Supporting Information

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SI Methods

Establishment and Characterization of CTL Lines. Human CTL lines were established from EBV-infected humanized NSG-HLA-A2/HHD mice as described previously (30). Briefly, human mononuclear cells isolated from recipient spleen were stimulated with X-irradiated autologous LCL in RPMI medium 1640 supplemented with 10% human AB serum and 10 U/mL IL-2 (Boehringer Mannheim). After 1 wk, CD8+ T cells were isolated using anti-hCD8 microbeads (Miltenyi Biotec). CD8+ T cells were then restimulated weekly with X-irradiated autologous LCL. After three restimulations, the cytotoxic activity of CD8+ T cells was examined by ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr ($Na_2{}^{51}CrO_4$; New England Nuclear) -labeled target cells and various numbers of effector cells in 200 μL of RPMI medium 1640 supplemented with 10% FCS were seeded into 96-well round-bottom plates. Following a 1-h incubation with anti-HLA class I framework monoclonal antibody (w6/32; American Type Culture Collection) or anti-HLA-DR monoclonal antibody (L243; American Type Culture Collection) at 10 μg/mL to assess HLA restriction of cytotoxicity, the effector cells were added. After 5 h, 100 μL of supernatant was collected from each well. The percentage of specific lysis was calculated as (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%).

Flow Cytometry and Cell Sorting. For phenotypical analysis and cell sorting, cells labeled with the following monoclonal antibodies (mAbs) were analyzed and/or sorted using FACSAria and FACSCanto-II (BD Biosciences): mouse anti-hCD3-V450, -hCD3-APC, -hCD4-PE-Cy7, hCD8-APC-Cy7, -hCD19-PE-Cy7, -hCD34-PE-Cy7, -hCD38-APC, -hCD45-APC and -V450, -hCD45-AmCyan and -APC-Cy7, -hCD45RO-APC, -hCD45RA-PE, -hCD45RA-PE-Cy7, -hCD56-FITC, -hCD56-PE, -HLA-DR-FITC, -hCCR7-PE-Cy7, -HLA-A2-PE, -hβ2 microglobulin-FITC, -hTCR-γ/δ-FITC, rat anti-mouse CD45-APC-Cy7 (all from BD Biosciences), mouse anti-hTCR-pan α/β-PE (Beckman Coulter), rat anti-hCCR7-FITC (eBioscience), and rat anti-mouse EpCAM-APC (BioLegend). T-cell receptor (TCR) Vβ characterization was performed using the TCR Vβ repertoire kit (IOTest Beta Mark; Beckman Coulter), which consists of mAbs specific for 24 distinct TCR Vβ families.

Cell Preparation. Recipient bone marrow (BM) was harvested from femurs and tibiae. Peripheral blood (PB) was collected from the retroorbital plexus. Thymic epithelial cells were isolated as described (1). Briefly, thymic fragments were digested for 60 min (15 min each, 4 times) at 37 °C in 0.125% (wt/vol) collagenase D (Roche) with 0.1% (wt/vol) DNase I (Sigma) in RPMI-1640 (Sigma). Pooled cells were resuspended in 5 mM EDTA in PBS containing 1% fetal calf serum (FCS) and incubated for 10 min at 4 °C to disrupt rosettes. Single-cell suspensions of BM, spleen, and thymus were filtered and used for subsequent analyses.

Cell-Cycle Analysis. CD34-enriched human cord blood (CB) cells were labeled with Hoechst 33342 and PyroninY (Sigma) followed by surface staining with anti-hCD34-PE-Cy7, -hCD38-APC, and –hCD45-APC-Cy7 mAbs and analyzed using FACSAria (BD Biosciences).

Intracellular Staining for Cytotoxic Molecules. After surface staining with anti-hCD3, -hCD4, -hCD8, and -hCD45 mAbs, splenocytes were permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained with mouse anti-hGranzyme A-FITC, -hGranzyme B-FITC, or -hPerforin-PE (all from BD Biosciences) mAbs and analyzed using FACSCanto-II (BD Biosciences).

Intracellular Cytokine Production Assay. Aliquots of 1×10^6 splenocytes were exposed to 25 ng/mL phorbol 12-myristate 13-acetate (Sigma), 10 μg/mL ionomycin (Sigma), and 1 μg/mL anti-CD28 (CD28.2; BD Biosciences) in RPMI-1640 supplemented with 10% FCS. After a 1-h incubation at 37 °C, 10 μg/mL brefeldin A (Sigma) was added followed by a 3-h incubation at 37 °C, and cells were stained with anti-hCD4-PE-Cy7, anti-hCD3-APC, anti-hCD8-APC-Cy7, and anti-hCD45-V450 mAbs. Subsequently, cells were permeabilized as described above and stained with mouse anti-hIFN-γ-FITC or -PE, -hIL-4-PE, -hIL-17A-FITC (eBioscience), mouse anti-hIL-21-PE (BD Biosciences), or mouse anti-hIL-22-PE (R&D Systems) mAbs and analyzed using FACSCanto-II (BD Biosciences).

Tetramer Assay. PE-labeled HLA-A*0201 + b2m + Epstein–Barr virus (EBV) LMP1 amino acids 159–167 (YLQQNWWTL) and BMLF1 amino acids 280–288 (GLCTLVAML) tetramer peptides were obtained from MBL. Single-cell suspensions of splenocytes from EBV-infected humanized NSG or NSG-HLA-A2/HHD mice were incubated with LMP1 or BMLF1 tetramer at 37 °C for 20 min, followed by anti-hCD3-V450, -hCD8-APC-Cy7, or -hCD45RO-APC labeling for 30 min at 4 °C. The cells were washed and analyzed using FACSCanto-II (BD Biosciences).

Generation of B-Lymphoblastoid Cell Lines. B-Lymphoblastoid cell lines (LCLs) were established by transformation of CB B lymphocytes with EBV. Autologous LCLs were generated from the same source of CB as the hematopoietic stem cells (HSCs) used for transplantation. B95-8 supernatant and 10^7 CB cells were incubated in RPMI medium 1640 supplemented with 10% FCS for 30 min at 37 °C. Cyclosporin A at 0.5 μg/mL was subsequently added and cells were cultured for 5–6 wk at 37 °C.

INF-γ ELISPOT Assay. EBV-specific T-cell responses were quantified by IFN-γ enzyme-linked immunospot (ELISPOT) assay. All experiments were performed in triplicate. MultiScreen-HA 96-well plates (Millipore) were coated with 5 μg/mL anti-IFN-γ mAb (Mabtech) for 3 h at room temperature and preincubated with RPMI-1640 supplemented with 10% FCS for 1 h at room temperature. Isolated human CD8+ T cells derived from recipient spleen were resuspended in media and 10^5 cells per well were stimulated with autologous LCLs at a ratio of 1:1. Target and responder cells were incubated for 18 h at 37 °C. Stimulation with anti-CD3 antibody (Mabtech) at 100 ng/mL and phytohemagglutinin (PHA; Sigma) at 10 μg/mL was used as positive control. Anti-HLA class I (eBioscience) or class II (Dako) mAb was added to the cell mixtures to demonstrate HLA-restricted cytotoxicity. Biotinylated anti-IFN-γ mAb (Mabtech) at 1 μg/mL was added and plates were incubated for 90 min at room temperature. Streptavidin-conjugated alkaline phosphatase (Mabtech) was added and incubated for 1 h at room temperature. Spots were developed using a tetramethyl benzidine substrate reagent set (Mabtech) for 5 min at room temperature. Individual spots were counted under a dissecting microscope (Carl Zeiss).

Histological Analysis. Hematoxylin/eosin (H&E) staining was performed using standard procedures. For immunofluorescence imaging, paraformaldehyde-fixed paraffin-embedded tissues were labeled using the following primary antibodies: rabbit anti-hCD3

polyclonal (Dako), mouse anti-hCD19 monoclonal (AbD Serotec), and mouse anti-LMP1 monoclonal (Dako). Nuclei were stained using DAPI. Laser-scanning confocal imaging was obtained using a Zeiss LSM 710 microscope (Carl Zeiss).

1. Gray DH, Chidgey AP, Boyd RL (2002) Analysis of thymic stromal cell populations using flow cytometry. *J Immunol Methods* 260:15–28.

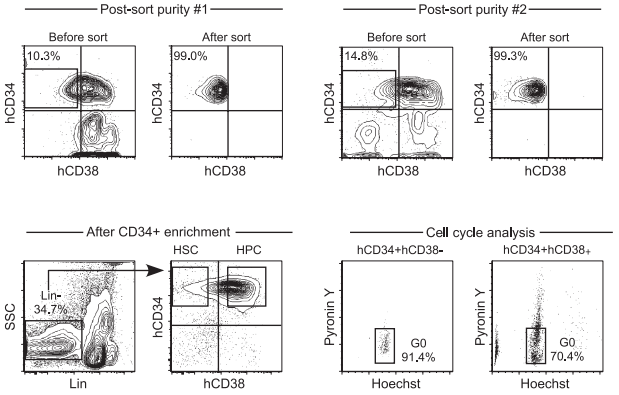

Fig. S1. (*Upper*) Human Lin–CD34+CD38– HSCs are isolated from CB by sorting. Pre- and postsort expression patterns of hCD34 and hCD38 for two independent sorting experiments are shown. (*Lower*) Representative contour plots demonstrating gating strategy used for isolation of CB HSCs (*Left*). Lin–CD34+CD38– HSCs were enriched for Hoechst(low)PyroninY(–) cell-cycle quiescent cells compared with Lin–CD34+CD38+ hematopoietic progenitor cells (*Right*).

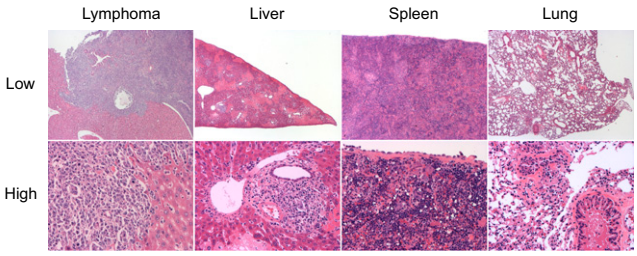

Fig. S2. Lymphoproliferative lesions develop in EBV-infected humanized NSG recipients. H&E-stained (upper panels: low-magnification images; lower panels: high-magnification images) tissue sections obtained from humanized NSG recipients infected with EBV demonstrate lymphoproliferative lesions in multiple organs. Lymphoma developed in the peritoneal cavity of NSG recipients. Lymphoproliferative lesions were identified in recipient liver and spleen, but not in the lung.

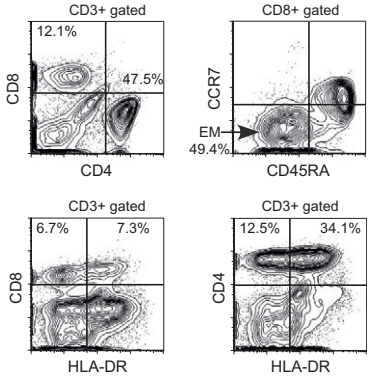

Fig. S3. Steady-state T-cell activation status in EBV-infected NSG-HLA-A2/HHD humanized mouse. The frequencies of effector memory CTLs and HLA-DR expression in CD8 and CD4 T cells are indicated.

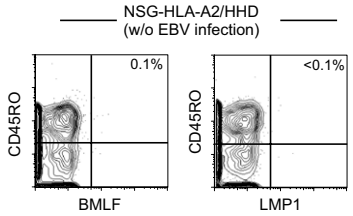

Fig. S4. CTLs specific for anti-EBV tetramers were undetectable in uninfected NSG-HLA-A2/HHD humanized mouse.

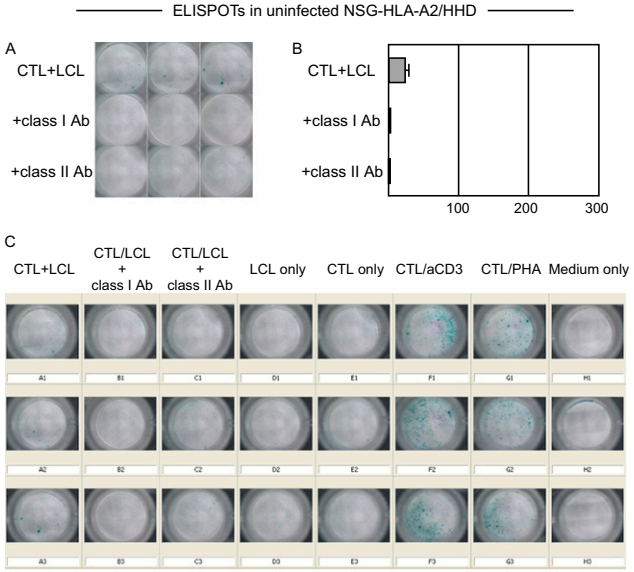

Fig. S5. CTLs from uninfected NSG-HLA-A2/HHD humanized mouse do not exhibit cytotoxicity against LCLs. EBV-specific T-cell responses were quantified by IFN-γ ELISPOT assay. A representative experiment performed in triplicate is shown. (*A*) Stimulation with autologous LCLs did not result in IFN-γ secretion by CD8+ splenocytes from uninfected NSG-HLA-A2/HHD humanized mouse (top row). The middle and bottom rows show wells with the addition of anti-HLA class I and anti-HLA class II antibodies, respectively. (*B*) Mean and SEM of the number of positive spots are shown. (*C*) A representative ELISPOT result showing negative (LCL only, CTL only, medium only) and positive (CTL+anti-CD3 antibody, CLT + PHA) controls is shown.