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Core-shell nanoparticles for targeted and combination antiretroviral activity in gut-homing T cells

Shijie Cao, BS¹, Yonghou Jiang, PhD¹, Hangyu Zhang, PhD^{1,2,3}, Nina Kondza, BS¹, and Kim A. Woodrow, PhD^{1,*}

¹Department of Bioengineering, University of Washington, Seattle 98195, USA;

²Department of Biomedical Engineering, Faculty of Electronic Information and Electrical Engineering, Dalian University of Technology 116023, Dalian, China;

³Research Center for the Control Engineering of Translational Precision Medicine, Dalian University of Technology 116023, Dalian, China

Abstract

A major sanctuary site for HIV infection is the gut-associated lymphoid tissue (GALT). The $\alpha 4\beta 7$ integrin gut homing receptor is a promising therapeutic target for the virus reservoir because it leads to migration of infected cells to the GALT and facilitates HIV infection. Here, we developed a core-shell nanoparticle incorporating the $\alpha 4\beta 7$ monoclonal antibody (mAb) as a dual-functional ligand for selectively targeting a protease inhibitor (PI) to gut-homing T cells in the GALT while simultaneously blocking HIV infection. Our nanoparticles significantly reduced cytotoxicity of the PI and enhanced its *in vitro* antiviral activity in combination with $\alpha 4\beta 7$ mAb. We demonstrate targeting function of our nanocarriers in a human T cell line and primary cells isolated from macaque ileum, and observed higher *in vivo* biodistribution to the murine small intestines where they accumulate in $\alpha 4\beta 7+$ cells. Our LCNPs show the potential to co-deliver ARVs and mAbs for eradicating HIV reservoirs.

Graphic Abstract

*corresponding author: Kim A. Woodrow, PhD, W.H. Foege N410D, 3720 15th Avenue NE, Seattle, WA 98122-5061, Phone: (+1) 206 685 6831, woodrow@uw.edu.

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We developed core-shell nanoparticles incorporating the $\alpha 4\beta 7$ monoclonal antibody (mAb) for selectively delivering therapeutic agents to gut-homing T cells in the gut-associated lymphoid tissue (GALT), while simultaneously blocking HIV infection. We demonstrate enhanced antiretroviral activity of a protease inhibitor and $\alpha 4\beta 7$ mAb combination *in vitro*, and show targeting function in rhesus macaque primary cells and in mice. These data demonstrate that our LCNP delivery system has the potential to co-deliver ARV drugs and mAbs to anatomical and cellular HIV reservoirs for the purpose of reducing reservoir size and potentially eradicating the virus.

Keywords

Targeted drug delivery; lipid-polymer hybrid nanoparticles; $\alpha 4\beta 7$ monoclonal antibody; GALT; gut-homing T cell; HIV-1

Background

Combination antiretroviral therapy (cART) achieves complete virus suppression to undetectable levels in peripheral blood but does not lead to complete eradication of HIV infection.¹ The existence of viral reservoirs is the main obstacle to HIV cure.^{2–4} The gut-associated lymphoid tissue (GALT) is one of the largest lymphatic tissues in the human body where elevated levels of viral replication and depletion of CD4⁺ T cells occur in HIV-infected patients.^{5–8} HIV reservoirs can be quickly established in the GALT, where the virus becomes resistant despite long-term cART.⁹ Several mechanisms have been proposed to explain persistent HIV infection within the environment of the GALT. First, infected CD4⁺ T cells are able to traffic to the GALT from other parts of the body mediated by the $\alpha 4\beta 7$ integrin gut homing receptor.¹⁰ $\alpha 4\beta 7$ mediates migration by binding to the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) expressed by endothelial cells of venules associated with the GALT.^{11, 12} Second, gut mucosal CD4⁺ lymphocytes are uniquely susceptible to HIV-1 infection,^{7, 13, 14} which may be explained by a higher expression of chemokine receptor CCR5 (a major coreceptor for HIV infection) and persistent activation and inflammation due to constant exposure to food and microbial antigens.^{13, 15} In addition, studies have shown that $\alpha 4\beta 7$ can serve as a receptor for the HIV-1 envelope protein gp120

and promote the upregulation of lymphocyte function-associated antigen-1 (LFA-1) to facilitate cell-to-cell spreading of HIV-1.¹⁶ Resting central memory $\alpha 4\beta 7^{+}$ CD4⁺ T cells have been shown to be the predominant target of SIV during acute infection.^{7, 17} These cells become latently infected and have the potential to traffic to and reside in the GALT because of the expression of $\alpha 4\beta 7$. Lastly, concentrations of antiretroviral drugs (ARVs) in lymphatic tissues, including the GALT, are lower compared to those in the peripheral blood.¹⁸ This may attribute to both early reservoir establishment and virus resistance in patients under suppressive cART.¹⁹ Thus, enhancement of ARV drug delivery to these $\alpha 4\beta 7$ -expressing gut-homing T cells and the GALT during the early phase of infection might be a strategy to reduce the reservoir size. Collectively, these findings suggest that the $\alpha 4\beta 7$ cell marker is a promising therapeutic for HIV reservoir eradication by targeting gut-homing T cells.

Nanoparticle technology has been used with great promise in enhancing the efficacy of antiretroviral therapies.^{20, 21} We have previously shown that nanocarriers (NCs) formulated with physicochemically diverse ARVs delivered in combination result in higher intracellular drug concentrations, and lead to more synergistically potent inhibition of HIV-1 infection in cell and tissue cultures.²² Nanoparticles surface conjugated with targeting ligand have been used to promote the accumulation of nanoparticles to specific cells or tissues.^{23, 24} A variety of nanocarrier-based delivery systems have been used to target anti-HIV drugs to cellular or anatomical viral reservoirs utilizing either peptides or antibodies as the ligands.^{25–32} Immunoliposomes (antibody-coupled liposomes) have been the most widely investigated for this purpose and offer great flexibility for conjugating with targeted ligands.^{33–35} However, liposomes lack structural integrity and consistent storage stability.³⁶ Lipid-polymer hybrid nanoparticles, that consist of a polymer core and a lipid shell, combine the advantageous properties of both polymeric nanoparticles and liposomes such as biocompatibility, biodegradability, multiple drug encapsulation, high drug loading, sustained drug-release profiles, high stability, functionalizable surfaces, *etc.*^{37–41}

Here, we developed a nanocarrier-based delivery system using the $\alpha 4\beta 7$ mAb for its dual function to target ARV drugs to gut-homing T cells and blocking HIV infection. We synthesized a core-shell nanoparticle consisting of a poly(lactic-co-glycolic) acid (PLGA) core and a phospholipid bilayer shell coated with an outer layer of polyethylene glycol (PEG) that was used for direct conjugation of $\alpha 4\beta 7$ mAbs (A4B7-LCNPs). Our LCNPs were designed such that the lipid shell, including the attached antibody, would delaminate from these nanoparticles and be available to block virus entry.⁴² A protease inhibitor was also encapsulated in targeted LCNPs to prevent infected cells from producing new HIV virions in the GALT. Our *in vitro* and *ex vivo* data show that tipranavir (TPV) loaded A4B7-LCNPs exhibit the dual function of targeting CD4⁺ $\alpha 4\beta 7^{+}$ cells and anti-HIV activity. We also found that A4B7-LCNPs accumulated with $\alpha 4\beta 7^{+}$ gut T cells of the small intestine after intravenous administration to mice. These data demonstrate that our LCNP delivery system has the potential to co-deliver ARV drugs and mAbs to anatomical and cellular HIV reservoirs for the purpose of reducing reservoir size and potentially eradicating the virus.

Methods

Description of materials, preparation of LCNPs and liposomes, conjugation of $\alpha 4\beta 7$ mAb to LCNPs, characterization of LCNP formulations, antibody conjugation efficiency, TPV loading analysis, lipid and antibody delamination and TPV release kinetics, storage stability, cytotoxicity analysis, *in vitro* cell binding assay, HIV-1 infection assay and antiviral activity of TPV loaded A4B7-LCNPs, *ex vivo* rhesus macaque ileum cell isolation and A4B7-LCNP targeting assay, *in vivo* mice small intestine targeting, biodistribution of targeted LCNPs in major organs, and *in vivo* gut-homing T cell targeting is detailed in Supplementary Materials.

Results

Synthesis and Characterization of Targeted LCNPs Loaded with Tipranavir

We modified the commonly used single-emulsion evaporation method to fabricate nanoparticles with PLGA core that facilitate incorporation of a lipid bilayer shell (Figure 1A).^{37, 43} We chose a lipid composition of neutral (1,2-Dioleoyl-sn-glycero-3-phosphocholine, DOPC), and cationic (1,2-dioleoyl-3-trimethylammonium-propane, DOTAP) lipids at equimolar content to obtain a positive net charge for stabilizing the negative PLGA core. In addition, we incorporated 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG-MAL) at 11.1% molar ratio of total lipids. The DSPE-PEG-MAL has a maleimide end group for conjugating to antibodies, and a polyethylene glycol (PEG) chain that was expected to stabilize our nanoparticles. As expected, DSPE-PEG-MAL incorporation led to a significant increase in the LCNP diameter, and altered the surface charge from positive to neutral (Table 1). This change in surface charge is also beneficial for reducing non-specific binding to cells. Both LCNPs with or without PEG were stable when stored in MilliQ water at 4 °C or 37 °C (Figure S1A, Supporting Information). However, LCNPs without PEG rapidly aggregated to sizes >1 μ M in PBS at 37 °C whereas PEG-coated LCNPs maintained their original size over 2 days (Figure S1B, Supporting Information). We observed aggregation of LCNPs after lyophilization during long-term storage, but this issue could be avoided by adding trehalose before freezing the LCNPs (Figure S1d, Supporting Information).

We used $\alpha 4\beta 7$ mAb conjugated to the lipid layer shell of LCNPs for the dual-function of targeting gut-homing T cells and inhibiting HIV transmission. The antibody was conjugated to the DSPE-PEG-MAL on the surface of LCNPs and led to a modest but significant increase in diameter (Table 1). Transmission electric microscope (TEM) showed a low contrast material associated with the A4B7-LCNPs surface (Figure 1B, C), which we attributed to be the lipid layer and antibodies surrounding the PLGA core. The surface density and efficiency of antibody conjugation to LCNPs was optimized using a Dylight 633-labeled $\alpha 4\beta 7$ mAb. The fluorescently-labeled antibody was conjugated to LCNPs at molar ratios from 100:1 to 2000:1 and then analyzed by flow cytometry to quantify the amount of mAb that would saturate the fluorescent signal on the LCNP. We observed that the mean fluorescent intensity (MFI) saturated at a molar ratio of 1000:1 (Figure 1C). We used Nanoparticle Tracking Analysis (NTA) to obtain the number concentration of LCNPs and TECAN fluorescent plate reader to analyze the conjugation efficiency, which is the

percentage of mAb conjugated to LCNPs relative to the input feed amount. Based on these values, we calculated the number of mAb per LCNP (Table 2). As expected, the efficiency of mAb conjugation was reduced as the input molar ratio of mAb to LCNPs was increased. We reached a conjugation efficiency of 30.8% for the 1000:1 input molar ratio of mAb to LCNPs. With this feed ratio, we achieved the highest surface density while maximizing conjugation efficiency since we expected to deliver $\alpha 4\beta 7$ mAb not only as a targeting ligand for gut T cells but also as a blocking agent to prevent HIV-1 from binding to $\alpha 4\beta 7^+$ cells. The loading of $\alpha 4\beta 7$ mAb on LCNPs at this feed ratio was 2.16 ± 0.10 wt% (w/w, weight of mAb relative to weight of LCNPs) measured by the micro bicinchoninic acid (BCA) assay.

A potential benefit of nanoparticles is the ability to incorporate physicochemically diverse drugs into the same delivery vehicle, which may increase drug potency through higher intracellular concentration and synergistic mechanisms.²² We employed the $\alpha 4\beta 7$ mAb to target gut-homing T cells and also used it to inhibit HIV transmission by blocking the $\alpha 4\beta 7$ receptor on uninfected T cells. We also decided to incorporate a protease inhibitor into the same nanoparticle with the aim of increasing drug concentrations in the GALT as well as inhibiting viral production from HIV infected gut-homing T cells. We chose tipranavir (TPV) due to its high potency, low solubility and reported toxicity.^{44, 45} Nanoparticle-based delivery systems have the potential to improve solubility and bioavailability of poorly water-soluble drugs and may also improve the safety profile of TPV.^{46, 47} To this end, we incorporated TPV into the LCNPs with initial drug input of 10 wt% (w/w, weight of TPV relative to weight of PLGA and lipids) which led to 8.0 ± 0.1 wt% TPV loading in LCNPs.

Encapsulation of TPV did not significantly change the size, polydispersity index (PDI) or ζ -potential of the A4B7-LCNPs (Table 1). After conjugation, we obtained a measured drug loading of 4.7 ± 0.2 wt% of TPV-loaded A4B7-LCNPs (TPV/A4B7-LCNPs), which translated to an encapsulation efficiency of 45.7 ± 3.4 %. The observed decrease in TPV loading of A4B7-LCNPs was mainly due to burst release of TPV from LCNPs during the antibody conjugation process and was consistent with the TPV release kinetics as described below. We also measured drug loading in our LCNP and conventional liposomes, and found a two-fold higher TPV loading and a 9-fold higher encapsulation efficiency in untargeted LCNPs compared to untargeted liposomes (Figure S2, Supporting Information). Our TPV/A4B7-LCNPs were stable in human serum over at least 14 days (Figure S1C, Supporting Information).

Release Kinetics of Lipids, $\alpha 4\beta 7$ mAb and TPV from LCNPs

Spontaneous delamination of the lipid shell from LCNPs has been previously documented and was used in our case to deliver the $\alpha 4\beta 7$ mAb.⁴² However, delamination could also lead to loss of targeting function. We characterized the delamination kinetics of our A4B7-LCNPs in PBS to ensure there would be sufficient antibodies remaining on the LCNPs to maintain nanoparticle targeting function over at least two days. We first characterized the delamination kinetics of DOPC and DSPE-PEG from LCNPs in PBS by using fluorescently-labeled versions of these lipids. We observed that DOPC and DSPE-PEG were stably associated with LCNPs at 4 °C but delaminated at 37°C, resulting in a 35% loss of both lipids after 48 hours (Figure S3, Supporting Information). We also directly measured Dylight

688 dye labeled $\alpha 4\beta 7$ mAb delamination and observed a faster delamination in human serum than in PBS (Figure 2A). Even after delamination, we measured that 60% of the $\alpha 4\beta 7$ mAb remained associated with the nanoparticles in human serum over 2 days, which is sufficient for maintaining targeting properties. In our case, delaminated antibodies that bind $\alpha 4\beta 7$ -expressing cells in the GALT was intended to further inhibit HIV transmission.

We also measured TPV release in sink conditions to inform potential drug bioavailability once dosed *in vivo*. Since TPV is rapidly bound to plasma proteins (>99%), including serum albumin and α_1 -acid glycoprotein,⁴⁸ we expected that BSA could be used as a solubilizing agent in the release media. In fact, we found that 50 mg/mL BSA in PBS increased TPV solubility by nearly 2500-fold. Using *in vitro* sink-conditions established with 50 mg/mL BSA in PBS (pH 7.4) or human serum, we observed rapid TPV release from A4B7-LCNPs of up to 80% after 24 hours (Figure 2B). Since we observed that nanoparticles reach the gut by 6 hours following intravenous administration as described below, and 40% of TPV remained associated with our LCNP at this time. We expect that this amount of delivered TPV is sufficient for antiviral effectiveness due to its high potency. A single dose of 600 mg/kg TPV/A4B7-LCNPs every two days would deliver a daily dose of ~800 mg TPV and ~140 mg $\alpha 4\beta 7$ mAb based on their loading and release profiles, which is comparable to their currently prescribed or reported dosing.^{49, 50}

A4B7-LCNPs Decrease Cytotoxicity of TPV

Encapsulation of hydrophobic drugs in biodegradable and non-toxic nanoparticles can protect drugs from degradation, increase their circulation half-life and exhibit improved pharmacokinetics profiles thereby lowering toxicity.⁵¹ Also, targeted nanoparticle-based delivery systems can increase the physiological concentration of drugs at target sites and minimize off-target binding. Here, we compared cytotoxicity of free TPV and LCNP-encapsulated TPV in the HUT-78 human T cell line. We chose HUT-78 cells for our *in vitro* studies since they exhibit high $\alpha 4\beta 7$ integrin expression compared with other T cells lines we tested (Figure S4A, Supporting Information), and their $\alpha 4\beta 7$ expression has also been confirmed by others.⁵² HUT-78 cells were treated with TPV, TPV/LCNPs or TPV/A4B7-LCNPs for two days and cell viability was measured by monitoring metabolic activity. Untargeted TPV/LCNPs and targeted TPV/A4B7-LCNPs were found to be less cytotoxic as measured by their higher half-maximal cytotoxic concentrations (CC_{50}), as 77.01 $\mu\text{g/mL}$ (95% confidence interval (CI) = 66.10 to 89.73, TPV/LCNP) and 62.94 $\mu\text{g/mL}$ (95% CI = 48.11 to 82.34, TPV/A4B7-LCNP) compared to that of free TPV as 32.01 $\mu\text{g/mL}$ (95% CI = 30.06 to 34.07) (Figure 3A). No cytotoxicity was observed for either LCNPs or A4B7-LCNPs vehicle controls (Figure S5, Supporting Information). Such reduced cytotoxicity might be explained by sustained release of TPV from LCNP formulations compared to the acute bolus of free drug.

Antiretroviral Activity of TPV Loaded A4B7-LCNPs In Vitro

We performed an anti-HIV-1 assay in HUT-78 cells to evaluate the activity of LCNPs after TPV encapsulation or chemical conjugation with the targeting antibody. Cells were pre-treated for one hour before being challenged with HIV-1_{SF2}. After incubation for 10 days, culture supernatant was collected and tested for HIV-1 antigen production using HIV-1 p24

ELISA. We dosed TPV/LCNPs and TPV/A4B7-LCNPs based on total TPV loading and the free TPV, $\alpha 4\beta 7$ mAb, and A4B7-LCNPs, were dosed at equivalent concentrations in all treatment groups. The potencies of TPV-LCNPs ($27.9 \pm 1.9\%$ HIV-1 inhibition) or A4B7-LCNPs ($34.3 \pm 5.5\%$ HIV-1 inhibition) were similar compared to free TPV ($22.5 \pm 4.9\%$ HIV-1 inhibition) or $\alpha 4\beta 7$ mAb ($32.4 \pm 6.7\%$ HIV-1 inhibition), respectively (Figure 3B). When free TPV and $\alpha 4\beta 7$ mAb are delivered in combination, they showed higher HIV-1 inhibition ($45.3 \pm 14.6\%$) but the difference was not statistically significant. However, we found that combination of TPV and $\alpha 4\beta 7$ mAb in our LCNPs (TPV/A4B7-LCNP, $52.5 \pm 3.8\%$ HIV-1 inhibition) led to a significantly higher potency than either free TPV or $\alpha 4\beta 7$ mAb, as well as formulated TPV/LCNP or A4B7-LCNP. We used the Bliss independent model to quantitate combined effects of TPV and $\alpha 4\beta 7$ mAb, and demonstrated that they displayed synergy in our TPV/A4B7-LCNPs ($f_{a_{xy}} = 0.05 > 0$).

Specific Targeting of A4B7-LCNPs to CD4+ $\alpha 4\beta 7$ + Cells from Rhesus Macaque Ileum

Since $\alpha 4\beta 7$ integrin mediates T cell migration to the GALT and also serves as a target of HIV, we were interested in investigating if our A4B7-LCNPs could specifically target these $\alpha 4\beta 7$ -expressing cells in the GALT. First, we used flow cytometry to analyze the binding of rhodamine B labeled A4B7-LCNPs to HUT 78 cells. Bare LCNPs without antibody or isotype IgG conjugated LCNPs (Iso-LCNPs) were used as the non-targeted control. To ensure that the rhodamine B remained conjugated with LCNPs, we performed *in vitro* release studies and found that less than 5% of rhodamine B released from A4B7-LCNPs over 24 hours in PBS at 37 °C. We observed that the association of A4B7-LCNPs to HUT-78 cells was significantly higher than bare LCNPs or Iso-LCNPs by comparing the MFI (Figure S4C, Supporting Information). While all LCNP formulations had nonspecific bindings to HUT-78 cells, including bare LCNPs and Iso-LCNPs, the MFI of HUT-78 cells treated with A4B7-LCNPs was two-fold higher than the cells treated with control LCNPs, which we expect is due to specific antibody- $\alpha 4\beta 7$ integrin interactions. We also performed a receptor blocking study to investigate the targeting specificity of our antibody-conjugated LCNPs, and observed significant but not a full reduction of cell binding after blocking with $\alpha 4\beta 7$ mAb. This can partially be explained by our observation that binding of the antibody to HUT-78 cells was reversible (Figure S4D, Supporting Information), which was indicated by a decrease in MFI over time following 30 min incubation with fluorescently-labeled $\alpha 4\beta 7$ mAb. We hypothesize that this could result in a competitive binding between A4B7-LCNP and free antibody.

Next, we tested the targeting function of A4B7-LCNPs for lamina propria lymphocytes (LPLs) isolated from the ileum of a rhesus macaque. Analysis of LPLs collected from the ileum showed a frequency of 36% CD4+ cells, 10% $\alpha 4\beta 7$ + cells and 6% of cells that were both CD4+ and $\alpha 4\beta 7$ + (Figure 4A). All cells were treated with either our A4B7-LCNPs or Iso-LCNPs control. LPLs treated with A4B7-LCNPs showed rhodamine B fluorescent was associated with most CD4+ $\alpha 4\beta 7$ + cells but not CD4- $\alpha 4\beta 7$ - cells (Figure 4B). Iso-LCNP control did not show any significant association with either CD4+ $\alpha 4\beta 7$ + or CD4- $\alpha 4\beta 7$ - cells (Figure 4C). Comparison of the geometric mean fluorescent intensity (GMFI) showed that our A4B7-LCNPs had up to four-fold higher association with CD4+ $\alpha 4\beta 7$ + cells compared to cells negative for both markers or cell populations treated with Iso-LCNPs

(Figure 4D). These results demonstrated that A4B7-LCNPs targeted gut-homing T cells through a receptor-mediated process and could be used to potentially target CD4+ α 4 β 7+ cells in gut, thereby diminishing off-target effects and enhancing bioavailability in target cells.

A4B7-LCNPs Enhance Accumulation in Mouse Small Intestine and Target α 4 β 7+ Cells

In order to understand if α 4 β 7 mAb could enhance LCNP accumulation in the gut by targeting α 4 β 7+ cells, we administered fluorescently-labeled A4B7-LCNPs into mice by tail vein injection. To avoid autofluorescence, especially from the feces in the gut, we used a near-infrared dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR), encapsulated into LCNPs. We performed *in vitro* release studies to ensure that the DiR remained associated with A4B7-LCNPs, and found that less than 7% of DiR released from A4B7-LCNPs over 24 hours in PBS at 37 °C. Compared to treatment with Iso-LCNPs, mice treated with A4B7-LCNPs showed significantly higher distribution of fluorescent particles in small intestines at 6 hours after administration (Figure 5A, B). There were no significant differences in nanoparticle distribution to other organs between A4B7-LCNP and Iso-LCNPs groups (Figure 5C). We also found that nanoparticles quickly accumulated in the small intestine within 6 hours after administration but were eliminated by 24 hours (Figure 5A). We performed histological examination of major organs after administering 50 mg/kg A4B7-LCNPs, Iso-LCNPs or the same volume of PBS dosed intravenously to mice. All the organs from three treatment groups displayed no morphological difference, indicating LCNPs were well tolerated. (Figure S7, Supporting Information).

We also measured nanoparticle binding to LPLs isolated from small intestines of mice at 12 hours. We observed that 19% of α 4 β 7+ cells but not α 4 β 7- from isolated mouse LPLs were associated with A4B7-LCNPs. In the control groups, no nanoparticle association was observed for any cell populations, indicating cell-specific binding of A4B7-LCNPs to gut-homing T cells when delivered to the small intestine (Figure 6). Such specific gut-homing T cell targeting *in vivo* might explain the observed higher accumulation of A4B7-LCNPs in the small intestine.

Discussion

Gut-homing T cells are attracting more attentions in the HIV field since they are actually infected at an early stage of infection and can traffic to the GALT within days of infection, leading to rapid virus replication, reservoir establishment and resistance to cART. Targeting these cells offers possible strategies to inhibit HIV transmission at specific reservoir sites and eradicate latent virus when combined with other therapeutics. In this work, we developed a core-shell nanoparticle surface modified with the α 4 β 7 mAb for selectively targeting therapeutics to gut-homing T cells, which are cells that play an important role in HIV infection and drug resistance. Our A4B7-LCNPs delivered TPV, a potent protease inhibitor, and used the α 4 β 7 mAb for its known capacity to inhibit HIV transmission, reduce virus load, and sustain virologic control.^{49, 50, 53, 54}

We designed and optimized our nanoparticles to meet the following criteria: (1) conjugation with antibodies for targeting gut-homing T cells, (2) encapsulation of hydrophobic drugs, (3)

ability to release both antibody and drug from the nanoparticle, (4) stability in physiological and storage conditions, and (5) biodegradability and biocompatibility. Our nanoparticles decorated with targeting antibodies were expected to significantly improve therapeutic effectiveness while reducing toxicity.⁵⁵ Since we also aimed to deliver antibody and antiretroviral drugs as therapeutic agents to inhibit HIV transmission, achieving appropriate antibody and drug release kinetics from LCNPs for targeting was important.

TPV and $\alpha 4\beta 7$ mAb co-delivered from our core-shell LCNPs retained and showed enhanced antiviral activity compared to the free drugs in preventing HIV-1_{SF2} infection of the HUT-78 human T cell line *in vitro*. While the mechanism for the antiviral activity of $\alpha 4\beta 7$ mAb is not fully understood, the $\alpha 4\beta 7$ integrin has been hypothesized to serve as a binding site for HIV-1 and facilitate cell-to-cell virus spreading through LFA-1.¹⁶ Therefore, blocking $\alpha 4\beta 7$ with a monoclonal antibody may inhibit the virus from binding and prevent infection. As described in our *in vitro* and *ex vivo* targeting studies, we hypothesize that this combined effect might enhance cell binding to A4B7-LCNPs and cause increased local concentrations of the drugs.

This work is significant because targeting gut-homing T cells with the $\alpha 4\beta 7$ mAb also enhanced biodistribution of LCNPs to the small intestines when delivered intravenously to mice compared to the isotype control mAb. Furthermore, we confirmed that A4B7-LCNPs accumulated in $\alpha 4\beta 7$ + cells in the small intestines. Quantification of the dose that actually reached the major organs using DiR-labeled LCNPs also showed biodistribution of A4B7-LCNP in small intestines, liver and spleen. However, we observed specific accumulation of our A4B7-LCNP to the small intestine whereas the liver and spleen showed non-specific accumulation of the nanoparticles (Figure S8, Supporting Information). Radiolabeled nanoparticles could be used in the future to obtain more accurate quantification of dose biodistribution since we observed that recovery efficiency using fluorescence was low and tissue- and dose-dependent. Other studies have also shown that liposomes, PLGA nanoparticles and silica nanoparticles accumulate in the liver and spleen and attribute this finding to clearance processes and the fact that these organs are highly vascular.^{56, 57} As such, future work will need to focus on increasing circulation half-life.

One of the biggest obstacles for curing HIV is the existence of HIV reservoirs, which cannot be cleared by current antiretroviral therapy. Targeting anti-HIV drugs to HIV reservoirs has been investigated for decades, but has focused mainly on targeting CD4+ T cells or macrophages, in the lymph nodes or brain.^{25, 26, 29–31, 58} Targeting gut-homing T cells has not been investigated although they are a major HIV reservoir.^{14, 17} In addition, studies have demonstrated that ARV drug concentrations in gut-associated lymphatic tissues (GALT) is 99% lower than what is found in the blood and can lead to reservoir persistence.¹⁸ Based on the TPV and $\alpha 4\beta 7$ mAb loading in our LCNPs and their release data, we expect to improve the currently prescribed dosing of these therapeutics by only requiring a single dose once every two days to administer both drugs.^{49, 50} In addition, our data indicates that co-delivery of the $\alpha 4\beta 7$ mAb and TPV together in a single LCNP had a modest but significant improvement in HIV inhibition compared to co-administration of the drugs separately. We also do not account for any improvement in efficacy that might arise from the targeting function of our formulations. The rapid accumulation of our A4B7-LCNPs carrying both

TPV and antibodies in the gut may provide a potential strategy to combat HIV-1 at an early stage and minimize HIV reservoir size. Moreover, our nanoparticles have the potential to target additional cellular or anatomical reservoirs when conjugated with other targeting ligands,⁵⁹ and deliver multiple agents such as latency reversing agents, HIV vaccines, neutralizing antibody, immune checkpoint inhibitors and gene-modifying oligonucleotide drugs for the eradication of HIV reservoirs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

$\alpha 4\beta 7$ mAb	monoclonal antibody against $\alpha 4\beta 7$ integrin
A4B7-LCNP	LCNP surface conjugated with $\alpha 4\beta 7$ mAb
APC anti-$\alpha 4\beta 7$	$\alpha 4\beta 7$ mAb conjugated to allophycocyanin
ARV	antiretroviral drug
cART	$\alpha 4\beta 7$ integrin combination antiretroviral therapy
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CCR5	C-C chemokine receptor type 5
DiR	1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide
DL	drug loading
DLS	dynamic light scattering
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOPC-NBD	1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DSPE-PEG-MAL	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]

DSPE-PEG-CF	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N'-carboxyfluorescein]
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GALT	gut-associated lymphoid tissue
HPLC	high-performance liquid chromatography
Iso-LCNP	LCNP surface conjugated with isotype control IgG mAb
LCNP	lipid-coated poly(lactic- <i>co</i> -glycolic) acid nanoparticles
LFA-1	lymphocyte function-associated antigen-1
LPAM-1	the mouse Peyer's patch adhesion molecule 1 or $\alpha 4\beta 7$ integrin
LPL	lamina propria lymphocytes
mAb	monoclonal antibody
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
NC	nanocarrier
NTA	nanoparticle tracking analysis
PDI	polydispersity index
PEG	polyethylene glycol
PLGA	poly(lactic- <i>co</i> -glycolic) acid
PVA	polyvinyl alcohol
STLV	simian T-cell lymphotropic virus
TEM	transmission electric microscope
TPV	tipranavir
TPV/LCNP	TPV loaded LCNP
TPV/A4B7-LCNP	TPV loaded LCNP surface conjugated with $\alpha 4\beta 7$ mAb

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Figure 1.

(A) Schematic illustration of the A4B7-LCNP fabrication process using a modified single-emulsion evaporation method. (B, C) TEM images of LCNPs (B) and LCNPs surface conjugated with $\alpha 4\beta 7$ mAb (A4B7-LCNPs) (C). (D) Mean fluorescence intensity (MFI) of LCNPs conjugated with fluorescent DyLight 633 labeled $\alpha 4\beta 7$ mAb measured by flow cytometry. Data represents mean $\pm$ SD, n=3.

Figure 2.

(A) Delamination of DyLight 680 labeled $\alpha 4\beta 7$ mAb from LCNPs in PBS at 37 °C or 4 °C, or in human serum at 37 °C over 48 hours. (B) Release kinetics of TPV from A4B7-LCNPs in PBS, PBS with 50 mg/mL BSA, or human serum at 37 °C over 48 hours. Data represents mean $\pm$ SD, n=3.

Figure 3.

LCNPs reduce cytotoxicity of TPV and enhance antiviral activity of TPV in combination with $\alpha 4\beta 7$ mAb. (A) Cell viability of HUT-78 cells after incubation with TPV, TPV/LCNP or TPV/A4B7-LCNPs at different concentrations for 2 days. (B) Anti-HIV activities of TPV, $\alpha 4\beta 7$ mAb, Iso mAb, a combination of free TPV and $\alpha 4\beta 7$ mAb, TPV and Iso mAb, TPV/LCNPs, A4B7-LCNPs or TPV/A4B7-LCNPs. HUT-78 cells were treated with free drugs or LCNP formulations for 1 hour followed by challenge with HIV-1_{SF2} for 10 days. HIV-1 p24 in the supernatant was measured by ELISA and percent inhibition was calculated as a reduction of HIV-1 production relative to untreated infected cell controls. Data represents mean $\pm$ SD, n=3, *p<0.05, ** p<0.005, ***p<0.0005, n.s., not statistically significant.

Figure 4.

A4B7-LCNPs specifically bind to CD4+ $\alpha 4\beta 7$ + cells from lamina propria lymphocytes (LPLs) isolated from rhesus macaque ileum. (A) Flow cytometry dot plot analysis of isolated LPLs stained with anti-CD4 FITC and anti- $\alpha 4\beta 7$ APC (orange dots) or their respective isotype controls (black dots), indicating CD4+ $\alpha 4\beta 7$ + or CD4- $\alpha 4\beta 7$ - cell subsets. (B, C) LPLs were treated with rhodamine B (Rhod B) conjugated A4B7-LCNPs (B) or Iso-LCNPs (C), and labeled with anti-CD4 FITC and anti- $\alpha 4\beta 7$ APC antibodies. Histogram curves represent Rhod B fluorescent signals from untreated LPLs (black), LCNP-treated CD4- $\alpha 4\beta 7$ - LPL subsets (gray), or LCNP-treated CD4+ $\alpha 4\beta 7$ + LPL subsets. (D) Corresponding bar graphs of geometric mean fluorescence intensity (GMFI) are presented for A4B7-LCNPs or Iso-LCNPs binding to different subsets of LPLs. Data represents mean $\pm$ SD, n=3, ***p < 0.0005.

Figure 5. $\alpha 4\beta 7$ mAb enhances LCNP accumulation in mouse small intestine compared to isotype control mAb. (A) Representative fluorescent images of mouse small intestines at 3, 6, 12, or 24 hours after intravenous administration of DiR loaded A4B7-LCNPs or Iso-LCNPs to mice. (B) Dot graph of total radiant efficiencies from Xenogen images of mouse small intestines at 6 hours post-administration of DiR loaded A4B7-LCNPs, Iso-LCNPs or PBS. (C) Dot graphs of total radiant efficiencies from Xenogen images of other organs (liver, kidney, spleen, lung and heart) and average radiant efficiencies from 100 μ L plasma. Data represents mean $\pm$ SD, *** $p < 0.0005$. $n = 8$ mice per group.

Figure 6.

A4B7-LCNPs target $\alpha 4\beta 7^+$ cells among lamina propria lymphocytes (LPLs) from mouse small intestines at 12 hours post-administration. (A) Flow cytometry dot plot analysis of LPLs isolated from mouse small intestines indicating cells stained with anti- $\alpha 4\beta 7$ APC (orange) or isotype control (gray). (B) Percentage of $\alpha 4\beta 7^+$ cells associated with rhodamine B conjugated A4B7-LCNP or Iso-LCNP analyzed by flow cytometry. (C-E) Representative histograms of flow cytometry analysis for $\alpha 4\beta 7^+$ (red) or $\alpha 4\beta 7^-$ (gray) LPL subsets associated with LCNPs. Data represents mean $\pm$ SD, n=4 mice per group.

Table 1.

Physical properties of LCNP formulations. *

Sample [†]	Size (d, nm)	PDI [‡]	ζ-potential (mV)
LCNP (No PEG)	142 ± 5 [§]	0.10 ± 0.03	29.3 ± 1.2 [#]
LCNP	172 ± 2 ^{§,}	0.08 ± 0.02	1.2 ± 2.0
A4B7-LCNP [¶]	204 ± 18	0.26 ± 0.10	2.1 ± 1.5
TPV/LCNP	178 ± 1	0.07 ± 0.01	4.4 ± 0.7
TPV/A4B7-LCNP [¶]	233 ± 31	0.29 ± 0.05	2.1 ± 0.3

* Samples were diluted in Milli-Q water for size and PDI measurements and in 10 mM NaCl solution (pH = 7.0) for ζ-potential measurements. Data presents mean ± SD of at least three independent preparations. LCNP, lipid-coated PLGA nanoparticles.

[†] Lipids composition for LCNP is 4:4:1 DOPC/DOTAP/DSPE-PEG-MAL (mol/mol/mol), except for LCNP (No PEG), which omits the DSPE-PEG-MAL but retains the other lipids at the same amounts (4:4 DOPC/DOTAP).

[‡] PDI: Polydispersity index.

[§] Significant difference at the 0.001 probability level.

^{||} Significant difference at the 0.05 probability level.

[¶] NS, nonsignificant difference between A4B7-LCNP and TPV/A4B7-LCNP groups in size, PDI and ζ-potential.

[#] Significant difference between LCNP (No PEG) and any of the other groups at the 0.0001 probability level.

Table 2.

Average content of antibody conjugated to the surface of LCNPs.

Sample [*]	Feed ratio of mAb to LCNP (mol/mol)	Conjugation efficiency [†]	Number of mAb per LCNP [‡]
A4B7-LCNP (100)	100	45%	45
A4B7-LCNP (200)	200	54%	108
A4B7-LCNP (500)	500	41.2%	206
A4B7-LCNP (1000)	1000	30.8%	308
A4B7-LCNP (2000)	2000	20.2%	405

^{*} A4B7-LCNP, lipid-coated PLGA nanoparticles surface conjugated with α4β7 mAb at different molar ratios (100:1 ~ 2000:1, mAb to LCNP)

[†] Conjugation efficiency = $\frac{\text{amount of mAb associated with LCNPs}}{\text{total amount of mAbs added}}$

[‡] Number of conjugated mAb per LCNP = $\frac{\text{number concentration of total mAb conjugated to LCNP}}{\text{number concentration of total LCNPs}}$ = concentration of mAb

$\times \frac{N_A}{\text{m.w. of mAb}} \times \frac{1}{\text{number concentration of LCNPs}}$

N_A : the Avogadro constant.

m.w. of mAb: the Avogadro constant.

m.w. of mAb: molecular weight of mAb, 15,000 g/mol.