BJD
British Journal of Dermatology

Glucocorticoids promote CCL20 expression in keratinocytes

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Linked Comment: R.F.L. O'Shaughnessy. Br J Dermatol 2021; **185**:1091–1092.

Summary

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.

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Accepted for publication
17 June 2021

Funding sources
This study was funded by the National Natural Science Foundation of China (No. 81502718) and the LEO Foundation (20-000506).

Conflicts of interest
The authors declare they have no conflicts of interest.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request. The data analysed from previously published genomic datasets were accessed through the National Institutes of Health Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/ under the reference numbers GSE72252, GSM1645719 and GSM7336674.

L.W., M.Y. and X.W. contributed equally to this project.

DOI 10.1111/bjd.20594

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What is the translational message?

- GCs can directly activate the expression of CCL20, which may help explain how topical steroids can worsen skin conditions such as perioral dermatitis and rosacea.

Rosacea is a common inflammatory skin disease that classically manifests with flushing, telangiectasias, and inflamed papules and pustules on the face.[1] 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.[2] A related inflammatory skin condition – perioral dermatitis – is frequently associated with the long-term use of topical steroids on the face.[3] 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.[4] 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.[5] In particular, the consistent elevation of CCL20 is characteristic of severe asthma in humans and mouse models.[6] CCL20 is a chemoattractant for T helper (Th) 17 T lymphocytes, neutrophils and dendritic cells.[7] In bronchial epithelia, GCs result in a paradoxical induction of CCL20, which may help to explain the molecular basis of steroid insensitivity in asthma.[8]

CCL20 is also consistently elevated in the papulopustular forms of rosacea.[9] While many clinical features of rosacea are characteristic of an exacerbated innate immune response,[10] a dense lymphocytic infiltrate is often present, implicating a role for adaptive immunity.[11] In the skin, CCL20 is expressed from keratinocytes and drives the Th17-polarized T-lymphocyte response observed in the inflammatory forms of rosacea.[9,12] 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 10^5 cells per well and incubated at 37 °C with 5% CO_2. 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 Easteep® 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.

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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 ($2{\cdot}5 \times 10^5$ 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 pGL4·24, yielding pGL4·24 *CCL20* 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 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,[13] but it is induced throughout the epidermis following epidermal injury or inflammation.[14] 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

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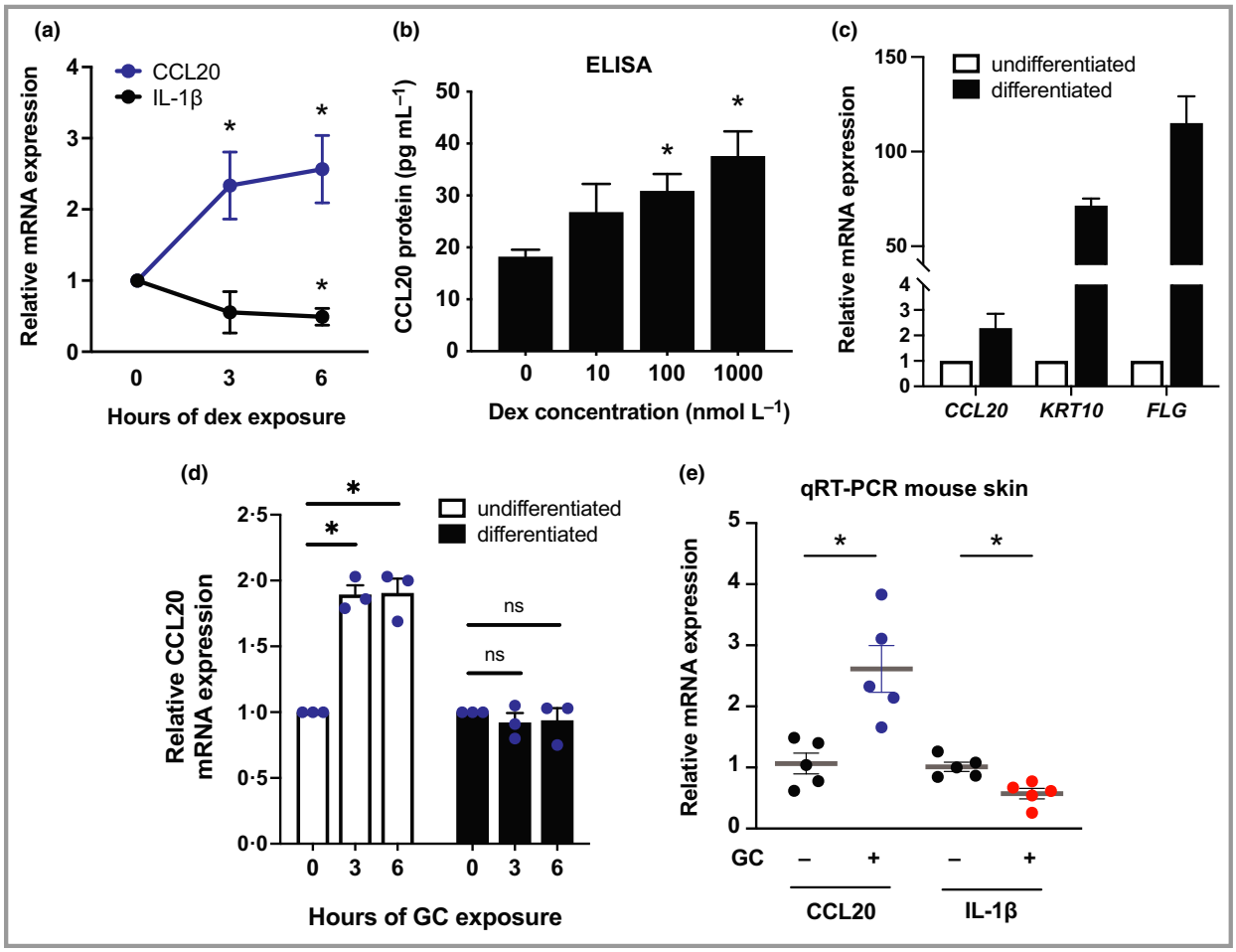

Figure 1 Glucocorticoids induce *CCL20* expression in keratinocytes. (a) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to measure mRNA expression in primary human keratinocytes after treatment with 100 nmol L^{-1} dexamethasone (dex; n = 5). Error bars indicate the SD. IL, interleukin. *P < 0·05 (two-sided t-test vs. 0 h timepoint). (b) Enzyme-linked immunosorbent assay (ELISA) to detect CCL20 protein from primary human keratinocytes treated for 24 h with a range of dexamethasone concentrations (n = 5). Error bars indicate the SD. *P < 0·05 (two-sided t-test vs. 0 nmol L^{-1} dexamethasone). (c) qRT-PCR to measure the mRNA expression of *CCL20*, *KRT10* and *FLG* in undifferentiated and differentiated keratinocytes. (d) qRT-PCR to measure *CCL20* mRNA expression in undifferentiated and differentiated keratinocytes following 100 mmol L^{-1} dexamethasone exposure. GC, glucocorticoid; ns, non-significant. *P < 0·05. (e) RNA expression of *CCL20* and *IL1B* from mouse skin treated with topical glucocorticoids (GC) for 2 weeks (n = 5). Grey lines denote the average and error bars the SEM. *P < 0·05 (two-sided t-test).

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 inductive 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.[9] 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).

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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 $P > 0{\cdot}05$). 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-α. However, as has been previously observed, the addition of dexamethasone resulted in further *CCL20* 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-α and dexamethasone. CCL20 protein expression increased in keratinocytes upon treatment with either dexamethasone or TNF-α. If treated with both, CCL20 was even further induced (Figure 2c). Viewed together, these results indicated that GCs do not repress CCL20 expression by TNF-α. Instead, GC treatment leads to additively induced *CCL20* production in keratinocytes.

Glucocorticoids inhibit tumour necrosis factor-α-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 (NFκB) 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-α stimulation of *CCL20* in human keratinocytes.

We applied chemical inhibitors to JNK, ERK, p38 and PI3K, and assessed their effect on TNF-α-mediated induction of *CCL20* in primary human keratinocytes (Figure 3a). *CCL20* induction by TNF-α 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-α-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-α 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-α stimulated phosphorylation of p65 (reflecting NFκB activation), as well as p38. TNF-α had modest effects on ERK phosphorylation and minimal effects on Akt (PI3K). Pretreatment with dexamethasone blocked TNF-α-induced phosphorylation of p65 and p38, indicating that GCs inhibited NFκB 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 NFκB and p38/MAPK, which is broadly consistent with the immunosuppressive effect of steroids. However,

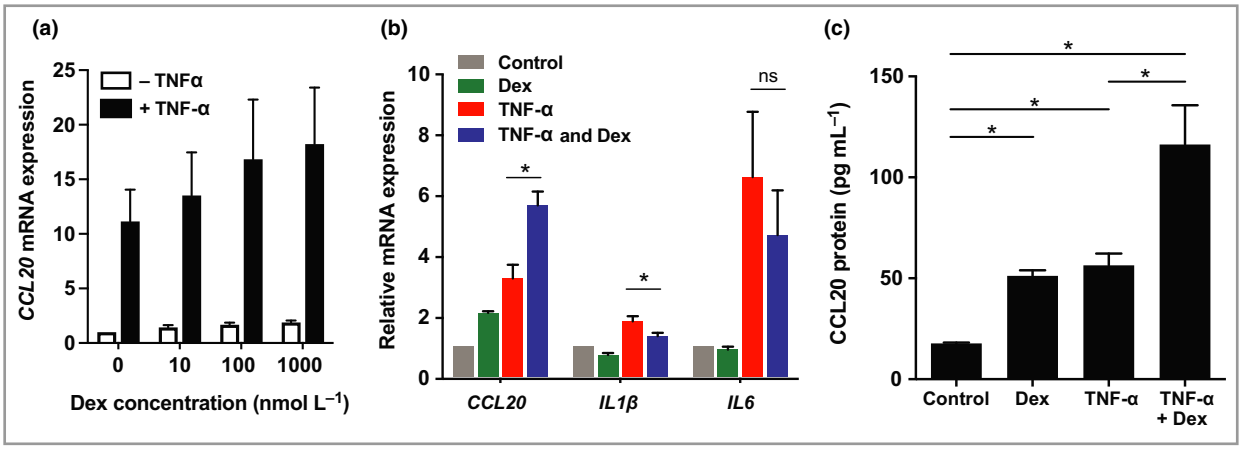

Figure 2 Tumour necrosis factor (TNF)-α and glucocorticoids additively stimulate *CCL20*. (a) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of *CCL20* following treatment with dexamethasone (Dex) at a range of doses (0, 10, 100 and 1000 nmol L^{-1}) (n = 3). Error bars denote the SEM. (b) qRT-PCR to assess mRNA expression of *CCL20*, *IL1B* and *IL6* following treatment of human keratinocytes with dexamethasone (Dex), TNF-α or both (n = 3). Error bars denote the SEM. ns, not significant. *$P < 0{\cdot}05$. (c) Enzyme-linked immunosorbent assay for CCL20 after treatment of human keratinocytes with dexamethasone, TNF-α or both. Cells were stimulated with TNF-α (10 ng mL^{-1}) for 24 h in the presence or absence of 1000 nmol L^{-1} dexamethasone (n = 3). Error bars denote the SEM. *$P < 0{\cdot}05$.

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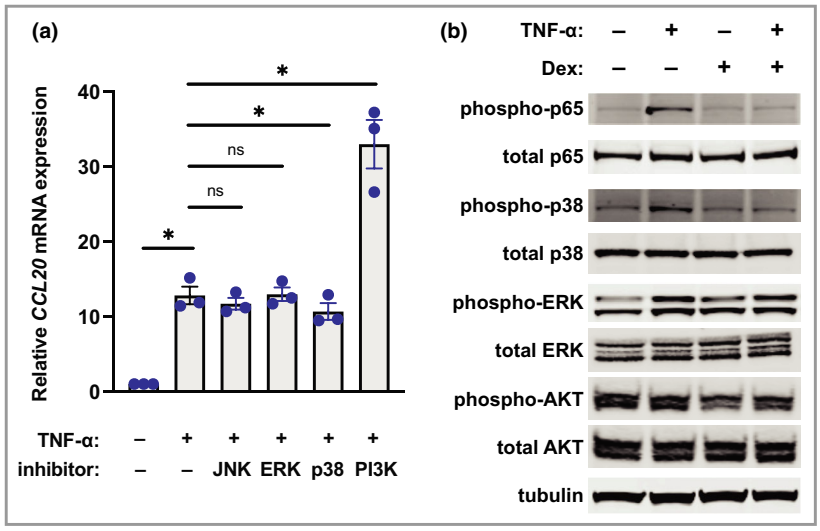

Figure 3 Glucocorticoids suppress tumour necrosis factor (TNF)-α-mediated stimulation of nuclear factor kappa B and mitogen-activated protein kinase (MAPK) pathways. (a) Quantitative reverse transcriptase polymerase chain reaction to assess CCL20 mRNA expression following treatment of human keratinocytes with TNF-α, with or without pathway inhibitors (n = 3–5). Error bars denote the SEM. *P < 0·05 (two-sided t-test). ERK, extracellular regulated kinase; JNK, Jun N-terminal kinase; ns, non-significant; PI3K, phosphoinositide 3-kinase. (b) Immunoblot of keratinocyte lysates following treatment with dexamethasone (Dex), TNF-α or both, to assess phosphorylation of sentinel kinases. ERK, extracellular regulated kinase.

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,[8] 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.[20] 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,[21] 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,[22] 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)[23] 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,[9] 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

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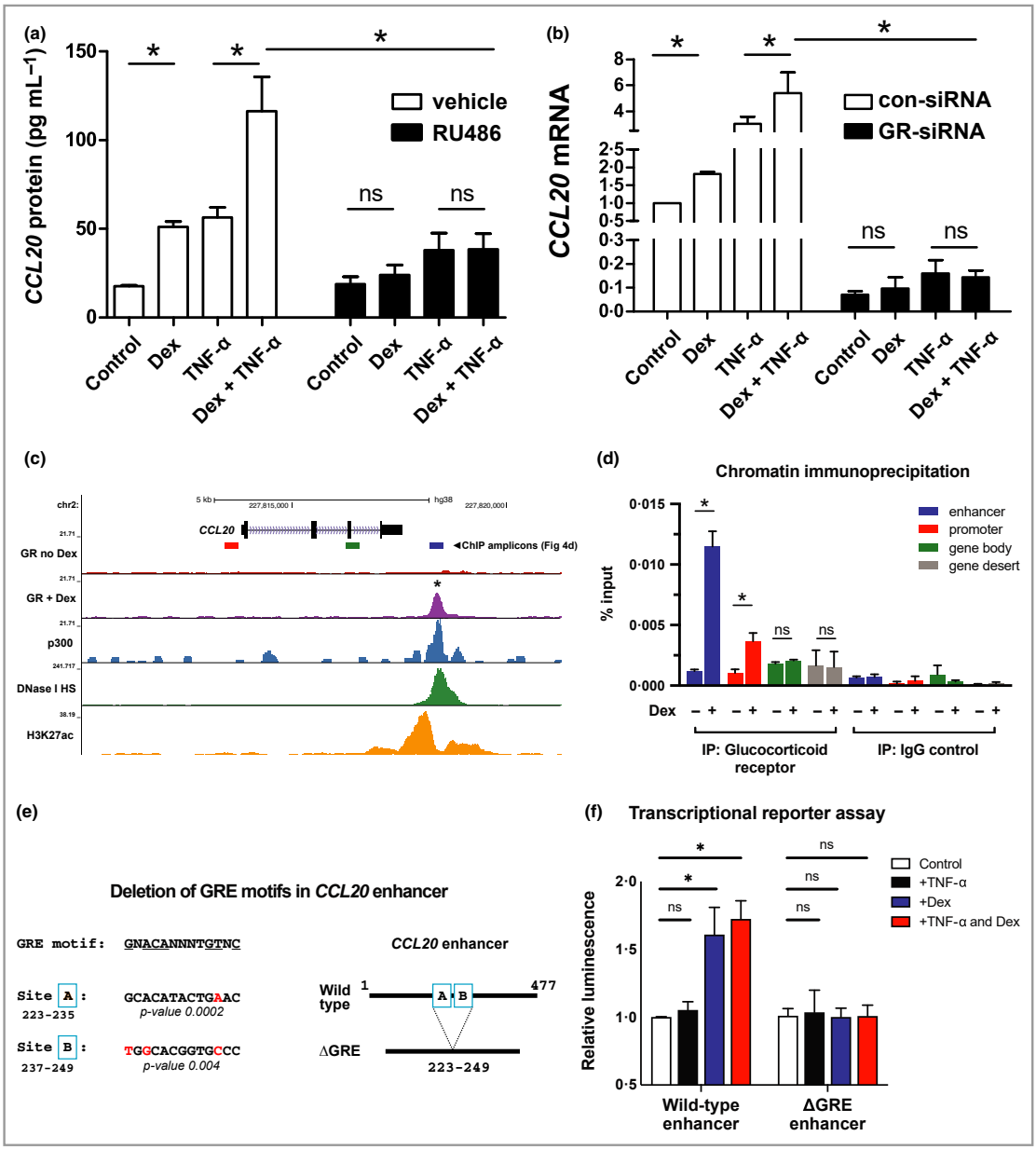

Figure 4 Activated glucocorticoid (GC) receptor (GR) directly induces CCL20 by binding its promoter and a downstream enhancer. (a) Enzyme-linked immunosorbent assay for CCL20. Human keratinocytes were treated with the GC antagonist mifepristone (RU486) then treated with dexamethasone (Dex), tumour necrosis factor (TNF)-α or both (n = 3). Error bars denote the SD. ns, not significant. *P < 0·05. (b) Quantitative reverse transcriptase 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. (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 GC response elements was generated and named ΔGRE. (f) Enhancer reporter assay. The wild-type and ΔGRE CCL20 enhancer sequence was cloned upstream of a minimal promoter driving firefly luciferase and co-transfected into 293T cells with *Renilla* luciferase 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).

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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.[24] We analysed the putative *CCL20* enhancer sequence using MEME suite tools to identify GR binding motifs.[25] Five GR binding motifs ($P < 0{\cdot}01$) were identified, with the best match located near the centre of the candidate enhancer (denoted as 'site A' in Figure 4e; $P = 0{\cdot}0002$). A second GR binding site was immediately adjacent to the first site (denoted as 'site B').

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 ΔGRE (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-α had no effect, arguing against its influence at the enhancer element. By comparison, the ΔGRE 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.[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-α. CCL20 potently attracts Th17 T lymphocytes and dendritic cells to epithelial surfaces such as the skin, and is characteristically elevated in rosacea.[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 NFκB and p38/MAPK, similarly to what has been observed in lung epithelia and other tissues.[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.

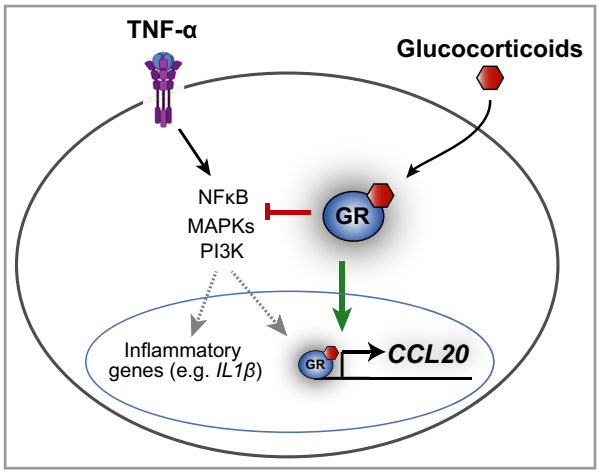

Figure 5 A proposed model for the effect of glucocorticoids (GCs) on the transcriptional regulation of *CCL20* in keratinocytes. Tumour necrosis factor (TNF)-α stimulation activates inflammation-related signalling pathways, including nuclear factor kappa B (NFκB), p38/mitogen-activated protein kinase (MAPK) and PI3K (phosphoinositide 3-kinase). Downstream signalling from these pathways influences the activity of inflammatory genes, including *CCL20*. Exposure to GCs activates the GC receptor (GR), which suppresses NFκB and p38/MAPK signalling to block proinflammatory gene expression. However, activated GR directly binds and activates *CCL20* though a proximal enhancer, counterbalancing other inhibitory effects of GR. This mechanism may contribute to the inflammation seen in skin conditions such as rosacea and perioral dermatitis, which are triggered or exacerbated by chronic steroid use.

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.[27]

The effect of GCs on skin inflammation is complex, with influences from other cytokines and chemokines, resident cell types and the skin microbiome.[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.[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.

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Acknowledgments

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

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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.

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