HIV-1 envelope proteins up-regulate \( N^6 \)-methyladenosine levels of cellular RNA independently of viral replication

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\( N^6 \)-methyladenosine (m\(^6\)A) modification of HIV-1 RNA regulates viral replication and protein expression. The m\(^6\)A modification is regulated by two groups of cellular proteins named writers and erasers that add or remove m\(^6\)A, respectively. HIV-1 infection of CD4\(^+\) T-cells increases m\(^6\)A levels of cellular mRNA, but the underlying mechanism is unknown. Here, we show that HIV-1 infection of CD4\(^+\) primary T-cells or Jurkat cells significantly increases m\(^6\)A levels of cellular RNA independently of viral replication. Compared with HIV-1–infected CD4\(^+\) T-cells, similar m\(^6\)A up-regulation was detected in total RNA from HIV-1–infected cells treated with a reverse-transcriptase inhibitor or with heat-inactivated HIV-1. Compared with mock controls, significantly increased m\(^6\)A levels were detected in total RNA from Jurkat cells infected by single-cycle HIV-1 pseudotyped with an HIV-1 envelope (Env) glycoprotein, but not with vesicular stomatitis virus glycoprotein G (VSV-G). Overexpression of HIV-1 Env in HEK293T cells did not affect m\(^6\)A levels of cellular RNA, suggesting that de novo synthesis of Env is not required for m\(^6\)A up-regulation. Interestingly, treatment of Jurkat cells with recombinant gp120 of HIV-1 Env significantly increased m\(^6\)A levels of cellular RNA, which was reduced by a gp120-neutralizing antibody. Preincubation of Jurkat cells with a CD4 receptor–neutralizing antibody blocked HIV-1–induced up-regulation of m\(^6\)A levels in cellular RNA. Moreover, HIV-1 infection or gp120 treatment did not alter the protein expression of m\(^6\)A writers and erasers in cells. Our findings suggest that HIV-1 gp120 binding to the CD4 receptor is required for m\(^6\)A up-regulation in cells.

During the life cycle of HIV-1, viral proteins and nucleic acids closely interact with many host proteins and nucleic acids to regulate viral replication (1–3). HIV-1 can alter many host proteins and nucleic acids to facilitate viral replication and impair cellular responses to infection. There are more than 100 post-transcriptionally modified nucleosides in all types of RNAs in eukaryotes (4–6). Among these RNA modifications, \( N^6 \)-methyladenosine (m\(^6\)A) modification of cellular mRNAs is the most abundant and important in controlling the cellular gene expression and physiological functions (4–6).

The RNA m\(^6\)A modification is regulated by two groups of cellular proteins named writers and erasers that add or remove m\(^6\)A, respectively (4, 6, 7). Methyltransferase-like 3, 14, and 16 (METTL3, 14, and 16) are m\(^6\)A writers that catalyze the addition of methyl group at sixth position in adenosine of mRNA (8). Another group of proteins named m\(^6\)A erasers include fat mass and obesity-associated protein (FTO) and AlkB family member 5 (AlkBHS5) that remove m\(^6\)A. These two groups of proteins (writers and erasers) orchestrate the mRNA fate and ultimately cells function through m\(^6\)A modifications (4, 6–8). However, it is unclear whether HIV-1 infection of cells alters protein expression levels or catalytic activities of the m\(^6\)A writers and erasers.

RNA m\(^6\)A modifications of several viruses have been discovered in late 1970s, including influenza virus, adenovirus, Rous sarcoma virus, and simian virus 40 (9–12). However, the functional significance of the m\(^6\)A modification in viral RNAs was not solved, mainly because of certain technical obstacles, such as isolation and sequencing of m\(^6\)A-modified mRNA (13). In 2016, three groups have reported that the m\(^6\)A modifications in HIV-1 RNAs are important for viral infection and protein expression (14–16). Lichinchi et al. (14) showed that HIV-1 infection of the human MT4 CD4\(^+\) T-cell line increased m\(^6\)A levels in cellular poly(A) RNAs by ~30% compared with uninfected cells, but the underlying mechanisms were not elucidated. To better understand HIV-1 and host interactions, it is significant to define the mechanisms by which HIV-1 induces up-regulation of m\(^6\)A levels in cellular RNA.

HIV-1 envelope (Env) glycoprotein on the virus surface binds to cellular receptors to initiate fusion of virus and cell membranes (17). The precursor polyprotein of HIV-1 Env is gp160, which is cleaved by a cellular protease into a surface unit (gp120) and a transmembrane unit (gp41) to form a trimer on virions. HIV-1 gp120 and gp41 are responsible for virus binding to target cells and membrane fusion, respectively. The binding of gp120 to the HIV-1 receptor CD4 and...
a co-receptor triggers conformational changes of gp41 to lead to membrane fusion and viral entry (18). HIV-1 gp120 proteins can be detected in the serum of HIV-1–infected patients and result in pathogenic effects (2, 19). Exposure of soluble gp120 exerts significant immunological and neurotoxic effects on different target cells (20, 21). However, it is unknown whether HIV-1 Env binding to its receptors can alter m6A levels of cellular RNA.

In this study, we show that HIV-1 up-regulates RNA m6A levels in primary CD4+ T-cells or the Jurkat T-cell line independently of viral replication. Significantly increased m6A levels were detected in total RNA from Jurkat cells infected by HIV-1 pseudotyped with an Env, but not VSV-G, indicating the important role of Env in m6A up-regulation. Interestingly, treatment of Jurkat cells with recombinant HIV-1 gp120 significantly increases m6A levels of cellular RNA. Up-regulation of m6A levels were observed in total RNA, mRNA, rRNA, and small RNA from gp120-treated or HIV-1–infected Jurkat cells. Furthermore, neutralizing antibodies to gp120 and CD4 inhibited m6A up-regulation of cellular RNA. Our findings suggest that HIV-1 gp120 interaction with the CD4 receptor is critical for HIV-1–induced m6A up-regulation of cellular RNA in CD4+ T-cells.

**Results**

**HIV-1 infection of CD4+ T-cells up-regulates m6A levels of cellular RNA**

To systematically analyze the effects of HIV-1 infection on cellular RNA m6A levels, CD4+ Jurkat T-cells were infected with replication-competent HIV-1NL4–3 using a range (0.5, 1, and 5) of multiplicity of infection (MOI). Mock-infected cells were used as a negative control. At 72 h post-infection (hpi), productive viral infection was evidenced by dose-dependent increases in released HIV-1 capsid protein p24 in infected cells based on viral input (Fig. 1A). To compare m6A levels of cellular RNA, m6A levels in total RNA from mock or HIV-1–infected cells were measured using a dot-blot assay (14). Total cellular RNA was stained with methylene blue (MB) as a loading control to normalize relative m6A levels. One representative experiment is shown. C, average results of relative m6A levels based on three independent experiments. The results are shown as means ± S.D. (n = 3). Dunnett’s multiple comparison test was used for statistical analysis. **,** **p** < 0.0005, compared with mock sample. D, MB staining and m6A dot-blot detection of a serial dilution of total cellular RNA from Jurkat cells infected with HIV-1NL4–3 (MOI = 1) or mock infected. Quantification of relative m6A levels is shown as representative results from three independent experiments (n = 3).
urated with HIV-1 infection at MOI of 1, which was used in the following experiments.

To analyze whether the m\text{6}A levels of cellular RNA could be quantified within a wide range, we performed MB staining and measured m\text{6}A levels of a 2-fold serial dilution of cellular RNA isolated from HIV-1–infected Jurkat cells (Fig. 1D).

Relative m\text{6}A levels in total RNA from HIV-1–infected cells were consistently higher (1.3–9-fold) than those from mock-infected cells, and the MB staining showed a clear signal with the sample of 200 ng of cellular RNA (Fig. 1D). Thus, we used 200 ng of cellular RNA in the following dot-blot assays for m\text{6}A quantification.

HIV-1 up-regulates RNA m\text{6}A levels in CD4+ T-cells independently of viral replication

To investigate whether HIV-1 replication is required for up-regulation of cellular RNA m\text{6}A levels and the underlying mechanism, we examined the effects of productive HIV-1 infection on RNA m\text{6}A levels and the expression of m\text{6}A writers and erasers in Jurkat cells (Fig. 2A–E) or primary CD4+ T-cells (Fig. 2F–J). Infection of Jurkat cells (Fig. 2A) or activated primary CD4+ T-cells (Fig. 2F) with HIV-1NL4–3 resulted in robust increases of HIV-1 p24 levels in supernatants at 72 or 96 hpi, respectively. As negative controls, treatment of infected cells with the HIV-1 reverse-transcriptase inhibitor nevirapine (NVP) significantly blocked HIV-1 replication, and treatment with heat-inactivated HIV-1 did not yield infection in Jurkat cells (Fig. 2A) or primary CD4+ T-cells (Fig. 2F). Productive HIV-1 infection increased cellular RNA m\text{6}A levels 6–8-fold compared with mock control. Interestingly, NVP treatment of infected cells and heat-inactivated HIV-1 also increased the cellular RNA m\text{6}A levels 4–8-fold compared with mock controls (Fig. 2B for Jurkat cells and Fig. 2G for primary CD4+ T-cells). The average results of three independent experiments...
showed that productive HIV-1 infection, NVP treatment, or heat-inactivated HIV-1 similarly increased cellular RNA m6A levels by 6–8-fold in Jurkat cells and by 3–5-fold in primary CD4+ T-cells (Fig. 2, C and H, respectively). Moreover, NVP treatment alone did not alter RNA m6A levels in these cells (Fig. 2, D and F). Similar results were observed using primary CD4+ T-cells from two additional donors (data not shown). These data suggest that HIV-1–induced up-regulation of cellular RNA m6A levels in CD4+ T-cells is independent on viral replication.

To explore the mechanisms by which HIV-1 increases m6A levels of cellular RNA, we examined the effect of HIV-1 infection on the expression levels of m6A writers (METTL3 and METTL14) and erasers (AlkBH5 and FTO) in CD4+ T-cells. No significant changes in protein levels of writers or erasers were observed after HIV-1 infection with or without NVP, or treatment with heat-inactivated HIV-1 in Jurkat cells (Fig. 2D) or primary CD4+ T-cells (Fig. 2I). Similar results were obtained using primary CD4+ T-cells from two additional donors (data not shown). Of note, METTL3 expression levels in mock control cells appeared slightly lower than that in other samples (Fig. 2, D and J). However, quantification of relative METTL3 levels from three independent experiments indicates no statistically significant difference among different samples (Fig. 2, E and J). These results suggest that HIV-1–induced up-regulation in m6A levels of cellular RNA is unlikely due to altered protein expression of the m6A writers or erasers.

Infection of Jurkat cells with Env- but not VSV-G-pseudotyped HIV-1 increases m6A levels of cellular RNA

Because HIV-1 Env is the only viral protein exposed on the virus surface (18) and treatment of CD4+ T-cells with heat-inactivated HIV-1 increased m6A levels of cellular RNA (Fig. 2), we hypothesized that Env might account for m6A up-regulation in cellular RNA. To test this hypothesis, we detected m6A levels in total RNA isolated from Jurkat cells infected by single-cycle HIV-1 pseudotyped with VSV-G (HIV-1/VSV-G) or with Env from HIV-1NL4–3 (HIV-1/NL4–3). Because HIV-1/VSV-G had higher infectivity compared with HIV-1/NL4–3 (Ref. 22 and data not shown), higher levels of GAPDH expression were detected in Jurkat cells infected with the same amounts of HIV-1/VSV-G relative to HIV-1/NL4–3 (Fig. 3A). Compared with mock-infected cells, significantly increased m6A levels (~3-fold) were detected in total RNA from Jurkat cells infected with HIV-1/NL4–3, but not with HIV-1/VSV-G (Fig. 3, B and C). These results suggest that Env in HIV-1 virions is likely responsible for m6A up-regulation of total RNA in infected cells.

HIV-1 Env overexpression in HEK293T cells does not alter m6A levels of cellular RNA

To examine the role of Env in up-regulating m6A levels of cellular RNA, we next investigated whether overexpression of different HIV-1 Env in HEK293T cells had any effect on m6A levels of intracellular RNA. Expression of X4-tropic Env of HIV-1NL4–3 strain in transfected cells was confirmed by immunoblotting, and both full-length gp160 precursor and the cleaved gp41 protein were evident (Fig. 4A). We observed similar m6A levels of cellular RNA from Env-expressing cells or vector control cells (Fig. 4B). Average results from three independent experiments confirmed no change in m6A levels of intracellular RNA from Env-expression cells compared with control cells (Fig. 4C). Similar results were obtained when R5-tropic Env of HIV-1AD8 strain was overexpressed in HEK293T cells (Fig. 4, D–F). Relative to mock control, vector and Env (AD8) expression samples showed a ~1.5-fold increase in m6A levels of cellular RNA, but no difference was observed between vector and Env expression samples (Fig. 4, E and F). Together, these data suggest that de novo synthesis of Env is not sufficient for m6A up-regulation in HIV-1–producing cells. HEK293T cells do not express the CD4 receptor, Env overexpression in these cells could not trigger potential downstream effects on m6A up-regulation because of the lack of CD4 on the cell surface.

Recombinant HIV-1 gp120, but not Gag protein, up-regulates m6A levels in CD4+ T-cells

Because treatment of CD4+ T-cells with heat-inactivated HIV-1 or infection with Env-pseudotyped HIV-1 up-regulated m6A levels of cellular RNA (Figs. 2 and 3, respectively) and de novo synthesis of Env was not sufficient for the up-regulation
(4), we questioned whether gp120 incubation with cells could be responsible for the effect. To address this question, we tested the effects of treatment of cells with purified recombinant HIV-1 gp120 on m6A levels of intracellular RNA. Recombinant HIV-1 Gag protein was used as a control for specificity. Jurkat cells were treated with recombinant Gag or gp120 protein (100 ng at 500 ng/ml) derived from X4-tropic HIV-1IIIB, and then m6A levels of intracellular RNA were measured at 72 h post-treatment. Interestingly, treatment with gp120, but not Gag protein, significantly enhanced m6A levels of intracellular RNA by 3-fold relative to mock control (Fig. 5, A and B). Moreover, we did not observe any cytotoxic effects by gp120 and Gag treatment of cells (data not shown). These data suggest that gp120 binding to CD4/CD110 T-cells can contribute to m6A up-regulation of cellular RNA.

Recombinant gp120 proteins from different HIV-1 isolates up-regulate cellular RNA m6A levels without affecting expression of writers and erasers in CD4+ T-cells

To examine whether gp120 from different HIV-1 isolates had similar effects on up-regulation of m6A levels in a dose-dependent manner, we further tested purified recombinant gp120 derived from two X4- and R5-tropic HIV-1 strains or primary isolates. Relative to mock controls, m6A levels of cellular RNA in Jurkat cells treated with different amounts of gp120 (1, 10,
HIV-1 gp120 proteins up-regulate m^6^A levels of cellular RNA

Figure 6. Recombinant gp120 proteins from different HIV-1 isolates up-regulate cellular RNA m^6^A levels without affecting expression of writers and erasers in CD4^+^ T-cells. Jurkat cells were treated with recombinant gp120 (1, 10, or 100 ng/sample at 5, 50, or 500 ng/ml) derived from X4-tropic HIV-1_{IIIB} or HIV-1_{CN54} (A–D, I, and J) or R5-tropic HIV-1_{CM235} (E–H, K, and L) at 37 °C for 2 h, washed and incubated for another 72 h, and then collected for RNA or protein analyses. Mock treated control cells (Ctrl) were used as negative controls. A, C, E, and G, relative m^6^A levels in total RNA (200 ng) from cells were measured by the dot-blot assay using m^6^A-specific antibodies and quantified by densitometry analysis. MB staining of RNA was a loading control to normalize relative m^6^A levels. One representative experiment is shown. B, D, F, and H, relative m^6^A levels of cellular RNA calculated from three independent experiments. The level of mock control was set as 1. The results are shown as means ± S.D. (n = 3). Dunnett’s multiple comparison test or unpaired t-test was used for statistical analysis. *, p < 0.05; **, p < 0.005; ***, p < 0.0005, compared with mock control. I–L, recombinant gp120 treatment does not affect protein expression of m^6^A writers and erasers. Cell lysates were detected for the m^6^A (METTL3 and METTL14) and erasers (AlkBH5 and FTO) by immunoblotting. β-Actin or GAPDH was a loading control.

and 100 ng) from X4-tropic HIV-1_{IIIB} or HIV-1_{CN54} increased by ~2–5-fold (Fig. 6, A and B) or ~9–12-fold (Fig. 6, C and D), respectively, at 72 h post-treatment. Similar results of m^6^A up-regulation in cellular RNA were observed when Jurkat cells were treated with recombinant gp120 derived from two isolates of R5-tropic HIV-1. Compared with mock controls, treatment with gp120 derived from HIV-1_{CM235} or HIV-1_{Bal} induced ~2–3-fold (Fig. 6, E and F) or ~3–6-fold (Fig. 6, G and H) increases in m^6^A levels of cellular RNA, respectively. Thus, recombinant gp120 derived from different HIV-1 isolates significantly up-regulate m^6^A levels of cellular RNA in CD4^+^ T-cells, despite variable efficiencies among gp120 from various HIV-1 strains. These data also indicate that the effect of m^6^A up-regulation is saturated when Jurkat cells were treated with gp120 (10 ng/sample at 50 ng/ml) from different HIV-1 isolates (Fig. 6, A–H).

To investigate the mechanisms by which recombinant gp120 treatment up-regulated the levels of m^6^A, we also detected protein expression of m^6^A writers and erasers in Jurkat cells treated with increased amounts of recombinant gp120 from two isolates of X4- and R5-tropic HIV-1. Jurkat cells treated with different recombinant gp120 proteins from these HIV-1 isolates showed similar expression of m^6^A writers and erasers compared with control cells at 72 h post-treatment (Fig. 6, I–L).
These data suggest that gp120-mediated m\textsuperscript{6}A up-regulation of cellular RNA is unlikely caused by altered expression levels of the writers and erasers.

m\textsuperscript{6}A levels of RNA species from gp120-treated or HIV-1–infected Jurkat cells

To investigate whether gp120 treatment can alter m\textsuperscript{6}A levels of cellular RNA species in T-cells, we first treated Jurkat cells with purified recombinant gp120 derived from two X4- and R5-tropic HIV-1 strains or primary isolates and performed the Northwestern blotting assay (14, 23) to detect m\textsuperscript{6}A levels in cellular RNA species separated by size. We observed that m\textsuperscript{6}A levels of cellular rRNA were significantly increased (∼3–8-fold) in gp120-treated Jurkat cells compared with mock controls (Fig. 7A). These results indicate m\textsuperscript{6}A up-regulation of highly abundant rRNA (∼80% of total cellular RNA) in gp120-treated cells.

Next, to investigate the effect of HIV-1 infection on m\textsuperscript{6}A levels of cellular RNA species in T-cells, we infected Jurkat cells with replication-competent HIV-1\textsubscript{NL4–3} isolated total RNA and mRNA, and precipitated the remaining rRNA and small noncoding RNA (sncRNA) from HIV-1–infected or mock-infected cells and then detected m\textsuperscript{6}A levels of these RNA species by the dot-blot assay. Productive HIV-1 infection of Jurkat cells was confirmed by p24 detection in the supernatants of infected cells (Fig. 7B). Compared with mock-infected cells, HIV-1 infection increased m\textsuperscript{6}A levels in total RNA, rRNA/sncRNA, and mRNA by 6.1-, 4.7-, and 1.6-fold, respectively (Fig. 7C). As expected, m\textsuperscript{6}A levels of cellular mRNA appeared to be more abundant than other RNA species in both mock- and HIV-1–infected cells (Fig. 7C, shorter exposure of the m\textsuperscript{6}A dot blot). These results suggest that HIV-1–mediated m\textsuperscript{6}A up-regulation of cellular RNA can occur in mRNA, rRNA, and sncRNA species in cells.

Neutralizing antibodies to gp120 and CD4 inhibit m\textsuperscript{6}A up-regulation of cellular RNA

Because HIV-1 gp120 specifically binds to the CD4 receptor on the target cell surface (24), we sought to examine whether gp120 interaction with CD4 is required for RNA m\textsuperscript{6}A up-regulation in Jurkat cells. We included a gp120-neutralizing antibody (b12) that targets the CD4-binding site of gp120 (25), and a soluble CD4 (sCD4) recombinant protein, comprised of the full-length extracellular domain of human CD4, to inhibit CD4 and gp120 binding. Treatment of Jurkat T-cells with gp120 and the b12 antibody inhibited gp120-induced up-regulation of
HIV-1 gp120 proteins up-regulate m^6A levels of cellular RNA

Figure 8. Neutralizing antibodies to gp120 or CD4 inhibit gp120- or HIV-1–induced m^6A up-regulation of cellular RNA in CD4^+ T-cells. A, recombinant gp120 derived from HIV-1cm was incubated with sCD4 and gp120 antibodies alone or in combination for 2 h, and then Jurkat cells were treated with gp120 for another 2 h. The cells were washed and incubated for 72 h before m^6A detection in total cellular RNA (200 ng) by the dot-blot assay and quantification by densitometry analysis. MB staining of RNA was used as a loading control to normalize relative m^6A levels. The level of PBS control was set as 1. B–D, Jurkat cells were pretreated with CD4 neutralizing antibody (SIM.2) with dilutions of 1:100 or 1:1000 for 30 min followed by HIV-1NL4–3 infection (MOI = 1) for 2 h and then washed and incubated for 72 h before the collection of supernatants and cells. Mock treated cells and anti-CD4 (1:100)–treated cells without HIV-1 infection were used as controls. B, HIV-1 p24 levels in supernatants were measured by ELISA. C, relative m^6A levels in total RNA (200 ng) from infected or control cells was measured by the dot-blot assay and quantified by densitometry analysis. The level of mock control was set as 1. MB staining of RNA was used as a loading control to normalize relative m^6A levels. B and D, average results of the relative m^6A levels were calculated from three independent experiments. The results are shown as means ± S.D. (n = 3). Dunnett’s multiple comparison test was used for statistical analysis. **, p < 0.0005; ***, p < 0.0005, compared with mock sample.

m^6A levels by ~3-fold, whereas sCD4 treatment did not result in an inhibitory effect compared with the PBS control (Fig. 8A), suggesting that gp120 binding to CD4 is important for up-regulation of m^6A levels of cellular RNA.

To further investigate the role of gp120 and CD4 binding in this process, we examined the effect of CD4 neutralization on cellular RNA m^6A levels during HIV-1 infection. Jurkat cells were preincubated with a CD4-neutralizing antibody (SIM.2) and then infected with HIV-1NL4–3. This CD4 antibody has been shown to bind to the CD4 receptor and inhibit HIV-1 entry into cells and syncytium formation (26, 27). As expected, pretreatment of cells with increasing amounts of the CD4 antibody led to a dose-dependent and significant reduction of supernatant p24 levels (Fig. 8B). Concordantly, we observed a dose-dependent reduction of m^6A levels in cellular RNA from Jurkat cells treated with the CD4 antibody (Fig. 8, C and D). These results suggest that CD4 interaction with Env is critical for HIV-1 infection-induced m^6A up-regulation of cellular RNA in T-cells.

Discussion

Previous studies in cellular RNA modifications highlighted the functional significance of m^6A modification in regulating transcriptome and gene expression (4–6). Recent technological advances facilitated functional studies of m^6A modification in viral RNAs from many pathogenic viruses (13, 28–31). Lichinchi et al. (14) reported that HIV-1 infection of a CD4^+ T-cell line increases m^6A levels of cellular RNA, but the underlying mechanism was unknown. The present study was designed to understand the mechanisms of the m^6A up-regulation in CD4^+ T-cells during HIV-1 infection. We show that HIV-1 infection of primary CD4^+ T-cells or Jurkat cells significantly up-regulates m^6A levels of cellular RNA independently of viral replication. Interestingly, HIV-1 gp120 treatment of CD4^+ T-cells up-regulates m^6A levels of cellular RNA, which is inhibited by neutralizing antibodies to gp120 and CD4, suggesting a novel mechanism that HIV-1 gp120 binding to CD4 is responsible for m^6A up-regulation in cells.

To more accurately and reproducibly quantify m^6A levels of cellular RNA using the dot-blot assay (14), we added the MB dye to stain the RNA as a loading control and normalize quantification of m^6A levels in cellular RNA based on densitometry analyses. MB dye binds to negatively charged phosphate groups of DNA or RNA to stain the nucleic acids. MB staining does not interfere with nucleic acid retention on membranes and is therefore used to verify the amount of RNA on hybridization membranes in Northern blotting (32).

Different early stages of HIV-1 infection, such as virus attachment and accumulation of reverse-transcription products in cells, can induce multiple signaling events, which can lead to many changes in cellular functions (33, 34). Using NVP treatment of HIV-1–infected cells and heat-inactivated HIV-1 as controls, we demonstrated that viral replication is not required for the up-regulation of m^6A levels of cellular RNA. These findings suggest the importance of extracellular HIV-1 and its attachment to cell surface for m^6A up-regulation of cellular RNA. Furthermore, infection of Jurkat cells with HIV-1 Env-pseudotyped single-cycle HIV-1, but not VSV-G-pseudotyped HIV-1, resulted in m^6A up-regulation of total RNA, confirming the specific role of HIV-1 Env in up-regulation of m^6A levels of cellular RNA. However, overexpression of X4- or R5-tropic Env in HEK293T cells did not affect cellular RNA m^6A levels. These data suggest that binding of extracellular gp120 could be more important than de novo synthesis of Env in up-regulation of intracellular RNA m^6A levels.

Binding of gp120 to CD4 and a co-receptor allows HIV-1 to enter cells by fusion. HIV-1 exploits CD4^+ T-cell signaling molecules to facilitate productive infection. It is possible that binding of gp120 and CD4 may indirectly increase the activity of m^6A writers or decrease the activity of m^6A erasers in T-cells, thereby up-regulating m^6A levels of cellular RNA species. Importantly, we found that treatment of Jurkat cells with recombinant gp120 derived from both R5- and X4-tropic
HIV-1 Env, but not Gag protein, significantly increased m6A levels of cellular RNA, which depended on gp120 and CD4 binding. This observation may have broad implications in HIV-1 infection, considering the importance of m6A modifications of cellular mRNA in modulating cellular gene expression and functions during HIV-1 infection.

Several HIV-1 proteins including gp120 can alter T-cell homeostasis by activating multiple transcription factors such as NF-κB, SP1, and AP-1 (2, 33). Accumulation of HIV-1–soluble gp120 in lymphoid tissues induces apoptosis (35). Binding of gp120 to its receptor and co-receptors (such as CXCR4 or CCR5) mediates chemotaxis, actin cytoskeleton changes (33). Moreover, recombinant gp120 can activate the viral replication in resting CD4+ T-cells in infected patients (36). In our experiments, we treated Jurkat cells with recombinant gp120 at 5, 50, and 500 ng/ml to mimic potential concentration of gp120 released from infected cells in vivo. Of note, all the recombinant gp120 or Gag proteins used in the study were expressed in mammalian cells or a baculovirus system to maintain important post-translational modifications, such as glycosylation of gp120.

Using LC-MS/MS quantification, Lichinchi et al. (14) detected that HIV-1 infection of a CD4+ T-cell line increases m6A levels of cellular mRNA by ~30%. Our Northwestern blotting analysis of m6A levels in cellular RNA suggests that tRNA have significantly up-regulated m6A levels (2.7–8.2-fold) in gp120-treated Jurkat cells relative to control cells. Furthermore, we detected up-regulation of m6A levels in mRNA and tRNA/snRNA isolated from HIV-1–infected Jurkat cells. Using a high-throughput sequencing method, a previous study did not identify m6A modification in tRNA of HEK293T and HeLa cells (37), indicating no m6A modification in tRNA of human cells. Thus, we did not isolate tRNA from HIV-1–infected or gp120-treated Jurkat cells for m6A analysis.

We did not observe any significant changes in the levels of m6A writer and eraser proteins in CD4+ T-cells infected with HIV-1 or treated with heat-inactivated HIV-1 or gp120 compared with control cells. It is possible that up-regulation of m6A levels in cellular RNA could be due to changes in the activity, post-translational modifications, or localization of these cellular enzymes rather than the levels of protein expression. However, there are no specific cell-based assays that we can detect the m6A methyltransferase activity of writers and demethylase activity of erasers. It is also plausible that additional m6A writers (such as METTL16) newly identified in cells (5, 8, 38, 39) contribute to HIV-1–induced m6A up-regulation of cellular RNA. We recently reported the role of m6A reader proteins that specifically bind to m6A-modified cellular or HIV-1 RNA in regulating HIV-1 infectivity and viral production (40). It is unclear whether HIV-1 infection of CD4+ T-cells or gp120 treatment alters protein expression of m6A readers and thereby affects viral gene expression and infectivity. We plan to explore these open questions in future studies.

HIV-1 replication is extensively affected by cellular gene expression (1), which can also be regulated by m6A modification (5, 31). Previous studies indicated that m6A modification of HIV-1 RNA enhances viral gene expression (14, 15) and that knockdown of the m6A writers (METTL3 and METTL14) in virus-producing cells significantly decreases HIV-1 Gag protein expression and viral production (16). Thus, it is possible that HIV-1 up-regulates m6A of cellular RNA to enhance viral gene expression and replication in host cells. Our mechanistic analysis of gp120-induced cellular RNA m6A up-regulation indicates that binding of gp120 to CD4 is required for m6A up-regulation of cellular RNA. It is likely that gp120–CD4 binding at the T-cell surface induces certain signaling alterations in cells to up-regulate m6A levels of cellular RNA. Our current studies lay the foundation for future investigations to further define the detailed mechanisms.

**Experimental procedures**

**Cell culture**

HEK293T cells, Jurkat cells, and the HIV-1 indicator cell line GHOST/X4/R5 were cultured as described (41). All the cell lines were tested for mycoplasma negative by PCR-based universal mycoplasma detection kit (American Type Culture Collection). The institutional review board at the Ohio State University has approved the in vitro experiments involving human blood cells from deidentified healthy donors. The consent requirements for the deidentified blood samples were waived by the institutional review board. Human primary CD4+ T-cells were isolated from healthy blood donors buffy coats (purchased from American Red Cross Blood Service, Columbus, OH) using anti-human CD4-coated magnetic particles according to the manufacturer’s instructions (BD Biosciences) as described (41). Isolated primary CD4+ T-cells were maintained in complete RPMI medium containing interleukin-2 (20 units/ml, PeproTech) and activated with phytohemagglutinin A (5 μg/ml, Sigma–Aldrich) as described (41).

**Plasmids and recombinant proteins**

The HIV-1 proviral DNA construct pNL4–3 (16) and the expression plasmid for an HIV-1 Gag–GFP fusion protein (42) were gifts from Dr. Vineet KewalRamani and Dr. Alan Rein (National Cancer Institute, respectively). The HIV-1 Env expression constructs pIIINL4env and pAD8 Env were gifts from Dr. Eric Freed (National Cancer Institute). The following recombinant proteins were obtained from the National Institute of Health AIDS Reagent Program (NARP): HIV-1 HxB Gag pr55 (catalog no. 3276) produced in SP9 cells from a baculovirus vector; HIV-1 X4 gp120 (catalog no. 11784), a full-length gp120 produced in a Chinese hamster ovary expression system and purified by immunoaffinity chromatography; HIV-1 C5N4 gp120 (catalog no. 7749), a subtype CRF07BC full-length protein produced in SP900-III insect cells using the baculovirus expression system (43), HIV-1 X4 gp120 (catalog no. 4961) expressed in HEK293 cell line and affinity-purified; HIV-1 CM235 gp120 (catalog no. 2968) produced in a baculovirus expression system; and human sCD4 (catalog no. 4615), a full-length extracellular domain of human CD4 (amino acids 1–370) produced in a mammalian (Chinese hamster ovary cells) expression system.

**HIV-1 infection assays and treatment with heat-inactivated HIV-1**

Replication-competent HIV-1NL4–3 stock was generated by calcium phosphate transfection of HEK293T cells with the
pNL4–3 as described (41). Single-cycle luciferase reporter HIV-1 was pseudotyped with VSV-G or Env from HIV-1NL4–3 and generated by transfecting HEK293T cells as described (40). The infectious units of virus stocks were evaluated by limiting dilution on GHOST/X4/R5 cells as described (44). Jurkat cells (7.5 × 10^6) were infected with HIV-1 for 72 h using an MOI of 0.5, 1, or 5. HIV-1 capsid p24 levels in supernatants were measured by an ELISA using anti-p24-coated plates (AIDS and Cancer Virus Program, National Cancer Institute, Frederick, MD) as described (16). Reverse-transcriptase inhibitor nevirapine (10 nm, obtained from the NARP, catalog no. 4666) or heat-inactivated HIV-1 was used as controls. Heat-inactivated HIV-1 was generated by incubating the virus at 65 °C for 1 h as inactivated HIV-1 was used as controls. Heat-inactivated HIV-1 was used by incubating the virus at 65 °C for 1 h as described (45). Activated primary CD4^+ T-cells were infected with HIV-1NL4–3 (MOI of 1), and cells were harvested at 96 hpi for total RNA extraction using RNAeasy kit (Qiagen). To eliminate potential DNA contamination in the RNA, on-column DNase treatment was included according to the manufacturer’s instruction during the RNA extraction.

**Antibodies and immunoblotting**

The antibodies and their catalog or clone numbers were as follows: GAPDH (clone 4G5, AbD Serotec), β-actin (clone AC-15, SC-69879, Santa Cruz), HIV immune globulin (46) (obtained from NARP, catalog no. 3957), human CD4 monoclonal Ab (SIM.2, obtained from NARP, catalog no. 723), METTL3 (15073-1-AP, Proteintech Group), METTL14 (HPA038002, Sigma–Aldrich), AlkBH5 (HPA007196, Sigma–Aldrich), FTO (ab124892, Abcam), gp160 (catalog no. 188, obtained from the NARP), and m6A polyclonal rabbit Ab (Synaptic Systems; 202 003). The cells were harvested and lysed in radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with the protease inhibitor mixture (P8340, Sigma–Aldrich). Total protein in cell lysates was quantified using a bicinchoninic acid assay kit (Pierce). Immuno blotting was performed as described (47). GAPDH was used as a loading control. Anti-HIV-1 gp120 mAb (lgG1 b12) (25) was obtained from the NARP (catalog no. 2640).

**Immunoblot analysis of m6A levels in RNA**

RNA was extracted from mock or HIV-1–infected cells by using RNA purification kit (Qiagen) or TRIzol (Invitrogen, catalog no. 15596026). The dot-blot assay of m6A detection in RNA samples was previously described (14) and performed according to the manufacturer’s instructions (Bio-Rad). Briefly, total RNA (make up to 100 μl using 1 mM EDTA) were mixed with 60 μl of 20 × saline-sodium citrate (SSC) buffer (3 mM NaCl, 0.3 M trisodium citrate) and 40 μl of 37% formaldehyde and incubated at 65 °C for 30 min. After 30 min, the samples were left on ice until use. Nitrocellulose membrane (162-0115, Bio-Rad) or nylon membranes (11209299001, Roche) were assembled in dot-blot apparatus with vacuum-on.

Equal amounts of RNA were transferred to nitrocellulose or nylon membranes to detect m6A, or loading control using methylene blue staining (MB119, Molecular Research Center). After the RNA solution completely absorbed to membranes, membranes were washed twice with 200 μl of 10× SSC buffer and UV cross-linked. Nitrocellulose membrane was blocked with 5% milk solution in TBS with Tween 20 (TBST) buffer (20 mM Tris, 0.9% NaCl, and Tween 20 0.05%) and used to detect m6A levels by immunoblot analysis using m6A-specific antibodies. Nylon membranes were washed once with TBST buffer for 5 min and stained with methylene blue (MB) for 2–5 s followed by two or three washes with water as described (32). Images were taken using Amersham Biosciences Imager 600 (GE Healthcare). Densitometry quantification of relative m6A levels was normalized to MB staining and performed using ImageJ software (National Institutes of Health).

**Treatment of Jurkat cells with recombinant gp120, sCD4, or Gag proteins**

Jurkat cells (7.5 × 10^6) were treated with either X4- or R5-tropic HIV-1 recombinant gp120 (1, 10, or 100 ng/sample at 5, 50, or 500 ng/ml) for 2 h and then washed with medium and incubated for 72 h. After 72 h, the cells were collected for the analysis of protein and RNA. The cells were also treated with Gag (pr55) as a negative control. For sCD4 and gp120 treatment, recombinant gp120 (100 ng. 0.5 μg/ml) was incubated with sCD4 (1 μg/ml) or gp120 monoclonal antibodies (b12, 2 μg/ml) alone or in combination for 2 h and then treated Jurkat cells with HIV-1NL4–3 gp120 (100 ng/ml) for an additional 2 h. The cells were washed and incubated for 72 h before RNA extraction and analysis. For CD4 antibody treatment, Jurkat cells were preincubated with CD4 antibody (SIM 2.0) for 10 min at 37 °C at dilutions of 1:1000 or 1:100 and then infected with HIV-1NL4–3 (MOI of 1).

**Transient overexpression of HIV-1 Env in HEK293T cells**

HEK293T cells (3 × 10^6) were transfected with mock or vector or HIV-1 Env expression plasmids (either from X4- or R5-tropic HIV-1 isolates) using the calcium-phosphate transfection method as described (41). At 24 h post-transfection, the cells were collected for cellular protein or RNA analysis.

**Northwestern blotting of m6A levels in cellular RNA**

Northwestern blotting of cellular RNA was performed to analyze m6A levels using the method as described (14, 23, 48). In brief, the RNA sample was denatured by heating at 65 °C for 10 min in RNA loading dye (New England Biolabs, catalog no. B0363A) and then was electrophoresed in 1% agarose gel containing 4.5% formaldehyde in 1× MOPS buffer (0.05 M sodium acetate, 0.01 M EDTA sodium salt). Separated RNA was transferred onto nitrocellulose membrane by capillary blotting with 10× SSC buffer overnight. After the RNA transfer, the membrane was UV cross-linked using a Stratalinker UV cross-linker (Stratagene). Nitrocellulose membrane was blocked with 5% milk in TBST buffer and used to detect m6A levels by immuno blot analysis using m6A-specific antibodies.

**Isolation of cellular mRNA, rRNA, and snRNA**

Total cellular RNA extracted from Jurkat cells with TRIzol was used to isolate mRNA using Oligotex mRNA mini kits (Qiagen, catalog no. 70022). Isolation of rRNA and snRNA was performed by isopropanol precipitation of the remaining RNA collected after mRNA isolation using the Oligotex mRNA col-
umn. The precipitated RNAs were quantified and used in the dot-blot assay to detect m<sup>6</sup>A levels using m<sup>6</sup>A-specific antibodies.

**Statistical analyses**

Dunnett’s multiple comparison test or unpaired t test was used for statistical analysis. p < 0.05 is considered significant. GraphPad Prism 5 (GraphPad Software) was used for statistical analysis and generating bar charts.

**Author contributions**—N. T. data curation; N. T. and L. W. formal analysis; N. T. investigation; N. T. methodology; N. T. writing-original draft; L. W. conceptualization; L. W. resources; L. W. supervision; L. W. funding acquisition; L. W. project administration; L. W. writing-review and editing.

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