Stem Cell Reports, Volume *18*

Supplemental Information

Generation of functional human T cell development in NOD/SCID/ IL2rγ^{null} humanized mice without using fetal tissue: Application as a model of HIV infection and persistence

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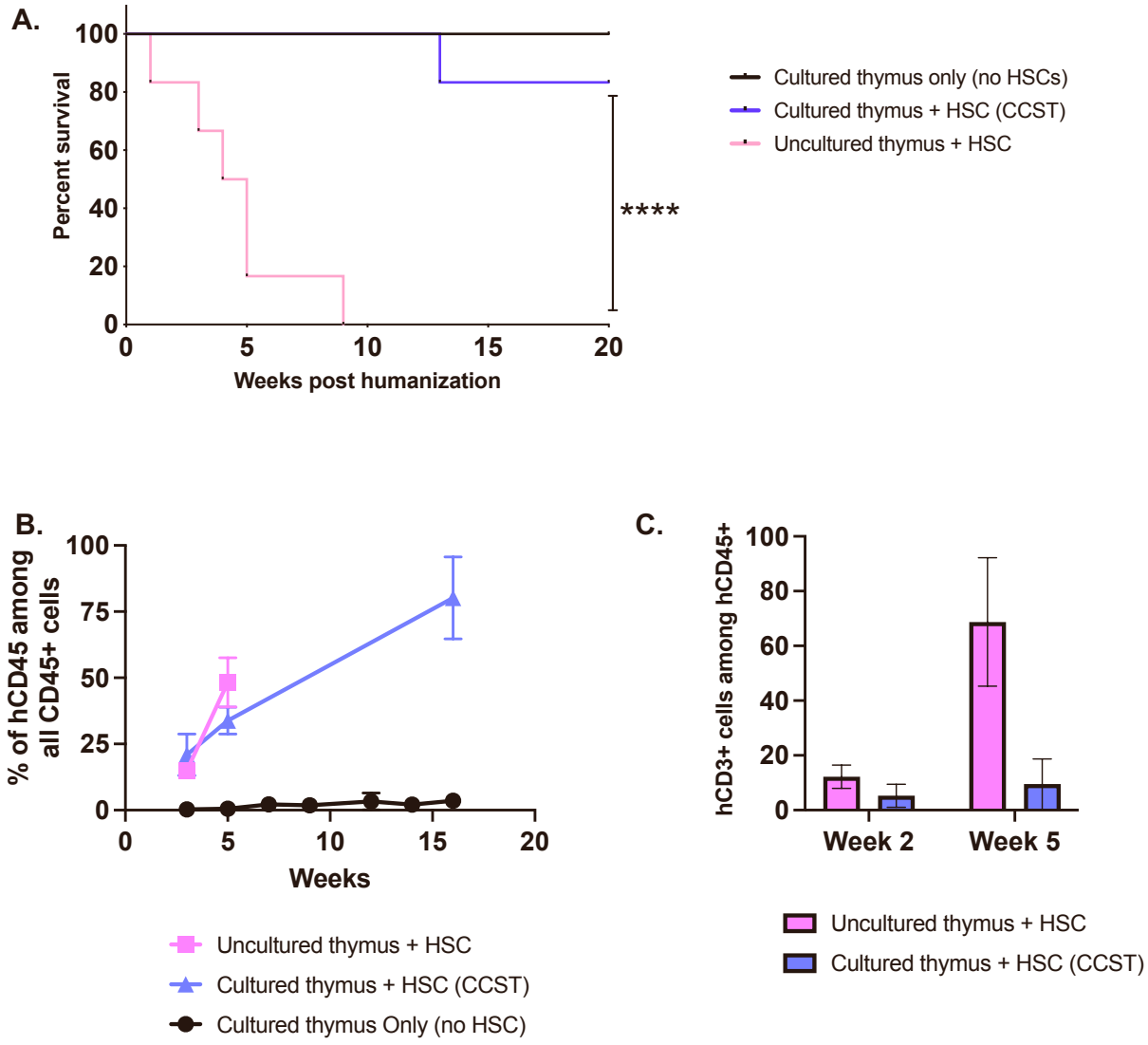
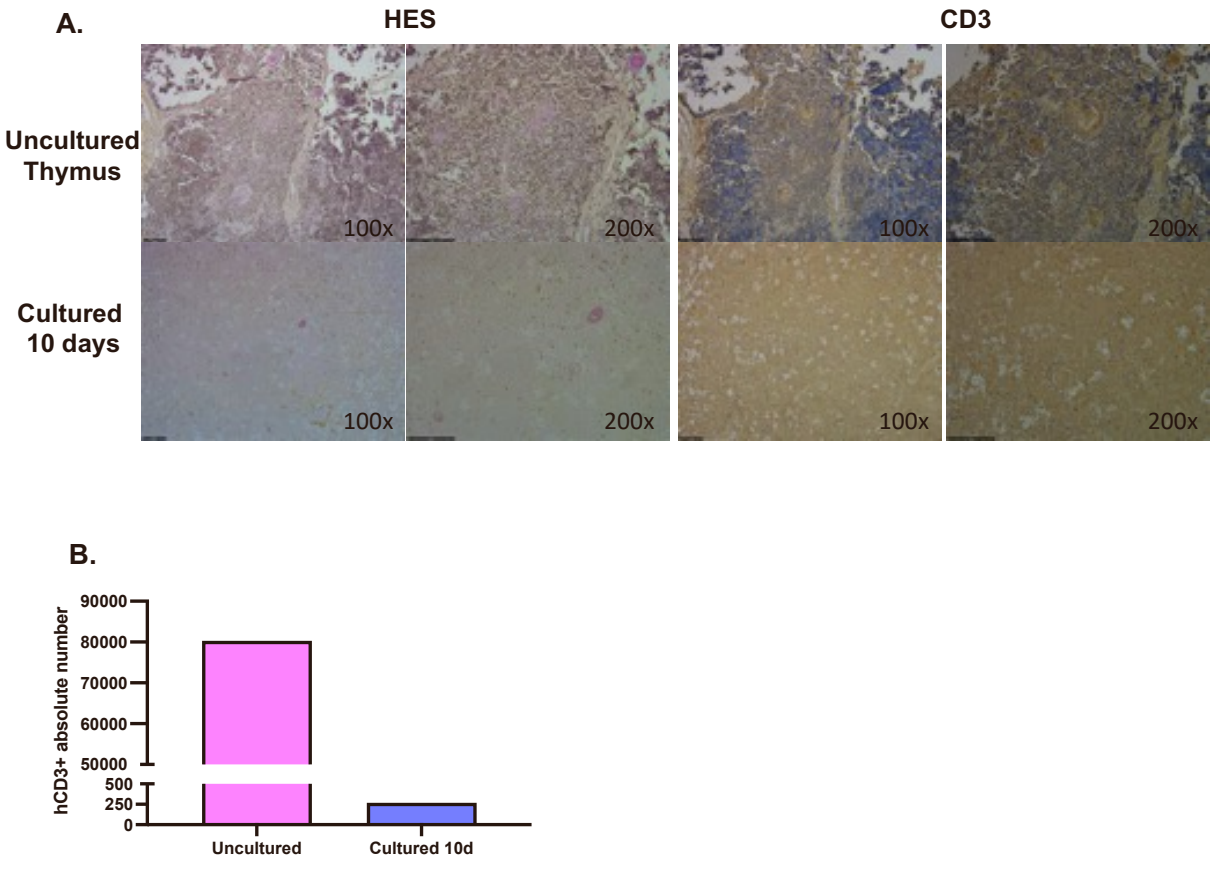
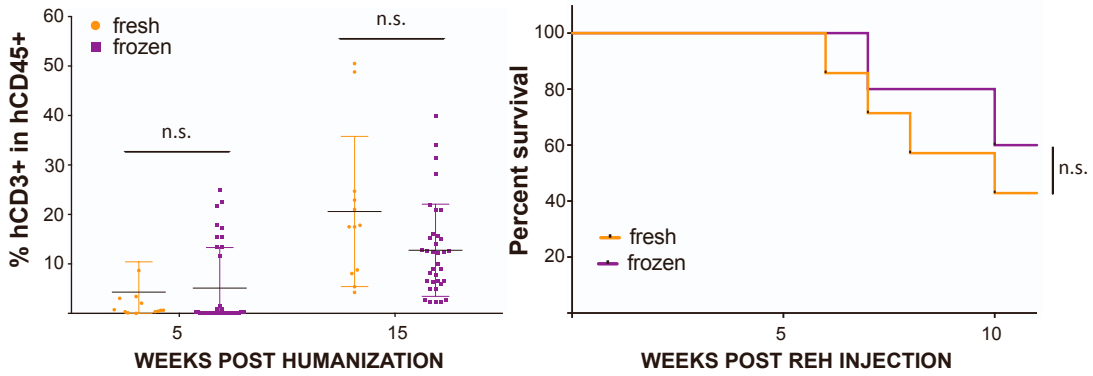
SUPPLEMENTAL MATERIAL



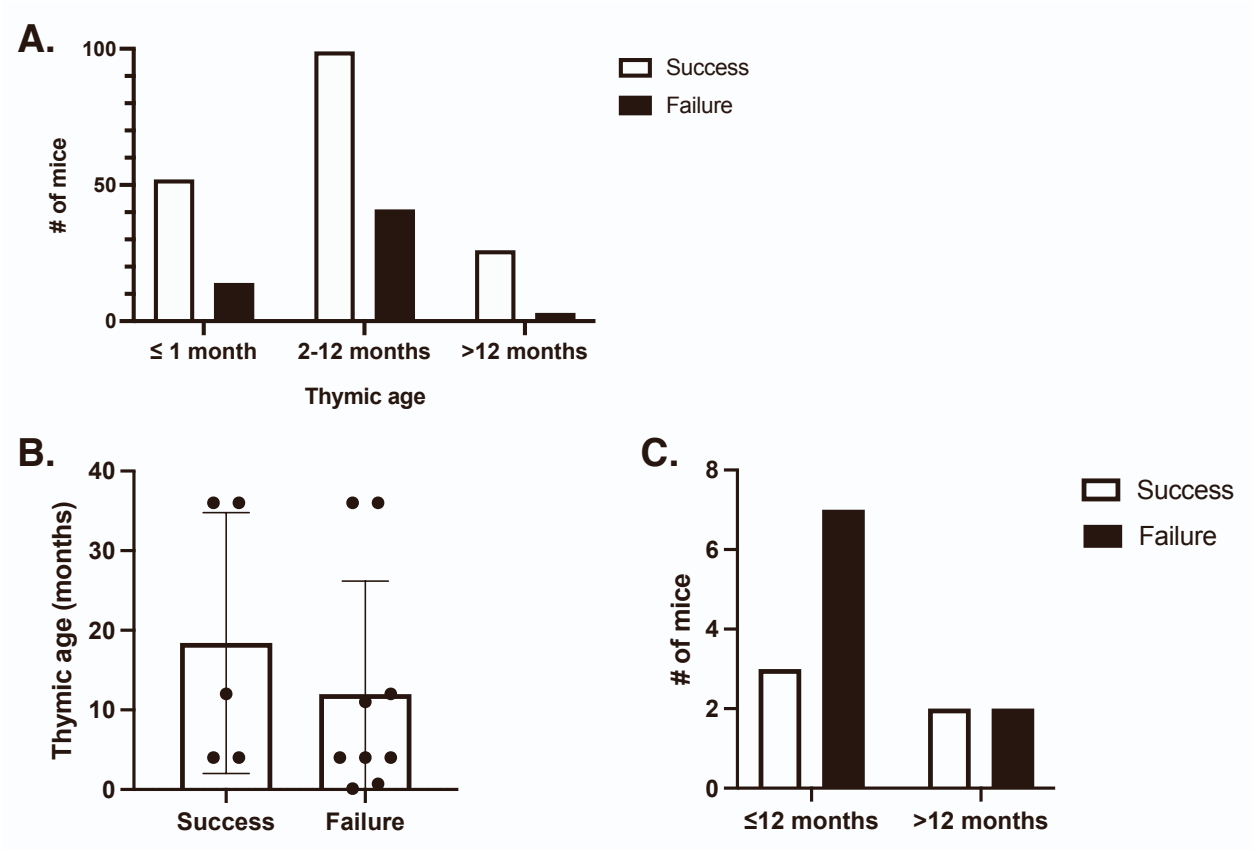
Supplemental Figure 1. Ex vivo Culture of Thymus Prior to Engraftment Improves Survival of Recipient Mice. A. Human pediatric thymus obtained from cardiac surgery was cut into small pieces and either implanted directly in the quadriceps muscle of sublethally irradiated-NSG mice humanized with $CD34^+$ from cord blood (uncultured, *n*=6) or put in culture (as described in Figure 1) for 7 days before implantation in the quadriceps muscle of humanized NSG mice (cultured, *n*=6). All mice that received uncultured thymus died within 9 weeks from severe GvHD symptoms, while all mice except one that received cultured thymus survived. These conditions were used in all subsequent experiments. Mice that were engrafted with a cultured thymus in the absence of HSC injection all survived (black line - *n*=4). Log-rank (Mantel-Cox) test, ****p<0.0001. **B.** Reconstitution data from mice humanized with either uncultured thymus (along with HSC injection – pink line), cultured thymus + HSC (CCST model – purple line) or cultured thymus only (without HSC -black line). Mice engrafted with uncultured thymus were followed until week 5, after which they all died as shown in panel A. **C.** Human T cell reconstitution (hCD3 proportion among hCD45) in mice that received an uncultured thymus piece and HSC (pink bars) vs mice engrafted with a cultured thymus and HSC (CCST model – purple bars).



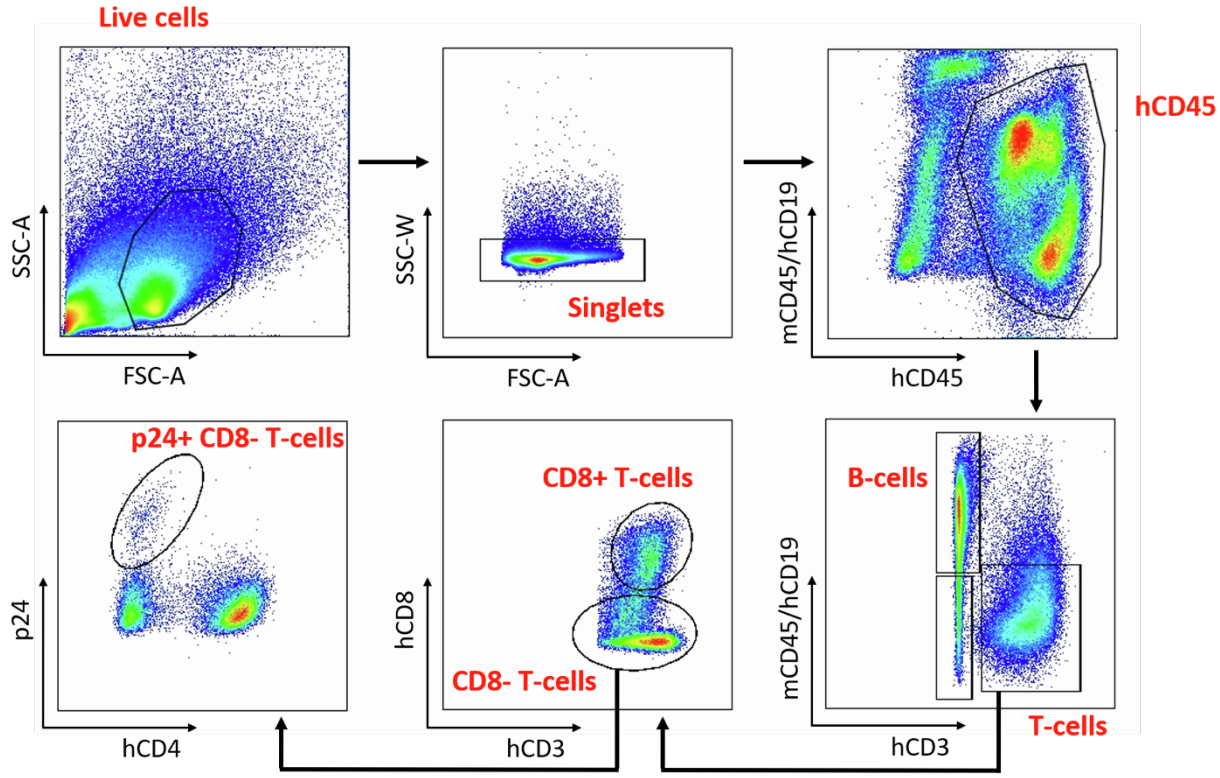
Supplemental Figure 2. Ex vivo Culture of Neonatal Thymus Decreases the Number of hCD3$^+$ cells. **A.** Hematoxylin-Eosin-Safran (HES - left panels) and immunochemistry staining for hCD3$^+$ (T cells – left panels) of uncultured (top line) or after 10 days of culture (bottom line). Images were taken at a magnification of 100x or 200x, bars represent 100um. **B.** Analysis by flow cytometry of the number of human CD3+ cells (hCD3+) in uncultured vs cultured (10 days) thymic pieces of similar sizes. An equal number of events, based on beads counts, were acquired in both conditions. Thymus pieces that were cultured showed a 298-fold reduction of hCD3+ cells in the tissues (80,291 hCD3$^+$ cells in uncultured vs 269 hCD3$^+$ cells in cultured thymus).



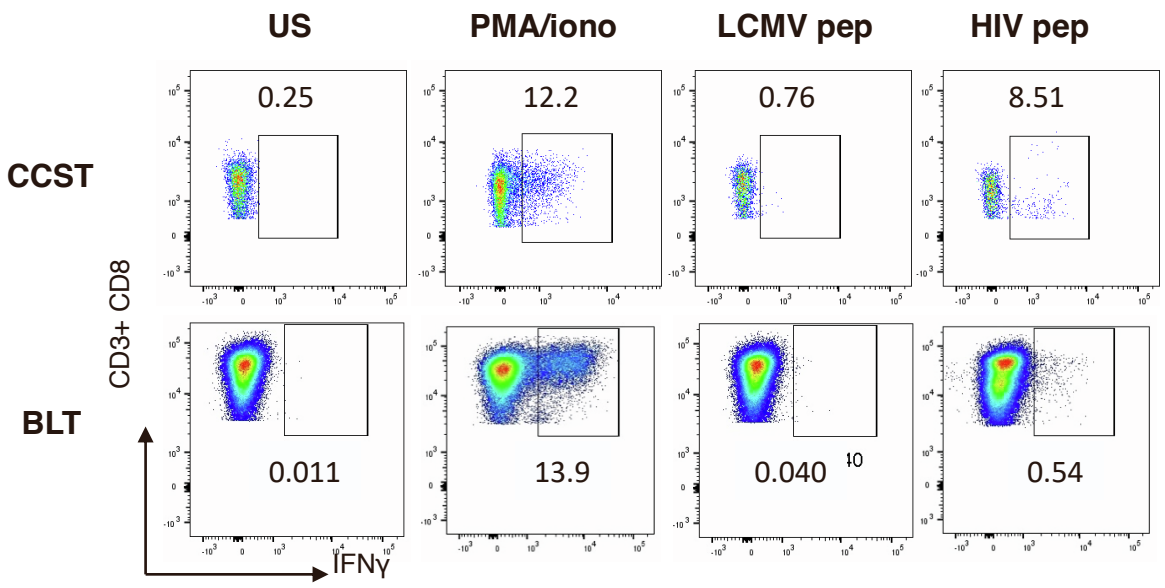
Supplemental Figure 3. Comparison of the different treatments used before transplanting a cardiac surgery thymus into a mouse. A. Following dissection, cardiac surgery thymus was put in culture for immediate use or cryopreserved for future use. CCST mice made with fresh or frozen tissues were not significantly different in the level of $hCD3^{+}hCD45^{+}$ cells (left panel, T-tests) or their ability to control REH leukemia challenge (right panel, Log-rank (Mantel-Cox) test). Each dot corresponds to a mouse. N.s: not significant.



Supplemental Figure 4. Impact of thymic age on the success of immune reconstitution and clearance of tumor cells. A. Success **(**n=177) or failure (n=58) of mice in reconstituting an immune system with early T cell output according to the age of the engrafted thymus. The data show that thymic age did not have an effect on the immune reconstitution (p=0.0732, Chi-Square test). Success was defined by early T cell output in immune reconstitution and functional assays (when available). **B.** Mean (+/- SD) of thymic age according to REH clearance (Success group, n=5) or death of the animal (failure group, n=9), p=0.4567, unpaired T-test. **C.** Effect of thymic age on mouse surviving REH challenge. p=0.4805, Two-sided Chi-Square test.



Supplemental Figure 5. Gating strategy for flow cytometry analysis to identify human immune cell subsets, including HIV-infected T-cells. Single cells were isolated from peripheral blood or tissues and stained with a mixture of fluorescent-labeled antibodies according to the cell surface staining protocol. Total human immune cell population was identified using anti-human CD45 antibodies and further characterized as B-cells ($hCD45^+CD19^+$), monocytes ($hCD45^+CD14^+$), T-cells ($hCD45^+CD3^+$) and their subtypes, $CD8^+$ T-cells ($hCD45^+CD3^+CD8^+$) and $CD4^+$ T-cells (identified as $hCD45^+CD3^+CD4^+$ in uninfected mice and $hCD45^+CD3^+CD8^-$ in HIV-positive mice as HIV downregulates CD4). To analyze T-cells infected with HIV, cells were permeabilized, and intracellular staining with anti-p24 antibody was performed. HIV-infected T-cells were subsequently gated as $hCD45^+CD3^+CD8^-p24^+$. Shown is the flow cytometry analysis of spleen cells from a representative HIV-infected CCST mouse.



Supplemental Figure 6. Interferon-ɣ production by CD8⁺ T cells from CCST and BLT mice upon antigenic stimulation. Splenocytes from infected CCST and BLT mice were kept untreated or stimulated with PMA/ionomycin, pooled Clade B HIV peptides (env, gag, pol, nef) (2 µg/mL) or LCMV peptides (GP61-80, GP276-286) (100 ng/mL). LCMV stimulation served as a specificity control for the assay and frequencies of IFNg-expressing human T-cells were measured intracellularly by flow cytometry. Shown is an example of cytokine staining in $CD8^+$ T cells.

Supplemental Table 1. HLA match and immune reconstitution

	Success n (%)	Failure n (%)	Chi-Square
No HLA match	3 (100%)	0 (0%)	0.9232
1 match	56 (95%)	3 (5%)	
2 or more matches	39 (95%)	2 (5%)	

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Allogeneic leukemic cell line challenge

Acute lymphocytic leukemia pre-B cells REH (ATTC®, Manassas, VA, USA) were modified to express luciferase using a pHUS Luciferase-GFP vector (constructed from the pHUS-GFP plasmid backbone and encoding for the firefly luciferase, an internal ribosome entry site (IRES) and eGFP). These cells were injected intravenously into mice (5,000 cells in 100 µl of $dPBS^{-/-}$). Mice were then monitored weekly by injecting 3 mg D-luciferin (Perkin Elmer, Waltham, MA, USA) intraperitoneally and imaging using an *in vivo* bioluminescence imaging system (LabeoTech, Montreal, QC, Canada) system. Images were analyzed using ImageJ (version 1.52p, NIH) to quantify the bioluminescence intensity.

T-cell proliferation assay

T cell capacity to proliferate upon stimulation with phytohemagglutinin-L (PHA-L) (Sigma Aldrich) was measured following guidelines of the Click-iT® EdU cell proliferation assay kit (Thermo Fisher Scientific). Briefly, 150-200 µl of peripheral blood was drawn from the saphenous vein and collected in heparinized tubes. Red blood cells were then lysed in a buffer containing 0.15 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA (prepared in distilled water) and white blood cells cultured with PHA-L (5 µg/mL) for 48 hours and then EdU (10 µM) for overnight. The following day, cells were harvested, stained with FITC hCD8a (clone RPA-T8, BioLegend) and hCD4 (clone RPA-T4, BD Biosciences), and then fixed/permeabilized using Click-iTTM fixative and saponin-based reagent and stained using the fluorescent dye picolylazide to reveal the incorporated EdU. Fixed cells were analyzed on the BD FACS Canto or FACS LSRFortessa system.

Histology

The specimens were fixed in buffered-formalin phosphate 10 % and embedded in paraffin. 4 µm sections were prepared, mounted on microscope slides, and stained with hematoxylin phloxin Safran. Immunohistochemistry staining was also performed on paraffin-embedded slices on the Autostainer Link 48 from Dako with the "ready to use antibodies" hCD3 (polyclonal rabbit),

hCD68 (clone KP1), human Cytokeratin 19 (clone AE1/AE3) and hCD19 (EPR5906; Abcam, Cambridge, UK).

Flow cytometry. Blood cells and single-cell suspensions from tissues were stained with a combination of fluorescently labelled Abs. Dead cells were excluded using a live/dead fixable violet dead cell stain kit (ThermoFisher). Surface-stained cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) as per the manufacturer's instructions and intracellularly stained with anti-p24 antibody or anti-IFNγ. Flow cytometry data were collected on a Fortessa flow cytometer (BD Bioscience) and analyzed by Flowjo software (Versions 9.9.3 and 10.1).

HIV Virus production. $5x10^6$ HEK 293T cells were transfected with 30 µg of CCR5-tropic pNL4.3.ADA.GFP proviral DNA (Dave et al., 2013) by the calcium phosphate method. Culture supernatant was collected at 60 h post-transfection. Viruses were concentrated by ultracentrifugation over a 20% sucrose cushion and tittered in HeLa TZMbl and CEM-CCR5 cell lines (NIH AIDS reagent program). $TCID_{50}$ was calculated using the Spearman-Karber method.

Ex vivo IFN-γproduction. Splenocytes from CCST and BLT mice were stimulated with PMA (50 ng/mL) plus ionomycin (500 ng/mL), or LCMV peptides (GP61-80, GP276-286) (100 ng/mL) for 1 hour or with pooled Clade B HIV peptides (env, gag, pol, nef) (2 µg/mL) (NIH AIDS Reagent Program) for overnight. Subsequently, Brefeldin-A (BioLegend) was added and cells were cultured for another 6 hours. Untreated splenocytes were used as controls. Frequencies of IFNγ-expressing human T cells were measured by flow cytometry as described below. Splenocytes from uninfected mice and LCMV stimulation were used to ensure the specificity of the assay.