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Canonical Wnts mediate CD8+ T cell non-cytolytic anti-HIV-1 activity and correlate with HIV-1 clinical status

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Abstract

CD8+ T cells do not rely solely on cytotoxic functions for significant HIV control. Moreover, the non-cytotoxic CD8+ T cell antiviral response is a primary mediator of natural HIV control such as that seen in HIV elite controllers (ECs) and long term non-progressors (LTNPs) that does not require combined antiretroviral therapy (cART). In this study we investigated the biological factors contributing to the non-cytotoxic control of HIV replication mediated by primary human CD8+ T cells. We report that canonical Wnt signaling inhibits HIV transcription in an MHC independent, non-cytotoxic manner, and that mediators of this pathway correlates with HIV controller clinical status. We show that CD8+ T cells express all 19 Wnts and CD8+ T cell conditioned media (CM) induced canonical Wnt signaling in infected recipient cells while simultaneously inhibiting HIV transcription. Antagonizing canonical Wnt activity in CD8+ T cell CM resulted in increased HIV transcription in infected cells. Further, Wnt2b expression was upregulated in HIV controllers vs. viremic patients, and in vitro depletion of Wnt2b and/or Wnt9b from CD8+ CM reversed HIV inhibitory activity. Lastly, plasma concentration of Dkk-1, an antagonist of canonical Wnt signaling, was higher in viremic patients with lower CD4 counts. This study demonstrates that canonical Wnt signaling inhibits HIV and significantly correlates with HIV controller status.

Introduction:

A small percentage of HIV+ patients exhibit delayed progression or do not progress to AIDS, maintaining high CD4+ T cell counts for several years to decades without cART. These individuals include long term non-progressors (LTNPs); defined as having a CD4+ T-cell count higher than 500/μL for many years despite plasma viremia, and elite controllers (ECs), which are <1% of the HIV+ population, and who maintain undetectable plasma HIV RNA without antiretroviral therapy (1–4). Uncovering a common mechanism of viral control among HIV controllers could be crucial in developing a therapeutic vaccine, and/or a

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functional cure that generates long-term viral suppression. To date, viral control displayed by LTNPs and ECs have been attributed to a multitude of antiviral responses, some of which are due to patient specific genetic features (5–9). However, numerous studies have uncovered commonalities among LTNPs and ECs, particularly in the anti-viral response of CD8+ T cells (10–14). Walker *et. al.* described a phenomenon where CD8+ T cells mediate a non-cytotoxic inhibition of HIV replication (10). Specifically, CD8+ T cells in HIV controllers; including LTNPs, ECs, and HIV+ patients prior to progression to AIDS, secrete soluble mediator(s) which interact with infected CD4+ cells, initiating a signaling cascade that culminates in the inhibition of HIV transcription (10, 15–18). This factor was termed the CD8+ T cell antiviral factor (CAF). While CAF activity has been documented by a number of groups (10, 19–27), its identity has remained elusive for over 30 years. Numerous factors including regulated-on-activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β (28), macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), I-309, angiogenin, RNase4 (29), prothymosin alpha (ProTα) (30), lymphotactin (31), α-defensins (32), and TOE1 (33) were thought to be CAF, yet these factors either did not share CAF's mechanism of action of inhibiting HIV at the transcriptional level or were not secreted by CD8+ T cells (34, 35). RANTES, MIP-1α, and MIP-1β inhibit CCR5 mediated HIV entry (28), and have been argued to be contributors to CAF activity though they have not been shown to inhibit HIV transcription it has been argued that CAF is a collection of antiviral factors with various mechanism of viral inhibition that is highly expressed in acute infection, LTNPs and ECs (36).

Other factors, namely MDC, TARC, I-309, angiogenin, and RNase 4 inhibit CXCR4 mediated viral entry (29). ProTα inhibits HIV infection in macrophages but not in CD4+ T cells (30) via induction of type I interferon (37). The chemokine lymphotactin, also secreted by CD8+ T cells, blocks HIV attachment and entry into host cells (31). α-defensins inhibit HIV transcription and were initially thought to be a component of CAF, but were later found to be secreted by neutrophils and monocytes but not CD8+ T cells and also were found to inhibit HIV via a mechanism distinct from that of CAF (34, 38). More recently the protein Target Of Egr1 (TOE1), which is secreted from CD8+ T cells, was described as an inhibitor of HIV transcription (33), however, CAF's processing and mechanism of action was determined to be different than that of TOE1 (35, 39–41).

β-catenin, a transcriptional co-regulator, inhibits HIV transcription in multiple target cells including peripheral blood mononuclear cells (PBMCs) and astrocytes (42–47). β-catenin is primarily regulated by Wnts which are a family of 19 small secreted glycoproteins that can interact with a family of 10 frizzled receptors and numerous co-receptors to regulate cellular development, survival and function contingent on the Wnt-frizzled receptor combination, cell type, or biological status (48–50). Pertaining to transcriptional inhibition of HIV, nuclear β-catenin is a part of a multiprotein transcriptional complex consisting of the nuclear matrix protein scaffold/matrix-associated region I (SMARI) and T-cell factor 4 (TCF-4). This complex pulls the DNA into the nuclear matrix and away from the transcriptional machinery interrupting RNA polymerase II (RNAPII) docking on the HIV LTR resulting in HIV transcriptional inhibition(45). Notably, CAF was also recently described to reduce the association of RNAPII with the HIV promoter (51).

Several functional and biological similarities exist between CAF and Wnts, namely they: 1) Inhibit HIV transcription by reducing the association of RNAPII with the HIV promoter (45, 51), 2) Are small proteins less than 50kDa that are heat stable for extended periods of time, 3) Are secreted by CD8+ T cells independent of MHC restriction, and 4) Exert non-cytotoxic effects on their signaling targets, which in this case are CD4+ HIV infected cells. Given these similarities, we evaluated here whether CD8 conditioned media from healthy donors infected in vitro would inhibit HIV transcription i.e. demonstrate CAF activity, whether canonical Wnts contribute to this antiviral activity, and whether canonical Wnts will positively correlate with HIV natural controller status (LTNP/EC). Indeed, we found that robust canonical Wnt signaling is a marker of natural HIV controller status and that inhibition of canonical signaling is associated with high level viremia, low CD4 count, and loss of CD8 CM mediated inhibition of HIV transcription. These studies have significant implication for exploiting canonical Wnt signaling as a potential therapeutic for HIV/AIDS, and/or a pathway to manipulate in functional cure efforts to achieve long-term viral suppression.

Methods

Ethics statement:

Research involving human subjects was conducted in accordance with institutional (IRBL06080703) and U.S. government guidelines on human research.

Human blood samples:

Blood was drawn both from healthy HIV sero-negative donors and HIV positive patients visiting the Mark Weiss Memorial Clinic for Infectious Diseases (Rush University Medical Center) and the Ruth M Rothstein CORE Center. Informed consents were signed by each participant prior to blood draw, and this study was approved by the Institutional Review Board of Rush University Medical Center and the Cook County Health and Hospitals System. In our cohort, HIV positive patients included 1) ECs who have had undetectable viral loads (<20, <40 or <75 copies/ml depending on assay) for 4 to 26 years with no antiretroviral therapy; they also had CD4+ T-cell counts higher than 500/μL. 2) Persons on therapy with undetectable viral loads, 3) Persons on therapy with detectable viral loads, and 4) Treatment naïve patients with detectable viral load. CD4+ T cell count and viral load were assessed for HIV+ donors on the day of the blood draw. For this application and for the results that follow ECs comprise the controller group (c), patients with detectable viral load comprise the viremic group (v), and patients that received therapy comprise the treated group (t).

PBMCs were isolated using ficoll hypaque density gradient centrifugation. Briefly, whole blood was diluted with DPBS (Corning, Manassas, VA, USA), and layered on top of lymphocyte separation medium (Corning, Manassas, VA, USA). The tube was then spun for 20 to 30 minutes without brake, followed by removal of the buffy layer to obtain PBMCs. CD8+ T cell isolation was performed using a negative magnetic isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany Cat#:130-096-495). CD8+ T cells and CD8+ T cell-depleted PBMCs (referred to as recipient cells) were cultured in complete RPMI media

supplemented with 10% heat inactivated fetal bovine serum (Gemini Bio-Products, CA, USA) 1% penicillin-streptomycin (Gibco Waltham, MA, USA), and 20U/ml IL-2 (NIH AIDS Reagent Program) in a 5% CO2 humidified atmosphere at 37°C. CD8+ T cells, CD4+ T cells and CD8 depleted PBMCs were activated with either soluble anti-CD3/anti-CD28 antibodies (BD Biosciences, Franklin Lakes, NJ) or anti-CD3/anti-CD28 beads (Life Technologies, Carlsbad, CA) for three days prior to usage. After activation, cells were spun down and the supernatant collected and either used immediately to treat HIV+ CD8 depleted PBMCs, or aliquoted and frozen at −80°C.

HIV infection:

CD8-depleted PBMCs from HIV sero-negative donors were infected with HIV clone BaL.26 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Mascola, Cat# 11446) at 2ng/1 million cells for 1hr to overnight depending on the experiment, washed extensively and incubated with CD8+ T cell conditioned media (CD8 CM), CD4 conditioned media (CD4 CM), recipient cell or CD8 depleted PBMC conditioned media (CD8 dep CM); collected prior to infection of the CD8 depleted PBMCs, or cRPMI for up to 6 days.

Quantitative real-time RT-PCR:

Total RNA was extracted using the RNeasy mini prep kit (Qiagen, Hilden, Germany), followed by incubation with DNase (Sigma-Aldrich) and retro-transcription (cDNA qScript, Quantabio, Beverly, MA, USA). Quantitative real-time RT PCR (q-RT-PCR) was performed using Ssofast Evagreen Supermix with Low ROX (Bio-Rad, Hercules, CA) in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using SDS software v2.0.1. Melting curve analysis was performed to ensure the amplification of a single product. The change in binding was calculated by relative quantification using the comparative threshold cycle (CT) method, with results reported both as fold change relative to control (ΔCT = CT Target – CT control; ΔΔCt = ΔCT Target – ΔCT experimental control; fold change relative to control = 2^−ΔΔCt) or ΔCT. Primers were designed using IDT (San Diego, CA, USA) PrimerQuest design software.

Alu PCR:

Seventy two hours post infection with $HIV_{Ba\text{-}L}$ (2ng/1 million cells) cells were collected and harvested for genomic DNA (Qiagen, Hilden, Germany) and checked for the presence of HIV DNA by PCR for LTR region using primers; F: 5’-TCA AGT GAG TGC CCG GTT-3’ and R: 5’-AGC TCC GGT TTC TCT TTC GCT −3’, with GAPDH primers GAPDH F: 5’-TGA CTT CAA CAG CGA CAC CCA CT-3’ and GAPDH R: 5’-ACC ACC CTG TTG CTG TAG CCA AAT-3’ as control. To quantify percentage of HIV infected CD8+ T cell depleted PBMCs harboring integrated HIV DNA, we employed Alu-PCR. In brief, genomic DNA was isolated from infected cells, quantified by UV spectrophotometry and by real time PCR of GAPDH DNA. Using similar a0mounts of gDNA, the first round Alu-PCR was performed with Alu1, Alu2 and LM667 primers, as described (52). AmpliTaq gold polymerase with GeneAmp 10X PCR buffer II was used according to manufacturer’s instructions (Applied Biosystems Foster City, CA). Reaction conditions were: hold-95× C for 10min; 12 cycles of 95 ×C for 30sec, 60 ×C for 20sec and 72 ×C for 170sec followed by

95 ×C hold for 10min. The products were run through a column PCR purification kit (Qiagen) to remove Taq, primers and dNTPs. Using 1/50 of this sample, the second-round nested real time PCR was performed employing above mentioned HIV LTR primers that amplify ~140bp LTR region. A minus Taq control and a minus template control were included throughout.

TZM-bl Assay:

HIV infectivity was measured using a luciferase reporter gene assay in TZM-bl cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (180–184). TZM-bl cells were plated at a density of 5000 to 7000 cells/100 μl in flat bottom 96 well tissue culture plate overnight. Twenty μl or 50 μl of experimental supernatant was added to the TZM-bl cells for an additional 24hrs, followed by collection and lysis of the TZM-bl cells. Cell lysates were combined with luciferase substrate (Promega, Madison WI) and luciferase activity was quantified as relative luminescence units (RLUs).

Depletion of Wnts in CD8+ T cell conditioned media:

Primary Wnt2b or Wnt9b antibody (Abcam, Cambridge, United Kingdom) was incubated with protein A/G magnetic beads (Thermo Fisher, Waltham, MA) for 1hr at 4°C in binding buffer. After incubation, the binding buffer was replaced with CD8 CM and further incubated for 2hrs at 4°C. The beads were then removed and the Wnt depleted CD8 CM was either incubated with HIV+ PBMCs-CD8, or frozen at −80°C.

Statistics:

All statistical analyses were either conducted or corroborated by the Rush Bioinformatics and Biostatistics Core. When the data were distributed normally, student t-test and ANOVA with post-hoc tests were used (using GraphPad Prism 5 Software (San Diego, CA)). When the data were not normally distributed, nonparametric analysis was performed. All tests assumed a two-sided significance level of 0.05.

Results:

A soluble factor secreted from CD8+ T cells inhibits HIV infection:

To verify that CD8+ T cells secrete a soluble factor(s) that inhibits HIV transcription, CD8+ T cell conditioned media (CD8 CM) from HIV seronegative donors or complete RPMI culture media (control) were added to HIV-infected CD8-depleted PBMCs (recipient cells). At day 6, HIV transcription and replication were measured by qRT-PCR for HIV *rev* and *nef* and TZM-bl assay to measure secreted infectious viral particles. HIV *rev* and *nef* transcripts were reduced by approximately 80% by CD8 CM (Fig 1A), while CD8-depleted PBMC CM or CD4+ T cell CM did not inhibit HIV *nef* transcription (Fig 1B, C). Finally, CD8 CM inhibited infectious HIV particles by greater than 2-fold (Fig 1D). These data verify that CD8 CM from healthy HIV seronegative donors secrete an MHC independent, anti-HIV factor(s) that inhibits HIV transcription and replication, and that this soluble factor(s) is not present in the CM of CD8-depleted PBMCs or CD4+ T cells.

CD8+ T cells express canonical Wnts:

To determine whether CD8 CM induces canonical Wnt signaling in recipient cells, we measured the transcription of *β-catenin* and two downstream targets of β-catenin; *axin2* and *cyclinD1*. *β-catenin*, *axin2* and *cyclinD1* were induced in the infected recipient cells by approximately 3-, 4- and 5- folds, respectively, in the presence of CD8 CM compared to control (Fig 2A), suggesting the presence of canonical Wnts in CD8 CM. Therefore, to determine whether CD8+ T cells are a source of canonical Wnts, we assessed Wnt mRNA expression in activated CD8+ T cells from healthy donors. CD8+ T cells expressed all 19 Wnt transcripts, with notable donor variability in their levels of expression (Fig. 2B). Together, these data indicate that CD8+ T cells express Wnts, which can mediate canonical Wnt signaling in HIV infected recipient cells.

Antagonizing canonical Wnts abrogates CD8 CM inhibitory activity:

The findings that 1) CD8 CM inhibited HIV transcription and simultaneously induced β-catenin expression in infected recipient cells (CD8 depleted PBMCs) and 2) CD8+ T cells produce canonical Wnts, suggests a relationship between upregulation of the canonical Wnt/β-catenin signaling pathway and inhibition of HIV transcription in infected cells. Given that there are no commercially available antibodies to neutralize Wnt activity, we used Dkk-1 to antagonize canonical Wnt signaling and assess its impact on CD8 CM activity. Dkk-1 competitively antagonizes canonical Wnts by binding to the low density lipoprotein 5/6 (LRP5/6) co-receptor resulting in the degradation of β-catenin (53–55). We treated HIV infected recipient cells with recombinant human Dkk-1 (rhDkk-1) during incubation with CD8 CM and found that Dkk-1 abrogated CD8 CM mediated HIV inhibition (Fig. 3A). Dkk-1 also increased baseline HIV transcription in non-treated recipient cells (Fig 3B). Likewise, the small molecule β-catenin inhibitor ADV also reversed CD8 CM inhibitory activity, and increased baseline HIV transcription in untreated recipient cells (Fig 3C, D). This observation is consistent with previously published reports that inhibition of β-catenin or Dkk-1 itself inhibit endogenous expression of Wnt/β-catenin leading to higher level of HIV replication (42, 56). Further, these data demonstrate that canonical Wnts secreted from CD8+ T cells inhibit HIV transcription and that this inhibition is reversed by antagonizing β-catenin activity in infected cells.

Wnt1 inhibits HIV transcription but not reverse transcription or integration:

To confirm that the presence of canonical Wnts in CD8 CM inhibits HIV transcription rather than reverse transcription or integration, we incubated infected cells with recombinant human Wnt1 protein (rhWnt1). Wnt1 induces β-catenin (57–59), and, is commercially available as an active recombinant human protein, unlike the majority of Wnts. We first assessed whether rhWnt1 would inhibit HIV transcription. Indeed, RhWnt1 significantly inhibited transcription of *nef* (2-fold decrease) (Fig. 4A), and simultaneously induced transcription of downstream β-catenin targets *axin2* and *cyclind1* by X- and X-fold, respectively (Fig. 4D). Neither reverse transcription (for *LTR* and *nef*), nor integration were inhibited by rhWnt1, as measured by quantification of *LTR* and *nef* from isolated DNA and Alu PCR, respectively (Fig. 4B, C). Together, these findings demonstrate that canonical Wnt

signaling, in this case mediated by rhWnt1, inhibit HIV transcription and not reverse transcription or integration.

Canonical Wnts are associated with HIV controller status:

To assess whether Wnts are associated with HIV controller status and clinical markers, we collected blood from a cohort of HIV controllers, as well as HIV+ patients on therapy with controlled viremia, and naïve or non-compliant patients with detectable viral load (VL) and low CD4 counts. Within the cohort, CD4 count ranged from 129 to 1598 count/ml, while viral load ranged from below assay limit of detection to 81,972 HIV RNA copies/ml (Table 1). We isolated CD8+ T cells from the PBMCs of HIV infected patients and measured the transcriptional profile of all 19 Wnts, which we then compared to CD4 count, CD4% and viral load. Of the 19 Wnts, increasing expression of Wnts2b, 3a, 9b, 10b and a trend in increase in Wnt8b was associated with a lower VL (Fig. 5A). Additionally, when patients were grouped by HIV status, i.e. controller vs viremic, there was significantly higher expression of Wnt2b in the controller group (Fig. 5B). Finally, Wnt2b expression was inversely proportional to CD4 percentage, which was lower in viremic patients (Fig. 5C). These data demonstrate that expression of Wnts2b, 3a, 9b and 10b which all signal through canonical β-catenin (60–65) are upregulated in HIV controllers.

Wnts2b and 9b depletion abrogate CD8 CM activity in a donor dependent manner:

From the Wnts identified to correlate with viral load (Fig 5A), we observed a positive correlation between Wnts 2b and 9b (Fig 6A). To assess whether the absence of these canonical Wnts would affect CD8 CM activity, we depleted either Wnt2b or Wnt9b in CD8 CM from seronegative donors via antibody mediated pulldown, followed by incubating the Wnt depleted CD8 CM with HIV infected recipient cells (Fig 6B). Donor 1 and donor 5 showed reversal in CD8 CM activity when Wnt2b was depleted, whereas donor 2 and donor 4 showed reversal in CD8 CM activity following depletion of Wnt9b. CD8 CM activity in donor 3 was reversed following depletion of either Wnt2b or 9b. These observations demonstrate that both Wnts2b and 9b can inhibit HIV transcription, and further suggests inhibition of HIV transcription can be mediated by different canonical Wnt ligands depending on the donor.

Canonical Wnt signaling antagonist Dkk-1 is associated with worsened HIV status:

Given that Dkk-1, an antagonist of canonical Wnt signaling detectable in patient plasma, inhibited CD8 CM activity in vitro (Fig. 3), and CD8 CM mediated HIV inhibition is relatively low to absent in patients with high VL and who progress to AIDS (66), we investigated whether Dkk-1 would be associated with a worsened HIV disease state. We measured plasma Dkk-1 concentration from HIV+ donors and compared the results to both VL and CD4 count. We observed a negative correlation between plasma Dkk-1 concentration and CD4 count (Spearman $r = -0.5106$, $p=0.018$) (Fig. 7A). Additionally, Dkk-1 concentration was approximately 2 times higher in viremic patients relative to patients with low to undetectable VL (Fig. 7B). These data demonstrate that inhibition of canonical Wnt signaling is associated with a worsened HIV status.

Discussion:

We demonstrated here that Wnts, specifically, Wnt2b, 3a, 8b, 9b and 10b are associated with a lower VL, and that antagonizing canonical Wnt signaling abrogates CD8 CM mediated inhibition of HIV transcription. Further, Wnt2b is associated with HIV controller status and CD4 percentage. These findings are reminiscent of components of CAF that control HIV infection via inhibition of LTR driven transcription. The identity of CAF has been controversial for over thirty years (10), but several studies have confirmed the existence of anti-HIV factors reminiscent of this unknown protein(s), including its association with disease progression and HIV status (15, 16, 18, 19, 22, 23, 66, 67). Importantly, CAF has been proposed to be a collection of antiviral cytokines and chemokines that inhibit HIV at various stages of the viral life cycle (36), and we argue that Wnts are a component of the CAF antiviral response. Expression of CAF is an indicator of a strong anti-HIV response, and defining CAF identity will inform current therapy, vaccine and cure efforts.

Several biological characteristics of Wnts may have precluded their earlier identification as CAF. Wnts are secreted palmitoylated glycoproteins that are hydrophobic in nature and thought to utilize secretory biological aids to signal to their recipient cells. Specifically, Wnts have been reported to rely on biological carriers such as exosomes (68), secreted wingless-interacting molecule (Swim) (69), and secreted frizzled related proteins (SFRPs) (69–71). SFRPs can also inhibit Wnt signaling (72–75). Additionally, some Wnts such as Wnts3a and 5a complex with afamin, a member of the albumin family of proteins and transporter of hydrophobic molecules (76–78). Both the potential association of Wnts with albumin in cell culture along with the similar molecular weight of both Wnts and albumin are obstacles in measuring Wnt expression in cell culture media via western blot or similar immunology techniques, as the relatively higher abundance of albumin may overshadow detection of secreted low abundance Wnts. These complexities in Wnt secretion may have also eluded extensive efforts to identify CAF. We also showed that while all donors evaluated express Wnts, there is considerable donor variability in the Wnt expression profile. This donor variability suggests that different Wnts may drive the suppression of HIV/ mediate CAF activity. Presumably, donor variability in canonical Wnt signaling has also contributed to the inability to identify CAF to date. Further, given that there is considerable redundancy in Wnts capable of activating β-catenin, there could be donor variability regarding which Wnt is inducing β-catenin mediated suppression of HIV transcription. Indeed, we found here that some donors lost CAF activity upon depletion of Wnt2b while others lost CAF activity when Wnt9b or both were depleted. Further, given that Wnts are secreted by a wide variety of cells, it may be that this CAF activity is not specific to CD8 T cells and it can be exerted by cells that secrete Wnts. It is also important to note that Wnts have other immune-regulatory functions, including regulation of T cell differentiation, phenotype and function. Wnts have been shown to promote central memory CD8+ T cell phenotype while inhibiting effector cell phenotype (79). In CD4 T cells, Wnt signaling has been shown to preferentially skew differentiation towards a Th2 phenotype (79), and canonical Wnt signaling has been shown to maintain CD4 expression on T cells (80). This multi-functionality of Wnts, however, does not negate its importance in the anti-HIV

response and might even suggests that Wnt signaling may be involved in generating or maintaining the phenotype of CD8+ T cells with CAF activity.

Due to the lack of commercially available neutralizing antibodies for Wnts, we used antibody-pull down to remove them from CD8 CM, and used Dkk-1 to compete with canonical Wnts for binding to LRP5/6 and as such antagonizing their activity. Repeated efforts to use loss of function studies that would target Wnts2b and 9b such as siRNA, lentiviral transduction or CRISPR Cas9 failed to yield high enough knockdown or knockout efficiencies in primary human CD8+ T cells, without significant cell death, that would allow for use of these strategies to further assess the link between Wnts and CAF activity. Nonetheless, the two strategies employed in our studies (Wnts depletion via pull down and antagonism through use of Dkk-1), as well as inhibition with the small molecule ADV, demonstrate that CAF activity is mediated by members of the canonical Wnt family.

CAF is maintained in HIV controllers and is lost in patients progressing to AIDS (66). Our observations demonstrate that Wnts, specifically Wnts2b, 3a, 8b, 9b and 10b are also associated with HIV controller status when compared to patients with high VL and low CD4 count. Interestingly, both Wnts2b and 9b exacerbate Sendai virus via canonical signaling (62) by inhibiting expression of the *IFNB1*, *IFIT1* and *TNF* genes. Also, it has been suggested prior that some key genes enriched in LTNPs are members of the Wnt signaling pathway(81).

Wnts 2b and 9b canonical activity are susceptible to Dkk-1 inhibition and indeed the presence of Dkk-1 resulted in higher levels of viral transcription, suggesting that Dkk-1 may exacerbate HIV infection. Our lab has demonstrated that interferon gamma (IFN-γ) enhances HIV infection, albeit in astrocytes, via STAT-3 mediated increase in Dkk-1 and subsequent decrease in β-catenin (56). Recent findings also implicate Dkk-1 as a biomarker of HIV induced neurocognitive impairment (NCI), where a higher Dkk-1 level was associated with NCI among HIV+ patients with suppressed plasma VL (82).

CD8+ T cells secrete a number of anti-HIV factors, while those factors contribute to HIV inhibition; they are not the originally described “CAF” as such because they do not share CAF’s mechanism of action, which is inhibiting HIV at the transcriptional level. It was also unclear if CAF is a single factor or a collection of factors that together inhibit HIV transcription. Our findings here demonstrate that canonical Wnts are responsible, at least in part, for the elusive CAF. While there is potentially significant heterogeneity in Wnts driving CAF activity, understanding this heterogeneity will help to further identify innate factors that lead to various clinical outcomes/statuses, particularly those that mediate LTNP and EC status. Lastly, given the recent challenges with the “shock and kill” strategy towards an HIV cure, engaging Wnts as mediators of long-term inhibitors of HIV transcription could inform novel therapeutic modalities for anti-HIV treatment including vaccine and functional cure strategies.

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Key points

- CD8+ T cells release Wnts that suppress HIV transcription
- Antagonizing Wnts abrogates CD8+ T cell non-cytotoxic anti-HIV activity
- Wnts correlate with HIV controller status.

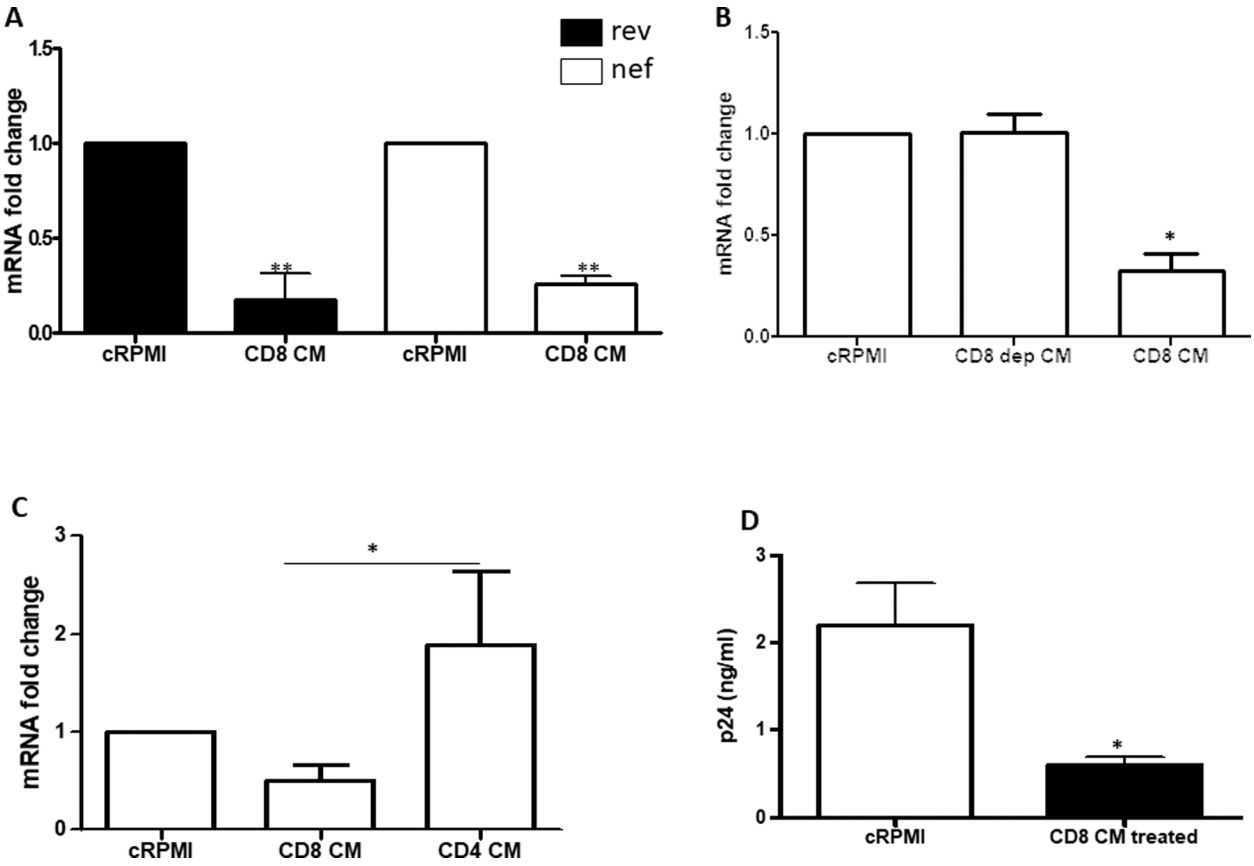

Fig. 1. A soluble factor secreted from CD8+ T cells inhibits HIV infection

(A) HIV infected CD8 depleted PBMCs (recipient cells) were activated with anti-CD3 and anti-CD28 antibodies and cultured in CD8 CM, (**B**) activated CD8 depleted PBMC conditioned media (**C**) activated CD4+ T cell conditioned media or cRPMI for 6 days. At day 6, the cells were collected and lysed for total RNA isolation followed by reverse transcription and qRT-PCR for HIV transcripts *rev*, *nef* or *gag*. (n=4, **p<0.01, *p<0.05 ANOVA, mean ± SEM). **(D)** Supernatant from HIV infected cells treated with CD8 CM or cRPMI for 6 days was collected and added to TZM-bl cells for 24 hours, the cells were then lysed and exposed to a luminescent β-gal substrate, data output was recorded in RLUs and converted to ng/ml by utilizing a known p24 standard in the assay. (n=4, * p<0.05, two-tailed t-test, mean ± SEM.

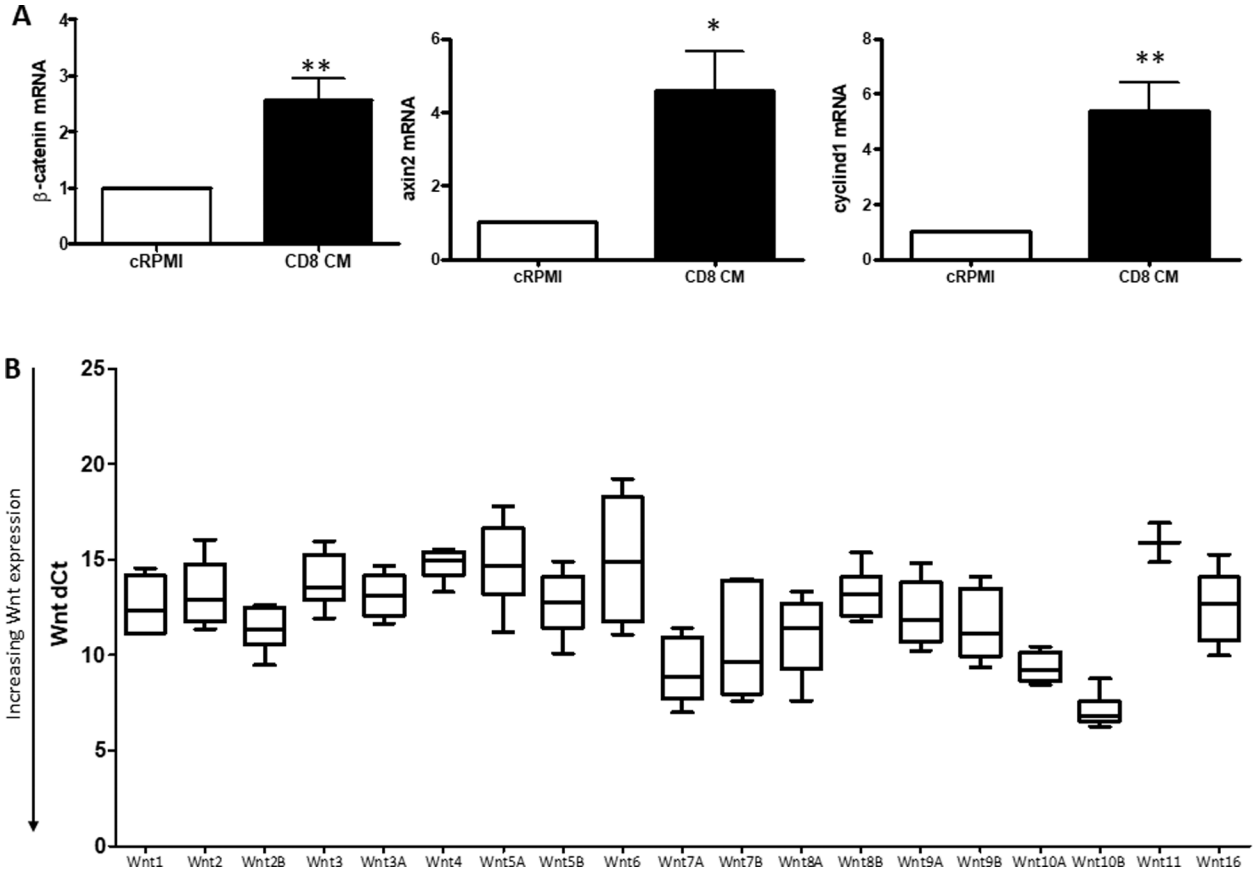

Fig. 2. CD8+ T cells express canonical Wnts

(A) HIV infected CD8 depleted PBMCs were cultured in CD8 CM or cRPMI for 6 days then the cells were collected and lysed for total RNA isolation followed by reverse transcription and q-RT-PCR for *β-catenin*, *axin2* and *cyclind1* (n=5, * $p<0.05$, ** $p<0.01$, two-tailed t-test, mean ± SEM). **(B)** CD8+ T cells isolated from PBMCs from healthy donors were activated and cultured for 3 days followed by RNA isolation, cDNA synthesis and q-RT-PCR for the 19 Wnts. Y-axis represents delta Ct normalized against GAPDH. Delta Ct is inversely proportional to Wnt expression (n=6 donors).

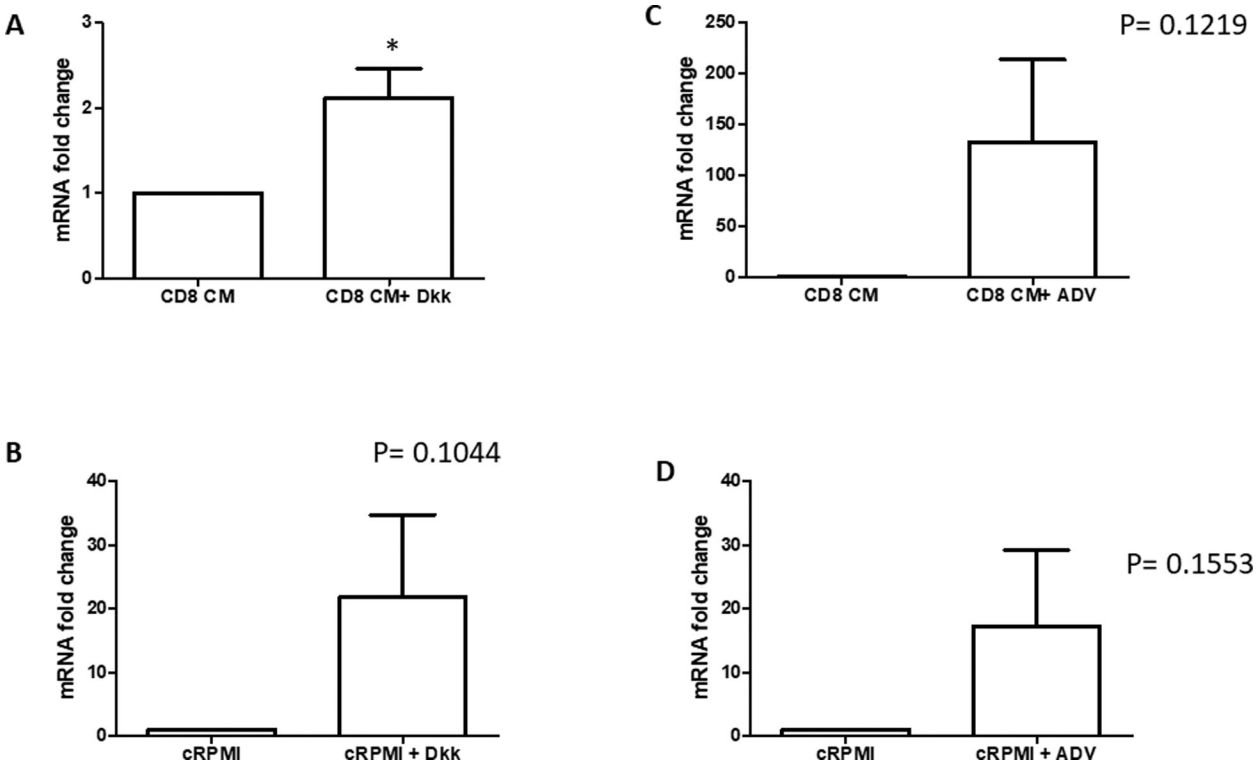

Fig. 3. Antagonizing canonical Wnts abrogates CD8 CM inhibitory activity.

(A) CD8 depleted PBMCs were infected with HIV before incubation of HIV infected cells with CD8 CM and 50ng/ml rhDkk-1 protein. RNA was isolated after 6 days, followed by cDNA synthesis and q-RT-PCR for *nef* expression (n=5, two-tailed t-test, mean ± SEM, * $p<0.05$). (**B**) CD8 depleted PBMCs were incubated with 50ng Dkk-1 (**C**) CD8 depleted PBMCs were incubated with either CD8 CM with or without the β-catenin inhibitor ADV or (**D**) ADV with or without cRPMI.

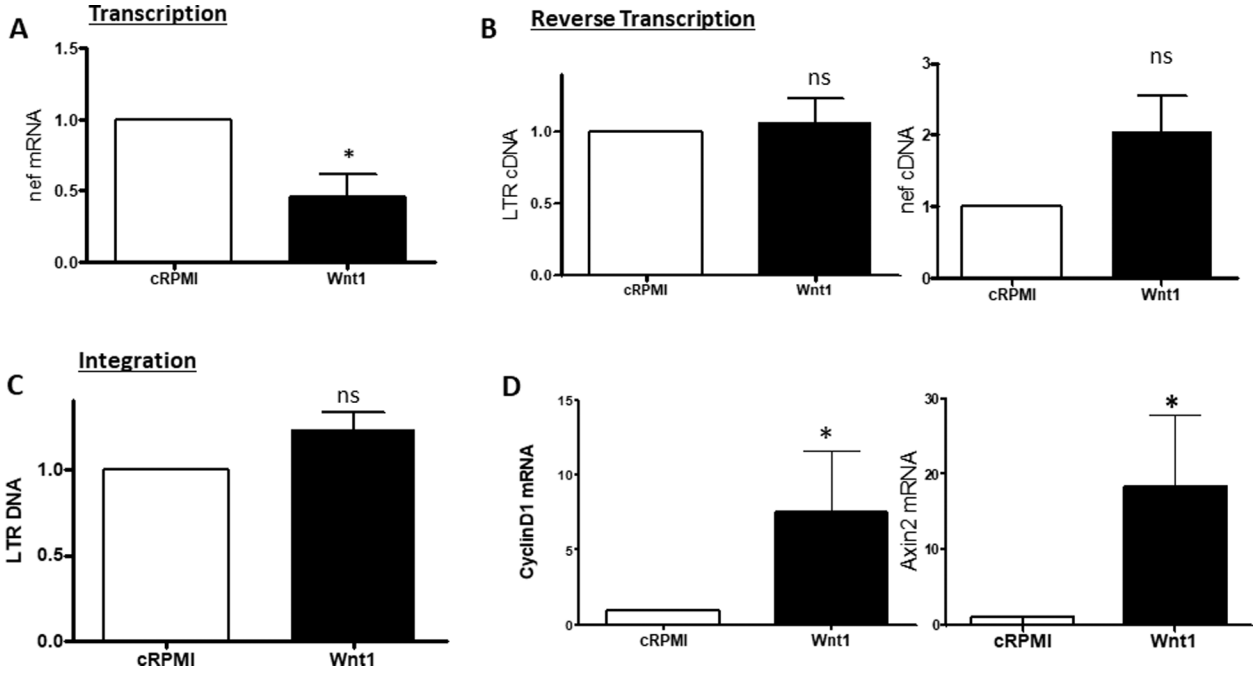

Fig. 4: Wnt1 inhibits HIV transcription but not reverse transcription or integration.

(A) CD8 depleted PBMCs were infected with HIV then incubated with 500ng/ml rhWnt1 in cRPMI for 72–96 hrs. and qRT-PCR was used to measure transcription of *nef* (n=5, * $p<0.05$, two-tailed t-test, mean ± SEM). **(B)** Similar to **(A)** with the exception that the CD8 depleted PBMCs were infected with HIV for 8 hrs. The cells were then washed and incubated with 500ng/ml rhWnt1 in cRPMI for 24 hours. The cells were lysed and DNA was isolated followed by qRT-PCR for *LTR* and *nef* (LTR: n=3, p=0.74, two-tailed t-test, mean ± SEM, *nef*: n=3, p=0.17, two-tailed t-test, mean ± SEM). **(C)** Infected CD8 depleted PBMCs were incubated with or without 500ng/ml rhWnt1 for 72–96rs hours prior to collecting and lysing the cells to isolate DNA for Alu PCR (*LTR:* n=3, p=0.15, two-tailed t-test, mean ± SEM). **(D)** qRT-PCR to measure cDNA from infected cells in (A) and (C) for expression of *axin2* and *cyclind1* (n=5, * $p<0.05$, two-tailed t-test, mean ± SEM).

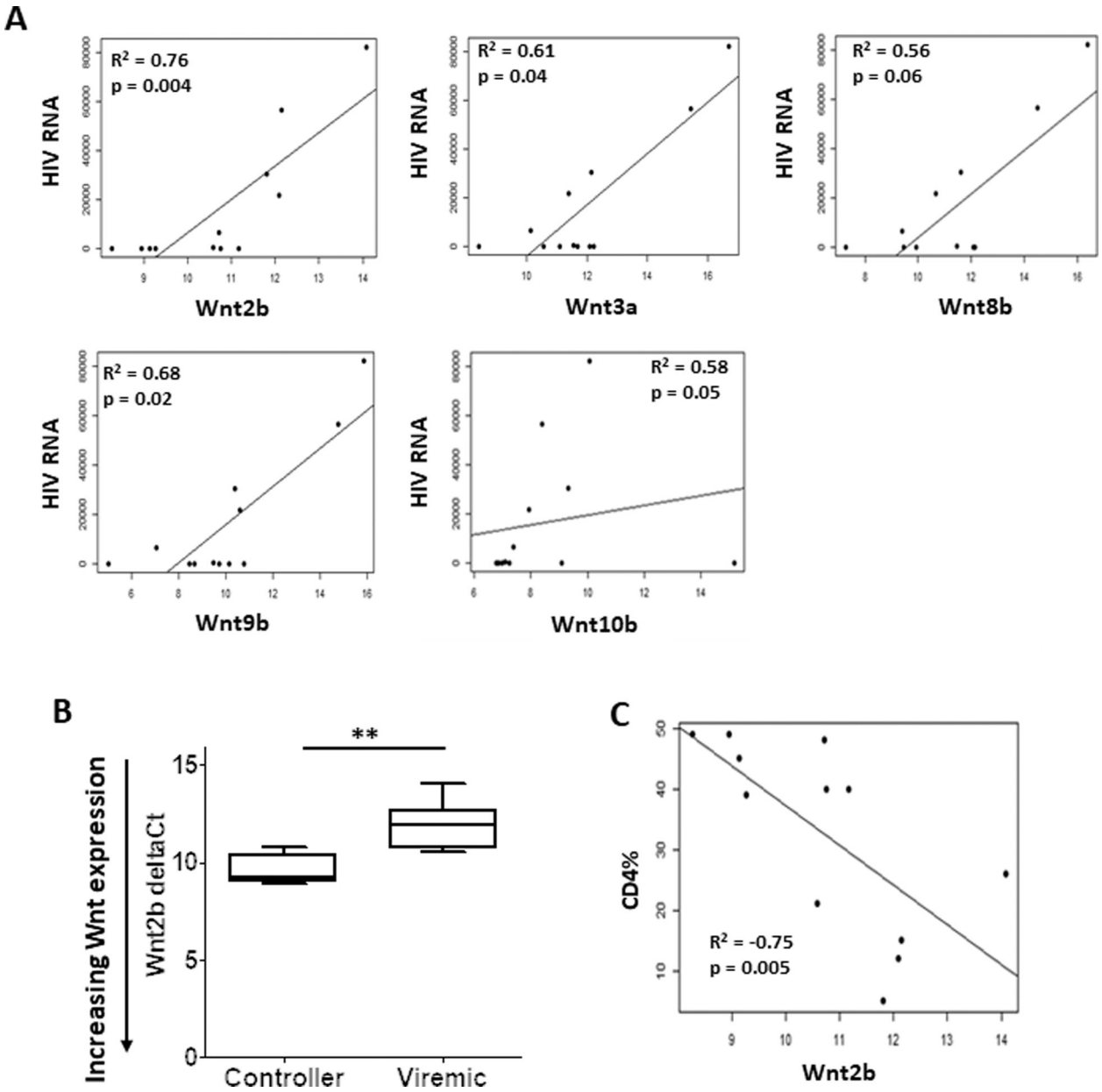

Fig. 5. Canonical Wnts are associated with HIV controller status

(**A)** q-RT-PCR was performed on RNA isolated from HIV+ patient CD8+ T cells for all 19 Wnts. Delta CT was calculated using control GAPDH expression. Lower delta Ct numbers denote higher levels of *Wnt2b, Wnt3a, 8b, 9b* and *Wnt10b* mRNA expression, and higher delta Ct numbers denote lower levels of mRNA expression. Delta Ct was plotted against viral load (n=12). **(B)** Similar to **(A)**, mRNA Wnt expression was measured for viremic patients and controllers and reported as delta Ct (n=12, ** $p<0.01$, two-tailed t-test, mean ±SEM)). **(C)** *Wnt2b* expression compared to CD4% (n=12).

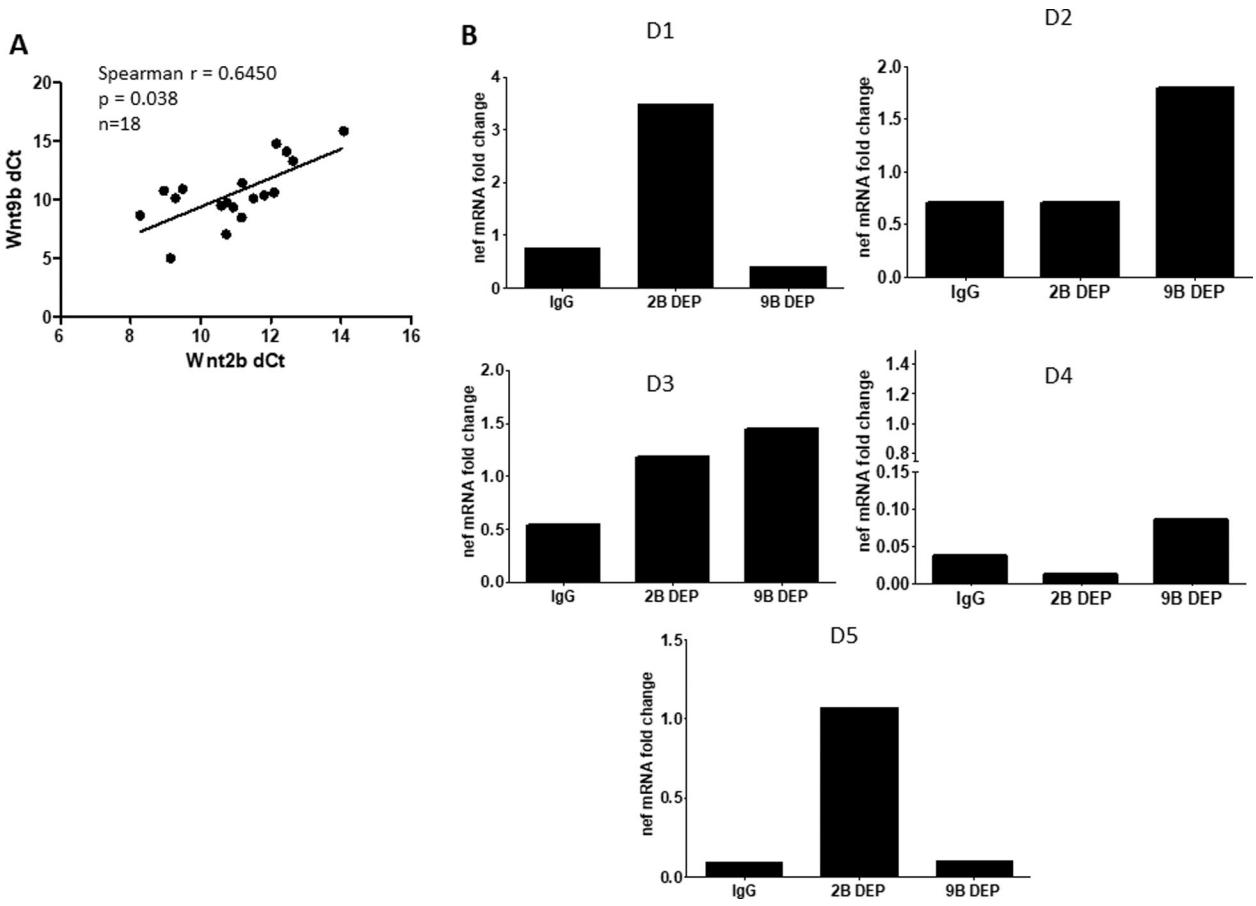

Fig. 6. Wnts2b and 9b depletion abrogate CD8 CM activity in a donor dependent manner

(A) relationship between Wnt2b and 9b RNA from CD8+ T cells of HIV+ donors. Delta CT was calculated using control GAPDH expression. Lower delta Ct numbers denote higher levels of Wnt expression (**B**) Primary antibodies for Wnt2b, Wntb9b and an IgG control were coupled with magnetic protein A/G beads. The coupled product was then incubated with CD8 CM to pulldown the respective Wnt, the Wnt depleted CD8 CM was then incubated with infected CD8 depleted PBMCs for 72–96hrs followed by measurement of HIV *nef* via q-RT-PCR in infected cells. D = donor.

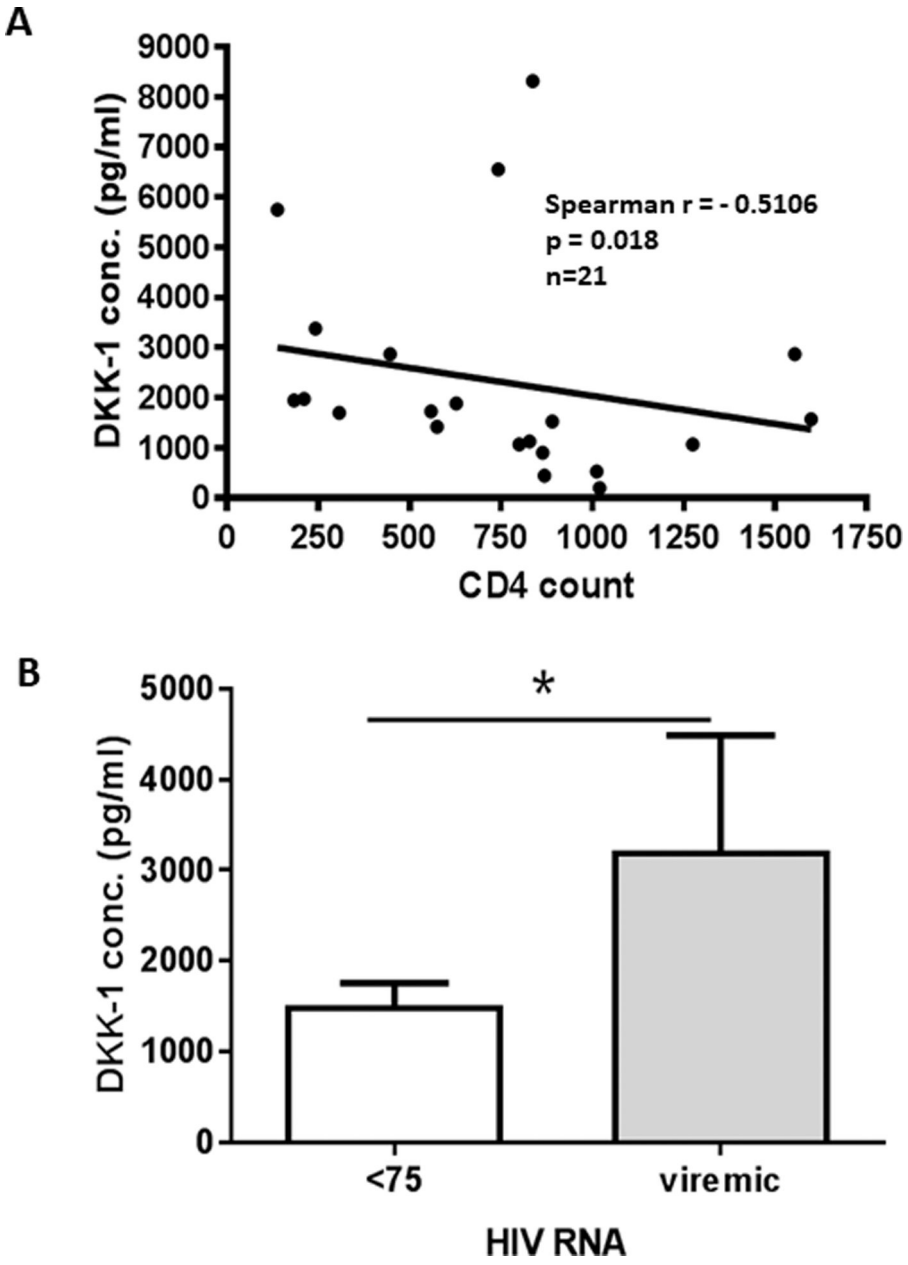

Fig. 7. Canonical Wnt signaling antagonist Dkk-1 is associated with worsened HIV status.

(A) Correlation between Dkk-1 plasma concentration and CD4 count (Spearman's rank-order correlation, n=21, r = −0.5106, * p <0.05). **(B)** Dkk-1 plasma concentration in patients with CD4 count below or above 500 (n=21, * p <0.05, two-tailed t-test, mean ± SEM). **(B)** Dkk-1 plasma concentration in patients with viral RNA copies below 75 copies/ml or viremic (n=21, * p <0.05, two-tailed t-test, mean ± SEM).

Table 1:

HIV+ Patient Cohort

Patient	status	CD4 count	CD4%	HIV RNA copies/ml
1	c	1274		<75
2	c	828		<75
3	v	139		1642
4	c	1019		<75
5	c	837		<75
6	c	559		<75
7	c	1598		<75
8	c	308		<75
9	c	575		<75
10	v	211		10253
11	c	869		<75
12	c	447		<75
13	c	1553		<75
14	c	243		<40
15	c	1012		52
16	c	742		<40
17	c	864	40	44
18	c	1100	49	<40
19	v	184	21	619
20	c	800	39	<40
21	c	628	45	<20
22	v	1518	48	6857
23	v	146	5	30274
24	v	129	12	21962
25	v	574	26	81972
26	v	214	15	56429
27	t	745	49	<40
28	t	1161	40	<20

Description of HIV+ cohort. C: controller, v: viremic, t: on therapy with undetectable viral load.