Molecular Medicine

Supplemental Data

High Immune Response Rates and Decreased Frequencies of Regulatory T Cells in Metastatic Renal Cell Carcinoma Patients after Tumor Cell Vaccination

Heike Pohla,[1,2] Alexander Buchner,[1,3*] Birgit Stadlbauer,[1] Bernhard Frankenberger,[2] Stefan Stevanovic,[4] Steffen Walter,[5] Ronald Frank,[6] Tim Schwachula,[7] Sven Olek,[7] Joachim Kopp,[8] Gerald Willimsky,[9,10] Christian G Stief,[1,3] Alfons Hofstetter,[1,3] Antonio Pezzutto,[8,11] Thomas Blankenstein,[9,10] Ralph Oberneder,[3,12*] and Dolores J Schendel[2*]*

Online address: http://www.molmed.org

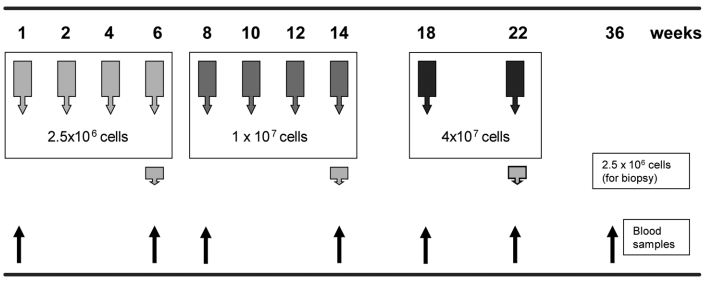
The Feinstein Institute for Medical Research North Shore LIJ

MATERIAL AND METHODS

ELISPOT

Heparin blood samples from patients were obtained at study wks 1, 6, 14, 22, and 36 (Supplementary Figure S1) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Leukosep® tubes (Greiner bio-one, Frickenhausen, Germany) following the manufacturer's instructions. For characterization of the patients see Table 1 and Supplementary Table S1). The median PBMC number obtained per ml blood was 1.1×10^6. Cryopreservation was done within 3 h of processing in freezing medium using Mr. Frosty® freezing container following the manufacturer's instructions and stored in the vapour phase of liquid nitrogen 24 h later. Freezing medium was 42.5% human serum albumin (20%, Octapharma GmbH, Langenfeld, Germany) and 10% DMSO in RPMI. PBMC were frozen in 1.8 ml cryogenic vials (Nalgene Nunc) at 5×10^6 cells in 1 ml per vial. Additional samples from five patients were taken on d 15 after the tenth vaccination and samples were available from two patients during longer follow-up.

PBMCs were thawed and washed in CTL wash supplemented medium (Cellular Technology Ltd. Europe, Bonn, Germany) containing Benzonase Nuclease (50 U/ml; Novagen Merck Biosciences). Recovery and viability of the cells were determined using trypan blue and a

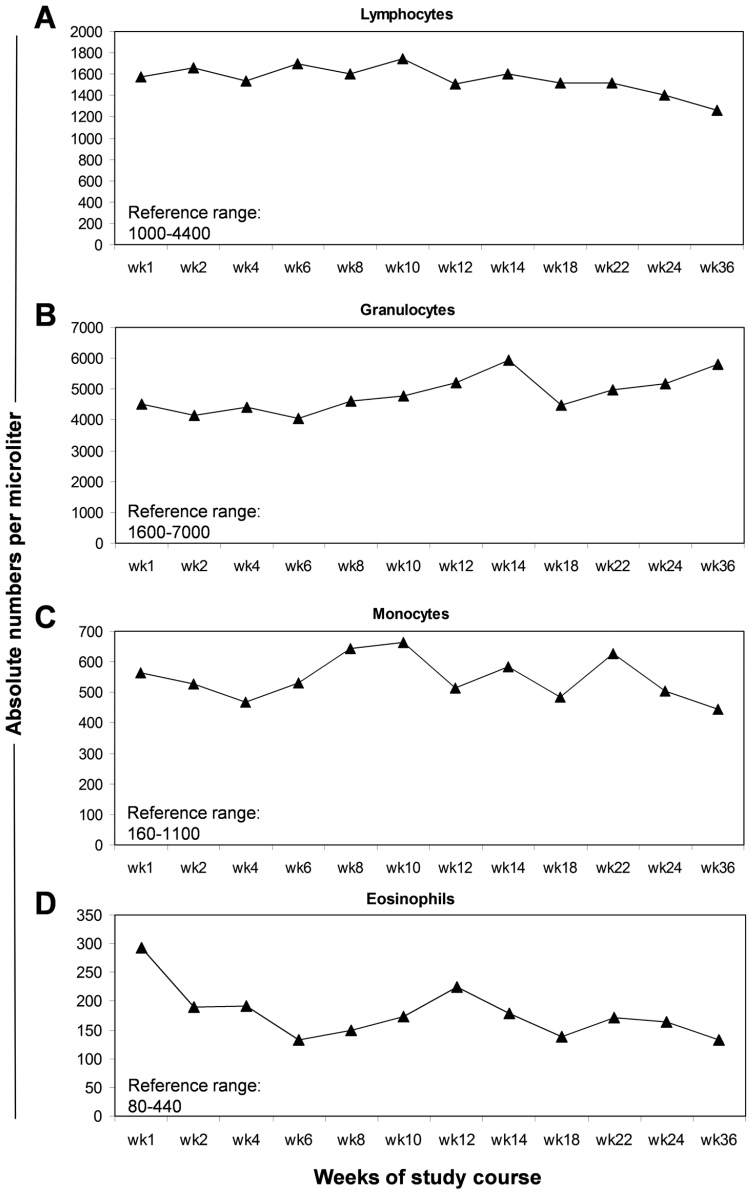


Supplementary Figure S1. Schematic view of the vaccination schedule. Blood samples and biopsies for immune monitoring are indicated.

Supplementary Table S1. Patient characteristics.

ID	Gender	Age (years) at first diagnosis	Age (years) at study entry	TNMG
MR-1	m	39	40	pT2pN0M0G2
MR-2	m	65	67	pT2pN0M1G3
MR-4	f	66	68	pT3bpN0M1G2
MR-5	m	60	62	pT3bpN0M1G2
MR-6	m	61	61	pT3bNxM1G2
MR-7	m	52	64	pT2pN0M0G2
MR-9	f	67	68	pT1pN0M1G2
MR-10	f	60	65	pT2NxM0G2
MR-11	m	46	49	pT3bNxM1G2
MR-13	m	50	54	pT1pN0M0G2
MR-14	m	61	61	pT3apN0M1G3
MR-15	m	50	58	pT2pNxM1G2

Abbreviation: m, male; f, female; TNMG, tumor size, node involvement, metastasis, grade



Supplementary Figure S2. Numbers of blood leukocytes during vaccination. The median of absolute numbers of lymphocytes (A), neutrophil granulocytes (B), monocytes (C), and eosinophils (D) per microliter from prevaccination (wk 1) to wk 24 and 36 in the follow-up for all patients are shown. Reference ranges for healthy individuals are indicated.

'Neubauer improved' hemocytometer. Median viability after thawing was 92%. Cells were rested in serum-free medium (CTL Test medium; Cellular Technology Ltd) for at least 2 h at 37°C, 5% CO_2 at a cell concentration of 2×10^6/ml in 50 ml polypropylene tubes with no more than 5 ml per tube. The cap was slightly loosened for gas exchange. Median cell recovery after resting was 83.1%.

For the ELISPOT the IFN-γ-ELISPOT^{PRO} kit (Mabtech, Nacka, Sweden) with precoated plates (monoclonal antibody 1-D1K), the monoclonal detection antibody 7-B-6-1-ALP conjugated and BCIP/NBT-plus substrate solution was used. Blocking of the plates was done with CTL Test medium. PBMC were seeded in quadruplicates at 1.5×10^5 cells per well and directly stimulated with selected peptides (each peptide, 5 μg/ml) in serum-free CTL Test medium, supplemented with CD28 (1 μg/ml; BD Biosciences, San Jose, CA) and recombinant IL-2 (Proleukin, 2U/ml; Chiron, Emeryville, CA) for 24 h. The peptides were selected from sequences of TAAs shown to be overexpressed in metastatic RCC lesions and/or the vaccine cells, using HLA-A*02:01 motif-based epitope predictions available on the web (http://www.syfpeithi.de), or as published in the literature. Several peptides were identified by elution from the RCC-26 cell line (Stefan Stevanovic, Tübingen) and tested for their expression on the vaccine cell line.

Peptides specific for the following antigens were used as described in Buchner *et al*., (3) for survivin, Cyclin D1, adipophilin, C-Met proto-oncogen, regulator of G protein signaling (RGS5), vascular endothelial growth factor (VEGF), NY-ESO-1, and additionally: carbonic anhydrase IX (CA IX, G250) (GLLFAVTSV; $HLSTAFARV_{217-225}$, (4), matrix metalloproteinase 7 (MMP7) (CLLPGSLAL, FLYAATHEL), preferentially expressed antigen in melanoma (PRAME) ($VLDGLDVLL_{100-108}$, $SLYSFPEPEA_{142-151}$, $ALYVDSLFFL_{300-309}$, $SLLQHLIGL_{425-433}$, (5), PRUNE2 ($ALFDGDPHL_{1-9}$, (6), insulin-like growth factor binding protein 3 (IGF-BP3) ($AALTLLVLL_{11-19}$, $LLDGRGLCV_{107-115}$), thymidylate synthetase (TYMS) ($VLEELLWFI_{84-92}$, $YMIAHITGL_{235-243}$, $RILRKVEKI_{280-288}$), ceruloplasmin (CP) ($YLFGMGNEV_{284-292}$, $RMFGNLQGL_{964-972}$, $YLMGMGNEI_{983-991}$) vimentin ($ILLAELEQL_{130-138}$, $DLERKVESL_{219-227}$), transcriptional intermediary factor 1 (TIF1) ($VIIDTLITKL_{268-277}$, $ALLHQLESL_{326-334}$, $FQDPVPLTV_{924-232}$), ORM1-like protein 3 (ORMDL3) ($VILPKLPQL_{134-142}$), chloride intracellular channel protein 1 (CLIC1) ($NLLPKLHIV_{179-187}$, $FLDGNELTL_{167-175}$, $FMVLWLKGV_{31-39}$), putative prostate cancer susceptibility protein 2 or heredity prostate cancer protein 2 (ELAC2, HPC2) ($GLSGMILTL_{120-128}$, $AIAPIIAAV_{270-278}$, $KLIPPLKAL_{726-734}$), tropomyosin (TPM1) ($QLEDELVSL_{38-46}$, $KLEKSIDDL_{248-256}$, $MLDQTLLEL_{273-281}$), AHNAK-related pro-

tein (SMPDFDLHL), B-cell translocation gene 1 protein (BTG1) (TLWVDPYEV$_{103-111}$), p53-induced gene 10 protein (PIG10) (YLPELLQTV$_{228-236}$), leukocyte receptor cluster (LRC) member 4, also known as malignant cell expression-enhanced gene/tumor progression-enhanced or membrane-bound O-acyltransferase domain-containing protein 7 (LENG4) (GLLPDVPSL$_{141-149}$). The HLA-A2 CEF peptide pool (PANATecs, Tübingen, Germany) was used as positive control. Culture medium without peptide was used as negative control.

Spots were counted with AID reader system ELR03 with software version 4.0 and 5.0 (Autoimmun Diagnostika [AID], Strassberg, Germany) and controlled by human audit. Spot parameters were established using the positive and negative control wells. Artifacts and faint small background spots observed in the negative control wells were excluded (see representative example, Supplementary Figure S7).

Responses were not considered positive, if spot number was equal or below 10 spots over background per 1.5×10^6 cells. Responses were considered to be positive, if the mean spot number of quadruplicates for a given peptide were at least two-fold over the mean background spot number. A response was considered to be vaccine induced, if the ratio of peptide mean to background mean for a given vaccination time point was at least two-fold over the corresponding prevaccination ratio.

The performance of ELISPOT assays was externally validated by participation in the immune assay proficiency panels of the international Cancer Immunotherapy Consortium (1) and the CIMT Association for Immunotherapy of Cancer (2). While our laboratory has not established GLP conditions, this study was performed using standard operating procedures (SOPs) covering the processing, freezing, storage, and thawing of cells and the ELISPOT assay. All conditions and reagents were standardized and pretested. The operator was the same throughout the clinical study.

Supplementary Table S2. Expression of tumor-associated antigens in metastatic RCC lesions, parental RCC-26, and the RCC-26/CD80/IL-2 vaccine.

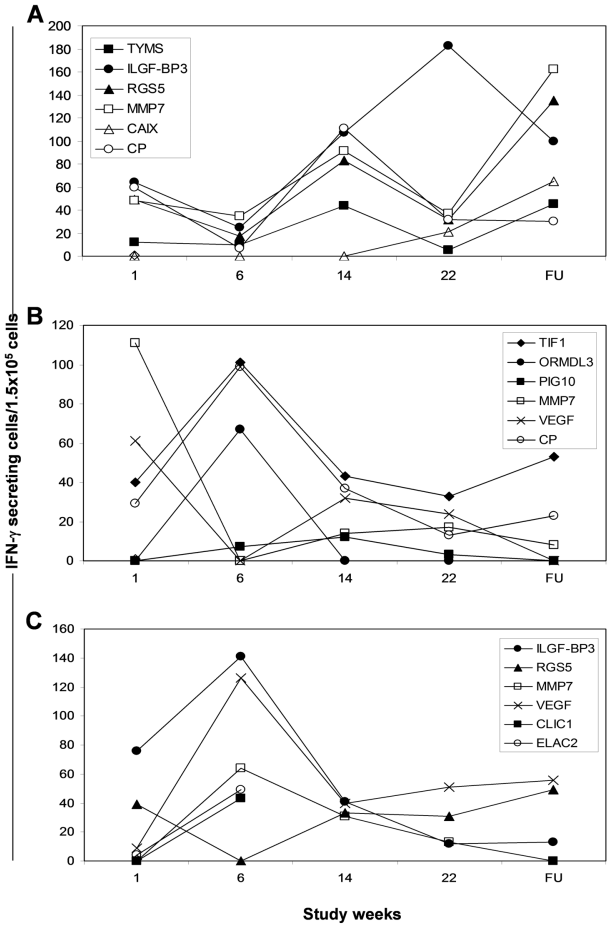
Antigens	Metastatic lesions		RCC-26 expression	RCC-26/CD80/IL2 expression
	% positive	expression		
TYMS	100	+++	+++	+++
IGFBP3	100	+++	+++	+++
CAIX (G250)	91	++	-	-
RGS5	100	+++	+	+
NY-ESO1	0	-	-	-
MMP7	66	+++	++	-
VEGF	100	+++	+++	+++
PRUNE2	97	+++	-	-
CP	88	+++	++	+++
Vimentin	100	+++	+++	+++
TIF1	100	+++	+++	++
ORMDL3	91	++	++	++
CLIC1	100	+++	+++	+++
ELAC2	97	++	++	++
TPM1	100	+++	+++	+++
AHNAK-rel.	100	+++	+++	+++
BTG1	100	+++	+++	+++
PIG10	69	++	++	++
LENG4	50	+++	++	++

Shown are percentages of samples positive for selected antigens in a panel of 32 metastatic RCC lesions and normalized levels of antigen expression in microdissected tumor cells from the lesions and in RCC-26 and the vaccine cells as determined by microarray analysis.
The scale for the model-based expression index is as follows: -, no expression; +, <100, ++, 100-999; +++, ≥1000.

Flow Cytometry

For surface immunostaining the following directly labelled monoclonal antibodies were purchased from BD Biosciences: CD3 (clone UCHT1, FITC and Horizon V500), CD4 (clone SK3, PE-Cy7 and PerCP-Cy5.5), CD8 (clone RPA-T8, Alexa Fluor 700), CD25 (clone M-A251, PerCP-Cy5.5), CD39 (clone TÜ66, APC), CD127 (clone hIL-7R-M21, PE), CD11b (clone ICRF44, Pacific blue), CD15 (clone HI98, FITC), CD14 (clone IV M_P9, APC-H7), CD33 (clone P67.6, PerCP-Cy5.5), CD124 (IL-4Rα, clone 25463, PE), HLA-DR (clone G46-6, APC); CD3 and CD19 (clone SK7 and clone SJ25C1, respectively, PE-Cy7, for dump channel in the MDSC analysis). For Treg analysis the monoclonal antibody FoxP3 (clone PCH101, eFluor450; eBiosciences, Frankfurt, Germany) was used. For discrimination of live/dead cells the LIVE/DEAD® Fixable Blue Dead Cell Stain Kit, for UV excitation (Molecular Probes®, Life Technologies, Carlsbad, CA, USA) was applied.

Thawing of cells was done as described above. Then, $1\text{-}2\times10^6$ cells were incubated first with the LIVE/DEAD Fixable Blue dye for 30 min at room temperature (RT), washed, and then stained with the directly labelled monoclonal antibodies for 30 min on ice in the dark, and washed. For intracellular staining with the FoxP3 antibody, the Fix/Perm buffer (eBiosciences) was used and the cells stained for 60 min on ice in the dark, and washed with the Perm buffer. Cells were finally washed with PBS and directly analyzed using the LSRII (BD Biosciences). Data were processed using FlowJo software (version 8.8.6; Tree Star, Ashland, Oregon, USA). Median acquired cell number for Treg analysis was $5\text{x}10^5$, for MDSC analysis 1.55×10^6. The PMT voltage was adjusted using un-



Supplementary Figure S3. Numbers of peptide-reactive T lymphocytes during vaccination. Shown are the IFN-γ-responses of patient MR-2 (A) to peptides TYMS, IGF-BP3, RGS-5, MMP7, CAIX, and CP, patient MR-13 (B) to peptides TIF1, ORMDL3, PIG10, MMP7, VEGF, and CP, and patient MR-14 (C) to peptides IGF-BP3, RGS-5, MMP7, VEGF, CLIC1, and ELAC2 measured by ELISPOT. Indicated is the mean of quadruplicates per $1.5x10^5$ cells per well after subtraction of background.

stained cells for all parameters. The gating strategy for Treg analysis is shown in Supplementary Figure S4 and for MDSC analysis in Supplementary Figure S5.

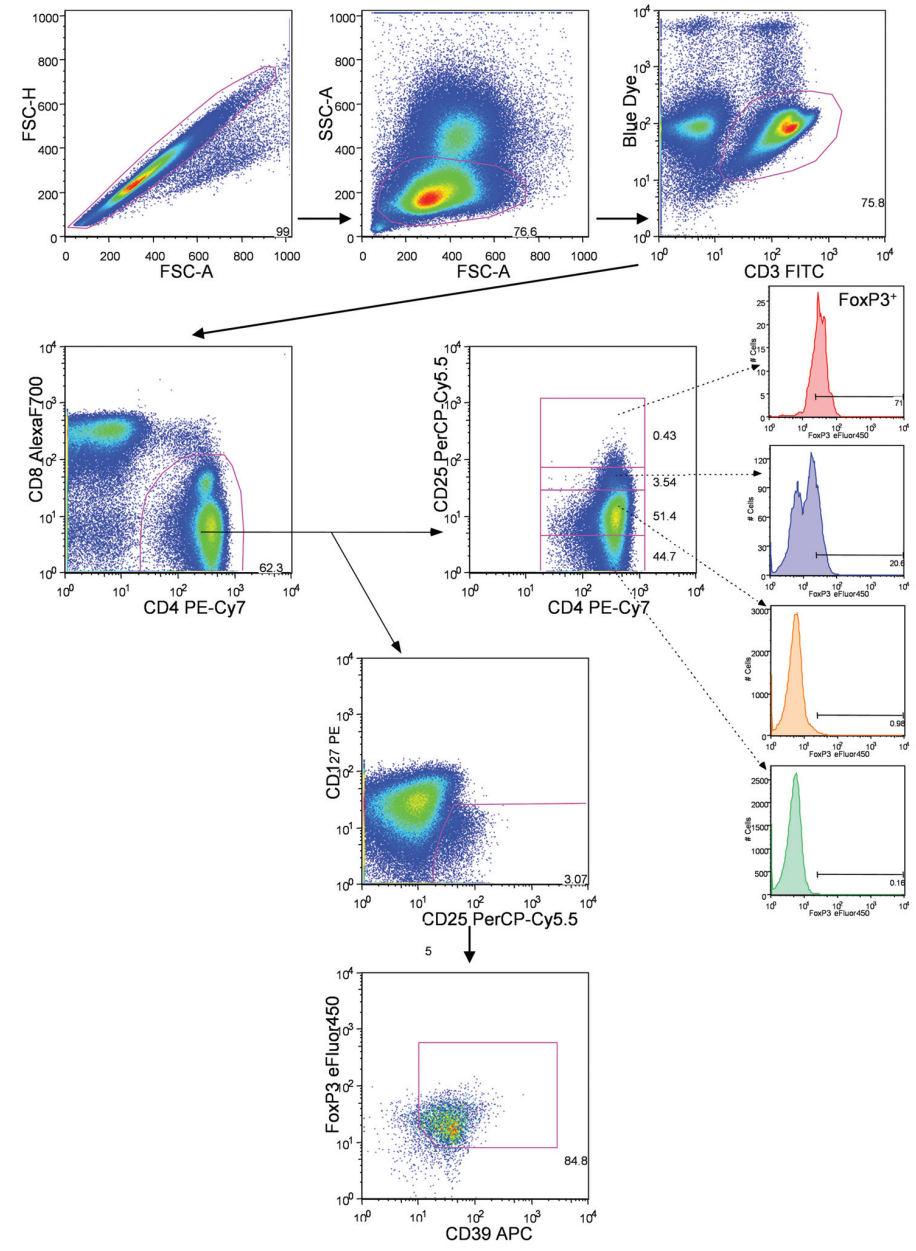
Cytokine/Chemokine Assays

Commercially available ELISA kits and protocols (R&D Systems GmbH, Wiesbaden, Germany) were used for VEGF, VEGF-C, VEGF-D, TGFβ1, and PGE_2. For all other cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, TNFα, IFNγ, GM-CSF, G-CSF) and chemokines CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CCL2 (MCP-1), CCL4 (MIP-1β), and CCL5 (RANTES) the Luminex (BioPlex, BioRad, Munich, Germany) and the multiplex cytokine bead array systems from BD Biosciences (CBA kit for chemokines and CBA Flex Set) were applied and performed according to the manufacturer's instructions.

Blood for serum samples were taken before and after first vaccination, and before and after 4th, 5th, 8th, 9th, 10th vaccinations at the time points when the dosage was enhanced and the skin biopsies were taken (Supplementary Figure S1). All blood samples were directly processed for serum preparation after 30-min incubation and stored immediately. PBMC ($5x10^5$) from the time point prevaccination (wk 1) and from wk 22 were cultured with the parental RCC-26 cell line and with the RCC-26/CD80/IL-2 vaccine cells ($5x10^4$ cells, 100 Gy irradiated) in supplemented RPMI 1640 containing 15% heat-inactivated pooled human serum. The culture supernatants (SN) were taken after 24 and 48 h following stimulation. Skin-infiltrating lymphocytes from biopsies (wks 6, 14, 22) were cultured for 10-19 d in supplemented Iscoves's medium containing 15% human serum and 20 U/ml IL-2, then $5x10^4$ cells were stimulated with 5×10^3 RCC-26 and vaccine cells in 150 µl medium per well, and measured for cytokines/chemokines in SN 24 and 48 h later. Culture SN and serum samples were frozen in aliquots at -20 °C and -80 °C, respectively, until use in assays. The four-parameter curve fit option was used to extrapolate values, which did not fall within the minimum and maximum limits of the standard curve. For the CBA chemokine assays, the BD FACSComp™ software (version 4.2) and the Calibrite beads were used for setting up the FACSCalibur. The BD CellQuest™ software (version 4.0.2) was used for data acquisition. The CBA Flex Set was analysed with the LSR II and for subsequent data analysis, the FCAP Array™ software (version v1.0.1) was applied. The Luminex 100 Reader (software version BioPlex Manager 4.1.1) was employed for the BioPlex assays.

Analysis of the Demethylation of the *Foxp3* Gene

Genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Bisulphite treatment of genomic DNA was performed as described (7) with minor modifications. A quantitative real-time (qRT) PCR-based methylation assay with methylation- and demethylation-specific amplification primers was used to analyze the *Foxp3* Treg-specific demethylated region (TSDR) DNA as described (8). Each sample was analyzed in triplicate using a LightCycler 480 System (Roche, Mannheim, Germany). Cycling condi-



Supplementary Figure S4. Gating strategy to determine the frequencies of $CD4^+CD25^{high}CD127^{low/-}Foxp3^+CD39^+$ Tregs. Patient MR-1 is shown as an example for gating strategy in flow cytometry. The gating was done in the following order: singlets – lymphocytes – viable $CD3^+$ cells – $CD4^+$ cells – $CD25^+$ $CD127^{low/-}$ cells – $FoxP3^+CD39^+$ cells. To demonstrate that most $FoxP3^+$ cells are in the $CD4^+CD25^{high}$ cell population, this gating is additionally shown.

Supplementary Table S3. Frequencies of $CD4^+CD25^{high}CD127^{low/-}Foxp3^+CD39^+$ Tregs before and after vaccination.

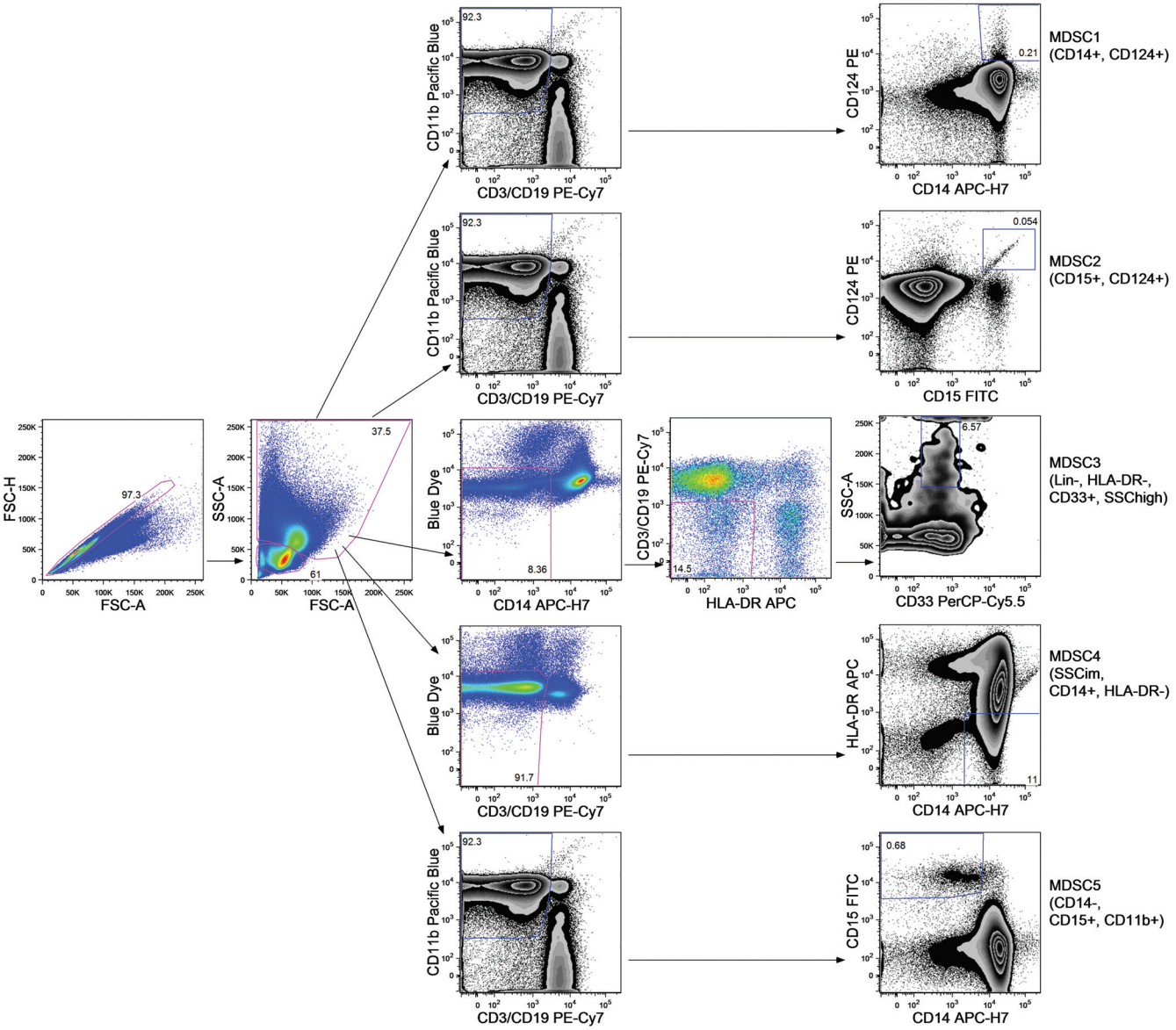
	Treg in (%) of viable CD3	
Patient	pre	post
MR-1	2.12 (54)[A]	1.62 (24)
MR-2	1.27 (21)	0.83 (7)
MR-4	2.02 (28)	1.75 (17)
MR-5	2.84 (20)	2.80 (26)
MR-6	3.46 (36)	3.25 (35)
MR-7	2.71 (22)	2.50 (29)
MR-9	3.45 (38)	3.72 (34)
MR-10	3.25 (33)	2.74 (28)
MR-11	0.93 (18)	1.50 (15)
MR-13	1.78 (22)	1.72 (18)
MR-14	3.56 (53)	2.37 (31)
MR-15	2.48 (32)	1.83 (24)
median	2.60 (30)	2.10 (25)
p value	0.065 (0.034)[B]	
HD median	2.71	

Abbreviations: HD, healthy donors; Median of eight healthy donors.
[A]In parentheses: absolute number of Tregs per μl blood.
[B]*p* Value of absolute number.

tions consisted of a 95°C preheating step for 10 min and 50 cycles of 95°C for 15 s followed by 1 min at 61°C. Titrated PCR products were generated using DNA from sorted naive and regulatory T cells for a standard curve. Amounts of methylated and unmethylated *Foxp3* DNA were estimated from calibration curves by linear regression on crossing points from the second-derivative maximum method. The proportion of unmethylated DNA was computed as the ratio of unmethylated *Foxp3* TSDR-DNA and the sum of methylated and unmethylated *Foxp3* TSDR-DNA. For female patients, this ratio was corrected with a factor of 2 due to the fact that one of the two TSDR alleles is methylated as a result of chromosome X inactivation.

Microarray Analysis

Tissue samples from 32 patients with clear-cell RCC metastases were collected, snap-frozen, and stored in liquid nitrogen after written informed consent had been obtained. The study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany. Cryostat sections were made and laser microdissection was used to isolate tumor cells (PALM MicroBeam, Zeiss, Munich, Germany). Total RNA was extracted from these cells and also from RCC-26 and the vaccine cells (RNeasy Micro kit; Qiagen, Hilden, Germany). RNA integrity was tested by capillary electrophoresis (2100 Bioanalyzer; Agilent, Santa Clara, CA). RNA amplification and hybridization



Supplementary Figure S5. Gating strategy to determine the frequencies of different MDSC subpopulations. Patient MR-15 is shown as an example for gating strategy in flow cytometry. The gating was done in the following order: singlets – non-lymphocytic cells - $CD11b^{+}CD3^{-}CD19^{-}$ cells – $CD14^{+}CD124^{+}$ cells for MDSC1, singlets – non-lymphocytic cells – $CD11b^{+}CD3^{-}CD19^{-}$ cells – $CD15^{+}CD124^{+}$ cells for MDSC2, singlets – non-lymphocytic cells – $CD3^{-}CD19^{-}DR^{low/-}$ cells – $SSC^{high}CD33^{+}$ cells for MDSC3, singlets – non-lymphocytic cells – viable $CD3^{-}CD19^{-}$ cells – $CD14^{+}DR^{low/-}$ cells for MDSC4, and singlets – non-lymphocytic cells – $CD11b^{+}CD3^{-}CD19^{-}$ cells – $CD15^{+}CD14^{-}$ cells for MDSC5. The lymphocyte gate was used to calculate the MDSC in % per lymphocytes.

on oligonucleotide microarrays (GeneChip Human Genome U133 Plus 2.0 array, Affymetrix, Santa Clara, CA) were performed according to the manufacturer's instruction. Normalized expression values were calculated for every transcript, using dChip software (9).

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Supplementary Table S4. Frequencies of different MDSC populations in peripheral blood samples pre- and postvaccination in (%) per lymphocytes.

	MDSC1 $CD14^+CD124^+$		MDSC2 $CD15^+CD124^+$		MDSC3 $Lin^-DR^-CD33^+SSC^{high}$		MDSC4 $SSCimCD14^+DR^-$		MDSC5 $CD14^-CD15^+CD11b^+$	
Patient	pre	post	pre	post	pre	post	pre	post	pre	post
MR-1	0.65	0.52	1.57	0.72	3.19	0.43	37.30	53.34	11.34	2.43
MR-2	0.78	0.39	1.45	1.02	0.35	0.53	6.69	0.86	2.64	4.14
MR-4	0.41	0.34	0.50	1.97	0.68	3.03	6.41	17.04	4.04	11.42
MR-5	0.26	0.35	0.30	0.75	0.06	0.23	13.00	11.27	0.42	2.04
MR-6	0.17	0.21	0.78	0.30	0.93	0.07	16.52	8.0	5.16	1.34
MR-7	0.26	0.33	0.19	0.20	0.20	0.13	18.05	16.43	1.53	1.02
MR-9	0.47	0.37	0.44	0.37	0.25	0.25	35.64	19.88	2.91	2.08
MR-10	0.39	0.45	0.14	0.19	0.10	0.16	2.65	4.17	0.57	0.80
MR-11	0.77	0.62	0.17	0.39	0.12	1.78	23.06	24.09	2.65	7.18
MR-13	0.74	0.44	0.30	0.12	0.20	0.11	5.14	7.99	0.77	0.35
MR-14	0.65	0.75	0.55	0.61	0.32	0.46	4.69	10.00	1.59	1.86
MR-15	0.84	0.54	0.77	1.02	1.81	1.02	11.26	15.16	4.07	2.46
median	0.56	0.42	0.47	0.50	0.29	0.34	12.13	13.22	2.65	2.06
p value	0.108		0.937		0.859		0.754		0.929	
HD median	0.84		1.08		1.27		10.32		2.03	

Abbreviations: HD, healthy donors; Median of five healthy donors.

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Supplementary Table S5. *Ex vivo* and *in vitro* cytokine secretion following stimulation with vaccine cells.

	without *in vitro* stimulation		stimulation with the vaccine		2nd restimulation	
Cytokine (pg/ml)	pre (wk 1)	post-2 (wk 22)	pre (wk 1)	post-2 (wk 22)	pre (wk 1)	post-2 (wk 22)
IL-2	0.00	0.00	106.32	145.75	105.86	51.27
IFN-γ	1.63	1.09	97.19	36.79	491.78	486.80
IL-4	0.00	0.00	0.15	1.21	1573.76	1309.13
IL-5	0.21	0.34	5.36	18.13	3819.11	3783.47
IL-10	138.12	128.17	112.67	70.93	781.52	845.56
IL-1β	564.34	480.37	522.95	340.14	0.00	0.00
IL-6	7558.10	8920.56	6712.94	6335.65	157.04	71.71
IL-8	>10000	>10000	>10000	9868.72	64.95	26.47
TNF-α	683.14	1687.44	368.52	781.38	222.50	161.92

PBMC were isolated prevaccination (wk 1) and 4 wks following the 9th vaccination at the day of the 10th vaccination (wk 22). Culture supernatants were measured for cytokine secretion with the CBA Flex Set 24 h after stimulation with the vaccine. The vaccine cell line secretes IL-2, IL-6, and IL-8. These background values are subtracted. Numbers are mean values.

Supplementary Table S6. Cytokine secretion of skin-infiltrating lymphocytes.

	Biopsy 1 (wk 6)			Biopsy 2 (wk 14)			Biopsy 3 (wk 22)		
Cytokine (pg/ml)	mean	min	max	mean	min	max	mean	min	max
CCL5[A] (RANTES)	7.82	0.00	32.40	61.68	0.00	251.70	0.00	0.00	0.00
CXCL9[A] (MIG)	142.68	12.60	1501.60	526.05	78.80	1504.40	434.60	12.60	1820.10
CXCL10[A] (IP-10)	70.85	0.00	357.50	337.52	1.50	1410.20	274.62	13.30	1036.80
CCL2[A] (MCP-1)	2171.63	124.30	>5000	2208.12	1011.60	3656.90	1591.10	132.80	>5000
CCL-4 (MIB-1β)	990.90	111.95	4976.35	859.00	79.30	2463.58	823.23	200.67	2253.38
CXCL8[A] (IL-8)	2115.12	646.10	>5000	2525.77	988.40	>5000	1959.05	566.80	4415.90
IL-2	3.94	0.00	22.49	4.79	0.00	14.07	2.61	0.65	5.23
IL-4	13.00	0.00	50.69	31.65	0.00	122.58	14.28	0.81	40.11
IL-5	9.24	0.06	82.64	4.07	0.68	11.19	5.28	0.35	14.23
IL-6	542.17	20.17	1718.31	1945.26	101.72	7325.70	497.14	119.39	1639.72
IL-10	5.00	0.17	28.65	10.11	0.23	49.07	11.91	2.46	51.61
IL-13	12.00	0.00	112.62	2.41	0.00	7.55	2.31	0.00	9.81
GM-CSF	11.72	0.00	68.83	13.84	0.00	58.65	5.86	0.00	14.38
G-CSF	19.24	2.13	75.33	71.78	0.00	331.08	16.76	3.59	36.91

Skin biopsies were taken 48 h later from the site, where $2.5x10^6$ cells have been injected intradermally into the inguinal region after the 4th (wk 6), 8th (wk 14), and 10th (wk 22) vaccination. Lymphocytes were grown out from small pieces of tissue for 10-12 or 17-19 d. Culture supernatants were measured for the chemokines RANTES, IP-10, MIG, IL-8, and MCP-1 with the CBA assay and for the other cytokines with the BioPlex 48 h later after changing the medium. Because of technical reasons IFN-γ was not measured. The vaccine cell line secretes IL-2, IL-6, GM-CSF, G-CSF, and the chemokines. These background values are subtracted. Numbers are mean values.
[A]24 h culture supernatant.

Supplementary Table S7. Cytokines pre- and postvaccination in patient sera.

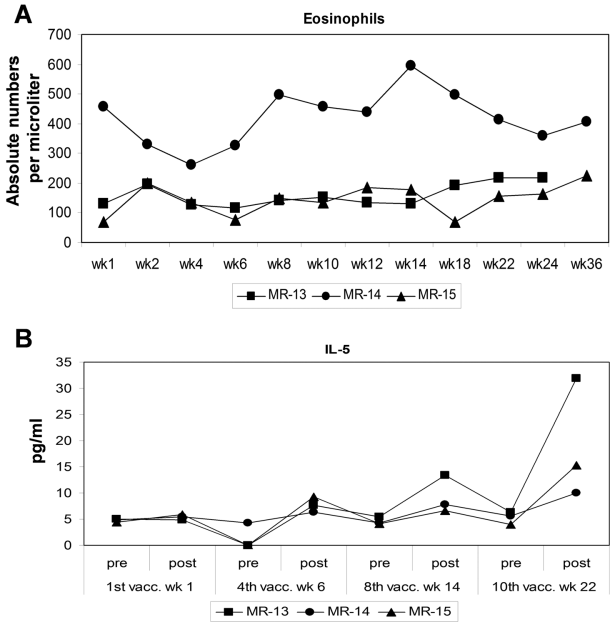
Cytokine	pre (wk 1)			post (4th vaccination—wk 6)			post (10th vaccination—wk 22)			Healthy donors*
(pg/ml)	mean	min	max	mean	min	max	mean	min	max	mean
IL-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IFN-γ	0.00	0.00	0.00	12.73	0.00	40.97	1.77	0.00	15.92	4.78
IL-12p70	0.62	0.00	6.84	3.67	0.00	12.35	1.57	0.00	7.08	0.61
IL-5	4.66	0.00	6.78	6.86	0.00	9.21	10.61	4.01	31.92	2.30
IL-10	1.54	0.00	10.81	5.45	0.00	10.51	2.10	0.00	7.11	1.05
IL-1β	3.43	0.00	8.06	1.68	0.00	5.47	3.89	0.00	7.92	0.66
IL-6	5.99	0.00	43.15	6.99	0.00	32.70	7.66	0.00	53.73	0.00
IL-8	8.61	0.00	25.25	11.46	0.00	23.28	8.04	0.00	18.25	2.01
TNF-α	0.61	0.00	6.75	1.13	0.00	6.76	0.00	0.00	0.00	0.54

Serum samples were taken prevaccination and 24 h after 1st and 48 h after 4th, and 10th vaccination.
Cytokines were measured with the CBA Flex Set assay. The values for IL-4 were negative. The mean values for 10 healthy control sera are indicated.

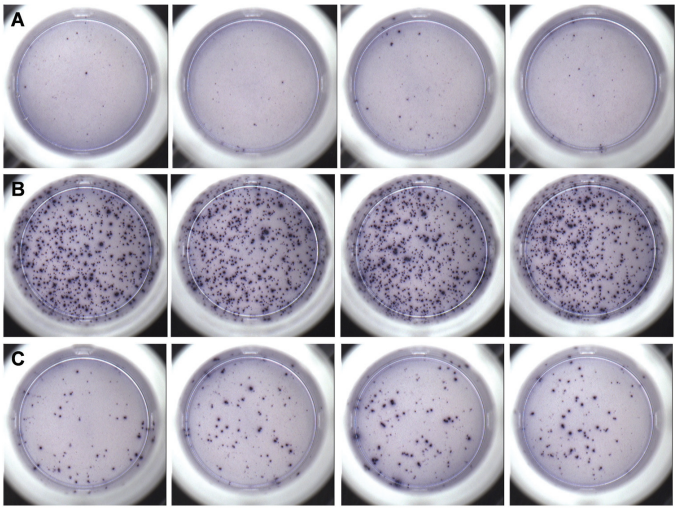
Supplementary Table S8. VEGF, TGF-β1 and PGE_2 in patient sera.

Cytokine (ng/ml)	pre (wk 1)	post-1 (wk 8)	post-2 (wk 18)	Healthy donors*
VEGF-A	1.127	1.416	0.733	0.220
VEGF-C	6.331	6.031	6.096	4.847
VEGF-D	0.261	0.283	0.268	0.297
TGF-β1	34.460	33.145	31.069	39.592
PGE_2	1.020	0.873	0.906	0.389

Serum samples were taken prevaccination and 24 h after the 5th (post-1) and after the 9th vaccination (post-2) when the dosage was increased.
Cytokines were measured with ELISA. The numbers are mean values.
*The mean values for healthy controls are according to the manufacturer.



Supplementary Figure S6. No correlation between numbers of eosinophils and serum IL-5. Eosinophil counts (A) per microliter blood and IL-5 levels (B) in pg/ml serum of patients MR-13, MR-14, and MR-15 are shown during the vaccination course. Eosinophils were estimated by the routine differential blood count and IL-5 was determined with the BioPlex assay.



Supplementary Figure S7. Representative ELISPOT examples of patient MR-4. (A) Quadruplicates of negative control wells (medium without peptide). (B) Quadruplicates of positive control wells (HLA-A2 CEF peptide pool). (C) Quadruplicates of test wells (tumor antigen peptide pool). Each well contains $1.5x10^5$ cells.