Combinatorial Signals from CD28 Differentially Regulate Human Immunodeficiency Virus Transcription in T Cells*

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Activation through the T-cell receptor and the costimulatory receptor CD28 supports efficient HIV transcription as well as reactivation of latent provirus. To characterize critical signals associated with CD28 that regulate HIV-1 transcription, we generated a library of chimeric CD28 receptors that harbored different combinations of key tyrosine residues in the cytoplasmic tail, Tyr-173, Tyr-188, Tyr-191, and Tyr-200. We found that Tyr-191 and Tyr-200 induce HIV-1 transcription via the activation of NF-κB and its recruitment to the HIV-long terminal repeat. Tyr-188 modifies positive and negative signals associated with CD28. Importantly, signaling through Tyr-188, Tyr-191, and Tyr-200 is required to overcome the inhibition posed by Tyr-173. CD28 also regulates P-TEFb activity, which is necessary for HIV-1 transcription processivity, by limiting the release of P-TEFb from the HEXIM1/7SK inhibitory complex in response to T-cell receptor signaling. Our studies reveal that CD28 regulates HIV-1 provirus transcription through a complex interplay of positive and negative signals that may be manipulated to control HIV-1 transcription and replication.

One of the major blocks to eradicating human immunodeficiency virus (HIV)2 infections with highly active anti-retroviral therapy has been the inability of this treatment to eliminate cellular reservoirs harboring latent provirus. (1–3). T cells are a major target for HIV-1 infection, and T-cell signal transduction has been demonstrated to impact multiple steps of HIV-1 replication, including provirus transcription (4–7). Characterizing T-cell signaling regulatory networks that govern T-cell function and HIV-1 transcription is critical for understanding the molecular mechanisms that directly contribute to the establishment, maintenance, and breaking of proviral transcription latency (8, 9).

HIV provirus transcription is controlled by the upstream long terminal repeat (LTR), which includes cis-elements that are recognized by cellular transcription factors, including NF-κB, AP-1, and NFAT that are induced in response to T-cell receptor (TCR)/CD28 engagement (10, 11). These transcription factors recruit coactivators, including histone acetyltransferases and the ATP-dependent chromatin-remodeling Swi/Snf complexes that influence the chromatin structure of integrated provirus (12–16). Furthermore, the LTR forms an RNA stem loop structure, TAR, which the HIV transactivator, Tat, binds. Tat enhances RNA polymerase II (pol II) processivity by recruiting P-TEFb to the HIV-LTR (17, 18). The availability of P-TEFb, which is negatively regulated through association with the HEXIM1/7SK RNA particle, is also controlled by cellular signals (19, 20). Therefore, it may be possible to manipulate specific signaling cascades to control HIV transcription and improve the efficacy of current anti-viral regimens.

Efficient T-cell activation requires signals from the TCR as well costimulatory molecules, including CD28, which enhances TCR activation, promotes cell survival and increases cytokine production (21–24). CD28 possesses no enzymatic activity and mediates signaling by recruiting other proteins to tyrosines and proline-rich motifs within its cytoplasmic domain. CD28 has four signaling tyrosine residues (Y) in the cytoplasmic tail of CD28 at positions Tyr-173, Tyr-188, Tyr-191, and Tyr-200, which are required for appropriate T-cell activation, induction of cytokine gene expression, cytoskeletal reorganization, and immunological synapse formation (25, 26). Key signal transduction events associated with CD28 include activation of Itk, Vav, and Rho/Rac GTases, protein kinase Cθ, and transcription factors such as NF-κB, AP-1, and NFAT (6, 27–32).

We have previously shown that signaling associated with CD28 positively and negatively regulates HIV-1 provirus transcription. Specifically, we demonstrated that Tyr-200 positively regulated HIV transcription by initiating Vav-1 and NF-κB signaling, whereas recruitment of PI3K to the Tyr-173 residue inhibited the ability of Tat to bind P-TEFb and HIV-1 transcription (30, 33). How these apparently opposing signals are coordinated to lead to induction of HIV-1 transcription, as well as the role of the other tyrosines in modulating HIV-1 transcription in response to CD28, has not been extensively investigated. Using chimeric CD28 receptors harboring mutations in different key tyrosines in the cytoplasmic domain, we show that CD28 induces HIV transcription through distinct but cooperative activities associated with the individual tyrosines.

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2The abbreviations used are: HIV, human immunodeficiency virus; HIV-1, HIV type 1; TCR, T-cell receptor; LTR, long terminal repeat; PI3K, phosphatidylinositol 3-kinase; NFAT, nuclear factor of activated T cells; AP-1, activator protein 1; NF-κB, nuclear factor-κB; P-TEFb, positive transcription elongation factor b; pol, polymerase; CsA, cyclosporin A; WT, wild type; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; IL-2, interleukin-2; 7SK RNP, 7SK RNA particle complex; TAR, transactivating response element.
EXPERIMENTAL PROCEDURES

Cell Lines and Primary Type Cells—Jurkat E6.1 T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.2 mM L-glutamine. Human embryonic kidney 293T cells were also obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Peripheral blood mononuclear cells were isolated from whole blood by Ficoll/histopaque gradient (Sigma-Aldrich), and CD4+ T cells were positively selected using the Dynal isolation kit (Invitrogen, 113.21D).

CD8/CD28 Chimeric Receptor Mutants—The 8WT, YFFF, FFYY, YFFY, and YYYF expression vectors have been described previously (25, 30, 33, 34), and these key residues are shown in Fig. 1. To generate receptors, FYF and YFFY, the plasmids corresponding to pMHneo FFYY and pMHneo YFFF were digested with Apal and HindIII (New England Biolabs). Two fragments were generated, a 400-bp fragment containing CD8α and the nucleotide sequence coding for tyrosine 173 of the cytoplasmic tail of CD28, and a 6.8-kb fragment containing the rest of CD8α and the pMHneo backbone. The 400-bp fragment from pMHneo FFYY and 6.8-kb fragment from pMHneo YFFF were digested with DpnI to eliminate donor plasmid and then recovered in complete RPMI. 48 h post-transfections, products were digested with DpnI to eliminate donor plasmid and then recovered in complete RPMI. 48 h post-transfections, clonal cell lines and selection protocol. We also generated clonal cell lines and these behaved identically to the CD8/CD28 pooled cell lines (Refs. 30, 33 and data not shown). The expression of the chimeric receptors was verified by Western blot (data not shown) and flow cytometry.

Flow Cytometry—For flow cytometry 2 x 10⁶ cells were washed and resuspended in 100 µl of staining media (phosphate-buffered saline containing 2% serum). Cells were incubated with 2 µl of anti-CD8α-phycoerythrin (BD 555635) and anti-CD28-FITC (BD 555725) or 1.0 µg/ml anti-human CD8α or 1.0 µg/ml anti-human CD28 antibodies (BD 555630) for 45 min on ice. Cells were washed three times with staining media and fixed with 2% paraformaldehyde. Fluorescence was measured using a BD Biosciences FACScan at the Flow Core Facility at Boston Medical Center.

Generation of HIV-1 Infectious Titers and Infections—0.5 x 10⁶ 293T cells were plated in a 6-well plate 24 h prior to calcium phosphate transfections, which were performed using 15 µg of pNL4-3-Luc(+)-Env(−) Nef(−) (35) or pHXB-PLAP-Env Nef(+) (36) (obtained from the National Institutes of Health AIDS Research and Reference Reagent Program) and 3 µg of pRSV-Rev, 3 µg of LTR VSV-G. 293T transfection efficiency for pNL4-3-Luc was assessed by determining luciferase activity using a luciferase kit (Promega Madison, WI), whereas p24 enzyme-linked immunosorbent assays were performed for the PHXB-PLAP virus. Supernatants were collected and filtered through a 0.45-µm disc prior to infection. Jurkat cells were infected with this virus for 12–16 h. Cells were then recovered and cultured in complete RPMI.

Activation of T Cells—Jurkat T cells were washed and resuspended in 5% fetal calf serum RPMI 1 x 10⁶ cells were plated in each well of a 24-well plate. Cells were either left unactivated, or activated with 0.1 µg/ml anti-human CD3 alone (BD 553356), anti-CD3, and 1.0 µg/ml anti-human CD28 (BD 55725) or 1.0 µg/ml anti-human CD8α antibodies (BD 55630) for 30 min. 5 µg/ml of goat anti-mouse antibody (Sigma M 4280) was added to cross-link the receptors. Following 8 h of stimulation, Jurkat cells were harvested, and luciferase activity was measured. In experiments using cyclosporin A (CsA), infected Jurkat T cells were recovered and activated in the presence of 500 ng/ml CsA or vehicle control.

Immunoprecipitation and Immunoblots—Jurkat T cells were serum-starved for 12–16 h, activated with antibodies as described above for 5 min prior to preparing protein extracts with lysis buffer (10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1.0 mM EDTA (pH 8.0), 2.0 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X-100, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture.
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III (Calbiochem). Lysates were precleared by incubating with protein A/G beads (Santa Cruz Biotechnology, sc-2003) for 30 min at 4 °C before incubating with primary anti-Vav (Santa Cruz Biotechnology, sc-132). Protein A/G beads were added to the antibody-lysate mix for 1 h at 4 °C, and beads were washed three times with lysis buffer and then suspended in SDS-PAGE loading buffer. The samples were heated for 5 min at 100 °C before being loaded onto a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by electrobolting. Western blot analysis was carried out using a phosphotyrosine antibody (BD Transduction Laboratories, 610024). The blot was stripped and reprobed with a Vav antibody. CyclinT1 (Santa Cruz Biotechnology, sc-8127) and HEXIM1 (Abcam, ab28016) immunoprecipitations were also carried out with the same protocol; however, nuclear extracts (described in the next section) instead of total protein extracts were used. In the immunoprecipitation experiments done with the PI3K inhibitor, 50 μM LY294002 (Promega) was introduced 30 min before activation to the culture. Western blots were quantified by densitometry. The ratio of CyclinT1 over HEXIM was calculated for all samples in Fig. 3A, and the ratio of HEXIM over CyclinT1 was calculated for Fig. 3 (B–D). The numbers depicted in the figures represent ratio of immunoprecipitation in each lane versus immunoprecipitation from the unactivated lane.

Electrophoretic Mobility Shift Assay—Jurkat cells were activated for 8 h, and nuclear extracts were isolated by resuspending 1 × 10^6 cells in low salt buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) for 15 min. 10% Nonidet P-40 was added to rupture the cell membranes, and the nuclei were pelleted and incubated in a high salt buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) to isolate the nuclear extract. Electrophoretic mobility shift assays were carried out by incubating 5 μg of protein from nuclear extracts with 4 μg of poly(dIdC) (Amersham Biosciences), 0.25 mM HEPES (pH 7.5), 0.6 mM KCl, 9.0% glycerol, 1.0 mM EDTA, 7.5 mM dithiothreitol, 50 mM MgCl2. Reaction mixtures were preincubated with 100-fold excess of specific or nonspecific competitors, or 0.5 μg of polyclonal antibodies against NF-κB subunits p50 (Santa Cruz Biotechnology, sc-7178) and p65 (Santa Cruz Biotechnology, sc-109). Samples were loaded onto a 6% polyacrylamide gel and electrophoresed at 120 V in 0.5× Tris-borate-EDTA. Probes for electrophoretic mobility shift assay were generated by annealing oligonucleotides representing the HIV-1 NF-κB sites (5’-AGCTCCTGGAAGATCCCAGCGGAGGACCCTTCGACTGCTGAGGACG-3’ and 5’-AGCTAAGGGACTTCCGCTGCTGCTGAGGACG-3’). Sp1 probe was used as nonspecific competitor (sense sequence 5’-GATCGCTGATTGGGGCCGGGGGAGC-3’ and antisense sequence 5’-GATCGCTGACCCGGGGGAGC-3’). Probes were generated by end filling with the Klenow fragment of E. coli polymerase in the presence of [a-32P]dCTP.

Transfection of LTR Reporter Constructs—Fifteen micrograms of pGL2 LTR luc and pGL2-mxB-LTR luc constructs (kindly provided by Dr. Suryaram Gummuluru (37)) were electroporated into 20 × 10^6 Jurkat E6.1 cells using the T280 BTX electroporator. The cells were recovered in 5% fetal calf serum RPMI for 16 h. 1 × 10^6 cells were either left untreated or activated with 0.1 μg/ml anti-human CD3 or 0.1 μg/ml anti-human CD28 and 1.0 μg/ml anti-human CD8, and luciferase assays were performed 6 h post activation as described above.

Chromatin Immunoprecipitation—1 × 10^8 cells were infected with pHXB-PLAP virus for 5 days. Cells were then activated with 0.1 μg/ml anti-CD3 and 1.0 μg/ml anti-CD8 or anti-CD28 antibodies for 30 min. 5.0 μg/ml of goat anti-mouse was then added, and cells were activated for 6 h. Cells were cross-linked using 11% formaldehyde solution (prepared from 37% formaldehyde, 10% methanol) in 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-HCl (pH 8) to the final concentration of 1% for 10 min at room temperature. The reaction is quenched by adding 2 μl glycine to a final concentration of 240 mM. Cells were washed with phosphate-buffered saline and resuspended in 1 ml of sonication buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride) and sonicated on ice for 30 cycles, 10 s on, 30 s off. 100 μl of sonicated chromatin was diluted 10-fold with dilution buffer and incubated with 1 μg of antibody pol II (sc-899) p65 (sc-109) for 16 h at 4 °C. Protein A/G beads were then added for 2 h. The beads were then washed twice each with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 0.1, 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and 10 mM Tris and 1 mM EDTA. Complexes were eluted with 1% SDS, 0.1 M NaHCO3. The complexes were reverse cross-linked at 65 °C for 4 h, followed by addition of protease K for 1 h at 45 °C. The DNA was extracted using phenol chloroform and precipitated with ethanol. Quantitative real-time PCR analysis was carried out using SYBR green reagents and the primers 5’-TGCAGTACATTCAGAAG-3’ and 5’-GAGGTTAAGCAGTGGGTTC-3’, which amplify the −150 to +76 region of HIV-LTR, and 5’-GACTAGAGCCCTGGAAAGCA-3’ and 5’-GCTTCTTCTGCATGAGG3’, which amplify the +5396 to +5531 region of HIV.

Statistical Analysis—Statistical analysis was carried out using Student t test. A two-tailed distribution was performed on paired samples, comparing CD3 responses to CD8 plus CD8 responses. Values of <0.01 were considered significant.

RESULTS

Specific Tyrosines within the CD28 Cytoplasmic Tail Regulate HIV-1 Transcription—To study the role of CD28 in regulating HIV transcription, we employed a strategy that was previously described (25, 30, 33), in which we generated CD8/28 chimeric receptors with mutations in key tyrosine residues. The chimeric receptors were designed such that the cytoplasmic domain of CD28 was fused to the transmembrane and extracellular domain of CD8α, which we refer to henceforth as CD8/CD28. This chimeric receptor forms a dimer similar to CD28 and functions identical to endogenous CD28 (25). In addition, the generation and expression of these CD8/CD28 chimeras allow their expression in cells along with endogenous CD28, and the direct comparison of mutant and WT CD28 signals in the same cells (25, 30, 33). As shown in Fig. 1A, we changed individual or
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FIGURE 1. Generation of stable cells lines expressing CD8/28 chimeric receptors. A, schematic representation of the CD8/28 chimeric receptor showing the extracellular domain and transmembrane domain of CD8α fused to the cytoplasmic domain of CD28 as well as a list of all the CD8/28 chimeric mutants used and mentioned in this study. B, expression of CD8/28 chimeric receptor (black line) and the endogenous CD28 (filled gray histogram) was determined by flow cytometry in either Jurkat T cells or Jurkat T cells stably expressing all the chimeric receptors used in this study. Cells were stained with phycoerythrin-conjugated CD8α antibody, and fluorescein isothiocyanate-conjugated CD28 antibody.

FIGURE 2. Specific tyrosine residues in the cytoplasmic domain regulate HIV-1 transcription. Jurkat cells expressing the indicated CD8/28 chimeric receptors were infected with NL4-3 luciferase virus for 16 h. Post infection cells were either left unstimulated, or stimulated with 0.1 μg/ml anti-CD3 plus 1.0 μg/ml anti-CD8α, or with 1.0 μg/ml anti-CD28, followed by 5 μg/ml secondary antibody (goat anti-mouse) to cross-link the receptors. Eight hours post-activation the cells were lysed, and luciferase activity was measured. The luciferase activity is shown as a percentage of endogenous response, wherein the CD3 plus CD28 response of each cell line was set to a 100%.

Previous studies have shown that the Tyr-173 residue negatively regulates HIV-1 transcription, whereas signaling through Tyr-200 was necessary for HIV-1 transcription (30, 33). We were interested in determining the functional interplay between these two apparently opposite activities mediated through Tyr-200 and Tyr-173, as well as the integration of signals downstream of other tyrosines within the CD28 cytoplasmic domain. We initially examined the ability of CD8 receptors with one functional tyrosine, Tyr-173 (YFFF), Tyr-188 (FYFF), Tyr-191 (FFYF), or Tyr-200 (FFFY), to support HIV-1 transcription. As expected, the YFFF receptor was unable to activate HIV transcription, consistent with our previous report that implicated Tyr-173 signaling as inhibitory (33). Furthermore, the FYFF receptor did not support HIV-1 transcription indicating that signals downstream of Tyr-188 were not sufficient to induce HIV-1 transcription. However, the FFYF and FFFY receptors activated HIV-1 transcription to levels comparable to the wild-type CD8/CD28 or the endogenous CD28 receptors, indicating that Tyr-191 and Tyr-200 positively regulate HIV transcription in the absence of other tyrosine residues in the signaling domain of CD28. These data suggest that Tyr-191 and Tyr-200 have distinct functions from Tyr-173 and Tyr-188. Our data also indicate that signaling through Tyr-191 and Tyr-200 are critical in governing the overall positive signaling associated with CD28 costimulation in HIV-1 transcription.

We were interested in understanding how the positive signals associated with Tyr-191 and Tyr-200 overcome the inhibition imposed by Tyr-173 within the context of the WT receptor. To address questions regarding how these signals are
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FIGURE 3. TCR signals negatively regulate CyclinT1-HEXIM1 interaction. A, Jurkat cells stably expressing the indicated CD8/28 chimeric receptors were serum-starved for 12–16 h prior to activating with 0.1 μg/ml anti-CD3 and 1.0 μg/ml anti-CD28, whereas wild-type Jurkat T cells were activated with 0.1 μg/ml anti-CD3 and 1.0 μg/ml anti-CD28 for 30 min before adding 5 μg/ml goat-anti-mouse for 4 h. Nuclear extracts were prepared, and CyclinT1 immunoprecipitation was carried out. The samples were run on a SDS-PAGE gel, Western blot analysis assessed the amount of CyclinT1, and an anti-HEXIM1 Western blot was done to determine the activity of CyclinT1. Blots were quantified in the panel below. B, CD4+ T cells were isolated from human peripheral blood and were left unstimulated or activated with 0.1 μg/ml anti-CD3, 0.1 μg/ml anti-CD28, or 1.0 μg/ml anti-CD28 alone. Receptors were crosslinked by adding 5 μg/ml goat-anti-mouse for 4 h. Nuclear extracts were prepared, and an anti-HEXIM1 immunoprecipitation was carried out. Western blot analysis determined the amount of HEXIM1 immunoprecipitated and the amount of CyclinT1 associated with HEXIM1. Blots were quantified in the lower panel. C, nuclear extracts were prepared from Jurkat cells activated through the CD3 and CD28 receptors as described above, and an anti-HEXIM1 immunoprecipitation was carried out. Western blot analysis determined the amount of HEXIM1 immunoisolated as well as CyclinT1 associated with HEXIM1 (quantified in the panel below). D, CD4+ T cells were treated with 50 μM LY294002 for 4 h prior to preparing nuclear extracts. HEXIM1 was immunoprecipitated, and Western blot analysis determined the levels of HEXIM1 and CyclinT1 (quantified in the panel below). For all panels, nuclear extracts (NE) were probed with the indicated antibodies as an input control. Data shown are from a single experiment, and quantification was performed from at least two independent experiments.

Whether Tyr-188 also modifies integrated or functionally cooperate to control the transcriptional response of HIV provirus we examined the activity of chimeric receptors that had a functional Tyr-173 combined with different distal tyrosines: Tyr-173 and Tyr-200 (YFFY), Tyr-173 and Tyr-188 (YYFF), and Tyr-173 and Tyr-191 (YYF). Signaling through YFFY and YYF did not support activation of HIV-1 transcription, suggesting that positive signals downstream of Tyr-191 and Tyr-200 were not sufficient to overcome the repressive activity associated with Tyr-173. Surprisingly, Tyr-188, which was not capable of inducing HIV-1 transcription when it was the only tyrosine within the CD28 cytoplasmic tail was able to partially overcome the repressive activity of Tyr-173 resulting in 70% activity compared with the endogenous CD28 receptor (Fig. 2). Therefore, none of the tyrosines individually were able to completely overcome the negative signals associated with Tyr-173, implying that the ability of CD28 to activate HIV-1 transcription is a combinatorial event that requires cooperative activities of Tyr-188, Tyr-191, and Tyr-200. These data also suggest that Tyr-188 has a modulatory role in CD28 costimulation by interacting and modifying the activity of other tyrosines within the cytoplasmic tail, including Tyr-173.

Our previous data examining the YYF receptor showed that Tyr-188 and Tyr-191 were not able to cooperate to activate HIV-1 transcription in the presence of Tyr-173 (33). To investigate what combination of tyrosine residues in CD28 could overcome the inhibition posed by Tyr-173, we studied the functions of the receptors containing the combination of Tyr-173, Tyr-188, and Tyr-200 (YFFY) and the combination of Tyr-173, Tyr-191, and Tyr-200 (YYFY). YYFY was compromised in its ability to activate HIV-1 transcription as it led to only 55% of the endogenous CD28 response. Activation of cells via YYFY receptor did not activate HIV-1 transcription (40% of endogenous receptor activation, similar to stimulation via the TCR alone) despite Tyr-191 and Tyr-200 being shown to be sufficient to activate HIV-1 transcription when present by themselves in the receptor. Even though Tyr-188 does not appear to directly activate HIV-1 transcription, this residue is indispensable for coordinating signals that induce HIV-1 transcription.
CD28 Signaling Does Not Activate P-TEFb—HIV-1 transcription is in part regulated by RNA pol II processivity (38, 39). In the absence of Tat, there is an accumulation of short initiated transcripts as a result of RNA pol II pausing and premature termination that occurs due to the lack of P-TEFb (40, 41). Tat enhances transcription processivity by binding the TAR element and recruiting P-TEFb to the HIV-LTR. P-TEFb is comprised of two subunits, CyclinT1 and Cdk9, and its availability to bind promoters is regulated by its association with the Hexim/7SK RNA particle complex (7SK RNP). Release of P-TEFb from the repressive 7SK RNP permits P-TEFb to target and activate RNA pol II complexes (19, 20). We examined if CD28 signaling regulates P-TEFb availability and activity and determined whether this influences HIV transcription. We assessed the ability of the different cell lines expressing chimeric receptors to modulate CyclinT1-HEXIM interactions. Of the chimeric receptors examined, FFYF, FYFF, and FFFY, mediated release of P-TEFb from HEXIM (Fig. 3, A and B). Furthermore, the All F receptor and activation with anti-CD3 alone induced release of P-TEFb. These data indicate that TCR signaling is sufficient for releasing P-TEFb from the 7SK RNP. This was also observed in primary CD4+ T cells isolated from peripheral blood, which showed a decrease in HEXIM1-associated CyclinT1 upon activation through the TCR (Fig. 3B).

PI3K is recruited to the YMNM motif in CD28, which includes Tyr-173. We have previously shown that PI3K inhibits P-TEFb-Tat interactions and HIV-1 transcription (33). Although CD28 signaling did not influence P-TEFb release from the HEXIM-7SK RNP complex, we wanted to determine if CD28 might negatively regulate P-TEFb by promoting the formation of P-TEFb-7SK RNP. Treating Jurkat T cells with the PI3K inhibitor LY294002 decreased CyclinT1-HEXIM interactions both before and after CD3 plus CD28 stimulation (Fig. 3C). Because Jurkat T cells have a defect in PI3K signaling due to a defect in phosphatase and tensin homolog (PTEN), we also assessed the effect of inhibiting PI3K in CD4+ T cells (42). Inhibition of PI3K in primary T cells decreased the association of CyclinT1 and HEXIM (Fig. 3D). Our data indicate that active PI3K signaling promotes interactions between CyclinT1 and HEXIM, thus sequestering P-TEFb from Tat and inhibiting HIV-1 transcription. These data suggest that CD28, through the recruitment of PI3K to Tyr-173, limits HIV-1 transcription by inhibiting the release of P-TEFb.

CD28-mediated Induction of HIV Transcription Requires NF-κB Activation—To gain a better understanding of the differential role of specific tyrosines in CD28 signaling, we examined if specific signaling events were associated with individual tyrosine residues. Vav, a guanine nucleotide exchange factor for the Rho GTPase, is known to be a target of T-cell activation and has been suggested to be downstream of CD28 signaling (43). We have shown that Vav is activated by the FFYF CD8/28 chimeric receptor and, in the absence of Tyr-200, Vav and Rac1, as well as NF-κB, are not efficiently activated (30). These observations indicate that Vav and Rac are key regulatory events for inducing HIV-1 transcription. Jurkat cells expressing CD8/28 FYFF, FFYF, and FFFY receptors were stimulated by cross-linking TCR and CD28 receptors, and Vav activation and tyrosine phosphorylation were assessed by immunoblotting. Following T-cell activation, FFYF and FFFY, which fully support HIV transcription, induced Vav phosphorylation (Fig. 4A). However, FYFF, which is compromised in its ability to activate HIV transcription, also activated Vav (Fig. 4A), indicating that, although activation of Vav is downstream of CD28 signaling, it is not sufficient to induce HIV-1 transcription mediated by T-cell activation. We also inhibited NFAT signaling with CsA. As expected, inhibition of NFAT led to a decrease in overall HIV-1 transcription, but CD28 signaling still enhanced HIV-1 transcription, with an ∼3-fold induction in both CsA-treated and untreated cells, indicating that the activation of NFAT is primarily regulated by the TCR (Fig. 4B). In addition, we examined the MAPK signaling pathways, ERK1/2, JNK, and p38, and did not detect differential activation of these molecules upon costimulation via the chimeric receptors FFYF, FFYF, and FFFY (data not shown).

A consequence of T-cell signaling is the activation of transcription factors, which in turn bind regulatory cis-elements within promoters and enhancers to regulate transcription. The HIV LTR has binding sites for multiple transcription factors, including NF-κB, NFAT, and AP-1 (44–46). We wanted to examine if the activities of the chimeric receptors on HIV transcription reflected differential activation and recruitment of transcription factors to the HIV LTR. Because costimulation was only modestly affected by CsA, and MAPK pathways were not differentially activated by the CD28 mutants, which are upstream of NFAT and AP-1, respectively, we focused on the
activation of NF-κB, which has been shown to be indispensable for HIV-1 transcription (46). Electrophoretic mobility shift assays were performed using nuclear extracts isolated from activated Jurkat T cells stably expressing CD8/28 chimeric receptor mutants to measure NF-κB binding activity following T-cell activation. Receptors that had Tyr-191 (FFFY) and Tyr-200 (FFFF) induced NF-κB binding, whereas FYFF,FFFY, or the All F receptors were unable to activate NF-κB, correlating with the ability of the former receptors to support HIV transcription (Fig. 5A). The specificity of NF-κB binding was confirmed using unlabeled competitor oligonucleotides (Fig. 5A). We also verified the identity of the NF-κB subunits by performing supershift assays in the presence of anti-p65 and anti-p50 antibodies (Fig. 5B). Anti-p50 antibody generated supershifted complexes and anti-p65 disrupted binding of NF-κB consistent with the conclusion that the activated NF-κB complexes are composed of p50 and p65 subunits. These data suggest that activation of NF-κB is critical for CD28-mediated HIV-1 transcription.

To verify a role of NF-κB in CD3 plus CD28-mediated HIV transcription, Jurkat T cells were transiently transduced with luciferase reporters under the control of either wild-type HIV LTR or a LTR harboring mκB binding sites (mκBLTR). LTR-luc was induced by ~4-fold when cells were activated through CD3 plus CD28 compared with cells either treated with anti-CD3 alone or not activated (Fig. 5C). Consistent with NF-κB being necessary for transcriptional activation, CD3 plus CD28 signaling did not induce the mκB-LTR (Fig. 5C). These data suggest that the activation and binding of NF-κB to the HIV LTR is indispensable for HIV transcription.

To verify that CD28 signaling leads to changes in NF-κB binding at the HIV-LTR of HIV-1 infected T cells, we performed chromatin immunoprecipitation experiments. Jurkat T cells expressing CD28 chimeric receptors were infected with pHBX2.2 and activated using anti-CD3, anti-CD8, or anti-CD28 antibodies. Chromatin was prepared, and p65 binding at the HIV-LTR was measured. Chromatin immunoprecipitation results indicate that recruitment of p65 to the HIV-LTR is compromised when costimulatory signals are initiated by the FFFY receptor (Fig. 6A), whereas recruitment of p65 to the LTR was comparable to the endogenous CD28 receptor when cells were activated through the FFFY and FFFY chimeric receptors. We also examined the distribution of RNA pol II at the HIV-LTR versus +5396 bp downstream of the transcriptional start site to determine RNA pol II processivity. RNA pol II was detected at both the LTR and downstream sequences in cells activated through the FFFY and FFFY receptors, whereas RNA pol II was not detected at the LTR and +5396 in activated FFYY cells (Fig. 6, B and C). Overall, the levels of pol II associated with downstream HIV sequences directly reflected levels of pol II at the LTR indicating that pol II recruitment and not RNA pol II processivity are regulated by signaling through the chimeric receptors. Taken together, these data indicate that the ability of CD28 to enhance NF-κB signaling and RNA pol II recruitment are primarily responsible for inducing HIV transcription in response to CD3 plus CD28 activation.

**DISCUSSION**

We have studied CD28 signaling and its impact on HIV transcription, with particular focus on the interplay between signals arising downstream from the four tyrosine residues present in its cytoplasmic domain. Our study reveals a complex interplay
of signals downstream of these tyrosine residues that are integrated to enhance HIV transcription.

Examining individual tyrosines we confirmed an inhibitory role for signals emanating from Tyr-173 and positive activity for Tyr-200 in CD28 signaling (25, 30, 33). In addition, Tyr-191 is sufficient to activate CD28-dependent HIV-1 transcription similar to Tyr-200. However, when different combinations of tyrosines were examined the complexity of CD28 signaling is revealed. Tyr-188, which does not induce HIV-1 transcription, modulates the negative activity of Tyr-173 as demonstrated by the ability of the YYFF mutants to induce HIV transcription. Tyr-188 also dampens the Tyr-200 response, because FYFY does not enhance HIV-1 transcription to the same level as WT CD28, whereas Tyr-188 does not alter Tyr-191 signaling capability. Therefore, we propose that by modulating positive and negative signals, Tyr-188 may set signaling thresholds for HIV transcription. The indispensable role of Tyr-188 for regulating HIV transcription is also supported by the observation that the YFYY receptor is unable to support HIV transcription. In addition, Tyr-191 and Tyr-200, which alone are sufficient for induction of HIV transcription, do not cooperate but rather neutralize each other when in receptors that contain both Tyr-191 and Tyr-200 emphasizing the need for all functional tyrosines in the receptor to assure proper CD28 signaling and function in HIV transcription. Overall, signals downstream to Tyr-191 and Tyr-200 cooperate in the presence of Tyr-188 to overcome the inhibitory signals posed by Tyr-173 to activate HIV-1 transcription. How Tyr-188 is modulating CD28 signaling is not clear, however, it is not simply altering the recruitment of PI3K to CD28, because similar levels of PI3K are associated with Tyr-173 in the context of Tyr-188 or the Phe-188 mutation (data not shown).

PI3K is recruited to the YMNM motif of CD28, and mutation of the Tyr-173 to Phe-173 abolishes the recruitment and activation of PI3K (47). Our previous studies have shown that Tyr-173 inhibits HIV-1 transcription in a Tat-dependent manner by negatively regulating the Tat-P-TEFb complex (33). Our current study indicates that PI3K diminishes the availability of P-TEFb by stabilizing interactions between CyclinT1 and HEXIM1. Therefore, PI3K-mediated signals appear to favor the sequestration of P-TEFb in the 7SK RNP, decreasing the availability of P-TEFb for recruitment to the HIV-LTR. Furthermore, we observed a minimal role for CD28 in activating P-TEFb, with release of P-TEFb being primarily controlled by TCR signaling. This is consistent with previous reports indicating that Ca2+ signaling released P-TEFb from 7SK RNP (48). Our data seem to be in conflict with recent data from Contreras et al. (49), who showed that PI3K/AKT increases active P-TEFb and HIV transcription induced by hexamethlene bisacetamide treatment. It is not apparent why there is a discrepancy between our data, although it could reflect differences in signals induced by hexamethlene bisacetamide versus CD28, cell model sys-

![CD28-mediated signals induce recruitment of NF-κB to proviral LTR](image-url)
CD28 Signals Regulate HIV Transcription

tems, or temporal differences in our assays. It should be noted that a recent paper by Chen et al. (48) has also suggested a minimal role for PI3K in P-TEFb activation.

Activation of NF-κB is indispensable for induction of HIV transcription. Residues that lead to NF-κB signaling such as Tyr-191 and Tyr-200 promote NF-κB binding, as well as recruitment of NF-κB and RNA pol II to the HIV-1 LTR, whereas Tyr-188 does not activate NF-κB or HIV-1 transcription. Furthermore, activation of Vav is not sufficient for activation of NF-κB, because Tyr-188 is capable of inducing Vav phosphorylation but not HIV-1 transcription. Our results would suggest that NFAT and MAPK pathways have a minimal role in CD28-mediated HIV transcription and would implicate roles for other signaling pathways. Protein kinase C θ (50–53), CARMA1 (54, 55), and Cot kinase (56) are downstream of CD28 and potential mediators of NF-κB signaling. The role of these factors and how they are integrated by CD28 signaling require additional investigation.

Recent studies have used similar approaches to examine the role of CD28 signaling in regulating T-cell activation and, in particular induction of IL-2 transcription. In summary, these reports suggest that the Tyr-173 residue and PI3K are dispensable for IL-2 transcription (25, 57–59), whereas Tyr-191 and Tyr-200 positively regulate IL-2 (25), correlating with CD28-mediated NF-κB activation. Furthermore, in general, mutations in the CD28 cytoplasmic tail that failed to activate IL-2 transcription did not support efficient HIV-1 transcription in response to CD3 plus CD28 signaling. However, IL-2 and HIV-1, despite being activated by overlapping signals in response to T-cell activation, do exhibit some unique responses to CD28 signaling. For example, the FYFY chimeric receptor does not robustly induce IL-2 transcription but is capable of fully activating HIV-1 transcription (data not shown). The responses of IL-2 and HIV-1 transcription to CD28 signaling may reflect differential requirements for gene activation, such as, a lower signaling threshold for induction of HIV-1 transcription.

In summary, we show that CD28-mediated induction of HIV-1 transcription is the result of coordinated positive and negative signals that ultimately lead to NF-κB activation. We propose a model (Fig. 7) wherein TCR signaling positively regulates P-TEFb, whereas CD28 signaling primarily leads to the activation of NF-κB and recruitment of RNA pol II. In addition, HIV-1 transcription can also be limited by CD28 signaling, which promotes the sequestration of functional P-TEFb in the 7SK RNP via PI3K, suggesting a negative feedback mechanism for CD28 to control T-cell activation. Our data, showing that NF-κB is limiting in HIV-1 transcription, and that it is a key target of CD3 plus CD28 signaling, is consistent with several groups that have demonstrated a role for NF-κB in the reactivation of latent HIV-1 transcription in cell lines and primary cell models (8, 9, 57, 60, 61). It is important to note that a recent report has suggested that NF-κB may have a minor role in reactivating HIV-1 expression in latently infected primary T cells that phenotypically resemble central memory cells, and that NFAT and p38 MAPK are the endpoints of the CD3 plus CD28 signaling cascade responsible for HIV-1 transcription (62). Whether these apparently contradic-
