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Research Article

Ah Receptor Activation Potentiates Neutrophil Chemoattractant (C-X-C Motif) Ligand 5 Expression in Keratinocytes and Skin

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ABSTRACT

Chemokines are components of the skin microenvironment, which enable immune cell chemotaxis. Traditionally, transcription factors involved in inflammatory signaling (eg, NFκB) are important mediators of chemokine expression. To what extent xenobiotics and their associated receptors control chemokine expression is poorly understood. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor known to mediate physiological responses in the skin through the regulation of genes involved in xenobiotic metabolism, epidermal differentiation, and immunity. Here, we demonstrate that AHR activation within primary mouse keratinocytes regulates the expression of a neutrophil directing chemokine (C-X-C motif) ligand 5 (*Cxcl5*). AHR-mediated regulation of *Cxcl5* is because of direct transcriptional activity upon treatment with AHR agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Additionally, AHR mediates enhanced induction of *Cxcl5* upon exposure to an agonist and the inflammatory cytokine interleukin 1 beta. This synergy is confined primarily to keratinocytes, as dermal fibroblasts did not achieve the same level of combinatorial induction. AHR-specific antagonists were able to reduce basal and induced levels of *Cxcl5*, demonstrating the potential for pharmacological intervention. Exposure of C57BL/6J mice to ultraviolet (UV) light followed by topical treatment with the AHR agonist formylindolo(3,2-b)carbazole (FICZ) significantly induced *Cxcl5* expression in skin compared with UV alone, and this response was absent in *Ahr*$^{-/-}$ mice. These results establish AHR as an important mediator of *Cxcl5*, with implications for the treatment of inflammatory skin diseases.

Key words: aryl hydrocarbon receptor; Cxcl5; inflammation; TCDD; Ah receptor.

The skin represents the primary barrier to the environment and external insult, and the cutaneous response to injury is critical for interpreting potential threats. Chemokines are one of many factors produced to aid in this protection. These low molecular weight cytokines form a gradient from the site of injury or infection to the blood stream by which immune cells can traffic to the appropriate tissue. The relative abundance of these inflammatory mediators is regulated through changes in the amount produced by resident immune cells and local epithelial cells (Griffith *et al.*, 2014). However, chemokine dysregulation

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can lead to an inappropriate infiltration of immune cells, resulting in inflammation and tissue damage (Proudfoot, 2002). Keratinocytes, the most abundant cell type of the epidermis, contribute to the formation of the immune microenvironment through the production of chemokines and other mediators (Nestle *et al.*, 2009).

Chemokines are classified according to the arrangement of cysteine residues at the N-terminus. One of these subclasses is characterized by the C-X-C motif, and further divided into Glu-Leu-Arg (ELR)$^+$ or ELR$^-$ CXC chemokines. The ELR$^+$ CXC chemokines induce neutrophil chemotaxis through specific interaction with the G-protein coupled receptors CXCR2 and CXCR1, and in the mouse, include CXCL1, CXCL2, CXCL5 (Bizzarri *et al.*, 2006). Though chemokines of this family perform a similar function, recent studies have identified nonredundant functions within the CXC family. For example, CXCL5 impairs chemokine scavenging through binding to the Duffy Antigen Receptor for Chemokines (DARC), causing an increase in plasma levels of CXCL1 and CXCL2 which in turn leads to an impairment of the chemokine gradient, decreased neutrophil chemotaxis to the lungs, and a subsequent increase in *Escherichia coli* numbers and heightened mortality in a mouse pneumonia model (Mei *et al.*, 2010). This demonstrates the importance of maintaining the appropriate levels of these factors during an infection.

CXCL5, also known as LPS-induced CXC chemokine (LIX) in mice, is produced by a variety of cells such as the epithelial cells of barrier tissues, including alveolar epithelial type II cells (Jeyaseelan *et al.*, 2005), enterocytes (Mei *et al.*, 2012), and keratinocytes (Guilloteau *et al.*, 2010). It has also been linked to a variety of skin pathologies. In human and rat skin, CXCL5 is the principal mediator of ultraviolet (UV)-induced pain (Dawes *et al.*, 2011) and *Cxcl5* expression is increased in keratinocytes after IL17 exposure, linking it to the pathogenesis of psoriasis (Nograles *et al.*, 2008). Additionally, *Cxcl5* expression can be induced by exposure to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Kim *et al.*, 2010). NFκB is likely an important regulator of *Cxcl5* expression in response to inflammation (Smith *et al.*, 2002), however, other factors that link *Cxcl5* induction to diverse stimuli remain unknown.

The aryl hydrocarbon receptor (AHR) has been implicated in regulating the inflammatory response in barrier tissues, including the intestine and skin (Li *et al.*, 2011). The prototypical AHR signaling pathway involves the activation of cytosolic AHR through ligand binding, translocation to the nucleus, and binding to its heterodimeric partner, the aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer subsequently binds to dioxin response elements (DREs) typically located upstream of the transcription start site of responsive genes (Beischlag *et al.*, 2008). Previous studies from our laboratory identified inflammation-related genes that are regulated through the canonical pathway in an enhanced or additive manner in the presence of inflammatory stimuli, including interleukin 6 (*IL6*) (Hollingshead *et al.*, 2008) in human tumor cell lines and chemokine (C-C motif) ligand 20 (*Ccl20*) in primary mouse peritoneal macrophages (Lahoti *et al.*, 2015). Furthermore, prostaglandin-endoperoxide synthase 2 (*Ptgs2*) is also regulated by inflammatory signaling and the AHR in a combinatorial manner (Yang and Bleich, 2004). An *in silico* search for putative DREs in chemokine genes revealed a series of overlapping putative DREs in the upstream promoter of *Cxcl5*. In addition, a potential role for AHR in the regulation of *Cxcl5* under proinflammatory conditions was demonstrated in a study in which a constitutively active form of AHR is expressed exclusively in mouse keratinocytes. These mice develop itchy skin lesions resembling atopic dermatitis, and a DNA microarray analysis revealed that *Cxcl5* was the most highly induced chemokine gene in the skin lesions of the mice (Tauchi *et al.*, 2005). In this study, we have determined that *Cxcl5* is regulated in an AHR and AHR ligand-dependent manner under basal and proinflammatory conditions in primary mouse keratinocytes, and in a UVB mouse exposure model. Our results further define how environmental factors may influence the inflammatory microenvironment in the skin under proinflammatory conditions.

MATERIALS AND METHODS

Animals and husbandry. All mouse lines were bred in-house after acquisition. C57BL/6J mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). *Ahr*$^{-/-}$ mice (B6.129-*Ahr*^{tm1Bra}/J) were a gift from Dr. Christopher Bradfield (University of Wisconsin, Madison, WI.) Animals were housed in specific pathogen-free conditions under a 12 h light/dark cycle with *ad libitum* access to standard chow and water in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University.

Chemicals and reagents. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was a kind gift from Dr. Stephen Safe (Texas A&M University). FICZ was purchased from Enzo Life Sciences (Farmingdale, NY), β-naphthoflavone (BNF) was purchased from INDOFINE Chemical Company, Inc (Hillsborough, NJ), and indolo[3,2-*b*]carbazole (ICZ) was purchased from Matrix Scientific (Columbia, SC). N-(2-(1*H*-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9*H*-purin-6-amine (GNF351) was provided by the Genomics Institute of the Novartis Research Foundation (San Diego, CA). 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH223191) was purchased from ChemBridge Corp (San Diego, CA), and 1-Allyl-3-(3,4-dimethoxyphenyl)-7-(trifluoromethyl)-1*H*-indazole (SGA360) was synthesized as described previously (Murray *et al.*, 2010). Recombinant mouse IL1B was purchased from PeproTech (Rocky Hill, NJ).

Mouse Cxcl5 *promoter analysis*. Promoter analysis of mouse *Cxcl5* was conducted using the FASTA sequence (NCBI) to examine the region 1- to 2-kb upstream of the transcription start site. This sequence was analyzed for potential DREs using the JASPAR CORE database.

Plasmids. The following oligonucleotides; 5′-CGATGCGTGCATGC ATGCGTGCGTGCGTGC GTGCGTGCGTGCGTGCGTGTGGGT AC-3′ and 5′-CCACACGCA CGCACGCACGCACGCACGCACGCA CGCACGCATGCATGCACGCATCGGTAC-3′ were annealed and inserted into a pGL3 promoter construct to generate pGL3promCxcl5 (promoter sequence containing DREs). The plasmid pcDNA3 was obtained from Invitrogen, and pSV40βgal was purchased from Promega.

Cxcl5 *reporter assay*. Hepa1 cells were seeded in 6-well plates and transfected with Genlantis GenePORTER 3000 Transfection Reagent (San Diego, CA) and various plasmids for 4 h, essentially as described by the manufacturer. The cells were subsequently placed in complete medium. Following overnight incubation, the cells were treated with AHR ligands for 6 h, then lysed in a buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton X-100,

combined with Luciferase Assay Substrate (Promega, Madison, WI). Luciferase activity was recorded using a Turner TD-20e luminometer and normalized to β-galactosidase activity.

Electrophoretic mobility shift assay. Gel shift assays were conducted as described previously (Chiaro *et al.*, 2008). Briefly, mouse AHR and mouse ARNT proteins were *in vitro* translated using T7 rabbit reticulocyte lysate (Promega). The proteins were combined and incubated with HEDG buffer (25 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.5), the indicated treatments, and 1×10^6 cpm of ^{32}P-labeled DRE probe (mouse *Cxcl5* oligonucleotides used: Forward Probe: 5′-ATGCGTGCA TGCATGCGTGCGTGC GTGCGTGCG TGCGTGCGTGCGTGCGTG-3′, Reverse Probe: 5′-CACGCACGCACGCACGCACGCACGCACGCACGCACGCATGCA TG CACGCAT-3′. The DRE present in the proximal region of *Cyp1a1* was used as a positive control. Lysate translated from empty plasmid was substituted for ARNT for the negative control. Lysate was resolved on a 6% nondenaturing DNA retardation gel (Invitrogen), fixed in a solution of 10% methanol, 10% glacial acetic acid, 10% glycerol, and 70% water for 20 min, vacuum dried and analyzed via autoradiography. Competition Electrophoretic Mobility Shift Assay (EMSA) using 10× nonradioactive probe and ^{32}P-labeled oligonucleotides was conducted in the presence or absence of 10× nonradioactive oligonucleotides by mass.

Isolation and culture of primary keratinocytes and dermal fibroblasts. Primary keratinocytes were isolated from neonatal pups as previously described (Dlugosz *et al.*, 1995) and cultured at 36 °C with 7% CO_2. Primary dermal fibroblasts were isolated from the dermal layer following keratinocyte isolation. Briefly, dermal fibroblasts were isolated using media supplemented with Collagenase I (0.35% wt/vol). The dermal layers were combined, diced, and incubated in a shaker at 37 °C for 30 min, followed by the addition of 200 U per dermis of DNAse in PBS (20 000 U/ml) and briefly swirled. DMEM, 10% FBS, and 100 IU/ml penicillin/0.1 mg/ml streptomycin were added to the cells after the addition of the DNase solution. Cells were kept at room temperature for 10 min with no additional shaking. The dermal solution was filtered into a new tube through a 100-μm filter, and centrifuged at 1100 rpm for 5 min. The cell pellet was resuspended in DMEM medium, and then centrifuged at 400 rpm for 5 min. The supernatant, which contains the fibroblasts, was collected and centrifuged again for 5 min at 400 rpm, after which the supernatant was again collected. Dermal fibroblasts were seeded and cultured at 37 °C with 5% CO_2. All primary cells were only utilized after initial plating and thus were not passaged prior to use in experiments.

RNA isolation and quantitative reverse transcription PCR. Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO), and was then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was conducted using PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primers used for real-time PCR are listed in Table 1. Ribosomal protein, large, P0 (*Rplp0*) was used to normalize gene expression.

Mouse LIX enzyme-linked immunosorbent assay. Quantification of secreted LIX (CXCL5) in mouse primary keratinocyte culture media was determined using the Quantikine Enzyme-Linked Immunosorbent Assay (ELISA) for Mouse LIX kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Western-blot analysis. Cytosolic protein was isolated from primary keratinocytes and primary dermal fibroblasts using a lysis buffer containing MENG buffer (20 mM MOPS, 2 mM EDTA, 0.02% sodium azide, 10% Glycerol, pH 7.4), 1% IGEPAL, and cOmplete Mini protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). The supernatant was collected after ultracentrifugation at 42 000 rpm for 30 min and stored in liquid nitrogen until further use. Samples were resolved by 8% tricine-SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Rpt1 antibody was used to probe for AHR (Perdew *et al.*, 1995), and β-actin antibody was purchased from Santz Cruz Biotechnology, Inc (Dallas, Texas). Biotin-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and ^{125}I-streptavidin were used for detection and radioactivity was visualized using BioMax film. ^{125}I-streptavidin was prepared as described previously (Narayanan *et al.*, 2012). ImageJ software was used for Western-blot quantification.

UV irradiation and treatment. Age-matched female C57BL/6J or $Ahr^{-/-}$ mice were intraperitoneally injected with avertin (240 mg/kg) prior to irradiation with a dose of 360 mJ/cm^2 UVB from UV bulbs (American Ultraviolet Light Co) covered with cellulose triacetate (Kodak) to allow only UV wavelengths between 280 and 320 nm. Output of the UV bulbs was measured using a UVX radiometer (UVP, Upland, CA). After UV exposure, the left ears of the mice were treated with 20 μl of 20% DMSO in acetone (vehicle), whereas the right ears received 100 ng of FICZ in vehicle. Mice received two further applications of the treatments at 8- and 16-h post-UV exposure, and were sacrificed by CO_2 asphyxiation 24 h after the initial exposure. The ears were collected, snap-frozen in liquid nitrogen and stored at − 80 °C until further analysis.

Statistical analyses. GraphPad Prism 5 software was used to conduct all statistical analyses. Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test, unless otherwise indicated in figure legends. Letters indicate compared columns with asterisks indicating statistical significance. Data represent mean ± SEM, with P value ≤ .05 (*), P value ≤ .01 (**), P value ≤ .001 (***). Alphabetical characters indicate statistical comparisons between different groups.

RESULTS

The Mouse Cxcl5 Promoter Contains Multiple Responsive DREs

In silico analysis of the mouse *Cxcl5* promoter revealed a sequence of nine tandem core DRE sequences (CGTG), which result in seven overlapping consensus DREs between 1- and 2-kb upstream of the transcription start site, as defined by Dension *et al.* (1998). In addition, a putative RelA binding element in the proximal promoter is shown (Figure 1A). Interestingly, this string of seven overlapping DREs composed of nine core DRE sequences is unique in the mouse genome. To determine if the DREs are responsive to AHR-mediated transcription, mouse hepatoma Hepa1 cells were transfected with a luciferase-driven reporter plasmid containing the mouse *Cxcl5* DRE sequence located adjacent to an SV40 promoter sequence. Treatment with the AHR ligands TCDD (2 nM) and ICZ (500 nM) significantly induced luciferase-driven reporter activity 1.8-fold compared with vehicle treated cells containing the mouse *Cxcl5* DRE sequence (Figure 1B), demonstrating the ability of the DREs to enhance transcriptional activity.

Table 1. Mouse Primer Sequences Used for qRT-PCR

Gene	Forward Primer	Reverse Primer
Cxcl5	TGCCCTACGGTGGAAGTCAT	AGCTTTCTTTTTGTCACTGCCC
Cxcl1	GCTGGGATTCACCTCAAGAA	TCTCCGTTACTTGGGGACAC
Cxcl2	AGACAGAAGTCATAGCCACTCTCAAG	CCTCCTTTCCAGGTCAGTTAGC
Cyp1a1	CTCTTCCCTGGATGCCTTCAA	GGATGTGGCCCTTCTCAAATG
Rplp0	CGTCCTCGTTGGAGTGACAT	TAGTTGGACTTCCAGGTCGC

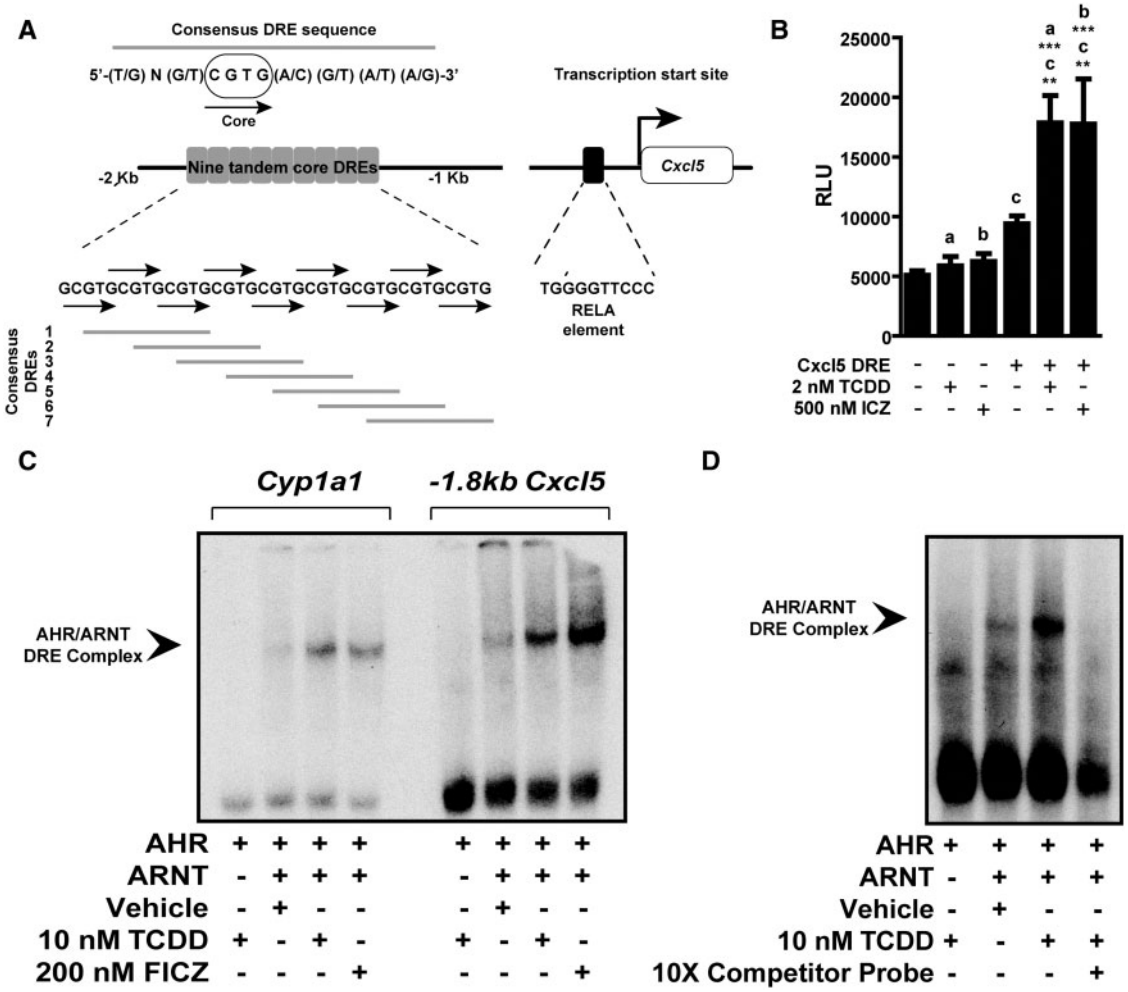

Figure 1. *Cxcl5* is an AHR-responsive gene. A, Scheme of the mouse *Cxcl5* promoter, depicting the sequence of nine overlapping DREs and a potential RelA binding site. B, Hepa1 cells were transfected with a plasmid expressing the mouse *Cxcl5* DRE linked to a luciferase reporter construct and treated with vehicle, TCDD, or ICZ for 6 h. C, An EMSA was performed with *in vitro* translated mouse AHR and ARNT. TCDD or FICZ was added to the translated proteins and 1×10^6 cpm of P^{32}-labeled DRE probe. The putative DRE present in the proximal region of *Cyp1a1* was used as a positive control. D, P^{32}-labeled oligonucleotide was used in presence or absence of 10× nonradioactive oligonucleotide by mass. The arrow indicates binding of the AHR/ARNT/DRE complex.

AHR-ARNT Complexes Bind Putative DRE Sequences Upstream of Cxcl5 *In Vitro*

An EMSA using *in vitro*-translated AHR and ARNT, was conducted to provide evidence that the induction of luciferase activity observed in Figure 1B is because of direct binding of the AHR/ARNT complex to the mouse *Cxcl5* DRE sequence (Figure 1C). Binding of the AHR to either the *Cyp1a1* or *Cxcl5* DRE sequence occurred only in the presence of ARNT, and was augmented in the presence of TCDD or FICZ. The presence of an AHR/ARNT mediated gel-shift in the absence of exogenous ligand appears to be because of constitutive receptor activation when in vitro translated AHR and ARNT are used. In the presence of 10× nonradioactive competitor probe, this binding is blocked (Figure 1D). These data demonstrate that the AHR/ARNT heterodimer is capable of directly binding to DREs contained in the promoter of mouse *Cxcl5 in vitro* and that the AHR-mediated increase of *Cxcl5* expression is most likely because of direct AHR/ARNT-DNA binding.

TCDD Treatment Results in the Induction of Cxcl5 *Expression in Mouse Keratinocytes*

To examine the response of primary keratinocytes to TCDD, cells isolated from $Ahr^{+/+}$ mice were exposed to 2 nM TCDD and

gene expression of *Cxcl5* was measured using qRT-PCR. TCDD results in an increase in *Cxcl5* transcript levels, as *Cxcl5* mRNA levels were significantly increased 20-fold in response to TCDD, and the prototypical AHR response gene *Cyp1a1* was induced >300-fold (Figure 2A). The AHR antagonist GNF351 (500 nM) significantly reduced TCDD-mediated induction of both genes (Figure 2A). To determine if this response is specific to *Cxcl5*, the expression of the ELR$^+$ CXC chemokines *Cxcl1* and *Cxcl2* was examined. Only *Cxcl5* was significantly induced upon TCDD exposure when compared with *Cxcl1* and *Cxcl2* (Figure 2A). These data support the concept that the DREs upstream of the mouse *Cxcl5* gene are functional and responsive to AHR-mediated transcriptional activation and that AHR-mediated regulation is specific to *Cxcl5*.

AHR Agonists Mediate an Enhanced Level of Cxcl5 *in Primary Keratinocytes in the Presence of IL1B*

Since TCDD alone is capable of inducing a robust induction of *Cxcl5* expression in primary mouse keratinocytes (Figure 2A), we wanted to determine if TCDD in combination with a relevant inflammatory cytokine would induce an enhanced increase in *Cxcl5* expression. IL1B induces secretion of CXCL5 in an *in vivo* model of peritonitis (Song *et al.*, 2013), In addition, UV mediated inflammation in skin leads to highly elevated levels of IL1B, and therefore selected as the model cytokine for these experiments (Brink *et al.*, 2000; Feldmeyer *et al.*, 2007). Each of the agonists used, including TCDD (2 nM), FICZ (100 nM), or BNF (5 μM), as well as IL1B (10 ng/ml) alone, significantly increased *Cxcl5* expression, whereas cotreatment with the agonists and IL1B induced an enhanced level of *Cxcl5*, with an approximately 6-fold (TCDD), 8-fold (FICZ), and 4-fold (BNF) induction compared with IL1B treatment only (Figure 2B, left panel). Consistent with previous studies, AHR agonists significantly induced *Cyp1a1* expression in keratinocytes (van den Bogaard *et al.*, 2015) (Figure 2B, right panel). In addition, the presence of IL1B with an AHR agonist repressed *Cyp1a1* expression; this is in contrast to the combinatorial effect on Cxcl5 expression. These data demonstrate that the enhanced increase in *Cxcl5* expression in the presence of IL1B is AHR-mediated and is not restricted to TCDD.

To further demonstrate the AHR dependence for enhanced induction of *Cxcl5*, primary keratinocytes from *Ahr*$^{+/-}$ or *Ahr*$^{-/-}$ mice were treated with 2 nM TCDD followed by treatment with IL1B (10 ng/ml). *Ahr*$^{+/-}$ or *Ahr*$^{-/-}$ primary keratinocytes were derived from littermates which would discount any possible effects because of genetics, housing, or microbome differences. TCDD combined with IL1B induces a significant 5-fold induction of *Cxcl5* expression in the *Ahr*$^{+/-}$ keratinocytes compared with IL1B alone, but fails to induce synergy in the *Ahr*$^{-/-}$ cells (Figure 2C). IL1B treatment alone induced *Cxcl5* expression to the same degree in both *Ahr*$^{+/-}$ and *Ahr*$^{-/-}$ keratinocytes and *Cyp1a1* induction was observed only in the *Ahr*$^{+/-}$ keratinocytes. These data demonstrate that the presence of activated AHR is crucial for the enhanced induction of *Cxcl5* expression in keratinocytes, but the AHR is not required for IL1B-mediated induction of *Cxcl5*.

AHR Regulates Cxcl5 *Expression in Keratinocytes but Not Dermal Fibroblasts*

The consequences of AHR activation are highly context-specific and dependent on factors such as pathological state, tissue, and cellular environment. To determine if AHR-dependent synergy observed in primary keratinocytes is cell-type specific, primary keratinocytes, and primary dermal fibroblasts were treated with 2 nM TCDD followed by IL1B (10 ng/ml). Combined exposure to TCDD and IL1B significantly induced *Cxcl5* expression in the dermal fibroblasts, but the extent of the induction did not reach the levels observed in primary keratinocytes (1.3-fold induction compared with 6.8-fold induction, respectively, when compared with IL1B exposure alone for each cell type) (Figure 3A). The difference in induction potential in these two skin-derived cell types may be because of the 2- to 3-fold higher levels of AHR protein in keratinocytes compared with dermal fibroblasts (Figure 3B). These data demonstrate that the AHR-mediated enhanced induction of *Cxcl5* expression in skin is likely to occur primarily in keratinocytes. In addition, the data would suggest that the level of AHR expression might play a role in the level of induction observed in the presence of IL1B. To address this question *Ahr*$^{+/+}$ *Ahr*$^{+/-}$ *Ahr*$^{-/-}$ keratinocytes were treated with IL1B for 4 h (Figure 3C). The results indicated that the level of Cxcl5 expression was much greater in *Ahr*$^{+/+}$ compared with *Ahr*$^{+/-}$ primary keratinocytes. Therefore, we focused our studies using only *Ahr*$^{+/+}$ keratinocytes.

Cxcl5 *Expression Is Repressed by AHR Inhibitors*

We investigated whether pharmacologic inhibition of AHR can reduce *Cxcl5* production because of the ability of AHR to induce its expression. Previous studies showed that these AHR inhibitors were not toxic to primary mouse keratinocytes (van den Bogaard *et al.*, 2015). To determine if pharmacological inhibition of AHR activation is effective in decreasing *Cxcl5* levels, primary mouse keratinocytes were treated with the AHR antagonists GNF351 (Smith *et al.*, 2011) or CH223191 (Kim *et al.*, 2006), followed by IL1B. Treatment with 500 nM GNF351 and 1 μM CH223191 significantly decreased levels of 10 ng/ml IL1B-induced *Cxcl5* 2.0- and 1.8-fold, respectively, compared with IL1B treatment alone (Figure 4A, left panel). Basal levels of *Cxcl5* expression were also significantly decreased by treatment with GNF351 or CH223191, two structurally diverse AHR antagonists, further supporting a role for the AHR in mediating Cxcl5 expression. Both antagonists also decreased *Cyp1a1* levels (Figure 4A, right panel).

Next, we examined whether exposure to SGA360, a selective aryl hydrocarbon receptor modulator (SAhRM), which in the presence of AHR agonists can antagonize AHR activity (Murray *et al.*, 2010) decreases *Cxcl5* expression. SGA360 (10 μM) exhibited a 3-fold reduction of IL1B-induced levels of *Cxcl5* after 24 h of exposure (Figure 4B, left panel). To determine the ability of AHR ligands to influence CXCL5 protein levels, an ELISA was performed using media collected from primary keratinocytes treated for 24 h. SGA360 (10 μM) significantly reduced both basal (2-fold reduction) and IL1B-induced (2.7-fold) CXCL5 protein levels, whereas 2 nM TCDD increased levels of CXCL5 protein in the media (Figure 4B, right panel). These data demonstrate that pharmacological inhibition of AHR is effective in decreasing gene expression of *Cxcl5* in primary mouse keratinocytes under basal and induced conditions, and that an AHR agonist and selective inhibitor can increase and reduce secreted CXCL5 protein levels, respectively.

AHR-Mediated Enhanced Induction of Cxcl5 *in Keratinocytes Is Enhanced Under High Calcium Culture Conditions*

It has been shown previously that exposing keratinocytes to high calcium-containing culture medium induces keratinocytes differentiation and enhances nuclear translocation of AHR (van den Bogaard *et al.*, 2015). As AHR-mediated induction of *Cxcl5* is regulated through the canonical DRE pathway, we wanted to determine if differentiation culture media (0.12 mM Ca^{2+}) would induce an augmented level of induction of *Cxcl5* compared with

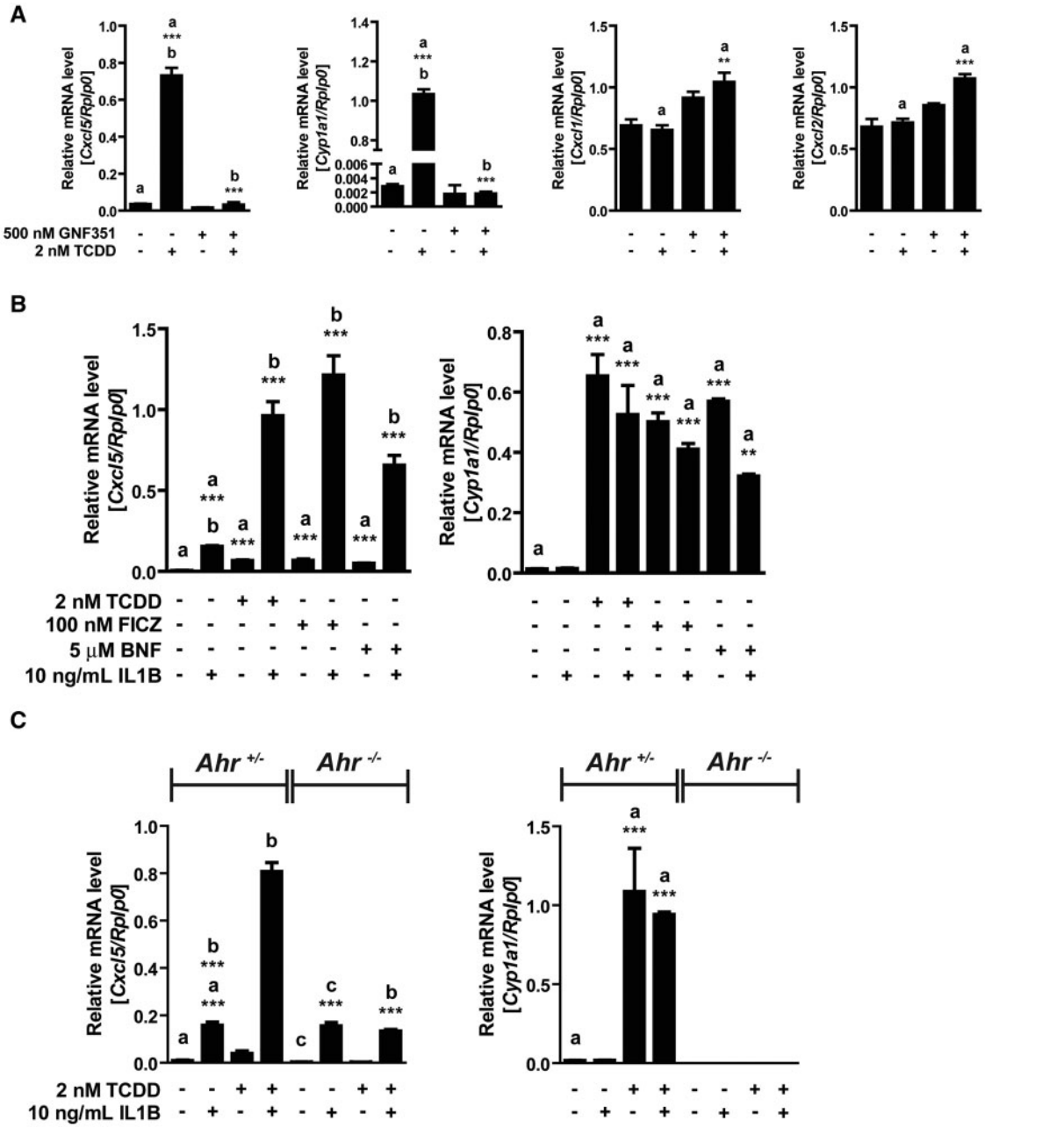

Figure 2. AHR agonists induce *Cxcl5* expression in primary mouse keratinocytes and mediate enhanced induction of *Cxcl5* in the presence of IL1B. A, $Ahr^{+/+}$ keratinocytes were pre-treated with vehicle or GNF351 for 8 h, followed by treatment with vehicle or TCDD for 4 h. B, $Ahr^{+/+}$ keratinocytes were treated with vehicle, TCDD, FICZ, or BNF for 1 h, followed by PBS or IL1B for 4 h. Statistical significance observed for *Cxcl5* with the treatments IL1B, TCDD, FICZ, and BNF, only compared with vehicle, was assessed when those treatments were compared with each other in the absence of agonist and IL1B combination treatment values. C, $Ahr^{+/-}$ or $Ahr^{-/-}$ keratinocytes were pretreated for 1 h with vehicle or TCDD followed by PBS or IL1B for 4 h. Expression levels were determined by qRT-PCR.

keratinocytes grown in proliferation media (0.05 mM Ca^{2+}). Exposure to differentiation culture media induced a statistically significant increase in TCDD (2 nM) and IL1B (10 ng/ml) exposed cells compared with keratinocytes exposed to proliferation media that were treated in the same manner (Figure 5, left panel). Additionally, the TCDD-induced increase in *Cyp1a1* was significantly enhanced under differentiation culture conditions compared with proliferation culture conditions (Figure 5, right panel). Therefore, media conditions, which promote enhanced differentiation of primary keratinocytes also contribute to a higher level of *Cxcl5* expression in an AHR-mediated manner.

AHR Mediates Cxcl5 *Expression After UVB Exposure* In Vivo

To determine the *in vivo* consequences of AHR activation under conditions of increased inflammatory signaling, mice were exposed to UVB (360 mJ/cm^2), followed by topical application of FICZ (100 ng directly after exposure, followed by reapplication every 8 h). UVB exposure significantly induced *Cxcl5* levels (2-fold) in the skin of $Ahr^{+/+}$ mice compared with sham irradiated mice, but when UV exposure was combined with FICZ treatment, the level of induction of *Cxcl5* was enhanced an additional 1.7-fold compared with UVB exposure alone (Figure 6A, left panel). UVB exposure alone did not significantly increase *Cyp1a1* expression in the skin of the exposed mouse ears,

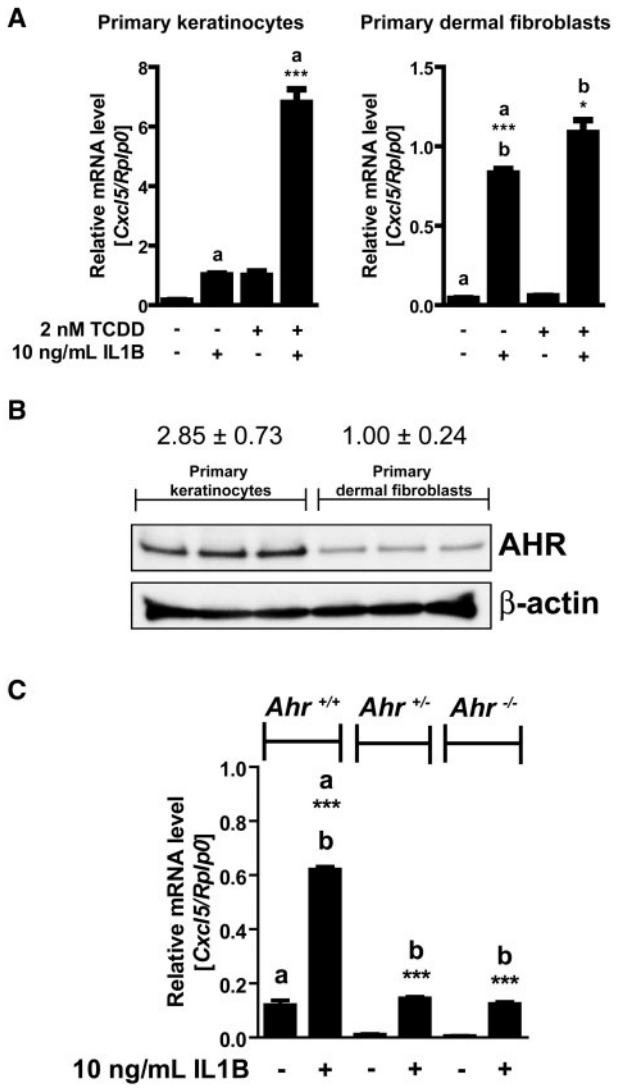

Figure 3. Keratinocytes are the primary mediators of AHR-dependent enhanced *Cxcl5* expression. A, Primary keratinocytes or primary dermal fibroblasts from $Ahr^{+/+}$ mice were treated for 1 h with vehicle or TCDD followed by treatment with PBS or IL1B for 4 h. Expression of *Cxcl5* was determined by qRT-PCR. B, Western blot depicting relative levels of AHR protein in primary keratinocytes or primary dermal fibroblasts from $Ahr^{+/+}$ mice. Quantification was conducted using ImageJ software and each value was normalized to β-actin. C, Comparison of IL1B-mediated *Cxcl5* expression in $Ahr^{+/+}$ $Ahr^{+/-}$ $Ahr^{-/-}$ keratinocytes.

whereas FICZ with or without UVB exposure significantly induced *Cyp1a1* (3-fold induction). Notably, UVB exposure significantly decreased FICZ-induced *Cyp1a1* induction by 36% (Figure 6A, right panel). This repressive effect has been observed in a number of model systems upon exposure to cytokines, which would be expressed 24 h after UV exposure (Barker *et al.*, 1992; Morgan, 1997; Reiners *et al.*, 1993). In $Ahr^{-/-}$ mice, neither UVB nor FICZ exposure significantly influenced *Cxcl5* in the exposed ears (Figure 6B, left panel). *Cyp1a1* expression was not influenced by UVB or FICZ exposure in $Ahr^{-/-}$ mice (Figure 6B, right panel). These data demonstrate that AHR is an important mediator of UVB-induced *Cxcl5* expression *in vivo*. The dose of UVB chosen for these studies has been utilized in skin carcinogenesis studies, often in a repetitive UV exposure protocol, and a single dose does not result in sunburn (Ming *et al.*, 2015). In our studies, invasion of neutrophils into skin after 24 h was not significantly observed by histological analysis (data not shown).

DISCUSSION

In this study, we have identified *Cxcl5* as a novel target of AHR-mediated gene expression in primary mouse keratinocytes. A physiological role for the AHR in the regulation of *Cxcl5* in the skin was previously suggested by the results of a DNA microarray conducted on mice expressing a constitutively active form of the AHR in keratinocytes. These mice developed inflammatory skin lesions resembling atopic dermatitis. Among the chemokines analyzed in the microarray, *Cxcl5* ranked as the most highly expressed (Tauchi *et al.*, 2005) but the authors did not investigate whether the increase in *Cxcl5* was because of direct AHR regulation. We hypothesized a direct role for AHR in *Cxcl5* regulation when the presence of nine adjacent overlapping DRE sequences was observed upstream of the transcription start site. We demonstrate here that AHR-mediated regulation of *Cxcl5* is most likely because of transcriptional activation by AHR via direct DRE binding, as opposed to an alternative pathway such as the interaction of AHR with another transcriptional mediator. It should be noted that the close proximity of the DRE sequences precludes the possibility that each DRE would allow simultaneous AHR/ARNT occupancy of each motif. Site-directed mutagenesis could be used to determine how many and which specific DREs contribute to the ability of AHR to induce *Cxcl5* expression. To our knowledge, a series of repetitive adjacent DRE elements has not been observed on other target genes.

In addition to the regulation of *Cxcl5* by AHR, this study provides further evidence for a model by which agonist-activated AHR potentiates inflammatory gene expression in an enhanced manner in the presence of additional stimuli, including the proinflammatory mediator IL1B, and under elevated calcium concentrations. Calcium levels in the epidermis regulate the terminal differentiation of keratinocytes (Bikle *et al.*, 2012), so the inflammatory status of the skin as well as the degree of differentiation could mediate the extent to which AHR agonists can induce *Cxcl5* expression. Our previous work established this combinatorial model of regulation for *IL6* in human tumor cell lines in the presence of IL1B (Hollingshead *et al.*, 2008) and for *Ccl20* in primary mouse peritoneal macrophages in the presence of LPS (Lahoti *et al.*, 2015), establishing this as a cross-species and cross-context phenomenon. The effect was first observed in human tumor cell lines that were minimally responsive to IL1B-mediated gene induction of *IL6* but found to robustly induce expression in the combined presence of TCDD and IL1B (Hollingshead *et al.*, 2008). The mechanism for this effect was determined to be because of the binding of AHR to nonconsensus DREs upstream of the *IL6* transcriptional start site, which in turn allowed a conformational change to occur, rendering the promoter more accessible for the IL1B-induced binding of NFkB factors to their response elements. In addition, the dismissal of histone deacetylase 1 (HDAC1), and reduction of HDAC3 on the promoter following combinatorial treatment with TCDD and IL1B enhanced acetylation of p65 bound to the *IL6* promoter (DiNatale *et al.*, 2010). Though additional work must confirm that a similar mechanism governs the regulation of *Cxcl5* in mouse keratinocytes, it is a probable explanation because of both the presence of a RelA binding site in the promoter of *Cxcl5*, and the sequential DREs discussed previously (Figure 1A). Interestingly, the DRE elements are localized distally from the RelA response element(s) in both the *IL6* and *Cxcl5* regulatory

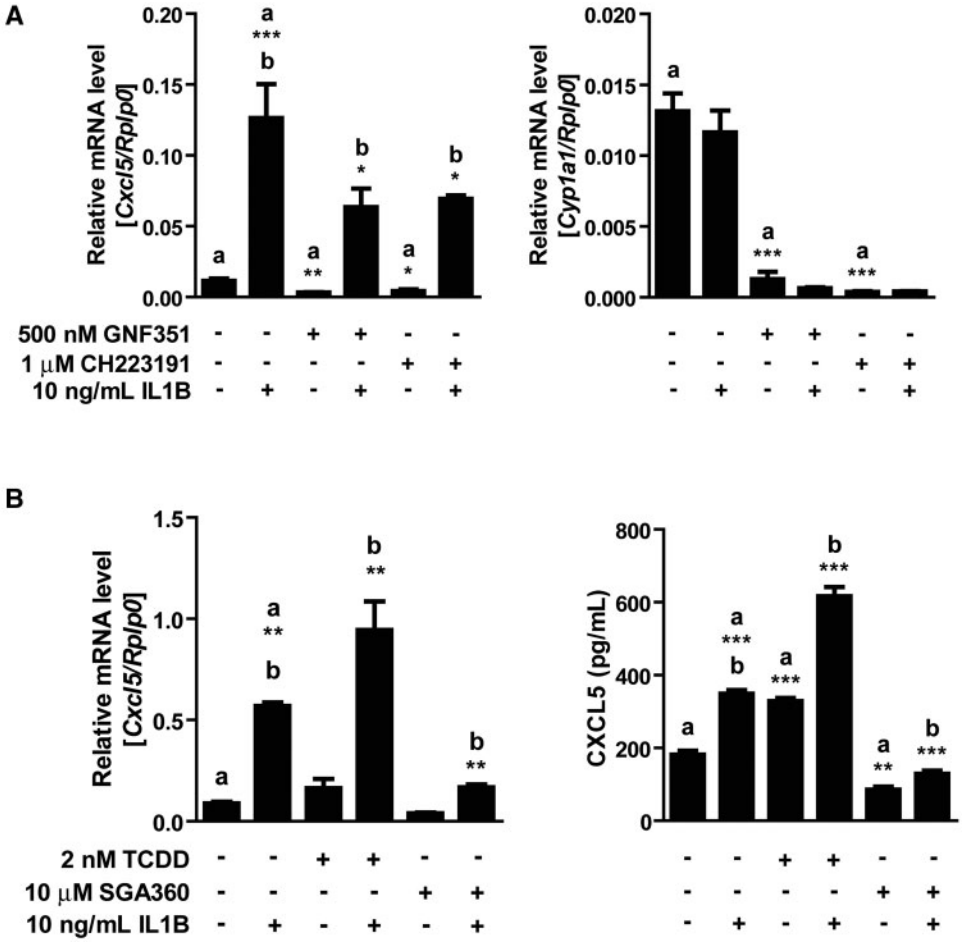

Figure 4. Antagonism of AHR activity represses basal and IL1B-induced *Cxcl5* levels in primary mouse keratinocytes. A, Keratinocytes from $Ahr^{+/+}$ mice were pretreated for 1 h with vehicle, GNF351 or CH223191 followed by treatment with PBS or IL1B for 4 h. Gene expression was determined by qRT-PCR. B, Keratinocytes from $Ahr^{+/+}$ mice were treated with vehicle, TCDD or SGA360 for 1 h, followed by treatment with PBS or IL1B for 23 h. Cells were redosed with vehicle or SGA360 at 12 h. Expression of *Cxcl5* was determined by qRT-PCR (left panel). Media was collected from keratinocytes and used for ELISA (right panel).

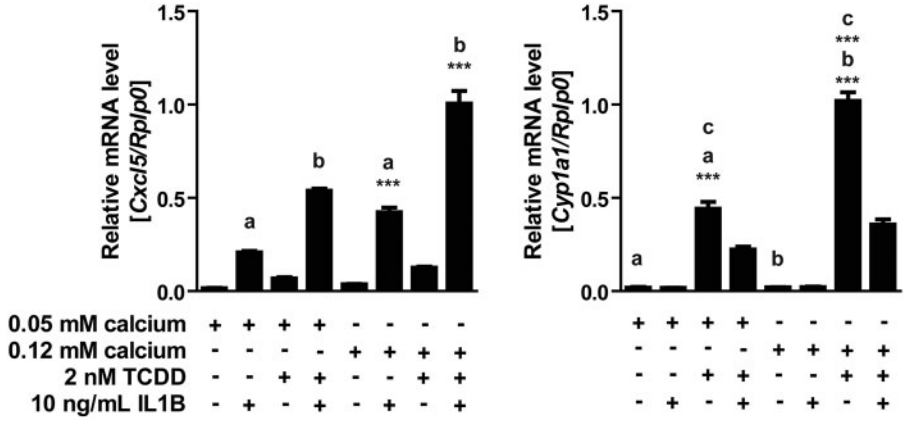

Figure 5. High calcium media enhances AHR-driven enhanced *Cxcl5* induction. $Ahr^{+/+}$ keratinocytes were exposed to either proliferation (0.05 mM Ca^{2+}) or differentiation media conditions (0.12 mM Ca^{2+}) for 7 h, followed by treatment with vehicle or TCDD for 1 h, then treatment with PBS or IL1B (4 h), for a total exposure time of 12 h. Expression of *Cxcl5* (left) and *Cyp1a1* (right) was determined by qRT-PCR.

regions. This observation promotes the likely scenario that other inflammation-related genes may be regulated in this manner and need to be identified and characterized in humans and mice. We therefore propose the term 'xenokine' to describe this subclass of inflammatory genes which exhibit increased gene expression in the presence of AHR agonists in combination with inflammatory stimuli. As more studies indicate an imperative role for AHR in immune regulation, identification of xenokine genes will aid in elucidating the role of AHR under specific pathological conditions. As further functions of AHR are

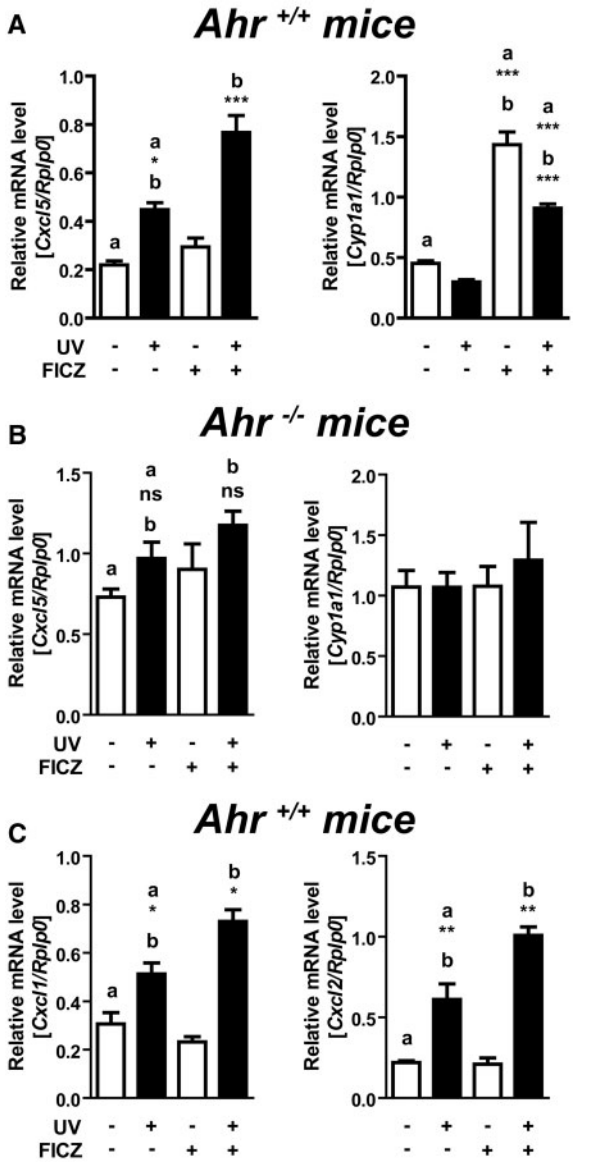

Figure 6. FICZ enhances UVB-mediated *Cxcl1*, *Cxcl2*, *Cxcl5* expression *in vivo*. Mice ($n = 4–5$) were anesthetized followed by sham or UVB exposure. UVB-exposed mice received a total dose of 360 mJ/cm^2 UVB. Immediately following exposure, the left ears (control) were treated with in DMSO/acetone (carrier solvent), and the right ears received 100 ng of FICZ in DMSO/acetone. Treatments were reapplied every 8 h, and the mice were sacrificed at 24 h after the initial exposure. Whole ears were collected and used for analysis. Expression of *Cxcl5* and *Cyp1a1* was analyzed by qRT-PCR in *Ahr*$^{+/+}$ (A) and *Ahr*$^{-/-}$ (B) mice. C, Expression of *Cxcl1* and *Cxcl2* in *Ahr*$^{+/+}$ mice.

defined, pharmacological intervention can be used to manipulate AHR activity.

Compounds which inhibit the binding of AHR to DRE sequences are effective in decreasing levels of *Cxcl5* induction, and this provides further evidence for a DRE-dependent mechanism of regulation. The two AHR antagonists GNF351 and CH223191 and the SAhRM, SGA360, were able to significantly reduce both basal and IL1B-induced *Cxcl5* levels. Though SGA360 was previously characterized as exhibiting SAhRM activity, in the case of *Cxcl5* inhibition, it is likely that its ability to antagonize DRE-dependent gene expression is responsible for its effects and it is therefore operating in the context of these experiments as a typical AHR antagonist. The reduction of basal levels of *Cxcl5* by these antagonists indicates the likely presence of an endogenous AHR agonist in keratinocytes. The characterization of tryptophan products such as indole, kynurenine, indoxyl sulfate, and FICZ as endogenous ligands of the AHR (Murray and Perdew, 2017), and their importance in a variety of processes, means that AHR control of xenokines could have implications in many biological functions. This study has also established for the first time a physiological, *in vivo* relevance for AHR-mediated regulation of xenokine gene expression, as mice exposed to UV and treated directly after with FICZ demonstrated a greater induction of *Cxcl5* gene expression in the skin than UV exposure alone. The ability of AHR antagonists to reduce levels of *Cxcl5* indicates that the development of therapeutics inhibiting AHR function could be useful in reducing excessive neutrophil chemotaxis in the skin after UVB exposure. Topical application of an AHR antagonist was able to reduce UVB-induced gene expression in the skin of humans (Tigges *et al.*, 2014), and whether this compound can inhibit *Cxcl5* expression *in vivo* could be used to examine this hypothesis.

Our UV alone in vivo treatments failed to induce *Cyp1a1* gene expression in skin, in contrast to reports in the literature that UV can mediate *Cyp1a1* expression (Fritsche *et al.*, 2007; Katiyar *et al.*, 2000; Mukhtar *et al.*, 1986). The reason for this is most likely because of the dramatically higher level of UV exposure used in these studies, although other differences in animal models used may also play a role. For example, studies in mice examining UV mediated AHR activation utilized an exposure regiment of 600 mJ/cm^2, whereas our study utilized 360 mJ/cm^2 (Fritsche *et al.*, 2007). The ability of the AHR after UV to mediate a robust induction of *Cxcl5* is likely because of combinatorial regulation through the AHR along with NFkB or other transcriptional factor(s) that respond to UV stimuli. This would suggest that AHR combinatorial regulation of gene expression is more sensitive measure of AHR activation compared with an AHR target gene that is solely regulated by the AHR, such as *Cyp1a1*. Furthermore, *Cyp1a1* is known to be repressed by inflammatory signaling and thus may be an additional reason for the lack of *Cyp1a1* induction observed after UV exposure in vivo (Morgan, 1997).

The AHR-mediated control of xenokine gene expression adds to an already complex narrative on the detrimental versus beneficial nature of cutaneous AHR. Locally produced AHR agonists in the skin include those derived from tryptophan upon UV exposure (Fritsche *et al.*, 2007), photooxidation products of skin surface lipids (Kostyuk *et al.*, 2012), and microbial products, such as those produced by the commensal and occasionally pathogenic yeast *Malassezia* (Magiatis *et al.*, 2013). Production of these ligands could lead to increased AHR transcriptional activity and therefore potentially excessive xenokine gene expression. Thus, the AHR acts as an epidermal sensor, and could influence both quantitative and qualitative aspects of the skin microbial population, as increased AHR ligands derived from a yeast infection combined with cytokines could lead to an enhanced response. However, these agonists could also lead to sustained expression of xenokine genes potentially mediating tissue toxicity, thus AHR inhibition could be of therapeutic interest. However, activation of the AHR also has beneficial properties with regards to the maintenance of a healthy skin phenotype and the response to infection. A recent study highlighted the importance of AHR ligands in the diet for the maintenance of proper skin barrier integrity. Upon the removal of dietary AHR ligands, transepidermal water loss (TEWL) was

similar to the impaired levels observed in *Ahr*$^{-/-}$ mice. When AHR is specifically knocked out in keratinocytes, increased levels of TEWL were observed, further demonstrating the protective role of AHR in the keratinocytes in this model. The addition of AHR ligands to the diet rescued barrier integrity in these mice (Haas *et al.*, 2016). Agonist-activated AHR is also involved in keratinocyte differentiation, a process that is essential for proper barrier function of the skin (Sutter *et al.*, 2011; van den Bogaard *et al.*, 2013, 2015). Impaired differentiation and inflammation are hallmarks of inflammatory skin diseases such as psoriasis and atopic dermatitis, and therefore agonist-driven AHR is postulated to have a protective effect under these disease conditions. The seeming contradictory nature of AHR activation in the skin in regards to disease treatment is well recognized, and has been addressed in a recent review (Haarmann-Stemmann *et al.*, 2015).

Additionally, previous studies have begun to characterize a role for AHR in neutrophil biology, particularly in the lungs. In an influenza model of lung inflammation, neutrophil recruitment to the lungs was enhanced upon TCDD exposure, which was because of AHR activation at the site of infection rather than AHR in the neutrophils (Teske *et al.*, 2008). However, earlier studies from the same group determined that the AHR-dependent increase in neutrophil infiltration to the lungs in this model was not because of enhanced levels of neutrophil chemo attractants in the bronchoalveolar lavage fluid, including CXCL5 (*aka* LIX) (Teske *et al.*, 2005). In contrast, it was observed in a different study that *Ahr*$^{-/-}$ mice had a more inflammation-inducible phenotype, in that they exhibited more lung inflammation upon cigarette smoke exposure when compared with controls, as well as enhanced neutrophilia upon endotoxin challenge (Thatcher *et al.*, 2007). A recent review explains this seeming contradiction by postulating that sustained exposure to TCDD in the influenza study may mimic an AHR-null phenotype (Stockinger *et al.*, 2014). However, the complexity which governs AHR activation precludes making an overarching conclusion about the role of the receptor in neutrophil recruitment; it is likely that cell-type, and context-specific effects are present.

Future studies are needed to determine the relevance of AHR activation in the skin and other barrier tissues for direct recruitment of neutrophils. Since the data presented is only in mice, future studies are needed to explore the role of AHR activation in human Cxcl5 expression. Whether the increased expression in the skin of *Cxcl5* leads to direct physiological consequences (ie, enhanced neutrophil chemotaxis to the skin) is the subject of current study in our laboratory. As specific effects of AHR are highly context-dependent, our observation of AHR-mediated control of *Cxcl5* expression could be specific to keratinocytes. Further work will elucidate whether the enhanced *Cxcl5* induction observed in our study is also present in other epithelial barrier cells, such alveolar epithelial type II cells or enterocytes.

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