



RESEARCH ARTICLE

Binding of HIV-1 virions to $\alpha_4\beta_7$ expressing cells and impact of antagonizing $\alpha_4\beta_7$ on HIV-1 infection of primary CD4⁺ T cells

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HIV-1 envelope glycoprotein is reported to interact with $\alpha_4\beta_7$, an integrin mediating the homing of lymphocytes to gut-associated lymphoid tissue, but the significance of $\alpha_4\beta_7$ in HIV-1 infection remains controversial. Here, using HIV-1 strain BaL, the gp120 of which was previously shown to be capable of interacting with $\alpha_4\beta_7$, we demonstrated that $\alpha_4\beta_7$ can mediate the binding of whole HIV-1 virions to $\alpha_4\beta_7$ -expressing transfectants. We further constructed a cell line stably expressing $\alpha_4\beta_7$ and confirmed the $\alpha_4\beta_7$ -mediated HIV-1 binding. In primary lymphocytes with activated $\alpha_4\beta_7$ expression, we also observed significant virus binding which can be inhibited by an anti- $\alpha_4\beta_7$ antibody. Moreover, we investigated the impact of antagonizing $\alpha_4\beta_7$ on HIV-1 infection of primary CD4⁺ T cells. In $\alpha_4\beta_7$ -activated CD4⁺ T cells, both anti- $\alpha_4\beta_7$ antibodies and introduction of short-hairpin RNAs specifically targeting $\alpha_4\beta_7$ resulted in a decreased HIV-1 infection. Our findings indicate that $\alpha_4\beta_7$ may serve as an attachment factor at least for some HIV-1 strains. The established approach provides a promising means for the investigation of other viral strains to understand the potential roles of $\alpha_4\beta_7$ in HIV-1 infection.

KEYWORDS HIV-1; integrin $\alpha_4\beta_7$; binding; infection; RNA interference; primary CD4⁺ T cells

INTRODUCTION

The predominant route of HIV-1 transmission is via sexual intercourse. During sexual transmission, HIV-1 viral particles or virus-infected cells have to overcome a series of obstacles to establish a successful infection including: mucus; the integrity of the genital or rectal epithelial barriers; host innate defense mechanisms; and the sparsity, activation state and susceptibility of mucosal CD4⁺ target cells (Borrow P, et al., 2010; Derdeyn C A,

et al., 2004; Keele B F, et al., 2008; Miller C J, et al., 2005; Temchura V, et al., 2014; Tsai L, et al., 2014; Zhang Z Q, et al., 2004). These obstacles collectively result in a relatively low transmission rate per exposure (Boily M C, et al., 2009; Wawer M J, et al., 2005). However, potential selective advantages acquired by HIV-1, such as enhanced binding to specific cell subtypes, might significantly increase the chances of a successful transmission event.

Recent studies indicate that HIV-1 envelope glycoprotein (Env) gp120 is capable of interacting with integrin $\alpha_4\beta_7$ to potentially increase viral infection (Arthos J, et al., 2008; Cicala C, et al., 2009; Darc M, et al., 2011; Li H, et al., 2011; Nawaz F, et al., 2011; Richardson S I, et al., 2013; Tjomsland V, et al., 2013). $\alpha_4\beta_7$ is expressed on lymphoid cells and mediates cell homing to gut-as-

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sociated lymphoid tissue (GALT), the dominant site of CD4⁺ T cell depletion shortly after HIV-1 acquisition and local propagation (Berlin C, et al., 1993; Mehandru S, et al., 2004; Sattentau Q, 2008; Veazey R S, et al., 1998). Lymphocytes expressing activated $\alpha_4\beta_7$ may be preferentially targeted by HIV-1 in GALT, facilitating the swift and profound gut pathogenesis of HIV/SIV infection (Mavigner M, et al., 2011; Wang X, et al., 2009). The close association of $\alpha_4\beta_7$ with primary mucosal portals of HIV-1 infection, specifically genital and rectal mucosa, suggests that $\alpha_4\beta_7$ interaction of viral gp120 may play an role in HIV-1 acquisition and establishment of a successful infection (Cicala C, et al., 2009; Haase A T, 2010; Martinelli E, et al., 2013; McKinnon L R, et al., 2011). However, the *bona fide* contribution of $\alpha_4\beta_7$ in HIV-1 infection has not been fully clarified. It is shown that a number of gp120s derived from different HIV-1 strains are capable of interacting with $\alpha_4\beta_7$ (Arthos J, et al., 2008; Li H, et al., 2011; Nawaz F, et al., 2011). The majority of previous studies investigating the binding capability of HIV-1 to $\alpha_4\beta_7$ mainly used the recombinant monomeric gp120s. Given that the structure of HIV-1 envelope protein is complex and the monomeric gp120 can be distinct significantly from the native trimeric gp120s anchored in the membrane of HIV-1 virion (Harris A, et al., 2011; Wyatt R, et al., 1998), whether whole HIV-1 virions also possess the $\alpha_4\beta_7$ reactivity is a topic of importance and remains to be further elucidated.

In the current study, after confirming the binding of HIV-1 BaL to various $\alpha_4\beta_7$ -expressing cells, we investigated the impact of antagonizing $\alpha_4\beta_7$ on HIV-1 infection of primary CD4⁺ T lymphocytes.

MATERIALS AND METHODS

Plasmids, cells, proteins and antibodies

Plasmid pNL4-3 BaL⁺ containing the backbone of NL4-3 and the Env of BaL (referred to as BaL), TZM-bl cell line and antibody Act-1 were from NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIH (Germantown, MD, USA). QT6, a fibrosarcoma cell line derived from Japanese quail, was from American Tissue Culture Collection (ATCC, Cambridge, MA, USA). Integrin expressing plasmids including human α_4 , α_E , β_7 and β_1 , mouse α_4 and β_7 , rat α_4 and β_7 were amplified from cDNA subtracted from PBMCs of corresponding species using primers listed in Table 1 (Table 1) and inserted into the expression vector pcDNA3.1(+), respectively. Antibody 2B4 and mouse anti-human IgG were from R&D Systems (Minneapolis, MN, USA). HP2/1 was from AbD Serotec (Oxford, UK). RPA-T4, anti-CD3-PE, anti-CD4-FITC, anti-CD8-FITC, anti-CCR5-PE, anti-human β_7 -PE, anti-mouse β_1 -PE, anti-rat β_7 -PE, anti-mouse IgG-APC and matched iso-

type control antibodies were from BD Pharmingen (San Jose, CA, USA). Leu3A, anti-human β_7 -APC and control IgG-APC were from Biolegend (San Diego, CA, USA). Reagents for ELISA assay were from Beckman Coulter (Brea, CA, USA), with a limit of sensitivity of 30 pg/mL.

Construction of $\alpha_4\beta_7$ expressing stable cell lines

The complete coding sequences of α_4 and β_7 were amplified from constructed α_4 and β_7 and subcloned into the lentiviral vectors pLJM1 (Addgene, Cambridge, MA, USA, with a substitution of CMV-EGFP into CMV-2a-RFP) and pLenti6.3/V5-DEST (Invitrogen, Grand Island, NY, USA, with an insertion of a MCS-IRES2-EGFP sequence after the CMV promoter), respectively. The constructed pLJM1-CMV- α_4 -2a-RFP or pLenti6.3-CMV- β_7 -IRES2-EGFP/V5-DEST vector was co-transfected with psPAX2 and pMG2.G (Addgene, Cambridge, MA, USA) into 293T cells to produce lentiviruses which were subsequently used to infect CHO cells at optimal multiplicity of infection. Cells with high and stable $\alpha_4\beta_7$ expression were selected by culture in the presence of antibiotics puromycin and blasticidine, and further purified by limited dilution (Wurm F M, 2004).

Preparation of PBMCs and T cell subsets

All human blood samples were collected under protocols approved by the Local Research Ethics Committee. PBMCs were isolated from single buffy coats, and stimulated with 20 U/mL interleukin-2 (R&D Systems, Minneapolis, MN, USA) and 1 μ g/mL phytohaemagglutinin (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. For $\alpha_4\beta_7$ activation, 10 nmol/L retinoic acid (RA, Sigma-Aldrich, St. Louis, MO, USA) was added to the medium at the beginning of the 3-day culture, followed by an additional 4-day culture in the presence of IL-2 and RA. CD4⁺ T cells were prepared from PBMCs by negative selection and cultured as PBMCs (Miltenyi, Bergisch Gladbach, NRW, Germany). CD8⁺ T cells with high or low/negative $\alpha_4\beta_7$ expression were sorted from activated PBMCs by gating on the CD8⁺ lymphocytes with corresponding β_7 expression.

Virus binding

For cell transfectants and CHO- $\alpha_4\beta_7$ cell lines, cells were detached by trypsin and recovered at 37°C for 2 hours. For CD8⁺ T cells, after sorting by flow cytometry, cells were cultured at 37°C for 2 days prior to binding assay. For each condition, 1×10^6 cell-line cells or 5×10^5 primary cells were incubated with 100 ng p24 of virus at 37°C for 2 hours (for transfectants) or 1 hour (for primary cells) with end-to-end rotation. Inhibitors were present as indicated and pre-incubated with the cells for 1 hour (for transfectants) or 30 min (for primary cells). The incubation medium was supplemented DMEM with the

addition of 1 mmol/L MnCl_2 . After incubation, the cells were washed 3 times using HEPES buffer supplemented with 1 mmol/L MnCl_2 and 100 $\mu\text{mol/L}$ CaCl_2 to remove unbound virus, lysed using 1% Triton X-100, then subjected to p24 antigen quantification.

RNA interference

Short-hairpin RNA interference was carried out as described previously (Qin X F, et al., 2003). In brief, shRNA oligo sequences targeting human integrin α_4 were downloaded from the RNAi Consortium (<http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/ProjTRC.shtml>), analyzed for suitability and potential efficacy according to general guidelines for RNAi design (Birmingham A, et al., 2007; Qin X F, et al., 2003; Tiscornia G, et al., 2003), synthesized, annealed and inserted into the lentiviral vector pLentiLox3.7 (referred to as pLL3.7) (Addgene, Cambridge, MA, USA). Scrambled shRNA and shRNA sequence targeting CCR5 were from Addgene or synthesized as described (Qin X F, et al., 2003) (Table 1), and subsequently inserted into the lentiviral vector pLL3.7. The constructed pLL3.7-shRNA or empty vector were co-transfected with psPAX2 and pMG2.G into 293T cells to produce lentivirus which were then titrated and used to transduce RA-treated CD4^+ T cells. 4-6 days post-transduction, the positively transduced cells (GFP^+) were analyzed for gene expression by FCM and sorted for downstream virus infection.

HIV-1 production, titration and infection

Pseudotyped reporter viruses were prepared as described (Hu Q, et al., 2005). In brief, 293T cells were co-transfected with plasmids expressing HIV-1 Env or VSV-G and pNL4-3.Luc.RE⁻ using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Infectious HIV-1 was produced by transfecting plasmid BaL into 293T cells using Lipofectamine 2000 according to the manufacturer's instructions. PBMC-originated viruses were produced by infection of PBMCs with 293T-derived viruses. All viral stocks were titrated by p24 ELISA.

For virus infection assays, 2×10^5 PBMCs or CD4^+ T cells were infected with 2 ng p24 of virus followed by extensive washes to remove unbound virus. Cells were cultured in supplemented RPMI-1640 for 12 days. Supernatants were collected every 3 days post-infection, lysed and then stored at -80°C until detection for p24 antigen.

Flow cytometry

For expression assays, 5×10^6 cells were used for each condition. In brief, cells were harvested, counted and washed once using PBS with 3% FBS. For integrin transfectants and $\alpha_4\beta_7$ -expressing cell lines, cells were washed

using HEPES buffer supplemented with 1 mmol/L MnCl_2 and 100 $\mu\text{mol/L}$ CaCl_2 . Cells were stained with indicated antibody for 30 minutes on ice, followed by staining with secondary antibody for another 30 minutes in some cases. The stained cells were washed twice, fixed with 500 μL of 1% paraformaldehyde in PBS and analyzed on a FACSAria III (BD, San Jose, CA, USA) cytometer.

For cell sorting, 5×10^7 cells were used for each sample. Cells were stained as described above except that cells were kept in ice-cold washing buffer and immediately sorted after staining.

Statistical analysis

Data are presented as mean \pm SD. The difference of mean value was analyzed by the two-tailed student's t test. $p < 0.05$ was considered statistically significant.

RESULTS

HIV-1 binds to cell transfectants mediated by gp120- $\alpha_4\beta_7$ interaction.

HIV-1 gp120 has been reported to interact with integrin $\alpha_4\beta_7$ expressed on primary lymphocytes and such interaction has been well studied (Arthos J, et al., 2008; Cicala C, et al., 2009; Darc M, et al., 2011; Jelacic K, et al., 2013; Li H, et al., 2011). However, whether gp120- $\alpha_4\beta_7$ interaction mediates the binding of HIV-1 virions to target cells remains to be further clarified (Arthos J, et al., 2008; Etemad B, et al., 2012). We initially performed experiments to examine whether expression of $\alpha_4\beta_7$ alone could mediate HIV-1 binding. We first sought to confirm HIV-1 binding to $\alpha_4\beta_7$ transfectants by cloning and co-expressing human α_4 and β_7 genes on 293T cells (Figure 1A). HIV-1 BaL bound to 293T cells transfected with $\alpha_4\beta_7$ to a significantly higher level than those transfected with pcDNA3.1. The $\alpha_4\beta_7$ -mediated virus binding was dependent upon its level of expression (Figure 1A). These results were confirmed using a second transduced cell line QT6, derived from Japanese quail (Figure 1B). In agreement with previous reports (Arthos J, et al., 2008; Nawaz F, et al., 2011), our data together shown that exogenously expressed $\alpha_4\beta_7$ can mediate HIV-1 binding to $\alpha_4\beta_7$ expressing cells.

$\alpha_4\beta_7$ is a heterodimer comprised of an α and a β subunits. It belongs to the integrin family which includes a large number of members. To date, 18 α and 8 β subunits and 24 different integrin heterodimers have been reported (Abram C L, et al., 2009; Yu Y, et al., 2012). Thus, we assessed whether $\alpha_4\beta_7$ is unique amongst integrins with respect to HIV-1 binding. We cloned two additional subunits α_E and β_1 , which can form $\alpha_E\beta_7$ and $\alpha_4\beta_1$ heterodimers *in vivo*, respectively. By transfection of 293T cells, we found that BaL virions also bound to $\alpha_4\beta_1$ transfectants, albeit to a lesser extent, whereas no significant

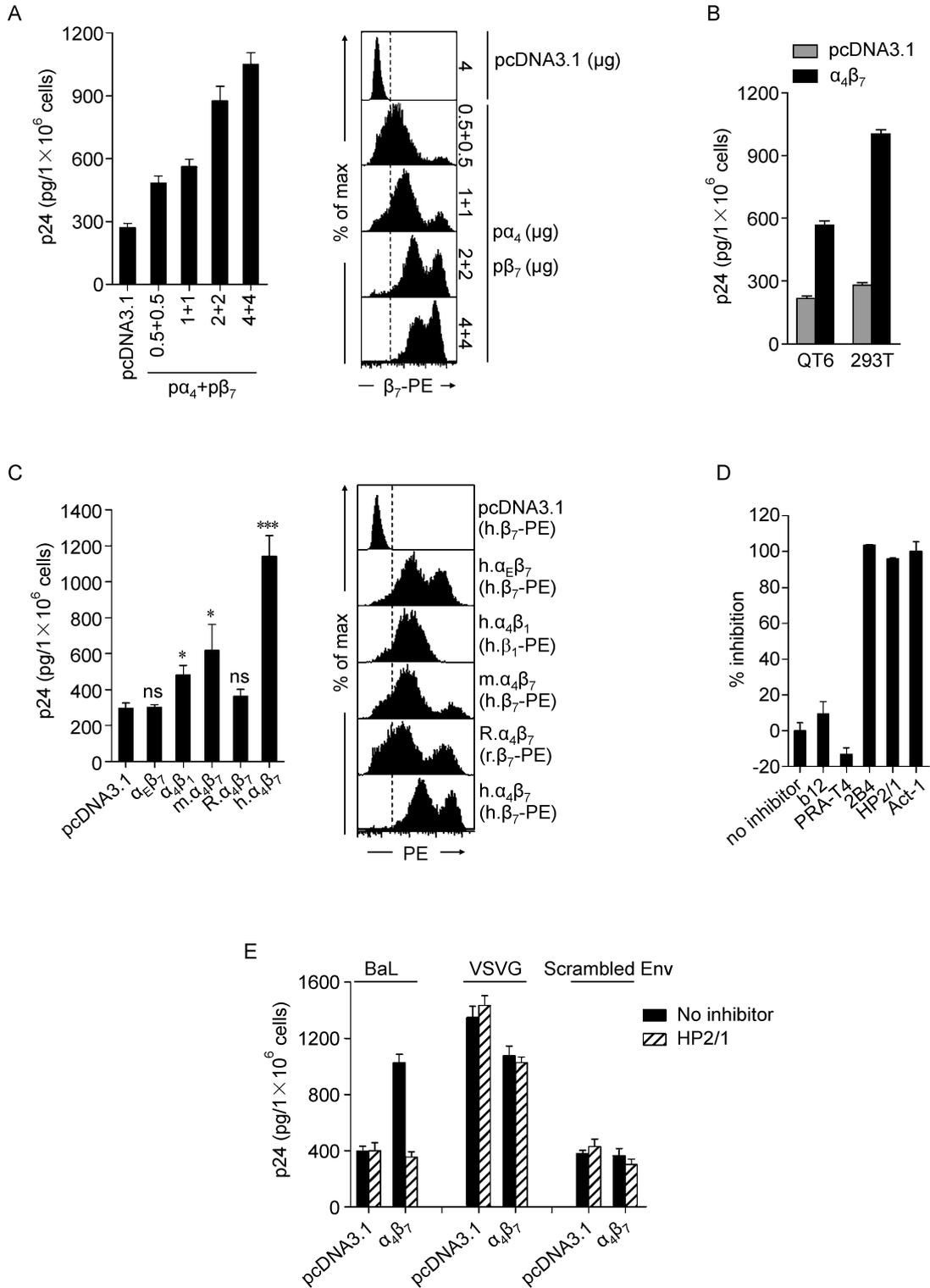


Figure 1. Binding of HIV-1 to cell transfectants mediated by gp120- $\alpha_4\beta_7$ interaction. A-D: 48 hours post-transfection, cells were detached and recovered at 37°C for 2 hours. For each condition, 1×10^6 cells were incubated with 100 ng p24 of HIV-1 BaL in complete DMEM medium with the addition of 1 mmol/L MnCl₂ at 37°C for 2 hours with rotation, followed by extensive washes to remove unbound virus. Cells were lysed with 1% Triton X-100 and subjected to p24 measurement. Antibodies (20 μ g/mL) were present as indicated and pre-incubated with the cells for 1 hour. A: Binding of BaL to 293T cells transfected with a serial dose of p α_4 + p β_7 or pcDNA3.1(+) control plasmid (left). The expression of $\alpha_4\beta_7$ was labeled by anti- β_7 -PE, assayed by flow cytometry and is shown on the right. B: Binding of BaL to 293T or QT6 cells transfected with p α_4 + p β_7 or pcDNA3.1(+). C: Binding of BaL to 293T cells transfected with plasmids expressing human $\alpha_E\beta_7$, human $\alpha_4\beta_1$, mouse $\alpha_4\beta_7$, rat $\alpha_4\beta_7$, human $\alpha_4\beta_7$, or pcDNA3.1(+) (left). The expression of the integrins was assayed by flow cytometry and is shown on the right. m, mouse; R, rat; h, human. D: Binding of BaL to 293T $\alpha_4\beta_7$ -transfectants in the presence of antibodies to gp120 CD4 binding site (b12), CD4 (RPA-T4), α_4 (2B4 or HP2/1) or $\alpha_4\beta_7$ (Act-1). Results are shown as % inhibition, with that in the absence of inhibitors set to 0%. E: Binding of HIV-1 pseudotyped with BaL, VSV-G or frameshift-mutated (scrambled) Env to 293T cells transfected with p α_4 + p β_7 or pcDNA3.1, in the presence or absence of anti- α_4 antibody HP2/1. All inhibitors were presented at 20 μ g/mL. Data shown are representative of at least three independent experiments with the bar graphs expressed as mean \pm SD. For virus binding assay, each condition was performed in triplicate. * $p < 0.05$ and *** $p < 0.001$, compared to virus binding using cells transfected with pcDNA3.1. ns, not significant.

binding was observed on $\alpha_E\beta_7$ transfectants (Figure 1C). To further identify whether $\alpha_4\beta_7$ mediated virus binding was species specific, we cloned mouse and rat $\alpha_4\beta_7$ and observed that BaL also moderately bound to mouse $\alpha_4\beta_7$, but not to rat $\alpha_4\beta_7$ (Figure 1C). Comparative integrin expression was determined by flow cytometry (FCM) (Figure 1C). The moderate binding to human $\alpha_4\beta_1$ was likely due to the shared subunit and structure similarity between $\alpha_4\beta_7$ and $\alpha_4\beta_1$ (Yu Y, et al., 2012), while binding to the murine ortholog reflects conserved sequence between human and mouse $\alpha_4\beta_7$ (Qi J, et al., 2012; Tidswell M, et al., 1997).

Subsequently, we measured the effect of inhibitors on virus binding. Results showed that monoclonal antibodies (mAbs, 20 μ g/mL) targeting α_4 (2B4, HP2/1) or $\alpha_4\beta_7$ (Act-1) almost completely blocked virus binding to $\alpha_4\beta_7$ transfectants, in agreement with a previous report that anti- $\alpha_4\beta_7$ inhibitors abolished gp120- $\alpha_4\beta_7$ interaction (Arthos J, et al., 2008). In contrast, no blocking effect was observed in mock control or in the presence of mAb to CD4 (RPA-T4) or gp120 CD4-binding site (b12) (Figure 1D).

To further explore the specificity of gp120- $\alpha_4\beta_7$ mediated virus binding, we produced pseudotyped HIV-1 using HIV-1 BaL Env, VSV-G or frameshift-mutated Env (scrambled Env), and observed that only BaL-pseudotyped HIV-1 bound to $\alpha_4\beta_7$ transfectants and the binding activity was significantly higher than that to pcDNA3.1 transfectants (Figure 1E). The pantropic VSV-G pseudotyped HIV-1 bound to cells regardless of $\alpha_4\beta_7$ expression, whereas scrambled Env pseudotyped HIV-1 lost the capability of binding to $\alpha_4\beta_7$ transfectants. In addition, only binding of BaL to $\alpha_4\beta_7$ transfectants was blocked by HP2/1, an anti- α_4 monoclonal antibody (Figure 1E). These results collectively indicate that the

interaction between gp120 and $\alpha_4\beta_7$ mediates HIV-1 binding.

HIV-1 binds to stable $\alpha_4\beta_7$ -expressing cell line and primary CD8⁺ and CD4⁺ T cells.

To further confirm the $\alpha_4\beta_7$ -mediated virus binding, we constructed a CHO cell line stably expressing $\alpha_4\beta_7$, using lentiviruses to introduce the α_4 and β_7 genes into the CHO genome. The expression of α_4 , β_7 and $\alpha_4\beta_7$ on the constructed CHO- $\alpha_4\beta_7$ was analyzed by fluorescence microscopy and FCM (Figure 2A and 2B). Compared to the parental CHO cells, HIV-1 BaL bound to CHO- $\alpha_4\beta_7$ cells at a significantly higher level which could be blocked by HP2/1 (20 μ g/mL) (Figure 2C). These results are consistent with those obtained from 293T transfectants (Figure 1).

The above experiments were conducted using cell lines. To assess whether $\alpha_4\beta_7$ -mediated HIV-1 binding can be reproduced in primary cells, we carried out virus binding assay using primary CD8⁺ T cells. This cell subset has little if any CD4 expression, eliminating the interference of CD4 mediated binding. PBMCs were stimulated for $\alpha_4\beta_7$ expression by retinoic acid (RA) (Mora J R, et al., 2003). Activated PBMCs from three different donors were subjected to sorting based on CD8 and β_7 expression by FCM (dot plot of Figure 2D). Compared to CD8⁺ T cells with low or negative β_7 expression ($\beta_7^{\text{low/-}}$), cells with higher β_7 expression (β_7^{hi}) mediated markedly higher level of virus binding (Figure 2D). CD4⁺ T lymphocyte is the primary target of HIV-1 infection *in vivo*. After RA treatment, we also observed significant virus binding to primary CD4⁺ T lymphocytes following blockade of CD4 with mAbs (2.5 μ g/mL RPA-T4 plus 2.5 μ g/mL Leu3A). This CD4 independent binding was inhibited by the HP2/1 mAb (5 μ g/mL) (Figure 2D). These results indicate that $\alpha_4\beta_7$ expressed on primary cells is

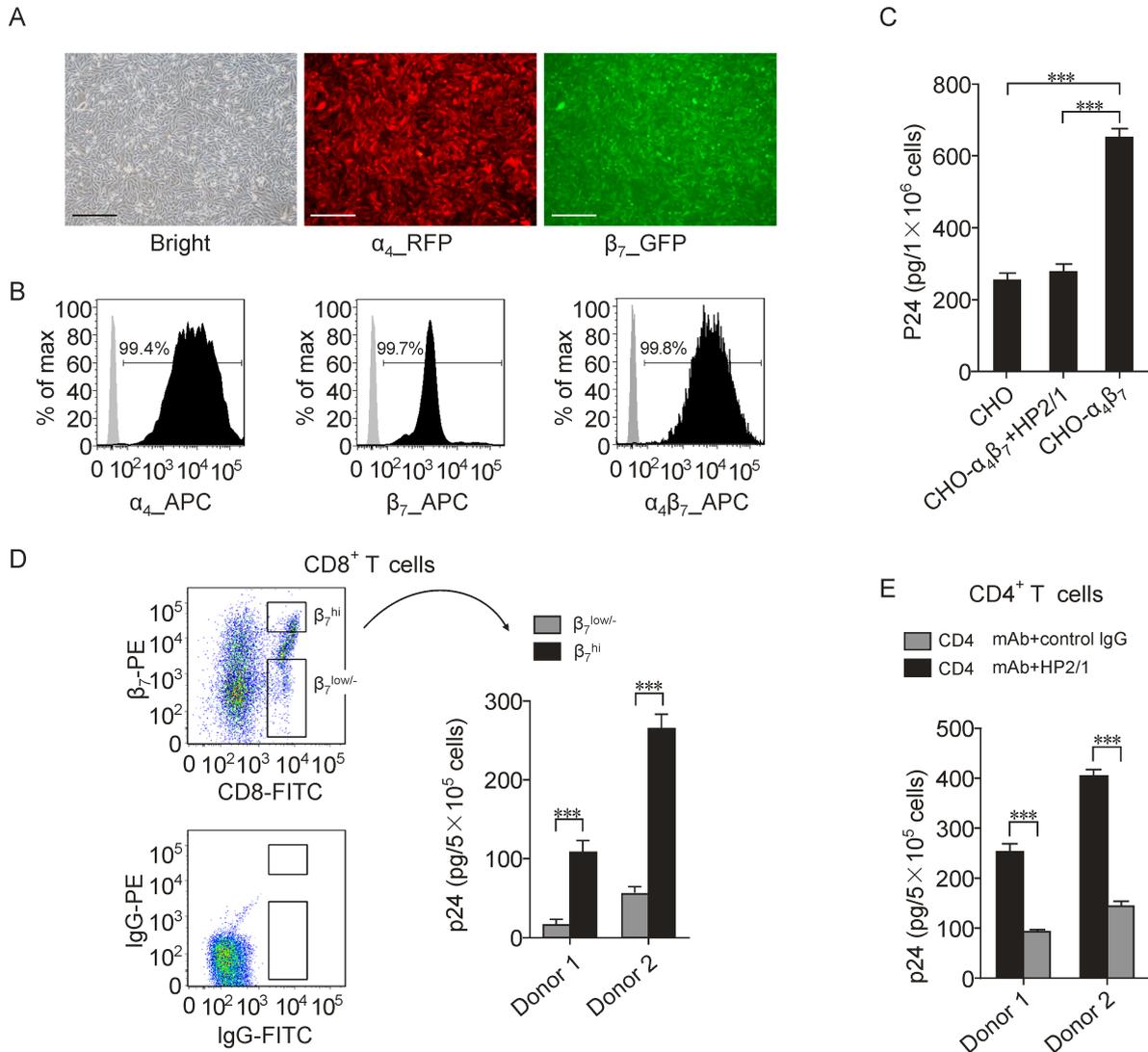


Figure 2. Binding of HIV-1 to a constructed CHO cell line stably expressing $\alpha_4\beta_7$ and RA-treated primary CD8⁺ and CD4⁺ T cells. A CHO- $\alpha_4\beta_7$ cell line with stable $\alpha_4\beta_7$ expression were constructed by transducing CHO cells with lentiviral vectors carrying the human α_4 and β_7 gene, followed by selection using antibiotic resistance and further purification using limited dilution. A: Visualizing of the constructed CHO- $\alpha_4\beta_7$ cells under a fluorescence microscopy. A fluorescent protein RFP or EGFP tag was bicistronically expressed with the α_4 or β_7 subunit, respectively. The black scale bar represents the length 100 μ m. B: Measurement of the α_4 , β_7 and $\alpha_4\beta_7$ expression on CHO- $\alpha_4\beta_7$ cells by FCM. The cells were correspondingly stained with anti- α_4 (2B4) and Alexa Fluor 647-conjugated goat-anti-mouse IgG, or anti- β_7 -APC, or Act-1 and allophycocyanin-conjugated rabbit anti-mouse IgG, followed by analysis using FCM. C: Binding of HIV-1 BaL to CHO cell lines with or without stable $\alpha_4\beta_7$ expression, in the presence or absence of anti- α_4 antibody HP2/1 (20 μ g/mL). D: Binding of HIV-1 BaL to primary CD8⁺ T cells with high (β_7^{hi}) or low or negative ($\beta_7^{low/-}$) $\alpha_4\beta_7$ expression. The cells were obtained by sorting of retinoic acid-treated PBMCs based on CD8 and β_7 expression. The gating strategy is shown by the dot plots. E: Binding of HIV-1 BaL to primary CD4⁺ T cells. The cells were separated from freshly-isolated PBMCs by negative magnetic selection and cultured for $\alpha_4\beta_7$ activation as PBMCs. The binding experiment was conducted as in described in the legend of figure 1 except that 5×10^5 cells were used for each condition of primary cells. For CD4⁺ T cells, the cells were incubated with 2.5 μ g/mL RPA-T4 plus 2.5 μ g/mL Leu3A at 37°C for 30 minutes to block CD4 receptor and in the presence of HP2/1 (5 μ g/mL) to block $\alpha_4\beta_7$ or IgG as a negative control. The bar graphs are expressed as mean \pm SD, with each condition performed in triplicate. For C, one out of three independent experiments is shown. For D and E, the experiments are performed using T cells derived from two different donors. *** $p < 0.001$.

capable of mediating virus binding.

Blockade or knockdown of $\alpha_4\beta_7$ expression on CD4⁺ T cells decreases HIV-1 infection

Because HIV-1 is capable of binding to primary T cells by interacting with $\alpha_4\beta_7$, we wonder if targeting this integrin could circumvent HIV-1 infection of CD4⁺ T cells which serve as the main target of HIV-1 infection. Initially, we conducted experiments using anti- $\alpha_4\beta_7$ antibodies. $\alpha_4\beta_7$ expression on CD4⁺ T cells treated with or without RA was analyzed by FCM (Figure 3A). HIV-1 BaL infection of RA-treated CD4⁺ T cells was partially inhibited by blocking $\alpha_4\beta_7$ with HP2/1 or Act-1 (5 μ g/

mL), while blockade of CD4 with RPA-T4 (5 μ g/mL) almost completely inhibited virus infection (Figure 3B). However, anti- $\alpha_4\beta_7$ antibodies could induce cell aggregation (Parrish N F, et al., 2012), with Act-1 being more potent than HP2/1 and the aggregates became remarkably larger when cells were cultured in the presence of higher concentrations of stimulators (i.e., IL-2 plus PHA or OKT3). The phenomenon of anti-integrin induced cell aggregation has been used as a common approach to investigate integrin function as reported by several studies (Andrew D P, et al., 1994; Ruegg C, et al., 1992; Zeller Y, et al., 2001). The formation of cell aggregates increases cell-cell contact, which in turn enhances viral infection

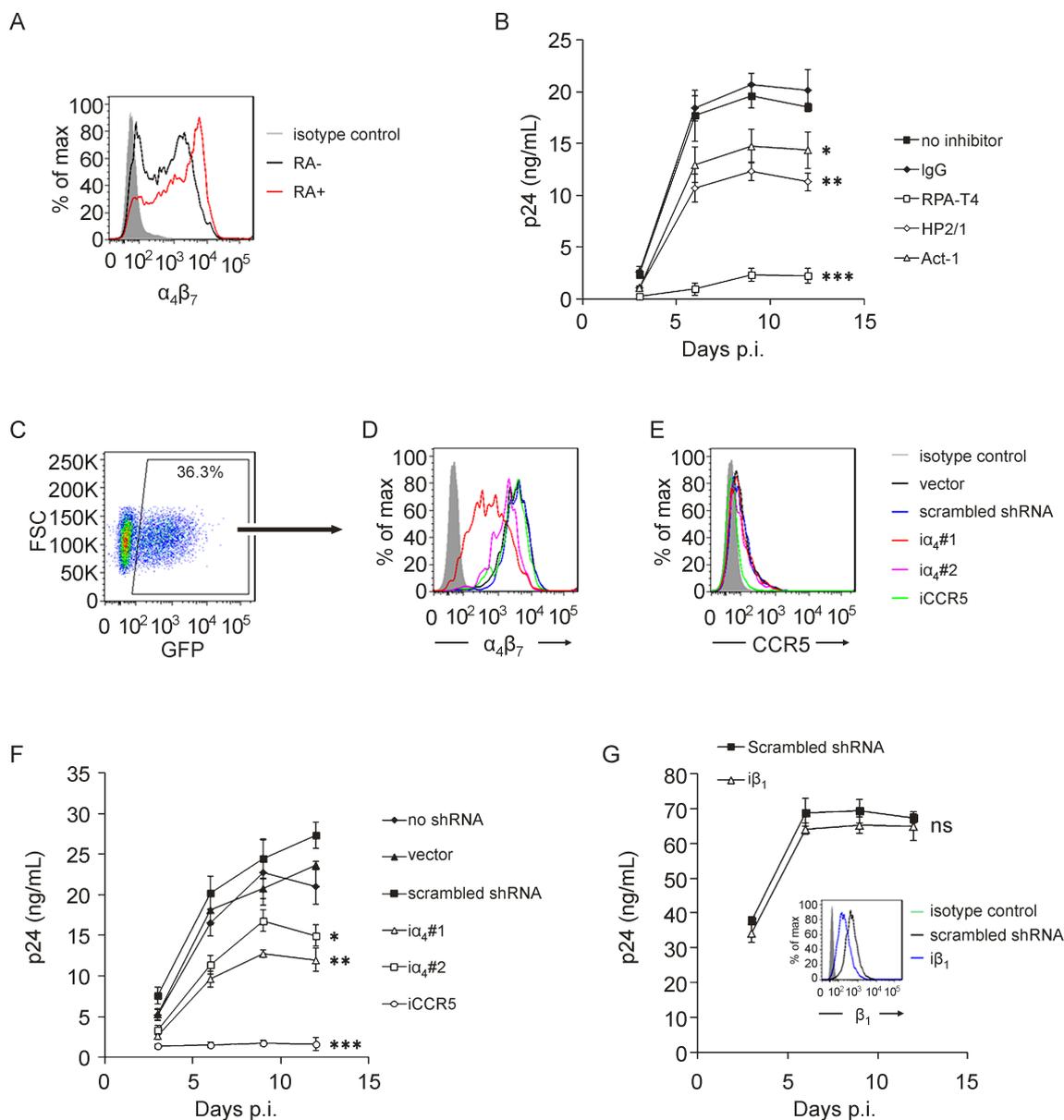


Figure 3. Impact of blocking $\alpha_4\beta_7$ by antibodies or down-regulating $\alpha_4\beta_7$ by RNA interference on HIV-1 infection of CD4⁺ T cells. A: $\alpha_4\beta_7$ expression on CD4⁺ T cells cultured w/ or w/o RA treatment was measured by FCM. B: Virus infection of RA-treated CD4⁺ T cells in the presence or absence of antibodies. After pre-incubation with anti- α_4 HP2/1, anti- $\alpha_4\beta_7$ Act-1, anti-CD4 RPA-T4 or control IgG (each 5 μ g/mL) for 1 hour at 37°C, 2×10^5 CD4⁺ T cells were infected with 2 ng p24 of HIV-1 BaL for 3 hours, followed by extensive washes to remove unbound virus. Antibodies were always present as indicated through the infection and subsequent culture procedure. Culture supernatants were collected every three days p.i. until 12 days p.i., lysed and stored at -80°C until p24 measurement. C-F: $\alpha_4\beta_7$ -activated CD4⁺ T cells were transduced with lentivirus carrying shRNA targeting integrin α_4 or HIV-1 coreceptor CCR5, non-targeting shRNA (scrambled) or vector alone. 4-6 days post transduction, the expression of $\alpha_4\beta_7$ (D) and CCR5 (E) were analyzed on positively transduced GFP⁺ lymphocytes (C). F: The positively transduced lymphocytes were sorted and subsequently infected with HIV-1 BaL. Virus infection and sample collection was conducted as described in (B). G: Infection of HIV-1 BaL in CD4⁺ T cells following transduction with shRNAs targeting β_1 or a non-targeting scrambled shRNA. The efficiency of β_1 -specific shRNA to down-regulating β_1 expression on CD4⁺ T cells was shown by the inset. Data shown are representative of three independent experiments using CD4⁺ T cells derived from different donors. For B and F, each condition was performed in sextuplicate and expressed as mean \pm SD. * $p < 0.05$ ** $p < 0.01$ and *** $p < 0.001$, compared to virus infection of CD4⁺ T cells in the presence of control IgG (B) or CD4⁺ T cells transduced with scrambled shRNA (F, G). ns, not significant.

(Alvarez R A, et al., 2011; Dale B M, et al., 2013; Liao Z, et al., 2000). This represents an important confounder and suggests that antibody engagement of $\alpha_4\beta_7$ is not an appropriate approach to determine the significance of $\alpha_4\beta_7$ in HIV-1 infection of primary lymphocytes (Parrish N F, et al., 2012).

We therefore chose an alternative method, short-hairpin RNA (shRNA) mediated interference, to investigate if disturbance of HIV-1- $\alpha_4\beta_7$ interaction affects HIV-1 infection. A panel of shRNAs were designed and constructed. The sequences of two α_4 shRNA candidates, a reported shRNA sequence targeting HIV-1 coreceptor CCR5 (iCCR5) (Qin X F, et al., 2003) and a non-targeting (scrambled) shRNA are shown in Table 1. The expression levels of $\alpha_4\beta_7$ and CCR5 on positively transduced cells (GFP⁺, Figure 3C) were monitored at various time points post transduction (p.t.). The knock-down effects of two α_4 s and the iCCR5 at 4 days p.t. (Figure 3E and 3F) remained constant up to 15 days p.t. (data not shown), consistent with the characteristics of shRNA-mediated interference. α_4 #1 and #2 knocked down $\alpha_4\beta_7$ expression with different efficiency without affecting CCR5 expression (Figure 3D and 3E), indicating that no significant off-target effect was produced. iCCR5 specifically down-regulated CCR5 expression to barely detectable levels (Figure 3E). Lentivirus infection did not cause evident damage to cell viability as measured by trypan blue staining.

The positively transduced GFP⁺ cells were sorted for HIV-1 infection. Knock-down of $\alpha_4\beta_7$ on CD4⁺ T cells by both α_4 #1 and #2 significantly reduced BaL infection (Figure 3F). Various efficacies between α_4 #1 and #2 in knock-down $\alpha_4\beta_7$ expression corresponded to an associ-

ated reduction in viral infection. Knock-down of CCR5 abrogated HIV-1 infection (Qin X F, et al., 2003), while viral infection was not significantly affected in groups transduced with scrambled shRNA or vector alone, when compared to groups without lentiviral transduction (sorted based on FSC and SSC parameters) (Figure 3F). Additionally, because integrin $\alpha_4\beta_1$, another member of integrin family, was observed to be capable of mediating low level of virus binding (Figure 1C), we determined whether interference of this integrin also affected HIV-1 infection. We found that although introduction of β_1 -specific shRNA resulted in a notable decrease of β_1 expression (the inset of Figure 3G), virus infection of CD4⁺ T cells was not significantly affected (Figure 3G). Collectively, the above results suggest that both blockade and knock-down of $\alpha_4\beta_7$ expression can decrease HIV-1 BaL infection.

DISCUSSION

The significance of $\alpha_4\beta_7$ in HIV-1 infection remains controversial. In particular, important concerns have been raised by recent studies regarding whether the ability of some gp120 proteins to engage $\alpha_4\beta_7$ can be recapitulated by HIV-1 virions (Parrish N F, et al., 2012). Our study therefore focused on the impact of gp120- $\alpha_4\beta_7$ interaction on HIV-1 binding and infection of cell line and primary cells. We demonstrated that expression of $\alpha_4\beta_7$ alone is enough to mediate HIV-1 binding. The binding was observed on $\alpha_4\beta_7$ -expressing transient and stable transfectants, and primary CD8⁺ and CD4⁺ T cells. Of note, we showed that blockade or down-regulation of $\alpha_4\beta_7$ expression on CD4⁺ T cells, the primary target of HIV-1

Table 1. Primers and shRNA oligos

Name	Sequence (5' to 3')	Usage
α_4 forward	GA ACTAGCTAGCG CATGGCTTGGGAAGCGAG ^a	PCR amplification of human
α_4 reverse	GCTCCTGC CTCGAGT CAATTTGAAAGAAGTCCTTAATC	α_4 from cDNA
β_7 forward	GAAACACG AATTCTT GGGATCTCGGGCATGGTGG	PCR amplification of human
β_7 reverse	CCTATTCTAG AGGGTAAGT GTCCCTCCCTCCTTCAGA	β_7 from cDNA
α_E forward	GA ACTAGCTAGCG CCTCCAGCAAGGATGTGGCTCTTC	PCR amplification of human
α_E reverse	TTATGC CTCGAGT CTCCAGTGGATAGCAGGTCC	α_E from cDNA
β_1 forward	AACGGA AATTCAAGAT GAATTTACAACCAATTTTCTG	PCR amplification of human
β_1 reverse	TAGCTCG TCTAGA AGTACTCATTTTCCCTCATACTTC	β_1 from cDNA
m. α_4 forward	GACCTAGCTAG CTGTT GAATGTTCTCCACCAAGAGCG	PCR amplification of mouse
m. α_4 reverse	TATGC CTCGAGG TCTTTCAGTCATCATTGCTTTTGTCT	α_4 from cDNA
m. β_7 forward	AATATG AATTCTG CTCCTCCTCAAGCACATGCCATG	PCR amplification of mouse
m. β_7 reverse	CACCTGG TCTAGA ACTGTCCCTCCAAGACAAGAATCCTAAGTC	β_7 from cDNA
r. α_4 forward	GACCTAGCTAG CTGTT GAATGTTCCCCACCAAGAGTG	PCR amplification of rat α_4
r. α_4 reverse	TTACCTCGAGAGT CCTCAGT CATCATTGCTTTTGTCTGT	from cDNA
r. β_7 forward	ACCTAGCTAG CGCCAT GGTGGATTCATCAACTGTTTC	PCR amplification of rat β_7
r. β_7 reverse	TTACCTCGAG GCTAAGT CAGTCAGCCTCCTGGGTCAG	from cDNA
ia $_4$ #1 forward	<u>TGCTCCGTGTTATCAAGATTATTTCAAGAGAATAATCTTGATAACA</u> <u>CGGAGCTTTTTTTG</u>	shRNA targeting α_4
ia $_4$ #1 reverse	<u>TCGACAAAAAAGCTCCGTGTTATCAAGATTATCTCTTGAAATAA</u> <u>TCTTGATAACACGGAGCA</u>	
ia $_4$ #2 forward	<u>TCGGGAGCAGTAATGAATGCAATTC AAGAGATTGCATTCACTACT</u> <u>GCTCCCGTTTTTTG</u>	shRNA targeting α_4
ia $_4$ #2 reverse	<u>TCGACAAAAAAGCGGGAGCAGTAATGAATGCAATCTCTTGAAATTG</u> <u>CATTCATTACTGCTCCCGA</u>	
i β_1 forward	<u>TGCCTTGCACTACTGCTGATAITTTCAAGAGAATATCAGCAGTAAT</u> <u>GCAAGGCTTTTTTTC</u>	shRNA targeting β_1
i β_1 reverse	<u>TCGAGAAAAAAGCCTTGCACTACTGCTGATAITTTCAAGAGAATAT</u> <u>CAGCAGTAATGCAAGGCA</u>	
iCCR5 forward	<u>TGAGCATGACTGACATCTACTTCAAGAGAGTAGATGTCAGTCAT</u> <u>GCTCTTTTTTTG</u>	shRNA targeting CCR5
iCCR5 reverse	<u>TCGACAAAAAAGAGCATGACTGACATCTACTCTCTTGAAAGTAGA</u> <u>TGTCAGTCATGCTCA</u>	
scrambled forward	<u>TCCTAAGGTTAAGTCGCCCTTTCAAGAGAAGGGCGACTTAACCT</u> <u>TAGGTTTTTTTG</u>	non-targeting shRNA
scrambled reverse	<u>TCGACAAAAAAGCTAAGGTTAAGTCGCCCTTCTCTTGAAAGGGC</u> <u>GACTTAACCTTAGGA</u>	

The underlined positions are enzymatic restriction sites, sense or anti-sense sequences of the oligos as implicated.

infection *in vivo*, resulted in decreased HIV-1 infection.

$\alpha_4\beta_7$ is not an indispensable receptor for HIV-1 entry (Arthos J, et al., 2008; Cicala C, et al., 2010; Cicala C, et al., 2009). We focused on investigating its role as an attachment factor. In agreement with previous reports (Arthos J, et al., 2008; Nawaz F, et al., 2011), we observed that *in vitro* cellular expression of human integrin $\alpha_4\beta_7$ conferred the capability for HIV-1 binding. Such binding likely increases the chance of HIV-1 binding to target cells. Our mutagenesis and pseudotyped virus experiments confirm that the $\alpha_4\beta_7$ -interacting site is located on the V2 loop of the envelope glycoprotein (unpublished data) (Arthos J, et al., 2008; McLellan J S, et al., 2011).

Recent electron cryotomography has shed lights on the three dimensional architecture of native Env spike (Liu J, et al., 2008), demonstrating that the V1/V2 domain is located on the top of Env spike. In addition, the recent low-resolution crystal structure of $\alpha_4\beta_7$ suggested that the long and wide groove between α_4 and β_7 subunits likely serves as the binding site for its ligands, including mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), Act-1 and a small antagonist (Yu Y, et al., 2012). Based on this finding, the crystal structure of a modeled full-length V1V2 loop was shown to be complementary to the groove of $\alpha_4\beta_7$, resembling the binding to its natural ligands (Spurrier B, et al., 2014). Although it

remains to be investigated, HIV-1 may utilize the V1V2 domain, protruding farthest away from viral membrane and nearest to the target cell, to interact with $\alpha_4\beta_7$ and therefore facilitate viral attachment with the maximum steric advantage.

Of interest, we found that human $\alpha_4\beta_1$ also exhibited the capability of mediating HIV-1 binding, albeit to a lesser extent. This is likely explained by that both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ belong to the α_4 integrin family and share common natural ligands including vascular cell adhesion molecule-1 (VCAM-1) and fibronectin, suggesting that structural similarity exists between them. Nevertheless, we observed that down-regulation of β_1 did not affect HIV-1 infection. This was likely due to a low level expression of $\alpha_4\beta_1$ on CD4⁺ T cells, and the observed marginal HIV-1 binding mediated by $\alpha_4\beta_1$ was unlikely to be sufficient to enhance viral infection. Given that $\alpha_4\beta_1$ is broadly distributed on many connective tissues in addition to lymphoid cells (Abram C L, et al., 2009; Yu Y, et al., 2012), targeting $\alpha_4\beta_1$ likely affected HIV-1 infection by changing cell-cell interactions. However, the physiological implications of interaction between gp120 and $\alpha_4\beta_1$ warrant further study.

By using anti- $\alpha_4\beta_7$ antibodies, we observed a decreased HIV-1 infection, demonstrating the potential significance of $\alpha_4\beta_7$. It is noteworthy that the presence of anti- $\alpha_4\beta_7$ antibodies could induce an obvious cell aggregation which varied from different antibodies used (Andrew D P, et al., 1994; Ruegg C, et al., 1992; Zeller Y, et al., 2001). Cell aggregation induced by $\alpha_4\beta_7$ antibodies promotes cell-cell contact, consequently facilitating cell-to-cell spread of HIV-1 infection. Therefore, $\alpha_4\beta_7$ antibodies likely have counteracting effects on HIV-1 infection. Here the negative impact of $\alpha_4\beta_7$ antibody blockade on virus binding to cells in suspension culture is likely offset by induced cellular aggregation, facilitating more efficient cell-to-cell spread of HIV-1. Whether virus infection was increased or decreased by antibody binding most likely depends on which one of the two effects is dominant. To avoid such confounders, we adopted shRNA-mediated interference to explore the impact of $\alpha_4\beta_7$ down-regulation on HIV-1 infection. We demonstrated that transduction of α_4 -specific shRNA significantly decreased HIV-1 infection. As a control, and consistent with a previous report (Qin X F, et al., 2003), CCR5-specific shRNA almost completely suppressed virus infection, confirming the indispensable role of CCR5 and the dispensable role of $\alpha_4\beta_7$ for HIV-1 infection.

Owing to the heterogeneity of HIV-1 strains, the conformation structure of Env trimer may vary markedly from strain to strain. It remains controversial that whether $\alpha_4\beta_7$ -utilization is a broad property across different HIV-1 strains, and the inconsistent observations from different groups may be attributed to differences in

viral strains, cell targets, and different experiment designs (Arthos J, et al., 2008; Nawaz F, et al., 2011; Parrish N F, et al., 2012; Perez L G, et al., 2014). In addition, given the structure flexibility of integrin $\alpha_4\beta_7$ and its multistep-regulated conformation (Luo B H, et al., 2007; Yu Y, et al., 2012), $\alpha_4\beta_7$ utilization by HIV-1 *in vivo* may be very complex. Study of transmitted/founder (T/F) HIV-1 strains which are isolated at very early stage of HIV-1 infection is of particular importance to dissect the mechanism of HIV-1 mucosal transmission and establishment of early infection (Keele B F, et al., 2008; Kishko M, et al., 2011). It is shown that the envelope glycoproteins of early-transmitted strains feature compact variable loops and less N-linked glycosylation sites compared to strains isolated during chronic infection (Chohan B, et al., 2005; Derdeyn C A, et al., 2004; Sagar M, et al., 2006). Whether T/F strains can efficiently utilize $\alpha_4\beta_7$ and identification of the sequence property of envelope proteins determining $\alpha_4\beta_7$ utilization are the other two interesting topics which remain to be further explored. In conclusion, our current study suggests that $\alpha_4\beta_7$ may serve as an attachment factor at least for some HIV-1 strains, providing a promising approach for investigating the potential roles of $\alpha_4\beta_7$ in HIV-1 infection.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflicts of interest. All human blood samples were collected under protocols approved by the Local Research Ethics Committee.

AUTHOR CONTRIBUTION

CL and QH conceived and designed the experiments. CL and JW performed the experiments. CL, RJS and QH analyzed the data. BW, TD, YL and RJS contributed to discussion. CL and QH wrote the paper.

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