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CIITA Enhances HIV-1 Attachment to CD4\(^+\) T Cells Leading to Enhanced Infection and Cell Depletion

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Activated CD4\(^+\) T cells are more susceptible to HIV infection than resting T cells; the reason for this remains unresolved. Induction of CIITA and subsequent expression of the MHC class II isotype HLA-DR are hallmarks of CD4\(^+\) T cell activation; therefore, we investigated the role of CIITA expression in T cells during HIV infection. CIITA-expressing SupT1 cells display enhanced virion attachment in a gp160/CD4-dependent manner, which results in increased HIV infection, virus release, and T cell depletion. Although increased attachment and infection of T cells correlated with HLA-DR surface expression, Ab blocking, transient expression of HLA-DR without CIITA, and short hairpin RNA knockdown demonstrate that HLA-DR does not directly enhance susceptibility of CIITA-expressing cells to HIV infection. Further analysis of the remaining MHC class II isotypes, HLA-DP and HLA-DQ, MHC class I isotypes, HLA-A, HLA-B, and HLA-C, and the class II Ag presentation genes, invariant chain and HLA-DM, demonstrate that these proteins likely do not contribute to CIITA enhancement of HIV infection. Finally, we demonstrate that in activated primary CD4\(^+\) T cells as HLA-DR/CIITA expression increases there is a corresponding increase in virion attachment. Overall, this work suggests that induction of CIITA expression upon CD4\(^+\) T cell activation contributes to enhanced attachment, infection, virus release, and cell death through an undefined CIITA transcription product that may serve as a new antiviral target. The Journal of Immunology, 2010, 185: 000–000.

Despite over 25 years of HIV research, mechanisms explaining the restriction of HIV infection to activated CD4\(^+\) T cells are unknown (1, 2). Upon activation, CD4\(^+\) T cells upregulate a number of surface receptors, including the MHC class II isotype HLA-DR (3, 4). Expression of HLA-DR on CD4\(^+\) T cells relies upon transcriptional induction by CIITA which is expressed after T cell activation (4). Interestingly, CIITA is thought to impact viral genome transcription from the HIV-1 long terminal repeat (the viral promoter) (5, 6). Despite its effect on HIV genome transcription, the role that CIITA plays during infection has not been fully explored.

Upon virus budding, HLA-DR is incorporated into the envelope of HIV virions (7). The effects of virion-associated HLA-DR on HIV infection are well-studied; enhanced virus-mediated bystander T cell apoptosis, aberrant signaling, and increased infectivity of virions (potentially through interaction with the HIV receptor, CD4) have been reported (8–10). The role of cellular HLA-DR in HIV infection is not clearly understood. However, twenty years ago, Mann et al. (11) suggested that HLA-DR was involved in HIV attachment to cells. Further, in vivo studies demonstrated that increased HLA-DR expression on CD4\(^+\) T cells correlated with disease progression to AIDS (12). Moreover, ex vivo HLA-DR expression is a marker of susceptible lymphoid tissues (13). In addition, in vivo studies monitoring HIV-resistant human cohorts indicate a general reduction in activated CD4\(^+\) T cells and HLA-DR surface expression, suggesting that HIV resistance may be associated with decreased T cell activation (14–16). Collectively, these studies provide evidence that the MHC class II pathway may play a role in HIV disease.

Because CIITA is believed to influence HIV genome transcription and its known transcription target HLA-DR is a marker for susceptible tissues, we wanted to determine what role CIITA plays in the infection of CD4\(^+\) T cells. In this study, we demonstrate that CIITA expression in the TCR-negative T cell line SupT1 renders them more susceptible to HIV infection through increased virion attachment. Increased virion binding was dependent upon the presence of viral envelope glycoproteins and cellular expression of CD4. However, CIITA-mediated enhancement of infection was independent of enhanced endocytosis of virus particles. Although increased attachment to CIITA-expressing SupT1 and activated primary CD4\(^+\) T cells correlated with HLA-DR surface expression, Ab blocking of HLA-DR and HLA-DRs knockdown by short hairpin RNA (shRNA) failed to reduce infection of CIITA-expressing cells. Further, flow cytometry and blocking studies demonstrated that HLA-DM, HLA-DP, HLA-DQ, invariant chain (li), and MHC class I are likely not responsible for increased HIV infection of CIITA-expressing cells. Therefore, this study strongly suggests that CIITA transcriptionally regulates expression of an HIV ligand on activated T cells that may serve as a novel antiviral target.

Materials and Methods

Cells, plasmids, and stable cell line generation

A293T, CD4\(^+\) T cell lines [SupT1 and BC7 (17)], DG75, and Raji cells were maintained as described previously (18). A293T cells were stably transfected with CIITA by cotransfecting PVU-I-linearized pDNA3.FLAG.
CIITA8 (pcCIITA) (19) and pCMV4His. Stable clones were selected in DMEM containing 5 mM t-histidinol (Sigma-Aldrich, St. Louis, MO). SupT1 and Jurkat cells stably expressing CIITA were generated by electroporation of pcCIITA into cells, followed by clonal selection in 600 μg/ml geneticin/48, as described previously (20). GHOST cells were maintained as described previously (21). All of the transfections were performed using FuGENE HD Transfection Reagent, as suggested by the manufacturer (Roche, Indianapolis, IN).

**Flow cytometry**

The following Abs were used at vendor-recommended concentrations: HLA-DR (L243-AF647 or PerCP), CXCR4-PE (12G5), CD4-allophycocyanin (RPA-T4), HLA-DQ-FITC (HLA-DQ1; Biologend, San Diego, CA), HLA-DM (Tal 18.1; Santa Cruz Biotechnology, Santa Cruz, CA), IL-2 (LN2) and HLA-ABC (W6/32; eBiosciences, San Diego, CA), HLA-DP (DP 11.1; Novus Biologicals, Littleton, CO), and goat anti-mouse AF488 (Invitrogen, Carlsbad, CA), and staining was performed as described (22) and analyzed using FlowJo 7.2.2 software (Tree Star, Ashland, OR). For all of the experiments, live cells were gated on prior to analysis.

**Virus production and infections**

Virus was produced by transfecting pNL4-3 provirus into either A293T or A293T-CIITA cells to produce either DR− or DR+ virions (18). Similar to previous studies, vesicular stomatitis virus envelope glycoprotein G (VSV-G) pseudotyped virions were produced by cotransfecting cells with pVSVG and pNL4-3Env using a 3:1 ratio of DNA to FuGene HD. HIV virions incorporating heat-stable Ag (HSA, also called mouse CD24) were produced by cotransfecting the above producer cells with pNL4-3Δp27HSA (23) and pNL4-3 (which provides Vpr in trans). Virus stocks were concentrated using Ambicon 100K m.w. cut off filters (Millipore, Billerica, MA) and stored at −80°C. Titers were determined as described previously (21). Infected cells were analyzed at indicated multiplicities of infection (MOIs) for 2 h in a final volume of 125 μl RPMI 1640 at 37°C/5% CO2. After being washed, infected cells were cultured at a concentration of 200,000 cells per milliliter at 37°C/5% CO2. Cell viability was analyzed by trypan blue exclusion assay.

**p24 ELISA**

Serial dilutions of the cell-free virus-containing supernatants were analyzed by p24 ELISA following the manufacturer’s directions (HIV-1 p24 Ag Capture Assay from the AIDS and Cancer Virus Program, SAIC-Frederick, Frederick, MD).

**Intracellular p24 staining**

A total of 10^6 cells were fixed and permeabilized with IntraPrep Permeabilization/Fixation Reagents and stained with FITC-conjugated anti-p24 (Beckman Coulter, Fullerton, CA), following the manufacturer’s instructions.

**Semiquantitative PCR and quantitative PCR**

A total of 1 μg DNA-free RNA was reverse-transcribed using the iScript cDNA synthesis kit, following manufacturer’s instructions (Bio-Rad, Hercules, CA). A total of 50 ng cDNA was used to amplify HLA DM, CIITA, gagA, and β-actin from each cell type to confirm expression in stable cell lines, using the following primers: CD74 (9) (24); HLA-DM forward 5'-AAGTTTCCCAGAAGTACCTG-3'; reverse 5'-CTGGAAAC-TGAGTTCATCG-3'; CIITA forward 5'-AACCCGCAACAGACACCAT-CACT3'-reverse 5'-GTCCAGTTCCCGATATTGCGATA-3'; gagdh forward 5'-CCAAAGGCTATCATCTGTCG-3'; reverse 5'-CAGGGG-CATCCACAGTCTC-3'. Quantitative PCR (qPCR) was performed using the Bio-Rad IQ5, where 10-fold dilutions (500 ng to 500 pg) were used as template and expression of HIV-1 gag or CIITA was normalized to gapdh. qPCR protocols and relative expression calculations were performed as described previously (25, 26). All of the primer sets anneal at 55°C, and primer sequences are as follows: CIITA (same as above); gag, forward 5'-TCTAAAGACCGAAGCACTCTACA-3', reverse 5'-TAGACCTCGGT-CCAAAGCTCTTA-3'; gagdh, forward 5'-GGGCAAAGCTGATCTGGT-3', reverse 5'-ACAGTCTCTGGGTGCACTAGT-3'.

**Binding assay**

HSA (CD24)-containing virions were incubated at room temperature for 30 min (27) at varying MOIs in 96-well plates in a final volume 125 μl medium. Cells were incubated with rat anti-mouse Ab (clone M1/69; Biologend) followed by goat anti-rat IgG conjugated to Alexa Fluor 647 (Invitrogen), and binding was assessed by FACS analysis for virion-associated CD24 bound to the cells.

Ab blocking and infection assay

A total of 2 × 10^6 cells were blocked for 30 min on ice with 1 μg of the following Abs: CD4 (RPA-T4), mouse IgG1, x control, HLA-DR (L243; Biologend), HLA-ABC (W6/32; eBiosciences), HLA-DRβ1 (HB10a or Tal 14.1), HLA-DRα4, (F-14; Santa Cruz Biotechnology), HLA-DRβ3 (DP 11.1; Novus Biologicals), and pan-MHC class II (Tu39; BD Pharmingen, San Diego, CA). After being washed, cells then were infected at an MOI of 0.08, and infection was determined at 3 d postinfection (d.p.i.) by intracellular p24 staining as described.

**GHOST cell transfections and infections**

GHOST cells, seeded at 30,000 cells in six-well dishes, were transfected with either pcDNA3.1, pcCIITA, or equal amounts of pcDRα and pcDRβ5 or pcDRαΔcyto and pcDRβ5Δcyto (28). Twenty-four hours later cells were transfected with HIV Gag-IGFP (29, 30), and 48 h posttransfection cells were analyzed for GFP and HLA-DR expression (as described) by flow cytometry.

**shRNA-mediated knockdown**

HLA-DRα-specific, empty vector and scrambled control HuSH pGFP–V-RS constructs (TG304088) were obtained from OriGene Technologies (Rockville, MD). HLA-DRα–specific construct (GI316346) was found to knock down HLA-DR surface expression in CIITA-expressing A293T cells and was used to stably knockdown HLA-DR in SupT1-CIITA B5 cells with scrambled (TR30013) and empty vector control (TR30007) plasmids. All stable clones were selected for in 0.5 μg/ml puromycin and monitored by flow cytometry for HLA-DR and CD4 expression.

**Primary cell experiments**

Primary, HL-DR− PBMCs from a donor whose HL-DR haplotype matches our virus-producer cells, A293T, were obtained from the Albany Medical Center Transplantation Immunology Laboratory. CD8+ cells were removed by negative selection using biotin-conjugated mouse anti-human CD8 (SK1) (Biologend), μMACS Streptavidin Microbeads (Miltenyi Biotec, Auburn, CA) and magnetic purification through MACS Separation Columns (Miltenyi Biotec). The remaining CD4+ cells were left unstir- mulated but maintained in Iscoves’ medium supplemented with human serum and rIL-2 (at 1, 3, and 5 day postinfection). The remaining cells were activated as described previously (31) and maintained in the same medium but supplemented with rIL-2 at 2 and 4 d poststimulation. Binding assays and flow cytometry were performed as described above.

**Results**

To assess the contribution of CIITA to HIV infection of CD4+ T cells, stable CIITA-expressing transfectants were produced using the CD4+CCR5+ TCR cell line SupT1. Among the CIITA stable cell lines produced, one clone, SupT1-CIITA B5, expressed surface HLA-DR (Supplemental Fig. 1B) at levels comparable to those of other T cells that endogenously express HLA-DR (e.g., Hut78). Expression of the other CIITA-dependent genes involved in MHC class II Ag presentation (i.e., HLA-DM and Ii) was also evident in SupT1-CIITA B5 cells (Supplemental Fig. 1A) and HLA-DR expression at the cell surface was unchanged at 3 d after HIV infection (Supplemental Fig. 1C).

**CIITA expression enhances virus release from SupT1 cells**

To quantify differences in productive HIV infection, infectious virus production from SupT1-CIITA B5 cells and the parental clone SupT1 was monitored using a GHOST assay, and virus particle release was quantified by p24 ELISA. At 2 d.p.i., no virus was detected after infection of either SupT1 or SupT1-CIITA. However, by 3 d.p.i., SupT1-CIITA B5 cells exhibited a significant increase in infectious virus production (17-fold) and released virus particles (16-fold; Fig. 1A) compared with those of control SupT1 cultures. Further, virus release from CIITA-expressing cells was greater than that of controls through 5 and 7 d.p.i. (Supplemental Fig. 2). These results demonstrate that CIITA expression significantly enhances viral release early in the infection cycle.
CIITA expression in CD4+ T cells enhances the kinetics of in-viability (Fig. 1B). By 7 d.p.i., both cell types showed an equal decline in viability (B5), indicating accelerated virus-induced cell death in CIITA-expressing SupT1-CIITA B5 cells and the parental clone SupT1 (Fig. 1C), and at 3 d.p.i., there was no difference in cell survival between CIITA cells (B5) and 3 d.p.i., postinfection productive infection was measured. Standard error is presented. *p ≤ 0.01, as determined by a two-sample unequal variance Student t test. n is the number of independent experiments.

CIITA expression enhances productive infection of SupT1 cells

The progression of HIV infection to AIDS is marked by a significant decline in functional CD4+ T cells. To monitor the effect of CIITA on T cell death during HIV infection, cell viability studies were performed in vitro using a trypan blue exclusion assay. At 2 and 3 d.p.i., there was no difference in cell survival between SupT1-CIITA B5 cells and the parental clone SupT1 (Fig. 1B), suggesting that CIITA does not contribute to cell death early in the infection cycle of these cells. However, by 5 d.p.i., SupT1-CIITA cells were significantly less viable (~2-fold; Fig. 1B), indicating accelerated virus-induced cell death in CIITA-expressing cells. By 7 d.p.i., both cell types showed an equal decline in viability (Fig. 1B). Collectively, these experiments suggest that CIITA expression in CD4+ T cells enhances the kinetics of infection and virus release and promotes T cell death at earlier times after HIV infection.

CIITA expression enhances productive infection of SupT1 cells

That CIITA-expressing cells are more permissive for HIV infection is one potential explanation for the increase in viral titers from SupT1-CIITA B5 cells. Intracellular expression of the HIV major capsid protein (p24) is a well established criterion of productive infection (32) and was used to assess productive infection of CIITA-expressing cells. Compared to that of SupT1 control cells, SupT1-CIITA B5 cells displayed a significant 2-fold increase in the percentage of p24+ cells at 2 d.p.i. (Fig. 1C). Notably, this percentage increased 6.5-fold by 3 d.p.i., because higher levels of p24 protein were present in the infected cells, demonstrating that CIITA-expressing SupT1 cells are more permissive to HIV infection. However, by 5–7 d.p.i., the difference in infection between these cells was no longer apparent, likely a consequence of increased cell death of SupT1-CIITA cells (Fig. 1B) and a second round of infection in the SupT1 cells. Collectively, these results suggest that CIITA expression renders cells more susceptible to initial infection, which may lead to the more rapid kinetics of virus release and cell death.

Enhanced HIV infection of SupT1 cells is gp120/41-dependent

Because SupT1-CIITA cells display greater productive infection by 2 d.p.i. in the absence of detectable, infectious virions in the culture supernatant, CIITA is likely acting during the first round of infection. Because CIITA is a transcriptional coactivator, we wanted to determine the extent to which CIITA may be contributing transcriptionally to increased virus production and p24 expression. However, CIITA also may be enhancing upstream steps (i.e., attachment/fusion), which may account for the increases in p24 expression and virus production. To account for these possibilities, we used a virion pseudotyping approach. Here, gp120/41 HIV envelope glycoproteins on the HIV virion were replaced with VSV-G, changing the route of viral infection from CD4/chemokine receptor-mediated plasma membrane fusion to endocytosis (i.e., endosomal membrane fusion) (33, 34). This method eliminates differences in viral entry; therefore, both CIITA-positive and CIITA-negative cells would be infected equally by pseudotyped virions, if the increased infection is mediated by virion–cell membrane fusion. Conversely, if the increased infection was due in part to increased virion uptake by another route, such as endocytosis, then the infection permissivity difference would be maintained. Interestingly, the number of SupT1-CIITA cells expressing p24 was only increased after infection with wild-type HIV-1 (Fig. 1D), indicating that CIITA-mediated enhancement of successful infection is HIV env-dependent and occurs at the early steps in the infection cycle.

Further, this result suggested that CIITA does not significantly enhance HIV genome expression. Indeed, qPCR demonstrated that increased Pr55ag gene expression was comparable (p ≥ 0.01; Table I) between wild-type and VSV-G pseudotyped HIV-infected SupT1-CIITA cells, as compared with that in SupT1 cells. Therefore, CIITA may modestly enhance HIV genome transcription from the HIV long terminal repeat (Table I). However, this enhancement does not coincide with an increase in the percentage of infected cells. (Fig. 1D). These results suggest that CIITA enhancement of HIV infection is likely independent of its transcriptional effects on the HIV genome. Rather, CIITA is most likely increasing infection at the initial stages of the infection cycle.

CIITA enhances attachment of HIV virions to SupT1 cells

The first stages of HIV infection are attachment and fusion to the plasma membrane. Recently, fusion has been shown to occur with the endosomal membrane after endocytosis of HIV particles (35). Studies monitoring the transcriptome of CIITA demonstrate that it coactivates transcription of the gene encoding Rab4B, a small GTPase that regulates endocytic recycling (36). Thus, CIITA might increase infection of SupT1 cells by enhancing the endocytic uptake of virus through upregulation of Rab4B (36).

Table I. CIITA-mediated transcriptional activation of the HIV genome

<table>
<thead>
<tr>
<th>Infecting Virus (MOI = 0.1)</th>
<th>Fold Change in gag* (SupT1-CIITA/SupT1 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT HIV-1</td>
<td>4.23 ± 2.14</td>
</tr>
<tr>
<td>VSV-G HIV-1</td>
<td>2.6 ± 0.23</td>
</tr>
</tbody>
</table>

* qPCR expression was normalized to gapdh using the Pfaffl method (26) after qPCR.

**n > 6.**
ever, in the above experiments, both SupT1 and SupT1-CIITA cells were infected equally with VSV-G pseudotyped HIV (Fig. 1D), strongly suggesting that CIITA does not enhance infection by increasing endocytic viral particle uptake. Further, these results indicate a requirement for gp120/41 on the virion, because the CIITA-mediated enhancement of infection was reversed upon VSV-G pseudotyping, suggesting that CIITA expression may influence an early env-dependent event, such as the first step in HIV infection, attachment.

To determine if CIITA expression enhances virion attachment to the cell surface, cells were incubated with HIV carrying mouse CD24 on the viral envelope, and the level of virion attachment was determined by measuring mouse CD24 at the cell surface by flow cytometry. To establish the background for these experiments, gates were set using mock-infected controls that were stained for mouse CD24 (mock; Fig. 2G, 2H). Using these gates, we then were able to determine the percentage of cells that were CD24hi (Quadrant IV) as an indicator of virus binding. SupT1 cells and SupT1-CIITA B5 cells (Fig. 2A–F) bound readily detectable amounts of virus at different MOIs. At an MOI of 5, ~21% of SupT1 cells were CD24hi, whereas 37% of CIITA-expressing cells were CD24hi at the same MOI (Fig. 2D, 2E, respectively). When the MOI was increased to 10, ~49% of SupT1 and 80% of CIITA-expressing cells were CD24hi (Fig. 2A, 2B, respectively), suggesting that CIITA expression leads to improved virion binding. These increases also can be seen when the data are plotted as a histogram (Fig. 2C, 2F); however, the peak representing binding to CIITA-expressing cells has a less dramatic increase, likely because SupT1 cells are a polyclonal population, unlike the B5 CIITA clone. Therefore, virus binding to SupT1 cells is likely heterogeneous, which can be seen in Fig. 2A (Quadrant IV). Nevertheless, when the percentage of CD24hi cells (Quadrant IV) is plotted against the MOI, the slope derived from the line of regression indicates that 2-fold more (p = 0.02) virus was bound to CIITA-expressing cells (Fig. 2F). Therefore, these results demonstrated that CIITA expression in SupT1 cells leads to enhanced attachment of virions.

**CIITA-mediated enhancement of HIV binding is CD4-dependent**

The VSV-G pseudotyping experiments demonstrated that CIITA enhancement of infection is dependent upon expression of the viral

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**FIGURE 2.** CIITA enhances attachment of HIV virions to SupT1 cells. A, B, D, E, G, H, Virions carrying mouse CD24 were incubated with either SupT1 (A, D, G) or SupT1-CIITA B5 (B, E, H) cells, and virion binding was determined by FACS analysis. Gates were set for each individual experiment using mock control to set background (G, H). C, F, and I, Histograms for MOI = 10 (C) and MOI = 5 (F) are shown as well, and the slope of the line of regression was determined for SupT1 (dashed line) and SupT1-CIITA (solid line) based upon the average percentage of cells (for three independent experiments) that are CD24hi (derived from scatter plots) graphed against MOI (I). Data are representative of three independent experiments unless indicated. *p ≤ 0.01, as determined by a two-sample unequal variance Student t test.
envelope glycoproteins (gp160-dependent), suggesting that gp120 binding to CD4 is involved in CIITA enhancement of infection. To confirm that increased attachment is dependent upon the gp120–CD4 interaction, binding assays were repeated using a CIITA stable clone of the CD4-negative, SupT1-derived BC7 cell line (Supplemental Fig. 5). As expected, BC7 had no detectable virus bound to cells (Fig. 3A). Similarly, on BC7-CIITA cells, virus binding was not increased despite the presence of CIITA, demonstrating that enhanced attachment of virus to CIITA-expressing cells remains CD4-dependent and cannot be overcome by the presence of CIITA. Similarly, stable CIITA clones produced in Jurkat cells demonstrated that the clone that expresses the most CIITA (approximately the same amount as SupT1-CIITA B5) does become the most productively infected (Supplemental Fig. 4). However, infection never reached the level of the SupT1-CIITA B5 clone, likely because CD4 expression was more than a log-fold less in Jurkat cells. Collectively, these results demonstrate that although CIITA enhances productive infection of T cells, this enhancement is dependent upon CD4 expression.

HLA-DR embedded in the virion envelope enhances virion infectivity by an unidentified mechanism (8). To ensure that increased infection at later points postinfection in CIITA-expressing cells was not due to the presence of HLA-DR on the virion, DR-positive and DR-negative virions were produced in wild-type and CIITA stable virus-producer cells and used to infect SupT1-CIITA cells. CIITA expression enhanced the infection of SupT1 cells regardless of whether virions were produced in DR-positive or DR-negative cells (Fig. 3B), leading us to conclude that CIITA-enhanced HIV attachment is independent of virion-associated MHC class II.

Increased virion attachment to CIITA-expressing SupT1 cells correlates with HLA-DR surface expression

A potential explanation for our results is the CIITA-dependent transcription of an HIV ligand. Because CIITA coactivates genes of the MHC class II Ag presentation pathway (37), we began our evaluation of potential CIITA-regulated HIV ligands by looking at HLA-DR. Analysis of three SupT1-CIITA clones (B5, C4, and B6) expressing progressively lower levels of surface HLA-DR (Supplemental Fig. 3) demonstrated a strong positive correlation (r = 0.999) between virus attachment and HLA-DR expression (Fig. 4A). Likewise, HLA-DR expression correlated strongly (r = 0.93) with intracellular p24 staining, indicating increased infection with increasing HLA-DR surface expression (Fig. 4B).

On the basis of these results, we reasoned that HLA-DR at the cell surface was likely enhancing binding to CIITA-expressing cells. However, blocking HLA-DR with various Abs did not reverse the increased infection of SupT1-CIITA cells, as blocking CD4 did (Fig. 4C). One potential explanation for this result is that these Abs simply did not block the HLA-DR–HIV interaction. However, blocking SupT1-CIITA B5 cells with L243 modestly enhanced infection, suggesting the possibility that surface HLA-DR moderately interferes with infection or that engagement of HLA-DR may indirectly promote infection.

Transient HLA-DR expression does not promote HIV infection

Despite multiple attempts, we were unable to select a SupT1 clone that stably expressed the HLA-DR α- and β-chains to directly assess virion binding to HLA-DR-expressing cells in the absence of CIITA expression. To determine if transient expression of the HLA-DR heterodimer could increase HIV infection, we instead used the osteosarcoma indicator cell line GHOST (CD4+CXCR4+CCR5*). When GHOST cells were transfected with CIITA, there was no significant increase in HIV infection (GFP+) of HLA-DR+ cells (Supplemental Fig. 5). However, these cells expressed limited surface HLA-DR (data not shown), suggesting that CIITA does not coactivate the MHC class II Ag presentation pathway efficiently in these cells. Surprisingly, when the HLA-DR heterodimer was expressed transiently in these cells, there was an ~50% reduction in HIV infection, suggesting that HLA-DR may impair infection of this cell type. However, because we previously had observed an increase in HIV infection of CIITA-expressing cells upon blocking with L243, we considered that enhanced infection may indicate increased endocytosis and/or signaling, thereby promoting viral uptake. Endocytosis and signaling through HLA-DR are dependent upon the cytoplasmic tails of the heterodimer (38–42); therefore, we transiently expressed HLA-DR α and β with truncated cytoplasmic tails (DRΔcyto) to inhibit any signaling through or endocytosis of HLA-DR at the cell surface. These mutations dramatically reduced HLA-DR surface expression in GHOST cells, while leading to a corresponding increase in HIV infection as compared with those cells transfected with wild-type HLA-DR (Supplemental Fig. 5). These results further suggest that HLA-DR may actually inhibit rather than promote infection of CIITA-expressing cells.

shRNA knockdown of HLA-DR in CIITA-expressing cells does not prevent enhanced infection

HLA-DR-mediated inhibition of infection may be specific to GHOST cells. To determine if this effect might be cell type-specific and to directly examine the contribution of HLA-DR expression to the infection of SupT1 cells, we used shRNA directed against the HLA-DR α-chain message to reduce surface HLA-DR expression on SupT1-CIITA B5 cells. A stable, HLA-DRα–specific shRNA clone (SupT1-CIITA B5Δ6C) was selected based on at least a log-fold reduction in surface HLA-DR, whereas all clones, including scrambled and empty vector controls, were selected for their maintenance of CD4 expression levels similar to that of the parental B5 clone (Supplemental Fig. 6, Supplemental Table I).

These clones provided a valuable tool for determining the role of HLA-DR expression in the enhanced infection of CIITA-expressing T cells. Despite downregulation of HLA-DR at the surface of the B5Δ6C clone, CIITA expression (Supplemental Table I) and productive infection of these cells (Fig. 4D) did not differ significantly compared with those of the wild-type SupT1-CIITA B5 clone. Further, CIITA expression and infection of this...
HLA-DR knockdown was still significantly greater than those of the SupT1-CIITA C4 clone, further suggesting that increased infection of SupT1 cells is due to CIITA and not HLA-DR. Even more interesting was that infectious virus release was enhanced from the B5D6C clone (Fig. 4E), although not significantly, above that of the parental SupT1-CIITA B5 line, despite both expressing similar levels of CIITA (Supplemental Table I). Interestingly, both the empty vector and the scrambled control clone exhibited reduced, though not significant, HIV infection and virus release. These results are likely due to the decrease in CIITA expression in the scrambled control cell line (Supplemental Table I), whereas the empty vector control clone showed reduced CXCR4 and CD4 expression (Supplemental Table I, Supplemental Fig. 6, respectively).

Together, Ab blocking, shRNA knockdown of HLA-DR in CIITA-expressing cells, and transient expression of HLA-DR, all suggest that increased infection of CIITA-expressing cells is due to a CIITA-dependent increase in viral binding that cannot be attributed to HLA-DR. Additional CIITA-upregulated cell surface proteins include the other MHC class II isotypes, HLA-DP and HLA-DQ, MHC class I (43, 44), the MHC class II accessory proteins HLA-DM (45), and Ii (46). We analyzed the surface expression of these proteins by flow cytometry to determine if any of these proteins could be playing a role in increased virion attachment. Although MHC class I expression was increased on SupT1-CIITA B5 cells over that on SupT1 cells, there was no difference in expression between the B5 and C4 clones (Supplemental Fig. 3), suggesting that increases in virion binding are likely not due to differences in MHC class I expression. HLA-DM and Ii expression was not detected on the surface of SupT1-CIITA B5 cells (Supplemental Fig. 3K, 3L, respectively). Therefore, these CIITA-dependent proteins are unlikely to be contributing to increased binding. Similarly, there was no change in expression of HLA-DQ or the HIV coreceptor CXCR4 (Supplemental Fig. 3N, 3J, respectively) between clones, suggesting that these molecules are also unlikely to be involved.

**Blocking of surface HLA-DP does not reduce enhanced infection of CIITA-expressing cells**

Surface expression of HLA-DP was greater on CIITA-expressing cells; specifically, SupT1-CIITA B5 expressed more than the C4 clone (Supplemental Fig. 3M). Therefore, we performed blocking experiments to determine if HLA-DP may play a role in increased infection of CIITA-expressing cells. Blocking SupT1-CIITA B5 cells with an HLA-DP–specific monoclonal or a pan-reactive MHC class II Ab did not return the level of HIV infection of CIITA-expressing cells to that of SupT1 cells (Fig. 4F), suggesting that HLA-DP and, for that matter, MHC class II are not involved directly in increased binding and infection of CIITA-expressing cells. Further, blocking with the pan-reactive MHC Ab slightly increased infection of cells, again suggesting that MHC class II may partially inhibit HIV infection. Collectively, we have assessed all known cell surface, MHC class II Ag presentation, and endocytosis proteins demonstrated to be transcriptionally activated by CIITA and have not identified the ligand that enhances virion binding and infection of cells. Yet, we have demonstrated the requirement for CD4 and gp120 in CIITA enhancement of T cell infection. Therefore, engagement of gp120
by CD4 not only induces the conformational change required to expose gp41 (fusion peptide) but may allow for an interaction with a CIITA-regulated, T cell-specific ligand that either stabilizes virus binding or promotes more efficient fusion.

Enhanced virion binding to primary CD4+ T cells corresponds to HLA-DR expression

To determine if these results could be reproduced in primary cells, HLA-DR CD8+ T lymphocytes from a healthy donor who has a syngeneic HLA-DR haplotype to cells used to produce virus were activated ex vivo using anti-CD3 and anti-CD28 cross-linking. At 4 d postactivation, HLA-DR expression increased seven times, and virus binding (mouse CD24) increased six times on activated primary cells, although CD4 expression remained similar between activated and quiescent primary lymphocytes (Fig. 5). Average increases in mean fluorescence intensity for virus binding and HLA-DR and CD4 expression between activated and unstimulated primary cells were 7.1-, 7.8-, and 1.98-fold, respectively. These experiments suggest that enhanced virus binding corresponds to HLA-DR expression on primary human CD4+ T cells. Because HLA-DR-expression is an established indicator of CIITA expression (47, 48) and we have demonstrated that HLA-DR is unlikely to be involved in enhanced infection of SupT1 cells, these data suggest that CIITA plays an important role in the infection of activated primary CD4+ T cells as well as CD4+ cell lines.

Discussion

Collectively, our results reveal for the first time, to our knowledge, that CIITA expression in SupT1 cells enhances HIV infection, not via an MHC II expression-dependent mechanism but by increasing virion attachment to cells in a gp120/CD4-dependent manner that is mediated by CIITA expression. Heightened virion attachment increases the number of HIV-infected cells, which, combined with CIITA-enhanced transcription of the HIV viral genome, leads to significantly higher viral titers and increased T cell depletion earlier in the infection cycle.

Our data shed new light on the role of CIITA and MHC class II in the HIV infection cycle. Prior to identification of the HIV coreceptors CXCR4 and CCR5, Mann et al. (11) suggested that HLA-DR might be involved in HIV binding to CD4+ T cells. Mannhalter et al. (49) disputed this finding, arguing that surface MHC class II did not boost HIV infection. However, our pseudotyped virus studies demonstrate that CIITA enhancement of infection is not due to increased endocytic uptake of virus particles. Further, our results suggest that increased infection is not related to other CIITA-regulated cell surface proteins: HLA-DP, HLA-DQ, MHC class I, or the MHC class II accessory proteins Li and HLA-DM.

Overall, our data demonstrate that CIITA may play a more significant role in activation-dependent infection of CD4+ T cells than previously thought. Indeed, human HIV-resistant cohorts demonstrate reduced expression of HLA-DR on CD4+ T cells (15), implying reduced CIITA expression or function. Thus, the absence of CIITA in resting CD4+ T cells may serve as an additional block to productive infection of quiescent cells. These results may extend into nonhuman primate species as well; a recent study comparing chimpanzees to humans suggests that chimpanzees are less susceptible to HIV infection, in part due to suboptimal CD4+ T cell activation and reduced surface expression of MHC class II (i.e., CIITA expression) (54). Therefore, our data reveal a novel aspect of CIITA function with respect to enhanced HIV infection of activated T cells in humans and potentially in nonhuman primate models of infection.

CIITA-mediated enhancement of infection may not be limited to newly activated, previously naïve CD4+ T cells. Activated memory cells also may be more susceptible, and these cells, specifically CD4+ memory T cells in HIV-infected patients, have a higher level of activation and HLA-DR expression than the same cells from uninfected patients (55). Further, HIV preferentially targets memory CD4+ T cells for infection, substantially depleting them from the GALT (56). Thus, future studies should help determine if CIITA plays a role in enhancing virus infection of memory T cells and whether using specific inhibitors of CIITA in T cells can prevent depletion of these cells from the GALT or promote their reconstitution after control of viral replication (56).

The recent Merck Phase IIb HIV vaccine trial showed that vaccinated subjects had an enhanced incidence of HIV infection,
cause activated, HIV-specific CD4+ T cells are preferentially adenovirus 5 (vaccine vector)-specific (58) but HIV-specific, be-

of infected (59). Our results provide important evidence that acti-

cated CD4+ T cells may be more susceptible to infection due

potentially through expansion of activated CD4+ T cells (57).

CD4+ T cell activation induced by the vaccine to avoid a potential CITA-mediated enhancement of infection.

Our data provide new insights into why activated CD4+ T cells are more susceptible to infection than resting cells. These findings may have implications for the design of new microbicides or chemotherapeutics. If CITA expression can be therapeutically uncoupled from T cell activation, then CITA inhibition could reduce viroin binding to activated CD4+ T cells in the vaginal, rectal, or gut mucosa and possibly reduce viral transmission by these routes of infection. Similarly, the viral burden in chronically infected patients might be reduced by such an uncoupling agent.

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Disclosures

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