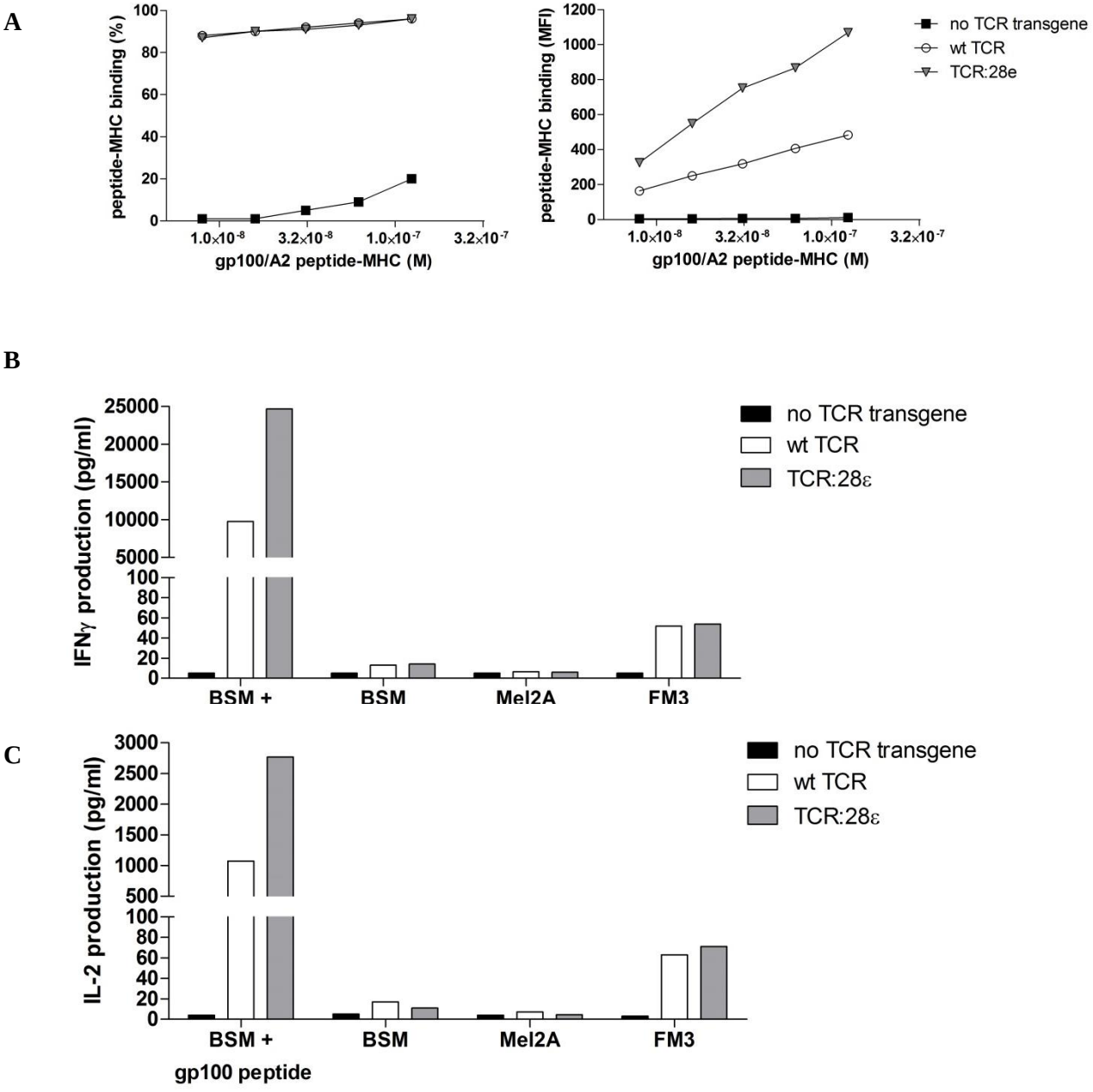
**Supplementary Figure 1**



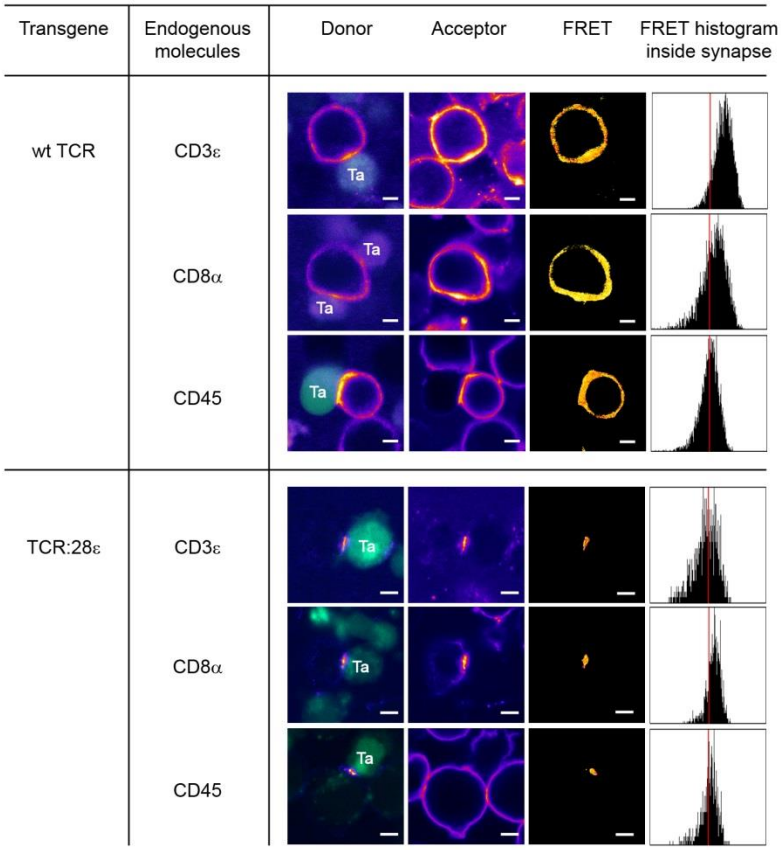

**Supplementary Figure 1: TCR:28ε T cells specific for gp100/HLA-A2 demonstrate enhanced binding of peptide-MHC and peptide-induced production of IFNγ and IL-2.**

Primary human T cells were transduced with empty virus particles or gp100/A2 TCRs as described in the legend to figure 2. In (**A**), T cells were stained with titrated amounts of peptide-MHC (range: $5x10^{-6}$ to $10^{-8}$ M). In (**B**) and (**C**), T cells were stimulated for 24h with

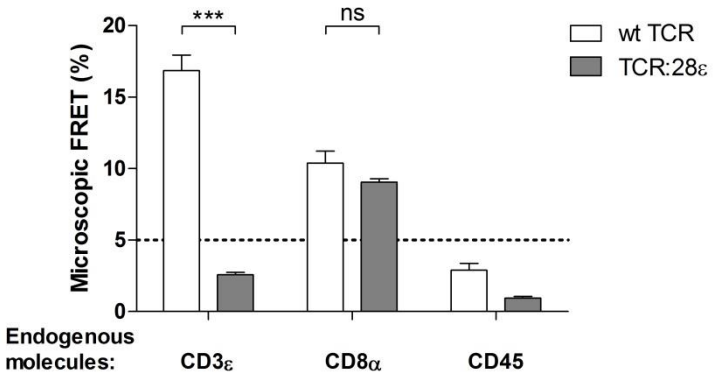
BSM B cells that were loaded or not loaded with gp100 wt peptide, or the melanoma cell lines MEL2A ($gp100^{+}/A2^{-}$) and FM3 ($gp100^{+}/A2^{+}$), and were subsequently analyzed for their production of (**B**) IFNγ or (**C**) IL-2. Representative data of flow cytometry stainings and IFNγ and IL-2 measurements are shown (n=2).

## Supplementary Figure 2

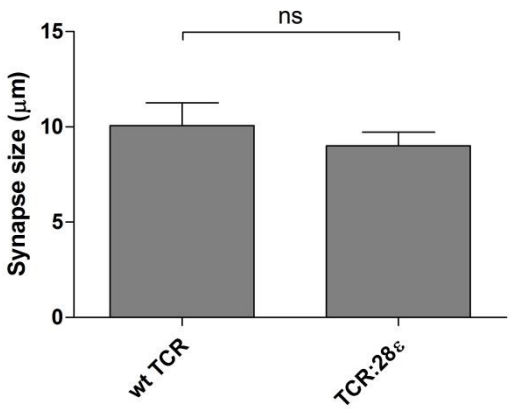
**A**




**B**

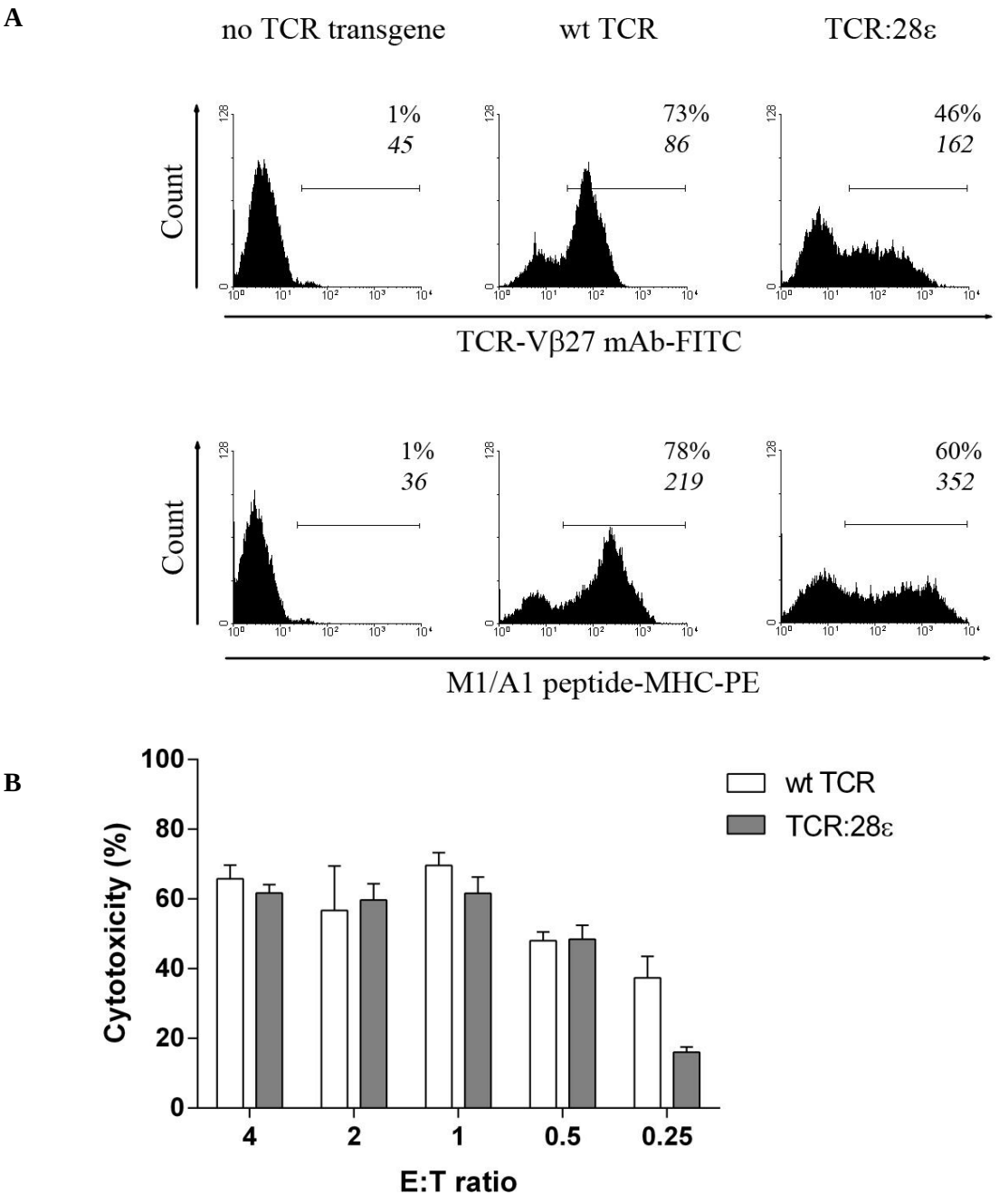



**C**



**Supplementary Figure 2: TCR:28ε induces formation of antigen-dependent immune-synapses.** Jurkat T cells were transduced with M1/A1 TCRs as described in the legend to figure 5, and analyzed for molecular associations between the TCRβ transgene and endogenous CD3ε, CD8α or CD45 molecules in immune synapses as described in the legend to figure 4. In (**A**) representative pictures are shown of immune synapses of T cells (magnification: 200x and with a white 5 μm bar), including FRET histograms of corresponding synapses. Mean % + SEM of microscopic FRET measured with Jurkat T cells are shown in (**B**), n=3 independent measurements of 15 cells per measurement. Data of wt TCR presented in (**A**) were modified from Roszik and colleagues (23). In (**C**) mean $\mu m^2$ + SEM of synapse sizes are shown, n=4 independent measurements. Synapses were defined as regions of contact between T cells and peptide-loaded target cells with an accumulation of TCR-Vβ9 molecules and their areas were calculated by ImageJ software. Mock T cells (no TCR transgenes) did not induce the formation of immune synapses (data not shown). Statistically significant differences between TCR:28ε and wt TCR were calculated with Student's *t*-test: ns: non-significant; *: $p<0.05$; ***: $p<0.0005$.

**Supplementary Figure 3**

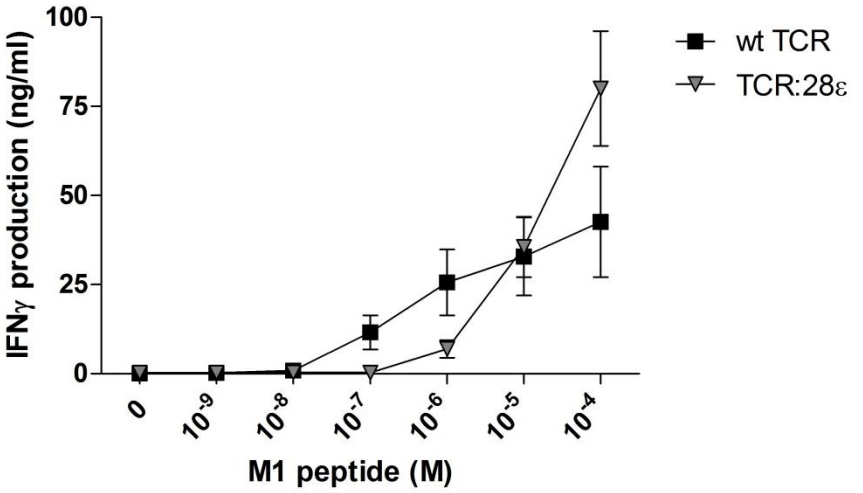



**Supplementary Figure 3: Murinized TCR:28ε is surface-expressed and mediates antigen-specific T cell functions following transduction in mouse T cells.** Murine splenocytes were transduced with gp100/A2 wt TCR and TCR:28ε, both murinized with respect to TCR-C domains and the 28ε cassette as well as codon-optimized. See *materials and methods* for details on the construction of TCR constructs. (**A**) At day 5 following T cell activation, T cells were analyzed for TCR-Vβ27 expression (upper row) and gp100/A2

peptide-MHC binding (lower row) by flow cytometry. Data are representative of n=3 separate transductions with similar results. (**B**) T cells were incubated with B16:gp100/A2 target cells and target cell lysis was measured by a $^{51}$Cr-release assay (E:T ratio of 40:1) after 4h incubation with target cells. The percentage of specific $^{51}$Cr-release was determined as follows: ((test counts – spontaneous counts) / (maximum counts – spontaneous counts)) x 100%. This was performed in triplicate and data are presented as mean % specific killing + SEM.

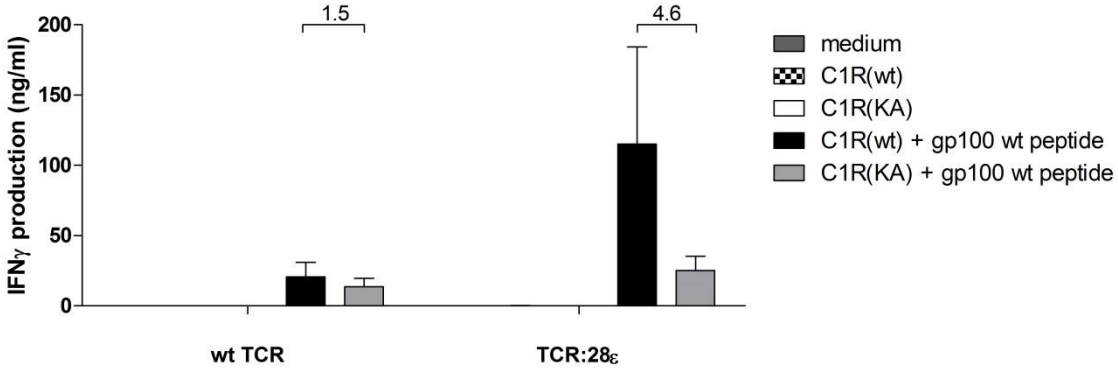
**Supplementary Figure 4**

**A**




| TCR variant | WT TCR | TCR:28ε |
|---|---|---|
| $EC_{50}$ (M) | $6{,}02 \times 10^{-7}$ | $9{,}86 \times 10^{-6}$ |
| Max (IFNγ production) | 42,6 ± 15.5 | 80 ± 16.1 |

**B**




**Supplementary Figure 4: TCR:28ε shows compromised binding ability for peptide.**

Primary human T cells were transduced with (**A**) M1/A1 TCRs or (**B**) gp100/A2 TCRs as described in the legend to figure 1 and (**A**) tested for their ability to produce IFNγ following stimulation with APD B cells that were either unloaded (no peptide) or loaded with titrated amounts of M1 peptide (range: $10^{-9}$ to $10^{-4}$ M). IFNγ was measured in supernatants from O/N cultures by ELISA, expressed as pg/ml, and presented as curves connecting mean values ± SEM, n=3 independent measurements. Half maximal effective concentrations of peptide

($EC_{50}$) were calculated using MasterplexReaderFit software and presented in insert. **(B)** T cells were O/N stimulated with C1R cells expressing wt HLA-A2 or the non-CD8α binding D227K/T228A mutant of HLA-A2 that were not loaded or loaded with gp100 wt peptide ($10^{-5}$ M final). IFNγ production was measured by ELISA and expressed in pg/ml, n=4 independent measurements. Fold difference (non-significant according to Student's *t*-test) between TCR:28ε and wt TCR T cells in response to peptide-loaded C1R(wt) and C1R(KA) target cells are indicated.