mRNA levels of CCL20 were increased in untreated and Etanercept-treated cultured KCs compared to the negative control, however, there was no significant difference between the culture conditions (Figure 3b). We also measured CCL20 protein in the supernatants and found protein levels were increased in untreated and Etanercept-treated KCs compared to the negative control (Figure 3c). There was also a greater increase of CCL20 protein in the supernatant of untreated KCs compared to KCs treated with Etanercept. Therefore, after 16 hours, CCL20 mRNA was significantly increased in cultured KCs compared to the negative control, but there was no significant difference in mRNA levels between the two culture conditions; however, CCL20 protein levels were significantly higher in untreated KCs compared to Etanercept-treated KCs, suggesting that KC production of TNF increases CCL20 protein production.

CCL20 mRNA and protein are increased in UVB-irradiated skin

We wanted to determine if *in vivo* KC injury could induce CCL20 expression, as predicted from our cell disassociation model. Accordingly, we examined skin biopsies of normal adult volunteers who were treated with 2MED of 312 nm ultraviolet B light (Judson *et al.*, 2010), a level of UVB that induces sunburn cells in the epidermis (Coven *et al.*, 1997). We

collected skin biopsies prior to irradiation and 24 hours after treatment, and measured CCL20 mRNA with qRT-PCR. We found CCL20 mRNA expression was increased after UVB irradiation in all ten patient biopsies compared to pre-treatment biopsies at a median upregulation of 34-fold (Figure 4a). We also performed immunohistochemistry staining for CCL20. Following treatment, there was increased CCL20 protein in the epidermis, in both KC cytoplasm and extracellular space (Figure 4b). Although there was neutrophil and monocyte/macrophage infiltration at the site of injury (Figures S4a, S4b), these data provide *in vivo* evidence that CCL20 is an early response cytokine to injury and that its presence precedes T cell infiltration (Figure S4c).

DISCUSSION

It is well established that early skin wounding, prior to T cell infiltration, is associated with an influx of neutrophils as well as monocytes/macrophages at the site of injury. Prior work has provided evidence for the up-regulation of cytokines, e.g., IL-8, that are likely regulators of the neutrophilic response (Koria *et al.*, 2003; Roupe *et al.*, 2010; Sorensen *et al.*, 2006). Our data suggest that KCs, as activated by cell disassociation, can synthesize a group of inflammatory molecules, but with a special prominence of CCL20, that have the capability to recruit monocytes/myeloid DCs and T cells into a focal skin region. Thus, cells of innate and adaptive immunity that are associated with wound healing, and also chronic skin inflammation, might both be initiated by an activated KC.

Following disassociation, sorted KCs upregulated their expression of numerous genes that could potentially direct innate immune processes, e.g., neutrophil recruitment into sites of wounded epidermis. These genes included IFN- κ , TNF, antimicrobial peptides (S100A7, S100A8, S100A9, RNASE7, NGAL, elafin) and other cytokines (IL-1 α , IL-8, CXCL1). All of the above genes were upregulated in TNF-treated KCs (with the exception of RNASE7) (Zaba *et al.*, 2009) as well as IL-1-treated KCs (with the exception of RNASE7 and IL-1A) (Nogralles *et al.*, 2008; Yano *et al.*, 2008). Therefore, the expression of these cytokines and antimicrobial peptides may be due to the secretion and subsequent autocrine stimulation by TNF and/or IL-1.

In addition to genes related to innate immune mechanisms, the range of DEG in sorted KCs included those that have the capability of activating and recruiting cells of the adaptive immune system, notably including CCL20, IL-15, and IL-23A, which have not been previously identified in wound healing studies. CCL20 was highly upregulated in sorted KCs compared to both cultured and steady state KCs. CCL20 recruits CCR6-positive T cells (Paradis *et al.*, 2008; Varona *et al.*, 2005) and immature DCs (Le Borgne *et al.*, 2006) to enter inflamed cutaneous tissue from the blood. CCL20 is also known to be upregulated in the epidermis of patients with chronic cutaneous inflammatory diseases such as psoriasis (Zaba *et al.*, 2008), and there is mounting evidence that the upregulation of CCL20 may also represent an acute response to injury by keratinocytes. For instance, Jennings *et al.* utilized electrical stimulation to mimic KC injury and demonstrated that *in vitro* KCs upregulated CCL20 in response to this stimulation (Jennings *et al.*, 2010). In addition, Enk *et al.* irradiated both cultured keratinocytes and the skin of human subjects with UVB and, using microarrays, found upregulation of CCL20 in both subsets (Enk *et al.*, 2006). We also

irradiated the skin of human subjects with UVB and found upregulated expression of CCL20 mRNA expression in all patients 24 hours after treatment. There was also increased CCL20 protein localized to the epidermis and this response occurred in the absence of infiltrating T cells. In conjunction with other studies, our findings provide further evidence that KCs may be able to upregulate and release CCL20 in response to epidermal perturbation by multiple stimuli.

In support of a handful of studies that have investigated the regulation of CCL20 in KCs, we established that TNF can regulate synthesis of CCL20 in KCs *in vitro* or in disassociated cells (Nakayama *et al.*, 2001; Zaba *et al.*, 2009). In addition, other studies have shown that IL-1 α and EGFR ligands are strong inducers of CCL20, particularly when both factors are used in combination (Johnston *et al.*, 2010; Nakayama *et al.*, 2001). As IL-1 α and EGFR ligands were also upregulated in sorted KCs and are known to be released during wound healing (Roupe *et al.*, 2010), there were multiple pathways that potentially contributed to CCL20 upregulation in our experiments.

Gene expression of IL-15 was also upregulated in sorted KCs as measured by microarray and RT-PCR. IL-15 is not a constitutive epidermal cytokine and accordingly, this gene did not amplify in our KC LCM samples. In contrast, human KCs upregulate IL-15 expression in culture and in chronic inflammatory conditions such as psoriasis (Han *et al.*, 1999). In murine wound healing studies, IL-15 has been shown to play a regulatory role, whereby IL-15 transgenic mice (under the control of an MHC class I inhibitor) exhibited accelerated wound healing (Kagimoto *et al.*, 2008). To this end, Kupper and Clark demonstrated that IL-15 and human fibroblasts supported the proliferation of regulatory T cells (Tregs) isolated from human skin, and that Tregs constituted a resident population of cutaneous T cells (Clark and Kupper, 2007). As Tregs are crucial for the induction and maintenance of immune tolerance, IL-15 produced by activated KCs (and fibroblasts) could serve to expand the population of resident cutaneous Tregs, thereby curbing inflammation during the wound healing process.

Gene expression of IL-23A was also upregulated in sorted KCs as measured by microarray and RT-PCR. IL-23A was upregulated in KCs cultured with TNF compared to negative controls, and this effect was inhibited when TNF was neutralized by Etanercept. IL-23 is known to be produced by DCs and macrophages (Lee *et al.*, 2004) and is required for the growth, survival and effector functions of Th17 cells (Zhou *et al.*, 2007), which are abundant in psoriatic plaques (Lowe *et al.*, 2008). As cutaneous injury can result in the formation of psoriatic plaques in susceptible individuals (the Koebner phenomenon), the elaboration of IL-23 by KCs could potentially contribute to the cytokine cascade resulting in the recruitment and activation of Th17 cells in psoriatic plaques (provided the p40 subunit of this cytokine is also synthesized by KCs in this setting).

As described above, the inflammatory response of sorted KCs broadly encompasses gene induction mediated by innate cytokines such as IL-1 and TNF and also cytokines of adaptive immunity such as IFN- γ and IL-17. Approximately 300 genes linked to IFN- γ signaling in KCs (Nograla *et al.*, 2008) were upregulated in response to cell disassociation. This effect was unexpected, since cells known to synthesize IFN- γ were not present in our model

system. It is possible that the expression of IFN- γ -related genes is due to activation by IFN- κ , which was upregulated in sorted KCs. IFN- κ , a member of the type I interferon family, utilizes the same receptor as other type I interferons and leads to the activation of the interferon-stimulated response element (ISRE) signaling pathway. As there are many overlapping genes in type I and type II-inducible genes, this may be why there was significant overlap between gene expression in sorted and IFN- γ -treated KCs. Our study provides evidence that IFN- κ upregulation may occur in KCs during acute inflammation, possibly as an early protective mechanism before T cells (source of IFN- γ) are recruited to an inflammatory site.

In summary, human epidermal KCs may have the ability to initiate a high-level protective immune response to cell disassociation, which we consider one form of epidermal injury. This response includes the upregulation of genes not only in innate immunity, but also genes regulating adaptive immunity. Our observations provide insight into the potential role of the KC in triggering or modulating cell-mediated inflammation. In the context of skin wounding and potential infection, CCL20 synthesis would provide the chemotactic stimulus to bring myeloid DCs and Th17 T cells into a site of skin injury and thus might help to eliminate organisms, e.g., Staph. aureus, where effective control may not be achieved by only innate immune mechanisms.

MATERIALS AND METHODS

Skin sample preparation for flow cytometry and epidermal cell cultures

Normal adult human skin was obtained (n=10). Patient consent was not required because discarded materials of surgery are considered exempt under Rockefeller University's IRB approved protocol. Epidermis and dermis of normal adult human skin were separated by dispase digestion. Epidermal cell suspensions were obtained following trypsin treatment. The protocols are described in detail in Supplemental Methods.

Flow cytometry

High speed cell sorting was performed to obtain populations of CD207⁻ c-kit⁻ cells, which were collected as KCs. The protocols and antibodies are described in detail in Supplemental Methods and Table S3.

Laser capture microdissection

Ten normal skin biopsies were obtained from patient volunteers under Rockefeller University's IRB approved protocol. Written, informed consent was obtained from all patients, and adhered to Helsinki Guidelines. LCM was performed following the manufacturer's protocol for CellCut system (Molecular Machines and Industries, Glatfbrugg, Switzerland), and are described in Supplemental Methods.

Keratinocyte cultures

KC *in vitro*—Normal adult human epidermal keratinocytes (PromoCell, Heidelberg, Germany) were equilibrated at 37° C and 5% CO₂ and grown in PromoCell KC Growth

Medium 2 (n=10). At less than 80% confluency, cells were washed with Dulbecco's PBS and harvested for RNA isolation.

Epidermal single cell suspensions from normal human skin samples—

Epidermal single cell suspensions from normal skin (n=3) were prepared as described above. Patient consent was not required because discarded materials of surgery are considered exempt under Rockefeller University's IRB approved protocol. Immediately after trypsinization, some cells for negative control, time 0, were lysed in RLT. Cell suspensions were cultured in media described above +/- etanercept (10 µg/ml, Enbrel; Amgen, Thousand Oaks, Calif) for 16 hours. The supernatants were collected for protein analysis and cells were harvested for RNA isolation.

KCs cultured with TNF and/or etanercept—Keratinocyte experiments were conducted as previously described (Zaba 2010 JACI). Briefly, primary pooled human keratinocytes (n=3; Yale Skin Diseases Research Center core facility) were grown to 80% confluence. TNF (10ng/ml, R&D Systems, Minneapolis, Minn) and/or etanercept (10 µg/ml, Enbrel; Amgen, Thousand Oaks, Calif) were then added for 24 hours and cells were harvested for RNA isolation.

RNA preparation and quantitative RT-PCR

Total RNA was extracted using RNeasy Micro Kit (Qiagen Valencia, CA) according to manufacturer's protocol with on-column DNase digestion. Quantification and purification methods are described Supplemental Methods. Quantitative RT-PCR was performed for epidermal cultures with etanercept, NHK cultures with etanercept and/or TNF, and UVB-treated biopsies using Taqman gene expression assays as previously described (Chamian *et al.*, 2005). For KC sorted and KC LCM, two step RT-PCR was performed and is described in detail in Supplemental Methods and Table S4. All data were analyzed by the Applied Biosystems PRISM 7700 software (Sequence Detection Systems, ver. 1.7) and normalized to hARP housekeeping gene.

Amplification and labeling of targets for cDNA microarray analysis

We performed microarray analysis using Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) for sorted KCs, KC LCM, and cultured KCs. The raw microarray data of this study were deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE30355 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30355>). The protocol is described in Supplemental Methods.

Luminex protein assay

Levels of CCL20 in supernatants from disassociated epidermal cells were measured using Human Cytokine Assay Ultra-Sensitive Kit following the manufacturer's protocol (Meso Scale Discovery, Gaithersburg, MD). This is an enzyme-linked immunosorbent assay (ELISA)-based electrochemiluminescence (ECL) assay.

Ultraviolet B radiation

We used UVB-treated skin samples archived from a previous study (Judson *et al.*, 2010). Archived materials included whole tissue RNA extracts and cryostat sections of skin biopsies. RT-PCR was performed as described above. Immunohistochemistry was as previously described (Zaba *et al.*, 2007) and antibodies are listed in Table S5.

Statistical analysis

Affymetrix CEL files were analyzed, and are described in detail in Supplemental Methods. A moderated t-test was used in the *limma* package framework (Bioconductor, Seattle, WA) to identify DEGs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DCs	dendritic cells
DEG	differentially expressed genes
EGFR	epidermal growth factor receptor
FCH	fold change
FDR	false discovery rate
GSEA	Gene set enrichment analysis
hARP	human acidic ribosomal protein
NHK	normal human keratinocytes
KC	keratinocyte
LC	Langerhans cell
LCM	laser capture microdissection
PDGF	platelet-derived growth factor
qRT-PCR	quantitative reverse transcriptase-PCR
TGF-β	transforming growth factor-beta
TNF	tumor necrosis factor
UVB	ultraviolet B light

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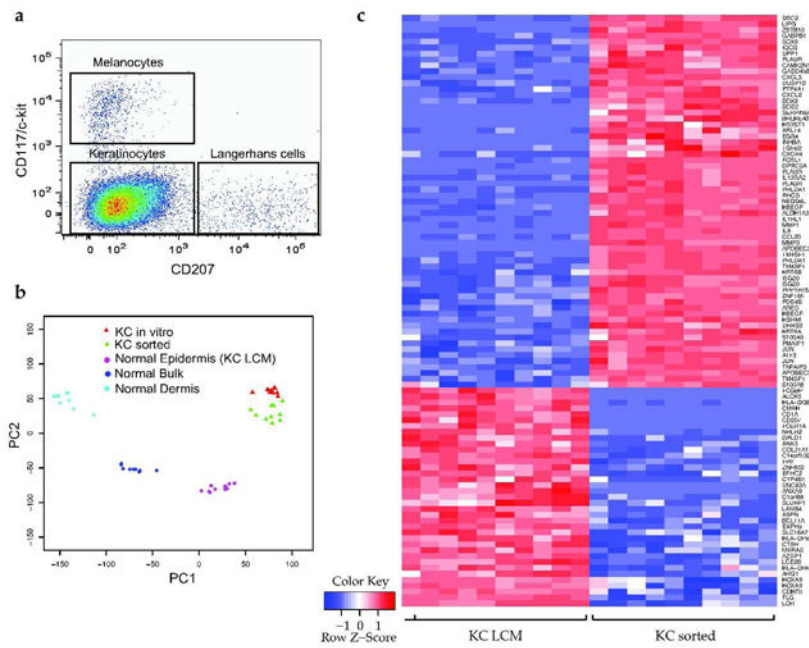


Figure 1. Isolation of a pure population of KCs by FACS and normal epidermis by laser capture microdissection

(a) Sorted KCs were isolated from epidermal single-cell suspensions with flow cytometry. Using antibodies for CD207 and c-kit, we identified three cell populations: CD207⁺ c-kit⁻ cells (Langerhans cells), CD207⁻ c-kit⁺ (melanocytes), and CD207⁻ c-kit⁻ cells. We collected CD207⁻ c-kit⁻ cells as KCs. (b) Principal components analysis of microarray data for sorted KCs (KC sorted), cultured KCs (KC *in vitro*), and tissue isolated by LCM, including normal epidermis (KC LCM), normal dermis, and bulk (dermis + epidermis). PC1 accounts for 39% of the variance. (c) Heat map of top 100 differentially expressed genes for sorted KCs (KC sorted) and normal epidermis (KC LCM).

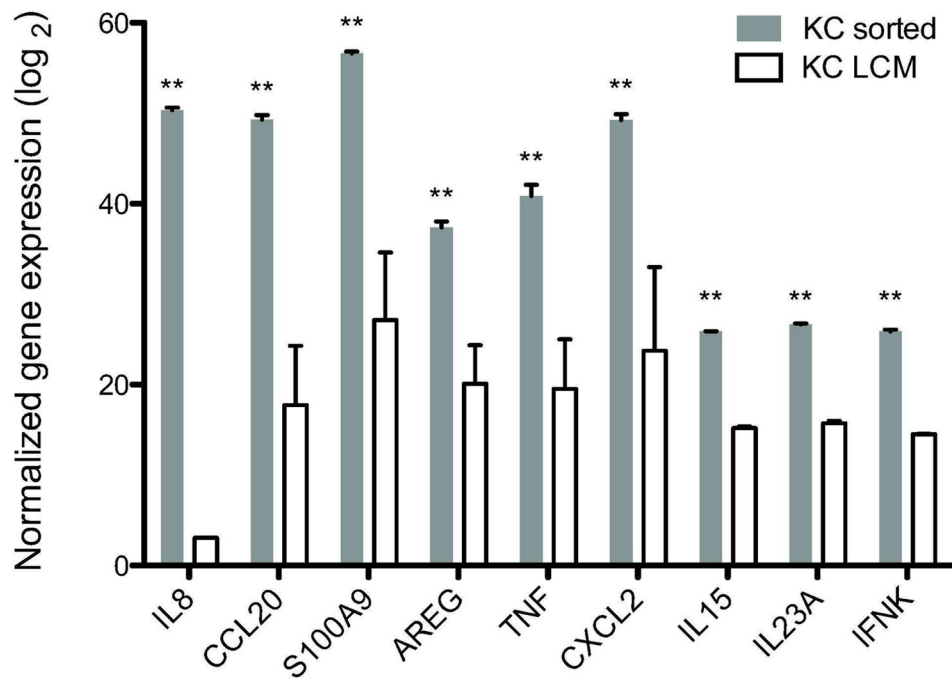


Figure 2. Differentially expressed genes in microarray for KC sorted vs. KC LCM are validated by quantitative RT-PCR

We performed quantitative RT-PCR using mRNA from sorted KCs (n=6) and KC LCM (n=6). All data was normalized to hARP (human acidic ribosomal protein) housekeeping gene. A two-tailed student's t-test confirmed the differential expression of all genes tested (* $p < 0.05$, ** $p < 0.01$). Error bar denotes mean \pm SD.

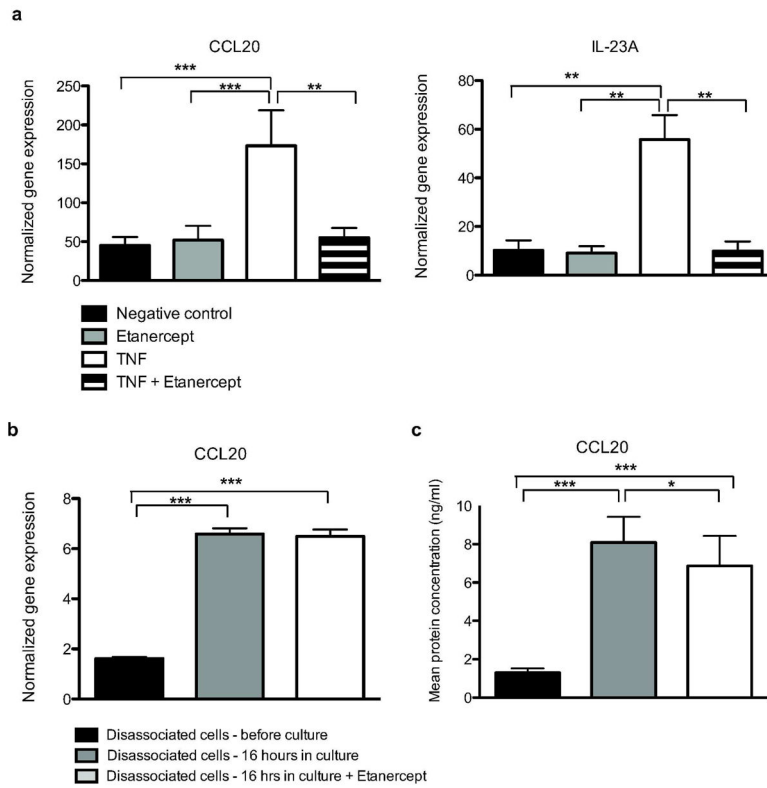


Figure 3. TNF regulates expression of CCL20 and IL23A by KCs

(a) Cell lines of NHK (n=3) were treated with 10 ng ml^{-1} TNF and/or Etanercept $10 \mu\text{g ml}^{-1}$. As measured by RT-PCR, CCL20 and IL-23A mRNA at 24 hours were increased compared to control ($p=0.005$, $p=0.0038$) and Etanercept neutralized TNF-induced expression of CCL20 and IL-23A ($p=0.0026$, $p=0.0037$) (b) Disassociated epidermal cells (n=3) were cultured for 16 hours +/- Etanercept. CCL20 mRNA expression was increased for both conditions compared to control. (c) Compared to control, CCL20 protein in supernatants of disassociated epidermal cells was increased in cells cultured +/- Etanercept ($p=0.0001$, $p=0.0002$). Etanercept inhibited CCL20 protein compared to cells cultured in media only ($p=0.0414$). Mean +/- SD. Statistical significance determined by two-tailed t-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$

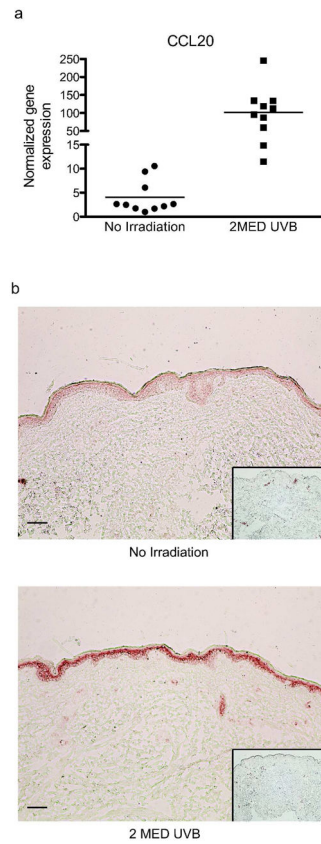


Figure 4. CCL20 mRNA and protein are increased in normal adult skin irradiated with ultraviolet B light

Archived material was used from a study in which we irradiated the skin of normal adults (n=10) with 2MED narrowband UVB light (312 nm). Biopsies were before irradiation and 24 hours later. **(a)** CCL20 mRNA expression, as measured by quantitative RT-PCR, was upregulated in 10/10 patients after UVB irradiation, with a mean 34-fold increase ($p = 0.002$). Lines denote the mean normalized gene expression. **(b)** Immunohistochemistry staining for CCL20 shows increased protein expression after UVB irradiation. The insert shows CD3 antibody staining as a control for non-specific staining of damaged epithelial cells. Scale bar = 1 μ m

Table 1

Selected DEGs for KC sorted vs. KC LCM

Gene	mRNA	Description	FCH ^{a, b}
IL1RL1	IL-1RL1	interleukin 1 receptor-like 1	2659.0
MMP1	MMP-1	matrix metalloproteinase 1 (interstitial collagenase)	2014.7
IL8	IL-8	Interleukin 8	2004.5
CCL20	CCL20	chemokine (C-C motif) ligand 20	1909.2
MMP3	MMP-3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	1296.4
KRT6B	KRT6B	keratin 6B	883.4
AREG	AREG	amphiregulin	430.9
HBEGF	HB-EGF	heparin-binding EGF-like growth factor	341.5
CXCL2	CXCL2	chemokine (C-X-C motif) ligand 2	322.8
KRT6A	KRT6A	keratin 6A	217.9
S100A9	S100A9	S100 calcium binding protein A9	187.5
CXCR4	CXCR4	chemokine (C-X-C motif) receptor 4	176.1
S100A8	S100A8	S100 calcium binding protein A8	129.1
CXCL3	CXCL3	chemokine (C-X-C motif) ligand 3	123.1
CXCL1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	59.3
S100A7	S100A7	S100 calcium binding protein A7	57.4
STAT3	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	51.3
RNASE7	RNase 7	ribonuclease, RNase A family, 7	47.9
LCN2	NGAL	lipocalin 2	40.6
IL1F9	IL-1F9	interleukin 1 family, member 9	35.6
PI3	elafin	peptidase inhibitor 3, skin-derived	35.5
IL1R2	IL-1R2	interleukin 1 receptor, type II	31.1
TNF	TNF	tumor necrosis factor	25.9
IL20	IL-20	interleukin 20	22.2
IL1A	IL-1 α	interleukin 1, alpha	18.6
TGFA	TGF- α	transforming growth factor, alpha	16.5
IL15	IL-15	interleukin 15	8.8
IL23A	IL-23A	interleukin 23, alpha subunit p19	8.7
IFNK	IFN- κ	interferon, kappa	6.6
HLADQB2	HLA-DQB2	major histocompatibility complex, class II, DQ beta 2	- 111.1
HLADPA1	HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	- 83.3
PNPLA3	PNPLA3	Patatin-like phospholipase domain containing 3	- 66.7
CD1A	CD1A	CD1a molecule	- 58.8
CD207	CD207	CD207 molecule, langerin	- 50.0
HLADPB1	HLA-DPB1	major histocompatibility complex, class II, DP beta 1	- 50.0

^aFCH = Fold-change in gene expression

^bFDR < 0.01 for all genes

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