# Supplementary Figure 1-8

## Multifunctional T Cell Analyses to Study Response and Progression in Adoptive Cell Transfer Immunotherapy

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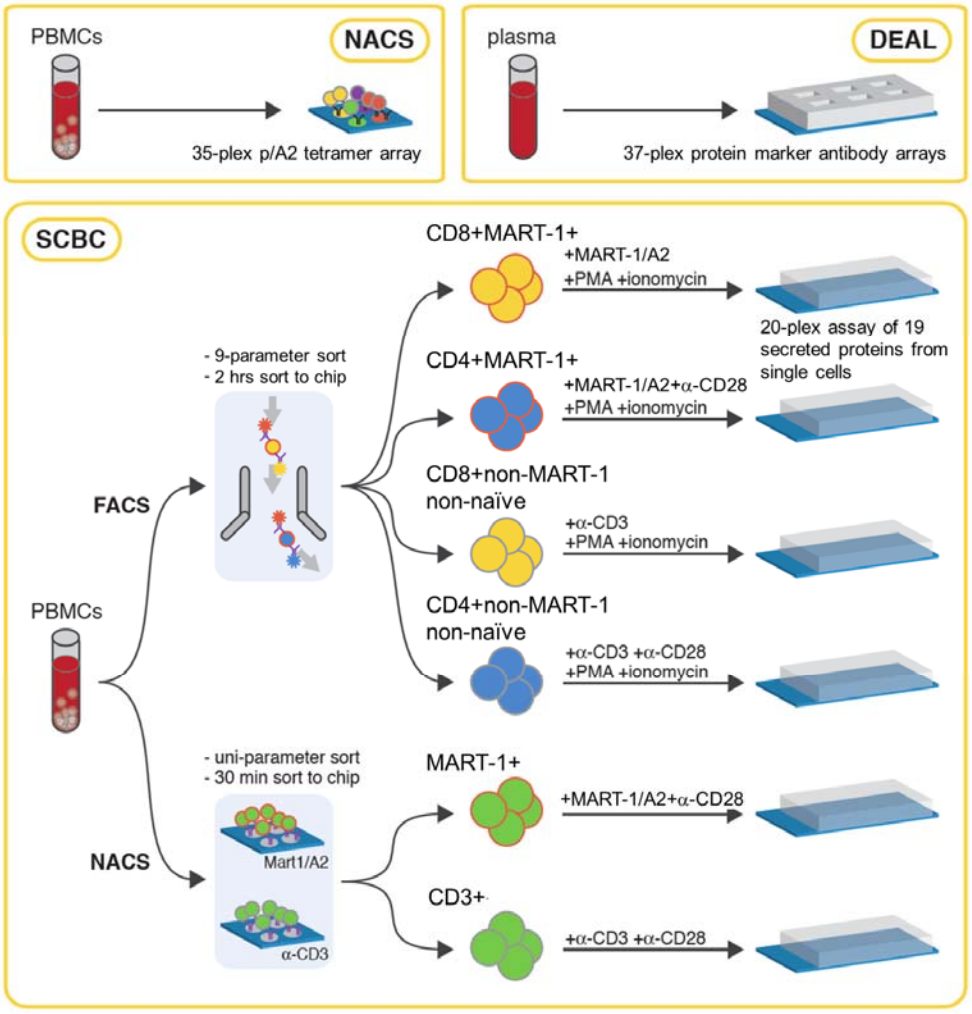
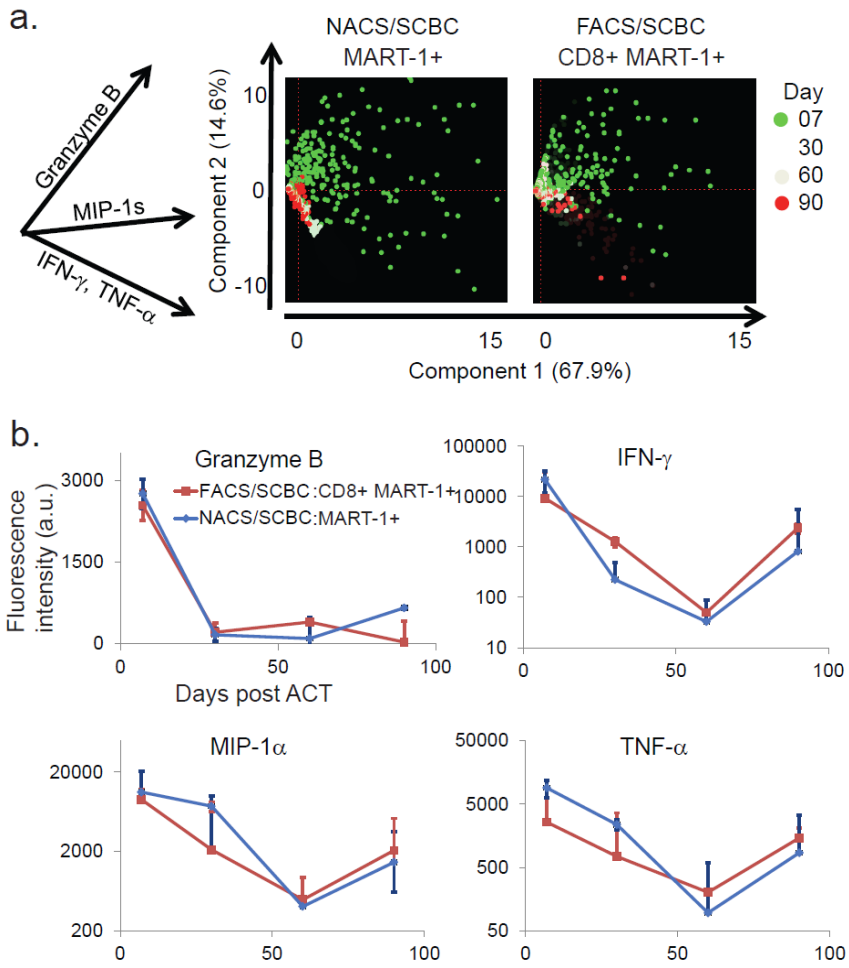
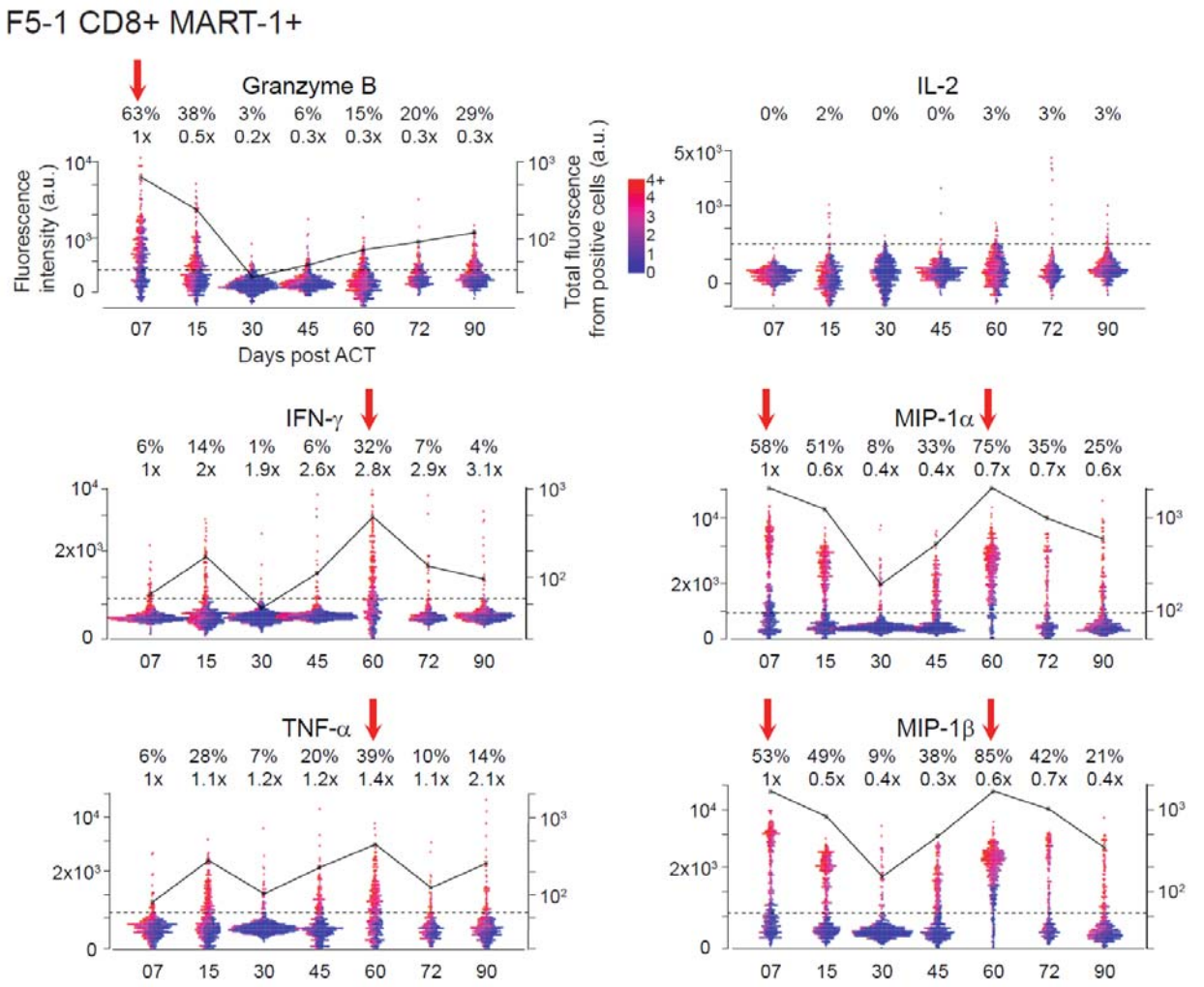


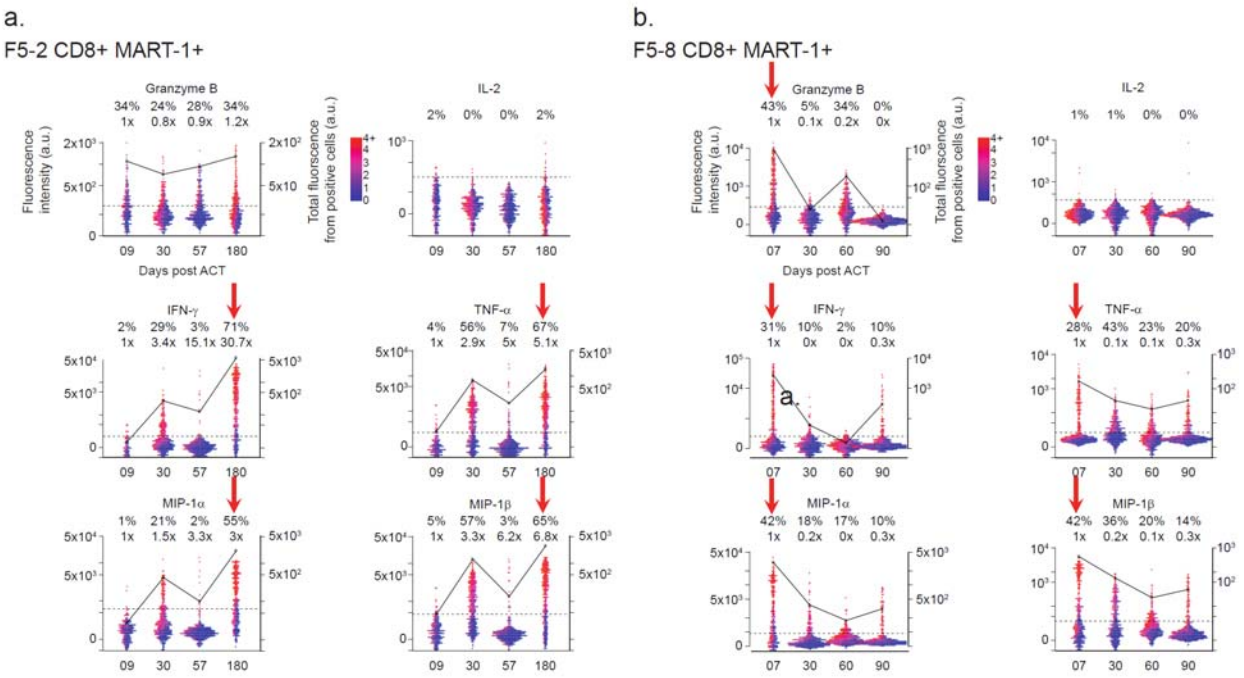

**Supplementary Figure 1. Scheme for the multiplexed immune monitoring study.** a. NACS peptide/MHC tetramer microarray assays are used to enumerate the abundance of 35 melanoma-antigen specific T cell populations in PBMCs. b. DEAL antibody microarrays are utilized to quantitatively measure the levels of 37 blood protein markers from blood plasma. c. SCBC microchips are coupled with multi-parameter FACS (top) or with NACS (bottom) to permit the quantitative analysis of the levels of 19 functional proteins secreted from phenotypically defined single T cells, based on 10 parameter (for FACS/SCBC) or 1 surface specificity (NACS/SCBC). For each sample, ~1400 experiments are conducted simultaneously. The FACS/SCBC permits study of highly refined phenotypically defined T cell populations, whereas NACS/SCBC assays select only for tumor-antigen-specific TCRs on the T cells, and so constitute a less-refined study.



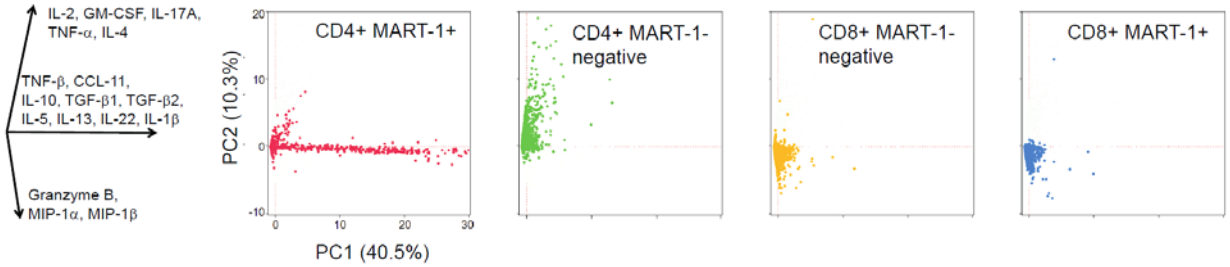

**Supplementary Figure 2. Comparison of results from NACS/SCBC and FACS/SCBC protocols for multiplexed single cell functional proteomic analysis.** a. PCA analysis showing the plot of the first two components of the time-dependent changes of CD8+ MART-1+ T cells (FACS/SCBC) and MART-1+ T cells (NACS/SCBC) from F5-8. b. Mean intensity for each protein from the polyfunctional T cells of CD8+ MART-1+ T cells (FACS/SCBC) and MART-1+ T cells (NACS/SCBC) from F5-8 over time. These plots indicate that the two experimental protocols revealed similar trends.



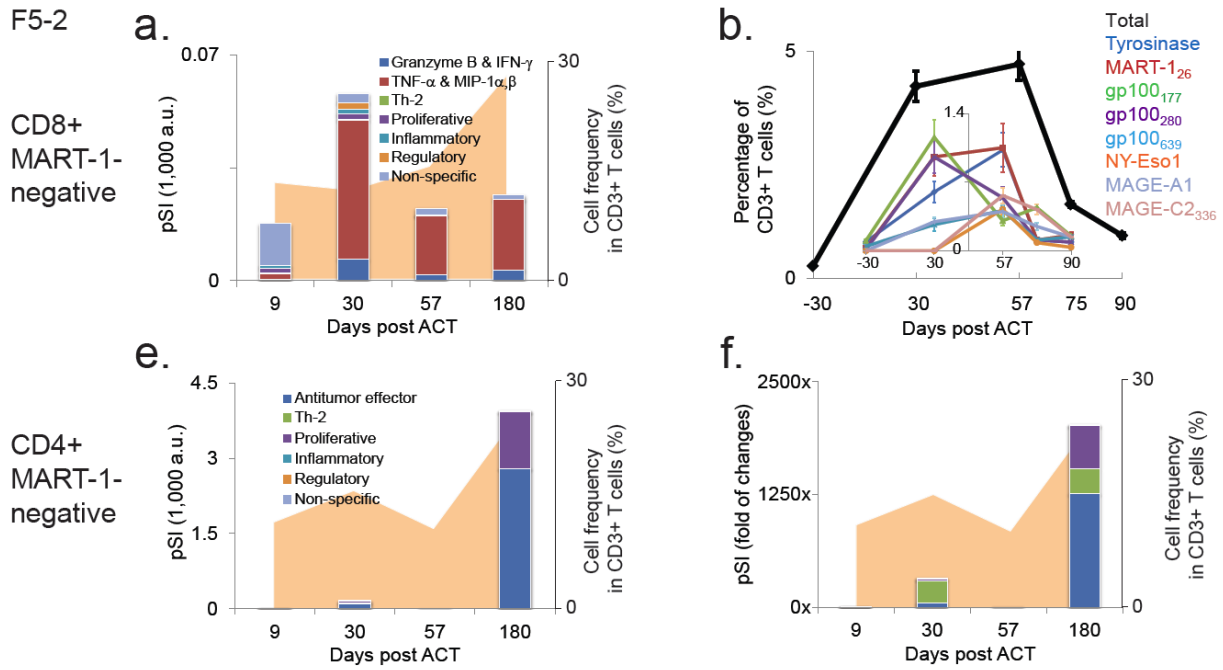

**Supplementary Figure 3. Changes of selected cytokines secreted by CD8+ MART-1+ T cells from F5-1.** One-dimensional scatterplots of six representative cytokines produced by single cells, separated by time points. The dotted line represents the gate that separates cytokine producing and non-producing cells. The percentages given above the plots denote the frequency of positive cells and the relative mean fluorescence intensity (MFI) of those cells relative to day 07. Each point represents a single cell assay. Those points are color encoded (from purple to red) to represent the numbers of different proteins produced by each cell. The black line shows the total functional intensity of the positive cells for the specific cytokine plotted, computed as the frequency of positive cells, times their MFI.



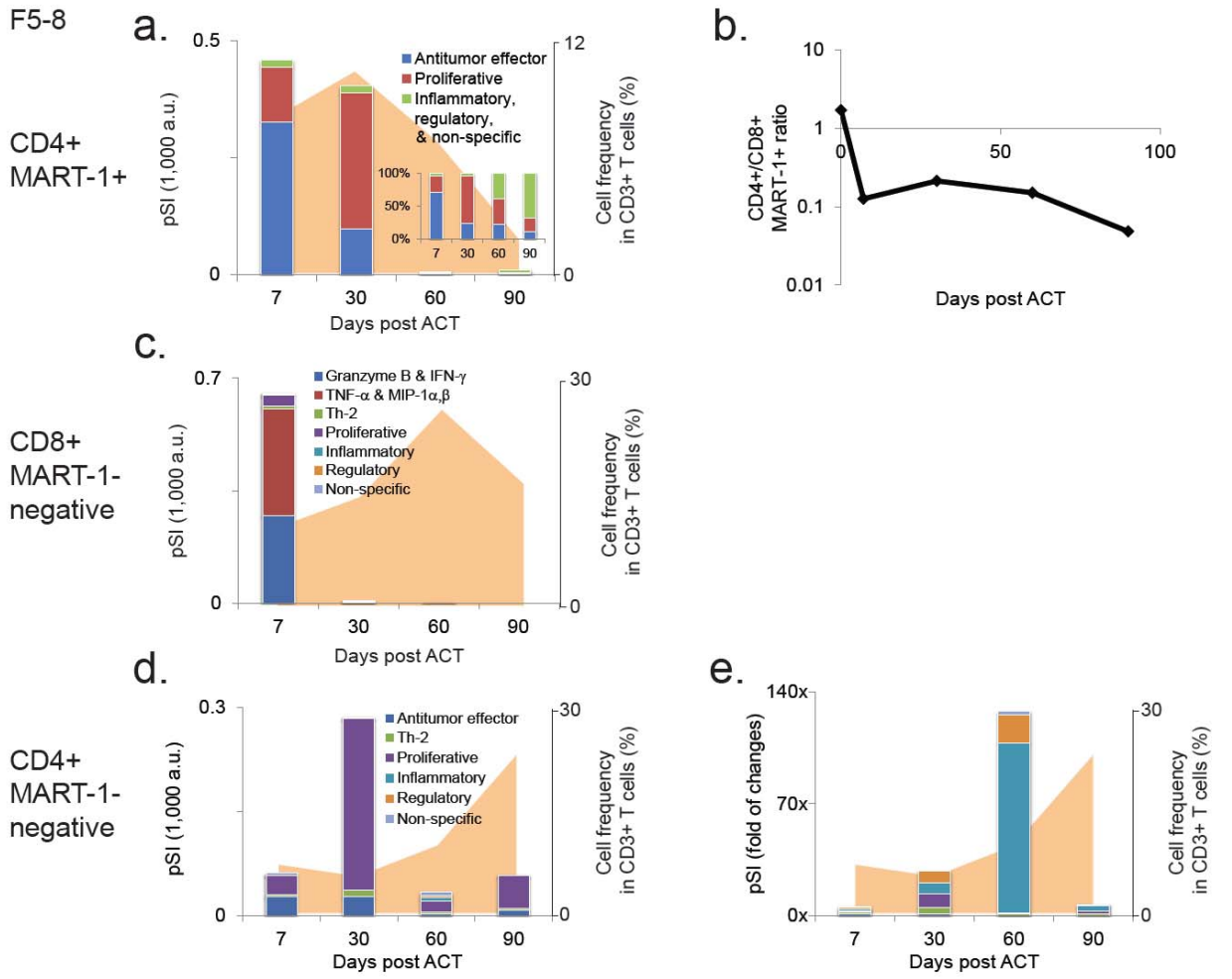

**Supplementary Figure 4. Changes of selected cytokines secreted by CD8+ MART-1+ T cells from Patients F5-2 and F5-8. a-b.** One-dimensional scatterplots of six representative cytokines produced by single cells, separated by time points for F5-2 and F5-8, respectively. The dotted line represents the gate that separates cytokine producing and non-producing cells. The percentages given above the plots denote the frequency of positive cells and the relative mean fluorescence intensity (MFI) of those cells relative to day 07. Each point represents a single cell assay. Those points are color encoded (from purple to red) to represent the numbers of different proteins produced by each cell. The black line shows the total functional intensity of the positive cells for the specific cytokine plotted, computed as the frequency of positive cells, times their MFI.



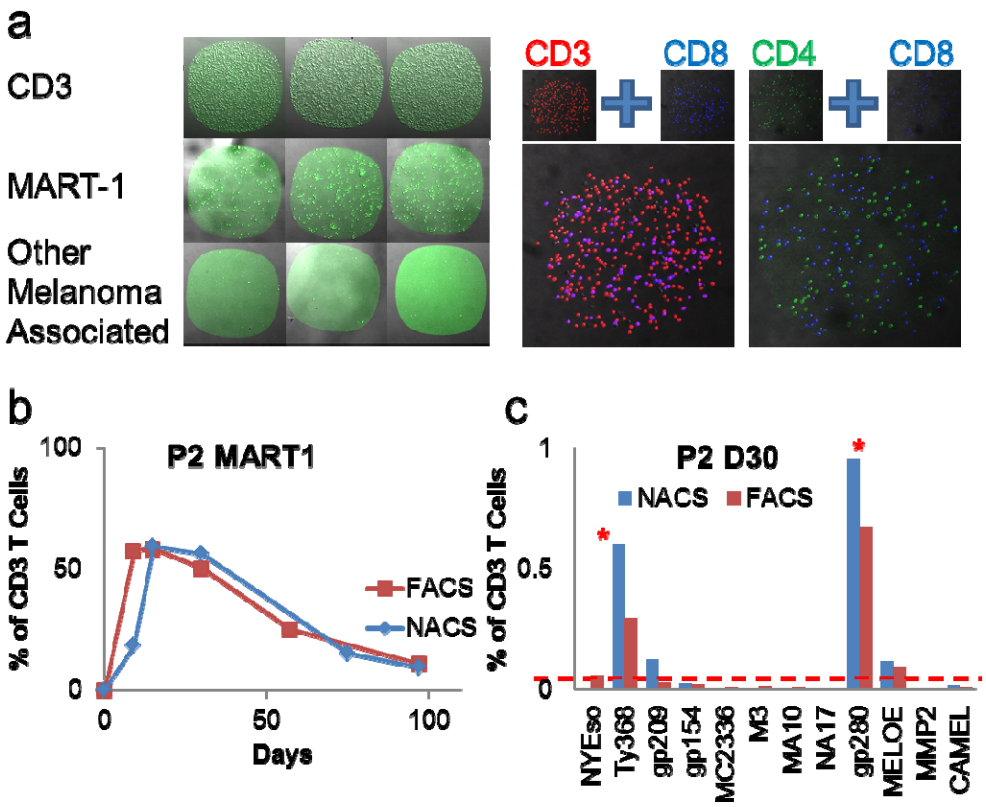

**Supplementary Figure 5. Differences between different cell types studied.** Cytokine secretion florescence intensity data, collected from the single cells functional assays, and collated for all time points and all three patients, was analyzed by principal component analysis. The first two components that jointly explain >50% of the data are plotted as the x- and y-axis. The four scatter plots highlight the individual single cell assays for each assayed T cell types. For each cell type, the points lie primarily along one direction. These different directions represent specific set of functions, which are given in the key at left. The ability of PCA to resolve the various phenotypes argues for the validity of the FACS/SCBC approach.




**Supplementary Figure 6. Functional changes of other T cell phenotypes for patient F5-2 over time.** **a.** Functional changes of the CD8+ MART-1-negative T cells, with cell frequency presented as the orange background. **b.** The frequency of antigen-specific T cells recognizing melanoma antigen other than MART-1 over the course of the therapy. The total frequency is plotted as the black line to provide an overall view of epitope spreading. The frequency of each antigen specificity detected is provided in the insert, denoted by different colors. **c.** Functional changes of the CD4+ MART-1-negative T cells, with cell frequency presented as the orange background. **d.** Relative functional changes of the CD4+ MART-1-negative T cells normalized to those observed at day 07, along with cell frequency.




**Supplementary Figure 7. Functional changes of other T cell phenotypes for patient F5-8 over time.** **a.** Functional changes of the CD4+ MART-1+ T cells, plotted as a bar graph in pSI. The total frequency of this phenotype is plotted as the orange background. Each cytokine function group is represented by a different color. The percent composition of the functions is provided in the insert. **b.** The frequency ratio of CD4+ MART-1+ to CD8+ MART-1+ T cells. **c.** Functional changes of the CD8+ MART-1-negative T cells, with cell frequency presented as the orange background. **d.** Functional changes of the CD4+ MART-1-negative T cells, with cell frequency presented as the orange background. **e.** Relative functional changes of the CD4+ MART-1-negative T cells normalized to those observed at day 07, along with cell frequency.




**Supplementary Figure 8. Validation of melanoma antigen specific T cell population enumeration assays.** a. Images of cells captured by CD3 antibody, MART-1/HLA-A0201 tetramer, and other melanoma-antigen/MHC tetramer on a multiplex microarray (left). Micrographs of immunofluorescence staining of CD3, CD4 and CD8 conducted after the cell capture (right). b. Comparison of the p/MHC tetramer microarray approach (NACS) with flow cytometry p/MHC tetramer staining for determining the abundance, over time, of the MART-1 T cells from patient F5-2. c. A comparison of NACS and FACS assays for the quantitation of the populations of other melanoma associated antigen-specific T cells from patient F5-2 on day 30 post-ACT.