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^3 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-antimicotic 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 pO2 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 10^6 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 10^9 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

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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-γ 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-γ ELISA: TIL (10.000 cells/well) were incubated with peptides (1µg/ml) in round-bottom 96-well microtiter plates in 200 µl TCM (T-Cell Medium: RPMI 1640 + 10 % human serum) for 3 - 7 days at 37°C with 5% CO_2. Culture supernatants (100µl/well) were harvested and analyzed for IFN-γ production in flat bottom 96-well multi plates using the IFN-γ ELISA kit from Mabtech AB (#3420-1A-6) according to the manufacturers instructions.

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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

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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 netMHCIIpan-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

name of the gene (TAA/NEO)	amino-acid sequence of the peptide	HLA restriction
PRPF8_Y471C	KKRCLFRSF	HLA-B*07:02
PRPF8_Y471wt	KKRYLFRSF	
SGIP1_G780C	CPSKPSPLV	HLA-B*55:01
SGIP1_G780C	GPSKPSPLV	
STAMBP_R170Q	MIQNQELEK	HLA-A*11:01
TRPS1_H590Y	YLGEITYPF	HLA-A*02:01
TRPS1_H590wt	HLGEITYPF	
NID2_P971A	CDEQGNFLALQCHGS	HLA-DRB1*04:08
NID2_P971wt	CDEQGNFLP LQCHGS	
ZNF512_A437S	FVSGKYKCL	HLA-C*03:03
ZNF512_A437wt	FVAGKYKCL	
GFM1_P534fs	NQVVQASMEK	HLA-A*11:01
DHCR7_I92fs	YLVGHLPGA	HLA-A*02:01
CNN1_A128D	VQSTLLALDSMAKTK	HLA-DRB1*04:08
AR_Var12	VKWAKALPDCERAAS	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
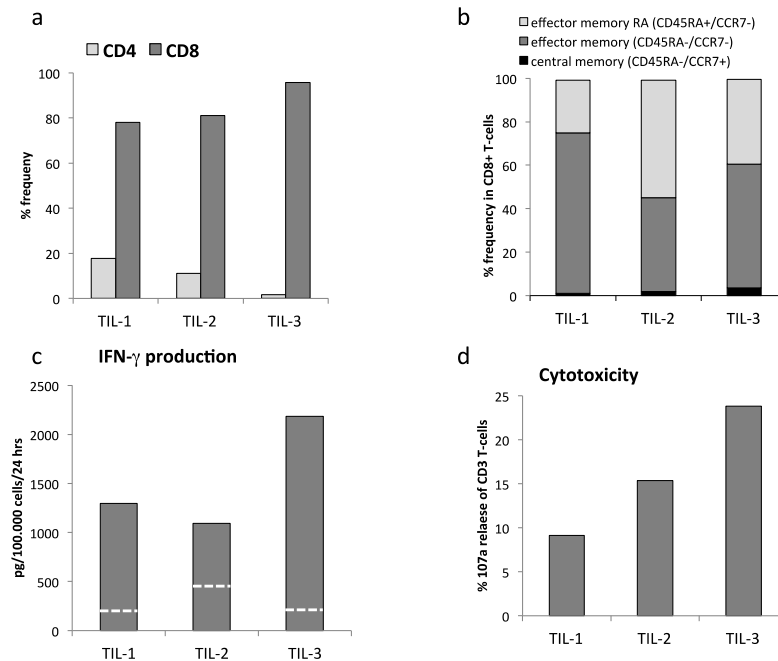

Figure legend: TIL were mostly CD8 positive T-cells (a) and the majority of CD8 cells were effector memory (EM) T-cells (CD45RA-/CCR7-) and effector memory RA (EMRA) T-cells (CD45RA+/CCR7-); Central memory (CM) T-cells (CD45RA-/CCR7+) (b) IFN-γ release of the TIL after 24-hour stimulation with OKT3. Dotted white line shows level of IFN-γ 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).

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