

1 SUPPLEMENTAL MATERIALS AND METHODS:

2 Cell lines

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

13

14 Cell culture media

15 T-cells were cultured in VST medium [RPMI 1640 (HyClone Laboratories Inc. Logan, Utah),
16 45% Click's (Irvine Scientific, Santa Ana, CA), 2mM GlutaMAX TM-I (Gibco by Life
17 Technologies, Grand Island, NY), and 10% Fetal Bovine Serum (HyClone Laboratories Inc.)].

18

19 Pepmixes

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

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

25

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

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

39

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

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

51

52 **Enzyme-linked immunospot (ELISpot) assay**

53 The frequency of T2 antigen-specific T cells within EBVST populations was measured in IFN γ
54 ELISpot assays. A 96-well MultiScreen HTS IP plate (EMD Millipore, Burlington, MA) was
55 coated with anti-human IFN γ mAb 1-D1K (Mabtech, Cincinnati, OH) and incubated overnight at
56 4°C. EBVSTs were plated at 1×10^5 per well in duplicate and stimulated with pepmixes, as
57 indicated in each experiment. T-cells cultured in EBVST medium were used as negative
58 controls. After 16-24 hours of incubation at 37°C with 5% CO $_2$, the plate was washed and
59 incubated with anti-human IFN γ mAb 7-B6-1- Biotin (Mabtech, Cincinnati, OH) for 2 hours –
60 48 hours at 37°C. The plate was washed again and Avidin-peroxidase-complex (Vector
61 Laboratories, Burlingame, CA) was added for 1 hour at room temperature. The plates were then
62 developed with 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO) substrate, dried
63 overnight, and sent to Zellnet Consulting (Fort Lee, NJ) for quantification or analyzed in the lab
64 using Mabtech IRIS – ELISpot reader (Mabtech, Cincinnati, OH). The spot-forming cells
65 (SFCs)/ 10^5 cells were used as a measure of the number of cells releasing IFN γ in response to
66 viral antigen pepmixes after subtracting negative control values.

67

68 **Intracellular staining (ICS):**

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

84

85 **Cytotoxicity assay**

86 The cytolytic specificity of the VSTs was evaluated in a standard 4-hour 51 Cr chromium release
87 assay. Autologous ATCs alone or pulsed with pepmixes were used as target cells by labeling
88 with 51 Cr sodium chromate for one hour at 37°C. The target cells were washed thrice, re-
89 suspended in VST medium, and used as target cells at an effector : target (E:T) ratios of 40:1,
90 20:1, 10:1, and 5:1. Target cells were cultured in VST medium or 1% Triton X-100 (Sigma-
91 Aldrich, St. Louis, MO) to achieve spontaneous and maximum release respectively. After 4

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

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98 **SUPPLEMENTARY FIGURE LEGENDS:**

99 **Supplemental figure 1**

100 **The efficiency of CD45RA depletion of PBMCs**

101 CD45RA expressing cells were depleted from the whole (W)-PBMCs of 14 healthy donors to
102 produce W-, CD45RA depleted (RAD)- and CD45RA+ PBMCs subsets and analyzed by flow
103 cytometry (A) Dot plot analysis showing CD45RA positive cells in W- and RAD-PBMCs in one
104 representative donor. (B) Frequency of CD45RA+ cells in W- and RAD-PBMCs from all 14
105 donors (mean ± SEM). (C) Representative dot plot showing CD3-, CD56+ NK-cells in W- and
106 RAD-PBMCs. (D) Graphical representation of the frequency of CD3- CD56+ NK cells (D) and
107 CD4+ CD8- and CD4-CD8+ lymphocytes (E) in W-, RAD- and RA+ PBMCs. A paired two-
108 tailed Student's *t*-test was used for statistical analyses $p < .05$, (.12(ns: non-significant), .033(*),
109 .002(**), <.001(***)). Data shown are plotted as mean ± SEM.

110

111 **Supplemental figure 2**

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

113 (A) EBV T2 antigen specificity for W- and RAD-EBVSTs for 16 donors. (B) Frequency of
114 polyfunctional T2-specific T-cells as measured in ICC assays. The irrelevant peptide is the
115 human tumor antigen, PRAME. (C) Frequency of CD3+ T-cells and CD3-CD56+ NK cells in
116 W- and RAD-EBVSTs (n=15). (D) PBMCs specific for adenovirus (hexon and penton), and
117 VZV (IE2, IE3, and ORF10) were generated from W- and RAD-PBMC as for EBVSTs. Antigen
118 specificity on day 16 as measured by ELISpot is shown for 7 donors. Statistical significance was
119 determined using paired two-tailed Student's *t*-test. $p < .05$, (.12(ns: non-significant),
120 .033(*),.002(**),<.001(***) Data shown are plotted as mean \pm SEM.

121

122 **Supplemental figure 3**

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

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

128

129 **Supplemental figure 4**

130 **Increased clonality of EBVSTs derived from CD45RA depleted PBMCs**

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

136 50 largest clonotypes in W- (top) and RAD (bottom) PBMCs and their derived EBVSTs for
137 donor 2. Ribbons indicate shared clonotypes (W-PBMCs (grey) , W-EBVSTs (blue), RAD-
138 PBMCs (grey) and RAD-EBVSTs (green). (E) Circos plot comparing the top 50 clonotypes of
139 W- (blue) and RA+ (purple) EBVSTs from donors 1 (left) and donor 2 (right) (F) Productive
140 Simpson's clonality of W-, RAD- and RA+ PBMC (black) and derived EBVST (red) TCR
141 repertoires. 0 indicates the most diverse possible repertoire (clonotypes size of 1), and 1
142 represents a monoclonal sample.

143

144 **Supplemental Table 1**

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

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148 **Supplemental figure 5**

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

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

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

164

165 **Supplemental figure 6**

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

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

179

180 **Supplemental figure 7**

181 **Lower expression of exhaustion markers on RAD-EBVSTs**

182 The surface expression levels of T-cell exhaustion markers were evaluated in W- and RAD-
183 EBVSTs on day 16. (A) Histogram plots show the mean fluorescence intensity (MFI) of TIM-3
184 and LAG-3 in W- and RAD-EBVSTs from 2 representatives of 6 EBV seropositive donor
185 EBVSTs. (B) Dot plot representing the TIM3+ and LAG-3+ T-cells and (C) Frequency of
186 KLRG1+ TIM3+ LAG-3+ CD3+ cells in W- and RAD-EBVSTs (n=6). Statistical significance
187 was determined by paired two-tailed Student's *t*-test. $p < 0.05$, (.12(ns: non-significant),
188 .033(*),.002(**) ,<.001(***) Data shown are plotted as mean \pm SEM.