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Stimulating the RIG-I pathway to kill cells in the latent HIV reservoir following viral reactivation

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The persistence of latent HIV proviruses in long-lived CD4+ T cells despite antiretroviral therapy (ART)[1–3] is a major obstacle to viral eradication[4–6]. Because current candidate latency-reversing agents (LRAs) induce HIV transcription, but fail to clear these cellular reservoirs[7,8], new approaches for killing these reactivated latent HIV reservoir cells are urgently needed. HIV latency depends upon the transcriptional quiescence of the integrated provirus and the circumvention of immune defense mechanisms[4–6,9]. These defenses include cell-intrinsic innate responses that use pattern-recognition receptors (PRRs) to detect viral pathogens, and that subsequently induce apoptosis of the infected cell[10]. Retinoic acid (RA)-inducible gene I (RIG-I, encoded by *DDX58*) forms one class of PRRs that mediates apoptosis and the elimination of infected cells after recognition of viral RNA[11–14]. Here we show that acitretin, an RA derivative approved by the US Food and Drug Administration (FDA), enhances RIG-I signaling *ex vivo*, increases HIV transcription, and induces preferential apoptosis of HIV-infected cells. These effects are abrogated by *DDX58* knockdown. Acitretin also decreases proviral DNA levels in CD4+ T cells from HIV-positive subjects on suppressive ART, an effect that is amplified when combined with suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor. Pharmacological enhancement of an innate cellular-defense network could provide a means by which to eliminate reactivated cells in the latent HIV reservoir.

Despite the suppression of HIV replication by ART, HIV persists as latent proviruses in CD4+ T cells, which enables viral rebound when ART is interrupted[1–3]. Latent proviruses produce little HIV RNA and protein expression, and they allow immune evasion. Ideal LRAs would reactivate latent proviruses without provoking broad T cell activation, enabling the killing of host cells by either viral cytopathic or immune responses. This is the basis for the 'shock-and-kill' therapeutic approach to eliminate the latent reservoir[4–9]. In clinical studies, however, SAHA used alone did not deplete reservoir cells, although it did induce viral expression[7]. Moreover, when therapy is delayed, the latent reservoir contains mutations in the virus, which allows for immune escape from cytotoxic T lymphocytes[8]. Consequently, alternative killing strategies are needed[9].

Cell-intrinsic innate immune defenses against viral infection involve the detection of pathogens through PRRs. RIG-I is a cytosolic PRR[10–15] that can sense HIV RNA[16] and activate innate antiviral signaling, which leads to the death of infected cells[17]. However, HIV infection impedes RIG-I signaling by sequestration and degradation[18,19], and RIG-I protein expression levels are lower individuals with chronic HIV infection than in healthy individuals[20]. RIG-I recognizes full-length HIV RNA more efficiently than short viral transcripts[16] that predominate during latent infection[2–6], which thereby limits RIG-I activity in individuals on suppressive ART.

RA induces RIG-I expression[11], potentially arming an innate immune defense[21,22]. RA also activates p300 acetyl transferase[23,24] and could lead to the stimulation of HIV transcription[6]. Acitretin is an FDA-approved RA derivative that has been used to treat patients with HIV who have psoriasis, and the drug is well tolerated[25,26]. We hypothesized that acitretin could be used to increase HIV transcription and to activate the RIG-I pathway to promote the preferential death of reactivated reservoir cells.

First, we assessed the effects of acitretin on viral expression from ACH-2 cells, a latent-HIV-infected human T cell line[27]. Treatment of ACH-2 cells with acitretin or SAHA for 72 h increased HIV transcription, as compared to treatment with DMSO (**Fig. 1a**). The effects of acitretin were partially blocked by curcumin, an inhibitor of p300 (ref. 28) (**Fig. 1a**). Acitretin also activated HIV RNA expression in latently infected CD4+ T cells from subjects with HIV on suppressive ART (**Fig. 1b**).

The induction of HIV transcription alone may not clear HIV reservoirs[4–9]. Because acitretin induces RIG-I, and RIG-I signaling can eliminate virally infected cells[10–15], we tested the ability of acitretin to promote the clearance of HIV-infected cells in a primary T cell model[29]. We used a single round viral construct, GM-HIV, containing a mutated *gag* gene (**Supplementary Fig. 1**) to infect unstimulated CD4+ T cells from healthy donors by spinoculation[29,30], and then treated cells with acitretin, SAHA, or DMSO. 1 d after treatment, both acitretin and SAHA induced HIV RNA expression (**Fig. 1c**). Next, we examined whether the induction of HIV

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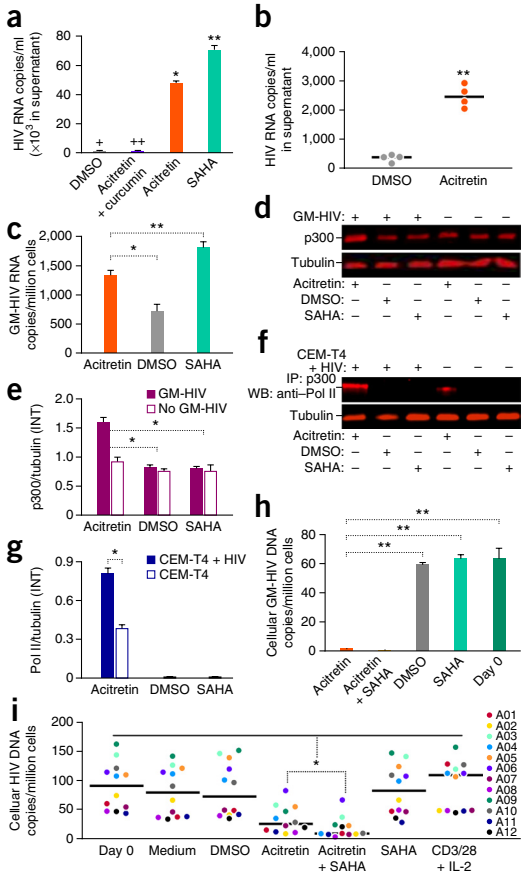

Figure 1 Acitretin induces HIV expression and reduces cellular HIV DNA in CD4⁺ T cells. (**a**) HIV copy number in the supernatant of a latent-HIV-infected T cell line (ACH-2) after 72 h of treatment. Both acitretin ($P < 0.05$) and SAHA significantly increased HIV transcription ($^{*}P < 0.01$), and curcumin, an inhibitor of p300, limited acitretin induction of HIV. ⁺HIV copy number, 486.6 ± 5.9; ⁺⁺HIV copy number, 379.6 ± 17.8. (**b**) HIV RNA copy number at day 6 in supernatants from cultures of CD4⁺ T cells from four aviremic subjects positive for HIV on ART. (**c**) Cellular GM-HIV RNA copies per million cells after 24 h of the indicated treatment of infected primary CD4⁺ T cells. (**d**) Immunoblot analysis of p300 and tubulin proteins from both GM-HIV-infected and uninfected CD4⁺ T cells (from the same donor) after 48 h of treatment. (**e**) The ratio of mean value intensities (INT) for p300 and tubulin from **d** ($n = 4$), confirming significantly higher expression of p300 in infected cells treated with acitretin than in cells treated with DMSO or SAHA. (**f**) Immunoblot analysis of co-immunoprecipitation of protein extracts of CEM-T4 cells with or without latent GFP-HIV virus, using antibody against p300 and western blot for RNA Pol II after 48 h of treatment. The association of p300 with RNA Pol II is enhanced by acitretin. (**g**) The ratio of RNA Pol II to tubulin from **f** ($n = 4$) is greatest with acitretin treatment of cells with GFP-HIV. (**h**) GM-HIV DNA content in cellular DNA after 72 h of treatment. (**i**) HIV DNA concentrations at day 0, and day 7 of treatments in CD4⁺ T cells from subjects positive for HIV on ART ($n = 12$). A01–A12 indicate each individual subject positive for HIV on ART. Values represent mean ± s.e.m. of duplicate samples from subjects with HIV (**b**,**i**) and triplicate samples from the ACH-2 (**a**) and GM-HIV infection model (**c**,**h**) representative of three independent experiments. Student's *t* test was used to compare experimental conditions (**a**–**c**,**e**,**g**–**i**); $^{*}P < 0.05$; $^{**}P < 0.01$.

RNA by acitretin was accompanied by p300 induction. Indeed, 48 h after acitretin treatment, p300 expression was increased in GM-HIV infected cells, as compared to that in uninfected cells (**Fig. 1d**,**e**), and the enhancement of p300 association with RNA polymerase II (Pol II) (**Fig. 1f**,**g**) was greater in HIV-infected CEM-T4 cells (a human lymphoblastoid T cell line)[14] than in uninfected cells. Furthermore, after 72 h of treatment, acitretin significantly reduced cellular GM-HIV DNA levels, and acitretin and SAHA in combination reduced cellular GM-HIV DNA to an undetectable level, as measured by real-time PCR (**Fig. 1h**).

We next tested whether acitretin reduces HIV DNA levels in samples from subjects with HIV on ART. Treatment of CD4⁺ T cells from

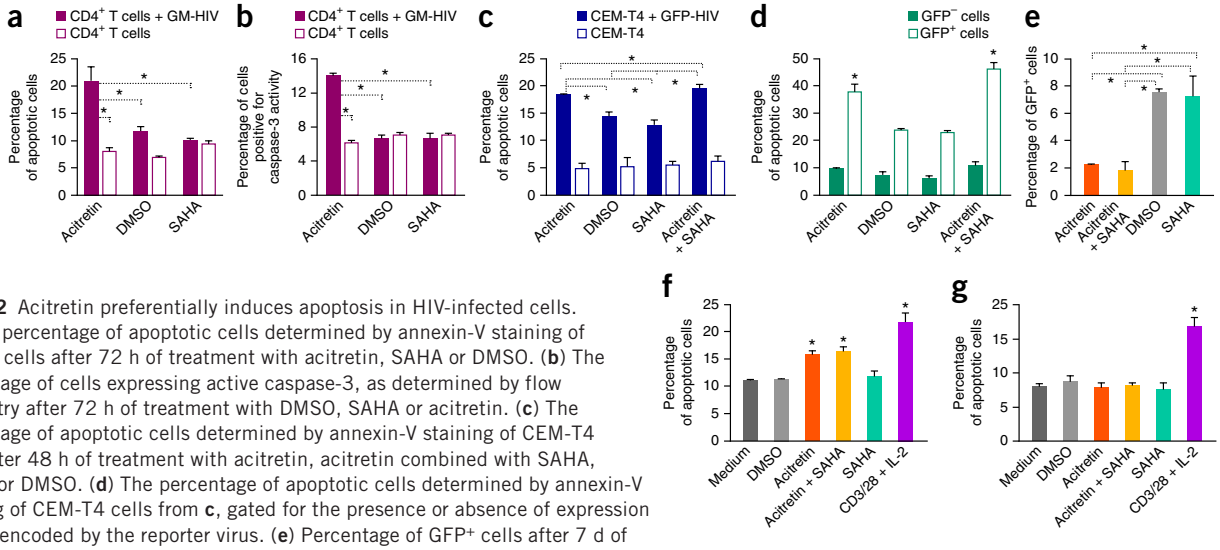

Figure 2 Acitretin preferentially induces apoptosis in HIV-infected cells. (**a**) The percentage of apoptotic cells determined by annexin-V staining of CD4⁺ T cells after 72 h of treatment with acitretin, SAHA or DMSO. (**b**) The percentage of cells expressing active caspase-3, as determined by flow cytometry after 72 h of treatment with DMSO, SAHA or acitretin. (**c**) The percentage of apoptotic cells determined by annexin-V staining of CEM-T4 cells after 48 h of treatment with acitretin, acitretin combined with SAHA, SAHA, or DMSO. (**d**) The percentage of apoptotic cells determined by annexin-V staining of CEM-T4 cells from **c**, gated for the presence or absence of expression of GFP encoded by the reporter virus. (**e**) Percentage of GFP⁺ cells after 7 d of treatment with acitretin, or acitretin combined with SAHA, or SAHA or DMSO, as determined by flow cytometry of infected CEM-T4 cells. (**f**) The average percentage of apoptotic cells, as determined by annexin-V staining of CD4⁺ T cells from subjects positive for HIV on ART ($n = 12$). After 7 d of treatment with acitretin or acitretin combined with SAHA, apoptosis was significantly increased, as compared to treatment with DMSO, SAHA, or medium ($P < 0.05$), except treatment with CD3/28 + IL-2. (**g**) The mean percentage of apoptotic cells, as determined by annexin-V staining of CD4⁺ T cells from four healthy control subjects. At day 7, acitretin or acitretin combined with SAHA did not increase apoptosis levels, except when treated with CD3/28 + IL-2 .Values represent mean ± s.e.m. of duplicate samples from experiments with cells from 12 subjects with HIV (**f**); values represent mean ± s.e.m. of triplicate samples for **a**–**e**, representative of three independent experiments; and values represent mean ± s.e.m. of triplicate samples for **g** from four CD4⁺ T cell experiments in healthy donors. Student's *t* test was used to compare experimental conditions for **a**–**g**; $^{*}P < 0.05$.

12 ART-suppressed subjects with HIV (**Supplementary Table 1**) with acitretin or a combination of acitretin and SAHA for 7 d reduced HIV DNA levels significantly more than did treatment with DMSO, SAHA, or anti-CD3 and anti-CD28 (anti-CD3/anti-CD28) beads ($P < 0.05$) (**Fig. 1i**). The reduction was greatest when acitretin was combined with SAHA. This reduction in HIV DNA concentration by acitretin was not due to the expansion of uninfected cells (**Supplementary Fig. 2**). Thus, acitretin facilitates the reduction of HIV DNA levels in CD4$^+$ T cells from subjects with HIV *in vitro*, an effect that has not been seen with any candidate LRAs studied to date.

In other models, RIG-I limits viral infection by inducing the apoptosis of infected cells[13–15]. To determine whether acitretin induces apoptosis in HIV-infected cells, we measured annexin-V staining and caspase-3 activity in GM-HIV-infected and uninfected CD4$^+$ T cells after 72 h of treatment. Acitretin treatment significantly ($P < 0.05$) increased the percentage of annexin-V$^+$ cells (**Fig. 2a**) and caspase-3 activity in the infected cells, as compared to SAHA and DMSO treatment and uninfected CD4$^+$ T cells (**Fig. 2b**), findings that are consistent with reports that the activation of RIG-I induces caspase-3-mediated apoptosis[13].

To more directly assess whether acitretin induces apoptosis preferentially in HIV-infected cells, and to test for potential synergistic activity with SAHA[31], cells were infected with GFP-HIV[29], and infected and uninfected cells were treated with acitretin, SAHA, or both. At 48 h, treatment with acitretin or with acitretin and SAHA in combination both resulted in significantly higher levels of apoptosis in the GFP$^+$ cells than in the total cells or the GFP$^-$ cells ($P < 0.05$) (**Fig. 2c**,**d**), which indicates preferential induction of apoptosis in GFP-HIV-infected cells. Furthermore, GFP$^+$ cells became significantly less frequent after treatment with acitretin or with acitretin and SAHA combined than with SAHA or DMSO (**Fig. 2e**). These results are consistent with a preferential induction of apoptosis in acitretin or acitretin and SAHA–treated HIV-expressing cells that leads to their elimination.

Next, we tested whether acitretin induces apoptosis in CD4$^+$ T cells from subjects positive for HIV on suppressive ART. Acitretin or acitretin and SAHA in combination each stimulated significantly higher levels of apoptosis than did treatment with DMSO, SAHA, or medium. No difference was seen in uninfected cells from healthy controls. Meanwhile, treatment with anti-CD3/anti-CD28 beads and interleukin (IL)-2 led to high levels of apoptosis in cells from both participants positive for HIV and healthy control participants (**Fig. 2f**,**g**). In contrast to treatment with anti-CD3/anti-CD28 beads, acitretin did not upregulate the activation markers CD69 and HLA-DR on CD4$^+$ T cells (**Supplementary Fig. 2h**). Kinetic studies showed that the induction of RNA expression occurred later with

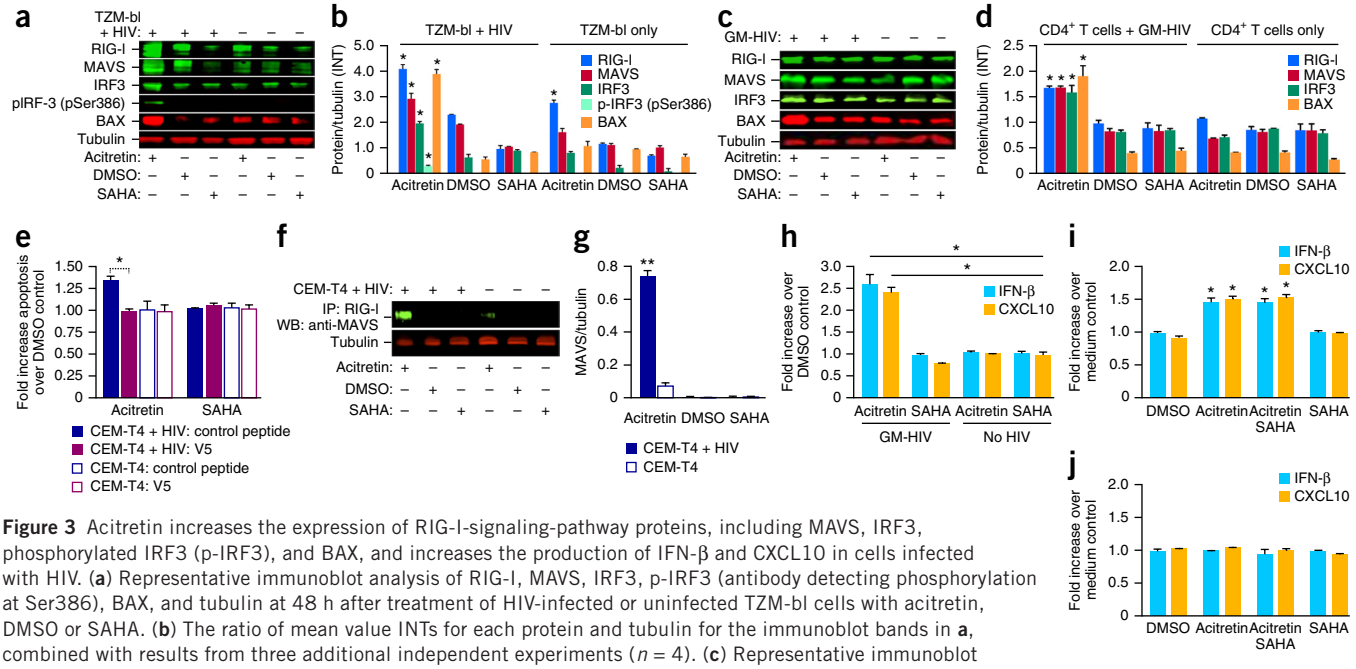

Figure 3 Acitretin increases the expression of RIG-I-signaling-pathway proteins, including MAVS, IRF3, phosphorylated IRF3 (p-IRF3), and BAX, and increases the production of IFN-β and CXCL10 in cells infected with HIV. (**a**) Representative immunoblot analysis of RIG-I, MAVS, IRF3, p-IRF3 (antibody detecting phosphorylation at Ser386), BAX, and tubulin at 48 h after treatment of HIV-infected or uninfected TZM-bl cells with acitretin, DMSO or SAHA. (**b**) The ratio of mean value INTs for each protein and tubulin for the immunoblot bands in **a**, combined with results from three additional independent experiments ($n = 4$). (**c**) Representative immunoblot analysis of RIG-I, MAVS, IRF3, BAX, and tubulin at 48 h after treatment of GM-HIV-infected and uninfected CD4$^+$ T cells with acitretin, DMSO or SAHA. (**d**) The ratio of mean value INTs of each protein and tubulin for the immunoblot bands in **c**, combined with results from three additional independent experiments ($n = 4$). RIG-I, MAVS, IRF3, p-IRF3, and BAX significantly increased when treated with acitretin, as compared to SAHA or DMSO treatment control, in HIV-infected cells ($P < 0.05$). RIG-I was also significantly increased by acitretin treatment in uninfected TZM-bl cells (**b**). (**e**) Fold increases in annexin-V$^+$ apoptotic cells in uninfected or HIV-infected CEM-T4 cells treated with acitretin or SAHA (versus DMSO) in the presence of the V5 peptide (a BAX inhibitor) or a control peptide. (**f**) Immunoblot analysis of co-immunoprecipitation of protein extracts of CEM-T4 cells with or without latent GFP-HIV virus, using an antibody against RIG-I for co-immunoprecipitation, followed by immunoblotting for MAVS, after 48 h of treatment with acitretin, DMSO, or SAHA. (**g**) The association of MAVS with RIG-I after acitretin treatment in **f** ($n = 4$) was higher in GFP-HIV-infected cells than in uninfected cells. (**h**) Fold increase over DMSO control in IFN-β and CXCL10 expression in supernatants from GM-HIV-infected and uninfected CD4$^+$ T cells 72 h after treatment. (**i**) Fold increase over medium control of IRF3-induced IFN-β and CXCL10 expression in the supernatants from cultures of CD4$^+$ T cells from subjects with HIV on ART ($n = 12$) on day 7 of treatment. (**j**) Fold increase over medium control of IRF3-induced IFN-β and CXCL10 expression in the supernatants from cultures of CD4$^+$ T cells from healthy control. All values represent mean ± s.e.m. of duplicate samples from subjects positive for HIV (**i**); values represent mean ± s.e.m. of triplicate samples for **e**,**h** from three independent experiments; and values represent mean ± s.e.m. of triplicate samples for **j** from four CD4$^+$ T cell experiments in healthy donors. Student's *t* test was used to compare experimental conditions (**b**,**d**,**e**,**g**–**j**); $^{**}P < 0.01$, $^{*}P < 0.05$.

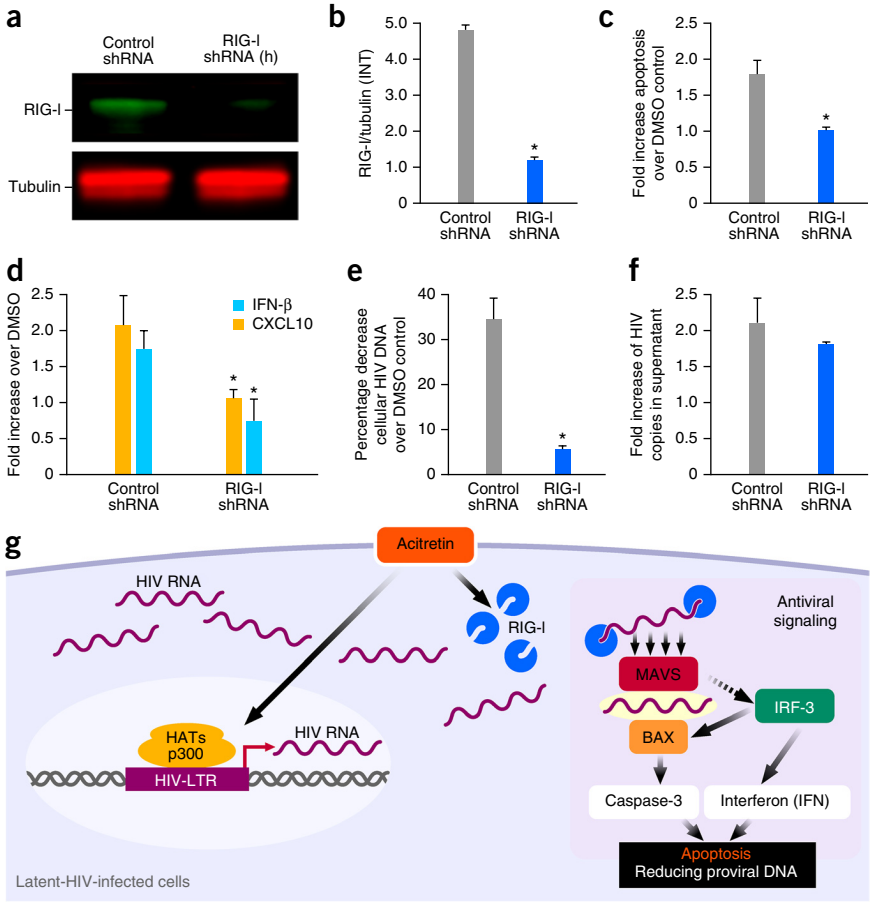

Figure 4 shRNA knockdown of RIG-I expression markedly inhibits acitretin enhancement of apoptosis, induction of IFN-β and CXCL10 production and preferential depletion of HIV-DNA$^+$ cells. (**a**) Immunoblot analysis of RIG-I and tubulin. (**b**) The ratio of mean value INTs of RIG-I to tubulin from **a** ($n = 4$); *DDX58*-shRNA (RIG-I shRNA) transfection significantly reduced RIG-I expression ($P < 0.05$). (**c**–**e**) Knockdown of *DDX58* significantly diminished ($P < 0.05$) acitretin-induced apoptosis at day 7 after treatment, as compared to the scrambled shRNA control (**c**); significantly reduced ($P < 0.05$) acitretin-induced CXCL10 and IFN-β release (**d**); and significantly impaired acitretin-induced decreases in HIV-DNA levels ($P < 0.05$) (**e**). (**f**) Knockdown of RIG-I expression, conversely, had no significant impact on acitretin-stimulated release of HIV RNA into supernatant at day 7. Fold increase of HIV copies in supernatant are from acitretin treatment, as compared to DMSO control, in both conditions. (**g**) Model depicting the preferential enhancement of RIG-I signaling by acitretin in latently infected HIV cells. Acitretin increases both *DDX58* and HIV RNA expression in the cytoplasm of latent-HIV-infected cells; recognition of HIV RNA by RIG-I initiates antiviral signaling. RIG-I–RIG-I interactions produce conformational changes that permit binding to MAVS, which in turn promotes IRF3 activation. Activated IRF3 activates two pathways: one inducing IFN production, and another resulting in the binding of BAX and the activation of caspase-3-dependent apoptosis in the virally infected cell. HATs, histone acetyltransferases. LTR, long-terminal repeat. All values represent mean ± s.e.m. of triplicate samples (**b**–**f**) representative of three independent experiments. Student's *t* test was used to compare experimental conditions (**b**–**f**); $^{**}P < 0.01$, $^{*}P < 0.05$.

acitretin than with SAHA, and that acitretin-induced apoptosis was greater for any given HIV-RNA level and time point than in the SAHA or DMSO conditions (**Supplementary Fig. 3**). These results support the conclusion that acitretin preferentially reduces HIV-containing cells by inducing apoptosis.

We examined whether the depletion of cells harboring HIV DNA and the induction of apoptosis by acitretin were dependent on RIG-I signaling. The binding of viral RNA to RIG-I leads to a conformational change in RIG-I (ref. 32) that enables binding of mitochondrial antiviral signaling protein (MAVS)[33] and the phosphorylation of interferon-regulatory factor 3 (IRF3) at serine 386 (refs. 34–37), which culminates in type I interferon (IFN) induction and caspase-3 activation[10–15]. To assess the effects of acitretin on RIG-I expression and signaling, we immunoblotted extracts from HIV-infected cells and uninfected TZM-bl cells—a HeLa cell line containing luciferase and β-galactosidase genes under control of the HIV-1 promoter—after 48 h of treatment with acitretin, or SAHA or DMSO, along with antibodies against RIG-I pathway proteins. Acitretin induces RIG-I in both infected and uninfected cells (**Fig. 3a**,**b**). However, only in HIV-infected cells did acitretin significantly enhance MAVS, IRF3 and phosphoserine 386–IRF3 levels ($P < 0.05$). Similar patterns were seen in latent GM-HIV-infected CD4$^+$ T cells (**Fig. 3c**,**d**). Acitretin treatment significantly increases the binding of MAVS to RIG-I (ref. 33) in latently HIV-infected CEM-T4 cells, as compared to uninfected cells (**Fig. 3**). These observations suggest the initial induction of RIG-I and viral RNA by acitretin, and the subsequent RIG-I sensing of viral RNA[12–15,32] promotes RIG-I association with MAVS[38]. RIG-I-induced apoptosis in virus-infected cells depends on the interaction of IRF3 with BCL-2-associated X protein (BAX), which triggers caspase-mediated apoptosis[12,13]. In the presence of acitretin, BAX expression was significantly increased only in HIV-infected cells, as compared to all other conditions, and pretreatment with a BAX inhibitor (V5) peptide[39], but not with a control peptide, diminished acitretin-induced apoptosis (**Fig. 3a**–**e**). These findings support a role for BAX signaling in acitretin-induced apoptosis[12,13].

Next, we measured type I IFN and CXCL10 chemokine production[18,33–37] after 72 h of acitretin treatment in GM-HIV-infected and uninfected primary CD4$^+$ T cells. Acitretin significantly increased IFN-β and CXCL10 secretion in infected cells, as compared to SAHA treatment, but not in uninfected cells (**Fig. 3h**). Similarly, IFN-β and CXCL10 levels in day-7 supernatants of T cell cultures from ART-suppressed subjects with HIV were higher after treatment with acitretin or with acitretin and SAHA combined, as compared to DMSO or SAHA alone (**Fig. 3i**). By contrast, no increase in IFN-β or CXCL10 production was detected in acitretin-treated or in acitretin and SAHA–treated cells from healthy volunteers (**Fig. 3j**). Acitretin produced dose-related changes in both apoptosis and IFN-β or CXCL10 production in infected cells (**Supplementary Fig. 4**). Taken together, these results indicate that acitretin stimulates preferential RIG-I-mediated signaling in HIV-infected cells, which leads not only to apoptosis, but also to type I IFN and chemokine production. The release of IFN and the subsequent activity of

IFN-stimulated genes might promote the death of some uninfected bystander, as well as infected, cells, which explains why the total number of dying cells is greater than the number of latently infected cells present (**Fig. 2a–c,f**).

Finally, to establish that the effects of acitretin treatment are mediated through RIG-I, short hairpin RNA (shRNA) was used to inhibit the expression of RIG-I in CEM-T4 cells. *DDX58* knockdown abrogated the induction of apoptosis, the production of CXCL10 and IFN-β, and the reduction in HIV DNA by acitretin treatment, but had no impact on the release of HIV virions in this latent-infection model[29] (**Fig. 4a–f**). These findings demonstrate the essential role of RIG-I in the observed acitretin effects. Further studies are needed to define the nature of HIV transcripts that bind and activate RIG-I (ref. 10).

In summary, our data support a model (**Fig. 4g**) whereby acitretin, at clinically achievable concentrations[40], increases HIV transcription, induces RIG-I expression, and augments RIG-I signaling, which culminates in the preferential apoptotic death of the reactivated reservoir cells. The addition of LRAs such as SAHA further enhances the effects of acitretin. Thus, one possible approach to inducing the death of reservoir cells could be to use combinations of LRAs and acitretin to preferentially 'shock and kill' $CD4^+$ T cells that harbor HIV. Such an approach might eliminate cells that produce both infectious and noninfectious forms of HIV.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.L. contributed to designing the research, performing the experiments, interpreting the data, and writing the paper. P. Kaiser assisted with experiments and contributed to interpreting the data and writing the paper. H.W.L. contributed to recruiting subjects with HIV for the study. P. Kim and S.A.Y. assisted with experiments. D.V.H. and W.C.G. provided key suggestions and contributed to interpreting the data. W.C.G. also contributed to writing the paper. J.K.W. contributed to recruiting subjects with HIV for the study, interpreting the data, and writing the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell lines and pNL4-3 plasmid. CEM-T4 cells (HIV$^-$, human CD4$^+$, lymphoblastic cell line), TZM-bl cells (HIV$^-$, HeLa cell line with luciferase, β-galactosidase reporters under control of the HIV promoter), and ACH-2 cells (latent-HIV T cell line derived from human T cells) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (courtesy of J.P. Jacobs, J.C. Kappes, X. Wu, T. Folks, and M. Martin, respectively).

Media and reagents. All T cells and T cell lines were cultured in RPMI 1600 supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) (all from Life Technologies, Grand Island, NY), and 10% heat-inactivated FCS (FCS) (Sigma-Aldrich, St. Louis, MO). TZM-bl cells were cultured in DMEM supplemented with the same additives as above. During cell culture after treatment or activation, 1 μM indinavir (IDV), 10 μM nevirapine (NVP), and 600 nM raltegravir (RAL) (NIH AIDS Reagent Program) were included to prevent the spreading of HIV infection. Working stocks of acitretin and SAHA were dissolved in DMSO (all from Sigma-Aldrich, St. Louis, MO). Anti-CD3 and anti-CD28 antibody beads (CD3/28) (Life Technologies, Grand Island, NY) were used at 1 bead per cell with human IL-2 (Chiron, Emeryville, CA) at 10 U/ml.

Cell culture and drug treatment. Cell treatment for both primary T cells and cell lines was performed by resuspending cells at 0.5 million cells per ml in complete RPMI (RPMI 1600 supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and 10% heat-inactivated FCS (FCS)) with antiretroviral drugs as indicated above. TZM-bl cells were used by plating approximately 2×10^5 cells/well of a 24-well tissue culture plate 1 d before infection. This results in approximately 75% cell confluence at the time of infection and further testing. In general, test drugs were used at the following final concentrations: SAHA (350 nM); acitretin (5 μM); equivalent DMSO (final DMSO concentration did not exceed 0.05%) input for negative control. Dose-response studies used acitretin varying from 1–25 μM using the DMSO (final DMSO concentration did not exceed 0.25%), which is equivalent to the 25 μM of acitretin comparator. Cells were maintained in the same drug concentration throughout the duration of experiments, which ranged from 3–7 d. Most assays employed acitretin at a fixed concentration of 5 μM (1,650 ng/ml). RAs are highly lipophilic, and thus, although serum concentrations peak at 500–700 ng/ml, tissue concentrations measured in patients on established therapy reach levels of 16,000 ng/ml or approximately 50 μM (ref. 40). The concentrations used in the current studies are therefore clinically achievable.

Generation of GM-HIV for single-round HIV infection. We excised the *gag* region from pNL4-3 by restriction digest with BssHII (711) and SpeI (1507), subcloning this region into the pcDNA3.1 TOPO TA vector (Life Technologies, Grand Island, NY). Using the Quikchange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), we mutated a region of *gag* from amino acids 1,404 to 1,432 (**Supplementary Fig. 1a**). The sequence-verified mutated *gag* was recloned into pNL4-3 to make pGM-HIV. To generate GM-HIV capable of only a single round of infection, we co-transfected HEK293T cells (a human kidney cell line (American Type Culture Collection, ATCC), Manassas, VA) with the pGM-HIV clone and a plasmid expressing wild-type *gag* (**Supplementary Fig. 1a**); only pGM-HIV with *gag*-expressing vector can produce p24 into supernatant (**Supplementary Fig. 1b**). To confirm the infectivity of GM-HIV, we first infected 0.5×10^6 TZM-bl cells with 1 ng of p24 supernatant from pGM-HIV with *gag*-expressing vector, the same volume of supernatant from pGM-HIV with empty vector, and 1 ng of p24 of HIV-1 (NL-4-3) as a positive control. After 3 d, HIV infectivity was measured using the Bright-Glo Luciferase assay system (Promega) and expressed as relative light units (RLU). Only supernatant from pGM-HIV with *gag*-expressing vector and HIV-1 (NL4-3) were able to infect TZM-bl cells (**Supplementary Fig. 1c**). Next, we infected phytohaemagglutinin (PHA)-stimulated 1×10^6 CD4$^+$ T cells with 5 ng of p24 in supernatant from pGM-HIV with *gag*-expressing vector, or an equal volume of supernatant from pGM-HIV with empty vector, by spinoculation at 2,000*g* for 2 h (ref. 29). The cells were washed with RPMI three times immediately after infection and once the next day to remove all residual inoculum. Subsequently, the cells were cultured in RPMI with IL-2 (10 U/ml), and then GM-HIV DNA and RNA concentrations were measured in cellular RNA and DNA extracts by real-time PCR at day 1 and day 7. GM-HIV was measurable only in cells infected with supernatant from pGM-HIV with *gag*-expression vector (**Supplementary Fig. 1d**).

Participants with HIV on ART. All participants positive for HIV were on combination ART and had had undetectable plasma viral loads (<50 copies/ml) for at least 1 year (median 5 years) (**Supplementary Table 1**). They and the healthy controls were recruited from the San Francisco Veterans Affairs Medical Center (SF-VAMC) Infectious Diseases Clinic to meet the predetermined number of study participants. The study was approved by the UCSF Committee on Human Research and the SF VAMC Human Subject Research subcommittee, and all research participants gave written informed consent.

Isolation and culture of CD4$^+$ T cells from HIV-infected participants and healthy donors. Peripheral blood mononuclear cells (PBMCs) were purified from whole blood (HIV$^+$, on ART) or PBMCs were isolated from leukoreduction filters (healthy donors) by density centrifugation. CD4$^+$ T lymphocytes were enriched by negative selection with the EasySep Human CD4 T cell enrichment kit (Stemcell, Vancouver, BC). The purity of CD4$^+$ T cells was assessed by flow cytometry and was typically >95%. Cells were rested overnight before additional use.

***In vitro* infection of primary CD4$^+$ T cells with GM-HIV for a primary T cell model of latent HIV infection.** Healthy donor CD4$^+$ T cells enriched by negative selection were cultured overnight, and then treated according to the method of Lassen[29] by first infecting the unstimulated, resting CD4$^+$ T cells with GM-HIV (5 ng p24 per 1×10^6 cells) by spinoculation at 2,000*g* for 2 h. Uninfected controls underwent mock spinoculation. The cells were washed with RPMI three times immediately after infection and once the next day to remove all residual inoculum. After the spinoculation of unstimulated CD4$^+$ T cells, the cells were cultured in RPMI for 10 d and integrated to establish latent proviral infection[29]. To confirm that the cells carried latent proviruses, we stimulated the cells with either PHA and IL-2 or anti-CD3/28 beads + IL-2 for 48 h, and then measured cellular GM-HIV RNA. Both PHA with IL-2 (PHA + IL-2) and CD3/28 + IL-2 treatment resulted in significantly increased cellular GM-HIV RNA relative to medium control (**Supplementary Fig. 1e**).

Generation of latent GFP–HIV-infected CEM-T4 cells. A GFP-reporter HIV construct (GFP-HIV)[29] was transfected into HEK293T (ATCC, Manassas, VA) cells to produce GFP-HIV virions. CEM-T4 cells (1×10^6) were infected with 1,000 pg of p24 for 5 h, and unbound virus was removed by washing the cells three times with RPMI. Subsequently, the cells were cultured in medium with Saquinavir (5 μM) for 10 d to prevent the spreading of infection and to allow for the attrition of productively infected cells[29]. To study whether the cells have latent proviruses, we stimulated the cells with 5 μM acitretin, 350 nM SAHA, or 500 nM prostratin for 72 h, and then harvested supernatant to measure the p24 production. Acitretin, SAHA, and prostratin all significantly increased p24 production, as compared to medium control, which demonstrated that the cells contained proviruses that respond to LRA stimulation (**Supplementary Fig. 3a**).

Assays for apoptosis. The following assays were performed in duplicate with the EasyCyte6HT-2L flow cytometer (Millipore, Billerica, MA), according to the manufacturer's instructions: (i) Guava ViaCount assay (Millipore, Billerica, MA) for absolute total cell counts and percentages of apoptotic cells; (ii) Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Life Technologies, Grand Island, NY) to detect apoptosis for all experiments except those using GFP-HIV virus (50,000 cells per sample at each time point) (**Figs. 1** and **3**); (iii) CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit (Life Technologies, Grand Island, NY) to detect caspase-3 and caspase-7 activity (50,000 cells per sample) (**Fig. 1**); (iv) Alexa Fluor 647 Annexin-V (Life Technologies, Grand Island, NY) to detect apoptosis in GFP-HIV-infected CEM-T4 cells (100,000 cells per sample) (**Fig. 2**), because GFP would interfere with Alexa Fluor 488 annexin-V apoptosis assay and caspase 3/7-green flow cytometry assay.

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Assay with BAX inhibitor-V5. Both GFP-HIV-infected and uninfected CEM-T4 cells were prepared as described above under 'Generation of latent GFP-HIV-infected CEM-T4 cells'. They were incubated with 200 μM of either BAX-inhibitor-V5 or BAX-Inhibiting Peptide Negative Control (Millipore, Billerica, MA) for 3 h, and then treated with the following drugs (final concentrations): SAHA (350 nM); Acitretin (5 μM); equivalent DMSO input. After treatment for 24 h (ref. 39), the cells were stained with Alexa Fluor 647 Annexin-V (Life Technologies, Grand Island, NY) to detect apoptosis (100,000 cells per sample) using the EasyCyte6HT-2L flow cytometer (Millipore, Billerica, MA), according to the manufacturer's instructions.

ELISA assays for cytokine. Supernatants from GM-HIV-infected and uninfected $CD4^+$ T cell cultures were collected 72 h after drug treatment and from HIV^+ or healthy donor $CD4^+$ T cells 7 d after drug treatment. The supernatants were analyzed with the following ELISAs: (i) Verikine Human IFN-β kit (BPL Assay Science, Piscataway, NJ) and (ii) human CXCL10/IP-10 kit (R&D Systems, Minneapolis, MN). Assays were done according to the manufacturers' protocols.

Immunoblot (western blot) analysis. Cellular protein was extracted with the Mammalian Cell Lysis Kit (Sigma-Aldrich, St. Louis, MO), separated on a 4–12% Bis-Tris gel (Life Technologies, Grand Island, NY), and transferred to an Immobilon-FL-PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with Odyssey Blocking buffer and incubated overnight with 1:200 dilution of primary antibodies against RIG-I, p300, MAVS, IRF3, BAX, Pol II, tubulin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or p-IFR3 (phospho-Ser 386) (GeneTex, Irvine, CA). The membranes were then incubated with 1:15,000 dilution of secondary antibodies: IRDye 800CW (green) for RIG-I, MAVS, and IRF3, p-IRF3 (pSer386) and IRDye680 (red) for BAX, p300, Pol II, and tubulin (all from LI-COR, Lincoln, NE). Finally, western blot membranes were analyzed with the Odyssey CLx Infrared Imaging System (LI-COR, Lincoln, NE). Protein expression is represented as mean band INTs normalized to results for tubulin.

Co-immunoprecipitation assays (IP) with RIG-I or p300. 30×10^6 of each cell sample were washed twice with cold PBS and lysed using the Mammalian Cell Lysis kit (Sigma-Aldrich, MO). The lysates were centrifuged at 4 °C for 10 min at 12,000*g* to pellet out cellular debris, and the clarified lysate was transferred to a fresh pre-chilled tube. 20 μl of lysates from each sample were saved for tubulin western blot to determine the amount of protein in the lysates. The remaining lysates were pre-cleared by incubation with protein A in Sepharose for 1 h at 4 °C, subjected to immunoprecipitation with 2 μg of anti-RIG-I polyclonal antibody (C-15), or 2 μg of anti-p300 polyclonal antibody (C-20) overnight at 4 °C, and then treated with 50 μl of protein G in Sepharose for 2 h. The immunoprecipitates were washed ten times with 1 ml of cold-wash buffer (1% NP-40, 1% triton X-100, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 10 mM $Na_4P_2O_7$, 50 mM NaF,1 mM $NaVO_4$, 0.1% 1-mercaptoethanol, 1 mM PMSF, 0.25 M NaCl) and then eluted from the protein G in Sepharose with 50 μl of elution buffer (1 M glycine, 0.25 M NaCl, pH 2.8). The eluate was neutralized with 2 μl of 1 M Tris-Cl (pH 9.5) and then subjected to 10% SDS–PAGE gel electrophoresis and transferred to nitrocellulose membranes. Membranes were treated with blocking buffer (Odyssey blocking buffer) for 1 h, and then incubated overnight in blocking buffer with either a 1:200 dilution of anti-MAVS (T-20) for RIG-I-immunoprecipitation or a 1:200 dilution of anti–RNA polymerase II (Pol II) (F-12)) for p300 immunoprecipitation at 4 °C. Detection was performed using a 1:15,000 dilution of secondary antibodies: IRDye 800CW (green) for MAVS, and IRDye680 (red) for Pol II for 2 h. The bands were analyzed using the Odyssey Infrared Imaging System (LI-COR, Nebraska).

Preparation and evaluation of cells with attenuated RIG-I expression. CEM-T4 cells were transfected with either RIG-I (*DDX58*)-shRNA (h) plasmid (sc-61480-SH), or control-shRNA plasmid (sc-108083) (both from Santa Cruz Biotechnology, Santa Cruz, CA) using FuGENE HD transfection reagent (Roche, Indianapolis, IN), according to the manufacturers' protocols. 72 h after transfection, puromycin at 10 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) was added to select for transfected cells for 7 d. Immunoblot analysis of protein prepared from both shRNA and control-plasmid transfected cells was performed to confirm RIG-I knockdown using anti-RIG-I (C-15). RIG-I-knockdown cells and control cells were infected with 1,000 pg of p24 of HIV NL4-3 for 5 h, and unbound virus was removed by washing the cells three times with RPMI. Subsequently, the cells were cultured in medium with Saquinavir (5 μM) for 7 d to prevent the spreading of infection and to permit a reversion to latency[29].

RIG-I (*DDX58*)-shRNA and control plasmid-transfected cells were then each treated with acitretin (5 μM) or the equivalent DMSO input (negative control) and maintained with an antiretroviral drug cocktail of 1 μM indinavir (IDV), 10 μM nevirapine (NVP), and 600 nM raltegravir (RAL) for 7 d.

Measurement of intracellular HIV-1 RNA, intracellular HIV-1 DNA, and HIV-1 RNA in supernatant. Total cellular RNA was extracted with the Rneasy Mini Kit (Qiagen, Valencia, CA). Genomic DNA was extracted with the DNA blood mini-kit (Qiagen, Valencia, CA). RNA and DNA concentrations were measured with an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). Supernatant HIV-1 RNA was extracted with the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA). HIV RNA copy numbers were measured with a locked nucleic acid (LNA) Taqman assay. Briefly, we performed a single-tube, one-step assay in a total volume of 25 μl using the TaqMan RNA-to-CT-1STEP KIT (Life Technologies, Grand Island, NY), primers G19-2-F-7Y (AGCAGCYATGCAAATGTTA) and G-20-R (AGAGAACCAAGGGGAAGTGA) (400 nM each), and a dual-labeled fluorescent LNA probe (6-FAM/+C+C+ATCA+A+T+G+A+G+G+A/BHQ1) (200 nM); '+' in front of nucleotides indicates an LNA. Cycling conditions were 50 °C for 30 min, 95 °C for 5 min, and then 50 cycles of 95 °C for 15 s and 59 °C for 1 min.

HIV DNA copy numbers were assessed with the same primer and probe combination and the TaqMan Gene Expression Master mix (Life Technologies, Grand Island, NY). Results were expressed as averaged HIV-1 RNA or DNA copies per million CD4 equivalents for cellular nucleic acids, and as RNA copies per ml for supernatant HIV RNA concentration. The limit of detection for all samples was ten copies per million cells.

For quantification of GM-HIV RNA and DNA, the same primers and methods were used but with the following probe: 6-VIC/AACGAAG TCAGTCGACCACACTCACTCTCAC/BHQ1.

Statistical analyses. ACH-2 cell, CEM-T4 cell, and GM-HIV-infected $CD4^+$ T cell assays were run in triplicate and repeated in at least three independent experiments. Experiments with primary $CD4^+$ T cells were run in triplicate and repeated with at least four different donors. Experiments with unstimulated $CD4^+$ T cells from subjects with HIV were run in duplicate. All immunoblot analysis was repeated from four independent experiments. Microsoft Excel 2008 was used for statistical analyses. Data are presented as means ± s.e.m. Significance was assessed by Student's *t* test; $P < 0.05$ was considered to be significant.