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The Aryl Hydrocarbon Receptor Negatively Regulates HIV Replication in Th17/Th22 Cells

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Introduction: ART fails to restore the depletion of Th17-polarized CCR6+CD4+ T-cells in PLWH. Novel Th17-targeted HIV remission/cure strategies are needed to restore Th17-mediated mucosal immunity. Autoimmunity studies demonstrated the existence of pathogenic and non-pathogenic Th17 cells and identified the aryl hydrocarbon receptor (AhR) as a marker of non-pathogenic Th17 cells. AhR is a ligand-dependent transcription factor that regulates the expression of several genes (IL-22, IL-10, integrin β7) and is involved in proteasomal degradation via its E3 ubiquitin ligase activity. We hypothesized that AhR negatively regulates HIV replication in non-pathogenic Th17 cells

Methods: PBMC of ART-treated PLWH (n=8; median CD4 counts: 598, plasma viral load < 40 HIV-RNA copies/ml) and uninfected controls (n=5) were used in this study. Total/CCR6+/CCR6- memory CD4+ T-cells were isolated by magnetic/flow cytometry sorting. Cells of uninfected donors were stimulated via CD3/CD28, exposed to HIV, and cultured for 9 days. Viral outgrowth assay (VOA) was performed with cells of ART-treated PLWH. AhR silencing was performed using Si RNA and CRISPR/cas9, with efficacy evaluated by real-time PCR, T7 endonuclease assay and Western blotting. AhR agonist (FICZ) and antagonist (CH223191) were used. Cell viability/proliferation, HIV replication, cytokines, and gene expression were quantified by ELISA, flow cytometry and/or real-time PCR.

Results: AhR mRNA/protein expression was induced by T-cell receptor triggering. CRISPR/cas9-mediated AhR silencing significantly inhibited IL-22, IL-17A, IL-10 and integrin β7 expression ($p<0.01$) and increased viral replication upon infection in vitro (n=5; $p=0.0315$). Similarly, CH223191 significantly down regulated IL-22, IL-17A, IL-10, production ($p<0.0001$); increased wild type HIV replication ($p=0.008$), as well as HIV-DNA integration/transcription upon single-round infection with HIV-VSVG pseudotyped viruses (n=5; $p=0.001$); and increased >2-fold HIV reactivation in VOA (n=8). At the opposite, FICZ significantly increased IL-22 and IL-10 production and inhibited viral replication in vitro and reactivation in VOA.

Conclusion: Our results identify the AhR as a novel negative regulator of HIV replication in Th17/Th22-polarized cells thus raising the interest in testing natural/synthetic AhR agonists/antagonists for HIV remission/cure strategies.

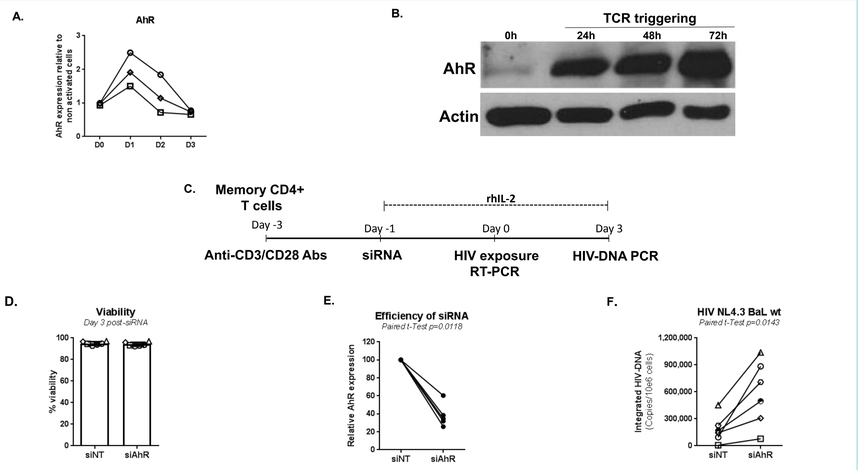


Figure 1: AhR interference increases HIV-1 replication in memory CD4+ T cells. Memory CD4+ T cells isolated from PBMCs of HIV-uninfected individuals were stimulated with anti-CD3/CD28 Abs. (**A**) AhR mRNA relative expression measured by RT-PCR; **(B)** AhR protein expression measured by Western Blotting before/after TCR triggering; **(C)** Experimental flow chart for siRNA and HIV infection. Briefly after 3 days of stimulation via CD3/CD28, cells were washed and nucleofected with non-targeting (NT) and AhR siRNA. Nucleofected cells were cultured in the presence of IL-2 for 24h and then exposed to wild type NL4.3BaL HIV for 3h. Infected cells were cultured in the presence of IL-2 for 3 days. Shown is **(D)** cell viability upon nucleofection monitored by Aqua vivid staining; **(E)** the yield of AhR silencing measured by RT-PCR and **(F)** levels of integrated HIV-DNA quantified by nested real-time PCR in cells nucleofected with NT and AhR siRNA. HIV-DNA copy numbers were normalized relative to CD3 expression. Results were generated with cells from 5 different donors. Each symbol represents 1 donor.

• AhR activation contributes to HIV latency and Th17/Th22 polarization in CD4+ T-cells

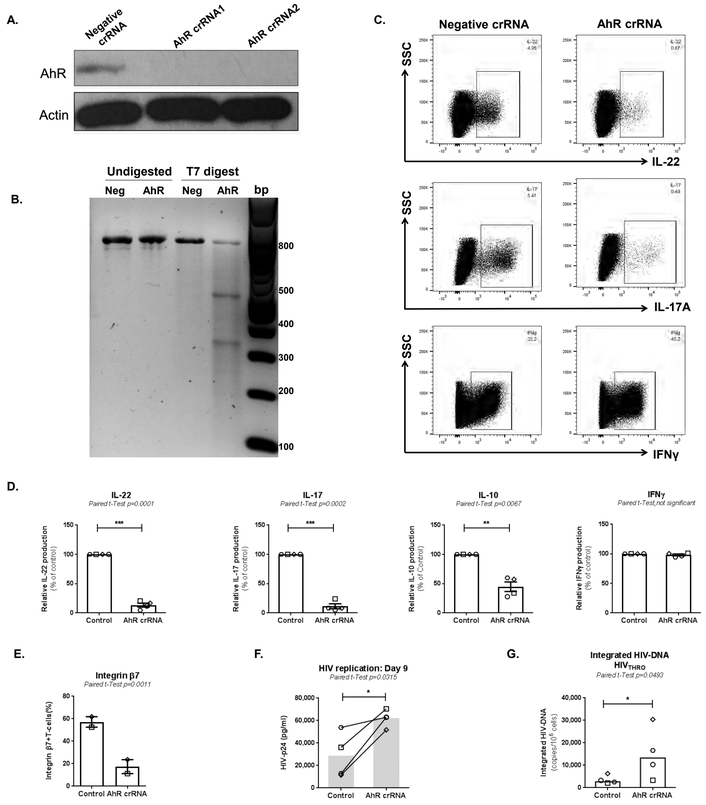


Figure 2: CRISPR/Cas9-mediated AhR silencing renders CD4+ T cells highly permissive to HIV infection. Memory CD4+ T cells isolated from PBMCs of HIV-uninfected individuals were stimulated with anti-CD3/CD28 Abs and electroporated with negative CRISPR (cr)RNA or with AhR-targeted crRNA. Four days after electroporation, cells were analyzed by **(A)**Western blotting for AhR protein expression and by **(B)** T7 endonuclease assay to validate the knock out at the protein and genetic levels respectively. At the same time, cells were exposed to transmitted founder (T/F) THRO HIV (50 ng/10e6 cells) and cultured up to 9 days in the presence of IL-2. Shown are **(C)** the expression of IL-22, IL-17A and IFNγ monitored by FACS in one representative individual, **(D)** levels of IL-22, IL-17, IL-10 and IFNγ production measured by ELISA, **(E)** integrin β7 expression monitored by FACS in 2 representative donor, **(F)** HIV-p24 levels measured by ELISA at day 9 post-infection as well as **(G)** levels of integrated HIV-DNA quantified by nested real-time PCR in cells nucleofected with negative and AhR crRNA. HIV-DNA copy numbers were normalized relative to CD3 expression. Results in **D, F, and G** were generated with cells from n=4 individuals.

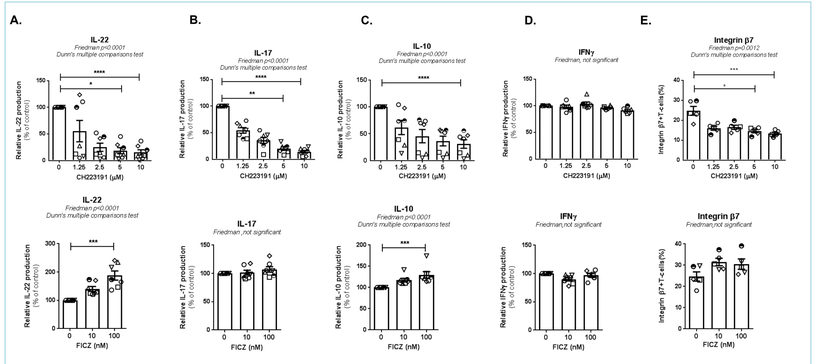


Figure 3: The AhR agonism/antagonism modulates cytokine and integrin β7 expression. (**A**) Memory CD4+ T cells were isolated and stimulated with anti-CD3/CD28 Abs in the presence or absence of the AhR antagonist CH223191 (1.25, 2.5,5 or 10 μM) or agonist FICZ (10 and 100 nM) for 3 days. Levels of **(A)** IL-22, **(B)** IL-17A, **(C)** IL-10 and **(D)** IFNγ were measured by ELISA in cell culture supernatants. Surface expression of **(E)** gut-homing molecule integrin β7 measured by FACS. Results were generated with cells from n=5-7 HIV-uninfected individuals. Each symbol represents 1 donor.

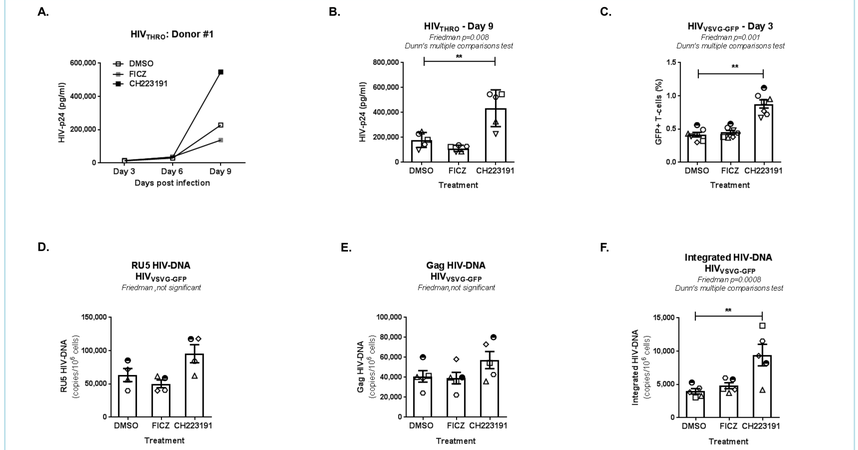


Figure 4:The AhR antagonism enhances HIV replication in CD4+ T cells. Memory CD4+ T cells were isolated and activated *via* CD3/CD28 and cultured in the presence or absence of the AhR antagonist CH223191 (10 μM) or agonist FICZ (100 nM) for 3 days. Cells were exposed to T/F THRO HIV (50 ng/10e6 cells) and further cultured for 9 days in the presence and absence of AhR drugs. Cells were also exposed to single-round VSV-G pseudotyped HIV encoding for GFP (50 ng/ml) and cultured for additional 3 days. Shown are **(A)**HIV-p24 levels quantified by ELISA in cell culture supernatants collected at days 3, 6, and 9 post-infection in n=1 donor, **(B)**statistical analysis of results obtained using n=5 donors; and the **(C)** frequency of HIV-GFP+ cells measured by FACS at day 3 post-infection.**(D-F)** RU5, Gag, and integrated HIV DNA levels quantified by real-time nested PCR at day 3 after infection. HIV-DNA copy numbers were normalized relative to CD3 expression. Results were generated with cells from 4-7 different donors. Each symbol represents one donor.

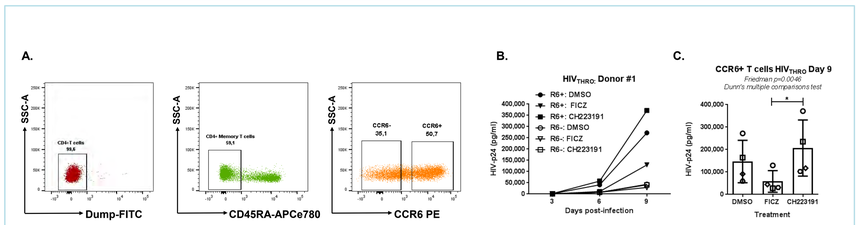


Figure 5: The AhR agonism/antagonism strongly modulate HIV replication in CCR6+ T cells. (A) Memory CCR6+ and CCR6–T cell subsets were sorted and stimulated via CD3/CD28 in presence/absence of AhR antagonist CH223191 (10 μM) or agonist FICZ (100 nM) for 3 days and exposed to T/F HIV THRO (50 ng HIV-p24/106 cells). Further, cells were cultured for additional 9 days in presence or absence of AhR drugs.**(B)** HIV-p24 levels quantified by ELISA in cell culture supernatant collected at day 3,6 and 9 post-infection (*n*=1) and **(C)** statistical analysis of results obtained using n=4 donors.

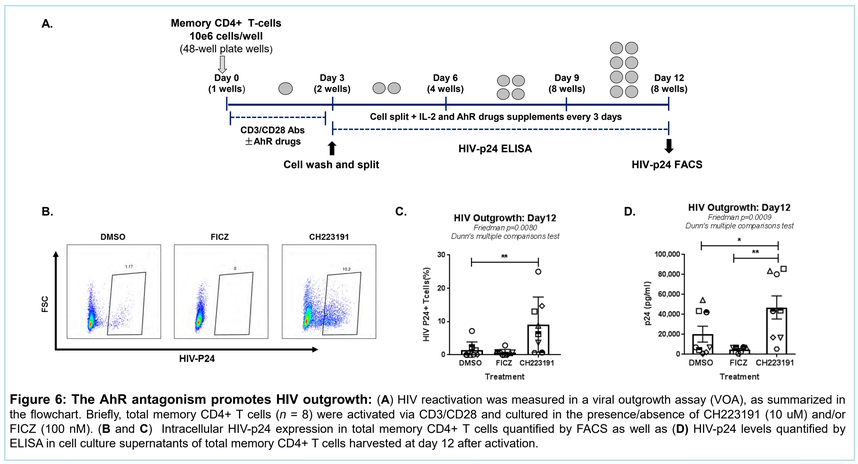


Figure 6: The AhR antagonism promotes HIV outgrowth: (**A**) HIV reactivation was measured in a viral outgrowth assay (VOA), as summarized in the flowchart. Briefly, total memory CD4+ T cells (*n* = 8) were activated via CD3/CD28 and cultured in the presence/absence of CH223191 (10 uM) and/or FICZ (100 nM). (**B** and **C**) Intracellular HIV-p24 expression in total memory CD4+ T cells quantified by FACS as well as (**D**) HIV-p24 levels quantified by ELISA in cell culture supernatants of total memory CD4+ T cells harvested at day 12 after activation.

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