Glucocorticoids promote CCL20 expression in keratinocytes

L. Wang,1,2 M. Yang,3 X. Wang,4 B. Cheng,2 Q. Ju,1 D.Z. Eichenfield2 and B.K. Sun2

1Department of Dermatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200127
2Department of Dermatology, University of California San Diego, La Jolla, CA, 92093, USA
3State Key Laboratory of Oncogenes and Related Genes, Renji-Med X Clinical Stem Cell Research Center, Ren Ji Hospital, Shanghai, 200127
4Department of Dermatology, Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200127, China

Background Glucocorticoids (GCs) are generally envisioned as immunosuppressive, but in conditions such as rosacea and perioral dermatitis they can lead to increased skin inflammation. In lung epithelia, GCs promote expression of the proinflammatory cytokine CCL20, which contributes to steroid-resistant asthma. In the skin, CCL20 stimulates inflammation by recruiting T helper 17 T lymphocytes and dendritic cells, and is elevated in papulopustular rosacea.

Objectives To understand if, and how, GCs affect CCL20 expression in human keratinocytes. CCL20 expression was assessed by quantitative reverse transcriptase polymerase chain reaction and enzyme-linked immunosorbent assay.

Methods Selective inhibition of candidate genes and signalling pathways was performed using RNA interference and chemical inhibitors. The binding of activated GC receptor to genomic DNA was determined by chromatin immunoprecipitation, and enhancer activity of genomic sequences was measured with a reporter assay.

Results We found that GC treatment increased CCL20 expression in human keratinocytes and murine skin, both in the undisturbed state and with tumour necrosis factor-α stimulation. GC repressed proinflammatory signalling pathways, including nuclear factor kappa B and p38/mitogen-activated protein kinase, but these inhibitory effects were opposed by the direct binding of activated GC receptor to the CCL20 enhancer, promoting CCL20 expression.

Conclusions Viewed together, these findings demonstrate a mechanism by which GCs induce expression of CCL20 in keratinocytes, which may contribute to the inflammation seen in steroid-exacerbated skin conditions.

What is already known about this topic?

- Glucocorticoids (GCs) are generally considered immunosuppressive but can actually worsen some inflammatory skin conditions such as rosacea and perioral dermatitis.
- In bronchial epithelia, GCs promote the expression of the proinflammatory cytokine CCL20, which contributes to steroid-insensitive asthma.
- Increased CCL20 is associated with the inflammation of rosacea, but whether GCs induce its expression in the skin is unknown.

What does this study add?

- This study demonstrates that activated GC receptor directly binds the CCL20 enhancer in human keratinocytes to promote its expression.
Glucocorticoids induce CCL20 in keratinocytes, L. Wang et al.

Rosacea is a common inflammatory skin disease that classically manifests with flushing, telangiectasias, and inflamed papules and pustules on the face. A wide spectrum of stimuli can exacerbate rosacea, including ultraviolet exposure, temperature, microbes and drugs. The inflammation of rosacea is often treated with topical or oral antibiotics, and its chronic nature typically requires long-term management and the avoidance of triggers.

Rosacea responds poorly to treatment with steroids. In general, steroids are envisioned as having potent immunosuppressive properties and have an essential role in the treatment of autoimmune and inflammatory diseases. However, clinicians have long recognized that topical steroids worsen rosacea with prolonged treatment or upon their withdrawal. A related inflammatory skin condition – perioral dermatitis – is frequently associated with the long-term use of topical steroids on the face. In fact, these two clinical entities (rosacea and perioral dermatitis) are histologically similar and considered by some clinicians to be variants of the same condition. Together they represent cardinal examples of skin conditions that are initiated or worsened by steroids. However, the molecular mechanisms that underlie their atypical proinflammatory response to steroids is not well understood.

Studies of other diseases may provide clues as to why some inflammatory skin conditions are responsive to steroids, while others are not. Asthma is a respiratory disease characterized by hyper-reactive airways and bronchial inflammation. A cornerstone of asthma therapy includes inhaled glucocorticoids (GCs), but a significant subset of individuals with asthma respond poorly to steroids. Studies of patient sputum and bronchial epithelial cells from steroid-insensitive patients demonstrated the elevation of proinflammatory cytokines. In particular, the consistent elevation of CCL20 is characteristic of severe asthma in humans and mouse models. CCL20 is a chemoattractant for T helper (Th) 17 T lymphocytes, neutrophils and dendritic cells. In bronchial epithelia, GCs result in a paradoxical induction of CCL20, which may help to explain the molecular basis of steroid insensitivity in asthma.

CCL20 is also consistently elevated in the papulopustular forms of rosacea. While many clinical features of rosacea are characteristic of an exacerbated innate immune response, a dense lymphocytic infiltrate is often present, implicating a role for adaptive immunity. In the skin, CCL20 is expressed from keratinocytes and drives the Th17-polarized T-lymphocyte response observed in the inflammatory forms of rosacea. Sparked by observations made in bronchial epithelia, the objective of this study was to determine whether GCs induce CCL20 expression in keratinocytes, towards a long-term goal of better understanding how steroids can worsen inflammation in skin conditions such as rosacea.

**Materials and methods**

**Cells and reagents**

Neonatal human epidermal keratinocytes (NHEKs) were a gift from Professor Ningli Li. In some experiments, NHEKs were harvested from discarded foreskin samples from elective circumcisions. Sources of reagents, plasmids and antibodies are provided in Appendix S1 (see Supporting Information).

**Mouse reagents and experimentation**

C57BL/6 (wild-type) mice were obtained from the Shanghai Laboratory Animal Center, Shanghai, China. All animals were housed and treated under institutional guidelines approved by the Institutional Animal Care and Use Committee at the University of Ruijin Hospital. Eight-week-old mice were divided into vehicle control and GC treatment groups. Mice in the GC group were treated with 10 mg topical halometasone daily for 14 days, applied to ear skin. Control groups received topical 10 mg Vaseline® (Unilever, London, UK) for 14 days.

**Cell culture**

NHEKs were cultured in a 50:50 mixture of Medium 154 and keratinocyte serum-free medium containing manufacturer supplements and 1× antibiotic antimycotic solution. Cells were seeded onto 12-well plates at 10⁴ cells per well and incubated at 37 °C with 5% CO₂. Cells were grown to ∼70% confluence and treated with dexamethasone, tumour necrosis factor (TNF)-α (10 ng mL⁻¹), Jun N-terminal kinase (JNK)/mitogen-activated protein kinase (MAPK) inhibitor SP600125 (1 μmol L⁻¹), extracellular regulated kinase (ERK)/MAPK inhibitor U0126 (1 μmol L⁻¹), & p38/MAPK inhibitor SB202190 (1 μmol L⁻¹), phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (5 μmol L⁻¹) and/or GC inhibitor RU486 (1 μmol L⁻¹) as indicated. Inhibitors were added 1 h before dexamethasone. In co-treatment experiments, dexamethasone was added 2 h before TNF-α. TNF-α was incubated for 3 h unless specified. Dimethyl sulfoxide (DMSO) was used as vehicle control for inhibitors and dexamethasone: water was used as control for TNF-α.

**Real-time quantitative polymerase chain reaction**

Total RNA from cells and tissues were extracted using an Eazyprep Super Total RNA Extraction Kit (Promega, Madison, WI, USA) or a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), following the manufacturers’ instructions.

© 2021 The Authors. British Journal of Dermatology published by John Wiley & Sons Ltd on behalf of British Association of Dermatologists
Quantitative polymerase chain reaction (PCR) was performed using SYBR Green Taq (Takara Bio, Kusatsu, Japan) or iTaq SYBR Supermix (Bio-Rad, Hercules, CA, USA) in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) or a CFX Connect (Bio-Rad). For quantitative PCR, RPL32 was used as an internal reference. Primer sequences are provided in Appendix S1.

**Enzyme-linked immunosorbent assay**

NHEKS were seeded onto 96-well culture plates at 2 × 10^4 cells per well and incubated for 24 h in the presence of TNF-α, dexamethasone and/or RU486 (1 μmol L⁻¹), as indicated. CCL20 was quantitated using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Immunoblotting**

NHEKS were seeded onto six-well plates (2.5 × 10^5 cells per well) and treated with stimuli as indicated. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was determined using a BCA assay. Lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked in Tris-buffered saline with 0.1% Tween 20 detergent (TBS-T) containing 5% bovine serum albumin. Membranes were incubated with antibodies (dilution 1:1000) overnight at 4°C. After four washes with TBS-T, membranes were incubated with secondary antibodies for 45 min and washed three times with TBS-T. Immunoblots were visualized on an Odyssey Imager (Li-Cor Biosciences, Lincoln, NE, USA).

**Small interfering RNA transfection**

NHEKS were seeded onto six-well plates at 3 × 10^5 cells per well for 24 h. Cells were transfected with small interfering RNA (siRNA; 10 μmol L⁻¹) using RNAiMAX (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were treated as indicated. GC receptor (GR) siRNA (4392420) and control siRNA (4390844) were from Life Technologies (Carlsbad, CA, USA).

**Luciferase assay analysis**

The CCL20 enhancer was amplified from genomic DNA by PCR (forward primer 5’-GACACACAGGATCACTGGAG-3’; reverse primer 5’-TCTTCTCAGGTCAATGACATCAAAT-3’) and cloned into the KpnI/BglII site of pGL4.10 Enhancer or pGL4.10 Enhancer. Deletion of GR binding motifs (denoted ΔGRE) was generated by inverse PCR. pGL4.24, pGL4.24 CCL20 enhancer or pGL4.24 CCL20 enhancer ΔGRE were transfected into HEK293T cells. pGL4.75-75 was used as a transfection control. After 42 h, cells were treated with dexamethasone (100 nmol L⁻¹), TNF-α (10 ng mL⁻¹) or both. Dexamethasone was added to the cell medium 2 h before TNF-α. Three hours after TNF-α stimulation, luciferase activity was assessed using the Dual-Glo™ Luciferase Assay (Promega).

**Chromatin immunoprecipitation**

NHEKS were cultured to 70–80% confluence on 15-cm plates and then treated with dexamethasone (100 nmol L⁻¹) or DMSO for 1 h. Cells were fixed in formaldehyde (1%) and disuccinimidyl glutarate (2 mmol L⁻¹). Chromatin was immunoprecipitated overnight at 4°C. The percentage input method was used to normalize chromatin immunoprecipitation (ChiP) data. Data from the National Institutes of Health Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) were used to generate genomic tracks for GR, histone 3 lysine 27 acetylation, P300 and DNAse I hypersensitivity. These data are available under study identifiers GSE72252, GSM1645719 and GSM733674.

**Statistical analysis**

Unless otherwise noted, mRNA and protein expression are presented as mean ± SEM. Comparisons between conditions were performed with two-sided t-tests. P-values < 0.05 were considered to be statistically significant. Statistical comparisons were conducted using SPSS software (version 10.1; SPSS, Chicago, IL, USA).

**Results**

**Glucocorticoids induce CCL20 expression in vitro and in vivo**

To assess if GCs affect CCL20 expression in the skin, we evaluated the impact of the GC dexamethasone on human keratinocytes and murine skin. We applied 100 nmol L⁻¹ dexamethasone to primary human keratinocytes and measured CCL20 mRNA expression over a time course using quantitative reverse transcriptase PCR (qRT-PCR). CCL20 mRNA expression was induced by 3 h of dexamethasone treatment, increasing >2-fold upon dexamethasone stimulation (Figure 1a). Induction of CCL20 contrasted with the suppression of interleukin (IL)-1β, another proinflammatory gene. To determine if CCL20 protein expression correlated with mRNA induction, as well as to assess dose dependence, we applied dexamethasone to primary human keratinocytes in a range of 0–1000 nmol L⁻¹ and measured CCL20 protein by enzyme-linked immunosorbent assay (ELISA). We observed a progressive induction of CCL20 with increasing doses of dexamethasone (Figure 1b). CCL20 is expressed more strongly in differentiated epidermal layers than in the undifferentiated basal layer, but it is induced throughout the epidermis following epidermal injury or inflammation. We corroborated these previous observations by differentiating keratinocytes in vitro by culturing at confluence for 96 h with the addition of 1-2 mmol L⁻¹ calcium. Expression of keratin 10 and filaggrin showed strong upregulation (Figure 1c), confirming the induction of
differentiation. In differentiated keratinocytes we observed an approximately twofold increase in CCL20 expression vs. the undifferentiated state, confirming previous observations. Next, we assessed how dexamethasone affected CCL20 expression in undifferentiated vs. differentiated keratinocytes. As noted in our initial observation (Figure 1a), CCL20 increased within 3 h of dexamethasone exposure in undifferentiated keratinocytes but did not change appreciably in differentiated keratinocytes (Figure 1d). This result suggested that the inducive effect of dexamethasone on CCL20 may be restricted to basal progenitor keratinocytes.

Finally, we examined whether GC led to similar results in mice. We applied the corticosteroid halometasone topically to the ear of mice for 14 days. qRT-PCR demonstrated a ~2.5-fold increase in CCL20 expression in murine skin treated with topical corticosteroids vs. vehicle control (Figure 1e). In contrast, the expression of IL1B decreased by ~40%. Together, these results demonstrated that GCs promote CCL20 expression in human keratinocytes and mouse skin, in contrast to its inhibitory effect on other proinflammatory genes.

Tumour necrosis factor-α amplifies the induction of CCL20 by glucocorticoids

The T-cell lymphocytic infiltrate in rosacea is characterized by Th1/Th17 polarization. We therefore aimed to understand whether GC induction of CCL20 is influenced by the cytokine microenvironment seen in rosacea. To evaluate this possibility, we pretreated keratinocytes with a series of GC doses (0–1000 nmol L−1) prior to stimulation with TNF-α, a signature Th1 cytokine. As anticipated, stimulation with TNF-α resulted in a robust induction of CCL20 expression (Figure 2a).
However, higher doses of GC did not suppress CCL20; instead, we observed a trend of increasing CCL20 with higher dexamethasone concentration, although these differences did not reach statistical significance (two-way ANOVA, multiple comparisons \( P > 0.05 \)). Nonetheless, these results sparked further interest in understanding why GCs appeared to potentiate the expression of the proinflammatory gene CCL20.

To determine if induction of CCL20 by dexamethasone was a unique characteristic of CCL20 among inflammatory genes, we compared the effect of dexamethasone on CCL20 to the proinflammatory genes IL1B and IL6. Pretreatment with dexamethasone inhibited the induction of IL1B and IL6 by TNF-\( \alpha \).

However, as has been previously observed, the addition of dexamethasone resulted in further upregulation, increasing by approximately sixfold over the untreated state (Figure 2b). This finding indicated that CCL20 responded distinctively to steroid exposure, compared to other inflammatory genes.

To determine if CCL20 protein expression corresponded to mRNA expression results, we performed an ELISA in the presence or absence of TNF-\( \alpha \) and dexamethasone. CCL20 protein expression increased in keratinocytes upon treatment with either dexamethasone or TNF-\( \alpha \). If treated with both, CCL20 was even further induced (Figure 2c). Viewed together, these results indicated that GCs do not repress CCL20 expression by itself, but rather, GC treatment leads to additively induced CCL20 production in keratinocytes.

Glucocorticoids inhibit tumour necrosis factor-\( \alpha \)-mediated activation of nuclear factor kappa B and p38/mitogen-activated protein kinase

We next sought to better understand how GCs led to the induction of CCL20. The activity of nuclear factor kappa B (NFkB) signalling is a well-established regulator of CCL20 expression.\(^{15}\) However, the potential involvement of other kinase signalling pathways has been suggested in recent studies. The activity of MAPK pathways has been shown to regulate CCL20 in response to CCN1 (cellular communication network factor 1).\(^{16,17}\) In addition, reports have linked PI3K signalling to CCL20 expression, although PI3K appears to induce or repress CCL20, depending on cell type and ligand.\(^{18,19}\)

We therefore assessed whether MAPK or PI3K signalling are involved in TNF-\( \alpha \) stimulation of CCL20 in human keratinocytes.

We applied chemical inhibitors to JNK, ERK, p38 and PI3K, and assessed their effect on TNF-\( \alpha \)-mediated induction of CCL20 in primary human keratinocytes (Figure 3a). CCL20 induction by TNF-\( \alpha \) was not affected by the JNK/MAPK (SP600125) or ERK/MAPK (U0126) inhibitor. By contrast, p38/MAPK inhibition (SB202190) repressed CCL20 moderately, while PI3K inhibition (LY294002) led to CCL20 upregulation. This panel of chemical inhibitors suggested that p38/MAPK and PI3K influence TNF-\( \alpha \)-mediated CCL20 activation in keratinocytes.

To further assess the effect of GCs on kinase signalling pathways, we performed immunoblotting to assess the effect of TNF-\( \alpha \) stimulation on p38 and PI3K signalling, with and without dexamethasone pretreatment. As positive and negative controls, we also assessed the phosphorylation of p65 and ERK. As expected, TNF-\( \alpha \) stimulated phosphorylation of p65 (reflecting NFkB activation), as well as p38. TNF-\( \alpha \) had modest effects on ERK phosphorylation and minimal effects on Akt (PI3K). Pretreatment with dexamethasone blocked TNF-\( \alpha \)-induced phosphorylation of p65 and p38, indicating that GCs inhibited NFkB and p38 activity. By contrast, there were no consistent effects of GCs on ERK and Akt phosphorylation.

Viewed together, these results indicate an inhibitory effect of GCs on NFkB and p38/MAPK, which is broadly consistent with the immunosuppressive effect of steroids. However,
NFκB and p38 are thought to be activators of inflammation genes. Therefore, these results appear to contradict the observed induction of CCL20 expression seen with GC treatment (Figures 1 and 2). Studies of bronchial epithelium have hinted that CCL20 activation by GCs may be independent of classical inflammatory signalling pathways, although it is still uncertain what these mechanisms are. We therefore wanted to explore whether GCs engaged NFκB- and p38-independent mechanisms to influence CCL20 expression in keratinocytes.

Glucocorticoids directly activate CCL20 by binding at its promoter and enhancer

In addition to their effects on inflammatory signalling pathways, GCs can control the transcription of gene targets through binding and activation of the GR. Activated GRs translocate from the cytoplasm to the nucleus and bind to genomic targets to repress or activate gene transcription. Because CCL20 was induced by GCs, we wanted to test the possibility that the GR directly binds and activates CCL20 in keratinocytes.

To test this hypothesis, we first assessed the effect of a GR chemical inhibitor, mifepristone (RU486), on the induction of CCL20 by TNF-α and dexamethasone (Figure 4a). Blocking the activity of GR prevented stimulation of CCL20 protein by dexamethasone, indicating a specific role for GR in the induction of CCL20. Consistent with this finding, TNF-α stimulation led to a modest induction of CCL20, and pretreatment with dexamethasone did not amplify this expression any further.

To corroborate this result, we also depleted the GR using RNA interference (Figure 4b). In comparison to chemical inhibition, genetic depletion of GR resulted in a similar but more severe phenotype. In GR-depleted keratinocytes, dexamethasone and TNF-α stimulation did not induce the expression of CCL20. In fact, CCL20 expression was markedly repressed even in the unstimulated state, which may be attributable to the essential function of the GR in keratinocytes, and the strong and acute loss of function caused by RNA interference.

These findings indicated that an activated GR is required for CCL20 expression but did not demonstrate if it functions directly. To assess if GR directly binds to the CCL20 genomic locus, we examined a GR chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) study performed in MCF7 cells, and identified a GR peak 800 nucleotides downstream of CCL20, which appeared after dexamethasone stimulation (Figure 4c [+Dex], asterisk).

We aligned the GR binding site with published ChIP-seq data performed in keratinocytes (Figure 4c) and noted that it coincided with enrichment for P300 (NIH GEO: GSM1645719), a DNase I hypersensitivity peak (ENCODE) and a bipartite histone 3-lysine 27 acetylation peak (NIH GEO: GSM733674). This epigenetic signature is characteristic of an enhancer and suggested that GR may bind this genomic site in keratinocytes to regulate CCL20 directly. We surveyed ChIP-seq datasets at the genomic neighbourhood (~10 kb upstream/downstream) of other proinflammatory genes elevated in rosacea, including IL1A, IL1B, IL6, IL17A and IL22. We did not identify GR binding peaks in the proximal genomic neighbourhoods of these genes, indicating that genomic binding by GR might be a relatively distinctive feature of CCL20 among proinflammatory genes.

Because the GR ChIP-seq results were derived from a different cell type, we performed ChIP to determine whether GR binds the CCL20 enhancer in human keratinocytes. We noted
Figure 4 Activated glucocorticoid (GC) receptor (GR) directly induces CCL20 by binding to its promoter and a downstream enhancer. (a) Enzyme-linked immunosorbent assay for CCL20. Human keratinocytes were treated with the GC antagonist mifepristone (RU486) then treated with dexamethasone (Dex), tumour necrosis factor (TNF)-α or both (n = 3). Error bars denote the SD. ns, not significant. *P < 0.05. (b) Quantitative reverse transcription polymerase chain reaction for CCL20 mRNA expression. Control (con-siRNA) or GR-targeted small interfering (siRNAs; GR-siRNA) were transfected into human keratinocytes, then treated with dexamethasone (Dex), TNF-α or both (n = 3). Error bars denote the SEM. ns, not significant *P < 0.05 (two-sided t-test). (c) Genome tracks displaying results from chromatin immunoprecipitation with sequencing (ChIP-seq) assays for GR with and without dexamethasone (GR no Dex, GR + Dex),22 p300 (GSM1645719), DNAse I hypersensitivity (DNase I HS, ENCODE) and histone 3 lysine 27 acetylation (H3K27ac, GSM733674). Coloured bars denote location of promoter (red), intergenic (green) and enhancer (blue) ChIP amplicons used in (d). *CCL20 enhancer. (d) Chromatin immunoprecipitation/polymerase chain reaction. Chromatin from primary human keratinocytes was immunoprecipitated with antibodies against GR or a non-specific IgG control. DNA enrichment was assessed at the CCL20 enhancer and promoter. Intergenic and distal gene desert regions were amplified as controls for specificity (n = 3). Error bars denote the SEM. ns, not significant. *P < 0.05 (two-sided t-test). (e) GC binding motifs in the CCL20 enhancer. Analysis of the CCL20 enhancer sequence using MEME suite tools identified the best GR motif match near the centre of the enhancer peak at positions 223–235, denoted as ‘site A’. A second adjacent GR motif was identified adjacent to site A and was noted as ‘site B’. Motif alignment and enrichment P-values are shown. Red text denotes mismatch from the canonical GR motif. Deletion of site A/B GR response elements was generated and named ΔGRE. (f) Enhancer reporter assay. The wild-type and AGRE CCL20 enhancer sequence was cloned upstream of a minimal promoter driving firefly luciferase and co-transfected into 293T cells with Rluciferase transfection controls. Cells were treated with dexamethasone (Dex), TNF-α or both (n = 3). Error bars denote the SD. ns, non-significant. *P < 0.05 vs. control (two-sided t-test).
enrichment for GR binding at the CCL20 enhancer that was induced by steroid (Figure 4d, +Dex). We also noted enrichment at the CCL20 promoter. GR binding was not detected in gene body or gene desert control regions, or with IgG antibody controls, supporting the specificity of GR binding to the CCL20 enhancer and promoter.

Activated GRs bind to enhancer elements at canonical DNA motifs.\textsuperscript{24} We analysed the putative CCL20 body controls, supporting the specificity of GR binding to the gene body or gene desert control regions, or with IgG anti-CCL20 antibody at the inflammation-related genes can also be directly regulated in

To test if GR binding to these sites mediated transcriptional activation, we cloned the 477 base pair (bp) CCL20 enhancer into a luciferase reporter appended to a minimal promoter. We also generated a 26-bp deletion that removed the two GR binding motifs, denoted AGRE (deletion of GC response elements; Figure 4e). The wild-type CCL20 enhancer stimulated an approximately fourfold increase in luciferase above the minimal promoter, indicating its positive effect on transcriptional activation (data not shown). The addition of dexamethasone increased the expression of the CCL20 enhancer reporter even further. Stimulation with TNF-\(\alpha\) had no effect, arguing against its influence at the enhancer element. By comparison, the AGRE enhancer showed no transcriptional response to dexamethasone, indicating that binding to the GR binding motifs is specifically required (Figure 4f). Collectively, these results demonstrated that GCs stimulate CCL20 expression in keratinocytes by activating the GR, which directly binds an enhancer 3’ downstream of the CCL20 locus at GR binding motifs.

**Discussion**

GCs are commonly thought of as strong immunosuppressants, but in some skin conditions they can worsen inflammation.\textsuperscript{26} In this study, we identified a molecular mechanism that may explain how GC promotes skin inflammation in specific contexts. We found that expression of the chemokine CCL20 in keratinocytes is upregulated by GC and that this induction is amplified in the context of TNF-\(\alpha\). CCL20 potently attracts Th17 T lymphocytes and dendritic cells to epithelial surfaces such as the skin, and is characteristically elevated in rosacea.\textsuperscript{9,12} We therefore propose that GCs exacerbate the inflammation in rosacea and perioral dermatitis, in part, by the stimulation of CCL20 expression.

At the molecular level, we found that GCs suppressed proinflammatory signalling pathways such as NFKB and p38/MAPK, similarly to what has been observed in lung epithelia and other tissues.\textsuperscript{8} However, we demonstrated that the suppressive effect of steroids on proinflammatory pathways is accompanied by direct locus binding and transcriptional activation of CCL20 (Figure 5). We speculate that other inflammation-related genes can also be directly regulated in the same manner as CCL20 in keratinocytes.

Systematically identifying GR targets in skin-resident cells is of interest because they may help to explain the pathogenesis of steroid-insensitive or steroid-induced skin conditions such as rosacea and perioral dermatitis. These studies could also provide insight into mechanisms of steroid resistance, tachyphylaxis and withdrawal – phenomena that are not completely understood but are important in a broad range of inflammatory diseases such as asthma, cystic fibrosis and rheumatoid arthritis, in addition to inflammatory skin conditions.\textsuperscript{27}

The effect of GCs on skin inflammation is complex, with influences from other cytokines and chemokines, resident cell types and the skin microbiome.\textsuperscript{28} In addition to direct transcriptional effects at target loci, GC and cytokine signalling reciprocally affect each other through direct protein interaction and post-translational modifications.\textsuperscript{29,30} GCs can also generate epigenetic changes that establish longer-term effects on skin inflammation. Consistent with this, perioral dermatitis typically manifests only after prolonged steroid use, indicating that steroid-induced molecular changes may take time to accumulate to the point where they manifest clinically.

GCs remain among the most essential and common medications used in dermatology and medicine. Continued study of their molecular mechanisms in the skin and other tissues will be critical to understand how they can be best used for treatment of autoimmune and inflammatory diseases.
Acknowledgments

We thank Professor Ningli Li for generous support of this work, and A. Otten and S. Takashima for presubmission review.

References

1. Steinhoff M, Schaub J, Leyden JJ. New insights into rosacea pathophysiology: a review of recent findings. J Am Acad Dermatol 2013; 69: S15–26.
2. Sneddon I. Adverse Effect of topical fluorinated corticosteroids in rosacea. BMJ 1969; 1: 670–3.
3. Sneddon I. Perioral dermatitis. Br J Dermatol 1972; 87: 430–4.
4. Rademaker M. Perioral dermatitis. In: Harper’s Textbook of Pediatric Dermatology (Hoeger P, Kinsler V, Yan A, eds), 1st edn. Chichester: Wiley-Blackwell, 2019; 338.
5. Bullens DM, Truyen E, Coteur L et al. Steroid dermatitis. In: Hoegers Textbook of Pediatric Dermatology (Hoeger P, Kinsler V, Yan A, eds), 1st edn. Chichester: Wiley-Blackwell, 2019; 338–42.
6. Bracke KR, D’hulst AI, Maes T et al. Lung inflammatory effects of chronic exposure to cigarette smoke. Respir Res 2014; 15: 11.
7. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. Cytokine Growth Factor Rev 2003; 14: 409–26.
8. Zijlstra GJ, Fattahi F, Rozeveld D et al. Glucocorticoids induce the production of the chemotactant CCL20 in airway epithelium. Eur Respir J 2014; 44: 616–70.
9. Buil T, Sulk M, Nowak P et al. Molecular and morphological characterization of inflammatory infiltrate in rosacea reveals activation of Th1/Th17 pathways. J Invest Dermatol 2015; 135: 2198–208.
10. Yamasaki K, Gallo RL. The molecular pathology of rosacea. J Am Acad Dermatol 2009; 55: 77–81.
11. Steinhoff M, Buddenkotte J, Aubert J et al. Clinical, cellular, and molecular aspects in the pathophysiology of rosacea. J Invest Dermatol Symp Proc 2011; 2: 11–12.
12. Homey B, Drieu-Noisjean M-C, Wiesnien Born A et al. Up-regulation of macrophage inflammatory protein-3α/CCL20 and CC chemokine receptor 6 in psoriasis. J Immunol 2000; 164: 6621–32.
13. Tohyama M, Shirakaya Y, Yamasaki K et al. Differentiated keratinocytes are responsible for TNF-a regulated production of macrophage inflammatory protein 3α/CCL20, a potent chemokine for Langerhans cells. J Invest Dermatol 2001; 117: 30–9.
14. Kennedy-Crispin M, Biclick E, Mitsuji H et al. Human keratinocytes’ response to injury upregulates CCL20 and other genes linking innate and adaptive immunity. J Invest Dermatol 2012; 132: 105–13.
15. Zhao L, Xia J, Wang X, Xu F. Transcriptional regulation of CCL20 expression. Microbes Infect 2014; 16: 864–70.
16. Reibman J, Hsu Y, Chen LC et al. Airway epithelial cells release MIP-3α/CCL20 in response to cytokines and ambient particulate matter. Am J Respir Cell Mol Biol 2003; 28: 648–54.
17. Li H, Li H, Huo R et al. Cytokine production by comparing p38 and JNK/AP-1 pathway in psoriasis. J Dermatol Sci 2017; 88: 46–56.
18. Vongsa RA, Zimmerman NP, Dwinell MB. CCR6 regulation of the actin cytoskeleton orchestrates human beta defensin-2 and CCL20-mediated resolution of colonic epithelial injury. J Biol Chem 2009; 284: 10034–45.
19. Rohani MG, Dijulio DH, An JY et al. PAR2-induced innate immune markers are negatively regulated by PI3K/Akt signaling pathway in oral keratinocytes. BMC Immunol 2010; 11: 53.
20. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J Allergy Clin Immunol 2013; 132: 1033–44.
21. Sevilla LM, Latorre V, Sanchis A, Pérez P. Epidermal inactivation of the glucocorticoid receptor triggers skin barrier defects and cutaneous inflammation. J Invest Dermatol 2013; 133: 361–70.
22. Swinstead EE, Miranda TB, Paakinaho O et al. Steroid receptors reprogram FoxA1 occupancy through dynamic chromatin transitions. Cell 2016; 165: 593–605.
23. Hofmann MM, Ernst J, Wilder SP et al. Integrative annotation of chromatin elements from ENCODE data. Nucl Acids Res 2013; 41: 827–41.
24. McDowell KC, Barrera A, D’ippolito MA et al. Glucocorticoid receptor recruits to enhancers and drives activation by motif-directed binding. Genome Res 2018; 28: 1272–84.
25. Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. Bioinformatics 2011; 27: 1017–18.
26. Ljubojeviae S, Basta-Juzbasiae A, Lipozeneiae J. Steroid dermatitis resembling rosacea: aetiopathogenesis and treatment. J Eur Acad Dermatol Venereol 2002; 16: 121–6.
27. Barnes PJ. Mechanisms and resistance in glucocorticoid control of inflammation. J Steroid Biochem Mol Biol 2010; 120: 76–85.
28. Holmes AD. Potential role of microorganisms in the pathogenesis of rosacea. J Am Acad Dermatol 2013; 69: 1025–32.
29. Caldenhoven E, Liden J, Wissink S et al. Negative cross-talk between RbA and the glucocorticoid receptor: a possible mechanism for the anti-inflammatory action of glucocorticoids. Mol Endocrinol 1995; 9: 401–12.
30. Iruzen E, Matthews JG, Takahashi A et al. p38 Mitogen-activated protein kinase–induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid-insensitive asthma. J Allergy Clin Immunol 2002; 109: 649–57.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:
Appendix S1. Plasmids, reagents, antibodies and primers.