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Supplementary Information for

Vitamin B12 and folic acid alleviate symptoms of nutritional deficiency by antagonizing aryl hydrocarbon receptor

Daniel J. Kim, Arvind Venkataraman, Priyanka Caroline Jain, Eleanor P. Wiesler, Melody DeBlasio, Jonathan Klein, Stephanie S. Tu, Seohyuk Lee, Ruslan Medzhitov, Akiko Iwasaki

Akiko Iwasaki
Email: akiko.iwasaki@yale.edu

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Supplementary Information Text

Cell Culture. For expansion, HepG2 cells were cultured in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin (P/S) on collagen-coated plates. For experiments, cells were plated on to collagen-coated plates in MEM media supplemented with 10% FBS and 1% P/S. After 24 h, cells were serum-starved overnight in MEM media supplemented with 0.5% FBS and 1% P/S. For treatments, media was first replaced with HBSS supplemented with 1% NEAA, 1% HEPES, 1% P/S to minimize background vitamin levels, and compounds were added at 1:1000 dilution.

CBC. Retro-orbital blood was collected with heparinized capillary tubes into EDTA-coated tubes. CBC was analyzed using Hemavet veterinary hematology analyzer (Drew Scientific).

Histology. Mouse liver sections were placed in histology cassettes and submerged in 10% NBF. Unstained and H&E slides were developed by Yale Pathology Tissue Services. Images were captured using a microscope with 10x objective lens (BX51; Olympus).

ChIP-qPCR. The procedure for ChIP-qPCR as described previously was performed with some modifications (1). Briefly, after 2 h treatment with 0.5 nM TCDD in the presence of 5000 pg/mL B12 (with 5 pM TCN2) and 50 ng/mL FA, cross-linking was performed by adding 1% formaldehyde to media for 5 min and stopped with 0.125 M glycine. Cells were subsequently lysed in cell lysis buffer (50 mM HEPES pH 7.4, 1 mM EDTA, 85 mM KCl, 10% glycerol, 0.5% Nonidet P-40, protease inhibitor) and centrifuged at 1200 g at 4C for 5 min. After suspension in nucleus lysis buffer (50 mM Tris·HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 5% glycerol, 1% Triton-X 100, 1% SDS, protease inhibitor), nuclear pellets were sonicated using a Bioruptor for 45 cycles (30 s on, 30 s off). Nucleosomes were immunoprecipitated by adding 3 ug anti-AhR antibody and rotating samples overnight at 4 °C. Samples were subsequently incubated with Dynabeads Protein G under rotation for 1 h at RT. Beads were washed twice with ChIP wash buffer 1 (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS, 2 mM EDTA), twice with ChIP wash buffer 2 (20 mM Tris·HCl, pH 8.0, 500 mM NaCl, 1% Triton-X 100, 0.1% SDS, 2 mM EDTA) twice, and once with ChIP wash buffer 3 (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA). After washing once in TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA), bound DNA was eluted from beads using ChIP Elute Kit (Takara) and analyzed with RT-qPCR with primers flanking an XRE in the promoter region of *CYP1A1* (Table S1) and normalized to 5% input.

RNA Extraction and RT-qPCR. For mice, tissues were homogenized in Buffer RLT using Lysing Matrix D Disruption Columns (MP Biomedicals) and MP Bio FastPrep-24 (MP Biomedicals). Samples were incubated with Proteinase K for 10 minutes and precipitated with ethanol. For tissue culture, cells were lysed in RLT buffer and precipitated with ethanol. Lysate was transferred to purification columns, and RNA was subsequently extracted using RNeasy Mini Kit (Qiagen). After elution with RNase-free water, cDNA was synthesized using iScript (Bio-Rad) and analyzed with RT-qPCR as previously described (2) using the primers listed in Table S1.

Western Blot. Protein samples were separated by SDS-PAGE on a 7.5% polyacrylamide gel run at 80V for 20 minutes and 110V for 55 minutes. Samples were transferred to a polyvinylidene difluoride membrane at 100V for 70 minutes. Membrane was blocked for 1 h in blocking buffer (5% milk in TBS-Tween, TBST) on orbital shaker and incubated overnight with anti-AhR (1:100 in 5% BSA/TBST) at 4C. Blot was washed three times in TBST and incubated on orbital shaker for 1 h in secondary antibody (1:1000 in 5% Milk/TBS-Tween). After three more washes in TBST, blot was visualized with SuperSignal West Femto Chemiluminescent Substrate (ThermoFisher, 34096) and ChemiDoc (Bio-Rad).

Fig. S1

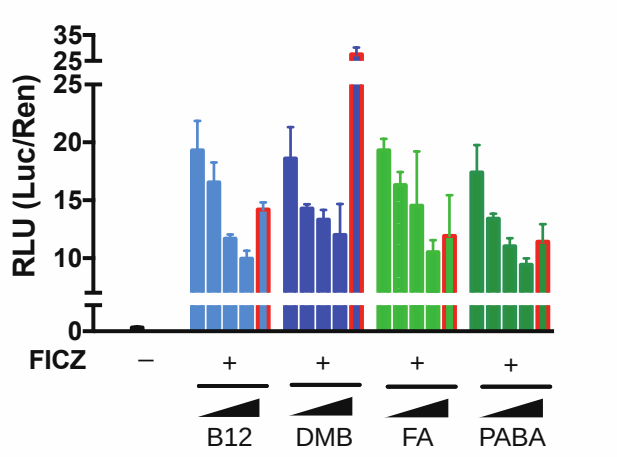

Fig. S2

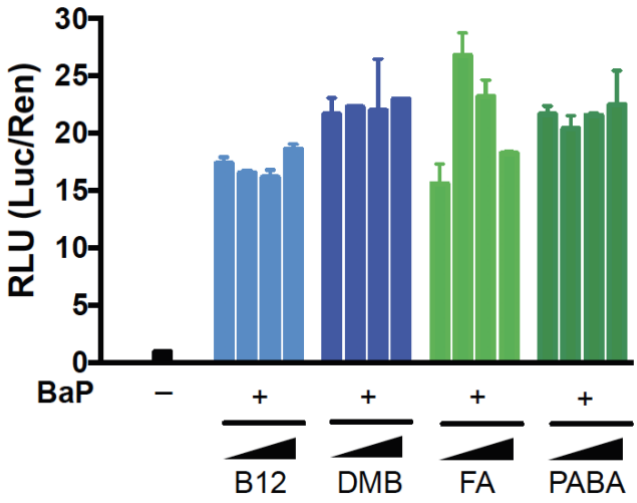

Fig. S3

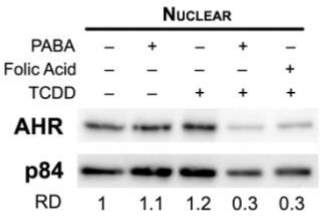

Fig. S4

Fig. S5

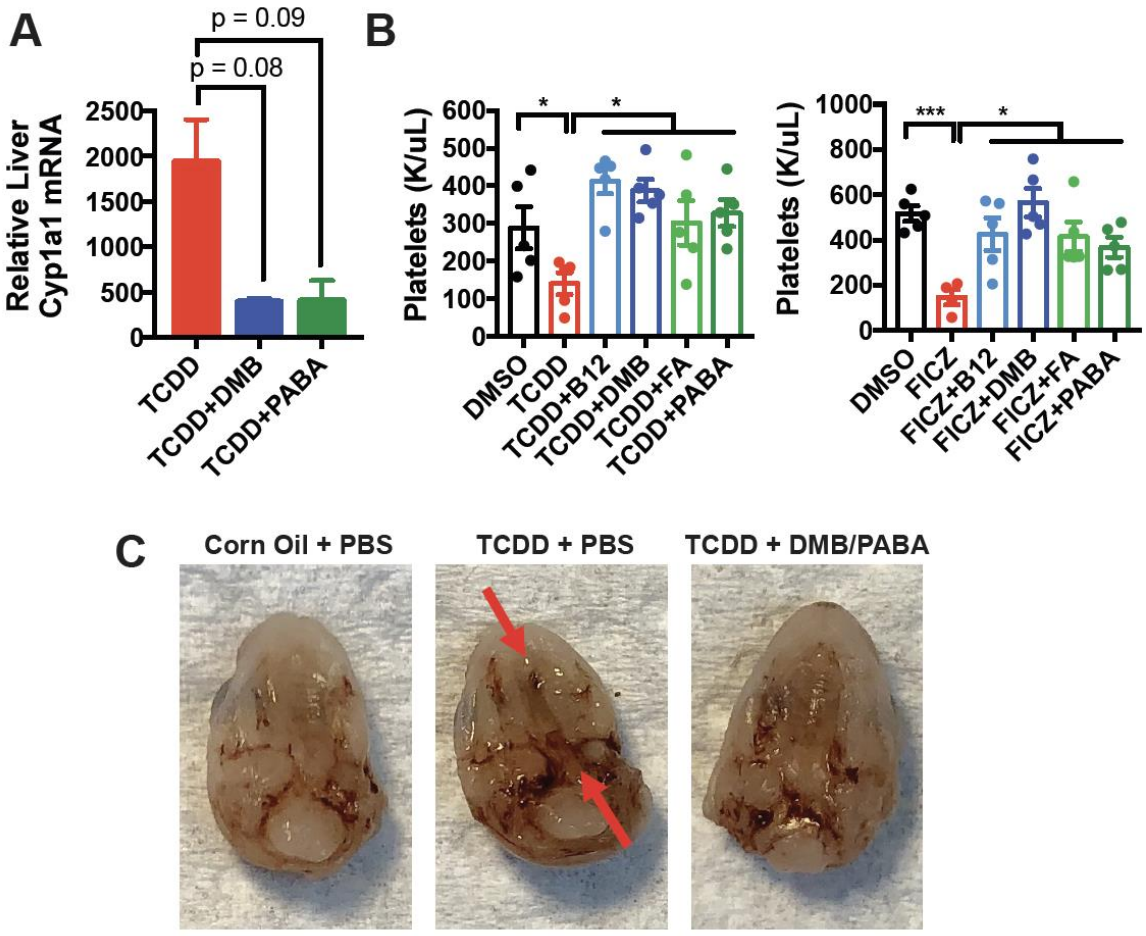
A
p = 0.09
p = 0.08
Relative Liver Cyp1a1 mRNA
2500
2000
1500
1000
500
0
TCDD
TCDD+DMB
TCDD+PABA
B
Platelets (K/uL)
600
500
400
300
200
100
0
*
*
DMSO
TCDD
TCDD+B12
TCDD+DMB
TCDD+FA
TCDD+PABA
Platelets (K/uL)
1000
800
600
400
200
0
***
*
DMSO
FICZ
FICZ+B12
FICZ+DMB
FICZ+FA
FICZ+PABA
C
Corn Oil + PBS
TCDD + PBS
TCDD + DMB/PABA

Fig. S6

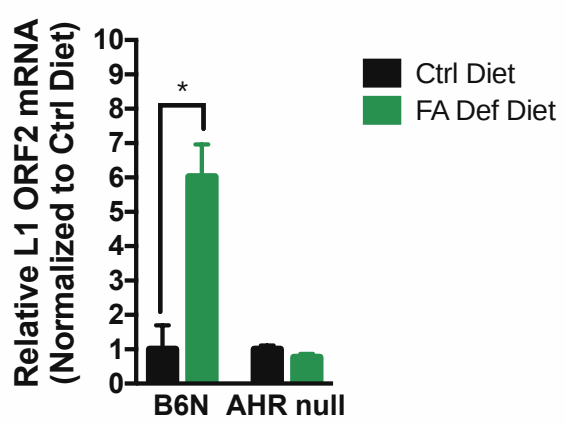
Relative L1 ORF2 mRNA
(Normalized to Ctrl Diet)
10
9
8
7
6
5
4
3
2
1
0
*
B6N
AHR null
Ctrl Diet
FA Def Diet

Fig. S7

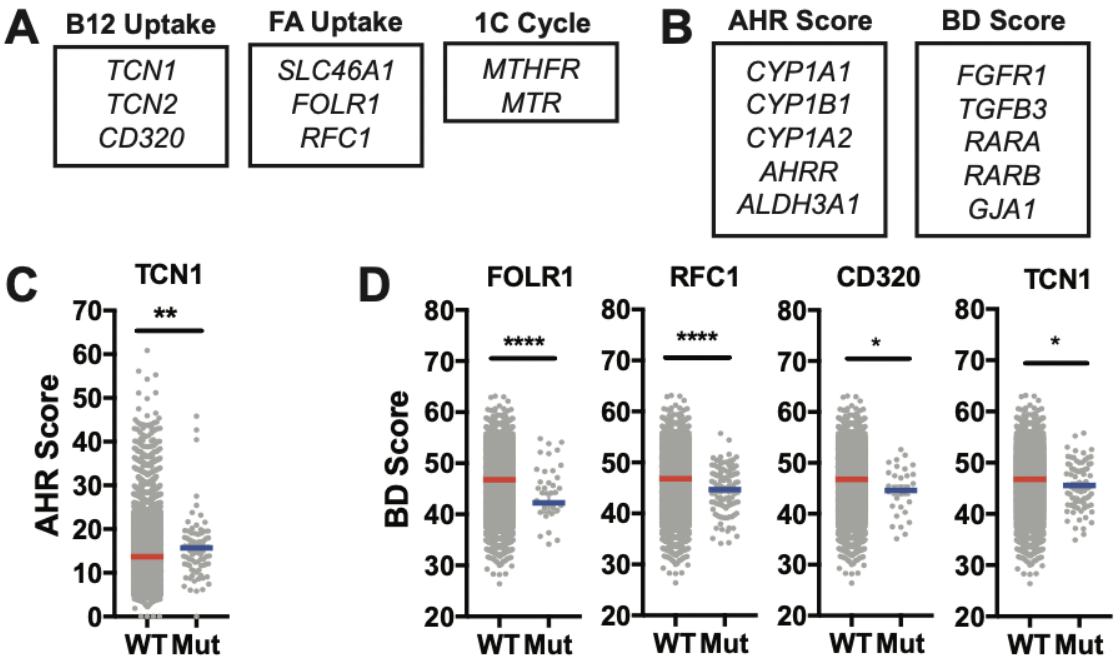
A
B12 Uptake
TCN1
TCN2
CD320
FA Uptake
SLC46A1
FOLR1
RFC1
1C Cycle
MTHFR
MTR
B
AHR Score
CYP1A1
CYP1B1
CYP1A2
AHRR
ALDH3A1
BD Score
FGFR1
TGFB3
RARA
RARB
GJA1
C
TCN1
AHR Score
**
WT Mut
D
FOLR1
RFC1
CD320
TCN1
BD Score
****
****
*
*
WT Mut

Table S1. RT-qPCR primers

Gene	Forward primer	Reverse primer
Mouse *Hprt1*	5'-CTGGTGAAAAGGACCTCTCG-3'	5'-TGAAGTACTCATTATAGTCAAGGGCA-3'
Mouse *Cyp1a1*	5'-GAAGGGCATAGGCAGCCAC-3'	5'-GCTCTGACCACCCAGAATCC-3'
Mouse *LINE1 Orf2*	5'-AGAAGACAGCCACAAGAACAGA-3'	5'-TATTGTGTGAGGCGCAATGT-3'
Human *HPRT1*	5'-CCTGGCGTCGTGATTAGTGAT-3'	5'-AGACGTTCAGTCCTGTCCATAA-3'
Human *CYP1A1*	5'-CCTGGCGTCGTGATTAGTGAT-3'	5'-AGACGTTCAGTCCTGTCCATA-3'
Human *CYP1A1* XRE	5'-CGTTGCAATCAGCACTAAGGC-3'	5'- GATTGAAGGATCGGAATGGATGG-3'

Supplementary Figure Legends

Fig. S1. Supraphysiologic concentrations of B12 and FA inhibit AhR activity less effectively than physiologic concentrations. Same experiment as in Fig. 1B with the inclusion of supraphysiologic concentrations, bordered in red: 50,000 pg/mL B12 (with 50 pM TCN2), 34 nM DMB, 500 ng/mL FA, or 1130 nM PABA.

Fig. S2. **B12 and FA are unable to suppress AhR activation by BaP.** HepG2 cells were transfected with pGL4.43 XRE-luc2P and pGL4.75 CMV-Ren plasmids for 24 h, pretreated with serial dilutions of B12 (with TCN2), DMB, FA, or PABA for 8 h (same concentrations as in Fig. 1C), and treated with 5 μM BaP for 12 h. Luminescence from cell lysates were measured by commercial kit and analyzed by microplate reader. Relative luciferase units (RLUs) were calculated by normalizing firefly luciferase signal with Renilla luciferase signal within each sample and further normalizing with DMSO-treated samples.

Fig. S3. FA and PABA suppress AhR nuclear localization induced by TCDD. HepG2 cells were treated with 0.5 nM TCDD in the presence of 50 ng/mL FA or 113 nM PABA for 24 h. Nuclear expressions of AhR and p84 were measured by western blotting. Relative densitometry (RD) of AhR blots were quantified by ImageJ.

Fig. S4. Overview of streptavidin bead pull-down experiment of biotinylated-B12/FA. Lysates from HEK293T cells overexpressing human AhR were incubated with streptavidin (SA) beads complexed with biotinylated-B12/FA in the presence of DMSO or serial dilutions of TCDD (10, 1, 0.1 nM). Bound protein was eluted and subjected to western blotting for AhR.

Fig. S5. DMB and PABA rescue mice from TCDD-induced thrombocytopenia and cleft palate. (A) WT B6 mice were intraperitoneally (i.p.) injected with PBS, 0.9 nmol/kg DMB, or 28.3 nmol/kg PABA and orally administered corn oil or 2 μg/kg TCDD. RNA was extracted from livers 5 h later. Liver *Cyp1a1* mRNA was measured by RT-qPCR and normalized by *Hprt1*. Data are means ± SE (n=2). (B) B6 mice were i.p. injected everyday with corn oil, 2 μg/kg TCDD, or 12.5 μg/kg FICZ alongside PBS, 12.5 μg/kg B12, 9.3 nmol/kg DMB, 2.5 mg/kg FA, or 5.7 μmol/kg PABA. On Day 7, platelets of retroorbital bleeds were measured by an automatic CBC analyzer. Same experiment as in Fig. 3B. Data are means ± SE (n=5). * $P < 0.05$, *** $P < 0.001$ versus DMSO- and TCDD-treated mice assessed by Student's t-test. (C) B6 female mice were mated with male mice overnight. After visualization of vaginal plugs (Day E0.5), mice were orally administered PBS or 37.2 nmol/kg DMB with 22.7μmol/kg PABA (20 times estimated daily intake of B12 and FA) everyday starting on Day E9.5. On Day E10.5, mice were orally administered a one-time dose of corn oil or 30 μg/kg TCDD. Embryos were collected on Day E18.5 and assessed for palatogenesis under dissecting microscope. Representative palates shown here. Red arrows indicate presence of cleft palate.

Fig. S6. AhR deficiency abrogates induction of LINE1 mRNA mediated by FA deficient diet. B6 and AhR null mice were fed control or FA-deficient diet for 8 weeks on wire-bottom cages. Relative brain LINE1 mRNA measured by RT-qPCR and normalized to *Hprt1* and control-diet-fed mice. * $P < 0.05$ versus control-diet-fed mice assessed by Student's t-test.

Fig. S7. Mutations in B12 and FA uptake proteins are associated with induction of AhR target genes and repression of pathways associated with birth defect. (A) Genes in B12 uptake, FA uptake, and 1C cycle examined for mutations. (B) Genes included in calculating AHR Score and BD Score. (C) AHR Score for TCGA samples with and without mutations in *TCN1*. (D) BD Score for TCGA samples with and without mutations in *FOLR1, RFC1, CD320,* and *TCN1*. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ versus non-mutated samples assessed by Student's t-test.

SI References

1. M. Taura, E. Song, Y. C. Ho, A. Iwasaki, Apobec3A maintains HIV-1 latency through recruitment of epigenetic silencing machinery to the long terminal repeat. *Proc Natl Acad Sci U S A* **116**, 2282-2289 (2019).
2. K. Hayashi, M. Sasai, A. Iwasaki, Toll-like receptor 9 trafficking and signaling for type I interferons requires PIKfyve activity. *Int Immunol* **27**, 435-445 (2015).