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Supplemental Material

DNA Methylation Patterns in $CD4^+$ T Cells of Naïve and Influenza A Virus-Infected Mice Developmentally Exposed to an Aryl Hydrocarbon Receptor Ligand

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Figure S1. Water consumption and body weight change in mice treated with DNA methylation altering drugs. Mice were developmentally exposed to peanut oil vehicle (Veh) or TCDD (1 μg/kg BW). At 21 days, 25 female offspring of vehicle dams, and 17 female offspring of TCDD dams were randomly assigned to 3 treatment groups: normal vivarium water (control), water containing S-adenosylmethionine (SAM; 0.5 mg/mL), or water containing Zebularine (Zeb, 0.2 mg/mL). (A) The amount of water consumed per mouse per day prior to infection. Water consumption was measured every 2 days. The amount of water consumed per mouse was calculated by recording the amount of water consumed per cage divided by the total number of mice within that cage (4-5 mice per cage). (B) Weekly weight gain from 3 weeks of age until infection. (C-E) Body weight after IAV infection in (C) all groups, (D) vehicle groups, and (E) TCDD groups was recorded for 9 days. No mice died from the infection. The number of mice in each group was as follows: control water vehicle (8), control water TCDD (5), SAM water vehicle (8), SAM water TCDD (6), Zeb water vehicle (9), and Zeb water TCDD (6). All data shown denote the mean ± SEM. Data were analyzed by 2-way ANOVA, and F values are shown in each graph.

Figure S2. $CD4^+$ T cell purity. $CD4^+$ T cells were isolated using MojoSort Mouse $CD4^+$ T cell kit (BioLegend, San Diego, CA). (A) Purity of isolated $CD4^+$ T cells was determined using flow cytometry. The FACS plots depict cell enrichment from two samples, and the number on each plot denotes the mean (±SEM) purity of $CD4^+$ T cells from infected offspring of dams treated with vehicle control or TCDD. (B) $CD4^+$ T cell purity for individual samples from vehicle and TCDD offspring that were naïve or infected.

Figure S3. Differential gene expression in $CD4^+$ T cell from mice exposed developmentally to TCDD or control and infected with influenza A virus. Pregnant mice were administered vehicle or TCDD (1 μg/kg body weight) or peanut oil vehicle control on gestational days (GD) 0, 7, 14, and 2 days after parturition (PND2). At 8-10 weeks of age, offspring were infected with influenza A virus (IAV; strain HKx31). Nine days after infection, $CD4^+$ T cells were purified, RNA isolated, and RNA-sequencing (RNA-seq) was performed. The number of differentially expressed genes (DEGs) was assessed and presented in a volcano plot.

Figure S4. Comparison of cellular functions and pathways during IAV infection in offspring developmentally exposed to vehicle control or TCDD. Pathways were ranked by p-value, and 20 pathways in $CD4^+$ T cells from infected vs. naïve offspring in (A) Vehicle and (B) TCDD exposure groups. Position of dots denotes relative rank in the other exposure group. The y-axis shows the pathway rank within the (A) Vehicle or (B) TCDD. A positive change in rank order indicated that pathway was found at a higher rank in the other exposure group. Open circles represent pathways that were ranked among the top 20 for both vehicle and TCDD exposure groups. In (A), filled circles represent pathways only in the vehicle. In (B), filled circles represent pathways only in the TCDD. Abbreviations: V, vehicle, T, TCDD, i, infected, n, naïve.

Figure S5. Correlation of changes in DNA methylation and gene expression. (A-F) Scatter plots show the correlation between change (Δ) in DNA methylation and fold change in differentially expressed genes (DEG) in $CD4^+$ T cells from (A-C) vehicle or (D-F) TCDD exposed offspring prior to and after infection. Correlations in (A,D) all genomic regions, (B,E) promoters, and (C,F) introns are shown. Black lines show the R2 and p-value for all DEGs (all circles). Blue lines show the R2 and p-value for DEGs with an inverse correlation between gene expression and DNA methylation change (open circles). The percentage of open circles is indicated above each plot.

Table S1: Pathways analysis of RNA-sequencing analysis of $CD4^+$ T cells

Vehicle: Infected vs. Naïve			TCDD: Infected vs. Naïve		
Pathways	**-log(p-value)**	**z-score**	**Pathways**	**-log(p-value)**	**z-score**
T Cell Exhaustion	8.9	2.412	T Cell Exhaustion	8.34	2.92
Mitotic Roles of Polo-Like Kinase	6.29	2	Th1 Pathway	9.31	1.616
Th1 Pathway	10.1	0.973	Th2 Pathway	8.39	1.461
Th2 Pathway	10.5	0.781	Oxidative Stress Response	4.86	0.447
ATM Signaling	7.01	0.756	iCOS-iCOSL Signaling	4.74	0.2
Apoptosis Signaling	6.57	0.365	ATM Signaling	5	0
DNA Damage Response	7.43	0	Type I Diabetes Signaling	5.33	0
Th1 and Th2 Activation	14.2	N/A	Th1 and Th2 Pathway	13.3	N/A
T Helper Differentiation	7.82	N/A	T Helper Differentiation	9.73	N/A
Chromosomal Replication	8.66	N/A	Chromosomal Replication	6.54	N/A
IL-10 Signaling	5.89	N/A	IL-10 Signaling	7.46	N/A
Cell Cycle: G2/M	10.2	-0.209	Signaling in Rheumatoid Arthritis	6.77	N/A
Leukocyte Extravasation	5.65	-0.438	Apoptosis Signaling	5.61	-0.2
NF-κB Activation by Viruses	6.15	-0.557	iNOS Signaling	8.45	-0.229
iNOS Signaling	7.01	-0.943	DNA Damage Response	6.09	-0.258
Death Receptor Signaling	5.84	-1.3	IL-6 Signaling	6.23	-0.686
Cell Cycle Control	8.46	-1.414	CD40 Signaling	4.87	-1.279
TNFR1 Signaling	6.11	-1.606	TNFR2 Signaling	4.73	-1.508
TNFR2 Signaling	6.23	-2.138	Cell Cycle: G2/M	4.78	-1.807
Sumoylation Pathway	7.13	-2.414	Cell Cycle Control	7.1	-2.324

Table S2. Numerical data and p-values for Figures 2 and 3

	Number (10^4)			Percentage (%)		
	Vehicle[a]	**TCDD**[a]	**p-value**[b]	**Vehicle**[a]	**TCDD**[a]	**p-value**[b]
PD1$^+$	14.53 ± 2.21	6.65 ± 1.81	**0.0086**	56.10 ± 2.21	46.93 ± 2.05	**0.0049**
CTLA$^+$	0.91 ± 0.20	0.46 ± 0.12	**0.0436**	3.41 ± 0.52	3.21 ± 0.27	0.3691
TIM3$^+$	1.06 ± 0.17	0.52 ± 0.13	**0.0142**	4.09 ± 0.18	3.80 ± 0.24	0.1769
KLRG1$^+$	0.185 ± 0.048	0.091 ± 0.017	0.0525	0.913 ± 0.096	0.704 ± 0.041	**0.0488**
p16$^+$	0.890 ± 0.186	0.977 ± 0.147	0.3605	4.606 ± 0.330	7.561 ± 0.813	**0.0040**
CD107a$^+$	3.557 ± 0.956	1.267 ± 0.530	**0.0342**	8.749 ± 0.716	5.973 ± 0.849	**0.0272**
Perforin$^+$	1.108 ±0.422	0.161 ± 0.063	**0.0333**	2.279 ±0.577	0.807 ± 0.060	**0.0217**

[a] Data presented are mean ±SEM.
[b] p-values calculated via one-tailed Student's t-test; bold font denotes p-values ≤ 0.05 (TCDD compared to Vehicle control)

Table S3. Numerical data and p-values for Figure 4

Day post primary infection	**I-A^{b}NP$_{311\text{-}325}{}^+$ CD4$^+$ T cells (10^4)**		
	Vehicle[a]	**TCDD**[a]	**p-value**[b]
Day 0	0.027 ± 0.008	0.029 ± 0.004	0.4134
Day 9	0.517 ± 0.149	0.147 ± 0.041	**0.0050**
Day 79	0.439 ± 0.090	0.286 ± 0.076	0.1099
Day 86	0.671 ±0.102	0.320 ± 0.045	**0.0055**

[a] Mean number of cells ±SEM
[b] p-values calculated via one-tailed Student's t-test; bold font denotes p-values ≤ 0.05 (TCDD compared to Vehicle control at a single point in time)

Table S4. ANOVA values global DNA methylation

Group 1[a]	**Group 2**[a]	**p-value**[b]
Vehicle Naïve	TCDD Naïve	0.9751
Vehicle Naïve	Vehicle Infected	**0.0002**
Vehicle Naïve	TCDD Infected	**<0.0001**
TCDD Naïve	Vehicle Infected	**0.0003**
TCDD Naïve	TCDD Infected	**<0.0001**
Vehicle Infected	TCDD Infected	**0.0118**

[a] Vehicle and TCDD refer to maternal treatment; naïve and infected refer to offspring that were not or were in infected with influenza A virus
[b] p-values from two-way ANOVA (developmental exposure*infection status) with Tukey HSD post-hoc test; bold font denotes p-values ≤ 0.05

Table S5. Z-scores and p-values corresponding to graphs in Figure 6

Figure 6B			Figure 6D		
Vehicle: Infected vs. Naïve			**TCDD: Infected vs. Naïve**		
Pathways	**-log(p-value)**	**z-score**	**Pathways**	**-log(p-value)**	**z-score**
NF-κB Activation by Viruses	8.61	-1.886	PPARα/RXRα Activation	7.13	-0.471
NF-κB Signaling	7.69	-1.225	NF-κB Signaling	7.09	-0.218
TNFR2 Signaling	7.54	-1	IL-6 Signaling	6.68	0.471
Lymphotoxin β Receptor Signaling	7.23	-1.508	NF-κB Activation by Viruses	6.68	-0.258
T Cell Receptor Signaling	7.13	N/A	Tec Kinase Signaling	6.46	1.414
Apoptosis Signaling	6.9	0.258	Th1 and Th2 Activation Pathway	6.45	N/A
T Cell Exhaustion Signaling Pathway	6.64	1.606	IL-10 Signaling	5.93	N/A
Th1 and Th2 Activation Pathway	6.43	N/A	Apoptosis Signaling	5.85	-1.387
CD40 Signaling	6.32	-1.387	iCOS-iCOSL Signaling in T Helper Cells	5.82	-0.775
LPS-stimulated MAPK Signaling	6.19	-2.324	Protein Kinase A Signaling	5.58	0
PEDF Signaling	6.19	-0.535	PKCθ Signaling in T Lymphocytes	5.37	-1.213
IL-6 Signaling	6.12	-0.943	GNRH Signaling	5.26	0
PKCθ Signaling in T Lymphocytes	6.03	-2.065	CD40 Signaling	5.17	-1.155
Type I Diabetes Mellitus Signaling	6.01	-1.387	iNOS Signaling	5.11	0
iCOS-iCOSL Signaling in T Helper Cells	5.99	-1	PPAR Signaling	4.86	-1.387

Table S6. Numerical data and p-values for Figure 7

	Group	Mean ± SEM	ANOVA p-value[a]	t-test p-value[f]
Total $CD4^+$ (10^5)	Vehicle Control	9.89 ± 1.53	N/A[b]	N/A[b]
	TCDD Control	3.75 ± 1.05	0.0122[c]	0.0069
	Vehicle SAM	7.68 ± 0.61	0.5143[d]	N/A
	TCDD SAM	5.06 ± 1.39	0.4337[e]	0.1273
I-$A^b$$NP_{311-325}^+$ $CD4^+$ cells (10^4)	Vehicle Control	1.15 ± 0.25	N/A	N/A
	TCDD Control	0.33 ± 0.11	0.0208[c]	0.0137
	Vehicle SAM	0.83 ± 0.12	0.5093[d]	N/A
	TCDD SAM	0.41 ± 0.10	0.3234[e]	0.0169
Activated $CD4^+$ (% of CD4s)	Vehicle Control	19.50 ± 1.84	N/A	N/A
	TCDD Control	11.10 ± 2.14	0.0322[c]	0.0151
	Vehicle SAM	21.60 ± 1.76	0.8260[d]	N/A
	TCDD SAM	14.00 ± 1.91	0.0412[e]	0.0131
Naïve $CD4^+$ (% of CD4s)	Vehicle Control	66.30 ± 2.35	N/A	N/A
	TCDD Control	74.10 ± 1.89	0.1478[c]	0.0259
	Vehicle SAM	63.2 ± 2.35	0.7516[d]	N/A
	TCDD SAM	71.20 ± 2.41	0.1055[e]	0.0370
Th1 (% of CD4s)	Vehicle Control	18.13 ± 2.15	N/A	N/A
	TCDD Control	12.54 ± 1.32	0.1779[c]	0.0498
	Vehicle SAM	17.01 ± 1.12	0.9626[d]	N/A
	TCDD SAM	13.71 ± 2.07	0.5600[e]	0.1990
Tfh (% of CD4s)	Vehicle Control	3.22 ± 0.53	N/A	N/A
	TCDD Control	1.68 ± 0.41	0.1571[c]	0.0440
	Vehicle SAM	3.87 ± 0.46	0.7183[d]	N/A
	TCDD SAM	2.11 ± 0.36	0.0655[e]	0.0107

[a] Two-way ANOVA (developmental exposure*water treatment) with Tukey post-hoc test
[b] N/A, not applicable (i.e., group not compared to itself)
[c] Vehicle control water to TCDD control water
[d] Vehicle control water to Vehicle S-adenosylmethionine (SAM) water
[e] Vehicle SAM to TCDD SAM
[f] Two-tailed t-test, Vehicle versus TCDD *within* water treatment group

Table S7. Numerical data and p-values for Figure 8

	Group	Mean ± SEM	ANOVA p-value[a]	t-test p-value[f]
Total $CD4^+$ (10^5)	Vehicle Control	9.89 ± 1.53	N/A[b]	N/A
	TCDD Control	3.75 ± 1.05	0.0431[c]	0.0069
	Vehicle Zeb	6.82 ± 1.53	0.3651[d]	N/A
	TCDD Zeb	2.84 ± 0.97	0.2239[e]	0.0477
I-$A^b$$NP_{311-325}^+$ $CD4^+$ cells (10^4)	Vehicle Control	1.15 ± 0.25	N/A	N/A
	TCDD Control	0.33 ± 0.11	0.0489[c]	0.0137
	Vehicle Zeb	0.77 ± 0.19	0.4469[d]	N/A
	TCDD Zeb	0.25 ± 0.09	0.2454[e]	0.0281
Activated $CD4^+$ (% of CD4s)	Vehicle Control	19.50 ± 1.84	N/A	N/A
	TCDD Control	11.10 ± 2.14	0.0496[c]	0.0151
	Vehicle Zeb	17.50 ± 2.02	0.8710[d]	N/A
	TCDD Zeb	18.00 ± 1.89	0.9987[e]	0.8789
Naïve $CD4^+$ (% of CD4s)	Vehicle Control	66.30 ± 2.35	N/A	N/A
	TCDD Control	74.10 ± 1.89	0.2282[c]	0.0259
	Vehicle Zeb	66.90 ± 2.66	0.9976[d]	N/A
	TCDD Zeb	67.6 ± 2.94	0.9981[e]	0.8758
Th1 (% of CD4s)	Vehicle Control	18.13 ± 2.15	N/A	N/A
	TCDD Control	12.54 ± 1.32	0.2009[c]	0.0498
	Vehicle Zeb	16.99 ± 1.51	0.9616[d]	N/A
	TCDD Zeb	15.39 ± 1.77	0.9204[e]	0.5061
Tfh (% of CD4s)	Vehicle Control	3.22 ± 0.53	N/A	N/A
	TCDD Control	1.68 ± 0.41	0.1365[c]	0.0440
	Vehicle Zeb	2.81 ± 0.41	0.8940[d]	N/A
	TCDD Zeb	2.55 ± 0.29	0.9770[e]	0.6210

[a] Two-way ANOVA (developmental exposure*water treatment) with Tukey post-hoc test
[b] N/A, not applicable (i.e., group not compared to itself)
[c] Vehicle control water to TCDD control water
[d] Vehicle control water to Vehicle Zebularine (Zeb) water
[e] Vehicle Zeb to TCDD Zeb
[f] Two-tailed t-test, Vehicle versus TCDD *within* water treatment group

Table S8. Genes in adult humans and mice that were differentially methylated following early life AHR activation

Gene name	Yucheng Methyl Diff % [a]	Feature[b]	TnVn Methyl Diff% [c]	Feature[b]	TiVi Methyl Diff %[d]	Feature[b]
Gng12	-8.6	TSS1500	-32.63	Intron	-47.30	Intergenic
Arl4c	12.7	3'UTR, 1exon	-91.67 49.31 45.00 -33.27	Intergenic Intergenic Intergenic Intergenic	29.92	TTS
Ahrr	17 -9.4 -5.8	Body Body Body	27.08 36.33	Intron Intron	--	
Myo1g	11 10.5 17.7 17.3	3'UTR Body Body Body	33.79	Intron	--	
Cntnap2	-2.7 -7.6	Body Body	-30.60 35.71 30.00 -50.51 -46.03 -29.78 -37.50 -37.23 -57.14 -36.00 57.34 29.83 -50.00 52.17 -77.78 27.83 43.72 32.93 -29.37 -37.73 -44.29 -28.63 -93.33 -58.31	Promoter-TSS Intergenic Intergenic Intergenic Intergenic Intergenic Intergenic Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron	55.28 -40.33 -43.67 -66.86 -68.97 59.31 43.33 -43.08 -80.00 -36.60 -72.81 -32.38 -31.82 -32.68 32.74 30.34 -26.29 -42.11 38.46 -26.47 31.21	Intergenic Intergenic Intergenic Intergenic Intergenic Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron Intron

Olfm1	9.6	Body	--		--	
Frmd4a			42.86	Intergenic	-26.09	Intron
	8.7	TSS200	-29.87	Intron	27.08	Intron
	7.8	TSS200	-30.95	Intron	30.43	Intron
	10.2	TSS200	-35.99	Intron	27.80	Intron
					-45.97	Intron
Cyp1a1	8.4	TSS1500	--		--	
Anpep	5.8	Unidentified	--		--	
Znf536 **(*Zfp536* in mice)**			66.67	Intergenic		
			54.71	Intergenic		
			52.02	Intergenic		
			-27.56	Intergenic	96.55	Intergenic
			-29.94	Intergenic	26.09	Intergenic
	14.4	5'UTR	-35.29	Intergenic	-36.39	Intergenic
	11.4	5'UTR	-41.18	Intergenic	-80.56	Intergenic
			-33.61	Intron	32.18	Intron
			-33.90	Intron		
			-26.36	Intron		
			-83.33	Intron		
Wwc3	-7.7	5'UTR	--		--	

[a] Genes with differential DNA methylation patterns in whole blood cells isolated from adults who were developmentally exposed to the AHR binding polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) (Su et. al. 2019). Negative sign denotes that DNA methylation was decreased in exposed individuals.

[b] Genomic feature (transcriptional start site [TSS], untranslated region [UTR])

[c] Percent change in DNA methylation in immunologically naïve (n) adult mice that were exposed developmentally to vehicle (V) or TCDD (T). Negative values denote DNA methylation was decreased in T compared to V.

[d] Percent change in DNA methylation in influenza A virus infected (i) adult mice that were exposed developmentally to vehicle (V) or TCDD (T). Negative values denote DNA methylation was decreased in T compared to V.

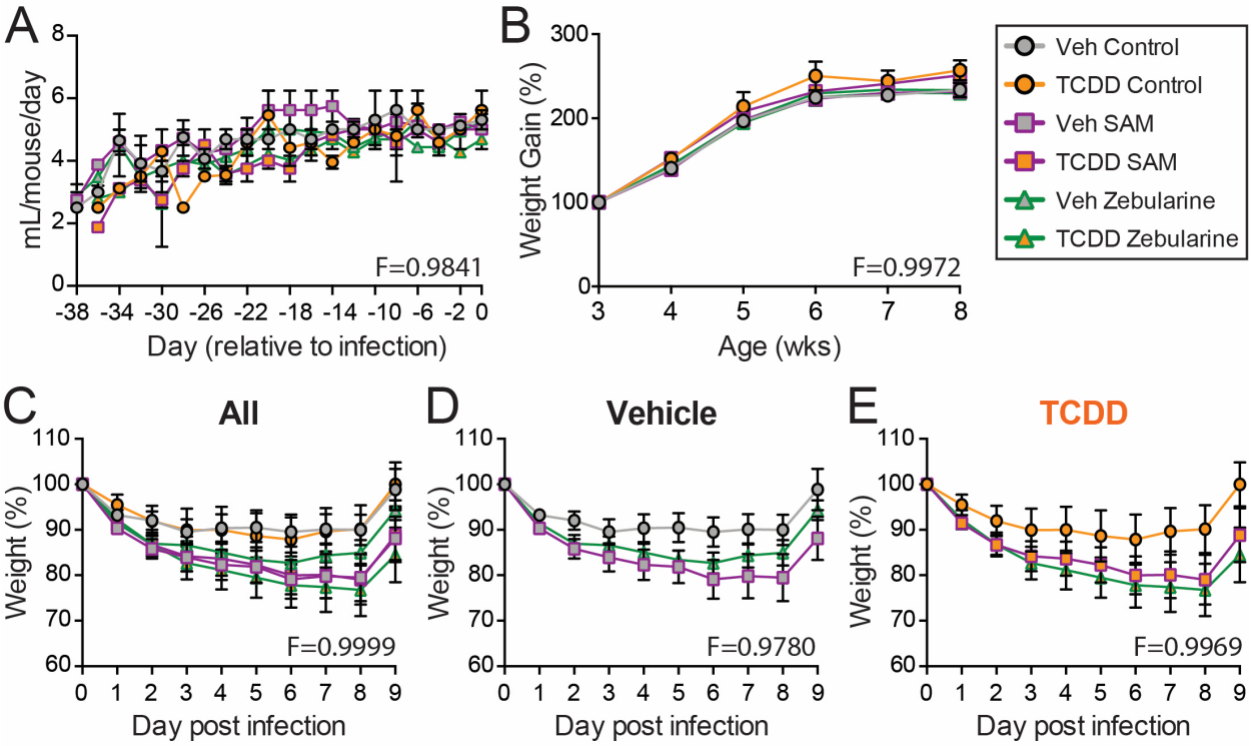
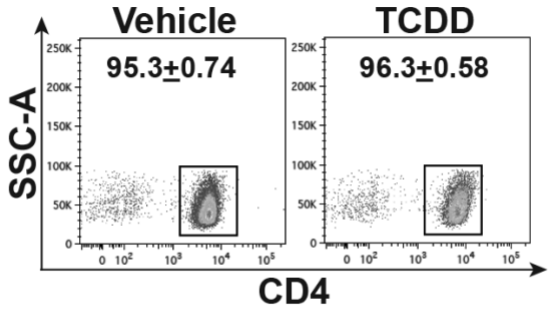


Figure S1: Water consumption and body weight change in mice treated with DNA methylation altering drugs. Mice were developmentally exposed to peanut oil vehicle (Veh) or TCDD (1 µg/kg BW). At 21 days, 25 female offspring of vehicle dams, and 17 female offspring of TCDD dams were randomly assigned to 3 treatment groups: normal vivarium water (control), water containing S-adenosylmethionine (SAM; 0.5 mg/mL), or water containing Zebularine (Zeb, 0.2 mg/mL). (A) The amount of water consumed per mouse per day prior to infection. Water consumption was measured every 2 days. The amount of water consumed per mouse was calculated by recording the amount of water consumed per cage divided by the total number of mice within that cage (4-5 mice per cage). (B) Weekly weight gain from 3 weeks of age until infection. (C-E) Body weight after IAV infection in (C) all groups, (D) vehicle groups, and (E) TCDD groups was recorded for 9 days. No mice died from the infection. The number of mice in each group was as follows: control water vehicle (8), control water TCDD (5), SAM water vehicle (8), SAM water TCDD (6), Zeb water vehicle (9), and Zeb water TCDD (6). All data shown denote the mean ± SEM. Data were analyzed by 2-way ANOVA, and F values are shown in each graph.

A



B

Sample #	Dev Expo	Naïve/Infect	% CD4+ T cells
1	Vehicle	Naïve	95.9
2	TCDD	Naïve	97.6
3	Vehicle	Naïve	95.9
4	TCDD	Naïve	97.3
5	Vehicle	Naïve	98.0
6	TCDD	Naïve	97.8
7	Vehicle	Naïve	97.5
8	TCDD	Naïve	97.5
9	Vehicle	Infected	95.8
10	TCDD	Infected	96.3
11	Vehicle	Infected	91.8
12	TCDD	Infected	94.6
13	Vehicle	Infected	94.4
14	TCDD	Infected	93.2
15	Vehicle	Infected	93.2
16	TCDD	Infected	95.8

Figure S2: CD4⁺ T cell purity. CD4⁺ T cells were isolated using MojoSort Mouse CD4⁺ T cell kit (BioLegend, San Diego, CA). (A) Purity of isolated CD4⁺ T cells was determined using flow cytometry. The FACS plots depict cell enrichment from two samples, and the number on each plot denotes the mean (±SEM) purity of CD4⁺ T cells from infected offspring of dams treated with vehicle control or TCDD. (B) CD4⁺ T cell purity for individual samples from vehicle and TCDD offspring that were naïve or infected.

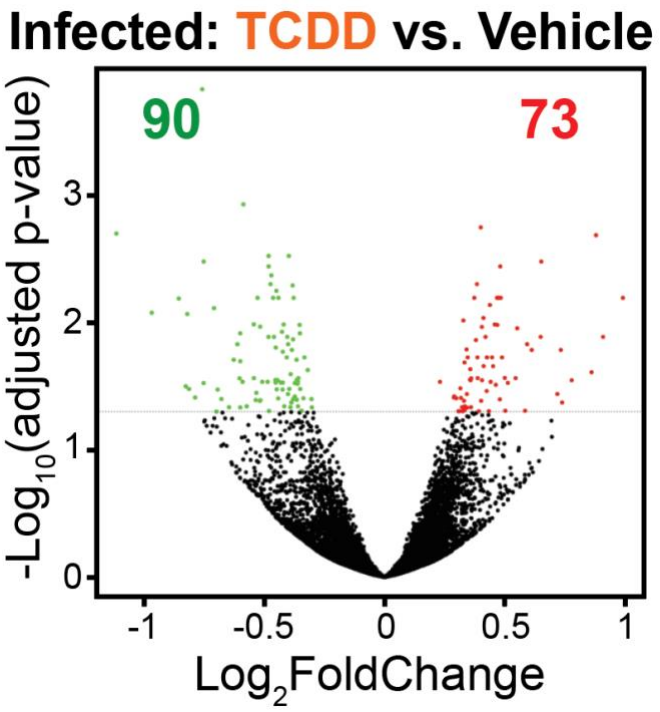


Figure S3: Differential gene expression in CD4⁺ T cell from mice exposed developmentally to TCDD or control and infected with influenza A virus. Pregnant mice were administered vehicle or TCDD (1 μg/kg body weight) or peanut oil vehicle control on gestational days (GD) 0, 7, 14, and 2 days after parturition (PND2). At 8-10 weeks of age, offspring were infected with influenza A virus (IAV; strain HKx31). Nine days after infection, CD4⁺ T cells were purified, RNA isolated, and RNA-sequencing (RNA-seq) was performed. The number of differentially expressed genes (DEGs) was assessed and presented in a volcano plot.

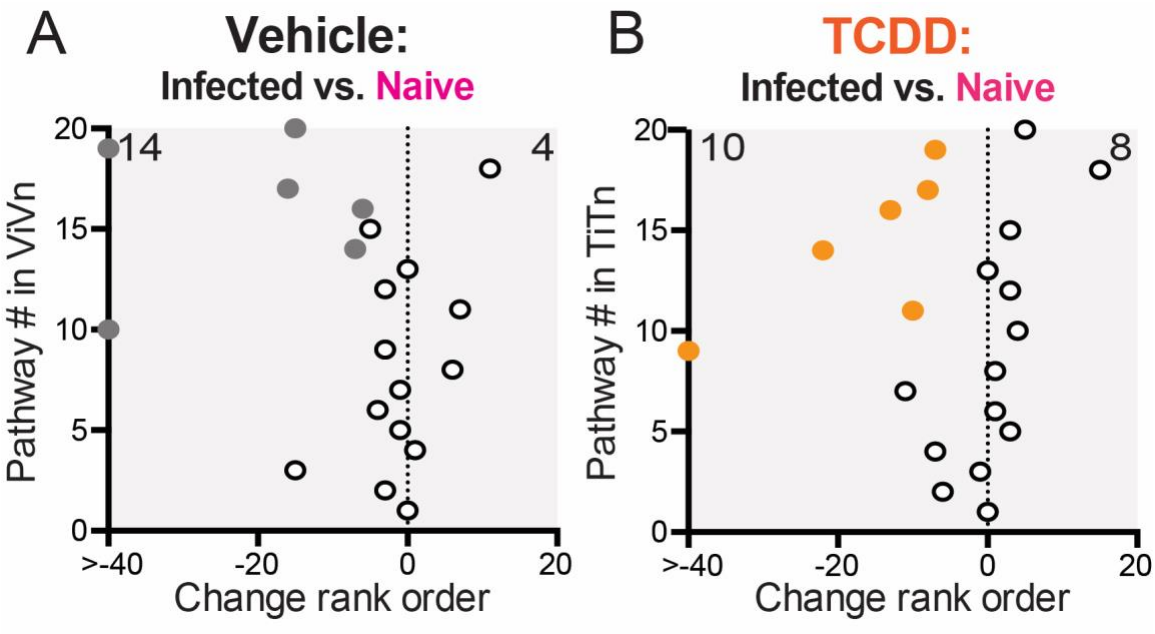


Figure S4: Comparison of cellular functions and pathways during IAV infection in offspring developmentally exposed to vehicle control or TCDD. Pathways were ranked by p-value, and 20 pathways in $CD4^+$ T cells from infected vs. naïve offspring in (A) Vehicle and (B) TCDD exposure groups. Position of dots denotes relative rank in the *other* exposure group. The y-axis shows the pathway rank within the (A) Vehicle or (B) TCDD. A positive change in rank order indicated that pathway was found at a higher rank in the other exposure group. Open circles represent pathways that were ranked among the top 20 for both vehicle and TCDD exposure groups. In (A), filled circles represent pathways only in the vehicle. In (B), filled circles represent pathways only in the TCDD. Abbreviations: V, vehicle, T, TCDD, i, infected, n, naïve.

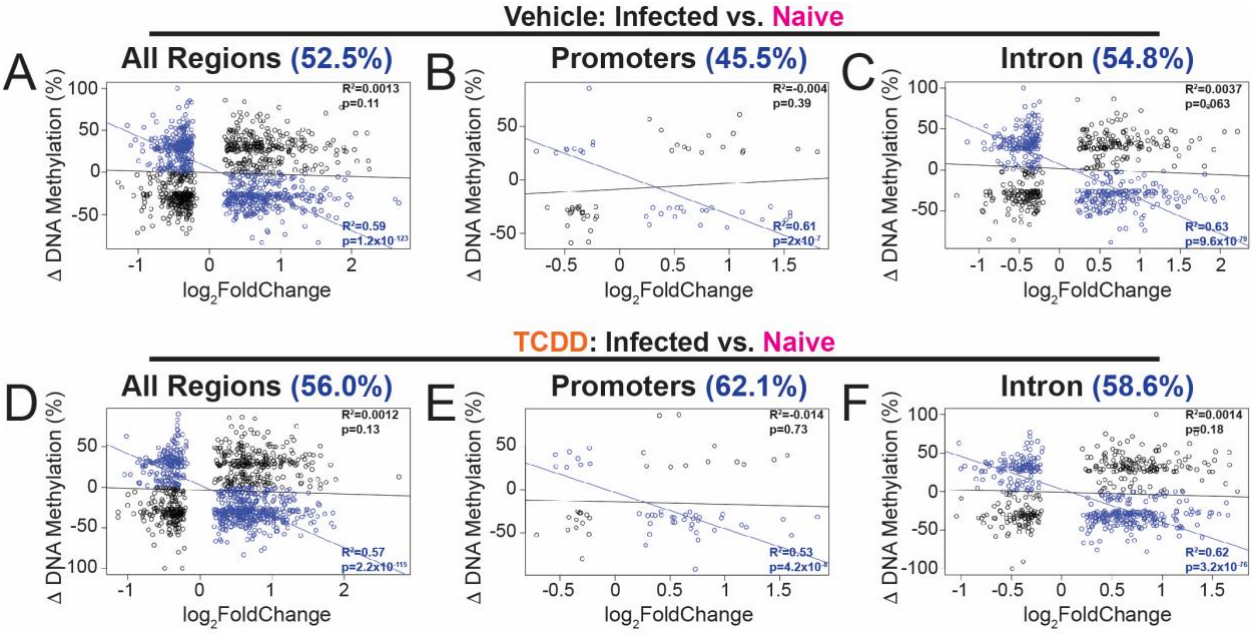


Figure S5: Correlation of changes in DNA methylation and gene expression. (A-F) Scatter plots show the correlation between change (Δ) in DNA methylation and fold change in differentially expressed genes (DEG) in CD4+ T cells from (A-C) vehicle or (D-F) TCDD exposed offspring prior to and after infection. Correlations in (A,D) all genomic regions, (B,E) promoters, and (C,F) introns are shown. Black lines show the R^2 and p-value for all DEGs (all circles). Blue lines show the R^2 and p-value for DEGs with an inverse correlation between gene expression and DNA methylation change (open circles). The percentage of open circles is indicated above each plot.