Supplementary Methods

Assessment of functionality and phenotype of $CD8^+$ T cells

Unless otherwise specified, all the fluorochrome-conjugated antibodies were obtained from BD Biosciences (Albertslund, Denmark). For functional evaluation, cells were acquired with a FACS Canto II (BD). $CD8^+$ T cells were initially gated within the live population (LIVE/DEAD® Fixable Near-IR, Thermo Scientific, Copenhagen, Denmark) of cells positive for CD3 (FITC, used only for blood samples) and CD8 (PerCP), and negative for CD4 (FITC, used only for TILs infusion products). Polyfunctionality was evaluated by enumerating the amount of $CD8^+$ T cells expressing TNF (APC), IFNγ (PE-Cy7) and/or CD107a (PE). Under these conditions, the majority of cytokine producing cells expressed both TNF and IFNγ simultaneously, with smaller fractions expressing only one of these cytokines. Analysis of expression on $CD107a^+$ cells appeared in general to be less sensitive, because of more significant overlap between control and stimulated samples as compared with cytokine expression (background of ~ 0.2-0.5% vs less than 0.1 % for $TNF^+/IFN\gamma^+$) especially for infusion products samples.

All melanoma cell lines were established internally at our laboratory. Melanoma cell lines were identified from their morphology and in vitro growth patterns. In some cases, when morphology or growth patterns were not typical, the melanocyte origin of the continuously growing cell lines was confirmed by PCR for melanocyte antigens. The cell lines were not otherwise authenticated.

For phenotypic assessment of polyfunctional tumor-reactive T cells, we used a BD LSRII machine equipped with 5-lasers. Polyfunctional tumor-reactive $CD8^+$ T cells were initially gated within the live population (LIVE/DEAD® Fixable Near-IR, Thermo Scientific, Copenhagen, Denmark) of cells positive for CD3 (FITC, used only for blood samples) and CD8 (PerCP), negative for CD4 (FITC, used only for TILs infusion products), and simultaneously expressing all three functional markers TNF (APC), IFNγ (PE-Cy7) and CD107a (BV421) – triple positive (TP) cells. Finally, phenotype gates using PD-1 (PE, from eBiosciences, San Diego, CA, USA), CD27 (QDOT655, Thermo Scientific), CD45RO (Alexa-Fluor700), CD57 (PE-CF594) and LAG-3 (R&D Systems, Abingdon, UK) were applied on TP cells. This panel was constructed in order to assess both the exhaustion (LAG-3 and PD-1) and differentiation status (CD27, CD45RO and CD57) of TP cells. It should however be noted that, in order to detect TP cells, surface marker expression was assessed

after activation with whole tumor cells, thus activation-induced expression cannot be differentiated from basal expression in this set of experiments. The 9-month sample was not analyzed here, and in those cases where a 12-month sample was unavailable, a 12 ± 3 months sample was used if available (patients M36 and M45, see Table 1).

For phenotypic assessment of T cells with defined antigen specificity, all the TIL infusion products were screened for specific recognition of over 200 different pMHC complexes, derived from known melanoma-associated antigens, as previously described (1) using combinatorial color-encoded MHC multimers (2). Three patients were selected for detailed study due to the high-frequency of T cells with defined antigen specificity in their TIL infusion products. Phenotype analysis was carried out as described above but samples were additionally stained with one or two peptide-MHC multimers, matching the predescribed specificities. Each MHC multimer was included in two different colors, in order to increase sensitivity. Samples were not stimulated with whole-tumor cells Peptide-MHC multimers were produced in house, as previously described (Andersen RS et al., Cancer Res 2012). The following fluorochromes and peptides were used: Melan-A/MART-1 derived peptide ELAGIGILTV, conjugated with APC or PE-Cy7 (pt M24) and APC or BV421 (pt M43); gp100/Pmel17 derived peptide YLEPGPVTA, conjugated with APC or BV421 (pt. M26); MAGE-A10 derived peptide GLYDGMEHL, conjugated with APC or PE-Cy7 (pt. M43).

High throughput sequencing of TCR-β chain clonotypes

Library preparation was carried out using the NEBNext Ultra Library Preparation kit (New England Biolabs, Herts, UK) for adaptor ligation. Purified, barcoded samples were then diluted and pooled to 4 nM. The library was further diluted to a final concentration of 6 pM in 50% PhiX control and run on a 2 x 300 cycle MiSeq sequencing run (Illumina, Cambridge, UK). TCR diversity was assessed by Shannon entropy and compositional similarity by Sorensen coefficients.

Shannon entropy (H) was calculated as:

$$H = -\sum_{i=1}^{n} p_i \ln(pi)$$

where p_i is the frequency of the ith clonotype in a population of n clonotypes.
Sorensen coefficients (QS) are given as:

$$QS = \frac{2(X \cap Y)}{X + Y}$$

where X is the number of clonotypes in the first sample (TIL) and Y is the number of clonotypes in the second sample (PBMC).
H values increase with TCR diversity; QS increases with repertoire overlap.

References

1. Andersen RS, Thrue CA, Junker N, Lyngaa R, Donia M, Ellebæk E, et al. Dissection of T-cell antigen specificity in human melanoma. Cancer Res [Internet]. 2012 [cited 2013 Jan 29];72:1642–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22311675
2. Andersen RS, Kvistborg P, Frøsig TM, Pedersen NW, Lyngaa R, Bakker AH, et al. Parallel detection of antigen-specific T cell responses by combinatorial encoding of MHC multimers. Nat Protoc [Internet]. 2012 [cited 2016 Jan 7];7:891–902. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22498709
3. Donia M, Andersen R, Kjeldsen JW, Fagone P, Munir S, Nicoletti F, et al. Aberrant expression of MHC Class II in melanoma attracts inflammatory tumor specific CD4+ T cells which dampen CD8+ T cell antitumor reactivity. Cancer Res [Internet]. 2015; Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-14-2956

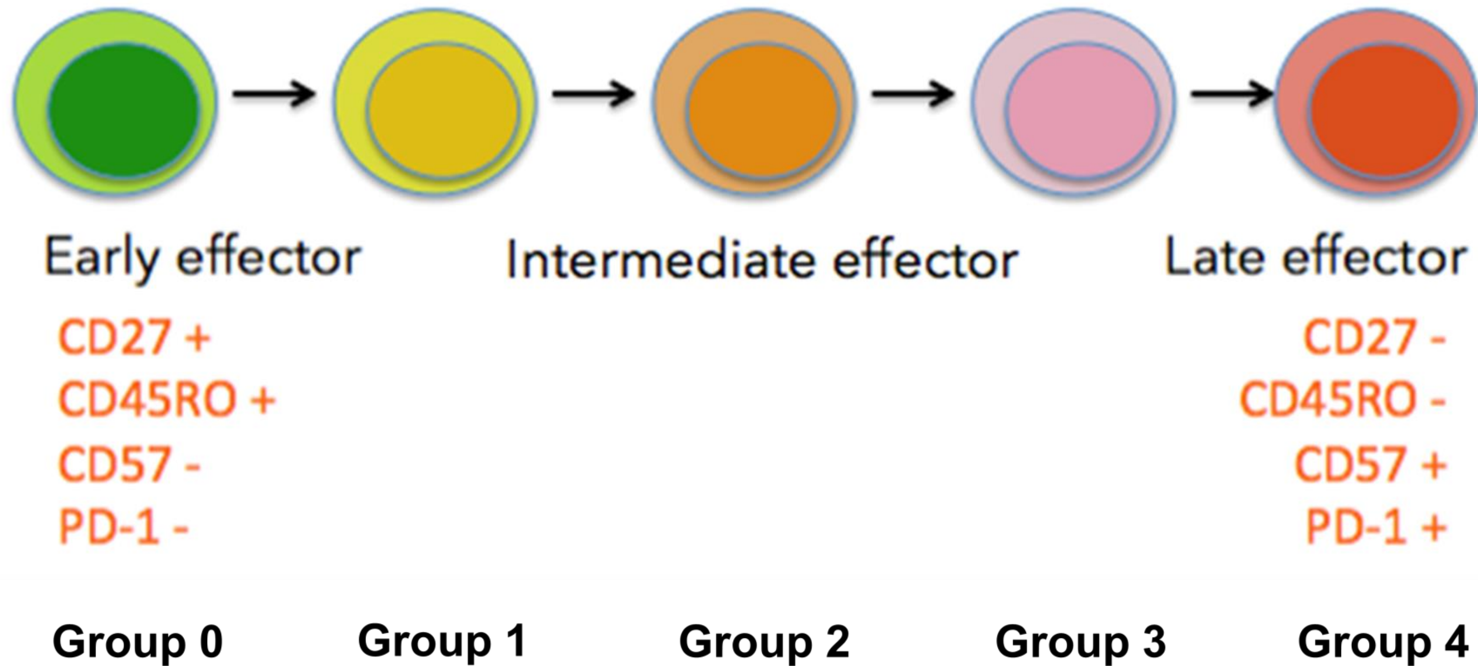
Supplementary Tables

Supplementary Table S1: Groups of the differentiation scoring system

Group 0	Group 1	Group 2	Group 3	Group 4
CD27^{+}CD45RO^{+}CD57^{-}PD-1^{-}	CD27^{+}CD45RO^{-}CD57^{-}PD-1^{-}	CD27^{-}CD45RO^{-}CD57^{-}PD-1^{-}	CD27^{+}CD45RO^{-}CD57^{+}PD-1^{+}	CD27^{-}CD45RO^{-}CD57^{+}PD-1^{+}
	CD27^{+}CD45RO^{+}CD57^{+}PD-1^{-}	CD27^{-}CD45RO^{+}CD57^{+}PD-1^{-}	CD27^{-}CD45RO^{+}CD57^{+}PD-1^{+}	
	CD27^{+}CD45RO^{+}CD57^{-}PD-1^{+}	CD27^{-}CD45RO^{+}CD57^{-}PD-1^{+}	CD27^{-}CD45RO^{-}CD57^{-}PD-1^{+}	
	CD27^{-}CD45RO^{+}CD57^{-}PD-1^{-}	CD27^{+}CD45RO^{-}CD57^{+}PD-1^{-}	CD27^{-}CD45RO^{-}CD57^{+}PD-1^{-}	
		CD27^{+}CD45RO^{-}CD57^{-}PD-1^{+}		
		CD27^{+}CD45RO^{+}CD57^{+}PD-1^{+}		

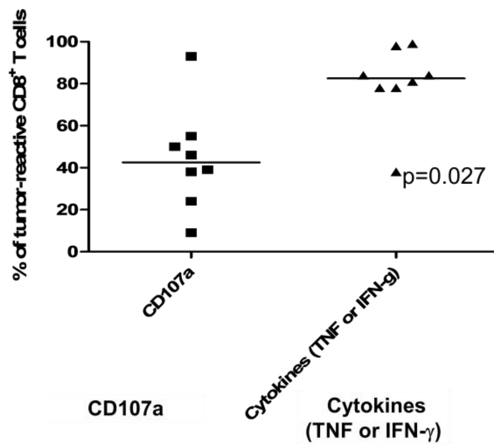
Supplementary Figures

Supplementary Figure S1: Differentiation Scoring System. Graphical representation of the differentiation scoring system used in the analyses reported in Figure 3A and Figure 3B.

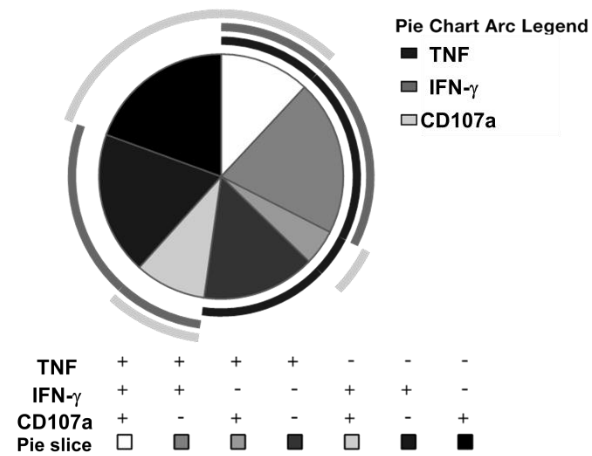


Supplementary Figure S2: Relative frequency of tumor-reactive CD8$^+$ T cells producing cytokines or mobilizing CD107a$^+$. The dataset presented in (3) was re-analyzed**. (A)** Frequency of cells producing cytokines (TNF and/or IFN-γ) or mobilizing CD107a among tumor-reactive CD8$^+$ T cells. (**B**) The relative distribution of TNF$^+$, IFN-γ$^+$ and CD107a$^+$ cells among tumor-reactive CD8$^+$ T cells is shown. (**C**) The same data shown in B are presented in a bar chart. (**D**) This pie chart shows the relative frequency of CD107a$^+$ CD8$^+$ T cells expressing cytokines.

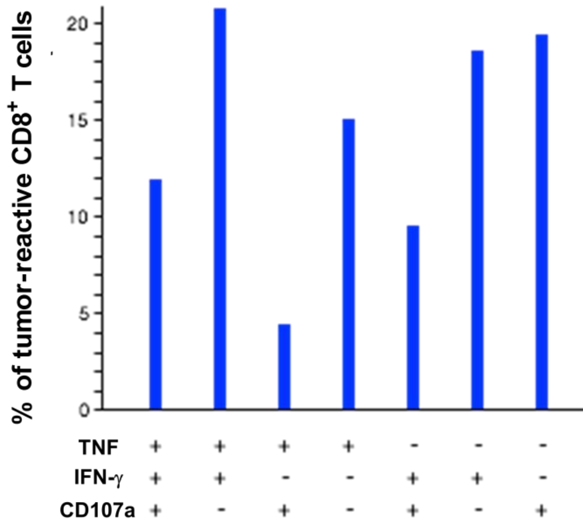
A



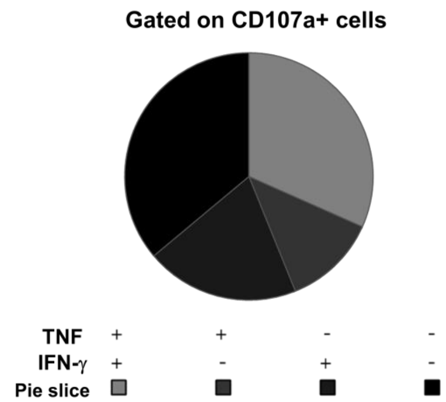
B



C

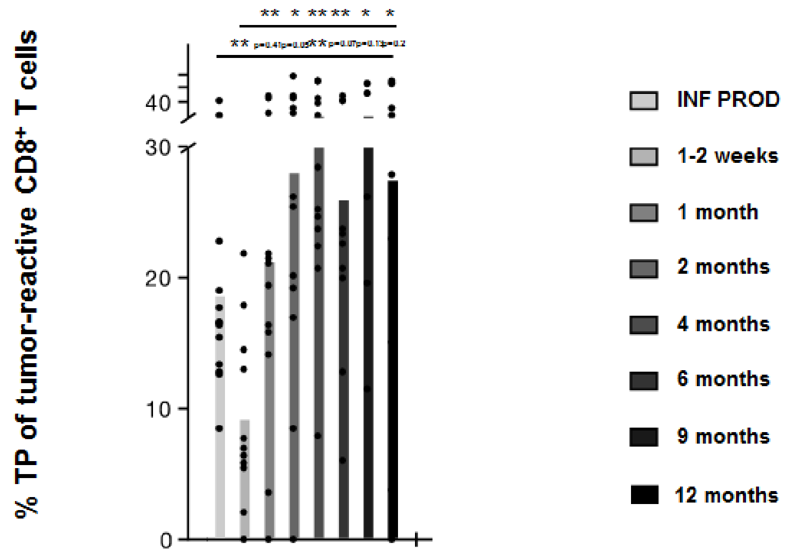


D

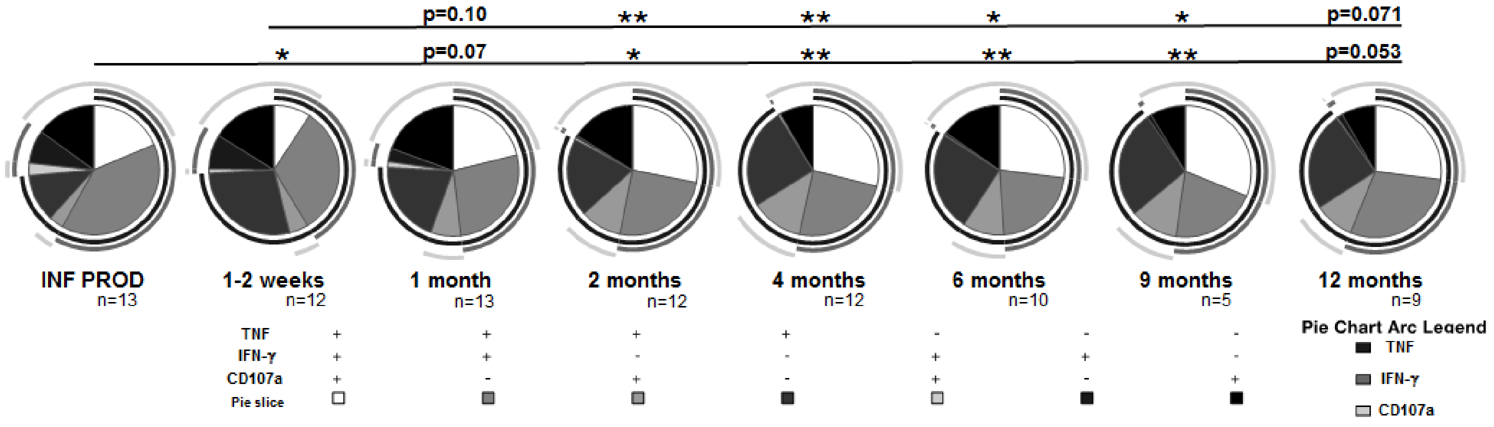


Supplementary Figure S3: Accumulation of polyfunctional tumor-reactive CD8$^+$ T cells after cell transfer (2). (A) The same data shown in Figure 1B are presented in a bar chart. (B) Pie charts showing the relative frequency of tumor-reactive CD8$^+$ T cells expressing combinations of TNF, IFN-γ and/or CD107a.

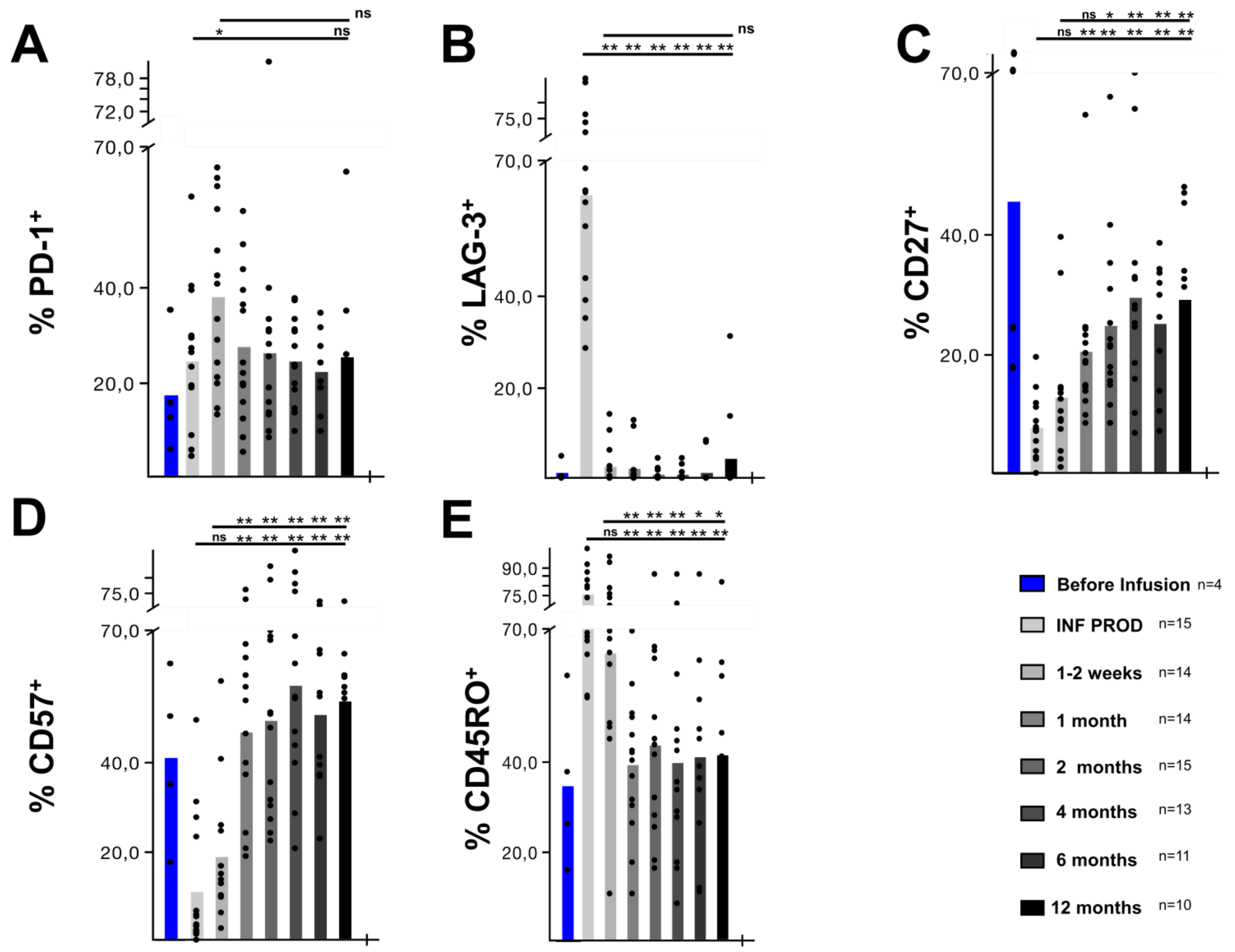
A



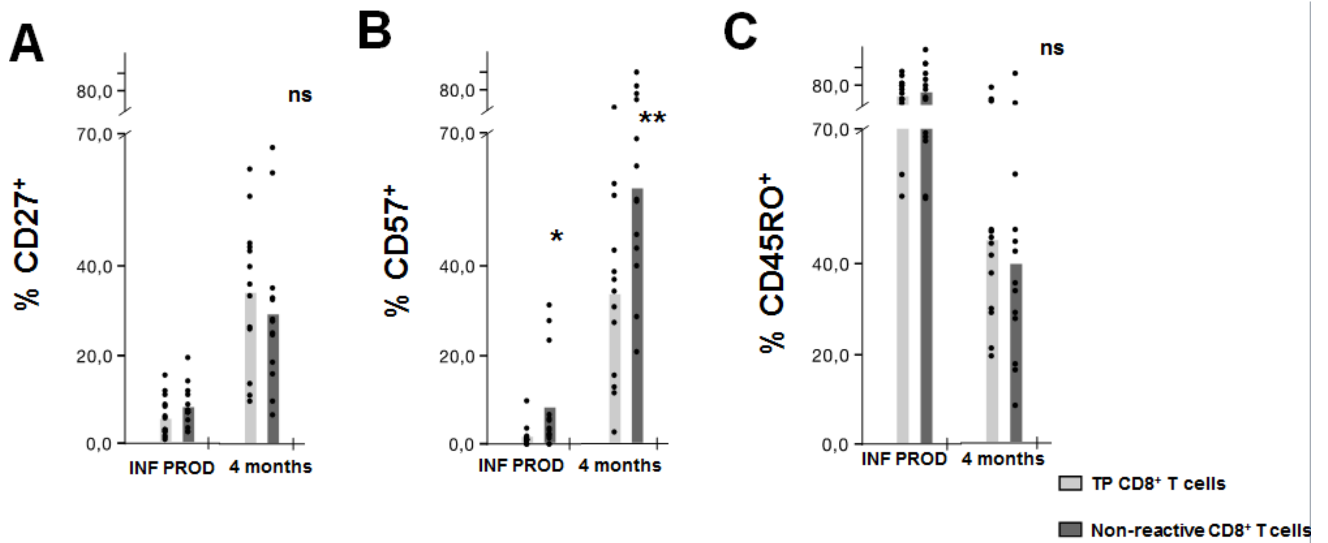
B



Supplementary Figure S4: Phenotype of non-tumor-reactive CD8+ T cells before and after cell transfer. Frequency (%) of cells positive for surface markers among non-tumor-reactive CD8+ T cells (A) PD-1, (B) LAG-3, (C) CD27, (D) CD57 and (E) CD45RO. Dots show individual patients and bars show mean values. * p<0.05; ** p<0.01

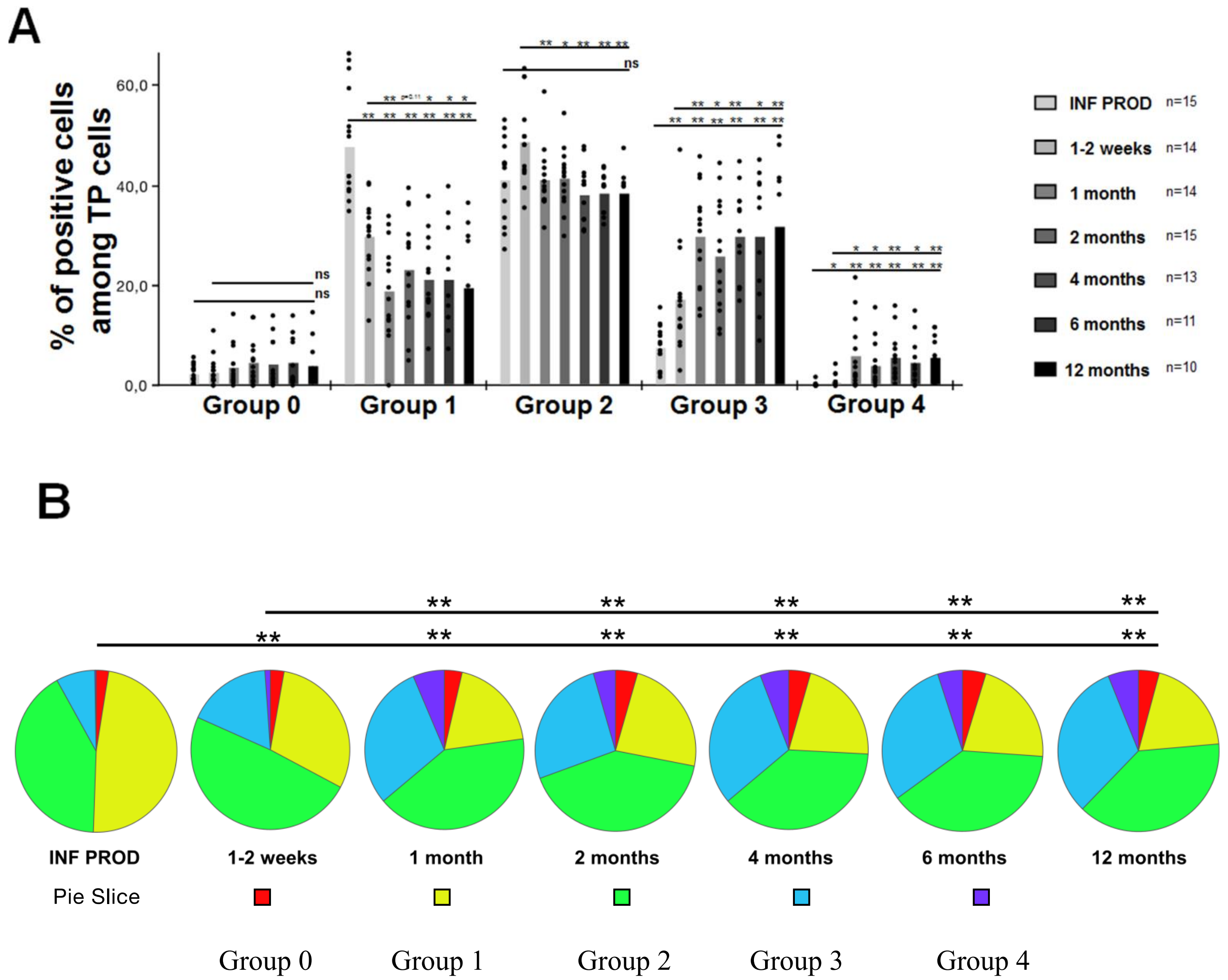


Supplementary Figure S5: Phenotype comparison of non-tumor-reactive CD8⁺ T cells and polyfunctional (triple positive, TP) tumor-reactive CD8⁺ T cells in TILs and after cell transfer. Frequency (%) of cells positive for surface markers in the infusion products (n=15) and peripheral blood 4 months after infusion (n=13), (A) CD27, (B) CD57 and (C) CD45RO. Dots show individual patients and bars show mean values. * p<0.05; ** p<0.01

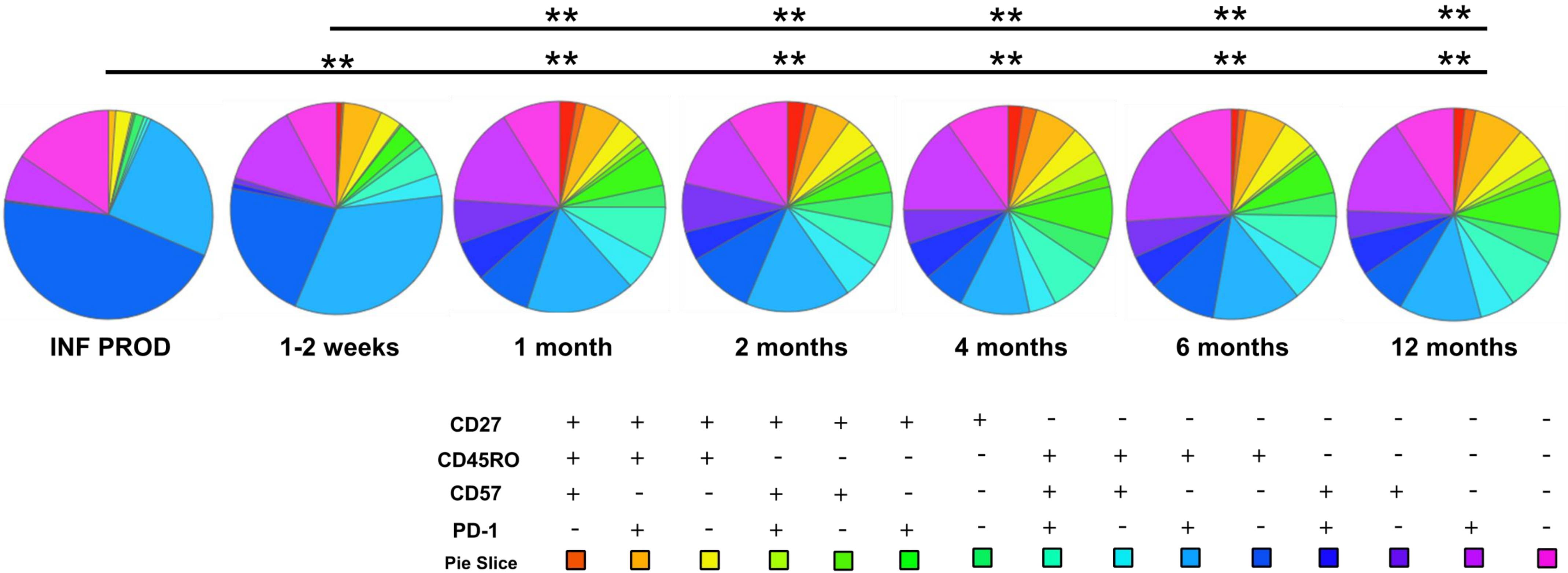


Supplementary Figure S6: Combinatorial phenotypic analysis of tumor-reactive CD8$^+$ T cells after cell transfer

(A) Frequency of cells in each differentiation scoring group. Dots show individual patients and bars show mean values. * $p<0.05$; ** $p<0.01$.(B) The same data are displayed in pie charts



Supplementary Figure S7: Phenotype changes of polyfunctional tumor-reactive CD8$^+$ T cells after cell transfer – combinatorial analysis. ** $p<0.01$



Supplementary Figure S8: Phenotype of CD8$^+$ T cells with defined tumor-antigen specificity – frequency of cells and combinatorial analysis. (**A**) Frequency of tumor-antigen specific CD8$^+$ T cells detected by peptide-MHC multimers at various time points in three selected patients. (**B**) Expression of PD-1 on tetramer positive and tetramer negative CD8$^+$ T cells. Lines represent median values. (**C**) Flow cytometry plots (upper panel) and combinatorial phenotype analysis (lower panel) from one representative patient (M26) is shown.