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CARD8 SENSITIZATION THROUGH DPP9 INHIBITION ENHANCES KILLING OF HIV-INFECTED CELLS

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Introduction: We recently reported that non-nucleoside reverse transcriptase inhibitors (NNRTIs) can induce the caspase recruitment domain-containing protein 8 (CARD8) inflammasome in HIV-1 infected cells.[1] As seen in **Figure 1,** NNRTIs induce premature protease activation and subsequent cleavage of the N-terminus of autoprocessed CARD8, targeting it for N-terminal proteasomal degradation. The freed C-terminus can either induce the inflammasome – when high concentrations are present – or be inhibited by Dipeptidyl Peptidase 9 (DPP9).[2]

Due to the binding affinity of human serum proteins for NNRTIs, highly effective intracellular concentrations may not be attained *in vivo*.[3] This work shows that using the DPP9 inhibitor Val-boroPro (VbP) can increase killing efficiency of NNRTIs by inhibiting DPP9-mediated cell rescue.

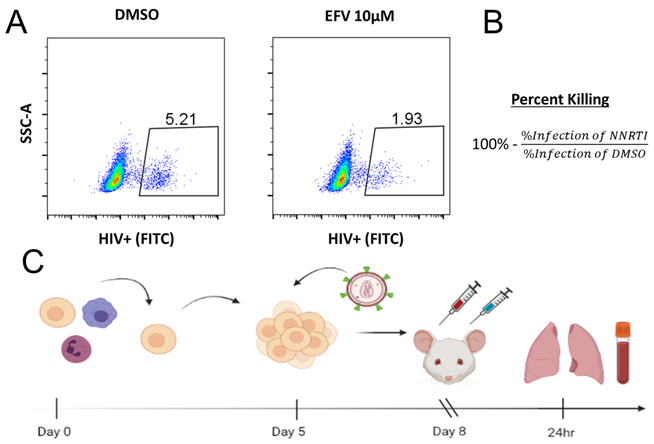

Figure 2: **A)** Representative gating of HIV infected CD4⁺ T cells treated with DMSO, or EFV. **B)** Calculation used to determine percent killing. **C)** Workflow of mouse experiments where CD4⁺ T cells were in vitro infected with a GFP reporter virus and transfused into mice prior to treatment with 60 µg VbP, 0.5 mg EFV, or combo.

Methods: For all in vitro killing assays three donors of primary CD4+ T cells were isolated, stimulated with αCD3/CD28 antibodies, and infected with pNL4-3-pol-Δenv-GFP reporter virus, or reporter virus with NNRTI resistance mutations introduced via PCR. Three days later, NNRTIs, VbP, or DMSO were added in regular media or media containing 50% human serum for two days prior to assessment of infection rate via flow cytometry as seen in **Figure 2A**. Percent killing was calculated as shown in **Figure 2B.** For mouse experiments primary CD4+ T cells were isolated and infected as above then $1x10^7$ cells were transfused into the MISTRG-6-15 mouse model and mice were treated for 24 hours prior to tissue and blood collection as seen in **Figure 2C.** Data analysis was done using Flowjo and GraphPad Prism.

1. Sharif, H. et al. Dipeptidyl peptidase 9 sets a threshold for CARD8 inflammasome formation by sequestering its active C-terminal fragment. Immunity (2021). doi:10.1016/j.immuni.2021.04.024
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4. Bednasz, C. J. et al. Efavirenz Therapeutic Range in HIV-1 Treatment-Naive Participants. Therapeutic Drug Monitoring 39, 596–603 (2017).
5. Aouri, M. et al. Population Pharmacokinetics and Pharmacogenetics Analysis of Rilpivirine in HIV-1-Infected Individuals. Antimicrobial Agents and Chemotherapy 61, (2017).

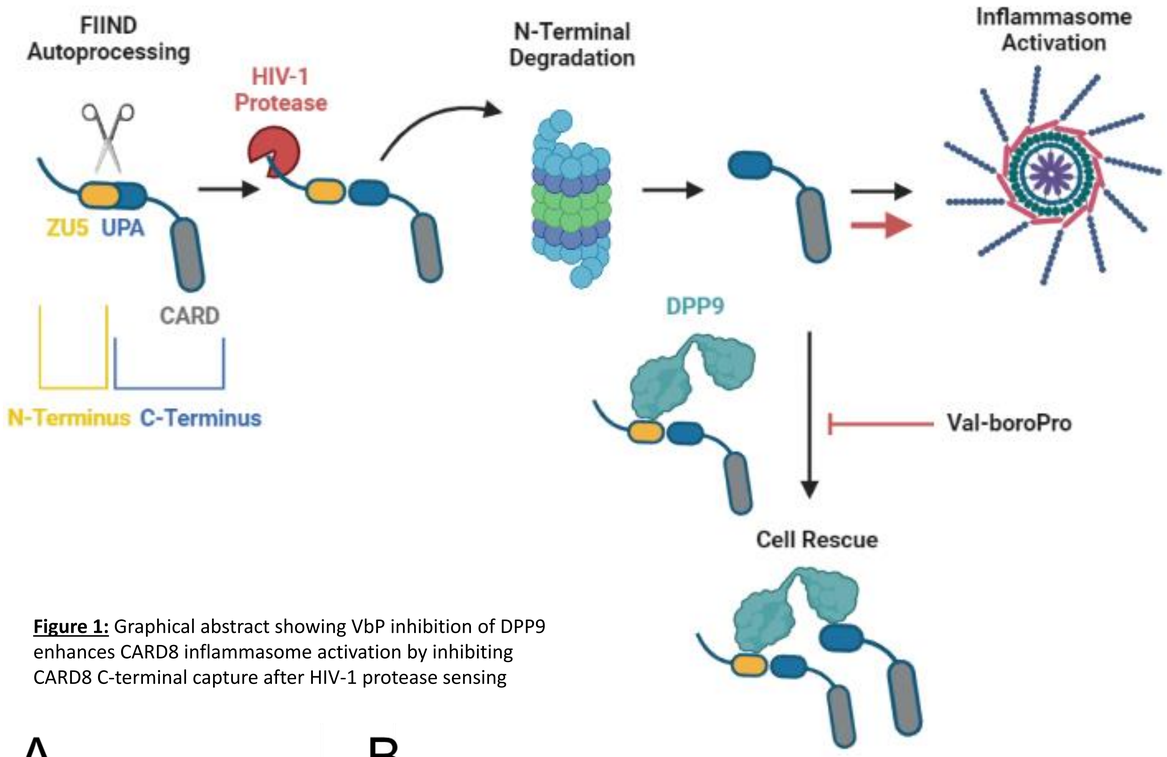

Figure 1: Graphical abstract showing VbP inhibition of DPP9 enhances CARD8 inflammasome activation by inhibiting CARD8 C-terminal capture after HIV-1 protease sensing

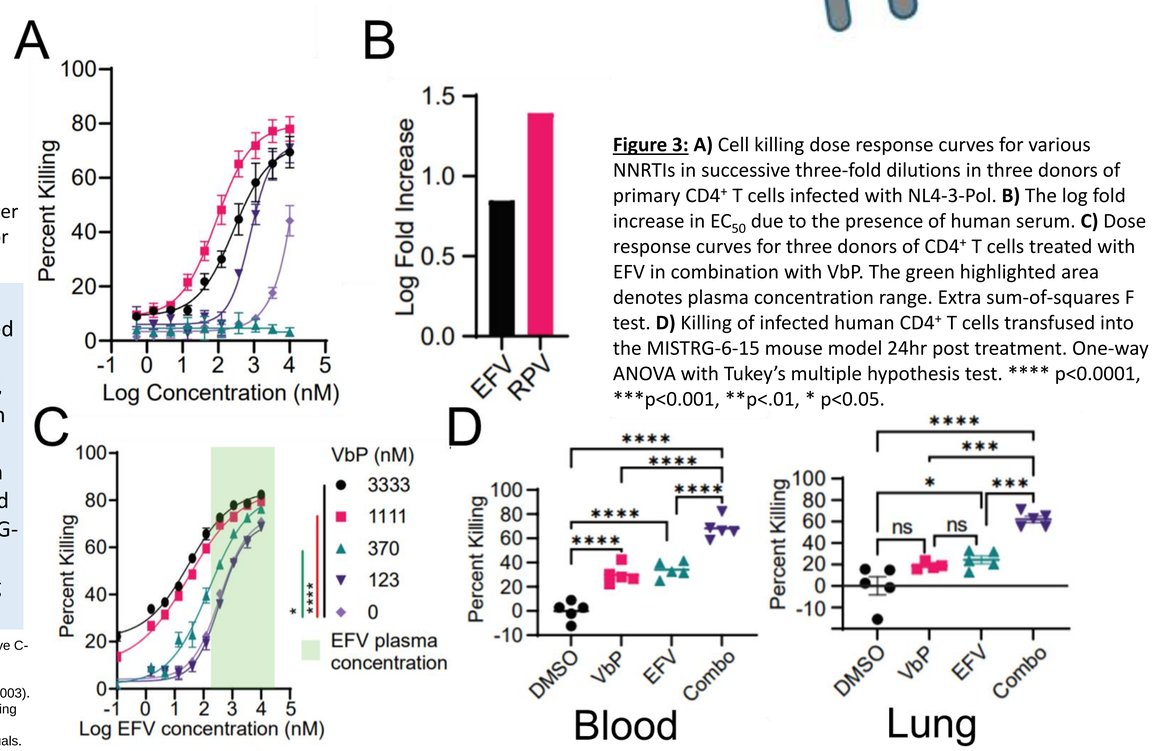

Figure 3: **A)** Cell killing dose response curves for various NNRTIs in successive three-fold dilutions in three donors of primary CD4⁺ T cells infected with NL4-3-Pol. **B)** The log fold increase in EC_{50} due to the presence of human serum. **C)** Dose response curves for three donors of CD4⁺ T cells treated with EFV in combination with VbP. The green highlighted area denotes plasma concentration range. Extra sum-of-squares F test. **D)** Killing of infected human CD4⁺ T cells transfused into the MISTRG-6-15 mouse model 24hr post treatment. One-way ANOVA with Tukey's multiple hypothesis test. **** p<0.0001, ***p<0.001, **p<.01, * p<0.05.

Results: To understand the potential utility of using NNRTIs as a "shock and kill strategy" we first assessed the killing efficiency at clinically relevant concentrations (**Figure 3A**).[4,5] Due to NNRTIs known binding affinity for human serum proteins, we also determined the effect that human serum had on NNRTI induced killing (**Figure 3B**). The steep reduction in killing efficiency highlights the need for an enhancement of NNRTI induction of pyroptosis. Utilizing the DPP9 inhibitor VbP, we were able to show that blocking DPP9's ability to inhibit the CARD8 inflammasome increases its sensitivity to triggers like HIV-1 protease (**Figure 3C**). We then tested this strategy *in vivo* and showed that EFV or VbP alone were able to kill HIV infected cells which was greatly improved with combination treatment as evidenced in blood and lung tissues. Additionally, we show that VbP sensitization can help ameliorate reductions in NNRTI efficacy due to resistance mutations (**Figure 4).**

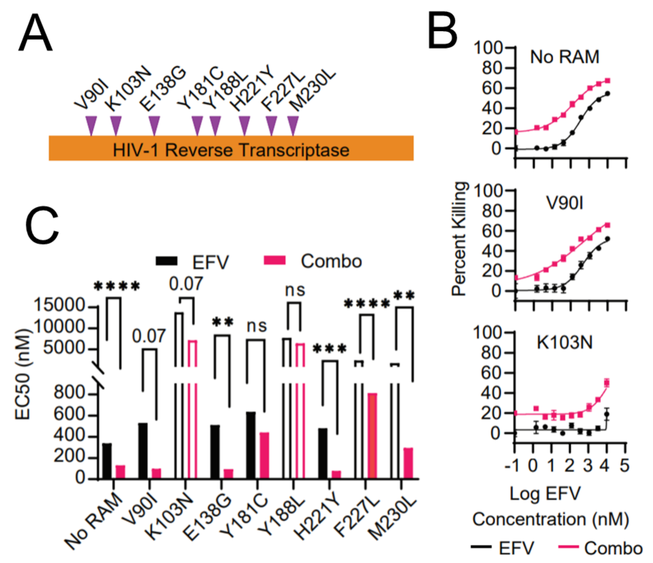

Figure 4: **A)** Graphical depiction of the location of NNRTI RAMS. **B)** Dose response curves for the selected NNRTI RAMs in one donor of CD4⁺ T cells treated with serial three-fold EFV dilutions with (red) or without (black) the presence of 1µM of VbP**. C)** EC_{50} values are shown for all viruses with or without the presence of VbP. Significance was calculated using extra sum-of-squares F test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Conclusions: Since NNRTIs offer a promising strategy for eradication of HIV-1 latent reservoirs, improving their in vivo cell killing potency is essential to treatment efficacy. This study proves that sensitization of the CARD8 inflammasome through DPP9 inhibition, *in vitro* and *in vivo*, can reduce the threshold of CARD8 activation and provide more effective clearance of HIV infected cells for clinically relevant scenarios. We also show that VbP can reduce the negative effects on killing due to NNRTI resistance mutations and bring the killing efficacy back to comparable levels as the control. Additionally, DPP9 inhibition through chemical means such as with VbP can induce targeted cell killing even without NNRTIs.