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Activation of HIV-1 reservoirs and induction of anti–HIV-1 T cells are critical to control HIV-1 rebound after combined antiretroviral therapy (cART). Here we evaluated in humanized mice (hu-mice) with persistent HIV-1 infection the therapeutic effect of TLR3 agonist and a CD40-targeting HIV-1 vaccine, which consists of a string of 5 highly conserved CD4+ and CD8+ T cell epitope-rich regions of HIV-1 Gag, Nef, and Pol fused to the C-terminus of a recombinant anti-human CD40 antibody (αCD40.HIV5pep). We show that αCD40.HIV5pep vaccination coadministered with poly(I:C) adjuvant induced HIV-1–specific human CD8+ and CD4+ T cell responses in hu-mice. Interestingly, poly(I:C) treatment also reactivated HIV-1 reservoirs. When administrated in therapeutic settings in HIV-1–infected hu-mice under effective cART, αCD40.HIV5pep with poly(I:C) vaccination induced HIV-1–specific CD8+ T cells and reduced the level of cell-associated HIV-1 DNA (or HIV-1 reservoirs) in lymphoid tissues. Most strikingly, the vaccination significantly delayed HIV-1 rebound after cART cessation. In summary, the αCD40.HIV5pep with poly(I:C) vaccination approach both activates replication of HIV-1 reservoirs and enhances the anti–HIV-1 T cell response, leading to a reduced level of cell-associated HIV-1 DNA or reservoirs. Our proof-of-concept study has significant implication for the development of CD40-targeting HIV-1 vaccine to enhance anti–HIV-1 immunity and reduce HIV-1 reservoirs in patients with suppressive cART.

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TLR3 agonist and CD40-targeting vaccination induces immune responses and reduces HIV-1 reservoirs

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Activation of HIV-1 reservoirs and induction of anti-HIV-1 T cells are critical to control HIV-1 rebound after combined antiretroviral therapy (cART). Here we evaluated in humanized mice (hu-mice) with persistent HIV-1 infection the therapeutic effect of TLR3 agonist and a CD40-targeting HIV-1 vaccine, which consists of a string of 5 highly conserved CD4+ and CD8+ T cell epitope-rich regions of HIV-1 Gag, Nef, and Pol fused to the C-terminus of a recombinant anti-human CD40 antibody (αCD40.HIV5pep). We show that αCD40.HIV5pep vaccination coadministered with poly(I:C) adjuvant induced HIV-1–specific human CD8+ and CD4+ T cell responses in hu-mice. Interestingly, poly(I:C) treatment also reactivated HIV-1 reservoirs. When administrated in therapeutic settings in HIV-1–infected hu-mice under effective cART, αCD40.HIV5pep with poly(I:C) vaccination induced HIV-1–specific CD8+ T cells and reduced the level of cell-associated HIV-1 DNA (or HIV-1 reservoirs) in lymphoid tissues. Most strikingly, the vaccination significantly delayed HIV-1 rebound after cART cessation. In summary, the αCD40.HIV5pep with poly(I:C) vaccination approach both activates replication of HIV-1 reservoirs and enhances the anti-HIV-1 T cell response, leading to a reduced level of cell-associated HIV-1 DNA or reservoirs. Our proof-of-concept study has significant implication for the development of CD40-targeting HIV-1 vaccine to enhance anti-HIV-1 immunity and reduce HIV-1 reservoirs in patients with suppressive cART.

Introduction

Combined antiretroviral therapy (cART) suppresses viral replication and improves survival and quality of life for those HIV-1–infected patients who can both access and tolerate cART. However, cART is not curative and must be continued for life (1-3). The HIV-1 reservoir persists indefinitely under suppressive cART, resulting in viral rebound in all HIV-1–infected individuals when cART is discontinued. In addition, cART does not fully restore immune function and lifelong treatment is associated with substantial side effects and non-AIDS related “end-organ diseases”(4). Therefore, there is a great need for the development of novel therapies to control or eliminate the persistent HIV-1 reservoir and thus reduce the need for lifelong cART.

During natural HIV-1 infection, a limited number of individuals (termed elite controllers or long-term nonprogressors) remain persistently infected for decades without marked depletion of CD4+ T cells (5-7). These patients exhibit strong HIV-1–specific polyclonal memory CD4+ T cell activity and maintain a highly polyfunctional cytotoxic T lymphocyte (CTL) response (8-10). The robust HIV-1–specific immune response observed in a large majority of these patients has been proposed to explain this natural functional cure, raising the possibility that therapeutic immunization in infected patients whose viral replication is suppressed by cART might result in similar control of viral replication after cART discontinuation (11-13).

Therapeutic immunization is intended to enhance the immune responses against HIV-1 by vaccination with a suitable immunogen. One way to enhance the immunogenicity of proteins is to increase their uptake by dendritic cells (DCs), which are specialized antigen-presenting cells (APCs) (14-16). Targeting DCs by fusing antigens to monoclonal antibodies (mAbs) directed against internalizing cell-surface receptors can substantially enhance protein immunogenicity (17-19). We and others have demonstrated that targeting HIV-1 Env gp140 or Gag p24 to various DC receptors including DEC-205, LOX-1, Langerin, DCIR, and CD40 induced potentially protective humoral and cellular immunity in both priming and viral vector boost settings (20-23). Recently, we tested in both nonhuman primates (NHPs) and in humans an epitope-based vaccine composed of 5 HIV-1 peptides which contain multiple and highly conserved T cell epitopes from HIV-1 Gag, Pol, and Nef that induce HIV-1–specific CD4+ and CD8+ T cell responses (24-29). Moreover, we have shown that administration of these T cell epitopes as HIV-1 lipopeptides either combined with a recombinant virus (CanaryPox) or as a component of an ex vivo DC therapeutic vaccine strategy, contributed to the control of viral replication after cART interruption (27, 30, 31).

CD40 is a potent activating receptor expressed by a range of APCs, including DCs (32). Thus, targeting CD40 offers the potential...
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**RESULTS**

**aCD40.HIV5pep plus poly(I:C) vaccination elicits HIV-1-specific human T cell responses.** We and others have shown that functional human immune systems, including human DCs and T cells, are developed in immunodeficient mice transplanted with human fetal liver-derived CD34+ cells and thymus, and these hu-mice can initiate T cell immunity in response to infection or vaccination (41–46). Hu-mice support HIV-1 infection and are proven relevant and robust models to study HIV-1 persistence, pathogenesis, and therapy (47, 48). In the present study, we first tested the immunogenicity of the aCD40.HIV5pep vaccine in vivo in hu-mice in the presence of the toll-like receptor 3 (TLR3) agonist poly(I:C) as adjuvant. Poly(I:C) treatment in vivo enhanced the expression of CD40 on human DCs (Figure 1, A and B), which potentially provided more binding targets for the aCD40.HIV5pep protein. In addition, poly(I:C) treatment also elevated the expression levels of HLA-DR and the costimulatory molecule CD86 on human DCs, which are important for antigen presentation and T cell activation. We also found that CD141+ DCs, which are important for antigen presentation and T cell activation.

**Materials and Methods**

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hu-mice vaccinated with poly(I:C) adjuvant produced IL-2 and TNF-α (Figure 1, C and D) after HIV-1 peptide, but not irrelevant HBV antigen (Supplemental Figure 2) after stimulation ex vivo, indicating vaccination-induced, antigen-specific T cell responses. T cells from mice vaccinated with poly(I:C) adjuvant also produced IFN-γ after peptide stimulation ex vivo (Figure 1E). Thus, T cells from mice vaccinated with poly(I:C) adjuvant also indicated vaccination-induced, antigen-specific T cell responses. HBV antigen (Supplemental Figure 2) after stimulation ex vivo, α (Figure 1, C and D) after HIV-1 peptide, but not irrelevant TNF-γ. hu-mice vaccinated with poly(I:C) adjuvant produced IL-2 and TNF-α, indicating its adjuvant effect. Poly(I:C) reactivates HIV-1 reservoirs ex vivo in CD4+ T cells from HIV-1-infected individuals treated with cART and in vivo infected hu-mice. HIV-1 persists during effective cART in part because its genome becomes stably integrated into latently infected cells. These latently infected cells do not express viral proteins and hence remain invisible to the immune system. We have reported before that, as in humans, cART efficiently suppresses HIV-1 replication in hu-mice, but cells harboring HIV-1 DNA persist (45). It is believed that to eliminate the viral reservoir, latent virus in infected cells needs to be reactivated to express HIV-1 proteins (53, 54). TLR agonists are potential reagents to reactivate HIV-1 expression (55–58). Thus, we tested whether the TLR3 agonist poly(I:C), in addition to its immune adjuvant activity, can activate the HIV-1 reservoir in vivo in infected hu-mice under cART. As shown in Figure 2A, cART treatment suppressed HIV-1 viremia in all infected hu-mice within 2 weeks. We treated infected hu-mice with poly(I:C) 3.5 weeks after initiating cART. Interestingly, poly(I:C) treatment in the presence of cART led to low “blips” of HIV-1 viremia within 3 days, which returned to undetectable levels after 1 week (Figure 2A). We detected increased levels of cell-associated HIV-1 RNA but not cell-associated HIV-1 DNA (Figure 2B) at the time point of virus rebound (8.5 weeks after infection), which suggested that the low blips of viremia in the plasma under cART were due to more active HIV-1 transcription after poly(I:C) treatment.

We further tested whether TLR3 agonist poly(I:C) can reactivate HIV-1 reservoirs in CD4+ T cells from cART-treated, HIV-1-infected individuals by virus outgrowth assay. We cultured resting memory CD4+ T cells of cART-treated individuals with undetectable plasma HIV-1 RNA (below 20 copies/ml) with autologous CD3-depleted blood mononuclear cells either irradiated or not irradiated in the presence of poly(I:C) as previously described (59). As controls, cells were stimulated with anti-CD3/anti-CD28 monoclonal antibody (mAb) (positive control) or left unexposed (negative control). Supernatants were collected at day 14 for HIV-1 RNA detection. The data indicated that poly(I:C) at either 5 µg/ml or 10 µg/ml induced significant production of HIV-1 RNA from 5 × 10^5 resting memory CD4+ T cells in the culture supernatants compared with untreated cultures (Figure 3, A and B). Compared with the anti-CD3/anti-CD28 mAb, the efficiency of reactivation of HIV-1 with poly(I:C) was around 20% at 5 µg/ml, and around 35% at 10 µg/ml by virus outgrowth assay (Figure 3, C and D). No difference was observed on the effect of HIV-1 reservoir reactivation from resting memory CD4 by poly(I:C) when cocultured with autologous CD3-depleted blood mononuclear cells either irradiated or not irradiated, suggesting that HIV-1 RNA was reactivated from resting memory CD4+ T cells (Figure 3, A–D). In addition, we also stimulated total leukocytes from lymphoid organs of HIV-1-infected hu-mice under cART ex vivo with poly(I:C) or with other TLR agonists. We found that poly(I:C) worked as efficiently as the TLR7 agonists R837 and R848 to reactivate HIV-1 RNA (Figure 3E), whereas CpG-B did not reactivate HIV-1 RNA compared with PBS-treated control (Figure 3E). As positive control, the inhibitor of histone deacetylases (SAHA) reactivated HIV-1 RNA more efficiently (10.4-fold more than control) than poly(I:C) (2.3-fold more than control) (Figure 3E). No change of cell-associated HIV-1 DNA was detected after stimulation with TLR agonists, suggesting that the increase in cell-associated RNA was due to active HIV-1 transcription, but not new infection during the culture (Figure 3F).

In summary, we found that in addition to its adjuvant activity in enhancing anti–HIV-1 T cell response to vaccination, poly(I:C) can also function as a reservoir-activating agent.

αCD40. HIV5pep with poly(I:C) vaccination rescues anti–HIV-1 T cell responses and reduces the size of the HIV-1 reservoir. We have previously reported that persistent HIV-1 infection in hu-mice led to T cell tolerance, and cART treatment rescued the number but not the function of T cells (45, 46). We next determined whether αCD40. HIV5pep plus poly(I:C) vaccination could rescue anti–HIV-1 T cell activity in infected hu-mice under cART. Hu-mice infected with HIV-1 received cART at 5 weeks postinfection (wpi) through 12 wpi. Hu-mice were vaccinated 2 times with αCD40.HIV5pep plus poly(I:C), at 8 wpi and 11 wpi (Figure 4A). Hu-mice treated with PBS or poly(I:C) alone were used as control. We monitored HIV-1 viremia during the treatment and found that αCD40.HIV5pep with poly(I:C) activated the HIV-1 reservoir in the same manner as treatment with poly(I:C) (Figure 4A). At 12 wpi we terminated the hu-mice and determined anti–HIV-1 T cell response levels as well as HIV-1 reservoir size in lymphoid organs. Results indicated that αCD40.HIV5pep with poly(I:C) rescued the ability of CD8+ T cells to produce both IFN-γ and TNF-α in response to HIV-1 peptide stimulation ex vivo (Figure 4, B and C). The IFN-γ- and TNF-α-expressing T cells also coexpressed the CD107a marker, signifying their killing ability (Supplemental Figure 3).
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Targeting of vaccine antigen to DCs is a promising strategy for boosting vaccine immunogenicity and inducing protective or therapeutic efficacy (18). We have shown before that αCD40.HIV-5pep can effectively expand HIV-1 antigen--specific multifunctional helper CD4+ and cytotoxic CD8+ T cells in the PBMCs of HIV-infected patients, and the expanded polyfunctional cytotoxic CD8+ T cells can control HIV-1 replication in vitro (39). Here we take advantage of hu-mice with functional human immune systems to test both the immunogenicity and efficacy of the αCD40.HIV5pep vaccine in vivo. We also tested the TLR3 agonist to induce CD40 expression on DCs to enhance the efficacy of the αCD40.HIV5pep vaccine. We found that αCD40.HIV5pep with poly(I:C) vaccination elicits both functional HIV-1 antigen--specific human CD8+ and CD4+ responses in vivo in naive hu-mice. Importantly, when administrated in a therapeutic setting in cART-suppressed mice, αCD40.HIV5pep with poly(I:C) vaccination restored the CD8+ T cells to produce IFN-γ and TNF-α in response to HIV-1 antigen stimulation. Our promising results in hu-mice provide the preclinical rationale to test the immunogenicity of the αCD40.HIV5pep vaccine in healthy volunteers and the therapeutic benefit of the vaccine to reverse anti–HIV-1 T cell function in HIV-1–infected patients.

It has been proposed that HIV-1 therapeutic vaccination should be combined with reservoir-activating agents. TLR agonists are potent vaccine adjuvants (60) and may also activate the HIV-1 reservoir or reverse HIV-1 latency (55–57). Whitney et al. recently reported that a TLR7 agonist given to SIV-infected ART-suppressed Rhesus macaques caused transient increases in the level of plasma virus (58). We found here that the TLR3 agonist poly(I:C) treatment led to transient production of plasma HIV-1 RNA blips (2/7) of poly(I:C)-treated hu-mice, but did not rebound in the vaccinated hu-mice (Figure 5, B–D and Table 2). By the second week (13 wpi), 100% (7/7) of the control-treated and 100% (7/7) of the poly(I:C)-treated hu-mice became HIV–1 positive, whereas only 25% (2/8) of vaccinated hu-mice showed detectable viremia in the blood (Figure 5, B–D and Table 2). We detected HIV-1 rebound in all vaccinated hu-mice (8/8) by the third week after cART cessation (14 wpi); however, the viremia in those hu-mice with rebounded HIV-1 was significantly lower in the vaccinated group than in the control groups (Figure 5, B–D and Table 2). By the fourth week (15 wpi), the vaccinated mice still showed lower viral load in the plasma compared with the control mice (Figure 5, B–D and Table 2).

Based on the findings described above, we conclude that αCD40.HIV5pep with poly(I:C) vaccination rescues anti–HIV-1 T cell responses, reduces cART-resistant HIV-1 reservoirs, and leads to better control of virus replication after discontinuation of cART.

**Discussion**

In humans, cART suppresses HIV-1 replication but is not curative due to the remarkably long half-life of the HIV-1 reservoir. One approach that has generated considerable enthusiasm is combining strategies to induce the production of virus from latently infected cells together with interventions that can enhance the ability of the host immune system to clear the virus-producing cells (53, 54). Using the humanized mouse model of persistent HIV-1 infection, we show here that a therapeutic vaccine targeting HIV-1 antigens to CD40 combined with poly(I:C) activated the cART-resistant HIV-1 reservoir, enhanced anti–HIV-1 T cell response, and thereby reduced the HIV-1 reservoir size.

Targeting of vaccine antigen to DCs is a promising strategy for boosting vaccine immunogenicity and inducing protective or therapeutic efficacy (18). We have shown before that αCD40.HIV-5pep can effectively expand HIV-1 antigen--specific multifunctional helper CD4+ and cytotoxic CD8+ T cells in the PBMCs of HIV-infected patients, and the expanded polyfunctional cytotoxic CD8+ T cells can control HIV-1 replication in vitro (39). Here we take advantage of hu-mice with functional human immune systems to test both the immunogenicity and efficacy of the αCD40.HIV5pep vaccine in vivo. We also tested the TLR3 agonist to induce CD40 expression on DCs to enhance the efficacy of the αCD40.HIV5pep vaccine. We found that αCD40.HIV5pep with poly(I:C) vaccination elicits both functional HIV-1 antigen--specific human CD8+ and CD4+ responses in vivo in naive hu-mice. Importantly, when administrated in a therapeutic setting in cART-suppressed mice, αCD40.HIV5pep with poly(I:C) vaccination restored the CD8+ T cells to produce IFN-γ and TNF-α in response to HIV-1 antigen stimulation. Our promising results in hu-mice provide the preclinical rationale to test the immunogenicity of the αCD40.HIV5pep vaccine in healthy volunteers and the therapeutic benefit of the vaccine to reverse anti–HIV-1 T cell function in HIV-1–infected patients.

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treatment in HIV-1–infected mice with cART treatment. This result indicates that poly(I:C) may work as a reservoir-activating agent in vivo. HIV-1 RNA in plasma became undetectable 1 week after treatment, probably due to inhibition by cART of reactivated HIV-1 replication. In addition, we proved that poly(I:C) can reactivate HIV-1 RNA in plasma became undetectable 1 week after the vaccination contributed to the reservoir reduction and virus control after cART discontinuation in HIV-1–infected cART-suppressed hu-mice. Of note, poly(I:C) treatment alone failed to reduce the HIV-1 reservoir or delay virus rebound, although it activated the HIV-1 reservoir in vivo. These data suggest that the anti–HIV-1 T cell response elicited or rescued by the vaccination contributed to the reservoir reduction and virus control after cART interruption. We also observed that even though the virus rebounded in all vaccinated mice at later time points after stopping cART, the plasma HIV-1 RNA levels were lower in vaccinated hu-mice compared with the PBS- or poly(I:C)-treated mice, indicating that vaccination enhanced virus control. It is important to point out that some cohorts of hu-mice that we used were reconstituted with HSCs from HLA-B57/B58+ donors, which may enhance vaccine responsiveness. It will be of interest to compare the relative response to HIV-1 vaccination in hu-mice with HLA-B57/B58+ and HLA-B57/B58− donors in future studies. It is also important to state that there are several limitations of the humanized mouse model, including short-term cART of HIV-1 infection and infection with a molecular clone of HIV-1. In addition, the human immunity developed in hu-mice is not fully functional as that found in immunocompetent hosts (42, 47). The lymphoid structures, such as B cell follicles, which have been identified as HIV-1 sanctuary sites for HIV-1 reservoir cells to escape from CD8+ cell-mediated killing in nonhuman primate models (63) and HIV-1–infected people (64), are not fully developed in hu-mice. The restored anti–HIV-1 T cell immune response by vaccination in hu-mice may not be robust enough to eliminate or fully control the HIV-1 reservoir. We believe that the effect of the vaccine would be better in immunocompetent hosts. We believe that our proof-of-concept study in hu-mice has important implication for the development of CD40-targeting HIV-1 vaccine to cure HIV-1 infection in humans.

Methods

Construction of hu-mice. NRG (NOD-Rag2−/−γc−/−) mice were obtained from the Jackson Laboratory. All mice were housed and bred in a specific pathogen-free environment. Hu-mice were generated as previously reported (65). Briefly, 6- to 8-week-old NRG mice were sublethally irradiated and anesthetized, and 1-mm3 fragments of human fetal thymus were implanted under the kidney capsule. CD34+ hematopoietic progenitor cells purified from fetal liver of the same donor were injected intravenously within 3 hours. Human immune cell engraftment was detected by flow cytometry 12 weeks after transplantation. We used different human donors for different cohorts of hu-mice in the study.

Production of αCD40.HIV5pep protein and vaccination. Recombinant anti-human CD40 antibody fused to 5 HIV peptide regions (αCD40.HIV5pep) was produced as previously reported (39), except that we humanized the mouse variable regions to reduce antigenic-implication for the development of CD40-targeting HIV-1 vaccine to cure HIV-1 infection in humans.

Table 1. αCD40.HIV5pep with poly(I:C) vaccination in the presence of cART reduces the size of the HIV-1 reservoir

<table>
<thead>
<tr>
<th>Donor</th>
<th>Mouse no.</th>
<th>Percentage of cell (preinfection)</th>
<th>Treatment</th>
<th>HIV-1 RNA in plasma (log10)</th>
<th>Cell-associated DNA/RNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CD45+</td>
<td>CD3+</td>
<td>5 wpi</td>
<td>8 wpi</td>
</tr>
<tr>
<td>1</td>
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<td>75.2</td>
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<td>65.1</td>
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<td>PBS</td>
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</tr>
<tr>
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<td>76.3</td>
<td>76.1</td>
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<tr>
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<td>70.9</td>
<td>Vax</td>
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<td>79.5</td>
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<td>5.6</td>
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<tr>
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<td>2754</td>
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<td>80.2</td>
<td>Vax</td>
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</tr>
<tr>
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<td>2755</td>
<td>63.1</td>
<td>75.9</td>
<td>Vax</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Humanized mice engrafted with human HSC and thymus were treated as in Figure 3A. aPercentage of human CD45+ of total cells in PBMCs. bPercentage of CD3+ from human CD45+ cells. cValue stands for copies per 106 human cells. dValue stands for relative level as defined in Methods. eDonor HLA class I type: HLA-A27, HLA-B7.27, HLA-B57.58. wpi, weeks postinfection. Vax, vaccination with αCD40.HIV5pep+poly(I:C). ND, not detectable.
CHO-S cells as previously described (39). The vaccines had low lipopolysaccharide levels of 0.04–0.07 ng/mg protein. For vaccination, hu-mice were intramuscularly (half-dose) and intraperitoneally (half-dose) injected with 10 μg sCD40–HIV5pep alone or co-injected with 50 μg poly(I:C). Control mice received PBS or poly(I:C) treatment.

**HIV-1 infection of hu-mice.** The R5 tropic strain of HIV-1 (JR-CSF) was generated by transfection of 293T cells with plasmid containing full-length HIV-1 (JR-CSF) genome. Hu-mice with stable human leukocyte reconstitution were anesthetized and infected with HIV-1 (JR-CSF) (10 ng p24 or 3,000 infectious units per mouse) through retro-orbital injection. Both male and female mice were used for all the experiments.

**Combination antiretroviral therapy (cART).** Food formulated with antiretroviral individual drugs was prepared as reported with elevated dose modifications (66). In brief, tablets of emtricitabine and tenofovir disoproxil fumarate (Truvada; Gilead Sciences) and raltegravir (Isentress; Merck) were crushed into fine powder and manufactured with TestDiet 5B1Q feed (Modified LabDiet 5058). The estimated daily drug doses were 768 mg/kg raltegravir, 250 mg/kg emtricitabine, and 1,560 mg/kg tenofovir disoproxil, and 1,040 mg/kg emtricitabine. Concentrations of drugs in the food were 4,800 mg/kg raltegravir, 250 mg/kg emtricitabine, and 166 mg/kg tenofovir disoproxil fumarate (Truvada; Gilead Sciences) and 1,560 mg/kg tenofovir disoproxil fumarate (Truvada; Gilead Sciences) and 1,040 mg/kg emtricitabine. For antigen-specific stimulation, splenocytes from vaccinated hu-mice were collected 10 days after the second vaccination and stimulated ex vivo with 5 specific epitopes of the HIV gag gene (5′-GGGCTCTCCTCTCCTCCTGGCTTCAGATTAAG-3′ and 5′-AGCTCCCTGCTTTGCCCATA-3′). The probe (FAM-AAAATTCG- GTTAAGGCCAGGGGA-3′) used for detection was ordered from Applied Biosystems and the reactions were set up following manufacturer’s guidelines and were run on the QuantStudio 6 Flex PCR system (Applied Biosystems). The detection limit of the real-time PCR reaction is 4 copies per reaction. Accordingly, due to the relatively small volume of each bleeding in mice (around 50–100 μl total blood), the limit of detection of the assay is 400 copies/ml plasma. We set the copy number that is below the detectable limit as 1.

**Cell-associated HIV-1 DNA detection.** To measure total cell-associated HIV-1 DNA, nucleic acid was extracted from spleen and bone marrow cells using the DNaseasy mini kit (Qiagen). HIV-1 DNA was quantified by real-time PCR. DNA from serial dilutions of ACH2 cells, which contain 1 copy of the HIV genome in each cell, was used to generate a standard curve.

**Cell-associated HIV-1 RNA detection.** To measure total cell-associated HIV-1 RNA, nucleic acid was extracted from spleen or bone marrow cells using the RNeasy plus mini kit (Qiagen). HIV-1 RNA was detected as described above. The HIV-1 RNA expression levels were normalized to human CD4 mRNA (5′-GGGCTCTCCTCTCCTCCTGGCTTCAGATTAAG-3′ and 5′-AGCTCCCTGCTTTGCCCATA-3′) and GCGCTTGAGACCAGCTT TC) controls and the result was calculated as fold change in gene expression.

**Antigen-specific T cell response detection.** For antigen-specific stimulation, splenocytes from vaccinated hu-mice were collected 10 days after the second vaccination and stimulated ex vivo with 5 specific HIV long peptides (39) plus sCD28 for 12 hours. Brefeldin A was added during the last 4 hours of stimulation and IL-2/TNF-α expression by CD4+ and CD8+ T cells was detected by intracellular staining. ELISPOT plates (96-well, Millipore, catalog 2EM004M99) were coated with 5 μg/ml mouse anti-human IFN-γ antibody (BD Pharmingen) overnight at 4°C. After blocking with complete RPMI for 2 hours at 37°C, 5 × 10^5 splenocytes were stimulated with the corresponding 5 specific
Humanized mice engrafted with human HSC and thymus were treated as in Figure 4A. A Percentage of human CD45+ of total cells in PBMCs. B Percentage of donor mouse no. of cell

Table 2. αCD40.HIV5pep with poly(I:C) vaccination in the presence of cART delays HIV-1 rebound after cART cessation

<table>
<thead>
<tr>
<th>Donor Mouse no.</th>
<th>Percentage of cell (preinfection)</th>
<th>Treatment</th>
<th>HIV-1 RNA in plasma (log10)</th>
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<tr>
<td></td>
<td>CD45- CD3+</td>
<td></td>
<td>4 wpi</td>
</tr>
<tr>
<td>1</td>
<td>1527</td>
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Humanized mice engrafted with human HSC and thymus were treated as in Figure 4A. A Percentage of human CD45+ of total cells in PBMCs. B Percentage of CD3+ from human CD45+ cells. C Donor HLA class I type: HLA-A2+, HLA-B7,27–, HLA-B57,58+. D Donor HLA class I type: HLA-A2+, HLA-B7,27–, HLA-B57,58+. wpi, weeks post HIV-1 infection. ND, not detectable. Vax, vaccination with αCD40.HIV5pep plus poly(I:C).

HIV-1 long peptides at 2 μg/ml, while addition of medium served as negative control. Plates were incubated at 37°C for 24 hours before washing with cold H2O twice and 5 times with PBS containing 0.05% (vol/vol) Tween 20. Biotinylated anti-human IFN-γ antibody (BD Pharmingen) was added at 1 μg/ml for 1 hour at 37°C and, after washing, a 1:3,000 dilution of Avidin-HRP (BD Pharmingen) was added for 1 hour at 37°C. After final washing, stable final substrate solution AEC (3-amino-9-ethylcarbazole) (BD Pharmingen) was added to the plate, incubated for 15 minutes at room temperature, and then the reaction was stopped by thorough rinsing with water. After drying, the number of spots in each well was counted with an automated ELISpot plate reader (CTL Immunospot).

Flow cytometry. For surface staining, single-cell suspensions prepared from spleens of hu-mice were stained with surface markers and analyzed on a CyAn ADP (Dako). For intracellular cytokine staining, cells were first stained with surface markers and then permeabilized with cytotox/cytperm buffer (BD Bioscience), followed by intracellular staining. FITC-conjugated anti-human CD40, PE-conjugated anti-human CD107a, CD141, PE/Cy5-conjugated anti-human CD4, CD86, CD1c, PE/Cy7-conjugated anti-human CD3, HLA-DR, PB-conjugated anti-human CD4, CD14, IL-2, APC-conjugated anti-human CD11c, TNF-α and APC/Cy7-conjugated anti-human CD45, and PE-conjugated anti-human HLA-A2 were purchased from Biolegend. Pacific orange-conjugated anti-mouse CD45, PE/Texas red-conjugated anti-human CD3, CD8, and LIVE/DEAD Fixable Aqua (LD7) Dead Cell Stain Kit were purchased from Invitrogen. FITC-conjugated anti-HIV-1 p24 was purchased from Beckman Coulter. FITC-conjugated anti-HLA-7,27 and biotinylated anti-HLA-57,58 were purchased from One Lambda, Thermo Fisher Scientific. Data were analyzed using Summit 4.3 software (Dako).

Sorting of resting memory CD4+ T cells. Cryopreserved blood mononuclear cells were thawed, and CD4+ T cells were enriched using the EasySep human CD4+ T cell enrichment kit (StemCell Technologies). CD4+ T cells were then stained with an Aqua LIVE/DEAD stain kit (4°C, 15 minutes) and then with anti-CD4-FITC, anti-CD45RA-ECD, anti-HLA-DR-PB, anti-CD25-PE-Cy7, and anti-CD69-PerCp-Cy5.5 (4°C, 25 minutes), and viable resting memory CD4+CD45RA-CD25-CD69-HLA-DR+ CD4+ T cell populations were sorted using a FACSARia fluorescence-activated cell sorter (Becton Dickinson). In all sorting experiments, the grade of purity of the sorted cell populations was greater than 97%.

Viral outgrowth assay (VOA). Different cell concentrations (5-fold limiting dilutions: 5 × 10^4, 10^4, 2 × 10^4, and 4 × 10^4 cells) of sorted viable resting memory CD4+ T cells (HLA-DR CD25 CD69+) of 3 aviremic cART-treated HIV-1-infected individuals were cultured with autologous CD3-depleted blood mononuclear cells (10^6 cells/ml) irradiated or not irradiated in the presence of poly(I:C) (5 or 10 μg/ml) as previously described (59). As controls, cells were stimulated with anti-CD3/anti-CD28 mAb–coated plates (10 μg/ml/72 hours) (positive control) or left unexposed (negative control) as previously described (59). All cell conditions were cultured in complete RPMI supplemented with IL-2 (50 U/ml) for 14 days. Medium was replaced at day 5 and resupplemented with cytokines and poly(I:C). Supernatants were collected
at day 14 and the presence of HIV-1 RNA was assessed by COBAS AmpliPrep/TaqMan HIV-1 Test (Roche) following 1:10 medium dilution in basement matrix buffer (RUWAG Handels AG) as previously described (59). RNA-units per million (RUPM) frequencies were calculated by conventional limiting dilution methods using Extreme Limiting Dilution analysis (http://bioinf.wehi.edu.au/software/elda/) as previously described (59).

Statistics. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software). Experiments were analyzed by 2-tailed Student’s t test, or by 1-way ANOVA and Bonferroni’s post hoc test or Gehan-Breslow-Wilcoxon test, according to the assumptions of the test, as indicated in the figure legends for each experiment. *P < 0.05, **P < 0.01, ***P < 0.001. All the data with error bars are mean ± SEM. A P value less than 0.05 was considered significant.

Study approval. Human fetal liver and thymus tissues (gestational age of 16 to 20 weeks) were obtained from elective or medically indicated terminations of pregnancies, through a nonprofit intermediary working with outpatient clinics (Advanced Bioscience Resources). Informed consent of the maternal donors was obtained in all cases, under regulations governing the clinic. The project was reviewed by the university’s Office of Human Research Ethics, which determined that the study with hu-mice does not constitute human subject research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)]. All animal studies were approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC ID 14-100). The human study was approved and performed by the institutional review board of Lausanne University Hospital, University of Lausanne (i.e., Centre Hospitalier Universitaire Vaudois), and all subjects gave written informed consent.

Author contributions
LS, GZ, LC, and YL conceived the study. LC, GZ, GP, MP, and LS designed the experiments. LC, QW, GL, RB, JM, HY, FY, and ZZ performed experiments. LC and LS analyzed and interpreted the data. SZ and GZ provided the aCD45.HIV5pep recombinant protein. LC, GZ, YL, and LS prepared the manuscript.

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