Supplementary Information

Supplementary Methods

Study cohort

In our study, a total of 1404 patients were included. Of these, 578 patients had confirmed infectious mononucleosis (IM), 412 adult or adolescent patients and 166 children. IM was clinically confirmed in all patients by the triad of fever, lymphadenopathy and tonsillitis and serologically by EBV-VCA-specific IgM, but non-detectable EBV-EBNA-specific IgG antibodies. In all IM patients, EBV-DNA was detected by EBV-PCR.

We further included 28 elderly patients with symptomatic EBV-reactivation. All elderly patients were >60 years old and were tested for EBV, due to a fever of unknown origin. All elderly patients had EBV-DNA detectable by PCR, as well as detectable EBV-VCA-specific IgM, as well as EBV-EBNA- and EBV-VCA-specific IgG antibodies.

Furthermore, 180 immunocompromised transplant recipients were included in the study. All had undergone either solid-organ- (SOT, N=149) or hematopoietic stem cell transplantation (HSCT, N=31) between 1999 and 2018, and were followed up for three years after transplantation.

HSCT recipients underwent matched unrelated donor (N=11), matched related donor (N=10) or autologous (N=10) HSCT. SOT recipients underwent kidney (N=86), liver (N=29), lung (N=20), heart (N=11), or heart-lung transplantation (N=1). All transplant patients were tested positive for EBV-DNA by EBV-PCR either due to unspecific symptoms, *i.e.* fever of unknown origin (N=144), or in case of PTLD diagnosis (N=36). EBV^+ PTLD occurred between 35-302 days post-infection in 16 HSCT and 20 SOT patients, and was diagnosed using histological and/or cytological findings according to published international guidelines [12-14].

In all non-PTLD patients an EBV-replication, exceeding >1000 copies/mL plasma, was detected by EBV-PCR. These patients had, however, no clinical evidence of PTLD (HSCT Non-PTLD, N=129 and SOT Non-PTLD, N=20).

We further included 206 healthy EBV-EBNA- and EBV-VCA-specific IgG positive individuals, who had never self-reported symptoms related to an IM in the past. At the time of sampling, none of the patients had positive EBV-PCR or VCA-specific IgM antibodies. Patients of the asymptomatic EBV-infection cohort were recruited using a questionnaire, sent to former patients of the Center for Virology, Medical University of Vienna. Details of the study cohort are shown in Table 1.

We then also included 412 control subjects (controls) in our study, who were selected independent of the EBV-status. Controls and asymptomatic EBV-infected individuals were matched to the adolescent/adult IM cohort in regard of the age and gender using case-control matching (SPSS 25).

From each patient, one plasma sample was available: From adolescent/adult IM and pediatric IM patients, the elderly and Non-PTLD patients during the EBV-viremic phase; from PTLD patients during the EBV-viremic phase, immediately (0-3 days) after the first diagnosis of PTLD. From asymptomatic EBV-infected individuals and controls, plasma samples were available, which was sent to the Center for Virology, Medical University of Vienna for routine vaccination titre controls. All plasma samples were part of the Biobank of the Center for Virology. From N=10 of all pediatric IM patients (6%) and N=10 of all adolescent/adult IM patients (2.4%), additional peripheral blood mononuclear cells (PBMC) were available after 10-23 and 1-8 years, respectively, after diagnosis of IM. From additional N=20 of all asymptomatic EBV-infected patients (9.7%), PBMC were also available.

For the functional assays, we also included 30 healthy, voluntary blood donors (EBNA EBV-IgG seropositive, N=24; EBNA EBV-IgG seronegative, N=6). From the healthy, voluntary blood donors, no information about the age, gender, or occurrence of an IM in the past were available.

HLA-E, *LMP-1* and *BZLF1* genotyping

Genomic and viral DNA was isolated from 200µL plasma using the NucliSens EasyMag extractor (bioMérieux). Nucleic acids were eluted in 50 µl nuclease-free H_2O. HLA-E genotyping was performed using a recently published TaqMan assay and HLA-E*0101- and HLA-E*0103-specific probes [1,2]. *LMP-1* variants were determined by nested PCR, followed by Sanger-Sequencing, as described before [3]. *BZLF1* peptide encoding sequences were analyzed by complete *BZLF1* gene amplification by nested PCR and subsequent Sanger-sequencing as previously described in detail [4]. DNA sequences were translated into protein sequences using the Expasy tool, developed by the Swiss-Prot group and supported by the SIB Swiss Institute of Bioinformatics (https://web.expasy.org/translate/).

EBV-detection and serology

Viral DNA was isolated from plasma samples using NucliSens EasyMag extractor. Nucleic acids were eluted in 50 µl nuclease-free H_2O. EBV-DNA was detected and quantified by TaqMan assay using recently published protocols [5]. EBV VCA IgM, EBNA IgG and VCA IgG antibodies were detected and quantified by ELISA (all: Euroimmune).

Isolation of primary cells

Peripheral blood mononuclear cells (PBMCs) from 30 voluntary and healthy blood donors, from additional 20 former IM patients and from additional 20 asymptomatic EBV-infected individuals were isolated from buffy-coats by Ficoll-Paque PLUS density (Cytiva) gradient centrifugation according to the manufacture's instruction. $CD56^+$ NK cells and $CD8^+$ T cells were then enriched by magnetic labelling using the human $CD56^+$ NK cell or the $CD8^+$ T Cell Isolation Kit according to the manufacturer's instruction (both: Miltenyi Biotec). Cells were stored frozen at −80 °C in $4x10^6$ viable cell per aliquots in 90% FCS + 10% DMSO (Thermo-Fisher). One $CD56^+$ NK cells or $CD8^+$ T cells aliquot of each blood donor was stained for CD56 and CD3 or CD8, CD4 and CD3, respectively, to ensure the purity of both cell subsets. Only samples with >95% $CD56^+CD3^-$ NK cells or $CD8^+CD4^-CD3^+$ T cells were included in the study.

HLA-E stabilization experiments

HLA-E surface stabilization was induced as previously described [6]. In brief, TAP-incompetent K562–HLA-E*0101/0101 and K562–HLA-E*0103/0103 (kindly provided by Thorbald van Hall, Leiden University Medical Center) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) + 10% FCS (both: Thermo-Scientific). $5x10^5$ cells/ml were incubated together with 300µM of *VMAPRTLIL* (positive control), *BZLF1*-dervied *SQAPLPCVL* or *LMP-1* peptides (Peptides&Elephants) in 1 ml Opti-MEM (Thermo-Fisher) for 16 h at 37°C. Peptide-pulsed cells were either stained for HLA-E surface expression by flow-cytometry analysis or washed with complete medium and used for *in vitro* stimulations.

Identification of *SQAPLPCVL*-specific, HLA-E-restricted CD8+ T cells

$CD8^+$ MACS-enriched cells were quickly thawed at 37°C, washed once, and rested overnight in RPMI, 10% FCS, 1% L-glutamine at 37°C. $CD8^+$ T cells were then harvested by centrifugation (400xg, 5 min) and then washed once with Opti-MEM I Reduced Serum Medium. $CD8^+$ T cells were then cultured together with peptide pulsed K562–HLA-E*0101/0101, K562–HLA-E*0103/0103 or K562 cells (Effector : Target, E:T, 1:2), 1x monensin and 1x brefeldin A (both: Biolegend) for 6h. $CD8^+$ T cells were then harvested, fixed with the FIX & PERM Cell Fixation & Cell Permeabilization Kit (Thermo-Scientific) and analysed by flow-cytometry, as described below. *SQAPLPCVL*-specific, HLA-E-restricted CD8+ T cells of each donor were identified as IFNγ positive cells and in comparison to cells, stimulated with K562–HLA-E*0101/0101 or K562–HLA-E*0103/0103 or K562 cells, but without peptides, respectively.

Creation of CR2-expressing cell lines

TAP-competent K562–HLA-E*0101/0101 and K562–HLA-E*0103/0103 (kindly provided by Thorbald van Hall, Leiden University Medical Center) were cultured in IMDM + 10% FCS. The cells were transfected with the CR2/CD21-receptor using the CD21 CRISPR activation plasmid (h) according to the manufacture's instruction (Santa Cruz Biotechnology). Successful CR2/CD21 insertion was confirmed by CD21 surface expression by flow-cytometry. K562-CR2-HLA-E*0101/0101 and K562-CR2-HLA-E*0103/0103 were afterwards sorted for the high expression of CD21, as described below.

Virus dissemination assays

For the virus dissemination assays, the marmoset B-lymphoblastoid cell line B95-8 was cultured in RPMl 1640 medium supplemented with 20 mM glutamine, 50pg/ml gentamicin, 0.2 pg/ml amphotericin B and 10% FCS (Thermo-Scientific). Cells were then cultured in the presence of 12-O-tetradecanoyl-phorbol-12-acetate (Biomol) for two weeks. EBV was harvested from the supernatant and enriched by ultracentrifugation (30000 U/min, 90 min, 10°C). The virus stocks, expressing the *BZLF1 SQAPLPCVL* and the *LMP-1 GGDPHLPTL* variant, were stored at -80°C in RPMI 1640 medium + 10% FCS + 10% DMSO (all: Thermo-Scientific). The multiplicity of infection (MOI) of the viral stocks was determined on K562–CR2-HLA-E*0101/0101 and K562–CR2-HLA-E*0103/0103 cells by flow-cytometry.

RMA-S/HLA-E/LFA-3 cells (kindly provided by Chiara Romagnani, DRFZ, Berlin) were maintained in RPMI-1640 + 20 mM glutamine + 10% FCS + 20µM β-mercaptoethanol + 100 U/ml penicillin-streptomycin (all Thermo Fisher) + 400µg/ml hygromycin B + 1mg/ml G418 (both InvivoGen). $2x10^6$ RMA-S/LFA3/HLA-E cells were incubated for 16h at 37°C, 5% CO_2, with 300µM of respective peptides (Peptides&Elephants) in 1ml serum-free OptiMEM (Thermo-Fisher). Cells were afterwards inactivated using 20 µg/mL Mitomycin C (Sigma Aldrich) at 37° C for 30 minutes. Peptide pulsed RMA-S/LFA3/HLA-E cells were co-cultured with 2mM CFSE (Thermo-Fisher) stained $CD56^+$ or $CD8^+$ MACS-enriched cells for 7 days in RPMI+10% FCS. Proliferating ($CFSE^{low}$) $NKG2A^+CD56^+$ NK cells, $NKG2A^+$ $CD8^+$ T cells or *SQAPLPCVL*-specific, HLA-E-restricted $NKG2A^+$ or $NKG2A^-$ CD8+ T cells were then sorted by FACS as described below. Proliferating *SQAPLPCVL*-specific, HLA-E-restricted $CD8^+$ T cells of each donor were identified as $CFSE^{low}$ in comparison to cells, stimulated with RMA-S/HLA-E/LFA-3, but without peptides.

Fresh K562-CR2-HLA-E*0101/0101 and K562-CR2-HLA-E*0103/0103 cells were then inactivated using 20 µg/mL Mitomycin C at 37° C for 30 minutes and then infected with the EBV B95-8 isolate (MOI=1) for 24h. The infected cells were then co-cultured together with the sorted NKG2A$^+$ CD56$^+$ NK cells, NKG2A$^+$ CD8$^+$ T cells or *SQAPLPCVL*-specific, HLA-E-restricted NKG2A$^+$ or NKG2A$^-$ T cells (E:T, 1:2). In some experiments, 300µM of respective peptides was also added. At indicated time points, the cells were harvested and analyzed by flow-cytometry, as described below.

CD8$^+$ proliferation Assay

For the CD8+ T cell proliferation assays, *SQAPLPCVL* pulsed TAP-deficient K562–HLA-E*0101/0101 or K562–HLA-E*0103/0103 cells were first inactivated using 20 µg/mL Mitomycin C (Sigma Aldrich) at 37° C for 30 minutes. The cells were then co-cultivated with CFSE stained MACS-enriched CD8$^+$ T cells for 7 days (E:T, 2:1). After three days, fresh peptide-pulsed K562–HLA-E*0101/0101 or K562–HLA-E*0103/0103 cells were added to the culture. Cells were then analysed after 7 days by flow-cytometry, as described below.

NKG2A$^+$ NK cells and NKG2A$^+$ CD8$^+$ T cell inhibition assay

For the NKG2A$^+$ NK cell inhibition experiments, MACS-enriched CD56$^+$ cells were quickly thawed at 37°C, washed, and pre-activated overnight in RPMI, 10% FCS, 1% L-glutamine (all: Thermo Fisher Scientific), 10 ng/ml IL-12 (PeproTec) and 100 ng/ml IL-18 (Biozym Scientific) at 37°C. CD56$^+$ NK cells were then harvested by centrifugation at 400xg for 5 minutes and washed once with Opti-MEM I Reduced Serum Medium (Gibco). The NK cells were then cultured together with peptide pulsed K562–HLA-E*0101/0101 or K562–HLA-E*0103/0103 cells (E:T,

1:2) and 5µL mouse anti-human CD107-APC-H7 (BD) for 6h. NK cells were then harvested, fixed with the FIX & PERM Cell Fixation & Cell Permeabilization Kit (Thermo-Scientific) and analysed by flow-cytometry, as described below.

For the $CD8^+$ T cells experiments, $CD8^+$ MACS-enriched cells were quickly thawed at 37°C, washed once, and pre-activated overnight in RPMI, 10% FCS, 1% L-glutamine and 20 ng/ml IL-2 (PeproTec) at 37°C. $CD8^+$ T cells were then harvested by centrifugation (400xg, 5 min) and then washed once with Opti-MEM I Reduced Serum Medium. $CD8^+$ T cells were then cultured together with peptide pulsed K562–HLA-E*0101/0101 or K562–HLA-E*0103/0103 cells (E:T, 1:2), 1x monensin and 1x brefeldin A for 6h. $CD8^+$ T cells were harvested, fixed with the FIX & PERM Cell Fixation & Cell Permeabilization Kit and analysed by flow-cytometry, as described below.

Flow-cytometry

The following conjugated mouse anti-human mAB were used for flow-cytometry: BV421-CD56 (NCAM 16.2), BV421-CD8 (RPA-T8), BV510-CD4 (M-T477), BV510-NKG2A (131411), APC/H7-CD107a (H4A3), APC/CD21 (all: BD), APC-CD3 (UCHT1), APC/Cy7-IFNγ (B27), APC-HLA/E (all: Biolegend), APC-Cy7-Granzyme B (Abcore), BZLF1-Alexa488 (Novusbio). Dead cells were identified using LIVE/DEAD Fixable Green Dead Cell Stain Kit (Thermo-Scientific) or 7-Aminoactinomycin D (7-AAD, Invitrogen). Flow-cytometry analysis was performed on a FACSCanto2 platform and FACSDiva Version 10.7.2 (BD). For cell sorting, dead cells were first removed using the Dead Cell Removal Kit (Miltenyi Biotec) and sorted with a FACSAria Fusion (BD).

Statistical analysis

The Chi-square test and Fisher's exact test were used to compare the distribution of the HLA-E and *LMP-1* variants. Outliers of the flow cytometry data were first identified using the ROUT method and then compared between the groups with the RM one-way ANOVA (with the Geisser-Greenhouse correction), the Kruskal-Wallis Test and the Dunn's post-test, Wilcoxon signed-rank test or the Mann-Whitney test. A p-value < 0.05 was considered statistically significant. Statistical differences were assessed with GraphPad Prism 9.

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Figure S1

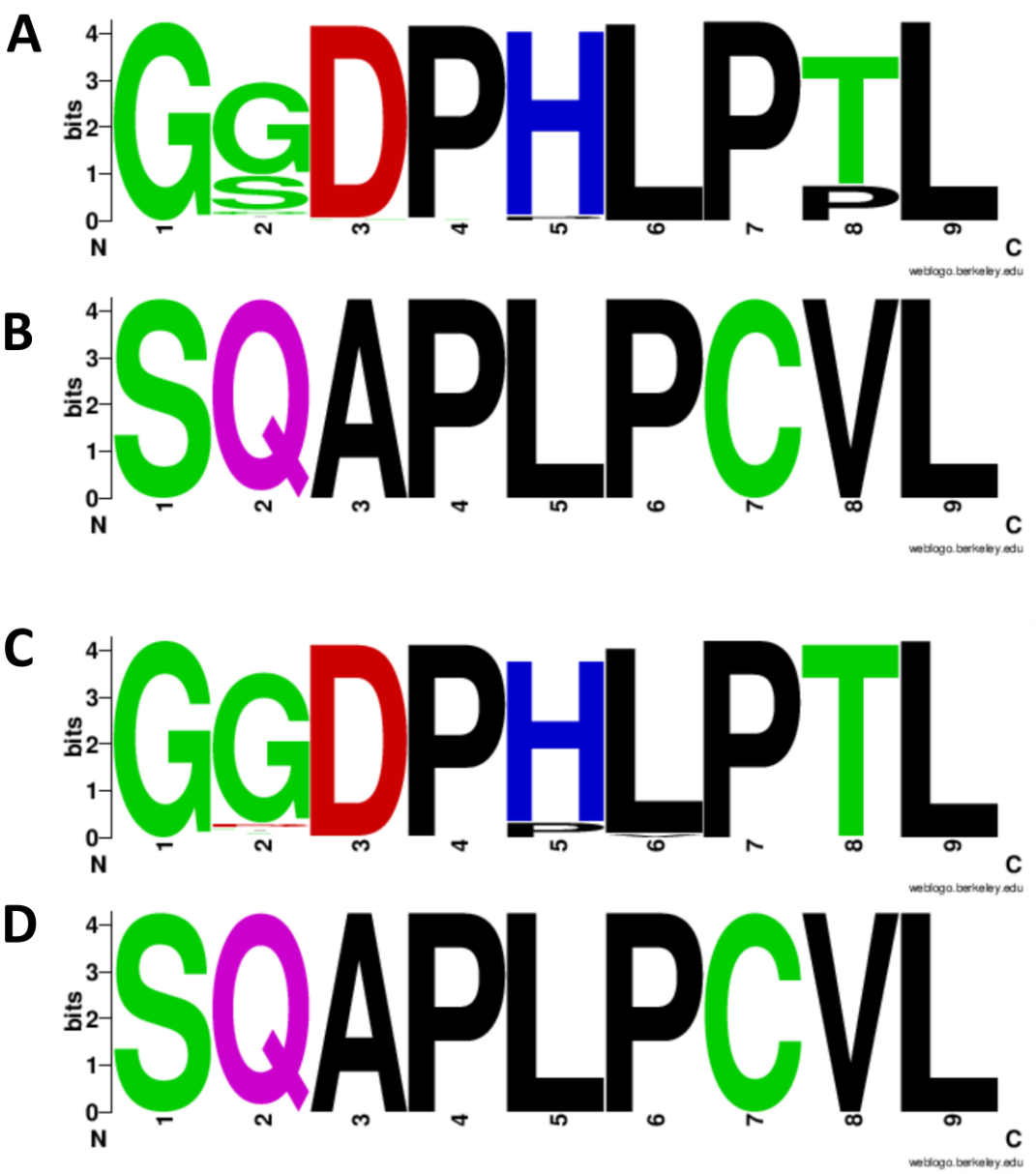

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Figure S1: Sequence logo alignment of the relative frequency of (A,C) *LMP-1* peptide variants and (B;D) *BZLF1*-dervied *SQAPLPCVL* from *N*=786 sequenced *BZLF1* and partially sequenced *LMP-1* genes. (A,B) Sequence logo alignment of the relative frequency of (A) *LMP-1* peptide variants and (B) *BZLF1*-dervied *SQAPLPCVL* from *N*=578 sequenced *BZLF1* and partially sequenced *LMP-1* genes from IM patients. (C,D) Sequence logo alignment of the relative frequency of (C) *LMP-1* peptide variants and (D) *BZLF1*-dervied *SQAPLPCVL* from *N*=208 sequenced *BZLF1* and partially sequenced *LMP-1* genes from elderly, NON-PTLD and PTLD patients with EBV reactivations. Sequence logos were created with the web-tool of the University of California, (https://weblogo.berkeley.edu/).

Figure S2

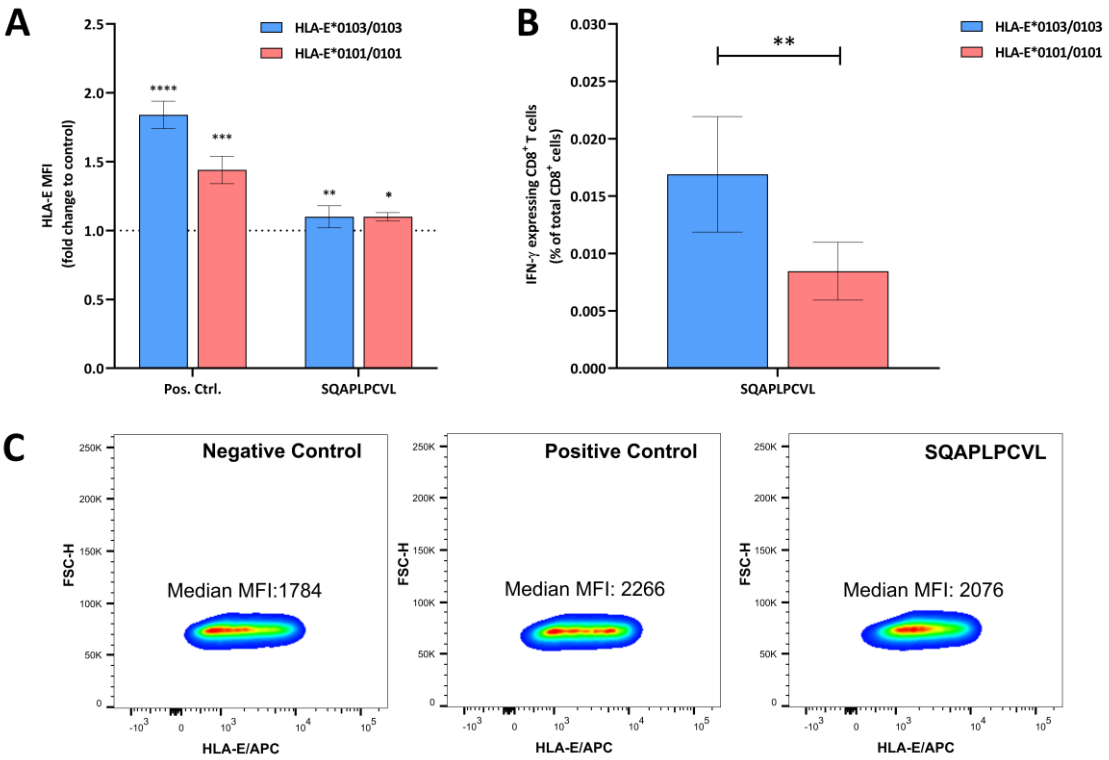

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Figure S2: *SQAPLOCVL*-mediated HLA-E stabilization and NKG2A$^+$ inhibition assay (A) HLA-E stabilization assay: K562-HLA-E*0103/0103 or K562-HLA-E*0101/0101 cells were incubated together with 300µM of the positive control (VMAPRTLIL) or the *BZLF1*-dervied *SQAPLPCVL* peptide. The surface expression of HLA-E was assessed after 16h of co-culture by flow-cytometry. Box plot represent the mean (±SD) of three independent replicates. Each peptide was compared to the negative control (dashed black line), *i.e.* K562-HLA-E*0103/0103 or K562-HLA-E*0101/0101 cells without peptides using the Mann-Whitney test. (B) Analysis of *SQAPLPCVL*-specific and HLA-E restricted CD8$^+$ T cell responses, evaluated between 12 healthy EBV-seropositive individuals by flow-cytometry. Enriched CD8$^+$ T cells were stimulated either with K562-HLA-E*0103/0103 or K562-HLA-E*0101/0101 cells and 300µM of the *SQAPLPCVL* peptide. CD8$^+$ T cells were then analyzed for the expression of IFNγ. Box bpot represent the mean (±SD) of 12 independent replicates. The percentage of IFNγ-expressing cells was compared between with K562-HLA-E*0103/0103 and K562-HLA-E*0101/0101 cells by the paired T-test. (C) Representative examples and median MFI of the negative control (without peptide), positive control (VMAPRTLIL) or *SQAPLPCVL*-induced HLA-E surface stabilisation on peptide pulsed K562-HLA-E*0103/0103 cells. **MFI:** mean fluorescence intensities

Figure S3

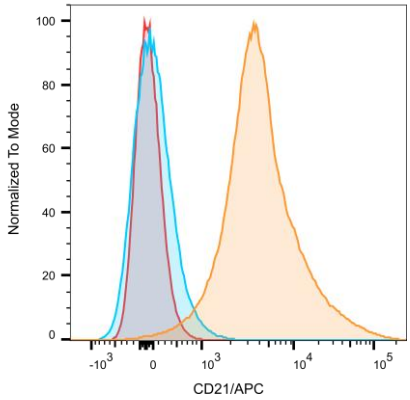

Figure S3: Representative example of established K562-CR2-HLA-E*0103/0103 cell line using CRISPR gene editing. Overlay of CR2-APC stained K562-CR2-HLA-E*0103/0103 cells (orange), CR2-APC stained K562-HLA-E*0103/0103 cells (blue) and unstained K562-CR2-HLA-E*0103/0103 cells.

Figure S4

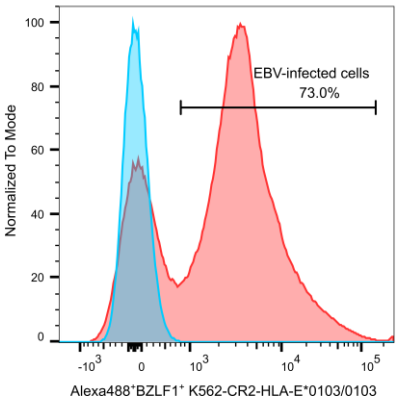

Figure S4: Representative example of *BZLF1* stained (red) and unstained (blue) EBV-infected K562-CR2-HLA-E*0103/0103 cells, obtained after 15 days of infection in the absence of *SQAPLPCVL*-specific and HLA-E restricted $CD8^+$ T cells.

Figure S5

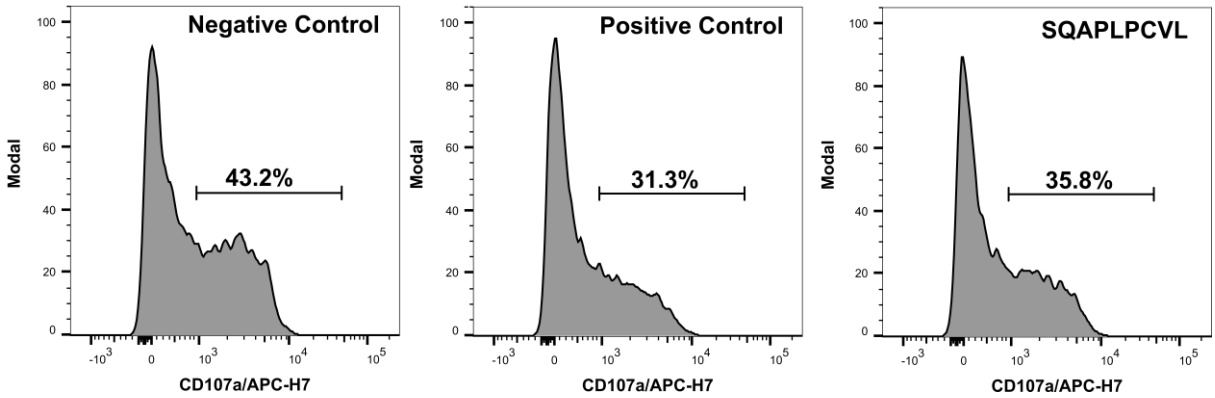

Figure S5: Representative example of the NKG2A$^+$ inhibition assay. K562-HLA-E*0103/0103 cells were first incubated together with 300µM of the negative control (without peptide), positive control (VMAPRTLIL) or the *BZLF1*-derived *SQAPLPCVL and* then incubated together with pre-activated CD56$^+$ NK cells from the same donor. The percentage of CD107 expressing NKG2A$^+$ NK cells was assessed by flow-cytometry.

Figure S6

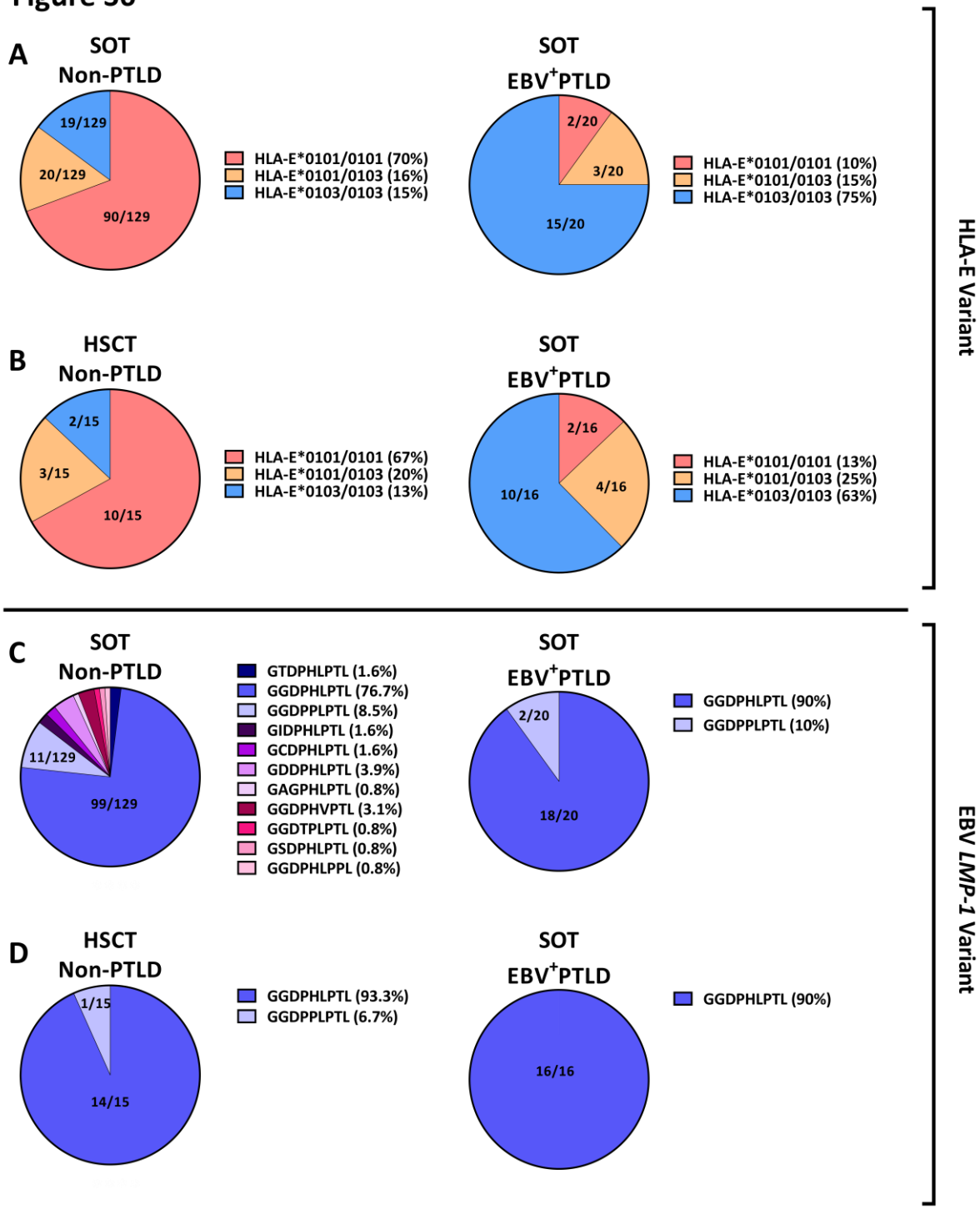

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Figure S6: Distribution of HLA-E and *LMP-1* peptide variants in transplant recipients with and without PTLD. (A-B) Distribution and comparison of HLA-E variants between (A) SOT Non-PTLD (*N*=129) and SOT PTLD (*N*=20) or (B) HSCT Non-PTLD (*N*=15) and HSCT PTLD (*N*=16). Fractions represent the relative frequency of HLA-E*0101/0101, HLA-E*0101/0103 and HLA-E*0103/0103. (C-D) Distribution and comparison of *LMP-1* peptide variants between (A) SOT Non-PTLD (*N*=129) and SOT PTLD (*N*=20) or (B) HSCT Non-PTLD (*N*=15) and HSCT PTLD (*N*=16). Fractions represent the relative frequency of the *LMP-1* peptide variants *GGDPHLPTL*, *GSDPHLPTL*, *GGDPHLPPL*, *GGDPPLPTL*, *GCDPHLPTL*, *GIDPHLPTL*, *GAGPHLPTL*, *GGDTPLPTL*, *GDDPHLPTL*, *GGDPHVPTL* and *GTDPHLPTL*. **HSCT**: hematopoietic stem cell transplantation, **PTLD**: post-transplant lymphoproliferative disorders, **SOT:** solid organ transplantation.

Figure S7

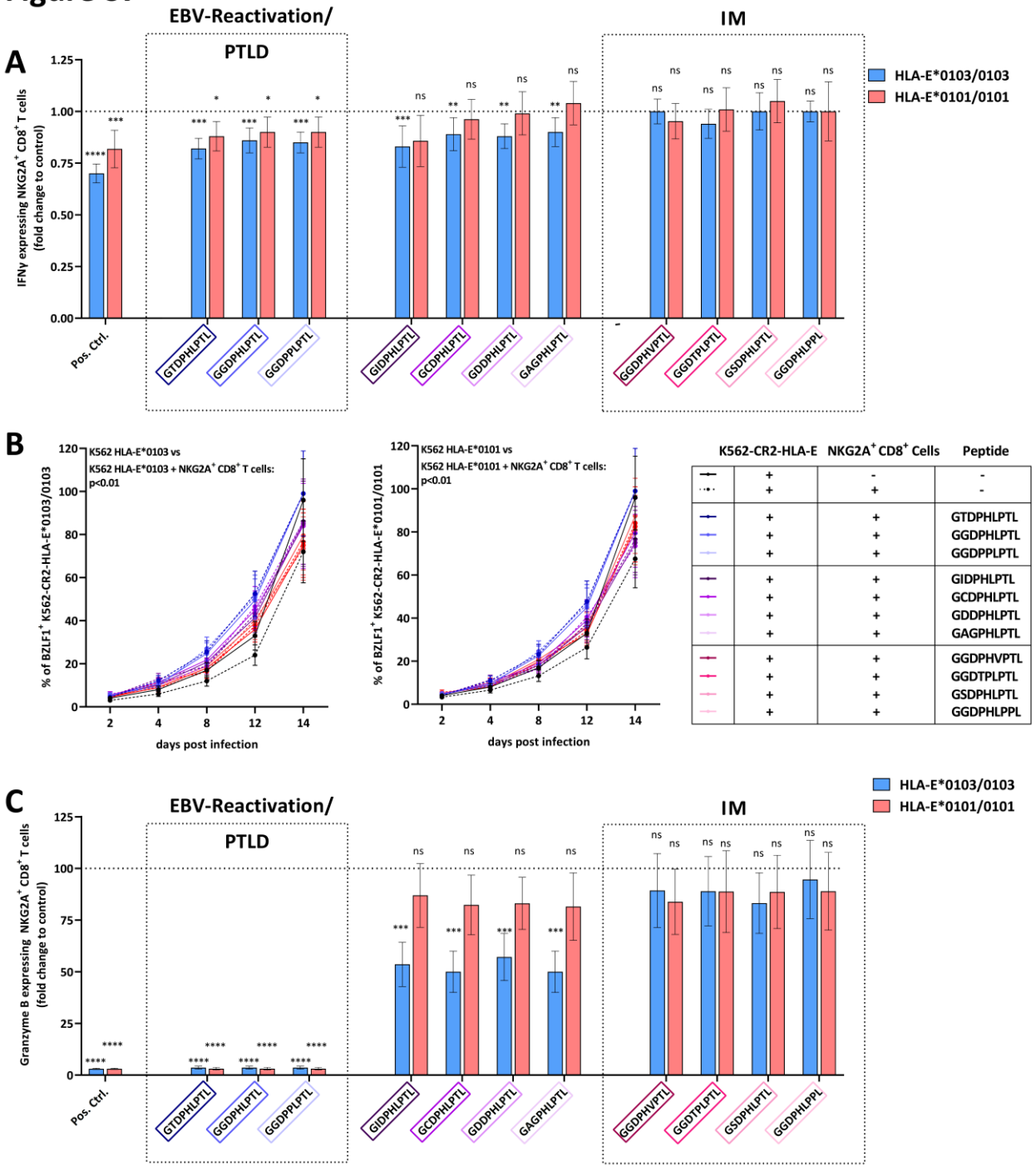

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Figure S7: *LMP-1*-dervied peptides are a potent Inhibitor of NKG2A$^+$ CD8$^+$ T cells. (A) NKG2A$^+$ inhibition assay: K562-HLA-E*0103/0103 or K562-HLA-E*0101/0101 cells were first incubated together with 300µM of the positive control (VMAPRTLIL) or the LMP-1 derived *GGDPHLPTL*, *GSDPHLPTL*, *GGDPHLPPL*, *GGDPPLPTL*, *GCDPHLPTL*, *GIDPHLPTL*, *GAGPHLPTL*, *GGDTPLPTL*, *GDDPHLPTL*, *GGDPHVPTL* and *GTDPHLPTL* peptides and then incubated together with pre-activated enriched NKG2A$^+$ CD8$^+$ T Cells. The percentage of IFNγ expressing NKG2A$^+$ CD8$^+$ T cells was assessed by flow-cytometry. Box plot represent the mean (±SD) of 12 independent biological replicates. Each peptide was compared to the negative control (dashed black line), *i.e.* K562-HLA-E*0103/0103 or K562-HLA-E*0101/0101 cells without peptides using the Mann-Whitney test. (B-C) EBV dissemination assay: K562-CR2-HLA-E*0103/0103 or K562-CR2-HLA-E*0101/0101 cells were infected with the EBV B95-8 isolate and cultured together with sorted NKG2A$^+$ CD8$^+$ T cells for 2, 4, 8, 12 or 14 days. The percentage of (B) *BZLF1*$^+$ K562-CR2-HLA-E*0103/0103 or K562-CR2-HLA-E*0101/0101 cells or (C) granzyme B-expressing NKG2A$^+$ CD8$^+$ T cells was assessed by flow-cytometry after 2, 4, 8, 12 or 14 days of co-culture. The dashed black line indicates the percentage of granzyme B-expressing CD8$^+$ T cells in the absence of any peptides. (B) RM one-way ANOVA (with the Geisser-Greenhouse correction) was used to analyze differences between groups. (C) Each peptide was compared to the negative control (dashed black line), *i.e.* K562-HLA-E*0103/0103 cells or K562-HLA-E*0101/0101 cells without peptides using the Mann-Whitney test. (B-C) Plots represent the mean (±SD) of 12 independent biological replicates. $p < 0.05$ was considered significant. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. **IFNγ:** Interferon γ, **Pos.Ctrl.:** positive control.

Supplementary References

1. Vietzen H, Pollak K, Honsig C, Jaksch P, Puchhammer-Stockl E. NKG2C Deletion Is a Risk Factor for Human Cytomegalovirus Viremia and Disease After Lung Transplantation. *J Infect Dis*. 2018;217(5):802-806.

2. Paquay MM, Schellekens J, Tilanus MG. A high-throughput Taqman approach for the discrimination of HLA-E alleles. *Tissue Antigens*. 2009;74(6):514-519.

3. Mbiribindi B, Pena JK, Arvedson MP, et al. Epstein–Barr virus peptides derived from latent cycle proteins alter NKG2A + NK cell effector function. *Scientific Reports*. 2020;10(1):19973.

4. Lorenzetti MA, Gantuz M, Altcheh J, De Matteo E, Chabay PA, Preciado MV. Epstein–Barr virus BZLF1 gene polymorphisms: malignancy related or geographically distributed variants? *Clinical Microbiology and Infection*. 2014;20(11):O861-O869.

5. Aberle SW, Puchhammer-Stöckl E. Diagnosis of herpesvirus infections of the central nervous system. *J Clin Virol*. 2002;25 Suppl 1:S79-85.

6. Heatley SL, Pietra G, Lin J, et al. Polymorphism in human cytomegalovirus UL40 impacts on recognition of human leukocyte antigen-E (HLA-E) by natural killer cells. *The Journal of biological chemistry*. 2013;288(12):8679-8690.