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Supplemental Information

Altered Peptide Ligands Impact the Diversity of Polyfunctional Phenotypes in T Cell Receptor Gene-Modified T Cells

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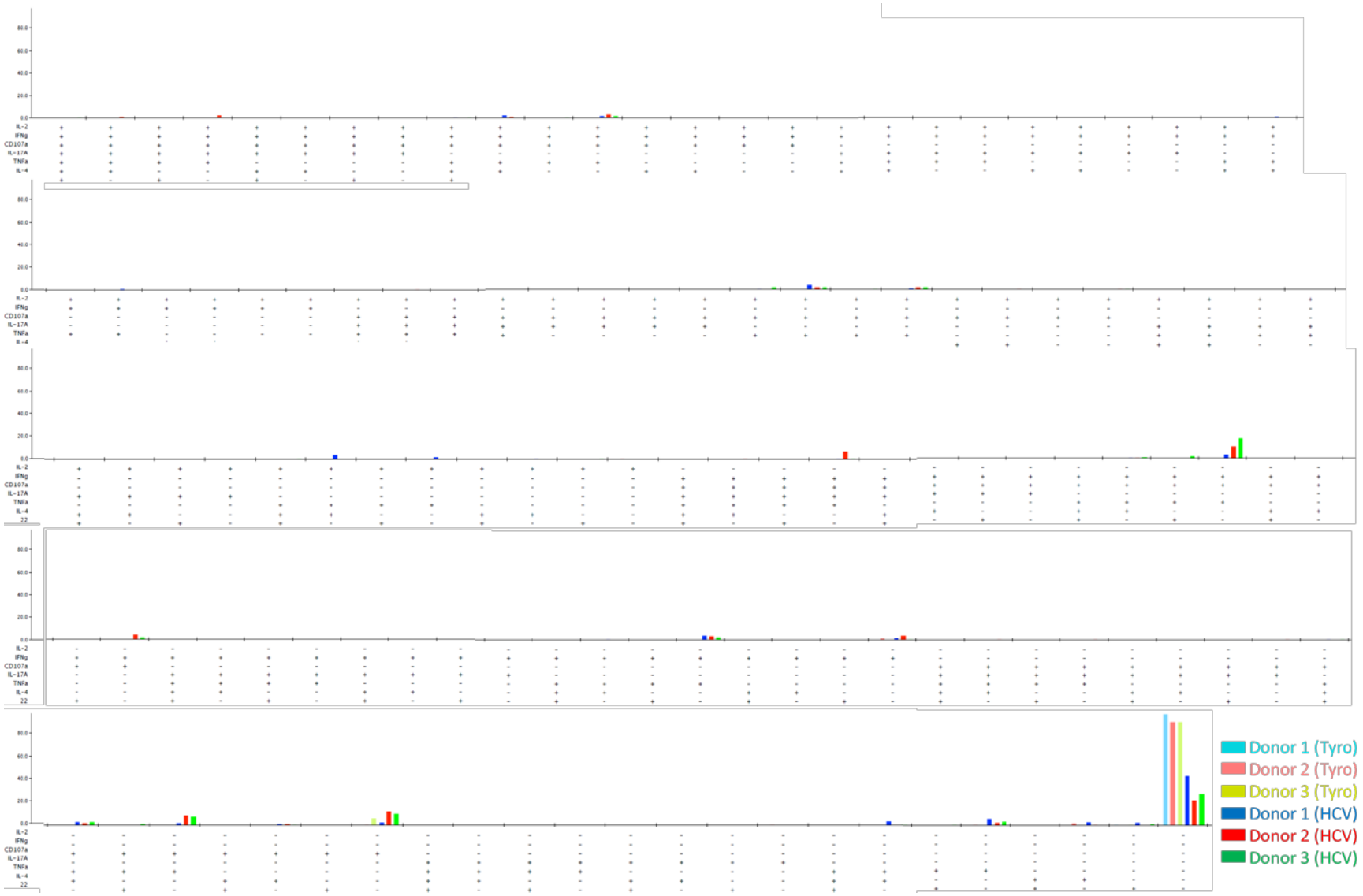
Supplementary Figure S1. SPICE-generated bar graphs comparing polyfunctional diversity of three PBL donor-derived T cells against WT HCV NS3:1406-1415 antigen. HCV1406 TCR-transduced T cells derived from PBL of three healthy donors were co-cultured for 5 hours with T2 cells loaded with 10 ug/mL of NS3:1406-1415 or tyrosinase:368-376 peptide. **(a)** $CD8^+$ or **(b)** $CD4^+$ T cells were evaluated CD107a, IFNγ, TNFα, IL-2, IL-4, IL-17A, and IL-22 expression by immunofluorescence. These complete graphs correspond to condensed versions in Figure 1c-d, which display phenotypes of >1% frequency in at least one donor. **(c)** and **(d)** represent respective plots generated without tyrosinase background subtraction displaying non-reactive populations.

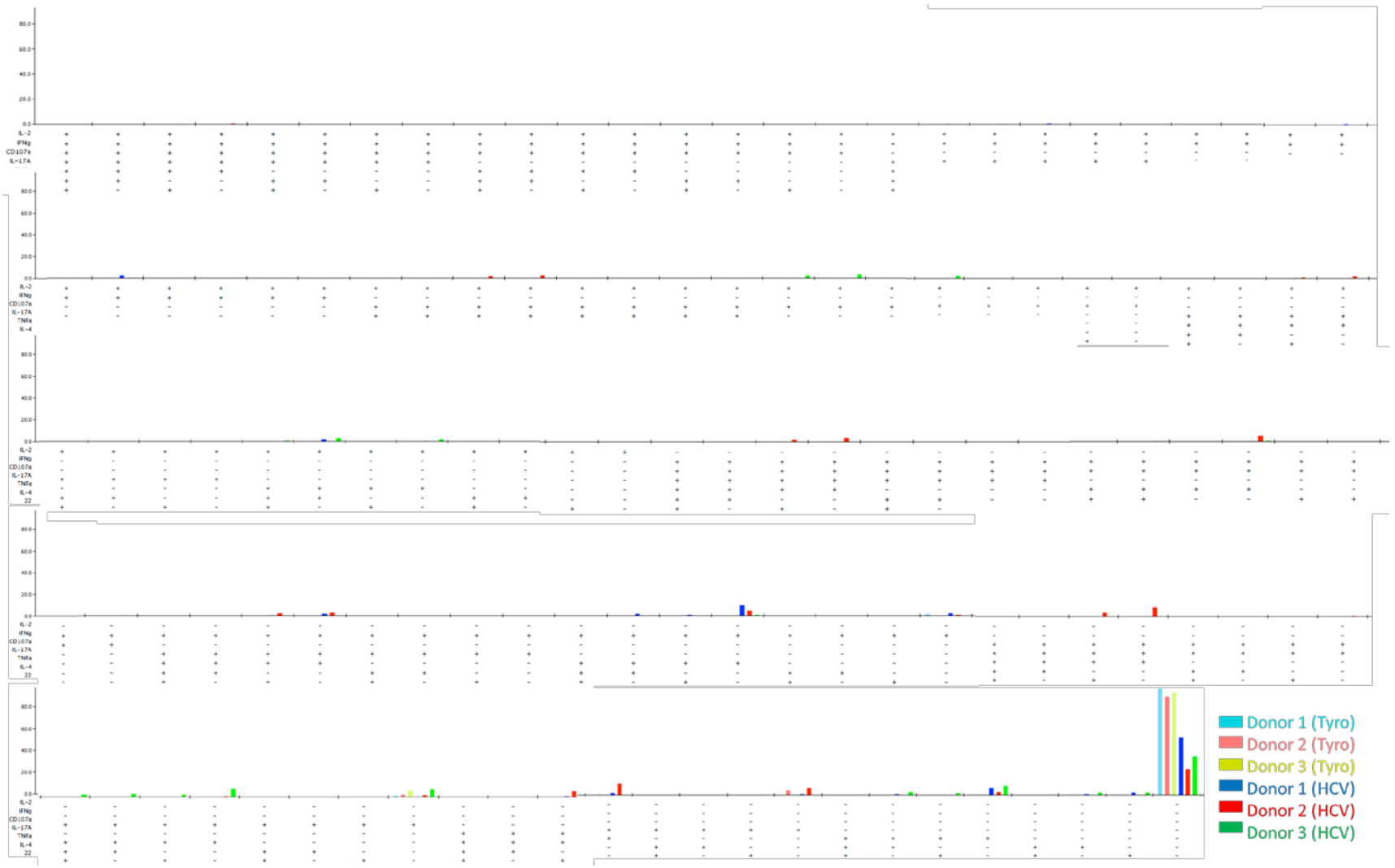
Supplementary Figure S1 (cont'd). SPICE-generated bar graphs comparing polyfunctional diversity of three PBL donor-derived T cells against WT HCV NS3:1406-1415 antigen. HCV1406 TCR-transduced T cells derived from PBL of three healthy donors were co-cultured for 5 hours with T2 cells loaded with 10 ug/mL of NS3:1406-1415 or tyrosinase:368-376 peptide. **(a)** $CD8^+$ or **(b)** $CD4^+$ T cells were evaluated CD107a, IFNγ, TNFα, IL-2, IL-4, IL-17A, and IL-22 expression by immunofluorescence. These complete graphs correspond to condensed versions in Figure 1c-d, which display phenotypes of >1% frequency in at least one donor. **(c)** and **(d)** represent respective plots generated without tyrosinase background subtraction displaying non-reactive populations.

C

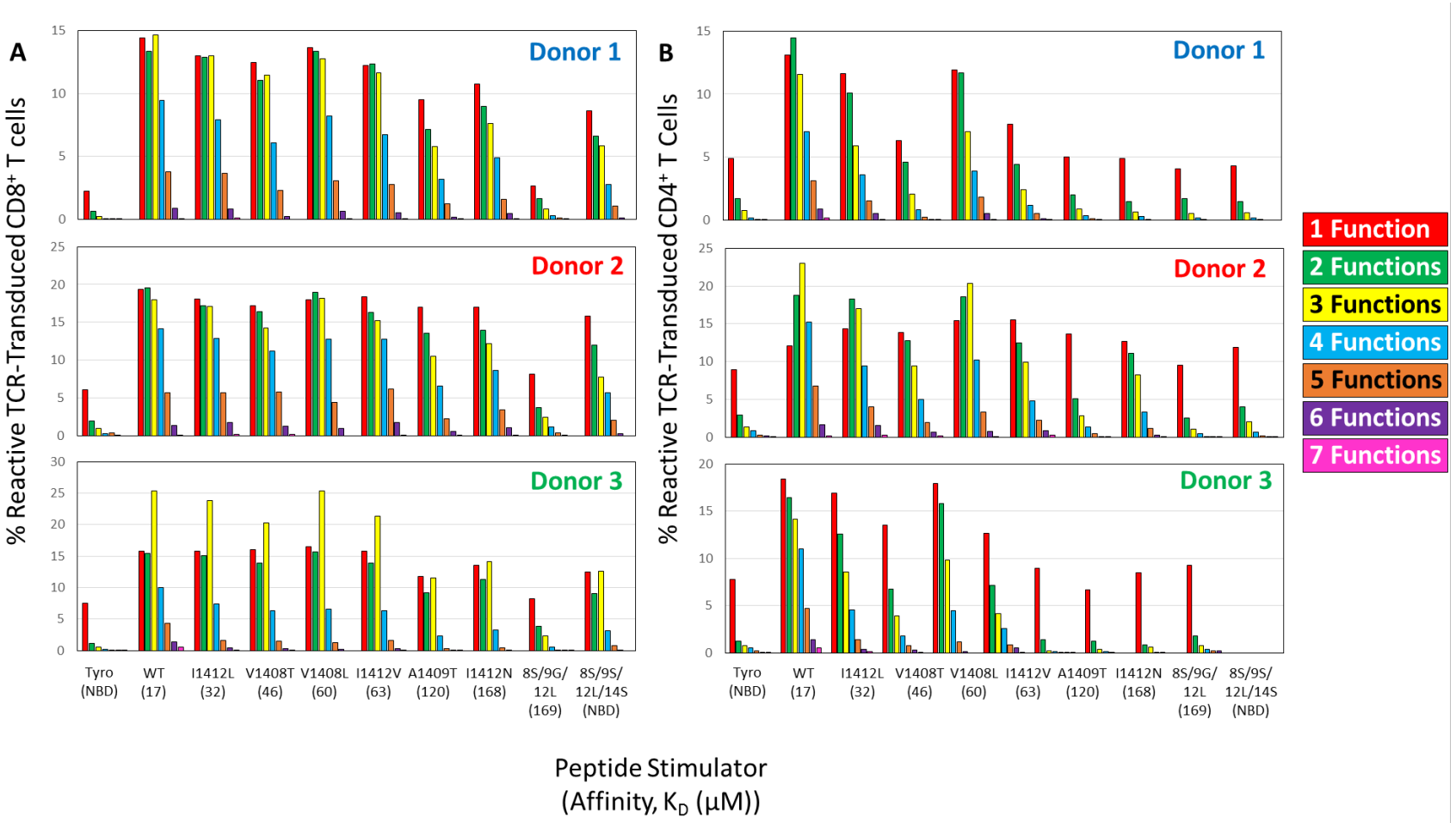


Supplementary Figure S1 (cont'd). SPICE-generated bar graphs comparing polyfunctional diversity of three PBL donor-derived T cells against WT HCV NS3:1406-1415 antigen. (c) frequency of polyfnctional $CD8^+$ T cells stimulated with tyrosinase (negative control) or HCV WT peptide-loaded T2 cells, generated without negative control (tyrosinase) background subtraction.

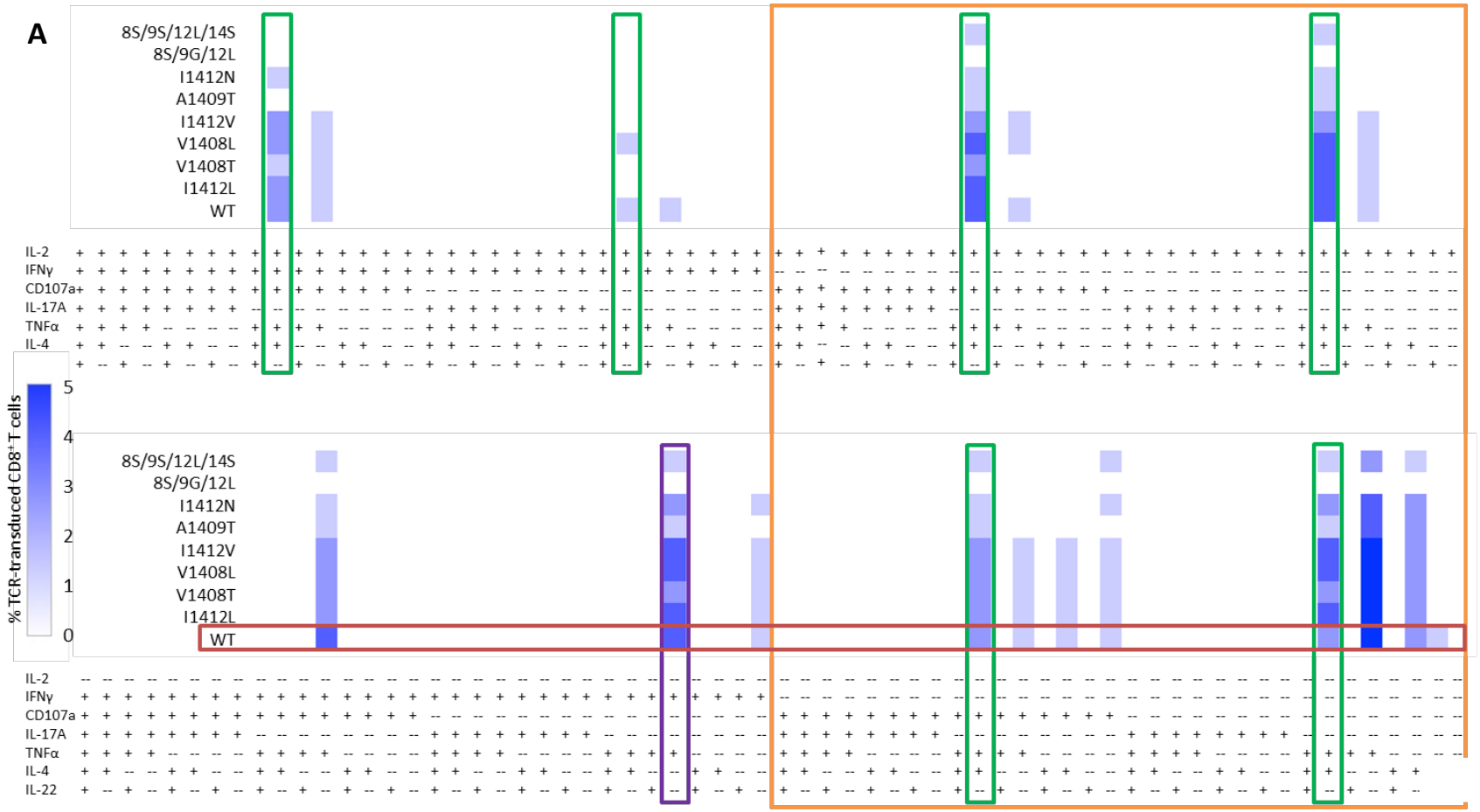
D



Supplementary Figure S1 (cont'd). SPICE-generated bar graphs comparing polyfunctional diversity of three PBL donor-derived T cells against WT HCV NS3:1406-1415 antigen. (c) frequency of polyfnctional $CD4^+$ T cells stimulated with tyrosinase (negative control) or HCV WT peptide-loaded T2 cells, generated without negative control (tyrosinase) background subtraction.

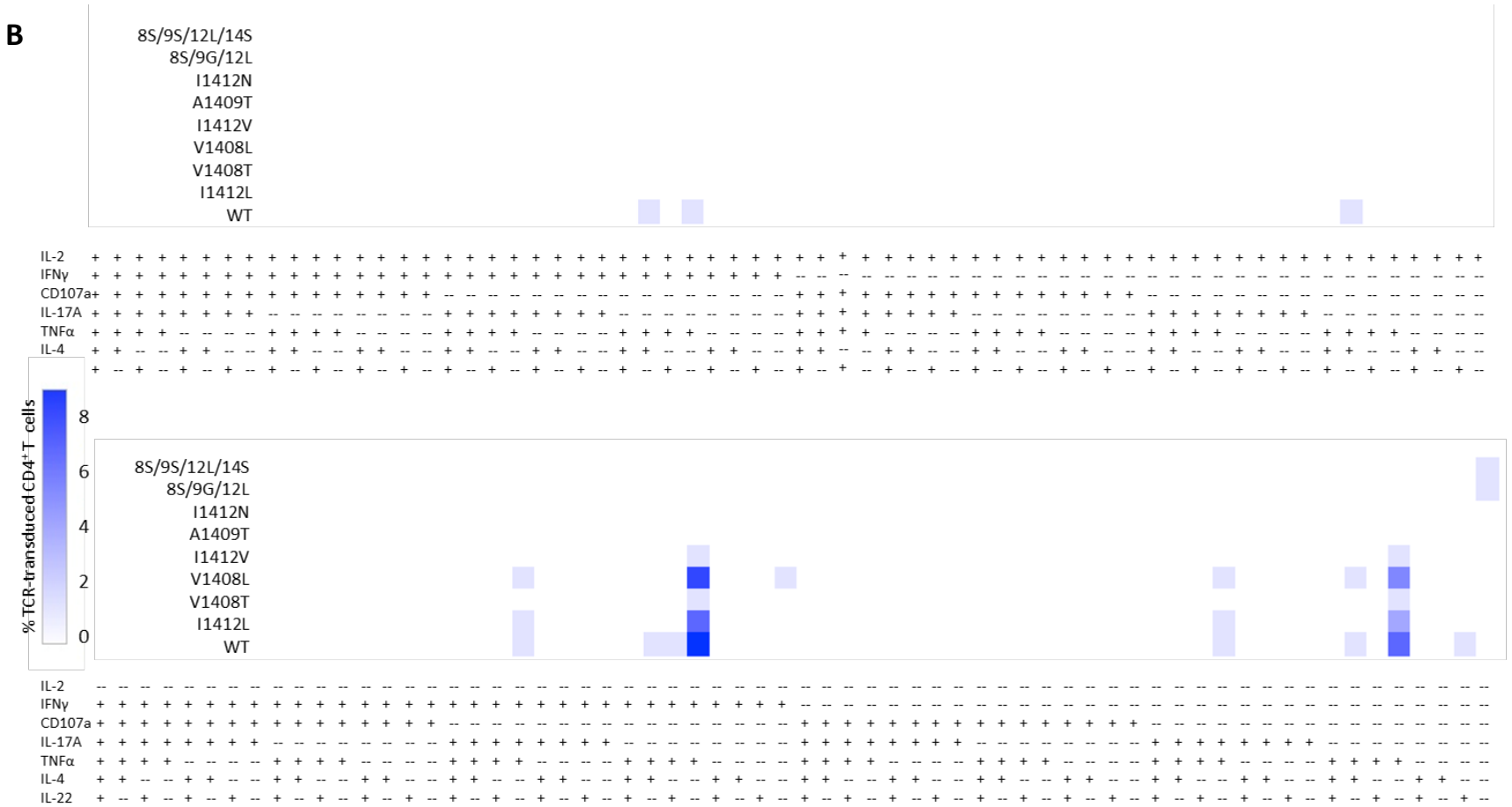


Supplementary Figure S2. Categorized polyfunctional phenotypes of HCV-stimulated TCR-transduced T cells. Includes negative control (tyrosinase) background reactivity for **(a)** $CD8^+$ and **(b)** $CD4^+$ T cells, corresponding to Figs. 2a-b.

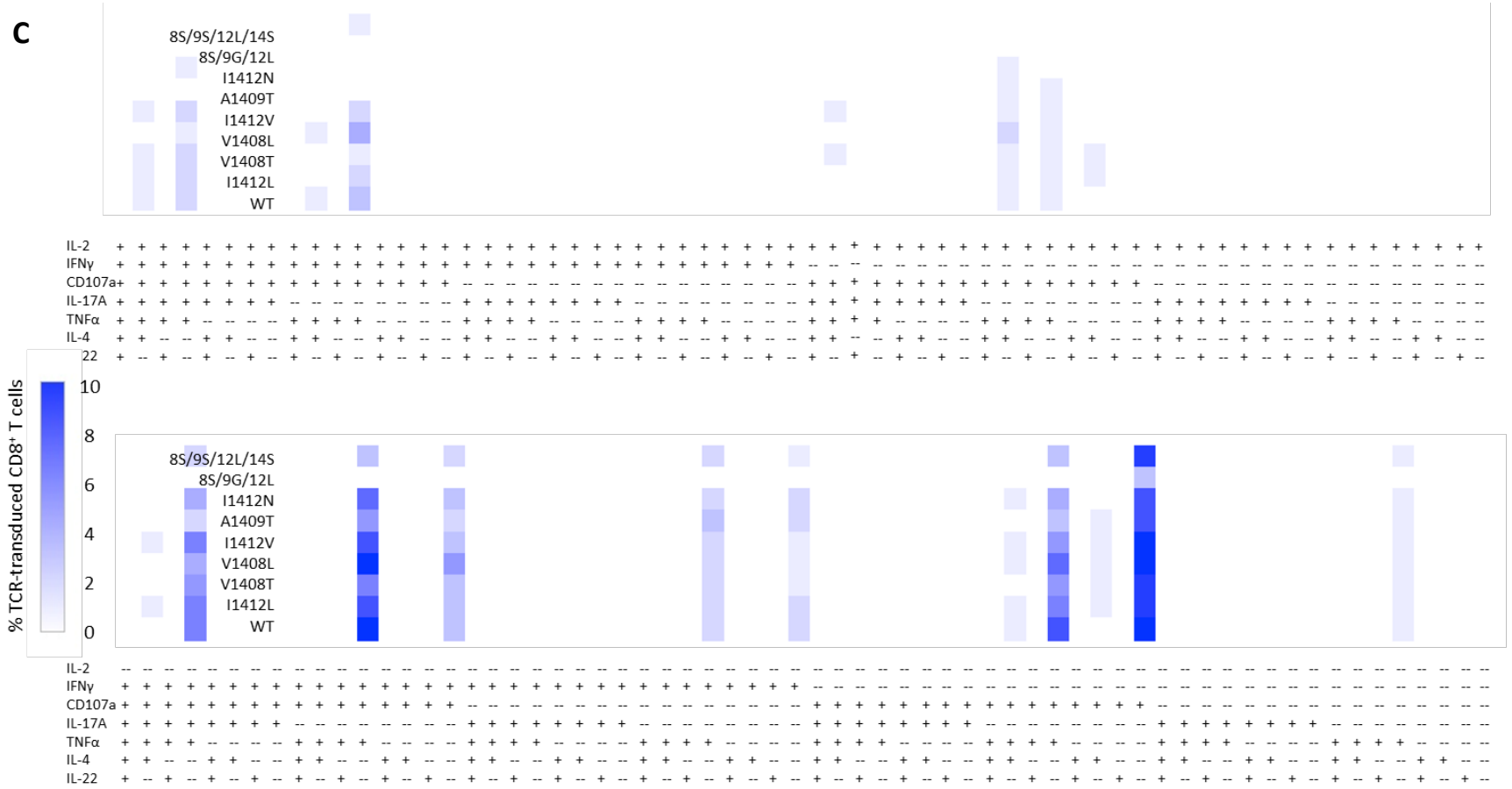


Supplementary Figure S3. SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. HCV 1406 TCR-transduced T cells were co-cultured for 5 hours with T2 cells loaded with 10 µg/mL of each WT and mutant HCV NS3:1406-1415 peptide. Cells were stained for CD3, CD4, CD8, CD34, and CD107a surface expression as well as intracellular IFN-γ, TNFα, IL-2, IL-4, IL-17A, and IL-22. Boolean gating for each functional marker was performed

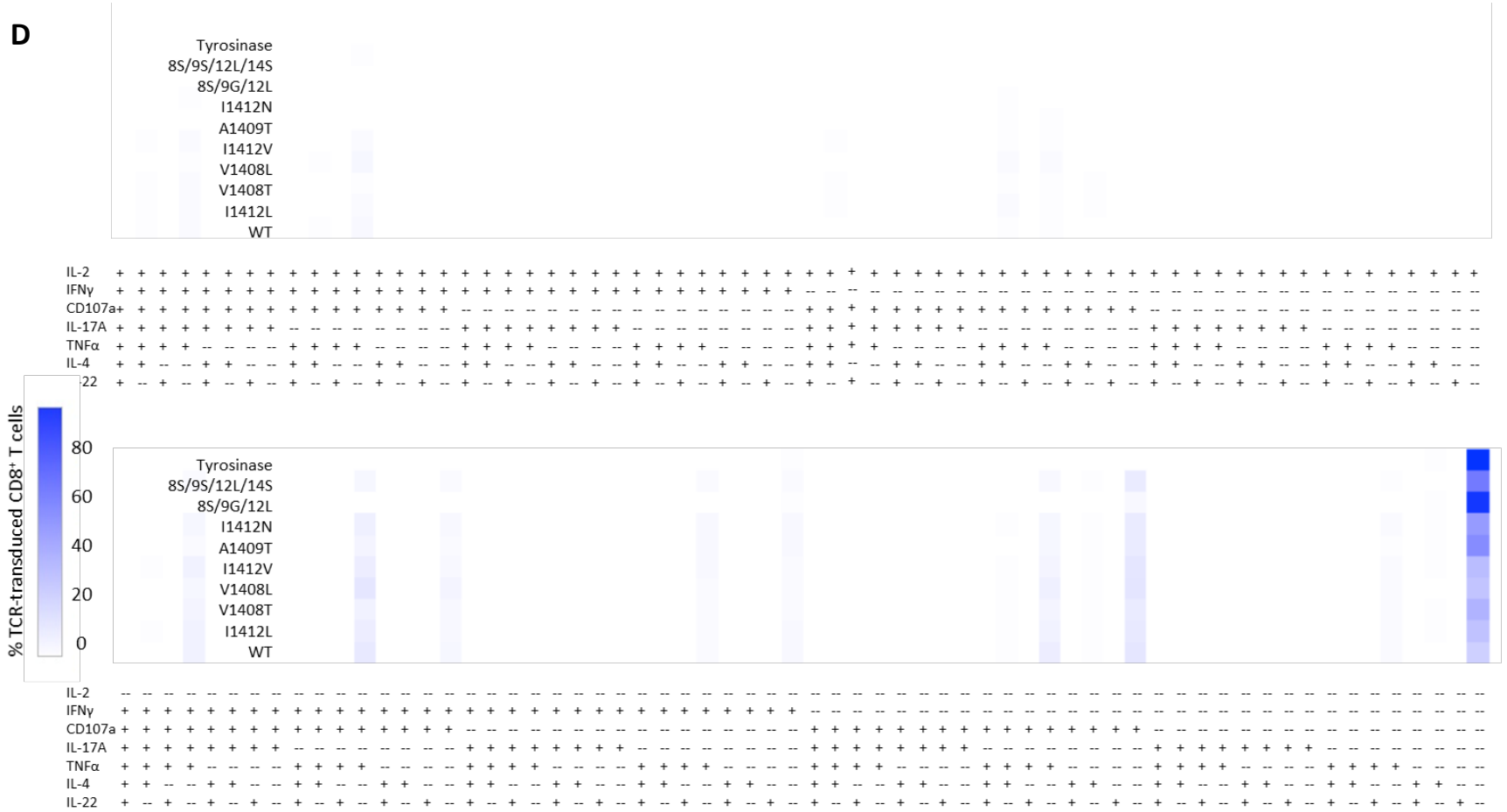
in FlowJo. Resulting multivariate datasets were formatted and background subtracted (tryosinase stimulation) in Pestle, and cool plot overlay was generated in SPICE. Evaluation along the x-axis (red box) determines frequency (shade of blue) of TCR-transduced cells for each of the 128 phenotypes. Each column is a separate phenotype denoted by +/- for each functional parameter. Evaluation along the y-axis (purple box) determines changes in frequency upon variant peptide stimulation for a given phenotype. Unique populations of simultaneously type 1 and type 2 cytokine producing cells are denoted in green boxes. Populations negative for IFNγ are surround by an orange box. TCR-pMHC interactions are ranked from bottom to top by decreasing affinity. Cool plots are representative of **(a)** Peptide-stimulated $CD8^+$ T cells, Donor 1; **(b)** Peptide-stimulated $CD4^+$ T cells, Donor 1; **(c)** Peptide-stimulated $CD8^+$ T cells, Donor 2; **(d)** Peptide-stimulated $CD8^+$ T cells (non-background subtracted), Donor 2; **(e)** Peptide-stimulated $CD4^+$ T cells, Donor 2; **(f)** Peptide-stimulated $CD4^+$ T cells, Donor 2 (non-background subtracted); **(g)** Peptide-stimulated $CD8^+$ T cells, Donor 3; **(h)** Peptide-stimulated $CD4^+$ T cells, Donor 3; **(i)** Tumor-stimulated $CD8^+$ T cells, Donor 2; **(j)** Tumor-stimulated $CD8^+$ T cells, Donor 2 (non-background subtracted); **(k)** Tumor-stimulated $CD4^+$ T cells, Donor 2; **(l)** Tumor-stimulated $CD4^+$ T cells, Donor 2 (non-background subtracted); **(m)** Tumor-stimulated $CD8^+$ T cells, Donor 3; **(n)** Tumor-stimulated $CD4^+$ T cells, Donor 3. Condensed plots in the text (Figure 3a-d) represent Supplementary Figure S1c,e,i,k, respectively.



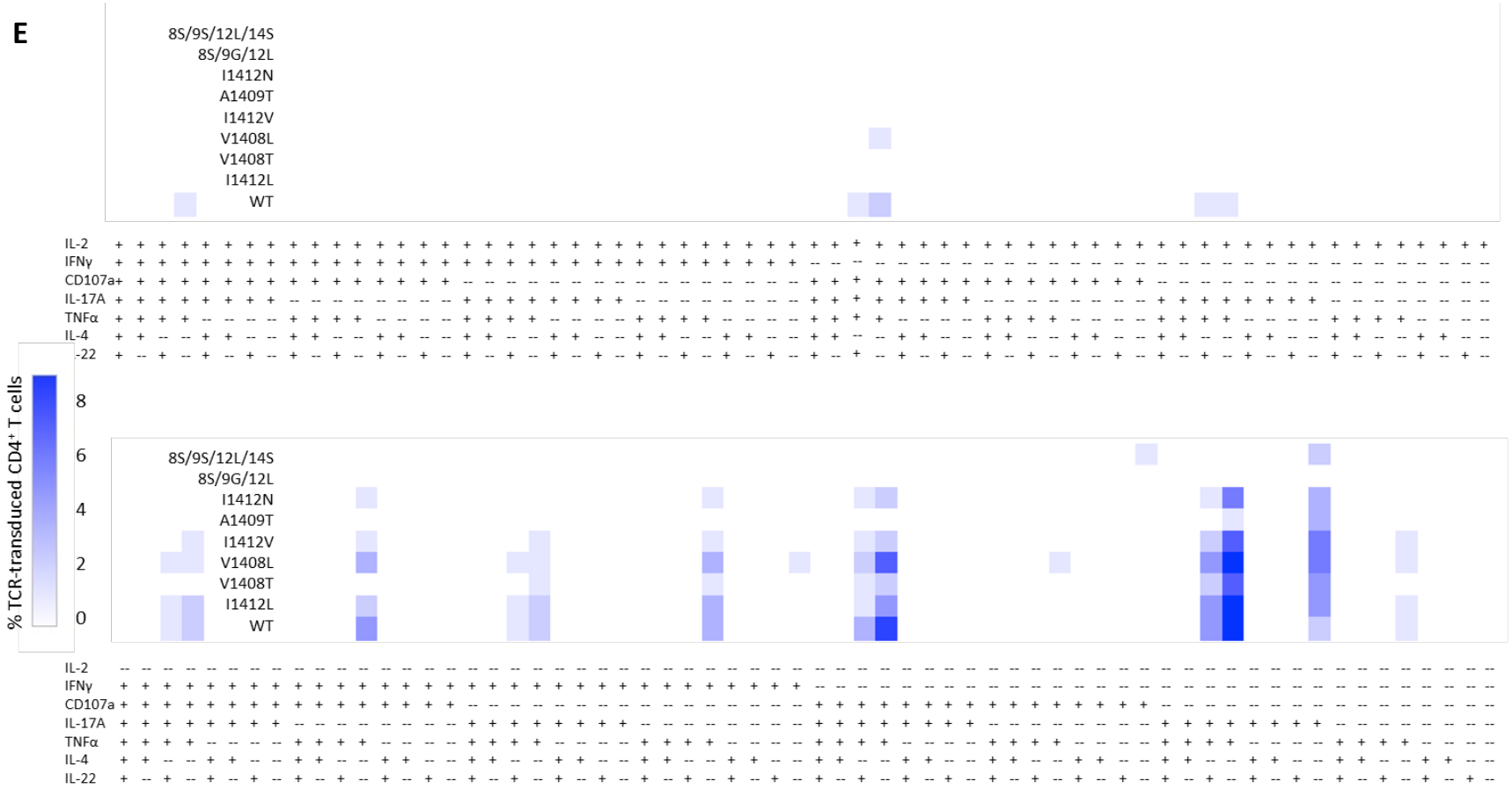
Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (b) Peptide-stimulated $CD4^+$ T cells, Donor 1



Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (c) Peptide-stimulated $CD8^+$ T cells, Donor 2. Full plot corresponds to Figure 3a.

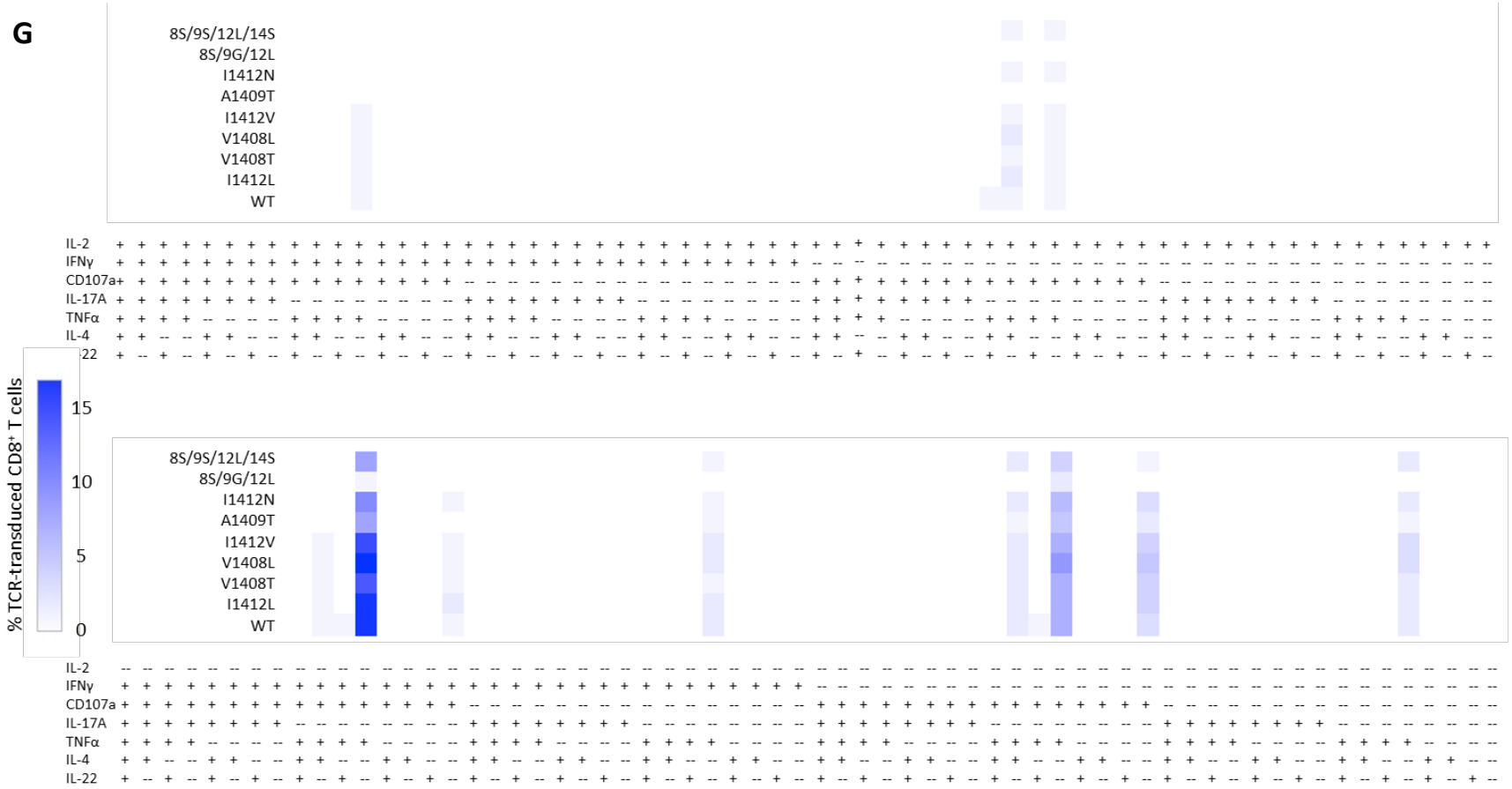


Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (d) Peptide-stimulated $CD8^+$ T cells, Donor 2. Displays negative control (tyrosinase)-stimulated T cells without background subtraction. Full non-background subtracted plot shown in Figure 3a.

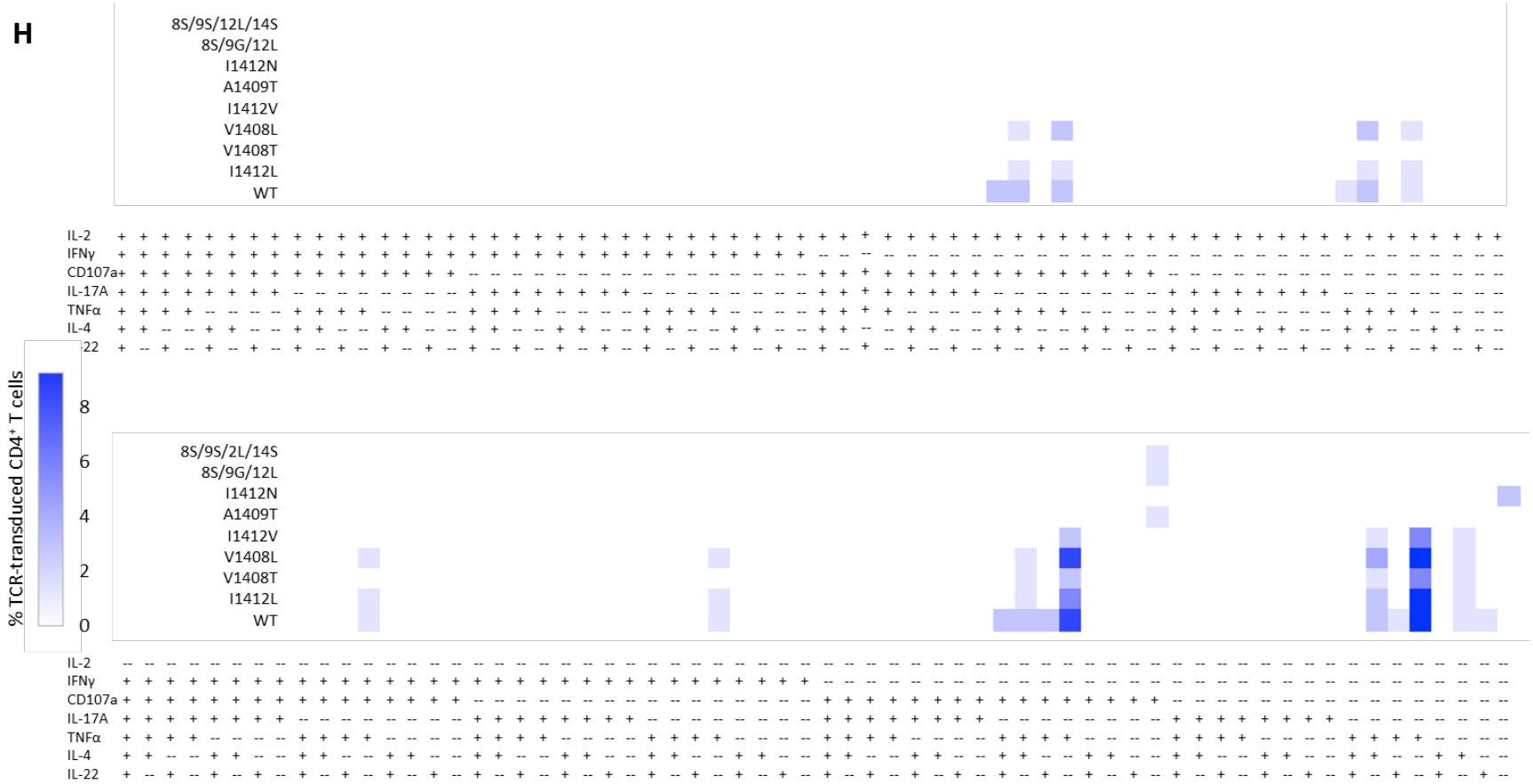


Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (e) Peptide-stimulated $CD4^+$ T cells, Donor 2. Full plot corresponds to Figure 3b.

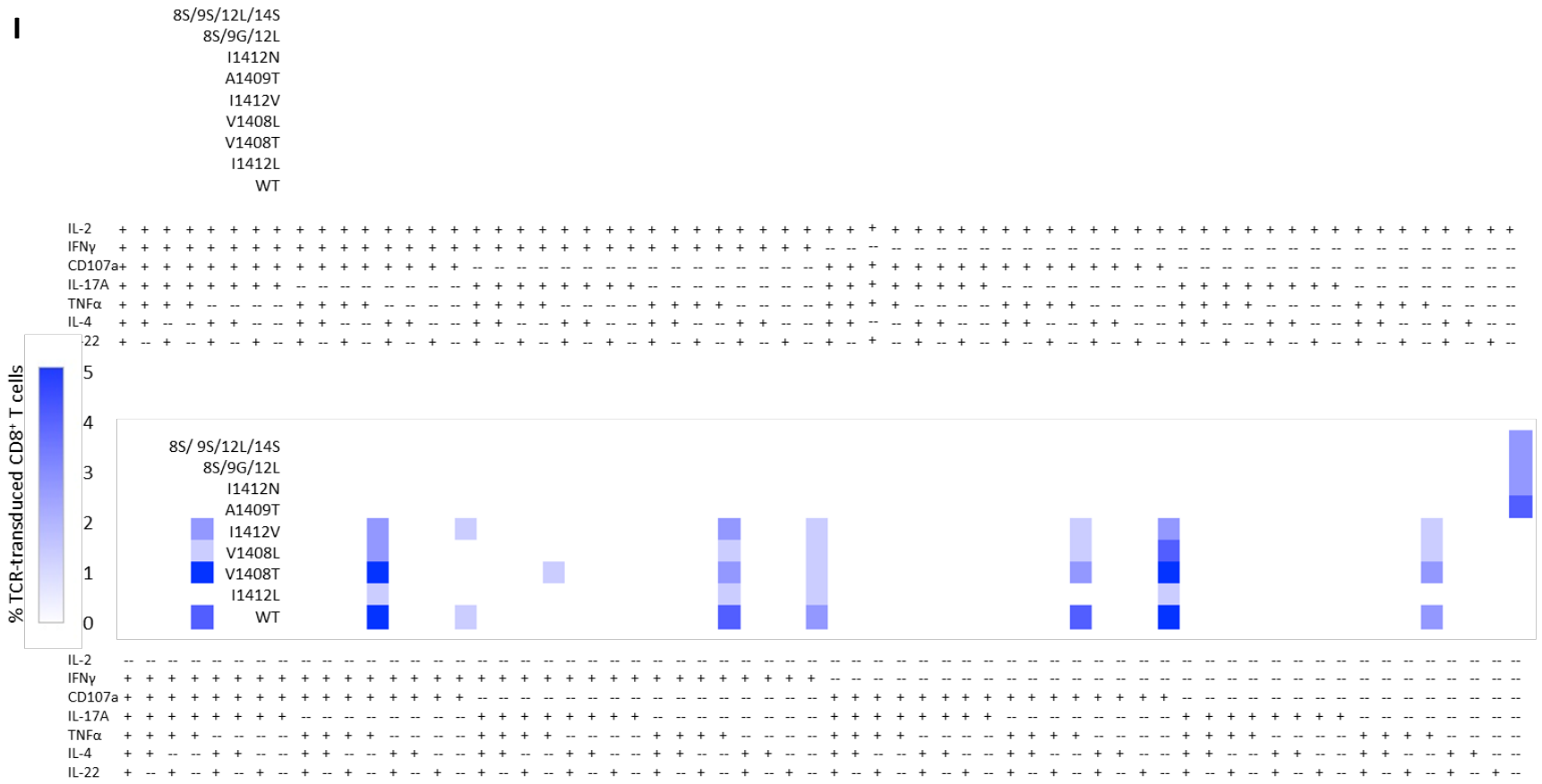
Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (f) Peptide-stimulated $CD4^+$ T cells, Donor 2. Displays negative control (tyrosinase)-stimulated T cells without background subtraction. Full non-background subtracted plot shown in Figure 3b.



Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (g) Peptide-stimulated $CD8^+$ T cells, Donor 3



Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (h) Peptide-stimulated CD4^{+} T cells, Donor 3

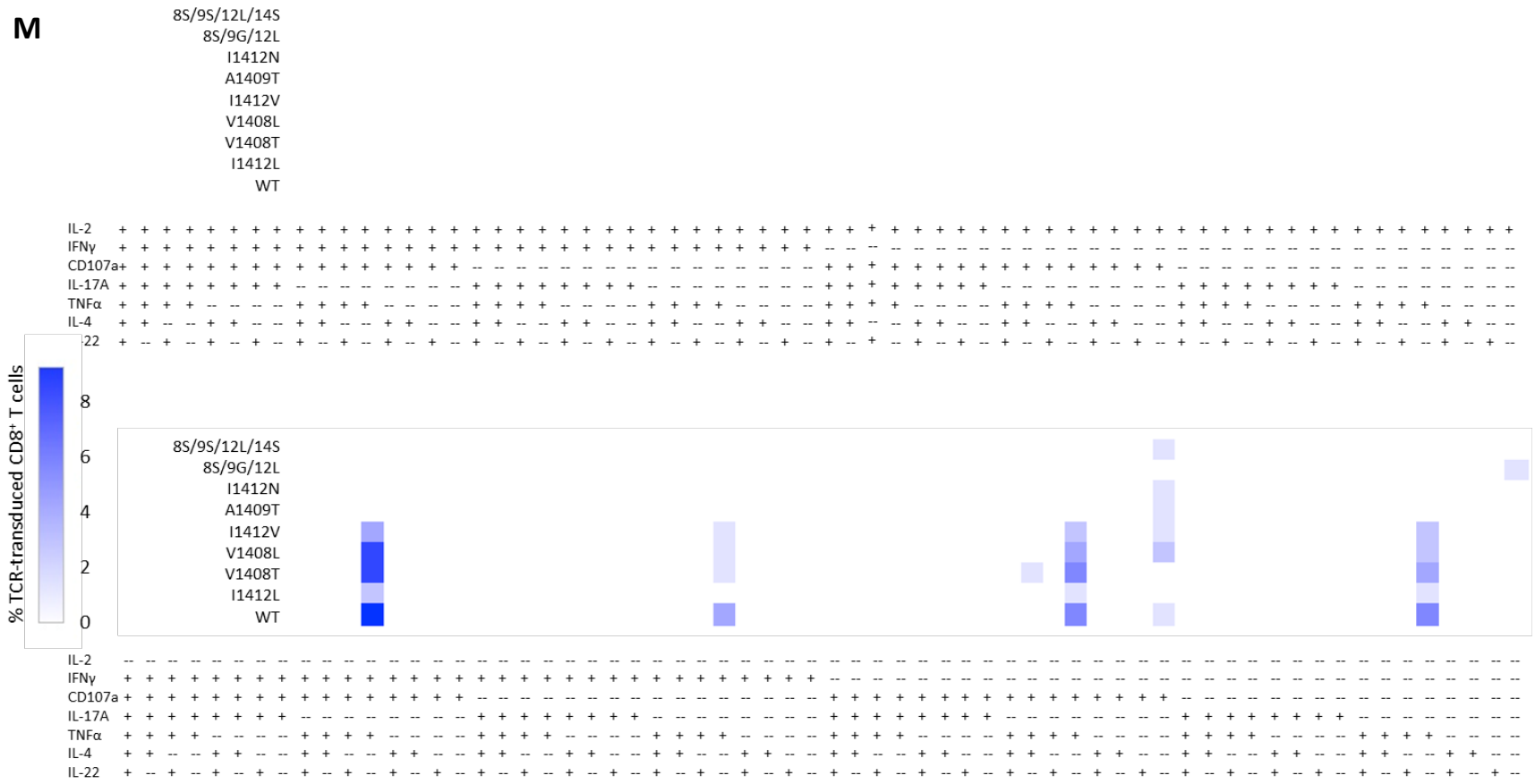


Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (i) Tumor-stimulated $CD8^+$ T cells, Donor 2. Full plot corresponds to Figure 3c.

Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (j) Tumor-stimulated CD8$^+$ T cells, Donor 2. Displays negative control (HepG2)-stimulated T cells without background subtraction. Full non-background subtracted plot shown in Figure 3c.

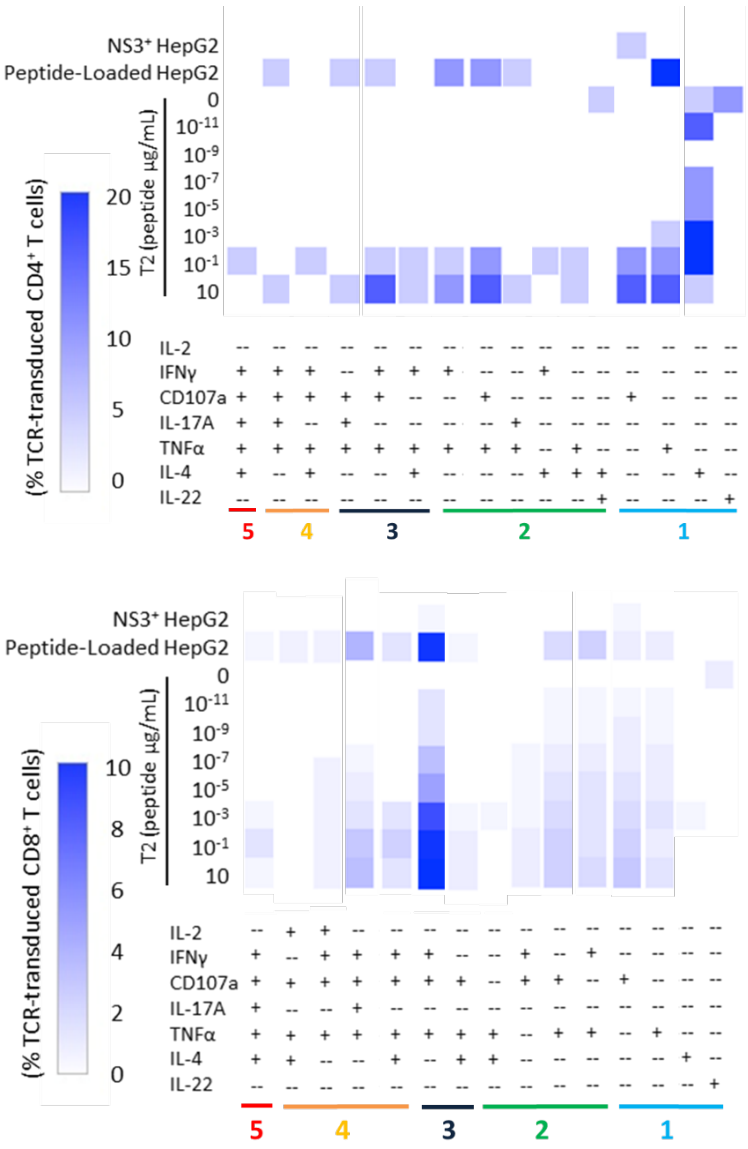
Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (k) Tumor-stimulated $CD4^+$ T cells, Donor 2. Full plot corresponds to Figure 3d.

Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (l) Tumor-stimulated $CD4^+$ T cells, Donor 2. Displays negative control (HepG2)-stimulated T cells without background subtraction. Full non-background subtracted plot shown in Figure 3d.



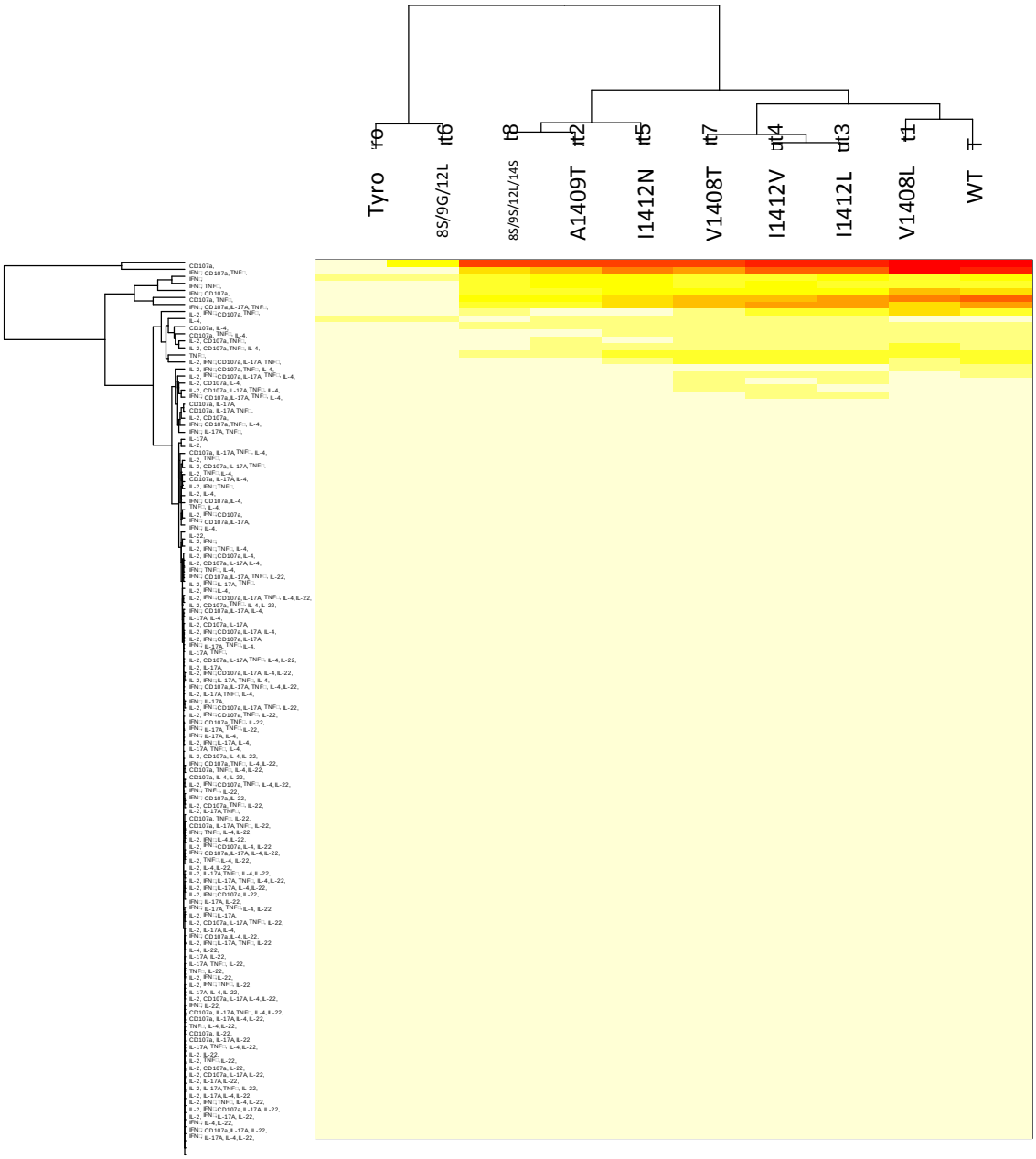
Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (m) Tumor-stimulated $CD8^+$ T cells, Donor 3

Supplemental Figure S3 (cont'd). SPICE-generated cool plots comparing changing frequencies of T cell polyfunctional phenotypes against APL peptide and tumor stimulations. (n) Tumor-stimulated CD4$^+$ T cells, Donor 3



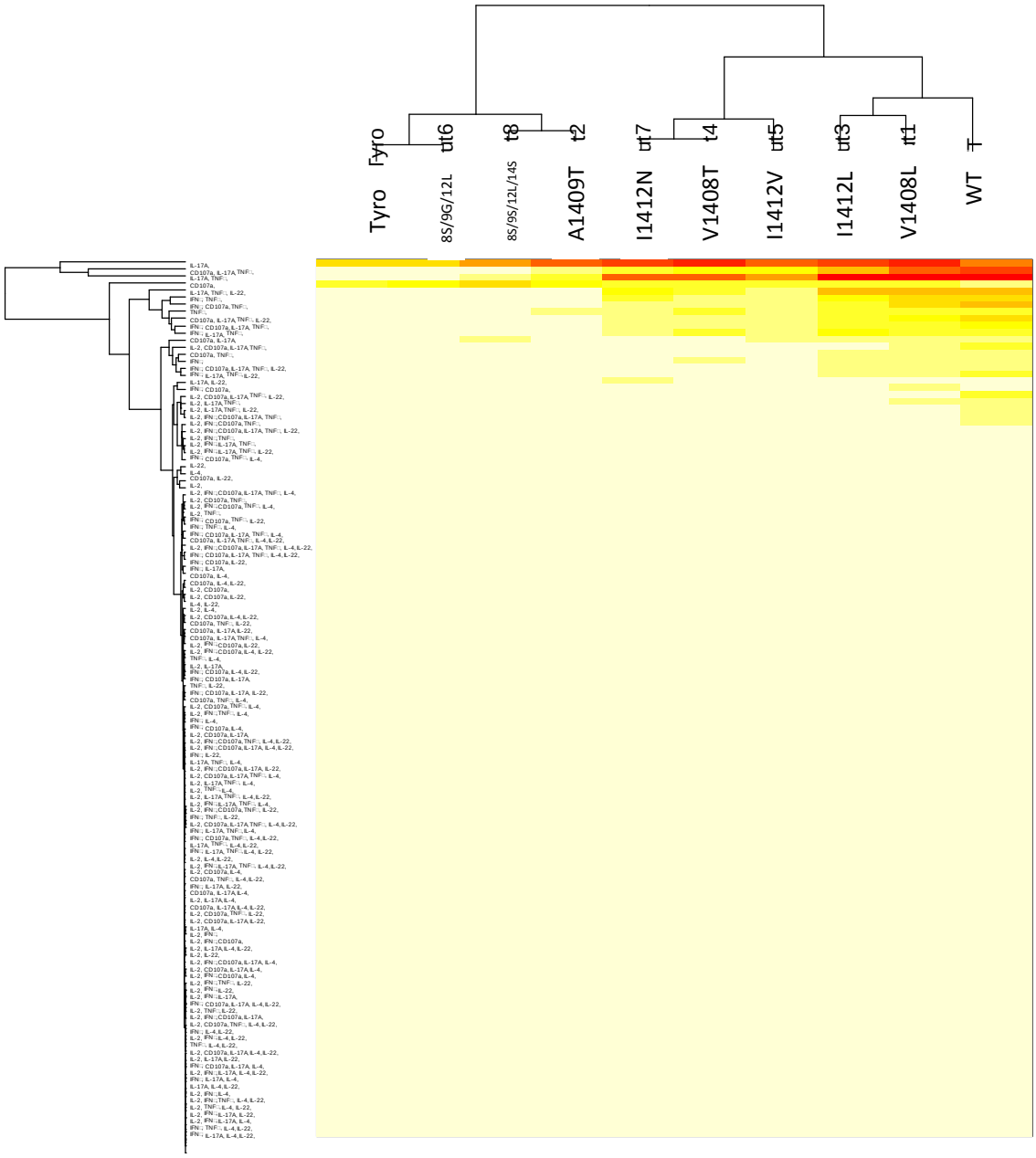
Supplementary Figure S4. Effects of peptide density on polyfunctional phenotypes. (a) $CD4^+$ and **(b)** $CD8^+$ HCV1406 TCR-transduced T cells were co-cultured with T2 cells loaded with WT HCV NS3:1406-1415 peptide ranging from 10 – 10^{-11} µg/mL. T cells were also co-cultured with HepG2 cells expressing naturally processed full length NS3 protein or HepG2 cells exogenously loaded with 10 µg/mL NS3:1406-1415 peptide. Cells were evaluated for cytokine production and CD107a expression by immunofluorescence. As peptide concentration decreased, higher-order phenotypes (3+ functions) generally disappeared earlier. Additionally, loading HepG2 with peptide rescued maximal function, suggesting lower, less polyfunctional reactivity against tumor lines is a direct effect of lower antigen density.

A



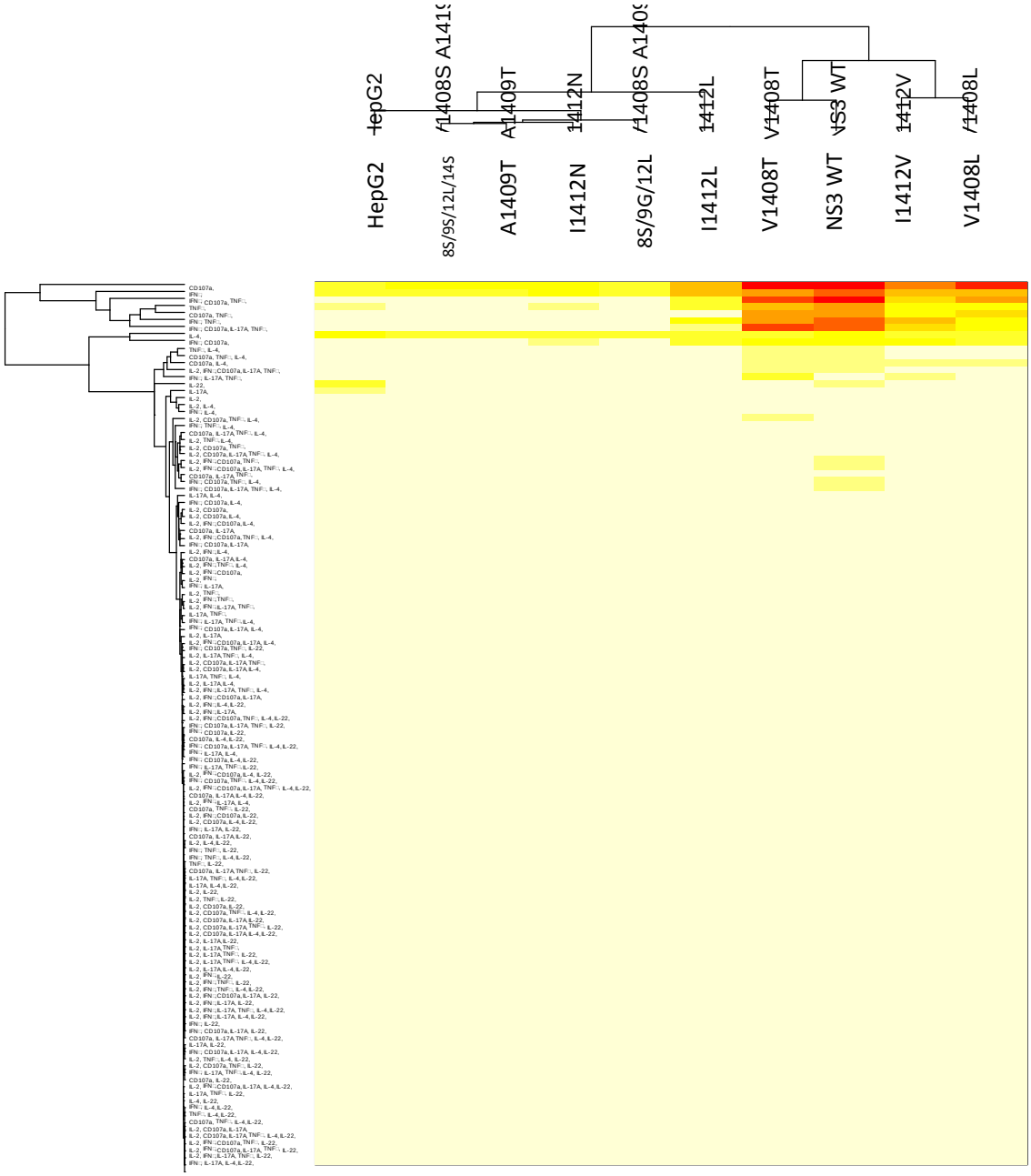
Supplementary Figure S5. Full hierarchical clustering maps. A hierarchical clustering analysis using FlowJo-generated Boolean gated frequencies demonstrates the functional relatedness between HCV NS3:1406-1415 APL stimulations. Full maps of **(a)** peptide-stimulated $CD8^+$ T cells, **(b)** peptide-stimulated $CD4^+$ T cells, and **(c)** tumor-stimulated $CD8^+$ T cells to Figure 4a-c, respectively.

B



Supplementary Figure S5 (cont'd). Full hierarchical clustering maps. (b) peptide-stimulated $CD4^+$ T cells. Full map corresponds to Figure 4b.

C



Supplementary Figure S5 (cont'd). Full hierarchical clustering maps. (c) tumor-stimulated $CD8^+$ T cells. Full map corresponds to Figure 4c.