

# Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor

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**HIV's considerable capacity to vary its HLA-I-restricted peptide antigens allows it to escape from host cytotoxic T lymphocytes (CTLs). Nevertheless, therapeutics able to target HLA-I-associated antigens, with specificity for the spectrum of preferred CTL escape mutants, could prove effective. Here we use phage display to isolate and enhance a T-cell antigen receptor (TCR) originating from a CTL line derived from an infected person and specific for the immunodominant HLA-A\*02-restricted, HIVgag-specific peptide SLYNTVATL (SL9). High-affinity ( $K_D < 400$  pM) TCRs were produced that bound with a half-life in excess of 2.5 h, retained specificity, targeted HIV-infected cells and recognized all common escape variants of this epitope. CD8 T cells transduced with this supraphysiologic TCR produced a greater range of soluble factors and more interleukin-2 than those transduced with natural SL9-specific TCR, and they effectively controlled wild-type and mutant strains of HIV at effector-to-target ratios that could be achieved by T-cell therapy.**

CTLs are crucial for the control of HIV infection. Unfortunately, HIV has an arsenal of mutational and nonmutational strategies that aid it in escaping from the CTL response mounted against it by its host<sup>1,2</sup>. One of the most worrying of these defenses, particularly for those working on vaccine design, is that HIV is readily able to vary the sequence of its HLA-I-restricted antigens<sup>3</sup>, allowing CTL escape by several mechanisms<sup>4</sup>. The most effective way for HIV to escape from CTL surveillance is to avoid displaying HLA-I-associated antigens on the surface of infected cells. Although this can be achieved in part by HIV Nef-mediated downregulation of HLA-I, such an escape strategy has the potential to leave infected cells prone to attack by natural killer cells<sup>2,5</sup>. HIV can also prevent the display of its antigens without affecting HLA-I expression by deleting its epitopes, altering the residues that anchor peptides to HLA-I, or by mutating to interfere with other aspects of the HLA-I presentation<sup>4</sup>. Some epitopes do not

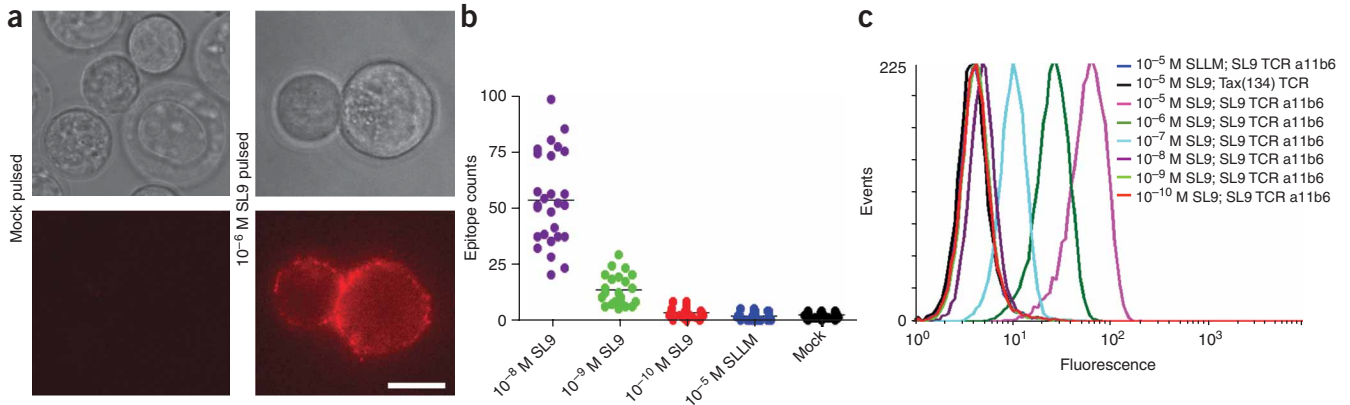
escape in this way but remain presented on the cell surface in mutated forms that interfere with recognition by antiviral TCRs<sup>1,4,6</sup>. However, studies both in the simian immunodeficiency virus macaque model and of natural HIV infection have ascertained that immunodeficiency viruses incur a fitness cost when they escape from some CTL responses<sup>7–10</sup>. Humans who target the virus through these epitopes show better viral control and an increased life expectancy<sup>9</sup>. As a result, there remains hope that at least three strategies of attacking HIV through its HLA-I-associated antigens may prove useful in containing the disease when used in combination with other therapies. First, HLA-I associated antigens from parts of the virus that are biologically constrained and therefore unable to mutate might be targeted. Second, interventions could be designed to target epitopes where escape results in a reduction in viral fitness. A third, 'disguise detection' strategy might target epitopes that remain on the surface of infected cells, albeit in mutated form. The success of this last strategy would be dependent on being able to also target the common variants that arise to escape from recognition by host TCRs.

Here, we test the feasibility of a 'disguise detection' strategy to control HIV infection using the HLA-A\*02-restricted, HIV p17 Gag-derived (amino acids 77–85) antigen SLYNTVATL (SL9). This antigen is an attractive candidate for targeting virally infected cells for several reasons. First, HLA-A\*02 is the most common HLA-I allele in Western populations, such that this epitope might be useful for targeting viruses in almost half of the population. Second, 75% of HIV-infected, HLA-A\*02<sup>+</sup> individuals mount a CTL response against SL9 (refs. 11–13), suggesting that the epitope is efficiently processed. Third, the SL9 peptide sequence may be under strict biological constraints, and residues within this peptide are known to be critical for p17 trimerization<sup>14</sup>. Indeed, a correlation has been noted between the presence of natural viral escape mutants in SL9 and lower viral load, suggesting that mutational strategies used to escape from SL9 CTLs result in a loss of viral fitness<sup>15</sup>. This concept is supported by the reversion to wild-type SL9 sequences once CTL pressure is lost<sup>15</sup>,

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**Figure 1** SL9-specific cell staining with high-affinity TCR. **(a)** Mock-pulsed (left panels) and SL9-pulsed (right panels) T2 cells stained with a11b6–TCR–biotin–streptavidin–PE and visualized by brightfield (top panels) or fluorescence (bottom panels). The entire three-dimensional surface of the cell was imaged by fluorescence microscopy. Fluorescence images in bottom panels show a single plane through the cell; brightfield image shows the locations of the cells. Scale bar, 10  $\mu$ m. **(b)** Total antigen on SL9-pulsed T2 cells, as determined by three-dimensional fluorescence microscopy through 20 z-planes. Cells were pulsed with indicated concentrations of SLYNTVATL (SL9) or SLLMWITQV (SLLM) or mock pulsed and then stained with a11b6–TCR–biotin–streptavidin–PE (see Methods). **(c)** Fluorescence-activated cell sorting analysis of T2 cells pulsed with a titration of SL9 peptide and stained with a11b6–TCR–biotin–streptavidin–PE. Control stains (SLLMWITQV peptide plus a11b6–TCR–biotin–streptavidin–PE, or SL9 peptide plus Tax(134)–TCR–biotin–streptavidin–PE<sup>19</sup>) are shown to indicate background staining.

indicating that the virus is continuously walking a tightrope between immune escape and fitness. Fourth, the common viral escape variants in SL9 interfere with TCR binding rather than HLA-A\*02 binding<sup>6,16</sup>; thus, these variant peptides are still presented on the surface of HIV-infected cells and are therefore available for targeting by ‘disguise detection’ strategies. Fifth, SL9 adopts a conformation that is distinct from that of other HLA-A\*02-bound peptides and which allows the potential for several backbone-directed hydrogen bonds with the TCR<sup>17</sup>. This potentially reduces the impact of substitutions in individual SL9 amino acid side chains on TCR engagement, increasing the likelihood that a TCR can be engineered to recognize many escape variants.

**RESULTS**

**Affinity enhancement of SL9-specific TCRs from a CTL line**

HIV-infected individual 868 makes a sustained and robust CTL response against the immunodominant HLA-A2-restricted SL9 epitope<sup>6,18</sup>. An SL9-specific CTL line (868 line) was grown from this subject in April, 1996 (refs. 6,18). Repeated attempts to generate SL9-specific T-cell clones from the 868 line by limiting dilution were unsuccessful. Instead, we used phage display to isolate a TCR from a T-cell line that was only ~14% tetramer positive for the SL9 antigen (**Supplementary Fig. 1** online). Flow cytometry of the starting CTL line confirmed that all SL9-tetramer<sup>+</sup> cells in this line expressed a TCR made from the same combination of variable genes as the TCR selected from the phage library (**Supplementary Fig. 1d**). This TCR seems to be the dominant SL9-specific TCR *in vivo*, as 10 of 18 SL9 tetramer<sup>+</sup>CD8<sup>+</sup> cells sorted from 868’s peripheral blood were found to express a TCR- $\beta$  variable 5-6 (TRBV5-6) TCR with an identical CDR3 sequence<sup>18</sup>. Surface plasmon resonance (SPR) analysis showed that a soluble, recombinant form of the 868 SL9 TCR bound to its cognate antigen with the highest affinity of a natural TCR for its ligand ever recorded ( $K_D$  = 143 nM by equilibrium binding and 85 nM by kinetic injection analysis; **Supplementary Figs. 1 and 2** and **Supplementary Tables 1 and 2** online). Despite the naturally high affinity of the 868 SL9 TCR, the average dwell time of interaction (<1 min) still places it well outside the range required to be useful in cell targeting as a monovalent molecule. We therefore selected for variants

of this TCR showing higher affinity<sup>19</sup> (**Supplementary Methods** online). Mutations selected in CDR2 $\beta$  or CDR3 $\alpha$  enhanced  $K_D$  values to between 5 and 16 nM; when variant  $\alpha$  and  $\beta$  chains were combined, the affinity was increased to  $K_D$  < 400 pM (**Supplementary Table 2**). The mean dwell time of the enhanced affinity a11b6 TCR on antigen was extended to a binding half-life at 25 °C of >2.5 h ( $K_{off}$  =  $7.12 \times 10^{-5}$  s<sup>-1</sup>).

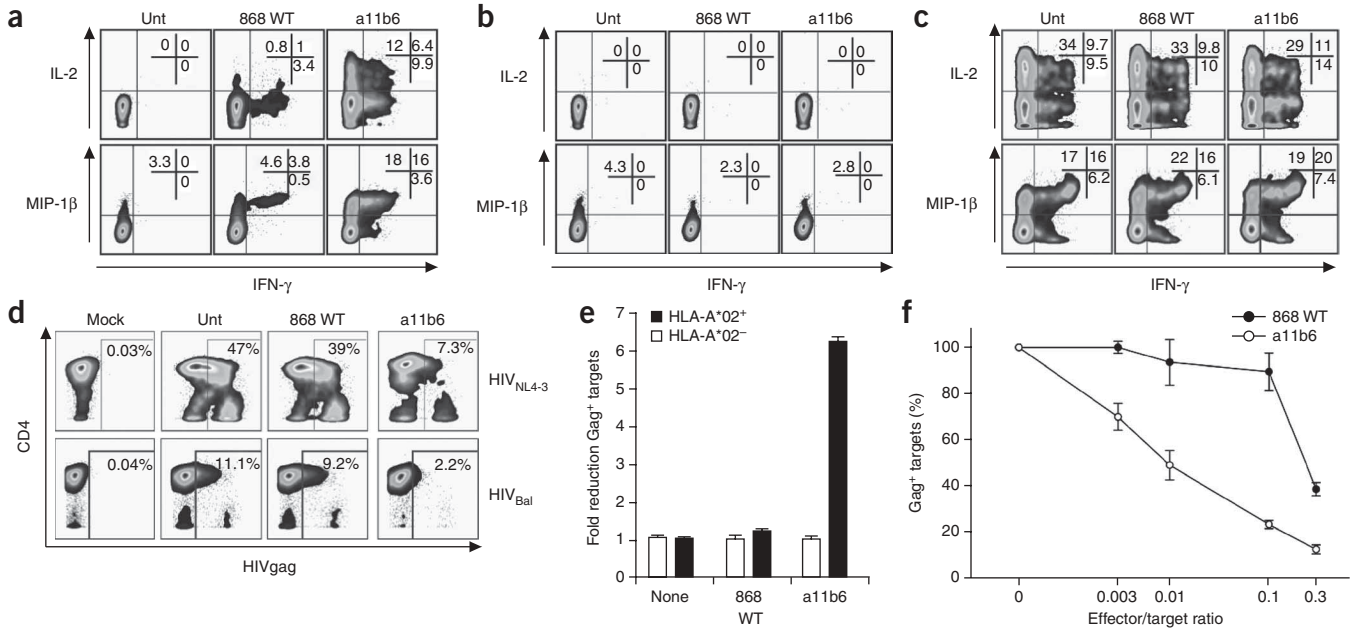
**Soluble high-affinity TCR binds to SL9 escape mutants**

Several natural variants of the SL9 peptide have been described in HLA-A\*02<sup>+</sup> individuals<sup>6,11,13</sup>. The SL9-specific CTL line from patient 868 is of particular interest as it can recognize these variants as either agonists or weak agonists<sup>6</sup>, making its TCR an attractive starting framework for generating a high-affinity targeting agent for HIV-infected cells. Indeed, SPR showed that both the parent and high-affinity-selected a11 and b6 mutant TCRs bound to the common natural variants of this antigen (**Supplementary Table 2**). Notably, at the time of culturing the 868 CTL line, 100% of the virus in patient 868 expressed a mutated SLYNTVATL sequence with a valine-to-isoleucine substitution at position 6 (11/12 sequenced proviruses encoded the sequence SLYNTI<sup>A</sup>AVL and 1/12 encoded SLYNTI<sup>A</sup>ATL)<sup>18</sup>. The SLYNTI<sup>A</sup>AVL and SLYNTI<sup>A</sup>ATL variants were also present at earlier time points. The isolated wild-type TCR showed the highest affinity for the SLYNTI<sup>A</sup>ATL variant (**Supplementary Table 2**), suggesting this may have been the founder antigen in patient 868. Furthermore, the order of affinities with which the SL9 antigenic variants bind the wild-type TCR is in exact accordance with their previously reported ability to induce activation of the 868 CTL line<sup>6</sup>. The a11b6 high-affinity TCR, or these mutated  $\alpha$  and  $\beta$  chains in combination with a wild-type chain, all showed a virtually identical affinity hierarchy for the natural HIV-SL9 peptide variants, indicating that parental TCR specificity and degeneracy was retained faithfully through the affinity maturation process (**Supplementary Table 2**).

**Soluble high-affinity TCR targets HIV-1 infected cells**

Fluorescence microscopy showed that the a11b6 TCR specifically targeted HLA-A\*02<sup>+</sup> cells pulsed with as little as 10<sup>-9</sup> M SL9 peptide



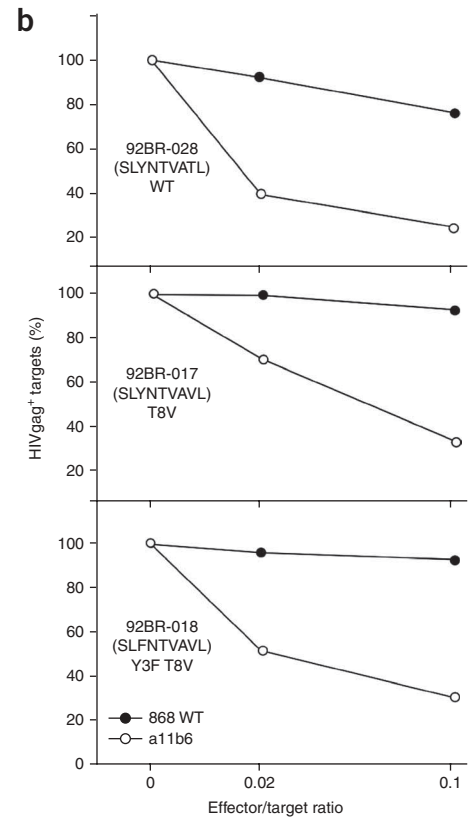
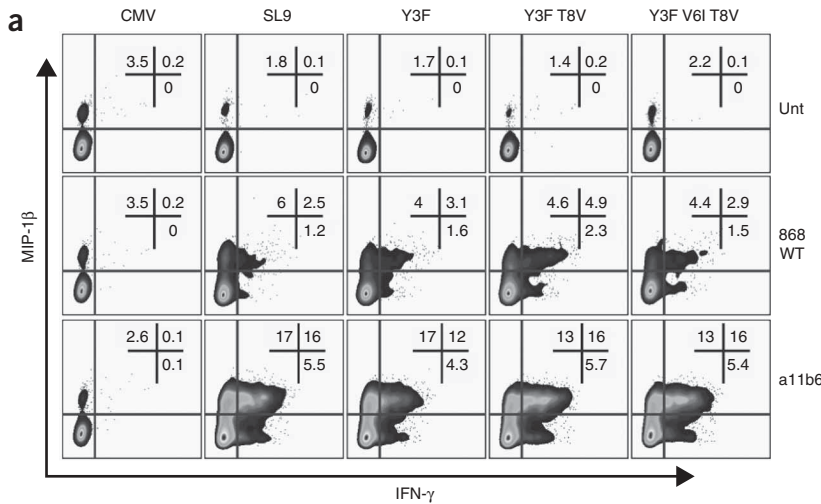


**Figure 2** CD8 T cells expressing supraphysiologic SL9-specific TCRs show an increased frequency of polyfunctional cells and an increased ability to control HIV-1 spread. (a–c) Expression of IFN- $\gamma$ , IL-2 and MIP-1 $\beta$ , measured by intracellular cytokine staining, in CD8<sup>+</sup> T cells transduced with the indicated TCR and then stimulated with HLA-A\*0201-expressing K562 targets loaded with 50 pg/ml SLYNTVATL (SL9) (a) or CMV pp65 peptide (NLVPMVATV) (b). TPA plus ionomycin stimulation for 5 h was used as a positive control (c). Unt, untransduced; WT, wild type. Numbers indicate percentage of cells in a given sector. (d) HIV-1 infection assessed by intracellular stain for HIVgag after coculture of HLA-A\*02<sup>+</sup> CD4 T-cell targets with the indicated SL9-specific TCR-transduced effectors at an effector-to-target ratio of 1:20. Cocultures infected with HIV<sub>NL4-3</sub> (top) or HIV<sub>Bal</sub> (bottom) are shown. Data are representative of three independent experiments. (e) Reduction of HIVgag<sup>+</sup> cells in HLA-A\*02<sup>+</sup> and HLA-A\*02<sup>-</sup> targets that were infected and mixed with the indicated SL9-specific TCR-transduced effectors at a 1:20 effector-to-target ratio. Error bars, s.e.m. of one experiment performed in triplicate; data are representative of two independent experiments. (f) Percentage of HIVgag<sup>+</sup> cells, compared between the indicated conditions and standardized to the addition of untransduced effectors. HIV-1 infection was established in an HLA-A\*02<sup>+</sup> CD4 T-cell culture and TCR-transduced effectors were added at the ratios shown. Error bars, s.e.m. of triplicate measurements; graph represents one of three independent experiments.

(Fig. 1). This concentration of exogenously applied peptide translates to a mean loading of <20 SL9 peptides per cell (Fig. 1). Comparison of CTL activation by SL9 peptide-pulsed and HIV-1-infected HLA-A\*02<sup>+</sup> T cells suggests that real infection generates surface amounts of SL9 peptide equivalent to incubation of cells with between 10<sup>-8</sup> M and 10<sup>-9</sup> M peptide for 1 h (data not shown). Direct microscopic visualization of soluble a11b6 TCR binding to cells pulsed with 10<sup>-9</sup> M peptide (Fig. 1b), and positive staining by fluorescence-activated cell sorting at 10<sup>-8</sup> M peptide and above (Fig. 1c), suggests that this reagent would successfully target HIV-infected cells. However, biological safety considerations required us to fix HIV-1-infected cells in order to examine them microscopically and, unfortunately, fixation of peptide-pulsed cells after a11b6 TCR staining was observed to reduce the sensitivity of antigen detection by >10-fold (data not shown). Therefore, we were unable to directly visualize and count SL9 antigen on the surface of HIV-infected cells with our systems. Instead, to test the ability of the a11b6 TCR to target natural amounts of cell-surface-expressed SL9 antigen, we examined its ability to compete with cognate CTL function. The a11b6 TCR used at 10 nM to 1  $\mu$ M inhibited activation of HLA-A2-SL9-specific CTLs by both SL9-pulsed target cells (Supplementary Fig. 3a–d online) and HIV-infected 174xCEM.T0 cells (Supplementary Fig. 3e,f). We therefore conclude that soluble a11b6 TCR can target antigen on the surface of HIV-infected cells. A previous study demonstrated that T cells transfected with an enhanced-affinity TCR showed enhanced peptide-dependent activation<sup>20</sup>. We next examined how a11b6 TCR would behave if expressed on the surface of a CD8 T-cell.

**a11b6 TCR promotes polyfunctional CD8 T-cell responses**

Lentiviral vectors expressing full-length wild-type and high-affinity TCR  $\alpha$  and  $\beta$  chains were used to transduce primary human CD8 T cells to produce a population of SL9 tetramer<sup>+</sup> cells (Supplementary Fig. 4 online). Analysis of cytokine production by TCR-transduced CD8 T cells (~40% of the cells were TCR transduced; Supplementary Fig. 5 online) after stimulation with SL9-loaded, HLA-A\*02-expressing K562 cells showed a higher overall number of cells producing cytokines in cells transduced with the a11b6 TCR (Fig. 2a and Supplementary Fig. 5). No response was observed when the TCR-transduced cells were stimulated with HLA-A\*02-expressing K562 cells in the absence of exogenous peptide (data not shown) or loaded with cytomegalovirus (CMV) peptide (Fig. 2b), suggesting that the transduced T cells remain specific for SL9 antigen. Cells transduced with the a11b6 TCR were also more likely to produce multiple cytokines in response to antigen than cells transduced with the wild-type TCR. This was not observed in response to stimulation with 12-O-tetradecanoylphorbol-13-acetate plus ionomycin, suggesting that the augmented functional response to antigen is due to the enhanced affinity of the TCRs (Fig. 2c and Supplementary Fig. 5). In addition, CD8 T cells transduced with a11b6 TCR made more interleukin (IL)-2 and interferon (IFN)- $\gamma$  in response to 3 pM SL9 peptide than cells transduced with 868 wild-type TCR in response to 3 nM peptide, suggesting that the dose-response curve for exogenously applied peptide was shifted by over 3 orders of magnitude by expression of enhanced-affinity TCR (data not shown).



**Figure 3** High-affinity TCR-transduced CD8 T cells can effectively recognize SL9 escape mutant peptides and suppress infection by SL9 escape HIV-1 variants. **(a)** Cytokine production by effector CD8 T cells after stimulation with HLA-A\*0201-expressing K562 targets loaded with a control peptide (from CMV pp65), SLYNTVATL (SL9) or naturally occurring escape mutant peptides SLFNTVATL (Y3F), SLFNTVAVL (Y3F T8V) or SLFNTI AVL (Y3F V6I T8V). Unt, untransduced. **(b)** Percentage of HIVgag<sup>+</sup> target cells infected with three primary isolates having mutated SL9 epitopes, compared between conditions and standardized to the addition of untransduced effectors, after coinubation of the indicated TCR-transduced CD8 T cells at a final effector-to-target ratios of 1:10 or 1:50. Data are representative of at least two independent experiments.

**Control of HIV spread at low effector-to-target ratios**

We then asked whether CD8 T cells expressing supraphysiologic SL9-specific TCRs could more effectively control HIV-1 replication. Activated HLA-A\*02<sup>+</sup> CD4 T cells were infected with provirus HIV<sub>NL4-3</sub>, and 2 d later these cells were mixed with SL9-specific TCR-transduced CD8 T cells at an effector-to-target ratio of 1:20. HIV infection was assessed by intracellular HIVgag staining 8 d later<sup>21</sup>. Infected CD4 T cells mixed with untransduced CD8 T cells showed robust HIVgag staining, whereas mock-infected cultures remained HIVgag negative (Fig. 2d). CD8 T cells expressing the SL9 wild-type 868 TCR exerted a modest effect in reducing the number of HIVgag-positive targets. In contrast, CD8 T cells expressing the a11b6 TCR genes were able to limit HIV-1 spread within these cultures. We also obtained similar results using cultures infected with HIV isolates HIV-1<sub>Bal</sub> and HIV-1<sub>SF162</sub> as targets (Fig. 2d and data not shown), suggesting that CD8 T cells transduced with high-affinity SL9-specific TCR can control replication of multiple HIV-1 isolates. To confirm that the HIV suppression was HLA-A\*02 restricted, we combined HIV-1-infected non-HLA-A\*02 target cells with TCR-transduced effectors. Using these mismatched cocultures, we observed no reduction in HIV replication (Fig. 2e), demonstrating that suppression of HIV replication by CD8 T cells expressing high-avidity SL9-specific TCRs is HLA-A\*02 restricted. To further quantify the antiviral effect of SL9-specific, TCR-transduced CD8 T cells, we used a wide range of effector-to-target ratios. We observed clear differences between cells transduced with the 868 and a11b6 TCRs at 1:100 and 1:10 effector-to-target ratios (Fig. 2f). These results suggest that HIV suppression by CD8 T cells is enhanced by increased TCR affinity to cognate peptide and that the benefits of supraphysiologic SL9-specific TCRs are manifest at the low effector-to-target ratios that could be achieved by TCR gene transfer *in vivo*. At the time of collection in HIV infection

assays, there were more SL9 TCR-positive cells in assays with the a11b6 TCR than with the 868 wild-type TCR, suggesting that cells transduced with high-affinity TCR might undergo greater cellular expansion in response to antigen. Direct comparison of the proliferation of cells expressing these TCRs in response to targets expressing HIVgag showed no substantial differences (Supplementary Fig. 6 online). T cells expressing the 868 wild-type TCR do not control virus as well as those with a high-affinity TCR (Figs. 2 and 3) and are therefore exposed to higher levels of HIV-1 Nef, Gag, Vpr and Vpu proteins during assays. All of these proteins are known to modulate T-cell activation and expansion<sup>22</sup>, and this could explain the differences in cell numbers observed at the end of culture, although the real reason for this difference remains unknown.

**High-affinity CD8 T-cells control SL9-escape HIV variants**

Next, we measured the response of SL9-specific TCR-transduced CD8 T cells to targets loaded with naturally occurring SL9 peptide mutants. Untransduced or TCR-transduced CD8 T cells were stimulated with HLA-A\*02-expressing K562 target cells loaded with CMV pp65 (NLVPMVATV) peptide as a negative control, SL9 (SLYNTVATL) peptide, or its Y3F (SLFNTVATL), Y3F T8V (SLFNTVAVL) or Y3F V6I T8V (SLFNTI AVL) mutants. The wild-type sequence and these escape mutations at positions 3, 6 and 8 accounted for 100% of 280 SL9-region sequences from an 18-patient cohort<sup>23</sup> and 92% of the viruses sequenced from a 107-patient cohort<sup>15</sup>. As above, we observed that a high-affinity TCR increased the magnitude of the cytokine response to SL9 (Fig. 3a). In accordance with results using the 868 CTL line<sup>6</sup>, CD8 T cells transduced with wild-type 868 TCR were able to recognize all the above escape mutant peptides when the peptides were loaded on targets. Indeed, the parent 868 CTL line selected for this study was capable of tolerating single alanine

substitutions at any amino acid side chain and, notably, even showed some recognition of the multiply substituted peptide SLYAAAAAL (ref. 6 and data not shown). The unusual SL9 escape variant SLHNTVATL was recognized only poorly by 868 CTLs, and viruses carrying this particular mutation would be likely to escape from recognition by the wild-type TCR<sup>24</sup>. SPR showed that a11b6 TCR bound to HLA A2-SLHNTVATL with a  $K_D$  of 97 nM (data not shown). This binding affinity is higher than any natural TCR–peptide–MHC interaction recorded by SPR and is more than sufficient to ensure recognition of the unusual SLHNTVATL escape sequence by CD8 T cells expressing the affinity-enhanced TCR.

Mutations within CTL epitopes can alter their processing and affect the amount of antigen naturally presented on the surface of infected cells. We thus felt it was important to examine whether TCR-transduced CD8 T cells could control HIV infection by isolates that contained CTL escape mutations in the SL9 region. CD8 T cells transduced with wild-type 868 TCR were unable to control infection by 92BR-017 or 92BR-018 HIV isolates that contain the common CTL SL9 escape mutations T8V and Y3F T8V, respectively (Fig. 3b). In contrast, CD8 T cells transduced with the high-affinity a11b6 TCR were able to control all viral isolates, including virus carrying the common Y3F T8V escape mutation that binds to this TCR with the weakest affinity (Fig. 3b; Supplementary Table 2).

## DISCUSSION

We used phage display to isolate a TCR specific for the HLA-A\*02-restricted, HIV-derived SL9 CTL epitope from a T-cell line derived from an infected individual. This approach, which bypasses the need for a T-cell clone, may be generically suitable for isolation of antigen-specific TCRs from polyclonal T-cell populations. The TCR isolated (868) was the dominant clone recognizing the SL9 antigen *in vivo*<sup>18</sup>. SPR binding analysis showed that a soluble, recombinant form of the 868 SL9 TCR bound to its cognate antigen with the highest affinity of a natural TCR for its ligand ever recorded (Supplementary Figs. 1 and 2; Supplementary Table 2). The high affinity of this TCR may explain its immunodominance *in vivo*<sup>18</sup>. The affinity of the 868 TCR is over tenfold higher than any human TCR–peptide–MHC interaction previously documented<sup>25</sup> and therefore substantially extends the affinity range described for natural TCR–peptide–MHC interactions. Despite the naturally high affinity of the 868 SL9 TCR, the average dwell time of interaction with antigen (<1 min) still places it well outside the range required to be useful in cell targeting as a monovalent molecule. We therefore subjected the 868 TCR to directed evolution<sup>19</sup> and produced a high-affinity ( $K_D < 400$  pM) TCR that bound with a half-life in excess of 2.5 h (Supplementary Table 2). This affinity and antigen-binding half-life is within the range of those reported for therapeutically applied antibodies (reviewed in ref. 26). High-affinity TCRs, like antibodies, are amenable to conjugation or fusion with a variety of immunostimulatory or cytotoxic agents and may thus offer the possibility of similar targeted approaches to new types of therapies. Antibody-mediated therapies for HIV are severely hampered by the extreme degree of variability of the main antigenic determinants on the surface of infected cells. In contrast, high-affinity TCRs can ‘see’ beyond these surface determinants and access a much wider range of viral targets, including some of restricted variability.

We also examined the use of supraphysiologic SL9 TCR for antigen recognition at the T-cell surface. CD8 T cells transduced with supraphysiologic TCR responded to SL9 antigen by producing a greater range of soluble factors and more IL-2 than those transduced with natural SL9-specific TCR. The enhanced polyfunctional phenotype of CD8 T cells transduced with high-affinity TCRs is of interest given the

inverse association between maintenance of polyfunctional effector CD8 T cells and HIV-1 disease progression<sup>27</sup>. It is also noteworthy that we observed a tenfold greater IL-2-producing cells by cells transduced with a11b6 supraphysiologic SL9-specific TCR after antigen stimulation than that in cells transduced with the wild-type TCR (Fig. 2a). Loss of IL-2 production is the most common functional defect of HIV-1-specific CD8 T cells<sup>27</sup>. IL-2 is considered important in mediating antigen-specific expansion in the absence of CD4 help<sup>28</sup>. Thus, the potential of high-affinity TCR to restore IL-2 production could have important consequences for adoptive T-cell therapy approaches to HIV. Indeed, CD8 T cells transduced with supraphysiologic TCR controlled HIV infection *in vitro* at effector-to-target ratios that could be achieved by T-cell therapy (Fig. 2). Crucially, CD8 T cells transduced with supraphysiologic SL9 TCR also recognized common immune escape variants of the SL9 epitope and, unlike cells bearing a natural receptor, controlled infection with strains of HIV carrying common SL9 escape variants. Our data suggest, at the very least, that HIV-1 will have to devise new ways to escape from CD8 T cells transduced with supraphysiologic SL9-specific TCR and show that it is possible to improve on nature when it comes to preventing HIV CTL escape. We conclude that this makes the use of supraphysiologic TCRs very attractive for adoptive T-cell therapy.

## METHODS

**Cell staining using a11b6 TCR.** T2 cells (American Type Culture Collection) were pulsed with either HIVgag<sub>77–85</sub> (SLYNTVATL) or the cancer-testis antigen NY-ESO-1(V)<sub>157–163</sub> (SLLMWITQV) peptides at the peptide concentration indicated for 90 min at 37 °C. After a wash step, cells were incubated for 30 min at 25 °C with SL9 a11b6-biotin monoclonal TCR (5 µg/ml) or Tax(134)-biotin monoclonal TCR (ref. 19) (5 µg/ml) in PBS containing 0.5% BSA. After incubation, cells were washed and further incubated with 5 µg/ml streptavidin conjugated to phycoerythrin (PE) (Pharmingen) in 0.5% BSA PBS, for 30 min at 25 °C in the dark. After two further washes, TCR-biotin–streptavidin–PE binding was examined by flow cytometry using an FC500 flow cytometer (Beckman Coulter) or three-dimensional microscopy (Zeiss). A Zeiss 200M/Universal Imaging system with a ×63 objective was used for single-molecule, wide-field fluorescence microscopy and data analysis as described<sup>29</sup>. As staining of cell-surface bound biotinylated complexes with an excess of streptavidin–PE has been shown to result in monomeric association of streptavidin–PE with target protein<sup>29</sup>, a single detected PE signal corresponds to a single TCR–peptide–HLA complex. To cover the entire three-dimensional surface of the cell, z-stack fluorescence images were taken (21 individual planes, 1 µm apart). Data was evaluated for at least 20 cells in each experimental condition.

**Primary T cells, cell lines and viruses.** Peripheral blood mononuclear cells, purified CD4 T cells, and purified CD8 T cells isolated from HIV-1 seronegative donors were obtained by University of Pennsylvania Center For AIDS Research Immunology Core. The purity of the negatively selected CD4 and CD8 T cells routinely exceeded 90%. T cells were cultured in X-Vivo 15 (Lonza) supplemented with 5% human serum (Valley Biomedical), 0.9% *N*-acetylcysteine (Roxane Laboratory), 2 mM GlutaMax and 25 mM HEPES buffer (Invitrogen). The HIV provirus pNL4-3 and the primary isolates 92BR-017, 92BR-018 and 92BR-028 were obtained through the AIDS Research and Reference Reagent Program. The sequence accession numbers for the gag-mutant viruses 92BR-017, 92BR-018, 92BR-028 are AAM98718, AAM98719 and AAM98720, respectively. HIV isolates Bal and SF-162 were obtained from the University of Pennsylvania Center For AIDS Research Virus and Molecular Core.

**HIV suppression.** HIV suppression was assessed by a modification of a previously described assay<sup>21</sup>. Briefly, purified CD4 T cells were activated with 5 µg/ml *Phaseolus vulgaris* leucoagglutinin (PHA-L, Sigma-Aldrich) and 300 IU/ml IL-2 (Chiron Therapeutics) for 72 h, followed by infection with HIV-1<sub>NL4-3</sub>, HIV-1<sub>Bal</sub> or HIV-1<sub>SF162</sub>. After a 48-h infection, cells were washed once with fresh medium. Infected CD4 T-cell blasts were plated as targets at a density of  $5 \times 10^5$  per well in a 24-well plate. TCR-transduced or untransduced

CD8 T cells were added at the specified ratio as effectors to a final volume of 2 ml per well. The cocultures were monitored and maintained for 7–10 d. Every 3 d, 0.5 ml of supernatant was collected and replaced with fresh medium. At day 8, intracellular HIVgag staining was performed on the cocultures to measure HIV infection. Cells were stained for TRBV5-6, CD4 and CD8, followed by intracellular HIVgag (KC57, Beckman Coulter) staining using the Caltag Fix & Perm buffers according to the manufacturer's protocol (Invitrogen). The number of HIVgag<sup>+</sup> target cells was determined by gating on viable, CD8<sup>+</sup>TRBV5-6<sup>+</sup> events.

**Intracellular cytokine staining.** The HLA-A\*02-expressing K562 artificial antigen-presenting cell line was generated as described previously<sup>30</sup> and loaded overnight with 2.5 µg/ml β2-microglobulin (Sigma-Aldrich) and 50 pg/ml SL9, SL9 escape variant peptides or CMV pp65 NLVPMVATV as a control peptide. SL9-specific TCR-transduced CD8 T cells were mixed with antigen-loaded, HLA-A\*02-expressing K562 cell-based artificial antigen-presenting cells at a 2:1 ratio in the presence of 0.5 µg/ml antibody to CD49d (eBiosciences) for 1 h, followed by 4 h in the presence of brefeldin-A (Golgiplug, BD Biosciences). Stimulation with TPA (3 µg/ml, Sigma-Aldrich) and ionomycin (1 µg/ml; Calbiochem) with brefeldin-A was used as positive control. Cells were washed in PBS and surface-stained using CD8 conjugated to the fluorophores PE and Cy7, and then fixed and permeabilized with the Caltag Fix & Perm kit (Invitrogen) and stained using IL-2-allophycocyanin, IFN-γ-FITC and macrophage inflammatory protein-1β (MIP-1β, CCL4)-PE, along with their respective isotype controls (BD Biosciences). Sequential gates of 50,000 viable (forward scatter versus side scatter), CD8<sup>+</sup> and single cytokine-positive events were generated for all conditions. Cytokine-positive events were analyzed by means of boolean gating in FlowJo (Tree Star Inc.) for production of cytokines singly and in all possible combinations.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

A.V.-R., M.M.S. and R.G.C. performed the TCR gene transfer experiments in the laboratories of C.H.J. and J.L.R. P.E.M., S.M.D. and Y.L. undertook the phage display selection. B.J.C., S.M.D. and R.M. made the MHC and TCR proteins. A.V., T.M. and D.K.C. performed the surface plasmon resonance. D.H.S. and M.A.P. performed the microscopic analyses. A.M. and B.L. undertook the experiments with primary T cells grown by A.K.S. R.E.P. secured the clinical materials. B.K.J., A.K.S. and J.L.R. conceived and wrote the study.

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1. Goulder, P.J. & Watkins, D.I. HIV and SIV CTL escape: implications for vaccine design. *Nat. Rev. Immunol.* **4**, 630–640 (2004).
2. Sewell, A.K., Price, D.A., Oxenius, A., Kelleher, A.D. & Phillips, R.E. Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. *Stem Cells* **18**, 230–244 (2000).
3. Phillips, R.E. *et al.* Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354**, 453–459 (1991).

4. Price, D.A. *et al.* The influence of antigenic variation on cytotoxic T lymphocyte responses in HIV-1 infection. *J. Mol. Med.* **76**, 699–708 (1998).
5. Cohen, G.B. *et al.* The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* **10**, 661–671 (1999).
6. Sewell, A.K., Harcourt, G.C., Goulder, P.J., Price, D.A. & Phillips, R.E. Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides. *Eur. J. Immunol.* **27**, 2323–2329 (1997).
7. Crawford, H. *et al.* Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B\*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *J. Virol.* **81**, 8346–8351 (2007).
8. Friedrich, T.C. *et al.* Reversion of CTL escape-variant immunodeficiency viruses *in vivo*. *Nat. Med.* **10**, 275–281 (2004).
9. Leslie, A.J. *et al.* HIV evolution: CTL escape mutation and reversion after transmission. *Nat. Med.* **10**, 282–289 (2004).
10. Martinez-Picado, J. *et al.* Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J. Virol.* **80**, 3617–3623 (2006).
11. Brander, C. *et al.* Lack of strong immune selection pressure by the immunodominant, HLA-A\*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection. *J. Clin. Invest.* **101**, 2559–2566 (1998).
12. Goulder, P.J. *et al.* Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. *J. Exp. Med.* **193**, 181–194 (2001).
13. Goulder, P.J. *et al.* Patterns of immunodominance in HIV-1-specific cytotoxic T lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-identical siblings with HLA-A\*0201 are influenced by epitope mutation. *J. Exp. Med.* **185**, 1423–1433 (1997).
14. Morikawa, Y., Zhang, W.H., Hockley, D.J., Nermut, M.V. & Jones, I.M. Detection of a trimeric human immunodeficiency virus type 1 Gag intermediate is dependent on sequences in the matrix protein, p17. *J. Virol.* **72**, 7659–7663 (1998).
15. Iversen, A.K. *et al.* Conflicting selective forces affect T cell receptor contacts in an immunodominant human immunodeficiency virus epitope. *Nat. Immunol.* **7**, 179–189 (2006).
16. Kan-Mitchell, J. *et al.* Degeneracy and repertoire of the human HIV-1 Gag p17<sub>77–85</sub> CTL response. *J. Immunol.* **176**, 6690–6701 (2006).
17. Martinez-Hackert, E. *et al.* Structural basis for degenerate recognition of natural HIV peptide variants by cytotoxic lymphocytes. *J. Biol. Chem.* **281**, 20205–20212 (2006).
18. Wilson, J.D. *et al.* Oligoclonal expansions of CD8<sup>+</sup> T cells in chronic HIV infection are antigen specific. *J. Exp. Med.* **188**, 785–790 (1998).
19. Li, Y. *et al.* Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat. Biotechnol.* **23**, 349–354 (2005).
20. Holler, P.D., Lim, A.R., Cho, B.K., Rund, L.A. & Kranz, D.M. CD8<sup>+</sup> T cell transfectants that express a high affinity T cell receptor exhibit enhanced peptide-dependent activation. *J. Exp. Med.* **194**, 1043–1052 (2001).
21. Darden, J.M. *et al.* A flow cytometric method for measuring neutralization of HIV-1 subtype B and E primary isolates. *Cytometry* **40**, 141–150 (2000).
22. McCune, J.M. The dynamics of CD4<sup>+</sup> T-cell depletion in HIV disease. *Nature* **410**, 974–979 (2001).
23. Edwards, C.T., Pfafferoth, K.J., Goulder, P.J., Phillips, R.E. & Holmes, E.C. Inpatient escape in the A\*0201-restricted epitope SLYNTVATL drives evolution of human immunodeficiency virus type 1 at the population level. *J. Virol.* **79**, 9363–9366 (2005).
24. Whelan, J.A. *et al.* Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J. Immunol.* **163**, 4342–4348 (1999).
25. Cole, D.K. *et al.* Human TCR-binding affinity is governed by MHC class restriction. *J. Immunol.* **178**, 5727–5734 (2007).
26. Molloy, P.E., Sewell, A.K. & Jakobsen, B.K. Soluble T cell receptors: novel immunotherapies. *Curr. Opin. Pharmacol.* **5**, 438–443 (2005).
27. Betts, M.R. *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8<sup>+</sup> T cells. *Blood* **107**, 4781–4789 (2006).
28. Zimmerli, S.C. *et al.* HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc. Natl. Acad. Sci. USA* **102**, 7239–7244 (2005).
29. Purhoo, M.A., Irvine, D.J., Huppa, J.B. & Davis, M.M. T cell killing does not require the formation of a stable mature immunological synapse. *Nat. Immunol.* **5**, 524–530 (2004).
30. Suhoski, M.M. *et al.* Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. *Mol. Ther.* **15**, 981–988 (2007).