VIROLOGY

Cofilin hyperactivation in HIV infection and targeting the cofilin pathway using an anti-$\alpha_4\beta_7$ integrin antibody

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A functional HIV cure requires immune reconstitution for lasting viremia control. A major immune dysfunction persisting in HIV infection is the impairment of T helper cell migration and homing to lymphoid tissues such as GALTs (gut-associated lymphoid tissues). ART (antiretroviral therapy) does not fully restore T cell motility for tissue repopulation. The molecular mechanism dictating this persistent T cell dysfunction is not understood. Cofilin is an actin-depolymerizing factor that regulates actin dynamics for T cell migration. Here, we demonstrate that blood CD4 T cells from HIV-infected patients ($n = 193$), with or without ART, exhibit significantly lower levels of cofilin phosphorylation (hyperactivation) than those from healthy controls ($n = 100$; ratio, 1.1:2.3; $P < 0.001$); cofilin hyperactivation is also associated with poor CD4 T cell recovery following ART. These results suggest an HIV-mediated systemic dysregulation of T cell motility that cannot be repaired solely by ART. We further demonstrate that stimulating blood CD4 T cells with an anti–human $\alpha_4\beta_7$ integrin antibody can trigger signal transduction and modulate the cofilin pathway, partially restoring T cell motility in vitro. However, we also observed that severe T cell motility defect caused by high degrees of cofilin hyperactivation was not repairable by the anti-integrin antibody, demonstrating a mechanistic hindrance to restore immune functions in vivo. Our study suggests that cofilin is a key molecule that may need to be therapeutically targeted early for T cell tissue repopulation, immune reconstitution, and immune control of viremia.

INTRODUCTION

Antiretroviral therapy (ART) has significantly extended the life span of HIV-infected patients, but it offers neither a cure nor full immune restoration. The natural course of HIV infection leads to multiple CD4 T cell defects (*1*), including the impairment of T cell migration and homing to lymphoid tissues such as GALTs (gut-associated lymphoid tissues) (*2*-*4*). Even with near-complete viral suppression with ART, normal levels of CD4 T cell repopulation to lymphoid tissues are rarely achieved (*2*, *3*, *5*, *6*), thereby dampening immune responses and preventing full immune reconstitution for lasting viremia control (*7*). In HIV-infected patients, the vast majority of circulating CD4 T cells are not HIV infected (0.2 to 16.4 HIV^+ cells per million) (*8*). Thus, the T cell migratory defect seen in patients likely results from a bystander effect from chronic signaling by viral (*9*, *10*) and/or inflammatory factors (*1*, *2*). However, the molecular mechanism dictating this persistent T cell dysfunction has not been fully elucidated. This has hindered the development of an effective therapy to restore T cell functions and to achieve persistent immune control of HIV.

In the human immune system, T cell activity is mainly regulated by receptor signaling. Persistent signaling through cytokine or chemokine receptors frequently leads to T cell polarization and commitment to distinct lineages such as T helper cell 1 (T_H1) and T_H2. In HIV infection, the virus infects T cells through gp120 binding to CD4 and the chemokine coreceptor CXCR4 (X4) or CCR5 (R5) (*11*, *12*). This binding also initiates aberrant signaling and may have pathogenic consequences (*9*, *10*, *13*, *14*). In particular, HIV signaling through CXCR4 has been shown to activate an actin depolymerizing factor, cofilin, to promote the actin dynamics necessary for viral nuclear entry in blood resting CD4 T cells (*9*, *15*).

Cofilin is an actin-binding protein that binds and depolymerizes filamentous actin (F-actin) to regulate actin treadmilling (*16*, *17*), a process in which monomeric actin (G-actin) is incorporated into F-actin at the (+) end and then dissociated from the (−) end. Cofilin-regulated actin treadmilling is the driving force in T cell motility and T cell migration to lymphoid tissues (*18*). Given that cofilin is a major part of the motility engine in T cells, we hypothesized that the T cell migratory defects seen in HIV-infected patients may directly result from cofilin dysregulation by persistent, pathogenic signaling occurring during HIV infection (*19*). Here, we examined cofilin phosphorylation in the peripheral resting CD4 T cells of HIV-infected patients in a large clinical study. Cofilin activity is regulated by cycles of serine 3 phosphorylation and dephosphorylation that control cofilin binding to F-actin for actin severing and depolymerization

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(*16*, *17*). We confirmed that blood CD4 T cells from HIV-infected patients exhibit significantly lower levels of cofilin phosphorylation (hyperactivation) than those from healthy controls. These results suggest a systemic cofilin-regulated T cell migratory impairment that needs to be therapeutically repaired for T cell tissue repopulation.

Integrins, such as the $\alpha_4\beta_7$ integrin, play an important role in T cell tissue repopulation. Integrins are transmembrane receptors that mediate cell–extracellular matrix and cell-cell interactions to facilitate T cell trafficking and homing to tissues (*20*). This homing process involves synergistic, bidirectional signaling with chemokine receptors (inside-out and outside-in signaling) (*21*): Stimulation of chemokine receptors triggers chemotactic signaling that leads to the assembly of an intracellular complex connecting integrins to the actin cytoskeletons for integrin activation (inside-out signaling), while binding of active integrins to ligands can trigger further signals that lead to actin cytoskeletal rearrangement for cell spreading, retraction, and migration (outside-in signaling). Given the central role of cofilin in regulating actin cytoskeletal rearrangement, we tested whether stimulating the $\alpha_4\beta_7$ integrin triggers synergistic outside-in signaling to modulate the cofilin pathway for repairing the T cell motility defect. Here, we demonstrate that stimulating $\alpha_4\beta_7$ with an anti–$\alpha_4\beta_7$ integrin antibody partially promotes CD4 T cell motility by modulating the cofilin pathway. Our results identify cofilin as a key molecule that may be therapeutically targeted to restore T cell motility necessary for T cell tissue repopulation, immune reconstitution, and immune control of viremia.

RESULTS

Cofilin activation through HIV gp120-CCR5 signaling

HIV binding to CXCR4 has been shown to activate cofilin to promote actin dynamics for viral nuclear entry (*9*, *15*). We investigated whether HIV-mediated CCR5 signaling can also lead to cofilin activation in blood CD4 T cells. In contrast to X4 viruses, which infect both memory and naïve CD4 T cells, R5 viruses infect a subpopulation of memory CD4 T cells (fig. S1A), leading to their early depletion in the gut and chronic immune activation (*22*, *23*). We quantified the CCR5 receptor on blood resting memory CD4 T cells and found that approximately 30% of resting memory CD4 T cells displayed the receptor on the surface (fig. S1B). To determine R5 HIV-mediated cofilin activation, we stimulated blood resting memory CD4 T cells with an R5 virus, HIV(AD8), or an R5 HIV envelope protein, gp120(BAL). As a control, we similarly stimulated blood resting CD4 T with an X4-tropic gp120 (IIIB). We observed cofilin activation by both the X4-tropic gp120 (IIIB) and the R5-tropic gp120(BAL), as well as HIV(AD8) (Fig. 1, A to C); this R5 HIV-mediated cofilin activation is also mediated through $G\alpha_i$, as pertussis toxin (PTX) largely abrogated late cofilin activation (Fig. 1B). In addition, maraviroc, a CCR5 blocker, also abrogated the R5-tropic gp120(BAL)–mediated cofilin activation (Fig. 1C). In conclusion, both X4 and R5 viruses activate cofilin by triggering $G\alpha_i$ signaling through gp120 binding to their respective chemokine coreceptors.

Cofilin hyperactivation in blood resting CD4 T cells of HIV-infected patients

Given that the CD4 T cells in patients with HIV are chronically exposed to viral proteins such as gp120 (*24*), particularly during the acute phase, persistent viral signaling may trigger cofilin dysregulation and cause a T cell migratory defect, as seen in patients with

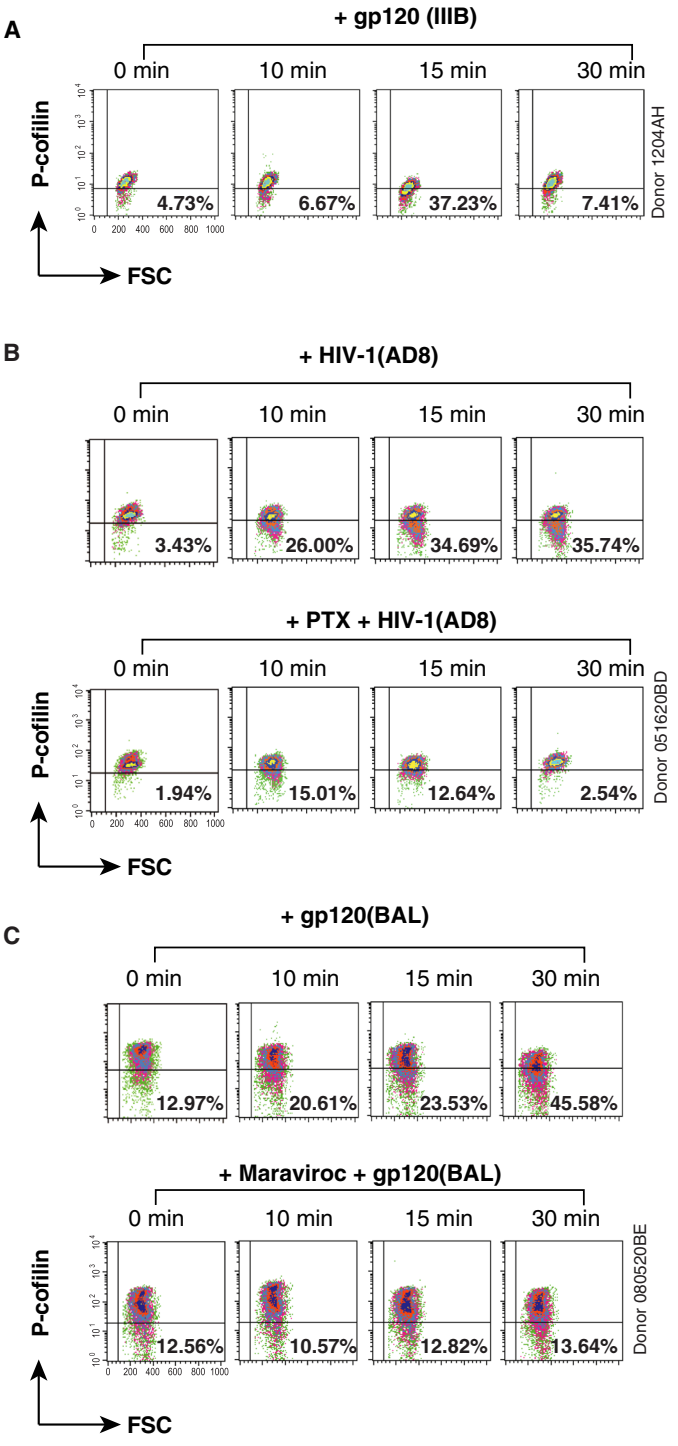

Fig. 1. HIV gp120-CCR5 signaling activates cofilin in memory CD4 T cells. (**A**) Resting CD4 T cells were treated with CXCR4-utilizing HIV gp120 (IIIB). Cofilin phosphorylation was measured with intracellular staining and flow cytometry using an anti-human p-cofilin antibody. (**B**) Resting memory CD4 T cells were infected with CCR5-tropic HIV-1(AD8) in the presence or absence of PTX. Cofilin phosphorylation was measured with intracellular staining and flow cytometry using an anti-human p-cofilin antibody. (**C**) Resting memory CD4 T cells were treated with CCR5-utilizing gp120(BAL) in the presence or absence of maraviroc. Cofilin phosphorylation was measured with intracellular staining and flow cytometry using an anti-human p-cofilin antibody. FSC, forward scatter.

HIV (*2*, *3*, *19*). We conducted a large clinical trial to examine cofilin phosphorylation in blood resting CD4 T cells (Fig. 2A). For this purpose, we developed a reverse-phase phospho-cofilin microarray that can quantify cofilin phosphorylation in a large number of clinical samples simultaneously (Fig. 2B) (*25*). Blood resting CD4 T cells from patients with HIV, with ART (HIV + ART, $n = 95$) or without ART (HIV, $n = 98$), or from healthy controls (HC, $n = 100$) (table S1) were purified by negative depletion, unstimulated, and then lysed. Blindly coded cell lysates were then profiled with the phospho-cofilin microarray (Fig. 2C). We observed a highly significant reduction in cofilin phosphorylation in patients with HIV (HIV = 0.968; HIV + ART = 1.139; HC = 2.254; $P < 0.001$). Unexpectedly, ART did not significantly restore cofilin phosphorylation (HIV = 0.968; HIV + ART = 1.139; $P = 0.981$). These results suggest that HIV-mediated cofilin hyperactivation may result from ART-irreversible, pathogenic polarization of T cells. This irreversibility appears to resemble the establishment of an early immune activation set point that dictates subsequent CD4 T cell depletion independent of viral load (*1*).

We further examined possible correlations between cofilin hyperactivation and viral load/CD4 count. In untreated patients, there was only a weak correlation between cofilin phosphorylation and viral load ($P = 0.043$, $r = -0.205$; Fig. 2D), and there was no correlation between cofilin phosphorylation and CD4 T cell counts ($P = 0.057$, $r = 0.193$; Fig. 2E). However, when ART-treated patients were categorized into immune responders (IRs) and immune nonresponders (INRs); the IRs had a significantly higher level of cofilin phosphorylation than the INRs (Fig. 2F). Both IRs and INRs had the viral load suppressed to the limit of detection after 1 year of treatment; the INRs had less than 20% recovery of CD4 T cells or a CD4 T cell count below 200, whereas the IRs had greater than 20% T cell recovery and a CD4 count above 500. Thus, higher levels of p-cofilin in ART-treated patients were associated with a better CD4 T cell recovery after ART. We also followed ART-naïve patients after their p-cofilin profiling. Some of these patients were subsequently treated with ART (table S2). Again, the IRs had significantly higher levels of cofilin phosphorylation than the INRs (Fig. 2G). These results demonstrate that pre-ART levels of p-cofilin can be used to gauge the degree of CD4 T cell damage and predict T cell recovery from ART.

Direct effects of cofilin hyperactivation on T cell motility

Cofilin hyperactivation has been shown to be associated with a migratory impairment of $CCR6^+$ and $CXCR3^+$ T_H cells, which are prevented from trafficking from the blood stream to peripheral organs even in patients with aviremic HIV on long-term ART (*2*). T cell migration is controlled by cycles of cofilin phosphorylation and dephosphorylation, which are regulated by the LIM domain kinase (LIMK) through serine 3 phosphorylation (*16*, *17*). To quantify the direct effect of cofilin hyperactivation on T cell migration, we used a recently discovered LIMK inhibitor, R10015 (*26*), to block cofilin phosphorylation in A3R5.7 CD4 T cells. We then performed CXCL12-induced T cell chemotaxis in the presence of cofilin hyperactivation. CXCL12 [stromal cell-derived factor 1 (SDF-1)] binding to CXCR4 triggers actin/cofilin dynamics for T cell migration (*9*). We observed an R10015 dosage-dependent inhibition of cofilin phosphorylation in A3R5.7 CD4 T cells (Fig. 3, A and B). We also observed a linear correlation between cofilin dephosphorylation and the impairment of CXCL12-mediated T cell chemotaxis (correlation coefficient $r = 0.999$, $P = 0.002$) (Fig. 3, C and D). At around 15 μM R10015, cofilin phosphorylation was reduced to around 50% in A3R5.7, a level approximate to what was seen in patients with HIV (Figs. 2C and 3A). A 50% reduction in cofilin phosphorylation resulted in a 20 to 40% decrease in cell migration for human A3R5.7 CD4 T cells (Fig. 3C). As a control, R10015 did not affect the surface density of the CXCR4 receptor (fig. S2). These results quantitatively measured the direct impact of cofilin hyperactivation on T cell motility.

Modulating the cofilin pathway using the anti-$\alpha_4\beta_7$ antibody Act-1

To modulate the cofilin pathway, we had previously identified a marine toxin, okadaic acid (OA), that can activate LIMK and enhance cofilin phosphorylation and actin polymerization (*27*). In a proof-of-concept study, OA was used to target the cofilin pathway to promote T cell migration both in vitro and in vivo in a murine model (*2*). The high toxicity of OA prevents its therapeutic use to restore T cell motility in humans. However, in a recent simian immunodeficiency virus (SIV)/macaque study, the use of an anti–$\alpha_4\beta_7$ integrin antibody unexpectedly promoted repopulation of CD4 T cells in a wide variety of immune tissues, including gastrointestinal tissues (GITs) (*7*). Integrins are cell surface adhesion molecules that mediate the cell-cell and cell-matrix interactions for T cell trafficking to tissues (*20*). This homing process involves synergistic, bidirectional signaling with chemokine receptors (inside-out and outside-in signaling) to initiate cell migration (*21*). Given that the anti–$\alpha_4\beta_7$ integrin antibody promoted T cell trafficking and homing to lymphoid tissues in SIV infection (*7*), we investigated whether stimulation of T cells with the anti-$\alpha_4\beta_7$ antibody would trigger an "outside-in" signaling to modulate the cofilin pathway and promote T cell motility.

In human peripheral blood, high levels of $\alpha_4\beta_7$ were detected in a subpopulation of resting memory CD4 T cells (*28*). In addition, culturing naïve T cells in interleukin-7 (IL-7) can greatly up-regulate $\alpha_4\beta_7$ on the cell surface (fig. S3) (*28*). We stimulated resting memory and IL-7–cultured naïve T cells with the anti–human $\alpha_4\beta_7$ antibody Act-1 and then quantified cofilin phosphorylation by intracellular staining and flow cytometry. We observed that Act-1 (1 μg/ml) can directly trigger cofilin phosphorylation and dephosphorylation in a time course (Fig. 4, C to E, H to J, and M to O), thereby demonstrating that the $\alpha_4\beta_7$ antibody is capable of modulating the cofilin pathway. The time-dependent fluctuation of cofilin phosphorylation and dephosphorylation, beginning at 10 min and then returning to the basal levels at 15 min, resembles the cycling pattern of actin polymerization and depolymerization during chemotaxis (Fig. 4, C and E, H and J, and M and O). When blood resting CD4 T cells were similarly stimulated with CXCL12, the CXCR4 ligand known to trigger cofilin activation (*9*), we observed a similar cycling pattern of cofilin phosphorylation and dephosphorylation (Fig. 4, B, G, and L). The $\alpha_4\beta_7$ antibody–mediated transient cofilin activity was also partially dependent on $G\alpha_i$ signaling, as PTX abrogated late cofilin activation (30 min) (Fig. 4, C and D, H and I, and M and N).

Targeting the cofilin pathway using Act-1 to restore T cell motility

Next, we investigated whether the $\alpha_4\beta_7$ antibody is capable of restoring T cell migration. First, we used R10015 to induce cofilin hyperactivation to inhibit T cell chemotaxis (Fig. 3); we titrated R10015 on blood resting CD4 T cells of individual donors to determine an active dosage (10 to 20 μM) that can induce a low degree of cofilin hyperactivation (less than 50% reduction in p-cofilin), causing a mild 20 to 40% decrease in cell migration (Fig. 5, A and B). At these

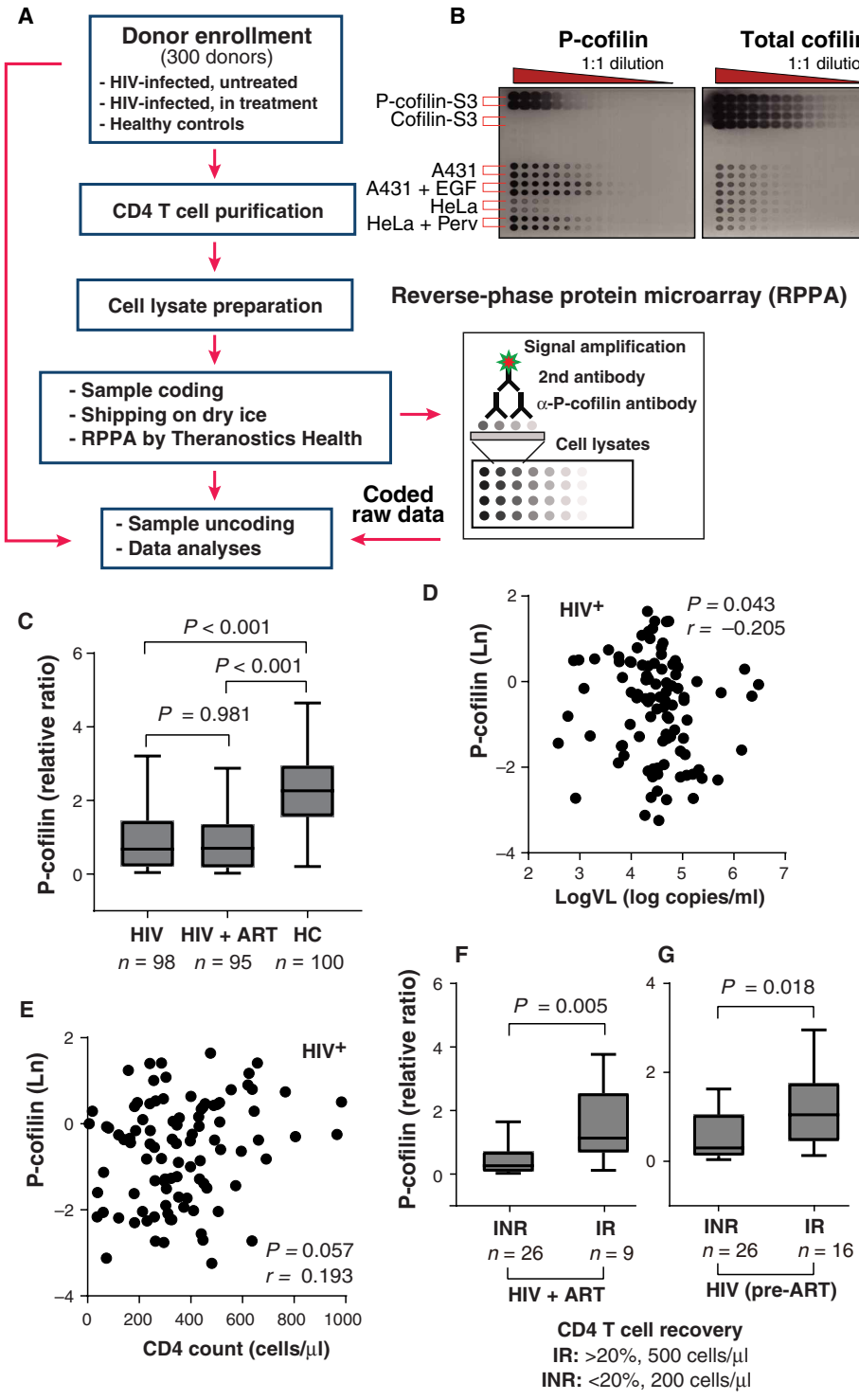

Fig. 2. Cofilin hyperactivation in HIV infection. (**A**) Flowchart of the clinical study. (**B**) Development of the reverse-phase cofilin microarray for profiling cofilin phosphorylation. Synthetic peptides or cell lysates were serially diluted (1:1) and printed onto the microarray slides, which were then stained with antibodies against either total cofilin (right) or phospho-cofilin (left). P-cofilin-S3, a synthetic cofilin peptide with serine 3 phosphorylated; cofilin-S3, a similar peptide with no serine 3 phosphorylation. A431 or HeLa cells were not treated or treated with human epithelial growth factor (EGF) or pervanadate (Perv). (**C**) Relative levels of p-cofilin in blood resting CD4 T cells from HIV-infected patients with ART (HIV + ART), without ART (HIV), or healthy control (HC) donors were profiled. Box plots show interquartile range, median, and range. There were no statistically significant differences in the total protein levels of the resting CD4 T cells from HC, HIV, and HIV + ART (see Materials and Methods). (**D** and **E**) The correlation between levels of p-cofilin and plasma viral load (D) and CD4 T cell count (E) in untreated patients was plotted using Spearman rank correlation tests (Ln, natural logarithm). (**F**) In ART-treated patients, IRs had significantly higher levels of cofilin phosphorylation than did INRs. (**G**) A subgroup of ART-naïve patients was subsequently treated with ART following p-cofilin profiling. IRs had significantly higher levels of cofilin phosphorylation than INRs.

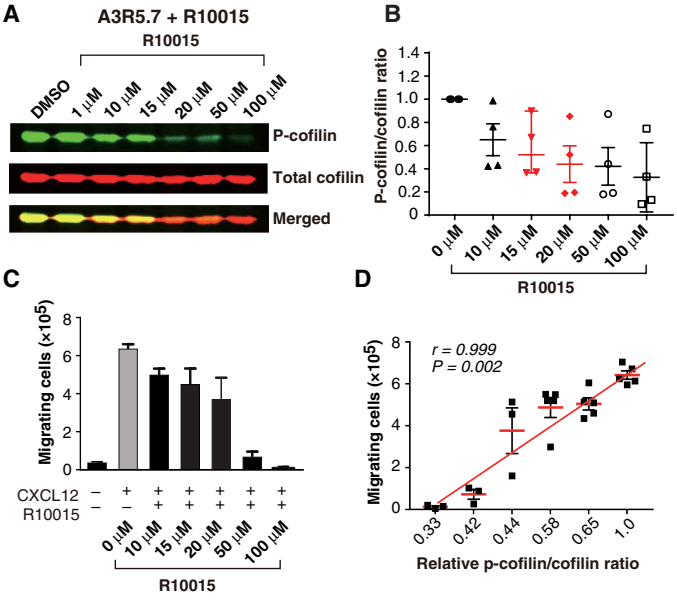

Fig. 3. Quantification of effects of cofilin hyperactivation on T cell migration. (**A**) A3R5.7 T cells were treated with different dosages of R10015 for 1 hour. Phosphocofilin and total cofilin were quantified by Western blot. (**B**) The relative ratio of p-cofilin/cofilin in response to R10015 treatment was plotted ($n = 4$ independent experiments). (**C**) R10015 inhibits cofilin phosphorylation and T cell chemotaxis in response to CXCL12. A3R5.7 cells were treated with different dosages of R10015 for 1 hour and then added to the upper chamber of a 24-well Transwell plate. The lower chamber was filled with CXCL12 (40 ng/ml), and cell migration to the lower chamber was quantified ($n = 3$ independent experiments). (**D**) The linear correlation between T cell migration and levels of cofilin phosphorylation. The *x* axis is the relative ratio of p-cofilin/cofilin derived from (B); the *y* axis is the number of migrating cells derived from (C).

dosages, R10015 did not affect the surface density of the $\alpha_4\beta_7$ receptor (fig. S4). Then, we used Act-1 to stimulate T cells in CXCL12-mediated chemotaxis. We found that the use of Act-1 (1 to 50 μg/ml) restored T cell migration in the presence of R10015 (Fig. 5B), demonstrating the capacity of the anti-$\alpha_4\beta_7$ antibody to rescue the migratory defects resulting from cofilin hyperactivation. As controls, we also tested whether Act-1 is a chemoattractant for CD4 T cells and whether Act-1 can promote T cell migration in the absence of R10015 (fig. S5). We found that Act-1 was not capable of attracting CD4 T cells by itself, and nor could it stimulate significantly higher levels of T cell migration toward CXCL-12 in the absence of R10015 (fig. S5).

We further investigated whether Act-1 specifically promoted the migration of the $\alpha_4\beta_7^+$ CD4 T cells by examining the cell subpopulation that migrated to the lower chamber of the Transwell plate (Fig. 5C). While R10015 inhibited the migration of all T cell subtypes, unexpectedly, the inhibitory effect was greater in the $\alpha_4\beta_7^{high}$ CD4 T cells, selectively diminishing their percentage in the migrating cell population (from 8.90 to 4.52%) (Fig. 5C). Act-1 effectively restored T cell migration and increased the percentage of migrating $\alpha_4\beta_7^{high}$ T cells from 4.52 to 12.0% (Fig. 5, C and D, and fig. S6). These results demonstrated that the motility of $\alpha_4\beta_7^+$ CD4 T cells is prone to inhibition through blocking cofilin phosphorylation; the $\alpha_4\beta_7^+$ CD4 T cells may have a higher sensitivity to cofilin hyperactivation likely because cofilin dysfunction can affect the outside-in and inside-out signaling from both the chemokine receptor and the integrin receptor. This cofilin-mediated migratory impairment can be rescued by the anti-$\alpha_4\beta_7$ antibody Act-1, which may act through synergistic signaling with chemokines to modulate the cofilin pathway. For comparison, the percentage of the $CCR7^+$ T cell subpopulation was similarly analyzed and was found to be unchanged by R10015 and Act-1 (fig. S7). These results demonstrated that Act-1 selectively restored and promoted the migration of the $\alpha_4\beta_7^{high}$ CD4 T cells (Fig. 5C), whereas it did not selectively increase the migration of $CCR7^+$ cells (fig. S7), suggesting that the signaling is likely transduced through the $\alpha_4\beta_7$ receptor rather than an unrelated receptor.

DISCUSSION

It has long been speculated that HIV binding to chemokine coreceptors may trigger aberrant G protein signaling and CD4 T cell pathogenesis (*11*). Nevertheless, this speculation has not been solidified by experimental and clinical data. In this large clinical trial, we have demonstrated cofilin dephosphorylation occurring in the blood CD4 T cells of HIV-infected patients. Cofilin has been identified as a direct downstream target of HIV-mediated G protein signaling through the chemokine coreceptors (*9*). Cofilin hyperactivation can directly affect T cell migration (*18*), as cofilin is a major driver of actin treadmilling for cell motility (*29*). In HIV infection, selective impairment of CD4 but not CD8 T cell homing (*2*, *4*) is a major hallmark of HIV-mediated T cell dysfunction (*3*), and this implies a direct role of cofilin dysregulation in HIV-mediated CD4 T cell pathogenesis. The molecular cue for this cofilin hyperactivation likely results from early viral signaling from HIV gp120 (*9*) and chronic immune activation later in the disease course (*2*). During acute HIV infection, gp120 levels are very high with active HIV replication. CD4 T cells are exposed to these high levels of gp120 for extended periods of time before ART initiation. During the asymptomatic phase with ART, gp120 in the peripheral blood is at a low level. However, in infected lymphoid tissues, gp120 can be present in high concentrations (>300 pg/ml) and is estimated to be in the range of 10 pg/ml to 10 ng/ml (*30*). In addition, chronic immune activation persists even with ART. Thus, the combined effects of gp120 and chronic immune activation may exacerbate and polarize CD4 T cells toward an ART-irreversible pathogenic lineage. This HIV-mediated T cell polarization may resemble the irreversibility of T cell differentiation and lineage commitment following persistent cytokine receptor signaling. For example, when T cells are stimulated with IL-12 or interferon-γ for an extended period of time, they are polarized to express a transcription factor, T-bet, the induction of which becomes irreversible. The ART irreversibility of cofilin hyperactivation also appears to resemble the establishment of the early immune activation set point that dictates subsequent CD4 T cell dysfunction and depletion independent of viral load (*1*).

While ART alone cannot repair cofilin dysfunction (Fig. 2C), the combined use of ART plus the anti–human $\alpha_4\beta_7$ integrin antibody Act-1 has been found to promote T cell motility and tissue repopulation (*7*). Although high dosages of anti-integrin antibodies can block integrin-ligand interaction and inhibit cell migration, these antibodies can also initiate an outside-in signal (Fig. 4). In particular, if antibody affinity is not optimal or antibody is used at a nonsaturating dosage, then such antibody-induced signaling may synergize with chemokine signaling to promote cell motility. In vivo, Act-1 unexpectedly promoted the repopulation of monkey CD4 T cells in a wide variety of immune tissues including GITs (*7*), suggesting that

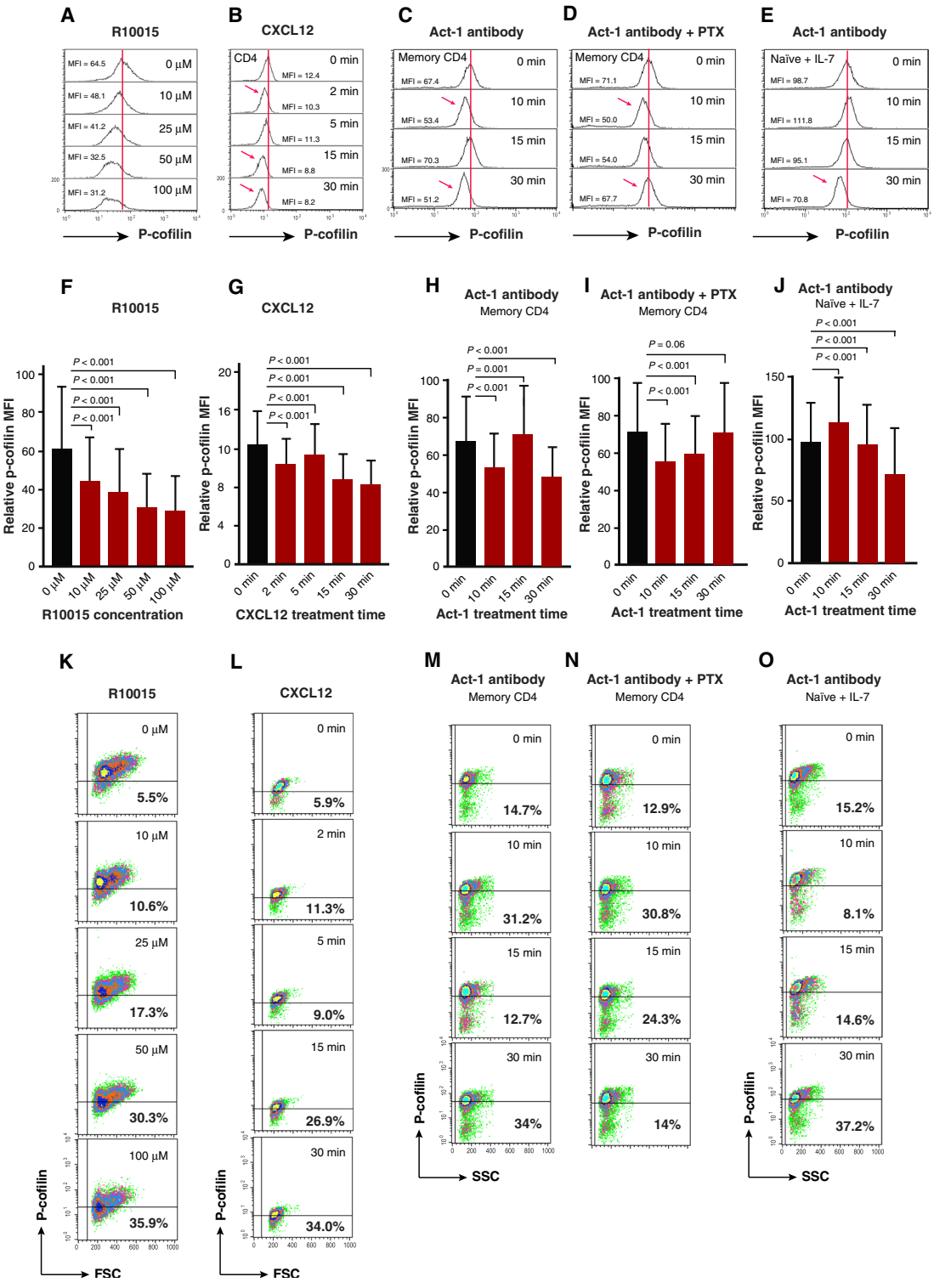

Fig. 4. Act-1 modulates the cofilin pathway through PTX-sensitive Gα_i signaling. (**A**) A3R5.7 T cells were treated with different dosages of R10015 for 1 hour. Cofilin phosphorylation was quantified with intracellular staining and flow cytometry. Shown are the histogram in (A) and the density plot in (**K**). (**B**) Resting CD4 T cells were stimulated with CXCL12 (50 ng/ml) for various times. Cofilin phosphorylation was quantified with intracellular staining and flow cytometry. Shown are the histogram in (B) and the density plot in (**L**). (**C** and **D**) Resting memory CD4 T cells were not treated (C) or treated with PTX (D) for 1 hour and then stimulated with Act-1 (1 μg/ml) for various times. Shown are the histograms in (C) and (D) and the density plots in (**M** and **N**). (**E**) Naïve CD4 T cells (cultured in IL-7) were also similarly stimulated with Act-1. Cofilin phosphorylation was quantified with intracellular staining and flow cytometry. Shown are the histograms in (E) and the density plots in (**O**). The mean fluorescence intensities (MFIs) of p-cofilin staining are also shown on the histograms. Statistical analyses of the MFI of p-cofilin staining in (A) to (E) are presented in (**F**) to (**J**). Results in (B) to (E) were representative of three independent experiments using blood CD4 T cells from three individual donors. SSC, side scatter.

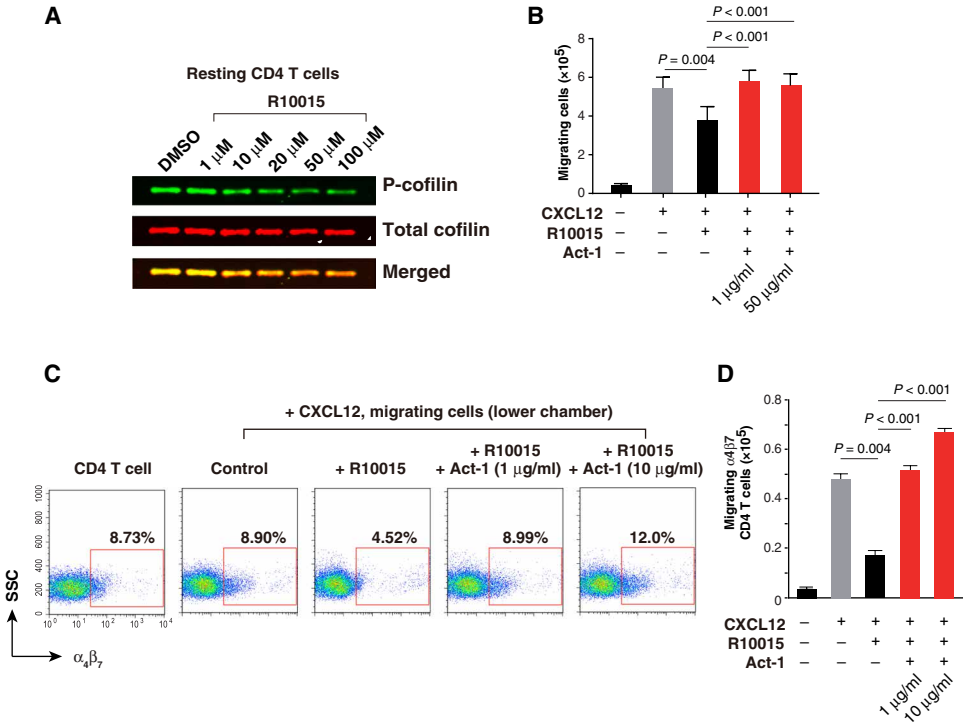

Fig. 5. Targeting the cofilin pathway using the anti–human α 4β7 antibody Act-1. (**A**) Resting CD4 T cells were treated with different dosages of R10015 for 1 hour. Phospho-cofilin and total cofilin were quantified by Western blot. (**B**) Act-1 promotes T cell chemotaxis. Resting CD4 T cells were pretreated with R10015 or dimethyl sulfoxide (DMSO) for 1 hour and then stimulated with Act-1 or a control mouse immunoglobulin G for an additional 15 min. Cells were then added to the upper chamber of a 24-well Transwell plate. The lower chamber was filled with CXCL12 (40 ng/ml). Cell migration to the lower chamber was enumerated. (**C**) Act-1 selectively promotes the migration of the $\alpha_4\beta_7^+$ CD4 T cells. Migrating T cells in the lower chamber were stained with the anti-$\alpha_4\beta_7$ antibody Act-1, followed by staining with Alexa Fluor 647–conjugated goat anti-mouse secondary antibodies. The percentage of the $\alpha_4\beta_7^{high}$ CD4 T cells was quantified with flow cytometry. (**D**) The number of migrating $\alpha_4\beta_7^{high}$ CD4 T cells in (C) was also enumerated. Results in (C) and (D) were representative of five independent experiments using blood CD4 T cells from five individual donors.

in this SIV infection setting, Act-1 was not effective at blocking T cell homing but rather may have triggered an outside-in signal to promote T cell chemotaxis.

Stimulation of integrin receptors has been known to trigger cytoskeletal signaling for cell adhesion and migration (*31*). Integrin signaling is connected to the actin cytoskeleton mainly through the activation of downstream Rho guanosine triphosphatases (GTPases), such as Rho, Rac, and CDC42, which then activate the cofilin kinase, LIMK, to regulate cofilin phosphorylation (*27*, *31*). The $\alpha_4\beta_7$ receptor has also been known to trigger actin signaling. Specifically, the cytoplasmic tail of the α_4 integrin has been shown to directly bind to a cytoskeletal and scaffolding protein, paxilin, that is involved in the activation of the Rho GTPase Rho, Rac1, and Cdc42 (*32*). Activation of these Rho GTPases can lead to the activation of LIMK to regulate cofilin phosphorylation. In addition, the cytoplasmic tail of the β_7 integrin also directly binds to filamin A, a large actin cross-linking protein that connects transmembrane receptors with the actin cytoskeleton (*33*). These previous studies provide possible signaling mechanisms by which Act-1 may modulate the cofilin pathway to promote $\alpha_4\beta_7$ T cell motility.

Given the time-dependent fluctuation of cofilin phosphorylation and dephosphorylation triggered by Act-1 stimulation (Fig. 4, C, H, and M), it is possible that Act-1 stimulation may result in the sequential activation of two pathways: one leading to the activation of the cofilin phosphatase (slingshot) and one leading to the activation of the cofilin kinase (LIMK). We also compared the degree of p-cofilin fluctuation from Act-1 stimulation (Fig. 4, C, H, and M) with that from CXCL12 stimulation (Fig. 4, B, G, and L) and observed comparable p-cofilin changes. However, biologically, they are likely very different in terms of the spatial and temporal regulation of cofilin phosphorylation. CXCL12 is a natural ligand to trigger cofilin activity for chemotaxis, whereas Act-1–mediated cofilin activity is likely aberrant and not geared toward the precise regulation of T cell motility. Thus, this Act-1–mediated cofilin activity may not have biological significance in enhancing normal T cell chemotaxis, but it could have therapeutic effects on promoting T cell migration when normal chemotactic signaling is defective.

A previous study by Teague and co-authors (*34*) has found that direct stimulation of T cells with Act-1 alone did not trigger T cell activation. However, when used with an anti-CD3 antibody, the two antibodies together resulted in T cell activation. We also confirmed that Act-1 alone cannot activate resting CD4 T cells (fig. S8). These results suggest that $\alpha_4\beta_7$ signaling can serve as an accessory or costimulatory signal, similar to what anti-CD28 costimulation does. It has been known that without anti-CD28 costimulation, the anti-CD3 stimulation alone cannot activate T cells by itself (*35*). Similarly, in the Teague study (*34*), without the anti-$\alpha_4\beta_7$ costimulation, the anti-CD3 stimulation alone also cannot activate T cells. Thus, both anti-CD28 and anti-$\alpha_4\beta_7$ stimulations share a similar accessory or costimulatory function. It has been known that the costimulatory function of anti-CD28 stimulation is to activate cofilin for the reorganization of the actin cytoskeleton, which is required for the formation

of the immunologic synapse (IS) (*36*, *37*); this cofilin-regulated IS formation stabilizes cell-cell contact for full T cell activation (*38*-*40*). These previous results demonstrate that the anti-$\alpha_4\beta_7$ stimulation can replace the costimulatory function of the anti-CD28 stimulation, likely through similar activation of cofilin.

In our in vitro assay system, stimulation of CD4 T cells with Act-1 can rescue the migratory defect caused by low levels of cofilin hyperactivation (Fig. 5). However, if cofilin hyperactivation had progressed to a high level (50% or more decrease of p-cofilin), then Act-1 was not effective in restoring T cell motility (fig. S9). In addition to gp120, another viral protein, Nef, has also been shown to alter the cofilin pathway to cause cofilin dysfunction (*41*). It is expected that for HIV-infected T cells, higher levels of HIV activity would be associated with greater degrees of cofilin dysfunction. Thus, our results suggest that early treatments of both HIV infection and immune dysfunction are important to prevent T cell polarization toward a pathogenic state in which cofilin-mediated T cell migratory impairment may become irreversible.

Early restoration of CD4 T cells in the gut has been shown to be associated with immune reconstitution and effective control of viremia without the need for ART (*7*, *42*). High levels of $\alpha_4\beta_7$ are associated with memory T cell migration preferentially into mucosal tissues such as GITs (*43*), in which CD4 T cells are severely depleted during HIV/SIV infection (*22*, *23*). A full CD4 T cell repopulation in the gut is rarely achieved with ART (*3*), and persistent cofilin hyperactivation is likely a major reason (*2*). A key T cell subpopulation for maintaining mucosal integrity is the T_H17 cells, which are depleted in both HIV infection and pathogenic SIV infection (*44*). Although ART restores the T_H17 cells in the bloodstream, it does not lead to full reconstitution of the T_H17 cells in the mucosal compartment (*5*). Virologically, T_H17 cells are highly permissive to HIV-1, and this permissiveness is linked to the expression of CCR6, CCR5, CCR9, and $\alpha_4\beta_7$ (*45*, *46*). Recently, it has been shown that $CCR6^+$ and $CXCR3^+$ T_H cells are impaired in trafficking from the blood stream to peripheral tissues (*2*), and these cells accumulate in the peripheral blood as a predominant viral reservoir (*47*). We also observed the accumulation of $\alpha_4\beta_7^{high}$ CD4 T cells in the peripheral blood of HIV-infected patients (HIV infection of 0.5 to 2 years, ART-free; fig. S10). Our results are consistent with a recent finding by Mavigner and co-authors (*3*), who have investigated the trafficking of $CD4^+$ T cells expressing the gut-homing receptors CCR9 and integrin $\alpha_4\beta_7$, and found that these T cells remain in the circulation rather than repopulate the mucosa of the small intestine. In our study, we directly quantified the peripheral $\alpha_4\beta_7^{hi}$ CD4 T cells with the anti-$\alpha_4\beta_7$ antibody Act-1, which has been shown to promote T cell homing to the gut and other lymphoid tissues (*7*). However, the complete homing identity of these peripheral Act-1 high-staining T cells remains to be fully elucidated.

Here, we have demonstrated that stimulating blood CD4 T cells with the anti-$\alpha_4\beta_7$ antibody can modulate the cofilin pathway and repair the T cell migratory defect caused by cofilin hyperactivation (Fig. 5). Our study has thus opened a new avenue for testing novel therapeutics that use such a "kick and go" strategy to restore T cell migration and tissue repopulation for immune reconstitution and immune control of viremia. Therapeutically, targeting T cell motility and the cofilin pathway would bring at least two major benefits (Fig. 6): For uninfected cells, the restoration of cofilin phosphorylation and CD4 T cell circulation and homing to GALTs and other lymphoid tissues may help immune reconstitution, and for infected cells, the circulation and homing of these latent HIV^+ cells to lymphoid tissues may lead to their reactivation and eventual containment by the restored immune system, reducing latent viral reservoirs persistent in the peripheral blood and in tissues. In sum, our study suggests that cofilin is a key molecule that needs to be therapeutically targeted to achieve a functional cure of HIV infection (*48*).

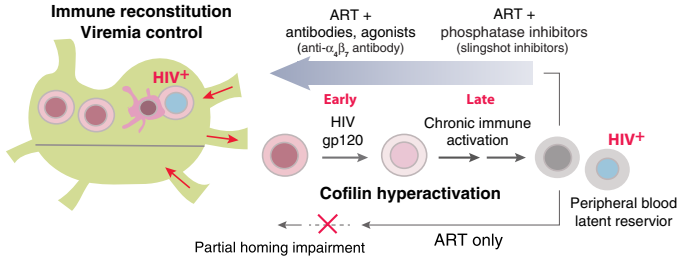

Fig. 6. Model of cofilin hyperactivation in HIV infection and therapeutic targeting of the cofilin pathway. Early HIV signaling through chemokine coreceptors (CCR5 and CXCR4) and late chronic immune activation may trigger cofilin hyperactivation, impairing CD4 T cell migration and homing to lymphoid tissues such as GALTs. ART alone is not sufficient to restore T cell motility. For early, low levels of cofilin hyperactivation, stimulation of the upstream regulators of cofilin through antibodies (e.g., stimulating chemokine or integrin receptors such as the $\alpha_4\beta_7$ receptor), receptor agonists, or activators of G proteins and GTPases may repair cofilin-mediated T cell migratory defect. For late, high levels of cofilin hyperactivation, it may need to use LIMK (the cofilin kinase) activators or the cofilin phosphatase (slingshot) inhibitors to directly inhibit cofilin dephosphorylation to restore actin dynamics. Therapeutically, targeting cofilin to restore T cell motility may bring two major benefits: (i) For uninfected cells, the restoration of CD4 T cell circulation and homing to lymphoid tissues such as GALTs may help immune reconstitution; (ii) for latent HIV^+ T cells, the restoration of T cell circulation and homing to lymphoid tissues may lead to their reactivation and eventual containment by the restored immune system, reducing latent viral reservoirs persistent in the peripheral blood and in tissues.

MATERIALS AND METHODS

Clinical study

All clinical study protocols were reviewed and approved by the Ethics Review Committee of China Medical University (CMU), Shenyang, P. R. China, and written informed consent from each participant in the study was obtained. We initially enrolled and evaluated 200 HIV-1–infected patients from the HIV patient cohort of the Key Laboratory of AIDS Immunology of the National Health and Family Planning Commission in The First Affiliated Hospital of CMU. Among the HIV-infected patients, 98 had no previous or current ART at the time of the p-cofilin profiling, and 102 had ongoing ART for over a year, but 4 of the ART-treated patients had a viral load greater than 1000 copies/ml and were excluded from the study for possible drug resistance. The CD4 T cell count and viral load of these subjects were measured every 3 months. One hundred age- and sex-matched HC were enrolled from the HIV voluntary counseling and testing center of CMU. A summary of the subjects is listed in tables S1 and S2. Of the ART-naïve patients, 65 eventually received ART at around 6 months after the p-cofilin profiling and were treated for more than a year. All of these patients receiving ART reached undetectable plasma HIV-1 RNA. ART-treated patients were further evaluated and categorized into IRs and INRs.

Both IRs and INRs were treated with ART for more than 1 year. IRs were those who had a CD4 T cell recovery greater than 20% and a CD4 T cell count more than 500 cells/μl; INRs had a CD4 T cell recovery less than 20% or a CD4 T cell count less than 200 cells/μl. For isolating blood resting CD4 T cells from study subjects, peripheral blood mononuclear cells (PBMCs) were freshly obtained from the subjects and purified by Ficoll-Hypaque density gradient centrifugation, followed by negative isolation of resting CD4 T cells as previously described (*9*, *49*). Briefly, monoclonal antibodies against human CD14, CD56, HLA-DR, CD8, CD11b, and CD19 (BD Biosciences, San Jose, CA) were used. Antibody-bound cells were depleted using Dynabeads Pan Mouse IgG (Thermo Fisher Scientific). Purified cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). One million resting CD4 T cells from each blood donor were lysed in 40 μl of SDS/T-PER extraction buffer [Novex Tris-Glycine SDS Sample Buffer, T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific), and 2.5% 2-mercaptoethanol (Sigma-Aldrich)]. Cell lysates were heated at 100°C for 8 min, immediately frozen and stored at −80°C, and then transported on dry ice to Theranostics Health (Gaithersburg, MD, USA) for p-cofilin reverse-phase protein microarray (RPPA) analyses. A total of 296 coded cell lysates were printed onto the microarrays and profiled; 3 lysates did not generate readable signals and were excluded from data analyses.

Reverse-phase protein microarray

Cofilin RPPA printing and analyses of cell lysates were provided by Theranostics Health (Gaithersburg, MD, USA). Details of RPPA have been published previously (*25*). The RPPA directly couples the phospho-cofilin detection antibody with highly sensitive amplification systems that can yield detection sensitivities to fewer than 1000 to 5000 molecules per spot with good linearity (correlation coefficient or $R^2 = 0.990$ to 0.999) and interexperiment precision ($R^2 = 0.973$). Published between-run and within-run analytical precision in our studies was 3 to 13% CV (coefficient of variation) (*50*). The RPPA technology has been developed and optimized for performance as a fluorescence-based calibrated assay, generally identical in design and analysis to standard ELISA (enzyme-linked immunosorbent assay) or standard clinical immunoassays. Each array consisted of patient cell lysates printed in triplicate two-spot dilutions (neat and 1:4), high and low controls printed in triplicate two-spot dilutions (neat and 1:4), and 6- to 10-point calibrators. The analyte concentration was determined by extrapolation to a nonparametrically determined curve fit of the calibration curve and reported in relative fluorescence units. For data normalization, each protein analyte value (relative p-cofilin value) was normalized to the total amount of protein printed on that spot with a fluorescent stain (Sypro Ruby Blot Stain; Molecular Probes, Eugene, OR) that binds to the amne group of proteins without bias. The protein loading value was also obtained by a calibrated assay technique. This total protein calibrator consisted of a protein lysate with a known concentration, which upon dilution spans the linear dynamic range of protein concentration. Each sample value was then extrapolated to the calibrator. The following were the quantified averages of the total protein levels from each test group: HIV (n = 98), 0.254; HIV + ART (n = 95), 0.259; and HC (n = 100), 0.252. There are no statistically significant differences in the total protein levels between the three groups (HC and HIV, $P = 0.77$; HC and HIV + ART, $P = 0.51$; HIV and HIV + ART, $P = 0.64$).

Purification of resting CD4 T subtypes from peripheral blood

All protocols involving human subjects were reviewed and approved by the George Mason University institutional review board. PBMCs were purified from the peripheral blood of HIV-negative donors by centrifugation in Lymphocyte Separation Medium (Corning, Corning, NY), and resting CD4 T cells were further purified by two rounds of negative selection as previously described (*9*, *49*). Briefly, for the first-round depletion, monoclonal antibodies against human CD14, CD56 and HLA-DR, DP, and DQ (BD Biosciences, San Jose, CA) were used. For the second-round depletion, monoclonal antibodies against human CD8, CD11b, and CD19 (BD Biosciences, San Jose, CA) were used. Antibody-bound cells were depleted using Dynabeads Pan Mouse IgG (Invitrogen, Carlsbad, CA). For further negative selection of the memory and naïve CD4 T cell subsets, monoclonal antibody against either CD45RA (0.02 μl per million cells) or CD45RO (0.1 μl per million cells) (BD Biosciences, San Jose, CA) was added during the second round of depletion. Purified cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA), penicillin (50 U/ml) (Invitrogen, Carlsbad, CA), and streptomycin (50 μg/ml) (Invitrogen, Carlsbad, CA). Cells were rested overnight before infection or treatment. For $\alpha_4\beta_7$ surface receptor up-regulation, resting CD4 T cells were also cultured in IL-7 (5 ng/ml) for 3 days.

Cell lines and viruses

A3R5.7 cells were acquired from the National Institutes of Health (NIH) AIDS Reagent Program. A3R5.7 cells were derived from A3.01, which naturally expresses CD4, CXCR4, and $\alpha_4\beta_7$. HIV-1(AD8) was provided by M. A. Martin. Virus stocks of NLENG1-ES-IRES(NL4-3), NLENG1-ES-IRES(Yu2), and HIV-1(AD8) were prepared by transfection of human embryonic kidney (HEK) 293 T cells with cloned proviral DNA as described (*9*, *49*). Viral titer ($TCID_{50}$) was determined on the Rev-dependent green fluorescent protein (GFP) indicator cell (*51*), Rev-A3R5-GFP (Virongy, Manassas, VA). For viral infection of resting CD4 T cells, cells were infected with envelope-negative GFP reporter HIV-1 virus NLENG1-ES-IRES, pseudotyped with NL4-3 or YU2 envelope. Infection was performed by spinoculation for 2 hours at 1200*g* of 400 virions particles per cell at 37°C, in the presence of DEAE Dextran (5 μg/ml; Sigma-Aldrich). After infection, cells were washed and incubated for 6 days with or without IL-7 (25 ng/ml; R&D Systems). T cells were stained with anti–CD45RO-Pacific Blue monoclonal antibody (BD Biosciences, San Jose, CA) and analyzed by flow cytometry for CD45RO and GFP expression. For treatment of resting CD4 T cells with HIV(AD8), cells were pretreated with or without PTX (100 ng/ml; Sigma-Aldrich) for 1 hour at 37°C and then treated with HIV(AD8) ($10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ per million cells) for various times. Cells were fixed and stained for intracellular p-cofilin. For treatment of resting CD4 T cells with HIV gp120 (IIIB) (Microbix Biosystems Inc., Toronto, Canada), cells were treated with 10 nM gp120 (IIIB) for various times. For treatment with HIV gp120(BAL) (from the NIH AIDS Reagent Program), resting memory CD4 T cells were treated with or without maraviroc (1 μM) (from the NIH AIDS Reagent Program) for 1 hour at 37°C and then treated with HIV gp120(BAL) (100 nM) for various times.

Western blotting for p-cofilin and cofilin

One million cells were lysed in NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA) followed by sonication. Samples were heated at 70°C

for 10 min, separated by SDS–polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membranes were washed in Tris-buffered saline (TBST) for 3 min and then blocked for 30 min at room temperature with 5% milk. The blots were incubated with a mouse anti-cofilin antibody (1:1000 dilution; BD Biosciences, San Jose, CA) and a rabbit anti–phospho-cofilin (serine 3) antibody (1:500 dilution; Cell Signaling) diluted in 3% milk-TBST and rocked overnight at 4°C. The blots were washed three times for 15 min and then incubated with DyLight 680 goat anti-mouse and DyLight 800 goat anti-rabbit antibodies (KPL, Gaithersburg, MD) (1:5000 diluted in blocking buffer) for 1 hour at 4°C. The blots were washed three times for 15 min and scanned with the Odyssey Infrared Imager (LI-COR Biosciences).

Intracellular p-cofilin staining and flow cytometry

One million cells were fixed, permeabilized, washed, and then stained with an anti-human p-cofilin antibody using an intracellular protein staining kit (a gift from Virongy, Manassas, VA) for 60 min at room temperature. Cells were washed twice and stained with Alexa Fluor 488–labeled chicken anti-rabbit antibodies (Invitrogen, Carlsbad, CA). Cells were washed twice and then analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). Multiple donors were used for intracellular p-cofilin staining.

Surface staining of CXCR4, CCR7, and $\alpha_4\beta_7$

Cells were stained with a phycoerythrin (PE)–labeled mouse anti-human CXCR4 antibody (BD Biosciences, San Jose, CA), a PE-labeled rat anti-human CCR7 antibody (BioLegend, San Diego, CA), or a mouse anti–human $\alpha_4\beta_7$ integrin antibody (Act-1) (obtained from the NIH AIDS Reagent Program), followed by secondary antibody staining with Alexa Fluor 647–labeled goat anti-mouse antibodies (Invitrogen, Carlsbad, CA). Cells were stained on ice in phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA) for 30 min, washed with cold PBS–0.5% BSA, and then analyzed on FACSCalibur (BD Biosciences, San Jose, CA).

Chemotaxis assay

A half million cells were resuspended into 100 μl of RPMI 1640 medium and then added to the upper chamber of a 24-well Transwell plate (Corning, Corning, NY). The lower chamber was filled with 600 μl of medium premixed with CXCL12 (40 ng/ml). The plate was incubated at 37°C for 2 hours, and then the upper chamber was removed and cells in the lower chamber were counted. To ensure accurate enumeration of cells, only Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter) was used. Where indicated, different concentrations of R10015 (*26*) or DMSO were added to cell culture and incubated for 1 hour at 37°C before adding cells to the upper chamber. Cells were also treated with the anti–human $\alpha_4\beta_7$ integrin antibody (Act-1) or the control mouse immunoglobulin G1 (IgG1) antibody for 15 min before adding cells to the upper chamber. Act-1 was also added to the lower chamber (1 μg/ml) with CXCL12 (40 ng/ml). Multiple donors were used for the chemotaxis assay.

Statistical analysis

Statistical calculations were performed using IBM SPSS statistics 23. Categorical data were described and analyzed by frequency and χ^2 test. For parametric comparison, two-tailed Mann-Whitney *U* test was used to assess differences between groups during p-cofilin profiling (Fig. 2). Spearman rank correlation tests were used to measure the correlations between variables. Unless otherwise stated, a *P* value less than 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/1/eaat7911/DC1

Fig. S1. Selective infection of memory CD4 T cells by CCR5-utilizing HIV-1.
Fig. S2. R10015 does not alter the CXCR4 receptor surface density.
Fig. S3. Surface expression of $\alpha_4\beta_7$ on different subsets of human blood resting CD4 T cells, as measured by surface staining and flow cytometry.
Fig. S4. R10015 does not alter the $\alpha_4\beta_7$ receptor surface density.
Fig. S5. Act-1 is not a chemoattractant.
Fig. S6. Act-1 selectively promotes the migration of the $\alpha_4\beta_7^{high}$ CD4 T cells.
Fig. S7. Act-1 does not selectively promote the migration of the $CCR7^+$ CD4 T cells.
Fig. S8. Act-1 stimulation does not activate resting CD4 T cells.
Fig. S9. Act-1 is not effective in rescuing T cell motility with high levels of cofilin hyperactivation.
Fig. S10. Accumulation of the $\alpha_4\beta_7^{high}$ CD4 T cells in the peripheral blood of HIV-infected patients.
Table S1. Characteristics of clinical study participants.
Table S2. Patient enrollment and grouping.

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wrote the manuscript. **Competing interests:** Y.W., H.S., and Y.F. are inventors on provisional patent applications related to this work filed by George Mason University [no. 62/593,335 (filed on 1 December 2017) and no. 62/620,598 (filed on 23 January 2018]. All the other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. The reverse-phase phospho-cofilin microarray datasets generated by Theranostics Health (Gaithersburg, MD, USA) are available upon request.

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