# HIV-1 reprograms resting CD4 T cells via Foxo1 and L-selectin suppression

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**Background:** Forkhead Box protein 01 (Foxo1, FKHR) is a transcription factor central to T cell biology. Foxo1 is necessary for the maintenance of T cell quiescence and for the expression of CD62L (L-selectin), an adhesion molecule that controls T cell migration into lymph nodes. TCR stimulation suppresses Foxo1 activity through PI3K/Akt-dependent phosphorylation of Foxo1 and its relocation to the cytoplasm. Foxo1 inactivation results in CD62L transcriptional repression and the activation or repression of a series of genes that regulate T cell activation, migration, growth and differentiation and survival.

**Methods**: Purified peripheral blood and tonsil CD4 T cells were infected with HIV-1 GFP reporter viruses (1-2). Blood and tonsil cells were analyzed by flow cytometry. Blood cells were analyzed by ImageStream (Amnis) for Foxo1 and NF-κB localization and TransAM ELISA (Active Motif) for Foxo1 sequence-specific DNA binding activity. RNA were analyzed by TaqMan qRT-PCR (3). Cell survival was measured by flow cytometry.

**Results:** HIV-1 down-modulated CD62L on productively infected naïve and memory (TCM and TEM) resting CD4 T cells (4). Partial T cell activation was further revealed by upregulation of the mRNA and surface expression of CD69, a Foxo1-suppressed gene. HIV-1-induced CD62L down modulation was inhibited by the PI3K inhibitors LY294002 and PI-103, indicating that activation of the PI3K/Akt pathway contributes to this effect. ImageStream analysis demonstrated relocation of Foxo1 to the cytoplasm in productively infected resting naïve and memory CD4 T cells, and TransAM ELISA demonstrated loss of Foxo1 transactivation activity. A series of Foxo1 regulated mRNA were decreased or increased in productively infected resting CD4 T cells, including CD62L, IL-7rα, Myc, KLF2, CCR5, Fam65b, S1P$_1$ (EDG1), CD52 and Cyclin D2, indicating a profound reprogramming of these cells by HIV-1. Application of the Foxo1 inhibitor AS1842856 accelerated *de novo* viral gene expression and enhanced infected cell viability. Finally, Foxo1 inhibition enhanced infected cell survival, while suppression of the Foxo1 inactivation pathway with an AKT inhibitor specifically enhanced the death of infected cells.

**Conclusions:** HIV-1 suppression of Foxo1 activity is apparently a strategy to promote viral replication in resting CD4 T cells. Foxo1 suppression both preserves infected cell viability and enhances virus expression. Dysregulation of Foxo1 may contribute to immune activation, viral load and HIV-1 pathogenesis. Inhibiting the PI3K/Akt pathway and Foxo1 inactivation may be therapeutically useful in promoting the elimination of HIV-1 reservoirs. As Foxo1 is an investigative cancer therapy target, the ongoing development of Foxo1 interventions may assist the quest to specifically suppress or activate HIV-1 replication *in vivo*.

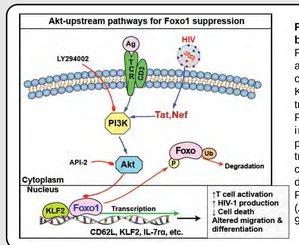



**Figure 1. Simplified schematic of Foxo1 inactivation by the PI3K/Akt pathway.** The transcription factor Foxo1 binds DNA in a sequence-specific manner and activates or suppresses many genes including activation of CD62L (L-Selectin, IL-7rα and the transcription factor KLF2. CD62L suppresses transcription of CD69. The transcription factor KLF2 frequently cooperates with Foxo1 to activate genes. T cell activation or HIV-1 infection can activate the kinase Akt, which phosphorylations nuclear Foxo1 resulting in its losing transactivation activity. Foxo1 then migrates to the cytoplasm where it can be degraded by a ubiquitin-dependent pathway or remain cytoplasmic. Inhibitors of PI3K are examined in Figure 3 and an inhibitor of Akt (API-2) is examined in Figure 8 and discussed in Figure 9.

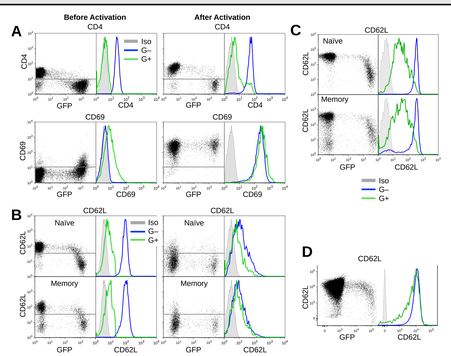



**Figure 2. Down-modulation of CD62L and upregulation of CD69 on productively infected naïve and memory resting CD4 T cells.** Resting IL-7 treated CD4+ T cells were infected with the GFP reporter virus NLENG1-IRES. Flow analysis was carried out on day 5 post infection on cells with and without activation by αCD3/CD28 beads added on day 3 p.i. Grey: isotype-matched IgG control antibody staining. Blue: GFP-negative cells. Green: GFP+ cells. **A.** CD4 down modulation and CD69 upregulation. **B.** Down modulation of CD62L on CD45RO-neg. and CD45 RO+ CD4 T cells. **C.** IL-4 also supports HIV-1-induced down-modulation of CD62L on both naïve and memory resting CD4+ T cells. **D.** CD62L down-modulation on HIV-1 infected naïve tonsil T cells 7 days after infection. Similar results were observed on CD45R0+cells (not shown).

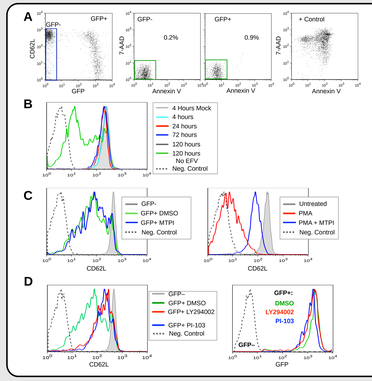



**Figure 3. CD62L down-modulation is not the result of apoptosis, virus contact or protease cleavage, but is facilitated by PI3K signaling.**

**A.** Cells infected with for 5 days, then were analyzed for GFP, CD62L expression, Annexin V and 7-AAD staining.
**B.** Contact with HIV-1 virions does not affect CD62L surface expression. Cells were treated with the reverse transcriptase inhibitor efavirenz (EFV) (2 μg/ml) and infected by spinoculation or spinoculated without virus (Mock). Surface CD62L expression was analyzed at the indicated times after infection. A positive control infection was performed without EFV as indicated. EFV completely prevented the appearance of GFP+ cells (not shown).
**C.** Metalloprotease inhibitor (MTPI) TAPI-1 (50 μM) does not inhibit HIV-1-induced CD62L surface down-modulation. Left: Control experiment demonstrating inhibition of PMA-induced CD62L downmodulation by MTPI. Cells were treated with PMA with and without MTPI and analyzed one hour later. Right: Cells infected with HIV-1 GFP reporter virus were treated with MTPI 1 day and again 3 hours prior to staining.
**D.** The PI3K inhibitors LY294002 and PI-103 suppress HIV-1-induced CD62L downmodulation. Infected cells were treated with LY294002 (5 μM), PI-103 (5 μM) or a carrier control (DMSO) 1 day and again 4 hours prior to staining for CD62L. Right: GFP expression is modestly suppressed by LY294002 and PI-103 (MFI = 1761 and 1471 respectively, vs.1981 for DMSO carrier control).

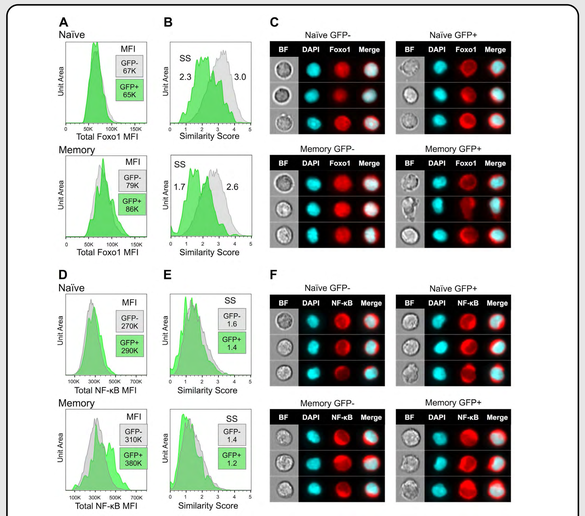


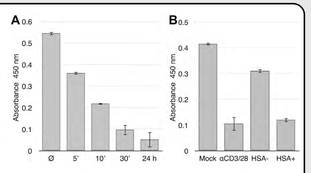

**Figure 4. ImageStream analysis of Foxo1 and NF-kB in HIV-1 infected cells.** HIV-1 GFP-reporter virus-infected resting CD4+ T cells were stained for CD45RA, intracellular Foxo1 or NF-kB and for DNA (DAPI) 7 days after infection, then analyzed by ImageStream (Amnis).

**A.** Total Foxo1 expression in naïve and memory GFP+ and GFP- cells. MFI = Mean Fluorescence Intensity.
**B.** Nuclear localization was analyzed in IDEAS software by comparison of Foxo1 distribution to nuclear DAPI staining using the Similarity Score (see materials and methods).
**C**. Representative ImageStream pictures of individual cells stained for Foxo1.
**D.** Total NF-kB expression in naïve and memory GFP+ and GFP- cells.
**E.** NF-kB nuclear Similarity Scores.
**F.** Representative ImageStream pictures of individual cells stained for NF-kB.

**Figure 5. Foxo1 activity by TransAM ELISA.**

**A.** Time course of Foxo1 inactivation following T cell activation with anti-CD3/CD28 beads. The ability of nuclear Foxo1 to bind a target DNA oligonucleotide was analyzed using a TransAM Foxo1 kit (Active Motif). Specificity was demonstrated by successful competition with a Foxo1 binding oligonucleotide but not with a negative control oligonucleotide (not shown).

**B.** Foxo1 activity in HIV-1 infected resting CD4 T cells, mock infected and beads activated cells. Cells were infected with HIV-1 HSA reporter virus for 7 days, then separated into HSA+ and HSA-negative subsets using an antibody to HSA and magnetic beads.



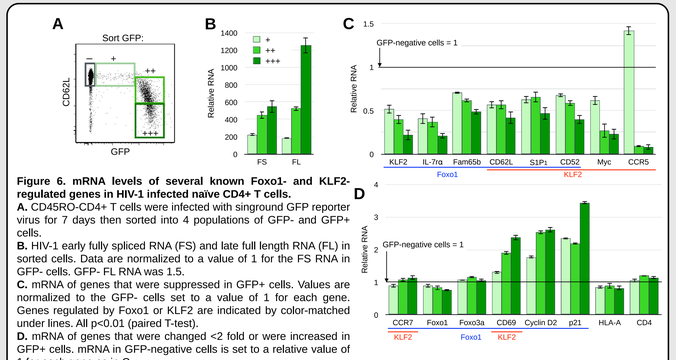




**Figure 6. mRNA levels of several known Foxo1- and KLF2-regulated genes in HIV-1 infected naïve CD4+ T cells.**
**A.** CD45RO-CD4+ T cells were infected with singround GFP reporter virus for 7 days then sorted into 4 populations of GFP- and GFP+ cells.
**B.** HIV-1 early fully spliced RNA (FS) and late full length RNA (FL) in sorted cells. Data are normalized to a value of 1 for the FS RNA in GFP- cells. GFP- FL RNA was 1.5.
**C.** mRNA of genes that were suppressed in GFP+ cells. Values are normalized to the GFP- cells set to a value of 1 for each gene. Genes regulated by Foxo1 or KLF2 are indicated by color-matched under lines. All p<0.01 (paired T-test).
**D.** mRNA of genes that were changed <2 fold or were increased in GFP+ cells. mRNA in GFP-negative cells is set to a relative value of 1 for each gene as in C.




**Figure 7. Foxo1 inhibitor AS1842856 accelerates and increases total HIV-1 production.**
AS1842856 (AS) was applied to cells 1 day before and 1 day after infection with single round GFP reporter virus. CD45RO- naïve T cells are shown.

**A.** AS1842856 accelerates the emergence of GFP+ cells.
**B.** AS1842856 increases HIV-1 expression measured as the mean fluorescence intensity (MFI) of GFP, which we have previously shown directly corellates with transcriptional activity.
**C.** AS1842856 accelerates the production of virion and increases total virus production.
**D.** Fold induction of each value in A-C plus the product of %GFP+ x GFP MFI (blue) as an indication of total HIV-1 gene expression in the system.
**E.** CD62L down modulation is accelerated in HIV-1 infected cells by Foxo1 inhibitor and lowered modestly in GFP-negative cells.
**F.** CD69 upregulation is synergistically increased in productively infected (GFP+) naïve and memory CD4 T cells by Foxo1 inhibitor AS1842856.

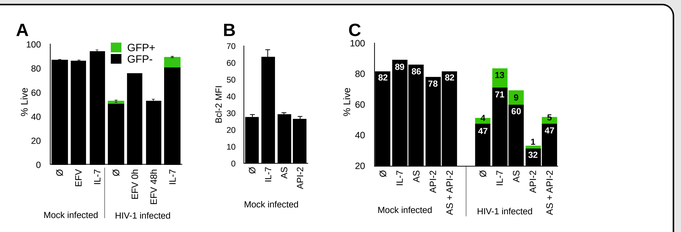



**Figure 8. Foxo1 inhibition is cytoprotective during HIV-1 infection, while AKT suppression is specifically toxic to HIV−1 infected resting CD4 T cells.**

**A:** HIV-1 infection is cytopathic, NNRTI efavirenz (EFV) partially rescues cells, while IL-7 rescues 100% and allows productive infection (green).
**B:** IL-7 induces Bcl-2 while AS1842856 does not. MFI = mean Bcl-2 fluorescence in flow cytometry.
**C:** AS1842856 rescues cells from HIV-1-induced death, AKT inhibitor API-2 specifically induces infected cell death and AS1842856 partially rescues this. Method: HIV−1-GFP infected or mock infected cells were treated with the indicated agents immediately after infection. Cells were analyzed for viability (SSC, FSC) and GFP expression on day 7 p.i. AS=AS1842856; API-2 (triciribine, 100nm) is a specific AKT inhibitor. Data are representative of 3 independent experiments.

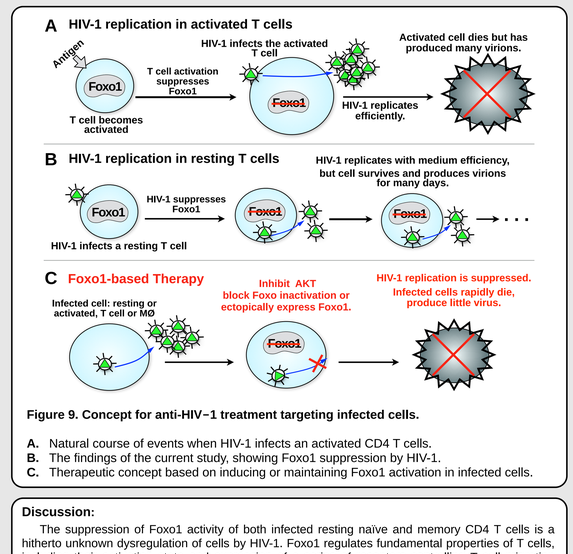



**Figure 9. Concept for anti-HIV−1 treatment targeting infected cells.**

**A.** Natural course of events when HIV-1 infects an activated CD4 T cells.
**B.** The findings of the current study, showing Foxo1 suppression by HIV-1.
**C.** Therapeutic concept based on inducing or maintaining Foxo1 activation in infected cells.

**Discussion:**

The suppression of Foxo1 activity of both infected resting naïve and memory CD4 T cells is a hitherto unknown dysregulation of cells by HIV-1. Foxo1 regulates fundamental properties of T cells, including their activation state and expression of a series of receptors controlling T cell migration such as CD62L; consequently the implications of this finding is thus wide ranging. The activation state of productively infected resting CD4 T cells was elevated, as they displayed increased CD69 and cyclin D2 mRNA expression and down-modulation of CD62L, all of which are Foxo1 target genes. A primary implication of this finding is that by raising the activation state of the infected cell, Foxo1 inactivation might enhance virus replication. Indeed, application of the Foxo1 inhibitor AS1842856 accelerated and increased virus expression. Interestingly, the herpesvirus Epstein-Barr Virus also suppresses Foxo1 activity (5), suggesting that Foxo1 inactivation might be a common viral strategy. Foxo1 is dysregulated in many cancers and the possibility of its therapeutic manipulation is avidly investigated. Control of Foxo1 activity might also provide a means to activate or suppress HIV-1 expression in resting T cells.

Importantly, inhibition of Foxo1 using AS1842856 rescued cells from death but without inducing Bcl-2. The specific AKT inhibitor API-2 (triciribine), a tricyclic nucleoside with anti-tumor and anti-HIV-1 properties, was non-toxic to uninfected cells but magnified death of infected resting T cells. AS1842856 also reduced API-2-induced cell death. AS1842856 bypasses AKT and directly binds to and inhibits Foxo1 in a phosphorylation-independent mechanism. Therefore, it may be useful to inhibit AKT as an adjuvant to curative therapies designed to eliminate HIV infected cells.The ability of PI3K inhibitors to partially inhibit CD62L down-modulation in infected cells, plus the relocalization of Foxo1 to the cytoplasm both implicate phosphorylation in HIV-1-induced Foxo1 inactivation. HIV-1 is known to activate PI3K and Akt by the interaction of envelope with resting T cells and through the activities of Tat and Nef. Each of these proteins has long been known to enhance both T cell activation and infection of resting T cells. Our findings likely add another consequence of HIV-1-induced Akt activation; however, Foxo1 phosphorylation has been difficult to document in our system. Studies are underway to define the kinetics of Foxo1 inactivation and the HIV-1 gene(s) and signaling pathway(s) responsible.

CD62L, KLF2, CD69, S1P$_1$, CCR5 and Fam65b expression were each affected by HIV-1 infection of resting CD4 T cells. Each of the genes has a profound effect on the behavior of T cells, particularly their migration, activation and survival. These potential effects might be moot if the cell were to rapidly die due to viral cytopathicity, but infected resting T cells survive for much greater lengths of time than infected activated T cells. While CD62L is required for entry into lymph nodes, S1P1 is required for egress in response to its ligand S1P present in blood. KLF2 and Foxo1 both induce S1P$_1$ expression. T cell responses to S1P$_1$ are impaired in HIV-1 infected individuals, perhaps contributing to lymphadenopathy. S1P$_1$ and CD69 are mutual antagonists and CD69 expression inhibits S1P1-dependent egress from lymphoid organs until later in the activation cascade, when CD69 levels decline. CD69 also regulates chemokine expression and T cell accumulation in the intestine. Thus, by increasing CD69 and suppressing both CD62L and S1P$_1$, HIV-1 could disrupt both lymphoid tissue entry and exit functions of infected cells.

Resting CD4 T cells are the dominant target of infection soon after transmission, the period when antiviral immunity is being established that will determine the outcome of infection. Much of the latent reservoir is also established early in infection. By manipulating the cells' activation state and the expression of receptors that support migration in and out of lymphoid tissues (and which also possess intracellular and extracellular signaling properties), HIV-1 could interfere with the establishment of antiviral immunity through suppression of Foxo1 activity.

**References:**


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