HIV-1 selectively targets gut-homing CCR6^{+}CD4^{+} T-cells *via* mTOR-dependent mechanisms

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ABSTRACT

Gut-associated lymphoid tissues (GALT) are enriched in $CCR6^+$ Th17-polarized $CD4^+$ T-cells that contribute to HIV-1 persistence during antiretroviral therapy (ART). This raises the need for Th17-targeted immunotherapies. In an effort to identify mechanisms governing HIV-1 permissiveness/persistence in gut-homing Th17 cells, we analyzed the transcriptome of $CCR6^+$ *versus* $CCR6^-$ T-cells exposed to the gut-homing inducer retinoic acid (RA) and performed functional validations in colon biopsies of HIV-infected individuals receiving ART (HIV+ART). Although both $CCR6^+$ and $CCR6^-$ T-cells acquired gut-homing markers upon RA exposure, the modulation of unique sets of genes coincided with preferential HIV-1 replication in RA-treated $CCR6^+$ T-cells. This molecular signature included the up-regulation of HIV-dependency factors acting at entry/post-entry levels, such as CCR5 and PI3K-Akt-mTORC1 signaling pathway. Of note, mTOR expression/phosphorylation was distinctively induced by RA in $CCR6^+$ T-cells. Consistently, mTOR inhibitors counteracted the effect of RA on HIV replication *in vitro* and viral reactivation in $CD4^+$ T-cells from HIV+ART individuals, *via* post-entry mechanisms independent of CCR5. Finally, $CCR6^+$ *versus* $CCR6^-$ T-cells infiltrating the colon of HIV+ART individuals expressed unique molecular signature including higher levels of CCR5, integrin β7, and mTOR phosphorylation. Together, our results identify mTOR as a druggable key regulator of HIV permissiveness in gut-homing $CCR6^+$ T-cells.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) causes the progressive depletion of $CD4^+$ T-cells thus impairing the ability of the immune system to fight pathogens. Although current antiretroviral therapies (ART) efficiently control viral replication and restore CD4 counts, the HIV eradication is not achieved (1-3). Major $CD4^+$ T-cell alterations occur in gut-associated lymphoid tissues (GALT) rapidly upon HIV transmission (4, 5) and these alterations persist despite viral suppressive ART (3, 6), even in patients treated during acute HIV infection (7). The GALT is considered an important site of HIV replication and viral reservoir persistence during ART (8). GALT-infiltrating $CD4^+$ T-cells represent optimal HIV targets likely due to their high expression of CCR5 (4, 5, 8), a major co-receptor for HIV entry (9-11), and integrin α4β7, a gut-homing molecule (12) identified as an HIV-binding molecule (13). Very recently, integrin α4β7 blocking Abs proved efficacy in controlling viral replication upon ART interruption in a SIV infection model (14), indicative that interfering with HIV replication in gut-homing $\alpha4\beta7^+CD4^+$ T-cells represents a potential curative strategy.

Among GALT-infiltrating $CD4^+$ T-cells, Th17 cells play a beneficial role in maintaining epithelial cell integrity at barrier surfaces *via* the production of effector cytokines under the control of lineage-specific transcription factors (15). Studies by our group and others demonstrated that Th17-polarized $CCR6^+CD4^+$ T-cells are highly permissive to infection (16-18), as a consequence of their expression of HIV dependency factors (HDFs) acting at entry and post-entry levels (19, 20), combined with the lack of HIV restriction mechanisms (21-23). Most recently, $CD4^+$ T-cells expressing the Th17

markers CCR6/RORγt were identified as the first targets of SIV during vaginal transmission (24); also, the detrimental role of viral permissive $CCR6^+$ T-cell infiltration into the gut was documented (25). The depletion of Th17 cells from the GALT upon HIV infection persists despite viral-suppressive ART (26-28), even when ART is intensified with integrase and CCR5 inhibitors (29). Other promising strategies tested for mucosal Th17 restoration include an ideal ART initiation at very early stages of infection (Fiebig I/II but not III) (7), the use of probiotics, and IL-21 supplementation (30-32). The non-restoration of GALT Th17 cells leads to significant damages of the intestinal barrier thus allowing the microbial translocation, a cause for chronic immune activation and occurrence of non-AIDS co-morbidities during ART (3, 6, 33). Despite their dramatic depletion, long-lived Th17 cells contribute to HIV reservoir persistence during ART (34, 35). Consistently, our group identified a subset of $CCR6^+CCR4^-CXCR3^-$ Th17 cells predominant in blood and lymph nodes and carrying replication-competent HIV reservoirs (36) and further demonstrated preferential HIV-DNA persistence in colon infiltrating $CCR6^+CD4^+$ T-cells during ART (37). These advances orient our search for immunotherapeutic strategies to limit HIV infection/persistence in mucosal Th17 cells in the ART era.

The homing of $CCR6^+$ Th17 cells into the GALT is mediated *via* the CCR6/CCL20 axis (28, 38), as well as the adhesion molecule integrin α4β7 (12) and chemokine receptor CCR9 (39). The imprinting for gut-homing is induced by *all-trans* retinoic acid (ATRA) (40), a vitamin A metabolite produced by GALT dendritic cells (41). Our previous studies demonstrated that ATRA increases HIV-1 permissiveness

preferentially in $CCR6^+$ compared to $CCR6^-$ T-cells (42). This effect coincided with the preferential ability of ATRA to up-regulate the expression of the HIV co-receptor CCR5 on $CCR6^+$ T-cells, indicative of a most efficient HIV entry. Nevertheless, exposure to single-round VSV-G-pseudotyped HIV entering cells by endocytosis provided evidence that ATRA also promotes HIV replication in gut-homing $CCR6^+$ T-cells *via* post-entry mechanisms (42). These findings led to the current hypothesis that CCR6 is a marker for Th17-polarized $CD4^+$ T-cells transcriptionally programmed to become HIV targets in the gut, where ATRA promotes the expression of unidentified HIV permissiveness factors, which may be manipulated to protect gut-homing Th17 cells from HIV.

In our search for new druggable molecular targets to prevent HIV replication/persistence in gut-homing Th17 cells, herein we performed a genome-wide transcriptional profiling to identify HDFs modulated by ATRA in memory $CCR6^+$ T-cells. Together, our studies *i)* provide an in-depth characterization of molecular mechanisms contributing to HIV replication/persistence in ATRA-exposed $CCR6^+$ Th17 cells; *ii)* identified mTOR as a druggable target modulated by ATRA in these cells; and *iii)* support the beneficial use of mTOR inhibitors in clinics to preserve mucosal Th17 cells from HIV infection/persistence during ART.

RESULTS

Transmitted/founder (T/F) HIV preferentially infects retinoic-acid treated CCR6$^+$CD4$^+$ T-cells. We previously reported that *all-trans* retinoic acid (ATRA) mediated-imprinting for gut-homing increases replication of the laboratory-adapted R5 NL4.3BaL HIV preferentially in CCR6$^+$ *versus* CCR6$^-$ T-cells (42). T/F HIV strains are isolated early upon seroconversion and are unique in their virulence/sensitivity to antiviral mechanisms (43). To determine whether T/F HIV also target gut-homing CCR6$^+$ T-cells for preferential replication, FACS-sorted memory CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* CD3/CD28 in the presence/absence of ATRA were exposed in parallel to T/F THRO (44) and NL4.3BaL HIV (Figure 1A). The relative frequency of CCR6$^+$ and CCR6$^-$ T-cells in peripheral blood mononuclear cells (PBMCs) before sort is depicted in Figure 1B-C. The dose of ATRA used (10 nM) (42) is consistent with physiological plasma levels (40) and had no influence on cell viability (data not shown). ATRA robustly increases replication of both NL4.3BaL and T/F THRO HIV in CCR6$^+$ T-cells, while the effects on CCR6$^-$ T-cells were only minor, as reflected by the quantification of early (RU5), late (Gag), and integrated (Alu/HIV) HIV reverse transcripts (Figure 1 D-E). Thus, T/F similar to NL4.3BaL HIV also target ATRA-treated CCR6$^+$ T-cells for preferential replication, suggesting a critical role played by gut-homing CCR6$^+$ T-cells during the early steps of mucosal HIV transmission.

CCR6$^+$ *versus* CCR6$^-$ T-cells exhibit higher proliferation, TNF-α production, and NF-κB DNA-binding activity in the presence/absence of ATRA. In search of post-entry permissiveness mechanisms, we first investigated whether ATRA modulated

proliferation and IL-2 production. In the absence of ATRA, $CCR6^+$ compared to $CCR6^-$ T-cells exhibited higher proliferation upon TCR triggering but a markedly lower production of IL-2 (Suppl. Figure 1A-C), consistent with previous studies (16), likely as a consequence of RORγt-mediated IL-2 inhibition (45). ATRA further decreased IL-2 production in $CCR6^+$ T-cells but had no significant effect on cell proliferation (Suppl. Figure 1A-C). Levels of CD25 (IL-2Rα) were similarly high on $CCR6^+$ and $CCR6^-$ T-cells exposed or not to ATRA (Suppl. Figure 1D-G), indicative that differences in IL-2 production are not related to IL-2 auto-consumption. Thus, increased HIV replication in $CCR6^+$ T-cells upon exposure to ATRA cannot be explained by enhanced cell proliferation and/or IL-2 production.

HIV permissiveness in $CCR6^+$Th17 *versus* $CCR6^-$Th1 cells is associated with higher NF-κB nuclear translocation (20). The pro-inflammatory cytokine TNF-α enhances HIV replication, in part by triggering the NF-κB pathway (46). This prompted us to investigate differences in TNF-α production and NF-κB-p65 activity. Upon TCR triggering, $CCR6^+$ *versus* $CCR6^-$ T-cells produced significantly higher levels of TNF-α in the absence of ATRA; although ATRA slightly decreased TNF-α production in $CCR6^+$ T-cells, levels remained significantly higher in ATRA-treated $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 2A). The NF-κB-p65 DNA-binding activity was higher in $CCR6^+$ *versus* $CCR6^-$ T-cell nuclei when cells were cultured in the presence/absence of ATRA; exposure to ATRA slightly decreased NF-κB-p65 DNA-binding activity in $CCR6^+$ T-cells (Suppl. Figure 2B), consistent with previous reports (47). Nevertheless, NF-κB-p65 DNA-binding activity remained significantly higher in ATRA-treated $CCR6^+$ *versus*

CCR6$^-$ T-cells (Suppl. Figure 2B). Together these results demonstrate that TNF-α production and NF-κB-p65 nuclear translocation/DNA-binding activity are significantly higher in memory CCR6$^+$ *versus* CCR6$^-$ T-cells upon TCR triggering, but ATRA has no positive impact on these differences.

ATRA promotes a unique transcriptional program associated with HIV permissiveness in CCR6$^+$ T-cells. To gain insights into mechanisms underlying the differential effects of ATRA on CCR6$^+$ *versus* CCR6$^-$ T-cells, their ability to integrate ATRA-mediated signals was investigated. CCR6$^+$ and CCR6$^-$ T-cells expressed similar levels of RA receptor alpha (RARα) mRNA *ex vivo* (Figure 2A) and ATRA up-regulated the expression of RA-inducible gene I (RIG-I)/Retinoic acid receptor responder protein 3 (RARRES3), a direct target of RA (48), in both cell subsets (Figure 2B). We hypothesized that CCR6$^+$ and CCR6$^-$ T-cell subsets respond to ATRA in distinct fashions, with the expression of HIV dependency factors (HDFs) being promoted preferentially in CCR6$^+$ T-cells. To test this hypothesis, a genome-wide transcriptional profiling was performed in CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA for four days; this time point coincided with the time of HIV exposure in Figure 1.

Differentially expressed genes were identified based on p-values (p) or adjusted p-values (adj. p) and fold change (FC) of gene expression. Results in Figure 2C and Suppl. Tables 1-4 illustrate transcriptional differences between CCR6$^+$ and CCR6$^-$ T-cells upon TCR triggering in the presence/absence of ATRA. In the absence of ATRA, up-regulated

transcripts (n=348; adj. p-values<0.05, FC cut-off 1.3) in $CCR6^+$ *versus* $CCR6^-$ T-cells included Th17 markers such as CCL20, IL-22, IL-17F, CTSH, KLRB1/CD161, IL-8, RORA, CSF2/GM-CSF, IL-26, RORC, CCR6, SGK1, PPARG, IL-23R, ARNTL, and PTPN13 (Suppl. Table 1). Transcripts up-regulated by ATRA in $CCR6^+$ T-cells (n=95; adj. p-values<0.05, FC cut-off 1.3) included RARRES3, CD38, DHRS3, UBD, LGMN, EVI5, LZTFL1, NAPSA, GBP4, HIC1, CCR9, CCR2, CXCR6, and ITGB7 (Suppl. Table 2). Transcripts up-regulated by ATRA in $CCR6^-$ T-cells (n=119; adj. p-values<0.05, FC cut-off 1.3) included NAPSA, CD38, EVI5, SAMD9, GALR2, DHRS3, ANKRD37, PTGER2, SLC26A4, and RARRES3, ARG2, APOBEC3B, RIPK2, and NFIL3 (Suppl. Table 3). The analysis of the n=319 transcripts over expressed in ATRA-treated $CCR6^+$ *versus* $CCR6^-$ T-cells revealed the preservation of a Th17 signature (*e.g.*, CCL20, IL-17F, CTSH, IL-22, KLRB1, CSF2, ANTXR2, GPR56, CXCR6, KLF2, RORC, RORA, S100A4, CCR6, MAP3K4, CD96, IL-26, FURIN, ABCB1, ARNTL, and PTPN13) (Suppl. Table 4). Finally, in the presence/absence of ATRA, $CCR6^+$ *versus* $CCR6^-$ T-cells show superior expression of the Th17 markers RORC, ARNTL, and CCR6, and inferior expression of the RORC nuclear repressor NFIL3 (49) (Suppl. Figure 3). Together these results demonstrate that $CCR6^+$ and $CCR6^-$ T-cells respond to ATRA in a unique transcriptional fashion and that ATRA exposure does not modify the Th17-polarisation profile of $CCR6^+$ T-cells.

An extended intelligent search within top modulated transcripts revealed that ATRA induced expression of gut-homing markers integrin $\alpha 4$, integrin $\beta 7$ and CCR9 (12) in both $CCR6^+$ and $CCR6^-$ T-cells. Of note, CXCR6, a minor HIV co-receptor (50, 51), was

significantly up-regulated by ATRA in $CCR6^+$ but not $CCR6^-$ T-cells (Suppl. Tables 2-4; Suppl. Figure 3). FACS analysis validated the microarray results for integrin β7 and CXCR6 expression **(**Suppl. Figure 4A-C). Although CCR5 transcripts were not differentially expressed in microarrays (data not shown), we confirm (42) that ATRA significantly up-regulates CCR5 preferentially on $CCR6^+$ T-cells **(**Suppl. Figure 4A and D). This coincided with the up-regulation of KLF2 (Suppl. Figure 3), a positive regulator of CCR5 transcription (52). The down-regulation of CCR5 ligands CCL3 and CCL3L1 by ATRA was mainly observed in $CCR6^+$ T-cells, indicative of a more efficient CCR5-mediated HIV entry (53). Although the T-cell activation marker CD38 was similarly up-regulated by ATRA in both subsets **(**Suppl. Figure 3), transcripts for MAP3K4, a Th17-specific kinase (54) we previously identified as a HDF (20), were expressed at superior levels and up-regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 3). Moreover, the transcripts for PPARγ, a transcriptional repressor of RORγt (55) and Th17-intrinsec negative regulator of HIV replication (19), was down-regulated by ATRA specifically in $CCR6^+$ T-cells (Suppl. Figure 3). Furthermore, $CCR6^+$ *versus* $CCR6^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA expressed decreased levels of interferon (IFN) stimulated genes, such as IFITM1, 2, and 3, and IRF8 (Suppl. Tables 1-4), consistent with the low Th17 ability to respond to IFN (23). Finally, although differences in the expression of SAMHD1, a key IFN-induced HIV restriction factor (22, 56, 57), were not observed in the microarrays, in the presence/absence of ATRA, levels of SAMHD1 mRNA measured by RT-PCR were significantly lower in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 5). This stresses the existence of false negatives in high-throughput transcriptional screenings. Together, these findings reveal

multiple HDFs acting at entry and post-entry levels to facilitate HIV replication in ATRA-treated $CCR6^+$ T-cells, as well as a state of antiviral immunity acting at different levels in $CCR6^-$ T-cells.

Among probe sets differentially modulated by ATRA in $CCR6^+$ and $CCR6^-$ T-cells, (p-value<0.05), only 466 transcripts were shared, while 1,285 and 1,538 transcripts were unique to $CCR6^+$ and $CCR6^-$ T-cells, respectively (Figure 2D). A meta-analysis using the NCBI HIV-1 interactions data-base allowed the identification of human genes previously described to enhance HIV-1 protein expression that were differentially regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 6). Among those transcripts, LGALS3, ADAM15, TRIM8, IL-1α, FOXO3, and ARNTL were up-regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 3 and 6).

Finally, Gene Set Variation Analysis (GSVA) identified pathways modulated by ATRA in $CCR6^+$ T-cells, including the Akt and PI3K signaling pathways (Figure 3), critical for the Th17 polarization (58-60). A custom pathway was created using the genes from BIOCARTA_AKT_PATHWAY and PID_PI3K_PLC_TRK_PATHWAY, genes that were differentially modulated by ATRA in $CCR6^+$ T-cells (Figure 4A). Of note, the mammalian target of rapamycin (mTOR) appeared up-regulated by ATRA in $CCR6^+$ T-cells in this gene interaction network representation (Figure 4A). Ingenuity Pathway Analysis (IPA) was further used to illustrate genes associated with the mTORC1 pathway and regulated by ATRA in $CCR6^+$ T-cells (Figure 4B).

Together, this genome-wide transcriptional analysis reveals unique molecular signatures associated with HIV permissiveness in Th17-polarized CCR6$^+$ T-cells upon exposure to ATRA and point to a critical role played by mTOR in this process.

ATRA induces mTOR expression and phosphorylation selectively in CCR6$^+$ T-cells. To explore the potential contribution of mTOR to HIV permissiveness in ATRA-treated CCR6$^+$ T-cells, the expression of total and phosphorylated mTOR was first investigated by western blotting in sorted CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA. Levels of total/phosphorylated mTOR were low to undetectable in TCR-activated CCR6$^+$ T-cells but significantly increased by ATRA treatment. In contrast, TCR-activated CCR6$^-$ T-cells expressed high levels of total/phosphorylated mTOR and ATRA did not modify this expression (Figure 5A-B). The molecular weight of the main mTOR band was 80 kDa, which corresponds to the β isoform of mTOR typically expressed in T-cells (61). To further validate these results, we performed semi-quantitative RT-PCR and visualized on gel the expression of mTOR mRNA corresponding to isoform β (2,118 Kb), using established primers (61). Levels of mTOR isoform β mRNA were significantly higher in CCR6$^+$ T-cells upon exposure to ATRA (Suppl. Figure 7). The findings that mTOR mRNA expression and mTOR phosphorylation are induced by ATRA preferentially in CCR6$^+$ T-cells point to the existence of mTOR-dependent HIV permissiveness mechanisms in gut-homing CCR6$^+$ T-cells.

mTORC1/2 inhibitors counteract the effect of ATRA on HIV replication. To determine the functional role of ATRA-mediated mTOR activity, two commercially available mTOR inhibitors were tested for their ability to counteract the effect of ATRA on HIV permissiveness: rapamycin, an inhibitor of mTORC1, and INK128, an inhibitor of mTORC1/2 (62). Memory $CD4^+$ T-cells were stimulated *via* the TCR and further exposed to HIV in the presence/absence of ATRA, rapamycin or INK128 (Figure 6A). Rapamycin and INK128 significantly reduced levels of early/late reverse transcripts and integrated HIV-DNA in ATRA-treated T-cells from five different donors, with INK128 having the most robust inhibitory effects, especially when added before and after infection (Figure 6B-D). Similarly, rapamycin and most significantly INK128 had a robust inhibitory effect on HIV replication measured by HIV-p24 ELISA at days 3, 6 and 9 post-infection (Figure 6E). Of note, the treatment with mTOR inhibitors in combination with ATRA significantly reduced the ability of cells to produce IL-17A but did not alter IFN-γ production, cell viability and proliferation (Suppl. Figure 8A-D). Similar experiments were further performed with FACS-sorted memory $CCR6^+/CCR6^-$ T-cells. INK128 significantly reduced HIV integration/replication in ATRA-treated $CCR6^+$ T-cells. Although levels of HIV integration/replication were lower in $CCR6^-$ compared to $CCR6^+$ T-cells, INK128 similarly acted on $CCR6^-$ T-cells (Figure 7A-B), consistent with the mTOR expression in these cells (Figure 5; Suppl. Figure 7). Together, these results support the role of ATRA-induced mTOR signaling pathway as a positive regulator of HIV permissiveness in gut-homing $CCR6^+$ T-cells.

mTORC1/2 inhibitors limit HIV replication at post-entry level in ATRA-treated T-cells. Studies in humanized mice demonstrated the ability of rapamycin and INK128 to inhibit HIV replication *via* the regulation of CCR5 expression (62). To investigate this possibility, we tested the effects of rapamycin and INK128 on CCR5 expression in memory $CD4^+$ T-cells stimulated *via* TCR in the presence/absence of ATRA. Although rapamycin and INK128 significantly reduced CCR5 expression in the absence of ATRA, no significant changes were observed in the presence of ATRA (Figure 8A-B). Similarly, rapamycin and INK128 had no significant effects on the expression of integrin β7 (Figure 8C-D), a gut-homing molecule induced by ATRA (12) that was also identified as an HIV-gp120 binding molecule (13). To further distinguish between HIV entry and post-entry mTOR-mediated mechanisms in the context of ATRA, we performed a single-round infection using replication-defective VSV-G-pseudotyped HIV and tested the effects of rapamycin or INK128 on HIV reverse transcription and integration. Both rapamycin and INK128 induced a partial decrease in levels of early (59±3 and 65±7% inhibition) and late (88±2 and 90±5% inhibition) HIV reverse transcript and an almost complete abrogation of HIV-DNA integration (98±0.1 and 99±0.1% inhibition, n=3) (Figure 8E-G). Together these results demonstrate that mTORC1/2 inhibitors limit HIV replication in gut-homing $CCR6^+CD4^+$ T-cells *via* post-entry mechanisms, during reverse transcription and prior to integration, without impact on CCR5- and/or integrin β7-mediated viral entry or cell homing into the gut.

ATRA promotes HIV reactivation in $CD4^+$ T-cells of ART-treated individuals in an mTOR-dependent manner. In a very recent study, we demonstrated that ATRA

facilitates HIV reactivation from latency in $CCR6^+CD4^+$ T-cells from ART-treated individuals (37). To determine whether HIV reservoir reactivation was dependent on mTOR activity, an optimized viral outgrowth assay (VOA) was performed in the presence/absence of ATRA and mTORC1/2 inhibitor INK128 (Figure 9A). Consistent with previous findings (37), ATRA significantly improved the efficacy of HIV reactivation in memory $CD4^+$ T-cells isolated from ART-treated individuals (Suppl. Table 5), as measured by intracellular HIV-p24 staining (Figure 9B-C) and soluble HIV-p24 in cell-culture supernatants at day 12 post-reactivation (Figure 9D). Of particular importance, INK128 inhibited HIV reactivation mediated by TCR triggering in the presence of ATRA (Figure 9B-D**).** Further, we tested the effects of INK128 on HIV reservoir reactivation in memory $CCR6^+$ and $CCR6^-$ T-cells isolated by FACS from ART-treated individuals (Suppl. Table 5). In the absence of ATRA, HIV reactivation was observed in $CCR6^+$ but not $CCR6^-$ T-cells in 1/3 donors (ART #3), while in the presence of ATRA HIV reactivation occurred in 3/3 donors preferentially (ART #3) or only (ART#4 and ART #6) in $CCR6^+$ T-cells. Of particular importance, INK128 dramatically reduced HIV reactivation in ATRA-treated $CCR6^+$ T-cells, as measured by intracellular HIV-p24 expression (Figure 9E-F) and soluble HIV-p24 production in cell-culture supernatants at day 12 post-reactivation (data not shown). Together, these results reveal mTOR as a key player in ATRA-mediated HIV reactivation in $CCR6^+$ T-cells of ART-treated individuals.

Colon-infiltrating $CCR6^+CD4^+$ T-cells express high levels of CCR5, integrin β7, and phosphorylated mTOR. The GALT is enriched in DCs metabolizing vitamin A into

ATRA (41). We further explored whether the molecular "*signature*" induced by ATRA *in vitro* including the up-regulation of CCR5, integrin β7 and mTOR phosphorylation on $CCR6^+$ T-cells can be observed as well in the GALT. To test this possibility, we had access to matched blood and sigmoid biopsies from n=10 HIV-infected individuals receiving ART (Suppl. Table 5; COL #1-10). Memory $CD4^+$ T-cells were identified using the gating strategy depicted in Figure 10A and further analyzed for the expression of CCR5, integrin β7, and phosphorylated mTOR in $CCR6^+$ *versus* $CCR6^-$ T-cells. The expression of CCR5 and integrin β7 was higher on cells from the colon *versus* the blood; with colon-infiltrating $CCR6^+$ *versus* $CCR6^-$ T-cells expressing significantly higher levels of CCR5 and integrin β7 (Figure 10B-E). Similarly, mTOR phosphorylation was mainly observed in the colon, with colon-infiltrating $CCR6^+$ *versus* $CCR6^-$ T-cells expressing the highest levels of phosphorylated mTOR in all individuals tested (Figure 10F-G). Thus, the activation of the mTOR pathway occurs almost exclusively in colon-infiltrating $CCR6^+$ T-cells and coincides with the highest levels of CCR5 and integrin β7 expression. These results provide a molecular explanation for the preferential HIV replication in colon $CCR6^+$ T-cells and point to mTOR as a new antiviral drug target in these cells (Figure 11).

Discussion

The role of *all-trans* retinoic acid (ATRA) in regulating tolerance and immunity *via* the modulation of regulatory and effector functions of $CD4^+$ T-cells, respectively, is well-established (41). Our current results support the idea that in the intestinal environment HIV takes advantage of ATRA-mediated signaling pathways for replication/persistence in $CCR6^+CD4^+$ T-cells. Briefly, we *i)* identified mTOR as a major regulator of HIV permissiveness in $CCR6^+CD4^+$ T-cells with gut-homing tropism mimicked *in vitro* by exposure to ATRA; *ii)* demonstrated the ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication *in vitro* and viral reservoir reactivation in T-cells from ART-treated individuals; and *iii)* revealed that $CCR6^+CD4^+$ T-cells infiltrating the colon of HIV-infected individuals on ART express a unique signature comprising high levels of CCR5, integrin β7, and phosphorylated mTOR, signature likely reflecting exposure to ATRA *in vivo* (Figure 11). Considering the strategic location of Th17 cells at portal sites of HIV/SIV entry (6, 24, 27, 28, 63), as well as their important role in maintaining mucosal immunity homeostasis in the context of a complex microbiota (6, 64, 65), our results point to the potential beneficial use of mTOR inhibitors in preventing HIV infection/persistence in gut-homing Th17 cells.

In this manuscript, we used the Illumina bead array technology to unveil post-entry HIV regulatory mechanisms modulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells. Among genes commonly modulated by ATRA in $CCR6^+$ and $CCR6^-$ T-cells, the RA-inducible gene-I (RIG-I) is essential for sensing of viral nucleic acids and promoting antiviral immunity (66). HIV is known to subvert RIG-I-mediated antiviral mechanisms (67); this

may explain robust HIV replication in RIG-I-expressing ATRA-treated $CCR6^+$ T-cells. Of particular importance, Li *et al*. recently demonstrated that acitretin, a RA derivative, increases HIV transcription in $CD4^+$ T-cells from ART-treated HIV-infected individuals and enhances RIG-I signaling thus leading to an antiviral response and the apoptosis of infected cells (68). Similarly, in a previous report (37) and in this manuscript, we demonstrated that ATRA significantly increases HIV reactivation in a viral outgrowth assay (VOA) without significant changes observed in T-cell survival/proliferation. Differences between our results and those published by Li *et al.* (68), may be explained by differences in the experimental design. Nevertheless, these findings commonly support the fact that ATRA promotes HIV reservoir reactivation.

A meta-analysis using the NCBI HIV interaction database revealed HDFs preferentially expressed by ATRA-exposed $CCR6^+$ T-cells. These HDFs include LGALS3, promoting HIV-1 budding *via* association with Alix and Gag p6 (69); ADAM15, involved in the nuclear import of the HIV-1 pre-integration complex (70); TRIM8, a regulator of NF-κB and STAT3-dependent signaling cascades (20, 71); and ARNTL (72), a component of the circadian clock machinery and regulator of Th17 development (49). All these HDFs contribute to robust HIV replication in gut-homing $CCR6^+$ T-cells.

Gene Set Variation Analysis (GSVA) of transcripts uniquely modulated by ATRA in $CCR6^+$ T-cells identified unique pathways, including Akt and PI3K. The network representation of Akt/PI3K pathways, previously linked to Th17 lineage polarization (58-60) and reported to be up-regulated in human lamina propria T-cells (73), pointed to

the up-regulation of mTOR, a metabolic sensor involved in the regulation of numerous cellular functions *via* the formation of two different signaling complexes, mTORC1 and mTORC2 (74-77). Considering the documented role of mTOR-mediated processes in the positive regulation of HIV replication (62, 78), we proceeded to the validation of mTOR expression at protein and mRNA levels, as well as functional validations, in cells from the blood and/or colon of HIV-infected individuals receiving ART and uninfected study participants.

The regulation of HIV permissiveness *via* mTOR-dependent mechanisms was demonstrated by the use of mTORC1 (rapamycin) and/or mTORC1/2 (INK128) inhibitors, that counteracted the effects of ATRA on HIV replication in $CCR6^+$ T-cells *in vitro* and inhibited HIV reservoir reactivation in an ATRA-based VOA performed with $CCR6^+$ T-cells from ART-treated individuals. Nevertheless, mTOR expression/phosphorylation is not always associated with HIV permissiveness. Indeed, $CCR6^-$ T-cells are relatively resistant to HIV despite high levels of mTOR expression, likely due to the existence of HIV restriction mechanisms acting at different levels including entry (low/undetectable CCR5 expression), reverse transcription (high SAMHD1 expression), and/or transcription (limited TNF-α production and NF-κB activity). Thus, mTOR favors HIV replication/reactivation only within the unique transcriptional context of ATRA-exposed $CCR6^+$ T-cells.

Evidence exists in the literature that mTOR regulates HIV replication *via* multiple mechanisms (78, 79). One possible mechanism is *via* the regulation of CCR5 expression

(62). However, CCR5 expression on ATRA-treated T-cells was not affected by mTOR inhibitors. This suggests that ATRA induces CCR5 expression *via* mTOR-independent mechanisms and that mTOR regulation of HIV permissiveness in gut-homing $CD4^+$ T-cells likely occurs at post-entry levels. Consistent with the evidence that mTOR regulates nucleotide biosynthesis essential for transcription (76, 80, 81), mTOR inhibitors may limit HIV reverse transcription by reducing the pool of nucleotides. Indeed, our single-round infection using VSV-G-pseudotyped HIV revealed restriction mechanisms acting during reverse transcription and prior to integration. Moreover, mTOR is a well-established regulator of autophagy (79, 82), a process that may be involved in the degradation of the incoming HIV upon fusion/uncoating (83, 84). By promoting the mTOR activity, ATRA may impair the autophagy process in $CCR5^+CCR6^+$ T-cells at mucosal sites. Furthermore, considering the fact that ATRA-treated $CCR6^+$ T-cells maintain their Th17 features (42) and that mTORC1 *via* the induction of the kinase S6K2 is involved in the nuclear translocation of RORC (60, 85), one other possibility is that mTOR inhibitors interfere with the RORC-mediated transcriptional program in Th17 cells, which is favorable to HIV replication (20). Consistent with this hypothesis, we demonstrated that decreased HIV replication in the presence of rapamycin and INK128 coincided with a significant reduction in IL-17A production by ATRA-treated $CD4^+$ T-cells.

Of particular importance, we found that $CCR6^+$ but not $CCR6^-$ T-cells infiltrating the colon of HIV-infected individuals receiving ART express a unique molecular signature including superior levels of CCR5, integrin β7 and phosphorylated mTOR expression,

likely as a consequence of ATRA exposure in the intestinal environment (12, 41). In line with our recent report that HIV-DNA persists in colon-infiltrating $CCR6^+$ T-cells during ART (37), it remains to be determined whether mTOR expression identifies a fraction of $CCR6^+$ T-cells enriched in HIV-DNA and whether mTOR activation contributes to residual HIV replication in colon-infiltrating $CCR6^+$ T-cells during ART.

While this manuscript was in preparation for submission, Besnard *et al.* reported the results of a shRNA screen that revealed mTOR as a regulator of HIV latency (86) via mechanisms involving CDK9 and NF-κB activation that controls Tat-dependent HIV transcription (86). Also, Martin *et al.* reported on the potential beneficial use of rapamycin in the context of HIV "*shock-an-kill*" strategies (87). These two reports (86, 87) further support the conclusions of our current study revealing the key role played by mTOR in regulating multiple post-entry and post-integration HIV replication steps in gut-homing Th17-polarized $CCR6^+$ T-cells.

In conclusion, our findings point to CCR6 as a "zip code" molecule expressed on the surface of $CD4^+$ T-cells transcriptionally programmed to become HIV targets upon recruitment into the intestine and provide the first detailed molecular explanation for preferential HIV/SIV replication and persistence in gut-homing $CCR6^+CD4^+$ T-cells (24, 25, 37, 42). Most significantly, we reveal the role of the mTOR pathway in regulating effector functions as well as HIV permissiveness in gut-homing $CCR6^+$ Th17 cells at multiple post-entry levels. Additionally, we demonstrate the ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication/reactivation in $CCR6^+$ T-cells. Our

findings raise new questions on the potential beneficial effects of mTOR inhibitors in decreasing HIV reservoirs and restoring the Th17-mediated immunity at the intestinal level during ART. This knowledge will open the path for new studies toward the implementation of new therapeutic strategies aimed at HIV functional cure.

EXPERIMENTAL PROCEDURES

Study subjects

Study subjects were recruited at the Montreal Chest Institute, McGill University Health Centre, and CHUM-Hôpital Saint-Luc (Montreal, Quebec). PBMCs were collected by leukapheresis (88) and cryopreserved until use. Sigmoid biopsies (≈32 biopsies/donor) were collected from HIV-infected individuals receiving antiretroviral therapy (ART) (Suppl. Table 5) during colonoscopy and processed using Liberase DL (Roche Diagnostics), as previously described (37, 89, 90). Matched peripheral blood (20 ml/donor) was collected the same day from biopsy donors.

Flow cytometry staining

Surface staining was performed with fluorochrome-conjugated Abs (Suppl. Table 6), as previously described (16). Cells extracted from sigmoid biopsies and matched PBMCs were stained on the surface with a cocktail of CD3-Alexa700, CD4-PE-Cy7, CD45RA-APC-eF780, CCR5-FITC, integrin β7-FITC, and CCR6-PE Abs, as well as non-T cell lineage Abs: CD326-BV650, CD8-PerCP-Cy5.5, CD19-PerCP-Cy5.5, CD66b-PerCP-Cy5.5 (37). Intracellular staining was subsequently performed with phosphorylated-mTOR (S2448)-eFluor-450 Abs (eBiosciences). The viability dye LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. Cells were analyzed using a LSRII cytometer, Diva version 6 (BD Biosciences, San Jose, CA), and FlowJo version 10.0.6 (Tree Star, Inc). Flow cytometry gates were defined using the *fluorescence minus one* (FMO) strategy (16, 91).

Magnetic (MACS) and fluorescence activated cell sorting (FACS)

Total or memory $CD4^+$ T-cells were enriched from PBMCs by negative selection using magnetic beads (MACS, Miltenyi), with a purity >95%, as previously described (42). Cells were then stained with CD45RA-APC-Cy7 and CCR6-PE Abs and a cocktail of FITC-conjugated Abs to exclude $CD8^+$ T-cells (CD8), NK (CD56), and B cells (CD19). The sorting gates were set on FITCneg memory (CD45RAneg) $CCR6^+/CCR6^-$ T-cells that were subsequently sorted by flow cytometry (BDAria III), with dead cells being excluded. Post-sort quality control analysis demonstrated >97% $CCR6^+/CCR6^-$ T-cell purity, as previously reported (42).

HIV-1 infection *in vitro*

The following HIV-1 molecular clones were used in this study: *(i)* replication-competent CCR5-using (R5) NL4.3BAL; *(ii)* single-round VSVG-HIV-GFP, an *env*-deficient NL4.3 provirus pseudotyped with the VSV-G envelope and encoding for *gfp* in place of *nef* (42, 92); and *(iii)* replication-competent Transmitted/Founder (T/F) THRO. The full-length T/F THRO HIV-1 Infectious Molecular Clone (Cat #11919) was obtained from Dr. John Kappes through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (44). HIV stocks were produced, titrated, and used to infect cells (25-50 ng HIV-p24/10^6 cells), as previously described (16, 19, 20, 36, 42).

Quantification of integrated and Gag HIV-DNA

Levels of integrated and Gag HIV-DNA were quantified in cell lysates by ultrasensitive nested real-time PCR (10^5 cells/test in triplicates; detection limit, 3 HIV-DNA copies), as previously described (16, 19, 20, 36, 37, 42, 93).

Quantification of early HIV reverse transcripts

Early HIV reverse transcripts (RU5) were quantified using a SYBR™ Green master mix (Qiagen). The external primers were: forward AA55 5'-CGTCTAGAGATTTTCCACAC-3' and reverse M667 5'-CTAACTAGGGAACCCACTG-3'. The internal primers were: forward SK29 5'-ACTAGGGAACCCACTGCT-3' and reverse SK30 5'-GGTCTGAGGGATCTCTAG-3' (94). The HIV and CD3 DNA were amplified together in the first PCR and separately during the second PCR (LightCycler; Roche). The first PCR products (diluted 1/10) were used as template for the second PCR: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, and 30 sec at 72°C; and final elongation of 15 min at 72°C. The ACH2 cells carrying one copy of integrated HIV DNA per cell (NIH AIDS reagent program) were used as a standard curve, as previously described (16, 19, 20, 36, 37, 42, 93).

Genome-wide transcriptional profiling

Genome-wide transcriptional profiling was performed in collaboration with Génome Québec (Montreal, Quebec, Canada) using the Illumina bead array technology, as previously described (20). Briefly, total RNA was isolated using the RNeasy kit (Qiagen) and quantified using the Pearl nanophotometer (Implen, Germany). The RNA quality was

determined using the Agilent 2100 Bioanalyzer chip. High quality RNA was reverse transcribed and hybridized on the Illumina HumanHT-12 v4 Expression BeadChip providing coverage for more than 47,000 transcripts and known spliced variants across the human transcriptome. The expression of differentially expressed genes was analyzed as previously described (19, 20, 36). The entire microarray dataset and technical information requested by Minimum Information About a Microarray Experiment (MIAME) are available at the Gene Expression Omnibus (GEO) database under accession number GSE93660. Differentially expressed genes (cut-off 1.3-fold; $p < 0.05$) were classified through Gene Ontology using the NetAffx web-based application (Affymetrix), while differentially expressed pathways were identified using *Ingenuity Pathway Analysis* (IPA) and *Gene Set Variation Analysis* (GSVA). Corresponding heat maps for biological function categories were generated using programming language R (19, 20, 36).

Real-time RT-PCR

One step SYBR Green real-time RT-PCR (Qiagen) was carried out in a LightCycler 480 II (Roche) according to manufacturer's recommendations, as we previously reported (16, 19, 20, 36, 42). Briefly, a standard curve was prepared with cDNA obtained upon reverse transcription of total RNA using specific QuantiTect Primers (Qiagen). The expression of each gene was normalized to the internal control 28S rRNA (forward 5′-CGAGATTCCTGTCCCCACTA-3′; reverse 5′-GGGGCCACCTCCTTATTCTA-3, IDT). Melting curve analysis performed after real-time amplification revealed the uniformity of thermal dissociation profile for each amplification product. Samples

without template or without reverse transcriptase were used as negative controls. Each RT-PCR reaction was performed in triplicate.

mTOR protein and mRNA quantification

Cells were lysed with RIPA buffer 1x (Cell Signaling) containing phosphatase inhibitors (PhosSTOP) and protease inhibitors (Complete Mini EDTA-free, Roche), for 10 minutes at 4°C and centrifuged at 14,000g for 10 min. Proteins were quantified in supernatants by Bradford assay (Biorad). Samples (15 µg protein/well) were loaded onto 7% acrylamide gels and SDS-PAGE migration was performed (90 min, 100 V). Proteins were transferred to PVDF membranes and further blotted with phosphorylated (D9C2) or total mTOR (7C10) Abs (Cell signaling). Proteins were revealed with ECL prime western blotting detection reagent (Amersham, GE Healthcare). In parallel, the expression of the mTOR mRNA was measured by semi-quantitative RT-PCR performed using the superscript III one-step RT-PCR system (Invitrogen, USA) and specific primers (forward: S1, ATGCTTGGAACCGGACCTGCCG ; reverse: AS1, TACCAGAAAGGGCACCAGCCAAT) (Integrated DNA Technologies, USA) that could amplify both mTOR isoforms α (7.647Kb Kb) and β (2.118 Kb) (61) (Suppl. Figure 7A). However, the RT-PCR conditions were set for the optimal amplification of the short β mTOR isoform, as follows: reverse transcription (30 min at 55 °C), 40 cycles of PCR amplification (denaturation, 15 secs at 94 °C; annealing, 30 secs at 63 °C; elongation, 130 secs at 68 °C) and final elongation (15 min at 68 °C). mTOR expression was normalized relative to 28S rRNA. Total RNA was extracted using the RNeasy kit

(Qiagen) and used for RT-PCR amplification of mTOR (250 ng/test) and 28S rRNA (5 ng/test).

Nuclear NF-κB quantification

The NF-κB DNA-binding activity was quantified using an activity-based ELISA kit (Assay Designs & Stressgen, Ann Arbor, Mi, USA) according to the manufacturer's protocol, as described (20). Briefly, nuclear extracts were obtained using the BD transfactor extraction kit (Clonetech Laboratories). Nuclear protein content was quantified by Nanodrop (Thermo Scientific). The active form of NF-κB-p65 was quantified in nuclear extracts (1 μg protein/test). The assay used streptavidin-coated plates with bound NF-κB biotinylated-consensus sequence to capture only the active form of NF-κB. The captured NF-κB was then incubated with a specific NF-κB-p65 Abs further detected using an HRP conjugated Abs. The assay was developed with a chemiluminescent substrate and the signal was detected using a luminometer. Results were expressed as Light Units. The specificity of the binding was demonstrated by the use of a wild type and mutated NF-κB Competitor Duplex included in the kit.

ELISA quantification

HIV-p24 levels were quantified in cell culture supernatants using a homemade ELISA, as described (16, 19, 20, 36, 37, 42). Cytokine levels in cell culture supernatants were quantified by ELISA assays specific for TNF-α, CCL20 (R&D Systems), IL-17, IFN-γ and IL-2 (eBioscience) according to the manufacturers' protocols.

Viral outgrowth assay

This viral outgrowth assay (VOA) was designed to allow optimal HIV reservoir reactivation from small numbers of primary $CD4^+$ T-cells (36, 37). Briefly, memory $CD4^+$ T-cells ($2x10^6$ cells/ml/well) or FACS sorted memory $CCR6^+/CCR6^-$ T-cells (10^6 cells/ml/well) were cultured in 48-well plates in the presence of immobilized CD3 and soluble CD28 Abs (1 µg/ml) for 3 days. Cells were further cultured in the presence of IL-2 (5 ng/ml) up to day 12. Every 3 days, cells were split and media was refreshed. The VOA was performed in the presence/absence of ATRA (10 nM) and/or INK128 (50 nM). HIV-p24 levels were quantified by ELISA in cell culture supernatants, while intracellular HIV-p24 expression was quantified by flow cytometry at day 12 post-reactivation.

Statistics

Statistical analyses were performed using the GraphPad Prism 6. RM One-way ANOVA or ordinary ANOVA with Tukey's or Dunnetts's post-test were used for multiple comparisons. Two-tailed paired t-test was used for matched pairwise analyses. A P-value less than 0.05 was considered significant. (*, p-value<0.05; **, p-value<0.01; ***p-value<0.001; ****p-value<0.0001). Details are included in Figure legends.

Microarrays accession number

The entire microarray dataset and technical information requested by Minimum Information About a Microarray Experiment (MIAME) are available at the Gene Expression Omnibus database under accession number GSE93660.

Study approval

This study used biological samples (blood and sigmoid biopsies) from HIV-infected and uninfected participants and was conducted in compliance with the principles included in the Declaration of Helsinki. This study received approval from the Institutional Review Board of the McGill University Health Centre and the CHUM-Research Centre, Montreal, Quebec, Canada. All human participants provided written informed consent for their participation in the study and agreed with the publication of the scientific results.

Competing interests

The authors declare no financial and non-financial competing interests.

Authors' contributions

DP performed research, analyzed data, and wrote the manuscript. YZ and PM performed research and contributed to manuscript writing. JPG performed microarray data analysis and generated figures. AG performed research. AF contributed to research design and manuscript writing. NG and TJH provided protocols and reagents and contributed to research design. JPR provided access to clinical samples/information, set up clinical research protocols, and contributed to manuscript writing. PA designed research, analyzed data, and wrote the manuscript. All authors reviewed and accepted the manuscript.

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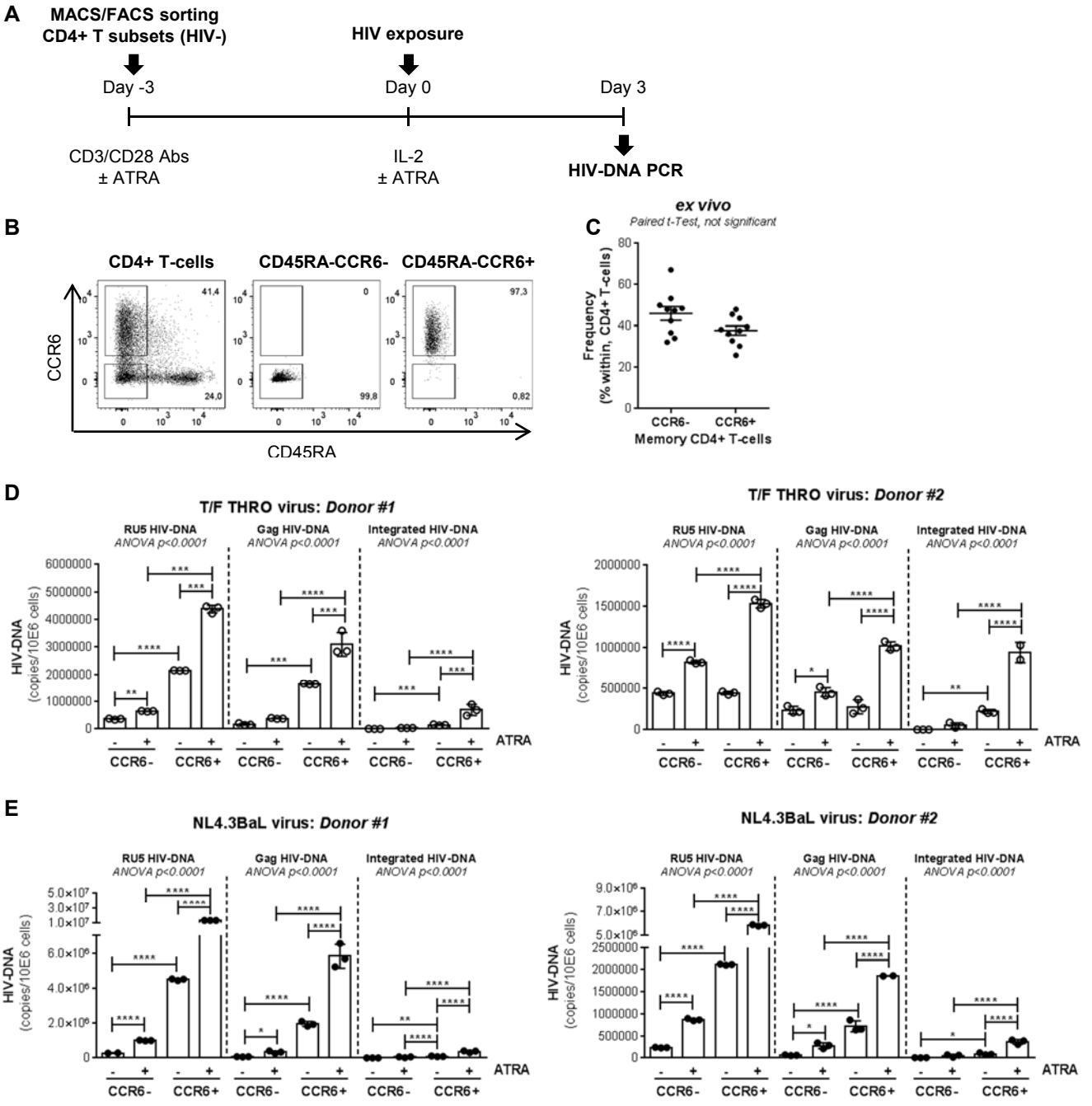


Figure 1: Replication advantage of transmitted founder HIV in CCR6$^+$ *versus* CCR6$^-$ T-cells upon exposure to ATRA. Memory CCR6+ and CCR6- T-cells were isolated by MACS (magnetic-activated cell sorting) and FACS from the PBMCs (peripheral blood mononuclear cells) of HIV-uninfected individuals and tested for their ability to replicate HIV *in vitro*. Shown is **(A)** the experimental flow chart, **(B)** the expression of CCR6 and CD45RA on total CD4$^+$ T-cells isolated using magnetic beads and on memory (CD45RA$^-$) CCR6$^+$ and CCR6$^-$ T-cells isolated by FACS, and **(C)** the frequency of CCR6$^-$ and CCR6$^+$ T-cells within memory CD4$^+$ T-cells in n=10 donors. Paired t-Test p-values are indicated on the graphs **(D-E)**. Sorted cells were stimulated *via* CD3/CD28 in presence/absence of ATRA (*all-trans retinoic acid*) (10 nM) for 4 days and exposed to **(D)** transmitted founder (T/F) THRO HIV or (E) laboratory-adapted NL4.3BaL HIV (25 ng HIV-p24/10^6 cells). Early (RU5 primers) and late (Gag primers) HIV-DNA reverse transcripts, as well as integrated HIV-DNA levels (Alu/HIV-LTR primers) were quantified by nested real-time PCR in matched subsets isolated from n=2 different donors at day 3 post-infection (mean±SD of triplicate wells). **(D-E)** One-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one triplicate value.

Planas *et al*., Figure 1

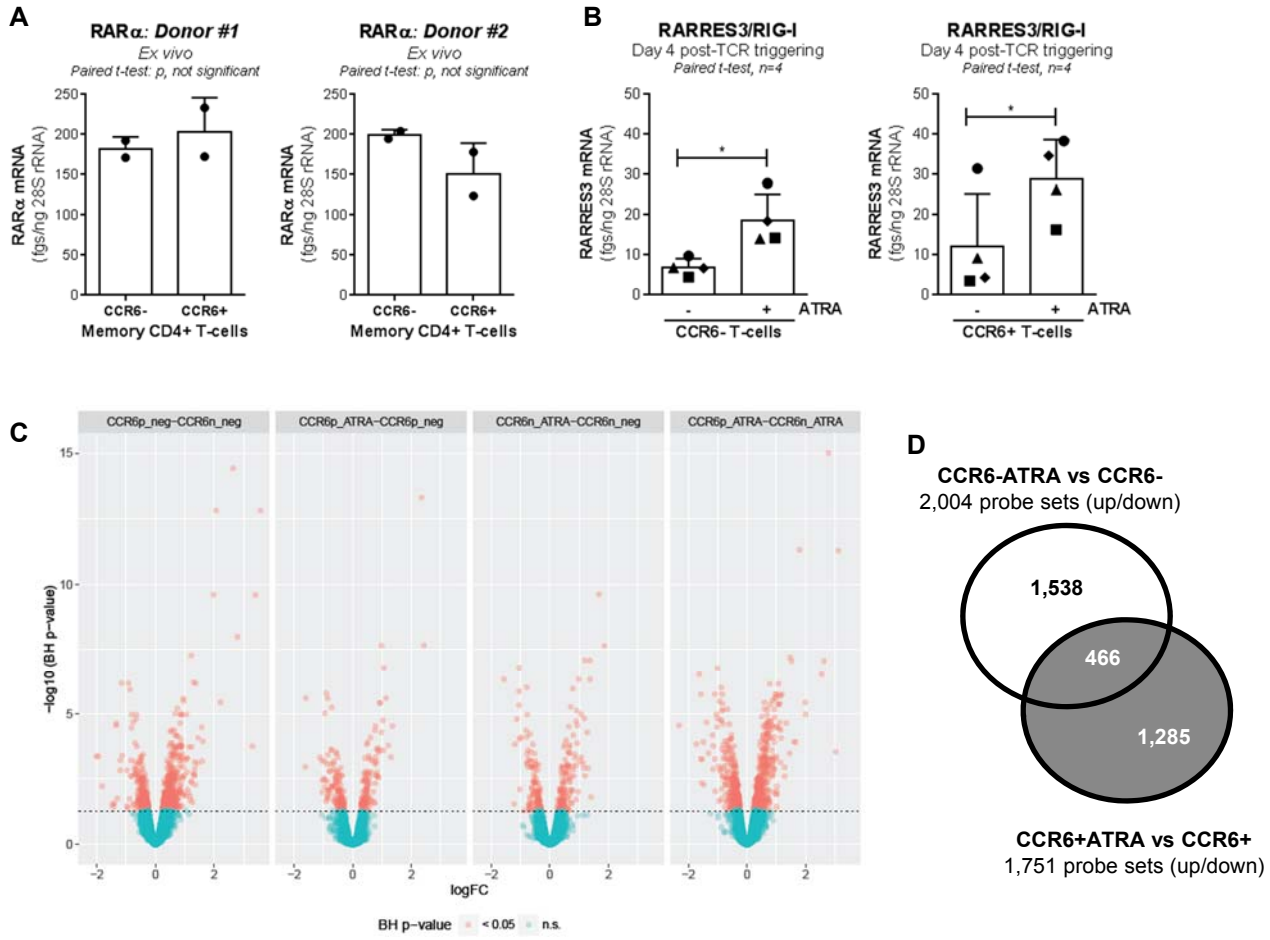


Figure 2: ATRA differentially modulates gene expression in CCR6$^-$ and CCR6$^+$ T-cells. Memory CCR6$^+$/CCR6$^-$ T-cells were sorted and stimulated as described in Figure 1. Shown is the SYBR green real-time RT-PCR quantification of RARα (retinoic acid receptor α) mRNA *ex vivo* (A; n=2, mean±SD) and RIG-I/RARRES3 (retinoic acid induced gene I) mRNA upon CD3/CD28 triggering in the presence/absence of ATRA (B; n=4, mean±SEM). **(A-B)** Paired t-Test p-values are indicated on the graphs. Each symbol represents **(A)** one duplicate value or **(B)** one different donor. **(C-E)** Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). **(C)** Shown are volcano plots for all probes in each linear model with the log2 FC on the x axis and the negative logarithm of the adjusted p-values for false discovery rate (FDR) on the y axis. The red/green color code is based on the 5% FDR threshold. **(D)** The Venn diagram depicts the number of probe-sets modulated by ATRA uniquely in CCR6$^+$ (grey) and CCR6$^-$ T-cells (white) (p-values<0.05), as well as 466 probe sets commonly modulated.

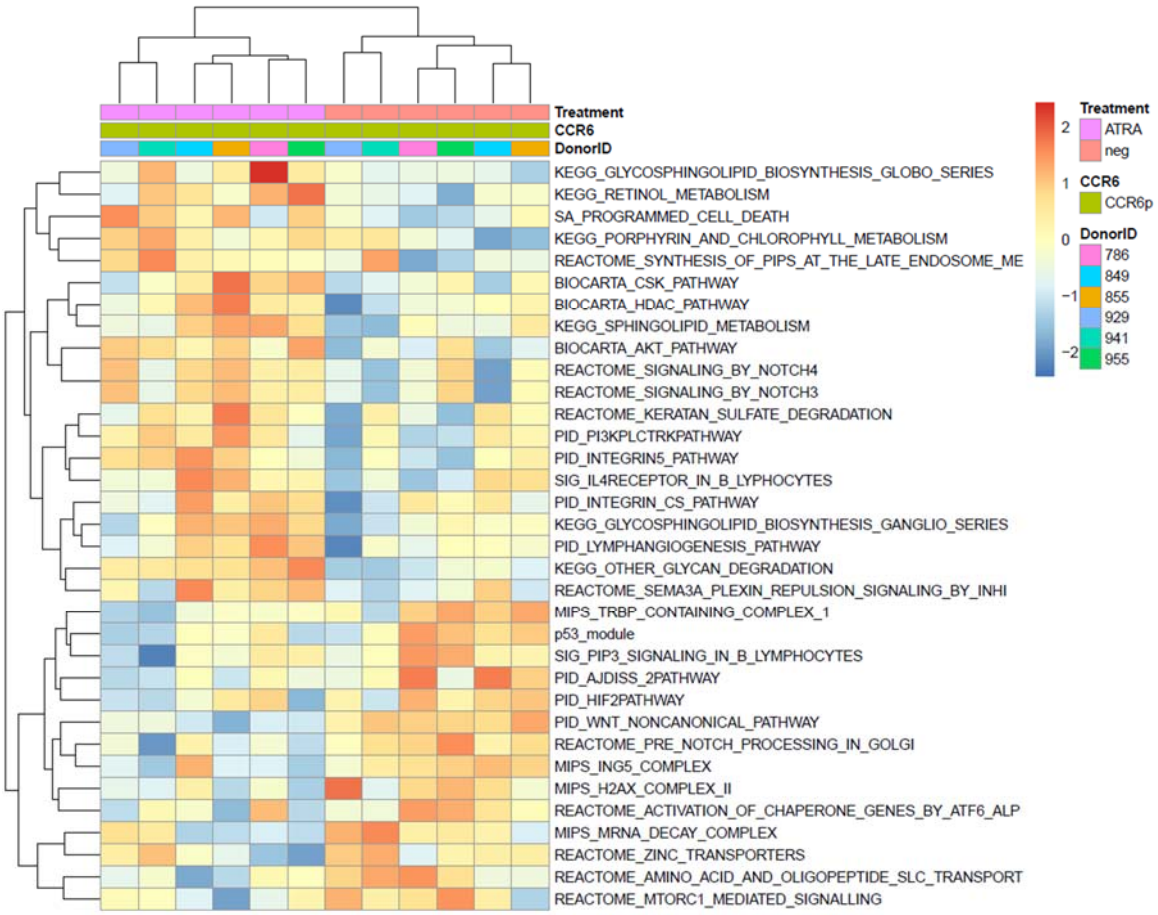


Figure 3: Canonical pathways modulated by ATRA in CCR6$^+$ T-cells. Memory CCR6$^+$/CCR6$^-$ T-cells were sorted and stimulated as described in Figure 1. Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling as described in Figure 2. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). The heat map depicts top 34 pathways up (red) and down (blue) modulated by ATRA in CCR6$^+$ T-cells, identified using gene-set variation analysis (GSVA; p-value<0.01;enrichment z-score).

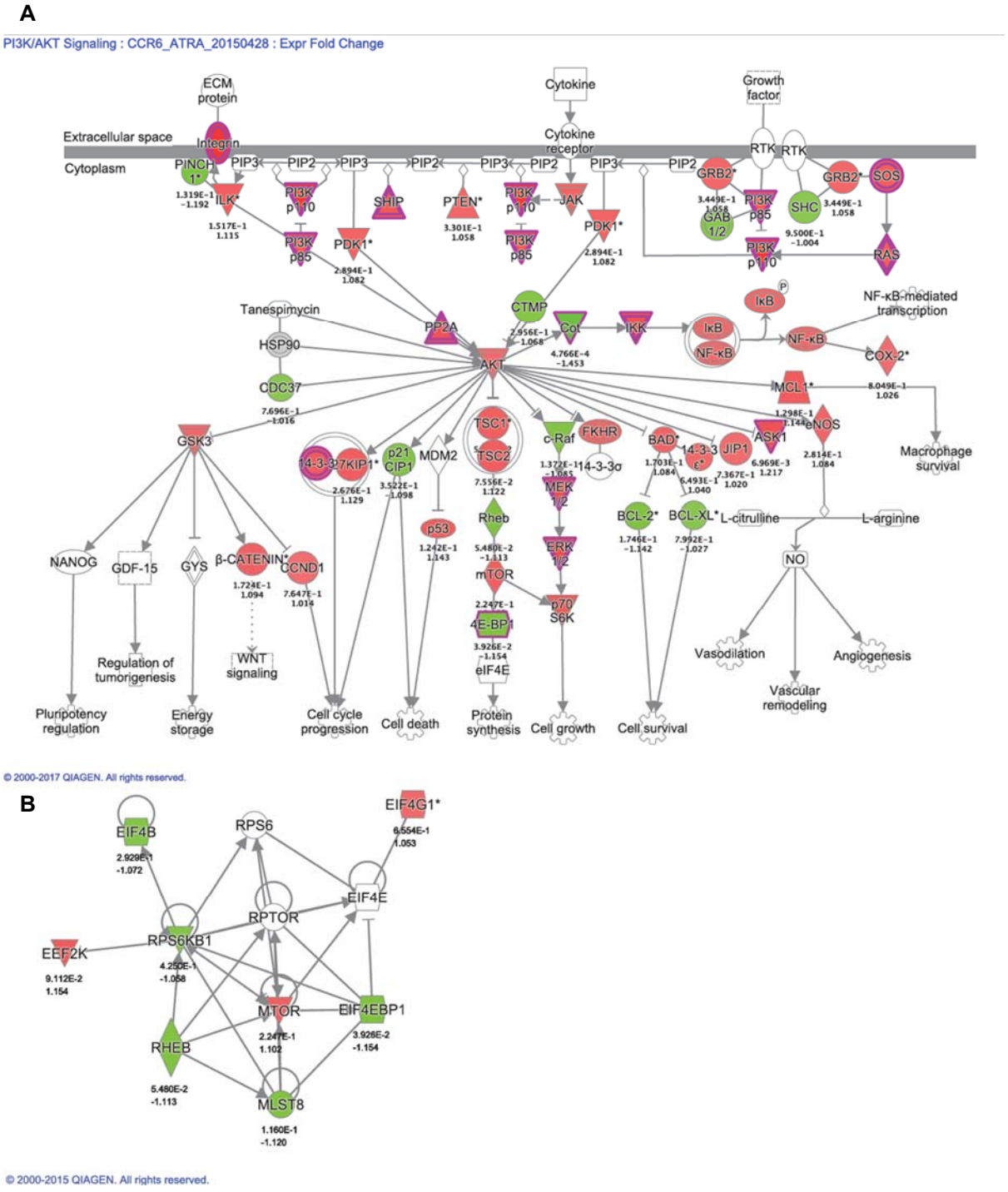


Figure 4: Components of the AKT/PI3K pathways modulated by ATRA in CCR6⁺ T-cells. Memory CCR6⁺/CCR6⁻ T-cells were sorted and stimulated as described in Figure 1. Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling as described in Figure 2. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). Shown are *(A)* combined BIOCARTA_AKT_PATHWAY and PID_PI3K_PLC_TRK_PATHWAY Canonical pathways significantly modulated by ATRA in CCR6⁺ T-cells, identified using Ingenuity Pathway Analysis®; and **(B)** Gene expression network generated using individual genes representing REACTOME_MTORC1_MEDIATED_SIGNALING, significantly modulated by ATRA in CCR6⁺ T-cells identified through GSVA. The color code is based on expression FC (red and green for up-regulated and down-regulated transcripts, respectively). Nodes representing individual genes are annotated with their corresponding p-values and FC.

Planas *et al.*, Figure 4

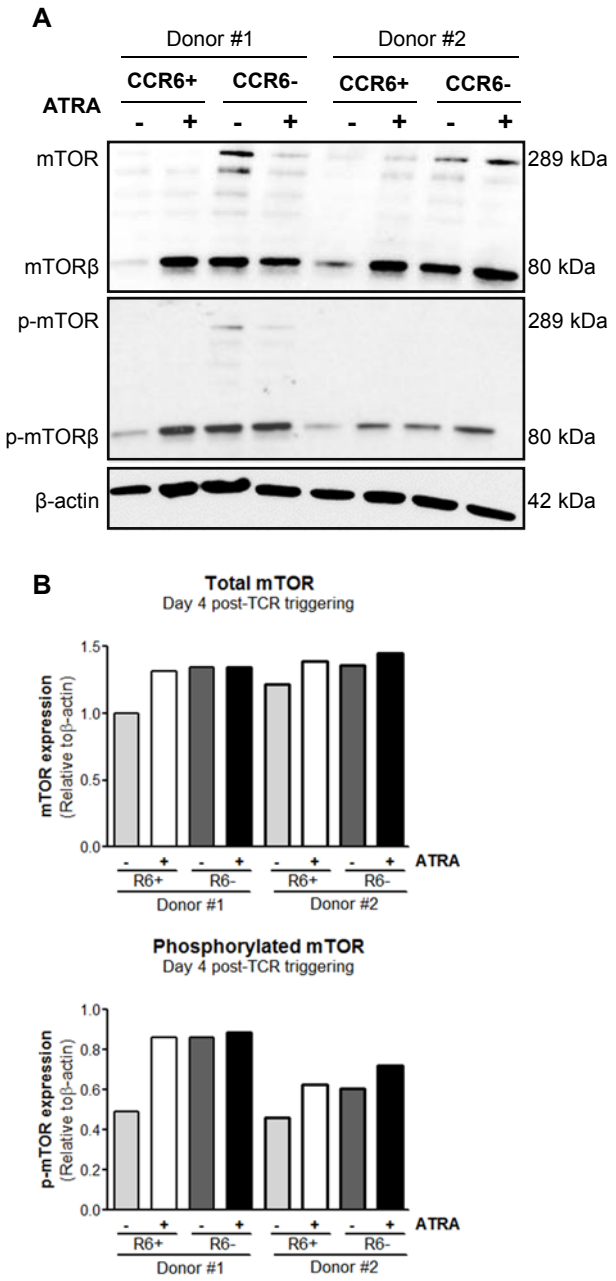


Figure 5: ATRA induces mTOR expression and phosphorylation selectively in CCR6⁺ T-cells. Memory CCR6$^+$ and CCR6$^-$ T-cell subsets were sorted and stimulated as described in Figure 1. **(A)** Cell lysates were used to visualize total and phosphorylated mTOR expression by Western Blotting. **(B)** Levels of total and phosphorylated mTOR expression were quantified by densitometry and normalized relative to β-actin levels. Of note, the molecular weight of the predominant mTOR band was 80 kDa, corresponding to the β isoform of mTOR (1). Shown are results obtained with cells from two different donors.

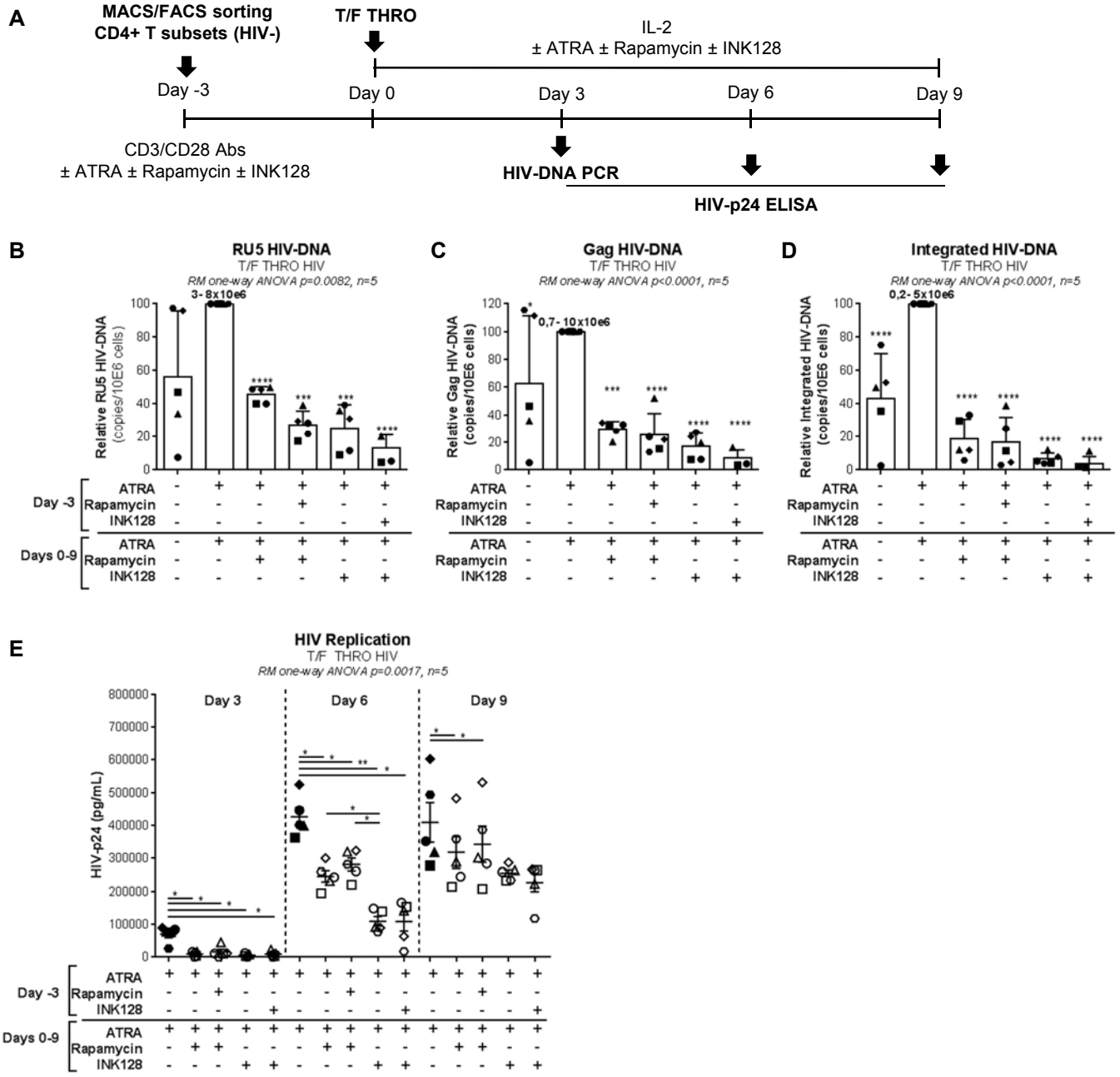


Figure 6: mTOR inhibitors counteract the effect of ATRA on HIV permissiveness in memory CD4+ T-cells. The ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication was tested in total memory $CD4^+$ T-cells **(A)**. Briefly, total memory $CD4^+$ T-cells were stimulated *via* CD3/CD28 in presence/absence of ATRA and/or rapamycin (10 nM) or INK128 (50 nM) for 4 days. Then, cells were exposed to replication competent T/F HIV THRO (25 ng HIV-p24/10^6 cells). Further, cells were cultured for 9 additional days in presence/absence of ATRA and/or rapamycin or INK128. Shown are the relative RU5, Gag, and integrated HIV-DNA levels quantified by real-time nested PCR at day 3 post-infection (with the range of absolute HIV-DNA copies/10^6 cells in ATRA-treated cells, considered 100%, being indicated on the figures) **(B-D)** and HIV-p24 levels quantified by ELISA in cell culture supernatants at days, 3, 6 and 9 post-infection **(E)** (n=5, mean±SEM) . The RM one-way ANOVA p-values **(B-E)**, with the significance of the Dunnetts's (relative to cells stimulated *via* the TCR in the presence of ATRA) **(B-C-D)** or Tukey's **(E)** multiple comparisons test are indicated on the figures. Each symbol represents one different donor.

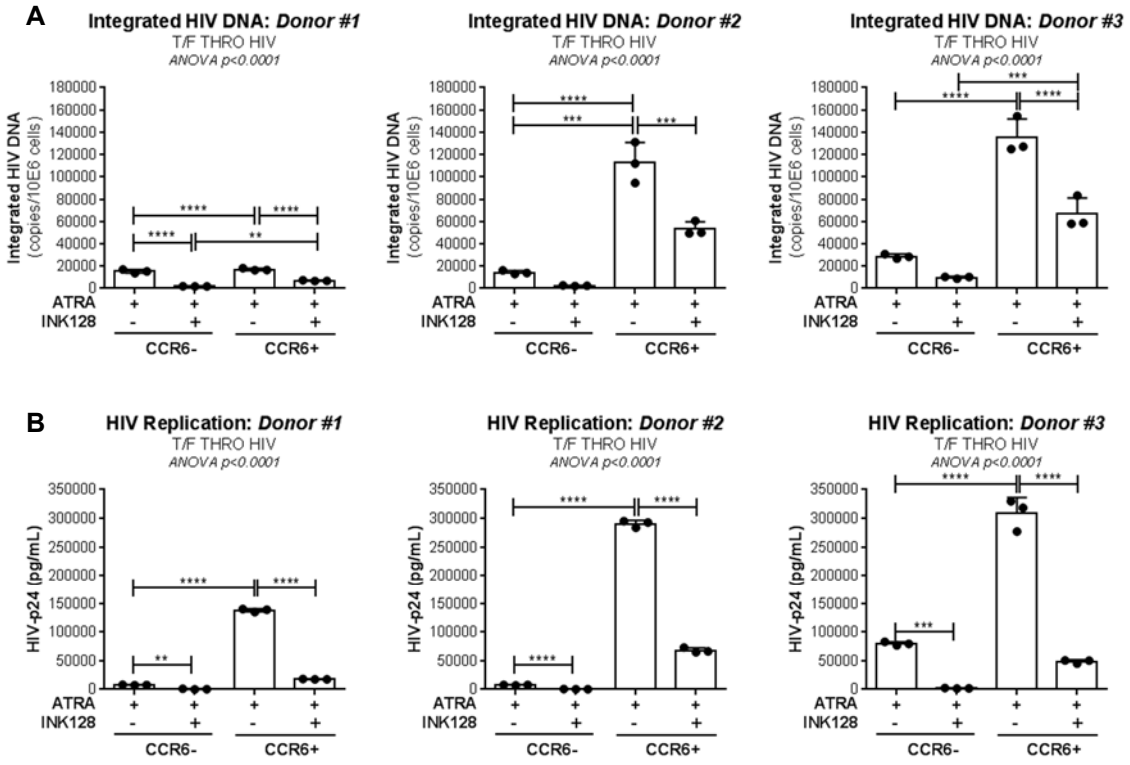


Figure 7: mTOR inhibitors counteract the effect of ATRA on HIV permissiveness in sorted CCR6+ and CCR6- T-cells. Memory CCR6+ and CCR6- T-cells were sorted as depicted in Figure 1 and stimulated *via* CD3/CD28 in presence of ATRA or ATRA and INK128 (50 nM) for 4 days. Then, cells were exposed to replication competent T/F HIV THRO (25 ng HIV-p24/10^6 cells). Further, cells were cultured for 9 additional days in presence of ATRA or ATRA and INK128. **(A-B)** Shown are absolute levels of integrated HIV-DNA at day 3 post-infection **(A)** and HIV-p24 levels in cell culture supernatants at day 6 post-infection **(B)** (n=3, mean±SD of triplicate wells), Ordinary one-way ANOVA with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one experimental triplicate value in individual donors.

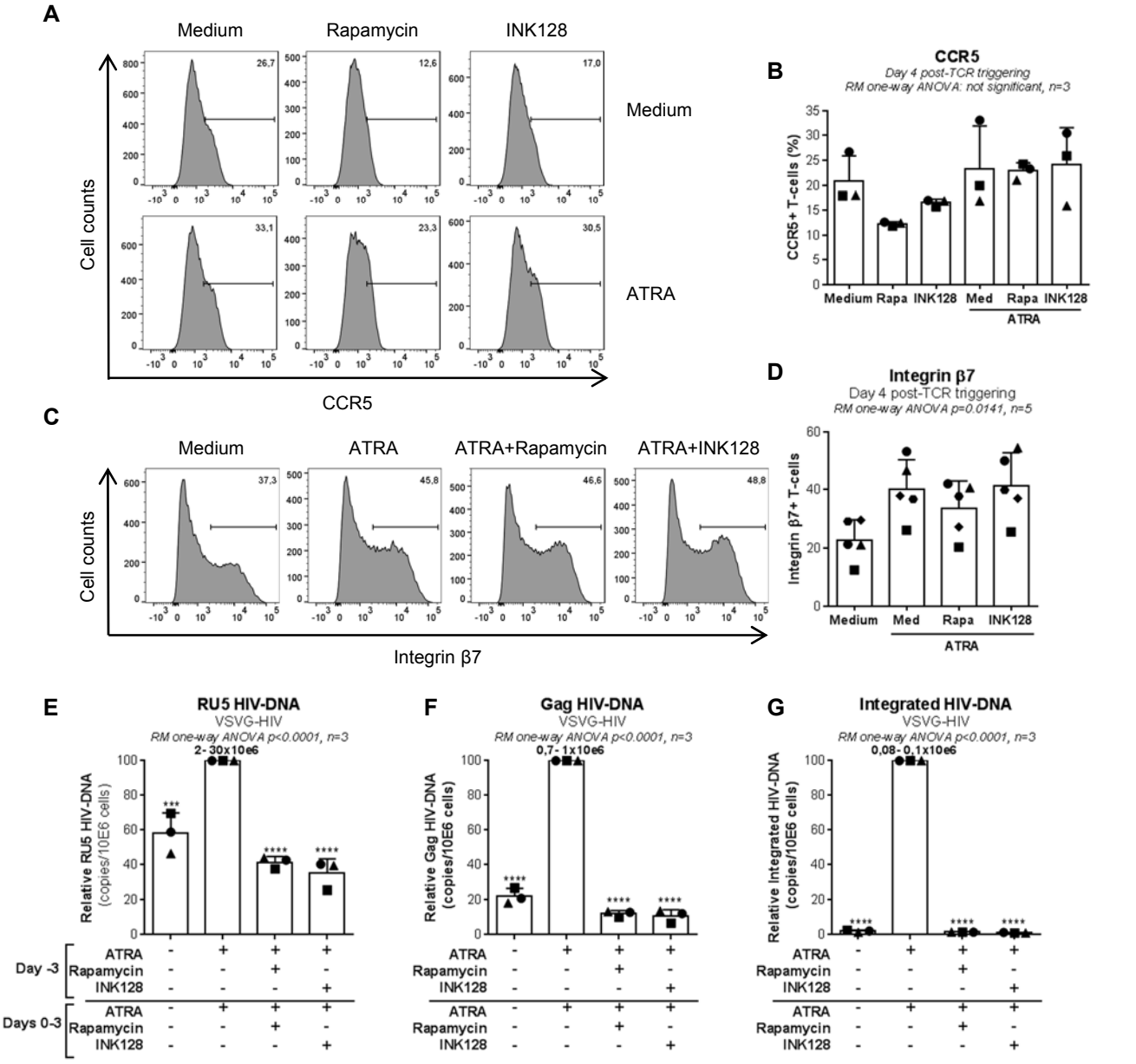
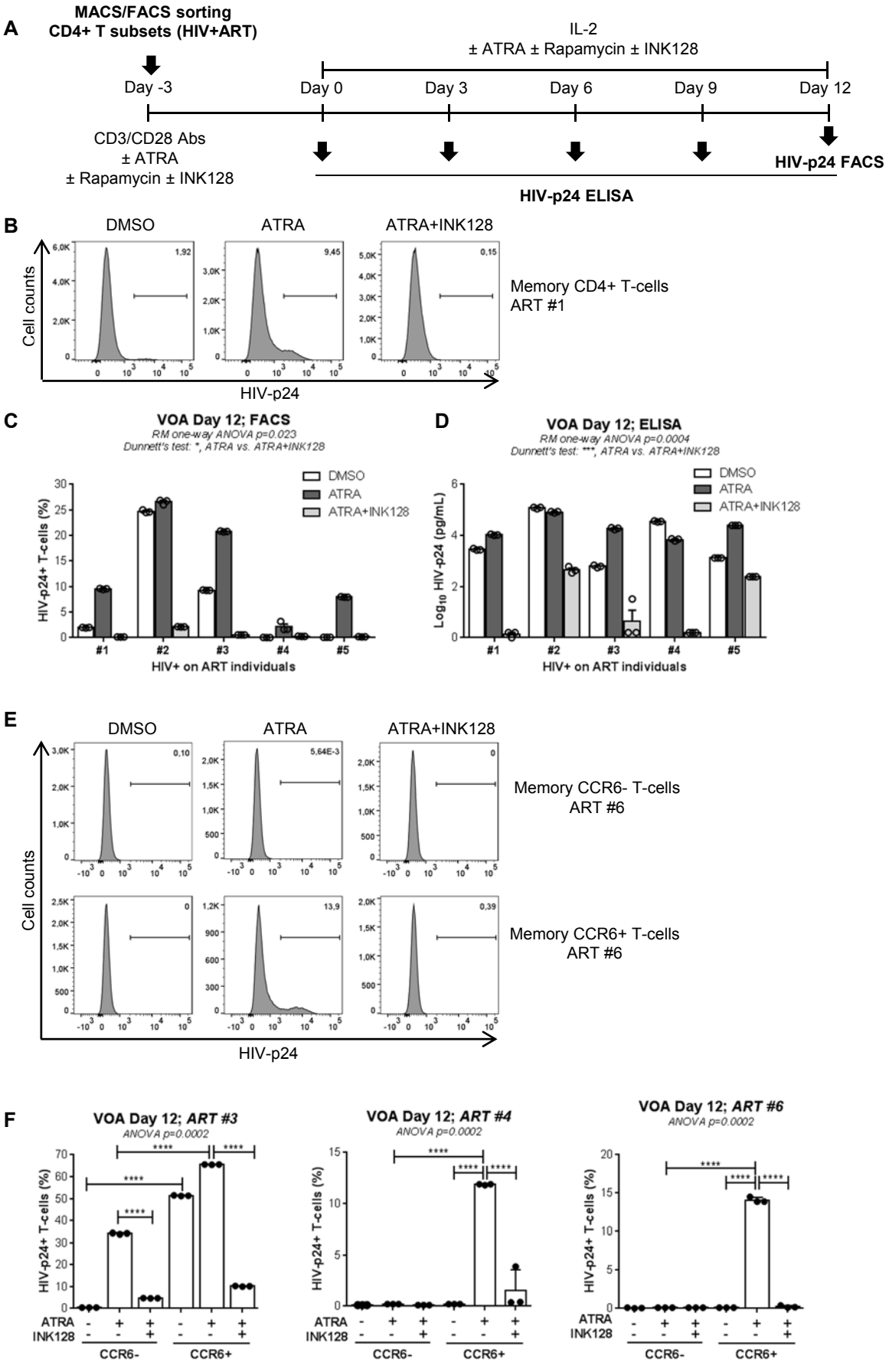


Figure 8: mTOR inhibitors limit HIV replication at post-entry levels in ATRA-treated CD4⁺ T-cells. (A-D) Memory CD4⁺ T-cells were first analyzed for the surface expression of CCR5 and integrin β7 at day 4 post-TCR triggering in the presence/absence of ATRA (10 nM), rapamycin (10nM), and/or INK128 (50nM). Shown are levels of CCR5 **(A)** and integrin β7 **(C)** expression in one representative donor and statistical analysis of CCR5 (**B**; n=3) and integrin β7 (**D**; n=5) expression in different donors (mean±SEM). **(E-G)** Then, stimulated cells were exposed to VSV-G-pseudotyped HIV (25 ng HIV-p24/10^6 cells) (single round infection) and cultured in presence/absence of ATRA and/or rapamycin or INK128 for 3 additional days. Levels of RU5 **(E)**, Gag **(F)** and integrated HIV-DNA **(G)** were quantified by nested real-time PCR in cells harvested at day 3 post-infection. **(E-G)** Shown are relative HIV-DNA levels (mean±SEM; n=3); the range of absolute HIV-DNA copies/10^6 cells in ATRA-treated CCR6⁺ T-cells (considered 100%) is indicated on the figure. The RM one-way ANOVA p-values with the significance of the Tukey's **(B-D)** or Dunnetts's (relative to cells stimulated *via* the TCR the presence of ATRA) **(E, F, G)** multiple comparisons tests are indicated on the graphs. Each symbol represents one different donor.



Planas *et al*., Figure 9

Figure 9: Drug-mediated mTOR blockade counteracts the enhancing effects of ATRA on HIV reactivation in a viral outgrowth assay. HIV reactivation was measured in a viral outgrowth assay (VOA), as summarized in the flowchart **(A)**. Briefly, total memory $CD4^+$ T-cells (**B-D**; n=5) and FACS-sorted memory CCR6+ and CCR6- T-cells (**E-F**; n=3) were isolated from PBMCs of HIV+ART individuals (Table S5; ART#1-6). Cells were activated *via* CD3/CD28 and cultured in presence/absence of ATRA (10 nM) and/or INK128 (50 nM). Shown is intracellular HIV-p24 expression in total memory CD4+ T-cells **(B-C)** and sorted memory CCR6+ and CCR6- T-cells **(E-F)** quantified by FACS, as well as HIV-p24 levels quantified by ELISA in cell culture supernatants of total memory CD4+ T-cells **(D)** harvested at day 12 post-activation. Results are depicted in individual donors. Each point represents one experimental triplicate (mean±SD of triplicate wells). The RM one-way ANOVA p-values with the significance of the Dunnetts's multiple comparisons test (relative to cells stimulated *via* the TCR the presence of ATRA) **(C-D)** and the ordinary one-way ANOVA with the significance of the Tukey's multiple comparisons test **(F)** are indicated in the figures.

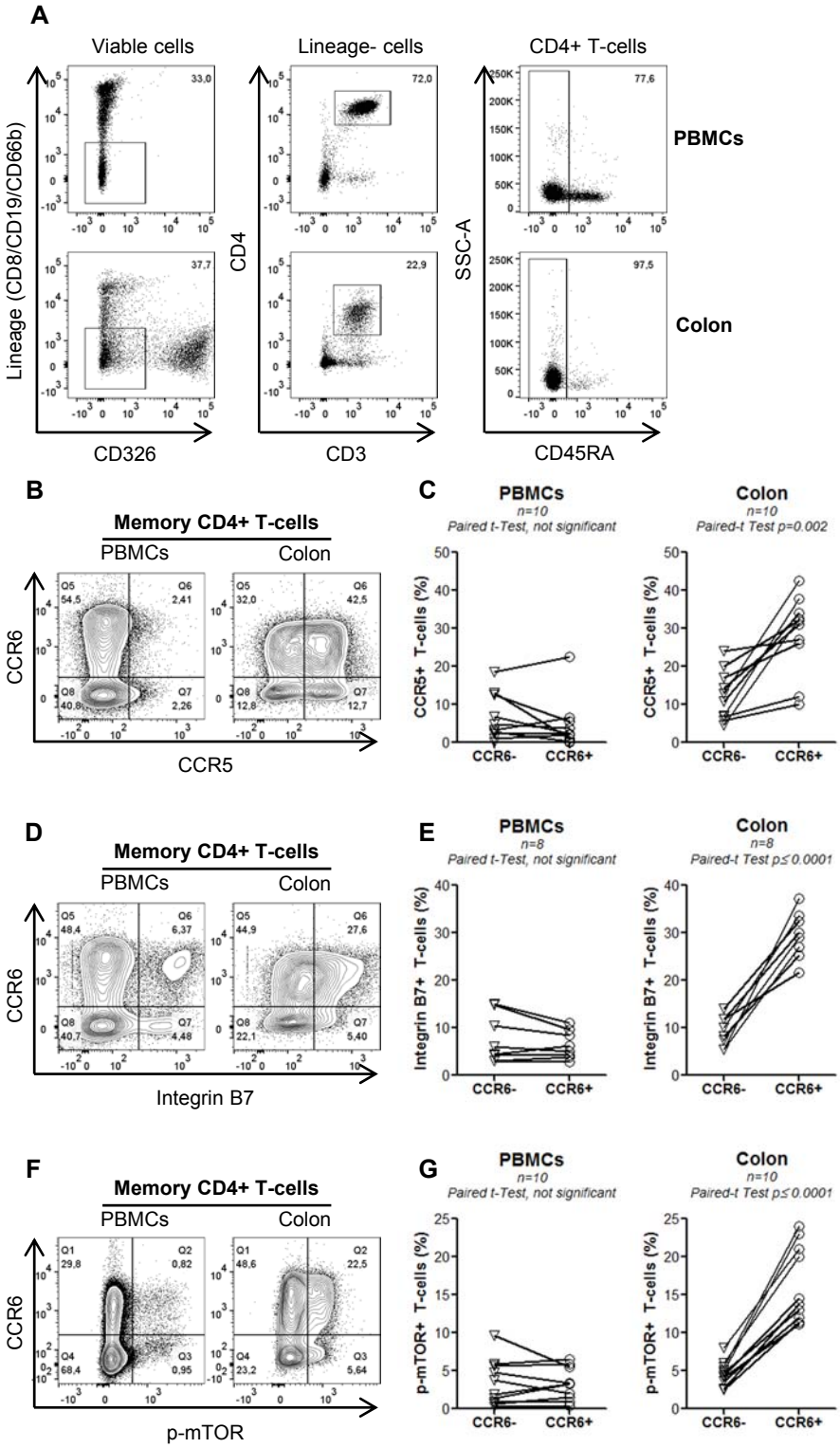


Figure 10: Memory CCR6^{+}CD4^{+} T-cells infiltrating the colon of HIV+ART individuals express high levels of CCR5, integrin β7, and phosphorylated mTOR. Matched blood and sigmoid biopsies were collected from HIV-infected individuals with undetectable plasma viral load (<40 HIV-RNA copies/ml) under ART (Table S5; COL#1-10). Cells were extracted and stained with a cocktail of specific Abs. Shown is the gating strategy for the flow cytometry identification of CD3^{+}CD4^{+}CD45RA^{-} T-cells **(A)**, as well as the surface expression of CCR5 **(B-C)** and integrin β7 **(D-E)** and the intracellular expression of phosphorylated mTOR **(F-G)** on CCR6^{+} and CCR6^{-} subsets from sigmoid biopsies *versus* PBMC in one representative donor (B, D, and F) and statistical analysis in all donors **(C, E, and G)**. Paired t-Test values are indicated on the graphs.

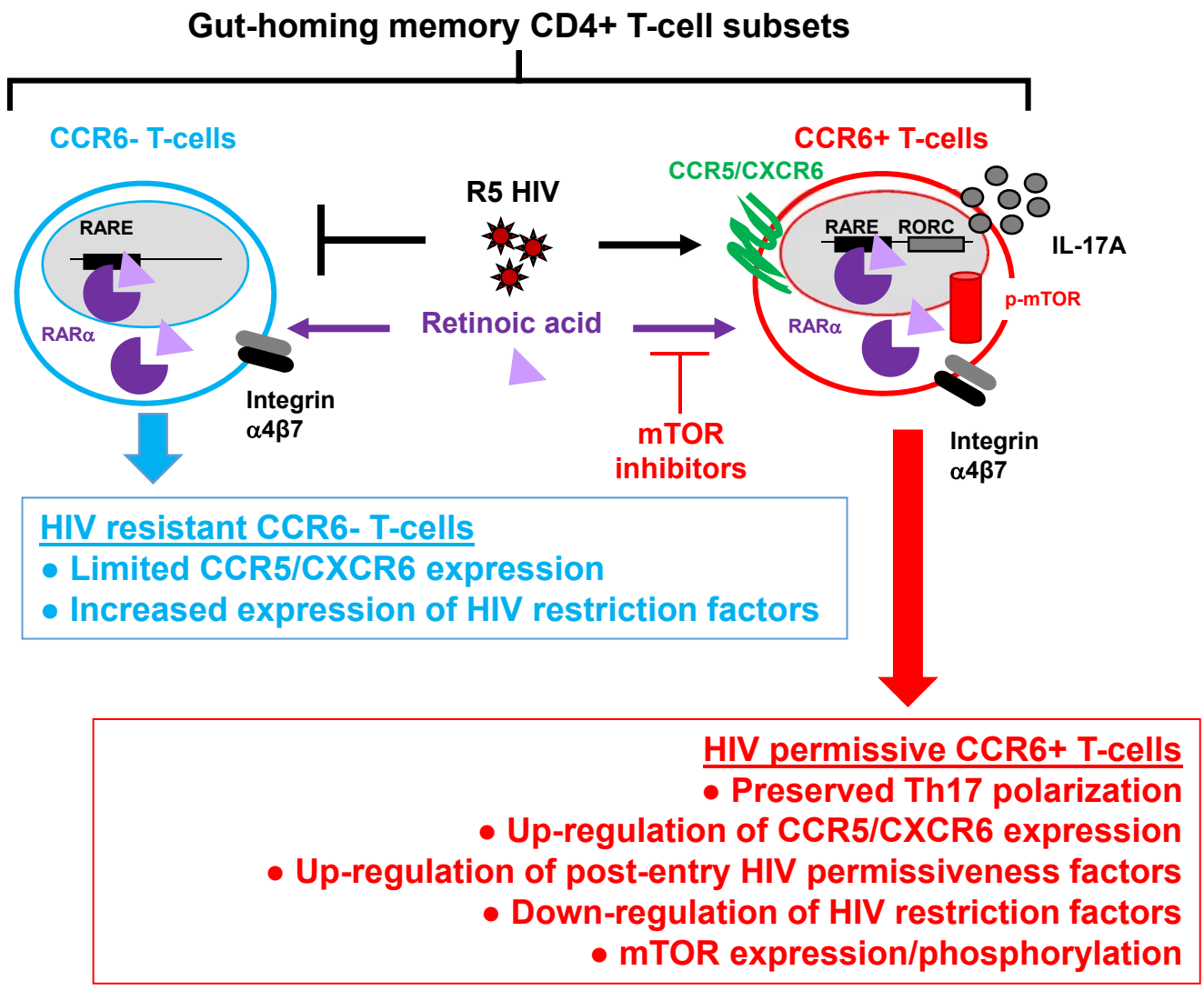


Figure 11: Mechanisms contributing to HIV replication in gut-homing CCR6⁺ T-cells. In this manuscript, we provide evidence that CCR6 is a marker for memory CD4⁺ T-cells programmed to become HIV targets, especially in the intestinal environment rich in retinoic acid, due to their expression of a unique Th17-polarized transcriptional program and a mTOR-governed metabolic state. We propose the use of mTOR inhibitors together with ART to protect gut-homing Th17 cells from HIV infection/persistence.