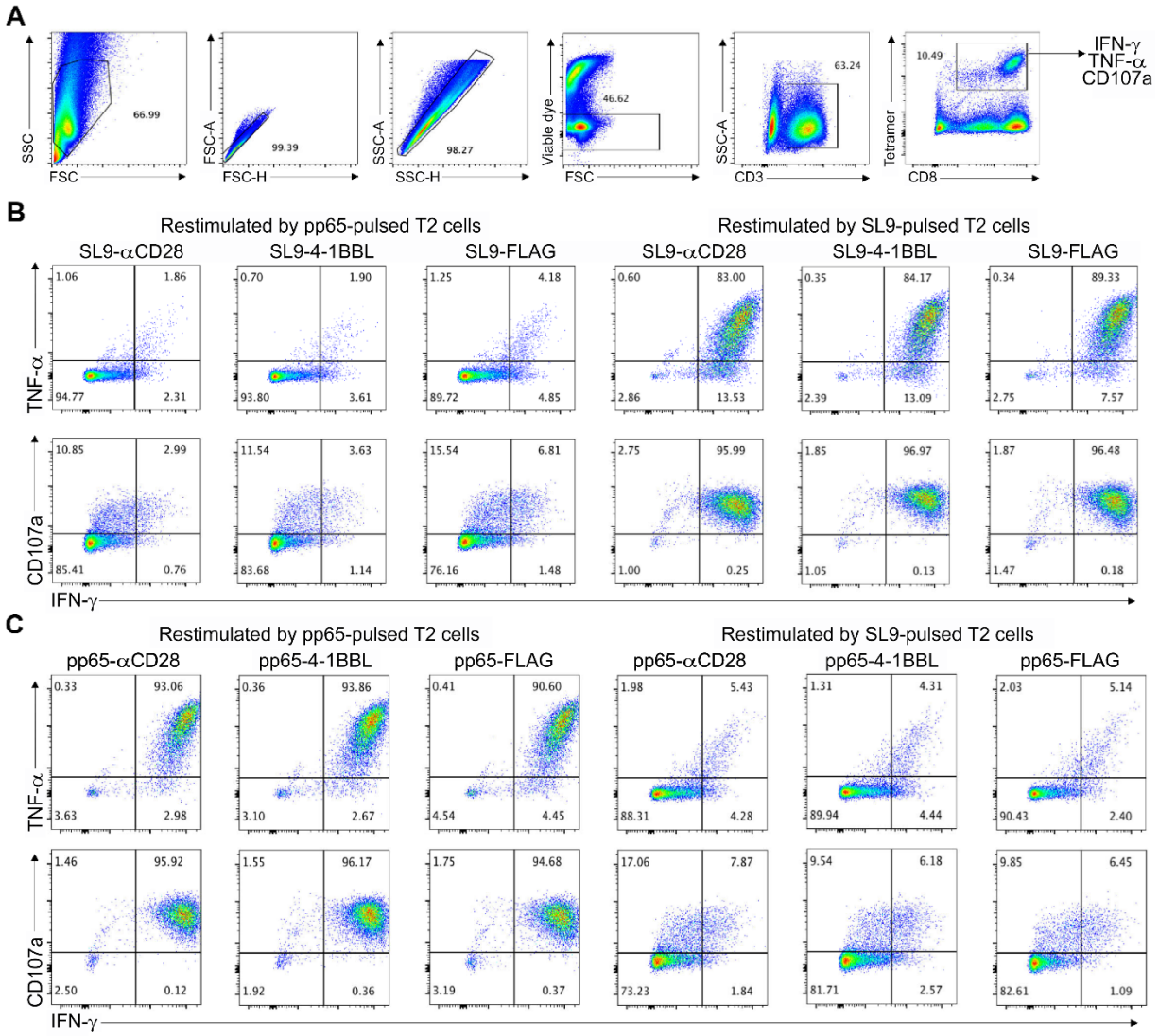
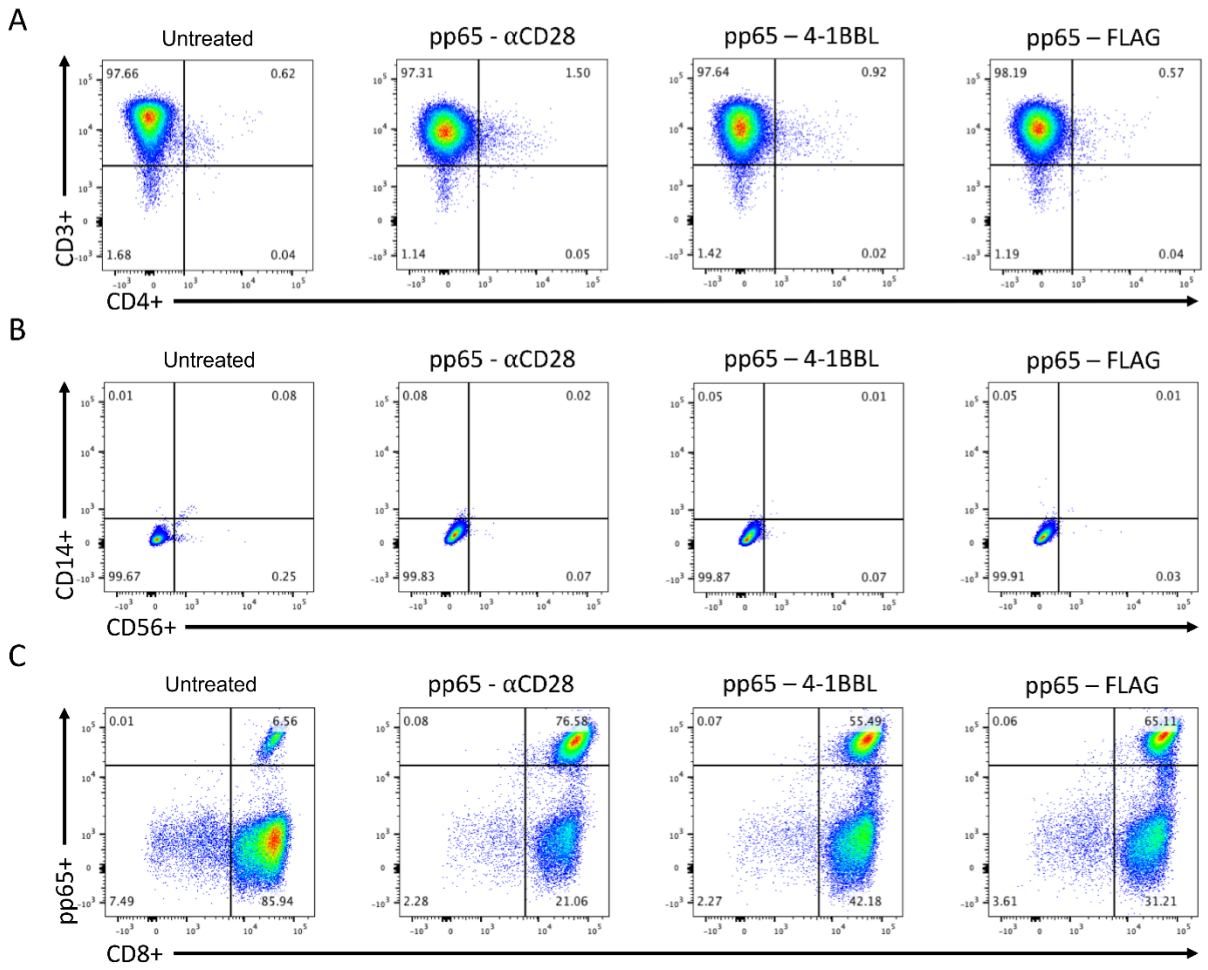
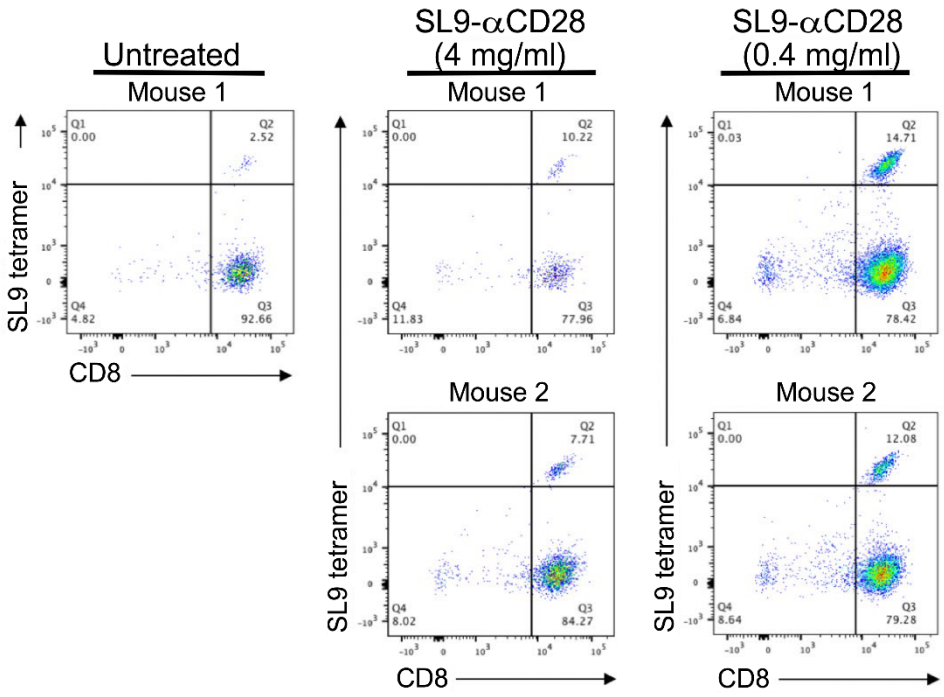

A
SSC
FSC
66.99
FSC-A
FSC-H
99.39
SSC-A
SSC-H
98.27
Viable dye
FSC
46.62
SSC-A
CD3
63.24
Tetramer
CD8
10.49
IFN-γ
TNF-α
CD107a
B
Restimulated by pp65-pulsed T2 cells
Restimulated by SL9-pulsed T2 cells
SL9-αCD28
SL9-4-1BBL
SL9-FLAG
SL9-αCD28
SL9-4-1BBL
SL9-FLAG
TNF-α
1.06
1.86
94.77
2.31
0.70
1.90
93.80
3.61
1.25
4.18
89.72
4.85
0.60
83.00
2.86
13.53
0.35
84.17
2.39
13.09
0.34
89.33
2.75
7.57
CD107a
10.85
2.99
85.41
0.76
11.54
3.63
83.68
1.14
15.54
6.81
76.16
1.48
2.75
95.99
1.00
0.25
1.85
96.97
1.05
0.13
1.87
96.48
1.47
0.18
IFN-γ
C
Restimulated by pp65-pulsed T2 cells
Restimulated by SL9-pulsed T2 cells
pp65-αCD28
pp65-4-1BBL
pp65-FLAG
pp65-αCD28
pp65-4-1BBL
pp65-FLAG
TNF-α
0.33
93.06
3.63
2.98
0.36
93.86
3.10
2.67
0.41
90.60
4.54
4.45
1.98
5.43
88.31
4.28
1.31
4.31
89.94
4.44
2.03
5.14
90.43
2.40
CD107a
1.46
95.92
2.50
0.12
1.55
96.17
1.92
0.36
1.75
94.68
3.19
0.37
17.06
7.87
73.23
1.84
9.54
6.18
81.71
2.57
9.85
6.45
82.61
1.09
IFN-γ

expansion by treatment with either **(B)** the indicated SL9-synTacs or **(C)** the indicated pp65-synTacs.



Supplemental Fig. 2. Treatment of highly purified CD8+ T cells with p65-synTacs markedly expands the pp65-specific CD8+ T cell population. Highly purified CD8+ T cells were isolated from HGLK055 by immunomagnetic depletion of CD4+ T cells, monocytes, neutrophils, eosinophils, B cells, stem cells, dendritic cells, NK cells, granulocytes, γ/δ T cells, and erythroid cells using a cocktail of biotin-conjugated antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCRγ/δ, and CD235a (Glycophorin A). The highly purified CD8+ T cells were treated with the indicated synTac (0.1 nM), cultured in complete RPMI media with added IL-2 (100 U/mL). After 12 days, the cells were analyzed by flow cytometry for the fraction of **(A)** CD3+ and CD4+ cells, **(B)** CD14+ and CD56+ cells, and (**C**) CD8+ and pp65-teramer+ cells.



Supplemental Fig. 3. *In vivo* SL9-αCD28-synTac dose response. NSG mice were intrasplenically injected with PBMCs from an HLA-A*0201 HIV-seropositive donor (donor 619) with reactivity to SL9 and were either untreated or immediately treated by intravenous injection with SL9-αCD28-synTac at a dose either of 4 mg/kg or 0.4 mg/kg. One week later, the mouse spleens were harvested and analyzed by flow cytometry. Dot plots are shown displaying SL9-specific CD8+ T cells from the untreated mouse (n = 1 mouse), and the mice treated with SL9-αCD28-synTac at a dose either of 4/mg/kg (n = 2 mice) or 0.4 mg/kg (n = 2 mice) after gating for viability and the expression of human CD45 and CD8.

Supplemental Table 1. Characteristics of synTac constructs used

synTac Constructs	Epitope	Costimulatory Domain	M.W. (kDa)
SL9-αCD28	HIV-SL9	αCD28 scFv	206
SL9-4-4BBL	HIV-SL9	Covalent 4-1BBL	268
SL9-FLAG	HIV-SL9	None, replaced by FLAG sequence	154.8
pp65-αCD28	CMV-pp65$_{495\text{-}503}$	αCD28 scFv	206
pp65-4-1BBL	CMV-pp65$_{495\text{-}503}$	Covalent 4-1BBL	268
pp65-FLAG	CMV-pp65$_{495\text{-}503}$	None, replaced by FLAG sequence	154.8

Supplemental Materials and Methods

Transduction of Jurkat/MA cells by the SL9-TCR-encoding lentiviral vector for evaluation of synTac binding and function. The lentiviral vector expressing SL9-specific TCR α and β chains was used to transduce Jurkat/MA cells as described [1]. To test synTac binding, SL9-TCR-transduced Jurkat/MA cells (0.5 x 10^6 cells) were incubated with SL9- or pp65-synTacs (1.56 nM) for 30 minutes at 4°C, washed twice with FACS buffer (2% FBS and 0.1% sodium azide in PBS), stained with fluorescent labeled anti-human IgG F(ab')2 fragment (Jackson ImmunoResearch Laboratories, West Grove, PA), washed twice and analyzed using an LSR-II flow cytometer (BD Biosciences, San Jose, CA). SynTac activation of SL9-specific TCR was determined by incubating SL9-TCR-transduced Jurkat/MA cells (5 x 10^5 cells/well) in triplicate in a 48-well plate with various concentrations of synTac constructs for 16 hours and quantifying of the NFAT-luciferase reporter gene activation using the Luciferase Assay System (Promega, Madison, WI) as described [2].

Tetramer staining to detect the expression of SL9- or pp65-specific T cell receptors. Cells were resuspended in FACS buffer and incubated with fluorochrome-labeled SL9 tetramer or pp65 tetramer (obtained from the NIH Tetramer Core Facility) for 40 minutes at room temperature, followed by staining with surface antibodies for 30 minutes at 4°C. DAPI was added to resuspended cells to gate out dead cells prior to acquiring labeled cells on LSR-II multi-laser flow cytometer.

Propagation of CMV luciferase virus and *in vitro* CMV inhibition assay. Towne strain of CMV was engineered to express luciferase (CMV-luc) under the promoter of a CMV late gene pp28 (UL99) and propagated as reported previously [3]. For assaying CMV

viral inhibition, an HLA-A*0201 human fetal lung fibroblast cell line MRC-5 (ATCC, Manassas, VA) was used as target cells by plating (41.7 x 10^3 cells/well) into a 24-well plate with 10% FBS-containing DMEM. One day later, MRC-5 cell culture media was replaced with serum-free DMEM media and CMV-luc virus was added at MOI = 3. After a 90-minute virus adsorption, the virus was removed. PBMCs that were untreated or treated with the indicated synTacs (0.1 nM) for 7-12 days to expand the pp65-specific CD8+ T cell population were used as effector cells, and added to each well at the indicated effector cell to target cell (E/T) ratios in DMEM with added FCS (4%). With the target-cell number fixed, synTac-treated PBMCs were added at different E/T ratios to each well and the cell number was not normalized for the number of expanded pp65-specific CD8+ T cells induced by synTac treatment. After 3 days of co-culture, cell culture media was removed, the cells were lysed with Luciferase Assay System lysis buffer (Promega) for 30 minutes while shaking and luciferase units were quantified using a Luminat Plus luminometer (Berthold Technologies, Oak Ridge, TN).

***In vitro* HIV inhibition by synTac-stimulated HIV-specific CD8+ T cells.** HIV-1-infected donor PBMCs were activated with PHA (4 µg/mL) and cultured in complete RPMI with added IL-2 (100 U/mL) for 48 hours. Activated cells were then washed, counted and resuspended at 10^6 cells/mL in complete RPMI with added IL-2 (100 U/mL) and mixed with Env-HIV-1_{Bal}-LucR virus (IMC-Bal) at MOI=1 and plated into 96-well U-bottom plates. The cells with added virus were spinfected for 90 minutes at 2,500 RPM (1,139 x g) at room temperature. Infected target cells were incubated for 24 hours before adding effector cells, which were untreated PBMCs or PBMCs that were treated with the indicated SL9-synTac (0.1 nM) to expand the SL-9-specific CD8+ T cell population, at the

indicated effector to target ratios. With the target cell number fixed, SL9-synTac-treated PBMCs was added to each well at different E/T ratios and the number was not normalized for the number of expanded SL9-specific CD8+ T cells induced by synTac treatment. Effector and target cells were then co-cultured for another three days before being lysed with the Renilla Luciferase kit (Promega) to quantify the luciferase activity (RLU). The percentage inhibition was calculated as (1-$Stimulated_{RLU}$/$Unstimulated_{RLU}$) x 100%.

Pharmacokinetic measurement of serum synTac concentration in mice. Mice were intravenously injected with synTac (4 mg/kg), serum samples were collected by submandibular bleeding and 4-1BBL synTac serum concentrations were measured using a 4-1BBL-specific ELISA using a standard curve generated using 4-1BBL standards. Briefly, Costar assay plates were coated with anti-human 4-1BBL antibody (2 μg/mL, BioLegend) at 4°C overnight, washed and blocked with buffer that contains 0.4% casein for 2 hours at 37°C. Serum samples and 4- 1BBL synTac standards were added to the wells and incubated for 1 hour at 37°C, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma-Aldrich, St. Louis, MO) for 1 hour. After SIGMA*FAST*™ OPD substrate (Sigma-Aldrich) was added, plates were read on a Wallac Victor plate reader (PerkinElmer, Waltham, MA) at 490nm.

Evaluation of *in vivo* inhibition of HIV infection by SL9-specific CD8+ T cells expanded by *in vivo* treatment of humanized mice with SL9-αCD28 synTac. NSG mice were intrasplenically injected with PBMCs (~32 X 10^6) from an HLA-A*0201 HIV-seropositive donor (619) and PBMC (14 x 10^6) that were depleted of CD8+ T cells by immunomagnetic sorting using CD8 microbeads (Miltenyi Biotec). One group of mice were untreated and another group were immediately intravenously injected with SL9-

αCD28-synTac (0.4 mg/kg). After 14 days, the mice were bled and HIV RNA in the plasma was quantified using a highly sensitive RT-qPCR assay as described[4]. Briefly, total RNA was extracted from 50-200 µl of mouse plasma using QIAamp MinElute Virus Spin Kit (QIAGEN, Germantown MD) and reverse transcribed to generate cDNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). RT-qPCR was performed using TaqMan Universal Master Mix II, no UNG (Applied Biosystems) with added cDNA (2 µl) and HIV *gag* sequence-specific primers 5'-TCAGCCCAGAAGTAATACCCATGT-3' (sense) and 5'-CACTGTGTTTAGCATGGTGTTT-3' and an HIV *gag* sequence-specific (antisense) probe (6FAM-ATTATCAGAAGGAGCCACCCCACAAGA-TAMRA) as described[5]. Cycle threshold values were calculated using a standard curve using samples with known copy numbers of absolute HIV DNA. Three days later, the spleens were harvested were evaluated by flow cytometry to quantify the population of CD8+SL9 tetramer+ cells.

Cytotoxicity assay. Cytotoxic activity of pp65-NLV-specific CD8+ T cells was assessed using DELFIA EuTDA Cytotoxicity Reagents (PerkinElmer) and peptide-loaded T2 cells as target cells. T2 cells were pulsed with 1µM of pp65-NLV (^{495}NLVPMVATV^{50}) peptide or irrelevant peptide in serum free media at room temperature for 40 minutes. Without washing the cells, BATDA reagent (2 µL) was directly added to the peptide-loading tube and incubated for another 20 minutes at 37°C. Cells were then washed three times in complete IMDM containing probenecid (2.5 mM) to reduce spontaneous release. Peptide-loaded T2 cells (50,000 cells) were added to each well of a 96-well U-bottom plate, followed by the addition of PBMCs (effector cells) that had been stimulated with synTacs at different effector-to-target ratios. After cells were co-cultured for 4 hours at

37°C, an aliquot of supernatant from each well (20 µL) was transferred to a microtiter plate supplied by the kit and Europium Solution (200 µL) was added to each well and shaken for 15 minutes. Time-resolved fluorescence was quantified on FLUOstar Omega (BMG LABTECH, Cary, NC) with excitation and emission wavelength of 355 nm of 620 nm respectively. The % specific lysis was then calculated using the formula: % specific lysis = (Experimental release – spontaneous release) / (Maximum release – spontaneous release) x 100%.

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