SUPPLEMENTAL MATERIALS AND METHODS:

Cell lines

EBV transformed B lymphoblastoid cell lines (EBV-LCLs) were generated by infecting PBMCs with the B95-8 strain of EBV in the presence of cyclosporin A (Sandoz, Germany). EBV-LCLs were cultured in RPMI 1640 (HyClone Laboratories Inc.), 2mM GlutaMAX TM-I, and 10% Fetal Bovine Serum (R-10 medium). The ULCL was generated by knocking out surface expression of HLA class I and II molecules. K562cs was derived from the HLA-negative, chronic erythroid leukemia cell line, K562 that lacks HLA class I and II molecules, by genetic modification with CD80, CD83, CD86, 4-1BB-L and was a gift from Dr. Carl June (University of Pennsylvania, Perelman School of Medicine). K562cs were cultured in R-10 medium and provided costimulation for VST expansion. In some experiments, an MHC class I and class II negative EBV-LCL (ULCL) was used for costimulation instead of K562cs.

Cell culture media

T-cells were cultured in VST medium [RPMI 1640 (HyClone Laboratories Inc. Logan, Utah), 45% Click's (Irvine Scientific, Santa Ana, CA), 2mM GlutaMAX TM-I (Gibco by Life Technologies, Grand Island, NY), and 10% Fetal Bovine Serum (HyClone Laboratories Inc.)].

Pepmixes

Overlapping peptide libraries - 15 mers overlapping by 11 amino acids) spanning the entire protein sequences of adenovirus (hexon and penton), varicella zoster virus (VZV) (IE61, IE62, IE63, and ORF10) were purchased from JPT technologies (Berlin, Germany). Lyophilized

pepmixes were reconstituted at 200 micrograms/ml in DMSO (Sigma-Aldrich, St Louis, MO) and stored at -80°C.

Addition of CD45RA+ T-cell subsets to RAD-PBMC

In experiments in which isolated $T_{EMRAs,}$ $T_{SCMs,}$ and T_N subsets were added back to RAD-EBVSTs, we first isolated CD14 expressing monocytes from PBMCs for use as antigen-presenting cells before further sorting of the RA+ subsets. CD14+ monocytes were added back to all cultures at a frequency of 20% to ensure an equal number of antigen-presenting cells in each group. When $T_{EMRAs,}$ $T_{SCMs,}$ and T_{NAIVE} subsets were added to RAD-PBMCs, the same number of each was added. Since T_{EMRAs} and T_{SCMs} have the lowest frequency in PBMCs, the number of cells sorted in those subsets determined the number of $T_{EMRAs,}$ $T_{SCMs,}$ and T_{NAIVE} cells used for each donor. To account for artifacts induced by cell sorting in these experiments, we generated W- and RA+ PBMCs by reconstituting the relevant separated subsets and found they expanded similarly to EBVSTs generated from standard W- and RA+ PBMCs (fig. S5). After sorting and recombination, PBMC subsets were combined with CD14+ monocytes and stimulated with EBV pepmixes. Expansion and specificity were analyzed as before.

VST generation: T-cells specific for adenovirus (AdV), varicella zoster virus (VZV) specific VSTs were generated in the same way as EBVSTs with pepmix cocktails containing a mastermix of pepmixes relevant for each virus (hexon and penton of adenovirus, and ORF10, IE61, IE62, IE63 of VZV) in the presence of IL-15 (at the indicated concentration) (R&D Systems, Minneapolis, MN) and IL-7 (10ng/ml) (PeproTech, Rocky Hill, NJ). The cells were split, and VST medium supplemented with cytokines was replenished as required. On day 9, the cells

received a second stimulation (S2) - with irradiated T2 pepmix-pulsed autologous ATCs and irradiated K562cs, at a responder T cell: pepmix-pulsed irradiated autologous ATCs: K562cs ratio of 1:1:5 in the presence of IL-7 and IL-15. In some instances, ULCLs were used as co-stimulatory cells instead of K562cs. The cells were then expanded for 7 to 9 days in cytokines and used for functional characterization.

Enzyme-linked immunospot (ELISpot) assay

The frequency of T2 antigen-specific T cells within EBVST populations was measured in IFNγ ELISpot assays. A 96-well MultiScreen HTS IP plate (EMD Millipore, Burlington, MA) was coated with anti-human IFNγ mAb 1-D1K (Mabtech, Cincinnati, OH) and incubated overnight at 4ºC. EBVSTs were plated at $1x10^5$ per well in duplicate and stimulated with pepmixes, as indicated in each experiment. T-cells cultured in EBVST medium were used as negative controls. After 16-24 hours of incubation at 37ºC with 5% CO_2, the plate was washed and incubated with anti-human IFNγ mAb 7-B6-1- Biotin (Mabtech, Cincinnati, OH) for 2 hours – 48 hours at 37°C. The plate was washed again and Avidin-peroxidase-complex (Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature. The plates were then developed with 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO) substrate, dried overnight, and sent to Zellnet Consulting (Fort Lee, NJ) for quantification or analyzed in the lab using Mabtech IRIS – ELISpot reader (Mabtech, Cincinnati, OH). The spot-forming cells (SFCs)/10^5 cells were used as a measure of the number of cells releasing IFNγ in response to viral antigen pepmixes after subtracting negative control values.

Intracellular staining (ICS):

VSTs (200 μL) were aliquoted into 96-well plates at 2 x 10^6 cells/ml and stimulated with 100ng of EBV pepmix (EBNA-1, LMP-1, LMP-2) or control pepmix (tumor antigen PRAME) in the presence of anti-human CD28 and anti-human CD49d (1μg/ml) (BD Biosciences, Franklin Lakes, NJ) at 37°C for an hour after which brefeldin A and monensin were added. The cells were then incubated overnight (16-hrs), then washed with PBS, and surface stained with CD3, CD4, and CD8 antibodies. After 15 mins, cells were washed and stained with fixable live dead stain – eBioscience Fixable Viability Dye efluor 780 (Thermofisher, Waltham, MA) for 20 mins at 4°C in the dark. After washing twice with PBS, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, Franklin Lakes, NJ) for 20 mins at 4°C in the dark. Cells were then washed twice with permeabilizing buffer (perm buffer) and incubated with IFN-γ and TNF-α antibodies (Becton Dickinson,) for 30 mins at 4°C in the dark. Cells were then washed twice and resuspended in permeabilizing buffer (Thermofisher, Waltham, MA) for flow cytometry. The cells were acquired using the Gallios Flow Cytometer or the BD FACSCanto II and results were analyzed using Kaluza software (Beckman Coulter, Inc) or FlowJo analysis software (FlowJo, LLC, Ashland, OR).

Cytotoxicity assay

The cytolytic specificity of the VSTs was evaluated in a standard 4-hour ^{51}Cr chromium release assay. Autologous ATCs alone or pulsed with pepmixes were used as target cells by labeling with ^{51}Cr sodium chromate for one hour at 37ºC. The target cells were washed thrice, re-suspended in VST medium, and used as target cells at an effector : target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1. Target cells were cultured in VST medium or 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) to achieve spontaneous and maximum release respectively. After 4

hours of co-culture, the supernatant was harvested and ^{51}chromium release was measured using a gamma counter. The percent specific lysis was calculated from the mean of triplicates as [(experimental release – spontaneous release) / (maximum release – spontaneous release)] × 100.

SUPPLEMENTARY FIGURE LEGENDS:

Supplemental figure 1

The efficiency of CD45RA depletion of PBMCs

CD45RA expressing cells were depleted from the whole (W)-PBMCs of 14 healthy donors to produce W-, CD45RA depleted (RAD)- and CD45RA+ PBMCs subsets and analyzed by flow cytometry (A) Dot plot analysis showing CD45RA positive cells in W- and RAD-PBMCs in one representative donor. (B) Frequency of CD45RA+ cells in W- and RAD-PBMCs from all 14 donors (mean ± SEM). (C) Representative dot plot showing CD3-, CD56+ NK-cells in W- and RAD-PBMCs. (D) Graphical representation of the frequency of CD3- CD56+ NK cells (D) and CD4+ CD8- and CD4-CD8+ lymphocytes (E) in W-, RAD- and RA+ PBMCs. A paired two-tailed Student's *t*-test was used for statistical analyses $p < .05$, (.12(ns: non-significant), .033(*), .002(**), <.001(***). Data shown are plotted as mean ± SEM.

Supplemental figure 2

Depletion of CD45RA positive PBMCs increases the antigen specificity of derived VSTs.

(A) EBV T2 antigen specificity for W- and RAD-EBVSTs for 16 donors. (B) Frequency of polyfunctional T2-specific T-cells as measured in ICC assays. The irrelevant peptide is the human tumor antigen, PRAME. (C) Frequency of CD3+ T-cells and CD3-CD56+ NK cells in W- and RAD-EBVSTs (n=15). (D) PBMCs specific for adenovirus (hexon and penton), and VZV (IE2, IE3, and ORF10) were generated from W- and RAD-PBMC as for EBVSTs. Antigen specificity on day 16 as measured by ELIspot is shown for 7 donors. Statistical significance was determined using paired two-tailed Student's *t*-test. $p < .05$, (.12(ns: non-significant), .033(*),.002(**),<.001(***) Data shown are plotted as mean ± SEM.

Supplemental figure 3

Expansion and specificity of EBVSTs generated from W-, RAD-, and CD45RA+ PBMCs

EBVSTs were generated from W-, RAD- and RA+ PBMC subsets, and their proliferation and antigen specificity were evaluated on day 16. (A) Fold expansion of W-, RAD- and RA+ EBVSTs from day 0 to day 16 (n=5). (B) EBV antigen specificity of W- vs. RAD- (D) and RA+ EBVSTs n=5. Data are plotted as mean ± SEM.

Supplemental figure 4

Increased clonality of EBVSTs derived from CD45RA depleted PBMCs

TCRVβ sequencing was performed on W-, RAD-, and RA+ PBMCs and their derived EBVSTs. (A) Illustration of the generation of W-, RA+ and RAD- subsets for TCRVβ immunosequencing. (B and C) Venn diagram representation of unique and shared TCR Vβ sequences observed in W-, RAD- and RA+ PBMCs (B) and their derived EBVSTs (C) for donor 2. N_c indicates the total number functional TCR templates. (D) Circos plots showing the

50 largest clonotypes in W- (top) and RAD (bottom) PBMCs and their derived EBVSTs for donor 2. Ribbons indicate shared clonotypes (W-PBMCs (grey) , W-EBVSTs (blue), RAD-PBMCs (grey) and RAD-EBVSTs (green). (E) Circos plot comparing the top 50 clonotypes of W- (blue) and RA+ (purple) EBVSTs from donors 1 (left) and donor 2 (right) (F) Productive Simpson's clonality of W-, RAD- and RA+ PBMC (black) and derived EBVST (red) TCR repertoires. 0 indicates the most diverse possible repertoire (clonotypes size of 1), and 1 represents a monoclonal sample.

Supplemental Table 1

Frequency of top 50 clones in W-, RAD-, and RA+ PBMCs and their subsequent derived EBVSTs

Supplemental figure 5

Top clonotypes in RAD-EBVSTs show a greater relative change in the frequency from PBMC compared to EBVSTs from W- or RA+ subsets

Violin plots show the relative change in frequency (over the 16 days of culture) of the top 10 ranked EBVST clonotypes in each other EBVST subset. For example, 5Aa compares the change in frequency of the top ten RAD-EBVST clonotypes in the RAD-EBVST subset with the change in frequency of the same clonotypes in the W- and RA+ EBVST subsets. The relative change was calculated by dividing the frequencies of the top EBVST clonotypes by their frequencies in PBMCs . (A) Donor 1 a-c, (B) Donor 2 d-f. Above the black dotted line are clonotypes whose frequency increased, below the dotted line the frequencies decreased. However, this does not take into account the fold expansion of each clonotype (shown in Figure 3f). Each clonotype is

represented by a solid circle, and lines connect the same clonotypes in other subsets. Black circles indicate clonotypes that were also detected in the PBMC of each subset. Red clonotypes were not present in the EBVST subset. Blue clonotypes were detected in the EBVST subset but not in the parental PBMC, I which case, the change in frequency was based on the assumption that the clonotype was of size 1 cell in the parental PBMC

Supplemental figure 6

The effects of various subsets on the antigen specificity of RAD-EBVSTs

To isolate subsets, NK-cells and T-regs were isolated from W-PBMCs as described in the methods section. T_{EMRAs} (CD45RA+, CCR7-), T_{SCMs} (CD45RA+, CCR7+, CD95+) and T_N (CD45RA+, CCR7+, CD95-) from EBV seropositive healthy donor were isolated using fluorescence-activated cell sorting (FACS). EBVSTs were generated from the PBMC subsets indicated and their antigen specificity was measured in ELIspot assays on day 16. (A) Bar graph compares the antigen specificity of EBVSTs derived from Whole (W)-, RAD, NK-depleted (NKD)- and natural Treg-depleted (TRD-) PBMCs from 5 PBMC donors. (B and C) CD45RA+ PBMCs were sorted into T_{SCM}, (T_S), T_{NAIVE} (T_N) and T_{EMRA} (T_E) subsets and stimulated alone or in combination using pepmix-pulsed, CD14+ PBMCs (n=6 from buffy coats). The RA+ subset was reconstituted from T_{SCM}, $T_{N,}$ and T_{EMRA} subsets. The reconstituted CD45RA+ subset was added to RAD-PBMCs to generate W-PBMCs. (B) Fold expansion of each subset alone and in combination. (C) Antigen specificity on day 16. Data are plotted as mean ± SEM.

Supplemental figure 7

Lower expression of exhaustion markers on RAD-EBVSTs

The surface expression levels of T-cell exhaustion markers were evaluated in W- and RAD-EBVSTs on day 16. (A) Histogram plots show the mean fluorescence intensity (MFI) of TIM-3 and LAG-3 in W- and RAD-EBVSTs from 2 representatives of 6 EBV seropositive donor EBVSTs. (B) Dot plot representing the TIM3+ and LAG-3+ T-cells and (C) Frequency of KLRG1+ TIM3+ LAG-3+ CD3+ cells in W- and RAD-EBVSTs (n=6). Statistical significance was determined by paired two-tailed Student's *t*-test. $p < 0.05$, (.12(ns: non-significant), .033(*),.002(**) ,<.001(***) Data shown are plotted as mean ± SEM.