

ORIGINAL ARTICLE

Atopic Dermatitis, Urticaria and Skin disease

Pharmacologic inhibition of hypoxia-inducible factor (HIF)-hydroxylases ameliorates allergic contact dermatitis

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Abstract

Background: When an immune cell migrates from the bloodstream to a site of chronic inflammation, it experiences a profound decrease in microenvironmental oxygen levels leading to a state of cellular hypoxia. The hypoxia-inducible factor-1 α (HIF-1 α) promotes an adaptive transcriptional response to hypoxia and as such is a major regulator of immune cell survival and function. HIF hydroxylases are the family of oxygen-sensing enzymes primarily responsible for conferring oxygen dependence upon the HIF pathway.

Methods: Using a mouse model of allergic contact dermatitis (ACD), we tested the effects of treatment with the pharmacologic hydroxylase inhibitor DMOG, which mimics hypoxia, on disease development.

Results: Re-exposure of sensitized mice to 2,4-dinitrofluorobenzene (DNFB) elicited inflammation, edema, chemokine synthesis (including CXCL1 and CCL5) and the recruitment of neutrophils and eosinophils. Intraperitoneal or topical application of the pharmacologic hydroxylase inhibitors dymethyloxalylglycine (DMOG) or JNJ1935 attenuated this inflammatory response. Reduced inflammation was associated with diminished recruitment of neutrophils and eosinophils but not lymphocytes. Finally, hydroxylase inhibition reduced cytokine-induced chemokine production in cultured primary keratinocytes through attenuation of the JNK pathway.

Conclusion: These data demonstrate that hydroxylase inhibition attenuates the recruitment of neutrophils to inflamed skin through reduction of chemokine production and increased neutrophilic apoptosis. Thus, pharmacologic inhibition of HIF hydroxylases may be an effective new therapeutic approach in allergic skin inflammation.

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1 | INTRODUCTION

Hypoxia is a common microenvironmental feature of inflamed tissues due to increased oxygen consumption, decreased blood flow, or a combination of both.^{1,2} Hypoxia activates an adaptive cellular transcriptional response driven by the hypoxia-inducible factor (HIF).³⁻⁶ HIF is a heterodimeric transcription factor formed by the association of oxygen-sensitive HIF- α subunits and a constitutively expressed HIF-1 β subunit. HIF- α subunits are synthesized at a high level in all cells but in the presence of oxygen are hydroxylated by three HIF-prolyl hydroxylases (PHD1-3) on proline residues marking them for ubiquitination by the von Hippel-Lindau E3 ligase complex and proteosomal degradation.⁵⁻⁷ A further hydroxylation of an asparagine residue within the HIF- α transactivation domain blocks its interaction with the CBP/p300 transcriptional co-activator.^{4,8} As well as conferring oxygen dependence on HIF, HIF hydroxylases have been implicated in the regulation of other inflammatory and fibrotic pathways including nuclear factor- κ B (NF- κ B) and extracellular signal-regulated kinase (ERK).⁹⁻¹⁴ Pharmacologic HIF hydroxylase inhibition has been reported to have beneficial effects in various in vivo models of inflammatory disease including colitis and chronic kidney disease.^{9,15,16} Furthermore, this pathway has been implicated in contact hypersensitivity to nickel, in allergy to UVB light, and in the regulation of T cell-mediated responses.¹⁷⁻²¹ Therefore, manipulation of the HIF pathway represents a potential strategy for the development of anti-inflammatory drugs. Little is known about the potential of therapeutic application of hydroxylase inhibitors in T cell-driven inflammatory skin disorders.

Allergic contact dermatitis (ACD) is a T cell-mediated inflammatory reaction to epidermal sensitization that causes an over-activation of the immune response upon repeated exposure to the sensitizing hapten.^{22,23} This allergic reaction involves an initial sensitization phase that leads to an elicitation or inflammatory phase.²⁴⁻²⁶ Current therapy for ACD is limited to treatment of the inflammatory episodes with anti-inflammatory agents such as corticosteroids.^{24,27} However, long-term systemic or topical treatment with glucocorticoids can result in severe side effects and recalcitrant cases are difficult to treat.

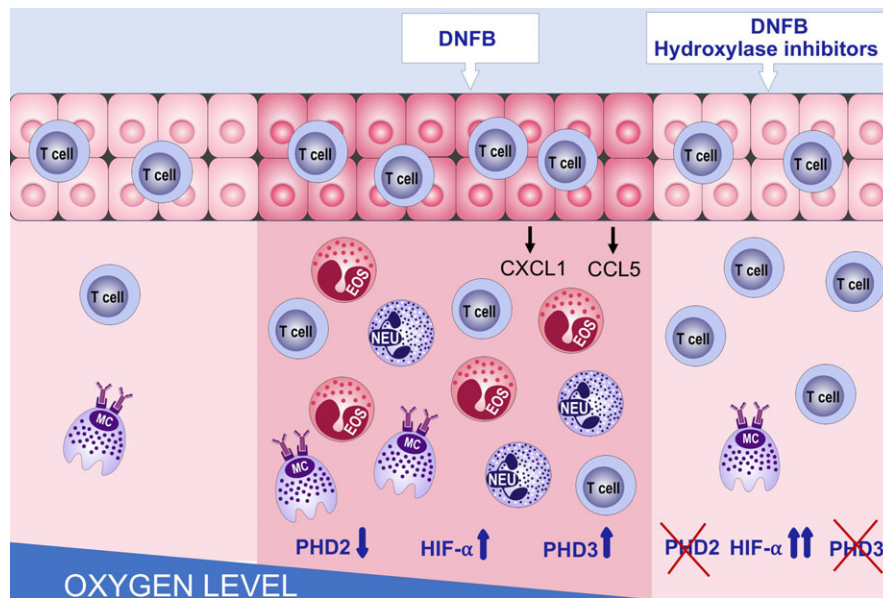
Because of its efficacy in the treatment of inflammation associated with colitis (which shares several key features with ACD), in this study we investigated whether the protective effects of pharmacologic hydroxylase inhibition extend to allergic skin inflammation. Of note, the protective effects of hydroxylase inhibition in the gut are largely

through the enhancement of epithelial barrier function.^{2,11} We report anti-inflammatory actions of hydroxylase inhibitors in allergic skin inflammation mediated through the regulation of chemokine production. Of note, the two hydroxylase inhibitors used in our study, DMOG and JNJ1935, showed similar effects on granulocyte infiltration. JNJ1935 is a more potent and PHD selective inhibitor than DMOG.^{6,4} A number of other hydroxylase inhibitors are currently in advanced clinical trials for the treatment of chronic kidney disease-associated anemia and have been shown in pre-clinical studies to be effective in the treatment of inflammatory bowel disease (IBD).¹⁰⁻¹⁶

Hydroxylase inhibitors are currently under advanced clinical investigation for the treatment of anemia making potential repurposing these compounds for other therapeutic applications such as allergic skin disorders a realistic short- to medium-term possibility.^{28,29}

2 | MATERIALS AND METHODS**2.1 | Reagents**

2,4-dinitrofluorobenzene was obtained from Sigma-Aldrich and diluted in Acetone:olive oil (4:1) at the indicated concentrations. TNF- α (PeproTech, Rocky Hill, NJ, USA), DMOG (Cayman Chemicals, Ann Arbor, MI, USA), and JNJ1935 (Merck Millipore, Darmstadt, Germany), SB203580 (p38 inhibitor), BAY-117082 (NF- κ B inhibitor), PD98059 (MEK inhibitor), and SP600125 (JNK inhibitor) were prepared according to the manufacturers protocols. For topical application, DMOG was reconstituted in acetone and then mixed with olive oil to a 4:1 concentration. For Western blot, anti-PHD1, 2, and 3 (Novus, Saint Charles, MO, USA; Diluted 1:500, 1:500, and 1:1000, respectively), anti-phosphoJNK (Santa Cruz, Dallas, TX, USA; Diluted 1:200), anti-phosphoERK (CST, Danvers, MA, USA; Diluted 1:1000), anti-HIF-1 α (BD, San Jose, CA, USA; Diluted 1:500), and anti- β -Actin (Sigma-Aldrich Diluted 1:10 000) were prepared in 5% skim milk powder in phosphate-buffered saline (PBS). For IFHC, anti-CD3 (abcam, Cambridge, MA, USA; Diluted 1:100), anti-Ly6G antibody (Biolegend, San Diego, CA, USA; Diluted 1:250), Anti-CXCR2 (abcam; Diluted 1:100), and anti-Rat Alexa Fluor[®] 488 conjugate and 568 conjugate (CST; Diluted 1:1000) were used in 5% goat serum in PBS. Human proteome profiler cytokine/chemokine quantikine array and ELISA kits for human CXCL1, CCL5, and IL-8 and mouse myeloperoxidase (MPO), CXCL1/KC and CCL5 kits were obtained from R&D systems (Minneapolis, MN, USA). Taqman probes for human CXCL1, CCL5, and CXCL8 and



GRAPHICAL ABSTRACT

Pharmacologic inhibition of HIF-hydroxylases, which mimics hypoxia, reduces inflammation in a mouse model of acute contact dermatitis. HIF-hydroxylase inhibition reduces inflammatory chemokine production by keratinocytes. Topical application of HIF-hydroxylase inhibitors may be an effective approach for the treatment of T-cell driven allergic skin inflammation.

mouse CXCL1, CCL5, IFN- γ , Glut1, CD4, CD8, granzyme B (GZMB), and TNF- α were from Applied Biosystems (Thermo Fisher Scientific, Cambridge, MA, USA).

2.2 | Animal studies

Ten- to 12-week-old wild-type female C57BL/6 mice were obtained from Charles River (Wilmington, MA, USA). All in vivo experiments were in compliance with regulations of the Health Products Regulatory Authority of Ireland according to protocols approved by the host animal research ethics committee. Mice were sensitized by applying 100 μ L of 0.5% DNFB to the shaved abdomen for two consecutive days. Four days after sensitization, mice were treated with vehicle or 8 mg/mouse intraperitoneal DMOG or 0.8 mg/mouse intraperitoneal JNJ1935 or topical DMOG, as indicated. For topical application, DMOG was applied to the mouse ear at an 8 mg/mouse concentration. One hour after treatment, mice were challenged by exposing the ears to 20 μ L of vehicle or 0.2% or 0.3% DNFB as indicated. Measurements of ear thickness were taken using a thickness gauge immediately after challenge and at times 8 and 24 hours following DNFB challenge. Mice were sacrificed and tissues collected 8 or 24 hours after challenge with 0.3% DNFB as indicated.

2.3 | Primary human Keratinocytes

Human primary adult keratinocytes were isolated from human donor surplus skin after plastic surgery. Cells from 8 different donors were used. Cells were maintained at 37°C in a humidified environment of 21% O₂ and 5% CO₂ (balance N₂) in keratinocyte KBM-gold medium

supplemented with human epithelial growth factor, bovine pituitary extract, transferrin, epinephrine, insulin, and hydrocortisone (Lonza, Visp, Switzerland). Cells were pre-equilibrated in supplement-free medium for 48 hours. For the characterization of the inflammatory profile, cells were treated with 50 ng/mL TNF- α for 24 hours. After treatment, the medium was collected for the measurement of secreted cytokines/chemokines. For the analysis of the effects of hydroxylase inhibitors on skin inflammation in vitro, cells were treated with 1 mmol/L DMOG or 100 μ mol/L JNJ1935 for 1 hour and then treated with TNF- α . For HIF transcription studies, the cells were transfected with the HRE-gaussia-luciferase reporter plasmid that contains the gaussia luciferase gene under the control of a promoter that includes tandem repeats of the HIF responsive elements (HRE) as previously described.³⁰ Transfection was achieved using lipofectamine 3000[®] (Roche, Penzberg, Upper Bavaria, Germany) following manufacturer's indications.

2.4 | Neutrophil apoptosis

Cells were stimulated with 1 μ mol/L all-trans retinoic acid (ATRA) to induce differentiation into neutrophils. Neutrophils were then treated with 1 mmol/L DMOG and samples collected at 2, 4, 8, and 24 hours and caspase 3/7 activity was measured.

2.5 | Histology

For histology, tissue was formalin-fixed and paraffin-embedded; 4 μ m sections were cut from paraffin-embedded tissues using a microtome (Leica, Wetzlar, Germany). Tissues were stained with

hematoxylin and eosin and imaged at 20X using the aperio Scan-scope console (Leica). For the analysis of T cells, sections were stained with anti-CD3 using Alexa Flour 568 secondary antibody. For the study of neutrophilic infiltration, sections were co-stained for Ly6G and CXCR2 using Alexa Fluor 488 and 568 secondary antibodies, respectively; 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Images at 20X, 40X, or 100X magnifications were obtained using a fluorescent microscope. CD3- or Ly6G-positive cells in one or two different fields were quantified at 20X using ImageJ image analysis software (National Institute of Health, Bethesda, MD) in a double-blinded fashion. Images of two 20X fields from each mouse were randomized in a double-blinded fashion, and CD3- or Ly6G-positive cells quantified and expressed as an average of the two fields. Giemsa staining was used to identify mast cells. Mast cell quantification was developed in a double-blinded fashion. Eosinophils were counted from H&E images in a double-blinded fashion.

2.6 | ELISA, chemokine/cytokine array, and Western blot

For protein studies, half mouse ears (10-15 mg) were homogenized in radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Sigma-Aldrich) using Qiagen stainless steel beads and the tissuelyser II (Qiagen, Hilden, Germany). Samples were centrifuged 2-3 times at 17968g at 4°C, and protein supernatants collected for ELISA and WB. For in vitro keratinocyte studies, medium supernatants were collected for ELISA and chemokine/cytokine array analysis was performed. For Western blot studies, cells were lysed in the same lysis buffer used for mouse ears and centrifuged to obtain protein supernatants. Equal amounts of protein per each sample were used in all cases.

2.7 | Quantitative real-time polymerase chain reaction

For RNA isolation, tissue was stored in RNA later (Qiagen) at 4°C overnight. Samples were homogenized in trizol (Thermo Fisher Scientific). For in vitro cultured keratinocytes, cells were lysed in trizol. RNAs were then extracted using a standard phenolic extraction protocol. Complementary DNA was synthesized and quantitative real-time polymerase chain reaction carried out as previously described.³¹

2.8 | Statistical analysis

Graph Pad Prism version 5.0 (GraphPad software, La Jolla, CA, USA) was used for all statistical analysis. One-way ANOVA followed by a Newman-Keuls post-test was used for the comparison of multiple groups. Student's *t* test was used for individual group comparisons. Differences were considered statistically significant when the *P*-value was ≤ 0.05 . There was a minimum of three experimental replicates per group.

3 | RESULTS

3.1 | Hydroxylase inhibition reduces inflammation in allergic contact dermatitis

Histologic analysis of DNFB-sensitized mouse ear crosssections revealed an increase in ear thickness following secondary DNFB challenge which was attenuated by DMOG treatment (Figure 1A,B). CXCL1 and CCL5 drive DNFB-induced dermatitis.^{22,32,33} DMOG treatment attenuated DNFB-induced CXCL1 and CCL5 expression (Figure 1C,D). We next investigated the effects of a second hydroxylase inhibitor JNJ1935 on DNFB-mediated inflammation. Ears of sensitized mice challenged with DNFB displayed a consistent increase in ear thickness and edema accompanied by increased IFN- γ , CXCL-1, and CCL-5 transcripts (Figure 1E-I). JNJ1935 treatment significantly reduced ear thickness, edema, and CXCL-1 expression. These data demonstrate that hydroxylase inhibition reduces inflammation in allergic contact dermatitis.

3.2 | DNFB-mediated inflammation induces transcriptional reprogramming of HIF hydroxylase expression

We next investigated the expression pattern of PHD1-3 in mouse ears. PHD1 was robustly expressed and did not differ in expression levels between vehicle- and DNFB-challenged ears (Figure 2A,B). PHD2 was expressed in the ears of vehicle-treated mice and was downregulated in DNFB-challenged ears (Figure 2A,C). PHD2 is the main regulator of HIF- α stability, and its degradation has been proposed as an alternative mechanism of HIF regulation triggered by TGF- β and endothelin.^{34,35} PHD3 expression levels were generally lower and not statistically significantly changed in response to DNFB challenge (Figure 2A,D). PHD3 has been purposed as a HIF-inducible protein implicated in inflammation through regulation of neutrophil survival.³⁶ Compared to vehicle, DNFB-challenged ears demonstrated increased expression of Glut1, a HIF-dependent gene (Figure 2E).

3.3 | Hydroxylase inhibition prevents neutrophil recruitment in ACD

We next analyzed the effects of hydroxylase inhibition on T cell infiltration. Mice challenged with DNFB demonstrated increased CD3-positive lymphocytes in the dermis which was not affected by DMOG treatment (Figure 3A-B). Furthermore, mice challenged with 0.3% DNFB for 24 hours did not have a detectable increase in CD4 levels, but demonstrated elevated CD8 expression implicating a dominance of a CD8+ cytotoxic T-lymphocyte infiltrate (Figure 3C-D). In addition, DNFB challenge increased the expression of granzyme B (GZMB), confirming the presence of CD8+ T cells (Figure 3E). However, treatment with JNJ1935 did not reduce the number of CD8+ T cells or granzyme B levels, suggesting that hydroxylase inhibition does not mediate its anti-inflammatory effects via altering T cell responses in this model (Figure 3D-E).

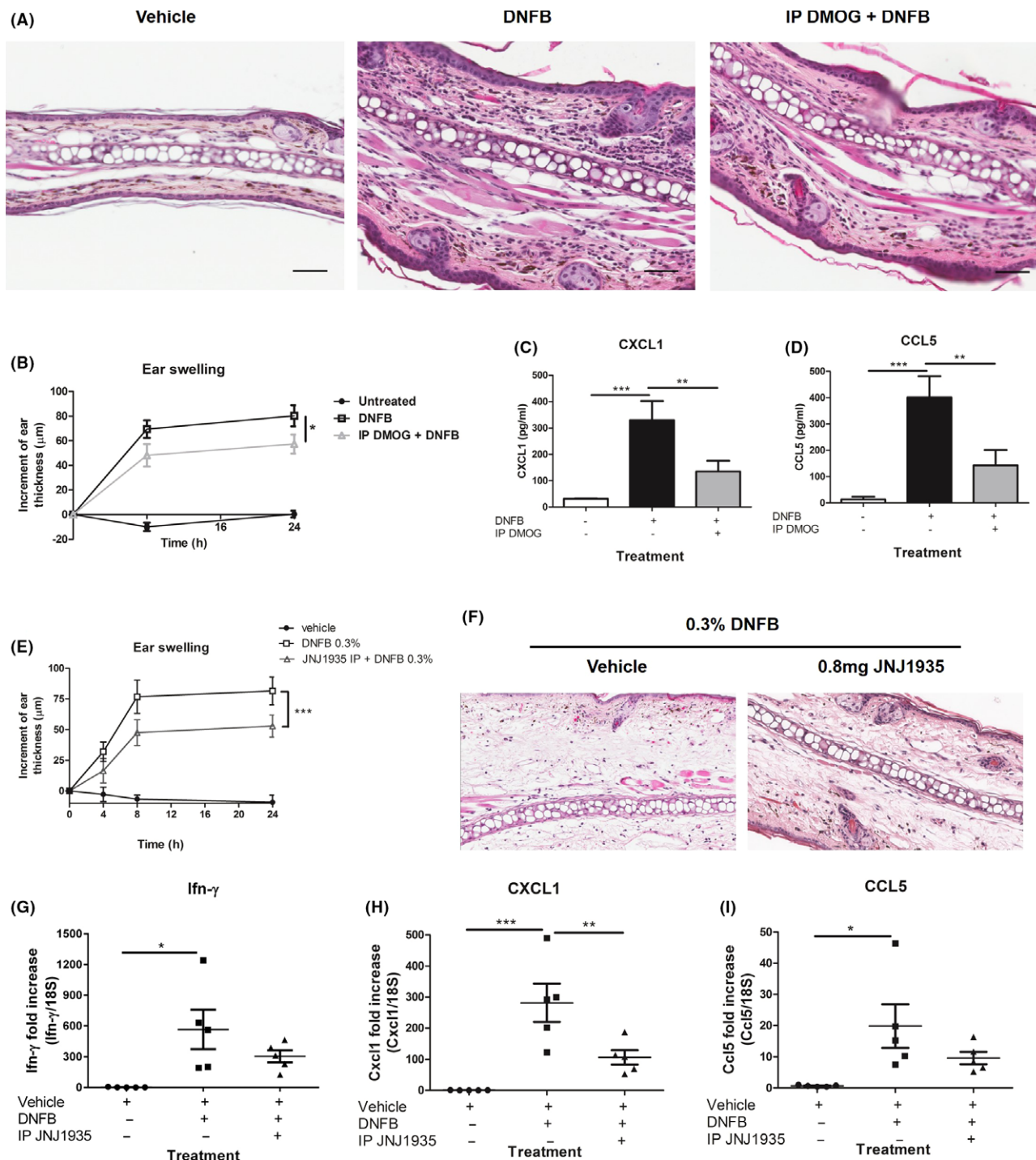


FIGURE 1 Hydroxylase inhibitors reduce inflammation in 2,4-dinitrofluorobenzene (DNFB)-induced acute dermatitis. A, Hematoxylin and eosin staining of mouse ear crosssections from mice treated or not with intraperitoneal injections of 8 mg/mouse DMOG and challenged with vehicle or DNFB ($n = 6$). B, Quantification of the increment in ear thickness in mice treated as described in A. C-D, ELISA of mouse CXCL1 and CCL5 from ear homogenates of mice treated as described in A ($n = 4$). E, Quantification of the increment in ear thickness in mice treated or not with intraperitoneal injections of 0.8 mg/mouse JNJ1935 and challenged with DNFB ($n = 5$). F, Hematoxylin and eosin staining of mouse ear crosssections from mice treated as described in E. G-I, qRT-PCR of IFN- γ , CXCL1, and CCL5 in mouse ears from mice treated as described in E ($n = 5$). *means $P \leq 0.05$; **means $P \leq 0.01$; ***means $P \leq 0.001$

Neutrophils also play a central role in DNFB-induced dermatitis.^{22,32} CXCL1 is the key chemokine involved in the recruitment of neutrophils via activation of the CXCR2 receptor.³⁷⁻³⁹ Since CXCL1

protein and RNA expression were reduced by hydroxylase inhibition, we next investigated its impact on DNFB-mediated neutrophil infiltration. Mouse ear cross sections were co-stained for the neutrophil

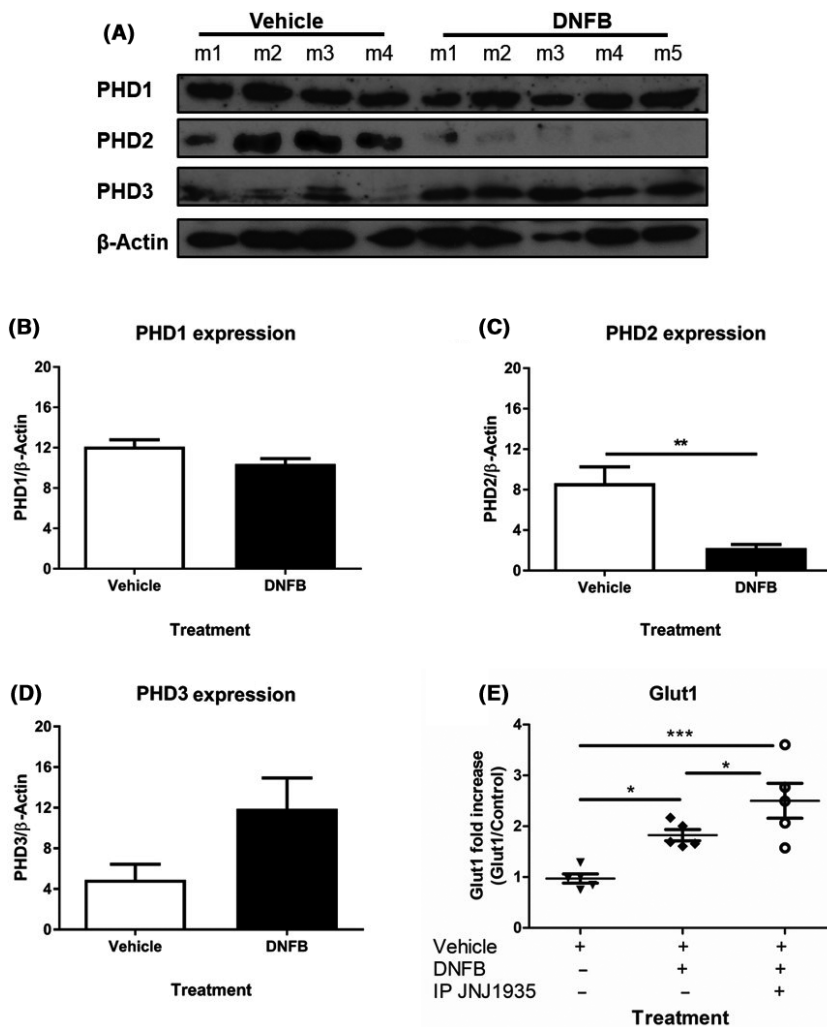


FIGURE 2 Differential profile of expression of HIF-prolyl hydroxylases in 2,4-dinitrofluorobenzene (DNFB)-induced dermatitis. A, Western blot of protein extracts of mouse ears sensitized with DNFB and then challenged with vehicle or DNFB for 8 h. B-D, Densitometry of PHD1 (B), PHD2 (C), and PHD3 (D) comparing mice treated as described in A (n $\geq$ 4). E, qRT-PCR of Glut1 in mouse ears challenged with vehicle or DNFB for 24 h and untreated or treated with 0.8 mg/mouse JNJ1935 (n = 5). *means $P \leq 0.05$; ***means $P \leq 0.001$

marker Ly6G and the chemokine receptor CXCR2 using double-immunofluorescence. In most cases, Ly6G and CXCR2 were co-expressed in infiltrating neutrophils suggesting the presence of CXCL1 sensitive neutrophils (Figure S1). Comparison between vehicle and DNFB-challenged ear crosssections showed a profound increase in neutrophil presence (Figure 3F). These neutrophils were concentrated in the dermis, showing the correlation between the dermal inflammatory reaction and neutrophil infiltration. Mice treated with intraperitoneal JNJ1935 demonstrated a lower number of infiltrating neutrophils in the dermis (Figure 3F). Quantification of Ly6G-positive cells showed higher number of neutrophils in DNFB-challenged ears compared to vehicle controls (Figure 3G). These Ly6G-positive cells were significantly reduced in mice treated with JNJ1935 (Figure 3G). To further analyze the effect of hydroxylase inhibition in neutrophil infiltration, we measured the amount of myeloperoxidase (MPO) in mouse ear protein homogenates. DNFB dramatically increased MPO levels compared to vehicle reflecting increased numbers of neutrophils (Figure 3H). In contrast, mice treated with JNJ1935 had significantly lower MPO values confirming its inhibitory effect on neutrophil-mediated DNFB-induced inflammation (Figure 3H). In addition to the effects detected on neutrophils, we also analyzed the effect of hydroxylase inhibitors on eosinophils. We

found that eosinophils were significantly increased in the ears of DNFB-sensitized and DNFB-challenged mice (Figure S2). This DNFB-induced increase in eosinophil numbers was inhibited in DMOG or JNJ1935 mice (Figure S2).

In order to extend our analysis of inflammatory infiltrates, we also investigated whether hydroxylase inhibitors affect mast cell infiltration. Mast cells were increased in the dermis of mice challenged with DNFB after sensitization but were not reduced by hydroxylase inhibition (Figure S2). Thus, our data indicate that hydroxylase inhibitors selectively affect eosinophil and neutrophil but not lymphocyte or mast cell infiltration in murine ACD.

Together, these results demonstrate that the anti-inflammatory effect of hydroxylase inhibitors in DNFB-induced dermatitis is associated with reduced neutrophil (but not T cell) infiltration into the inflamed tissue.

3.4 | Hydroxylase inhibition reduces chemokine production in human primary keratinocytes

Keratinocytes play an important role in skin inflammatory diseases through secretion of chemokines and cytokines which recruit

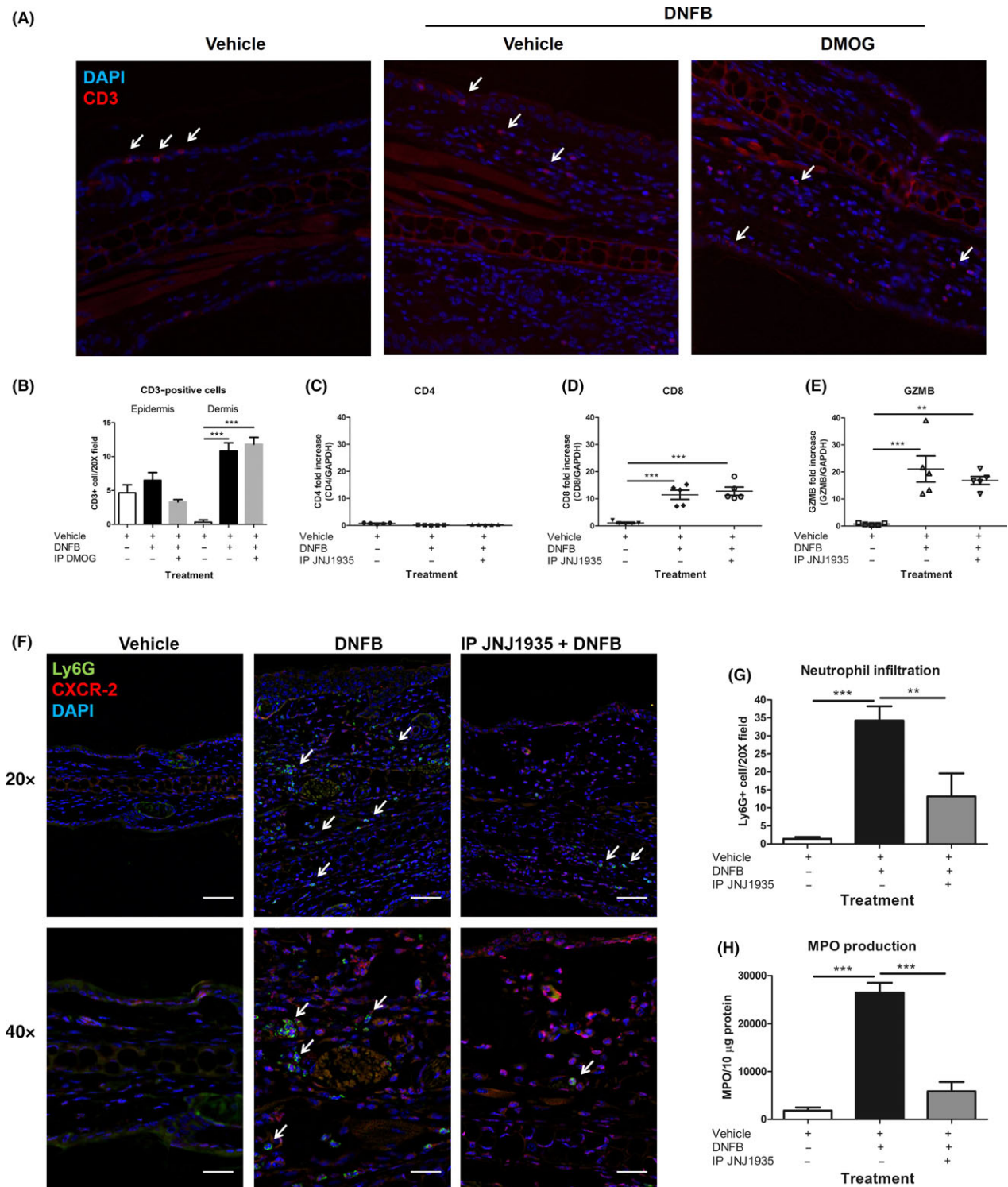


FIGURE 3 Hydroxylase inhibitors reduce 2,4-dinitrofluorobenzene (DNFB)-mediated Ly6G/CXCR2-positive neutrophil infiltration. A, Representative images of immunofluorescence of mouse ear crosssections stained for CD3 of mice treated or not with 8 mg/mouse IP DMOG and challenged with DNFB for 24 h. B, Epidermal and dermal quantification of CD3-positive staining in mouse ear crosssections from mice treated as described in A. C-E, qRT-PCR of CD4, CD8, and GZMB in mouse ears challenged with vehicle or DNFB for 24 h and untreated or treated with 0.8 mg/mouse JNJ1935 ($n = 5$). F, Representative images of immunofluorescence of mouse ear crosssections stained for Ly6G/CXCR2 in mice treated or not with intraperitoneal injections of 0.8 mg/mouse JNJ1935 before challenge with topical vehicle or DNFB (20X and 40X) ($n = 6$). G, Quantification of Ly6G positive neutrophils in the dermis of mice treated as in F ($n = 6$). H, ELISA of mouse MPO from ear homogenates of mice treated as described in F ($n = 4$). *means $P \leq 0.05$; **means $P \leq 0.01$; ***means $P \leq 0.001$

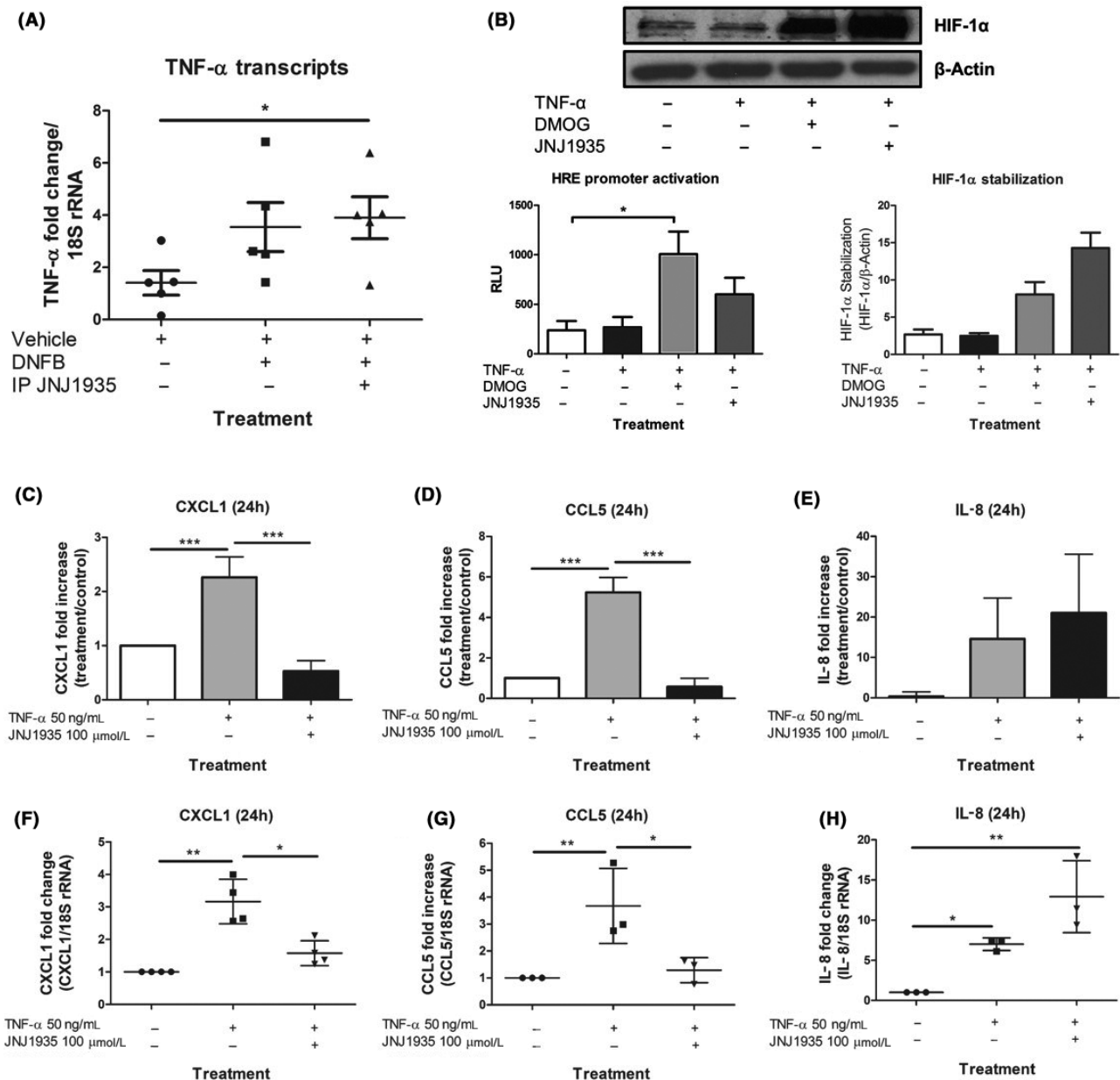


FIGURE 4 Hydroxylase inhibitors reduce TNF- α induced production of chemokines in human primary keratinocytes. A, qRT-PCR of TNF- α in ears of mice treated or not with intraperitoneal injections of 0.8 mg/mouse JNJ1935 and challenged with vehicle or 2,4-dinitrofluorobenzene (n = 5). B, Above, representative Western blot of HIF-1 α and β -Actin in primary human keratinocytes unstimulated or stimulated with TNF- α and untreated or treated with 1 mmol/L DMOG or 100 μ mol/L JNJ1935 in C (n = 4). B, Below left, relative luciferase units from primary human keratinocytes transfected with the HRE-Gluc HIF reporter and treated as described (n = 4). B, Below right densitometry of Western blots as described (n = 4). C-E, Supernatants obtained from human primary keratinocytes treated with 100 μ mol/L JNJ1935 or vehicle for 1 h and then stimulated with 50 ng/mL TNF- α were analyzed by ELISA to determine the amount of CXCL1 (C), CCL5 (D), and IL-8 (E). F-H, RNA extracts from cultured human primary keratinocytes treated likewise were analyzed by qRT-PCR to evaluate the relative amounts of CXCL1 (F), CCL5 (G), and IL-8 (H) transcripts (n = 3). *means $P \leq 0.05$; **means $P \leq 0.01$; ***means $P \leq 0.001$

neutrophils and activate downstream inflammatory pathways.⁴⁰ In ACD, keratinocytes both respond to and express TNF- α .²⁴ In our experiments, sensitized mice challenged with DNFB expressed increased levels of TNF- α mRNA which were not affected by hydroxylase inhibition (Figure 4A). In vitro stimulation of primary human keratinocytes with TNF- α led to the production of a range of cytokines and chemokines including CXCL1, IL-8, and CCL5

(Figure S3).⁴¹ Treatment of primary human keratinocytes with either DMOG or JNJ1935 induced HIF stabilization and HIF-dependent transcriptional responses demonstrating responsiveness of this cell type to hydroxylase inhibition (Figure 4B). Treatment with JNJ1935 reduced TNF- α -induced secretion of CCL5 and CXCL1, but not IL-8 (Figure 4C-E). Moreover, TNF- α increased the expression of CCL5 (up to 4-fold), CXCL1 (up to 4-fold), and

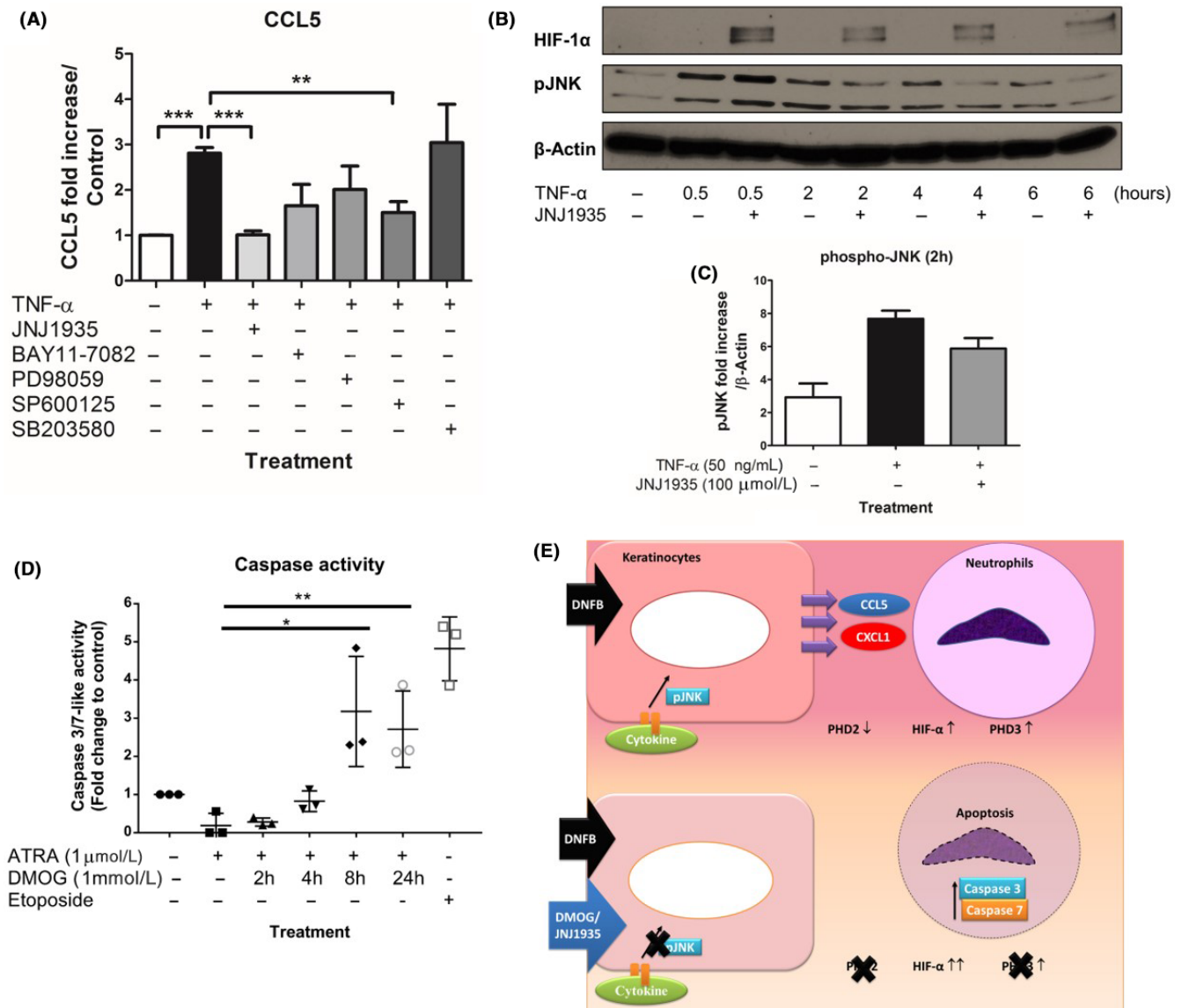


FIGURE 5 Hydroxylase inhibitors reduce JNK phosphorylation in keratinocytes and increase caspases 3/7 activity in neutrophils. A, Supernatants obtained from human primary keratinocytes treated with vehicle or JNJ1935 or BAY11-7082 or PD98059 or SP600125 or SB203580 for 4 hours and then stimulated with 50 ng/mL TNF-α were analyzed by ELISA to determine the amount of CCL5 ($n = 3$). B, representative Western blot of HIF-1α, pJNK, and β-Actin in primary human keratinocytes untreated or treated with JNJ1935 for 1 h and then stimulated with 50 ng/mL of TNF-α for 0.5, 2, 4, or 6 h. C, Densitometry of pJNK from Western blots described in B ($n = 3$). D, Caspase 3/7 activity in HL-60 cells differentiated to neutrophils with 1 μmol/L ATRA and untreated or treated with 1 mmol/L DMOG for 2, 4, 8, or 24 h. Etoposide was used as apoptotic control. E, Scheme indicating the potential multifactor mechanism whereby hydroxylase inhibition reduces chemokine production in keratinocytes while it increases apoptosis in neutrophils. **means $P \leq 0.01$; ***means $P \leq 0.001$

CXCL8 (IL-8) (up to 10-fold) mRNA transcripts compared with vehicle-treated cells. JNJ1935 caused a significant reduction of CCL5 and CXCL1 RNA but did not affect the expression of IL-8 (Figure 4F-H). Similarly, stimulation with IL-1β induced production of CCL5, CXCL1, and IL-8 from keratinocytes and treatment with JNJ1935 reduced IL-1β-induced CCL5 and CXCL1 but did not affect IL-8 (data not shown). These results show an inhibitory effect of hydroxylase inhibitors on the expression of CXCL1 and CCL5 from primary human keratinocytes.

3.5 | TNF-α-mediated JNK phosphorylation is reduced by hydroxylase inhibition

The production of inflammatory chemokines in response to DNFB is controlled by the NF-κB and MAPK pathways.^{24,42} We next investigated whether the anti-inflammatory effects of hydroxylase inhibition were due to the blockade of MAPK/NF-κB pathways. Using TNF-induced CCL5 expression as a readout, we compared the effects of JNJ1935 to that of inhibitors of the NF-κB, ERK, JNK, and p38 pathways. As previously

shown, JNJ1935 reduced CCL5 secretion (Figure 5A). Only, the JNK inhibitor SP600125 caused a significant reduction of TNF- α -mediated CCL5 production (Figure 5A). Furthermore, TNF- α transiently increased JNK phosphorylation peaking at 0.5 and 2 hours after stimulation in a manner that was attenuated by JNJ1935 treatment (Figure 5B,C).

Another possible mechanism whereby hydroxylase inhibition could reduce neutrophil infiltration is by triggering apoptotic cell death. In order to investigate this, we used HL60 cells. As seen in Figure 5D, we found DMOG to induce activation of caspase 3/7 in a time-dependent manner. This pro-apoptotic action was comparable to that induced by the positive control etoposide. Therefore, in addition to reducing JNK-mediated chemokine production, hydroxylase inhibitors also increase

neutrophil apoptosis (Figure 5E). From these data, we hypothesize that the anti-inflammatory effects of hydroxylase inhibition are mediated at least in part through attenuation of the JNK pathway in this model and the induction of neutrophil apoptosis.

3.6 | Topical administration of DMOG prevents DNFB-mediated neutrophil infiltration

Intraperitoneal administration of hydroxylase inhibitors may cause erythropoiesis. We next investigated whether topical application of the hydroxylase inhibitor DMOG has anti-inflammatory actions in DNFB-treated mice. Hematoxylin and eosin (H&E) staining and

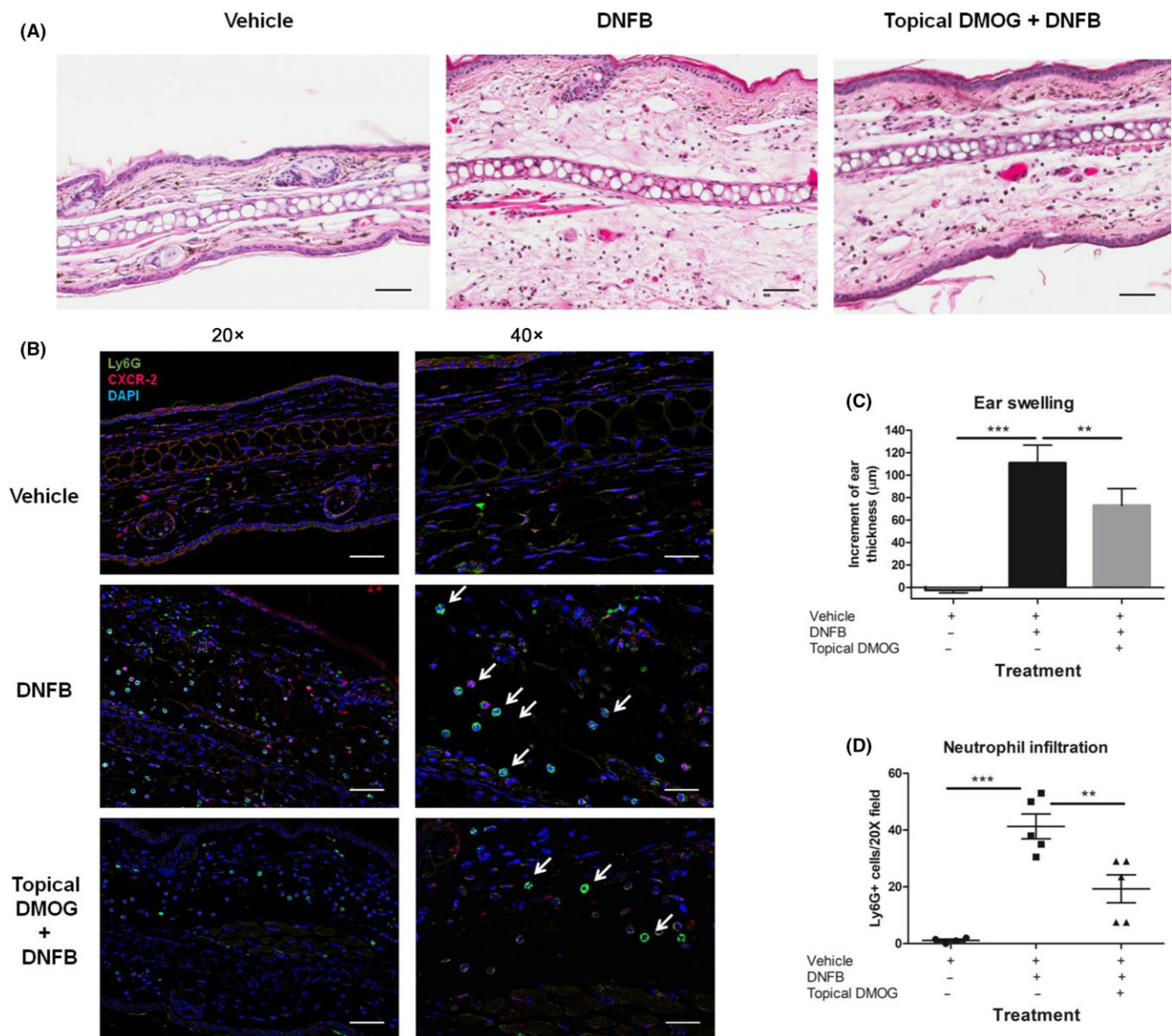


FIGURE 6 Topical administration of DMOG reduces acute inflammation and neutrophil infiltration in 2,4-dinitrofluorobenzene (DNFB)-induced dermatitis. A, Representative images of hematoxylin and eosin stained mouse ear crosssections from mice untreated or treated with topical administration of 8 mg/mouse DMOG for 1 hour and then challenged with vehicle or DNFB. B, representative images of mouse ear cross-sections stained for Ly6G/CXCR2 in mice treated as described in A. C, ear thickness quantification (reflecting ear swelling) at 8h post-challenge in mice treated as described in A. D, quantification of Ly6G positive cells from staining described in B. (n=5), **means $P \leq 0.01$; ***means $P \leq 0.001$

immunofluorescence for Ly6G/CXCR2 of mouse ears revealed severe DNFB-induced dermal edema and neutrophil infiltration following challenge (Figure 6A-D). Mice treated with topical DMOG displayed significantly diminished ear thickness and neutrophil infiltration (Figure 6A-D). These results show that topical application of hydroxylase inhibitors has anti-inflammatory properties.

4 | DISCUSSION

In this study, we investigated the impact of hydroxylase inhibitors on allergic skin inflammation.

Inflammatory skin disorders including AD, psoriasis, or ACD are common dermatological diseases, and thus, improved therapies with high efficacy and a low side effect profile are still an unmet clinical need.^{24,43} ACD is caused by a severe inflammatory response upon contact with a hapten to which an individual has become sensitized. Hapten challenge triggers a multistep inflammatory response in which T cells and neutrophils are key effectors.^{22,25} This response is orchestrated by the release of cytokines and chemokines leading to further inflammation and tissue edema. The DNFB model of ACD is a common model for the study of allergic skin inflammation.^{24,25} Challenge of mouse ears of DNFB-sensitized mice induced a severe inflammatory reaction with formation of edema in the dermis. Dermal edema was accompanied by an increase in CXCL1 and CCL5 expression. These chemokines are crucial for the recruitment of neutrophils and T lymphocytes, respectively.⁴⁴⁻⁴⁶ Intraperitoneal treatment with hydroxylase inhibitors reduced ear edema in a manner, which was associated with diminished expression and release of CXCL1 and CCL5. In addition, DNFB-induced inflammation differentially regulated the expression of oxygen-sensing hydroxylases. Firstly, our studies showed that PHD2 was downregulated in DNFB-challenged skin. PHD-2 is the predominant PHD isoform expressed throughout the body, and its suppression is sufficient to stabilize HIF- α .⁴⁷ The downregulation of PHD2 has been purposed as a mechanism of regulation of the HIF pathway mediated by different proinflammatory stimuli.^{34,35} In line with this evidence, our studies showed that DNFB is capable of increasing the expression of the HIF target gene *Glut1*. This further indicates that DNFB-induced inflammation is related to the establishment of a hypoxic response and modulation of the HIF pathway. This supports the concept that the HIF pathway plays an active role in allergic skin disease.

T cells are key drivers of the allergic response that governs DNFB-mediated inflammation.^{22,48,49} In our studies, the expression of CD4 was not detected 24 hours after challenge in mouse ears. In contrast, CD8 and GZMB were significantly upregulated at the challenged site suggesting a predominance of CD8+ T cells. Hydroxylase inhibitors did not affect the expression of T cell markers investigated, suggesting that the anti-inflammatory properties of the drugs are not through reducing lymphocyte recruitment. Multiple studies have described the role of CXCL1-mediated neutrophil recruitment in the initiation stages of DNFB-induced ACD.^{32,46,50} Importantly, the presence of neutrophils is central to the establishment of the T cell-

mediated response that regulates contact hypersensitivity, highlighting the importance of neutrophils in ACD.^{22,51} Mice sensitized and challenged with DNFB had a large amount of infiltrating Ly6G/CXCR2-positive neutrophils in the dermis. CXCR2 is the main receptor responsible for CXCL1-dependent recruitment of neutrophils.³⁷ Studies using models of arthritis have highlighted the importance of this axis. In these studies, blockade of CXCR2 as well as knock out of CXCL1 had anti-inflammatory effects through reduced neutrophil recruitment.^{37,52} In the context of skin inflammation, CXCR2-/- mice were protected against DNFB-induced dermatitis through reduced recruitment of polymorphonuclear cells, whereas other studies correlate reduced CXCL1 with reduced inflammation.^{38,50} In line with this evidence, hydroxylase inhibitor-mediated reduction of CXCL1 expression and secretion was associated with a reduction of neutrophil infiltration, confirming a repression of the CXCL1-induced neutrophil response during acute ACD. Of note, we also found similar effects of hydroxylase inhibitors on eosinophils, indicating that the effects on neutrophils might be extended to other granulocyte populations.

Beyond the formation of physical barriers, the active role of epithelial cells in inflammatory diseases is well documented.^{7,50,53-55} In the case of the skin, a number of studies show the importance of keratinocytes and endothelial cells in the elicitation of inflammation.^{50,56-59} TNF- α is a strong stimulus for keratinocytes and plays important roles in skin inflammation. In our studies, TNF- α expression was increased in DNFB-induced ACD but was not affected by hydroxylase inhibitors. TNF- α stimulation of primary human keratinocytes isolated from multiple donors increased the expression and release of multiple cytokines and chemokines including CXCL1, CCL5, or IL-8 supporting a fundamental role of the keratinocytes in orchestrating skin inflammation. Hydroxylase inhibitors blocked the production and expression of CXCL1 and CCL5 but did not affect that of IL-8. These *in vitro* findings were in line with our *in vivo* observations and support the anti-inflammatory actions of these drugs via reduced recruitment of inflammatory cells such as neutrophils.

Several different pathways including NF- κ B and MAPK pathways have been reported to regulate inflammation in different cell and *in vivo* models. In our *in vitro* model, TNF-mediated CCL5 production was reduced in cells treated with the JNK inhibitor SP600125 in a manner similar to JNJ1935. Further, JNJ1935 reduced TNF α -induced JNK phosphorylation suggesting a potential effect of hydroxylase inhibition on the JNK pathway. Another key aspect to consider for the translation of these inhibitors into new skin therapies is that of their route of administration. In many cases, the topical route can be exploited for the development of therapies for skin disease. In our studies, topical administration of DMOG achieved similar results to those seen with intraperitoneal administration, reducing ear edema, and the recruitment of neutrophils. Thus, our results support a model whereby hydroxylase inhibition attenuates CXCL1 and CCL5 production and consequently blocks downstream events leading to neutrophil recruitment during skin inflammation. Of note, a similar mechanism was recently proposed in the context of pulmonary infection. Increased susceptibility of myeloid-deficient

HIF1 α mice to infection was due to decreased production of CXCL1 and increased neutrophil apoptosis, resulting in decreased neutrophil infiltration.⁶⁰ For instance, hydroxylase inhibition might regulate chemokine production causing inhibition of proinflammatory immune responses.

Several authors have implicated oxygen-sensing hydroxylases in the regulation of inflammatory cell survival. Of note, Walmsley and colleagues described a key role of PHD3 in the induction of neutrophil survival in vitro and in vivo.³⁶ Given the increased expression of PHD3 in DNFB-challenged skin found in our studies, we investigated whether hydroxylase inhibitors induced neutrophil apoptosis. In line with a role for hydroxylases in the regulation of neutrophil survival, hydroxylase inhibition with DMOG induced time-dependent cell death in cultured neutrophils. Current therapies for ACD are based on the use of anti-inflammatory drugs such as topical/systemic glucocorticosteroids, or immunomodulating ultraviolet light therapy.^{61–63} However, all therapies are still limited for different reasons, whether as to efficacy in severe cases or due to various side effect profiles (eg, skin atrophy, infections, skin dryness, or increased cancer risk after long-term repeated phototherapy). In addition, glucocorticosteroids can in some cases induce allergic contact dermatitis,⁶³ and systemic approaches induce harmful, partly irreversible adverse events under long-term therapy. Here, we have shown for the first time that hydroxylase inhibitors may exert a beneficial effect for the treatment of allergic contact dermatitis and probably other chronic inflammatory skin diseases in which neutrophils and eosinophils are involved.

CONFLICTS OF INTEREST

CTT is a member of the Scientific Advisory Board of Akebia Therapeutics. LCD is an employee at Almirall, S.A.

AUTHOR CONTRIBUTIONS

CTT, MS, and MCM wrote the manuscript. MCM and all other authors contributed to experimental data and reviewed the manuscript.

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SUPPORTING INFORMATION

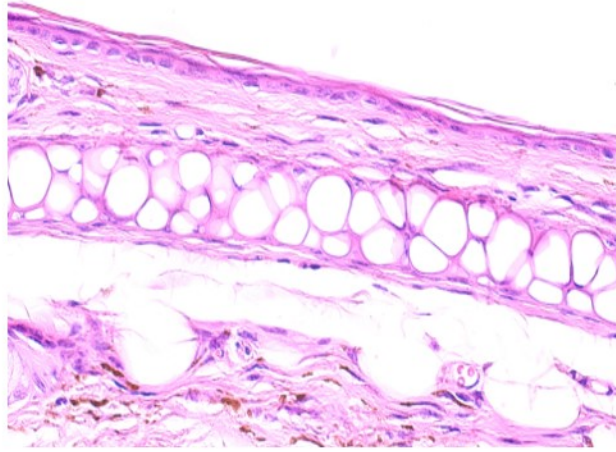
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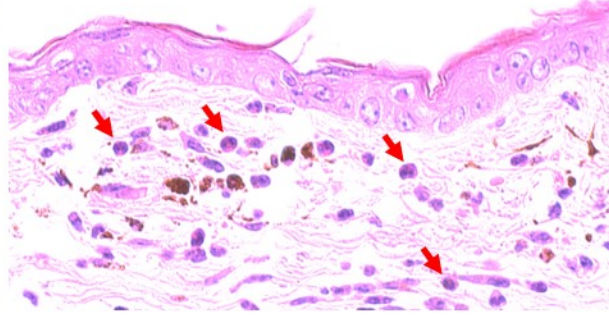
DNFB

A

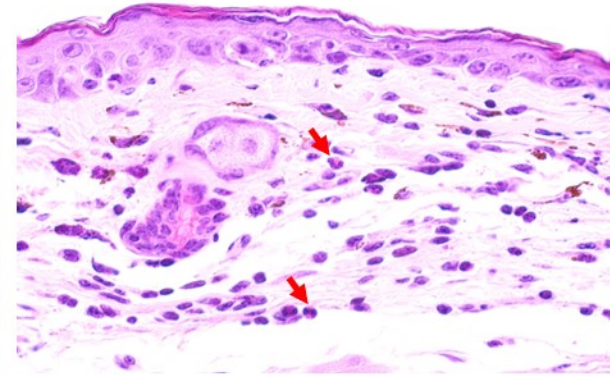
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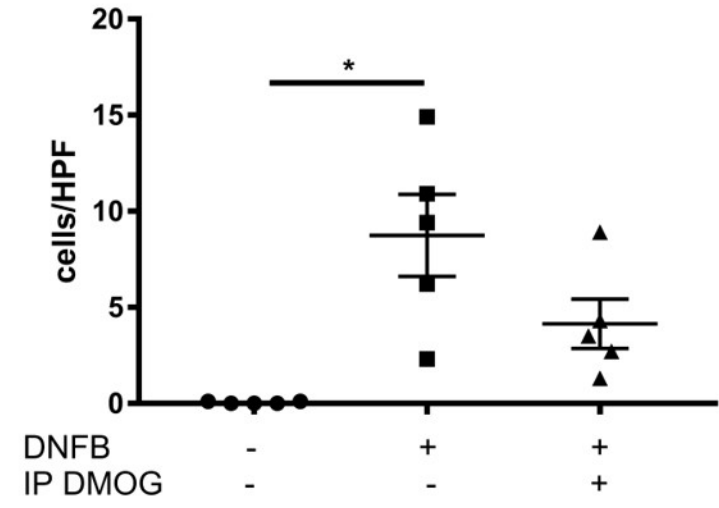


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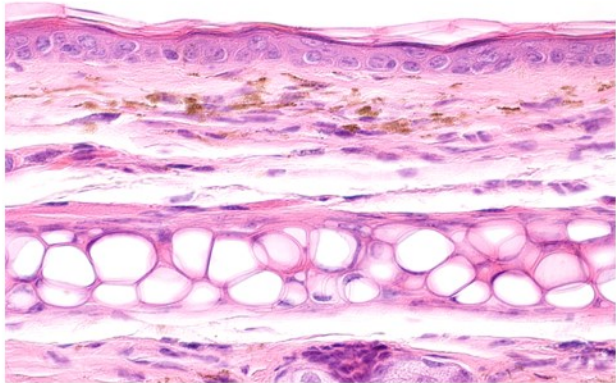
B

Eosinophils



C

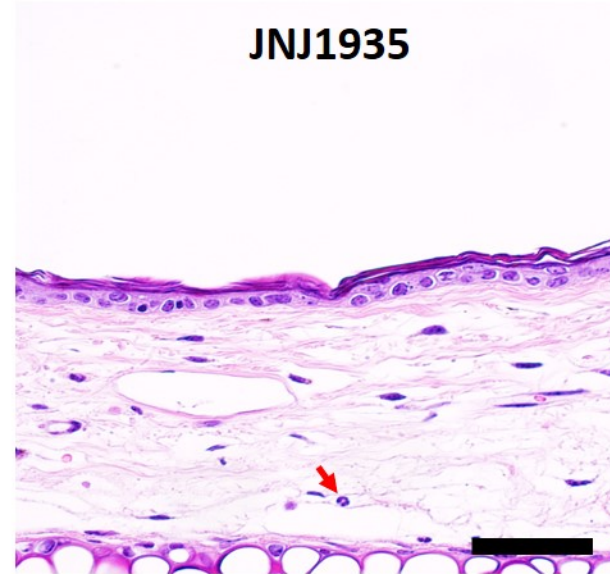
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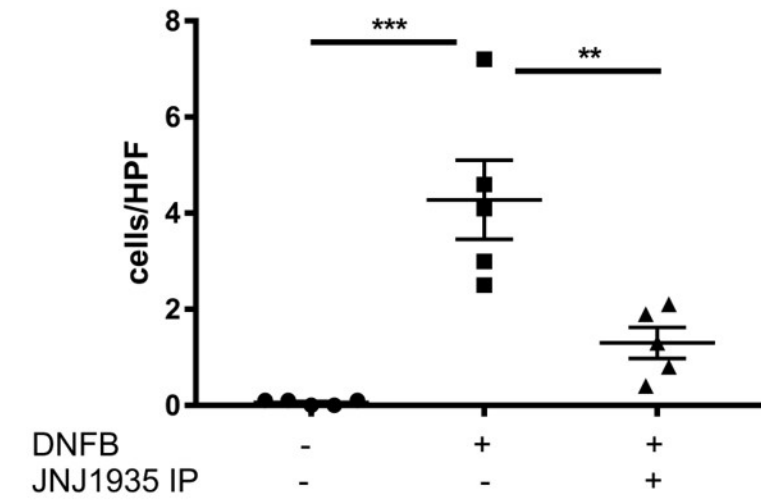


JNJ1935



D

Eosinophils

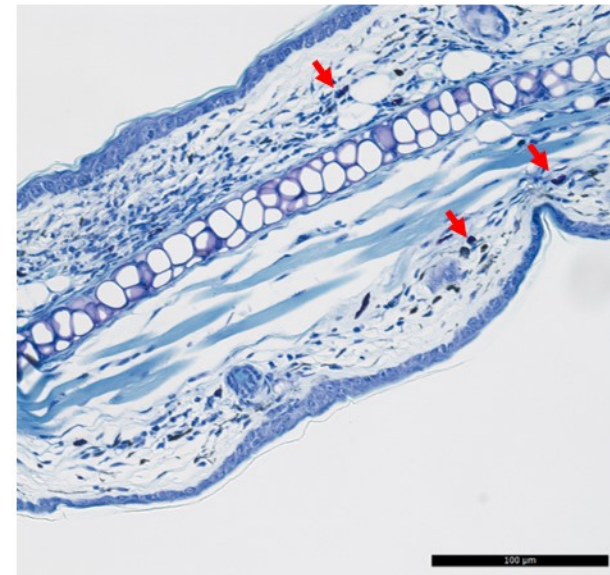
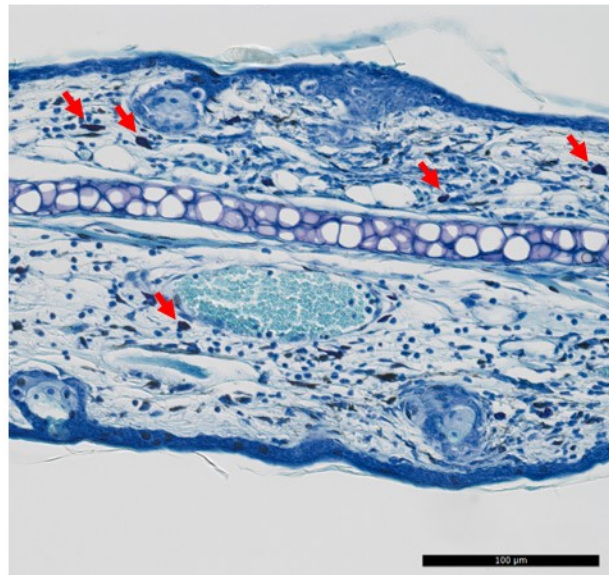
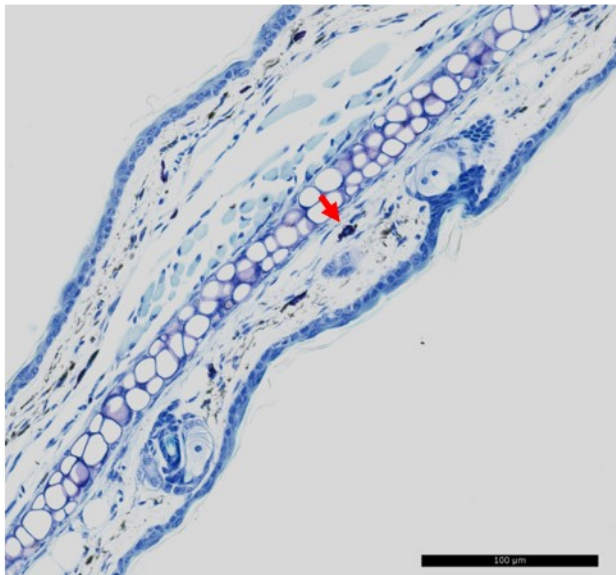


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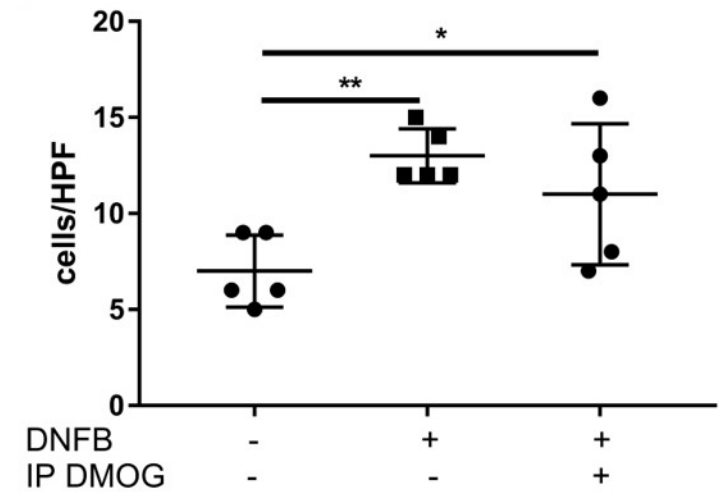
DMOG

E



F

Mast cell

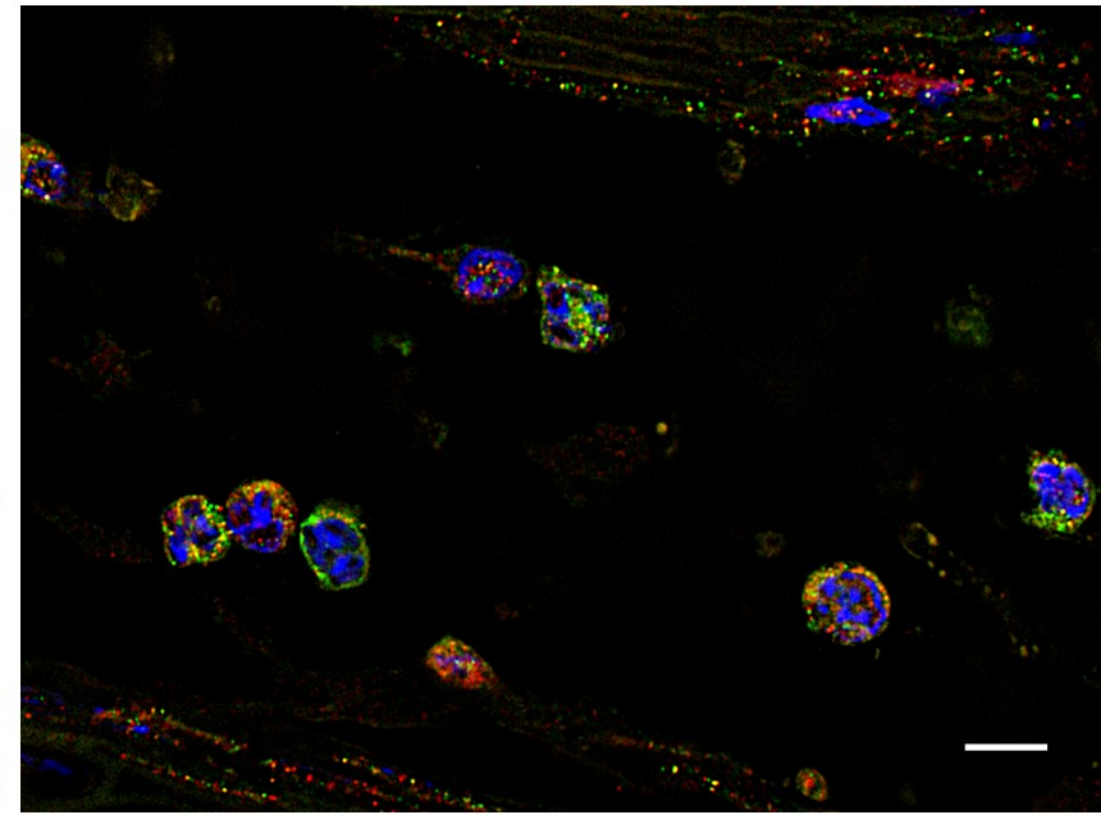
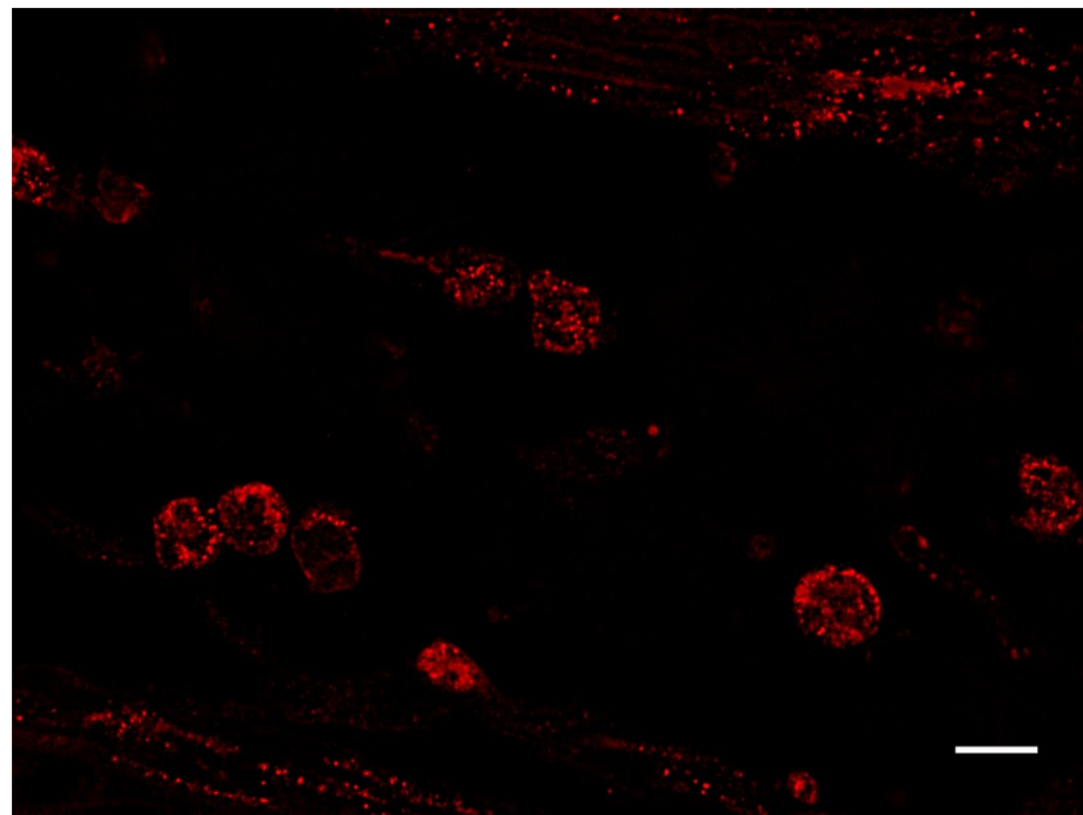
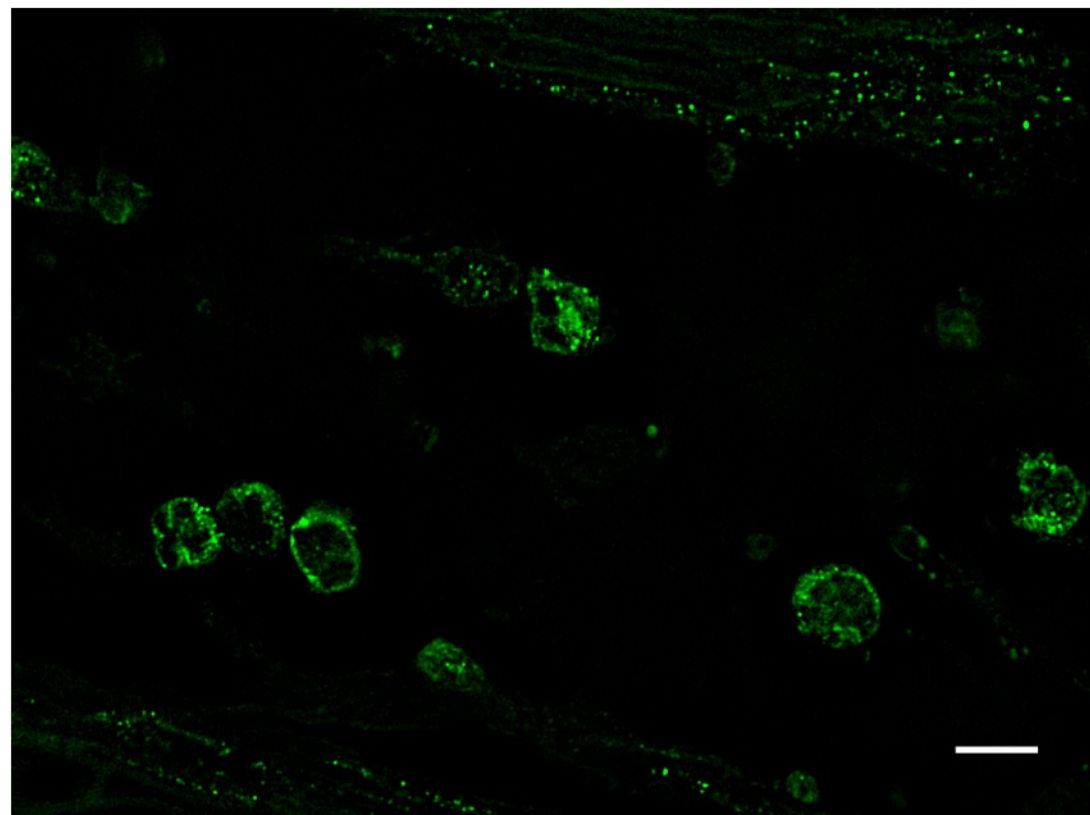


Ly6G

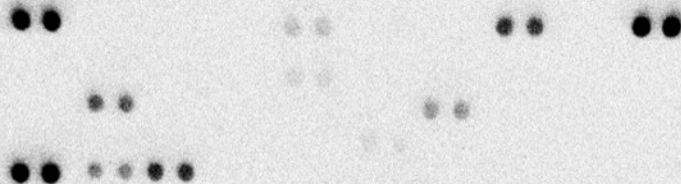
CXCR-2

Merged

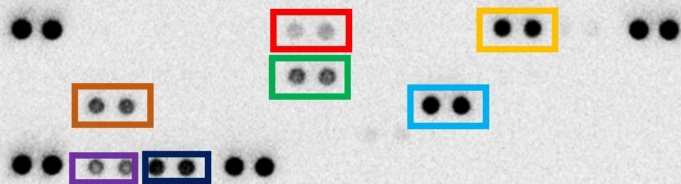
DNFB



Control



TNF- α (50ng/ml)



CCL5 CXCL1 GM-CSF IL1Ra IL8 MIF Serpin-E1

Supplementary figure 1. *Colocalization of CXCR2 and Ly6G.* Representative images of immunofluorescence of mouse ear cross-sections stained for Ly6G/CXCR2 at 24 hours post DNFB challenge (100X).

Supplementary figure 2. *Hydroxylase inhibition attenuates eosinophilic but not mast cell infiltration in DNFB induced ACD.* (A) haematoxylin and eosin staining of mouse ear cross-sections from mice treated or not with intraperitoneal injections of 8mg/mouse DMOG and challenged with vehicle or DNFB (n=6). (B) eosinophil quantification in mouse ear cross-sections from mice treated and stained as described in A. (C) haematoxylin and eosin staining of mouse ear cross-sections from mice treated or not with intraperitoneal injections of 0.8mg/mouse JNJ1935 and challenged with vehicle or DNFB (n=5). (D) eosinophil quantification in mouse ear cross-sections from mice treated and stained as described in C. (E) Giemsa staining of mouse ear cross-sections from mice treated as in A. (F) Mast cell quantification in tissues stained and treated as described in E. * means $p \leq 0.05$; ** means $p \leq 0.01$; *** means $p \leq 0.001$.

Supplementary figure 3. *TNF- α induces a pro-inflammatory profile in human primary keratinocytes.* Representative image of proteome profiler cytokine/chemokine array from supernatants of primary human keratinocytes un-stimulated or stimulated with 50ng/ml TNF- α .