Supplementary MATERIALS AND METHODS

The reporting of obtained data on immune responses is in line with MIATA (Minimal Information About T cell Assays; www.miataproject.org) guidelines. Assays were validated and performed using standard operating protocols (SOPs) by experienced and trained personnel. Generation of DC Vaccine was conducted in a laboratory that operates under GMP principles. Immunomonitoring was conducted in a laboratory that operates under GLP prinicples. The immunomonitoring laboratory regularly participates in external proficieny panels for ELIspot, peptide-HLA multimer stainings and intracellular cytokine secretion (ICS) assays conducted by different organizers.

Generation and Administration of the DC Vaccine

Standardized DCs were generated from an initial apheresis essentially as described previously *(121)*. In short, PBMCs isolated on Lymphoprep ™ (Nycomed Pharma) gradient were plated on NUNC Cell factories ™ (Nalgene NUNC International) in RPMI 1640 (Cambrex, Belgium) supplemented with 2mM glutamine (Cambrex, Belgium), 20µg/ml gentamycin (Refobacin7, Merck, Germany) and 1% heat inactivated autologous plasma. Adherent cells were cultured in 800 U/ml GM-CSF (available as a drug; Leukomax, Sandoz, Germany) and 250 U/ml IL-4 (GMP quality of Strathmann, Germany) for 6 days, then matured by adding 10 ng/ml TNF-a (available as drug; Beromun, Boehringer Ingelheim, Germany) 2ng/ml IL-1b (GMP quality of ACM, Germany), 1000 U/ml IL-6 (GMP quality of Novartis, Germany) and 1µg/ml prostaglandin E2 (available as drug; Minprostin E2, Pharmacia Upjohn, Germany). Added cytokines were either licensed drugs or produced according to GMP standards.

For cohort 1 on day 6 a small proportion of cells was additionally separated and pulsed with 10µg/ml endotoxin free KLH (endotoxin-free keyhole limpet hemocyanin; obtained from Calbiochem, and biosafety tested in analogy to GMP guidelines) in order to be able to inject a separate batch of 4 mio DC loaded with KLH at vaccination # 1.

Mature DC were harvested on day 7, divided into eight fractions and loaded with six HLA class I and six class II peptides at a concentration of at least 10 and 20µg/ml, respectively, for 4 hours (six separate batches, one for each class I peptide to avoid non-controllable competition between peptides and 2 batches for 2 groups of HLA class II peptides). 6 HLA-A1 and 6 HLA-A2 peptides were used for loading cells with HLA class I peptides: HLA-A1 (MAGE-1 =

EADPTGHSY, MAGE-3 = EVDPIGHLY, Tyrosinase 243-251 = KSDICTDEY, Tyrosinase 146-156 = SSDYVIPIGTY), FluNP = CTELKLSDY, FluPB1 = VSDGGPNLY), HLA-A2.1 (MAGE-10 = GLYDGMEHL, gp100 ana = IMDQVPFSV, MelanA ana = ELAGIGILTV, NY-Eso1 ana = SLLMWITQV, IMP = GILGFVFTL, FluBNP = KLGEFYNQMM). Patients positive for both respective HLAs received cells simultaneously loaded with peptides for both HLA types. All peptides used were produced in GMP quality by Clinalfa, Läufelfingen, Switzerland.

As HLA class II restricted peptides in contrast to HLA I restricted ones are rather promiscuous in binding to HLA molecules, cells were pulsed with class II peptides irrespective of the actual expression of HLA-DP4, HLA-DR11, HLA-DR13 or HLA-DR4 by the patient's cells. One half of the cells for HLA class II loading was pulsed with Tyrosinase.DR4 (SYLQDSVPDSFQD), MAGE-3.DR13 (LLKYRAREPVTKAE) and MAGE-3.DP4 (KKLLTQHFVQENYLEY) peptides, while the other half of cells was pulsed with gp100.DR4 (WNRQLYPEWTEAQRLD), MAGE-3.DR11 (TSYVKVLHHMVKISG) and NY-Eso1.DP4 (SLLMWITQCFLPVF).

To one half of the class I and class II loaded DC of patients in cohort 1 additionally 1 µg/ml soluble trimeric CD40L in GMP quality (kindly provided by Immunex Corporation, Seattle, WA, USA) *(122)* was added (in patients with uneven recruitment numbers to batch 1 to 4, in patients with even recruitment numbers to batch 5 to 8; see Figure 1 B, Loading of DC).

In Cohort 2 all cells loaded with HLA class I peptides (batches 1 to 3, 5 to 7) were simultaneously loaded with 2 µg/ml KLH in full GMP quality (Immucothel®, Biosyn, Germany). Thereafter cells were frozen in batches of 12 mio cells in human serum albumin (pharmaceutical grade, Aventis, France) or (from patient CD40L-50-AR onwards) in 20% autologous human serum, 10% DMSO (Sigma-Aldrich, USA) and 5% glucose (pharmaceutical grade, Fresenius, Germany) at a concentration of 12 mio cells/ml in the gas phase of liquid nitrogen for later use.

Release criteria for DC included typical morphology (>95% non-adherent veiled cells), phenotype (>95% HLA-DR+++, CD86+++, CD40+, CD25+, > 75% CD83++) and negative microbial tests.

For vaccinations cryopreserved DC were thawed in a 37° C water bath, diluted with cold PBS and loaded into 10 ml syringes for deep intradermal injections.

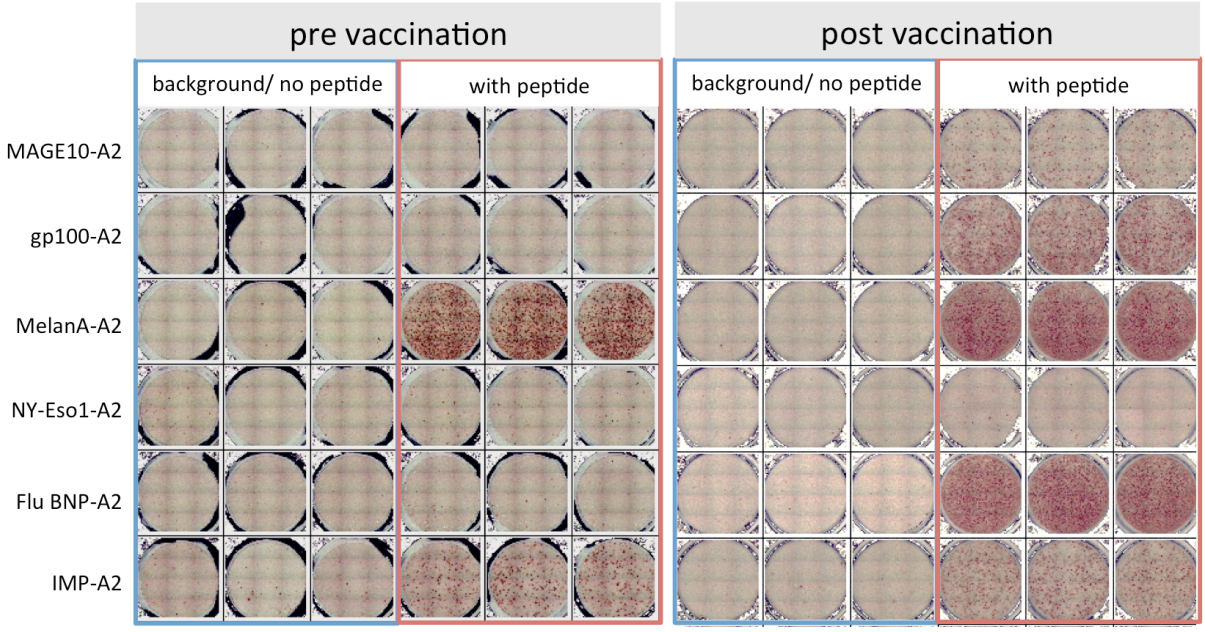
PBMC sample collection, preparation and storage

At indicated vaccination and leucapheresis timepoints (see Figure 1) PBMC were isolated from blood or aphereses by density gradient centrifugation (Lymphoprep) within 30 min to 1 hour after sampling. Cells were either used directly after isolation for analysis of immune responses by ELIspot, or frozen for later analyses in freezing media containing 5% glucose (Glucosteril 40%, Fresenius), 15.5% Human serum albumin (HSA 20%, Baxter) and 10% DMSO (Sigma). Vials were stored in the gas phase of liquid nitrogen until use.

In vitro stimulation ELIspot analysis

Freshly isolated PBMC were seeded in a 24 well plate ($1x10^6$/well) in RPMI1640 medium (supplemented with pretested 10% heat-inactivated human serum, glutamine, gentamycin, HEPES, pyruvate, nonessential amino acids) and corresponding vaccine or control peptides (5 µg/ ml). IL-2 (5 U/ ml) and IL-7 (10 ng/ ml) were added. Then, cells were incubated at 37°C for 7 days. On day 7, cells were harvested, counted, and then seeded ($3x10^5$/well), in RPMI1640 medium supplemented with 5% heat-inactivated human serum, in triplicate to nitrocellulose-bottomed 96 well plates (MAHA S4510), that were pre-coated with the primary anti-IFNγ mAb in PBS (1-D1K, Mabtech, Stockholm). For the detection of antigen-specific T cells, 10µg/ml of the respective peptide was added. Two negative controls were performed: cells were either left untreated or were stimulated with HIV-derived peptides (for HLA-A1: GSEELRSLY and for HLA-A2: ILKEPVHGV). For a positive control, cells were either stimulated with PHA (5 µg/ ml)/ SEA (20 ng/ ml) or with a mixture of Influenza, CMV or EBV-derived peptides (for HLA-A1: VSDGGPNLY, CTELKLSDY, DSELEIKRY, LTEWGSGNRTY and for HLA-A2: GILGFVFTL, CLGGLLTMV, LLDFVRFMGV, GLCTLVAML, NLVPMVATV), according to patient´s HLA type. Following incubation at 37°C for 20 hrs. wells were washed 6 times, incubated with biotinylated 2^{nd} mAb to IFNγ (clone 7-B6-1, Mabtech) for 2 hrs, washed and stained with 100µl/well ABC Vectastain Elite (Vector Laboratories, Burlingame, CA, USA) washed and stained with 100µl/well 3-Amino-9-Ethylcarbazole (Sigma Aldrich). Plates were evaluated using the Carl Zeiss Vision Axioplan 2 automated reader system, software KS ELIspot 4.13.0. Spot parameters were established using SOPs. Representative pictures of wells from patient 57 see below. Results were audited. Positive reactivity to an experimental antigen was defined as more than 10 spots and 2.5 fold above background (corresponding negative/

unstimulated control). Responses were not considered positive if the antigen-specific reactivity level was below the Limit of Detection (LOD = 10 spots per well) for this assay protocol.



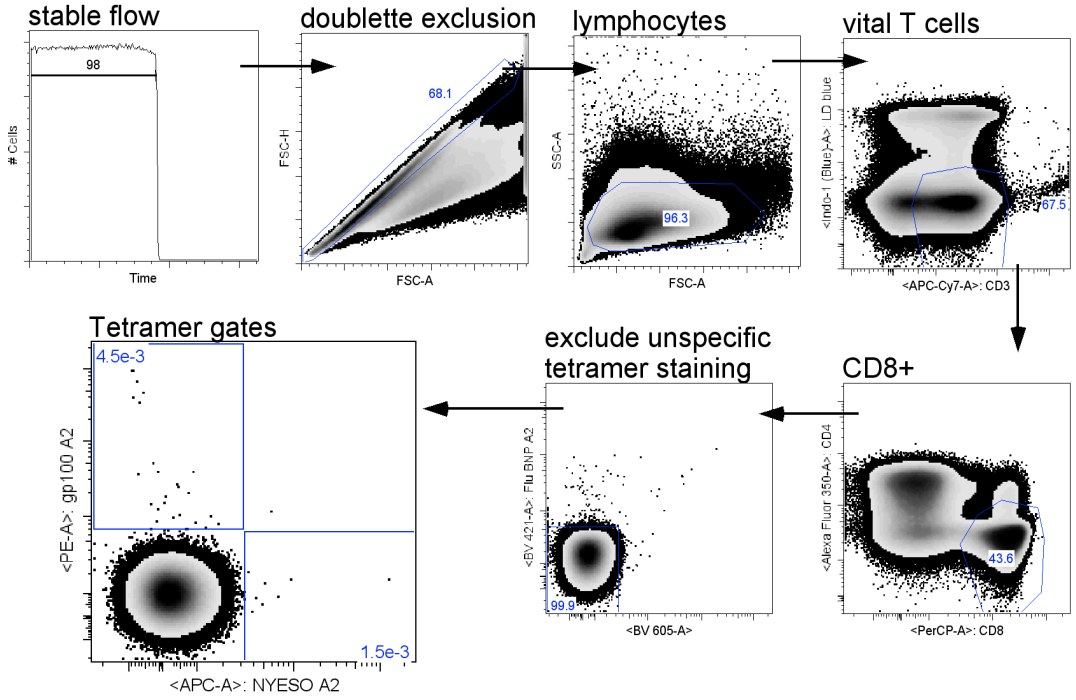
Ex vivo Class I Peptide/HLA-tetramer staining

For the ex vivo peptide/HLA-multimer staining, between 10 and 15 million thawed and washed PBMC were used per staining. Cells were first stained with the dead-cell stain Live/Dead blue (Invitrogen) in PBS. Then, the cells were spun down and stained simultaneously with four different peptide/HLA-tetramers labeled with PE, APC, BV421 or BV605 (all from TCmetrix), corresponding to the patient´s HLA type and peptides used for vaccination. After 20 minutes at room temperature, surface-staining antibodies (CD3 – APC-H7, CD4 – BUV395, CD8 - PerCP, CD45RA – PE-Cy7 all from BD) were added for an additional 20 minutes. The cells were then washed with PBS, fixed and permeabilized according to manufacturers instructions (fix and perm solutions from eBioscience). Intracellular staining was performed with GranzymeB – AlexaFluor700, Perforin - FITC and ki67 - BV711 antibodies (all from BD) in permwash for 25 minutes, after which the cells were washed again and re-suspended in PBS. Cells were analyzed on a BD FACS Fortessa.

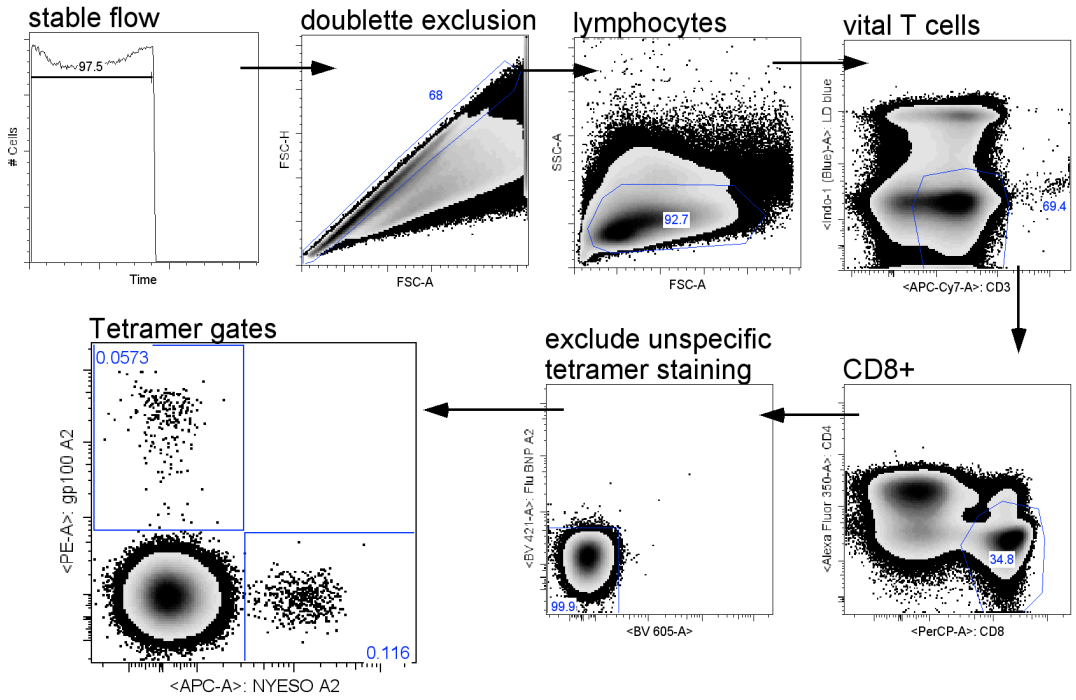
Examples of stainings and the gating strategy are shown below. The standard cut-off criteria for a positive response by pHLA-multimer staining were, a minimum of 10 cells detected in the multimer-gate, a minimum percentage of 0.01% of the $CD8^+$ T cells, and that the pHLA-

multimer positive cells formed a population that was clearly separated from the pHLA-multimer negative one.

Patient 45, pre vaccination



Patient 45, post vaccination



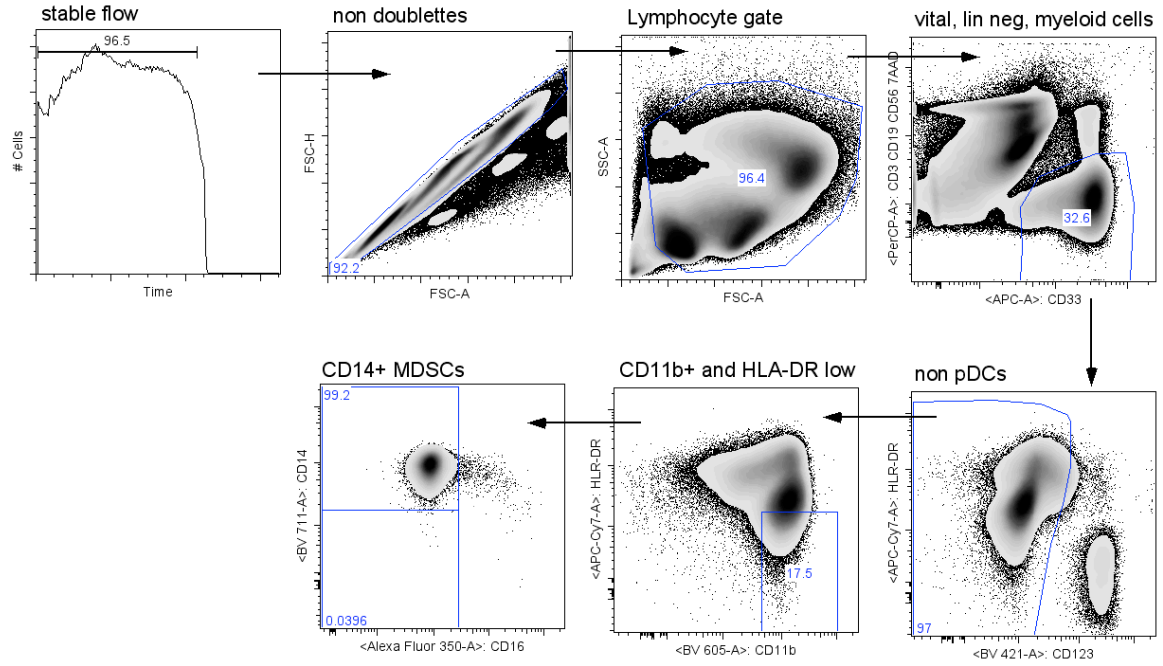
Ex vivo Class II Peptide/HLA-tetramer staining

For the ex vivo peptide/class II-HLA-multimer stainings between 5 and 15 million thawed and washed PBMC were used. Cells were first stained with the dead-cell stain Live/Dead aqua (Invitrogen) in PBS. The cells were then spun down and stained with PE-labelled tyrosinase peptide/HLA-DR4 tetramer (SYLQDSVPDSFQD-HLA-DR4 from Beckman Coulter) in RPMI1640 medium (supplemented with pretested 10% heat-inactivated human serum, glutamine, gentamycin, HEPES, pyruvate, nonessential amino acids). After 45 minutes at 37˚C the surface-staining antibodies (CD4 – V450, CD8 - PerCP, CD127 – APC-Cy7 and CD14 – PacificOrange, all from BD) were added for an additional 15 minutes at room temperature. The cells were then washed with PBS, fixed and permeabilized (eBioscience solutions as described). Intracellular staining was performed with FoxP3-antibody (AlexaFluor700 labeled, from eBioscience) for 30 minutes at 4˚C, after which the cells were washed again, resuspended in PBS and analyzed on a BD FACS AriaII. Cells were pre-gated on stable flow, non-doublettes, lymphocyte gate, vital CD14 negative cells, CD4 positive and CD8 negative. Examples of stainings are shown in Figure 4C.

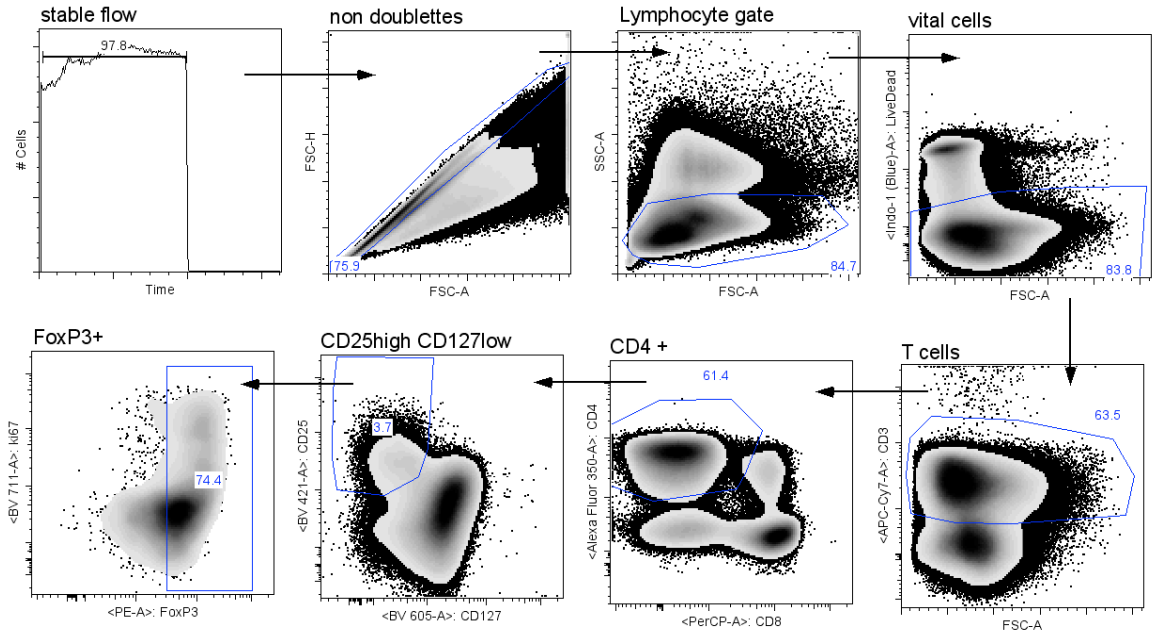
Analysis of MDSC and Treg

For the staining of myeloid suppressor cells (MDSCs) and Tregs, between 3 and 5 million thawed and washed PBMC were used. The MDSC-Panel contained a lineage-/ dump channel with PerCP-conjugated antibodies for CD3, CD19, CD56 (all from Biolegend) and 7AAD as dead cell marker. In addition, CD11b-BV605, CD11c-BV510, CD14-BF711, CD16-BUV395, CD33-APC, CD80-AlexaFluor700, CD83-FITC, CD124-PE, CD274-PE-Cy7 and HLA-DR-APC-Cy7 (all from BD) were stained together in PBS for 20 minutes. Then, cells were washed twice with PBS. For the Treg-staining, cells were first stained with the dead-cell stain Live/Dead blue (Invitrogen), followed by surface staining for CD3-APC-H7, CD4-BUV-395, CD25-BV421, CD45RA-BV510, CD127-BV605, CD279-PE-Cy7 (all from BD) and CD8 – PerCP (Biolegend), and then intracellular staining with FoxP3-PE (eBioscience), CTLA4-APC (BD), GranzymeB-AlexaFluor700 (BD) and ki67-BV711 (BD) antibodies in permwash. Then, cells were washed twice with PBS. Samples were analyzed on a BD FACS Fortessa.

Gating strategy for MDSCs (shown for patient 20, pre vaccination)



Gating strategy for Tregs (shown for patient 07, pre vaccination)



MLPC limiting dilution

For the mixed lymphocyte/peptide culture limiting dilution assay with subsequent readouts, we used a described in Godelaine et al. *(56)* with minor modifications. Briefly, on day 0 approximately 60 mio PBMC (prepared from aphereses) were thawed in warm complete medium consisting of Iscove's modified Dulbecco's medium (Lonza, Verviers, Belgium), heat inactiviated human pooled serum (Lonza, Verviers, Belgium), L-Arginine (Merck, Darmstadt, Germany), L-Asparagine (Merck, Darmstadt, Germany), L-Glutamine (Merck, Darmstadt, Germany), Methyltryptophan (Sigma, St Louis, USA) and Gentamycin (PAA, Pasching, Austria). After thawing, cells were counted with an automated cell counter (Casy, Schäfer Systems). An aliquot of 1-2 mio PBMC was used for flow cytometric analysis of CD3, CD4 and CD8. The remaining cells were divided equally into different tubes (5-10 mio each) and incubated separately with the HLA Class I restricted vaccination peptides (MAGE-A1, MAGE-A3, Tyrosinase, MelanA, gp100, MAGE-A10, NY-ESO1), according to the patient´s HLA, at a concentration of 10µM, for 1 hour at 37°C. Then, cells were pooled, re-counted and washed. The median recovery after thawing and resting was 62% of the originally thawed cells. Cells were then transferred to a 96 well plate (1 to 2 $x10^5$ cells per well) in complete medium containing IL-2 (5U/ml; Roche, Switzerland) and IL-7 (10ng/ml; TEBU, France). On day 7, a re-stimulation was performed by repeating the peptide loading procedure (day 0) with freshly thawed autologous PBMC irradiated with 100Gy. Subsequently, 1.6 $x10^5$ irradiated cells (stimulator/ feeder cells) were added to each well of the initial peptide loaded PBMC with 5 U/ml IL-2. On day 9, the cells were divided into an additional 96 well plate, and fed with 100µl complete medium with IL-2 (5U/ml final concentration) per well. Division and addition of medium and IL-2 of plates was repeated on day 11, if sufficient cells were present. On day 14, each well of one of the 96 well plates was harvested separately, and analyzed using one of the following assays.

MLPC-Tetramer readout

For the tetramer readout of the MLPC limiting dilution assays, each sample was stained at room temperature with one PE-coupled peptide/HLA-tetramer (10nM 15 min for class I and 100nM 60 min for class II, tetramers provided by the De Duve Institute, Brussels, Belgium) and CD8- FITC (BD), respectively. After two washing steps, the cells of each well were acquired on a BD FACS

Calibur, or a BD FACS Canto II instrument. A well was considered positive if ≥ 0.1% of the CD8-positive cells were tetramer-positive, and formed a clearly separated population.

MLPC-Chromium-release readout

The cells in the cognate wells of one or two of the remaining 96 well plates, were analyzed for their lytic capacity in a standard 4 hour ^{51}Cr-release assay. As targets we used peptide-loaded T2A1 cells (peptide 10μg/ml for 1 hour at 37°C). As negative control T2A1 cells without peptide and K562 cells were used. In all assays an excess of non-labeled K562 cells was used to block NK activity. In some cases, enough cells were obtained to perform the assay with two different peptide concentrations (10μM versus 10nM), in order to obtain an estimate of the functional avidity of the cells. In some patients with positive immune responses against the vaccination peptides, the peptide-stimulated cells were also tested for the lysis of autologous (patient 31-WO) or HLA-matched (patient 46-MM) melanoma cells.

MLPC-Intracellular cytokine production readout

Cells from one corresponding 96 well plate were washed with MLPC medium and stimulated overnight (37°C, 5% CO2) in a 96 well plate in MLPC medium with the corresponding peptide in the presence of brefeldin A, monensin and CD107a-FITC antibody (BD). The next day, cells were washed with PBS and stained with dead-cell stain Live/Dead aqua (Invitrogen, USA) according to the manufacturer's instructions. The cells were then spun down, the supernatant was discarded and the cells were resuspended in PBS containing surface-staining antibodies reactive for CD8 - PerCP, CD4-PE, and CD14-Pacific Orange (all from BD). After 20 minutes at 4˚C cells were washed with PBS and fixed and permeabilized with fix/perm solution and perm/wash (both eBioscience, USA) according to the manufacturer's instructions. Intracellular staining was performed in perm/wash with IL2-APC, TNF-PE-Cy7 and IFNγ-AlexaFluor700 antibodies (all from BD), for 30 minutes. Cells were once again washed and resuspended in PBS. Each well was then acquired separately on a BD FACS Canto.

Analysis of flow cytometry data

Data acquired with the FACS Calibur (MLPC limiting dilution tetramer readout) was analyzed with BD Cellquest software. FlowJo (9.1) was used to analyze data acquired from BD FACS Canto and Fortessa.

Analysis of MDSC and Treg

For the staining of myeloid suppressor cells (MDSCs) and Tregs thawed PBMC were surface/ intracellular stained with the two following panels: MDSC-Panel (lineage-/ dump channel with PerCP-conjugated antibodies for CD3, CD19, CD56 and 7AAD + CD11b, CD11c, CD14, CD16, CD33, CD80, CD83, CD124, CD274 and HLA-DR) and Treg-Panel (Live/Dead blue, CD3, CD4, CD8, CD25, CD45RA, CD127, CD279 and intracellular FoxP3, CTLA4, GranzymeB and ki67). Stained samples were analyzed on a BD FACS Fortessa.

Luminex/ Cytokine Bead Array analysis

PBMC were thawed, washed and rested for two hours at 37°C in RPMI1640 medium (supplemented with 10% heat-inactivated human serum, glutamine, gentamycin, HEPES, pyruvate, nonessential amino acids). Then the vaccination-peptides were added at 5 µg/ml. The next day IL-2 (5 U/ ml) and IL-7 (10 ng/ ml) were added and half of the medium was replaced every three to four days by fresh medium containing IL-2. After 14 days of prestimulation, cells were harvested, counted, and then seeded ($3x10^5$/well) in 96-well roundbottom plates. Then peptides (5 µg/ml) were added and cells were incubated at 37°C for 24 hours. As negative control cells were incubated without peptide and as a positive control CD3/ CD28 T cell expander beads (Dynal/ Life Technologies) were added. Supernatants were analyzed by using the Human Th1/Th2 & Chemokine 20-plex Kit supplemented with simplex kits for IL-13, IL-10 and IL-17A (ProcartaPlex,eBioscience) according to manufacters instructions on a MagPix instrument (Luminex).

Table S1) Patient characteristics

Patients still alive are marked in grey.

LDH: n = normal, e = elevated
Metastases at study entry: LN = lymph node; m = multiple (> 3 metastases in respective organ); DD = radiologist discussing differential diagnosis

FE: = fully evaluable (patient received at least 4 vaccinations and underwent 2nd apheresis)
PFS = progression free survival
OS = overall survival:
* = death not disease-related
+ = continuing

Treatment prior or after start of trial:
S = surgery
I = immunotherapy (IFN-α, IL-2)
C = chemotherapy
CI = chemoimmunotherapy
R = radiotherapy
RC = radiochemotherapy
PV = peptide/IFA vaccination
DCV = Dendritic Cell vaccination (other than trial vaccine)
So = Sorafenib
KI = BRAF and/or MEK inhibitor
Ipi = Ipilimumab
ILP = isolated limb perfusion
U = Unknown

~~Crossed-Text~~ indicates metastases that were excised or destroyed by radiation therapy *before* start of the trial

Patient code	Sex	Age	AJCC stage	N/M classifi-cation	Tumor bearing	Prior treatment	LDH	Sites of metastases at trial start	F.E	PFS (months)	OS (months)	Number of vaccs	Treatment after trial start
01	f	48	IV	M1c	y	S, C	e	LN, Liver, Lung	no	1	7	2	PC
02	f	58	IV	M1b	y	S, R, I, C, CI	n	Skin m, Lung	no	3	10	1	C, R
03	m	34	IV	M1b	y	S	n	Skin m, Lung m	yes	1	35	18	PC, R, S, ILP
04	f	58	III	N2c	n	S	n		yes	9	176 +	31	ILP
05	m	36	IV	M1b	y	S	n	LN m, Lung m	no	2	3	2	
06	m	52	IV	M1c	n	S, R, I	n	~~Liver~~	yes	17	117 *	23	C, S
07	f	60	IV	M1a	y	S, ILP	n	LN mesenterial	yes	29	45	20	C, PC
08	m	67	III	N3	n	S, R, I, C	n		yes	87	87 *	10	
09	f	56	IV	M1a	y	S, I	n	Skin m, LN	yes	3	47	8	CI, R, DCV, S
10	m	20	IV	M1c	y	S, I, C	n	Lung, Pancreas	yes	10	23	7	RC, DCV
11	f	60	IV	M1c	y	CI, S, R, I	n	Lung m, Liver, Bone	yes	4	15	7	C
12	m	65	IV	M1a	y	S	n	LN paraaortal, kidney (DD)	yes	167	172 +	24	
13	m	55	IV	M1b	y	S, I	n	Lung, LN paraaortal, Mamma (DD)	yes	167	172 +	28	S
14	f	34	III	N2a	n	S	n		yes	166	171 +	10	
15	m	48	IV	M1c	y	S	n	Lung, Liver m	yes	32	54	27	PC, R, So, PV, S
16	m	63	III	N2c	n	S, I	n		yes	6	113	16	R, PV, S
17	f	49	IV	M1a	y	S	n	LN visceral	yes	24	169 +	15	S
18	f	48	IV	M1b	y	S, I,	n	Lung m	no	3	47	4	U
19	m	66	IV	M1b	y	S, R	n	Skin m, Lung m	yes	5	17	14	S
20	f	22	III	N1b	n	S, I	n		yes	164	169 +	11	
21	f	25	IV	M1a	y	S, I, R, H, C	n	Skin m, LN m, LN visceral	yes	1	104	8	C, So, PV
22	f	21	IV	M1a	y	S	n	LN visceral	yes	5	168 +	41	S
23	m	52	IV	M1b	y	S	n	Lung (DD)	no	2	11	3	U
24	f	69	IV	M1c	y	S	n	Mamma, ~~Muscle~~	yes	4	50	4	S, U
25	m	37	IV	M1c	y	S	n	Intestines, LN visceral	no	2	20	4	RC, PC, S
26	f	33	IV	M1a	n	S	n		yes	69	95	18	
27	m	68	IV	M1c	y	S, R	e	Lung m, LN visceral, Adrenal gland	yes	20	29	15	R
28	m	44	IV	M1c	y	S, I	n	Lung m, Adrenal gland	yes	4	22	4	R, C
29	f	41	III	N1b	y	S	n	LN	yes	16	165 +	12	PV, S, R
30	m	21	III	N1a	n	S	n		yes	3	163 +	17	S, DCV
31	m	64	IV	M1c	n	S	e		yes	23	63	25	C, PC, So, PV
32	f	44	III	N3	y	S, I, C, R	n	Skin m	yes	1	51	18	C, R
33	f	72	IV	M1c	y	S, R, C	n	Muscle, Kidney, ~~CNS~~, Lung, Liver	yes	51	51	8	
34	f	63	IV	M1b	y	S, C, R	n	Skin m, Mammae, Lung m (DD)	yes	27	41	20	C, R
35	m	44	IV	M1c	y	S, R	n	Muscle/Soft tissue, LN m, Pleura	yes	5	64	9	C, So, DCV, S
36	m	66	IV	M1b	y	S	n	Skin, Lung	yes	16	155 +	57	S, R, Ipi, DCV
37	m	53	IV	M1a	n	S, I	n		yes	150	155 +	17	
38	f	40	IV	M1c	y	S, CI, R, I	e	Intestines, LN visceral	yes	5	50	21	R, C, PCT, So
39	f	37	III	N1b	n	S	n		yes	148	153 +	20	
40	m	45	IV	M1c	y	S, I	e	Lung, Thyroid gland (DD), ↑LDH	yes	4	18	5	U
41	f	78	IV	M1a	n	S	n	~~LN~~	yes	1	27	10	C, PV
42	f	62	IV	M1c	y	S, ILP	e	Skin, LN, Lung m, ↑LDH	yes	1	23	8	C, S, PV
43	m	22	IV	M1a	n	S, R, I	n		yes	32	115	20	DCV, R, PC, S, So, Ipi, KI
44	f	64	III	N2c	y	S, C	e	Skin	yes	1	43	8	CI, ILP
45	f	38	III	N1b	n	S	n		yes	57	101	21	S, R, DCV
46	f	26	IV	M1c	y	S, I, PC, R	n	Lung ~~m, CNS~~	yes	6	41	26	C, So, R
47	f	32	III	N1a	n	S	e		yes	145	150 +	13	
48	f	65	III	N1a	n	S	e		yes	34	150 +	15	R, S, DCV
49	f	75	IV	M1c	y	S, R	e	LN visceral	yes	4	15	5	R, C
50	f	53	III	N1b	n	S, I, C	n		yes	144	149 +	15	
51	m	62	IV	M1c	y	S	n	LN, Lung m, Liver m, Bone, Spleen	yes	4	12	5	R, C
52	m	75	IV	M1b	y	S, R	n	Soft tissue, LN (DD), Lung (DD)	no	4	26	2	R, C
53	m	50	IV	M1c	y	S	n	LN, Lung m, Bone	yes	3	9	4	
54	m	74	IV	M1c	y	S, I	n	Bone	yes	4	9	7	R, C, S
55	f	41	IV	M1a	n	S	n		yes	142	147 +	14	
56	m	40	IV	M1c	y	S, R	n	LN, LN visceral, ~~Bone~~	yes	3	18	13	R, C, S
57	m	36	IV	M1a	n	S, I	n		yes	135	140 +	19	
58	m	44	IV	M1c	y	S, I, PC	e	LN, Lung m, Liver m	no	2	6	3	R, PC
59	m	30	III	N3	y	S	n	Skin	no	1	19	4	R, S
60	m	54	IV	M1a	n	S, I	n		yes	135	140 +	17	PV
61	f	31	IV	M1c	n	S, R	n	~~CNS~~	yes	2	144 +	14	S, DCV
62	m	39	III	N2b	y	S	n	LN	yes	2	36	22	R, C, So, PV

Table S2) Quantification of intratumoral T cells (Lymphocyte Score) in accessible metastases before and after vaccination

A melanoma immunoscore (lymphocyte score) was determined by semiquantitative assessment of the distribution and intensity of tumor infiltrating CD3+ T cells as recently described (see reference 45 and Materials and Methods). The score ranges from 0 (no T cells) to 6 (very strong infiltration of T cells into the tumor), whereby a favorable prognosis has been shown for score 3-6 in contrast to an unfavorable prognosis for score 0-2. In some patients the score as shown in the table represents the mean score of several tumor samples from different sites or slightly different timepoints.

unfavorable score 0 - 2
favorable score 3 - 6
not done

Patient	Pre-vaccination	induction	maintenance
02	2.0		
03	0.0	1.5	2.0
06	2.0		
07	4,0		2.3
16	2.0		4.0
17		3.0	
20	2.0		
22		2.0	3.0
24	2.0	4.0	
29	6.0		2.5
33	2.0		
36		3.0	2,8
38	4.0	1.0	4.0
39	2.0		
41	0.0		
42		3.0	2.0
43	2.0		2.3
44	0.0	2.0	
55	2.0		
56		5.0	3.0
58		2.0	
59		2.0	
60	2.0		
61		2.0	
62	6.0		

Table S3) Peptide-loading of DC

Class I Peptides were grouped and loaded onto different batches of DCs to avoid competition for the same HLA molecules. In cohort 1 in addition the two peptide groups were alternately loaded onto batches of DCs generated in the presence or absence of soluble trimeric-CD40 Ligand to allow comparison of the immunogenicity of these DCs. Patients in cohort 1 with uneven recruitment numbers received peptides of group A on mature DC pulsed with soluble trimeric CD40L and peptides of group B on DC that were not treated with soluble CD40L, whereas patients with even recruitment numbers received peptides of group B on CD40L pulsed DC and peptides of group A on DC that were not treated with soluble CD40L. In cohort 2, mature DC of patients were not loaded with soluble CD40L, but all DC loaded with HLA class I peptides were pulsed with 2µg/ml KLH during maturation.

HLA A1 positive patients received	C	Seqence													N	Group
$10x10^6$ DC FluNP.A1	C	T	E	L	K	L	S	D	Y							A
$10x10^6$ DC Flu PB1.A1 aa 591-599	V	S	D	G	G	P	N	L	Y							B
$10x10^6$ DC MAGE-3.A1	E	V	D	P	I	G	H	L	Y							A
$10x10^6$ DC MAGE-1.A1	E	A	D	P	T	G	H	S	Y							B
$10x10^6$ DC Tyrosinase.A1 243-251 ana	K	S	D	I	C	T	D	E	Y							A
$10x10^6$ DC Tyrosinase.A1 aa 146-156	S	S	D	Y	V	I	P	I	G	T	Y					B
$10x10^6$ DC Tyrosinase.DR 4 450-462 ana	S	Y	L	Q	D	S	V	P	D	S	F	Q	D			A
+ MAGE-3.DR13 aa 121-134	L	L	K	Y	R	A	R	E	P	V	T	K	A	E		A
+ MAGE-3.DP4 aa 243-258	K	K	L	L	T	Q	H	F	V	Q	E	N	Y	L	E	Y	A
$10x10^6$ DC gp100.DR4 aa 44-59	W	N	R	Q	L	Y	P	E	W	T	E	A	Q	R	L	D	B
+ MAGE-3.DR11 aa 281-295	T	S	Y	V	K	V	L	H	H	M	V	K	I	S	G		B
+ NY-Eso-1.DP4	S	L	L	M	W	I	T	Q	C	F	L	P	V	F			B

HLA A2 positive patients received	C	Seqence													N	Group
$10x10^6$ DC IMP.A2	G	I	L	G	F	V	F	T	L							A
$10x10^6$ DC Flu BNP.A2 aa 85-94	K	L	G	E	F	Y	N	Q	M	M						B
$10x10^6$ DC MAGE10.A2 aa 256-262	G	L	Y	D	G	M	E	H	L							A
$10x10^6$ DC NY-Eso-1.A2 ana	S	L	L	M	W	I	T	Q	V							B
$10x10^6$ DC MelanA.A2 aa 26-35 ana	E	L	A	G	I	G	I	L	T	V						A
$10x10^6$ DC gp100.A2 aa 209-217 ana	I	M	D	Q	V	P	F	S	V							B
$10x10^6$ DC Tyrosinase.DR 4 450-462 ana	S	Y	L	Q	D	S	V	P	D	S	F	Q	D			A
+ MAGE-3.DR13 aa 121-134	L	L	K	Y	R	A	R	E	P	V	T	K	A	E		A
+ MAGE-3.DP4 aa 243-258	K	K	L	L	T	Q	H	F	V	Q	E	N	Y	L	E	Y	A
$10x10^6$ DC gp100.DR4 aa 44-59	W	N	R	Q	L	Y	P	E	W	T	E	A	Q	R	L	D	B
+ MAGE-3.DR11 aa 281-295	T	S	Y	V	K	V	L	H	H	M	V	K	I	S	G		B
+ NY-Eso-1.DP4	S	L	L	M	W	I	T	Q	C	F	L	P	V	F			B

Figure S1) Representative time courses of ivsELIspot results from select patients with long-term follow-up.

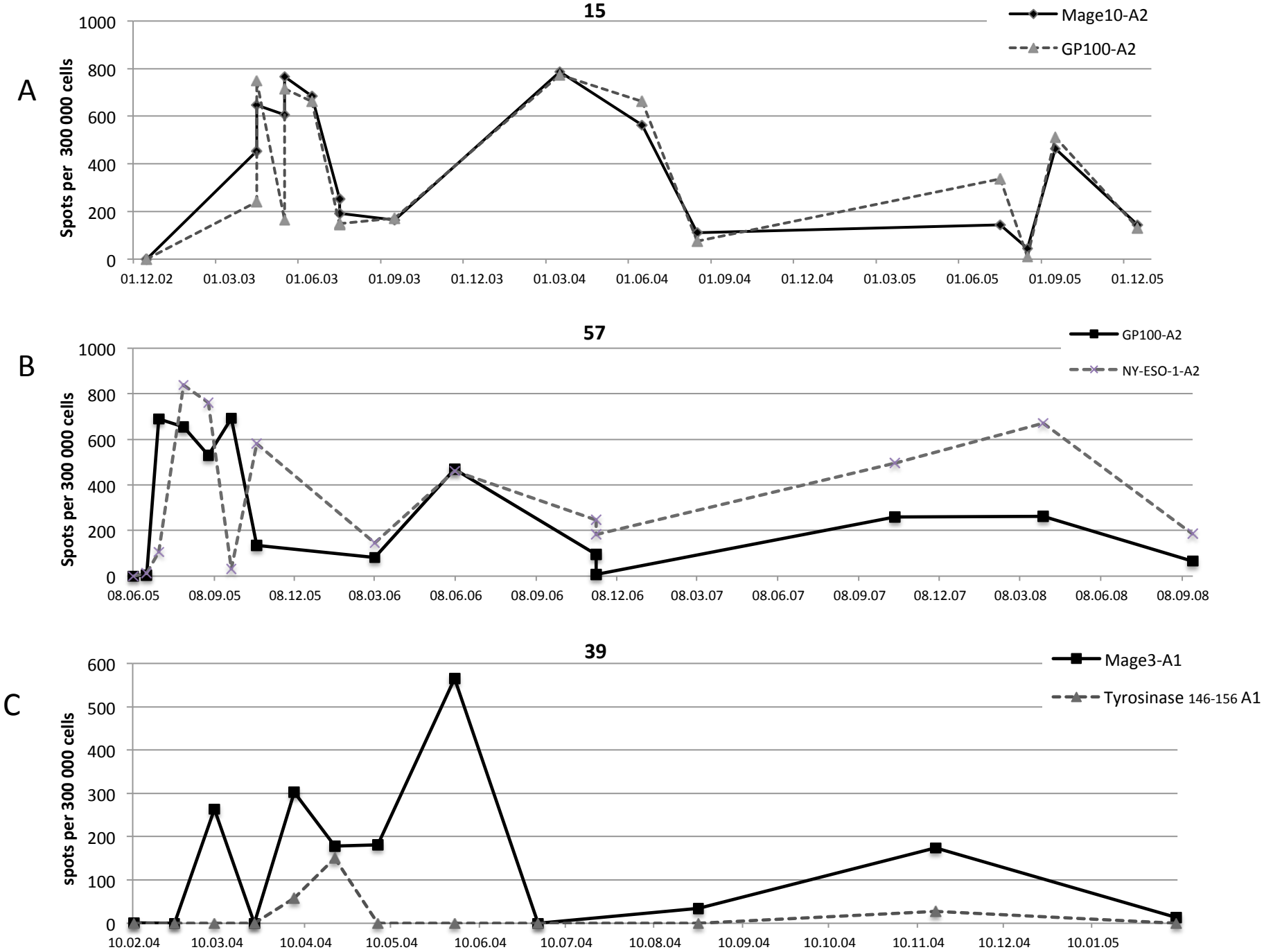
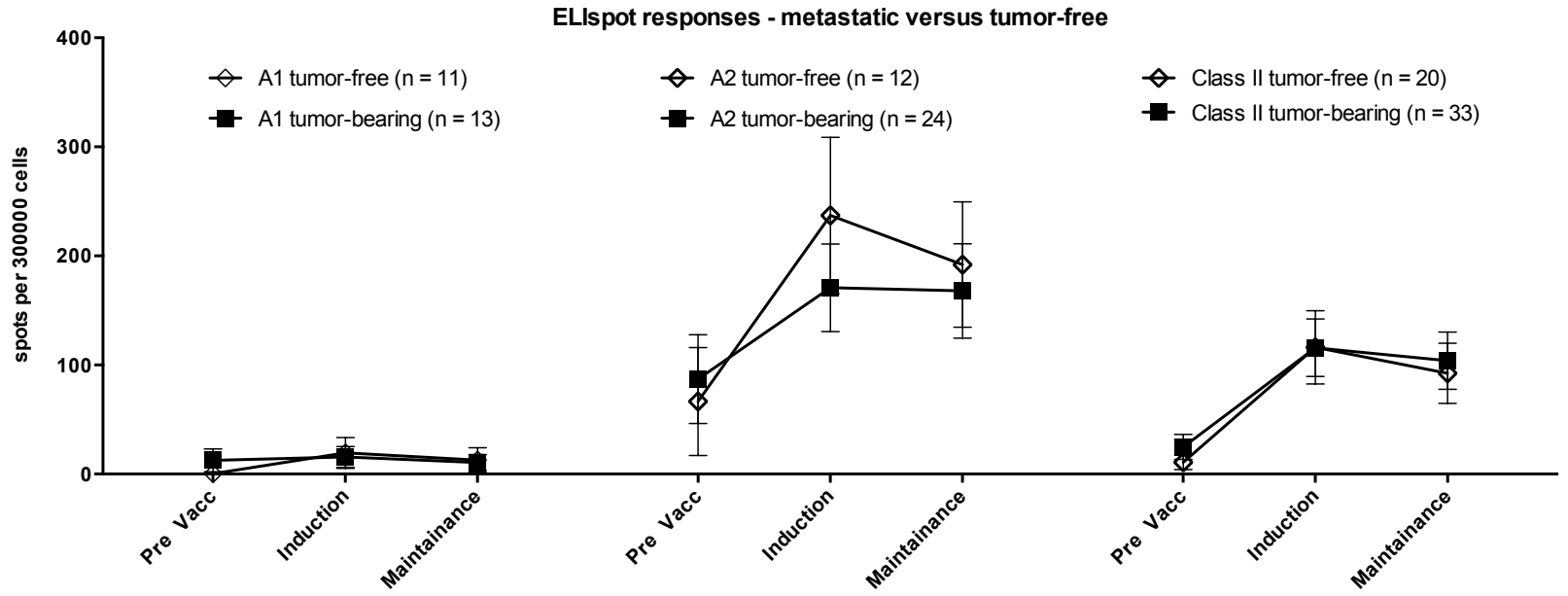


Figure S2 A,B) Immune responses of tumor-bearing and tumor-free patients

Immune responses of all patients to vaccine peptides before and during induction and maintenance phase of DC vaccination as measured by in vitro stimulated IFN gamma ELIspot are shown. Data from several timepoints are combined (1 or 2 before and 2-6 timepoints for induction and maintenance phase, respectively). Plots show the means with whiskers approximating the 95% CI. Immune responses are shown for **(A)** metastatic versus tumor-free patients, **(B)** stage III versus stage IV patients and **(C)** for patients surviving up to 60 months after start of treatment versus patients surviving longer than 60 months.

A



B

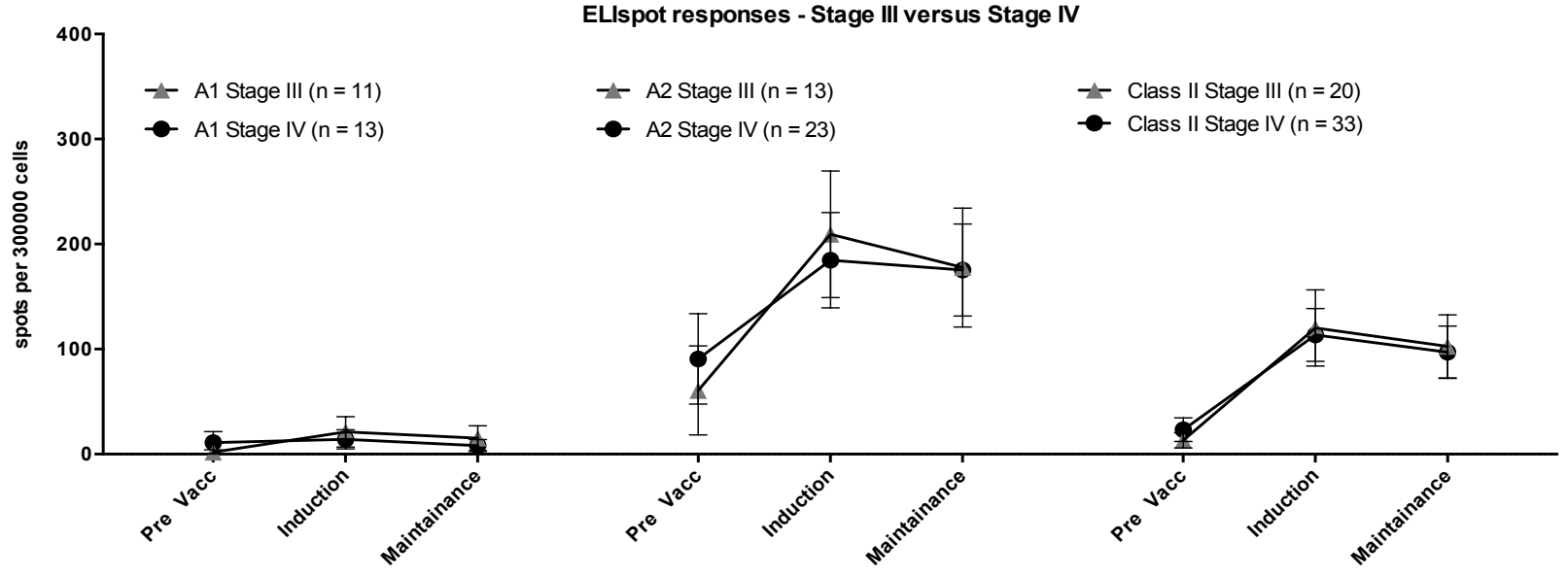


Figure S2 C) Lack of correlation between vaccine-specific immune responses and long-term survival

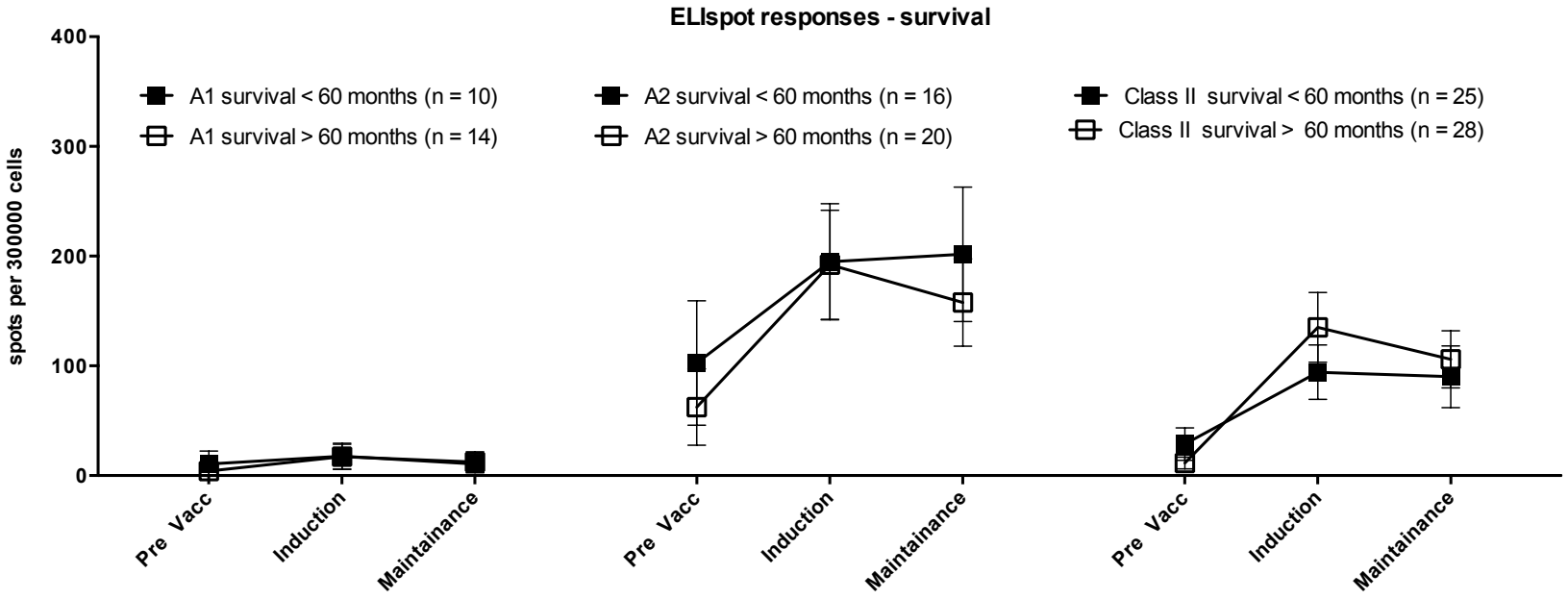


Figure S3 Frequencies of regulatory T cells (Tregs) and the portion of proliferating cells as well as frequencies of MDSCs pre- and post DC-vaccination measured in 50 patients. In the graphs mean and standard deviation are shown. For statistical analysis a two-tailed unpaired t test with 95% confidence level was used.

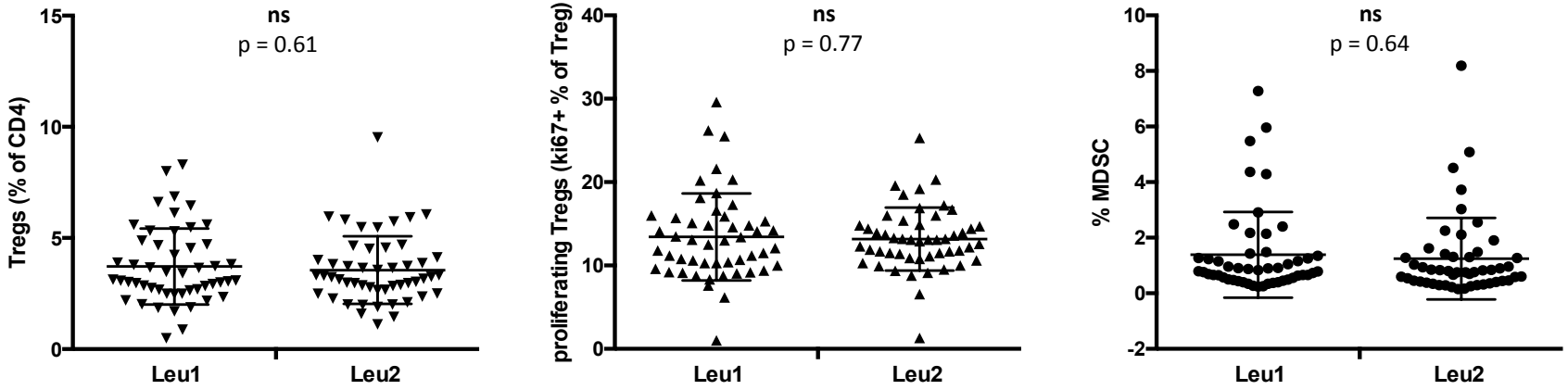


Figure S4 A-C) Functional capacity of vaccine-specific CD8+ T cells

Vaccine-specific CD8+ T cells were repeatedly stimulated under limiting-dilution conditions in a MLPC assay. (**A**) The number of tetramer-positive wells (out of 96 wells plated, each containing 200,000 PBMCs) after two rounds of stimulation is shown pre- and post-vaccination. (**B**) To determine the fraction of polyfunctional T cells the different tetramer-positive wells (containing T cell lines / clones) were functionally analyzed for intracellular cytokine-production (IFNγ, TNFα, IL-2) and lytic capacity (CD107a staining). (**C**) To determine the range of TCR affinities the tetramer-positive wells were tested for their ability to lyse target cells loaded with a high 10 µM as well as a 10 nM peptide concentration. Each vertical line represents a single well containing a certain (low or high) percentage (green triangles) of tetramer positive CD8+ T cells with varying capability to lyse targets loaded with 10µM versus only 10nm peptide depending on the affinity of the involved TCR.

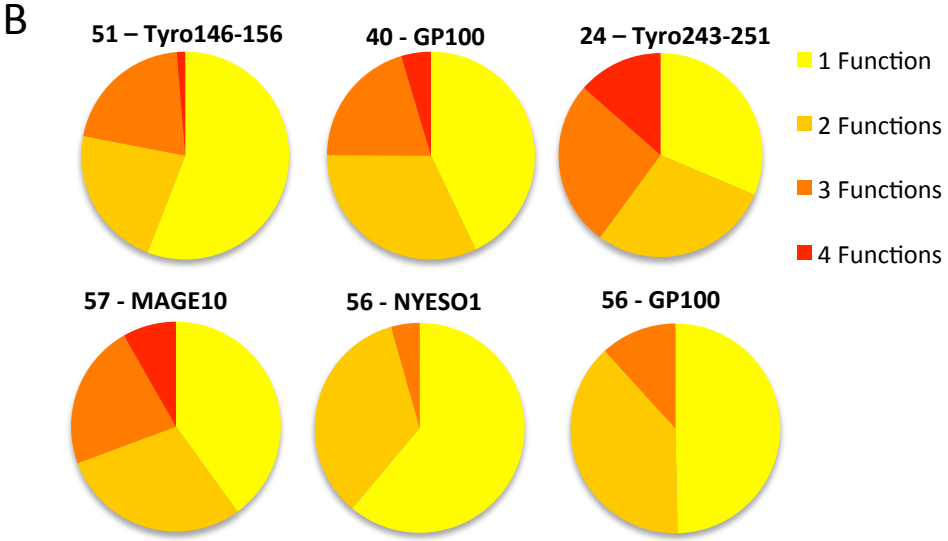
A

Patient ID	Mage1 A1 pre	Mage1 A1 post	Mage3 A1 pre	Mage3 A1 post	Tyro 243-251 A1 pre	Tyro 243-251 A1 post	Tyro 146-156 A1 pre	Tyro 146-156 A1 post
24	0	0	0	3	0	0	0	36
26	0	0	0	0	0	0	0	1
14	0	0	0	0	0	0	0	69
34	0	0	0	0	0	0	0	0
51	0	0	0	0	0	32	0	3
20	1	1	1	0	0	0	0	1

	Mage10 A2 pre	Mage10 A2 post	gp100 A2 pre	gp100 A2 post	NY ESO A2 pre	NY ESO A2 post	MelanA A2 pre	MelanA A2 post
20	0	22	4	90	52	84	70	95
27	0	11	1	85	0	74		
28	0	0	0	32	0	28		
37	0	50	0	95	0	96	0	90
40	0	7	0	23	0	8	0	73
54	0	26	0	96	2	96	23	96
56	0	3	0	37	0	44	58	96
57	0	39	0	96	0	83	31	96
04	0	48	3	75	1	96		
07	0	0	2	23	0	73		
13	1	12	8	49	1	52		
15	4	96	10	96	4	96		

n.d.	0	1 - 30	31 - 60	61 - 96

B



C

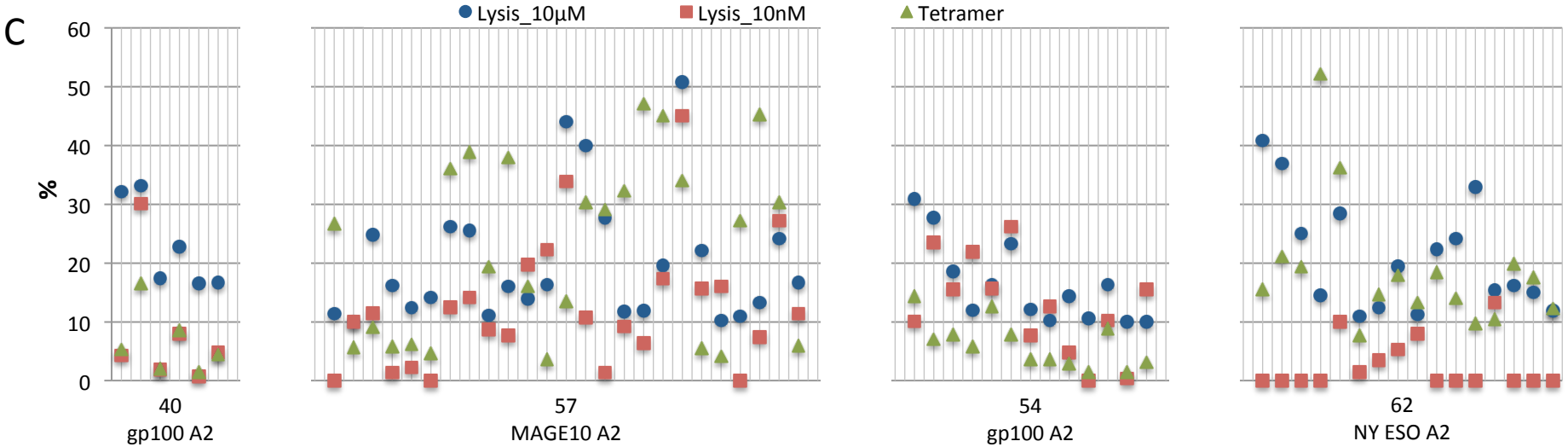
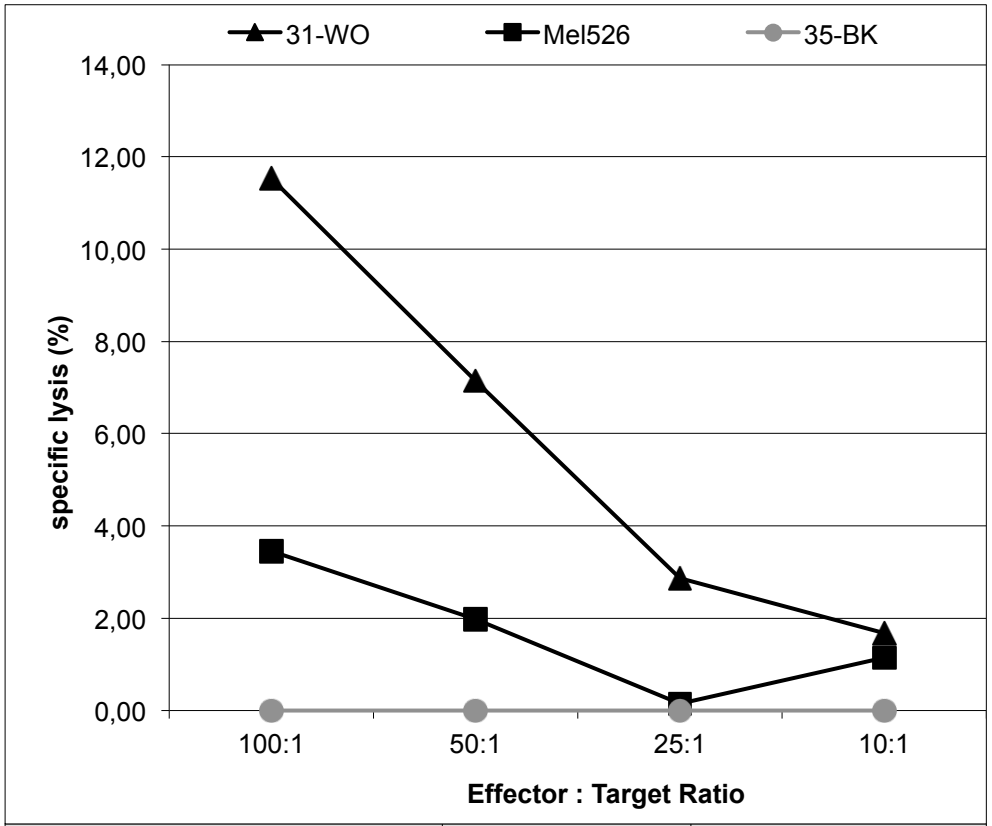
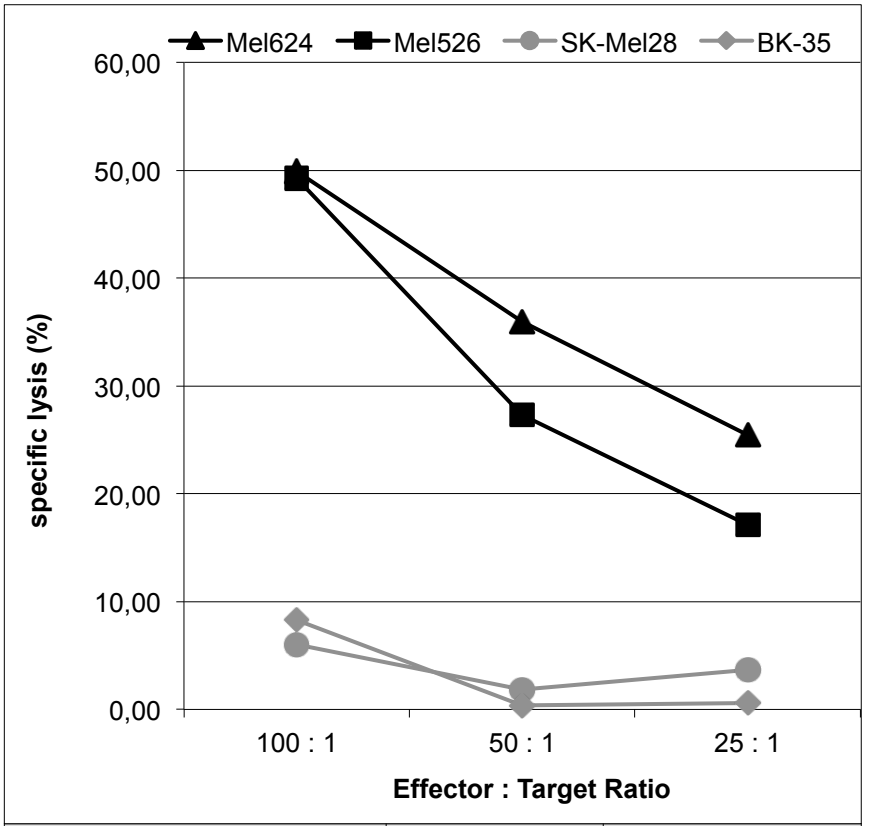


Figure S4 D) Killing of autologous or HLA-matched but not HLA-mismatched tumor cell lines by patient´s post-vaccination CD8+ T cells that have been pre-stimulated in vitro with the vaccine peptides.

D



Description	Name	HLA
autologous tumor cells	WO-31	A2, A26
HLA-matched target	Mel526	A2, A3
HLA-missmatched target	BK-35	A1, A24



Description	Name	HLA
HLA-matched target	Mel624	A2, A3
HLA-matched target	Mel526	A2, A3
HLA-missmatched target	SK-Mel28	A11, A26
HLA-missmatched target	BK-35	A1, A24

Figure S5) Luminex-assay was performed with post-vacc samples from 45 patients. PBMC were prestimulated with either class I or class II peptides for 14 days. Then cells were restimulated with peptides and supernatants were analyzed after 24 hours. Depicted are the mean cytokine profiles of all patients after stimulation with either class I or class II peptides or as positive control with CD3/ CD28 beads.

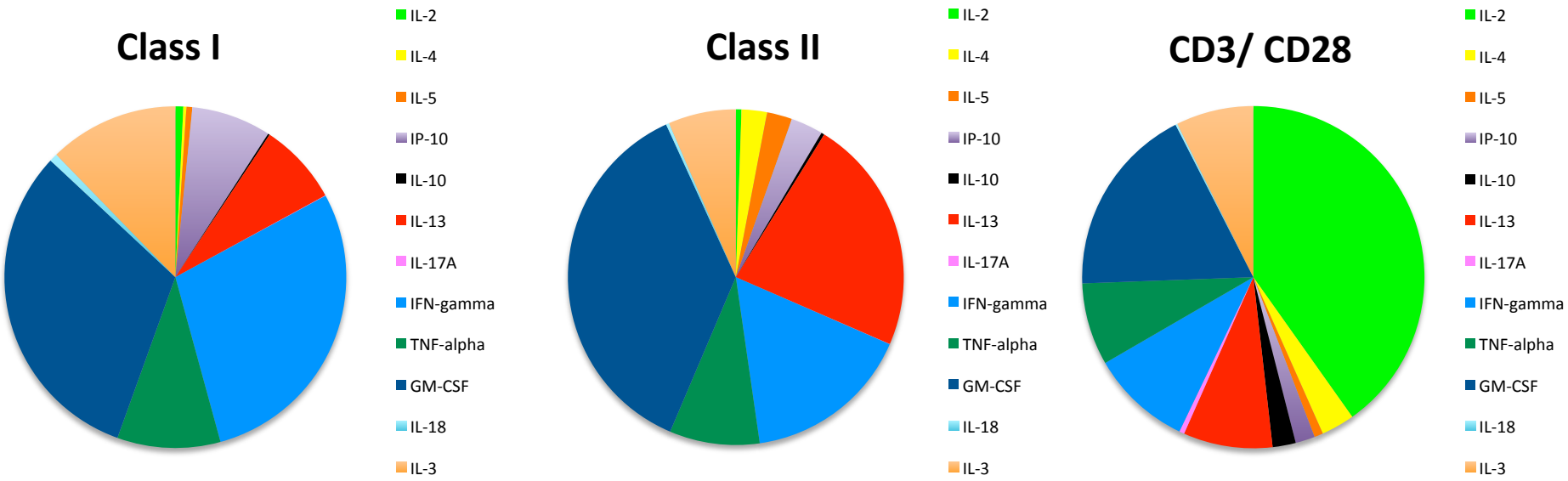
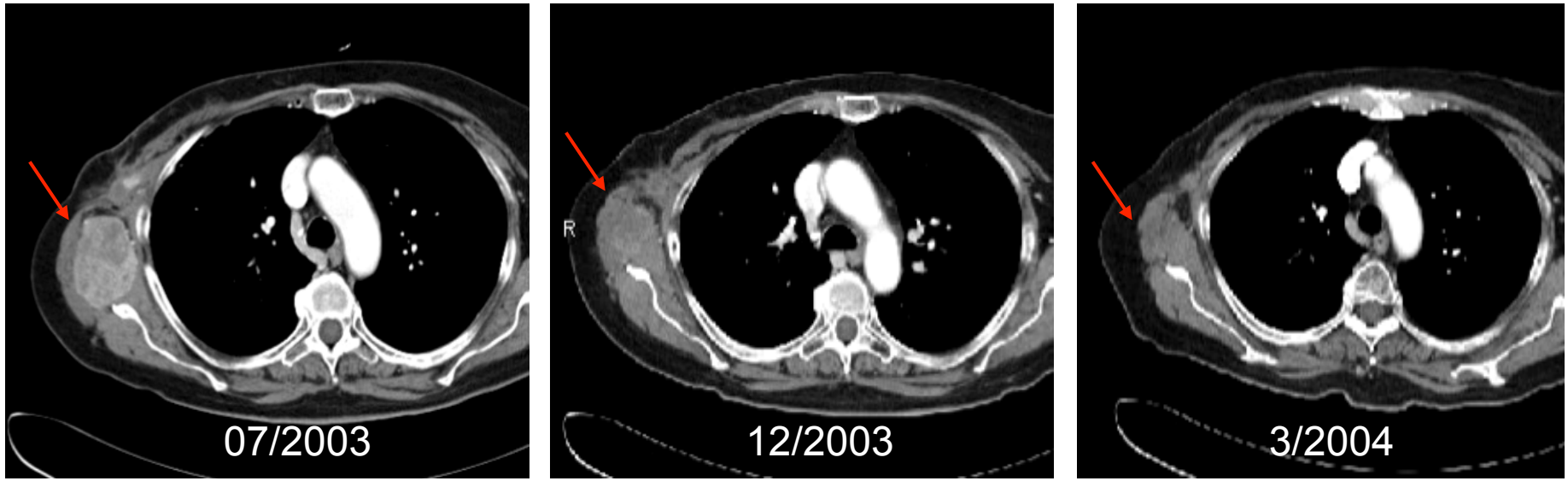
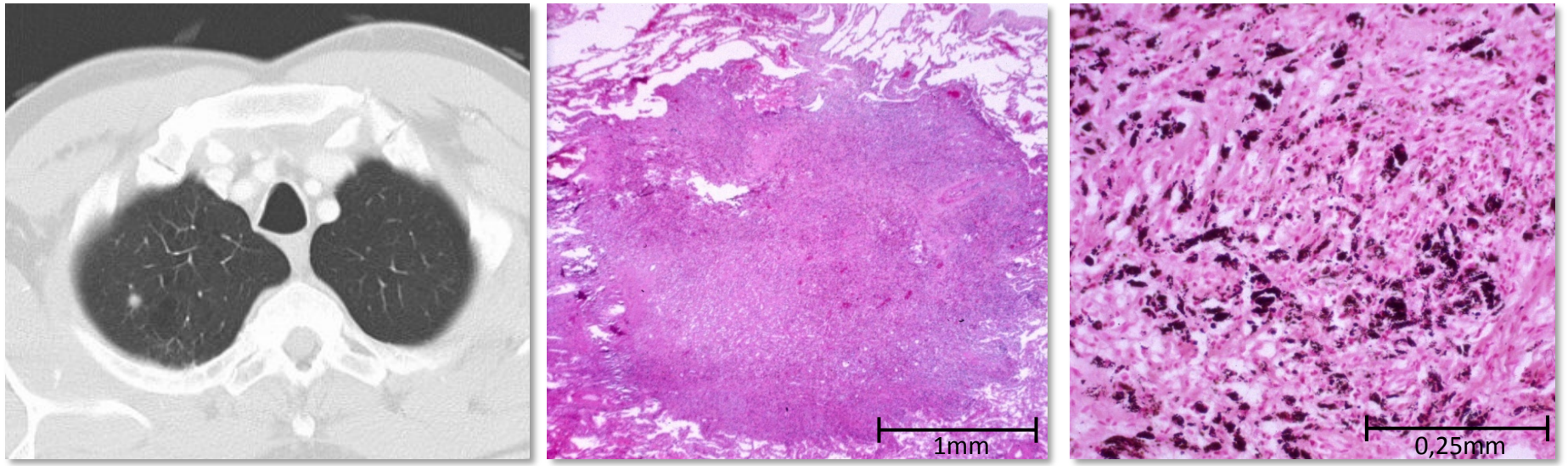


Figure S6) (A) Regression of metastases over time. (B) Granuloma formation.



(A) Slow regression of a subscapular muscular metastases in patient 33 resulting in a complete regression as two other metastases (one in front of, one behind the right kidney had slowly regressed leaving behind scars)



(B) Multiple lung metastases in patient 06 regressed, and residual metastases (left) upon resection revealed granulomas (middle) with melanin-containing (Fontana staining) macrophages, i.e. melanophages, yet few if any tumor cells left (picture on the right).

Figure S7 A) Long term immunologic memory to vaccine tumor antigens

Responses to vaccine tumor antigens can be measured by in vitro stimulation (ivs) ELIspot even after long vaccination intervalls. For each vaccine antigen, the responses pre vaccination and the maximum response observed at any timepoint is shown, followed by responses detected at least 3 or 6 months after the last vaccination. Plots show datapoints from all available patients and the means with whiskers approximating the 95% CI. This is shown for **(A)** the HLA-A2 binding peptides and **(B)** for some of the HLA-Class II binding peptides

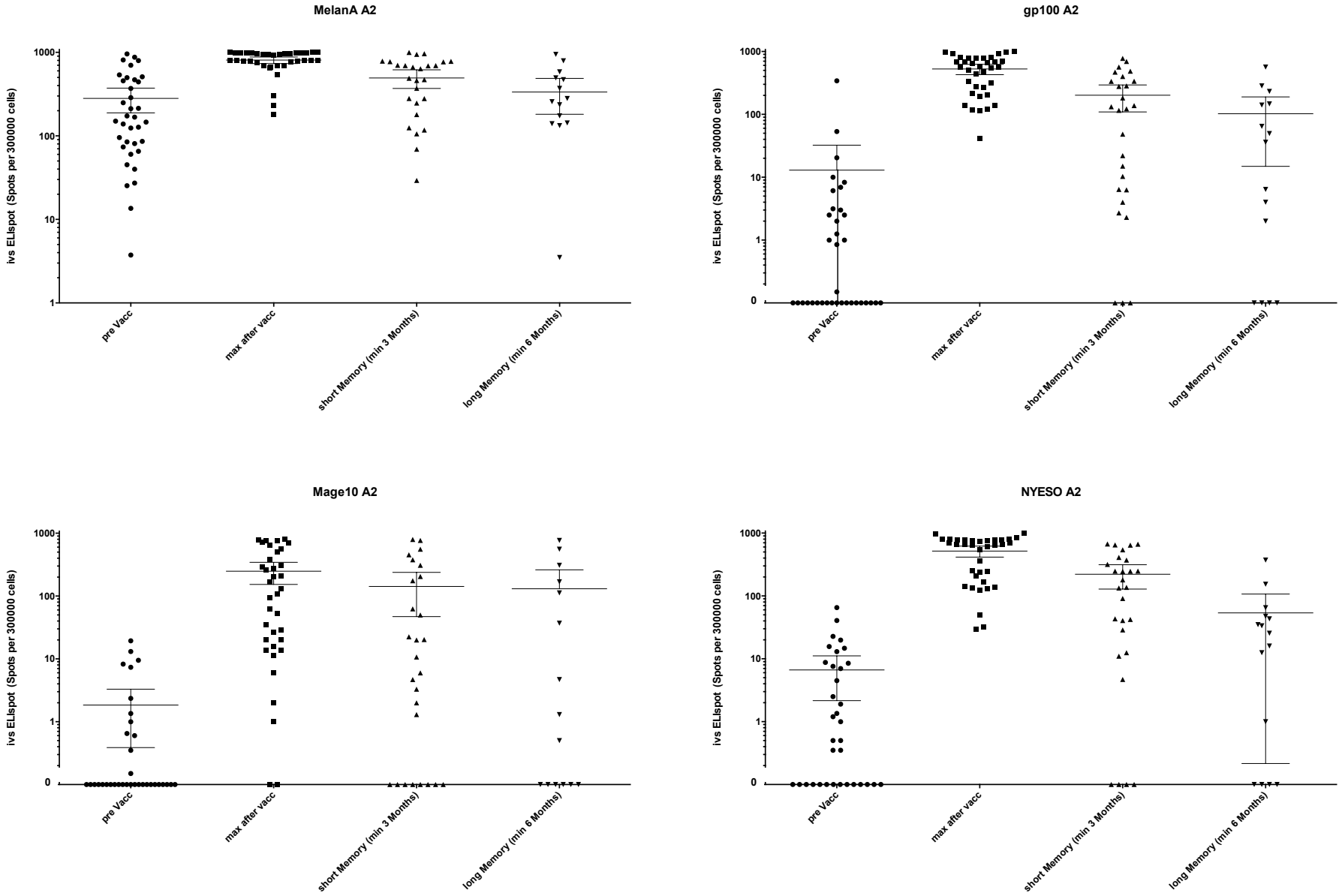
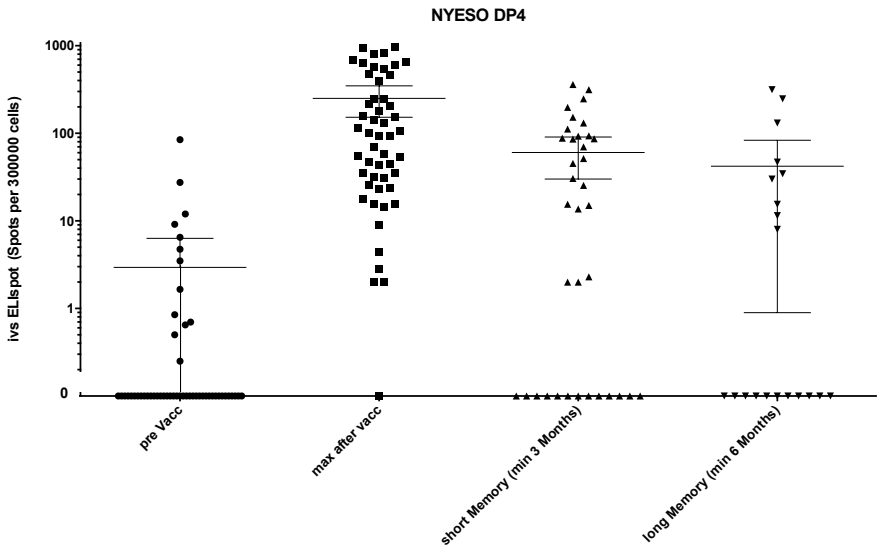
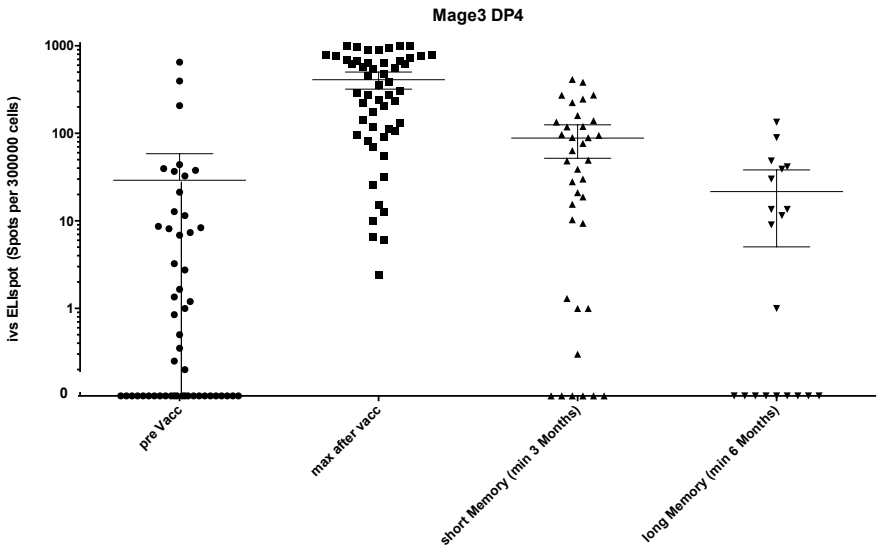
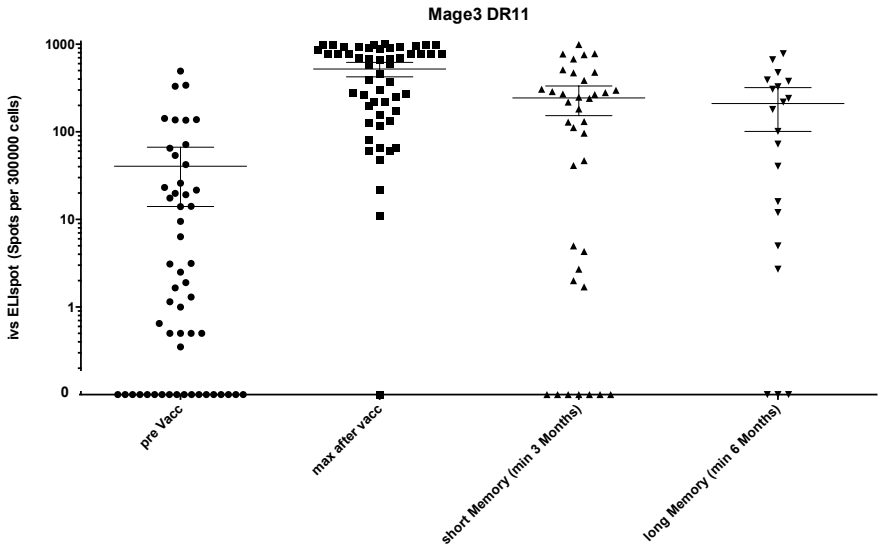


Figure S7 B) Long term immunologic memory to vaccine tumor antigens







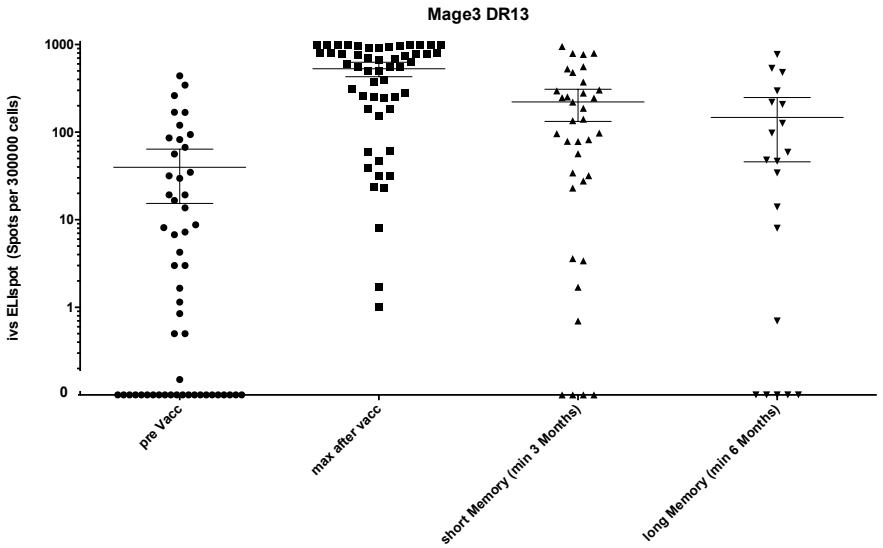


Figure S8) Lack of correlation between non-specific immune responses and survival

ex vivo ELIspot responses against the non-specific antigen KLH are plotted against the survival of the respective patient and are shown (A) before vaccination, (B) in the induction phase and (C) during maintenance phase.

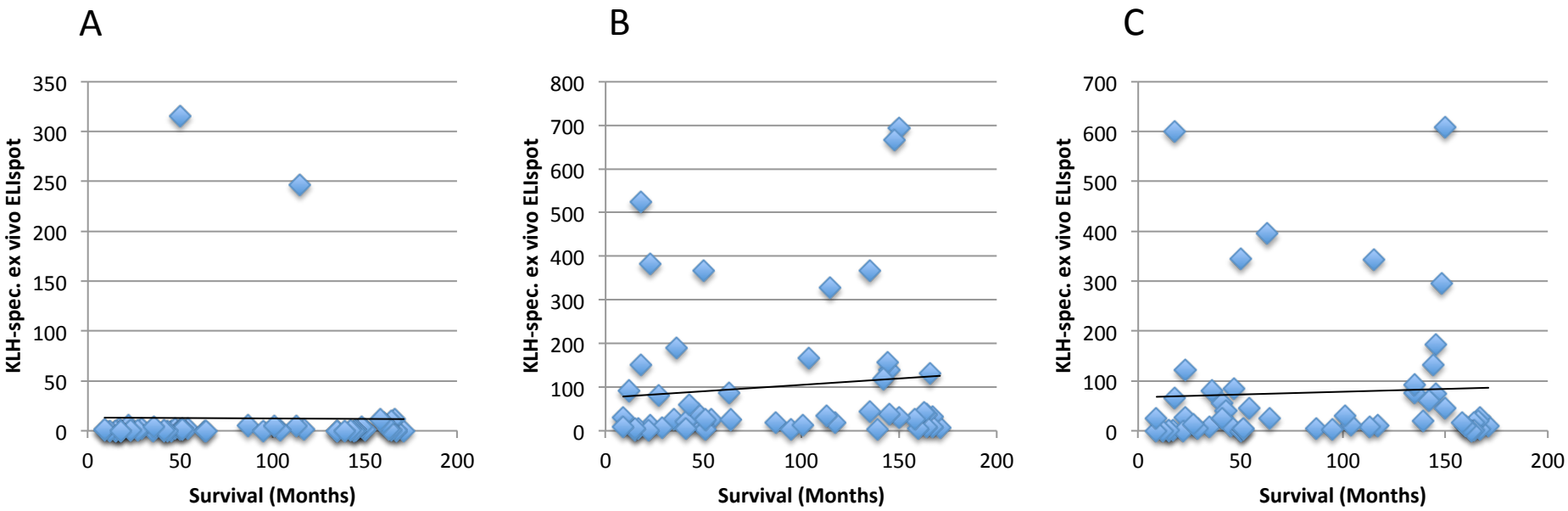


Figure S9) Immunity to non-vaccine antigens

Frequency of CMV-pp65 − A2 specific T cells did not significantly change during vaccination, as measured by ex vivo Tetramer staining (n = 28).