Establishment of HIV-1 latency in resting CD4$^+$ T cells depends on chemokine-induced changes in the actin cytoskeleton

Paul U. Cameron[a,b,c,1], Suha Saleh[b,1], Georgina Sallmann[b], Ajantha Solomon[b], Fiona Wightman[b], Vanessa A. Evans[b], Genevieve Boucher[d], Elias K. Haddad[d], Rafick-Pierre Sekaly[d], Andrew N. Harman[e], Jenny L. Anderson[f], Kate L. Jones[f], Johnson Mak[f,g], Anthony L. Cunningham[e], Anthony Jaworowski[b,c,f], and Sharon R. Lewin[a,b,f,2]

[a]Infectious Diseases Unit, Alfred Hospital, Melbourne, Victoria 3004, Australia; Departments of [b]Medicine and [c]Immunology, Monash University, Melbourne 3004, Australia; [d]Laboratoire d'Immunologie, Centre de Recherche de Centre Hospitalier de L'Universitie de Montreal, Saint-Luc, Quebec, Canada; [e]Westmead Millenium Research Institute, Westmead 2145, Australia; [f]Centre for Virology, Burnet Institute, Melbourne 3004, Australia; and [g]Department of Biochemistry and Molecular Biology, Department of Microbiology, Monash University, Clayton 3800, Australia

Edited by Malcolm A. Martin, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and approved August 23, 2010 (received for review March 8, 2010)

Eradication of HIV-1 with highly active antiretroviral therapy (HAART) is not possible due to the persistence of long-lived, latently infected resting memory CD4$^+$ T cells. We now show that HIV-1 latency can be established in resting CD4$^+$ T cells infected with HIV-1 after exposure to ligands for CCR7 (CCL19), CXCR3 (CXCL9 and CXCL10), and CCR6 (CCL20) but not in unactivated CD4$^+$ T cells. The mechanism did not involve cell activation or significant changes in gene expression, but was associated with rapid dephosphorylation of cofilin and changes in filamentous actin. Incubation with chemokine before infection led to efficient HIV-1 nuclear localization and integration and this was inhibited by the actin stabilizer jasplakinolide. We propose a unique pathway for establishment of latency by direct HIV-1 infection of resting CD4$^+$ T cells during normal chemokine-directed recirculation of CD4$^+$ T cells between blood and tissue.

Eradication of HIV-1 with highly active antiretroviral therapy (HAART) is currently not possible because of latent infection of long-lived resting memory CD4$^+$ T cells (1, 2). HIV-1 latency in resting CD4$^+$ T cells may occur as preintegration or postintegration latency. Preintegration latency refers to unintegrated HIV-1 DNA that is unstable and will either degrade or will integrate into the host cell genome, usually following cell activation (3–6). In postintegration latency, integrated HIV-1 DNA is found in cells that are not actively producing viral particles. Reverse transcription and integration of HIV-1 is inefficient in resting peripheral blood CD4$^+$ T cells (4, 7), whereas postintegration latency can be established in resting CD4$^+$ T cells in lymphoid tissue explants or in the tissues of HIV-1–infected individuals or SIV-infected macaques in vivo (8–11). It is currently not understood how postintegration latency is established in resting CD4$^+$ T cells.

A recent study showed that HIV-1 itself can facilitate nuclear localization in resting CD4$^+$ T cells via HIV-1 viral envelope signaling through the HIV-1 coreceptor CXCR4 (12, 13). Following CXCR4 signaling, a pathway involving dephosphorylation of cofilin and changes in filamentous actin (F-actin) was critical for entry of HIV-1 into the nucleus of a resting CD4$^+$ T cell. However, in this model, integration into the genome did not occur. In addition, this study did not provide the rationale for how latency is established following infection with CCR5-using (R5) viruses, which are identified in vivo far more frequently than CXCR4-using (X4) viruses (14–16).

We have previously shown that the chemokines CCL19 and CCL21, ligands for the CCR7 receptor, can dramatically increase the frequency of HIV-1 DNA integration and latent infection in resting CD4$^+$ T cells (17). These chemokine receptors are critical in guiding the migration of naive and memory T cells across high endothelial venules and endothelium into lymphoid tissue (18) and are important in the clustering of T cells with dendritic cells (DCs) (19). We therefore hypothesized that multiple chemokines, in addition to CXCR4 ligands may facilitate HIV-1 entry and integration in resting CD4$^+$ T cells and that this was mediated via activation of actin.

We now show that the exposure of resting CD4$^+$ T cells to the chemokines CCL19, CXCL10, and CCL20, all of which regulate T-cell migration, allows for efficient HIV-1 nuclear localization and integration of the HIV-1 provirus, that this occurs in the absence of significant changes in gene expression, and is mediated via cofilin dephosphorylation and changes in actin polymerization. This data provide a unique mechanism for establishing latent HIV-1 infection that depends on chemokines involved in cell migration and is independent of antigen presentation or cell activation.

Results

Chemokine-Mediated Viral Entry and Integration in Resting CD4$^+$ T Cells. We tested the effects of chemokines binding to CCR7 (CCL19), CXCR3 (CXCL9 and CXCL10), CCR6 (CCL20), CCR8 (CCL1), and CCR2/CCR3 (CCL13) as described in Fig. 1*A*. Incubation of resting CD4$^+$ T cells with ligands to CCR7, CXCR3, and CCR6, followed by infection with the X4 virus NL4.3 or the R5 virus AD8 led to high levels of viral integration and minimal production of reverse transcriptase (RT) in supernatant, consistent with latent infection (Fig. 1 *B* and *C*). Following incubation with chemokines CCL1 or CCL13 (ligands for CCR8 and CCR2/CCR3, respectively), HIV-1 integration was not observed. In keeping with our previous observations with CCL19 (17), there were no changes in the expression of surface markers of T-cell activation, including CD69, HLA-DR, and CD25, following incubation with these additional chemokines for 3 d (Fig. 1*D*). In addition, there was little or no change in the expression of chemokine receptors after culture with each chemokine (Fig. 1*E*).

Chemokine Receptor Expression on Resting CD4$^+$ T Cells and After Culture. To determine whether the changes in viral integration seen with these chemokines were associated with differences in gene expression, we initially performed microarray analyses of resting CD4$^+$ T cells following 6, 18, and 72 h of culture with

Author contributions: P.U.C., A.J., and S.R.L. designed research; S.S., G.S., A.S., F.W., V.A.E., G.B., A.N.H., J.L.A., and K.L.J. performed research; P.U.C., S.S., G.S., G.B., E.K.H., R.-P.S., A.N.H., J.L.A., J.M., A.L.C., A.J., and S.R.L. analyzed data; and P.U.C., S.S., and S.R.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]P.U.C. and S.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: sharon.lewin@med.monash.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1002894107/-/DCSupplemental.

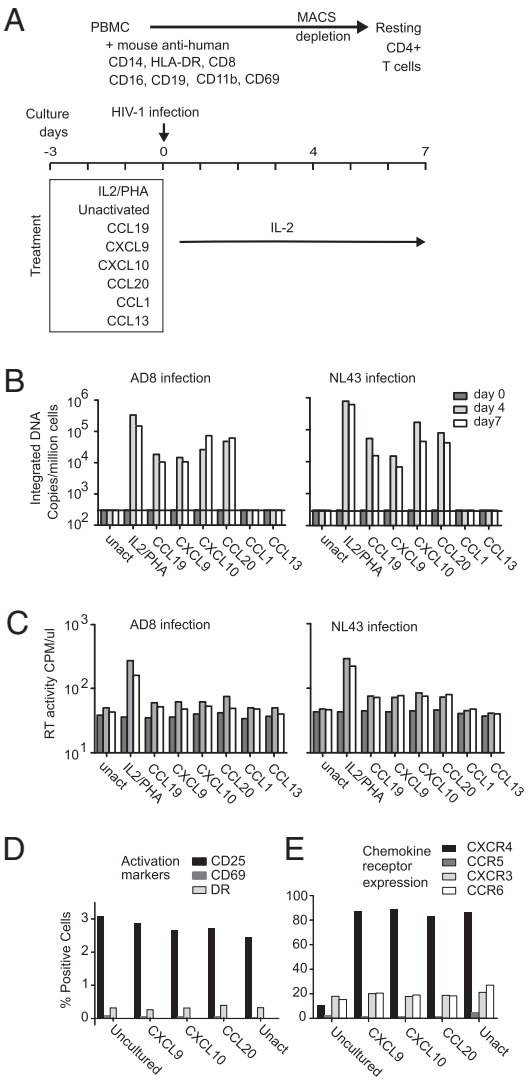

Fig. 1. Multiple chemokines facilitate HIV-1 latent infection in resting CD4$^+$ T cells. (*A*) Schematic of the experimental protocol used for isolation of CD4$^+$ T cells and culture with chemokines. Resting CD4$^+$ T cells were cultured for 3 d without activation (unactivated), or with PHA/IL-2, or the chemokines CCL19 (100 nM, ligand for CCR7), CXCL9, or CXCL10 (100 nM, ligands for CXCR3), CCL20 (100 nM, ligand for CCR6), CCL1 (100 nM, ligand for CCR8), and CCL13 (100 nM, ligand for CCR2, CCR3). Cells were infected with HIV-1 NL4.3 and AD8 (2 h) and cultured with media containing IL-2 (10 IU/mL) up to 7 d postinfection. (*B*) Integrated HIV-1 DNA was quantified using Alu-LTR PCR on cell lysates and (*C*) RT activity was quantified in culture supernatants collected at days 0, 4, and 7 following infection (AD8, *Left*; NL4.3, *Right*). Results are representative of two similar experiments for each virus. (*D*) Flow cytometry analysis of cells cultured for 3 d with the relevant chemokine or media alone (unactivated) for expression of the T-cell activation markers CD25, CD69, and HLA-DR) and (*E*) chemokine receptors. Uncultured, expression on resting CD4$^+$ T cells present in fresh blood; unact, unactivated cultured cells.

CCL19 and compared gene expression to unactivated cells or PHA/IL-2 activated cells (data from the 72-h comparison are summarized in Table S1). Compared with unactivated CD4$^+$ T cells, the PHA/IL-2 activated CD4$^+$ T cells had many genes that were significantly up- (n = 2,426) or down-regulated (n = 2,195) when the lower probability was applied (Table S1). In contrast, only a limited number of genes were found to be up- (n = 87) or down-regulated (n = 157) during CCL19 activation and none were significantly different from unactivated cells when corrections for false discovery rate (FDR), or higher criteria for fold change (>2), or cutoff for significance (P < 0.01) were applied. These findings suggested that there were only limited changes in gene expression associated with exposure to CCL19 and that rapid changes in signaling pathways may be most important in controlling HIV-1 nuclear localization and DNA integration, in keeping with long-lived cells that are continually trafficking and recirculating in vivo (20).

We next used the gene array data to assess the comparative level of gene expression for known chemokine receptors in the unactivated and CCL19-activated CD4$^+$ T cells (Fig. 2*A*). Similar levels of chemokine receptor gene expression were seen when CD4$^+$ T cells were cultured with chemokines for 6, 18, or 72 h. The chemokine receptors CCR7, CXCR4, and CCR6 were expressed at the highest level in both resting and CCL19-activated cells, followed by lower expression of CXCR3 and CCR4 (Fig. 2*A*). We confirmed these observations with flow cytometry to measure surface expression of the chemokine receptors before and after culture with each chemokine for up to

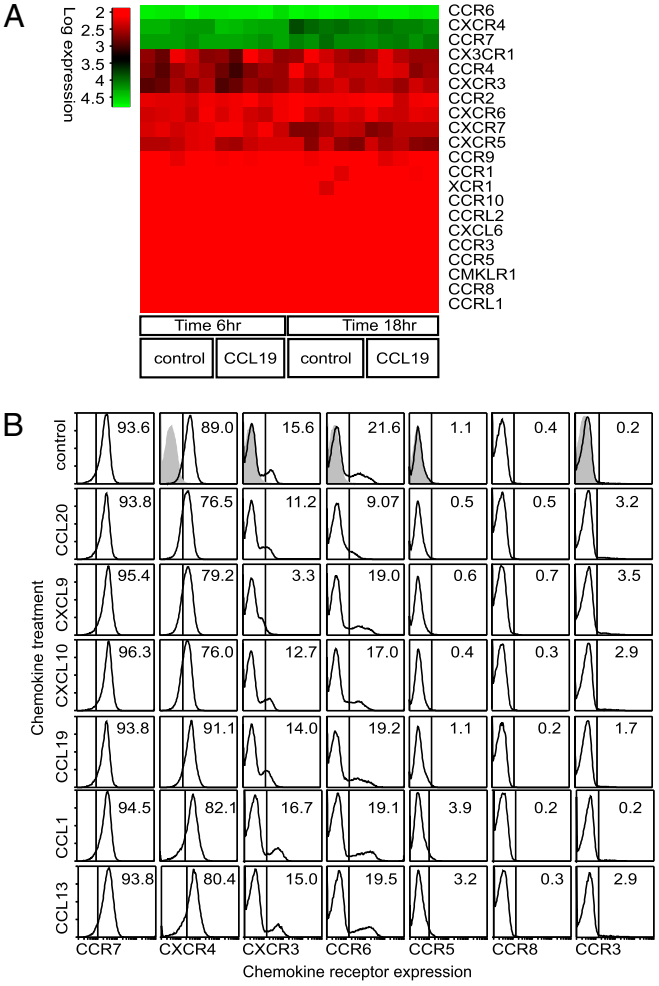

Fig. 2. Chemokine receptor gene array and surface expression on resting CD4$^+$ T cells and following culture with multiple chemokines. (*A*) Heatmap of log signal intensity of chemokine receptor gene expression in resting CD4$^+$ T cells cultured for 6 or 18 h with or without the chemokine CCL19. (*B*) Surface expression of chemokine receptors on CD4$^+$ T cells following incubation with chemokines. Each chemokine is indicated on the *y* axis and the expression of the chemokine receptor in each histogram on the *x* axis. Expression was determined by flow cytometry in resting CD4$^+$ T cells after 3 d of culture in media alone (control) or with chemokines as indicated for each row. Isotype controls are shown as gray histograms.

72 h (Fig. 2*B*). Nearly all resting CD4$^+$ T cells (defined as negative for HLA-DR and CD69) expressed CXCR4 and CCR7, whereas subpopulations of resting CD4$^+$ T cells expressed CXCR3 and CCR6. Levels of CCR5, CCR8, and CCR3 were not detected above background. Together these data on chemokine receptor expression indicate that the ability of a chemokine to condition resting CD4$^+$ T cells to allow HIV-1 nuclear localization and integration (Fig. 1*B*) was related to the level of surface expression of the chemokine receptor.

Modulation of Efficiency of HIV-1 Nuclear Entry and Integration by Chemokines. To determine where in the HIV-1 life cycle chemokines may have their effect, we used quantitative PCR to determine the frequency of strong stop DNA (early reverse transcripts), full-length DNA (complete reverse transcripts), and two nuclear forms of viral DNA: 2-long terminal repeat (LTR) circles and integrated HIV-1 DNA. We compared the quantity of each viral species after infection of resting CD4$^+$ T cells activated with either CCL19 or PHA/IL-2 or left unactivated (Fig. 3*A*). Comparison of the frequency of each of these forms of viral DNA was used to calculate the efficiency of early reverse transcription, nuclear import, and viral integration. There was little difference in the frequency of early reverse transcripts (Fig. 3*A*) or in the production of full-length viral transcripts (Fig. 3*B*) using each culture condition. Efficiency of nuclear entry was low for resting cells (0.8%) compared with cells cultured with PHA/IL-2 (24.5%) or CCL19-treated cells (8.5%; Fig. 3*C*). The efficiency of viral DNA integration was also low (<4%) in resting cells compared with PHA/IL-2 (22%) and CCL19-treated cells (25%; Fig. 3*D*).

We then examined the kinetics of appearance of each of the forms of the viral genome (Fig. 3*E*). Accumulation of strong stop DNA and full-length transcripts was similar in all three culture conditions. Appearance of 2-LTR circles was delayed and was lower in unactivated cells compared with either CCL19-treated cells or PHA/IL-2–activated cells. Integrated DNA was not present in unactivated cells. These findings demonstrated that CCL19 alters resting CD4$^+$ T cells to allow enhanced HIV-1 nuclear localization and integration but that CCL19 had little impact on earlier stages of the HIV-1 life cycle including viral entry or reverse transcription.

Finally we examined whether the addition of the chemokine had an effect on HIV integration in a resting cell that contained unintegrated HIV DNA. We did this using two approaches. First we assessed the effects of CCL19 if added to resting CD4$^+$ T cells before infection (−24 or −1 h), at the time of infection (0 h), or following infection (1, 2, or 6 h). Integration was only established if CCL19 was added before or at the time of infection (Fig S1). In addition we tested the effect of CCL19 on resting CD4$^+$ T cells isolated from HIV-infected treatment-naive individuals (n = 5; median HIV RNA = 38,012 copies/mL and CD4 count = 684 cells/μL). Consistent with our in vitro studies, we found no increase in integrated HIV DNA following incubation with CCL19 (Fig. S2). These data demonstrate that CCL19 can facilitate HIV nuclear localization and integration by inducing changes in the target cell but that the changes must occur before HIV infection.

Reversible Effects of Chemokines on Viral Integration. If HIV-1 entry and subsequent proviral integration is enhanced in resting CD4$^+$ T cells by exposure to chemokines during T-cell recirculation and migration through lymphoid tissue, we hypothesized that the chemokine-induced changes in resting CD4$^+$ T cells should be transient and reversible. We therefore quantified HIV-1 integration in resting CD4$^+$ T cells following addition and removal of CCL19 at different time points before infection with HIV-1 (Fig. 4*A*). Purified resting CD4$^+$ T cells were cultured for 72 h before infection. CCL19 was added to the cultures for 3, 12, 24, 48, and 72 h before infection to test the onset rate of the effect of the chemokine pretreatment on efficient HIV-1 integration (Fig. 4*A Left*, "washin"). We also tested whether the effect of CCL19 was reversible by removing CCL19 at various time points before infection (Fig. 4*A Right*, "washout"). Exposure of the resting CD4$^+$ T cells to CCL19 for as little as 3 h showed an increase in HIV-1 integration compared with unactivated CD4$^+$ T cells (Fig. 4*B*) but the effect of CCL19 was lost when it was removed more than 3 h before infection (Fig. 4*B*). These experiments confirmed that the effect of CCL19 on resting CD4$^+$ T cells was rapidly reversible and depended on continued presence of CCL19.

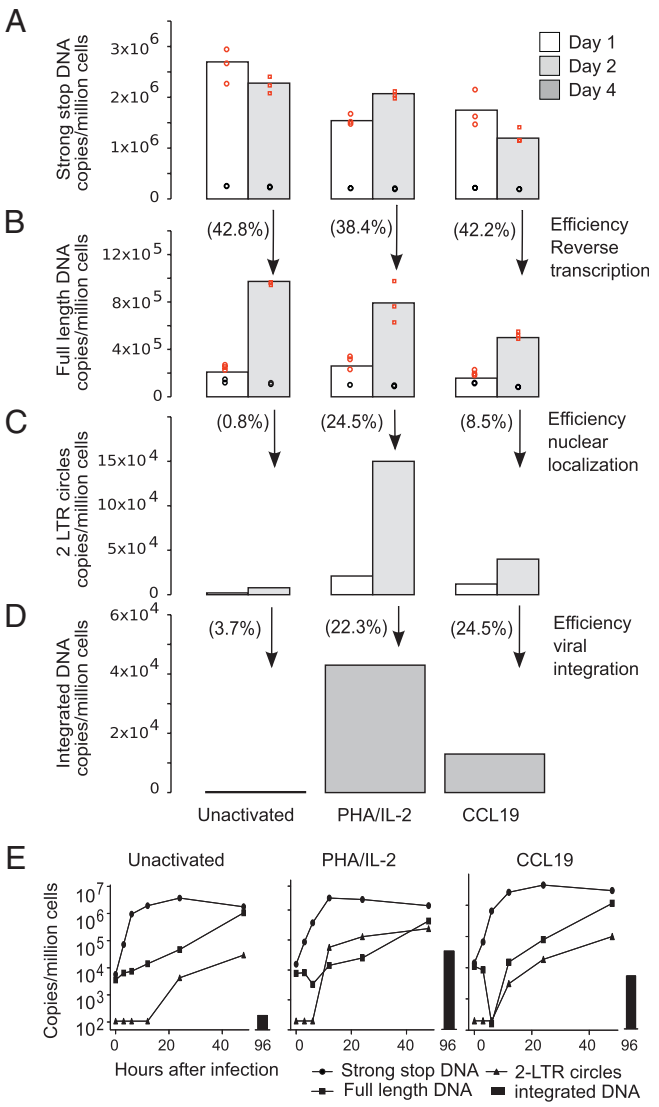

Fig. 3. Incubation of resting CD4$^+$ T cells with CCL19 increases HIV-1 nuclear localization and integration. Resting CD4$^+$ T cells were cultured for 3 d with media alone (unactivated) or with PHA/IL-2 or with the chemokine CCL19, and infected with HIV NL4.3. Quantitative PCR for (*A*) early reverse transcripts (strong stop DNA), (*B*) full-length DNA, (*C*) the nuclear products 2-LTR circles, and (*D*) HIV-1 integrated DNA was performed and quantified as copies/million cells. The efficiency of each step in reverse transcription, nuclear localization, and viral integration is shown in parentheses below each graph. The efficiency of each step was calculated as described in *Materials and Methods*. Replicates (*A* and *B*) (red open circles) and the mean (open column) from one experiment are shown. Results from one of two experiments are shown. Infection with heat-inactivated virus as control for background strong stop and full-length DNA is shown (black circles). (*E*) Kinetics of entry and reverse transcription following incubation with PHA/IL-2 or CCL19 or in unactivated cells. Strong stop DNA, full-length DNA, and 2-LTR circles were quantified 3, 6, 12, 24, and 48 h postinfection and integrated DNA was quantified at 96 h. Representative results from one of two experiments are shown.

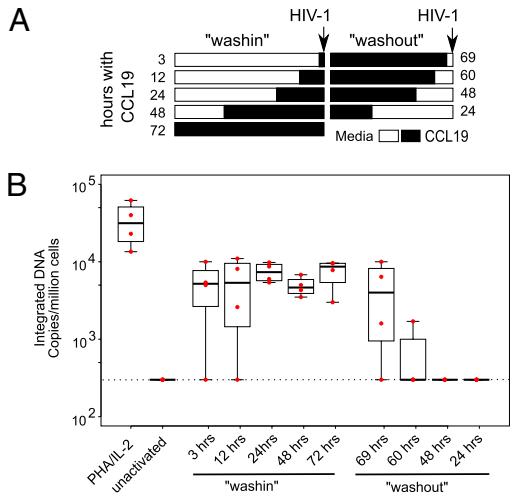

Fig. 4. Kinetics of CCL19 induced latent HIV-1 infection in resting CD4+ T cells. Resting CD4+ T cells were cultured for a total of 3 d and incubated with CCL19 for different durations during this time, before infection with HIV-1 NL4.3. Resting CD4+ T cells were also incubated with PHA/IL-2 or left unactivated for 3 d before infection. Integrated HIV-1 DNA was quantified in cells collected 4 d postinfection. (*A*) The method for addition ("washin" experiments) and removal ("washout" experiments) of CCL19 from cultures of resting CD4+ T cells before HIV-1 infection is shown. (*B*) Integrated DNA copies per million cells following different culture conditions are shown as red circles (four separate experiments) and summarized as box plots (median, 25th and 75th percentiles, and range). Dotted line represents the limits of detection of integrated HIV-1 DNA.

Chemokine Effects on Cytoskeletal Rearrangements and Cortical Actin. We next determined whether these chemokines were acting at the level of cortical actin to allow for increased nuclear localization, as previously shown for CXCR4 ligation following infection with X4 HIV-1 (12). Actin depolymerization is mediated by dephosphorylation of cofilin so we first used immunoblotting to detect phosphorylated and dephosphorylated cofilin following chemokine treatment. A decrease in phosphorylated cofilin was demonstrated after addition of the chemokine CCL19 and with positive controls phorbol 12-myristate 13-acetate (PMA) and SDF-1 (ligand for CXCR4; Fig. 5 *A* and *B*). The effect of these chemokines on cofilin dephosphorylation was evident within minutes and was sustained (Fig. 5 *A* and *B*). Corresponding changes in F-actin levels using the probe Alexa Fluor 488 phalloidin was determined in parallel using flow cytometry and wide-field fluorescent microscopy. A transient uniform increase in F-actin was seen by both assays within 1 min (Fig. 5 *C*, *D*, and *E*) followed by a decrease to baseline by 30 min. Using microscopy, we observed persisting changes in distribution of F-actin with some polarization evident by 30 min after chemokine treatment. As expected, incubation with latrunculin A, which blocks actin polymerization, markedly reduced F-actin.

Furthermore, when actin depolymerization was inhibited using jasplakinolide (Jas), we observed a dose-dependent decrease in integrated DNA in both the PHA/IL-2 and CCL19-treated resting CD4+ T cells (Fig. 5*G*; $P = 0.03$). Taken together these data show that early modification of the cytoskeleton in resting T cells by chemokines facilitates enhanced nuclear localization of the HIV-1 preintegration complex.

Discussion

We have previously described a model of postintegration latency in resting CD4+ T cells in vitro and showed that the CCR7 ligands, CCL19 and CCL21, conditioned resting CD4+ T cells to allow for efficient HIV-1 integration while maintaining low levels of viral expression (17). We now show that (*i*) other chemokines including CXCL9, CXCL10, and CCL20 mediated the same effect as CCL19 and CCL21; (*ii*) CCL19 and these other chemokines did not directly activate the resting CD4+ T cell or substantially alter expression of other chemokine receptors; (*iii*) CCL19 treatment of resting CD4+ T cells allowed for both efficient nuclear localization and integration; (*iv*) the effect of CCL19 was rapid and

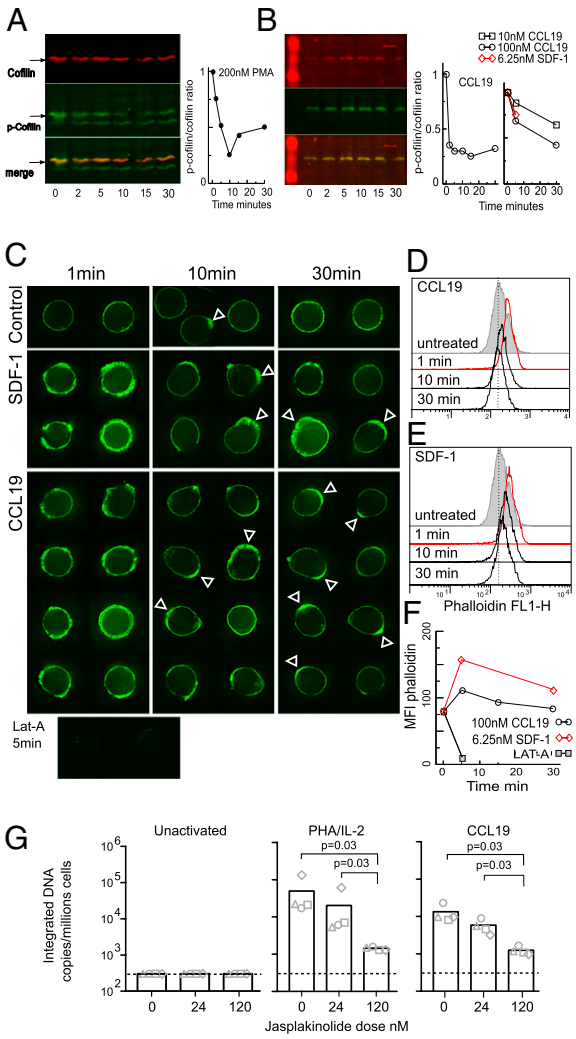

Fig. 5. Chemokine-induced changes in cofilin phosphorylation and actin depolymerization. Western blot with total cofilin (red) or phosphorylated cofilin (p-cofilin, green) was performed using lysates of resting CD4+ T cells after culture with (*A*) 200 nM PMA or (*B*) 100 nM CCL19. The antibody-stained immunoblots were visualized using fluorescence imaging (*Left*). Lanes are labeled with time of culture with PMA or CCL19. The signal intensity for each channel was used to calculate a corrected ratio of p-cofilin to cofilin (*Right*). *B, Right,* shows the corrected ratio of p-cofilin to cofilin following incubation with 10 nM or 100 nM CCL19 and 6.25 nM SDF-1. (*C–F*) Changes in F-actin were measured using Alexa Fluor 488 conjugated phalloidin labeling of resting CD4+ T cells fixed at various times after incubation with CCL19, SDF-1 or Lat-A. Control cells were CD4+ T cells incubated with Alexa Fluor 488 conjugated phalloidin without permeabilization. The mean fluorescence intensity (MFI) data points from the histograms are summarized in the line plots. (*C*) Cells were imaged using wide-field fluorescence microscopy (DeltaVision) and *z* sections from the center of cells are shown for each condition. Polar distribution of F-actin at later time points is indicated (white arrowhead). (*D* and *E*) Changes in the mean fluorescence intensity (MFI) by flow cytometry for the cells corresponding to those shown in *C*. (*F*) Kinetics of chemokine-induced changes in MFI. (*G*) Resting CD4+ T cells were cultured with Jas for 24 h at either 24 or 120 mM before incubation with PHA/IL-2, or CCL19, or left unactivated. After 2 d, cells were infected with HIV-1 NL4.3 and cell lysates tested for integrated HIV-1 DNA 4 d after infection. The mean (black open bar) of four separate experiments (each marked by a different gray symbol) is shown.

reversible and did not involve significant changes in gene expression; and (*v*) CCL19 induced dephosphorylation of cofilin and transient changes in actin polymerization were critical for nuclear localization in resting $CD4^+$ T cells (12). Taken together our observations suggest that ligation of chemokine receptors highly expressed on resting $CD4^+$ T cells can provide a signaling pathway for induction of postintegration latent infection. This pathway would be active during chemokine-induced $CD4^+$ T-cell migration in vivo and would allow viral integration independent of $CD4^+$ T-cell activation or antigenic stimulation.

The chemokine receptors CCR7, CXCR3, and CCR6 are critical in guiding the migration of naive and memory T cells across high endothelial venules and endothelium into lymphoid tissue and are important in the clustering of T cells with DCs (19). Several previous studies have shown that ligation of chemokine receptors during HIV-1 infection of macrophages and activated T cells (21–23) leads to changes in cellular signaling. In addition, HIV-1 itself may facilitate nuclear localization in resting $CD4^+$ T cells via HIV-1 viral envelope signaling through the HIV-1 coreceptor CXCR4 (12, 13). Our work clearly demonstrates that multiple chemokines, in addition to CCR7 and CXCR4 ligands, may efficiently facilitate HIV-1 nuclear entry as well as integration in resting $CD4^+$ T cells. We used a concentration of chemokines (100 nM) that approximates the concentration in tissue in vivo in murine models (24) and that has been shown to induce chemotaxis in vitro (25–27). In HIV infection, plasma levels of CCL19, CCL21, and CXCL10 are all significantly elevated in plasma (28–31) and it is highly likely that the concentrations of these chemokines are even higher in lymphoid tissue and mucosal surfaces, as recently reported in SIV-infected macaques (32).

Our data suggest that the effect of each chemokine is limited only by the expression level of the corresponding chemokine receptor on the resting $CD4^+$ T cell. Using gene array and flow cytometry, we showed that CCR7, CXCR4, CXCR3, and CCR6 were the most highly expressed chemokine receptors on resting $CD4^+$ T cells and that there were minimal changes to gene expression following incubation with CCL19. The chemokines CCL19, CCL20, CXCL9, and CXCL10 that interact with CCR7, CCR6, and CXCR3, respectively, but do not bind directly to HIV-1, are important for T-cell trafficking into lymphoid and mucosal tissue (18, 33, 34). Here we show that these chemokines condition resting $CD4^+$ T cells resulting in high levels of HIV-1 integration with minimal viral production. In our model, the chemokine effects that allowed viral integration in resting $CD4^+$ T cells were short lived with onset of the effect within 3 h and complete loss of the effect when the chemokines were absent for longer than 3 h. This is consistent with the rapid onset of chemokine effects on intracellular signaling and cytoskeletal changes (35, 36). The longevity of resting T cells is also consistent with minimal permanent changes during recirculation and repeated chemokine exposure (18, 37).

We demonstrated that the main effect of chemokine exposure was the increase in both HIV-1 nuclear localization and integration. We observed similar kinetics of early viral events for both R5 and X4 viruses including entry and reverse transcription in fully-activated, CCL19-treated and unactivated $CD4^+$ T cells. Our findings were consistent with some studies that found efficient reverse transcription in resting $CD4^+$ T cells following HIV-1 infection via spinoculation (7, 38). However, our results differed from other work that has suggested that HIV-1 infection of resting unactivated $CD4^+$ T cells is blocked due to incomplete or inefficient reverse transcription as a result of low levels of intracellular deoxyribonucleotide triphosphates (dNTPs) in these cells (5, 6, 39). Culture of resting $CD4^+$ T cells for 3 d before infection may have removed the restriction of reverse transcription seen in freshly isolated cells (40).

We found that the main difference following infection of CCL19- or PHA/IL2-treated cells was the rapid kinetics and higher peak production of 2-LTR circles and integration. In contrast to 2-LTR circles, integration was completely absent in unactivated $CD4^+$ T cells. Yoder et al. showed that ligation of CXCR4 by HIV-1 can itself modulate the actin cytoskeleton to allow for efficient nuclear localization in resting $CD4^+$ T cells but integration was not observed (12). In contrast, we were able to clearly differentiate between nuclear localization (2-LTR circles and integrated DNA) and HIV-1 integration using quantitative PCR. Ligation of CXCR4 by HIV-1 gp120 may potentially lead to enhanced nuclear localization but not efficient integration. Whereas it is unknown whether R5 viruses mediate a similar effect via CCR5, latent infection of resting $CD4^+$ T cells with R5 viruses in vivo is well described (8, 10, 41–43). In our model, chemokine treatment of resting $CD4^+$ T cells resulted in latency with both R5 and X4 viruses, even though there was minimal expression of CCR5 on these cells, which is consistent with the presence of infected CCR5-negative resting T cells in vivo (10). We conclude that ligation of chemokine receptors that are highly expressed on resting $CD4^+$ T cells but not used as coreceptors for HIV-1, leads to enhancement of both nuclear localization and integration of both X4 and R5 HIV-1 in resting $CD4^+$ T cells.

Chemokine ligation of G-coupled chemokine receptors activates several downstream pathways (reviewed in refs. 44, 45) including those involved in motility (Rho and Pyk2 pathway of cofilin dephosphorylation), endocytosis (cdc42/Rac pathway), maturation, chemotaxis (Erk1/2, JNK pathway), and cell survival (PI3K, NF-κB pathway) (44, 45). We found that ligation of CCR7 and other chemokine receptors led to cofilin dephosphorylation similar to that previously described with SDF-1 and gp120 ligation of CXCR4 (12, 13). It is likely that pathways other than cofilin and actin may be important for chemokine-induced postintegration latency, particularly with respect to HIV-1 integration. In activated T cells, integrase function depends on the activation of JNK by the PI3K pathway (46). Other components of the PI3K pathway may also potentially affect the efficiency of HIV-1 integration in chemokine-treated resting $CD4^+$ T cells via activation of NF-κB, which may be required at low levels to allow viral integration and latent infection in resting $CD4^+$ T cells (47). The Cdc42/Rac pathways activated by chemokine binding are important in actin polymerization, endocytosis (48), and in viral fusion (49) but their effect on enhancing migration of the preintegration complex into the nucleus and integration is unknown.

So how are these findings relevant to the establishment or maintenance of latently infected $CD4^+$ T cells in vivo? In the absence of HAART and in the presence of active viral replication, latently infected cells may potentially arise by multiple mechanisms—including direct infection of resting $CD4^+$ T cells following stimulation with chemokines, as we propose in this paper or alternatively by reversion of actively infected cells to resting latently infected central memory T cells, as demonstrated by several other in vitro models (50–54). However, in the setting of effective HAART and HIV RNA <50 copies/mL, we propose that direct entry in resting $CD4^+$ T cells may make a more significant contribution to replenishment of the pool of latently infected cells for several reasons. First, in the setting of effective HAART, there are a limited number of activated infected $CD4^+$ T cells and therefore a reduced source of new latently infected cells. Second, it is likely that resting $CD4^+$ T cells are exposed to the highest concentration of chemokines in tissues such as the gastrointestinal tract and lymphoid tissue where penetration of HAART may be limited and where low-grade viral replication continues to occur allowing for ongoing transfer of virus to circulating resting $CD4^+$ T cells (42, 43, 55).

In summary, we have shown that chemokines that bind to receptors CCR7, CXCR3, and CCR6 expressed on resting $CD4^+$ T cells, can induce latent infection in resting $CD4^+$ T cells in the absence of T-cell activation. The chemokine effect was transient and was associated with a significant increase in efficiency of nuclear localization and integration of HIV-1 compared with infection of unactivated $CD4^+$ T cells. CCL19 treatment induced changes in the actin cytoskeleton that may facilitate migration of the preintegration complex to the nucleus. Together these observations suggest that the establishment of HIV-1 viral latency depends only on the physiologically normal process of resting

$CD4^+$ T-cell recirculation through chemokine-rich environments such as lymphoid tissue.

Materials and Methods

Isolation of CD4 T Cells and Flow Cytometry. Resting $CD4^+$ T cells were isolated from the peripheral blood as previously described (17). The resulting T cells consisted of naive and central memory T cells that express CCR7 (56) and purity was routinely >95% when assessed by flow cytometry. Flow cytometry (FACScalibur analyzer, BD Biosciences) was used to determine expression of chemokine receptors and activation markers on the sorted cells at the time of isolation and after 3 d of culture. Cells were labeled with antibodies to the chemokine receptors CCR5, CXCR4, CXCR3, CCR6, CCR8, and CCR7 and the activation markers CD69, HLA-DR, and CD25 (BD Biosciences). Cells were gated for CD4-expressing cells and expression was compared with isotype controls using fluorescence-minus-one strategy (57).

See *SI Materials and Methods* for additional methods.

ACKNOWLEDGMENTS. This work was supported by National Health and Medical Research Council of Australia Program Grant 358399 and Project Grant 491154. S.R.L. was supported by National Health and Medical Research Council of Australia Practitioner Fellowship 478100.

1. Chun TW, et al. (1997) Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* 387:183–188.
2. Finzi D, et al. (1997) Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278:1295–1300.
3. Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA (1990) HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J* 9:1551–1560.
4. Zack JA, et al. (1990) HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. *Cell* 61:213–222.
5. Zack JA, Haislip AM, Krogstad P, Chen IS (1992) Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. *J Virol* 66:1717–1725.
6. Zhou Y, Zhang H, Siliciano JD, Siliciano RF (2005) Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells. *J Virol* 79:2199–2210.
7. Swiggard WJ, et al. (2005) Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. *J Virol* 79:14179–14188.
8. Zhang Z, et al. (1999) Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* 286:1353–1357.
9. Eckstein DA, et al. (2001) HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. *Immunity* 15:671–682.
10. Mattapallil JJ, et al. (2005) Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434:1093–1097.
11. Kreisberg JF, Yonemoto W, Greene WC (2006) Endogenous factors enhance HIV infection of tissue naive CD4 T cells by stimulating high molecular mass APOBEC3G complex formation. *J Exp Med* 203:865–870.
12. Yoder A, et al. (2008) HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. *Cell* 134:782–792.
13. Yu D, Wang W, Yoder A, Spear M, Wu Y (2009) The HIV envelope but not VSV glycoprotein is capable of mediating HIV latent infection of resting CD4 T cells. *PLoS Pathog* 5:e1000633.
14. van't Wout AB, et al. (1994) Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J Clin Invest* 94:2060–2067.
15. Liu R, et al. (1996) Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86:367–377.
16. Keele BF, et al. (2008) Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci USA* 105:7552–7557.
17. Saleh S, et al. (2007) CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: A novel model of HIV-1 latency. *Blood* 110:4161–4164.
18. Förster R, et al. (1999) CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99:23–33.
19. Adema GJ, et al. (1997) A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 387:713–717.
20. Mackay CR, Marston WL, Dudler L (1990) Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med* 171:801–817.
21. Davis CB, et al. (1997) Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J Exp Med* 186:1793–1798.
22. Weissman D, et al. (1997) Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* 389:981–985.
23. Arthos J, et al. (2000) CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. *J Virol* 74:6418–6424.
24. Luther SA, et al. (2002) Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol* 169:424–433.
25. Kim CH, Pelus LM, White JR, Broxmeyer HE (1998) Differential chemotactic behavior of developing T cells in response to thymic chemokines. *Blood* 91:4434–4443.
26. Smit MJ, et al. (2003) CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* 102:1959–1965.
27. Hirota K, et al. (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 204:2803–2812.
28. Crane M, et al. (2009) Immunopathogenesis of hepatic flare in HIV/hepatitis B virus (HBV)-coinfected individuals after the initiation of HBV-active antiretroviral therapy. *J Infect Dis* 199:974–981.
29. Stacey AR, et al. (2009) Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J Virol* 83:3719–3733.
30. Damås JK, et al. (2009) Enhanced levels of the CCR7 ligands CCL19 and CCL21 in HIV infection: Correlation with viral load, disease progression and response to highly active antiretroviral therapy. *AIDS* 23:135–138.
31. Damås JK, et al. (2009) Homeostatic chemokines CCL19 and CCL21 promote inflammation in human immunodeficiency virus-infected patients with ongoing viral replication. *Clin Exp Immunol* 157:400–407.
32. Durudas A, et al. (2009) Elevated levels of innate immune modulators in lymph nodes and blood are associated with more-rapid disease progression in simian immunodeficiency virus-infected monkeys. *J Virol* 83:12229–12240.
33. Cook DN, et al. (2000) CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 12:495–503.
34. Qin S, et al. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 101:746–754.
35. Brown MJ, et al. (2003) Chemokine stimulation of human peripheral blood T lymphocytes induces rapid dephosphorylation of ERM proteins, which facilitates loss of microvilli and polarization. *Blood* 102:3890–3899.
36. Grabovsky V, et al. (2000) Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med* 192:495–506.
37. Debes GF, et al. (2005) Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol* 6:889–894.
38. Agosto LM, et al. (2007) HIV-1 integrates into resting CD4+ T cells even at low inoculums as demonstrated with an improved assay for HIV-1 integration. *Virology* 368:60–72.
39. Korin YD, Zack JA (1999) Nonproductive human immunodeficiency virus type 1 infection in nucleoside-treated G0 lymphocytes. *J Virol* 73:6526–6532.
40. Gao WY, Cara A, Gallo RC, Lori F (1993) Low levels of deoxynucleotides in peripheral blood lymphocytes: A strategy to inhibit human immunodeficiency virus type 1 replication. *Proc Natl Acad Sci USA* 90:8925–8928.
41. Pierson T, et al. (2000) Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. *J Virol* 74:7824–7833.
42. Chun TW, et al. (2005) HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. *J Clin Invest* 115:3250–3255.
43. Chun TW, et al. (2008) Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *J Infect Dis* 197:714–720.
44. Burkhardt JK, Carrizosa E, Shaffer MH (2008) The actin cytoskeleton in T cell activation. *Annu Rev Immunol* 26:233–259.
45. Wu Y, Yoder A (2009) Chemokine coreceptor signaling in HIV-1 infection and pathogenesis. *PLoS Pathog* 5:e1000520.
46. Manganaro L, et al. (2010) Concerted action of cellular JNK and Pin1 restricts HIV-1 genome integration to activated CD4+ T lymphocytes. *Nat Med* 16:329–333.
47. Duverger A, et al. (2009) Determinants of the establishment of human immunodeficiency virus type 1 latency. *J Virol* 83:3078–3093.
48. McGavin MK, et al. (2001) The intersectin 2 adaptor links Wiskott Aldrich Syndrome protein (WASp)-mediated actin polymerization to T cell antigen receptor endocytosis. *J Exp Med* 194:1777–1787.
49. Harmon B, Ratner L (2008) Induction of the Galpha(q) signaling cascade by the human immunodeficiency virus envelope is required for virus entry. *J Virol* 82:9191–9205.
50. Contreras X, et al. (2009) Suberoylanilide hydroxamic acid reactivates HIV from latently infected cells. *J Biol Chem* 284:6782–6789.
51. Yang HC, Shen L, Siliciano RF, Pomerantz JL (2009) Isolation of a cellular factor that can reactivate latent HIV-1 without T cell activation. *Proc Natl Acad Sci USA* 106:6321–6326.
52. Marini A, Harper JM, Romerio F (2008) An in vitro system to model the establishment and reactivation of HIV-1 latency. *J Immunol* 181:7713–7720.
53. Bosque A, Planelles V (2009) Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood* 113:58–65.
54. Yang HC, et al. (2009) Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest* 119:3473–3486.
55. Yukl SGS, Li Q PLUS Study Group (2010) Differences in HIV burden throughout the gut of patients on suppressive ART: Implications for HIV persistence. *J Infect Dis*, in press.
56. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708–712.
57. Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: A guide for the perplexed. *Nat Immunol* 7:681–685.