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# Host Factor Transcriptional Regulation Contributes to Preferential Expression of HIV Type 1 in IL-4–Producing CD4 T Cells

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HIV type 1 (HIV-1) replicates preferentially in IL-4–producing CD4 T cells for unclear reasons. We show increased HIV-1 expression is irrespective of viral tropism for chemokine receptors as previously suggested, but rather transcription of the HIV-1 long terminal repeat (LTR) is increased in IL-4–producing CD4 T cells. Increased expression of HIV-1 message is also confirmed in IL-4–producing CD4 T cells from HIV-1–infected individuals *ex vivo*. In exploring a transcriptional mechanism, we identify a novel c-maf (required for IL-4 expression) transcription factor binding site just upstream of the dual NF- $\kappa$ B/NFAT binding sites in the proximal HIV-1 LTR. We demonstrate that c-maf binds this site *in vivo* and synergistically augments HIV-1 transcription in cooperation with NFAT2 and NF- $\kappa$ B p65, but not NFAT1 or NF- $\kappa$ B p50. Conversely, small interfering RNA inhibition of c-maf reduces HIV-1 transcription in IL-4–producing T cells. Thus, c-maf increases HIV-1 expression in IL-4–producing CD4 T cells by binding the proximal HIV-1 LTR and augmenting HIV-1 transcription in partnership with NFAT2 and NF- $\kappa$ B p65 specifically. This has important implications for selective targeting of transcription factors during HIV-1 infection because, over the course of HIV-1 progression/AIDS, IL-4–producing T cells frequently predominate and substantially contribute to disease pathology. *The Journal of Immunology*, 2012, 189: 2746–2757.

**H**IV type 1 (HIV-1) infection is characterized by viremia, progressive depletion of CD4 T lymphocytes, and the development of AIDS. Although the CD4 T cell count provides an indication of the intactness of the CD4 T cell compartment and its ability to prevent opportunistic infections, the peripheral blood viral load provides an indication of how rapidly the CD4 T cell compartment will be depleted as a result of pathological processes driven by viral replication (1). The profound con-

tribution of highly active antiretroviral therapy is to interrupt HIV replication with a resultant decrease in viral load and an increase in CD4 T cell count and function (2). Therefore, the viral load assay is one of the most sensitive and reliable parameters to assess highly active antiretroviral therapy effectiveness, despite the fact that use of immunological criteria is suggested for making treatment decisions about changing from a first- to a second-line regimen under the current World Health Organization HIV-1 treatment guidelines in resource-limited settings (3–5). Similarly, failure of intermittent antiretroviral therapy is marked by a strong viral rebound followed by more severe and longer depletion of CD4 T cells and disease progression (6, 7). Notably, viral replication substantially contributes to the viral load in an infected individual, and thus plays a critical role in HIV-1/AIDS pathogenesis.

HIV-1 largely infects and replicates in activated human CD4 T cells. In particular, HIV-1 shows a preferential replication in Th2 and Th0 subsets of activated effector T cells without clearly defined reasons. Early studies found that HIV-1 infection might induce a shift in the relative proportion of different CD4 Th cell subsets, and the virus preferentially replicated in CD4 T cells that produced Th2-type cytokines (8, 9). This preferential replication phenomenon by HIV-1 was later confirmed in several studies by using primary HIV-1 isolates capable of binding either the CCR5 or the CXCR4 chemokine receptors required as coreceptors for viral entry into CD4 T cells (10–14). However, these studies were conducted *in vitro* using conditionally polarized Th0, Th1, and Th2 clones derived from HIV-1 patients or healthy donors, and thus not necessarily reflecting infection of CD4 T cell subsets *in vivo*. Therefore, analysis of HIV-1 expression levels *ex vivo* in different CD4 T cell populations from infected individuals will be much more relevant to understanding HIV-1/AIDS progression (15).

Because of the significance of viral replication in HIV-1/AIDS pathogenesis, it is important to understand the mechanism un-

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; Ct, cycle threshold; hIFN- $\gamma$ , human IFN- $\gamma$ ; hIL-4, human IL-4; HIV-1, HIV type 1; LTR, long terminal repeat; MARE, Maf recognition element; MFI, mean fluorescence intensity; siRNA, small interfering RNA.

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derlying HIV-1 preferential replication in Th2 and/or Th0 effector CD4 T cells. Initially, one of the possible explanations put forward was related to a difference on T cell subsets in expression of chemokine receptors required for HIV-1 infection. Namely, Th1 cells express more CCR5 (16) and consequently are more easily infected by R5-tropic HIV-1; conversely, Th2 cells express a high density of CXCR4 (17) and are more susceptible to X4-tropic HIV-1 infection. However, over the time course of infection, R5 viral replication surprisingly increased in Th2 cells and drastically decreased in Th1 cells. By comparison, X4 viruses consistently maintained a high level of replication in Th2 cells and a low level in Th1 cells (10, 11). In addition, a proposed blockade of CCR5 by associative ligands, such as RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ , does not provide a satisfactory explanation because they seem to be generated and secreted constitutively, and a variety of chemokines might play different roles during HIV-1 replication (10, 18, 19). Thus, differential chemokine receptor expression does not appear to explain increased HIV-1 expression in IL-4–producing CD4 T cells.

An alternative hypothesis is that HIV-1 gene transcription influences differential viral replication in host CD4 T cell subsets (20). Along this line, in this study, we investigate whether a Th2-specific transcriptional mechanism affects HIV-1 replication. Specifically, we focus on a Th2-restricted factor, c-maf, exploring a potential mechanism that augments HIV-1 gene expression in IL-4–producing CD4 T cells. The proto-oncogene, c-maf, is the cellular counterpart of oncogenic v-maf (21). The c-maf protein belongs to a large family of basic region leucine zipper domain transcription factors and exerts its transcriptional role through binding to a Maf recognition element (MARE) (22). c-maf is expressed in Th2 but not Th1 clones, and plays a critical and selective role in transcriptional activation of IL-4 (in conjunction with NFAT), IL-10, and IL-21 cytokine gene transcription (23–26). NFAT transcription factors, especially NFAT2, are also involved in the positive regulation of IL-4 gene expression (27). Moreover, NFAT2 is capable of inducing a highly permissive state for HIV-1 replication in primary CD4 T cells (28). NFAT proteins, as well as their relatively homologous Rel family members, NF- $\kappa$ B RelA (p65) and p50, have been clearly shown to directly bind to and augment transcription of the HIV-1 long terminal repeat (LTR) via the dual proximal NF- $\kappa$ B/NFAT binding sites (28, 29). Moreover, they have even been reported to be essential for enhancing HIV-1 transcription (30–32) and likely play a role in governing latency maintenance (33). In this article, we show the transcription factor, c-maf, contributes to increased HIV-1 transcription in IL-4–producing CD4 T cells via binding to the LTR in cooperation with NFAT2 and NF- $\kappa$ B p65 specifically. Our studies provide new insight into the preferential replication of HIV-1 in IL-4–producing CD4 T cells and further suggest means to disrupt this increased HIV-1 expression or, alternatively, augment transcription of latently infected cells.

## Materials and Methods

### Plasmids and reagents

The original expression vectors for c-maf from Dr. Xiaojing Ma (34) and for NFAT2 (29) have been described. Luciferase reporter plasmids pLTR-Luc and pIL-4-Luc were previously described (35). The pLTR-GFP, pIFN- $\gamma$ -GFP, and pIL-4-GFP constructs were generated by subcloning the 3' HIV-1 LTR segment (598 bp), human IFN- $\gamma$  (hIFN- $\gamma$ ) promoter segment (646 bp), and human IL-4 (hIL-4) promoter segment (805 bp), respectively, into pEGFP-N1 to substitute the CMV promoter (Clontech). Similarly, the pc-maf-GFP plasmid was generated with the minimum c-maf-binding promoter, in which two tandem MARE consensus sequences (5'-TGCTGACTCAGCA-3') were inserted just upstream of the minimal c-maf-binding fragment (position -206 to +30) of the hIL-10 promoter (34). rIL-2 was supplied by the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD). rIL-4 was obtained from R&D Systems. Fluorescence-

conjugated anti-IL-4, anti-IFN- $\gamma$ , anti-CXCR4, and anti-CCR5 Abs were purchased from BD Bioscience, as was 7-amino-actinomycin D. Fluorescence-conjugated anti-HIV-1 core Ag (p24) Ab was purchased from Beckman Coulter.

### Cell isolation and expansion of IL-4–producing CD4 T cells

Institutional Review Board approval for protection of human subjects was obtained from the University of Pennsylvania and the University of Alabama at Birmingham. Primary human peripheral blood CD4 T cells from healthy donors were isolated by negative selection using a proprietary Ab mix (StemCell Technologies) as described previously (36). The primary T cells were activated with PHA (200 ng/ml) for 3 d in the presence of IL-4 (10 ng/ml) and irradiated (3000 rad) syngeneic PBMCs to polarize IL-4–producing CD4 T cells (37). The activated cells were cultured for another 8–12 d in the presence of rIL-2 (30 IU/ml) and rIL-4 (10 ng/ml) with fresh culture media replacement every 2–3 d.

### Transient transfection and reporter gene assays

Freshly isolated human CD4 T cells were transiently transfected with expression vector(s), alone or in combination with GFP or luciferase reporter plasmids, using Human T cell Nucleofector kits (Lonza), as described elsewhere (38). In primary infection experiments, the transfected cells were activated with PHA (1.5  $\mu$ g/ml) and rIL-2 (20 U/ml) for 3 d followed by HIV-1 infection. In c-maf knockdown experiments, the polarized CD4 T cells were transiently transfected with a small interfering RNA (siRNA) expression vector or its control as previously detailed (39). The cells were then cultured in the presence of rIL-2 (30 U/ml) before analysis. All GFP reporter expression was detected by flow cytometry.

### Electricity mobility shift assay and in vitro footprinting

Nuclear extracts were prepared from polarized human CD4 T cells. Oligonucleotide probes were labeled with  $\gamma$ -[ $^{32}$ P]-ATP (Table I). Electricity mobility shift assay was performed as previously described (36). Baculovirus-generated recombinant mafK and NFAT2 were prepared and studied for binding to the proximal HIV-1 LTR by in vitro footprinting as previously described (29).

### Chromatin immunoprecipitation and real-time PCR

Cytokine polarized human CD4 T cells were specifically infected with the HIV-1 NL4-3-GFP strain, which was kindly provided by Dr. David N. Levy (New York University). Before the chromatin immunoprecipitation (ChIP) assay, HIV-1-infected cells (GFP $^{+}$ ) were live sorted by flow cytometry after activation with PMA and ionomycin for 3 h. The ChIP assay was performed on the sorted GFP $^{+}$  cells according to the product guidelines (Upstate Biotechnology). Purified DNA precipitated by anti-NFAT1, -NFAT2, -NF- $\kappa$ B p65, -NF- $\kappa$ B p50, -c-maf, -Oct1 Abs, or IgG (Santa Cruz Biotechnologies) was subjected to real-time PCR. The amplicon was designed to overlap with the dual NF- $\kappa$ B/NFAT sites and the nearby upstream MARE site within the proximal HIV-1 LTR (Table I). The reaction was performed with the TaqMan Universal PCR Master Mix (Applied Biosystems). Fold transcription factor binding relative to negative control Ab precipitation was calculated by the formula, fold binding =  $2^{(\Delta Ct)}$ , where  $\Delta Ct$  is calculated as cycle threshold (Ct) of Ab control minus Ct of the transcription factor Ab of interest.

### HIV-1 infection and p24/gag detection

The HIV-1 virus strains, NL4-3, 89.6, Bal-1, were supplied by the Center for AIDS Research of the University of Pennsylvania. The NA420/BB3 HIV-1 strain was kindly provided by Dr. Phillip Smith (University of Alabama at Birmingham, Birmingham, AL). The activated (or polarized) CD4 T cells were infected with viral stock (p24, 50–100 ng/10 $^6$  cells) in the presence of DEAE-Dextran (4  $\mu$ g/ml) for 2 h or overnight at 37°C. The cells were then cultured in the presence of rIL-2 for an additional 3–5 d before analysis. Intracellular p24/gag staining and analysis was performed as previously described (40).

### Intracellular cytokine analysis and cell proliferation assays

Cytokine-polarized CD4 T cells were HIV-1 (or mock) infected and then stimulated with PMA (25 ng/ml) and ionomycin (1.5  $\mu$ M) for 4–6 h at 37°C in the presence of brefeldin A (10  $\mu$ g/ml) followed by intracellular staining according to the manufacturer's suggestions (BD Pharmingen). For proliferation assays during HIV-1 infection, cytokine-polarized cells were first labeled by using CFSE before viral infection. Two days later, the CFSE-labeled, HIV-1-infected cells were stimulated with anti-CD3 (5  $\mu$ g/ml) and anti-CD28 (5  $\mu$ g/ml) Abs in the presence of irradiated syngeneic PBMCs

for another 3 d before analyzing intracellular cytokine production, CFSE dilution, and HIV-1 p24 expression. Flow cytometry data were collected using a BD Calibur or LSR II flow cytometer, and analyzed using FlowJo software (Tree Star).

#### *Isolation of IL-4<sup>+</sup> cells and detection of HIV-1 mRNA from HIV-1-infected individuals*

Sixty milliliters of peripheral blood was collected from HIV-1-infected individuals with high viral titers (viral loads were between 65,000 and 950,000 copies/ml; CD4 counts ranged between 127 and 350/ $\mu$ l). CD4<sup>+</sup> cells were then purified by negative selection using a proprietary Ab mix as previously described (35), followed by stimulation of PMA plus ionomycin overnight to allow for IL-4 expression. IL-4<sup>+</sup> and IL-4<sup>-</sup> cells were then separated by using IL-4 cell enrichment and detection kits (Miltenyi Biotech) according to the product guidelines. RNA was isolated from each population of cells and converted to cDNA with SuperScript III (Invitrogen) reverse transcriptase. Two rounds of PCR amplification were carried out to determine the HIV-1 mRNA abundance as described previously (41). In brief, the first round of PCR was performed on a conventional PCR machine with the setting as follows: 94°C for 3 min, followed by 21 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. These products were subsequently used as templates for the second, seminested, real-time PCR, performed on the iQ5 multicolor real-time detection machine (Bio-Rad). The TaqMan detection chemistry was used, and real-time PCR settings were as follows: 50°C for 2 min, then 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Real-time RT-PCR analyses were also performed for 18S RNA, IL-4, and c-maf mRNA levels. Primers and probes used for detection are listed in Table I. Differences in message levels between IL-4<sup>+</sup> and IL-4<sup>-</sup> cells in individual patients were calculated using the Wilcoxon matched paired test (GraphPad Prism 5), and statistical significance was set at  $p < 0.05$ .

## Results

### *HIV-1 preferentially replicates in IL-4-producing CD4 T cells*

The majority of previous studies examining HIV-1 infectivity and replication were carried out using in vitro separately derived Th1 and Th2 cell lines from HIV-1-infected individuals, healthy donors, or umbilical cord blood samples. HIV-1 infection and replication efficiency was then compared by monitoring p24/gag levels in culture supernatants (8, 9). There are, however, major shortcomings of these types of studies: 1) the infection conditions differed for the different environments used to support the Th1 and Th2 cell lines, and 2) assaying the p24 level in the bulk culture supernatant does not provide data at an individual cell level within a bulk population.

To study the fidelity of HIV-1 preferential replication in Th2 cells and to overcome the two aforementioned problems, we generated in vitro, using a combination of IL-2 and IL-4, an admixture of Th1-like (IFN- $\gamma$ <sup>+</sup>, IL-4<sup>-</sup>), Th2-like (IFN- $\gamma$ <sup>-</sup>, IL-4<sup>+</sup>), and Th0-like (IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>) CD4 T cells in one bulk population. After 8–10 d of differentiation in vitro, the cells were infected with HIV-1 for 3–5 d in the presence of IL-2 and IL-4. The production of cytokines and the expression of virus protein, p24, were measured by intracellular staining. Notably, IL-4 single-positive (Th2) or IL-4 plus IFN- $\gamma$  double-positive (Th0) cells generate a higher percentage of p24 than IFN- $\gamma$  single-positive (Th1) or IFN- $\gamma$  and IL-4 double-negative cells (Fig. 1A). This increased expression in IL-4<sup>+</sup> cells is not due to a relative increased rate of apoptosis in IFN- $\gamma$ <sup>+</sup> cells after HIV-1 infection, because HIV-1 infection increased the rate of apoptosis (as detected by 7-amino-actinomycin D) virtually identically (~1.5-fold) in both IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> CD4 T cells (Supplemental Fig. 1). Therefore, IL-4-producing CD4 T cells preferentially support NL4-3 replication.

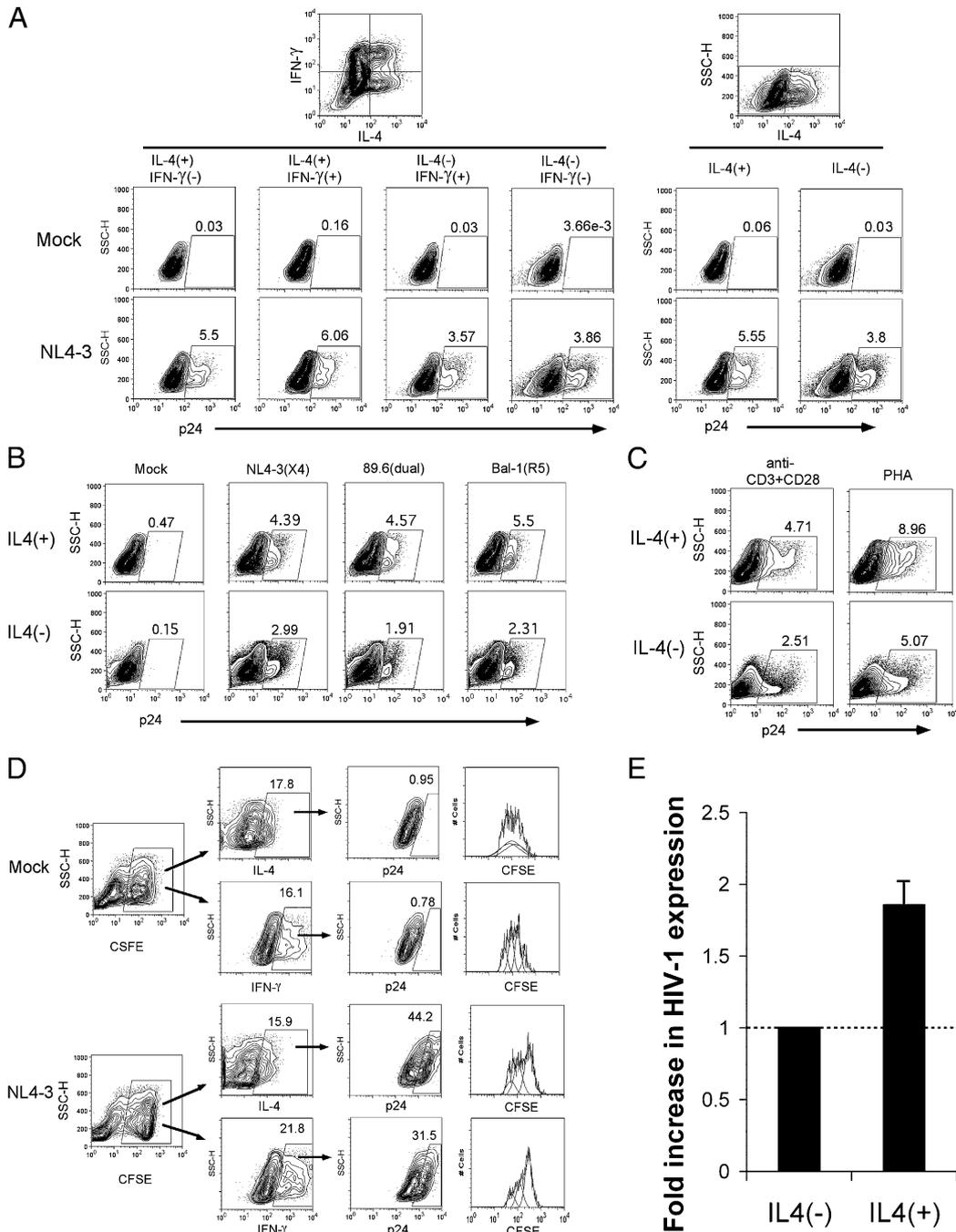
To further exclude the potential influences of viral tropisms on viral replication described elsewhere (42), an X4-tropic strain, NL4-3, a R5-tropic strain, Bal-1, and a X4R5 dual tropic strain, 89.6, were compared for their abilities to infect an admixture of polarized Th0, Th1, and Th2 CD4 T cells. To our surprise, IL-4-producing cells consistently and preferentially support HIV-1 viral expression irrespective of the viral tropism (Fig. 1B). Furthermore,

this preference is consistent using different initial stimuli (anti-CD3 plus anti-CD28 mAb versus PHA) to polarize the host cells (Fig. 1C). As a complementary alternative approach, we also show that surface CXCR4 expression is similar between IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> CD4 T cells, whereas CCR5 expression is higher on IFN- $\gamma$ <sup>+</sup> cells after T cell activation (Supplemental Fig. 2A). Nevertheless, both X4- and R5-tropic HIV-1 strains are expressed at similar levels in CXCR4<sup>+</sup> and CCR5<sup>+</sup> CD4 T cells (Supplemental Fig. 2B) despite increased expression in IL-4<sup>+</sup> relative to IFN- $\gamma$ <sup>+</sup> CD4 T cells (Supplemental Fig. 2C). Thus, differences in chemokine receptor expression or in HIV-1 viral tropism do not explain increased HIV-1 expression in IL-4<sup>+</sup> CD4 T cells.

To exclude the possibility that high-level expression of HIV-1 in IL-4-producing T cells is a result of a relative rapid growth during HIV-1 infection, we labeled cytokine-polarized CD4 T cells by using CFSE to allow for tracking of cell proliferation during HIV-1 infection. As shown in Fig. 1D, HIV-1 infection does not result in increased proliferation of IL-4-producing CD4 T cells. Thus, our data demonstrate that HIV-1 strains of any tropism are capable of replicating in either Th1 or Th2 cells, but IL-4-producing cells preferentially support replication of all varieties of HIV-1 tropism. Averaging the results of several experiments ( $n = 12$ ) reveals that IL-4-producing cells support HIV-1 replication approximately twice as well as IL-4<sup>-</sup> cells (Fig. 1E). This increased expression of HIV-1 did not appear to be related to either increased proliferation or decreased HIV-1-driven apoptosis of IL-4<sup>+</sup> relative to IFN- $\gamma$ <sup>+</sup> CD4 T cells.

### *Higher levels of HIV-1 gag mRNA are detected in IL-4-producing CD4 T cells from HIV-1-infected individuals*

To substantiate the increased difference in p24 production between IL-4-producing and -nonproducing CD4 T cells seen in vitro, we assessed HIV-1 levels in peripheral blood CD4 T cells from HIV-1-infected individuals ex vivo. CD4 T cells from peripheral blood in HIV-1-infected individuals were stimulated ex vivo with PMA and ionomycin to allow for production of cytokines. IL-4<sup>+</sup> and IL-4<sup>-</sup> cells were then sorted by an IL-4 detection and enrichment method, and used for RNA purification. HIV-1 gag mRNA was detected by real-time RT-PCR. Similarly, IL-4 mRNA and c-maf mRNA were also quantified. In these assays, 18S RNA levels served as an internal control and all other Ct values were normalized to it. Analyses were performed on peripheral blood samples from five different HIV-1-infected individuals with notably increased HIV-1 levels in their blood. There was a remarkable and statistically significant ( $p = 0.01$ ) increase in HIV-1 message in IL-4<sup>+</sup> cells as assessed by Ct values (mean  $\pm$  SEM is  $26.89 \pm 1.29$  for IL-4<sup>+</sup> cells, and  $30.33 \pm 1.24$  for IL-4<sup>-</sup> cells;  $\Delta$ Ct = 3.44 or ~10.8-fold increase in HIV-1 mRNA between IL-4-producing versus non-producing cells) for the group as a whole (Fig. 2A). As predicted, based on the importance of c-maf for IL-4 expression (24), c-maf mRNA levels were detected to any significant level only in the IL-4<sup>+</sup> cells (Fig. 2B). Likewise, IL-4 mRNA was essentially limited to IL-4-producing cells (Fig. 2C). Thus, HIV-1 mRNA levels are ~10-fold higher, on average, in c-maf-expressing, IL-4-producing CD4 T cells from peripheral blood of HIV-1-infected individuals. Because the cells were sorted based on IL-4 expression, the relative levels of IL-4 and associated c-maf are markedly higher in the IL-4<sup>+</sup> cells compared with the IL-4<sup>-</sup> cells (e.g.,  $2^{10-40}$ ) as expected. However, because the difference in HIV-1 was expected to be only near 2-fold (based on the in vitro data, Fig. 1) between IL-4<sup>+</sup> and IL-4<sup>-</sup> cells, it is difficult to demonstrate a linear correlation among the three genes. Nevertheless, using a Wilcoxon matched paired test, with an  $n = 5$ , IL-4<sup>+</sup> cells demonstrate greater HIV-1 viral RNA levels than IL-4<sup>-</sup> cells for each patient studied with a  $p$  value of 0.0313.

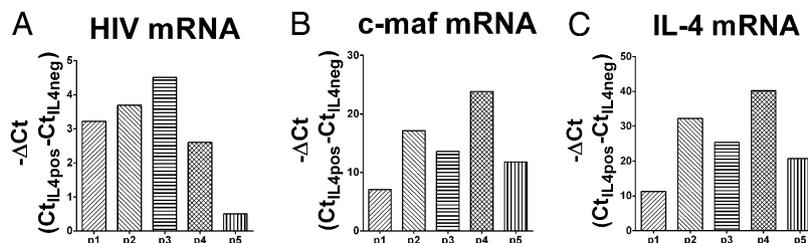


**FIGURE 1.** HIV-1 preferentially replicates in IL-4-producing CD4 T cells. IL-4 polarized human CD4 T cells were infected with HIV-1 and tested for cytokine production and viral core protein, p24, expression by intracellular staining and flow cytometric analysis. (A) Mimic and HIV-1 NL4-3-infected cells were analyzed for IL-4 and IFN- $\gamma$  along with p24 expression by three-color flow cytometry. HIV-1 p24 expression is depicted for the different subsets of cytokine expression patterns. (B) HIV-1 p24 expression was compared between IL-4<sup>+</sup> and IL-4<sup>-</sup> subsets postinfection with three different HIV-1 strains (NL4-3, 89.6, Bal-1) with different tropisms for chemokine receptor expression. (C) Primary human CD4 T cells were stimulated by anti-CD3 and anti-CD28 Abs plus IL-4, or by PHA plus IL-4, for 3 d. Both were then cultured in the presences of IL-2 and IL-4 to allow the development of IL-4-producing CD4 T cells, and infected with the HIV-1 NL4-3 strain. HIV-1 p24 expression was compared between IL-4<sup>+</sup> and IL-4<sup>-</sup> subsets in each of polarized cells with initially different stimulations. (D) IL-4 polarized CD4 T cells were labeled with CFSE, followed by mock or HIV-1 NL4-3 strain infection. The cells were further stimulated with anti-CD3 and anti-CD28 Abs in the presence of irradiated syngeneic PBMCs. Cytokine production, cell proliferation (e.g., CFSE dilution), and p24 expression were detected by flow cytometry. (E) The average (from 12 separate infections) fold increase ( $1.86 \pm 0.189$ ) in HIV-1 expression in IL-4<sup>+</sup> cells versus IL-4<sup>-</sup> cells is shown.

*Increased HIV-1 LTR transcriptional activity is detected in IL-4-producing CD4 T cells*

To explore a role for differential HIV-1 LTR transcriptional regulation in IL-4<sup>+</sup> versus IL-4<sup>-</sup> CD4 T cells, we infected IL-4 polarized human CD4 T cells with a GFP-expressing recombinant

lentivirus driven by the HIV-1 LTR. A CMV-driven GFP lentiviral infection served as a relative control. As shown in Fig. 3A, both CMV and LTR transcriptional activities are higher in IL-4-producing T cells than in non-IL-4-producing cells. Nevertheless, the promoter activity ratio for IL-4<sup>+</sup> relative to IL-4<sup>-</sup> CD4 T cells



**FIGURE 2.** Higher levels of HIV-1 mRNA are detected in IL-4<sup>+</sup> CD4 T cells from HIV-1-infected individuals. **(A)** The mRNA levels of HIV-1 *gag* in IL-4<sup>+</sup> and IL-4<sup>-</sup> CD4 T cells from HIV-1-infected patients' peripheral blood were determined by real-time RT-PCR. Before real-time RT-PCR, HIV-1-specific seminested primer PCR was carried out after reverse transcription of total RNA extracts. 18S RNA acted as the internal control. The Ct values of HIV-1 *gag* mRNA in IL-4<sup>-</sup> CD4 T cells were normalized to that in IL-4<sup>+</sup> cells by correcting them according to the 18S Ct values. The *p* value is 0.01 when comparing with IL-4<sup>+</sup> cells. The  $-\Delta\text{Ct}$  calculated [ $-(\text{Ct of IL-4}^+ - \text{Ct of IL-4}^- \text{ cells})$ ] values are shown for each individual patient ( $\Delta\text{mean} = 3.44$ , where  $p1 = \text{patient 1}$ ,  $p2 = \text{patient 2}$ , and so on;  $n = 5$ ). Similarly, the Ct values of *c-maf* mRNA **(B)** and IL-4 mRNA **(C)** were calculated, and the *p* values are 0.001 and 0.0002, respectively, when comparing IL-4<sup>+</sup> and IL-4<sup>-</sup> cells. The  $-\Delta\text{Ct}$  calculated [ $-(\text{Ct of IL-4}^+ - \text{Ct of IL-4}^- \text{ cells})$ ] values of *c-maf* mRNA and IL-4 mRNA are shown for each individual patient ( $\Delta\text{mean} = 15.51$  for *c-maf* mRNA,  $\Delta\text{mean} = 25.89$  for IL-4 mRNA, where  $p1 = \text{patient 1}$ ,  $p2 = \text{patient 2}$ , and so on;  $n = 5$ ).

driven by the HIV-1 LTR ( $8.62 \pm 1.40$ ) is significantly ( $p = 0.0179$ ) higher than that from the CMV immediate early promoter ( $3.4 \pm 0.90$ ; Fig. 3B). Interestingly, the LTR activity ratio calculated in these *in vitro* experiments (8.6-fold greater in IL-4<sup>+</sup> cells) is very close to the difference in HIV-1 mRNA levels detected in the *in vivo* patient samples (Fig. 2A;  $\Delta\text{Ct} = 3.44$ , mRNA level in IL-4-producing cells is  $2^{3.44} = 10.8$  times greater than in non-IL-4-producing cells). Therefore, HIV-1 LTR-directed transcription is substantially higher in IL-4<sup>+</sup> than IL-4<sup>-</sup> cells and correlates with increased HIV-1 mRNA levels.

#### *c-maf*, NFAT2, and NF- $\kappa$ B p65 preferentially bind the proximal HIV-1 LTR

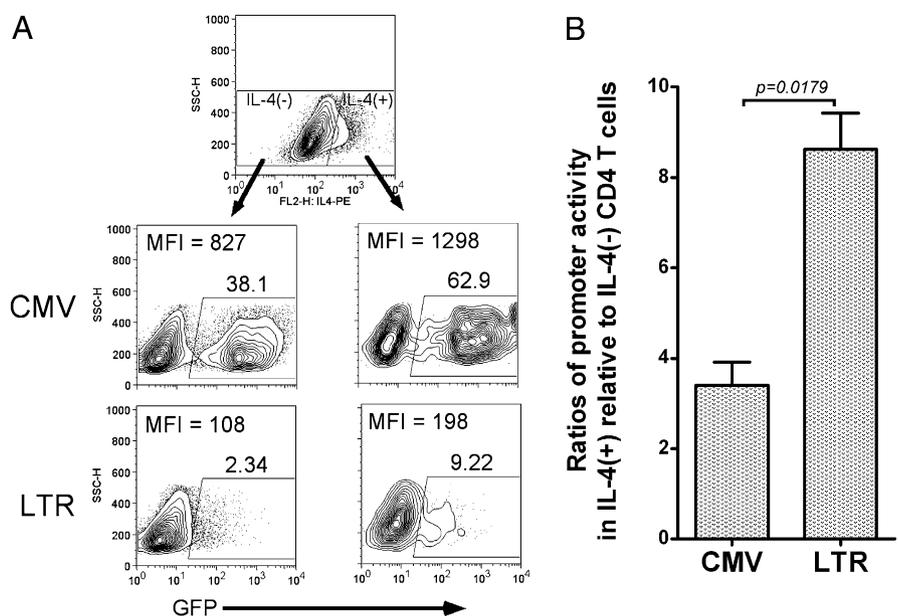
Because increased HIV-1 expression in IL-4<sup>+</sup> cells correlated with increased HIV-1 mRNA levels and transcription rates, we next focused on factors critical to IL-4 transcription. In particular, the Th2-restricted transcription factor, *c-maf*, binds to a MARE in the proximal IL-4 promoter and cooperates with a neighboring NFAT binding site to specifically enhance transcription of IL-4 (24, 35). In comparison with a *c-maf* consensus-binding sequence (43), 5'-TGCTGACTCAGCA-3', a novel homologous sequence, 5'-TGCTGACATCGAG-3', was identified and located just upstream (5') of

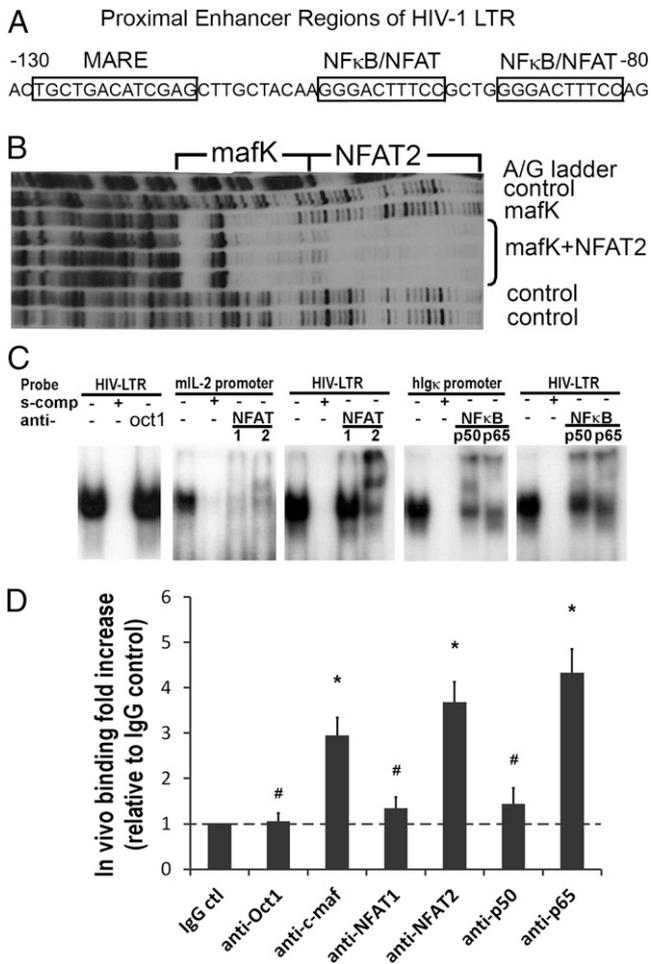
the dual NF- $\kappa$ B/NFAT sites in the HIV-1 proximal LTR (Fig. 4A). We next explored the ability of *c-maf* to bind the HIV-1 LTR.

*c-maf* belongs to a large family of *maf* proteins, all of which share a very homologous DNA-binding domain (44). To facilitate preparation of recombinant protein, we tested the ability of *mafK*, a small *maf* family member with a virtually identical DNA binding domain to *c-maf*, to bind to the proximal HIV-1 LTR by *in vitro* footprinting. *mafK* clearly protected DNA residues from DNase I digestion (i.e., bound the LTR *in vitro*) corresponding to the predicted MARE (Fig. 4B). Moreover, rNFAT2 in conjunction with *mafK* extended the protected residues through the NF- $\kappa$ B/NFAT region (Fig. 4B). Interestingly, even though there are 10 residues between the predicted MARE and the NF- $\kappa$ B/NFAT region, the site protected by *mafK* is adjacent to the NFAT2 binding site. This suggests that the two factors may interact and potentially cooperate when binding to the proximal HIV-1 LTR.

The ability of NFAT and NF- $\kappa$ B proteins present in polarized human CD4 T cells to bind in conjunction with *c-maf* on the proximal HIV-1 LTR was then explored. Prior work, including our own, has shown that rNF- $\kappa$ B family members, p65 and p50, and NFAT family members, NFAT1 and NFAT2, are all capable of binding to the HIV-1 LTR NF- $\kappa$ B/NFAT sites *in vitro* (29, 31). In

**FIGURE 3.** HIV-1 LTR transcriptional activity is increased in IL-4<sup>+</sup> CD4 T cells. **(A)** IL-4 polarized human CD4 T cells were infected by rGFP-expressing lentiviruses driven by either the immediate-early CMV promoter or HIV-1 LTR. GFP expression and IL-4 production were detected by flow cytometry. Results from one representative experiment of five are depicted demonstrating that both CMV and LTR promoters show higher transcriptional activity in IL-4<sup>+</sup> CD4 T cells than in IL-4<sup>-</sup> cells, but HIV-1 LTR transcriptional activity was notably higher than CMV promoter activity in IL-4<sup>+</sup> cells relative to IL-4<sup>-</sup> cells. **(B)** The mean ratios of transcriptional promoter activities of IL-4<sup>+</sup> cells relative to IL-4<sup>-</sup> cells were calculated (percentage positive  $\times$  MFI in IL-4<sup>+</sup> cells divided by percentage positive  $\times$  MFI in IL-4<sup>-</sup> cells) and presented as bar graphs. The HIV-1 LTR shows a higher relative ratio (IL-4<sup>+</sup>/IL-4<sup>-</sup>) of transcriptional activity than CMV (mean  $\pm$  SEM of ratio for CMV promoter:  $3.4 \pm 0.90$ ; for HIV-1 LTR:  $8.62 \pm 1.40$ ;  $p = 0.0179$ , Student *t* test;  $n = 5$ ).





**FIGURE 4.** c-maf, NFAT2, and NF-κB p65 preferentially bind the proximal HIV-1 LTR. **(A)** The dual NF-κB/NFAT binding sites and the predicted c-maf binding site (MARE) in the proximal HIV-1 LTR are depicted. **(B)** The in vitro footprint of the HIV-1 LTR DNase I digestion shows a mafK-induced footprint of the LTR just upstream and adjacent to the NFAT2 footprint. **(C)** Nuclear extracts prepared from activated IL-4 primed human CD4 T cells bind the HIV-1 LTR NF-κB/NFAT dual-site probe (panels 1, 3, 5), the mouse proximal IL-2 promoter NFAT site (panel 2), and the human Iγk promoter NF-κB site (panel 4) in gel shift assays (see *Materials and Methods* for details). NFAT1 and NFAT2 bind the IL-2 promoter similarly (panel 2), whereas NFAT2 preferentially binds over NFAT1 to the HIV-1 proximal LTR (panel 3). Likewise, NF-κB p65 and p50 bind the Iγk promoter NF-κB site similarly (panel 4), but NF-κB p65 binds preferentially, relative to NF-κB p50, to the proximal HIV-1 LTR (panel 5). Oct1 protein acts as a control that does not bind to the proximal HIV-1 LTR (panel 1). Results are representative of three independent experiments. **(D)** Real-time PCR was performed to quantify transcription factors bound to the integrated HIV-1 LTR in vivo as detected by ChIP. The results reveal c-maf, and NFAT2 over NFAT1, and NF-κB p65 over p50 preferentially bind to the HIV-1 LTR in vivo. Rabbit IgG and anti-Oct1 Abs are negative controls. The relative fold increases of transcription factor binding are calculated in comparison with the rabbit IgG control. #*p* > 0.05 when comparing with IgG control, \**p* < 0.05 when comparing with IgG control; *n* = 5.

the present studies, the LTR NF-κB/NFAT dual-site probe (primers and probes used for detection are listed in Table I) was incubated with activated IL-4 polarized CD4 T cell nuclear extracts in the presence of specific anti-transcription factor Abs during the gel shift assays. Interestingly, NF-κB p65 is preferentially present in the shifted complexes relative to NF-κB p50, and NFAT2 preferentially binds the LTR over NFAT1 (Fig. 4C). This preferential binding of NF-κB p65 and NFAT2 is unique to the HIV-1

LTR and not a function of the particular supershifted Abs, as NF-κB p50, NF-κB p65, NFAT1, and NFAT2 contained in the identical nuclear extracts were all relatively equally supershifted by the same Abs when tested for binding to NF-κB and NFAT sites derived from the Iγk and IL-2 promoters, respectively (Fig. 4C). Thus, the preferential binding of NF-κB p65 and NFAT2 to the HIV-1 LTR may reflect subtle differences in the HIV-1 LTR DNA binding or flanking sequences, and/or cooperative protein–protein interactions that can only be detected in physiologically relevant CD4 T cell nuclear extracts.

Binding of c-maf to the HIV-1 LTR in vitro was also explored by gel shift assay. A gel retarded band, from nuclear extracts from IL-4-primed CD4 T cells, migrated identically comparing a consensus MARE probe and a probe containing the putative HIV-1 LTR MARE (Supplemental Fig. 3). These bands were both fully competed with excess unlabeled self-probe, and they were both partially diminished in intensity (decreased by 48% for the consensus MARE probe, and decreased by 40% for the HIV-1 MARE probe, by densitometry) using a blocking anti-c-maf Ab (Supplemental Fig. 3). This suggests that c-maf present in IL-4-primed CD4 T cell nuclear extracts is capable of binding the HIV-LTR MARE in vitro just upstream of the dual NF-κB/NFAT sites.

To confirm the binding of c-maf and the individual NF-κB and NFAT family members to the HIV-1 LTR in vivo, we infected IL-4-primed human CD4 T cells with the NL4.3-GFP strain (45). ChIP assays were then performed using the purified GFP<sup>+</sup> (infected) cells with anti-c-maf, -NF-κB, and -NFAT Abs, and immunoprecipitated DNA was quantified by real-time PCR. We previously demonstrated that octamer transcription factors do not bind the HIV-1 LTR in vitro (36), so anti-Oct1 was chosen as a negative transcription factor Ab control, in addition to the isotype control Ab. As shown in Fig. 4D, the fold binding to the HIV-1 LTR for each transcription factor was calculated according to the respective Ct value of the specific transcription factor Ab relative to the IgG control Ab. Similar to in vitro binding results (Fig. 4B, 4C, Supplemental Fig. 3), there was clear evidence for binding to the HIV-1 LTR in vivo for c-maf, NF-κB p65, and NFAT2, but not for Oct1, NF-κB p50, and NFAT1 (Fig. 4D). Not only did this imply a role for c-maf in HIV-1 regulation, but it confirmed the preferential binding of unique NF-κB (p65 over p50) and NFAT (NFAT2 over NFAT1) transcription factor family members to the HIV-1 LTR.

#### *c-maf cooperates with NFAT2 and NF-κB p65 to augment HIV-1 LTR transcription*

The functional significance of c-maf alone, c-maf plus NFAT2, or c-maf plus NF-κB p65 to HIV-1 expression was next explored. First, primary human CD4 T cells were transiently transfected with an HIV-1 LTR-driven GFP reporter plasmid and cotransfected with c-maf, NFAT2, NF-κB p65, c-maf plus NFAT2, or c-maf plus NF-κB p65 expression vectors. It is evident that c-maf alone increases HIV-1 LTR activity (mean fluorescence intensity [MFI]: 127 to 399) without increasing cell survival (<10% difference in cell number after transfection). The increased HIV-1 LTR transcriptional activity is further and significantly enhanced when coexpressing NFAT2 (MFI: 438) or NF-κB p65 (MFI: 776) (Fig. 5A, top panel), suggesting a cooperative interaction between c-maf and NFAT2 and NF-κB p65.

Was this cooperative transcriptional activation by these factors relatively unique to the HIV-1 LTR? This possibility was explored by comparing hIL-4 promoter-, hIFN-γ promoter-, and HIV-1 LTR-driven GFP reporter gene activity in primary human CD4 T cells. As expected, c-maf notably increases HIV-1 LTR and hIL-4 promoter (MFI: 35.9–123) transcriptional activity, but only modestly augments hIFN-γ promoter activity (MFI: 62–133).

Table I. Listing of oligonucleotides used in this study

Electricity Mobility Shift Assay Probes (5' → 3' Direction)	
NFAT binding sequence (mouse IL-2 promoter)	TCGAGCCCAAAGAGGAAAATTTGTTTCATG
NF-κB binding sequence (human IκB promoter)	ATGTGAGGGGACTTCCCAGGC
c-maf binding sequence (hIL-4 promoter)	AATTGCTGACTCAGCATTA
HIV-1 LTR-NFAT/NF-κB dual-site sequence	CAAGGGACTTTCCGCTGGGGACTTCCCAGGG
HIV-1 LTR-MARE	AACTGCTGACATCGAGCTTGTACAAGGGACTTCCGCT
Real-Time PCR for HIV-1 gag, 18S, c-maf, and IL-4 (5' → 3' Direction)	
HIV-1 seminested, forward primer	CTTAGGCATCTCCTATGGCAGGAA
HIV-1 seminested, reverse primer	GGATCTGTCTCTGTCTCTCTCCACC
HIV-1 forward primer	ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA
HIV-1 reverse primer	The same as seminested reverse primer
HIV-1 probe	FAM-TTCCTTCGGGCTGTCCGGTCCC-BHQ2
18SRNA forward primer	TTTCGATGGTAGTCGCCG
18SRNA reverse primer	TGGATGTGGTAGCCGTTT C
18SRNA probe	FAM-CCACGGGTGACGGGGAATCAGG-BHQ2
c-maf forward primer	GTCAGCAAGGAGGAGGTGAT
c-maf reverse primer	TTCTTCTCCGACTCCAGGAC
c-maf probe	FAM-ATGCCAGTCCTGCCGCTTC-BHQ2
IL-4 forward primer	CCA CGG ACA CAA GTG CGA TA
IL-4 reverse primer	CCC TGC AGA AGG TTT CCT TCT
IL-4 probe	FAM-TCT GTG CAC CGA GTT GAC CGT AAC AGA C-BHQ
Real-Time PCR for ChIP (5' → 3' Direction)	
HIV-1 LTR forward primer	TTGACAGCCGCCTAGCATT
HIV-1 LTR reverse primer	CACGCCTCCCTGGAAAGTC
HIV-1 LTR probe	FAM-CATCACGTGGCCCGAGAGCTGC-BHQ
siRNA and Scrambled Control (5' → 3' Direction)	
c-maf-specific siRNA	<u>CGGCGAAGCTTTTTCCAAAAAAGAGACCGACCGCATCATCA-</u> <u>CTACACAAATGATGATGCGGTCTCCGGTGTTCGTC-</u> <u>CTTCCACAAG (AF055376, start position 918) (core oligo 19 bp)</u>
Scrambled sequence control	<u>CGGCGAAGCTTTTTCCAAAAAAGCACAACTAACGTCGCACG-</u> <u>CTACACAAACGTGCGACGTTAGTTGTGCCGGTGTTCGTC-</u> <u>TTCCACAAG (core oligo 19 bp)</u>

Underlined bases are the core oligonucleotide (oligo) binding sequences for corresponding transcription factors or for c-maf siRNA and its scrambled control.

Increased HIV-1 LTR and IL-4 promoter transcription can be augmented in the presence of either NFAT2 or NF-κB p65 (Fig. 5A, top and middle panels). The activity of each promoter was calculated [%(+ cells × MFI)] and averaged from five independent experiments and is shown in Fig. 5B. NF-κB p65, but not NFAT2, increased individual promoter transcription. However, both significantly cooperate with c-maf to increase LTR and IL-4, but not IFN-γ promoter transcription. These results demonstrate that c-maf in cooperation with NFAT2 or NF-κB p65 notably augments HIV-1 LTR and hIL-4 promoter, but not hIFN-γ promoter, transcription.

#### *Inhibiting c-maf expression dramatically decreases HIV-1 LTR activity*

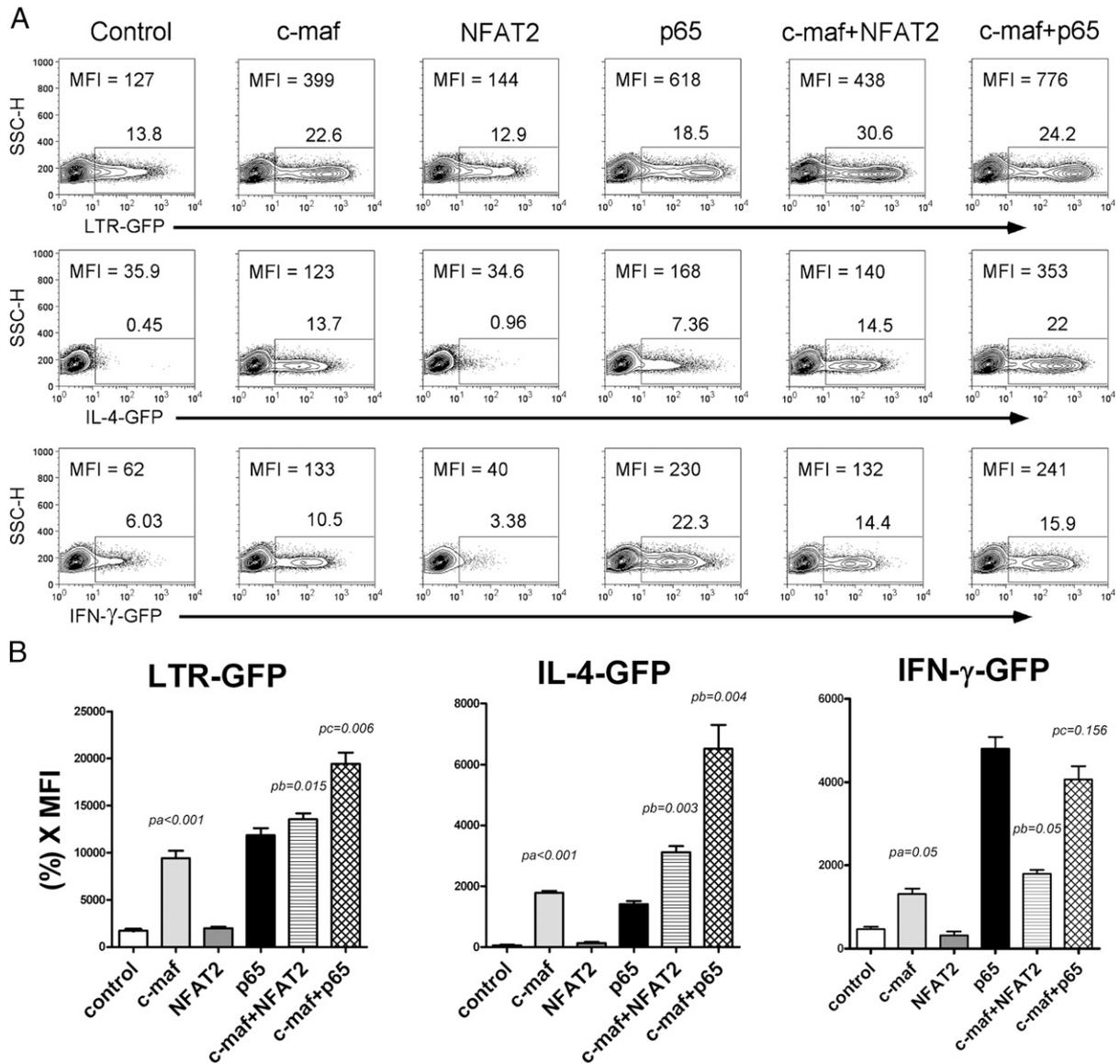
The role of endogenous c-maf during HIV-1 transcription was tested by introduction of c-maf-specific siRNA into IL-4 polarized CD4 T cells using our own published protocol (39). We first determined the efficiency and specificity of c-maf-specific siRNA designed by ourselves by verifying decreased expression of endogenous c-maf in IL-4 polarized CD4 T cells at both the mRNA and protein levels, and a decreased activity of a c-maf-responsive reporter plasmid, when transfecting c-maf siRNA into those cells (Supplemental Fig. 4A–C, respectively). Therefore, the c-maf-specific siRNA can be used to assess the role of endogenous c-maf during HIV-1 infection.

To first test HIV-1 LTR transcriptional activity, we cotransfected IL-4-producing CD4 T cells with an HIV-1 LTR-GFP reporter plasmid plus the c-maf-specific siRNA expression vector or scrambled control. Compared with control siRNA, HIV-1 transcriptional

activity is notably diminished in the presence of c-maf siRNA. Moreover, increasing doses of the c-maf-specific siRNA demonstrate dose-dependent inhibition of HIV-1 transcription (Fig. 6A). Similarly, c-maf-specific siRNA inhibits hIL-4 promoter- but not hIFN-γ promoter-driven transcription in IL-4-producing CD4 T cells (Fig. 6B, *left panel*). When visualized as percentage inhibition of transcription averaged over several experiments, it is clear that c-maf-specific siRNA inhibits both HIV-1 LTR- and hIL-4 promoter-driven transcription, but not transcription of the hIFN-γ promoter (Fig. 6B, *right panel*). Thus, the c-maf-specific siRNA is specific to its target genes, and the results with its use suggest that endogenous c-maf present in IL-4-producing CD4 T cells is not only important for optimal IL-4 gene transcription but for that of HIV-1 transcription as well.

#### *c-maf alone and c-maf in cooperation with NFAT2 or NF-κB p65 augment HIV-1 expression*

Based on c-maf binding to the proximal HIV-1 LTR and its ability to increase HIV-1 LTR transcription, we next examined the effect of c-maf on HIV-1 replication. To generate stable c-maf expression, we used a c-maf-expressing retrovirus to infect Jurkat T cells before the infection of HIV-1 NL4-3-GFP. HIV-1 expression, as detected by GFP, was analyzed 2–4 d post HIV-1 infection. In comparison with three different control expression retroviruses (empty, DsRed, CD28), c-maf clearly led to increased HIV-1 infection at all three time points (Fig. 7A). Using another model system, we transfected HEK-293T cells with increasing amounts of a c-maf expression plasmid followed by infection with HIV-1 NLENG-vsvg-GFP. In comparison with the control vector, c-maf was capable of increasing



**FIGURE 5.** c-maf synergizes with NFAT2 and NF-κB p65 to augment HIV-1 and hIL-4 promoter transcription. **(A)** Primary human CD4 T cells were transfected with single or dual-expression vector(s) together with HIV-1 LTR, hIL-4, or hIFN-γ promoter GFP reporter plasmids. GFP expression was detected by flow cytometry after 24 h. Expression of c-maf alone is capable of increasing HIV-1 LTR and hIL-4 promoter activity, but not hIFN-γ promoter activity. This effect is further and significantly enhanced when cotransfecting c-maf and NFAT2, or c-maf and NF-κB p65. A typical result of five independent experiments is shown. **(B)** Promoter reporter activity was calculated as GFP percentage multiplied by MFI (%\*MFI) and is expressed as means ± SEM from five independent experiments. c-maf alone and in cooperation with NFAT2 or NF-κB p65 statistically significantly increases HIV-1 LTR and hIL-4 promoter activity (<sup>a</sup>c-maf-transfected cells in comparison with control, <sup>b</sup>c-maf and NFAT2 dual-transfected cells in comparison with c-maf alone, <sup>c</sup>c-maf and NF-κB p65 dual-transfected cells in comparison with NF-κB p65 alone; n = 5).

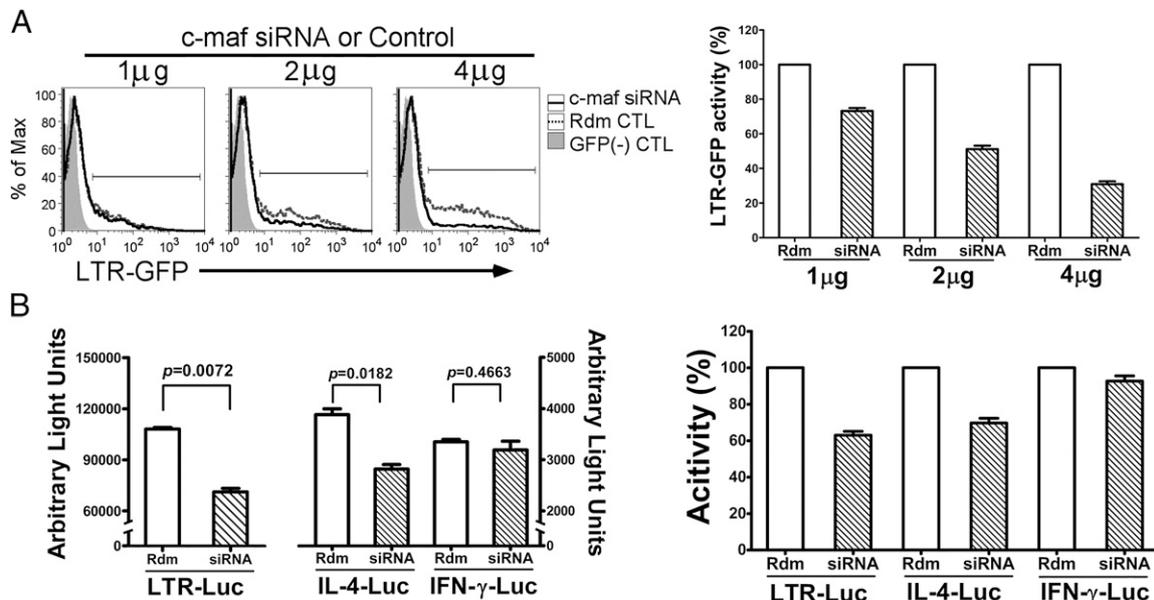
HIV-1 expression in a dose-dependent manner (Fig. 7B). Thus, enhanced c-maf expression led to increased HIV-1 expression in two different cell lines.

Because c-maf is known to cooperate with NFAT in driving IL-4 transcription (46), we explored the ability of c-maf to cooperate with NFAT2 in driving HIV-1 expression. HEK-293T cells were transfected with c-maf alone, NFAT2 alone, or cotransfected with c-maf and NFAT2 expression vectors. Although NFAT2 did not increase HIV-1 expression alone, NFAT2 clearly augments c-maf-enhanced HIV-1 expression, particularly at day 2 postinfection (Fig. 7C). Most importantly, we wished to confirm this effect in primary human CD4 T cells. CD4 T cells were first transfected with control, c-maf, NFAT2, or NF-κB p65 expression vectors before HIV-1 NL4-3 infection. HIV-1 expression was analyzed at 3–5 d postinfection. Although none of the transcription factors in

isolation dramatically augments HIV-1 expression in primary CD4 T cells (Fig. 7D, top row), when all three expression vectors (c-maf, NFAT2, and NF-κB p65) were cotransfected, the combined overexpression of these three factors notably augments HIV-1 viral expression (Fig. 7D, bottom row). Thus, there does appear to be a cooperative/synergistic effect of c-maf, NFAT2, and NF-κB p65 on increasing HIV-1 expression in primary human CD4 T cells.

**Discussion**

In exploring a previously noted shift from a Th1 to Th2/Th0 phenotype occurring during HIV-1 infection and progression to AIDS, investigators found a bias of HIV-1 replication in different CD4 T cell subsets or clones (8, 9). Focusing on HIV-1 infection and expression in individual cells from a bulk culture, we confirm that HIV-1 preferentially replicates in either Th2 or Th0, namely,



**FIGURE 6.** Inhibition of endogenous c-maf expression decreases HIV-1 LTR and IL-4 promoter transcription. **(A)** IL-4 polarized CD4 T cells were cotransfected with c-maf-specific siRNA plus a HIV-1 LTR GFP reporter. GFP expression was detected 24 h later by flow cytometry. GFP expression in c-maf siRNA-transfected cells is decreased compared with the scrambled sequence control siRNA, and the inhibition is in a dose-dependent manner relative to the amount of transfected siRNA. Representative flow cytometry histograms of GFP expression at the different siRNA (solid line represents c-maf; dashed line represents scrambled control) concentrations are shown on the left. The percentage LTR transcriptional activity, relative to the scrambled control, was calculated using the formula [(MFI × GFP% in the c-maf siRNA sample)/(MFI × GFP% in scrambled control sample) × 100%], and a summary graph of the means ± SEM LTR activities in the presence of escalating amounts of c-maf-specific siRNA is depicted on the right ( $n = 3$ ). **(B)** IL-4 polarized CD4 T cells were cotransfected with scrambled control or c-maf-specific siRNA plus various luciferase reporter plasmids as depicted. Transcriptional activities (relative light units) are shown as means ± SEM of three similar experiments on the left. Percent transcriptional activities in the presence of c-maf siRNA for the LTR and IL-4 and IFN- $\gamma$  promoters, relative to scrambled control, are shown on the right.

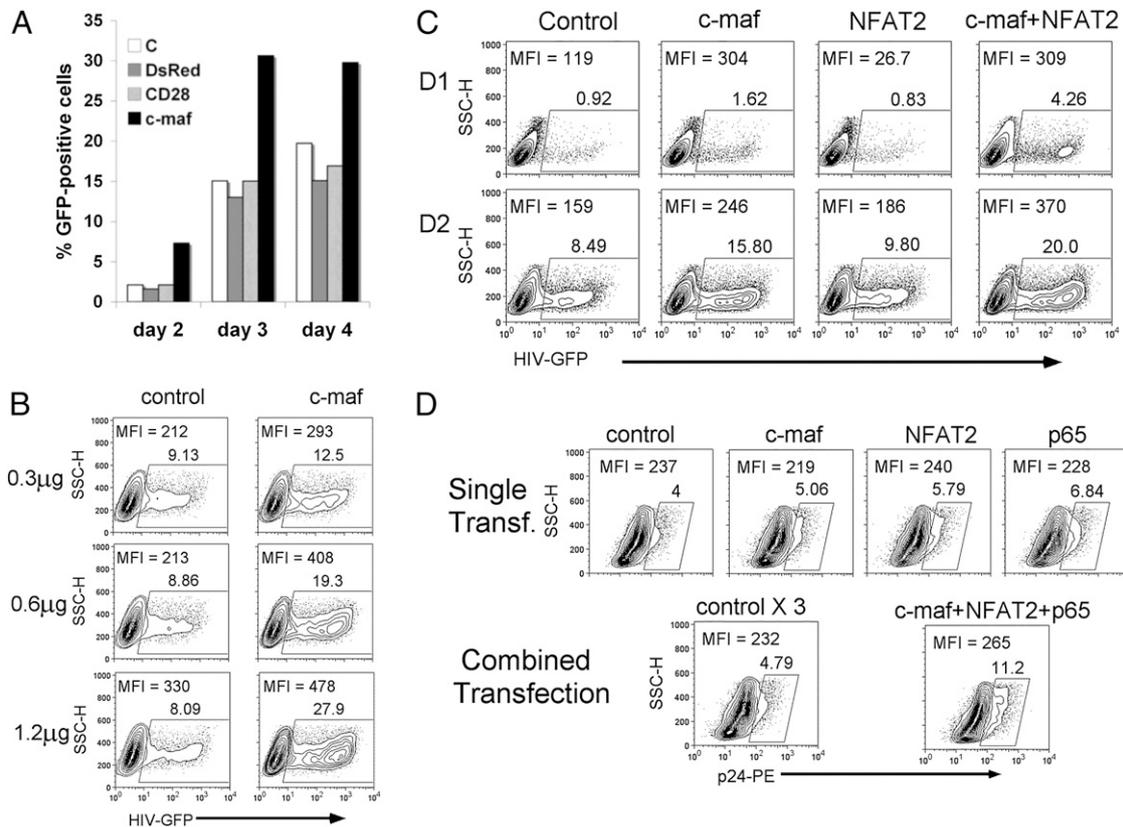
IL-4-producing, cells. Attempting to explain this phenomenon, others have previously reported that cloned Th1 cells are more susceptible to HIV-1-induced apoptosis (47). However, using cytokine primed bulk CD4 T cells, we demonstrate that preferential HIV-1 expression is not secondary to a rapid growth of IL-4-producing cells (Fig. 1D), nor is it related to an increased rate of HIV-1-triggered apoptosis of non-IL-4-producing cells (Supplemental Fig. 1). Moreover, we show that increased HIV-1 expression in IL-4<sup>+</sup> cells is independent of viral tropism (Fig. 1B) and the level of chemokine receptor expression (Supplemental Fig. 2). These results are consistent with the majority of previous reports (10–14, 18), but differ from others where the HIV-1 R5 strain alone or both R5 and X4 strains were found to replicate (equally) in Th1 and Th2 cells (48–50). Although our in vitro infection is not identical to the infection occurring in vivo, by using primary human CD4 T cells in bulk culture, it more closely resembles the natural infection process than cell lines or clones. Therefore, we believe our results are pathophysiologically relevant.

The findings of high levels of the ex vivo HIV-1 mRNA in IL-4-producing CD4 T cells from infected individuals, and increased HIV-1 LTR activity in IL-4-polarized human CD4 T cells in vitro, stimulated exploration of whether an IL-4-restricted transcriptional mechanism was involved in optimal HIV-1 gene expression. Accordingly, we identified a functional binding site for the Th2-restricted transcription factor, c-maf, in the proximal region of the HIV-1 LTR adjacent to the dual NF- $\kappa$ B/NFAT sites. In addition, NF- $\kappa$ B factors, p65 over p50, and NFAT factors, NFAT2 over NFAT1, were shown to preferentially bind to the LTR dual NF- $\kappa$ B/NFAT sites. It has been reported that both p65 and NFAT2 are increased in hIL-4 expression (51, 52). In this regard, a synergistic effect between c-maf and NFAT2, or between c-maf and p65, specifically increased HIV-1 LTR and hIL-4 promoter, but not

hIFN- $\gamma$  promoter, transcriptional activity. Taken together, c-maf contributes to increased HIV-1 transcription in IL-4-producing T cells via binding to the proximal LTR in cooperation with specific NFAT (NFAT2) and NF- $\kappa$ B (p65) family members.

Based on our findings, as well as a recent report describing a physical interaction specifically between NFAT2 and NF- $\kappa$ B p65, which synergistically promotes cardiac hypertrophy and ventricular remodeling (53), we postulate that a similar cooperative interaction among c-maf, NFAT2, and NF- $\kappa$ B p65 contributes to increased HIV-1 transcription in IL-4<sup>+</sup> CD4 T cells. Moreover, this synergism is likely an important mechanism contributing to the frequently observed preferential replication of HIV-1 in IL-4-producing CD4 T cells. Nevertheless, HIV-1 may lower c-maf expression, as recently suggested by Ahmad and colleagues (54), thus placing more importance on a cooperative interaction of transcription factors needed to promote HIV-1 transcription.

HIV-1 gene transcription and expression regulate viral replication, and consequently influence AIDS progression. Along these lines, it has been reported that HIV-1 LTR transcriptional activity is increased 3- and 10-fold in neonatal cord blood T lymphocytes and macrophages, respectively, in comparison with adult blood cells (55). The authors (55) conclude that it is increased viral gene expression derived from enhanced LTR transcription, rather than differences in cell proliferative capacities, cells surface receptor expression, viral RNA genome reverse transcriptional activity, or translocation of the preintegration complex into the nucleus, that directly results in a higher level of viremia and faster disease progression in neonates than in adults. They further identify a complex transcriptional mechanism regulating control of HIV-1 gene transcription and expression in neonatal mononuclear cells (20). In this study, we find an 8.6-fold higher HIV-1 LTR transcriptional activity and almost 2-fold higher HIV-1 p24 expression



**FIGURE 7.** c-maf alone or in cooperation with NFAT2 and NF- $\kappa$ B p65 increases HIV-1 expression. **(A)** Jurkat T cells were infected with recombinant retroviruses that express nothing, deRed2, CD28, or c-maf before infection of a HIV-1-GFP reporter virus. GFP expression was analyzed at 2, 3, and 4 d postinfection. c-maf significantly increases HIV-1 replication compared with the controls. One representative result of three independent experiments is shown. **(B)** HEK-293T cells were transfected with increasing amounts of c-maf expression or control plasmid 12–16 h before HIV-1-GFP virus infection. GFP expression was detected by flow cytometry 24 h postinfection. c-maf increases HIV-1 infection in a dose-dependent fashion. Results from one of three representative experiments are shown. **(C)** HEK-293T cells were transfected with control, c-maf, NFAT2, or c-maf plus NFAT2, expression plasmids, 12–16 h before HIV-1-GFP virus infection. GFP expression was detected by flow cytometry at days 1 and 2 postinfection. c-maf notably increases HIV-1 expression, and this is augmented in the presence of NFAT2 cotransfection. One typical result of three independent experiments is shown. **(D)** Freshly isolated human peripheral blood CD4 T cells were transfected with c-maf, NFAT2, or NF- $\kappa$ B p65 alone, or the combination of all three factors. The control plasmid was also transfected in 1 $\times$  or 3 $\times$  quantities of DNA to serve as a negative control. Cells were subsequently infected with the HIV-1 NL4-3 virus, and HIV-1 expression was detected by flow cytometry (intracellular p24 staining) at day 3 postinfection. HIV-1 p24 expression is modestly enhanced when individual transcription factors are expressed; however, HIV-1 expression is notably augmented when all three factors are coexpressed compared with the 3 $\times$  control. Results are representative of one of three similar experiments.

in IL-4-producing CD4 T cells compared with non-IL-4-producing T cells. Therefore, one may postulate that viral production in Th2 and Th0 cells substantially contributes to viral load and viremia during HIV-1 infection and AIDS progression. Over a decade or more of infection, even 2-fold differences in viral production will cause substantial differences in total viral burden. Considering the hypothesis that a Th1 to Th2 (or Th0) shift occurs under certain circumstances during HIV-1 infection (8, 9, 56), it seems reasonable that relatively high levels of IL-4 will help maintain the stability of the Th2 compartment and concomitantly inhibit the generation of the Th1 compartment. This scenario predicts facilitation of HIV-1 proliferation via the virus taking advantage of Th2 cell-specific transcriptional machinery to optimize its replication whereas avoiding Th1-mediated antiviral immune responses. Moreover, individuals who resist HIV-1 infection by secreting an IL-4 variant with a deletion of exon 2, a natural antagonist of IL-4, further support pivotal roles of IL-4 and Th2 cells during HIV-1 infection (57, 58). Taking together these previous reports and this study, we propose that the preferential support of HIV-1 replication by IL-4-producing T cells, along with the relative cell stability in the presence of IL-4, are critical pathophysiologic consequences of HIV-1 infection and

AIDS progression. Our findings are of significant importance in helping to increase our current understanding of HIV-1 biology and AIDS pathogenesis.

### Acknowledgments

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### Disclosures

The authors have no financial conflicts of interest.

### References

- Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley. 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* 272: 1167–1170.
- Hart, J. E., C. Y. Jeon, L. C. Ivers, H. L. Behforouz, A. Caldas, P. C. Drobac, and S. S. Shin. 2010. Effect of directly observed therapy for highly active antiretroviral therapy on virologic, immunologic, and adherence outcomes: a meta-analysis and systematic review. *J. Acquir. Immune Defic. Syndr.* 54: 167–179.
- Bongaarts, J., and M. Over. 2010. Public health. Global HIV/AIDS policy in transition. *Science* 328: 1359–1360.
- Bunupuradah, T., T. Puthanakit, P. Kosalaraksa, S. Kerr, P. Boonrak, W. Prasitsuebsai, P. Lumbiganon, T. Mengthaisong, C. Phasomsap, C. Pancharoen, et al. 2011.

- Immunologic and virologic failure after first-line NNRTI-based antiretroviral therapy in Thai HIV-infected children. *AIDS Res. Ther.* 8: 40.
5. Kantor, R., L. Diero, A. Delong, L. Kamle, S. Muyonga, F. Mambo, E. Walumbe, W. Emonyi, P. Chan, E. J. Carter, et al. 2009. Misclassification of first-line antiretroviral treatment failure based on immunological monitoring of HIV infection in resource-limited settings. *Clin. Infect. Dis.* 49: 454–462.
  6. Ledergerber, B., J. D. Lundgren, A. S. Walker, C. Sabin, A. Justice, P. Reiss, C. Mussini, F. Wit, A. d'Arminio Monforte, R. Weber, et al; PLATO Collaboration. 2004. Predictors of trend in CD4-positive T-cell count and mortality among HIV-1-infected individuals with virological failure to all three antiretroviral-drug classes. *Lancet* 364: 51–62.
  7. Trotta, M. P., A. Cozzi-Lepri, A. Ammassari, J. Vecchiet, G. Cassola, P. Caramello, V. Vullo, F. Soscia, A. Chioldera, N. Ladisa, et al; ICONA Foundation Study. 2010. Rate of CD4+ cell count increase over periods of viral load suppression: relationship with the number of previous virological failures. *Clin. Infect. Dis.* 51: 456–464.
  8. Maggi, E., M. Mazzetti, A. Ravina, F. Annunziato, M. de Carli, M. P. Piccinni, R. Manetti, M. Carbonari, A. M. Pesce, G. del Prete, et al. 1994. Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science* 265: 244–248.
  9. Romagnani, S., E. Maggi, and G. Del Prete. 1994. HIV can induce a TH1 to TH0 shift, and preferentially replicates in CD4+ T-cell clones producing TH2-type cytokines. *Res. Immunol.* 145: 611–617, discussion 617–618.
  10. Moonis, M., B. Lee, R. T. Bailer, Q. Luo, and L. J. Montaner. 2001. CCR5 and CXCR4 expression correlated with X4 and R5 HIV-1 infection yet not sustained replication in Th1 and Th2 cells. *AIDS* 15: 1941–1949.
  11. Ofori, H., and P. P. Jagodzinski. 2004. Increased in vitro replication of CC chemokine receptor 5-restricted human immunodeficiency virus type 1 primary isolates in Th2 lymphocytes may correlate with AIDS progression. *Scand. J. Infect. Dis.* 36: 46–51.
  12. Ofori, H., J. Prokop, and P. P. Jagodzinski. 2003. Replication at the level of reverse transcription of HIV-1SF2 strain in Th1 and Th2 cells. *Biomed. Pharmacother.* 57: 15–19.
  13. Vicenzi, E., P. Panina-Bodignon, G. Vallanti, P. Di Lucia, and G. Poli. 2002. Restricted replication of primary HIV-1 isolates using both CCR5 and CXCR4 in Th2 but not in Th1 CD4(+) T cells. *J. Leukoc. Biol.* 72: 913–920.
  14. Vyakarnam, A., P. M. Matear, S. J. Martin, and M. Wagstaff. 1995. Th1 cells specific for HIV-1 gag p24 are less efficient than Th0 cells in supporting HIV replication, and inhibit virus replication in Th0 cells. *Immunology* 86: 85–96.
  15. Rubbo, P. A., E. Tuailon, K. Bolloré, V. Foulongne, A. Bourdin, N. Nagot, P. Van de Perre, C. Desgranges, D. Israël-Biet, and J. P. Vendrell. 2011. The potential impact of CD4+ T cell activation and enhanced Th1/Th2 cytokine ratio on HIV-1 secretion in the lungs of individuals with advanced AIDS and active pulmonary infection. *Clin. Immunol.* 139: 142–154.
  16. Loetscher, P., M. Uguccioni, L. Bordoli, M. Baggiolini, B. Moser, C. Chizzolini, and J. M. Dayer. 1998. CCR5 is characteristic of Th1 lymphocytes. *Nature* 391: 344–345.
  17. Jagodzinski, P. P., and W. H. Trzeciak. 2000. Differential expression of chemokine receptor CXCR4 in Th1 and Th2 subtypes of CD4+ lymphocytes. *Folia Histochem. Cytobiol.* 38: 21–23.
  18. Annunziato, F., G. Galli, F. Nappi, L. Cosmi, R. Manetti, E. Maggi, B. Ensoli, and S. Romagnani. 2000. Limited expression of R5-tropic HIV-1 in CCR5-positive type 1-polarized T cells explained by their ability to produce RANTES, MIP-1alpha, and MIP-1beta. *Blood* 95: 1167–1174.
  19. Ansari, A. W., H. Heiken, D. Meyer-Olson, and R. E. Schmidt. 2011. CCL2: a potential prognostic marker and target of anti-inflammatory strategy in HIV/AIDS pathogenesis. *Eur. J. Immunol.* 41: 3412–3418.
  20. Sundaravaran, V., R. Mehta, D. T. Harris, J. A. Zack, and N. Ahmad. 2010. Differential expression and interaction of host factors augment HIV-1 gene expression in neonatal mononuclear cells. *Virology* 400: 32–43.
  21. Nishizawa, M., K. Kataoka, N. Goto, K. T. Fujiwara, and S. Kawai. 1989. v-maf, a viral oncogene that encodes a "leucine zipper" motif. *Proc. Natl. Acad. Sci. USA* 86: 7711–7715.
  22. Blank, V., and N. C. Andrews. 1997. The Maf transcription factors: regulators of differentiation. *Trends Biochem. Sci.* 22: 437–441.
  23. Bauquet, A. T., H. Jin, A. M. Paterson, M. Mitsdoerffer, I. C. Ho, A. H. Sharpe, and V. K. Kuchroo. 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat. Immunol.* 10: 167–175.
  24. Ho, I. C., M. R. Hodge, J. W. Rooney, and L. H. Glimcher. 1996. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell* 85: 973–983.
  25. Kim, J. I., I. C. Ho, M. J. Grusby, and L. H. Glimcher. 1999. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10: 745–751.
  26. Xu, J., Y. Yang, G. Qiu, G. Lal, Z. Wu, D. E. Levy, J. C. Ochando, J. S. Bromberg, and Y. Ding. 2009. c-Maf regulates IL-10 expression during Th17 polarization. *J. Immunol.* 182: 6226–6236.
  27. Yoshida, H., H. Nishina, H. Takimoto, L. E. Marengère, A. C. Wakeham, D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity* 8: 115–124.
  28. Kinoshita, S., L. Su, M. Amano, L. A. Timmerman, H. Kaneshima, and G. P. Nolan. 1997. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. *Immunity* 6: 235–244.
  29. Cron, R. Q., S. R. Bartz, A. Clausell, S. J. Bort, S. J. Klebanoff, and D. B. Lewis. 2000. NFAT1 enhances HIV-1 gene expression in primary human CD4 T cells. *Clin. Immunol.* 94: 179–191.
  30. Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 326: 711–713.
  31. Jacqué, J. M., B. Fernández, F. Arenzana-Seisdedos, D. Thomas, F. Baleux, J. L. Virelizier, and F. Bachelier. 1996. Permanent occupancy of the human immunodeficiency virus type 1 enhancer by NF-kappa B is needed for persistent viral replication in monocytes. *J. Virol.* 70: 2930–2938.
  32. Chêne, L., M. T. Nugeyre, F. Barré-Sinoussi, and N. Israël. 1999. High-level replication of human immunodeficiency virus in thymocytes requires NF-kappaB activation through interaction with thymic epithelial cells. *J. Virol.* 73: 2064–2073.
  33. Duverger, A., J. Jones, J. May, F. Bibollet-Ruche, F. A. Wagner, R. Q. Cron, and O. Kutsch. 2009. Determinants of the establishment of human immunodeficiency virus type 1 latency. *J. Virol.* 83: 3078–3093.
  34. Cao, S., J. Liu, L. Song, and X. Ma. 2005. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J. Immunol.* 174: 3484–3492.
  35. Cron, R. Q., S. J. Bort, Y. Wang, M. W. Brunvand, and D. B. Lewis. 1999. T cell priming enhances IL-4 gene expression by increasing nuclear factor of activated T cells. *J. Immunol.* 162: 860–870.
  36. Zhang, M., A. Genin, and R. Q. Cron. 2004. Overexpression of octamer transcription factors 1 or 2 alone has no effect on HIV-1 transcription in primary human CD4 T cells. *Virology* 321: 323–331.
  37. Calabresi, P. A., R. Allie, K. M. Mullen, S. H. Yun, R. W. Georgantas, III, and K. A. Whartenby. 2003. Kinetics of CCR7 expression differ between primary activation and effector memory states of T(H)1 and T(H)2 cells. *J. Neuroimmunol.* 139: 58–65.
  38. Cron, R. Q. 2003. CD154 transcriptional regulation in primary human CD4 T cells. *Immunol. Res.* 27: 185–202.
  39. Yin, J., Z. Ma, N. Selliah, D. K. Shivers, R. Q. Cron, and T. H. Finkel. 2006. Effective gene suppression using small interfering RNA in hard-to-transfect human T cells. *J. Immunol. Methods* 312: 1–11.
  40. Selliah, N., and T. H. Finkel. 2001. HIV-1 NL4-3, but not IIIB, inhibits JAK3/STAT5 activation in CD4(+) T cells. *Virology* 286: 412–421.
  41. Pasternak, A. O., K. W. Adema, M. Bakker, S. Jurriaans, B. Berkhout, M. Cornelissen, and V. V. Lukashov. 2008. Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. *J. Clin. Microbiol.* 46: 2206–2211.
  42. Suzuki, Y., Y. Koyanagi, Y. Tanaka, T. Murakami, N. Misawa, N. Maeda, T. Kimura, H. Shida, J. A. Hoxie, W. A. O'Brien, and N. Yamamoto. 1999. Determinant in human immunodeficiency virus type 1 for efficient replication under cytokine-induced CD4(+) T-helper 1 (Th1)- and Th2-type conditions. *J. Virol.* 73: 316–324.
  43. Kataoka, K., M. Noda, and M. Nishizawa. 1994. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site and forms heterodimers with both Fos and Jun. *Mol. Cell. Biol.* 14: 700–712.
  44. Motohashi, H., J. A. Shavit, K. Igarashi, M. Yamamoto, and J. D. Engel. 1997. The world according to Maf. *Nucleic Acids Res.* 25: 2953–2959.
  45. Kutsch, O., E. N. Benveniste, G. M. Shaw, and D. N. Levy. 2002. Direct and quantitative single-cell analysis of human immunodeficiency virus type 1 reactivation from latency. *J. Virol.* 76: 8776–8786.
  46. Hodge, M. R., H. J. Chun, J. Rengarajan, A. Alt, R. Lieberson, and L. H. Glimcher. 1996. NF-AT-driven interleukin-4 transcription potentiated by NIP45. *Science* 274: 1903–1905.
  47. Acronero, P., M. Radrizzani, D. Delia, F. Gerosa, R. Kurrel, and M. P. Colombo. 1997. Differential susceptibility to HIV-GP120-sensitized apoptosis in CD4+ T-cell clones with different T-helper phenotypes: role of CD95/CD95L interactions. *Blood* 89: 558–569.
  48. Mikovits, J. A., D. D. Taub, S. M. Turcovski-Corrales, and F. W. Ruscetti. 1998. Similar levels of human immunodeficiency virus type 1 replication in human TH1 and TH2 clones. *J. Virol.* 72: 5231–5238.
  49. Salgame, P., M. X. Guan, A. Agahtehrani, and E. E. Henderson. 1998. Infection of T cell subsets by HIV-1 and the effects of interleukin-12. *J. Interferon Cytokine Res.* 18: 521–528.
  50. Vicenzi, E., P. P. Bordignon, P. Biswas, A. Brambilla, C. Bovolenta, M. Cota, F. Sinigaglia, and G. Poli. 1999. Envelope-dependent restriction of human immunodeficiency virus type 1 spreading in CD4(+) T lymphocytes: R5 but not X4 viruses replicate in the absence of T-cell receptor restimulation. *J. Virol.* 73: 7515–7523.
  51. Ranger, A. M., M. R. Hodge, E. M. Gravalles, M. Oukka, L. Davidson, F. W. Alt, F. C. de la Brousse, T. Hoey, M. Grusby, and L. H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. *Immunity* 8: 125–134.
  52. Li-Weber, M., P. Salgame, C. Hu, I. V. Davydov, O. Laur, S. Klevenz, and P. H. Krammer. 1998. Th2-specific protein/DNA interactions at the proximal nuclear factor-AT site contribute to the functional activity of the human IL-4 promoter. *J. Immunol.* 161: 1380–1389.
  53. Liu, Q., Y. Chen, M. Auger-Messier, and J. D. Molkenin. 2012. Interaction between NFκB and NFAT coordinates cardiac hypertrophy and pathological remodeling. *Circ. Res.* 110: 1077–1086.
  54. Iannello, A., M. R. Boulassel, S. Samarani, O. Debbeche, C. Tremblay, E. Toma, J. P. Routy, and A. Ahmad. 2010. Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study. *J. Immunol.* 184: 114–126.

55. Sundaravaradan, V., S. K. Saxena, R. Ramakrishnan, V. R. Yedavalli, D. T. Harris, and N. Ahmad. 2006. Differential HIV-1 replication in neonatal and adult blood mononuclear cells is influenced at the level of HIV-1 gene expression. *Proc. Natl. Acad. Sci. USA* 103: 11701–11706.
56. Clerici, M., and G. M. Shearer. 1993. A TH1→TH2 switch is a critical step in the etiology of HIV infection. *Immunol. Today* 14: 107–111.
57. Becker, Y. 2005. The molecular mechanism of human resistance to HIV-1 infection in persistently infected individuals—a review, hypothesis and implications. *Virus Genes* 31: 113–119.
58. Becker, Y. 2007. The spreading of HIV-1 infection in the human organism is caused by fractalkine trafficking of the infected lymphocytes—a review, hypothesis and implications for treatment. *Virus Genes* 34: 93–109.

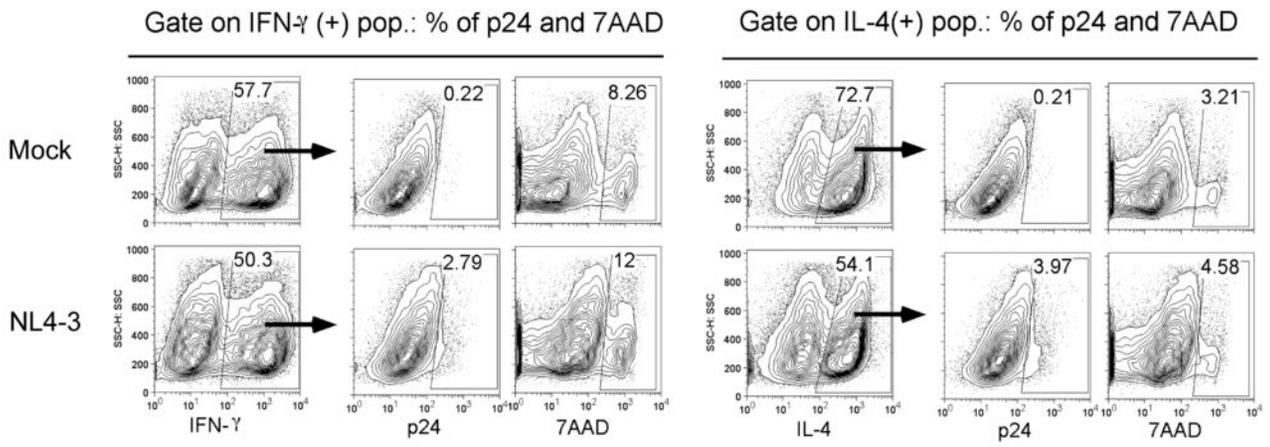
**Supplemental Figure 1. HIV-1 infection leads to a similar increased rate of apoptosis in both IFN- $\gamma$ (+) and IL-4(+) CD4 T cells.** Freshly isolated human peripheral blood CD4 T cells were cultured in the presence of IL-4 and IL-2 for 9 days, and then infected with the HIV-1 NL4-3 strain for 4 days (see Materials and Methods for details). The cells were then stimulated with PMA and ionomycin to allow the production of cytokines before harvesting and intracellular staining. HIV-1 infection (p24 expression), cytokine production, and apoptosis (7AAD) were analyzed by flow cytometry. Increased expression of HIV-1 p24 is noted in IL-4(+) cells compared to IFN- $\gamma$ (+) cells (3.97% vs. 2.79%, respectively). HIV-1 infection leads to a similarly increased rate of cell death in both IFN- $\gamma$ (+) cells (1.45-fold increase) and IL-4(+) cells (1.43-fold increase). Representative results of one of three independent experiments are shown.

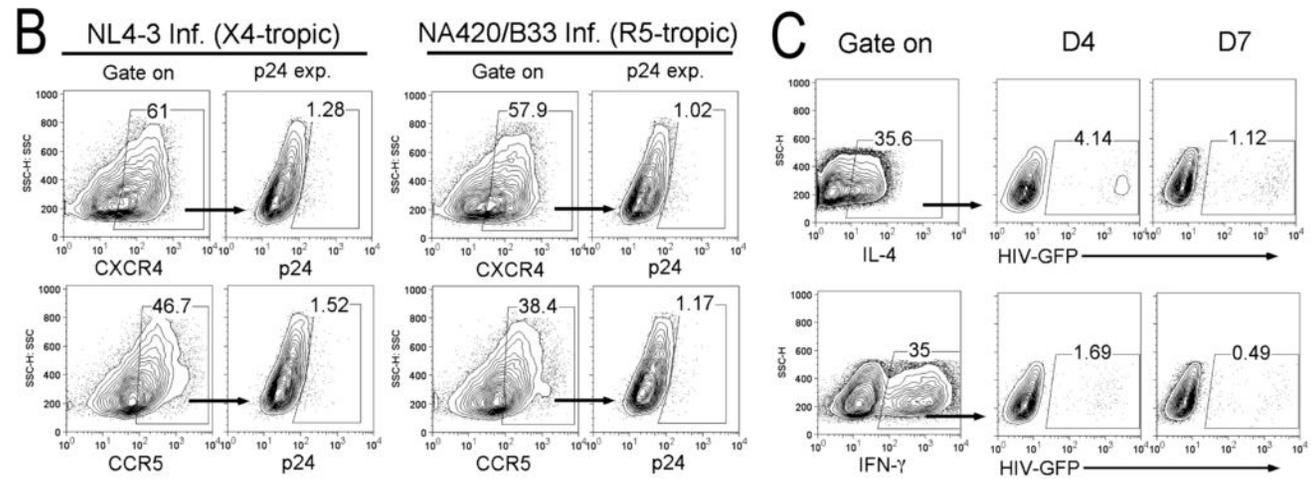
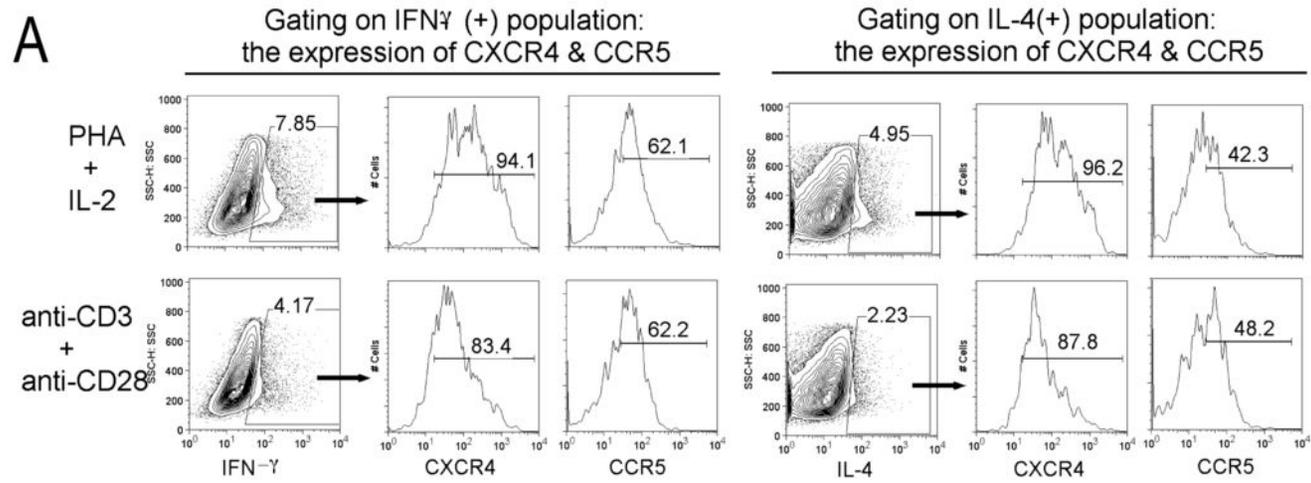
**Supplemental Figure 2. HIV-1 preferentially replicates in IL-4(+) CD4 T cells regardless of viral tropism for chemokine receptor expression.** (A) Freshly isolated human CD4 T cells were cultured in the presence of IL-4 and IL-2 for 9 days (see Materials and Methods for details). The cells were then stimulated with PHA (10  $\mu$ g/ml) plus IL-2 (100 ng/ml) (upper panel) or anti-CD3/CD28 antibodies (5  $\mu$ g/ml for each) (lower panel) overnight. Cytokine production (IFN- $\gamma$ , IL-4) and chemokine receptor expression (CXCR4, CCR5) were detected by flow cytometry. As shown, CXCR4 expression is similar between IFN- $\gamma$ (+) cells and IL-4(+) CD4 T cells using either method of T cell activation. By comparison CCR5 expression is higher on IFN- $\gamma$ (+) cells than IL-4(+) cells using the same stimuli. (B) X4-, and R5-tropic strains of HIV-1 show a similar infection level in CXCR4(+) and CCR5(+) CD4 T cells. IL-4-primed CD4 T cells were

infected with the HIV-1 NL4-3 (X4-tropism) strain or the NA420/B33 (R5-tropism) strain for 4 days. HIV-1 p24 expression was detected by intracellular staining and flow cytometry, and plotted according to CXCR4(+) CD4 T cells and CCR5(+) CD4 T cells. (C) HIV-1 preferentially replicates in IL-4(+) CD4 T cells. IL-4-primed CD4 T cells were infected with the NL4-3 GFP-expressing strain. 3TC was freshly added into culture media at day 3 and day 6 post infection to block a potential internal novel infection. p24 expression and intracellular IFN- $\gamma$  and IL-4 production were tested by intracellular staining and flow cytometry at days 4 and day 7 post-infection. Three independent experiments were performed for each assay above, and one representative result is depicted for each analysis.

**Supplemental Figure 3. c-maf binds to the HIV-1 LTR DNA *in vitro*.** Nuclear extracts prepared from polyclonally activated IL-4 primed human CD4 T cells interact with a c-maf consensus or an HIV-1 LTR MARE oligonucleotide probe. A specific gel retarded band seen with the c-maf consensus probe migrates identically to a similar band detected with the HIV-1 LTR MARE probe. These bands are inhibited by pre-incubation with their respective unlabeled excess self-oligonucleotides. In addition, the c-maf antibody partially blocks band formation (as measured by densitometry) for both the consensus MARE (48% inhibition) and the HIV-1 LTR MARE (40% inhibition). Results are representative of 3 similar experimental results.

**Supplemental Figure 4. siRNA specific for c-maf reduces c-maf expression.** IL-4 polarized human CD4 T cells were transfected with c-maf specific siRNA or randomized sequence control expression vectors. c-maf gene expression was determined 24-48 hours following siRNA transfection. **(A)** Total RNA was purified from transfected CD4 T cells. Real-time RT-PCR was used to measure c-maf mRNA levels. Densitometry measurements reveal that c-maf, but not the GAPDH control, mRNA levels are decreased substantially (~30-fold) in c-maf specific siRNA transfected CD4 T cells compared to cells receiving randomized sequence control siRNA. **(B)** c-maf specific siRNA transfected CD4 T cells were lysed and analyzed by Western blot to measure c-maf protein levels. In comparison with randomized sequence control siRNA transfection, the level of c-maf protein is reduced to approximately one quarter of the control in c-maf specific siRNA transfected CD4 T cells, whereas there was no effect on the level of the  $\beta$ -actin control protein by c-maf specific siRNA. **(C)** IL-4 polarized CD4 T cells were co-transfected with c-maf specific siRNA and a c-maf promoter-driven GFP reporter plasmid (see Materials and Methods). GFP expression was detected by flow cytometry after 24 hours. c-maf transcriptional activity is notably decreased in c-maf specific siRNA transfected CD4 T cells compared to randomized sequence siRNA transfected control cells. All results in this figure are representative of 3 separate experiments.





Probe	<u>consensus</u>			<u>LTR-MARE</u>		
s-comp	-	+	-	-	+	-
$\alpha$ -c-maf	-	-	+	-	-	+

