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Novel Cellular Targets of AhR Underlie Alterations in Neutrophilic Inflammation and Inducible Nitric Oxide Synthase Expression during Influenza Virus Infection

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The underlying reasons for variable clinical outcomes from respiratory viral infections remain uncertain. Several studies suggest that environmental factors contribute to this variation, but limited knowledge of cellular and molecular targets of these agents hampers our ability to quantify or modify their contribution to disease and improve public health. The aryl hydrocarbon receptor (AhR) is an environment-sensing transcription factor that binds many anthropogenic and natural chemicals. The immunomodulatory properties of AhR ligands are best characterized with extensive studies of changes in CD4+ T cell responses. Yet, AhR modulates other aspects of immune function. We previously showed that during influenza virus infection, AhR activation modulates neutrophil accumulation in the lung, and this contributes to increased mortality in mice. Enhanced levels of inducible NO synthase (iNOS) in infected lungs are observed during the same time frame as AhR-mediated increased pulmonary neutrophilia.

In this study, we evaluated whether these two consequences of AhR activation are causally linked. Reciprocal inhibition of AhR-mediated elevations in iNOS and pulmonary neutrophilia reveal that although they are contemporaneous, they are not causally related. We show using Cre/LoxP technology that elevated iNOS levels and neutrophil number in the infected lung result from separate, AhR-dependent signaling in endothelial and respiratory epithelial cells, respectively. Studies using mutant mice further reveal that AhR-mediated alterations in these innate responses to infection require a functional nuclear localization signal and DNA binding domain. Thus, gene targets of AhR in non-hematopoietic cells are important new considerations for understanding AhR-mediated changes in innate anti-viral immunity. *The Journal of Immunology*, 2013, 190: 659–668.
Previous work has revealed that AhR activation affects neutrophils indirectly but does not alter well-known mediators of neutrophil migration, including soluble neutrophil chemotractions and cytokines, adhesion molecules on neutrophils or structural cells of the lung, vascular permeability, or numbers of circulating neutrophils (17, 25, 26). Collectively, these findings suggest that AhR modulates a novel pathway that regulates neutrophil migration during influenza virus infection. One possible novel AhR target gene is inducible NO synthase (iNOS). Concomitant with exacerbating neutrophil accumulation, AhR activation increases iNOS expression in lungs of influenza virus–infected mice (27). Thus, it is possible that AhR-mediated increases in iNOS levels influence neutrophil recruitment to the lung during infection. Circumstantial evidence suggests a possible causal relationship between elevated iNOS levels and pulmonary inflammation in other model systems, although this relationship has not been examined in the context of a respiratory viral infection (28–31).

We report our investigation of whether a cause-and-effect relationship exists between AhR-mediated increases in iNOS levels and neutrophil migration to the lung during influenza virus infection. Further, prior work has established that AhR-mediated increases in neutrophilia and iNOS levels in the infected lung are mediated by AhR-regulated events extrinsic to bone marrow–derived cells (25, 27). Therefore, we used Cre/loxP technology to delineate whether AhR signaling intrinsic to endothelial cells or lung epithelial cells directly contributes to altered neutrophil recruitment and iNOS levels in the infected lung. Our results expand the repertoire of AhR target cells that need to be considered as we evaluate immune modulation by AhR agonists.

## Materials and Methods

### Animals and treatment

C57BL/6 mice (female, 5–6 wk of age) were purchased from either The Jackson Laboratory or the National Cancer Institute, and B6.Cg-Tg(Tek-cre)12FlvJ (Crelox) mice were purchased from The Jackson Laboratory. Breeding stock for AhrNls/nls and Ahrd/dbd mutant mice (32, 33) as well as mice expressing the Ah receptor conditional allele (34) were provided by Dr. Christopher Bradfield (University of Wisconsin) and maintained at the University of Rochester Medical Center. B6.Ahrfx/fx mice, maintained at the University of Rochester Medical Center, were used as controls for Ahrfx/fx and Ahrfxfxfx mutant mice. Mice that express the Cre transgene under control of the surfactant protein C (SPC) (CreSPC) mice were provided by Dr. Michael O’Reilly (University of Rochester) (35). All mice used were backcrossed onto the C57BL/6 genetic background.

For some experiments, the Ah receptor mutant mice were crossed with either the Crelox or CreSPC mice to generate offspring hemizygous for the Ah receptor and heterozygous for the Ah mutant allele (CreAhfxlox). CreAhfxlox mice were generated by crossing CreAhfxlox mice with Ahfxlox mice. Ahfxlox littermates that do not express Cre were used as experimental controls. Male mice were used to transmit the Crelox transgene to prevent Cre-mediated deletion of floxed alleles in the germline (36). Female mice were used to transmit the Crelox transgene to prevent Cre-mediated deletion of floxed alleles in the germline (37). Endothelial specific deletion of the Ahr was accomplished by crossing Crelox mice with the Ahrlox strain. Importantly, Tek expression occurs during mouse fetal development in both endothelial cells and hematopoietic progenitors (38). Therefore, in adult mice, Ahr deletion occurs in the endothelium and hematopoietically derived cells (34). Respiratory epithelial cell–specific deletion of the Ahr was accomplished using Crelox mice crossed with the Ahlox strain. Type II epithelial cells in adult mice produce surfactant protein C. However, Sftpc gene expression is first detected in the fetal developing lung, and therefore Cre-mediated floxed gene deletion is not limited to type II epithelial cells, but rather occurs throughout the respiratory epithelium (39, 40). Thus, using this approach, Ahr expression is conditionally ablated from the lung epithelium.

Mice were housed in pathogen-free microisolator cages and maintained on a 12-h light/dark cycle and provided food and water ad libitum. To activate AhR, mice were gavaged with 10 μg TCDD/kg body weight (≥98% pure; Cambridge Isotope Laboratories, Andover, MA) dissolved in aniseole and diluted in peanut oil, unless otherwise noted. Vehicle control–treated mice received the peanut oil–anisole solution in which the TCDD is dissolved. One day after gavage, mice were anesthetized with Avertin (2,2,2-tribromoethanol) and infected intranasally with 120 hemagglutinating units influenza virus, strain A/HKx31 (x31, H3N2). In vehicle–treated mice, this inoculum of virus has been shown to have little to no lethality (41). All procedures involving laboratory animals and infectious agents were conducted in accordance with protocols that were preapproved by the University of Rochester Institutional Animal Care and Use and Institutional Biosafety Committee.

### Collection and preparation of cells

Mice were sacrificed 7 d postinfection, and left and right lungs were separated at the bronchi. For all experiments, the same side was removed and snap frozen in liquid nitrogen for immunoblotting or iNOS activity assays. The other side was collagenase-digested as previously described to obtain lung-derived immune cells (16, 17, 25, 27). A single-cell suspension of lung-derived immune cells was incubated with fluorochrome-conjugated mAbs specific for CD45, CD8, CD11b, and Gr-1 (eBioscience, San Diego, CA, or BD Pharmingen, San Jose, CA). Nonspecific staining was blocked by preincubating the cells with anti-mouse CD16/CD32 CD16/CD32 Abs and rat IgG. A combination of isotype-matched, labeled Abs and fluorescence minus one controls was used to define nonspecific fluorescence staining and to establish gating parameters. Data were collected using a FACSCanto and analyzed using FlowJo software (Tree Star, Ashland, OR). A separate aliquot of total lung-derived immune cells (30,000) was spun onto microscope slides using a cytosping device (Thermo Fisher Scientific, Rockford, IL), fixed, and stained with H&E. Neutrophils and other leukocytes were enumerated by differential cell counting (200 cells/slide).

### iNOS inhibition and activity assay

To inhibit iNOS activity in vivo during infection, mice were injected (i.p.) with the NOS inhibitor Nω-[iminomethylaminomethyl]-t-ornithine (L-NMMA, 2 mg/mouse; Cayman Chemical, Ann Arbor, MI) every 12 h, starting 5 d postinfection until sacrifice at 7 d postinfection. This dosing scheme was previously shown to block iNOS activity in vivo during influenza virus infection (28). To confirm efficacy of L-NMMA treatment, iNOS activity in the lung was determined using the NO Synthase Assay Kit (EMD Calbiochem, San Diego, CA) with [3H]arginine (PerkinElmer, Waltham, MA).

### In vivo neutrophil depletion

Neutrophils were depleted during infection using either a rat monoclonal anti–Gr-1 Ab, which recognizes Ly-6G/C (purified from the RB6-8C5 hybridoma cell line), or rat monoclonal IAb, which recognizes Ly-6G Abs (BioXCell, West Lebanon, NH). Mice were injected (i.p.) with 300 μg anti–Gr-1 Ab or IAb control 2 h prior to infection and 4 d after infection with influenza virus, as described previously (17). This Ab has been used by our laboratory and by others to deplete neutrophils in vivo (17, 42–44). In separate experiments, mice were injected (i.p.) with 500 μg IAb or Ab control 1 d prior to infection with influenza virus and every 72 h until sacrifice at 7 d postinfection. Rat IgG was used as a control for both Abs. Depletion efficacy was determined by flow cytometry and/or differential cell counts and was found to be greater than 80% in the TCDD-treated groups. Flow cytometric analyses showed little to no depletion of CD8+ T cells from mice that received the anti–Gr-1 or IAb treatment (data not shown).

### Immunohistochemistry

Lungs were perfused with 10% neutral buffered formalin, embedded in paraffin, and sectioned as previously described (27). Slides were incubated with 1% saponin for 30 min at 25°C, rinsed, and endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 min. Nonspecific binding was minimized using 3% normal goat serum (Vector Laboratories, Burlingame, CA). Slides were incubated overnight with rabbit anti-mouse AhR IgG (Enzo Life Sciences, Farmingdale, NY) followed by HRP-conjugated goat-anti-rabbit P(ab)2 fragments. AhR staining was visualized with 3,3′-diaminobenzidine, and slides were counterstained with hematoxylin (Vector Laboratories). Negative controls included no primary Ab and isotype control IgG. Slides were counterstained with hematoxylin and coverslipped using aqueous mounting medium (Serotec, Raleigh, NC).

### Immunoblotting

Frozen lung lobes were homogenized in cold homogenization buffer containing protease inhibitors (50 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0.6% Igepal, 10 μg/ml aprotinin and leupeptin, and 20 μg/ml PMSF) using a 12-gauge needle and cycle and provided food and water ad libitum. To activate AhR, mice were gavaged with 10 μg TCDD/kg body weight (≥98% pure; Cambridge Isotope Laboratories, Andover, MA) dissolved in aniseole and diluted in peanut oil, unless otherwise noted. Vehicle control–treated mice received the peanut oil–anisole solution in which the TCDD is dissolved. One day after gavage, mice were anesthetized with Avertin (2,2,2-tribromoethanol) and infected intranasally with 120 hemagglutinating units influenza virus, strain A/HKx31 (x31, H3N2). In vehicle–treated mice, this inoculum of virus has been shown to have little to no lethality (41). All procedures involving laboratory animals and infectious agents were conducted in accordance with protocols that were preapproved by the University of Rochester Institutional Animal Care and Use and Institutional Biosafety Committees.
a Tissue Tearor (Biospec Products, Bartlesville, OK). The protein concentration of clarified homogenates was determined by bicinchoninic acid protein assay (Thermo Fisher Scientific). Samples were boiled in SDS-PAGE sample buffer for 5 min, and 50 μg protein was subjected to SDS-PAGE. After transfer onto polyvinylidene fluoride membranes, blots were blocked with 5% nonfat dry milk and incubated with anti-iNOS (Cayman Chemical), anti-CYP1A1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AhR (Enzo Life Sciences), or anti-β-actin (Sigma–Aldrich, St. Louis, MO) Abs. After incubation with appropriate HRP-conjugated secondary Abs, blots were developed with enhanced chemiluminescent reagent (Western Lightning Plus ECL; Thermo Fisher Scientific) and exposed to x-ray film. Immunoblot films were scanned, and blot band density was analyzed using ImageJ v.1.38x.

Endothelial cell isolation and confirmation of Ahr gene excision

Endothelial isolation was performed as described (45), with the following modifications. Whole lungs were perfused with 5% BSA in HBSS, minced with surgical scissors, and digested with collagenase (1 mg/ml; Worthington Biochemical, Lakewood, NJ) and DNase I (30 μg/ml; Roche) in RPMI 1640 (Life Technologies) for 1 h at 37°C with constant shaking. The digestion was quenched with 10% FBS and 10 mM HEPEs in RPMI 1640. After filtration to remove clumps and debris and lysis of erythrocytes using an ammonium chloride lysis solution (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA), the cells were sorted using a FACSAria (BD Biosciences) after staining with fluorochrome-conjugated Abs for CD31 and CD45 to distinguish endothelial cells and leukocytes. DNA was isolated from whole lung and sorted cell populations with Ahr<sup>ΔNOS</sup> excision being determined as previously described (34).

Statistical analyses

All statistical analyses were conducted using StatView or JMP software (SAS, Cary, NC). Mean differences between treatment groups were analyzed using one-way ANOVA, followed by Bonferroni–Dunn or Tukey–Kramer post hoc test. Differences between two treatment groups on the same day postinfection were analyzed using a two-tailed Student t test. A p value ≤ 0.05 was considered significant.

Results

AhR nuclear translocation and binding to DNA are required to mediate infection-associated increases in neutrophil recruitment and iNOS expression

Agonist binding to cytosolic AhR results in nuclear translocation and interaction of the AhR with aryl hydrocarbon response elements (AhRE) within AhR target genes, such as the xenobiotic metabolizing enzymes Cyp1a1 and Cyp1b1 (46). Alternative pathways of AhR activation have been reported, in which the AhR affects cell signaling or gene expression independently of nuclear pathways of AhR activation have been reported, in which the AhR upregulation of responses, including aspects of neutrophil chemotaxis and the previously described (34).

To determine whether increases in iNOS levels and neutrophil accumulation require AhR binding to DNA via its intrinsic DNA binding domain, mice expressing AhR protein with a mutated DNA binding domain (AhR<sup>ΔDBD</sup>) were used. This mutated AhR associates with co-chaperone proteins in the cytoplasm, binds ligand, and translocates into the nucleus upon ligand binding; however, it cannot bind to DNA and induce the expression of Cyp1a1 (33). Upon activation with TCDD, neither neutrophil number nor iNOS levels were elevated in the lungs of AhR<sup>ΔDBD</sup> mice (Fig. 1A, 1B). In separate experiments, mice that express a mutated AhR protein that lacks the nuclear localization signal (AhR<sup>ΔNLS</sup>) were used.

FIGURE 1. AhR requires a functional DNA binding domain to enhance infection-associated pulmonary neutrophilia and iNOS levels. AhR<sup>ΔDBD</sup> and B6 wild-type (WT<sup>d</sup>) mice were administered either TCDD or peanut oil (VEH) by gavage 1 d prior to infection (infected intranasally) with 120 hemagglutinating unit influenza A virus (HKx31). B6 WT<sup>d</sup> and AhR<sup>ΔDBD</sup> express the low-affinity AhR<sup>Δ</sup> allele and thus require a dose of 100 μg/kg TCDD, which elicits an equivalent dose-effect on immune endpoints as observed when a 10 μg/kg dose of TCDD is given to wild-type, AhR<sup>Δ</sup> allele–expressing mice (data not shown). Mice were sacrificed 7 d postinfection. (A) Bars represent the average percentage of neutrophils from the whole lung (n = 4 to 10 mice per treatment group). *p ≤ 0.05 (a significant difference compared with the vehicle control group of the same genotype). (B) SDS-PAGE was performed on lung homogenates from TCDD- or VEH-treated WT<sup>d</sup> or AhR<sup>ΔDBD</sup> mice infected with influenza virus, and blots were probed with Abs against iNOS and Cyp1A1, with β-actin as a loading control. Two representative samples from each treatment group are shown (n = 4 to 10 mice per group); results were similar in all animals within each genotype and treatment group. Data in this figure are representative of two independent experiments.

This defective AhR protein binds ligand in the cytoplasm but cannot translocate to the nucleus (32). Similar to the AhR<sup>ΔDBD</sup> mutants, TCDD-treated and infected AhR<sup>ΔNLS</sup> mutant mice do not exhibit increased iNOS levels in their lungs or enhanced pulmonary neutrophilia (Supplemental Fig. 1). These results indicate that AhR nuclear translocation and binding to DNA via its intrinsic DNA binding domain are required for AhR-dependent increases in iNOS levels and neutrophil recruitment to the infected lung.

Exacerbation of infection-associated lung neutrophilic inflammation and enhanced iNOS levels are independent consequences of AhR activation

The temporal association between AhR-mediated elevation in pulmonary neutrophil number and iNOS levels in the infected lung, combined with the reported causal relationship between iNOS and neutrophil recruitment in other model systems (30, 31, 55), suggests a causal relationship may exist. To determine if the AhR-mediated increase in iNOS is necessary for or drives the enhanced neutrophil recruitment to the infected lung, we used the Nos inhibitor L-NMMA. We have previously reported that increases in iNOS expression in TCDD-treated, infected mice are not detected until day 5 postinfection (27). Therefore, mice were treated with L-NMMA beginning on the 5th day of virus infection. Consistent with prior reports of the efficacy of L-NMMA during influenza virus infection (28), this treatment inhibited iNOS activity in the lung (Fig. 2A); however, it did not alter iNOS protein levels (Fig.
2B). Although iNOS activity was inhibited during infection, AhR activation still increased pulmonary neutrophilia to the same extent as in mice given PBS control (Fig. 2C). These results demonstrate that AhR-mediated increases in iNOS activity do not directly contribute to the AhR-driven increase in the number of neutrophils observed in the infected lung.

It is also possible that AhR-mediated increases in iNOS result from excessive neutrophil accumulation in the lung during infection. To determine whether this is the case, neutrophils were depleted in vivo over the course of infection using the rat mAb 1A8, which specifically recognizes the Ly6G Ag on neutrophils. The consequence of this depletion on AhR-mediated increases in iNOS was examined. This treatment results in >80% depletion of Gr-1<sup>hi</sup> cells [neutrophils (56, 57)] in TCDD-treated mice (Fig. 3A, 3B).

However, this reduction in neutrophils did not affect AhR-mediated increases in iNOS levels. That is, the amount of iNOS protein was equivalent upon AhR activation, regardless of 1A8 treatment (Fig. 3C, 3D). Similar results were obtained using the RB6-8C5 rat mAb instead of the 1A8 Ab (data not shown). These results indicate that enhanced iNOS is not caused by AhR-mediated increases in neutrophilia in the infected lung. Collectively, these findings show that AhR activation increases lung neutrophilia and iNOS levels independently of one another, indicating that they are two separate targets of AhR activation during influenza virus infection.

**AhR activation in endothelial cells drives increased iNOS expression, whereas enhanced neutrophil recruitment is controlled by AhR within the respiratory epithelium**

Our laboratory has previously shown that AhR-mediated enhancement of neutrophil recruitment and elevated iNOS levels during influenza virus infection are not intrinsic to AhR activation in cell lineages of hematopoietic origin (25, 27). These observations suggest that activation of the AhR in nonimmune cells is responsible for these alterations. When considering possible cellular targets, it is important to bear in mind that we do not observe systemic changes in neutrophil number or iNOS levels (17, 27). Instead, AhR-mediated increases in neutrophilic inflammation and iNOS are limited to the site of Ag challenge. Thus, we wondered whether AhR in cells of the lung drives these events during infection. It has long been known that relative to other tissues, whole-lung homogenates have high levels of AhR protein, as measured by immunoblotting (58–60). Moreover, AhR activation induces known AhR target genes such as Cyp1a1 and Cyp1b1 in whole-lung tissue, which demonstrates that AhR within the lung can be induced to a transcriptionally active state (61). We show in this study, using immunohistochemistry, that AhR is broadly found throughout the lung, including in both large and small airways as well as alveolar regions (Fig. 4A–C). Given that influenza viruses infect respiratory epithelial cells and downregulate expression of many host proteins, the consequence of infection on AhR levels in the lung is an important consideration. Also, AhR ligands have been shown to downregulate AhR levels in cultured cells (62, 63). Therefore, we next determined whether infection and AhR activation singly, or in combination, modulate AhR levels in the lung. As shown in Fig. 4, neither influenza virus infection nor TCDD treatment, singly or together, significantly affected AhR protein levels in the lung on any given day. However, when examined over the entire course of infection, TCDD treatment slightly, but significantly, reduced AhR protein levels when all days were examined together (p = 0.0235). We also examined whether ligand activation altered AhR protein levels in the lung in the absence of infection and found that it did not (data not shown). Thus, AhR protein is found abundantly in the lung, is transcriptionally active, and AhR protein levels are not significantly modulated by infection, but they are slightly reduced by ligand exposure.

With this knowledge, we considered two candidate lung cell lineages within which altered AhR signaling could lead to increased neutrophil recruitment and iNOS levels: endothelial cells and respiratory epithelial cells. Endothelial cells are generally considered the primary barrier that circulating neutrophils must traverse before gaining access to the infected lung (64). Respiratory epithelial cells also play a role in neutrophil migration, via the expression of inflammatory cytokines that influence adhesion molecule expression, as well as by the production of neutrophil chemoattractant factors. Furthermore, respiratory epithelial cells are one of the cell types in which AhR-mediated increases in
iNOS levels are observed (27). To determine whether the AhR influences iNOS expression and neutrophil recruitment in these cell lineages, Cre recombinase–mediated excision of the AhR was performed.

Conditional AhR deletion in endothelial cells was accomplished by crossing Ahrfx/fx mice with mice expressing Cre recombinase under direction of the Tie2 tyrosine kinase promoter (CreTek), which is expressed in endothelial cells throughout development and adulthood (36). Tie2 is also expressed in hematopoietic progenitors during development (38). Therefore, in CreTekAhrfx/fx mice, Ahr excision is expected to occur in both endothelial cells and hematopoietically derived cells. We confirmed that the Ahr was selectively deleted from endothelial cells (CD31+CD452) and neutrophils (Gr1+CD11b+) in CreTekAhrfx/fx mice, whereas endothelial cells and neutrophils in Ahrfx/fx mice retained Ahr expression (Fig. 5A, 5B). Histological examination of lungs revealed no

![Image of Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Neutrophil depletion does not affect AhR-mediated increases in lung iNOS levels during infection. C57BL/6 mice (6–8 mice per treatment group) were treated with peanut oil (VEH) or TCDD and infected as described for Fig. 2. Mice were injected (i.p.) with 500 μg 1A8 or rat IgG control every 72 h starting 1 d prior to infection and sacrificed on day 7 postinfection. (A) Representative dot plots show the percentage of Gr-1hi lung-derived immune cells [neutrophils (56, 57)] in mice with and without 1A8. Numbers in the upper right corner of each dot plot indicate the mean percentage (±SEM) of Gr-1hi cells in each group. (B) Graphs depict the average percentage (±SEM) of Gr-1hi cells in each treatment group. (C) SDS-PAGE was performed on lung homogenates, and blots were probed with Abs against iNOS, with β-actin as a loading control. Two representative samples from each treatment group are shown. (D) Densitometry was performed on all samples in each treatment group using ImageJ software (6–8 mice per treatment group) with bars representing the average iNOS/β-actin expression (±SEM) for each treatment group. *p ≤ 0.05 (a significant difference from the rat IgG–treated vehicle control), **p ≤ 0.05 (a significant difference from the rat IgG, TCDD–treated group), ***p ≤ 0.05 (a significant difference from the 1A8-treated vehicle control).

![Image of Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** AhR levels in lung are unaltered by influenza virus infection and only slightly reduced by ligand activation. AhR in the lungs of naive untreated mice was examined using immunohistochemistry. Representative images show AhR in cells of the (A) large airway, (B) alveolar regions, and (C) small airways. Original magnification ×400. AhR protein levels were examined in whole-lung homogenates on days 1, 3, 5, 7, and 9 postinfection (infected intranasally) with influenza virus (HKx31), and in vehicle- and TCDD-treated wild-type mice. (D) An immunoblot of representative samples collected 3 and 7 d postinfection from vehicle (VEH) and TCDD-treated mice (2 per group/time). (E) The bar graph depicts densitometric analysis of all samples (n = 6 to 8 mice per treatment group per day), which was performed using ImageJ software. AhR band density, relative to β-actin for each sample, was determined, and the mean value (±SEM) for each point in time and group are presented.
observational anomalies in lung development in adult naïve CreTek AhRX/− mice (data not shown). Upon infection, TCDD-treated CreTek AhRX/− mice had elevated numbers of neutrophils in the lung, similar to AhRX/− mice that were given TCDD (Fig. 6A). In contrast, iNOS levels were not enhanced in CreTek AhRX/− mice treated with TCDD, whereas AhR activation elevated iNOS levels in infected AhRX/− mice (Fig. 6B). Excision of AhR from neutrophils in CreTek AhRX/− mice did not affect the migration of neutrophils into the lung after TCDD treatment. This is consistent with our prior report that upon infection of AhRX/− → AhRX+/+ bone marrow chimera, AhR activation still enhanced pulmonary neutrophilia and iNOS levels when hematopoietic cells lack AhR protein (25). Taken together, these findings indicate that AhR-mediated events in endothelial cells drive increases in iNOS during infection but are not responsible for enhanced neutrophil accumulation associated with AhR activation.

We next sought to determine whether AhR activation within the respiratory epithelium regulates the number of neutrophils in the lung during infection. AhR deletion from respiratory epithelial cells was accomplished by crossing AhRX/− mice with mice expressing Cre recombinase under control of the surfactant protein C promoter. CreSftpc mice express Cre in type I and II respiratory epithelial cells, as well as airway epithelial cells, during development and into adulthood (35, 39). Similar to CreTek AhRX/− mice, no differences in tissue architecture were observed upon histopathological examination of lungs from naïve, adult AhRX/− and CreSftpc AhRX/− mice (data not shown). As expected, infected AhRX/− mice treated with TCDD had a greater number of neutrophils in the lung compared with vehicle-treated AhRX/− mice controls. However, upon infection with influenza virus, increased neutrophil recruitment to the lung was not observed when the AhR was activated in CreSftpc AhRX/− mice (Fig. 7A). However, and in contrast to CreTek AhRX/− mice, in which AhR-mediated elevation of iNOS was no longer observed, ablation of AhR in the respiratory epithelium did not alter the ability of AhR activation to increase iNOS levels in the lung (Fig. 7B). These results demonstrate that the AhR-mediated increase in neutrophil frequency in the lung is dependent on AhR activation within the respiratory epithelium; whereas AhR modulates iNOS levels in the infected lung via events within endothelial cells. These novel observations further support the idea that AhR-mediated increases in iNOS and neutrophil recruitment occur independently of one another by separately targeting endothelial and respiratory epithelial cells.

FIGURE 5. CreTek AhRX/− mice do not express the AhR in endothelial cells from the lung. Lungs from CreTek AhRX/− and AhRX/− mice were digested with collagenase, and endothelial cells were isolated as described in Materials and Methods. (A) A representative dot plot shows the identification of CD31+CD45− and CD31+CD45+ cells (endothelial cells), which were isolated from AhRX/− and CreTek AhRX/− mice by sorting (FACS Aria). (B) DNA from whole-lung tissue, endothelial cells, and neutrophils (Gr1+CD11b+) was digested with collagenase, and endothelial cells were isolated as described in Materials and Methods. *Bars represent the mean percentage (± SEM; n = 6 to 9 mice per treatment group) in each treatment group. **Bars depict the average number of Gr-1+ cells (endothelial cells), which were isolated from AhRX/− and CreTek AhRX/− mice by sorting (FACS Aria). (A) Neutrophils (CD45+Gr1+ cells) were identified by flow cytometric analysis of lung-derived immune cells. Bars represent the mean percentage (±SEM; n = 6 to 9 mice per treatment group) in each treatment group. *p < 0.05 (a significant difference compared with vehicle-treated mice of the same genotype). (B) Lung homogenates were subjected to SDS-PAGE and probed with Abs against iNOS and β-actin. Two samples per treatment group are shown and are representative of findings in all mice in each group (n = 6 to 9 mice per treatment group).

FIGURE 6. Conditional deletion of the AhR in endothelial cells does not attenuate excessive neutrophil recruitment to the lung but prevents increased iNOS during infection. CreTek AhRX/− and AhRX/− littermates were treated with TCDD (100 μg/kg) or vehicle (VEH) control 1 d prior to infection with HKx31. Similar to AhRX/− mice, AhRX/− mice express the low-affinity AhR allele, thus requiring 10-fold more TCDD than AhRX/− mice. Mice were sacrificed 7 d postinfection. (A) Neutrophils (CD45+Gr1+ cells) were identified by flow cytometric analysis of lung-derived immune cells. Bars represent the mean percentage (±SEM; n = 6 to 9 mice per treatment group) in each treatment group. *p < 0.05 (a significant difference compared with vehicle-treated mice of the same genotype). (B) Lung homogenates were subjected to SDS-PAGE and probed with Abs against iNOS and β-actin. Two samples per treatment group are shown and are representative of findings in all mice in each group (n = 6 to 9 mice per treatment group).

FIGURE 7. AhR-mediated enhancement of infection-associated pulmonary neutrophilia requires AhR in the respiratory epithelium. CreSftpc AhRX/− and AhRX/− were treated and infected as described for Fig. 5. Mice were sacrificed 7 d postinfection. (A) Bars depict the average number of Gr-1+ cells, as determined by flow cytometric analysis of lung-derived immune cells (±SEM; n = 6 to 8 mice per treatment group). Similar observations were obtained using differential cell counts of H&E-stained, lung-derived immune cells. An * indicates a significant difference compared with vehicle-control of the same genotype (p ≤ 0.05). (B) Lung homogenates were subjected to SDS-PAGE and probed with Abs against iNOS and β-actin. Two samples per treatment group are shown and are representative of findings in all mice in each group (n = 6 to 8 mice per treatment group).
**Discussion**

A central tenet of immunobiology is that immune responses need to be balanced, with neither too little nor too much of a particular mediator, and each effector cell or soluble factor needs to be present in the right place, for the correct amount of time: neither too long nor too brief. However, there are numerous examples in which poorly controlled responses contribute to disease. Several recent reports suggest that the AhR is a very important molecule that integrates responses of the immune system with cues from the host’s environment, thereby influencing the kinetics, magnitude, and/or direction of an immune response (8). Indeed, it has long been known that AhR ligands have tremendous influence on lymphocyte subset differentiation and function, which affect a wide range of disease models (11, 15, 65–67). However, the influence of AhR on other components of the immune system has received less attention. Through the work reported in this study, we have expanded the repertoire of AhR-sensitive target cells that influence the function of the immune system to include endothelial cells and, at least within the lung, epithelial cells. Our data further emphasize that AhR-mediated regulation of immunobiology is complex and that in addition to direct effects on immune cells, AhR activation in nonhematopoietic cells acts in a paracrine manner to modulate aspects of leukocyte function.

The current study focuses on how activation of the AhR modulates two anti-viral innate responses in the lung during influenza virus infection. Although examined in only a handful of studies, neutrophil recruitment to the site of Ag challenge is consistently modulated by AhR activation, regardless of the Ag or target organ (17–19). Also, changes in neutrophil number have been reported in AhR-deficient mice (49, 68). Thus, AhR may be an important modulator of neutrophil recruitment or accumulation in tissues. However, AhR activation alone, in the absence of infection or other secondary stimuli, does not affect neutrophilic inflammation. Therefore, it is likely that AhR signaling integrates with, modifies, or impedes the “message” delivered by other host-derived signals.

In the case of neutrophilic inflammation during infection with influenza viruses, our data indicate that AhR modulates gene expression and signaling events within respiratory epithelial cells, which in turn regulate the recruitment or retention of neutrophils in the infected lung. When considering this approach and the data obtained, it is important to consider that conditional deletion of the AhR from the respiratory epithelium was accomplished using CreSftpc mice. In adult mice, surfactant protein C is produced by type II respiratory epithelial cells. However, during fetal mouse development, Sftpc gene expression is first observed on embryonic day 10.5 within the undifferentiated respiratory epithelium in the distal developing lung buds (40). Therefore, in CreSftpc,Ahr+/− mice, AhR deletion is not limited to type II epithelial cells, but is broadly abrogated from the respiratory epithelium. This has been validated in other systems, for example using CreSftpc,Bcl-XΔt/Δt mice to delete Bcl-X in respiratory epithelial cells (39). Several groups have reported that different lung-specific cell lineages are likely sensitive to AhR expression and activation, including Clara cells, bronchial epithelial cells, and fibroblasts (69–71). However, there is limited information regarding how cell type–specific AhR activation in vivo impacts immune responses. Our data reveal that respiratory epithelial cells are direct AhR target cells and suggest that AhR-mediated gene expression in this nonhematopoietic lineage is an important consideration when seeking to understand how AhR modulates immune responses.

The discovery that AhR-mediated events within lung epithelial cells directly influence neutrophil recruitment during infection has many implications for human health. Lung epithelial cells are major producers of cytokines and chemoattractant factors that control the emigration of leukocytes, including neutrophils, to sites of infection (72). We previously reported that in the context of viral infection, AhR activation does not alter levels of inflammatory cytokines, such as IL-1 and TNF-α (26), or neutrophil chemoattractants, including KC (CXCL1), MIP-1α, MIP-2, LIX, IL-6, and C5a (17). Further, increased neutrophil migration is not the result of AhR-mediated increases in an unknown soluble neutrophil chemoattractant (25), nor does it appear to be due to alterations in level of cell adhesion molecules such as ICAM (ICAM-1, CD54), PECAM (CD31), VCAM-1 (CD106), CD38, E-selectin, and P-selectin (Ref. 17 and B.P. Lawrence, unpublished observations). Additionally, we have found no difference in the level of SP-A, Clara cell secretory protein, vascular endothelial growth factor, leukotriene B4, or tissue inhibitor of metalloproteinase 1 at any point in time examined in lung tissue or lavage fluid from TDCC-treated, infected mice compared with the levels detected in infected, vehicle-treated mice (data not shown). These data indicate to us that the relationship between epithelial cell–specific AhR activation and neutrophil recruitment during influenza virus infection is a complicated and intricately regulated process. Although we have discovered that many of the pathways and mediators known to affect neutrophil migration and retention to the lung are not altered by AhR activation, there remain numerous potential molecular targets of AhR in the respiratory epithelium. Possible targets include genes encoding enzymes that regulate lipid mediators of inflammation, matrix metalloproteinases, antimicrobial peptides, mucins, and components of the extracellular matrix (72–75). Therefore, the influence of AhR activation on these lung epithelium–derived targets and the relationship with neutrophil recruitment remains unknown and is of considerable interest.

The novel findings reported in this study also add to growing appreciation that AhR in endothelial cells broadly influences health and disease. For example, AhR expression within endothelial cells regulates the closure of the ductus venosus in the liver during development (34). Further, endothelial-specific AhR deletion has been associated with elevated hypotension due to reduced responsiveness to angiotensin II and decreased expression of renin and angiotensin receptor 1 (76). Through the use of CreAhr+/−,Ahr+/− mice, we found that AhR-mediated increases in iNOS are mediated through activation of the AhR within the endothelium. At first glance this may seem confusing, as within the infected lung, AhR-dependent increases in iNOS are mediated through activation of the AhR within the endothelium. However, although macrophages are one of the principal cell types that expresses iNOS in the influenza virus–infected lung, we previously demonstrated using Ahr−/−→Ahr+/− bone marrow chimeric mice that elevated macrophage iNOS is driven by an AhR-dependent mechanism that is extrinsic to hematopoietic cells (27). Further, although we identified two putative AhRE within the inos transcriptional regulatory region, we have found no evidence that ligand-activated AhR binds to either of these sites using chromatin immunoprecipitation assays (B.P. Lawrence, unpublished observations). Yet, our results obtained using AhrbdΔ/Δ and AhrbdΔ mice show that the AhR must translocate to the nucleus and bind DNA via its cognate DNA binding domain to mediate changes in iNOS levels. Collectively, these findings indicate that within the context of infection with influenza virus, AhR activation within endothelial cells likely alters expression of genes that encode iNOS-inducing factors, which then drive enhanced iNOS expression in respiratory epithelial cells and macrophages through paracrine signaling mechanisms.
A number of pathways regulate iNOS transcription, including the JNK, JAK-STAT, and p38 MAPK pathways (77). These pathways are largely activated through cytokine induction; therefore, it is possible that AhR activation within the endothelium could increase production of iNOS-inducing cytokines or factors, which act upon macrophages and epithelial cells. Endothelial specific events have also been shown to modulate immune responses. Stimulation of endothelial S1P,R alters immune responses to H1N1 influenza virus by suppressing cytokine production and immune cell recruitment (78). S1P signaling has also been shown to attenuate iNOS levels and NO production in the lungs of mice undergoing intestinal ischemia–reperfusion mediated acute lung injury (79). However, a relationship between AhR activation and S1P signaling is yet to be defined. Another potential endothelial cell–derived factor that has been shown to modulate iNOS expression is endothelin-1 (ET-1). Increased iNOS expression has been linked to ET-1 expression in a rat model of acute respiratory distress syndrome and in rat astrocytes (80, 81). In mice, ET-1 is produced in the lung, with most of its expression originating from endothelial cells (82). Further, endothelin receptors A and B are widely expressed in the lung. AhR knockout mice have elevated levels of plasma ET-1, indicating that the AhR could play a role in ET-1 signaling (83). Through gene expression profiling studies of whole-lung tissue obtained from influenza virus–infected mice, we found that AhR activation increases ET-1 transcript levels 2-fold compared with vehicle-treated, infected mice (data not shown). Thus, it is possible that AhR-mediated increases in ET-1 could be one of the factors responsible for elevations in iNOS levels during infection.

In summary, through the work reported in this study, we have expanded the repertoire of direct AhR target cells that influence immune function to include endothelial cells and, at least within the lung, epithelial cells. We have shown that these are separate AhR targets cells and that AhR-mediated effects in them lead to independent downstream events in the infected lung. These findings provide the opportunity to probe pathways specific to these cell lineages and will likely lead to the discovery of novel, AhR-dependent regulatory signals that modulate neutrophil migration and iNOS expression. The idea that AhR activation leads to simultaneous but independent events within multiple cell types, which collectively influence disease outcome, has broad consequences for health. Although this work was conducted in the context of infection with influenza virus, increased inflammation due to excessive neutrophil accumulation and iNOS expression can be detrimental in other disease states as well. The implications of this include a better understanding of how the AhR regulates the balance between appropriate and excessive inflammation during infection, which will translate to a broader knowledge of AhR-dependent factors that regulate these innate immune mediators in other diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


