SUPPLEMENTAL METHODS

Flow cytometry immunofluorescence analysis

The following fluorophore-conjugated antibodies were used for staining of surface costimulatory molecules and co-activatory/inhibitory receptors for 20 min at 4°C; CD27 (APC-Alexa780; eBioscience), CD70 (FITC; BD Biosciences), HVEM (APC; Biolegend), BTLA (PE; BD Biosciences), CD28 (FITC; BD Biosciences), CD8α (PerCP-Cy5.5; Biolegend), CD8β (PE; Beckman Coulter), PD-1 (PerCP-eFl710; eBioscience), PD-L1 (PE-Cy7; BD Bioscience), PD-L2 (APC; Biolegend), Granzyme B (FITC; Biolegend) and Perforin (APC, Biolegend). Appropriate isotype controls were used to define negative populations.

Calcium flux assays upon NY-ESO-1 peptide loaded T2 cells

$15x10^4$ T2 cells pulsed with increasing concentrations of $NY\text{-}ESO_{157\text{-}165}$ peptide were used as APCs and brought in contact with $5x10^4$ (E:T ratio 1:3), 2 μM Indo1-AM loaded TCR-transduced primary $CD8^+$ T cells through a 10 seconds 1400 rpm centrifugation before immediate cytometric recording. Intracellular Ca^{2+} flux was recorded under UV excitation and constant temperature of 37ºC using a thermostat device on a LSR II SORP (BD Biosciences) flow cytometer. Indo-1(violet)/Indo-1(blue) 405/525 nm emission ratio was analyzed by FlowJo kinetics module software (TreeStar).

Microarray analysis

Total RNA was isolated using NucleoSpin® RNA II extraction protocols (Macherey-Nagel, Bietlenheim, Germany) and RNA was of high quality and integrity, as verified through Agilent 2100 Bioanalyser platform (Agilent Technologies, Waldbronn, Germany). 1μg of RNA was used for T7-based amplification and Cy3 labeling. Samples were hybridized to Agilent Whole Human Genome Oligo Microarrays 4x44K and scanned using the Agilent microarray scanner system (Agilent). The Agilent Feature Extraction Software was used for readout and processing of image

files. Background correction, filtering of data, and quantile normalization were done using R and the *Agi4x44PreProcess* software package as described in the package manual. The Limma software package was used to identify differentially expressed genes and to calculate the $\log_2$ fold change. Gene probes were considered significant if their P value corrected for a FDR of 0.05 was $P < 0.05$. Fold change expression between 0 hr and 6 hr detected in T cells variants generating optimal function (G50A, A97L, DMβ and TMβ) were converted into UniProt IDs using BioMart and then classified into broad gene ontology (GO) terms using the GOTermFinder (http://go.princeton.edu/cgi-bin/GOTermFinder). The $\log_2$ fold changes of probes assigned to the same GO-term were averaged in absolute value to yield a general measure of expression change independent of its direction.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. *Calcium mobilization assays in primary $CD8^+$ T cells engineered with affinity-optimized TCR variants*

A. A representative kinetic analysis of Ca^{2+} mobilization in $CD8^+$ T cells transduced with affinity-optimized TCR variants after stimulation with T2 cells (APC) loaded with increasing peptide concentrations. Acquisition of Ca^{2+} influx was performed over time in the following order: (i) without APC, (ii) with APC loaded with increasing concentrations of NY-ESO-1 $_{157-165}$ peptide (from 0 to 5 μM) and (iii) with ionomycin as a positive control. **B.** The mean Ca^{2+} influx values for all independent experiments (n > 3) for each engineered $CD8^+$ T cells following stimulation at the indicated peptide concentration (no peptide, 0.001 to 1 μM). Ca^{2+} mobilization obtained after stimulation with NY-ESO-1-expressing Me 290 or Me 275 (HLA-$A2^+$/NY-ESO^+) tumor cell lines in TCR WT-transduced T cells are highlighted as a shaded gray box on the graphs, and indicate that the 0.01 μM peptide-loaded T2 stimulation condition resembles closely to the natural antigen presentation by tumor cell lines. Of note, no Ca^{2+} flux is detected upon stimulation of untransduced CD8+ T cells (no TCR) or upon stimulation of WT NY-ESO-1-transduced T cells with Flu-specific peptide (WT/Flu). Maximal Ca^{2+} flux after ionomycin stimulation indicates equal capacity to mobilize calcium in all T cell variants. Importantly, similar data were obtained independently of stimulation with either peptide-pulsed APCs (as shown here) or directly with A2/peptide multimers (Figure 1A, see main manuscript).

Supplemental Figure 2. *Surface levels of TCR expression and TCR/CD8 down-regulation in engineered SUP-T1 and CD8$^+$ T cells*

A. Percentage of SUP-T1 cells expressing affinity-optimized NY-ESO-1-specific TCRs as detected by NY-ESO-1$_{157-165}$-specific multimer staining (left panel). Surface expression levels (in MFI) of TCR β-chain BV13.1 (middle) and total αβTCR (right) are shown for all SUP-T1 transduced cells. **B.** Surface staining of CD8 and TCR β-chain BV13.1 on CD8$^+$ T cells engineered with TCR variants in the absence (un-stimulated) or following stimulation with 0.1 μg/ml unlabeled HLA-A2/NY-ESO-1$_{157-165}$ specific multimers for 4 hours. Representative dot-plots showing the proportion of reduced CD8 expression (CD8 low) compared to CD8 high expression for all engineered T cells. Of note, increased CD8 down-modulation was observed for T cells expressing optimal TCR affinities (e.g. A97L, DMβ and TMβ), in contrast to supraphysiological T cells (e.g. TMα, wtc51m). **C and D**. Down-regulation of TCR expression on engineered CD8$^+$ T cells was assessed in the absence (-) or presence (+) of 10 μM peptide-loaded T2 (**C**) or 0.1 μg/ml unlabeled A2/ NY-ESO-1$_{157-165}$ specific multimers (**D**) for 4 hours. TCR down-regulation was revealed by reduced multimer fluorescence (**C**) or TCR β-chain BV13.1 staining (**D**). Importantly, similar results were obtained independently following stimulation with either peptide-pulsed T2 cells (Supplemental Fig. 2C) or A2/peptide multimers (Figure 1E, main manuscript).

Supplemental Figure 3. *Global gene expression profiles of CD8$^+$ T cells engineered with affinity-optimized TCR variants*

A genome-wide microarray analysis was performed on primary CD8$^+$ T cells expressing TCR variants following low dose (0.002 μg/ml) of HLA-A2/ NY-ESO-$1_{157\text{-}165}$ specific multimer stimulation as described in the main manuscript. We identified 524 genes enriched between 0 hr and 6 hr in T cells transduced with optimal TCR variants (G50A, A97L, DMβ, TMβ), which could be further classified according to GO terms (Figure 2, main manuscript). Using the same list of enriched genes, we also assessed the average $\log_2$ gene expression variance for each engineered T cell variant at baseline (un-stimulated) and per GO term. Variance is defined as the difference from the mean on a $\log_2$ scale. These results show that in contrast to stimulated CD8$^+$ T cells, no major changes in the GO terms (immune response, T cell activation, cell proliferation, signaling, gene expression and apoptosis) were observed within un-stimulated T cells and among the various TCR-transduced T cells. However, it should be noted that a marginal trend in gene expression over-representation was sometimes found for the T cells bearing highest TCR affinities (e.g. TMα and wtc51m). Moreover, we also noticed small fluctuations, particularly when we compared the T cell variants for the GO term "gene expression" and "cell proliferation", likely reflecting differences in culture conditions occurring during the expansion of these cells before performing the micro-array experiments.

Supplemental Figure 4. *Levels of ZAP-70 and ERK 1/2 protein expression and phosphorylation in engineered T cells with affinity-optimized TCR variants*

A. TCR-transduced SUP-T1 T cells and $CD8^+$ T cells were stimulated with 10 μg/ml HLA-A2/NY-ESO-$1_{157-165}$ multimers at 37^oC for the indicated time-points and assessed for total ZAP-70 and total ERK1/2 levels by Western blotting, in parallel to the data presented in Figure 3A and C (main manuscript). Tubulin (SUP-T1) or actin ($CD8^+$) expression levels were used as loading controls between samples and time-points. TCR-untransduced cells; Ø. n.d., not done. **B.** To allow direct comparison between TCR-engineered SUP-T1 cells, intensity of total ZAP-70 and total ERK1/2 levels were quantified and normalized to α-tubulin. Data from 3 independent experiments are presented as min-to-max bar graphs with average mean lines. TCR variants are presented in order of increased affinity. Baseline represents the un-stimulated T cells. **C.** Example of another representative western blot analysis on stimulated $CD8^+$ T cells. ZAP-70 and ERK1/2 phosphorylation levels as well as total ZAP-70 and ERK1/2 are depicted at the indicated time-points following antigen-specific stimulation.

Supplemental Figure 5. *Levels of SHP-1 phosphorylation and of total SHP-2 protein expression in engineered T cells with affinity-optimized TCR variants*

A. Actin (for $CD8^+$ T cells) or α-tubulin (for SUP-T1 cells) expression levels were used as loading controls between samples and time-points, and were performed in parallel to the data presented in Figure 6B and C (main manuscript). **B.** TCR-transduced $CD8^+$ T cells and SUP-T1 cells were stimulated with 10 μg/ml HLA-A2/NY-ESO-$1_{157-165}$ multimers at 37°C for the indicated time-points and assessed for SHP-1 phosphorylation levels. TCR-untransduced cells; Ø. **C.** Un-stimulated at baseline (0h) and Log_2 fold change (0-6 hr difference) expression levels of *SHP2* transcripts as detected in microarray analysis. **D and E.** TCR-transduced $CD8^+$ T cells (**D**) and SUP-T1 cells (**E**) were stimulated with 10 μg/ml A2/NY-ESO-$1_{157-165}$ multimers for the indicated time-points and assessed for total SHP-2 protein expression levels by Western blotting. We used for SHP-2 protein expression analysis the following antibody: rabbit anti-SHP-2 (C-18) (Santa Cruz Biotechnology, Inc). Actin (for $CD8^+$ T cells) or α-tubulin (for SUP-T1 cells) expression levels were used as loading controls between samples. Baseline represents the un-stimulated T cells. For $CD8^+$ transduced T cell samples, all lines were run on the same gel, but were noncontiguous.

Supplemental Table 1. Relative affinities of the sequence-optimized A2/NY-ESO-1 specific TCR variants

TCR AV23.1-BV13.1[a]	CDR modification	Relative affinity[b]
wtc51m[c]	CDR2β	1427
QMα	CDR2+3β; CDR2α	153
TMα	CDR2β; CDR2α	54
TMβ	CDR2+3β	24
DMβ	CDR2β	11
A97L	CDR3β	8
G50A	CDR2β	5
WT[d]	none	1
V49I	CDR2β	N/A

[a]The NY-ESO-1-specific TCR AV23.1-BV13.1 was optimized by in silico modeling, through amino acid substitutions in CDR2α and/or CDR2β and/or CDR3β loops as previously described (1).

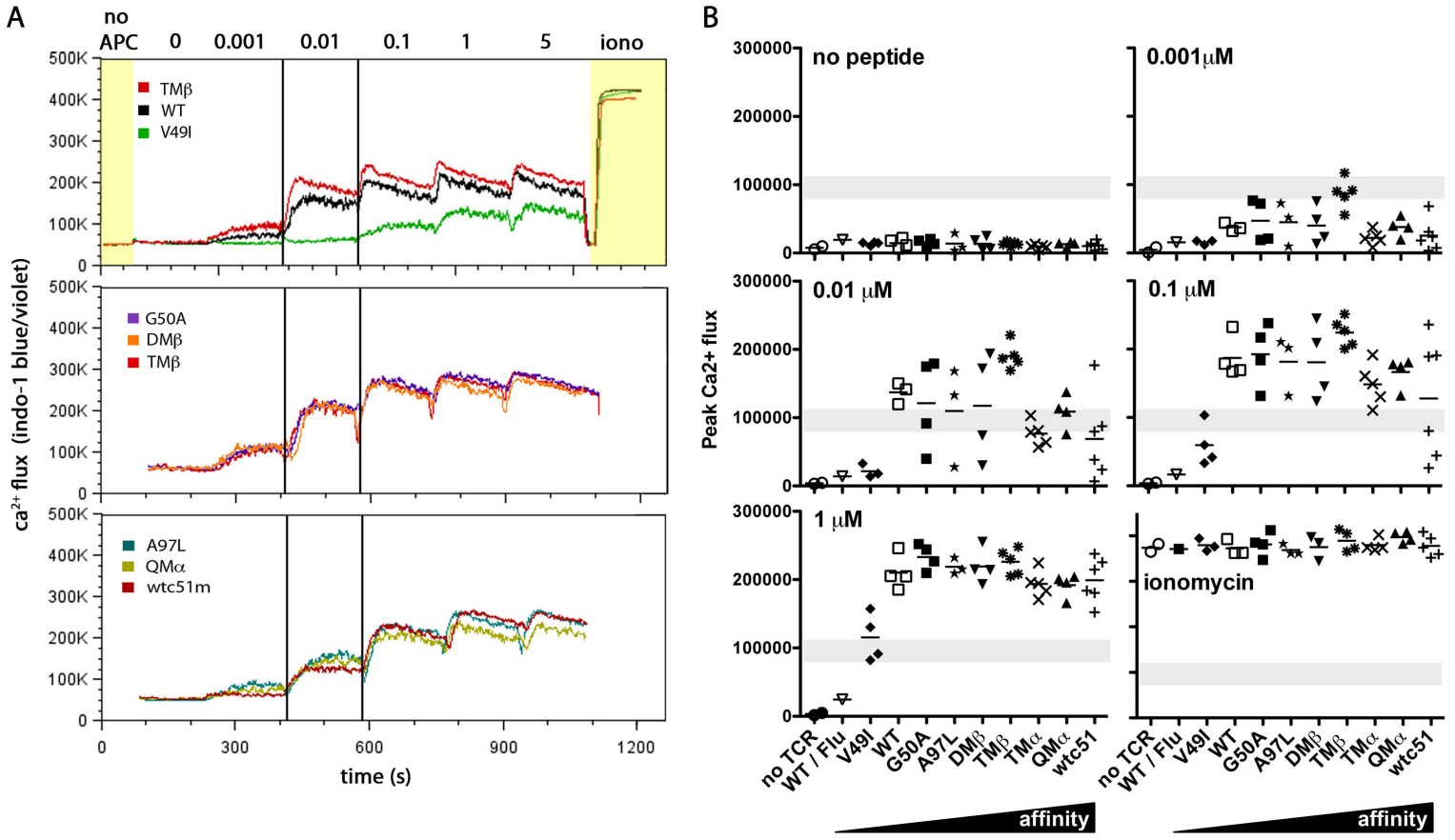
[b]Sequence-optimized TCR affinities are indicated in relative fold-increase from the WT TCR (K_D, 21.4 μM), as characterized recently (2). A sub-physiological, weak-binding TCR (V49I), with an estimated affinity of > 100 μM to pMHC was included. N/A, not applicable.

[c]The modified wtc51 TCR variant, comprises the WT TCR sequences with four amino acid replacements within the CDR2β loop (3), resulting in the drastic increase of its affinity towards pMHC.

[d]Wild-type (WT) TCR isolated from melanoma patient LAU 155 (4).

SUPPLEMENTAL REFERENCES

1. Zoete, V., Irving, M.B., and Michielin, O. 2010. MM-GBSA binding free energy decomposition and T cell receptor engineering. *J Mol Recognit* 23:142-152.
2. Irving, M., Zoete, V., Hebeisen, M., Schmid, D., Baumgartner, P., Guillaume, P., Romero, P., Speiser, D., Luescher, I., Rufer, N., et al. 2012. Interplay between T cell receptor binding kinetics and the level of cognate peptide presented by major histocompatibility complexes governs CD8+ T cell responsiveness. *J Biol Chem* 287:23068-23078.
3. Dunn, S.M., Rizkallah, P.J., Baston, E., Mahon, T., Cameron, B., Moysey, R., Gao, F., Sami, M., Boulter, J., Li, Y., et al. 2006. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity. *Protein Sci* 15:710-721.
4. Derre, L., Bruyninx, M., Baumgaertner, P., Ferber, M., Schmid, D., Leimgruber, A., Zoete, V., Romero, P., Michielin, O., Speiser, D.E., et al. 2008. Distinct sets of alphabeta TCRs confer similar recognition of tumor antigen NY-ESO-1157-165 by interacting with its central Met/Trp residues. *Proc Natl Acad Sci U S A* 105:15010-15015.



Supplemental Figure 1
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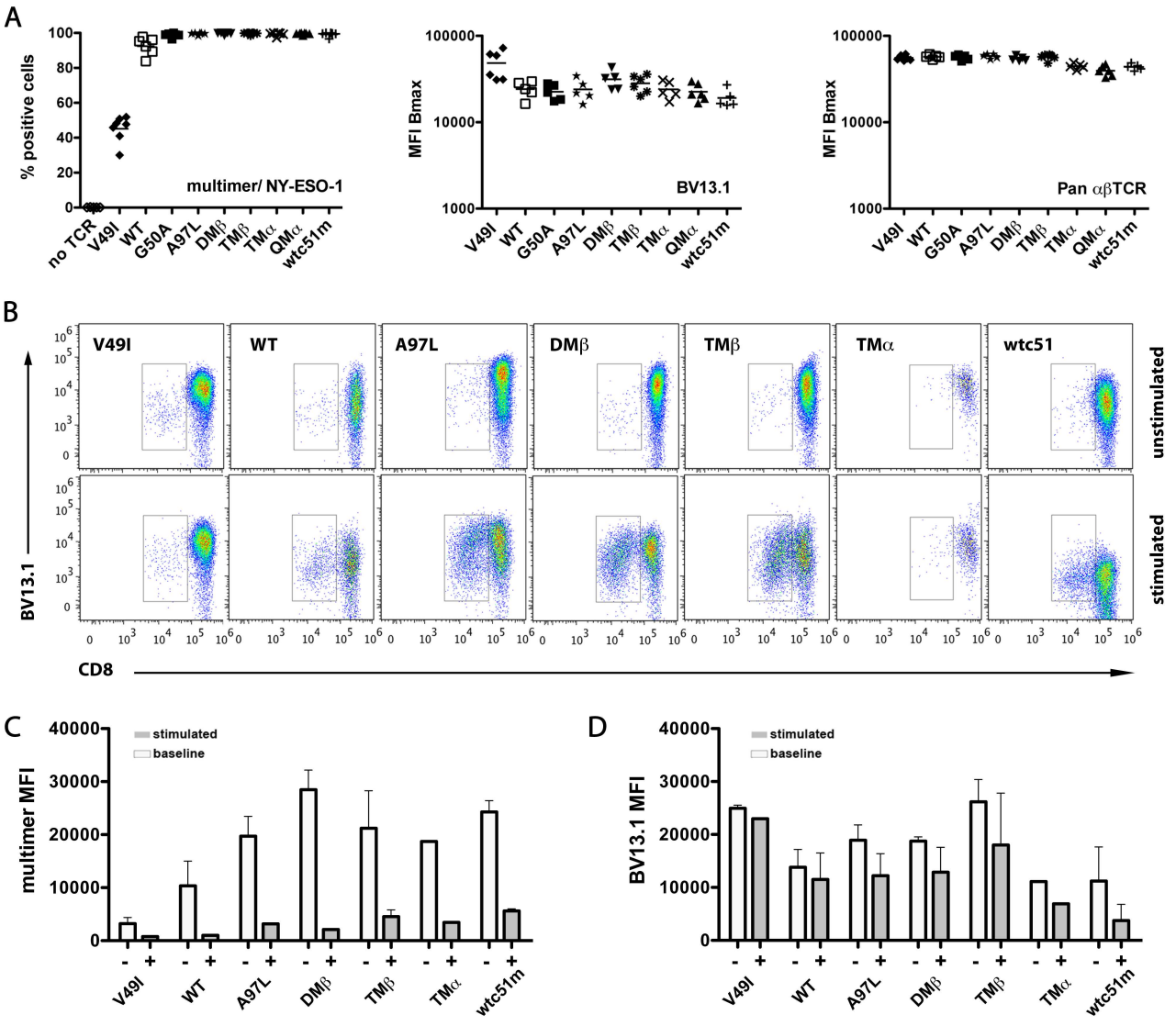

Supplemental Figure 2
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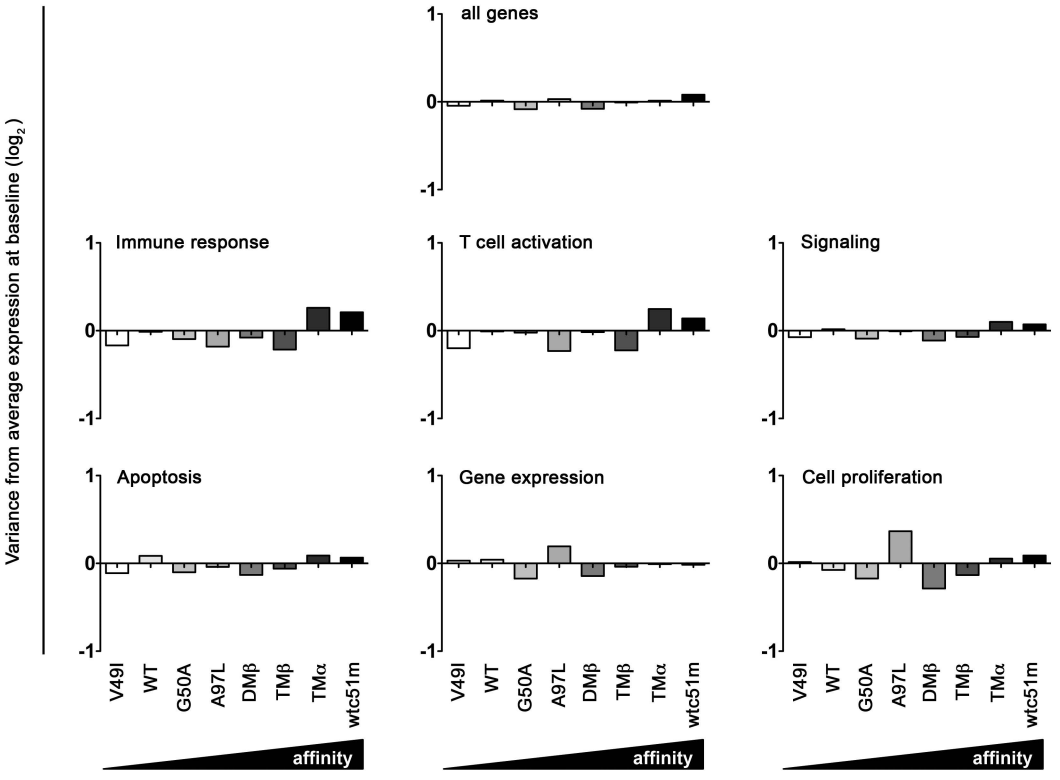

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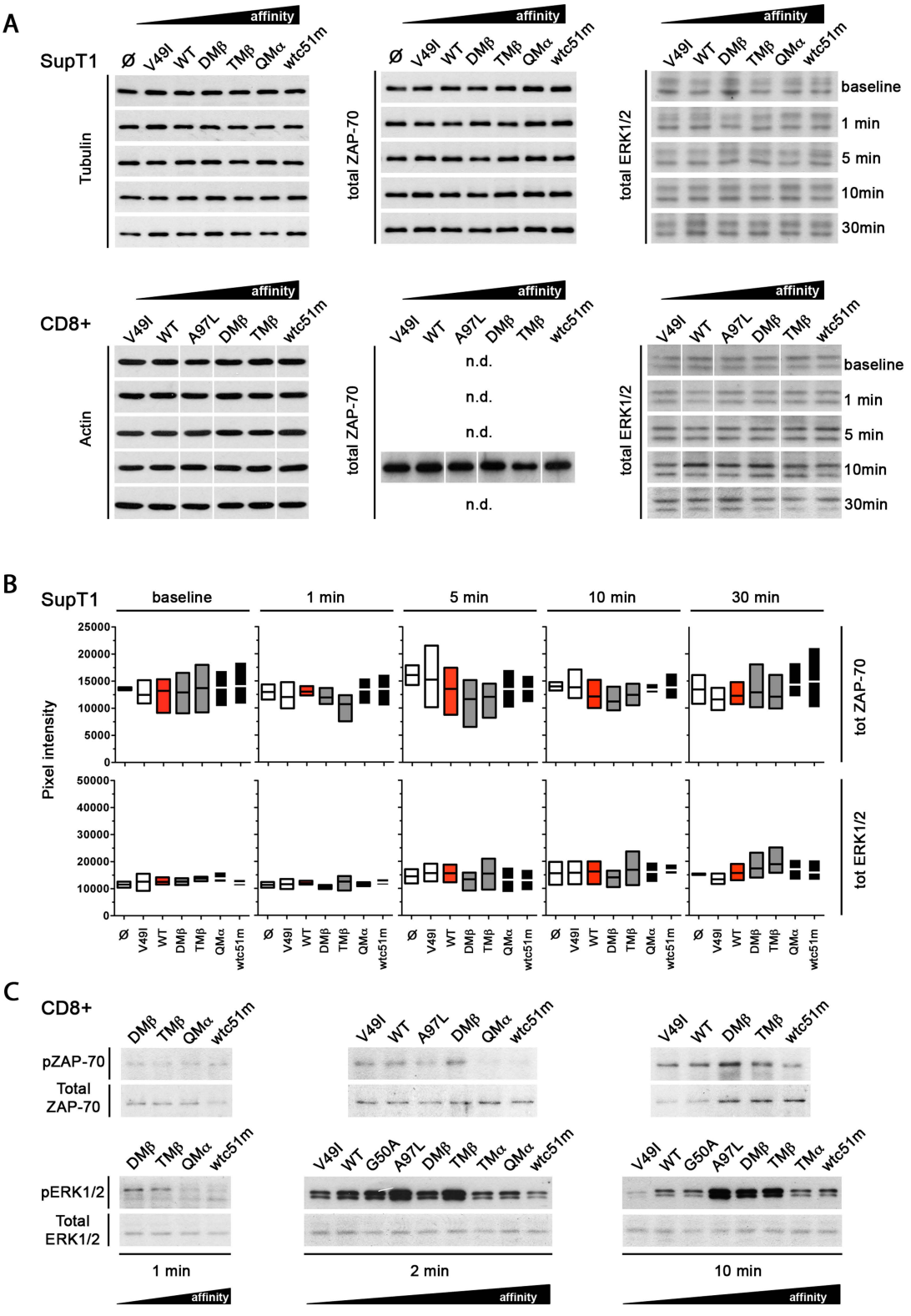

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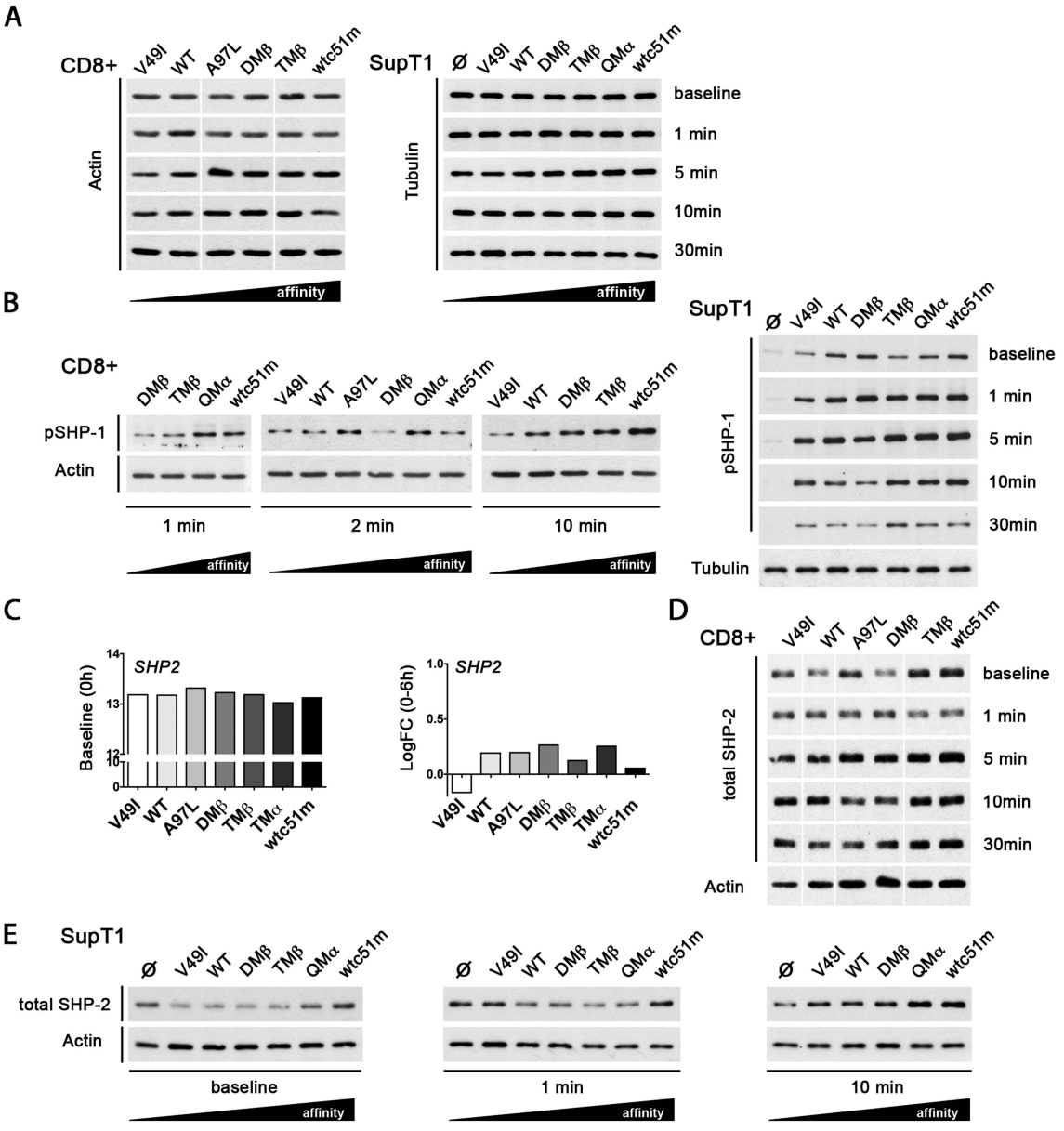

Supplemental Figure 5
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