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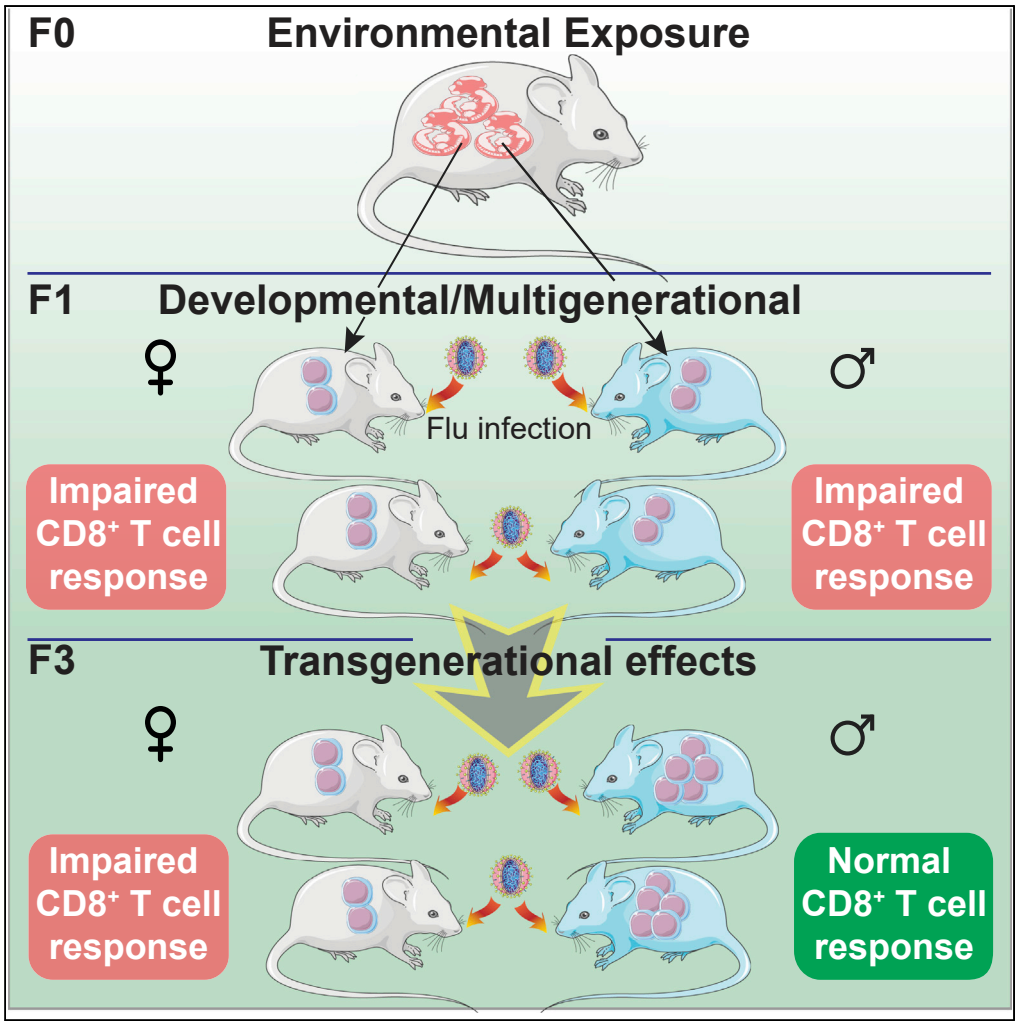

Christina M. Post, Lisbeth A. Boule, Catherine G. Burke, Colleen T. O'Dell, Bethany Winans, B. Paige Lawrence

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HIGHLIGHTS

Maternal AHR activation causes transgenerational effects on an immune response

CD8 T cell response to influenza virus is decreased in male and female F1 offspring

Transgenerational effects were observed in infected lungs of female F3 offspring

Both maternal and paternal F1 generations contribute to the immune effects in F3

Post et al., iScience *20*, 168–183
October 25, 2019
https://doi.org/10.1016/j.isci.2019.09.014

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The Ancestral Environment Shapes Antiviral CD8$^+$ T cell Responses across Generations

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SUMMARY

Recent studies have linked health fates of children to environmental exposures of their great grandparents. However, few studies have considered whether ancestral exposures influence immune function across generations. Here, we report transgenerational inheritance of altered T cell responses resulting from maternal (F0) exposure to the aryl hydrocarbon receptor ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Since F0 exposure to TCDD has been linked to transgenerational transmission of reproductive problems, we asked whether maternal TCDD exposure also caused transgenerational changes in immune function. F0 exposure caused transgenerational effects on the CD8$^+$ T cell response to influenza virus infection in females but not in males. Outcrosses showed changes were passed through both parental lineages. These data demonstrate that F0 exposure to an aryl hydrocarbon receptor (AHR) agonist causes durable changes to immune responses that can affect subsequent generations. This has broad implications for understanding how the environment of prior generations shapes susceptibility to pathogens and antiviral immunity in later generations.

INTRODUCTION

It has become increasingly clear that early life development is a critical and unique window of vulnerability during which environmental exposures influence cellular programming in ways that shape health and disease later in life. There is also growing evidence that maternal stressors and environmental factors affect not just the offspring's health, but also that of subsequent generations as well (Heindel, 2018; Rattan et al., 2018; Rissman and Adli, 2014; Skinner, 2014; Skinner et al., 2010; van Steenwyk et al., 2018; Walker and Gore, 2011). Exposure during pregnancy results in the developing fetus (F1) and its gametes (F2) being exposed. Changes in the F1 and F2 progeny are referred to as developmental and multigenerational effects, respectively. The F3 generation is the first generation with no exposure to the original insult to the pregnant mother; therefore changes in this generation represent transgenerational inheritance (Skinner, 2008). Recent reports of transgenerationally inherited adverse health effects of environmental exposures underscore the importance of this phenomenon to human health and disease (Ferey et al., 2019; Gillette et al., 2018; Klukovich et al., 2019).

The majority of studies demonstrating transgenerational effects of environmental agents have examined their consequences on the reproductive, nervous, or endocrine systems (Babenko et al., 2015; Bohacek et al., 2013; Guerrero-Bosagna and Skinner, 2014; Heindel, 2018; Nilsson and Skinner, 2015; Stegemann and Buchner, 2015). In contrast, research on transgenerational inheritance rarely includes assessment of whether maternal exposures impinge on the function of the immune system. Yet, a properly functioning immune system is fundamentally important to individual and public health. Even slight alterations can reduce defenses against infections or diminish vaccine efficacy (Dallaire et al., 2006; Glynn et al., 2008; Stolevik et al., 2013; Winans et al., 2011). Thus, the consequences of maternal and early life exposures that alter the function of the immune system are broad reaching. Moreover, when it has been examined, developmental exposures to a range of common pollutants, pharmaceuticals, as well as maternal diet have been associated with changes in immune function later in life (Boule and Lawrence, 2016; Dietert, 2009). For instance, higher levels of dioxins and polychlorinated biphenyls (PCBs) in breast milk, cord, and infant blood correlate with increased respiratory tract infections and reduced responses to vaccination (Hochstenbach et al., 2012; Jusko et al., 2016; Miyashita et al., 2011; Stolevik et al., 2013). Animal studies reveal parallel events, showing that maternal and early life exposures durably change immune responses in the

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https://doi.org/10.1016/j.isci.2019.09.014

offspring (Winans et al., 2011; Boule and Lawrence, 2016). For example, in mouse models, maternal treatment with the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) perturbs T cell responses to influenza A virus (IAV) infection in offspring (Boule et al., 2014; Vorderstrasse et al., 2004; Winans et al., 2015). TCDD and other dioxins, as well as PCBs, are persistent organic pollutants. Moreover, many dioxins and PCBs, including TCDD, activate the aryl hydrocarbon receptor (AHR), an environment-sensing transcription factor that plays important, albeit not fully understood, roles in the development and function of the immune system (Rothhammer and Quintana, 2019; Stevens et al., 2009). In addition to pollutants, the AHR has a multitude of ligands ranging from pharmaceuticals to compounds in foods we eat to microbial by-products (Hubbard et al., 2015; Murray et al., 2014; Nguyen and Bradfield, 2008); thus, we are regularly exposed to AHR ligands. Therefore, it is important to understand how exposure to AHR ligands impacts the immune system, including in subsequent generations. TCDD is the most potent and specific AHR agonist and provides a valuable tool to study how developmental activation of the AHR affects the immune system.

In addition to being expressed in cells of the immune system, the AHR is expressed in the reproductive tract, and there have been numerous studies examining the effects of TCDD on the reproductive system (Bruner-Tran et al., 2017; Lew et al., 2011; You et al., 2018). Developmental exposure to TCDD affects both the male and female reproductive systems. For instance, reduced sperm counts in males and higher incidence of endometriosis in females are among the effects observed in F1 offspring (Bruner-Tran et al., 2017; Pilsner et al., 2017). In addition to impacting germ cells and adult F1 offspring, there is also a growing literature base connecting F0 exposure to TCDD with transgenerational effects on both the male and female reproductive systems (Bruner-Tran et al., 2014; Bruner-Tran and Osteen, 2011; Manikkam et al., 2012). Taken together with studies showing that F0 TCDD exposure affects immune responses in F1 offspring (Boule et al., 2014, 2015a; 2015b; Hogaboam et al., 2008; Meyers et al., 2018; Vorderstrasse et al., 2004, 2006; Winans et al., 2015), we hypothesized that F0 TCDD exposure also causes transgenerational effects in the immune system.

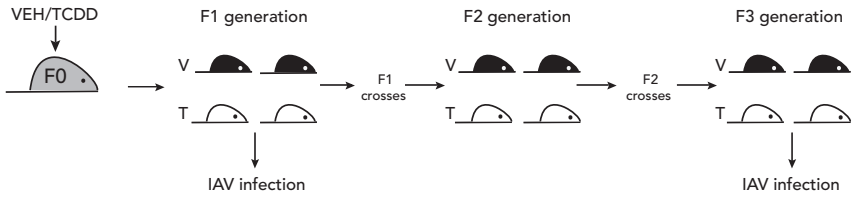
Influenza A virus (IAV) is a common human pathogen that infects the respiratory system and causes infections that range in severity from moderate to fatal. Every year IAV infections are among the major causes of global morbidity and mortality (Iuliano et al., 2018; Reed et al., 2015). Mouse models of infection with human IAV provide an experimental system with strong relevance to human health, and the murine immune response to IAV closely resembles that of humans (Schmidt and Varga, 2018). In particular, successfully fighting acute primary IAV infection is $CD8^+$ T cell dependent (Topham et al., 1997). The T cell response to IAV occurs in two distinct sites: the lung-draining lymph nodes, such as the mediastinal lymph nodes (MLNs), and the respiratory tract. After encountering IAV in the respiratory tract, dendritic cells (DCs) emigrate to lymph nodes that drain the lung, where they activate naive $CD8^+$ T cells. When they receive the correct signals, virus-specific $CD8^+$ T cells undergo clonal expansion and differentiate into cytotoxic T lymphocytes (CTL), which migrate to the site of infection (i.e., lung), where they directly kill virally infected cells through the release of cytotoxic granules (Kohlmeier and Woodland, 2009).

In this study, we determined whether maternal (F0) exposure to the AHR agonist TCDD causes transgenerational effects on $CD8^+$ T cell responses to IAV infection. Specifically, following maternal exposure to a low, environmentally relevant dose of TCDD (Birnbaum and Tuomisto, 2000; DeVito et al., 1995), we determined the frequency of responding $CD8^+$ T cells in male and female offspring in the F1 and F3 generations, using well-established metrics of their activation, expansion, and differentiation into effector cells. By outcrossing F1 generation mice, we also sought to define whether the transgenerational effects on the immune system are preferentially passed through the maternal or paternal lineage. Delineating which aspects of the $CD8^+$ T cell response to infection are affected across generations furthers our understanding of how environmental AHR ligands cause durable changes to T cell responses and also expands current knowledge of how developmental exposures can impinge on immune responses later in life.

RESULTS

To generate transgenerationally exposed cohorts, F0 dams were administered either vehicle or TCDD orally. The dose of TCDD used in this study does not cause overt toxicity to the dam or pups, nor does it alter immune organ cellularity in naive (unchallenged) offspring (Vorderstrasse et al., 2004; Winans et al., 2015). At maturity, non-sibling F1 offspring were bred to create the F2 generation, and then non-sibling, non-cousin F2 offspring were bred to create the F3 generation (Figure 1A, see Transparent Methods). Consistent with prior reports (Vorderstrasse et al., 2004, 2006), maternal treatment with TCDD

A



B

Generation	F1		F2		F3	
F0 exposure	Vehicle	TCDD	Vehicle	TCDD	Vehicle	TCDD
Pregancy metric (d)	18.8 ± 0.2	18.8 ± 0.1	26.5 ± 2.3	22.6 ± 0.1	23.1 ± 1.2	23.7 ± 1.2
Number of pups/litter	5.9 ± 0.4	5.3 ± 0.5	6.6 ± 0.5	5.9 ± 0.4	6.0 ± 0.3	6.4 ± 0.4
Sex ratio (M:F)	0.9 ± 0.1	1.4 ± 0.3	1.3 ± 0.4	1.1 ± 0.3	1.2 ± 0.2	1.3 ± 0.3

Figure 1. Breeding Schematic and Reproductive Endpoints

(A) Transgenerational breeding strategy: Pregnant C57/Bl6 dams were treated (p.o.) with either vehicle or TCDD (1 μg/kg) on GD0, 7, 14, and PND2. Separate sires were used for each dam. No culling of litters was performed, and littermates were housed in same-sex groups. Offspring sex was determined, and offspring were randomly assigned to groups for breeding or infection. 15–20 pairs of non-sibling F1 offspring were bred to create the F2 generation. F2 offspring were used to create the F3 generation.

(B) The table summarizes pregnancy metrics, litter size, and sex ratio of offspring from the F1, F2, and F3 generations. Pregnancy metrics: F1 generation, time to parturition, which is defined as the number of days from vaginal plug identification to birth; F2 and F3 generations, the number of days from pairing to giving birth. Data are shown as mean ± SEM. A Student's t test was used to compare TCDD with vehicle groups for each endpoint within each generation.

at this dose and on this schedule did not alter the time to parturition (time from vaginal plug discovery to birth), litter size, or sex ratio in F1 offspring (Figure 1B). Similarly, F0 exposure did not significantly change fertility and duration of pregnancy, as there were no differences in the time between pairing and birth in the F2 or F3 generations, nor were there differences in litter size or sex ratio among the offspring (Figure 1B).

F0 Exposure to TCDD Has Transgenerational Effects on the $CD8^+$ T cell Response to IAV in the Lungs of Female F3 Offspring

IAV infects epithelial cells in the respiratory tract, and $CD8^+$ T cells are the principle cell type responsible for viral clearance during acute primary infection. In particular, in the lung, virus-specific cytotoxic $CD8^+$ T cells eliminate IAV infected cells (Kohlmeier and Woodland, 2009; McGill and Legge, 2009; Schmidt and Varga, 2018). To assess how F0 AHR activation affects the overall quality of the $CD8^+$ T cell response to IAV across generations, we examined metrics of clonal expansion and effector function in F1 and F3 offspring. At maturity, female mice that were exposed developmentally (F1 generation offspring) or transgenerationally (F3 generation offspring) were infected with IAV. Nine days after infection, which is the peak day of this response (Lawrence et al., 2000, 2006), the percentage and number of $CD8^+$ T cells in the lungs of TCDD lineage offspring were significantly reduced in F1 generation offspring, but not in the F3 generation (Table S1). The percentage of viral nucleoprotein (NP)-specific $CD8^+$ T cells was reduced by about 60% in female mice that were developmentally exposed to TCDD (Figure 2A). Similarly, the number of NP^+CD8^+ T cells was significantly decreased in TCDD lineage F1 offspring compared with infected vehicle F1 offspring (Figure 2B). In the F3 generation, the percentage of NP^+CD8^+ T cells was not affected by F0 AHR activation (Figure 2C). However, F0 AHR ligand exposure caused a significant decrease in the number of NP^+CD8^+ T cells in the lungs of F3 female TCDD lineage offspring (Figure 2D). To assess how F0 AHR activation affects the overall magnitude of the $CD8^+$ T cell response to IAV, we also determined the percentage and number of CTL ($CD44^{hi}CD62L^{lo}CD8^+$ T cells), a population that includes NP-specific as well as $CD8^+$ T cells specific for other viral peptide epitopes. In both the F1 and F3 generations, the proportion of $CD8^+$ T cells that were $CD44^{hi}CD62L^{lo}$ was not influenced by F0 AHR activation (Figures 2E and 2G). However, the number of CTL was significantly reduced in F1 TCDD lineage offspring (Figure 2F) and 60% reduced in F3 offspring (Figure 2H). These results demonstrate that AHR activation in F0 dams has transgenerational effects on $CD8^+$ T cell clonal expansion during the response to IAV.

A successful $CD8^+$ T cell response to IAV infection not only involves a significant increase in the magnitude of the $CD8^+$ T cell population in the lung, but also indicates that the cells have gained their effector

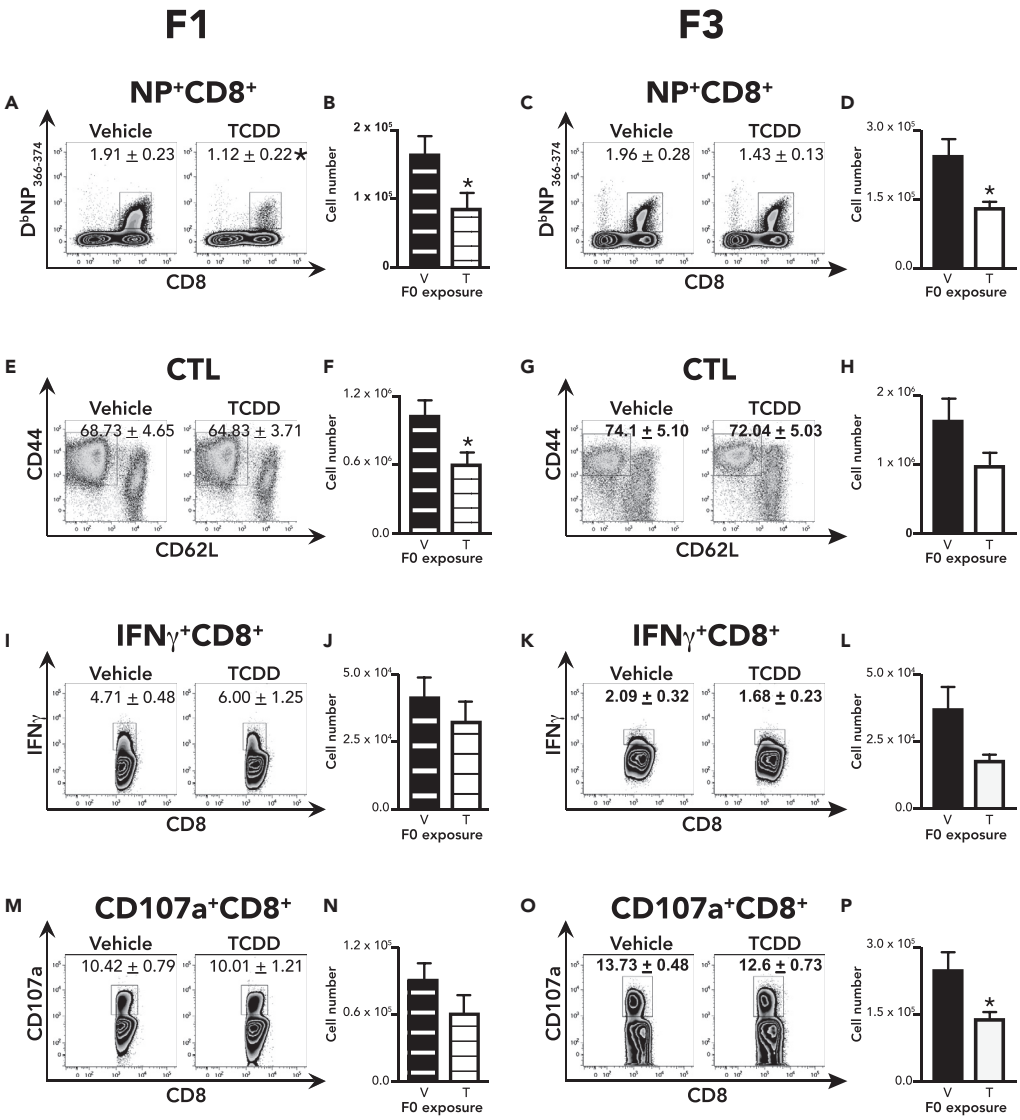


Figure 2. F0 Exposure to TCDD Has Transgenerational Effects on the CD8$^+$ T Response to IAV in the Lung of Female Offspring

At maturity (8–10 weeks of age) 7–9 female mice from each exposure group were infected with IAV, and the CD8$^+$ T cell response in the lung was measured 9 days later.

(A) Flow plots depict the mean percent of NP$^+$CD8$^+$ T cells in vehicle (left) and TCDD (right) F1 offspring.

(B) The graph indicates the mean number of NP$^+$CD8$^+$ T cells in vehicle and TCDD F1 offspring.

(C) Flow plots depict the mean percent of NP$^+$CD8$^+$ T cells in F3 vehicle (left) and TCDD (right) lineage offspring.

(D) The mean number of NP$^+$CD8$^+$ T cells in F3 vehicle and TCDD lineage offspring.

(E) The flow plots display the mean percent of CTL in vehicle (left) and TCDD (right) F1 offspring.

(F) The mean number of CTL in vehicle and TCDD F1 offspring.

(G) Flow plots depict the mean percent of CTL in F3 vehicle (left) and TCDD (right) lineage offspring.

(H) The mean number of CTL in vehicle and TCDD lineage F3 offspring.

(I) The flow plot depicts the mean percent of IFNγ$^+$CD8$^+$ T cells in the lung of F1 offspring.

(J) The mean number of IFNγ$^+$CD8$^+$ T cells in the lung of F1 offspring.

(K) The flow plots show the mean percent of IFNγ$^+$CD8$^+$ T cells in the lung of F3 offspring.

(L) The mean number of IFNγ$^+$CD8$^+$ T cells in the lung of F3 offspring.

(M) The flow plots depict the mean percent of CD107a$^+$CD8$^+$ T cells in the lung of F1 offspring.

(N) The mean number of CD107a$^+$CD8$^+$ T cells in the lung of F1 offspring.

(O) The flow plots depict the mean percent of CD107a$^+$CD8$^+$ T cells in F3 offspring.

Figure 2. *Continued*

(P) The mean number of CD107a$^+$CD8$^+$ T cells in the lung of F3 offspring. Full gating strategy is depicted in Figure S1. NP$^+$CD8$^+$ T cells were derived from the leukocyte gate; all others were generated from the CD8$^+$ T cell gate (Figure S1). Data are represented as mean ± SEM; * denotes $p \leq 0.05$ (Student's t test). The numerical values that correspond to the bar graphs as well as p values for each comparison are listed in Table S1.

functions. These include secretion of antiviral cytokines and degranulation, which enable CD8$^+$ T cells to kill IAV-infected cells and clear the infection. To assess how F0 TCDD exposure impacts CD8$^+$ T cell effector function, we determined the frequency and number of CD8$^+$ T cells that make the antiviral cytokine IFNγ and that express CD107a, which is an indicator of degranulation. In the F1 generation, IFNγ production by CD8$^+$ T cells in the lung was largely unaffected by F0 AHR activation (Figures 2I and 2J). However, in the F3 offspring, there was a 50% decrease in the number of IFNγ$^+$CD8$^+$ T cells in the lung (Figures 2K and 2L). A similar pattern was observed for CD107a$^+$CD8$^+$ T cells: there were no significant differences in either the percentage or number of CD107a$^+$CD8$^+$ T cells between vehicle and TCDD lineage offspring in the F1 generation (Figures 2M and 2N). However, in the F3 generation, the number, but not proportion, of CD107a^{+}CD8$^+$ T cells was significantly reduced when the AHR was activated in F0 dams (Figure 2P and 2O). Thus, our findings indicate that F0 exposure to the AHR agonist TCDD has transgenerational effects on both CD8$^+$ T cell effector function and clonal expansion during IAV infection. Interestingly, the transgenerational effects appear more robust with regard to the reduction of clonal expansion than effector function, suggesting that these pathways may be differentially regulated by F0 AHR activation.

F0 Exposure to TCDD Has Developmental but Not Transgenerational Effects on the CD8$^+$ T cell Response to IAV in the Lungs of Male Offspring

In parallel to experiments performed using female mice, we measured the CD8$^+$ T cell response in their male siblings in the F1 and F3 generations. F0 exposure did not significantly modify the percent of NP$^+$CD8$^+$ T cells in the lungs of male F1 or F3 offspring (Figures 3A and 3C, Table S1). The number of NP$^+$CD8$^+$ T cells was reduced by 45% in the F1 generation (Figure 3B) but was not significantly different from vehicle lineage in the F3 generation (Figure 3D). The percentage of CTL in the lungs of TCDD lineage F1 offspring was reduced by approximately 75% compared with vehicle (Figure 3E). Likewise, the size of the CTL population was smaller in TCDD lineage F1 offspring compared with vehicle (Figure 3F). However, in F3 generation male TCDD lineage offspring, neither the percentage nor the number of CTL was affected by F0 exposure to TCDD (Figures 3G and 3H). In the F1 generation, the proportion of CD8$^+$ T cells that produced IFNγ was increased by F0 AHR activation (Figure 3I); however, the number of IFNγ$^+$CD8$^+$ T cells was reduced in F1 TCDD lineage offspring (Figure 3J). In the F3 generation, however, the percentage of IFNγ$^+$CD8$^+$ T cells was not altered by F0 TCDD exposure (Figure 3K). Moreover, similar to the F1 generation, the number of IFNγ$^+$CD8$^+$ T cells was reduced by 60% in TCDD lineage offspring compared with vehicle (Figure 3L). F0 AHR activation had a minimal impact on the CD107a$^+$CD8$^+$ T cell population in lungs of male offspring. Specifically, the proportion of CD8$^+$ T cells that expressed CD107a was not different in either F1 or F3 generation offspring (Figures 3M and 3O). The number of CD107a$^+$CD8$^+$ T cells in F1 generation offspring was reduced by 40% in offspring of TCDD-treated dams (Figure 3N); however, the number of CD107a$^+$CD8$^+$ T cells in TCDD F3 generation offspring was not different from vehicle F3 lineage mice (Figure 3P).

In summary, these results indicate that F0 exposure to TCDD affects important aspects of the CD8$^+$ T cell response to IAV in the lungs of both male and female F1 offspring. However, in the F3 generation, F0 exposure had a minimal impact on the CD8$^+$ T cell response to IAV in male offspring. Conversely, F0 exposure caused transgenerational effects on the CD8$^+$ T cell response to infection in the lungs of female F3 offspring, findings that suggest that transgenerational inheritance in the immune system may be sex specific.

In the MLN, F0 AHR Activation Alters the CD8$^+$ T cell Response of Female and Male F1 but Not F3 Generation Offspring

Although the effector functions of CD8$^+$ T cells to IAV infection are predominantly executed in the respiratory tract, their activation begins in the lymph nodes, where CD8$^+$ T cells interact with antigen-presenting cells expressing viral peptides. This stimulates naive CD8$^+$ T cells to clonally expand and differentiate into armed effectors before migrating to the lung. Therefore, we determined whether F0 TCDD exposure also caused transgenerational changes in CD8$^+$ T cell clonal expansion or differentiation in the MLN in response

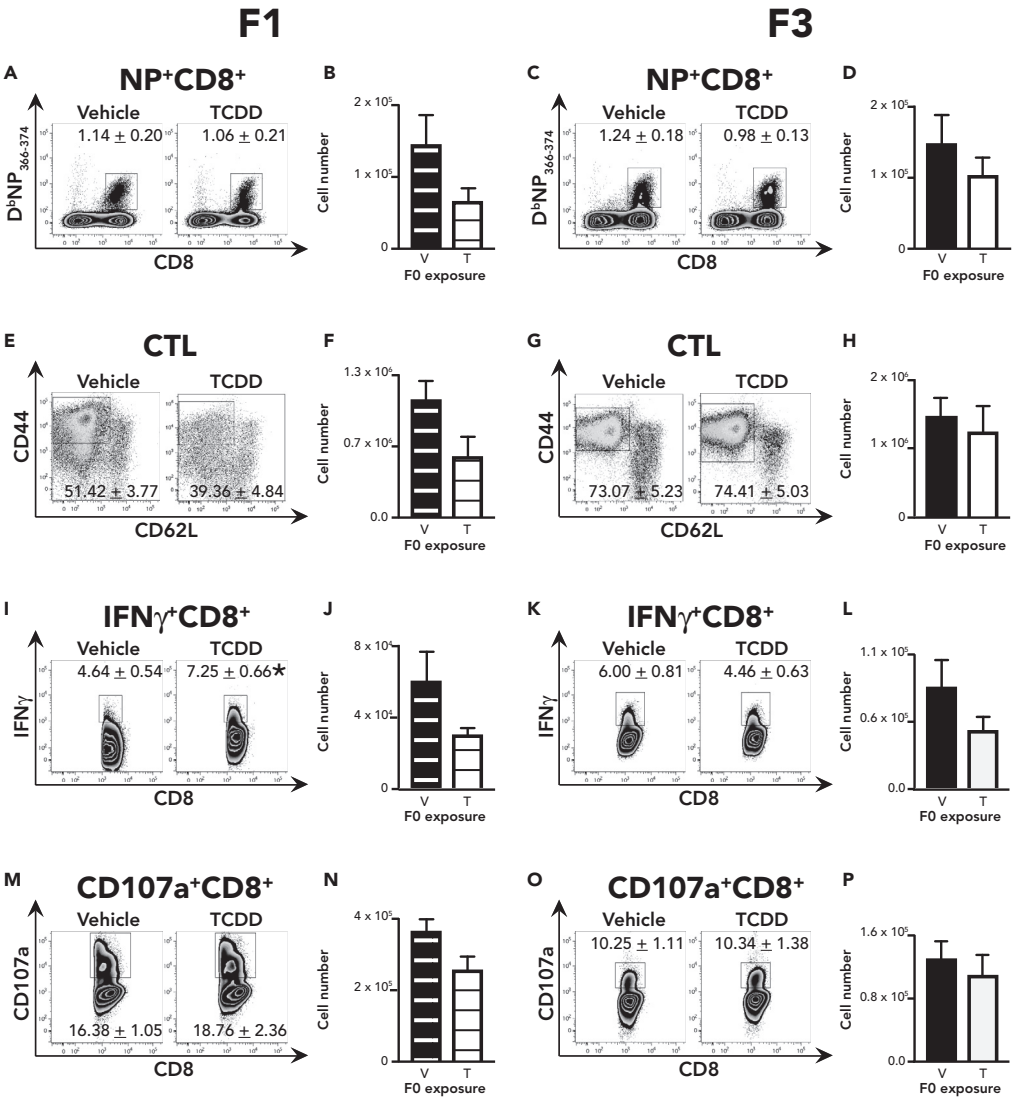


Figure 3. F0 Exposure to TCDD Has Developmental but Not Transgenerational Effects on the CD8$^+$ T Response to IAV in the Lung of Male TCDD Lineage Offspring

7–8 male mice from each treatment group were infected with IAV, and the CD8$^+$ T cell response in the lung was measured 8 days later.

(A) Flow plots depict the mean percent of NP$^+$CD8$^+$ T cells in vehicle (left) and TCDD (right) F1 offspring.

(B) The mean number of NP$^+$CD8$^+$ T cells in vehicle and TCDD F1 offspring.

(C) Flow plots depict the mean percent of NP$^+$CD8$^+$ T cells in F3 vehicle (left) and TCDD (right) lineage offspring.

(D) The mean number of NP$^+$CD8$^+$ T cells in F3 vehicle and TCDD lineage offspring.

(E) The flow plots display the mean percent of CTL in vehicle (left) and TCDD (right) F1 offspring.

(F) The mean number of CTL in vehicle and TCDD F1 offspring.

(G) Flow plots depict the mean percent of CTL in F3 vehicle (left) and TCDD (right) lineage offspring.

(H) The mean number of CTL in vehicle and TCDD lineage F3 offspring.

(I) The flow plot depicts the mean percent of IFNγ$^+$CD8$^+$ T cells in the lung of F1 offspring.

(J) The mean number of IFNγ$^+$CD8$^+$ T cells in the lung of F1 offspring.

(K) The flow plots show the mean percent of IFNγ$^+$CD8$^+$ T cells in the lung of F3 offspring.

(L) The mean number of IFNγ$^+$CD8$^+$ T cells in the lung of F3 offspring.

(M) The flow plots depict the mean percent of CD107a$^+$CD8$^+$ T cells in the lung of F1 offspring.

(N) The mean number of CD107a$^+$CD8$^+$ T cells in the lung of F1 offspring.

(O) The flow plots depict the mean percent of CD107a$^+$CD8$^+$ T cells in F3 offspring.

Figure 3. *Continued*

(P) The mean number of CD107a$^+$CD8$^+$ T cells in the lung of F3 offspring. Full gating strategy is depicted in Figure S1. NP$^+$CD8$^+$ T cells were derived from the leukocyte gate; all others were generated from the CD8$^+$ cell gate (Figure S1). Data are shown as mean + SEM; * denotes $p \leq 0.05$ (Student's t test). Numerical values for the bar graphs as well as p values that correspond to the comparisons in each panel are shown in Table S1.

to IAV infection. Across all metrics, maternal exposure reduced the CD8$^+$ T cell response to IAV in the MLN of male and female F1 offspring. Thus, offspring of TCDD-treated dams exhibited significant reductions in the number of NP$^+$CD8$^+$ T cells (Figures 4A and 4E), CTL (Figures 4B and 4F), IFNγ^+ (Figures 4C and 4G), and CD107a$^+$CD8$^+$ T cells (Figures 4D and 4H and Table S2). Despite the strong effect that F0 AHR activation had on the CD8$^+$ T cell response in F1 offspring, we did not observe any statistically significant differences in these measures of CD8$^+$ T cell expansion or effector function in the MLN of male or female F3 offspring (Figure 4, Table S2).

Transgenerational Effects on the CD8$^+$ T cell Response to IAV Are Transmitted through Both Parental Lineages

To determine whether the reduced CD8$^+$ T cell responses in the lungs of female F3 generation offspring are preferentially transmitted through the maternal or paternal F1 lineage, we performed outcrosses of F1 mice. The outcrosses generated a group of offspring that had only maternal lineage TCDD exposure and another group of offspring with only paternal lineage TCDD exposure (Figure 5A). As controls, we also carried F1 siblings forward by incrossing F1 males and females from the same F0 exposure groups (i.e., F1 VEH x F1 VEH; F1 TCDD x F1 TCDD). At maturity, we infected F3 offspring from all four crosses and measured CD8$^+$ T cell clonal expansion and effector function. Consistent with our prior observations (Figure 2D), in the F3 generation, the number of virus NP-specific CD8$^+$ T cells was significantly decreased in the incrossed TCDD lineage relative to incrossed vehicle lineage offspring (Figure 5B, black and white bars). Maternal TCDD lineage exposure replicated the significant decrease (Figure 5B, black and light gray bars). Paternal lineage exposure also decreased the number of NP$^+$CD8$^+$ T cells, but the effect was not statistically significant (Figure 5B compare the black and dark gray bars; see also Table S4). TCDD exposure through either the paternal or maternal lineage significantly reduced the number of CTL (Figure 5C). The average number of IFNγ^+CD8$^+$ T cells was not significantly different among the outcrossed groups and control; however, the two incrossed groups were significantly different (Figure 5D, Table S4). Similar to viral NP-specific CD8$^+$ T cells, the number of CD107a$^+$CD8$^+$ T cells was significantly reduced in both the TCDD incrossed and maternal lineages, whereas paternal TCDD lineage exposure caused a subtler decrease in the number of CD107a$^+$CD8$^+$ T cells, which did not reach statistical significance (Figure 5E). When considered together, these changes in CD8$^+$ T cell clonal expansion and effector function do not appear to be transmitted solely through a single parental lineage, as F3 effects were observed in both the maternal and paternal TCDD lineage exposures separately. Although both F1 parental lineages affected the CD8$^+$ T cell response to IAV in the F3 generation, these data suggest that exposure through the maternal line had a more potent effect on the CD8$^+$ T cell response than paternal lineage TCDD exposure and that clonal expansion may be more sensitive to the effects of F0 AHR activation than acquisition of CTL effector function.

Developmental AHR Activation Influences the CD8$^+$ T cell Response to Infection via Targeting Hematopoietic Cells

The AHR is widely expressed in hematopoietic and non-hematopoietic cells. To determine whether AHR signaling within the hematopoietic compartment contributes differentially to the effects of developmental exposure in the CD8$^+$ T cell responses of female and male offspring, we used conditional knockout mice that lack *Ahr* in all hematopoietic cells (Bennett et al., 2018). To create littermates that do and do not express the *Ahr* in hematopoietic cells, we crossed nulliparous $Ahr^{fx/fx}$ females with $Ahr^{fx/fx}Vav1^{cre}$ male mice. The resulting male and female $Ahr^{fx/fx}$ and $Vav1^{cre}Ahr^{fx/fx}$ offspring of control or TCDD-treated dams were infected with IAV at maturity. Similar to wild-type B6 mice, female $Ahr^{fx/fx}$ F1 offspring that were developmentally exposed to TCDD exhibited reduction in the percentage (Figure 6A) and number (Figure 6B) of NP$^+$CD8$^+$ T cells compared with vehicle-exposed $Ahr^{fx/fx}$ mice. However, when *Ahr* was excised from hematopoietic cells, maternal exposure did not affect the number of NP$^+$CD8$^+$ T cells in female offspring (Figure 6B). Although there was no difference in the percentage (Figure 6C), infected male $Ahr^{fx/fx}$ offspring of dams treated with TCDD had a statistically significant reduction in the number of NP$^+$CD8$^+$ T cells compared with male $Ahr^{fx/fx}$ offspring of vehicle control dams (Figure 6D). Yet, the number of NP$^+$CD8$^+$

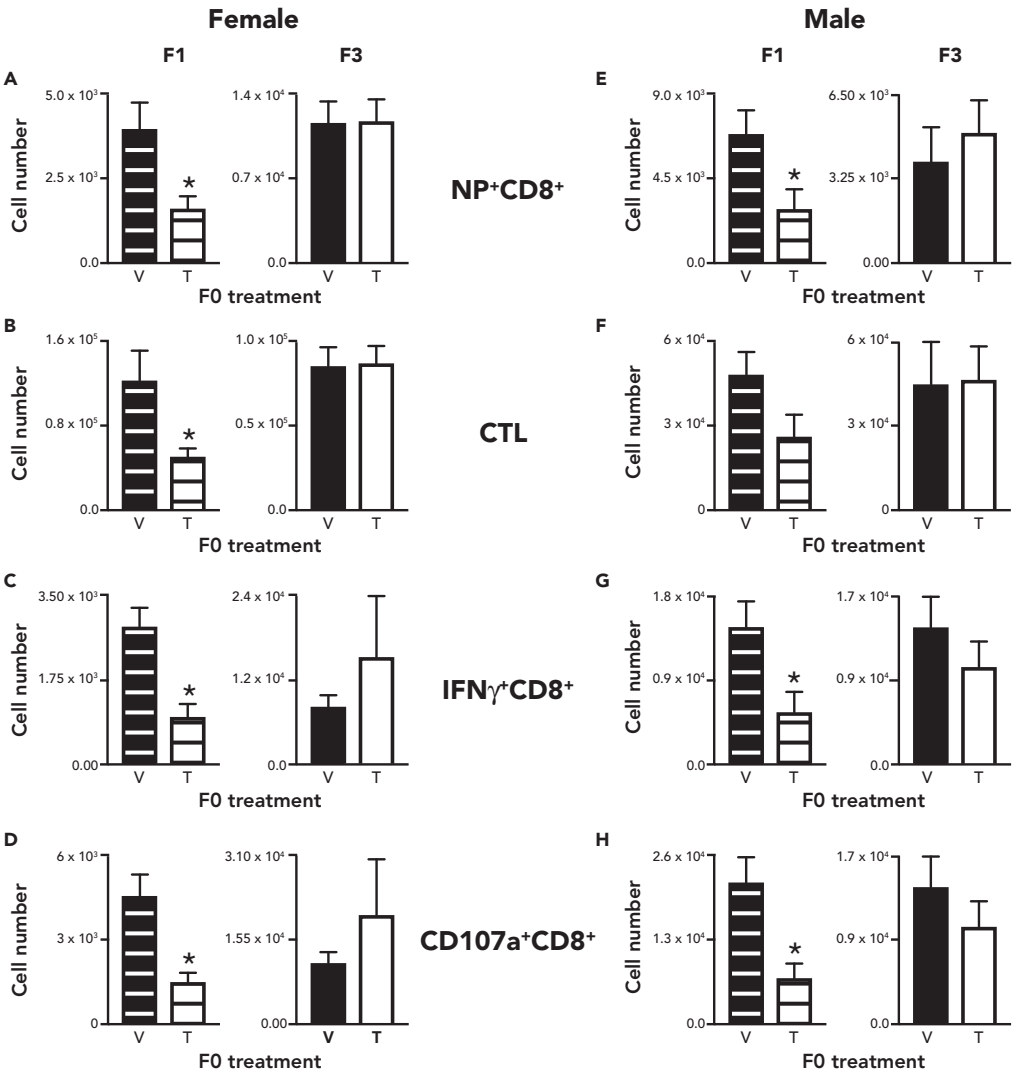


Figure 4. In the MLN, F0 AHR Activation Alters the CD8⁺ T Cell Response of Female and Male F1 but Not F3 Generation Offspring

F1 and F3 generation offspring were generated as shown in Figure 1A. 7–9 adult F1 and F3 generation offspring from each treatment group were infected with IAV, and MLN cells were harvested on day 8 (males) or 9 (females) post infection. The graphs depict the mean number of cells from vehicle and TCDD offspring found in the MLN on day 8 or 9 post IAV infection.

(A) The number of NP^+CD8^+ T cells in female F1 (left) and F3 (right) offspring.

(B) The number of CTL in female F1 (left) and F3 (right) offspring.

(C) The number of $IFN\gamma^+CD8^+$ T cells in female F1 (left) and F3 (right) offspring.

(D) The number of $CD107a^+CD8^+$ T cells in female F1 (left) and F3 (right) offspring.

(E) The number of NP^+CD8^+ T cells in male F1 (left) and F3 (right) offspring.

(F) The number of CTL in male F1 (left) and F3 (right) offspring.

(G) The number of $IFN\gamma^+CD8^+$ T cells in male F1 (left) and F3 (right) generation offspring.

(H) The number of $CD107a^+CD8^+$ T cells in male F1 (left) and F3 (right) offspring. Black-striped bars, VEH F1; white-striped bars, TCDD F1; black bars, VEH F3; white bars, TCDD F3.

Data are shown as mean +SEM. * denotes p value ≤ 0.05 (Student's t test). Numerical values for all the bar graphs as well as p values for each comparison are shown in Table S2.

T cells in TCDD exposed *Vav1*^{cre}*Ahr*$^{fx/fx}$ offspring was not significantly different from that of *Vav1*^{cre}*Ahr*$^{fx/fx}$ male offspring of control dams (Figure 6D). Thus, the decreased number of NP^+CD8^+ T cells in TCDD-exposed F1 offspring requires AHR-mediated signaling in the hematopoietic cells. In addition to

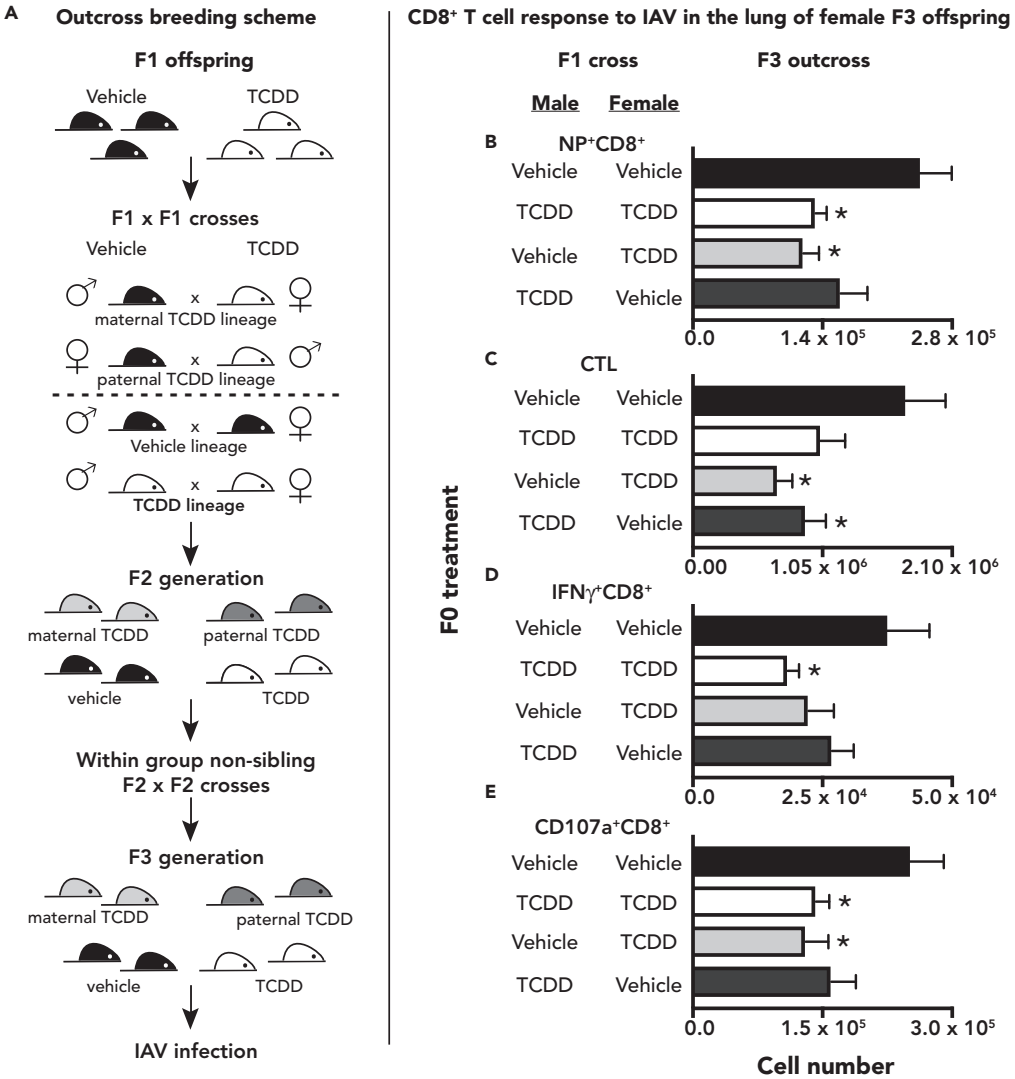


Figure 5. Transgenerational Effects on the CD8$^+$ T Cell Response to IAV in the Lung Are Transmitted through Both Parental Lineages

(A) Outcross breeding scheme: F1 offspring were generated as in Figure 1A. A portion of F1 offspring from both vehicle and TCDD treatment groups was used to perform outcrosses. Vehicle males were paired with TCDD females to create the maternal TCDD lineage. For the paternal TCDD lineage, vehicle females were paired with TCDD males. Vehicle x vehicle and TCDD x TCDD incrosses were also made. The resulting F2 offspring were paired to other non-sibling F2 offspring to create the F3 generation. At 8–10 weeks of age, 7–9 male and 7–9 female offspring from different dams were infected with IAV. MLNs and lungs from males were harvested on day 8 post infection and from females on day 9.

(B–E) The CD8$^+$ T cell response in the lung of female F3 outcrossed offspring. The designations to the left of each graph denote the F0 treatment group of the male and female F1 offspring involved in each F1 cross. The graphs depict the mean number of cells found in the lung of F3 female offspring: (B) NP$^+$CD8$^+$ T cells, (C) CTL, (D) IFNγ^+CD8$^+$ T cells, and (E) CD107a$^+$CD8$^+$ T cells. Black bars, VEH; white bars, TCDD; light gray bars, maternal TCDD lineage; dark gray bars, paternal TCDD lineage.

Data are shown as mean ± SEM. * denotes p value $\leq$ 0.05 determined by a two-way ANOVA followed by a Dunnett's test with the control as the VEH x VEH lineage. The numerical values for the bar graphs are listed in Table S3, and the p values generated using two-way ANOVA and Dunnett's test are shown in Table S4. The outcross experiment was performed once.

measuring the expansion of virus-specific CD8$^+$ T cells, we compared differentiation into CTL. Regardless of maternal exposure, the percentage of CTL was similar in female *Ahr*$^{fx/fx}$ and *Vav1*^{cre}*Ahr*$^{fx/fx}$ offspring (Figure 6E). Yet, compared with female offspring of control-treated dams, there was a statistically significant

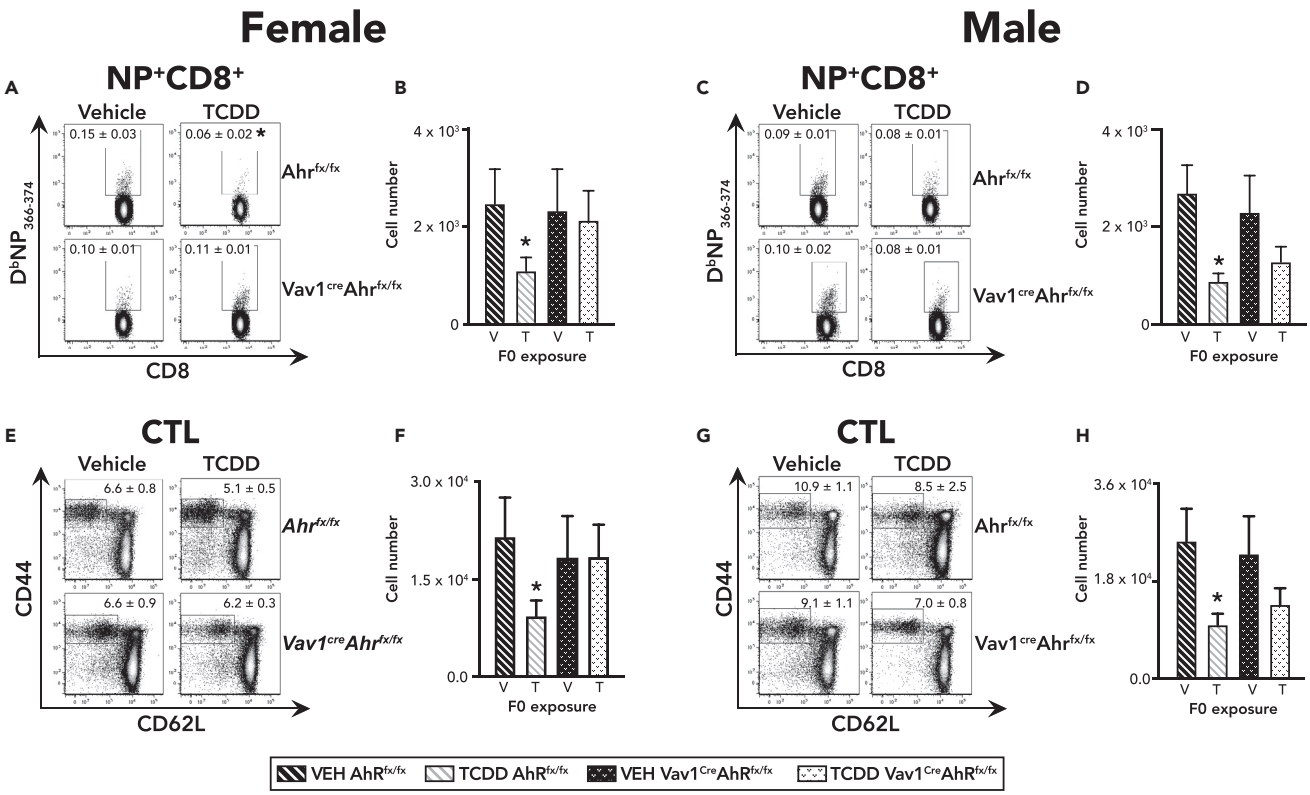


Figure 6. Lack of AHR in Hematopoietic Cells Has Differential Effects on CD8+ T Cell Expansion in Female and Male Offspring during IAV Infection

At maturity, 9–11 male or female developmentally exposed *Vav1^{cre}Ahr$^{fx/fx}$* and *Ahr$^{fx/fx}$* offspring were infected with IAV. MLN cells were harvested and stained as described in the Transparent Methods section.

(A) The percentage of NP$^+$CD8$^+$ T cells in IAV-infected female vehicle and TCDD-exposed *Ahr$^{fx/fx}$* (top row) and *Vav1^{cre}Ahr$^{fx/fx}$* offspring (bottom row). The number on the plots denotes the mean percentage of NP$^+$CD8$^+$ T cells.

(B) The number of NP$^+$CD8$^+$ T cells from vehicle (V) and TCDD (T)-exposed *Ahr$^{fx/fx}$* and *Vav1^{cre}Ahr$^{fx/fx}$* offspring on day 9 post IAV infection.

(C) The percentage of NP$^+$CD8$^+$ T cells in male exposed *Ahr$^{fx/fx}$* and *Vav1^{cre}Ahr$^{fx/fx}$* offspring on day 9 post IAV infection.

(D) The number of NP$^+$CD8$^+$ T cells from male *Ahr$^{fx/fx}$* and *Vav1^{cre}Ahr$^{fx/fx}$* offspring of vehicle and TCDD treated dams.

(E) The percentage of CTL (CD44^{hi}CD62L^{lo}CD8$^+$ T cells) in female *Ahr$^{fx/fx}$* (top row) and *Vav1^{cre}Ahr$^{fx/fx}$* offspring (bottom row) on day 9 post IAV infection. The number on the plots denotes the mean percentage of CTL.

(F) The number of CD44^{hi}CD62L^{lo} CD8$^+$ T cells in female *Ahr$^{fx/fx}$* and *Vav1^{cre}Ahr$^{fx/fx}$* offspring.

(G) The percentage of CTL in male *Ahr$^{fx/fx}$* (top row) and *Vav1^{cre}Ahr$^{fx/fx}$* offspring (bottom row). The number on the plots denotes the mean percentage of CTL.

(H) The number of CTL in male *Ahr$^{fx/fx}$* and *Vav1^{cre}Ahr$^{fx/fx}$* offspring 9 days after infection. All flow plots are derived from the CD8$^+$ T cell gate (Figure S1). All data are presented as mean ± SEM. * denotes p value ≤0.05, compared with control offspring with the same genotype (ANOVA followed by Tukey HSD).

reduction in the number of CTL in female *Ahr$^{fx/fx}$* mice that were developmentally exposed to TCDD (Figure 6F). Lack of *Ahr* in hematopoietic cells eliminated this difference in the number of CTL (Figure 6F). In IAV-infected male offspring, there was also no significant difference in the percentage of CTL (Figure 6G), but the number of CTL in TCDD-exposed *Ahr$^{fx/fx}$* males was significantly less than in vehicle-exposed *Ahr$^{fx/fx}$* offspring (Figure 6H). When *Vav1^{cre}Ahr$^{fx/fx}$* TCDD-exposed offspring were infected, the number of CTL was not significantly different from that of *Vav1^{cre}Ahr$^{fx/fx}$* offspring (Figure 6H). However, similar to NP$^+$CD8$^+$ T cells from *Vav1^{cre}Ahr$^{fx/fx}$* males, the number of CTL was 1.7-fold lower in TCDD-exposed offspring compared with males of vehicle-treated dams means. Thus, when the AHR is triggered during development, it effects hematopoietic cells in a manner that leads to changes in CD8$^+$ T cell responses later in life, although the consequences may be slightly different between sexes.

DISCUSSION

Recent studies reveal that maternal exposures can cause changes in biological processes that span generations. For example, maternal exposure to endocrine disrupting chemicals causes transgenerational

changes in metabolism as well as altered reproductive and nervous system functions (Heindel, 2018; Rattan et al., 2018; Rissman and Adli, 2014; Skinner, 2014; Skinner et al., 2010; van Steenwyk et al., 2018; Walker and Gore, 2011). Other studies have shown that maternal and early life exposures affect immune responses in the F1 generation (Boule and Lawrence, 2016; Winans et al., 2011). Although a recent study indicates that maternal exposure to diesel exhaust particles affects asthma risk across generations (Gregory et al., 2017), no prior studies have directly examined whether maternal exposure to AHR-binding chemicals causes transgenerationally inherited changes in immune responses. The work reported in the present study evaluated whether maternal (F0) exposure affects a key immune defense in a transgenerational manner. We found that F0 exposure caused transgenerational effects on the $CD8^+$ T cell response to influenza A virus infection; however, not all of the effects observed in the F1 generation were transmitted equally to the F3 generation, and the transgenerational effects observed were organ and sex specific.

The major finding of our study was that F0 exposure to TCDD caused transgenerational changes to the $CD8^+$ T cell response to infection. Interestingly, changes in the F3 generation were observed in the infected lung but not in the MLN. This is in contrast to the F1 generation, in which the T cell response to infection is altered in both MLN and lung. Although the site of IAV infection is the respiratory tract, the MLN is the primary site where naive $CD8^+$ T cells become activated, clonally expand, differentiate, and acquire effector functions before migrating to the lung. In addition to recognizing and eliminating infected cells in the lung, newly emigrated $CD8^+$ T cells can also receive additional signals from lung resident DCs. This provides greater stimulation to the $CD8^+$ T cells, prompting them to undergo additional rounds of proliferation and enhancing their cytotoxicity (McGill and Legge, 2009). As such, DC-$CD8^+$ T cell interactions in the lung are important for an optimally effective $CD8^+$ T cell response to IAV (Lawrence et al., 2005; McGill et al., 2008). Thus, a possible explanation for transgenerational differences in the $CD8^+$ T cell response in the lung, but not the MLN, is that there are durable effects of F0 exposure on DC functions that bolster $CD8^+$ T cell responses in the infected lung. A recent report demonstrates that developmental exposure to TCDD alters DC function in adult F1 offspring, including their ability to activate naive IAV-specific $CD8^+$ T cells (Meyers et al., 2018). Other studies provide further evidence that early life exposures influence the function of DCs. For example, offspring of dams sensitized with ovalbumin or diesel exhaust particles (DEPs) are predisposed to airway inflammation later in life, in part due to changes in DCs, and some effects of DEP exposure span generations (Fedulov et al., 2008; Gregory et al., 2017). Thus, changes in DC function potentially carry across generations.

In addition to influencing leukocyte functions, F0 exposure may trigger changes to the programming of lung epithelial cells, and the consequences of this may differ across generations in a manner that influences the quality of the $CD8^+$ T cell response in the infected lung. For example, lung epithelial cells produce thymic stromal lymphopoietin (TSLP), a cytokine that, when produced during an IAV infection, acts on DCs in the lung and enhances the DC-$CD8^+$ T cell interactions necessary for continued expansion of $CD8^+$ T cells (Yadava et al., 2013). Furthermore, activation of the AHR influences *Tslp* gene expression (Jeong et al., 2018). Therefore, it is possible that the reduced $CD8^+$ T cell response in the lung is due to ineffective DC-$CD8^+$T cell interactions, which arise from changes to the DCs themselves and also from aberrant signaling from the lung epithelium that is programmed by F0 AHR activation.

Another important observation in our study was that the transgenerational effects were sex specific. This was somewhat surprising given that in the F1 generation both male and female offspring of TCDD-treated dams had significantly suppressed $CD8^+$ T cell responses to IAV infection. Yet, in the F3 generation, differences in these same metrics of the $CD8^+$ T cell response to infection were observed in female offspring but not in their male littermates. A simple explanation is that immunomodulatory programming is passed on only maternally. However, results from the outcross experiment indicate that there are consequences of F0 AHR activation in male F1 offspring that contribute to generation-spanning changes in T cell responses. Specifically, paternal lineage TCDD exposure transmitted altered $CD8^+$ T cell responses to female F3 offspring. The difference in the consequences of F0 exposure in the male F1 and F3 generation could indicate that direct (F0, F1) and indirect (F3) exposures have differential effects on programming in the male germ line. AHR activation by TCDD affects the male and female reproductive system (Bruner-Tran et al., 2014; Bruner-Tran and Osteen, 2011; Karman et al., 2012; Manikkam et al., 2012), and the AHR is broadly expressed in many tissues, including immune and non-immune cells. Within the developing hematopoietic system, the *Ahr* is expressed in fetal liver and thymus and is transcriptionally active. For instance, AHR activation stimulates *Cyp1a1* gene expression in fetal liver, hematopoietic stem and progenitor cells, thymus,

and also peripheral T cells of neonatal mice that were directly exposed to TCDD (Ahrenhoerster et al., 2014; Majora et al., 2005; Singh et al., 2012; Winans et al., 2015). Yet, the transcriptional activity triggered by early life exposure is transient, as evidenced by the observation that, in adult offspring exposed perinatally, the AHR activity induced by earlier exposure does not persist (Gehrs and Smialowicz, 1997; Ishimaru et al., 2009; Winans et al., 2015). Although the mechanisms by which triggering the AHR lead to generation-spanning alterations in immune function remain to be elucidated, these results suggest that direct exposures (F0, F1) may lead to immunomodulatory effects that are easily discernable but are transient, whereas indirect effects are more subtle. Variations in AHR-mediated changes in gene expression in distinct cell types, as well as in developing male and female fetuses and neonates, may underlie some of the differences observed in our study. Based on a combination of mRNA and protein expression, different cells and tissues express different levels of the *Ahr* (Esser and Rannug, 2015; Li et al., 1994; Nishihashi et al., 2006). For example, the lung and liver generally express higher levels compared with immune cells (Li et al., 1994). When it has been compared directly, male and female rodents express the *Ahr* at similar levels (Lee et al., 2015; Nault et al., 2017). A limitation of current knowledge is that, in many studies of *Ahr* expression and AHR signaling, only one sex has been used. Moreover, in fetal tissues, sex is generally not reported, perhaps owing to technical challenges, such as the need to pool tissue from more than one fetus to obtain sufficient material for assays. Nonetheless, distinct consequences of *direct* exposure to TCDD in male and female mice have been described (Lee et al., 2015; Pohjanvirta et al., 2012; Prokopec et al., 2015). For instance, *Ahr* expression levels and induction of known AHR target genes were similar in male and female mice, whereas TCDD treatment elicited changes to the overall transcriptome profile in liver that were different in males and females (Lee et al., 2015; Prokopec et al., 2015). Also, the immune responses of both males and females are similarly influenced by direct exposure to AHR agonists, whereas differences in the magnitude have been observed, although which sex is more sensitive (i.e., showed significant effect at lower dose) depended on the antigen challenge (Mustafa et al., 2008; Sugita-Konishi et al., 2003; Vorderstrasse et al., 2006). Therefore, different levels of *Ahr* within a particular cell or tissue are not likely sufficient to explain differential responses. Instead, differences reflect a combination of the manner in which AHR signaling converges with other signaling pathways that are specific to cell type and sex and, in the case of immune responses, the specific antigen challenge.

Further support for the idea that the observed consequences of developmental and transgenerational exposures reflect complex interplay comes from our finding that the effects of developmental exposure were not identical in male and female offspring. For instance, in female $Vav1^{cre}Ahr^{fx/fx}$ offspring, excision of *Ahr* from hematopoietic cells fully eliminated the effect of maternal TCDD treatment on the response to IAV. In male $Vav1^{cre}Ahr^{fx/fx}$ mice, the effect appeared blunted, suggesting that in males there may be hematopoietic and non-hematopoietic effects of developmental exposure that contribute to the attenuated $CD8^+$ T cell response. The AHR is expressed widely throughout the body and is highly expressed in barrier organs such as the lung (Esser and Rannug, 2015). Therefore, it is possible that AHR activation in non-hematopoietic cells, such as developing lung epithelial cells, sets off an alternative programming that indirectly affects the $CD8^+$ T cell response to infection in male TCDD-exposed offspring. A related potential explanation is that sex differences in the immune response to infection contribute to the observed differences. Although the overall cellular and molecular defense mechanisms are the same, there are differences in the timing and magnitude of the immune response to IAV in males and females (Vom Steeg and Klein, 2019). For example, during a primary IAV infection, females generally mount adaptive immune responses that are larger in magnitude, yet males have less mortality than females (Klein et al., 2012). However, during secondary challenge with a heterosubtypic virus, females have lower viral titers in their lungs, which is thought to be due to their greater ability to produce antibodies than males (Fink et al., 2018; Lorenzo et al., 2011). The mechanisms that explain sex differences in the response to IAV are not completely understood; however, steroid sex hormones are a contributing factor (Robinson et al., 2011). Given that AHR signaling influences endocrine tissues and endocrine-mediated pathways, it is likely that F0 AHR activation affects myriad different pathways that could potentially influence the programming and function of the immune system.

Although the totality of mechanisms that drive transgenerational inheritance are not fully known, changes in epigenetic regulatory marks are one of the means for conveying changes across generations. Non-coding RNA and histone marks are increasingly recognized as facilitators; however, DNA methylation has been the focus of many studies of transgenerational inheritance (Baxter and Drake, 2019; Blake and Watson, 2016; Boskovic and Rando, 2018; Nilsson and Skinner, 2015). Several pieces of evidence link developmental

exposure to the AHR agonist TCDD with altered DNA methylation in transgenerational inheritance of disease phenotypes. For example, in a transgenerational study using rats, developmental exposure to TCDD caused differentially methylated regions (DMRs) in the promoters of sperm DNA isolated from F3 offspring (Manikkam et al., 2012). Developmental exposure to TCDD also altered DNA methylation in the female reproductive tract (Bruner-Tran et al., 2012). A comparison of progesterone receptor (PR) methylation levels in female offspring revealed that developmental TCDD exposure caused durable hypermethylation of the PR gene (Bruner-Tran et al., 2012). Specifically, in F1 offspring developmentally exposed to TCDD, the PR gene was 60% hypermethylated compared with control offspring, and it remained 40% hypermethylated in F3 offspring (Bruner-Tran et al., 2012). Furthermore, in preimplantation embryos treated with TCDD, there was a reduction in gene expression of the imprinted gene *H19* that corresponded with an increase in DNA methylation and increased methyltransferase activity (Wu et al., 2004). Another study found that male F1 and F3 rats developmentally exposed to TCDD had altered levels of DNMT3a and DNMT3b in liver tissue (Ma et al., 2015). Hence, there is evidence that developmental exposure to TCDD alters DNA methylation patterns in F1 and F3 offspring. Additionally, separate studies have shown that developmental exposure of mice to TCDD alters DNA methylation patterns in $CD8^+$ T cells in F1 offspring (Winans et al., 2015). DMRs were observed across all genomic features in $CD8^+$ T cells from IAV-infected and from naive mice, suggesting that F0 TCDD exposure influences DNA methylation marks in T cells. In contrast to the liver, changes in overall DNA methylation in $CD8^+$ T cells were not associated with alterations in *Dnmt* expression levels (Winans et al., 2015). This suggests that, in $CD8^+$ T cells, changes in DNA methylation levels may not be mediated by direct modulation of DNMT expression or that developmental exposure is causing broad changes in the cellular machinery that regulates DNA methylation. Nonetheless, these overall findings indicate that altered DNA methylation likely plays an important role in shaping how exposures in the womb and shortly after birth mold the immune system's function later in life, and potentially across generations.

Few studies of transgenerational inheritance have examined the effects of environmental exposures on the immune system, and no prior studies of developmental exposure to AHR agonists have examined transgenerational effects on immune cells. The work presented here demonstrates that the immune system, like other organ systems, is vulnerable to transgenerational effects caused by environmental exposures. These studies also emphasize the importance of examining both sexes and considering that differences at the site of infection and secondary lymphoid organs may not always be the same. The discovery that maternal exposure to an AHR agonist caused effects on T cells that persisted across generations expands our knowledge of how developmental exposures impact the immune system. Given that early life exposure to environmental chemicals that bind AHR is associated with greater incidence and severity of infectious disease in human populations, the broad implications of these new findings indicate that environmental cues have a tremendous impact on public health, not just in the present but also for future generations.

Limitations of the Study

Although we report novel and exciting findings about transgenerational effects in the immune system, only a single immune challenge was used. This constrains conclusions about sex specificity and whether T cell responses in other peripheral tissues would also be affected across generations. Future studies with additional immune challenges, such as other types of viruses and assessment of antitumor immunity (which also requires $CD8^+$ T cells), will be important extensions of this work.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

All the FCS (flow cytometry standard) files used to generate the data in this paper, as well as FCS files from an independent transgenerational cohort, has been deposited in the NIH FigShare repository (DOI: http://doi.org/10.35092/yhjc.c.4649441)

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.09.014.

ACKNOWLEDGMENTS

We thank Dr. Brian Rudd (Cornell University) and Dr. Deborah Cory-Slechta (University of Rochester) for thoughtful discussion and critical feedback on our manuscript. We gratefully acknowledge Dr. Sally Thurston (University of Rochester) for her input regarding the overall design of the transgenerational breeding strategy. We are also appreciative of Dr. Timothy Bushnell and the outstanding team at the University of Rochester Flow Cytometry Core. We thank Rachel Garcia for assistance with the graphical abstract. Graphic images are from Servier Medical Art (https://smart.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License Agreement (https://creativecommons.org/licenses/by/3.0/). The authors are supported by grants from the United States National Institutes of Health (R01-ES0004862, R01-ES023260, T32-ES07026, T32-AI007285, T32-HL066988, and P30-ES01247).

AUTHOR CONTRIBUTIONS

Conceptualization, B.P.L.; Methodology, C.M.P., B.W., L.A.B., and B.P.L.; Investigation, C.M.P., B.W., C.G.B., L.A.B., and C.T.O.; Writing – Original Draft, C.M.P. and L.A.B; Writing – Review & Editing, C.G.B, L.A.B., and B.P.L.; Visualization, C.M.P. and L.A.B.; Project Administration, C.T.O and B.P.L.; Funding Acquisition and Supervision, B.P.L.

DECLARATION OF INTERESTS

The authors declare that they have no actual or potential competing financial or non-financial interests.

Received: May 7, 2019
Revised: August 5, 2019
Accepted: September 11, 2019
Published: October 25, 2019

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Supplemental Information

The Ancestral Environment Shapes Antiviral CD8^{+} T cell Responses across Generations

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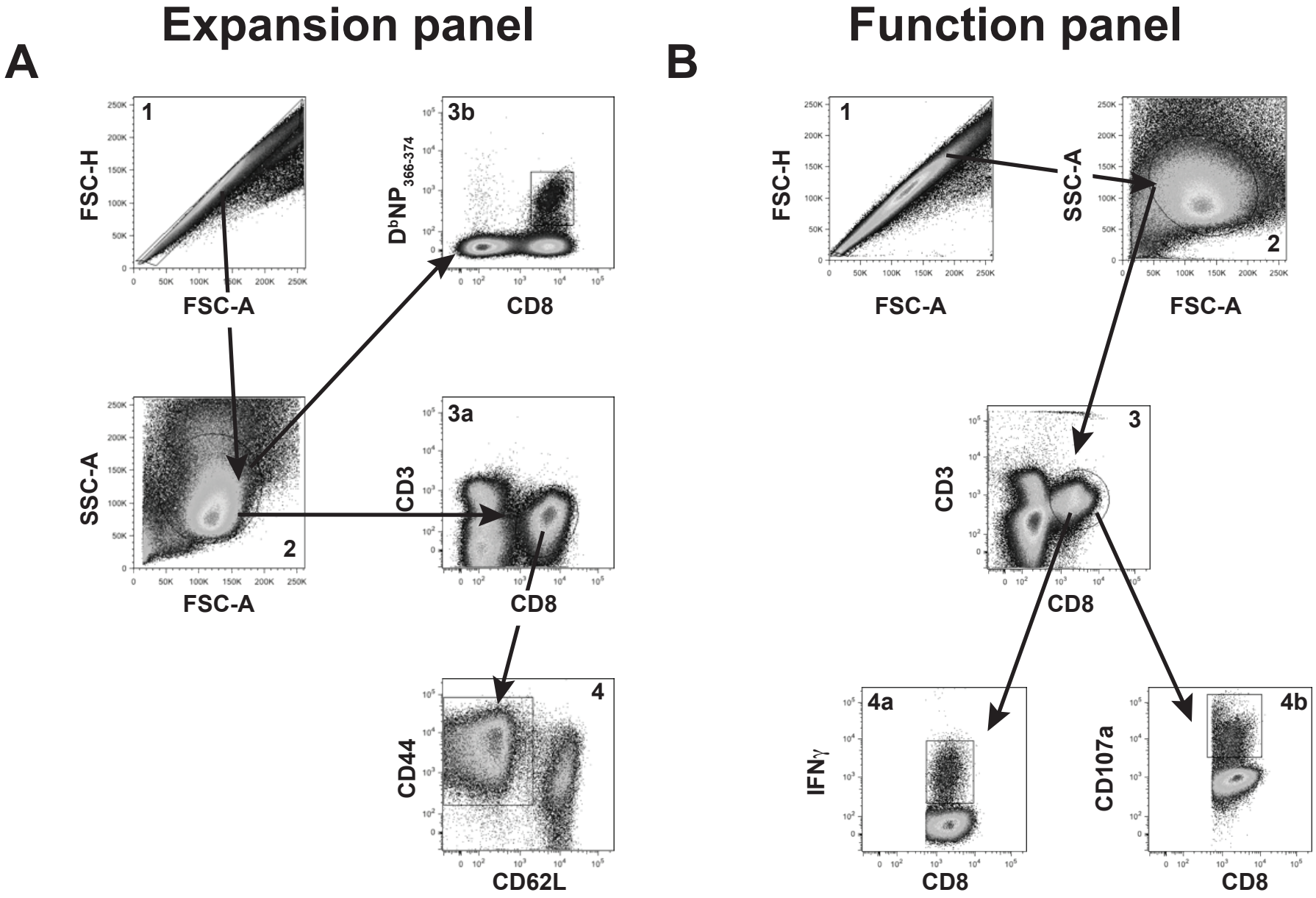
A
Expansion panel
1
FSC-H
FSC-A
3b
DbNP366-374
CD8
2
SSC-A
FSC-A
3a
CD3
CD8
4
CD44
CD62L
B
Function panel
1
FSC-H
FSC-A
SSC-A
2
FSC-A
3
CD3
CD8
4a
IFNγ
CD8
4b
CD107a
CD8

Figure S1: Gating strategy for the expansion and function panels, related to figures 2-6. (**A**) Gating strategy for $CD8^+$ T cell expansion and differentiation panel. Doublets and dead cells were excluded (plot 1). $CD3^+CD8^+$ T cells (plot 3a) and NP^+CD8^+ T cells (plot 3b) were derived from the leukocyte gate (plot 2). CTL ($CD44^{hi}CD62L^{lo}$, plot 4) were identified based on expression levels of CD44 and CD62L after gating on $CD8^+$ T cells. (**B**) Gating strategy for the function panel. This panel was gated in a similar manner as the expansion panel. After gating to exclude doublets and dead cells from the leukocyte population (plots 1 and 2), $CD8^+$ cells were identified (plot 3). From within these cells, $IFN\gamma^+CD8^+$ T cells (plot 4a) and $CD107a^+CD8^+$ T cells (plot 4b) were identified.

Table S1. Percentage and number of $CD8^+$ T cell populations in the lungs of female and male offspring, related to Figures 2 and 3.

	Female F1 generation offspring						Female F3 generation offspring					
	% $CD8^+$			# of cells (x 10^5)			% $CD8^+$			# of cells (x 10^5)		
	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value
[1]$CD8^+$	17.3 ± 1.10	12.46 ± 1.17	0.0125	14.49 ± 1.45	9.28 ± 1.52	0.0308	16.97 ± 1.74	14.76 ± 1.70	0.3825	22.05 ± 3.63	13.51 ± 2.08	0.0699
[1]NP^+CD8^+	1.91 ± 0.23	1.12 ± 0.22	0.0292	1.63 ± 0.26	0.84 ± 0.22	0.0398	1.96 ± 0.28	1.43 ± 0.13	0.1264	2.44 ± 0.35	1.30 ± 0.12	0.0177
CTL	68.73 ± 4.65	64.83 ± 3.71	0.5265	10.16 ± 1.29	5.94 ± 0.98	0.0248	74.1 ± 5.10	72.04 ± 5.03	0.7789	17.07 ± 3.23	10.21 ± 2.00	0.1013
$IFN\gamma^+CD8^+$	4.71 ± 0.48	6.00 ± 1.25	0.3712	0.42 ± 0.07	0.33 ± 0.07	0.3970	2.09 ± 0.32	1.68 ± 0.23	0.3258	0.37 ± 0.08	0.18 ± 0.02	0.0584
$CD107a^+CD8^+$	10.42 ± 0.79	10.01 ± 1.21	0.7799	0.91 ± 0.14	0.61 ± 0.16	0.1938	13.73 ± 0.94	12.6 ± 0.73	0.3588	2.49 ± 0.39	1.40 ± 0.15	0.0334
	Male F1 generation offspring						Male F3 generation offspring					
	% $CD8^+$			# of cells (x 10^5)			% $CD8^+$			# of cells (x 10^5)		
	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value
[1]$CD8^+$	14.7 ± 0.89	9.51 ± 1.38	0.0100	17.7 ± 2.43	5.93 + 1.35	0.0020	16.95 ± 1.65	15.28 ± 1.78	0.5052	19.23 ± 3.47	15.82 ± 4.46	0.5572
[1]NP^+CD8^+	1.14 ± 0.20	1.06 ± 0.21	0.7902	1.43 ± 0.41	0.65 ± 0.18	0.1128	1.24 ± 0.18	0.98 ± 0.13	0.2698	1.46 ± 0.40	1.01 ± 0.25	0.3628
CTL	51.42 ± 3.77	39.36 ± 4.84	0.0868	10.67 ± 1.72	5.51± 1.83	0.0739	73.07 ± 5.23	74.41 ± 5.03	0.8563	14.7 ± 2.74	12.46 ± 3.96	0.6456
$IFN\gamma^+CD8^+$	4.64 ± 0.54	7.25 ± 0.66*	0.0102	0.60 ± 0.17	0.30 ± 0.04	0.1236	6.00 ± 0.81	4.46 ± 0.63	0.1612	0.83 ± 0.23	0.47 ± 0.12	0.1925
$CD107a^+CD8^+$	16.38 ± 1.05	18.76 ± 2.36	0.3949	3.71 ± 0.35	2.61 ± 0.39	0.0688	10.25 ± 1.11	10.34 ± 1.38	0.9622	1.29 ± 0.23	1.09 ± 0.26	0.5610

[1] Percentage is of total lung cells. Data are displayed as mean ± SEM; p values were calculated using a Student's t test.

Table S2. Percentage and number of $CD8^+$ T cell populations in the MLN of female and male offspring, related to Figure 4.

	Female F1 generation offspring						Female F3 generation offspring					
	% $CD8^+$			# of cells (x 10^5)			% $CD8^+$			# of cells (x 10^5)		
	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value
[1]$CD8^+$	12.37 ± 1.60	14.69 ± 1.35	0.2924	4.12 ± 2.05	9.74 ± 0.60	0.0415	12.24 ± 0.56	11.82 ± 1.12	0.7442	8.28 ± 1.07	9.81 ± 2.13	0.5363
[1]NP^+CD8^+	0.064 ± 0.02	0.05 ± 0.01	0.7247	0.04 ± 0.008	0.02 ± 0.004	0.0286	0.19 ± 0.04	0.15 ± 0.02	0.4786	0.12 ± 0.02	0.12 ± 0.02	0.9584
CTL	12.23 ± 0.57	11.79 ± 0.31	0.5104	1.22 ± 0.28	0.50 ± 0.08	0.0506	10.33 ± 0.66	10.53 ± 1.85	0.9217	0.85 ± 0.11	0.87 ± 0.10	0.9156
$IFN\gamma^+CD8^+$	0.44 ± 0.11	0.28 ± 0.06	0.2525	0.03 ± 0.004	0.01 ± 0.003	0.0033	1.18 ± 0.44	0.90 ± 0.22	0.5755	0.08 ± 0.02	0.15 ± 0.09	0.4519
$CD107a^+CD8^+$	0.88 ± 0.05	0.61 ± 0.04	0.0015	0.05 ± 0.008	0.01 ± 0.003	0.0061	1.36 ± 0.29	1.32 ± 0.16	0.9536	0.11 ± 0.02	0.20 ± 0.10	0.4286
	Male F1 generation offspring						Male F3 generation offspring					
	% $CD8^+$			# of cells (x 10^5)			% $CD8^+$			# of cells (x 10^5)		
	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value	Vehicle	TCDD	p value
[1]$CD8^+$	13.03 ± 0.96	13.8 ± 1.88	0.7243	7.77 ± 1.29	4.38 ± 1.31	0.0986	11.76 ± 0.57	9.92 ± 1.6	0.3126	5.00 ± 1.20	4.0 ± 1.01	0.5234
[1]NP^+CD8^+	0.12 ± 0.02	0.10 ± 0.02	0.4400	0.07 ± 0.01	0.03 ± 0.01	0.0385	0.076 ± 0.01	0.12 ± 0.02	0.0786	0.04 ± 0.01	0.05 ± 0.01	0.5546
CTL	6.35 ± 0.67	5.89 ± 0.36	0.5610	0.48 ± 0.08	0.26 ± 0.08	0.0817	7.68 ± 1.12	11.9 ± 1.78	0.0718	0.45 ± 0.15	0.46 ± 0.12	0.9352
$IFN\gamma^+CD8^+$	1.62 ± 0.19	1.16 ± 0.17	0.0969	0.15 ± 0.03	0.05 ± 0.02	0.0289	5.91 ± 2.18	5.85 ± 3.42	0.9893	0.14 ± 0.03	0.1 ± 0.26	0.3385
$CD107a^+CD8^+$	2.37 ± 0.28	1.48 ± 0.17	0.0262	0.22 ± 0.04	0.07 ± 0.02	0.0115	0.89 ± 0.27	1.63 ± 0.28	0.0797	0.05 ± 0.02	0.07 ± 0.02	0.5913

[1] Percentage is of total lung cells. Data are displayed as mean ± SEM; p values were calculated using a Student's t-test.

Table S3. Percentage and number of $CD8^+$ T cell populations in the lungs of female and male F3 offspring, related to Figure 5.

Female	VEH x VEH		TCDD x TCDD		VEH x TCDD		TCDD x VEH	
	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)
NP^+CD8^+	1.96 ± 0.28	1.96 ± 0.28	1.43 ± 0.13	1.43 ± 0.13	1.35 ± 0.20	1.17 ± 0.17*	1.48 ± 0.27	1.60 ± 0.29
CTL	74.1 ± 5.10	74.1 ± 5.10	72.04 ± 5.03	72.04 ± 5.03	62.15 ± 4.19	6.72 ± 1.32*	64.24 ± 4.54	8.99 ± 1.76*
$IFN\gamma^+CD8^+$	2.09 ± 0.32	2.09 ± 0.32	1.68 ± 0.23	1.68 ± 0.23	2.36 ± 0.44	0.22 ± 0.05	2.15 ± 0.22	0.26 ± 0.04
$CD107a^+CD8^+$	13.73 ± 0.94	13.73 ± 0.94	12.6 ± 0.73	12.6 ± 0.73	13.31 ± 1.18	1.28 ± 0.26*	12.75 ± 1.13	1.58 ± 0.28

Male	VEH x VEH		TCDD x TCDD		VEH x TCDD		TCDD x VEH	
	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)	% of $CD8^+$ T cells	# of cells (x 10^5)
NP^+CD8^+	1.24 ± 0.18	1.46 ± 0.40	0.98 ± 0.13	1.01 ± 0.25	1.06 ± 0.14	1.31 ± 0.41	1.53 ± 0.23	1.18 ± 0.21
CTL	73.07 ± 5.23	14.7 ± 2.74	74.41 ± 5.03	12.46 ± 3.96	75.81 ± 2.99	10.6 ± 2.27	74.85 ± 1.84	15.0 ± 2.68
$IFN\gamma^+CD8^+$	6.00 ± 0.81	0.83 ± 0.23	4.46 ± 0.63	0.47 ± 0.12	5.47 ± 0.78	0.40 ± 0.10	4.25 ± 0.73	0.66 ± 0.11
$CD107a^+CD8^+$	10.25 ± 1.11	1.29 ± 0.23	10.34 ± 1.38	1.09 ± 0.26	9.23 ± 0.56	0.90 ± 0.17	9.27 ± 1.13	1.16 ± 0.16

Data are displayed as mean ± SEM; * denotes $p \leq 0.05$ determined by 2-way ANOVA followed by a Dunnett's post-hoc test w/ VEH x VEH as the control group.

Table S4. Outcross experiment 2-way ANOVA effect test and Dunnett's test p values, related to Figure 5.

ANOVA	NP^+CD8^+	CTL	$IFN\gamma^+CD8^+$	$CD107a^+CD8^+$
2-way ANOVA p value	**0.0096**	0.0140	0.0953	**0.0309**
Paternal lineage exposure* maternal lineage exposure p value	0.0620	0.0110	0.5210	0.0833
Maternal lineage exposure p value	**0.0057**	**0.0400**	**0.0308**	**0.0229**
Paternal lineage exposure p value	0.1648	0.2876	0.1691	0.1771

Dunnett's post-hoc test	NP^+CD8^+	CTL	$IFN\gamma^+CD8^+$	$CD107a^+CD8^+$
TCDD incrossed vs. vehicle incrossed	**0.0155**	0.0918	**0.0485**	**0.0414**
Maternal TCDD vs. vehicle incrossed	**0.0050**	**0.0056**	0.1226	**0.0177**
Paternal TCDD vs. vehicle incrossed	0.0571	**0.0284**	0.3293	0.0761

Table S1: Percentage and number of $CD8^+$ T cells in the lungs of female and male F1 and F3 offspring. The data in the top of the table correspond to the data from lungs of female offspring shown in Fig. 2. The data in the bottom of the table correspond to the data from the lungs of male F1 and F3 offspring depicted Fig. 3.

Table S2: Percentage and number of $CD8^+$ T cell populations in the MLN of female and male F1 and F3 offspring. The data in the top of the table correspond to the data that are presented in Fig. 4A-D (MLN cells from female offspring). The data in the bottom of the table correspond to data from the MLN of male offspring that are presented in Fig. 4E-H.

Table S3: Percentage and number of $CD8^+$ T cells in the lungs of female and male outcrossed F3 offspring. The data in the top of Table S3 correspond to the female F3 outcross data shown in Fig. 5. The percent and number of $CD8^+$ T cell populations in the male littermates are shown in the bottom of the table.

Table S4: Outcross experiment 2-way ANOVA effect test and Dunnett's post-hoc test p values. The data in this graph are the numeric values that correspond to the statistics shown on the graphs in Fig.5. A 2-way ANOVA was used to compare the effect of paternal F0 lineage exposure vs. maternal F0 lineage exposure on the $CD8^+$ T cell response to IAV in F3 offspring. A Dunnett's post-hoc test was performed using the VEH x VEH group as the control. Bold letters indicate $p \leq 0.05$.

TRANSPARENT METHODS

Mice

Male and female C57BL/6 (B6 were purchased from Jackson Labs (Bar Harbor, ME). Original breeding stocks of B6.Cg-Tg A2Kio/J (Vav1^{Cre}) mice were obtained from Jackson Laboratories, and initial Ahr$^{fx/fx}$mice breeding pairs were provided by Dr. Christopher Bradfield (University of Wisconsin). Female *Ahr*^{fxfx} mice were crossed with male *Vav1*^{cre} transgenic mice to create a cohort of *Vav1*^{cre}*Ahr*$^{fx/fx}$ mice that were maintained at the URMC. Genotyping of conditional *Ahr* knockout mice was done by PCR using to detect the *Cre* transgene under the *Vav1* promoter (5'-AGATGCCAGGACATCAGGAACCTG-3'; 5'-ATCAGCCACACCAGACACAGAGATC-3', IDT), and *Ahr*$^{fx/fx}$ sense and antisense primers [OL4062, OL4064 and OL4088, (Integrated DNA Technologies (IDT), Coralville, IA)] (Franchini et al., 2019; Walisser et al., 2005).

F0 exposure and transgenerational breeding strategy

Adult (8-week-old) B6 mice were paired as 1 female and 1 male per cage to create unique F0 pairs. On gestational day 0 (GD0, day of vaginal plug) pregnant females were separated from the males, and singly housed for the remainder of their pregnancy. On GD0, GD7, GD14, and post-natal day 2 (PND2), wild-type C57Bl/6 F0 dams received either 1 µg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) ($\geq$99% pure, Cambridge Isotope Laboratories, Woburn, MA) per kg body weight or peanut oil vehicle control (VEH) by gavage. For experiments using conditional knockout mice, nulliparous *Ahr*$^{fx/fx}$ females were housed with *Vav1*^{cre}*Ahr*$^{fx/fx}$ males, and 10 µg TCDD/kg was administered on GD0, GD7, GD 14 and PND2. The higher dose of TCDD was used because Ahr$^{fx/fx}$ mice carry the *d* allele of the *Ahr*, which has a 10-fold lower binding affinity for TCDD compared to B6 mice, which carry the *b* allele (Poland et al., 1994; Walisser et al., 2005). TCDD was dissolved in anisole and diluted in peanut oil. The vehicle control consisted of peanut oil containing an equivalent concentration of anisole (0.01%). The half-life of TCDD in mice is 7-10 days, thus treatment every 7 days results in sustained activation of the AHR throughout gestation and into lactation, a period of time that encompasses the immune system's development . All offspring were weaned at 21 days of age and separated into cages with same sex littermates. At 8 weeks of age, F1 non-siblings from the same treatment group were randomly paired to create F2 lineages derived from F0 exposure to VEH or TCDD. Non-sibling and non-cousin F2 generation offspring were randomly paired for breeding to create the F3 generation. Vaginal plugs were identified for F1 generation (GD0), but not for the F2 and F3 generation breeding. The breeding scheme is diagrammed in Fig. 1A. In some studies, outcrosses were created by pairing F1 male VEH lineage offspring with F1 female TCDD lineage offspring (maternal TCDD lineage outcross), and by pairing F1 male TCDD lineage offspring with F1 female vehicle lineage offspring (male TCDD lineage outcross). Non-sibling F2 offspring from each cross were paired within the same outcross group to generate F3 outcrossed mice as shown in Fig. 6A. In all studies reported here, the only exposure to TCDD was via oral administration to the F0 dams. Thus, F1, F2 and F3 generation mice were never given TCDD (or VEH) directly. We obtained similar findings in F1 and F3 offspring from two independently created transgenerational cohorts of mice. Data from one of the two cohorts are presented in the figures. All animal treatments were conducted with prior approval of Institutional Animal Care and Use Committee of the University of Rochester. The University has accreditation through the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were treated humanely and with due consideration to alleviation of any distress and discomfort. All guidelines from the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals were followed in handling of vertebrate animals.

Influenza A virus infection

Influenza A virus (IAV) strain A/HKx31 (HKx31; H3N2) was prepared, titered, and stored as previously described (Warren et al., 2000). At maturity (7-10 weeks of age), male and female offspring from the F1 and F3 generation were anesthetized with an intraperitoneal (i.p.) injection of avertin (2,2,2-tribromoethanol; Sigma Aldrich, Milwaukee, WI), and then infected intranasally (i.n.) with 120 hemagglutinating units (HAU) IAV, diluted in endotoxin-free PBS (Warren et al., 2000). For experiments using B6 mice, there were 7-12 male and 7-12 female mice from each exposure group at each point in time. For experiments using conditional knockout mice, there were 9-11 female and 9-11 male mice in each group. Same sex mice in each exposure group were from different F0 dams. In all experiments both IAV administration and organ collection were initiated in the morning. All work with infectious agents was conducted with prior approval of the Institutional Biosafety Committee of the University of Rochester, following guidelines of the NIH/CDC.

Isolation of immune cells and flow cytometry

Single cell suspensions from the mediastinal lymph nodes (MLN, lung-draining lymph nodes) and lung-derived immune cells were prepared as previously described . For all samples, non-specific staining was blocked by incubating cells with an anti-mouse CD16/32 mAb. Two million cells per sample were incubated with combinations of previously determined optimal concentrations of fluorochrome-conjugated antibodies against the following cell surface antigens: CD3α PE-CF594/PE-Cy5 (145-211), CD8α FITC/PerCP-Cy5.5 (53-6-7), CD44 BV711/eF450 (IM7), CD62L BV605/PE-Cy7 (MEL-14). Fluorochrome conjugated MHC class I tetramers specific to an immunodominant nucleoprotein peptide epitope of IAV ($D^b/NP_{366-374}$) were used to identify and enumerate virus specific $CD8^+$ T cells. Cells were fixed using 2% formaldehyde and analyzed directly by flow cytometry, or permeabilized to detect additional differentiation or functional attributes. To detect intracellular IFNγ, cells were incubated in the presence of rmIL-2 (R&D Systems), IAV nucleoprotein peptide ($NP_{366-374}$: ASNENMETM) and brefeldin A (BFA, Sigma Aldrich) for 1 hour at 37°C prior to cell surface staining. For permeabilization, fixed cells were placed in staining buffer containing 1% saponin (Sigma Aldrich) prior to incubation with IFNγ APC (XMG1.2). To detect de-granulation, anti-CD107a (LAMP-1, 1D4B) was added and the cells were incubated for an additional 4 hours (Betts et al., 2003). Cellular viability was above 90% and was assessed using either fluorescent or non-fluorescent approaches, depending on the availability of required open channels on the flow cytometer. All antibodies were purchased from eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). Fluorescence minus one (FMO) controls were used to determine non-specific fluorescence and define gating parameters. 500,000 events per sample were collected using an LSRII flow cytometer (BD Biosciences, San Jose, CA), and analyzed using the FlowJo software program (TreeStar, Ashland, OR). The gating strategies used to define CTL, NP-specific, IFNγ, and CD107a positive CD8 cells are depicted in Fig. S1. All the FCS (flow cytometry standard) files used to generate the data in this paper, as well as FCS files from an independent transgenerational cohort, has been deposited in the NIH FigShare repository (DOI: http://doi.org/10.35092/yhjc.c.4649441).

Statistical analyses

The dam is defined as the statistical unit for all experiments; therefore, the number of animals in each experimental group refers to the number of different F0 dams. We used 1 offspring of each sex from each dam for experiments whenever possible. All offspring that were crossed to create a new generation were from a different initial dam-sire pairing. Likewise, for infection, within each exposure group, offspring of the same sex were from different dams. Data were analyzed using JMP software (Version 14, SAS, Cary, NC). Differences between vehicle and TCDD offspring within sex and within a generation were analyzed using a two-tailed Student's t test. For the outcross experiment, a two-way analysis of variance (ANOVA) of paternal F0 exposure vs. maternal F0 exposure was performed, followed by a Dunnett's test with the

control defined as the VEH x VEH lineage. A two-way ANOVA followed by a Tukey HSD *post-hoc* test was used to compare differences between independent variables (e.g. multiple genotypes and F0 exposure groups). Differences were considered statistically significant when p values were less than or equal to 0.05. Error bars on all graphs represent the standard error of the mean (SEM).

Supplemental References

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