

# Tumor-infiltrating lymphocytes mediate complete and durable remission in a patient with NY-ESO-1 expressing prostate cancer

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## ABSTRACT

Adoptive transfer of autologous tumor-specific lymphocytes represents a viable treatment method for patients with advanced malignancies. Here, we report a patient's case with metastatic hormone-refractory New York esophageal squamous cell carcinoma 1 (NY-ESO-1) expressing prostate cancer treated with in vitro expanded tumor-infiltrating lymphocytes (TILs) in conjunction with IL-2 and immune-checkpoint blockade. Complete and durable tumor remission was observed after three TIL infusions consisting of  $1.4 \times 10^9$ ,  $2.0 \times 10^9$ , and  $8.0 \times 10^9$  T cells, respectively, lasting now for more than 3.5 years. Immunological correlates to the clinical development were the decrease of tumor-driven NY-ESO-1 serum antibody and the drop of prostate-specific antigen to  $<0.01 \mu\text{g/L}$ . TILs were reactive against cancer-testis antigen NY-ESO-1, individual tumor mutational proteins (eg, PRPF8, TRPS1), and the androgen receptor splice variant 12.

## INSIGHT

This case demonstrates clinical efficacy of prostate cancer-derived tumor-infiltrating lymphocytes (TILs) and highlights the immunological impact of the cancer-testis antigen New York esophageal squamous cell carcinoma 1 (NY-ESO-1).

## INTRODUCTION

Advanced prostate cancer is associated with poor prognosis and remains incurable; more than 70 000 men die from this disease each year in Europe. Apart from Sipuleucel-T, a Food and Drug Administration (FDA)-approved therapeutic cancer vaccine, effective immunotherapeutic approaches for prostate cancer still remain challenging. Immune-checkpoint inhibitors anti-programmed death protein 1 (PD-1), anti-programmed death-ligand 1 (PD-L1), anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4), that are effective in different malignancies show only limited activity in prostate cancer,

partially due to the relatively low mutational burden in this disease. Response rates, for example, with pembrolizumab, are reported with 5% and 3% only.<sup>1</sup>

Adoptive therapy with TILs has shown remarkable results in the treatment of individual patients with different types of cancer in the last decades.<sup>2</sup> The recognition of multiple individual neo-antigens as well as shared tumor-associated antigens (TAAs) linked to enhanced tissue homing capacity and strong immune effector functions jointly contribute to the clinical efficacy of TIL in patients with solid tumors.<sup>3</sup> Currently, only few clinical studies investigating adoptive transfer of T cells in metastatic hormone-refractory prostate cancer (mHRPC) are listed on [www.clinicaltrials.gov](http://www.clinicaltrials.gov), all in form of chimeric antigen receptor (CAR) T cells.<sup>4</sup>

New York esophageal squamous cell carcinoma 1 (NY-ESO-1) is a highly immunogenic cancer-testis antigen, inducing simultaneous cellular and humoral immune responses in a high percentage of patients with advanced NY-ESO-1 expressing tumors and has therefore been considered a promising target for cancer immunotherapy. Serum antibodies against NY-ESO-1 have been found to increase with disease progression and to decrease with disease regression, making the antibody a useful biomarker to monitor disease activity and treatment response. Several studies also indicate that patients with pre-existing NY-ESO-1 antibodies show an increased clinical benefit from immune checkpoint blockade.<sup>5–8</sup>

Here, we report a patient's case with advanced HRPC who experienced complete and durable tumor remission after treatment with in-vitro expanded TIL that recognized NY-ESO-1 as well as multiple mutational tumor antigens.



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## CASE PRESENTATION

A patient in the 70s was first diagnosed with prostate cancer in December 2004. He underwent radical prostatectomy and pelvic lymphadenectomy. The disease was classified pT2c N0 Mx G3 R1, Gleason Score 9 (= 4 + 5). Radiotherapy was administered postoperatively to a single bone metastasis with a total dose of 67 Gy. He received various antihormonal treatments until the disease progressed significantly in May 2018 infiltrating urethra, para-urethral soft tissue, penis root, and lymph nodes. At that time, the patient refused standard chemotherapy and was therefore evaluated for experimental TIL treatment on the basis of a single patient compassionate use treatment. A first biopsy from soft tissue metastasis was taken in June 2018 and transported to Zellwerk GmbH in order to isolate and expand TIL (method of TIL preparation is described in the online supplemental file). A first TIL infusion (TIL-1) was administered in September 2018 consisting of  $1.4 \times 10^9$  T cells, followed by a second TIL infusion (TIL-2) of  $2 \times 10^9$  T cells in November 2018. In order to generate a new batch of TIL, the tumor was re-biopsied in January 2019 and a third TIL infusion (TIL-3) was administered in March 2019 at a dose of  $8 \times 10^9$  T cells. NY-ESO-1 expression in both tumor biopsies was confirmed by RT-PCR and immunohistochemistry. Prior to each TIL infusion the patient received conditioning chemotherapy with cyclophosphamide on day -1 at a dose of 60 mg/kg (TIL-1) and on day -4 at 30 mg/kg (TIL-2, TIL-3). TIL infusions were supported by interleukin-2 (IL-2), given four and five times every 12 hours at 600 000 IU/kg, starting 12 hours after each TIL infusion. One week before TIL-3 infusion, checkpoint blockade with pembrolizumab (1 mg/kg) was initiated and continued for a total of four doses. An overview of the treatment schedule is provided in [figure 1A](#). Prostate-specific antigen (PSA) levels decreased after each of the three TIL infusions. A complete decrease of PSA down to 0.01 µg/L was first observed 2 months after the third TIL infusion in parallel with the development of a complete remission of all tumor manifestations. Concomitantly, the high pre-TIL NY-ESO-1 serum antibody titer continuously decreased over the course of TIL treatment ([figure 1B,C](#)). MR images show that the tumor burden had reduced significantly 2 months after administration of TIL-3. The patient's most recent imaging studies show complete and sustained tumor remission lasting for now 3.5 years ([figure 1D](#)).

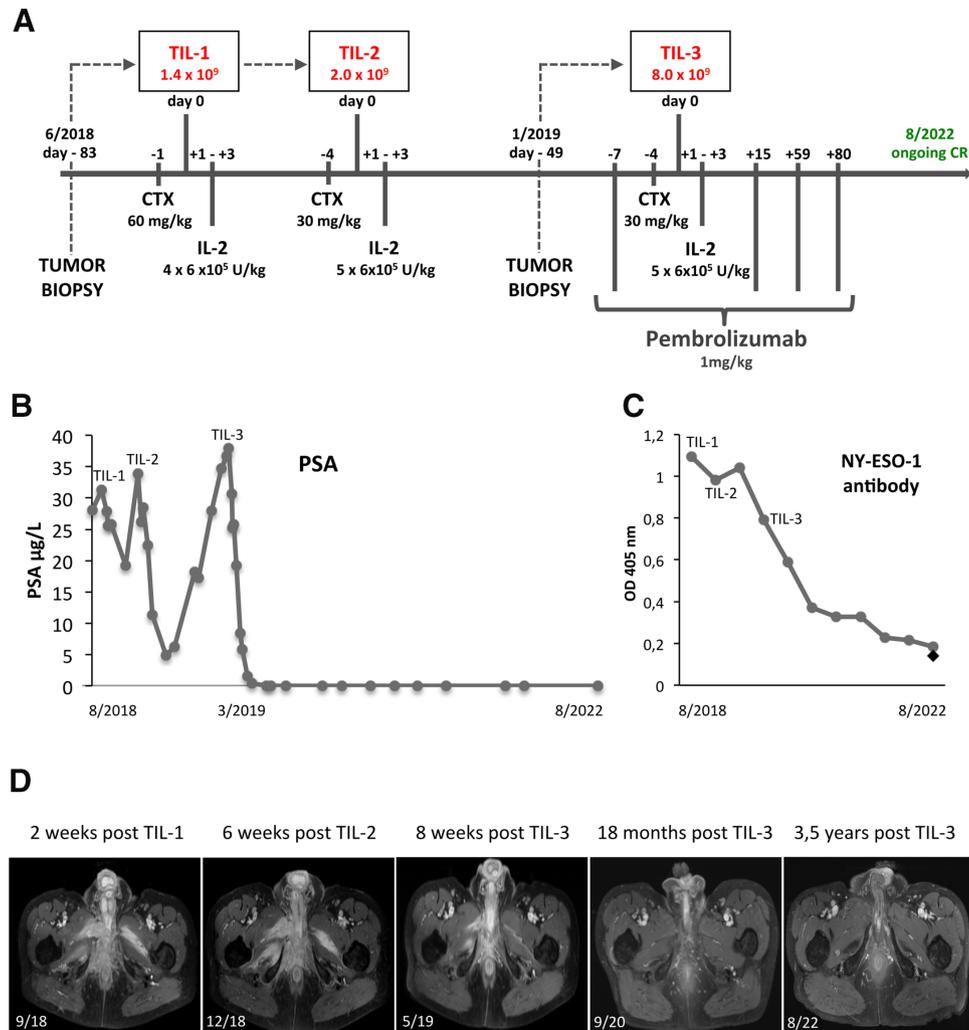
## Specificity of TIL

Based on the antigenic profile of the tumor and the patient's human leukocyte antigen (HLA) type (A2, A11, B7, B55, C3, C7, DR4) we used a panel of immunogenic 'hot spot' NY-ESO-1 peptides known to be recognized by T cells of patients with NY-ESO-1 expressing tumors and a panel of 10 individual HLA-matched peptides derived from mutational antigens identified by whole exome sequencing (WES). This peptide panel represents a selection of nine peptides of 77 non-synonymous coding

neo-antigens detected in the tumor by exome sequencing and of the frameshift androgen receptor (AR) alternative splice variant AR-V12, found by OncoPrint RNA sequencing (online supplemental table). TILs were incubated with these peptides and cell culture supernatant was analyzed for interferon gamma (IFN-γ) in ELISA assay. Specific reactivity for NY-ESO-1 was found in all three TIL infusions, in particular for the HLA-A2 restricted peptide NY-ESO-1 p157-165, for NY-ESO-1 20mer peptides p81-100 and p91-110 that include 9mer peptides p92-100 and p96-104, known to be presented on HLA-Cw3 and for NY-ESO-1 24mer peptide p119-143 ([figure 2A](#)). In addition, TIL showed specific cytotoxicity against NY-ESO-1/HLA-A2 expressing tumor cells in standard chromium-51 ( $Cr^{51}$ ) release assay ([figure 2B](#)). Reactivity was also demonstrated for several mutational peptides: KKRCLFRSF derived from gene PRPF8 (TIL 1,2,3), YLGEITYPF from gene TRPS1 (TIL 1,3) FVSGKYKCL derived from gene ZNF512 (TIL 1,3), NQVQASMEK from gene GFM1 (TIL 2,3), CPSKPSPLV from gene SGIP1 (TIL3) and peptide VKWAKALPDCERAAS derived from a part of the unique C-terminus of the V12 splice variant AR-V12 (TIL 1,2,3) ([figure 2C](#)). Given that TIL-1 and TIL-2 infusions were generated from the same pre-expansion culture and TIL-3 from a separate one, these results show that the immunological phenotype of TIL comprised of NY-ESO-1-specific T cells in all three TIL infusions and of four and six tumor-specific T cells in TIL-1, TIL-2 and TIL-3, respectively. T cells of TIL-3 infusion were also analyzed for specific recognition of the wild type peptides derived from genes PRPF8 and TRPS1, respectively. IFN-γ production was significantly higher for the mutant versus the wild type peptide ([figure 2D](#)).

## Phenotype and functional activity of the TIL

Flow cytometric analysis revealed approximately 78%, 81%, and 96% CD8+ T cells and 18%, 11%, and 1.7% CD4+ T cells in the first (TIL-1), second (TIL-2), and third (TIL-3) infusions, respectively. TILs were further characterized for the expression of co-stimulatory molecules CD27/CD28 and differentiation markers CD45RA/CCR7. The majority of the TILs were effector memory T cells (CD45RA-/CCR7-), with 24%, 54%, and 39% effector memory RA (CD45RA+/CCR7-) and <10% central memory (CD45RA-/CCR7+) T cells. Functional activity was evaluated by IFN-γ release after a 24-hour stimulation of  $10^5$  T cells with OKT3. IFN-γ release of the TIL reached approximately 1298 pg in TIL-1, 1095 pg in TIL-2, and 2184 pg in TIL-3, respectively. Cytotoxic potential was demonstrated by flow cytometric evaluation of CD107a expression after activation by phorbol 12-myristate 13-acetate (PMA). 9.1% of TIL-1, 15.4% of TIL-2, and 23.8% of TIL-3, mostly CD8+ T cells, were CD107a+, exhibiting cytotoxic potential (online supplemental figure). CD25hi CD127- FoxP3+ regulatory CD4+ T cells (Tregs) were <0.13% in all TIL preparations.

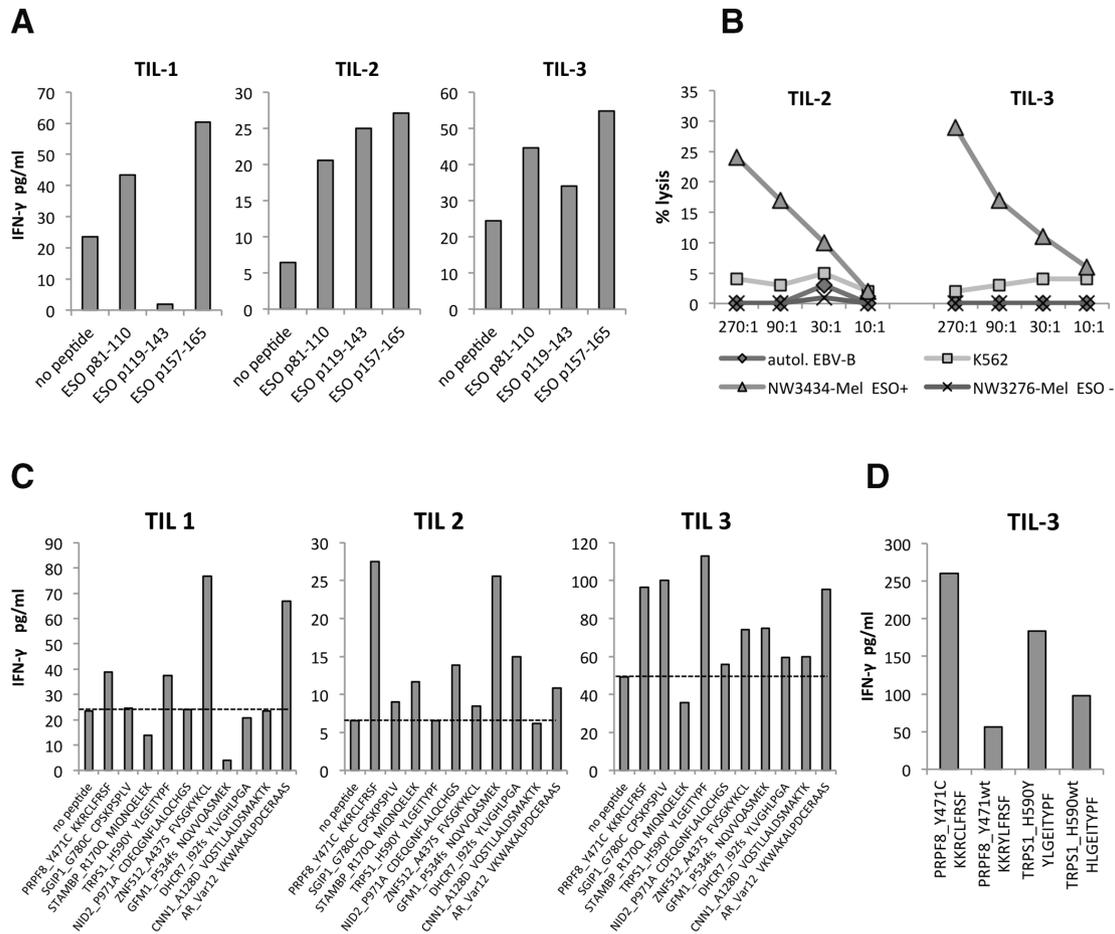


**Figure 1** TIL treatment and correlation with immunological and clinical responses. (A) Treatment schedule. Time point of each TIL infusion was defined as day 0. TIL-1 and TIL-2 infusions were generated from the same initial biopsy, TIL-3 infusion from an independent second tumor biopsy. The patient received conditioning preparative non-myeloablative lymphodepleting chemotherapy (CTX) with cyclophosphamide (60 mg/kg) on day -1 (TIL-1) and (30 mg/kg) on day -4 (TIL-2 and TIL-3) before intravenous infusion of the TIL supported by administration of IL-2 (Aldesleukin) at 600 000 IU/kg. IL-2 was infused four and five times every 12 hours starting 16 hours after each TIL infusion. Four doses of pembrolizumab (1 mg/kg) were administered within the third TIL treatment (TIL-3) on days -7, +15, +59, and +80. (B) PSA values: After TIL-1 treatment PSA decreased from 33 µg/L to 18 µg/L within a period of 5 weeks and recurred. After TIL-2 treatment PSA decreased from 34 µg/L to 5 µg/L within a period of 7 weeks and recurred. After TIL-3 treatment PSA levels continuously decreased from 38 µg/L to <0.01 µg/L within 8 weeks and remained there. (C) NY-ESO-1 serum antibody. The patient showed a high antibody titer before TIL treatment. During and after the course of TIL treatments the antibody continuously decreased and is currently detectable only at a residual level. ELISA OD values are shown at 1:400 serum dilution. (◆): cut off OD in healthy donor. (D) MR images over the course of TIL treatment. The tumor has significantly regressed 8 weeks after TIL-3 infusion and has completely resolved 18 months after TIL-3 infusion. The patient continued to demonstrate complete response (CR) 3.5 years after TIL-3 treatment. CR, complete remission; MR, magnetic resonance; NY-ESO-1, New York esophageal squamous cell carcinoma 1; OD, optical density; PSA, prostate-specific antigen; TIL, tumor-infiltrating lymphocyte.

## DISCUSSION

We report here the clinical and immunological efficacy of in vitro expanded TIL in a patient with metastatic prostate cancer. TIL administered three times at relatively low cell numbers led to complete tumor regression lasting now for more than 3.5 years. In contrast to other groups who are engaged in TIL therapy, we followed alternative strategies for TIL expansion, mode, and number of application as well as for conditioning regimens that may have contributed to this extraordinary result.

First, we used a modified culture method for isolation and expansion of TIL, utilizing IL-2, IL-15, and IL-21-containing medium, and a Good Manufacturing Practice (GMP) compliant closed perfusion bioreactor system.<sup>9,10</sup> We suppose that these cytokines in combination with the culturing procedure led to the expansion of a high percentage of CD8+ cytotoxic T cells (78%–96%) and a relatively low number of CD4+ T cells (11%–1.7%), with the majority of TILs being effector memory T cells (CD45RA–/CCR7–). In addition,



**Figure 2** Specificity of TIL. (A) NY-ESO-1 reactivity measured by IFN-γ secretion was detectable in all three TIL infusions, in particular for the HLA-A2 restricted epitope NY-ESO-1 p157-165, for NY-ESO-1 20mer peptides p81-100 and p91-110, and for NY-ESO-1 24mer peptide p119-143. (B) Specific cytotoxicity of TIL-2 and TIL-3 against NY-ESO-1/HLA-A2-expressing tumor cells in standard chromium-51 (Cr<sup>51</sup>) release assay. (C) TIL reactivity specific for several mutational peptides measured by IFN-γ secretion: KKRCFLRSF derived from gene PRPF8 (TIL 1,2,3), YLGEITYPF from gene TRPS1 (TIL 1,3), FVSGKYKCL derived from gene ZNF512 (TIL 1,3), NQVVQASMEK from gene GFM1 (TIL 2,3), CPSKPSPLV from gene SGIPI (TIL 3) and peptide VKWAKALPDCERAAS derived from a part of the unique C-terminus of the V12 splice variant AR-V12 (TIL 1,2,3). (D) IFN-γ secretion of TIL-3 was significantly higher for the mutant versus the wild type peptides derived from PRPF8 and TRPS1 genes. Values represent IFN-γ secretion by 10 000 TIL and of three independent tests. AR, androgen receptor; HLA, human leukocyte antigen; IFN-γ, interferon gamma; NY-ESO-1, New York esophageal squamous cell carcinoma 1; TIL, tumor-infiltrating lymphocyte; IFN-γ, interferon gamma

this culture method did not promote the outgrowth of Tregs (<0.13%).

We decided to administer repeated transplants with three escalating doses of relatively low numbers of TIL (1.4×10<sup>9</sup>, 2.0×10<sup>9</sup>, and 8.0×10<sup>9</sup>), instead of a single high-dosed transplant of several-fold 10<sup>10</sup> T cells. We hypothesized that repeated transfers of TIL preparations might improve the clinical outcome by prolonging TIL-tumor interactions in vivo and by reducing treatment-related toxicities.

Second, we used a modified preparatory chemotherapy regimen that differs from those employed in TIL studies reported by the US National Cancer Institute, using a single low-dose infusion of cyclophosphamide aiming to reduce regulatory T cells without a higher impact on general lymphodepletion. This helped to significantly reduce treatment-related toxicity in comparison with the widely used high dose cyclophosphamide and fludarabine

regimen that has considerable side effects. The single dose of cyclophosphamide mediated mild lymphopenia and moderate neutropenia but did not cause complete lymphodepletion in the patient. In order to protect the infused TIL from potential toxic effects, we administered cyclophosphamide 4 days before TIL-2 and TIL-3 transfer.

We observed PSA and radiological response after the first and second TIL treatments lasting for 5 weeks and 7 weeks, respectively. The only partial response might be explained by the low number of TIL, by an impaired persistence of transferred T cells, or by an inadequate migration potential of the transferred T cells into the tumor and tumor microenvironment. To improve the outcome, a significantly higher cell number (fourfold to TIL-2) was infused in TIL-3. In addition, we combined TIL-3 treatment with peri-interventional pembrolizumab. As compared with the first and second TIL treatments,

PSA values now completely decreased down to  $<0.01$   $\mu\text{g/L}$  and remained negative since then. Closely correlated to the decrease of PSA, the high pre-TIL NY-ESO-1 serum antibody titer continuously decreased over the course of TIL treatment in parallel with disease regression.

We were able to identify NY-ESO-1-specific T cells in each of the three TIL transplants. Additional TIL specificities were identified for peptides derived from the individual tumor mutational proteins PRPF8, TRPS1 and for the AR splice variant-12. AR-V12 induces a ligand-independent expression of PSAs (KLK-3, kallikrein related peptidase 3; KLK-2, kallikrein related peptidase 2; FOLH1, folate receptor alpha) whose overexpression was observed at the mRNA level. The corresponding proteins represent potential immune targets; antigen-specific T cell interaction may have contributed to the durable clinical response in this patient. The peptide panel used for the identification of TIL specificities did not represent the full range of TAA and neo-antigens expressed in the tumor. T cells reactive against other target antigens, for example, melanoma-associated antigen (MAGE) that was also expressed in the tumor, may have also contributed to the clinical outcome.

It is unclear to which degree tumor remission has been mediated by either TIL treatment or checkpoint inhibition. Clinical responses after TIL-1 and TIL-2 treatments were not complete and not durable. Checkpoint blockade has not shown convincing treatment results in prostate cancer probably due to the low tumor mutation burden (TMB) and lower expression of PD-L1. In our case clinical remission became complete and durable after combined administration of TIL-3 and checkpoint inhibition, suggesting a potential role of PD-1 blockade for the sustained effect of the transplanted TIL-3 preparation.

In the present report we demonstrate that prostate cancer-derived TIL can be successfully isolated and effectively expanded in vitro. TILs were reactive against NY-ESO-1 and several individual tumor antigens in this case. Complete and durable tumor response may have resulted from the combination of TIL treatment with immune-checkpoint blockade that might have helped to overcome PD-1/PD-L1 mediated T cell inhibition in the tumor microenvironment. With respect to different immune escape mechanisms, TIL treatment targeting several tumor antigens may be advantageous over monospecific immunotherapeutic strategies like cancer vaccines or CAR T cell treatment.

In conclusion, adoptive transfer of TIL represents a promising immunotherapeutic approach also for tumors with low TMB like mHRPC. Further investigation in larger patient populations in clinical studies, including the precise analysis of tumor antigen and T cell receptor repertoire, are warranted.

**Contributors** JK designed and performed experiments, analyzed data and wrote the manuscript. DK was involved in treating the patient and data collecting. KB and CW performed experiments. ES, DG and HH manufactured the TIL product and wrote parts of material and methods. H-BP performed and interpreted WES and RNA-sequencing data. AA supervised the treatment, wrote the manuscript and was involved in data analysis. EJ conceptualized and supervised the treatment of the patient, analyzed data and wrote the manuscript.

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## REFERENCES

- 1 von Amsberg G, Alsdorf W, Karagiannis P, *et al*. Immunotherapy in advanced prostate Cancer—Light at the end of the tunnel? *Int J Mol Sci* 2022;23:2569.
- 2 Zacharakis N, Chinnasamy H, Black M, *et al*. Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med* 2018;24:724–30.
- 3 Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 2015;348:62–8.
- 4 Tschernia NP, Norberg SM, Gulley JL. Car T cells reach clinical milestone in prostate cancer. *Nat Med* 2022;28:635–6.
- 5 Gnjatich S, Nishikawa H, Jungbluth AA, *et al*. Ny-Eso-1: review of an immunogenic tumor antigen. *Adv Cancer Res* 2006;95:1–30.
- 6 Thomas R, Al-Khadairi G, Roelands J, *et al*. NY-ESO-1 based immunotherapy of cancer: current perspectives. *Front Immunol* 2018;9:947.
- 7 Jäger E, Stockert E, Zidianakis Z, *et al*. Humoral immune responses of cancer patients against “Cancer-Testis” antigen NY-ESO-1: Correlation with clinical events. *Int J Cancer* 1999;84:506–10.
- 8 Yuan J, Adamow M, Ginsberg BA, *et al*. Integrated NY-ESO-1 antibody and CD8<sup>+</sup> T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A* 2011;108:16723–8.
- 9 Luo X-H, Meng Q, Liu Z, *et al*. Generation of high-affinity CMV-specific T cells for adoptive immunotherapy using IL-2, IL-15, and IL-21. *Clinical Immunology* 2020;217:108456.
- 10 Bröker K, Sinelnikov E, Gustavus D, *et al*. Mass production of highly active NK cells for cancer immunotherapy in a GMP conform perfusion bioreactor. *Front Bioeng Biotechnol* 2019;7:194.

## Supplemental material

### Methods

**TIL production:** Surgical tumor biopsies were taken in 6/2018 (TIL-1 and TIL-2 source material) and in 1/2019 (TIL-3 source material) and transported at the Zellwerk GmbH, Berlin, Germany. Tissue samples were sliced into pieces of approx. 8 mm<sup>3</sup> and placed in a GMP-compliant and completely closed perfusion bioreactor system, operated on a platform of GMP Breeder, Control Unit and belonging software for advanced therapeutic medicinal products (ATMPs). TIL expansion started in a 30MM perfusion bioreactor (phase 1) and continued in a 500M perfusion bioreactor (phase 2) in CellGenix GMP DC culture media supplemented with 10% human serum and 1% antibiotic-antimycotic solution (Pen, Strep, Amphotericin; Sigma-Aldrich). Culture medium for TIL-1 and TIL-2 expansion was supplemented with IL-2 (1000 IU/ml), IL-15 (180 IU/ml) and IL-21 (1 IU/ml) (Miltenyi Biotech, Bergisch Gladbach, Germany), while the second phase of TIL-3 expansion was carried out in IL-2 (1000 IU/ml) medium only. The culturing process is automatically steered by an algorithm based on pH-, hyperoxic pO<sub>2</sub> and temperature providing fresh media to the upgrowing cells. In a short initiation phase, anti-human CD3 antibody (30 ng/ml), clone OKT3, Miltenyi Biotech) and a low amount of allogenic feeder cells (1-2 x 10<sup>6</sup> PBMC, mixture from 4 donors), irradiated with 55-Gy, were added once. Outgrowth of TIL from tumor slices takes place under controlled circulation of the medium in the bioreactor vessel and maintenance of a proven ratio of circulating to freshly supplied medium. Numbers of cells were routinely counted and glucose and lactate concentrations assessed. At a density of 1-3 x 10<sup>9</sup> TILs in the 30MM bioreactor, TIL were separated from tissue material by transferring the cells by gravity over the mesh port into a single use 500MM perfusion bioreactor. During this phase the cultivation was continued until harvest of the TILs. Thus, no

additional stimulus was made causing changes in the phenotypes formed during phase 1. Culture time in phase 1 was 22 days for TIL-1 and TIL-2, and 23 days for TIL-3. Culture time in phase 2 was 12 days for TIL-1, 14 days for TIL-2 and 25 days for TIL-3.

Cells were washed twice with 0,9 % saline solution, resuspended in 5 % albumin solution and transferred into infusion bags. Bags were transported to the patient in a biospecimen handling unit at ambient temperature 15°- 22°C. Back-up TIL samples, if any, were suspended in 90% human AB serum with 10% dimethyl sulfoxide (DMSO) for storage in liquid nitrogen.

**Flow cytometry and functional characterization of TIL:** TIL phenotype, Treg-cells and CD107a induction was determined using Beckman Coulter DURAClone IM T-cell subset and DuraClone IM Treg tube kits. CD107a induction was performed using Beckman Coulter DURActive 1 for cellular activation and stained with anti-human CD107a PE antibody (clone H4A3), anti-human CD3 PE-Cy7, anti-human CD4 V450 and anti-human CD8 APC-Cy7 antibodies. Acquisition of events was performed in a CytoFlex flow cytometer from Beckman Coulter. IFN- $\gamma$  production was measured after stimulation of 100.000 TIL with OKT3 (30ng/ml) for 24 hours followed by cytokine quantification of the culture supernatant by enzyme-linked immunosorbent assay (ELISA kit from Mabtech AB #3420-1A-6).

**IFN- $\gamma$  ELISA:** TIL (10.000 cells/well) were incubated with peptides (1 $\mu$ g/ml) in round-bottom 96-well microtiter plates in 200  $\mu$ l TCM (T-Cell Medium: RPMI 1640 + 10 % human serum) for 3 - 7 days at 37°C with 5% CO<sub>2</sub>. Culture supernatants (100 $\mu$ l/well) were harvested and analyzed for IFN- $\gamma$  production in flat bottom 96-well multi plates using the IFN- $\gamma$  ELISA kit from Mabtech AB (#3420-1A-6) according to the manufacturers instructions.

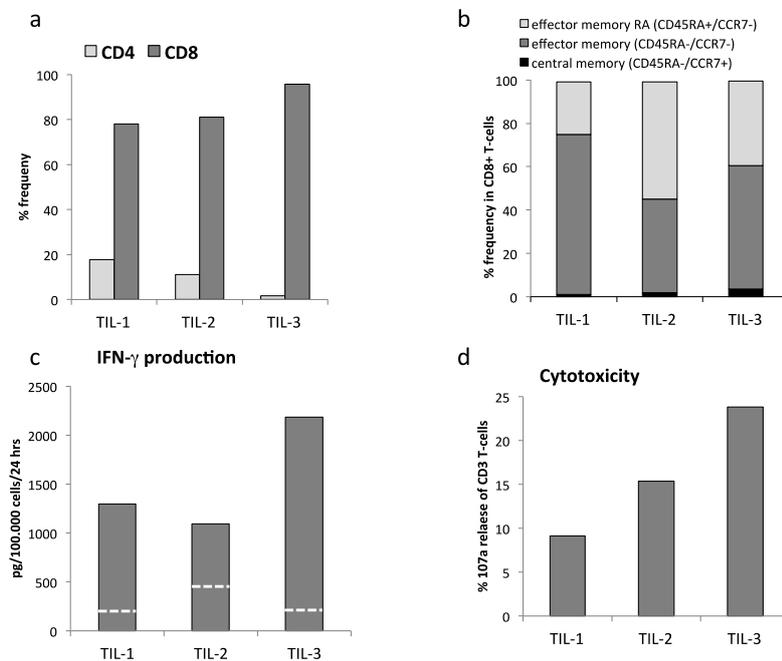
**Nucleic acid isolation, whole-exome sequencing and neoepitope selection:** Genomic DNA from tumor tissue and corresponding normal blood was purified by DNeasy Blood & Tissue Kit (Qiagen, Cat #: 69504). All DNA isolations included the optional RNase-A digestion step. DNA exome sequences were enriched from tumor and normal DNA using the Ion Torrent AmpliSeq™ Exome RDY Kit (Thermo Fisher Scientific, Carlsbad, CA, Cat #: A38264) and the Ion AmpliSeq™ Library Preparation Kit plus (Thermo Fisher Scientific, Cat #: 4488990). Barcoded libraries were quantified by Ion Library TaqMan™ Quantification Kit (Thermo Fisher Scientific, Cat #: 4468802). Tumor- and normal DNA-derived libraries were adjusted at a molecular ratio of 2:1. Sequencing was done using Torrent Suite™ Software 5.6 and 200 bp OT2 kit on Ion GeneStudio™ S5 System using a Chip 540 delivering 80-90 million reads per exome pair. The reads were aligned to the human genome reference sequence (hg19) using integrated torrent suite algorithm. The typical average coverage was 120x in the tumor versus 60x in normal DNA. Output BAM files were screened to identify point mutations, small insertions, deletions and stop codons by Ion Reporter Software using AmpliSeq Exome tumor-normal pair workflow with filter focus on Ion AmpliSeqExome HiQ region. Minimal requirements for mutations to be included in further analysis were: coverage of more than 49x in tumor tissue and more than 19x in normal DNA with a frequency of mutation >10% in the tumor with no single affected read in the accompanying normal DNA sample. Corresponding protein sequences were translated by an algorithm developed in-house for SNPs which allows constructing 31-mer wildtype and mutated peptides, respectively by placing the mutation at the center of the sequence. Indels were separately translated, by using the “codong\_change.pl” function in the ANNOVAR software, which was also used to annotate mutations and to exclude variances with significant presence in the human genome database (kaviar\_20150923, with genomic variances in 64K exomes). For the evaluation of expressed genes, total RNA was extracted from tumor slices

using the RNeasy FFPE Kit (Qiagen, Cat #: 73504), transcribed, amplified and cyanine-3-stained according to the Gene Expression FFPE Workflow Guide (Agilent Technologies, Cat #: G4112-90000). Stained cDNA was hybridized to Human GE 4x44 v2 Microarray slides (Agilent, Cat #: G4845) and scanned on a DNA Microarray Scanner (Agilent, Cat #: G2505C). Mutation-containing peptides corresponding to higher mRNA expressions were favored for downstream selection. HLA typing (HLA A/B/C and HLA-DRB1 loci at least at 4 -digit resolution) was done using EDTA blood at the Institute for Medical Diagnostics (IMD), Berlin-Potsdam, Germany. 9-mer peptides carrying putative HLA class-I neoepitopes were selected by a prediction score from the netMHC-4.0 and IEDB MHC-I--2.17 software packages. For the HLA class II locus, 15 to 17-mer peptides were selected using the netMHCIpan-3.1 and IEDB MHCII-2.17.3) (using rank score of the method “IEDB recommended” in IEDB algorithm resp.) programs. Peptides carrying mutations in the predicted core region of HLA class II are preferred. Peptides were synthesized and HPLC-purified by Intavis Bioanalytical Instruments and Peptide Services (Cologne, Germany) with a purity threshold of at least 90% (mostly > 98%) using MALDI-MS and RP-HPLC (214 nm).

**Table: Peptides used for identification of TIL specificity**

<b>name of the gene (TAA/NEO)</b>	<b>amino-acid sequence of the peptide</b>	<b>HLA restriction</b>
PRPF8_Y471C	KKR <b>C</b> LFRSF	HLA-B*07:02
PRPF8_Y471wt	KKRYLFRSF	
SGIP1_G780C	<b>C</b> PSKPSPLV	HLA-B*55:01
SGIP1_G780C	<b>G</b> PSKPSPLV	
STAMPB_R170Q	MIQNQELEK	HLA-A*11:01
TRPS1_H590Y	<b>Y</b> LGEITYPF	HLA-A*02:01
TRPS1_H590wt	HLGEITYPF	
NID2_P971A	CDEQGNFL <b>A</b> LQCHGS	HLA-DRB1*04:08
NID2_P971wt	CDEQGNFLP LQCHGS	
ZNF512_A437S	FV <b>S</b> GKYKCL	HLA-C*03:03
ZNF512_A437wt	FVAGKYKCL	
GFM1_P534fs	<b>NQVVQASMEK</b>	HLA-A*11:01
DHCR7_I92fs	<b>YLVGHLPGA</b>	HLA-A*02:01
CNN1_A128D	VQSTLLAL <b>D</b> SMAKTK	HLA-DRB1*04:08
AR_Var12	VKWAKALP <b>D</b> CERAAS	HLA-DRB1*04:08
NY-ESO-1	SLLMWITQC	HLA-A2
NY-ESO-1	p81-100 (p92-100 minimal epitope)	HLA-B35, Cw3
NY-ESO-1	p91-110 (p96-104 minimal epitope)	HLA-B35, Cw3
NY-ESO-1	p113-143 (p124-135 minimal epitope)	HLA-A2

## Supplemental Figure : TIL Phenotype and Function



**Figure legend:** TIL were mostly CD8 positive T-cells (a) and the majority of CD8 cells were effector memory (EM) T-cells (CD45RA-/CCR7-) and effector memory RA (EMRA) T-cells (CD45RA+/CCR7-); Central memory (CM) T-cells (CD45RA-/CCR7+) (b) IFN- $\gamma$  release of the TIL after 24-hour stimulation with OKT3. Dotted white line shows level of IFN- $\gamma$  release without stimulation (c). PMA induced CD107a expression in TIL showing cytotoxic potential. Un-stimulated controls showed 0.18, 0.07 and 0.19 % CD107a expression in CD3+ T-cells of TIL-1, TIL-2 and TIL-3, respectively (d).

## Supplemental Figure : TIL Phenotype and Function

