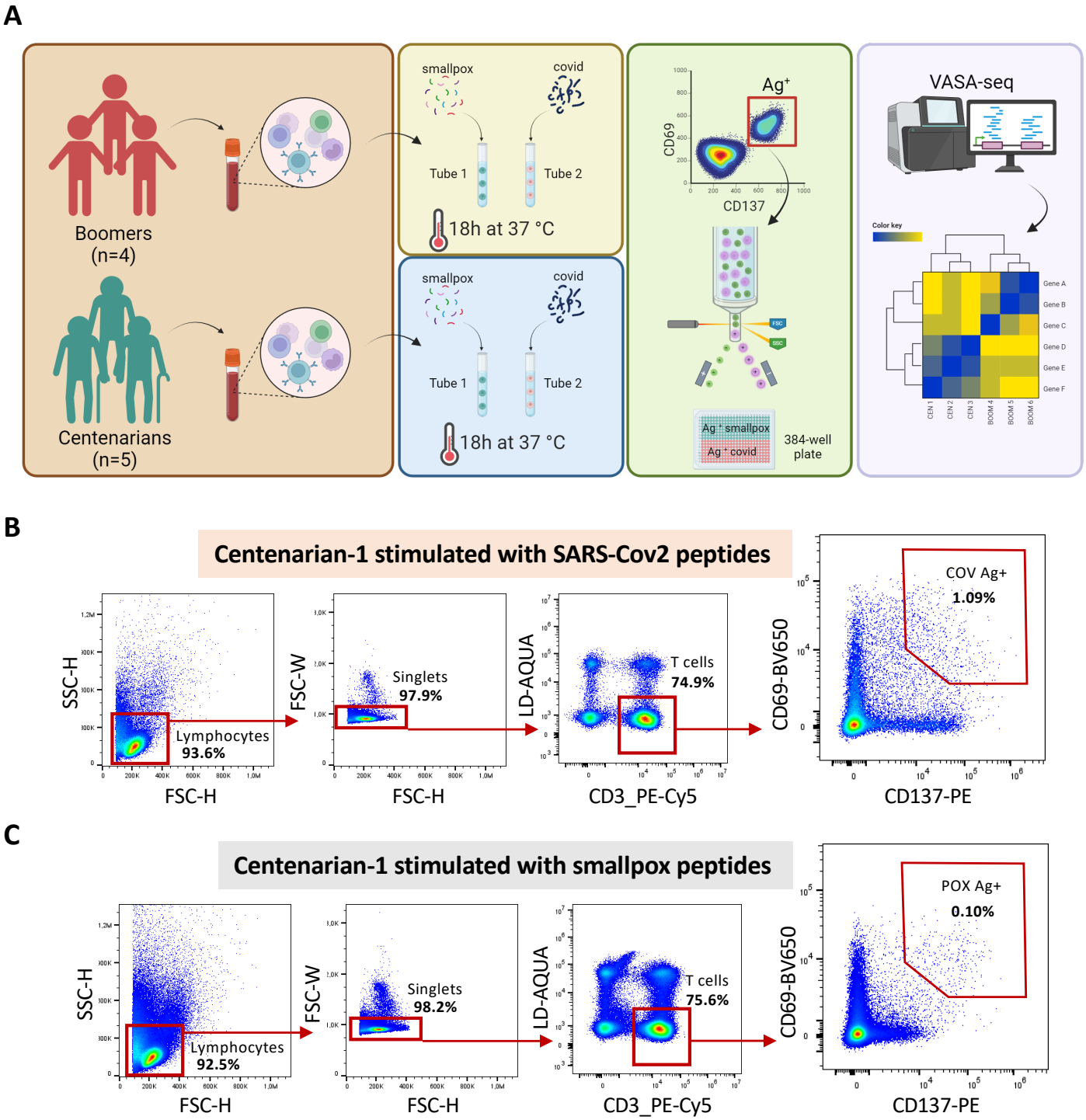
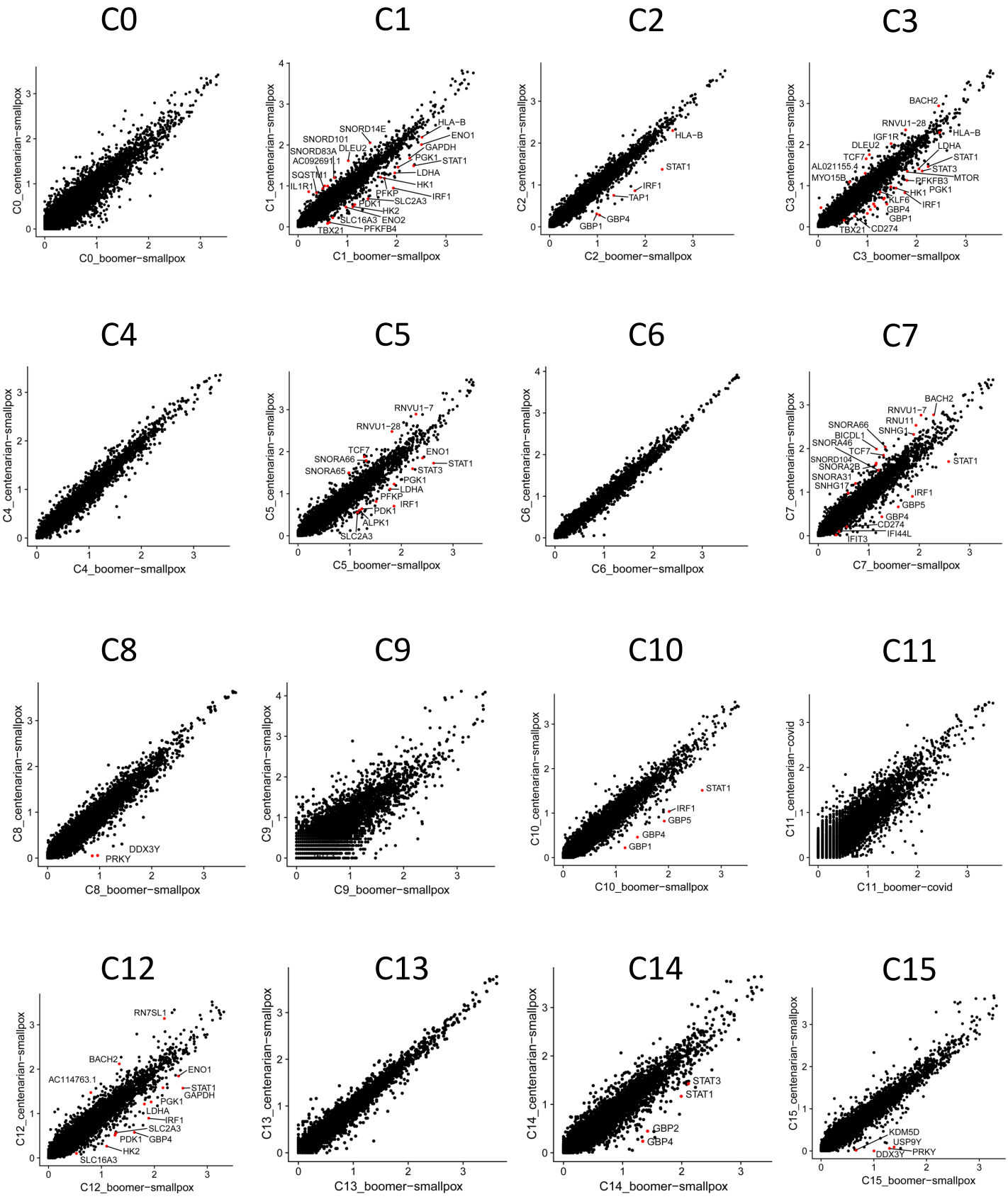
Supplementary figures

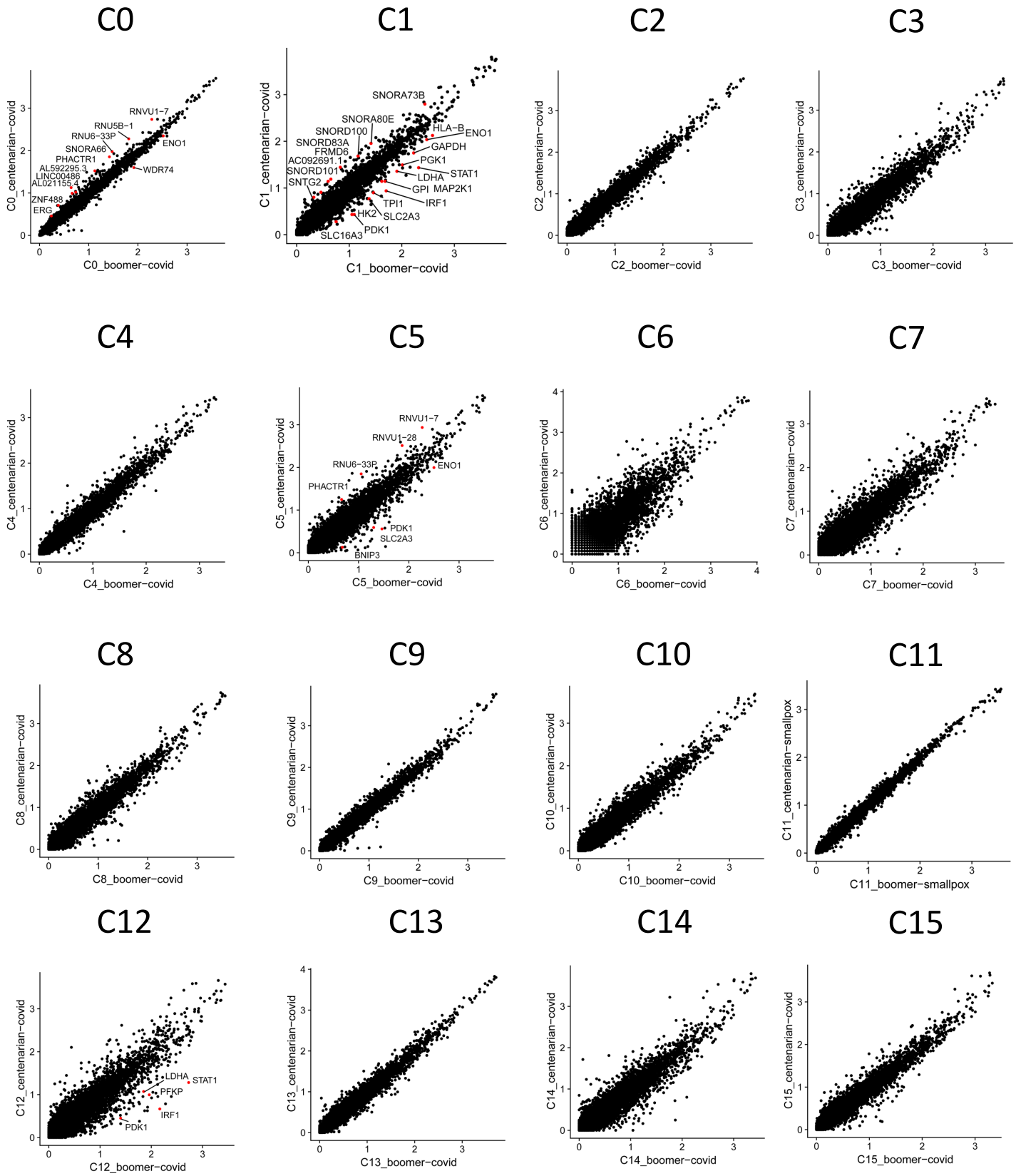
De Biasi, S., Lo Tartaro, D. et al.



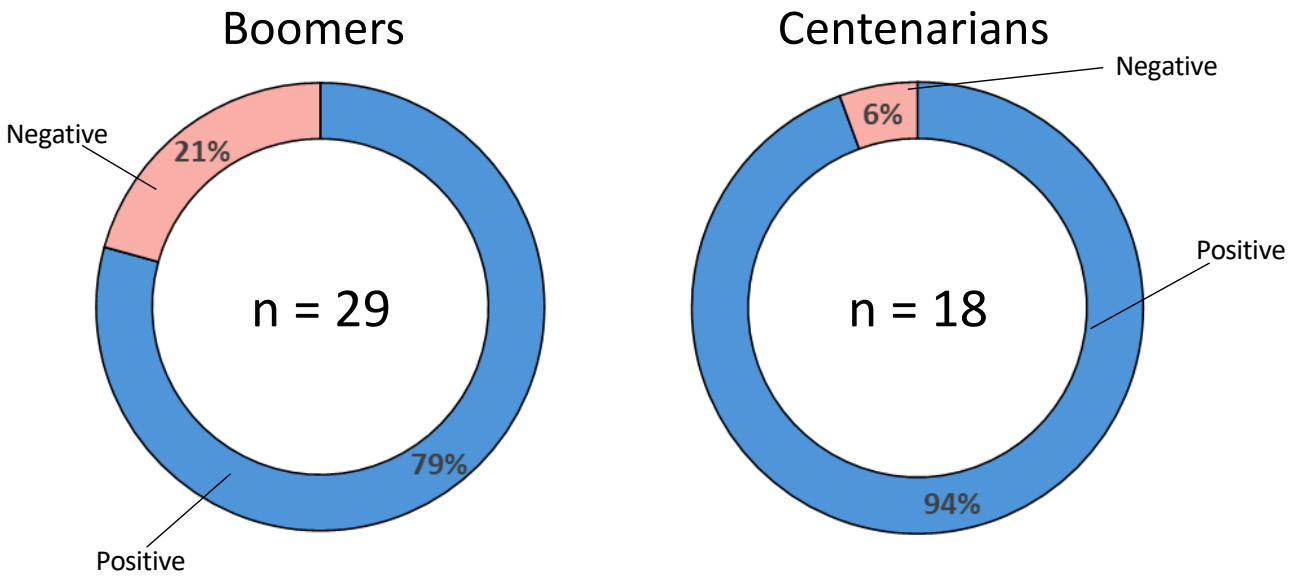
Supplementary Fig 1. FACS sorting of Ag⁺ T cells for high-throughput single-cell total RNA sequencing using VASA-seq (384-well plate). A) Experimental workflow. **B**) Gating strategy used to isolate total Ag⁺ T cells after 18 hours of in vitro stimulation with SARS-CoV-2 peptides. Lymphocytes were identified based on forward (FSC-H) and side (SSC-H) scatter characteristics. Doublets were excluded using FSC-H versus FSC-W gating. Viable CD3⁺ T cells were selected by excluding Live/Dead (LD) positive cells. Within this population, total antigen-specific (Ag⁺) T cells—both CD4⁺ and CD8⁺—were identified based on co-expression of CD137 and CD69. **C**) Gating strategy used to isolate total Ag⁺ T cells after 18 hours of in vitro stimulation with smallpox peptides. Lymphocytes were identified based on forward (FSC-H) and side (SSC-H) scatter characteristics. Doublets were excluded using FSC-H versus FSC-W gating. Viable CD3⁺ T cells were selected by excluding Live/Dead (LD) positive cells. Within this population, total antigen-specific (Ag⁺) T cells—both CD4⁺ and CD8⁺—were identified based on co-expression of CD137 and CD69.



Supplementary Fig 2. Identification of differentially expressed genes (DEGs) between smallpox-specific T cells from Boomers and Centenarians. Scatter plots display DEGs for each cluster, with genes highlighted in red indicating significance ($p < 0.05$) and a $\log_2$ fold change > 0.25.

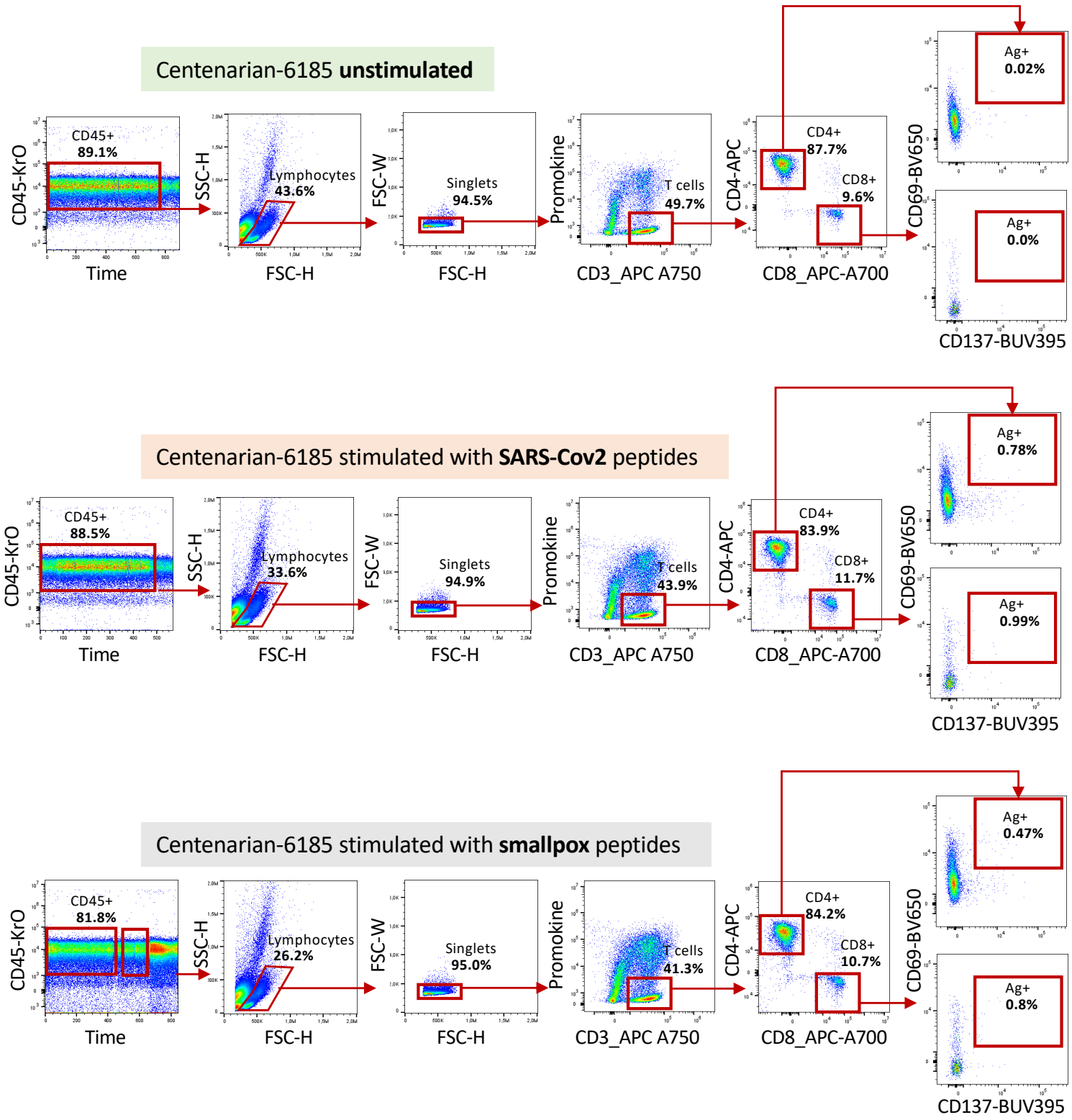


Supplementary Fig 3. Identification of differentially expressed genes (DEGs) between SARS-CoV-2-specific T cells from Boomers and Centenarians. Scatter plots display DEGs for each cluster, with genes highlighted in red indicating significance ($p < 0.05$) and a $\log_2$ fold change > 0.25.

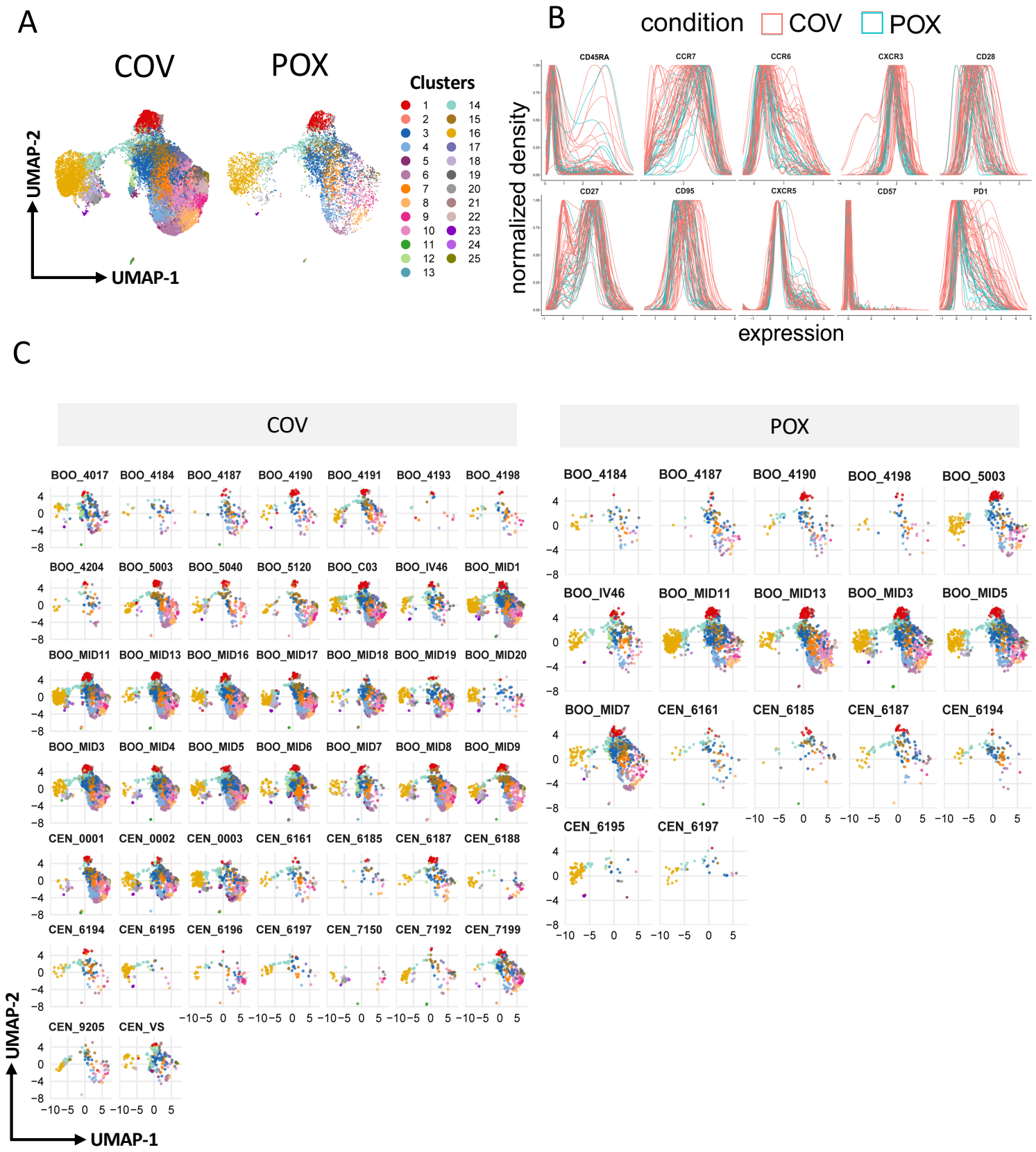


Sample_ID	Group_ID	CMV IgG CLIA (U/ml)	Results
MIDDLE 1	BOOMERS	139	positive
MIDDLE 3	BOOMERS	121	positive
MIDDLE 4	BOOMERS	108	positive
MIDDLE 5	BOOMERS	98	positive
MIDDLE 6	BOOMERS	135	positive
MIDDLE 7	BOOMERS	80	positive
MIDDLE 8	BOOMERS	< 12	negative
MIDDLE 9	BOOMERS	102	positive
MIDDLE 11	BOOMERS	149	positive
MIDDLE 13	BOOMERS	82	positive
MIDDLE 16	BOOMERS	< 12	negative
MIDDLE 17	BOOMERS	73	positive
MIDDLE 18	BOOMERS	> 180	positive
MIDDLE 20	BOOMERS	104	positive
MIDDLE 19	BOOMERS	158	positive
4198	BOOMERS	155	positive
4193	BOOMERS	118	positive
4190	BOOMERS	20	positive
4191	BOOMERS	75	positive
5120	BOOMERS	147	positive
5003	BOOMERS	91	positive
4189	BOOMERS	116	positive
4187	BOOMERS	< 12	negative
C03	BOOMERS	< 12	negative
5040	BOOMERS	170	positive
4204	BOOMERS	< 12	negative
4017	BOOMERS	> 180	positive
4184	BOOMERS	82	positive
IV46	BOOMERS	< 12	negative
7160	CENTENARIANS	102	positive
6161	CENTENARIANS	104	positive
6185	CENTENARIANS	109	positive
6188	CENTENARIANS	170	positive
6137	CENTENARIANS	108	positive
7192	CENTENARIANS	114	positive
6194	CENTENARIANS	108	positive
6196	CENTENARIANS	166	positive
6197	CENTENARIANS	87	positive
7199	CENTENARIANS	106	positive
9205	CENTENARIANS	74	positive
V.S.	CENTENARIANS	160	positive
CEN_001	CENTENARIANS	11	negative
CEN_002	CENTENARIANS	127	positive
CEN_003	CENTENARIANS	174	positive
CEN_004	CENTENARIANS	127	positive
CEN_005	CENTENARIANS	123	positive
CEN_006	CENTENARIANS	> 180	positive

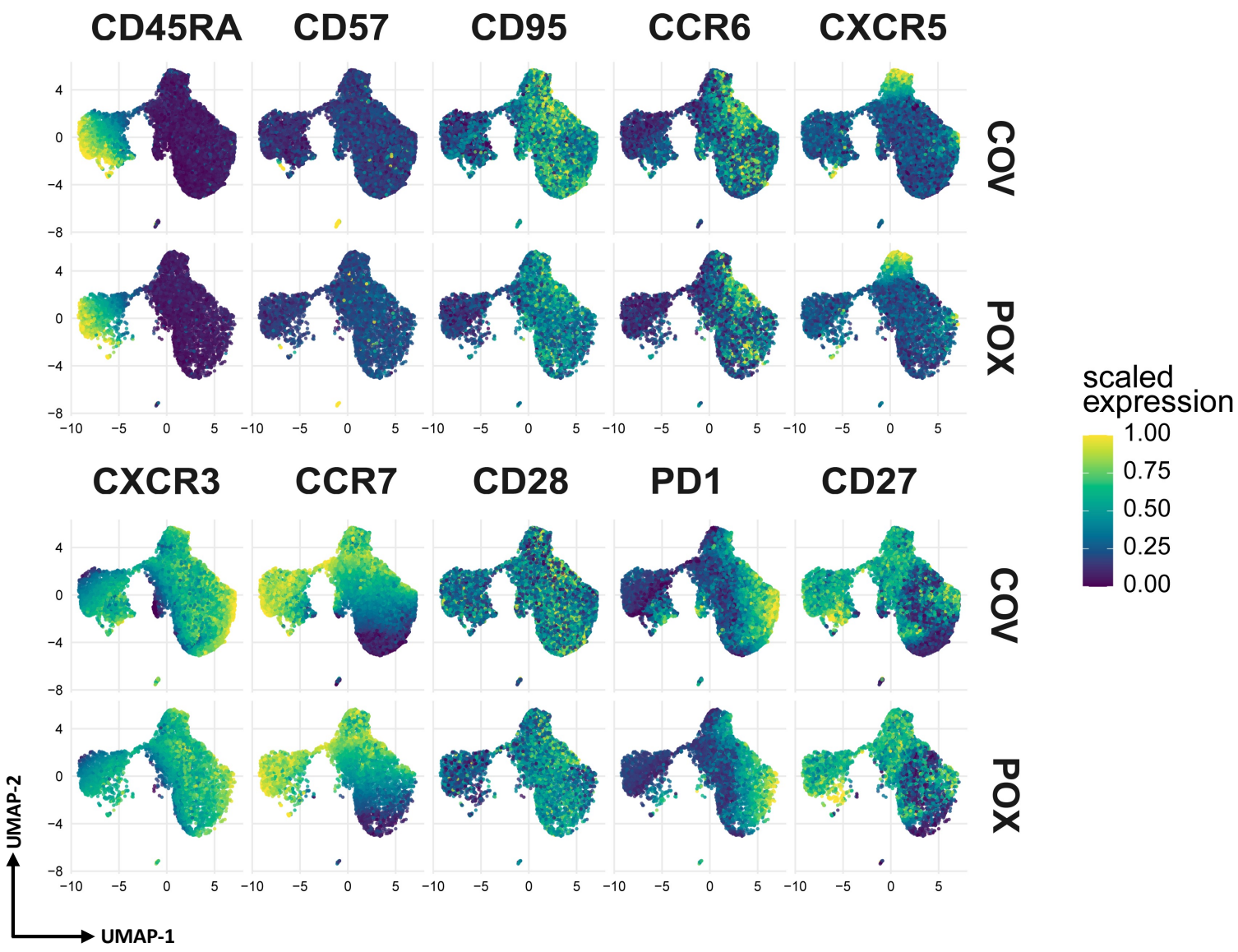
Supplementary Fig. 4. CMV serostatus in boomers and centenarians. Ring plot illustrating the proportion of CMV-IgG–positive and CMV-IgG–negative individuals in both cohorts. The accompanying table reports results for participants stratified as boomers and centenarians based on CMV IgG antibody levels measured by CLIA. Values greater than 12 U/mL were classified as positive.



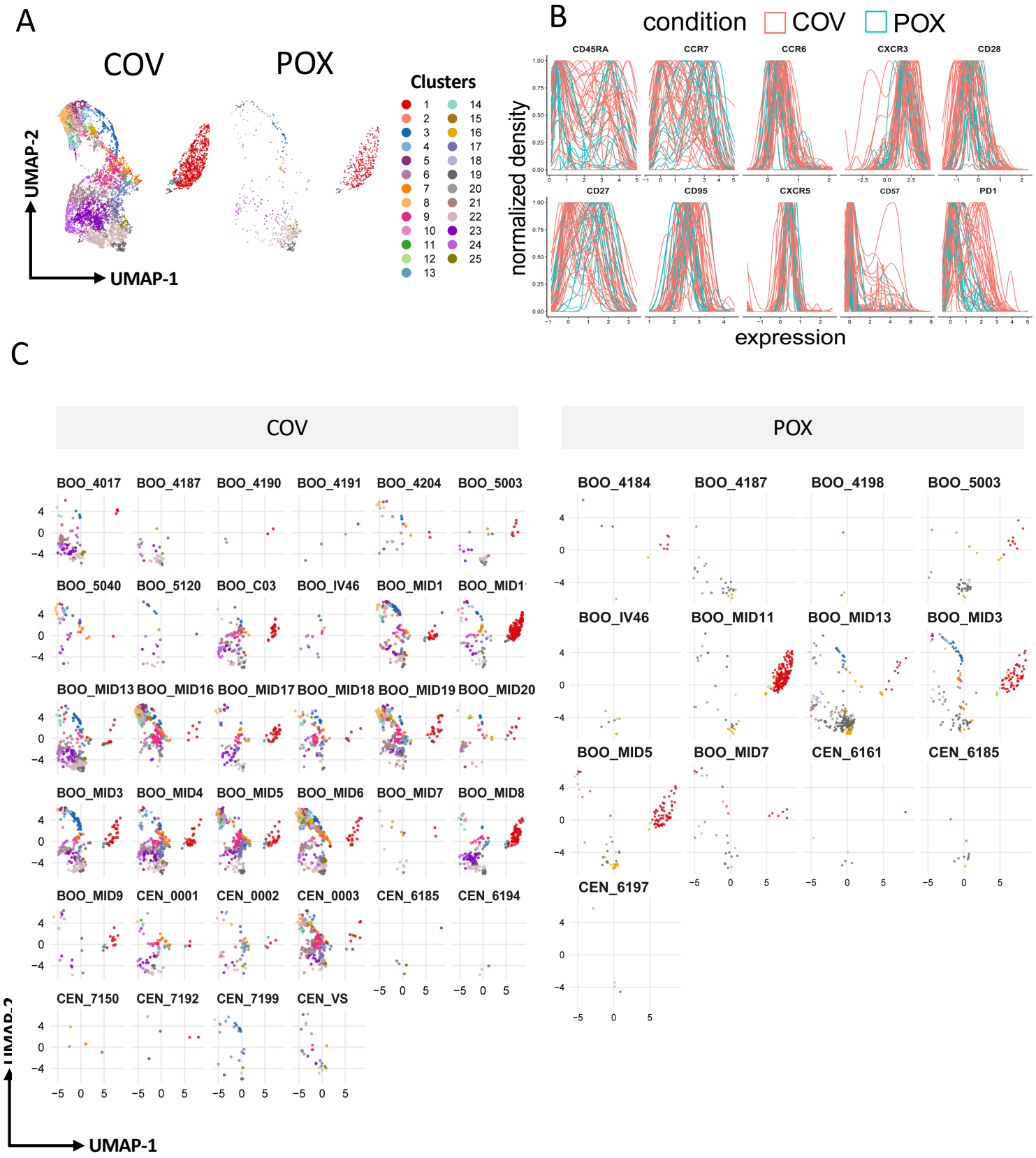
Supplementary Fig 5. Gating strategy for identifying antigen-specific CD4⁺ and CD8⁺ T cells (AIM assay). A) Unstimulated sample from centenarian 6185. **B)** SARS-CoV-2 peptide-stimulated sample from centenarian 6185. **C)** Smallpox peptide-stimulated sample from centenarian 6185. For all conditions, initial gating was performed to exclude flow instabilities by plotting CD45 against Time. Lymphocytes were then identified based on forward and side scatter (FSC-H vs SSC-H). Doublets were excluded using an FSC-H vs FSC-Width plot. Viable T cells were gated as CD3⁺ and negative for Promokine (viability dye). Within the live CD3⁺ population, CD4⁺ and CD8⁺ T cells were identified. Antigen-specific (Ag⁺) T cells were defined by the co-expression of activation markers CD69 and CD137 within both CD4⁺ and CD8⁺ subsets."



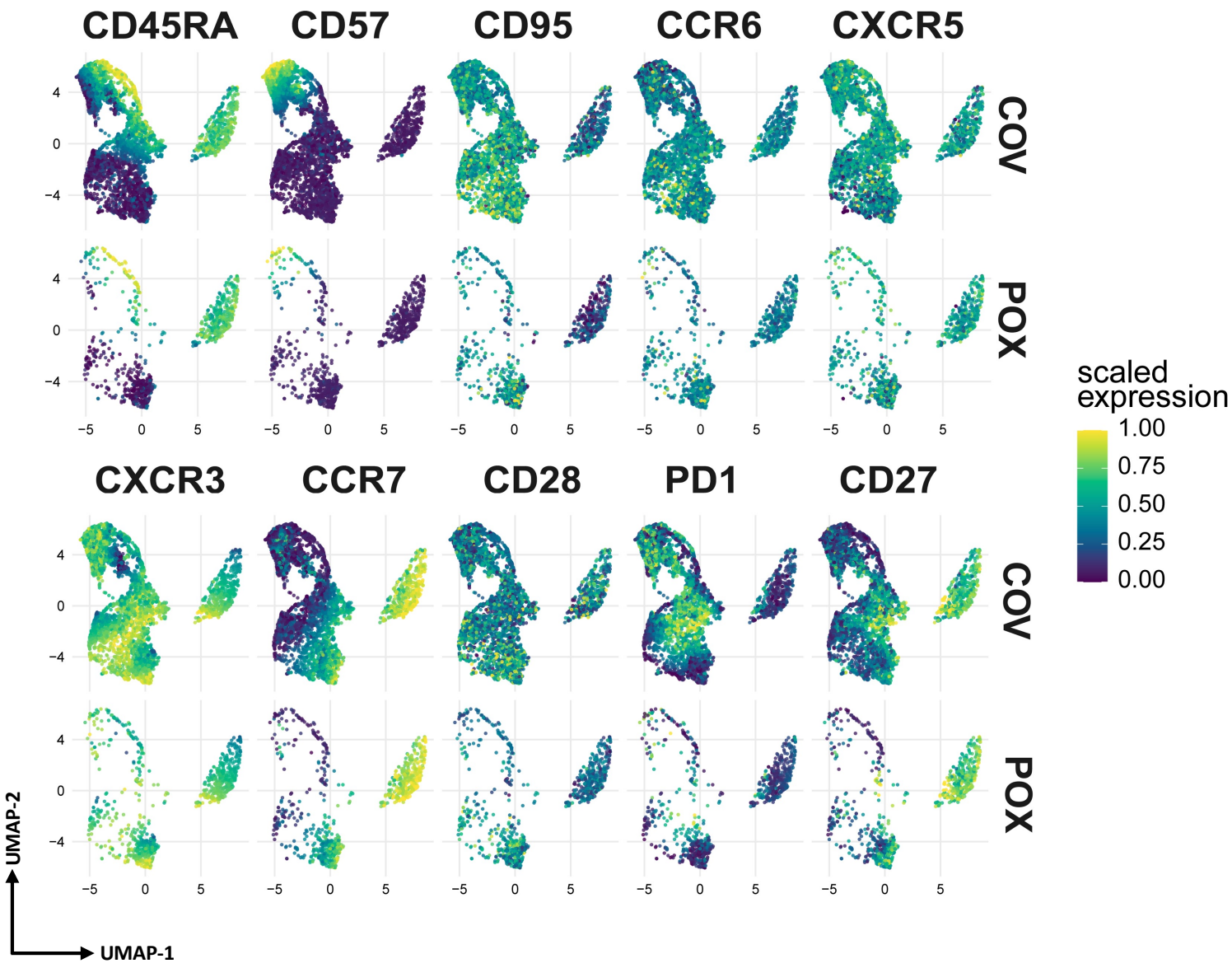
Supplementary Fig 6. Quality control (QC) of the clustering process for CD4⁺ T cells. A) UMAP distribution divided by condition (COV and POX). **B)** Histograms showing the expression of the indicated markers according to condition (COV and POX) in each patient. **C)** UMAP showing cell distribution in each patient divided by condition.



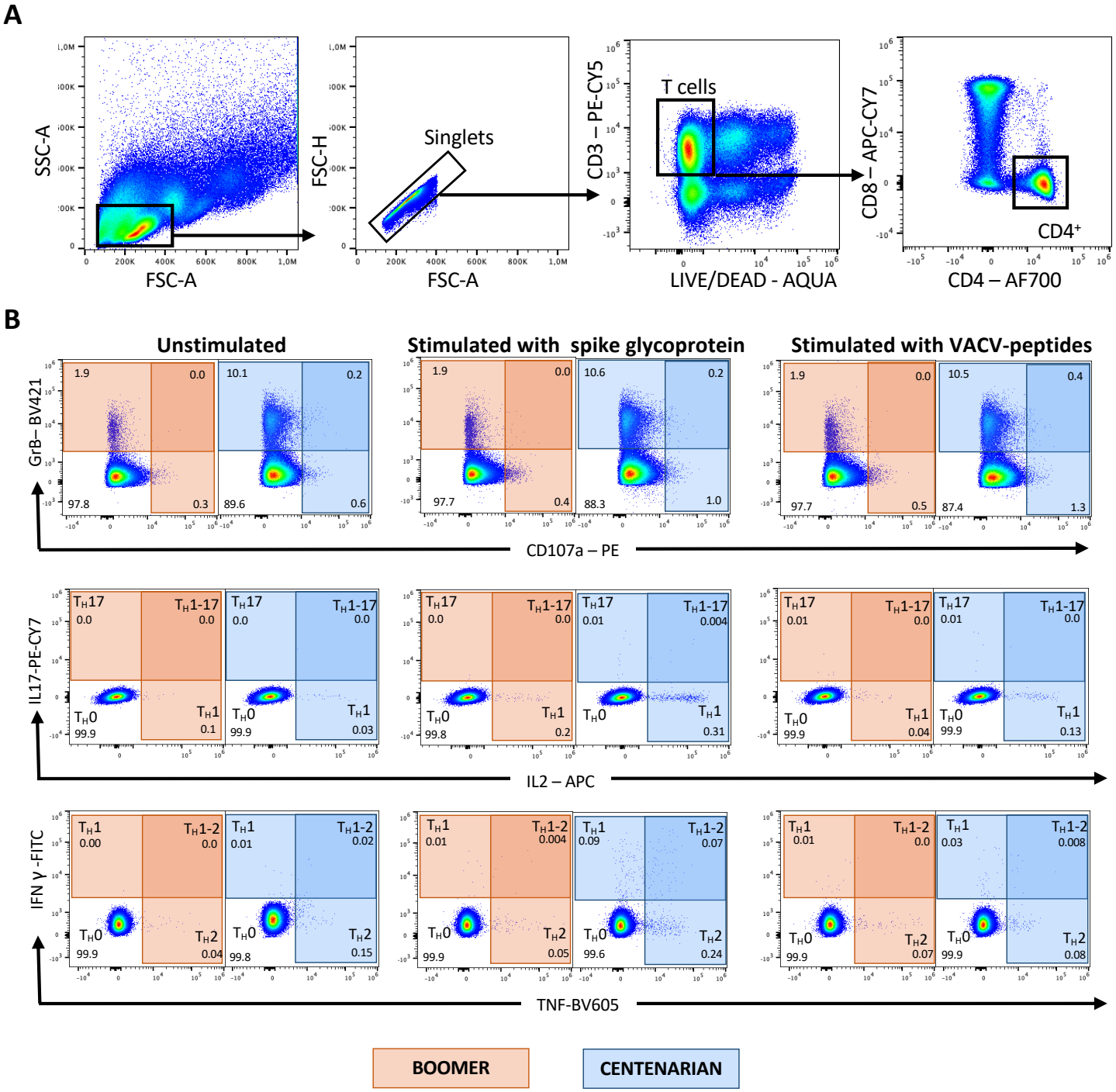
Supplementary Fig 7. Quality control (QC) of the clustering process for CD4⁺ T cells. UMAP representation of the relative expression of the sixteen (16) markers used for CD8⁺ T cell phenotyping.



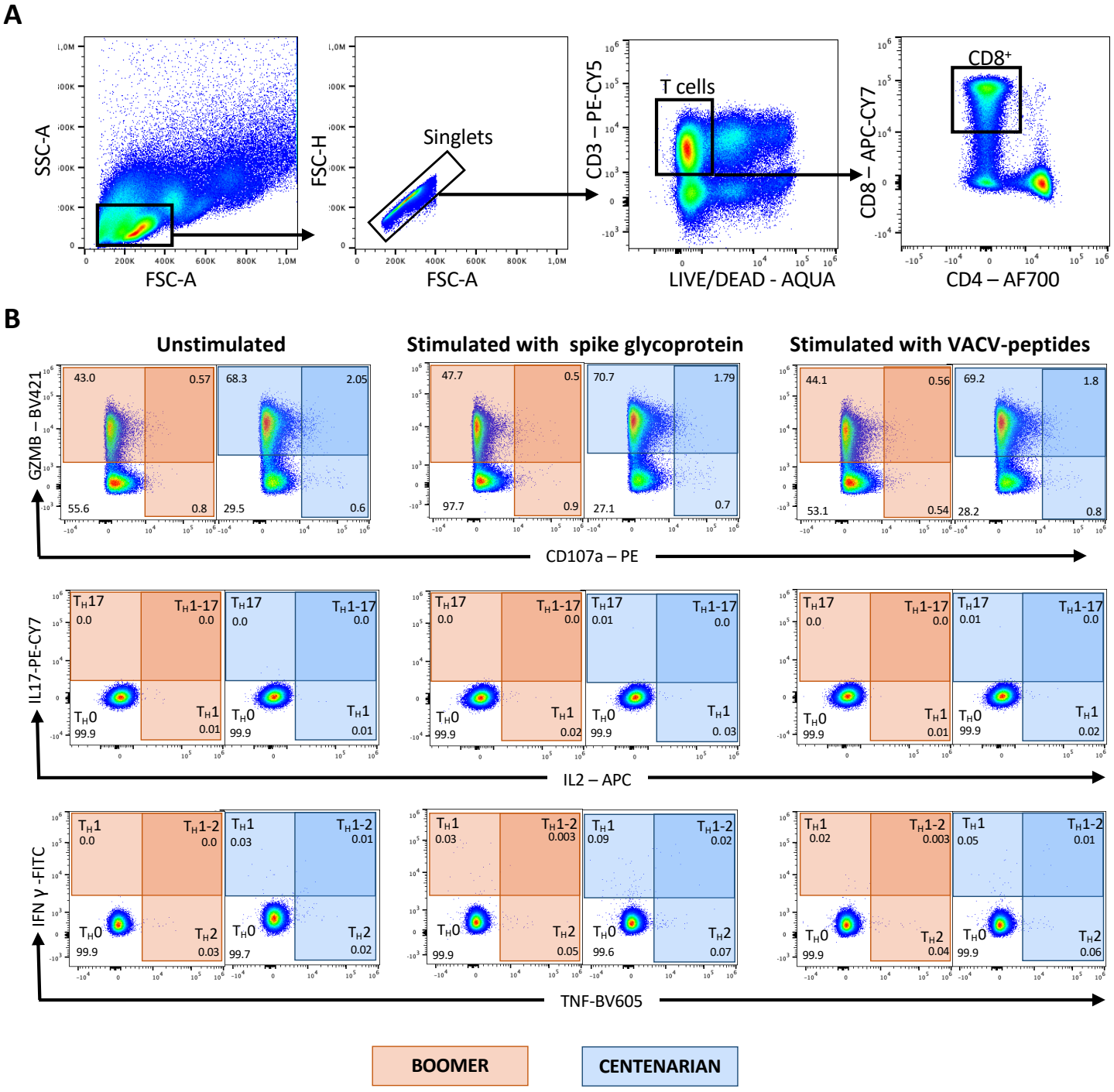
Supplementary Fig 8. Quality control (QC) of the clustering process for CD8⁺ T cells. A) UMAP distribution divided by condition (COV and POX). **B)** Histograms showing the expression of the indicated markers according to condition (COV and POX) in each patient. **C)** UMAP showing cell distribution in each patient divided by condition.



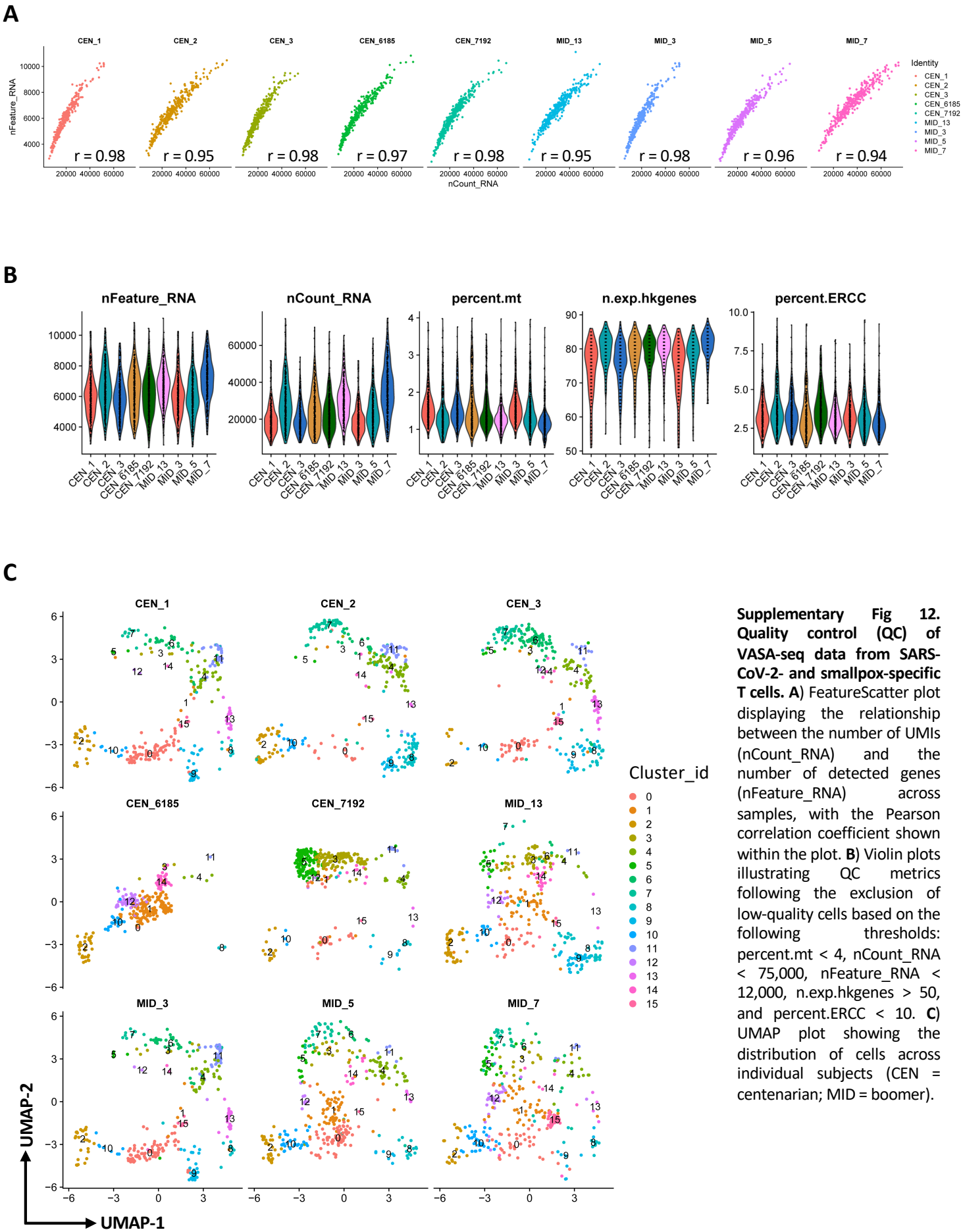
Supplementary Fig 9. Quality control (QC) of the clustering process for CD8⁺ T cells. UMAP representation of the relative expression of the sixteen (16) markers used for CD8⁺ T cell phenotyping.



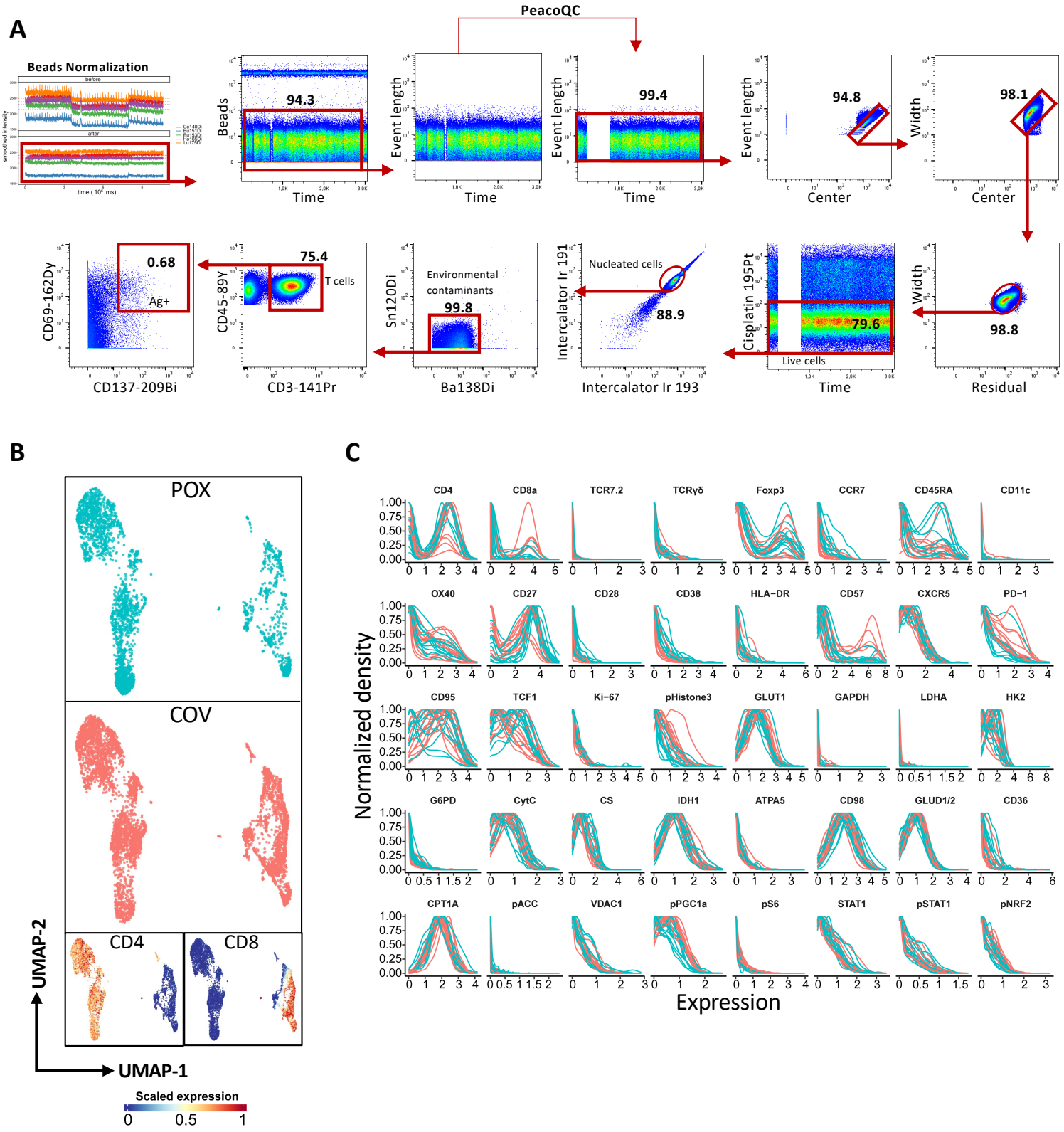
Supplementary Fig 10. Intracellular Cytokine Staining (ICS) of CD4⁺ T Cells. A) The gating strategy used to assess intracellular cytokine production in CD4⁺ T lymphocytes is shown. Leukocytes were first selected based on forward and side scatter (FSC/SSC) characteristics. Doublets were excluded using FSC-H vs FSC-A gating. Viable CD3⁺ T cells were identified by excluding LIVE/DEAD-positive events. Within this population, CD4⁺ and CD8⁺ T cells were further delineated. **B**) The quadrant plot displays the percentages of CD4⁺ T cells expressing IFN-γ, TNF, CD107a, Granzyme B (GrB), IL-2, as well as IL-17.



Supplementary Fig 11. Intracellular Cytokine Staining (ICS) of CD8⁺ T Cells. A) The gating strategy used to assess intracellular cytokine production in CD8⁺ T lymphocytes is shown. Leukocytes were first selected based on forward and side scatter (FSC/SSC) characteristics. Doublets were excluded using FSC-H vs FSC-A gating. Viable CD3⁺ T cells were identified by excluding LIVE/DEAD-positive events. Within this population, CD4⁺ and CD8⁺ T cells were further delineated. **B**) The quadrant plot displays the percentages of CD8⁺ T cells expressing IFN-γ, TNF, CD107a, Granzyme B (GrB), IL-2, as well as IL-17.



Supplementary Fig 12. Quality control (QC) of VASA-seq data from SARS-CoV-2- and smallpox-specific T cells. A) FeatureScatter plot displaying the relationship between the number of UMIs (nCount_RNA) and the number of detected genes (nFeature_RNA) across samples, with the Pearson correlation coefficient shown within the plot. **B**) Violin plots illustrating QC metrics following the exclusion of low-quality cells based on the following thresholds: percent.mt < 4, nCount_RNA < 75,000, nFeature_RNA < 12,000, n.exp.hkgenes > 50, and percent.ERCC < 10. **C**) UMAP plot showing the distribution of cells across individual subjects (CEN = centenarian; MID = boomer).



Supplementary Fig 13. Quality control (QC) of single-cell metabolic regulomes (scMEP) analysis of Ag⁺ T cell . A) Gating strategy illustrating the manual pre-processing of CyTOF data used to identify smallpox-specific (POX) and SARS-CoV-2-specific (COV) T cells ($CD3^+CD69^+CD137^+$). Raw mass cytometry data were bead-normalized to minimize acquisition-related variability. Following normalization, beads, flow instabilities (PeacoQC), aggregates, doublets, and dead cells were removed. Non-biological events and artifacts were excluded using Gaussian parameters (Center, Width, Offset, Residual). Live, nucleated cells were identified via Cisplatin-195Pt and Intercalator signals, while unused channels (Sn120Di, Ba138Di) were checked for environmental contamination. T cells were gated as $CD3^+CD45^+$, and antigen-specific cells (Ag^+) were defined as $CD69^+CD137^+$. **B**) UMAP projection showing cell distribution by sample type (POX vs. COV) and by CD4/CD8 expression (in red positive expression). **C**) Histogram plots displaying marker expression profiles stratified by sample type.