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Research Article

Aryl Hydrocarbon Receptor Activation Synergistically Induces Lipopolysaccharide-Mediated Expression of Proinflammatory Chemokine (c–c motif) Ligand 20

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ABSTRACT

The Ah receptor (AHR) is directly involved in the regulation of both innate and adaptive immunity. However, these activities are poorly understood at the level of gene regulation. The chemokine (c–c motif) ligand 20 (CCL20) plays a nonredundant role in the chemoattraction of C–C motif receptor 6 expressing cells (eg, T cells and others). A survey of promoter regions of chemokine genes revealed that there are several putative dioxin responsive elements in the mouse *Ccl20* promoter. The addition of an AHR agonist along with lipopolysaccharide (LPS) to cultured primary peritoneal macrophages results in synergistic induction of both *Ccl20* mRNA and protein, compared with each compound alone. Through the use of macrophage cultures derived from $Ahr^{-/-}$ and $Ahr^{nls/nls}$ mice, it was established that expression of the AHR and its ability to translocate into the nucleus are necessary for AHR ligand-mediated synergistic induction of *Ccl20*. Gel shift analysis determined that a potent tandem AHR binding site ~3.1 kb upstream from the transcriptional start site can efficiently bind the AHR/ARNT (aryl hydrocarbon receptor/AHR nuclear translocator) heterodimer upon activation with a number of AHR agonists. Furthermore, studies reveal that LPS increases AHR levels on the *Ccl20* promoter while decreasing HDAC1 occupancy. The level of *Ccl20* constitutive expression in the colon is greatly attenuated in $Ahr^{-/-}$ mice. These studies suggest that the presence of AHR ligands during localized inflammation may augment chemokine expression, thus participating in the overall response to pathogens.

Key words: aryl hydrocarbon receptor; AHR agonists; chemokine (c–c motif); ligand 20; macrophages; lipopolysaccharide; chemokine.

Chemokines are a diverse superfamily of low molecular weight chemotactic cytokines, which mediate leukocyte migration and hence play crucial roles in immune homeostasis and inflammation (Murdoch and Finn, 2000). The innate immune system provides the first line of defense to pathogens, primarily via recognition of foreign molecules through pattern recognition receptors (Kawai and Akira, 2010). Such signaling drives the production of a distinct set of chemokines and cytokines that dictate the specificity of the adaptive immune response (Luster, 2002). Chemokines are classified by a number of criteria, including their arrangement of conserved cysteine residues in the N-terminus. Four configurations stratify this superfamily into the C, C-C, CXC, and CX3C subfamilies (Murdoch and Finn, 2000; Zlotnik and Yoshie, 2000). Chemokines can also be functionally classified as homeostatic versus inflammatory (Comerford and McColl, 2011). Inflammatory chemokines are expressed at low levels basally, but are induced at the mRNA level by proinflammatory cytokines and pathogen-derived insults (Ye and Young,

1997). Chemokines mediate directed chemotaxis by binding and signaling through transmembrane G protein–coupled receptors.

The chemokine (c–c motif) ligand 20 (CCL20) is a member of the C–C family of chemokines that bind a single cognate receptor, C–C motif receptor 6 (CCR6), expressed on the surface of immature dendritic cells, B cells, T_H17 cells, and Treg cells (Le Borgne *et al.*, 2006; Schutyser *et al.*, 2003; Yamazaki *et al.*, 2008). Basal *Ccl20* expression varies across tissue types, but can be markedly induced by inflammatory cytokine challenge or pathogen-derived insults such as LPS (Hosokawa *et al.*, 2005; Schutyser *et al.*, 2000; Sierro *et al.*, 2001). As with many chemokines, inducible expression is regulated at the transcriptional level by NF-kB and AP-1 transcription factors (Kanda *et al.*, 2009). Its pleiotropic nature has made the delineation of the cell-specific recruitment difficult to determine; however, it is likely that effector cell types recruited by Ccl20 is highly context dependent (Comerford *et al.*, 2010). CCL20 has been implicated in a multitude of human pathologies, including cancer of a variety of tissues, atherosclerosis, psoriasis, rheumatoid arthritis, and multiple sclerosis (Harper *et al.*, 2009; Hirota *et al.*, 2007; Lahoti *et al.*, 2013; Rubie *et al.*, 2010). As such, a detailed understanding of mechanisms underlying *Ccl20* expression is warranted.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and member of the basic helix-loop-helix, Per-Arnt-Sim family of proteins. Historically, AHR has been studied as a mediator of polycyclic aromatic hydrocarbon metabolism and resulting carcinogenesis. In addition, the toxicities associated with dioxin are almost completely mediated by the AHR (Bunger *et al.*, 2003). Prior to ligand activation, AHR resides in the cytoplasm complexed with a number of chaperones and cochaperones, including the 90 kDa heat shock protein (HSP90) (Petrulis and Perdew, 2002). Upon agonist binding the AHR translocates to the nucleus, disassociates from HSP90 and heterodimerizes with its binding partner, AHR nuclear translocator (ARNT). This heterodimeric complex is capable of binding to dioxin-response elements (DREs) and transactivating a number of genes involved in a wide variety of functions, including xenobiotic metabolism, eg, cytochrome P450 family 1 member 1A (*Cyp1a1*) (Beischlag *et al.*, 2008). Recently, AHR has been shown to interact with other transcription factors to drive cytokine production and developmental programing in the immune system (Apetoh *et al.*, 2010; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). In particular, the AHR can physically interact with the transcription factor NF-kB, leading to gene regulation at many inflammatory gene promoters (DiNatale *et al.*, 2010; Vogel *et al.*, 2013, 2014).

Here we employ an *ex vivo* murine macrophage system to explore the cooperation between the AHR and inflammatory signaling to drive *Ccl20* gene expression. We show that activation of AHR with ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or activation of NF-kB via toll-like receptor 4 (TLR4) agonist, lipopolysaccharide (LPS), induces *Ccl20* gene expression. Combinatorial treatments with both stimuli synergize to drive *Ccl20* gene expression in primary macrophages. These observations contribute to the complexity of chemokine gene expression, suggesting that the presence of AHR ligands may affect the recruitment of T cells and other CCR6 expressing cells to tumors or sites of infection.

MATERIALS AND METHODS

Reagents. TCDD was kindly provided by Dr Stephen Safe (Texas A&M University, College Station, Texas). Indolo[3,2*b*]carbazole (ICZ) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, Missouri). Recombinant mouse IL1B was acquired from PeproTech (Rocky Hill, New Jersey). LPS was acquired from Santa Cruz (Dallas, Texas).

Mouse experiments. C57BL6/J (Ahr^b), *Ahr* null ($Ahr^{-/-}$), *Ahr* heterozygous ($Ahr^{+/-}$), $Ahr^{nls/nls}$ (Ah^d allele) mice, which have the nuclear localization sequence (NLS) disrupted (kindly provided by Dr Christopher Bradfield, University of Wisconsin-Madison), and Ah^d congenic (Ahr^d) mice obtained from Jackson (Bar Harbor, Maine) were housed on corncob bedding in a pathogen-free, temperature- and light-controlled facility and given access to food and water *ad libitum*. *Ahr* null ($Ahr^{-/-}$) and *Ahr* heterozygous ($Ahr^{+/-}$) mice were generated through the breeding of an $Ahr^{+/-}$ female (Ah^b allele) with an $Ahr^{-/-}$ male mice. The Ah^b and Ah^d alleles exhibit high affinity and 10-fold lower affinity for TCDD, respectively. Mouse experiments utilized eight 6- to 8-week-old mice and were performed humanely with techniques approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

Primary peritoneal macrophage isolation from mice. For the isolation of primary peritoneal macrophages, mice were IP injected with 3 ml of 3% thioglycolate media on day 1. After 72 h mice were euthanized through asphyxiation. Primary peritoneal macrophages were isolated by peritoneal lavage using ice-cold phosphate buffered saline (PBS). Cells were recovered by centrifugation and resuspended in macrophage cell culture medium (Zhang *et al.*, 2008).

Cell culture. Primary peritoneal macrophages were maintained at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (Gibco, Carlsbad, California) supplemented with 10% fetal bovine serum (Hyclone Labs, Logan, Utah), 100 IU/ml penicillin and 100 μg/ml streptomycin, 2mM L-glutamine, and 1 mM sodium pyruvate (Sigma).

Primary keratinocyte isolation from mice. Keratinocytes from less than 3-day-old Ahr^b, $Ahr^{-/-}$, and $Ahr^{+/-}$ mice were isolated and cultured in primary keratinocyte medium as described previously (Dlugosz *et al.*, 1995).

RNA extraction and quantitative RT-PCR. Upon treatments, total RNA was isolated from macrophages as previously described using Trizol (Invitrogen Carlsbad, California) (Murray *et al.*, 2010b). Total RNA was converted into cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, California). Gene expression was measured using quantitative RT-PCR as previously described (Murray *et al.*, 2010b), utilizing the primers described in Supplementary Table S1.

CCL20 ELISA. CCL20 ELISA was performed on macrophage culture supernatants. Harvested supernatants were briefly spun at 5000 rpm for 3 min to remove cellular debris. ELISA was performed as per the manufacturer's protocol (Cayman Chemicals).

Electromobility shift assay. Electrophoretic mobility shift assays (EMSA) were performed using *in vitro*-translated mouse AHR and ARNT proteins or nuclear extracts from peritoneal macrophages exposed to vehicle or TCDD as described previously (Flaveny *et al.*, 2009; Singh *et al.*, 1996). *Ccl20* oligonucleotides spanning the AHR binding site are described in Supplementary Table S2.

Preparation of cytosolic and nuclear fractions. Cytosolic and nuclear fractions were prepared from primary peritoneal macrophages

isolated from Ahr^{b} and $Ahr^{-/-}$ mice as described previously (Lahoti *et al.*, 2013). Protein samples were resolved on 8% polyacrylamide gel and transferred onto polyvinylene difluoride membranes. Primary and secondary antibodies are described in Supplementary Table S3.

Chromatin immunoprecipitation assays. Primary macrophages were isolated from C57BL6/J mice and plated in 150-mm cell culture plates. Macrophages were treated with 5 ng/ml lipopolysaccharide (LPS) for 45 min or 10nM TCDD for 30 min followed by 5 ng/ml LPS for 45 min. Each treatment was performed in triplicate. Following treatment, the medium was removed and cells were chemically cross-linked using 1% formaldehyde in warm PBS for 10 min at room temperature. Cross-linking was terminated by adding glycine to a final concentration of 0.125 M for 5 min at room temperature. Cells were washed twice with cold PBS and collected in harvest buffer (100 mM Tris–HCl, pH 8, 10 mM dithiothreitol). The cells were centrifuged at 2000 rpm for 5 min, washed with PBS, and resuspended in lysis buffer (1% lithium dodecyl sulfate, 50 mM EDTA, protease inhibitor cocktail, 50 mM Tris–HCl, pH 8). Chromatin was sheared with the Bioruptor water bath sonicator (Diagenode, Sparta, New Jersey) to an average size of 0.5–1 kb and centrifuged at 10 000 rpm for 10 min. The sheared chromatin was diluted to 50 μg/ml in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8) and 500 μl chromatin was incubated for 4 h at 4°C with 2 μg of the following antibodies: rabbit anti-AhR (a kind gift from Dr Pollenz, University of South Florida), anti-HDAC1 (Santa Cruz Biotechnology, California), and rabbit IgG (Santa Cruz Biotechnology) as control. Immunoadsorbed complexes were captured on Protein G magnetic beads (New England BioLabs, Massachusetts) for 2 h at 4°C and washed 3 times with RIPA (1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 140 mM NaCl, 10 mM Tris–HCl, pH 8) followed by one wash with TE8 (1 mM EDTA, 10 mM Tris–HCl, pH 8). DNA was eluted in 200 μl elution buffer (100 mM $NaHCO_3$, 1% SDS) at 65°C overnight. Eluted DNA was purified using the chromatin immunoprecipitation assays (ChIP) DNA Clean & Concentrator kit (Zymo Research, California) and analyzed by quantitative real-time PCR.

RESULTS

Treatment With an AHR Agonist in Combination With LPS Synergistically Induces Ccl20 Gene Expression in Macrophages

A previous report suggested that expression of a constitutively active AHR in skin of transgenic mice induced *Ccl20* expression. However, this study did not identify whether the AHR directly regulated *Ccl20* gene expression (Tauchi *et al.*, 2005). Numerous reports suggest that *Ccl20* gene expression can be significantly induced by microbial factors such as LPS, contributing to human pathologies such as sepsis (Crane-Godreau and Wira 2005; Scapini *et al.*, 2002). Stimulated macrophages have been shown to secrete CCL20 and contribute to the chemotaxis of inflammatory T cells to a given site (Li *et al.*, 2013). Therefore the role of AHR activation on *Ccl20* expression in the absence or presence of LPS was examined utilizing an *ex vivo* mouse macrophage system. Our initial data suggested that activation of AHR in primary peritoneal macrophages by prototypical AHR ligand TCDD and/or gram-negative bacterial cell wall product LPS stimulated a dose-dependent increase in *Ccl20* mRNA expression (Figs. 1A and 1B). This suggests that there are multiple mechanisms capable of inducing *Ccl20* gene regulation. Furthermore, the addition of both TCDD and LPS in macrophages resulted in an 8-fold synergistic induction of *Ccl20*, indicating a complex interplay between activation of AHR and TLR pathways that was previously poorly described (Fig. 1B). We also observed a time dependency in synergy as maximum induction of *Ccl20* expression was identified at 4 h post TCDD and LPS coexposure (Supplementary Fig. 1S). The reason for the reduced level of synergism observed at 6 h is not known. In addition, macrophages coexposed to different AHR agonists, such as ICZ, combined with LPS, initiated a similar increase in *Ccl20* expression (Fig. 1C).

AHR/LPS-Mediated Synergistic Induction of Ccl20 Is a Direct Transcriptional Event

To further study the role of agonist-mediated AHR–ARNT activation on *Ccl20* gene transcription, macrophages were pre-exposed to protein translation inhibitor cycloheximide (CHX; 10 μg/ml) for 1 h. In the absence of CHX, TCDD and LPS synergistically induced expression of *Ccl20* (Fig. 1D). Pre-exposure to CHX had no effect on the synergistic induction of *Ccl20* mRNA mediated by TCDD and LPS cotreatment (Fig. 1D). The failure of CHX to alter the TCDD/LPS synergistic effect on the induction of *Ccl20* mRNA expression is consistent with the assertion that AHR-mediated induction of *Ccl20* is a direct primary transcriptional event. It is important to note that the increased AHR-mediated transcriptional activation of *Cyp1a1* has been observed in the presence of CHX (Joiakim *et al.*, 2004; Lusska *et al.*, 1992; Ma and Baldwin, 2002).

Synergistic Induction of Ccl20 Expression by AHR Activation Can Also Be Observed in IL1B-Stimulated Macrophages and in LPS-Treated Primary Keratinocytes That Express AHR

Previous studies have shown that cells stimulated with IL1B can also induce *Ccl20* gene expression (Lahoti *et al.*, 2013; Shindo *et al.*, 2014). Thus, we investigated whether IL1B and TCDD cotreated macrophages could synergistically induce *Ccl20* mRNA expression. Similar to the results obtained with LPS, cells exposed to IL1B lead to potentiation of TCDD-mediated expression of *Ccl20* (Fig. 2A). Studies have shown that CCL20 plays a critical role in psoriasis and higher levels of CCL20 protein were observed in keratinocytes from psoriatic patients (Harper *et al.*, 2009; Kennedy-Crispin *et al.*, 2012). We wanted to investigate whether the synergy in *Ccl20* mRNA expression obtained in macrophages could also be observed in primary mouse keratinocytes. The data revealed a 5-fold induction of *Ccl20* in primary keratinocytes from Ahr^{b} mice exposed to LPS and TCDD compared with either agent alone (Fig. 2B). These results also reveal that the observed synergy is AHR dependent, as keratinocytes from $Ahr^{+/-}$ and $Ahr^{-/-}$ mice largely failed to respond. In addition, lack of AHR expression attenuated LPS-mediated induction of *Ccl20*, suggesting that AHR contributes to the induction of *Ccl20* even in the absence of exogenous ligand.

LPS Mediates an Increase in AHR Protein Levels

Macrophages exposed to LPS resulted in a time-dependent increase in *Ahr* transcription, with a maximum level of induction obtained after 3 h of LPS exposure (Fig. 3A). In addition, AHR protein levels were enhanced in a time-dependent manner (Fig. 3B). Next, we wanted to test whether LPS-mediated up-regulation in AHR protein levels influenced target gene expression. Thus, macrophages from Ahr^{b} and $Ahr^{-/-}$ mice were treated with LPS for 24 h, followed by TCDD exposure for 3 h. The results established that the induction of both *Cyp1a1* and *Ccl20* mRNA expression by TCDD was dependent on AHR expression (Fig. 3C). In addition, synergistic induction of *Ccl20* was observed in macrophages from Ahr^{b} mice when exposed to

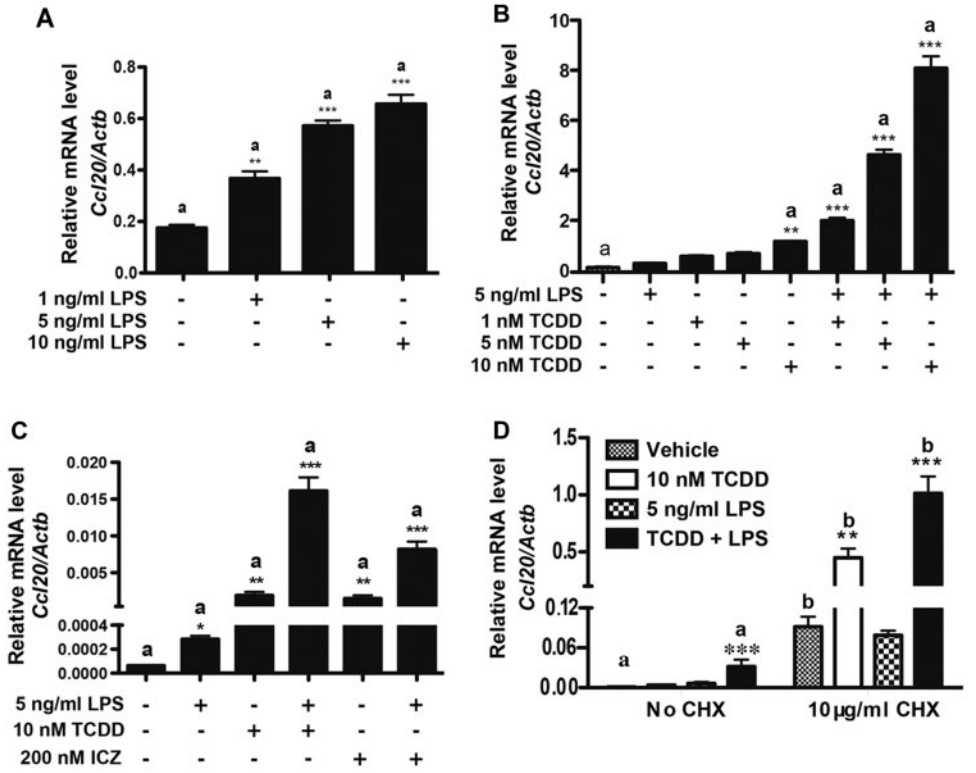

FIG. 1. AHR activation coupled with LPS exposure synergistically induces *Ccl20* in primary macrophages isolated from Ahr^b mice. The level of *Ccl20* expression was assessed through qRT-PCR analysis. A, Macrophages were treated with increasing concentrations of LPS for 3 h. B, Macrophages were pretreated with increasing concentrations of TCDD for 1 h, followed by 5 ng/ml LPS for 4 h. C, Macrophages isolated from Ah^b mice pretreated with either 10 nM TCDD or 200 nM ICZ for 1 h, followed by 5 ng/ml LPS for 4 h. D, Macrophages were pretreated with TCDD for 1 h followed by 5 ng/ml LPS for 4 h. Where indicated, CHX was added 10 min prior to the addition of TCDD. Data represent mean mRNA levels normalized to *Actb* mRNA expression ± SEM (n = 3/treatment group; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$).

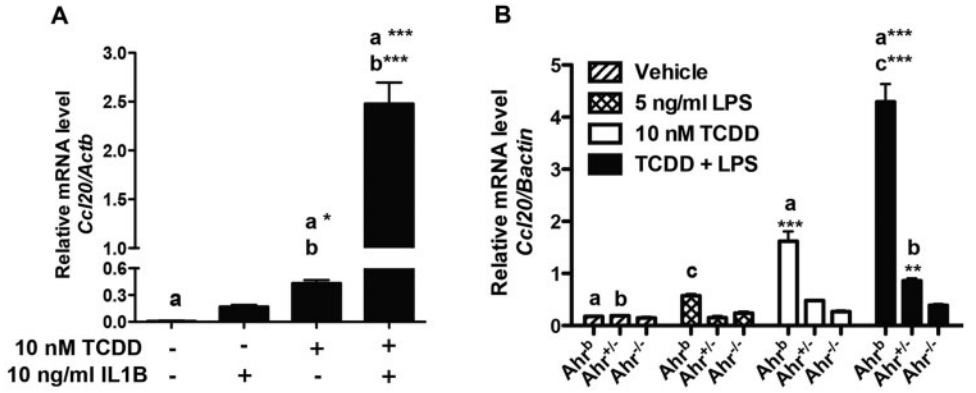

FIG. 2. IL1B and TCDD synergize to induce *Ccl20* in primary macrophages and LPS/TCDD cotreatment synergistically induces *Ccl20* in primary keratinocytes dependent on AHR expression. A, Macrophages were treated with TCDD for 1 h, followed by 5 ng/ml LPS for 4 h. B, Primary mouse keratinocytes derived from Ahr^b, $Ahr^{+/-}$, and $Ahr^{-/-}$ newborn mice were treated with TCDD for 1 h, followed by 5 ng/ml LPS for 4 h. Data represent mean mRNA levels normalized to *Actb* mRNA expression ± SEM (n = 3/treatment group; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$).

LPS for 24 h followed by TCDD exposure for 8 h. Interestingly, this result suggests that AHR expression is required for optimal LPS-mediated induction of *Ccl20*.

The Synergistic Induction of Ccl20 Mediated by AHR Agonist and LPS Is AHR Dependent in Primary Macrophages

To further delineate the level of AHR dependency on *Ccl20* gene expression, macrophages were isolated from Ahr^b and $Ahr^{-/-}$ mice and exposed to TCDD and LPS using a short-term treatment regimen. Results indicated that *Cyp1a1* expression was almost absent from $Ahr^{-/-}$ macrophages (Fig. 4A). Furthermore, Western blot analysis demonstrated the absence of AHR expression in macrophages from $Ahr^{-/-}$ mice, in contrast to macrophages from Ahr^b mice (Fig. 4B). This lack of AHR in macrophages resulted in a complete loss of AHR agonist-mediated induction of *Ccl20* transcription (Fig. 4C). Furthermore, despite being equally sensitive to LPS-mediated induction in Ahr^b, $Ahr^{-/-}$ macrophages proved to be refractory to combinatorial agonist/LPS-mediated synergistic induction of *Ccl20*. To determine the effect of AHR dependency on CCL20 protein

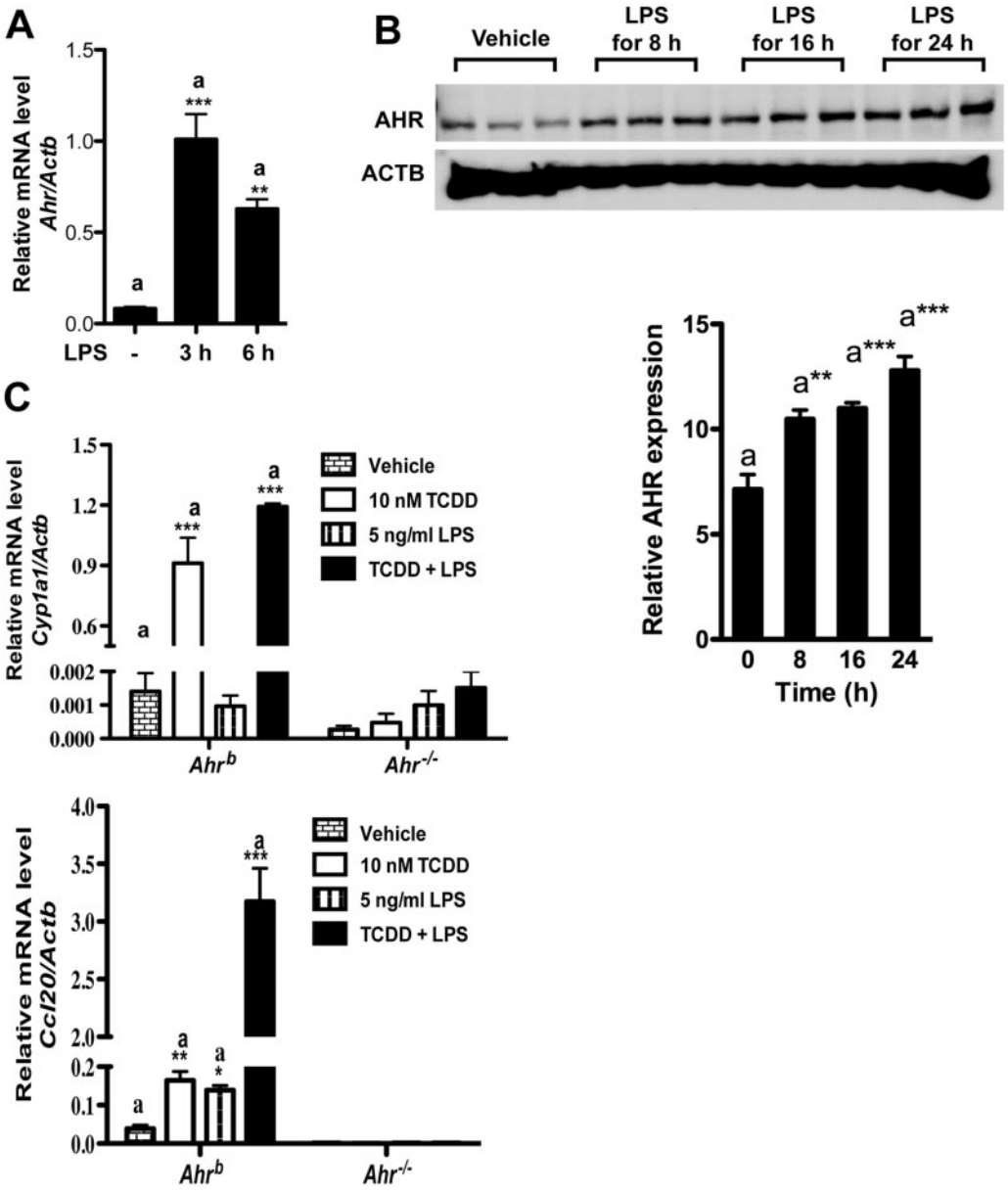

FIG. 3. After long-term exposure to LPS in macrophages TCDD treatment still mediated synergistic induction of Ccl20 in an AHR-dependent manner. Primary macrophages from Ahr^b and $Ahr^{-/-}$ mice were isolated via peritoneal lavage. A, Relative levels of *Ahr* mRNA in cultured macrophages after LPS treatment for 3 or 6 h. B, Time-dependent increase in AHR protein expression in macrophages after LPS treatment for the indicated times. C, Primary macrophages derived from Ahr^b mice were pretreated with LPS for 24 h, followed by TCDD treatment for 3 h; *Cyp1a1* and *Ccl20* relative mRNAs were assessed. Data represent mean mRNA levels normalized to *Actb* mRNA expression ± SEM ($n = 3$/treatment group; *$P < .05$, **$P < .01$, ***$P < .001$).

levels, macrophages from Ahr^b and $Ahr^{-/-}$ mice were exposed to TCDD and LPS for 8 h. An ELISA was performed on cell culture supernatant; results obtained were similar to the data observed at the mRNA level, with a 4-fold induction of CCL20 protein levels upon combinatorial treatment compared with treatment with TCDD or LPS alone (Fig. 4D). Overall, the results suggest that synergistic induction of *Ccl20* by exposure to TCDD and LPS is dependent on AHR expression and activation.

AHR Agonist-Mediated Induction of Ccl20 Is Dependent on Nuclear Translocation of the AHR

Whether AHR activation of *Ccl20* transcription requires the liganded AHR to reside in the nucleus was examined utilizing macrophages isolated from $Ahr^{nls/nls}$ mice. These mice carry a mutation in the composite NLS/DNA binding domain sequence; thus the mutated AHR fails to both translocate into the nucleus and bind to DREs (Bunger *et al.*, 2008). Because these mice carry the Ah^d allele, they were compared with Ah^d congenic mice (Ahr^d). Treatment of $Ahr^{nls/nls}$ macrophages with TCDD resulted in essentially no increase in *Cyp1a1* gene expression relative to the dramatic increase observed in macrophages derived from Ahr^d mice (Fig. 5). Although mouse genotype had no effect on LPS-mediated induction of Ccl20, combinatorial treatment of macrophages with LPS and an AHR agonist resulted in synergistic induction of *Ccl20* in Ahr^d macrophages. In contrast, only a modest induction of *Ccl20* was observed in $Ahr^{nls/nls}$ macrophages, consistent with the requirement for nuclear localization to induce *Ccl20* transcription.

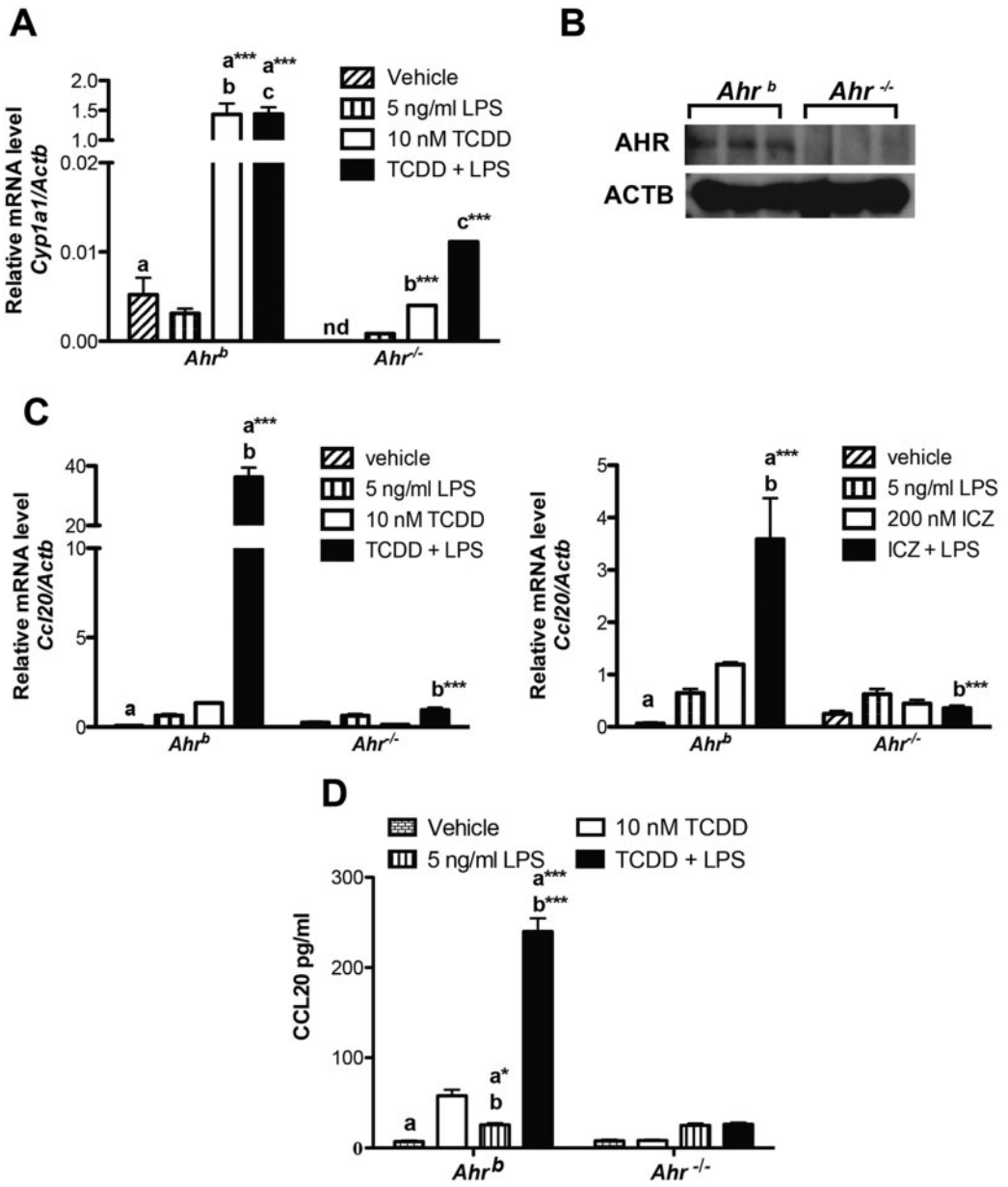

FIG. 4. Both mRNA and protein expression are synergistically induced in primary macrophages by short-term combinatorial treatment with LPS and AHR ligands in an AHR-dependent manner. Primary macrophages were isolated via peritoneal lavage. A, *Cyp1a1* mRNA levels are induced in macrophages derived from Ahr^b but not in $Ahr^{-/-}$ mice after treatment with TCDD in the presence or absence of LPS. B, Protein blot analysis of AHR expression in macrophages derived from Ahr^b and $Ahr^{-/-}$ mice. C, Effect of 1 h pretreatment with an AHR agonist followed by 4 h treatment with LPS on *Ccl20* mRNA levels in macrophages derived from Ahr^b and $Ahr^{-/-}$ mice. D, Macrophages were pretreated with 10 nM TCDD for 1 h followed by 5 ng/ml LPS or vehicle for 8 h. ELISA was performed on supernatants collected from Ahr^b and $Ahr^{-/-}$ macrophages. Data represent mean mRNA levels ± SEM ($n = 3$/treatment group; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$).

AHR Activation Does Not Influence LPS-Mediated NF-kB Translocation

Macrophages isolated from Ahr^b and $Ahr^{-/-}$ mice and maintained in cell culture were treated with LPS or LPS-TCDD for 1 h and p65 was localized using indirect immunofluorescence microscopy (Supplementary Fig. 2S). No difference in p65 translocation into the nucleus was observed whether the AHR is expressed or upon ligand activation.

Agonist-Mediated AHR Activation Promotes Binding of the AHR–ARNT Protein Complex to DREs Present on Ccl20 Promoter

AHR agonist-mediated gene activation is primarily associated with binding of heterodimerized AHR–ARNT protein complex to the DREs present on the gene promoters. The induction of *Ccl20* gene transcription by TCDD or ICZ in an AHR-dependent fashion suggests that the agonists could invoke binding to DREs present on the *Ccl20* promoter (Fig. 1C). To determine whether the same mechanism of gene activation is utilized here, we examined the 5 kb upstream promoter of the *Ccl20* gene relative to the transcription start site. Four putative DREs along with the NF-kB binding site on the *Ccl20* promoter were identified by *in silico* analysis (Fig. 6A). To examine the functional significance of the identified DREs, gel shift analysis was performed. In vitro-translated mouse AHR and ARNT proteins were exposed to TCDD, leading to the formation of AHR–ARNT complexes. These were then mixed with radiolabeled

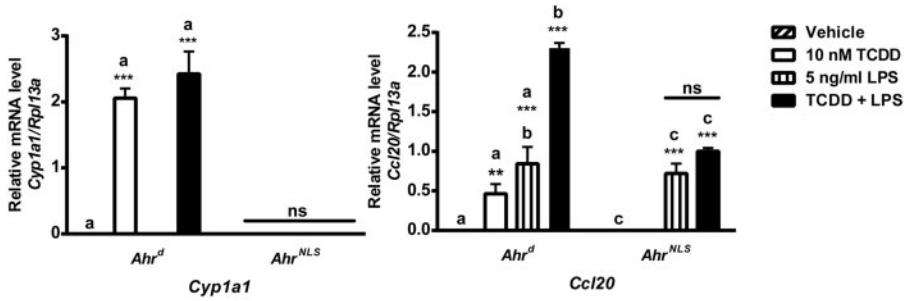

FIG. 5. Synergistic induction of *Ccl20* levels requires AHR nuclear translocation. Primary macrophages were isolated from Ahr^{d} and Ahr^{nls} mice via peritoneal lavage. *Cyp1a1* and *Ccl20* mRNA levels were assessed 4 h after the various indicated treatments. Data represent mean mRNA levels ± SEM ($n = 3$/treatment group; $^{**}P < .01$, $^{***}P < .001$). The term "ns" means no significant difference between data bars under the line.

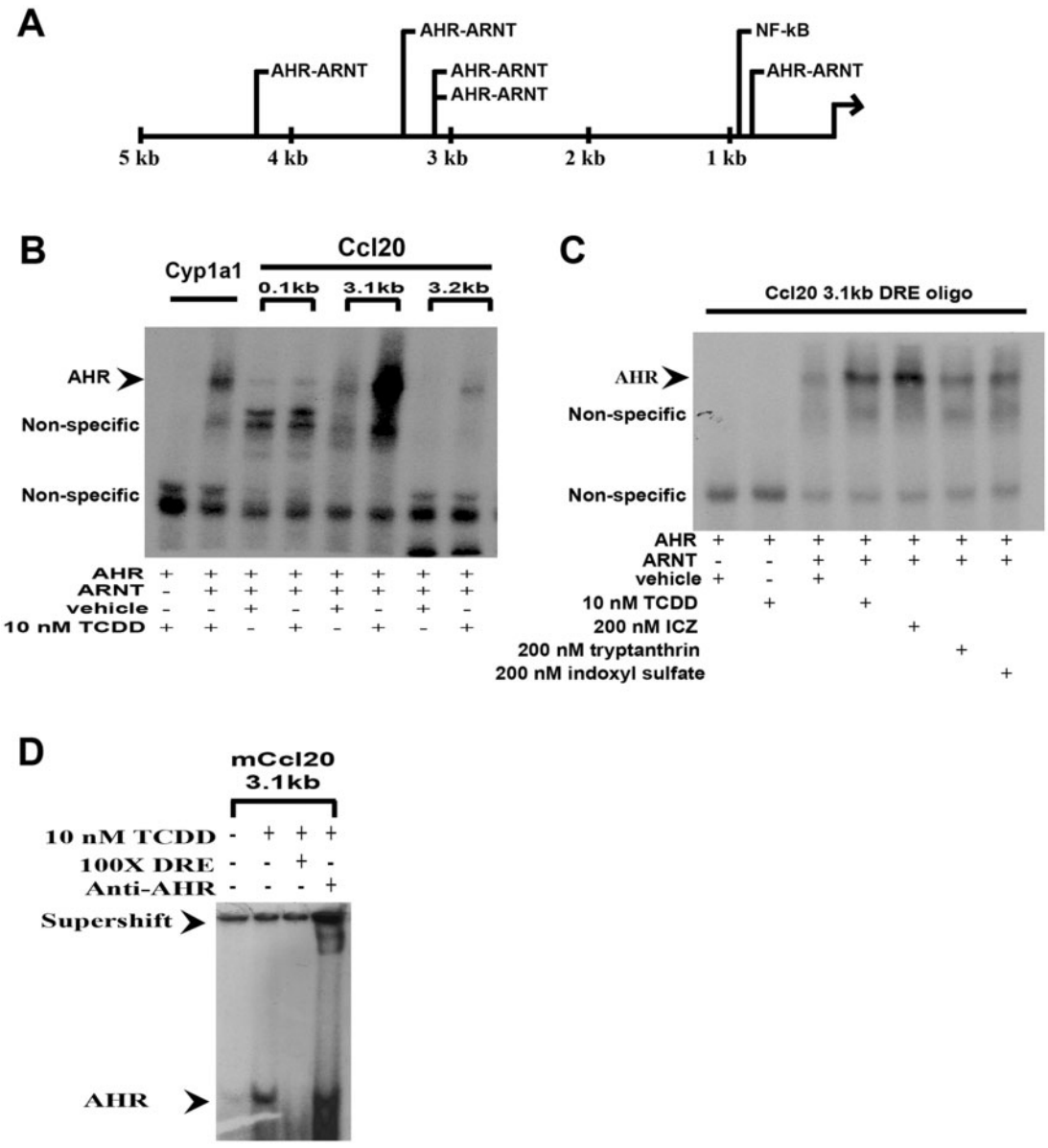

FIG. 6. DREs present in the *Ccl20* promoter are capable of binding AHR/ARNT heterodimer in EMSA. A, The 5 kb promoter of mouse *Ccl20* with putative DRE and NF-kB binding sites. B, Mouse AHR and ARNT proteins were generated using *in vitro* translations. Oligonucleotides spanning DREs at 0.1, 3.1, and 3.2 kb were used in EMSA. C, EMSA analysis of the ability of various AHR agonists to induce AHR/ARNT binding to *Ccl20* DRE. D, Nuclear extracts were prepared from cultured macrophages from Ahr^{b} mice treated with vehicle or 10 nM TCDD for 1 h. EMSA and AHR antibody supershifts were performed.

oligonucleotides corresponding to 3 different DRE-containing regions of the *Ccl20* promoter. Gel shift analysis suggests that TCDD-mediated AHR–ARNT protein complexes can bind to the DREs present on the *Ccl20* promoter, with maximum binding observed at the 3.1 kb putative DRE site (Fig. 6B). Other AHR agonists were tested and ICZ, tryptanthrin, and indoxyl sulfate were all capable of inducing AHR binding to the 3.1 kb oligonucleotide (Fig. 6C), suggesting that many AHR agonists can promote binding of AHR–ARNT to a DRE present on the *Ccl20* promoter. To further study these events in macrophages from Ahr^b mice, we exposed the macrophage cultures to TCDD and nuclear proteins were isolated. The isolated proteins were incubated with ^{32}P-radiolabeled sequences spanning DREs at 0.1, 3.1, and 3.2 kb. The data suggest that nuclear protein extracts of primary peritoneal macrophages from Ahr^b mice exposed to TCDD could promote DRE binding of the AHR–ARNT protein complex (Fig. 6D). The addition of nonradiolabeled DREs blocked binding to the radiolabeled oligonucleotide. In addition, AHR antibody addition induced an AHR–protein supershift of each oligonucleotide AHR complex. Overall, the data demonstrate the ability of AHR agonists to induce AHR–ARNT protein complex formation, which then binds DREs present in the *Ccl20* promoter. Furthermore, the DRE at 3.1 kb is particularly efficient at binding the liganded AHR–ARNT complex.

LPS Promotes AHR Recruitment to the Ccl20 Promoter in Macrophages

ChIP assays were performed to further examine the mechanism of AHR-mediated synergistic induction of *Ccl20* transcription. Treatment of macrophages with LPS leads to an increase in AHR occupancy on both the *Cyp1a1* and *Ccl20* promoters (Fig. 7). In contrast, LPS treatment led to a significant decrease in HDAC1 presence on the *Cyp1a1* and *Ccl20* promoters. Surprisingly, combinatorial treatment with TCDD and LPS actually yielded an apparent lower level of AHR occupancy. The reason for this result has not been elucidated. Nevertheless, these results indicate that the AHR can be recruited to the *Ccl20* promoter and is associated with synergistic Ccl20 induction.

Ccl20 Expression Is Repressed in the Colon of $Ahr^{-/-}$ Mice

The data in Figure 3C would suggest that the AHR expression might play a role in the constitutive and LPS-inducible expression of Ccl20, to examine this *in vivo* in a tissue that is exposed to bacterial inflammatory signaling molecules. The level of expression of Ccl20 was examined in the colon of $Ahr^{-/-}$ and $Ahr^{+/-}$ mice and there was a dramatic decrease in *Ccl20* mRNA levels in $Ah^{-/-}$ mice (Fig. 8).

DISCUSSION

Expression of the AHR plays an important role in both the innate and adaptive immune status of the gastrointestinal tract (Quintana and Sherr, 2013; Stange and Veldhoen, 2013). In particular, the AHR participates in the postnatal expansion of intestinal innate lymphoid cells and the differentiation of T cells (Kiss *et al.*, 2011; Quintana *et al.*, 2008). Intestinal exposure studies have revealed that $Ahr^{-/-}$ mice are highly susceptible to either dextran sodium sulfate or *Citrobacter rodentium* challenge (Furumatsu *et al.*, 2011). These studies clearly indicate a critical role for AHR in intestinal immune homeostasis. In a normal healthy gut the resident macrophage population exhibits a non-inflammatory phenotype characterized by low levels of TLR2 and TLR4, CD14, CD80 and CD89 (Smith *et al.*, 2001). However, during a bacterial challenge a subset of macrophages, characterized by the expression of CX3CR1, expands into inflammatory macrophages that respond to the infection (Bain *et al.*, 2013). These cells in turn produce chemokines to attract other immune cells to the infection site. One key chemokine, CCL20, is expressed in cells of epithelial origin, macrophages and Th17 cells (Li *et al.* 2013). CCL20 expression is induced by inflammatory mediators, such as LPS and other bacterial components, and is the primary ligand for the chemokine receptor CCR6 (Hausmann *et al.*, 2012; Ito *et al.*, 2011). The recruitment of CCR6+ cells (eg, macrophages, dendritic cells, and T cells) to the site of injury is critical for an effective response to bacterial invasion. Our results suggest that the expression of CCL20 in the colon is partial dependent of AHR, supporting the notion that the presence of AHR agonists would likely enhance the overall response to an intestinal challenge.

However, in the absence of a strong agonist, the AHR presumably in the cell cytoplasm can inhibit inflammatory signaling, perhaps leading to enhanced resolution of inflammation. Support for this concept can be found in several studies in macrophages, where the absence of AHR expression leads to an enhanced inflammatory phenotype (Beamer *et al.*, 2012; Kimura *et al.*, 2009; Masuda *et al.*, 2011; Sekine *et al.*, 2009). In addition, the use of selective Ah receptor modulators that exhibit essentially no DRE-mediated activity elicit a potent anti-inflammatory activity (Murray *et al.*, 2010a). After long-term exposure to inflammatory mediators, we have shown that AHR protein levels are increased 2-fold in primary macrophages. Results are similar to the increased levels of AHR observed in peritoneal exudate cells from LPS-treated mice (Bessede *et al.*, 2014). Recent studies have demonstrated that there are several functional NF-kB elements in the AHR promoter (Vogel *et al.*, 2014). These observations are consistent with the AHR either mediating a sustained level of inflammatory signaling or a feedback loop that leads to anti-inflammatory activity dependent on whether AHR agonists are present.

The *Il6* promoter has been extensively characterized in terms of the ability of NF-kB and AHR to synergistically induce transcription (DiNatale *et al.*, 2010). A cluster of putative DREs is located ~3 kb upstream from the transcriptional start site of *Il6*; occupation of this site by AHR/ARNT results in the dismissal of HDAC1 and subsequent acetylation of NF-kB, leading to dramatically enhanced transcription. However, this cluster of DREs in the Il6 promoter, when considered in the context of a reporter vector with a heterologous promoter, was only modestly capable of driving transcription in a transient cell transfection experiment. Interestingly, at 3.1 kb upstream from the transcriptional start site of the *Ccl20* promoter there are potent tandem DREs that are highly efficient with regard to AHR heterodimer binding in the context of gel shift analysis. As in the case of Il6, these DREs failed to mediate transcription in the context of a reporter vector in the presence of an AHR agonist (data not shown). After LPS exposure in primary macrophages, the AHR is clearly recruited and HDAC1 is dismissed from the *Ccl20* promoter. This was an unexpected result, as generally only AHR ligand treatment causes a significant increase in promoter occupancy. Interestingly, this result is similar to the ability of calcium-induced signaling in primary keratinocytes to lead to retention of the AHR in the nucleus (van den Bogaard *et al.*, 2015). Indeed, LPS can induce calcium signaling in macrophages, thus the possible connection between calcium signaling and AHR activation in the macrophages should be investigated (Ren *et al.*, 2014). It was also intriguing to find that the addition of an AHR ligand apparently decreased the level of occupancy when in combination with LPS—the reason for this result is not

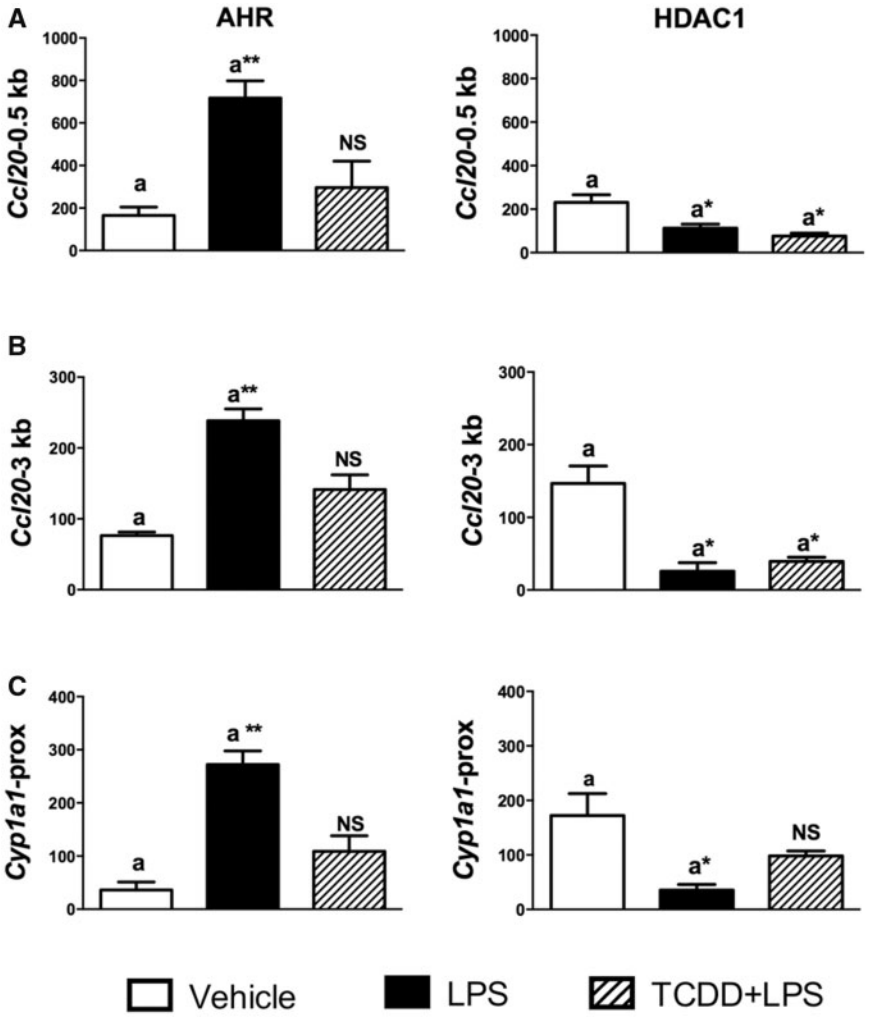

FIG. 7. LPS mediates AHR occupancy onto the *Ccl20* promoter and HDAC1 dismissal. ChIP analyses were performed using primary macrophage cultures treated with TCDD or vehicle for 60 min followed by LPS or vehicle treatment for 30 min. ChIP assays were performed using either anti-AHR or anti-HDAC1 immunoprecipitations and qPCR was performed using primers localized near the 0.5 kb (A) and 3.0 kb (B) region upstream from the transcriptional start site of *Ccl20*, as well as primers to the proximal promoter region of the *Cyp1a1* promoter (C).

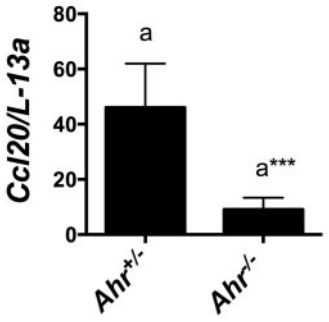

FIG. 8. Ccl20 expression is attenuated in the colon of $Ahr^{-/-}$ mice. The level of Ccl20 expression in the colon of $Ahr^{-/-}$ and $Ahr^{+/-}$ was assessed using qRT-PCR. Data represent mean mRNA levels normalized to *L-13a* mRNA expression ± SEM ($n = 3$/treatment group; ***$P < .001$).

known. However, the high level of transcriptional activity that occurs with combinatorial treatment may result in epitope access issues in the ChIP assays. Nevertheless, the presence of the AHR on the promoter appears to participate in the synergistic activation of *Ccl20* transcription. A pictorial scheme depicting the proposed mechanism of combinatorial activity at the *Ccl20* promoter is shown in Supplementary Figure 3S. The fact that *Il6* and *Ccl20* exhibit similar modes of regulation prompts the question of how many cytokine/chemokine genes are in part regulated by the AHR.

Activation and heterodimerization of the AHR with ARNT during innate inflammatory signaling likely evolved as a means to respond to the presence of ligands produced either through dietary consumption, endogenous production, or tryptophan metabolism by flora (Fukumoto *et al.*, 2014; Magiatis *et al.*, 2013). Interestingly, the human AHR exhibits dramatically enhanced activation potential for indolic derivatives, such as indirubin, relative to the mouse AHR (Flaveny *et al.*, 2009). In the case of a yeast skin infection in humans, AHR ligands (eg, indirubin, indolo[3,2*b*]carbazole, and malassezin) are produced that lead to the host inflammatory and barrier responses. This could explain why the AHR plays a role in the differentiation of T cells as a means to enhance the host response to infection. In addition, the enhanced production of CCL20 mediated by AHR activation could play an important role in the recruitment of T cells within the gastrointestinal tract. For example, in the presence of inflammation mediated by invasive bacteria, the production

of AHR ligands would further increase chemokine expression and thus the recruitment of immune cells to the site of infection. Furthermore, the CCL20–CCR6 axis may also play a critical role in intestinal tumorigenesis, where AHR activation would likely enhance the tumorigenesis process through the generation of an inflammatory environment (Nandi *et al.*, 2014). In this environment, IDO1 is likely up-regulated, which could lead to the production of AHR ligands to further enhance response to a toxic insult. The AHR has also been shown to contribute to inflammatory signaling in other chronic diseases, such as rheumatoid arthritis (Kobayashi *et al.*, 2008). Synoviocytes isolated from rheumatoid arthritis patients during joint replacement exhibit significant constitutive *Ccl20* expression, which is in part mediated by AHR activity (Lahoti *et al.*, 2013). In conclusion, these studies yield further insight into the mechanisms that mediate participation of the AHR in inflammation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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