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Supplemental information

Activation-neutral gene editing of tonsillar CD4 T cells for functional studies in human *ex vivo* tonsil cultures

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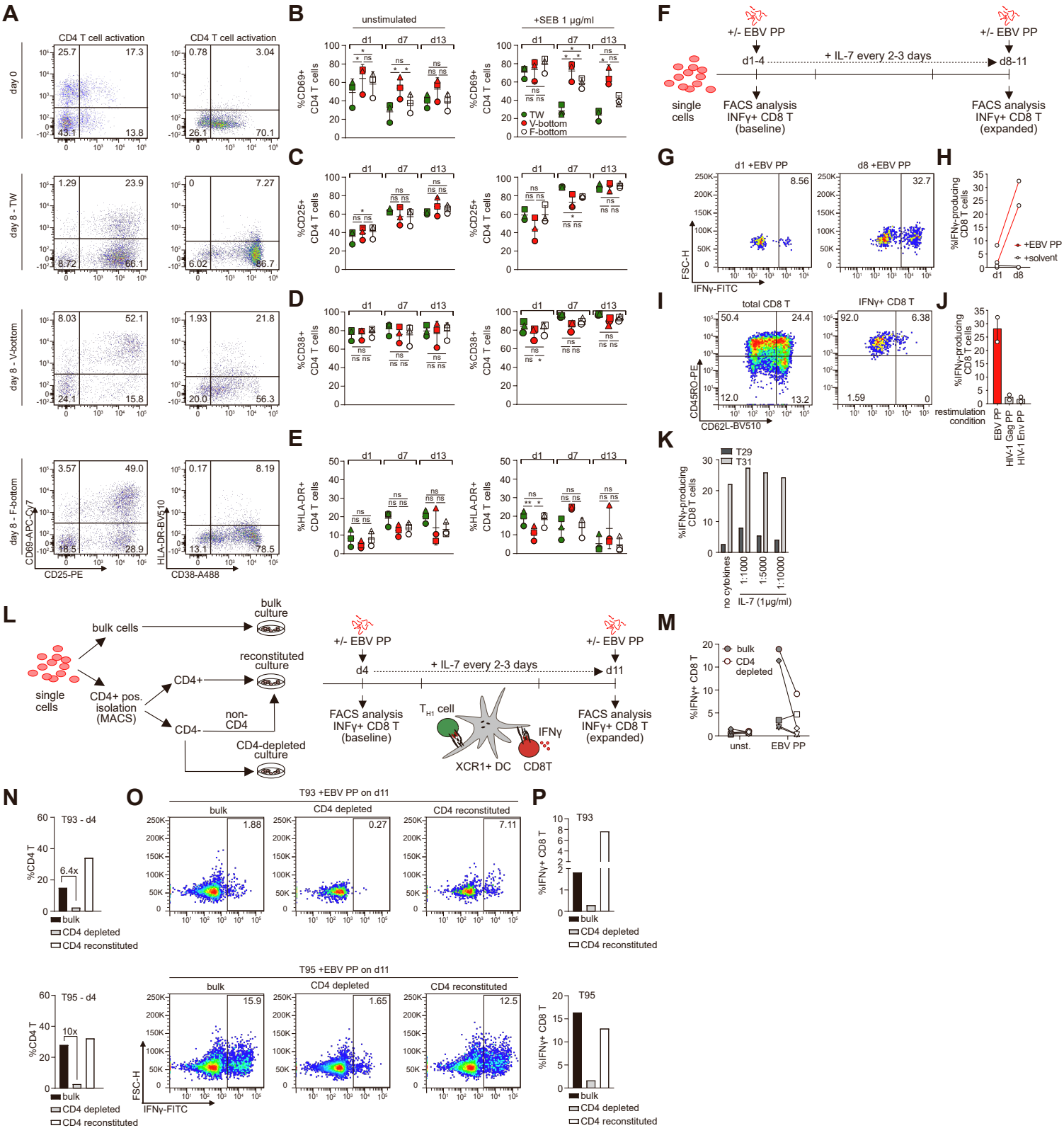
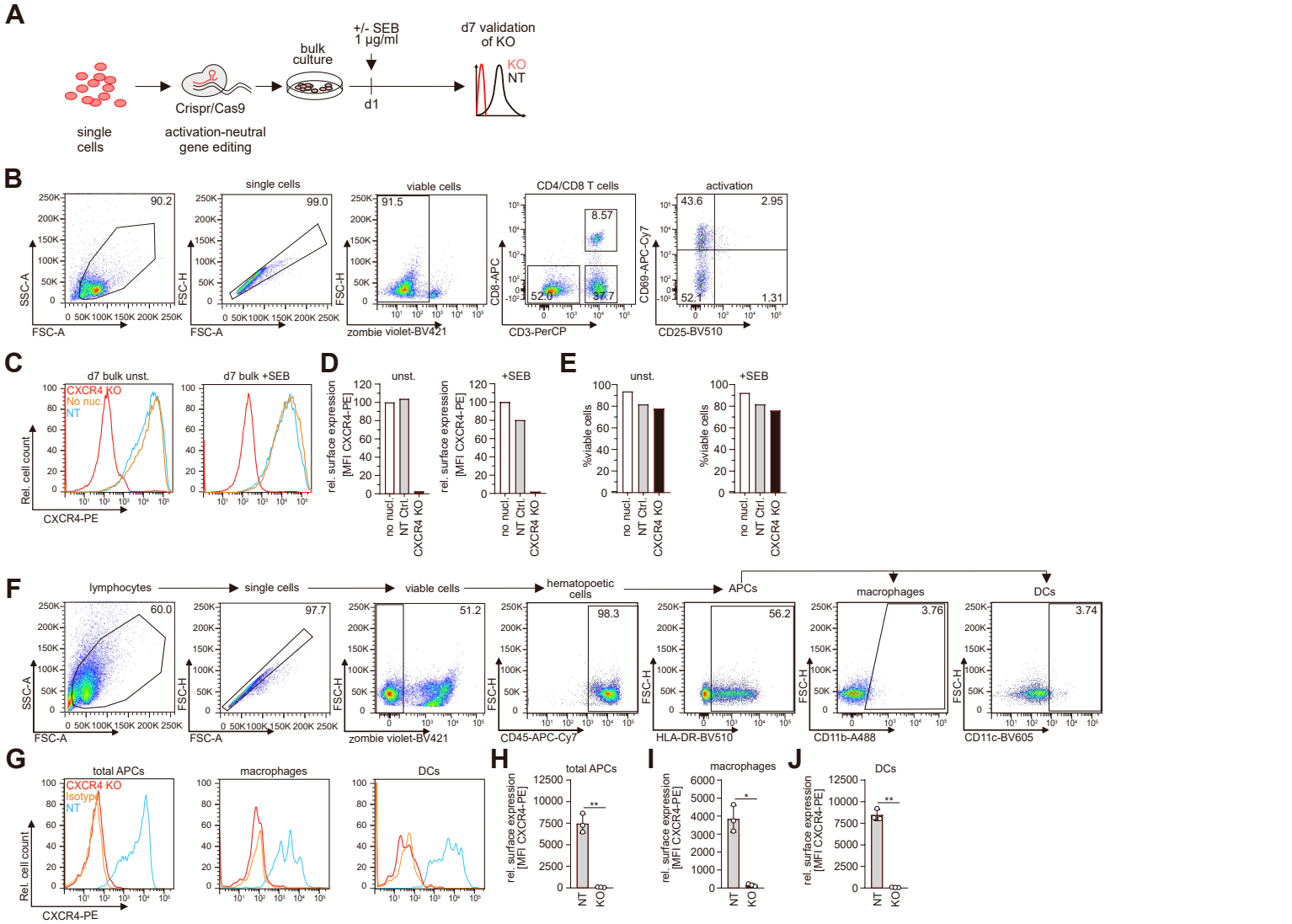


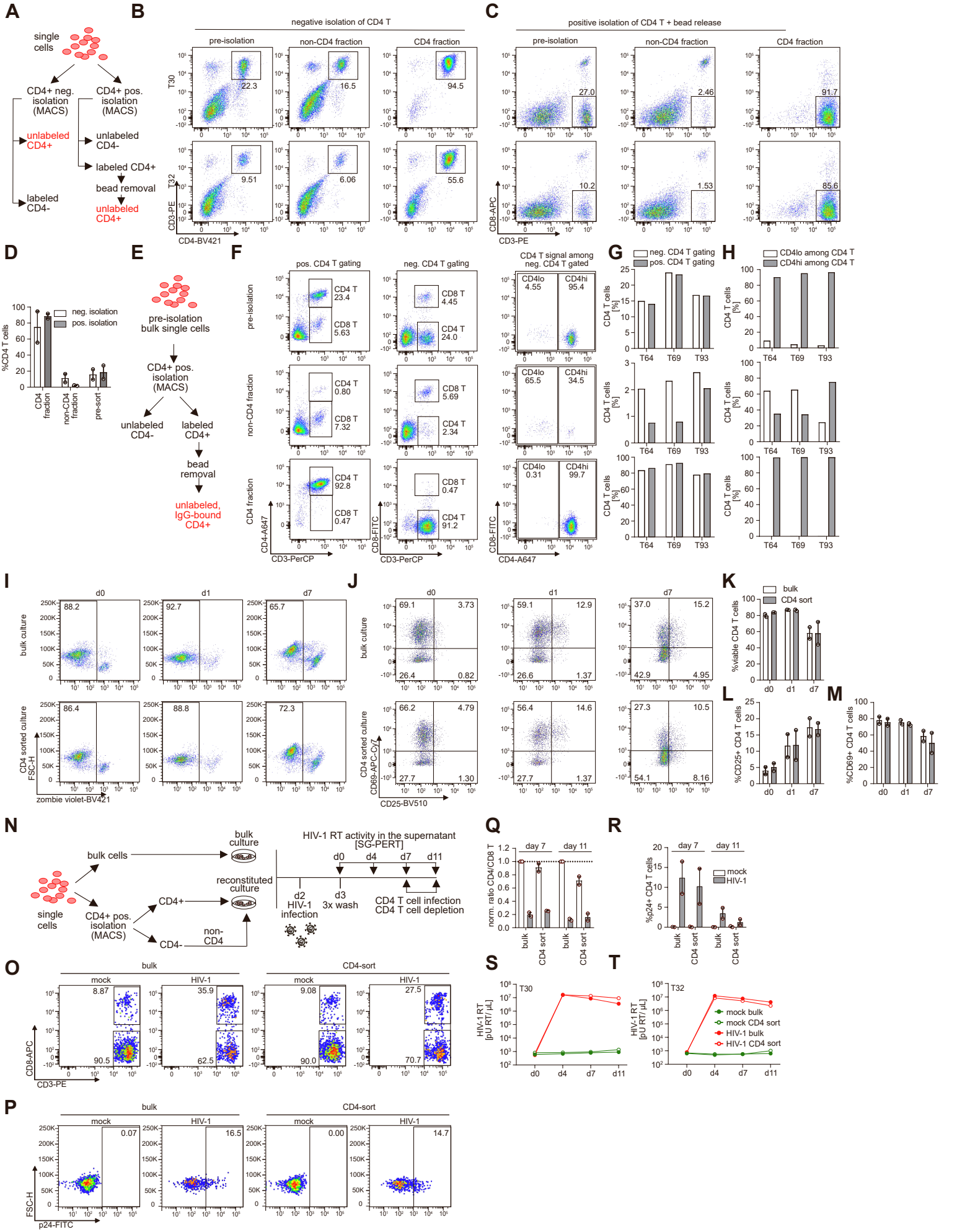
Morath et al. 2023, Fig. S1, related to Fig. 1 and Fig.2

Figure S1. Tonsil CD4 T cells can respond to superantigen and provide antigen-specific help to memory CD8 T cells. Related to Figure 1 and Figure 2. (A) Representative dot plots of CD4 T cell surface activation marker expression showing frequency of cells positive for CD25 and CD69 (left panel) or CD38 and HLA-DR (right panel) on day 0 and day 8 of culture as indicated with (+SEB 1 µg/ml) exogenous activation in transwell, V- or F bottom plate culture. (B)-(E) Quantification of A without (left panel) or with (right panel) exogenous activation for three tonsils in TW (green), V-bottom (red) or F-bottom (white) setups. Shown are means with SD. Each symbol represents one donor. Statistical significance was assessed by repeated measures one-way ANOVA for all datasets except for CD25 expression in unstimulated condition in C, where data was not normally distributed and Friedman test was used (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). (F) Schematic of the experimental set-up for assessment of EBV-specific memory CD8 T cell reactivation in tonsil culture as in Fig. 2G. (G) Representative dot plots of tonsil CD8 T cells showing IFNγ-production upon EBV peptide stimulation on day 1 (baseline) and day 8 (expanded). (H) Quantification of G for two tonsils, showing increase of EBV-specific CD8 T cells that are identified by IFNγ-production over the culture period. Solvent control is water. (I) Representative dot plots of tonsil CD8 T cells after stimulation with EBV peptide pool, showing typical distribution of memory subsets among total or IFNγ+ tonsil CD8 T cells measured by CD45RO and CD62L expression, confirming an effector memory phenotype of the reactivated CD8 T cell response. (J) Quantification of IFNγ-production among CD8 T cells after 7 days of culture with EBV peptide pool and upon restimulation with EBV peptide pool or unspecific/background IFNγ-production upon restimulation with HIV-1 Gag and HIV-1 Env peptide pools, validating EBV-specificity of the expansion and reactivation process. (K) Quantification of IFNγ+ CD8 T cells upon restimulation with EBV peptide pool after 7 days of culture with EBV peptide pool and additional supplementation of IL-7 at indicated concentrations for two tonsils, respectively. (L) Schematic of the experimental set-up for assessment of CD4 T cell help to EBV-specific memory CD8 T cell reactivation. Briefly, tonsil single cell suspensions were seeded as bulk cultures or subjected to CD4 T cell positive isolation and the CD4-depleted fraction was seeded with or without reconstitution of CD4 T cells. The cultures were stimulated with EBV peptide pool on day 4 and expansion was assessed 7 days later. (M) Quantification of IFNγ+ CD8 T cells on day 11 after 7 days of culture with (EBV PP) or without (unst) EBV peptide pool for bulk cultures (grey) or CD4-depleted cultures (white). Each symbol represents one donor. (N) Frequency of CD4 T cells among tonsil cells upon initial peptide stimulation on day 4 for bulk, CD4-depleted and CD4-reconstituted cultures for two tonsils, respectively. (O) Dot plots showing IFNγ-production of tonsil CD8 T cells upon EBV peptide pool restimulation on day 11 after 7 days of culture with EBV peptide pool for bulk, CD4-depleted and CD4-reconstituted cultures for the two tonsils from N. (P) Quantification of O for the two tonsils. Cells in F-P were cultured on F-bottom plates.



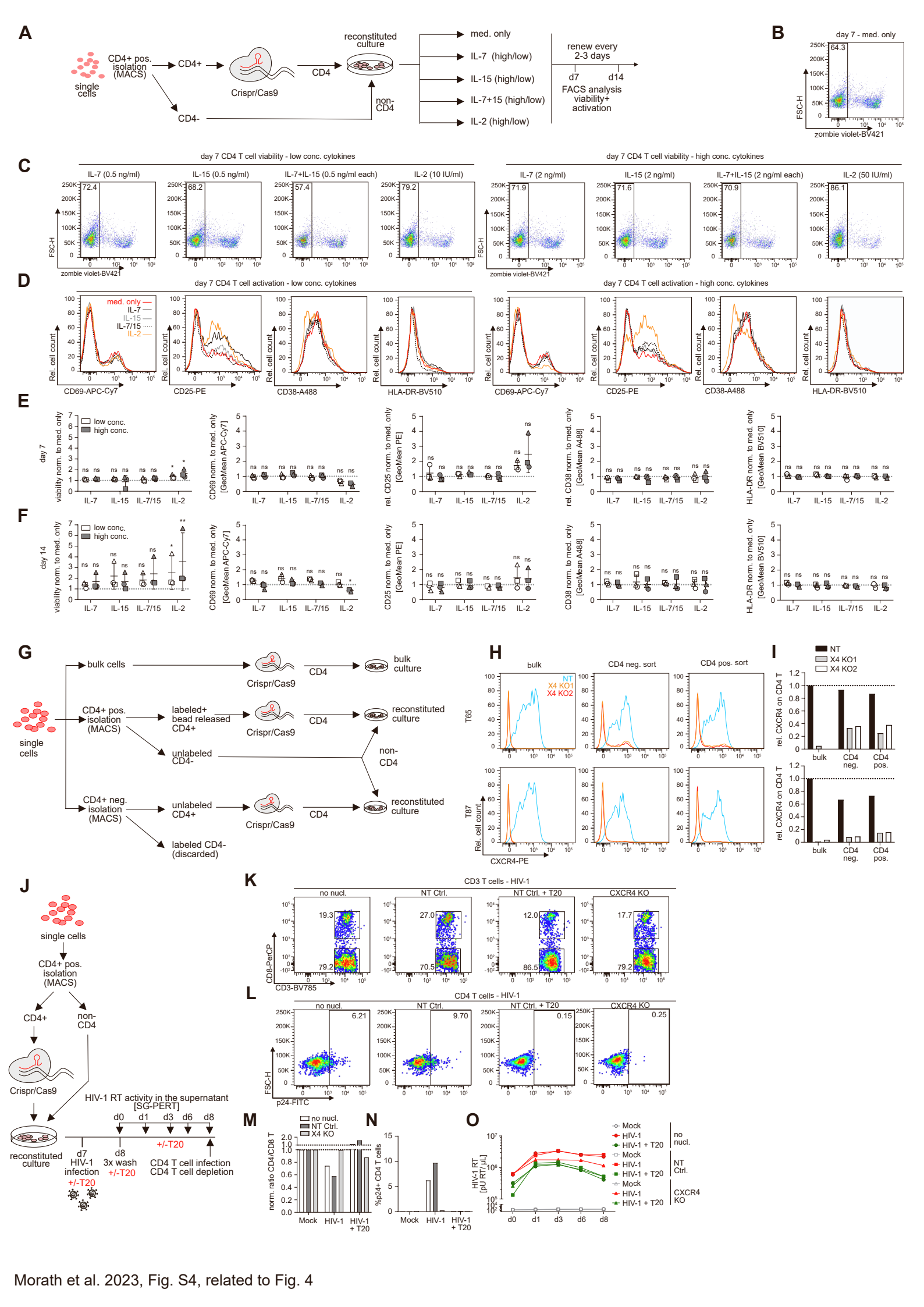
Morath et al. 2023, Fig. S2, related to Fig, 3

Figure S2. Crispr/Cas9 KO of bulk tonsil cells does not require exogenous activation and is efficient across APCs and myeloid cell populations. Related to Figure 3. (A) Schematic of the experimental set-up. (B) Representative dot plots showing gating strategy and typical activation phenotype of tonsil CD4 T cells upon seeding in the absence of exogenous activation, measured by CD25 and CD69 expression. (C) Histograms showing CXCR4 surface expression on CD4 T cells in tonsil bulk cultures without (left) or with (right) SEB stimulation at 1 µg/ml on day 7 post nucleofection. SEB was added 1 day post nucleofection. Superimposed are histograms for the non-targeting control (NT) and no nucleofection (no nucl.) condition. (D) Quantification of C for one tonsil without (unst.) or with (+SEB) exogenous activation on day 7 post nucleofection. (E) Frequency of viable CD4 T cells for conditions in C. Cells in A-E were cultured in 24-well TW-plates. (F) Representative dot plots and gating strategy for identification of myeloid subsets among tonsil cell cultures. (G) Representative histograms showing CXCR4 surface expression on total APCs, macrophages or dendritic cells (DCs) on day 7 post nucleofection with CXCR4-targeting RNP (CXCR4 KO) with superimposed histograms for the non-targeting RNP (NT) condition and isotype control. (H)-(J) Quantification of G for NT or CXCR4 KO in total APCs, macrophages or DCs. Cells were cultured in 96-well F-bottom plates. Each dot represents one tonsil. Shown are means with SD. Statistical significance was assessed by paired t-test (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).



Morath et al. 2023, Fig. S3, related to Fig. 4

Figure S3. Positive isolation of tonsil CD4 T cells is efficient and does not affect phenotype or functionality. Related to Figure 4. (A) Schematic of the experimental set-up for CD4 T cell positive or negative isolation for comparison of sorting efficiency with indicated fractions resulting from the two procedures. (B) and (C) Dot plots of CD4 negative (B) or positive (C) isolation fractions before (pre-isolation) and after sorting (non-CD4 fraction and CD4 fraction) for two tonsils. Indicated are frequencies of CD4 T cells based on CD3 and CD4 surface staining for negative isolation (B) or CD3 and CD8 surface staining for positive isolation (C). (D) Quantification of B and C for the two tonsils. Shown are means with range. Each dot represents one tonsil. (E)-(H) Assessment of the residual CD4 T cells left in the non-CD4 T cell fraction after positive isolation. (E) Schematic of the experimental set-up for positive isolation, indicating the resulting populations of antibody-bound, bead-released CD4 T cells and unlabeled non-CD4 T cells. (F) Representative dot plots showing tonsil cells pre-isolation, the non-CD4 T cell fraction post-isolation and CD4 T cell fraction post-isolation stained with CD3, CD8 and CD4 antibodies but showing either positive CD4 T cell gating based on CD3 and CD4 staining (left panel) or negative CD4 T cell gating based on CD3 and CD8 staining (middle panel). The right panel shows CD4 signal among negatively gated CD4 T cells based on CD3 and CD8 staining in the indicated fractions pre- and post-isolation. The CD4 antibody that was used in this experiment is the OKT4 clone which has a distinct binding site from the CD4-binding antibodies used in the positive isolation procedure. (G) Quantification of left and middle panel in F, indicating frequency of CD4 T cells based on the two gating strategies in the same samples. (H) Quantification of the right panel in F, indicating true frequency of CD4-positive cells among negatively gated CD4 T cells. (I)-(M) Validation of the activation-neutral CD4 T cell positive isolation procedure. (I) and (J) Representative dot plots showing viability (I) or activation (J) by surface expression of CD25 and CD69 of CD4 T cells on day 0, day 1 or day 7 in untouched bulk cultures or after CD4 T cell positive isolation and reconstitution of the culture. (K)-(M) Quantification of I and J for two tested tonsils at the respective timepoints. Shown are means with range. Each dot represents one tonsil. (N) Schematic of the experimental workflow to assess impact of the CD4 positive isolation on HIV-1 infection dynamics. Briefly, tonsil cells were thawed and infected with HIV-1 NL4.3SF2Nef at $1.5x10^5$ BCU per $2x10^6$ cells one day after CD4 positive isolation and reconstitution or bulk culture seeding. On the following day (d0), cells were washed 3x and infection was assessed by analysis of CD4 T cell infection and depletion by flow cytometry analysis upon final harvest. Further, production of HIV-1 virions in the supernatant was measured by SG-PERT throughout the infection course. (O) and (P) Representative dot plots showing CD4 and CD8 T cells subgated from CD3 T cells (O) as well as p24 signal in CD4 T cells (P) in bulk or CD4 T cell-sorted mock or HIV-1 infected conditions, respectively on day 7 post infection. (Q) and (R) Quantification of O and P for two tonsils with the ratio of CD4 vs. CD8 T cells in Q normalized to the mock condition for the respective bulk cultures. Shown are means with range. Each dot represents one tonsil. (S) and (T) HIV-1 RT activity in the supernatant of mock or HIV-1 infected bulk or CD4 T cell-sorted cultures at indicated timepoints for the two tonsils from O-R, respectively. Cells in I-T were cultured in 24-well TW-plates.



Morath et al. 2023, Fig. S4, related to Fig. 4

Figure S4. Crispr/Cas9 KO of tonsil CD4 T cells is highly efficient independent of the isolation strategy or RNP preparation and cytokine supplementation of the culture post KO is not required. Related to Figure 4. (A) Schematic of the experimental workflow. Briefly, tonsil cells were thawed, CD4 T cells were sorted, nucleofected with NT Ctrl. RNP and merged with non-CD4 T cells and cultured in transwell plates. Reconstituted cultures were kept in tonsil medium without further supplements (med. only) or with IL-7, IL-15 (separate or in combination) or with IL-2 with a low or high concentration as indicated. (B) and (C) Representative dot plots of the med. only (B) or cytokine-supplemented cultures with low (left panel) or high (right) concentrations on day 7 showing viability of the CD4 T cells based on zombie violet staining. (D) Representative histograms of CD4 T cells showing surface expression of activation markers CD25, CD69, CD38 or HLA-DR in low (left panel) or high (right panel) cytokine conditions. Superimposed are histograms for the med. only, IL-7, IL-15, IL-7+IL-15 or IL-2 cultures as indicated. (E) and (F) Quantification of C and D for day 7 (E) or day 14 (F) of culture. For each donor, values were normalized to the med. only condition. Each symbol represents one donor. Shown are means with SD. Statistical significance was assessed by Friedman test for normalized data in E and F (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). (G) Schematic of the experimental set-up for determining nucleofection efficiency in bulk or CD4 negatively vs. positively-isolated CD4 T cells. Briefly, tonsil cells were thawed and either nucleofected as bulk cultures or processed for CD4 negative or CD4 positive isolation with subsequent bead release. CD4 T cells resulting from the two separation procedures were nucleofected and merged with the unlabeled, CD4-depleted fraction resulting from the CD4 positive isolation while labeled non-CD4 T cells resulting from the CD4 T cell negative isolation were discarded. (H) Histograms showing CXCR4 surface expression on CD4 T cells in either bulk nucleofected cultures or CD4 negatively and CD4 positively-isolated and nucleofected populations on day 7 post nucleofection for two tonsils. Two individually prepared RNP complexes (X4 KO1 and X4 KO2) were tested side by side in each condition to test for variability of the complex quality in addition to CD4 T cell sorting strategy and to estimate overall robustness of the workflow. Superimposed are histograms for the respective non-targeting control (NT) condition. (I) Quantification of CXCR4 surface levels on CD4 T cells as shown in H for the two tested tonsils and the two RNP preparations normalized to the bulk NT values for each tonsil. Cells were cultured in 96-well plates. (J)-(O) Validation of the inhibitory effect of CXCR4 KO in CD4 T cell sorted cells in the context of HIV-1 infection by comparison with T20 entry inhibitor of HIV-1. Data is derived from the same experiment and donor as shown in Figure 4 G and H as T20 inhibition was tested in parallel, hence the controls are identical. Briefly, tonsil CD4 T cells were positively isolated, nucleofected and after 7 days, infected with HIV-1 NL4.3SF2Nef at $1.5x10^5$ BCU per $2x10^6$ cells in the presence or absence of T20/Enfuvirtide throughout the culture period. (K) and (L) Dot plots showing CD4 and CD8 T cells subgated from CD3 T cells (K) as well as p24 signal in CD4 T cells (L) on day 8 post infection in the non-nucleofected condition or NT Ctrl (untreated or with T20 treatment) or CXCR4 KO condition as indicated. (M) and (N) Quantification of K and L for one tonsil with the ratio of CD4 vs. CD8 T cells in M normalized to the mock condition for the respective conditions. (O) HIV-1 RT activity in the supernatant of mock or HIV-1 infected CD4 T cell-sorted cultures for the different conditions at indicated timepoints for one tonsil. Cells in J-O were cultured in 24-well TW-plates.

		pre-isolation			post-isolation			
		Starting total cell count	**% non-CD4 T (cell count)**	**% CD4 T (cell count)**	**total cell count selected fraction**	**% CD4 T in selected fraction**	**Total cell count non-selected fraction**	**% residual CD4 T in non-selected fraction**
CD4 negative isolation	**T30**	2,50E+07	77,7 (1,94E+07)	22,3 (5,58E+06)	2,85E+06	94,5	NA	NA
	T32	2,50E+07	90,5 (2,26E+07)	9,5 (2,38E+06)	1,50E+06	55,6	NA	NA
	T65	1,25E+07	86,3 (1,08E+07)	13,7 (1,71E+06)	3,38E+06	43,6	NA	NA
	T87	1,25E+07	78,9 (9,86E+06)	21,1 (2,64E+06)	2,69E+06	66,9	NA	NA
CD4 positive isolation	**T30**	4,73E+07	72,5 (3,43E+07)	27,5 (1,30E+07)	7,91E+06	91,3	2,74E+07	2,4
	T32	5,00E+07	89,9 (4,50E+07)	10,1 (5,05E+06)	3,29E+06	85,5	3,36E+07	1,5
	T65	2,50E+07	85,5 (2,14E+07)	14,5 (3,63E+06)	1,70E+06	90,5	1,13E+07	2,2
	T87	2,50E+07	76,3 (1,91E+07)	23,7 (5,93E+06)	2,50E+06	91,5	1,06E+07	3,7

Table S1. Comparison of cell yields and population purity obtained from positive or negative isolation of tonsil CD4 T cells. Related to Figure 3. Table summarizing cell yields from negative and positive isolation performed in parallel with starting material from the same tonsils as indicated. Frequency of CD4 and non-CD4 T cells is based on flow cytometry analysis following CD3 and CD4 staining for negative isolation or CD3 and CD8 staining for positive isolation, respectively.