Nuclear Factor of Activated T Cells Is a Driving Force for Preferential Productive HIV-1 Infection of CD45RO-expressing CD4+ T Cells*

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Gilles A. Robichaud§§§, Benoît Barbeau§§§, Jean-François Fortin**, David M. Rothstein‡‡, and Michel J. Tremblay§§§

From the ‡Centre de Recherche en Infectiologie, Hôpital du Centre Hospitalier de L’Université Laval, Centre Hospitalier Universitaire de Québec, and Département de Biologie médicale, Faculté de Médecine, Université Laval, Ste-Foy, Québec G1V 4G2, Canada, the **Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California 94305-5175, the §§Department of Medicine, Yale Medical School, New Haven, Connecticut 06520

Human immunodeficiency virus type-1 (HIV-1) preferentially replicates in CD4-expressing T cells bearing a “memory” (CD45RO+) rather than a “naïve” (CD45RA+/CD28+) phenotype. Yet the basis for the higher susceptibility of these cells to HIV-1 infection remains unclear. Because the nature of the CD45 isoform itself can affect biochemical events in T cells, we set out to determine whether these isoforms could differently modulate HIV-1 long terminal repeat (LTR) activity and thereby replication. Through the use of CD4+ Jurkat T cells specifically expressing distinct CD45 isoforms (i.e. CD45RABC or CD45RO), we demonstrated that a difference in CD45 isoform expression conferred preferential replication of HIV-1 to CD45RO-expressing T cell clones following a physiological CD3/CD28 stimulation. Closer analysis indicated that higher HIV-1 LTR activation levels were consistently observed in CD45RO-positive cells, which was paralleled by more pronounced nuclear factor of activated T cells (NFAT) activation in these same cells. Specific involvement of NFAT1 was revealed in studied Jurkat clones by mobility shift analyses. In addition, preferential activation of the LTR and viral replication in CD45RO T cells was FK506- and cyclosporin A-sensitive. These results underscore the importance of NFAT in HIV-1 regulation and for the first time identify the role of the CD45 isoform in limiting productive HIV-1 replication to the human CD4 memory T cell subset.

The human immunodeficiency virus type-1 (HIV-1),1 the etiological agent of acquired immunodeficiency syndrome (AIDS), exhibits tropism for CD4+ T lymphocytes. Overlapping signal transduction requirements between T cell gene expression and activation of HIV-1 regulatory sequences tightly link virus replication to T cell activation. CD45 is a protein-tyrosine phosphatase intimately involved in T cell activation. Alternative splicing of three exons, commonly referred to as A, B, and C, generates multiple CD45 isoforms (1, 2). The higher and lower molecular weight (M.) isoforms are differentially expressed on subsets of CD4+ T cells having distinct functional repertoires (3, 4). Antigenic exposure results in a shift in the expression of CD45 from high (i.e. CD45RA) to low (i.e. CD45RO) M. isoforms (5). Although these changes in CD45 isoform expression may not be permanent, they have been used in identification of previously activated or “memory” CD4 cells, often in combination with other phenotypic changes (e.g. loss of CD62L expression or up-regulation of CD44) (5–11). Previous studies have shown that expression of distinct CD45 isoforms alters T cell activation signaling and IL-2 production, raising the possibility that CD45 isoform expression, in and of itself, may contribute to the distinct functions of CD45RO and CD45RA CD4+ T cells (4, 12–15).

Recent studies revealed that naïve and memory CD4+ T cell subsets are equally susceptible to the early events in HIV-1 life cycle (i.e. binding, fusion, and entry) (16). However, CD45RO T lymphocytes consistently support a greater level of HIV-1 replication upon physiological stimulation than do CD45RA T cells (16–22). Despite considerable efforts directed toward the identification of the factors driving preferential productive HIV-1 infection in CD45RO cells, its molecular basis remains unresolved.

HIV-1 replication is intimately connected with the activity of its promoter region, positioned in the 5’ long terminal repeat (LTR) region and more specifically within a sequence corresponding to the HIV-1 enhancer segment (–104/–81) (23–25). One important factor binding to this region is the nuclear factor kappa B (NF-κB) (26–29). Recent reports indicate that this factor might also be acting in synergy with the nuclear factor of

1 The abbreviations used are: HIV-1, human immunodeficiency virus type-1; AIDS, acquired immunodeficiency syndrome; IL-2, interleukin-2; LTR, long terminal repeat; NF-κB, nuclear factor kappa B; TCR, T cell receptor; PBS, phosphate-buffered saline; PHA, phytohemagglutinin A; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; Iono, ionomycin; dsDNA, double-stranded DNA.
activated T cells (NFAT) to positively modulate HIV-1 LTR activation (30, 31), an observation that was confirmed in primary human T cells (32, 33).

We have recently demonstrated that CD45 expression profoundly alters the transcriptional activation of NFAT, resulting in marked differences in HIV-1 LTR activity (34). Because CD45 isoforms can differentially modulate different T cell signaling events (4, 12–15, 35–37), we set out to define whether expression of CD45RO (as compared with CD45RA) might specifically activate transcription factors driving HIV-1 replication. In this present study, we demonstrate that physiological stimulation of CD45RO-expressing T cells leads to increased activation of NFAT, HIV-1 gene transcription, and virus replication compared with that seen in CD45RA-expressing T cells. Based on these findings, we propose a new model by which HIV-1 replication is accentuated in CD4+ memory T cells through an NFAT-dependent signal transduction pathway that is seen following engagement of both T cell receptor (TCR)/CD3 complex and CD28.

MATERIALS AND METHODS

Cell Lines Used in this Study—The lymphoid T cell lines used include a parental CD4+ CD45wt Jurkat cell clone (JKF) and a CD45-negative clone (J-AS-1) derived by stably transfecting JKF with an antisense gene targeting the 5′ non-coding region of CD45. J-AS cells were reconstituted with an expression vector encoding the CD45RA isoform containing exons A, B, and C (clones JABC-1, JABC-2, and JABC-3) or an expression vector encoding the CD45RO isoform, which lacks alternative exons (clones J10; J10-3, and J10-1). Both expression vectors had limited overlap with the sequence targeted by the CD45 antisense mRNA (13). We also used a second CD45-deficient Jurkat cell line, J45.01 (provided by Dr. A. Weiss, Howard Hughes Medical Center, San Francisco, CA) (38). These cells were cultured in medium made of RPMI 1640 supplemented with 20% fetal bovine serum (HyClone Laboratories, Logan, UT), glutamine (2 mm), penicillin G (100 units/ml), and streptomycin (100 μg/ml) and were maintained at 37 °C under a 5% CO2 humidified atmosphere. DT30 cells are derived from the mastocytoma P815 cell line and stably express cell surface human B7.1 (39). Such cells also express murine Fcy receptors and are thus capable of binding and cross-linking soluble antibody. This cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). DT30 cells were grown in RPMI 1640 medium supplemented with 10% commercial fetal bovine serum and were fixed in 1% paraformaldehyde, washed extensively with phosphate-buffered saline (PBS), and then stored frozen at a density of 2 × 10^7/ml in PBS.

Plasmids and Antibodies—The pLTR-LUC plasmid was kindly provided by Dr. K. L. Calame (Columbia University, New York, NY) and contains the luciferase reporter gene under the control of the complete HXB2-derived HIV-1 LTR (40). The pNL4-3-Luc contains the luciferase reporter gene under the control of the complete nef gene and has been provided by Dr. D. Baltimore (Rockefeller University, New York, NY) (41). The pRLCM vector (Stratagene) and contains five consensus NF-κB-binding sites upstream of the luciferase reporter gene under the control of the complete HIV-1 LTR (42). This plasmid was a generous gift from Dr. W. C. Greene (The J. E. Glusdon Institute, San Francisco, CA). pNL4-3-Luc-CD28-Luc is commercially available (Stratagene) and contains five consensus NF-κB-binding sites upstream of the luciferase reporter gene under the control of the complete HIV-1 LTR (42). A LUC-reporter plasmid designated as B-TATA-LUC plasmid contains the HIV-1 enhancer region (43). Anti-CD3 OKT3 (specific for the chain of the CD3 complex) and anti-CD4 OKT4 hybridomas were obtained from the ATCC. Antibodies from these hybridomas were purified with protein G affinity columns according to the manufacturer's instructions (Amerham Biosciences, Inc., Uppsala, Sweden). Purified anti-CD28 antibodies (clone 9.3) were a generous gift from Dr. J. A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) (47). Purified goat anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Antibodies directed against specific isoforms of CD45RA/BC and CD45RO (clones J10261A and UCHL-1, respectively) were purchased from BD PharMingen (Mississauga, Ontario, Canada).

Transfections—Transient transfections were performed using the DEAE-dextran method as follows. Cells (5 × 10^6) were first washed once in TS buffer (137 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM KCl, 0.6 mM Na2HPO4, 0.5 mM MgCl2, and 0.7 mM CaCl2) and resuspended in 0.5 ml from the indicated plasmid(s) and 500 μg/ml DEAE-dextran (Amerham Biosciences, Inc.) (final concentration). The cell/Ts/plasmid/DEAE-dextran mix was incubated for 25 min at room temperature. Thereafter, cells were diluted at a concentration of 1 × 10^6/ml using complete culture medium supplemented with 100 μg chloroquine (Sigma Chemical Co.) and transferred into six-well plates. After 45 min of incubation at 37 °C, cells were centrifuged, resuspended in complete culture medium, and incubated at 37 °C for 24 h. To minimize variations in plasmid transfection efficiencies, cells were transfected in bulk and were next separated into various treatment groups. Viral entities (i.e. HXB-LUC particles) were generated by calcium phosphate transfection in 293T cells as described previously (48). Virus stocks were normalized for virion content using an in-house plaque assay protocol (49). These particles under-grew only one freeze-thaw cycle before initiation of infection studies.

Stimulation, Reporter Gene Assays, and Cell Viability Assay—Transiently transfected cells were seeded at a density of 10^5 cells per well (100 μl) in 96-well flat-bottom plates. Cells were either left unstimulated or were treated in a final volume of 200 μl with phytohemagglu-tinin-P (PHA-P at 3 μg/ml, Sigma), phorbol 12-myristate 13-acetate (PMA at 20 ng/ml, Sigma), ionomycin (Iono at 1 μM, Calbiochem) or TNF-α (10 ng/ml, R & D Systems). Treatment with anti-CD3 antibody (clone OKT3 at 3 μg/ml) and DT30 (2 × 10^6 DT30/10^5 target cells) was also used to mimic physiological stimulation. Next, cells were incubated at 37 °C for 8 h. For some experiments, prior to the addition of the stimuli, cells were either left untreated or were pretreated with FK506 (10 ng/ml, Fujisawa, Osaka, Japan) or cyclosporin A (CsA) (100 ng/ml, Sigma) for 15 min at 37 °C. In these conditions, cell viability was determined by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphosphoryl)-2-(4-sulfophenyl)-2H-tetrazolium) assay as previously described (50). Luciferase activity was determined following a previously described protocol (51).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were left untreated or were incubated for the indicated times at 37 °C with the combination of either PMA (20 ng/ml/Iono (1 μM)) or anti-CD3 antibody (clone OKT3 at 3 μg/ml/anti-CD28 antibody (clone 9.3 at 1 μg/ml) along with a goat anti-mouse IgG (5 μg/ml) in a final volume of 5 ml. Incubation with the various stimulating agents was terminated by the addition of ice-cold PBS, and nuclear extracts were prepared according to the described microscale protocol (52). EMSA was performed using the bichromonic assay with a commercial protein reagent kit (Pierce, Rockford, IL). EMSA was performed with 10 μg of nuclear extracts incubated for 20 min at room temperature in 20 μl of 1× binding buffer (100 mM Hepes, pH 7.9, 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl, 2 μg of poly(dI-dC), 10 μg of nuclear-free bovine serum albumin fraction V) containing 0.8 ng of γ-32P-labeled double-stranded DNA (dsDNA) oligonucleotide. The following dsDNA oligonucleotides were synthesized in-house and used as probes and/or competitors: the distal NFAT-binding site from the murine IL-2 promoter (5′-TGGGAGGGAAAAGAAAAATTGTCTGAG-3′); the consensus NF-κB-binding site (5′-ATGTGAGGGGACTTTCCGCTGGGGACTTTCCAGGG-3′); and the enhancer region (107–77) from the HIV-1 NL4-3 strain (5′-CAAGGGACTTTCCGCTGGGGACTTTCCAGGG-3′). NFAT DNA-protein complexes were resolved from free labeled DNA by electrophoresis in native 4% (w/v) polyacrylamide gels. The gels were subsequently dried and autoradiographed. Cold competition assays were carried out by adding a 100-fold molar excess of unlabeled dsDNA oligonucleotide simultaneously with the labeled probe. Supershift assays were performed by preincubation of nuclear extracts with 1 μl of antibody in the presence of the appropriate competitor for 30 min on ice prior to the addition of the labeled probe.

Intracellular Calcium Mobilization Assay—Measurements of intracellular calcium mobilization were performed with the Indo-1AM cell dye (Molecular Probes, Eugene, OR) according to a previously described protocol (34).
cells) were first inoculated with HXB-LUC virions (100 ng of p24) in a total volume of 1 ml of complete medium and seeded in 12-well dishes. After an overnight incubation, untreated and anti-CD3/anti-CD28-treated cells were incubated for up to 7 days during which aliquots of 100 μl of cell-free supernatant (p24 analysis) and 100 μl of cell suspension (luciferase assay) were retrieved at the indicated time points. Again, for some experiments, prior to the addition of the stimuli, cells were either left untreated or were pretreated with FK506 (10 ng/ml) or CsA (100 ng/ml) for 15 min at 37°C. Quantification of the viral p24 core protein was performed through the p24 enzyme-linked immunosorbent assay assay. Assessment of luciferase activity was performed as described above.

RESULTS

CD45RO Isoform Promotes Higher HIV-1 LTR Activity through NFAT Factor

Three CD45RA+ clones (J[ABC]-1, J[ABC]-2, and J[ABC]-3) and three CD45RO+ clones (J[O]-1, J[O]-2, and J[O]-3), as well as wild type (JKF) and CD45-deficient Jurkat cells (J-AS) were analyzed. All clones expressed comparable amounts of cell surface CD3, CD4, and CD45. Two clones that exhibited the closest levels of CD45 expression were initially tested (i.e. J[ABC]-1: 94% positive, mean 2.5; J[O]-2: 97% positive, mean 2.8) (data not shown).

We first conducted viral infections in the J[ABC]-1 and J[O]-2 cell clones with HXB-LUC virus particles and quantified viral replication by measuring virus-encoded luciferase activity. Our data showed that untreated CD45RA and CD45RO cell clones demonstrated similar levels of viral production over time (Fig. 1A). However, strikingly, upon CD3/CD28-stimulation, HIV-1 replication was more pronounced in CD45RO cells compared with CD45RA cells. It should be noted that, at early time points after viral infection (i.e. 24 h), virus-encoded reporter gene activity was similar in both CD45RA- and CD45RO-positive cells, suggesting no differences between the two cell clones in their susceptibility to the early steps in the virus life cycle.

Quantification of viral p24 antigen in the cell-free supernatants yielded similar results (data not shown). Multinucleated giant cell formation (syncytia) was exclusively observed in anti-CD3/anti-CD28-treated CD45RO cells (Fig. 1B), again confirming a higher HIV-1 replication process in CD45RO T cells.

FIG. 1. Enhanced HIV-1 transcription in CD45RO T cells undergoing physiological stimulation. A, J[ABC]-1 (squares) and J[O]-2 cells (circles) were infected with HXB-LUC virus particles (100 ng of p24) for 24 h and were either left untreated (filled symbols) or were treated (empty symbols) with anti-CD3 (3 μg/ml)/anti-CD28 (1 μg/ml) in the presence of a goat anti-mouse IgG (5 μg/ml). Cells were then assessed for virus-encoded luciferase activity over time (relative light units, RLU). Results are presented in terms of luciferase activity from the calculated means ± S.D. of three different lysed cell samples in the same experimental setting. B, virally infected cells from the above experiment were photographed under a phase contrast microscope 4 days following initiation of the cultures (magnification of 100×). Arrows point to syncytia.

CD45RO Expression Augments HIV-1 Replication after T Cell Stimulation—Previous in vitro and in vivo studies indicate that productive HIV-1 replication preferentially occurs in memory (CD45RO+) rather than in “naive” (CD45RA+/CD62L+) CD4+ T cells. The objective of this study was to examine the direct effect of the CD45 isoform on viral replication. We thus utilized a model whereby different individual CD45 isoforms were expressed on the same cellular background. Jurkat cells differing only in their CD45 isoform expression were generated by stably transfecting a CD45-deficient Jurkat cell line with vectors expressing either the largest CD45 isoform encoding exons A, B, and C (CD45RA) or the smallest CD45 isoform lacking alternative exons (CD45RO).
These results thus indicated that the CD45-reconstituted Jurkat T cell clones behaved in a similar fashion as primary human CD45RA-/CD45RO-positive T lymphocytes in terms of susceptibility to HIV-1 infection.

**Higher HIV-1 LTR Activation Is Present in CD45RO-expressing T Cells**—We next focused on the identification of the causal factor of this increase in HIV-1 replication in CD45RO-positive cells. We assessed whether this difference in HIV-1 replication susceptibility was paralleled by a difference in the regulation of virus transcription using a vector containing the luciferase gene regulated by the HIV-1 LTR (i.e. pLTR-LUC). After transfection, each Jurkat cell clone was activated with various stimuli, including the mitogenic lectin PHA or the PMA/ionomycin (PHA/Iono) combination. In addition, cells were also activated with a more physiologically relevant stimulus consisting of DT30 cells (expressing human B7.1 and the Fcy receptor) plus anti-CD3. As expected, the HIV-1 LTR was less responsive in CD45-negative J-AS cells than in wild type JKF cells following exposure to the described stimuli (data not shown). However, when the single-isoform transfectants were transfected and compared (selected at random for each independent experiment), the CD45RO clones consistently supported a higher level of HIV-1 LTR activity than did CD45RA clones regardless of the variation in fold inductions between the different experiments (Fig. 2, A–C). To confirm these results in a proviral DNA context, transfections were conducted in the J[ABC]-1 and J[O]-2 cell clones with the pNL4-3-Luc ”Env” construct in which the luciferase reporter gene had been inserted into the HIV-1 NL4-3 proviral DNA. Again, the CD45RO T cell clone showed a greater level of responsiveness to the tested stimuli than in the CD45RA T cell clone (Fig. 2D).

**Higher Stimulus-induced HIV-1 LTR Activity in CD45RO T Cells Does Not Involve NF-κB**—We next determined whether the higher LTR activity in CD45RO T cells might be mediated through the enhancer region. We thus transfected the J[ABC]-1 and J[O]-2 T cell clones with the pSB-TATA-LUC plasmid containing the luciferase gene under the control of the HIV-1 enhancer region. The enhancer region was again more active in CD45RO- than in CD45RA-expressing T cells following stimulation (Fig. 3A). In fact, a 2.7-fold increase in luciferase activity following stimulation with anti-CD3/DT30 was apparent when we compared the enhancer activity of cells expressing CD45RO versus CD45RA. These results were confirmed for each of the other single-isoform transfectants (data not shown).

We next assessed whether NF-κB could be responsible for the higher level of LTR activation observed in CD45RO T cells. This was achieved using TNF-α, a potent inducer of NF-κB. As depicted in Fig. 3B, CD45RA- and CD45RO-expressing clones gave similar levels of activation of the HIV-1 LTR activity upon TNF-α treatment. To more convincingly demonstrate that NF-κB was not involved in the higher level of HIV-1 LTR activity seen in CD45RO T cells, J[ABC]-1 and J[O]-2 cells were transfected with the pNF-κB-LUC plasmid and then treated with the same stimuli described above. NF-κB activity did not vary between CD45RA- and CD45RO-expressing cells regardless of the stimulating agents that were used (Fig. 3C). The same results were also obtained using the other single isoform transfectants (data not shown). To corroborate these data, EMSA analysis was performed with nuclear extracts from activated CD45RA- and CD45RO-positive Jurkat cell clones incubated with an NF-κB probe. An anti-CD28 antibody (clone 9.3) was used instead of the DT30 cell line to avoid contamination from DT30 nuclear proteins. As depicted in Fig. 3D, no apparent quantitative differences between CD45RA- and CD45RO-expressing cells were observed when comparing the NF-κB complex from anti-CD3/anti-CD28-treated cells (lanes 2 and 5) or PMA/Iono-treated cells (lanes 3 and 6). The specificity of the signal was confirmed by competition experiments with specific and nonspecific oligonucleotides (lanes 7 and 8, respectively). Thus, the level of NF-κB activation could not explain the increased induction of the HIV-1 LTR enhancer observed in the CD45RO cell subset. We consequently decided to assess the activity of another HIV-1 enhancer-binding factor, i.e. NFAT.

**Stimulus-induced Enhancement of HIV-1 Transcriptional Activity in CD45RO T Cells Is Linked to NFAT**—Both J[ABC]-1 and J[O]-2 T cell clones were next transfected with the pNFAT-
FIG. 3. Greater stimuli-induced activation of HIV-1 LTR activity in CD45RO-expressing T cells is independent of NF-xB.

A

B

C

D

Stimulation of CD45RO T Cells Promotes Higher NFAT1

Binding to the HIV-1 LTR Enhancer—EMSA analyses were next performed to better delineate the identity of the transcription factors that bind to the HIV-1 enhancer region upon CD3/CD28 cross-linking. Using nuclear extracts from anti-CD3/anti-CD28-treated Jurkat cell clones incubated with a probe made of the HIV-1 enhancer, we observed a more intense HIV-1 enhancer binding activity in nuclear extracts from CD45RO than from CD45RA T cells (Fig. 5A, compare lanes 3 and 8). Previous findings from our group have demonstrated that this

transfected with pxB-TATA-LUC (4), pLTR-Luc (5), or pNF-xB-LUC (C) and were treated with PMA (20 ng/ml/Iono (1 μg), PHA (3 μg/ml), anti-CD3 (3 μg/ml)DT30 cells (2 × 10⁶ DT30/10⁴ transfected Jurkat cells). In B, cells were treated with TNF-α (10 ng/ml). After 8 h of stimulation, cells were assessed for luciferase activity. Results are presented as -fold induction of luciferase activity over untreated samples from the calculated means ± S.D. of three different lysed cell samples in the same experimental setting. The -fold differences between CD45RA- and CD45RO-expressing cells are indicated above each column pair. These results are representative of three different experiments. For D, CD45RA (clone J[ABC]-1) and CD45RO (clone J[O]-1) cells were either left untreated or were stimulated with anti-CD3 (3 μg/ml) or PMA (1 μg/ml) for 1 h. Nuclear extracts from CD45RA (lanes 1–3) and CD45RO cells (lanes 4–8), which were either untreated (lanes 1 and 4), CD3/CD28-treated (lanes 2 and 5) or PMA/Iono-treated (lanes 3 and 6) were incubated with a NF-xB-labeled probe to be finally analyzed on a 4% native polyacrylamide gel. Competitions were also conducted to demonstrate the specificity of the shifted complexes (lanes 7 and 8). The arrow on the left indicates the NF-xB-specific complex.
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HIV-1 pathogenesis is regulated by a complex interplay between viral and cellular factors in infected host cells. A better understanding of the role of NFAT in HIV-1 LTR regulation is crucial. Our study demonstrates that CD45RO-expressing T cells promote higher HIV-1 LTR activity compared to CD45RA-expressing cells.

**Fig. 4.** CD45RO promotes NFAT activity upon stimulation through CD3 and CD28. A, CD45RA (clone J[ABC]-1, □) and CD45RO (clone J[O]-2, ■) cells were transiently transfected with pNFAT-LUC and were next treated with PMA (20 ng/ml)/Iono (1 μM), PHA (3 μg/ml), and anti-CD3 (3 μg/ml)/DT30 cells (2 × 10⁵ DT30/10⁵ of target cells) for 8 h. Cells were then assessed for luciferase activity. B, cells were either left untreated or were stimulated with anti-CD3 (3 μg/ml)/anti-CD28 (1 μg/ml) in the presence of a goat anti-mouse IgG (5 μg/ml) for 0, 5, 10, 20, 30, and 45 min. Nuclear extracts from CD45RA (lanes 2–7) and CD45RO cells (lanes 8–15), which were either left untreated (lanes 2 and 8) or CD3/CD28-treated (lanes 3–7 and 9–15), were incubated with a NFAT-labeled probe to be finally analyzed on a 4% native polyacrylamide gel. Competitions were also conducted to demonstrate the specificity of shifted complexes (lanes 14 and 15). The arrow on the left indicates the NFAT-specific complex. C, J45.01 (CD45-negative) cells were co-transfected with pNFAT-LUC as well as with HIV-1 enhancer-bound complex results from superimposed signals from both NF-κB and NFAT (34, 53). To define the relative contribution of these two factors, reciprocal competition experiments were performed (i.e. NFAT competition for NF-κB complex isolation and vice versa). Isolation of the NF-κB complex by NFAT competition revealed little change in the intensity of the signal between both cell clones (lanes 5 and 10). However, following NF-κB competition, the NFAT-related complex was more intense in extracts from CD45RO cells (compare lanes 4 and 9). The specificity of both signals was confirmed through competition experiments with cold enhancer oligonucleotide (lanes 6 and 11). Supershift assays were also performed to identify the most prominent NFAT family member(s) binding to the HIV-1 enhancer sequence in nuclear extracts from stimulated single-isofrom transfectants. After NF-κB competition, supershift experiments were conducted and indicated that, in the presence of anti-NFAT1 or pan anti-NFAT (directed against all NFAT members), protein-DNA complexes were supershifted or abolished in both CD45RA (lanes 4 and 6) and CD45RO cells (lanes 9 and 11) (Fig. 5B). These data suggest that NFAT1 could be responsible for the increased HIV-1 LTR activity observed upon activation of CD45RO-expressing CD4+ T cells.

**Higher HIV-1 LTR Activation and HIV-1 Replication in CD45RO-expressing T Cells Is Reduced by an FK506 Treatment**—To clearly demonstrate the role of NFAT in this preferential HIV-1 LTR activity and virus production in CD45RO-expressing cells, we tested FK506 and CsA, two commonly used inhibitors of calcineurin and thus NFAT activation. Again, CD45RO-positive Jurkat cells demonstrated increased reporter activity (pLTR-LUC) compared with CD45RA-expressing cells following stimulation (Fig. 6A). However, pre-treatment with either FK506 or CsA reduced the preferential activation seen in CD45RO cells, resulting in levels of LTR activation similar to those seen in CD45RA cells (Fig. 6A and data not shown). No changes in cell viability or TNF-α-mediated HIV-1 LTR activation were observed.

The effect of FK506 on viral replication was also evaluated in the two cell clones. Based on the experiment described in Fig. 1A, two time points were selected; one when viral expression from stimulated CD45RA and CD45RO CD4 cells was similar (i.e. at 24 h) and a second when viral expression was significantly different (i.e. at 72 h). At 24 h, viral expression measured by luciferase activity was similar in both anti-CD3/anti-CD28-stimulated CD45RA and CD45RO cells, whether or not they were pretreated with FK506 (Fig. 6B). However, at 72 h, the FK506 treatment reduced virus-encoded reporter gene activity in treated CD45RO-bearing cells, which was not paralleled by a change in cell viability (data not shown). Moreover, no significant changes in luciferase activity were measured in stimulated CD45RA-positive cells upon FK506 treatment. Similar results were obtained in CsA-pretreated infected cells (data not shown). These results further support the role of NFAT in viral production.

**DISCUSSION**

HIV-1 pathogenesis is regulated by a complex interplay between viral and cellular factors in infected host cells. A better understanding of the role of NFAT in HIV-1 LTR regulation is crucial. Our study demonstrates that CD45RO-expressing T cells promote higher HIV-1 LTR activity compared to CD45RA-expressing cells.
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Fig. 5. The CD45RO isoform induces higher NFAT1 binding to the HIV-1 LTR enhancer. CD45RA (clone J[ABC]-1) and CD45RO (clone J[O]-2) cells were either left untreated or stimulated with anti-CD3 (3 µg/ml)/anti-CD28 (1 µg/ml) in the presence of a goat anti-mouse IgG (5 µg/ml) for 5 min. A, nuclear extracts from CD45RA (lanes 2–6) and CD45RO cells (lanes 7–11), which were either left untreated (lanes 2 and 7) or CD3/CD28-treated (lanes 3–6 and 8–11) were incubated with an HIV-1 enhancer-labeled probe (Enh) to be finally analyzed on a 4% native polyacrylamide gel. The arrows on the left indicate the NFAT- and NF-xB-specific complex upon the HIV-1 enhancer probe. B, nuclear extracts from CD45RA (lanes 2–6) and CD45RO cells (lanes 7–11), which were either left untreated (lanes 2 and 7) or CD3/CD28-treated (lanes 3–6 and 8–11) were first incubated with various NFAT antibodies directed against NFAT members for 30 min on ice and then incubated with an HIV-1 enhancer-labeled probe (Enh) in the presence of cold NF-xB oligonucleotide to be finally analyzed on a 4% native polyacrylamide gel. The arrows on the left indicate the shifted and supershifted (SS) NFAT-specific complexes upon the HIV-1 enhancer probe.

understanding of these complex interactions will allow a rational development for new classes of therapeutic inhibitors against HIV-1 replication. In this regard, understanding the basis for the preferential replication of HIV-1 in human CD4+ T cells bearing the CD45RO memory phenotype takes on particular significance. We now show for the first time that, among a number of differences between naive and memory T cells, it is most likely the expression of distinct CD45 isoforms that regulates differential activation of the HIV-1 LTR and productive virus replication. Moreover, we have determined that the molecular basis for this observation resides in the preferential activation of NFAT in T cells expressing the lower $M_r$ CD45RO isoform.

We made use of a unique Jurkat model whereby endogenous CD45 has been specifically inhibited by stable expression of an antisense gene and then reconstituted by expression of either the CD45RA or the CD45RO isoform. Initial studies indicated that this Jurkat model was behaving in a similar fashion to naive and memory primary CD4+ T cells with respect to the preferential replication observed in CD45RO-expressing cells after T cell activation. Indeed, luciferase activity and cell supernatant p24 levels were consistently higher in CD45RO clones than in their CD45RA counterparts treated with anti-CD3/anti-CD28. This increase in luciferase activity and p24 levels extended beyond 4 days and is likely indicative of a re-infection phenomenon occurring in a more pronounced fashion in CD45RO-expressing Jurkat cells due to a more abundant
production of HXB-LUC particles. These results have also been previously reported by other groups using primary naive T cells in vivo (16, 18, 20, 22, 54). Also, syncytium formation in HIV-1-infected cells occurred exclusively in CD45RO T cells activated by anti-CD3/anti-CD28 treatment. However, in this case, the positive modulation of the number of syncytia in CD45RO-expressing Jurkat clones should be mostly representative of an increase in gp120 at the surface of the initially infected cell line. These latter results are supportive of previous data from Helbert and co-workers (19) who observed that syncytium formation was restricted to CD45RO-positive (memory) T cells.

Preferential replication of HIV-1 in CD45RO CD4 T cells is known to be independent from viral entry, cellular proliferation rates, viral tropism, and activation status of NF-κB and AP-1 (16–19, 21). Our results have also demonstrated similar degrees of activation-induced nuclear translocation of NF-κB in CD45RA and CD45RO Jurkat cells. Further analysis by Ostrowski and colleagues (21) has demonstrated that, although CD45RA-positive CD4 + T lymphocytes are inherently resistant to productive HIV-1 infection in vivo, these cells are not devoid of HIV-1 proviral DNA. We have corroborated these results in our Jurkat-derived single isoform transfectants showing no differences in susceptibility to the early steps in the HIV-1-replicative cycle.

EMSA, calcium flux, and luciferase assays in the single-isoform transfectants strongly implicate the interaction of NFAT with the HIV-1 enhancer as a key element in the enhanced HIV-1 LTR activity in T cells expressing CD45RO. We previously reported augmented anti-CD3-mediated IL-2 production in CD45RO-positive cells in this same model, which is likely to result from increased NFAT activation in these cells (13). Such differences in NFAT activation might contribute to the greater responsiveness of memory T cells to antigenic stimulation (4). The preferential involvement of the NFAT1 family member in positive regulation of the HIV-1 LTR was surprising, based on previous results from Macian and Rao (55) that have suggested a negative role played by this factor on HIV-1 gene regulation. In contrast, we and others (33, 34, 53) have previously suggested an important positive role played by NFAT1 in HIV-1 replication.

Interestingly, our data have also shown that differential activation of NFAT in CD45RO and CD45RA cells occurs even after stimulation with PMA/ionomycin. Although these results are surprising, we have recently demonstrated that the absence of CD54 expression in Jurkat cells led to greater NFAT activation following PMA/ionomycin stimulation (34). It is thus conceivable that, in our Jurkat cell system, the type of CD45 isoform expressed on the cell surface could also alter the strength of NFAT activation even in the context of stimuli bypassing the proximal signaling machinery.

The importance of NFAT activation in the CD45RO-mediated increase in HIV-1 LTR activity has also been suggested by the use of the immunosuppressive and NFAT-inhibiting agents FK506 and CsA. Indeed, induction of the HIV-1 LTR after activation of CD45RO-positive T cells was reduced to the same level of LTR activation in treated CD45RA-positive cells. Interestingly, LTR activation of the CD45RA-expressing cells resulted in little reduction of luciferase activity following treatment with FK506. These data might indicate that NFAT is not induced to sufficiently high levels to participate in the positive modulation of HIV-1 transcription. In addition, they also provide evidence that NF-κB activation by the different tested activators, which is responsible for the remnant LTR induction observed in FK506-treated cells, is not sensitive to FK506 in our Jurkat cell clones. In fact, some of our data have demonstrated that FK506 had no significant effect on the activation of an NF-κB-reporter construct in our model (data not shown). We have also assessed viral replication in anti-CD3/anti-CD28-activated Jurkat cell clones. The greater level of HXB-LUC replication observed in CD45RO-expressing cells was partly reduced by the addition of FK506 reaching comparable luciferase activity levels to the one obtained in infected CD45RA cells. These experiments confirm the importance of NFAT in the differential susceptibility of HIV-1 replication in cells expressing CD45RA and CD45RO. However, the fact that FK506 did not totally abolish the difference in HIV-1 replication between CD45RO and CD45RA T cell clones might be reminiscent of a possible sub-optimal FK506 treatment for HIV-1 LTR inhibition in the context of full-length proviral DNA. It can also be proposed that other FK506-resistant cellular components might be specifically acting during HIV-1 replication and might allow greater level of NFAT activation in CD45RO T cells. Nonetheless, taken together, our data indicate that the NFAT transcription factor plays a major role in productive HIV-1 replication.

Previous published observations support such an important link between NFAT and HIV-1 gene expression/replication in primary human cells, although none of these studies have looked at the involvement of the different CD45 isoforms in this relationship (32, 33, 56). Our results confirm initial suggestions (30, 57), that a cellular factor (in this case, NFAT1) might underlie preferential HIV-1 replication in memory T cells. The mechanism for this higher level of NFAT activation in CD45RO-positive cells is unclear. It is not presently well understood how the expression of distinct CD45 isoforms alters signal transduction. The various isoforms differ only in their extracellular domains and have identical and equally active PTPase domains (58). Therefore, it has been speculated that the different isoforms might be differentially regulated by interaction with distinct ligands. However, no specific ligand for CD45 or its isoforms has been confirmed (59). On the other hand, differences in the extracellular domains of CD45 might contribute to isoform-specific interactions of CD45 with other molecules on the surface of the same cell, directing the cytoplasmic PTPase domains next to distinct substrates. This hypothesis is supported by co-capping data (4). In agreement, our current data and previous studies involving antibody-mediated stimulation of cell lines suggest that ligands on other cells need not be present to induce isoform-specific signaling (12, 13). Alternatively, it is possible that the shorter CD45RO isoform (in comparison to the CD45RA isoform) exhibits a longer time delay in their exclusion from immunological synapses (37, 60, 61). Interestingly, a recent study is shedding light on the role played by the different CD45 isoforms in signal transduction events mediated through the TCR-CD3 complex. It was found that CD45RO, but not CD45RBC or CD45RABC, forms heterodimers with CD4 and CD8 at the cell surface of HPB-ALL T lymphoid cells, an association that correlates with an increased TCR-CD3-mediated signal transduction intensity (62). Our observations are thus perfectly in line with this work.

Here we have shown for the first time that NFAT activation is augmented in memory compared with naive CD4 + T cells and that this contributes toward augmented HIV-1 replication in T cells expressing CD45RO. Moreover, our findings underscore the importance of NFAT in HIV-1 regulation following physiological T cell activation through CD3 and CD28. It now becomes crucial to assess new therapeutic avenues aimed at modulating the NFAT transcription factor in hopes of limiting HIV-1 pathogenesis.

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