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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-κB/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-κB p65, but not NFAT1 or NF-κB 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-κB 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.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; Ct, cycle threshold; hNF-γ, human IFN-γ; 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-1α, and MIP-1β, 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-κB 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-κB/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-κB 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-γ-GFP, and pIL-4–GFP constructs were generated by subcloning the 3′ HIV-1 LTR segment (598 bp), human IFN-γ (hIFN-γ) promoter segment (464 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′-TGCGACCTCAAGCA-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-γ, 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 rL-2 (30 IU/ml) and rL-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 μg/ml) and rL-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 rL-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 γ-32P-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-κB p65, -NF-κB p50, -c-maf, -Oct1 Abs, or IgG (Santa Cruz Biotechnologies) was subjected to real-time PCR. The amplon was designed to overlap with the dual NF-κB/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(ΔCt), where ΔCt 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/106 cells) in the presence of DEAE-Dextran (4μg/ml) for 2 h or overnight at 37°C. The cells were then cultured for the presence of rL-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 μM) for 4–6 h at 37°C in the presence of brefeldin A (10 μg/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 μg/ml) and anti-CD28 (5 μg/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 LSRII flow cytometer, and analyzed using FlowJo
software (Tree Star).

Isolation of IL-4+ 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 μl). CD4+
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+ and IL-4− cells were then
separated by using IL-4 cell enrichment and detection kits (Miltenyi
Biotec) according to the product guidelines. RNA was isolated from each
culture supernatant (8, 9). There are, however, major shortcomings
of these types of studies: 1) the infection conditions differed for
both environments used to support the Th1 and Th2 cell lines,
and to overcome the two aforementioned problems, we generated
Th0-like (IFN-γ−, IL-4−) cells in individual patients were calculated
using the Wilcoxon matched paired test (GraphPad Prism 5), and statistical
significance was set as 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 cul-
ture supernatants (8, 9). There are, however, major shortcomings
of these types of studies: 1) the infection conditions differed for
both 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-γ+, IL-4−), Th2-like (IFN-γ−, IL-4+), and Th0-like
(IFN-γ−, IL-4−) 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 intra-
cellular staining. Notably, IL-4 single-positive (Th2) or IL-4 plus
IFN-γ double-positive (Th0) cells generate a higher percentage of
p24 than IFN-γ single-positive (Th1) or IFN-γ and IL-4 double-
negative (Fig. 1A). This increased expression in IL-4+ cells is not due to a relative increased rate of apoptosis in IFN-γ+ cells
after HIV-1 infection, because HIV-1 infection increased the rate
of apoptosis (as detected by 7-amino-actinomycin D) virtually iden-
tically (∼1.5-fold) in both IFN-γ+ and IL-4− CD4 T cells (Supple-
mental Fig. 1). Therefore, IL-4–producing CD4 T cells preferentially
support NL-4-3 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 X4/R5 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-γ+ and
IL-4+ CD4 T cells, whereas CCR5 expression is higher on IFN-γ−
cells after T cell activation (Supplemental Fig. 2A). Nevertheless,
both X4- and R5-tropic HIV-1 strains are expressed at similar levels
in CXCR4+ and CCR5+ CD4 T cells (Supplemental Fig. 2B) de-
spite increased expression in IL-4+ relative to IFN-γ− 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+ 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.

Assessing the results of several experiments (n = 12) reveals that IL-
4–producing cells support HIV-1 replication approximately twice as
well as IL-4− 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+ relative to IFN-γ− 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+ and IL-4−
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+ cells as
assessed by Ct values (mean ± SEM is 26.89 ± 1.24 for IL-4+ cells,
and 30.33 ± 1.24 for IL-4− cells; △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+ 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–pro-
ducing CD4 T cells from peripheral blood of HIV-1–infected in-
dividuals. 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+ cells compared with the IL-4− cells (e.g., 210−240) 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+ and IL-4− cells, it is difficult to demonstrate a linear cor-
relation among the three genes. Nevertheless, using a Wilcoxon
matched paired test, with an n = 5, IL-4− cells demonstrate greater
HIV-1 viral RNA levels than IL-4− cells for each patient studied
with a p value of 0.0313.
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+ versus IL-4− 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+ relative to IL-4− CD4 T cells
Higher levels of HIV-1 mRNA are detected in IL-4+ CD4 T cells from HIV-1–infected individuals. (A) The mRNA levels of HIV-1 gag in IL-4+ and IL-4– 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– CD4 T cells were normalized to that in IL-4+ cells by correcting them according to the 18S Ct values. The p value is 0.01 when comparing with IL-4+ cells. The −ΔCt calculated [−(Ct of IL-4− − Ct of IL-4+ cells)] values are shown for each individual patient (Δmean = 3.44, where p1 = patient 1, p2 = 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+ and IL-4– cells. The −ΔCt calculated [−(Ct of IL-4− − Ct of IL-4+ cells)] values of c-maf mRNA and IL-4 mRNA are shown for each individual patient (Δmean = 15.51 for c-maf mRNA, Δmean = 25.89 for IL-4 mRNA, where p1 = patient 1, p2 = patient 2, and so on; n = 5).

c-maf, NFAT2, and NF-κB p65 preferentially bind the proximal HIV-1 LTR

Because increased HIV-1 expression in IL-4+ 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-κB/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-κB/NFAT region (Fig. 4B). Interestingly, even though there are 10 residues between the predicted MARE and the NF-κB/NFAT region, the site protected by mafK is adjacent to the NF-κB/NFAT 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-κB 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-κB family members, p65 and p50, and NFAT family members, NFAT1 and NFAT2, are all capable of binding to the HIV-1 LTR NF-κB/NFAT sites in vitro (29, 31). In

**FIGURE 3.** HIV-1 LTR transcriptional activity is increased in IL-4+ 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+ CD4 T cells than in IL-4– cells, but HIV-1 LTR transcriptional activity was notably higher than CMV promoter activity in IL-4+ cells relative to IL-4– cells. (B) The mean ratios of transcriptional promoter activities of IL-4+ cells relative to IL-4– cells were calculated (percentage positive × MFI in IL-4+ cells divided by percentage positive × MFI in IL-4– cells) and presented as bar graphs. The HIV-1 LTR shows a higher relative ratio (IL-4+/IL-4−) of transcriptional activity than CMV (mean ± SEM of ratio for CMV promoter: 3.4 ± 0.90; for HIV-1 LTR: 8.62 ± 1.40; p = 0.0179, Student t test; 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 and p50 bind the IgG promoter NF-κB site similarly (panel 2), whereas NFAT2 preferentially binds to NFAT1 to the proximal HIV-1 LTR (panel 3). Likewise, NF-κB p65 and p50 bind the IgG 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; \( n = 5 \).

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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 IgG 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 IgG 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 supershifting 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 IgG 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\(^{+}\) (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).
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 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–produced CD4 T cells using our own published protocol (39). We first determined the efficiency and specificity of c-maf-specific siRNA designed by ourselves. We then transfected 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 NL4-3–GFP. In comparison with the control vector, c-maf was capable of increasing expression of HIV-1 LTR and hIL-4 promoter-driven transcription in IL-4–producing CD4 T cells (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.

**Table I. Listing of oligonucleotides used in this study**

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<tr>
<th>Oligonucleotide Name</th>
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<tr>
<td>NFAT binding sequence (mouse IL-2 promoter)</td>
<td>TGGAGCCCAAGGAAAATTTGTTCATG</td>
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<tr>
<td>NF-κB binding sequence (human lgs promoter)</td>
<td>ATTTGGAAGGGACTTCTCCAGGC</td>
</tr>
<tr>
<td>c-maf binding sequence (hIL-4 promoter)</td>
<td>AAACTGCTGACTCAGATTAC</td>
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<tr>
<td>HIV-1 LTR-NFAT/NF-κB dual-site sequence</td>
<td>CAAGGCGTTTCCGCTGGAATTTCCAGGG</td>
</tr>
<tr>
<td>HIV-1 LTR-MARE</td>
<td>AACTGCTGACTCAGGCTTTTCAAGGAGTCAGATTAC</td>
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</table>

Real-Time PCR for HIV-1 gag, 18s, c-maf, and IL-4 (5’ → 3’ Direction)

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>Sequence (5’ → 3’ Direction)</th>
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<tbody>
<tr>
<td>HIV-1 LTR forward primer</td>
<td>CTTAGGCGATCTTCAATGCGAGAA</td>
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<tr>
<td>HIV-1 LTR reverse primer</td>
<td>GGAATCTGTCTGTCCTCTCCACC</td>
</tr>
<tr>
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</tr>
<tr>
<td>c-maf forward primer</td>
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</tr>
<tr>
<td>c-maf reverse primer</td>
<td>CCCCGAAGCCAAGAGT</td>
</tr>
<tr>
<td>IL-4 forward primer</td>
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<tr>
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<td>GCCGCAACAAGAGT</td>
</tr>
<tr>
<td>IL-4 probe</td>
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</table>

Real-Time PCR for ChIP (5’ → 3’ Direction)

<table>
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<tr>
<th>Oligonucleotide Name</th>
<th>Sequence (5’ → 3’ Direction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 LTR forward primer</td>
<td>TTAGGCGCGGCTGAGATT</td>
</tr>
<tr>
<td>HIV-1 LTR reverse primer</td>
<td>AACGCTCCGAGAAGT</td>
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<tr>
<td>HIV-1 LTR probe</td>
<td>CTAGTCAGACTCATCAAGTTTCTCTATCAAAGCA</td>
</tr>
<tr>
<td>c-maf–specific siRNA</td>
<td>CGGCGAAGCTTTTTCCAAAAAA</td>
</tr>
<tr>
<td>Scrambled sequence control</td>
<td>CGGCGAAGCTTTTTCCAAAAAA</td>
</tr>
</tbody>
</table>

Underlined bases are the core oligonucleotide (oligo) binding sequences for corresponding transcription factors or for c-maf siRNA and its scrambled control.
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,
c-maf AUGMENTS HIV-1 EXPRESSION IN IL-4+ T CELLS

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+ 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-kB/NFAT sites. In addition, NF-kB factors, p65 over p50, and NFAT factors, NFAT2 over NFAT1, were shown to preferentially bind to the LTR dual NF-kB/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-γ 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-kB (p65) family members.

Based on our findings, as well as a recent report describing a physical interaction specifically between NFAT2 and NF-kB p65, which synergistically promotes cardiac hypertrophy and ventricular remodeling (53), we postulate that a similar cooperative interaction among c-maf, NFAT2, and NF-kB p65 contributes to increased HIV-1 transcription in IL-4+ 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.
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


Supplemental Figure 1. HIV-1 infection leads to a similar increased rate of apoptosis in both IFN-γ(+) 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-γ(+) cells (3.97% vs. 2.79%, respectively). HIV-1 infection leads to a similarly increased rate of cell death in both IFN-γ(+) 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 µg/ml) plus IL-2 (100 ng/ml) (upper panel) or anti-CD3/CD28 antibodies (5 µg/ml for each) (lower panel) overnight. Cytokine production (IFN-γ, IL-4) and chemokine receptor expression (CXCR4, CCR5) were detected by flow cytometry. As shown, CXCR4 expression is similar between IFN-γ(+) cells and IL-4(+) CD4 T cells using either method of T cell activation. By comparison CCR5 expression is higher on IFN-γ(+) 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-γ 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 β-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.
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![Image of gel electrophoresis](image.png)