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Supplemental Material to:

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Tumor-associated macrophages subvert T cell function and correlate with reduced survival in clear cell renal cell carcinoma

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Supplementary Table S1: Summary of Taq Man Assays used for retrospective screen

	Target	Gene Symbol	Taq Man Assay ID
A	CD45	PTPRC	Hs00236304_m1
	CD3	CD3G	Hs00962186_m1
	CD4	CD4	Hs00181217_m1
	CD8	CD8A	Hs00233520_m1
	CD68	CD68	Hs00154355_m1
	MHC class-I	HLA-C	Hs03044135_m1
	MHC class-II	HLA-DRA	Hs00219575_m1
	CTLA-4	CTLA4	Hs00175480_m1
	FoxP3	FOXP3	Hs01085834_m1
	Tim-3	HAVCR2	Hs00262170_m1
	PD-1	PDCD1	Hs00169472_m1
	PD-L1	CD274	Hs00204257_m1
	BTLA	BTLA	Hs00699198_m1
	HVEM	TNFRSF14	Hs00187058_m1
	CD200	CD200	Hs01033303_m1
	IDO	IDO1	Hs00984148_m1
	Arginase 1	ARG1	Hs00968979_m1
	NKG2D	KLRK1	Hs00183683_m1
	MICA	MICA	Hs00741286_m1
	MICB	MICB	Hs00792952_m1
	IL-10	IL10	Hs00961622_m1
	TGF-b	TGFB1	Hs00998133_m1
	IL-17a	IL17A	Hs00174383_m1
	TNF-a	TNF	Hs00174128_m1
	IFN-g	IFNG	Hs00174143_m1
	IL-2	IL2	Hs00174114_m1
	Perforin	PRF1	Hs00169473_m1
	Granzyme B	GZMB	Hs00188051_m1
	LT-a	LTA	Hs00236874_m1
	LT-b	LTB	Hs00242739_m1
	LT-bR	LTBR	Hs00158922_m1
B	iNOS	NOS2	Hs01075529_m1
	CD163	CD163	Hs00174705_m1
	MR (CD206)	MRC1;MRC1L1	Hs00267207_m1
	FN-1	FN1	Hs01549976_m1
	IRF-4	IRF4	Hs01056533_m1
	IRF-5	IRF5	Hs00158114_m1
	IL-12	IL12B	Hs01011518_m1
	EGF	EGF	Hs01099999_m1
	EGFR	EGFR	Hs01076078_m1
	CSF-1	CSF1	Hs00174164_m1
	CSF-1R	CSF1R	Hs00911250_m1
	Mena	ENAH	Hs00403109_m1
C	IL-4	IL4	Hs00174122_m1
	IL-13	IL13	Hs00174379_m1
D	c-MYC	MYC	Hs00905030_m1

Taq Man Assays used for initial screen (A), for TAM-related screen (C), and to further characterize sorted CD4+ T cells (C) and myeloid fractions (D)

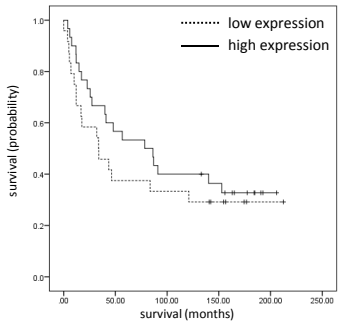
Supplementary Table S2: Patients' characteristics of received FFPE material

Histology	clear cell renal cell carcinoma	
Patient number	n=54	
Age (years)	40-86 (66.3 ± 7.2)	
Survival time (months)	0-213 (80.9 ± 64.2)	
Pathological Stage	pT1	2
	pT1a	3
	pT1b	16
	pT2	4
	pT3	8
	pT3a	6
	pT3b	13
	pT3c	1
	pT4	1

Information is only displayed for those patients who died of tumor-related causes

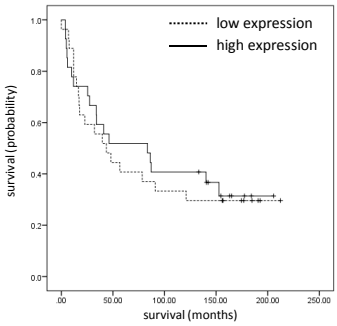
A

Perforin



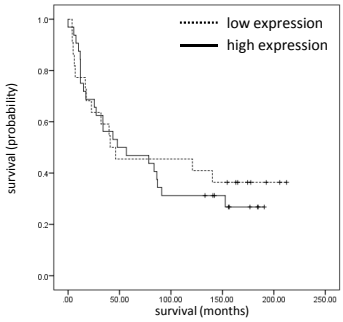
Log-rank test p=0.384
Cox regression: p=0.009, HR=1.550, 95% CI=1.177-2.151

CD4



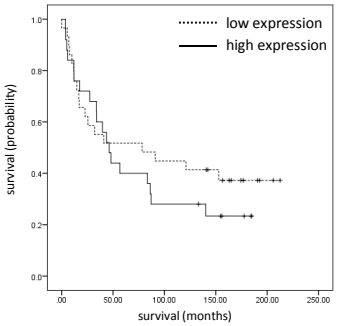
Log-rank test p=0.750
Cox regression: p=0.979, HR=0.933, 95% CI=0.588-1.678

CD8



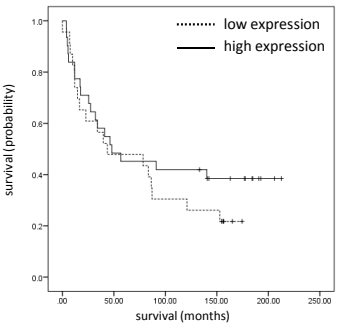
Log-rank test p=0.695
Cox regression: p=0.998, HR=1.000, 95% CI=0.788-1.267

MHC class-I



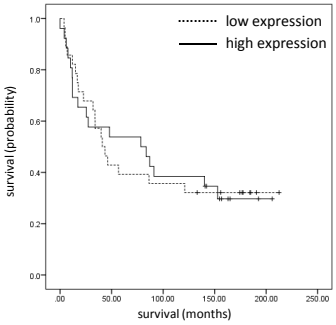
Log-rank test p=0.412
Cox regression: p=0.276, HR=0.885, 95% CI=0.710-1.103

MHC class-II



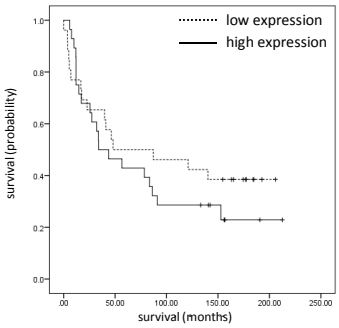
Log-rank test p=0.340
Cox regression: p=0.478 HR=0.899 95% CI=0.670-1.207

IDO



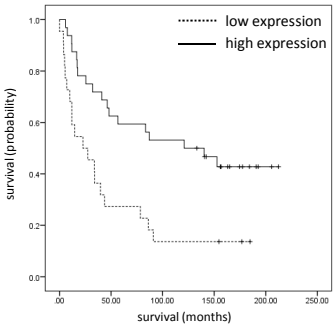
Log-rank test p=0.987
Cox regression: p=0.409, HR=1.077, 95% CI=0.903-1.284

IFN-γ*



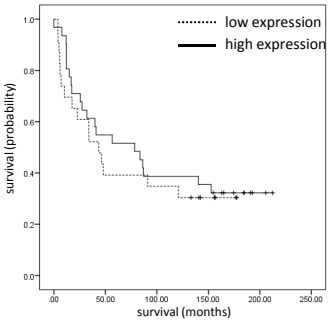
Log-rank test p=0.384
Cox regression: p=0.745, HR=0.973, 95% CI=0.827-1.146

TNF-α*



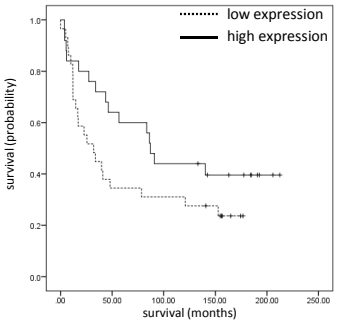
Log-rank test p=0.002
Cox regression: p=0.626, HR=1.035, 95% CI=0.902-1.186

Granzyme B*



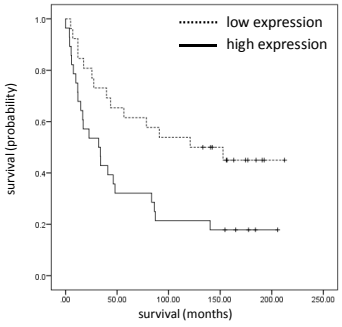
Log-rank test p=0.580
Cox regression: p=0.983, HR=0.998, 95% CI=0.841-1.184

HVEM



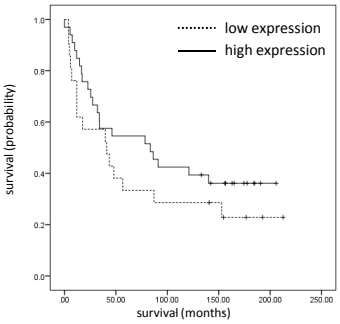
Log-rank test p=0.113
Cox regression: p=0.076, HR=1.380, 95% CI=0.967-1.970

LT-βR



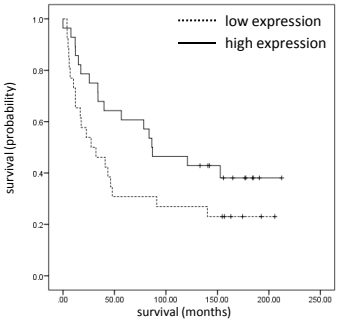
Log-rank test p=0.012
Cox regression: p=0.795, HR=0.963, 95% CI=0.722-1.283

LT-β*



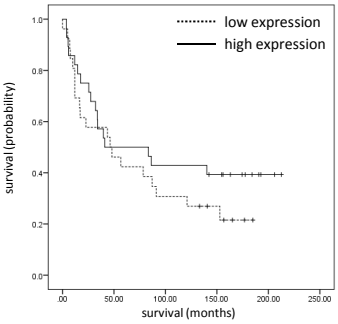
Log-rank test p=0.248
Cox regression: p=0.324, HR=1.129, 95% CI=0.887-1.436

NKG2D



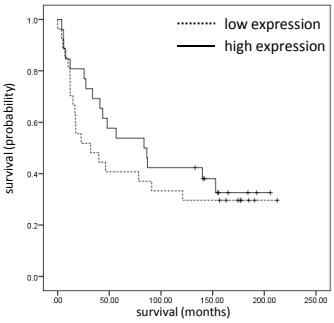
Log-rank test p=0.084
Cox regression: p=0.119, HR=1.337, 95% CI=0.928-1.926

MICA



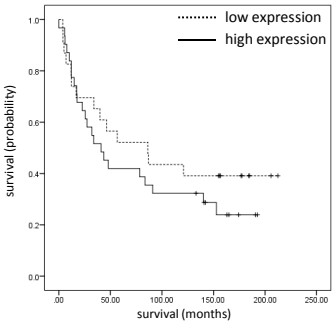
Log-rank test p=0.293
Cox regression: p=0.686, HR=0.949, 95% CI=0.737-1.222

MICB



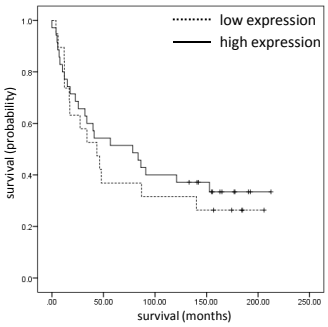
Log-rank test p=0.399
Cox regression: p=0.893, HR=1.011, 95% CI=0.858-1.192

CTLA-4*



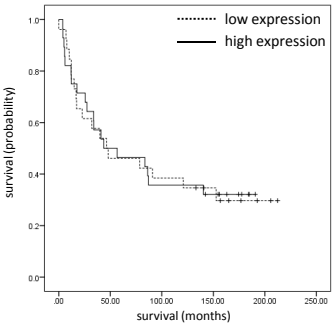
Log-rank test p=0.346
Cox regression: p=0.211, HR=0.923, 95% CI=0.814-1.046

IL-10*



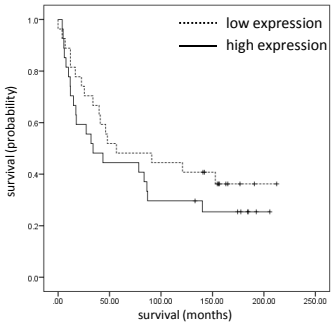
Log-rank test p=0.568
Cox regression: p=0.154, HR=0.871, 95% CI=0.721-1.053

TGF-β



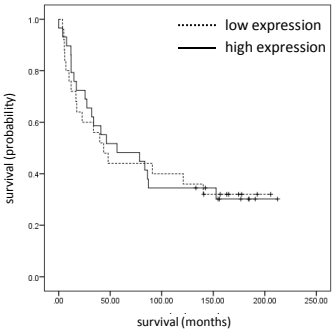
Log-rank test p=0.950
Cox regression: p=0.771, HR=1.054, 95% CI=0.740-1.501

PD-L1



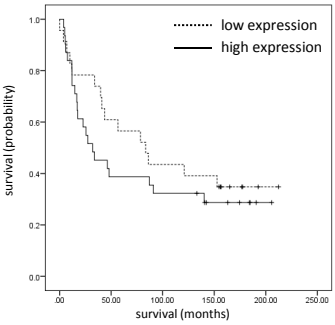
Log-rank test p=0.281
Cox regression: p=0.109, HR=0.820, 95% CI=0.644-1.045

PD-1*



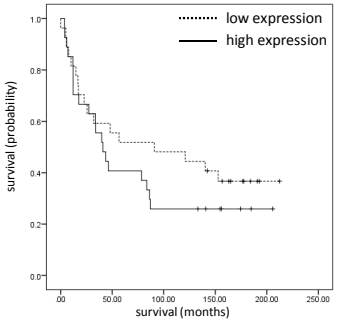
Log-rank test p=0.913
Cox regression: p=0.504, HR=0.952, 95% CI=0.826-1.099

Tim-3*



Log-rank test p=0.377
Cox regression: p=0.513, HR=0.947, 95% CI=0.806-1.114

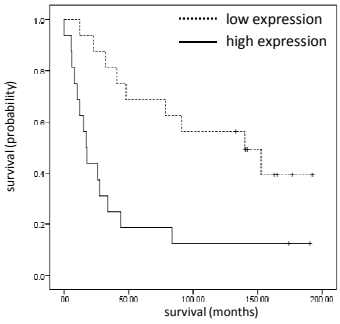
CD200



Log-rank test p=0.330
Cox regression: p=0.713, HR=0.968, 95% CI=0.815-1.150

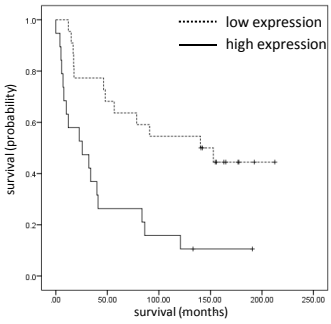
B

CTLA-4



Log-rank test p=0.003
Cox regression: p=0.008, HR=0.556, 95% CI=0.361-0.856

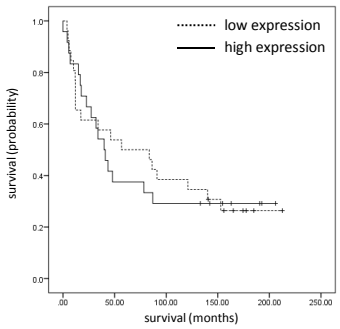
IL-10



Log-rank test p=0.001
Cox regression: p=0.009, HR=0.588, 95% CI=0.396-0.874

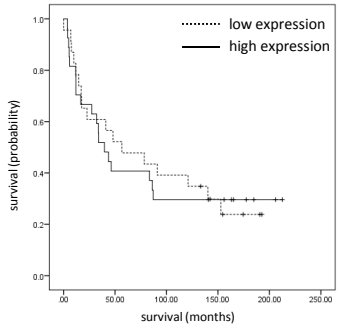
C

MR



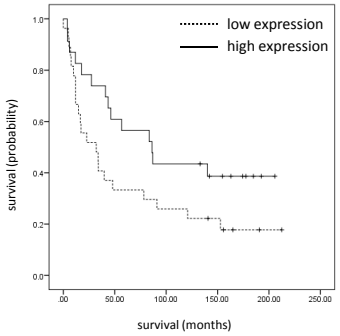
Log-rank test p=0.878
Cox regression: p=0.364, HR=1.109, 95% CI=0.887-1.387

EGF



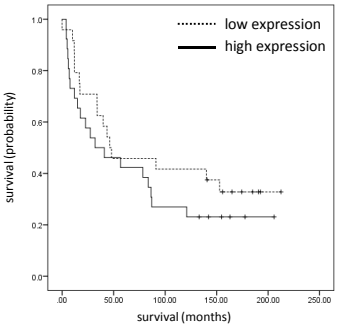
Log-rank test p=0.884
Cox regression: p=0.688 HR=1.027 95% CI=0.902-1.169

Mena



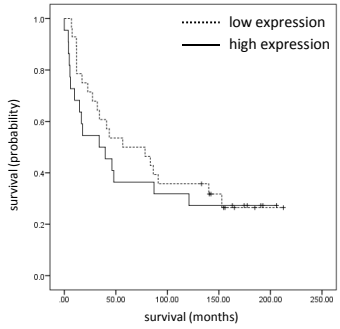
Log-rank test p=0.459
Cox regression: p=0.071, HR=0.771, 95% CI=0.581-1.023

CSF-1



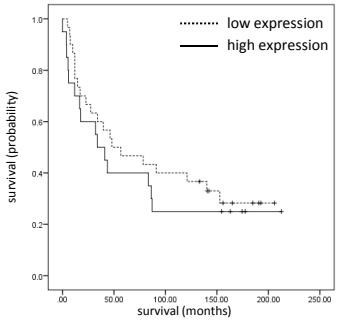
Log-rank test p=0.283
Cox regression: p=0.432, HR=0.860, 95% CI=0.590-1.253

CSF-1R



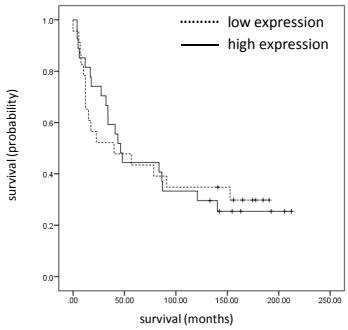
Log-rank test p=0.505
Cox regression: p=0.317, HR=0.816, 95% CI=0.549-1.215

EGF-R



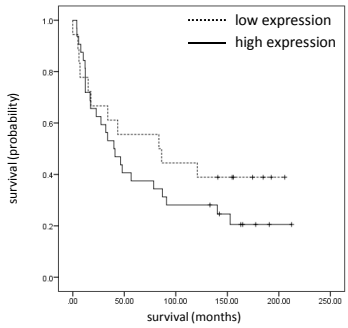
Log-rank test p=0.070
Cox regression: p=0.219, HR=1.18, 95% CI=0.906-1.536

IRF-5



Log-rank test p=0.989
Cox regression: p=0.542, HR=0.882, 95% CI=0.588-1.322

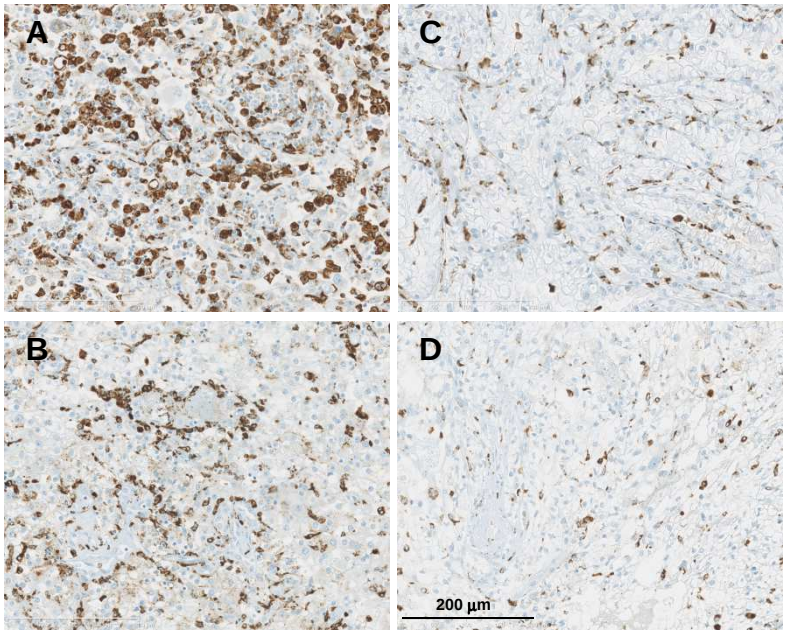
IL-12*



Log-rank test p=0.285
Cox regression: p=0.395, HR=0.959, 95% CI=0.872-1.056

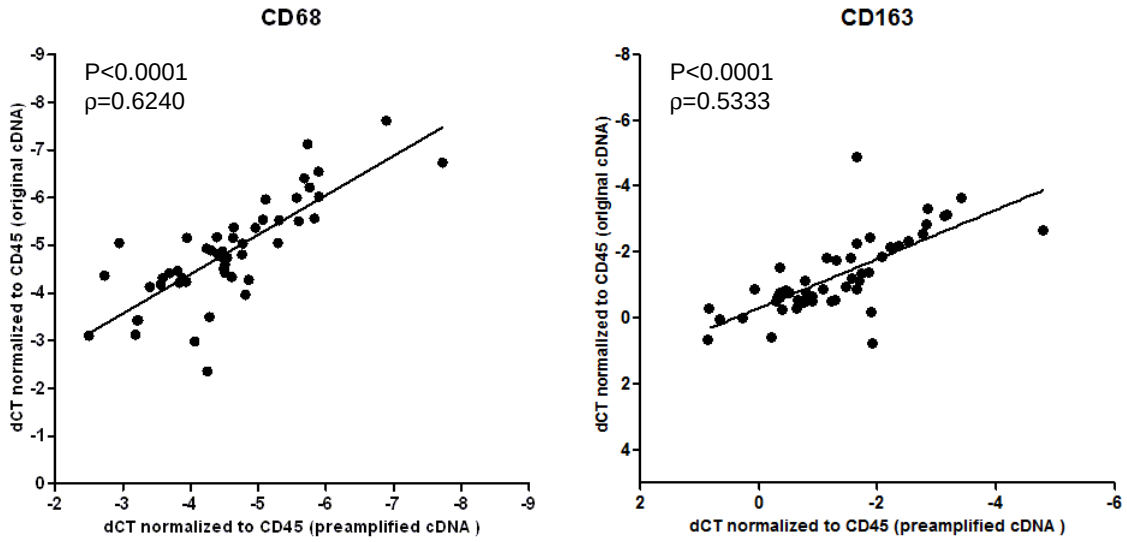
Supplementary Figure S1: Immune profile of ccRCC correlates with survival

54 ccRCC paraffin-embedded tumor samples were subjected to a retrospective qRT-PCR analysis for 43 different immune response-related genes. ΔCt levels of CD45 (A) were calculated by normalization to the endogenous control (18s rRNA), ΔCt levels of all other genes were calculated by normalization to CD45. Transcripts were quantified using original cDNA (A, B) or preamplified cDNA (C). Survival analysis was performed using the Cox proportional hazard model and, after dichotomizing the data based on the mean-expression level, also with the log-rank test of the Kaplan-Meier estimator. Kaplan-Meier curves are shown to represent the relation of the expression of the different target genes with survival and the results of both statistical tests are displayed underneath the plot. Patients that were still alive at time of analysis are marked with a tick. Some genes (marked with an asterisk) were only detected in very low amounts, and in some samples no signal was observed for those genes. Survival analysis was therefore performed once including the no-signal samples by giving an estimated value (Ct=40), and once after excluding the no signal samples from the analysis. The results with or without the no-signal samples were similar for all genes except for IL-10 and CTLA-4. Plots calculated after inclusion of all samples are displayed (A, C), and for CTLA-4 and IL-10 also after exclusion of the no-signal samples (B). Expression of Arginase-1, IL-2, LT-α, BTLA and IL-17 was not detected in the majority of samples and therefore not considered for survival analysis.



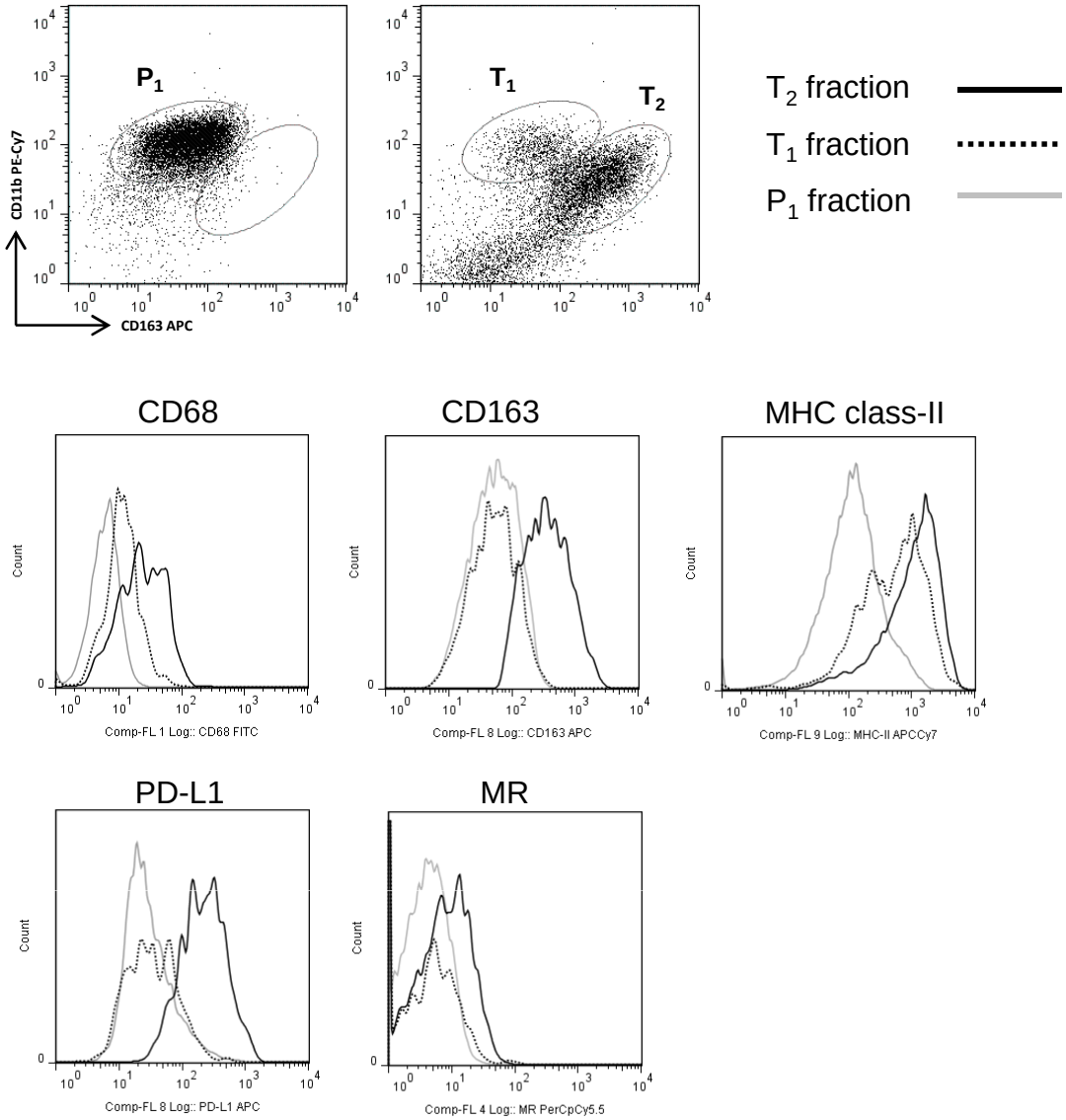
Supplementary Figure S2: Correlation between CD68 expression measured by qRT-PCR and by immunohistochemistry

Tumor sections of selected patients were stained for CD68 by immunohistochemistry (brown staining). Scans of microscopy slides at a magnification of 20x are shown. qRT-PCR analysis of the corresponding paraffin punches normalized to the endogenous control 18s rRNA revealed following ΔCt values (A): 14.07, (B): 14.19, (C): 15.63, (D): 18.47.



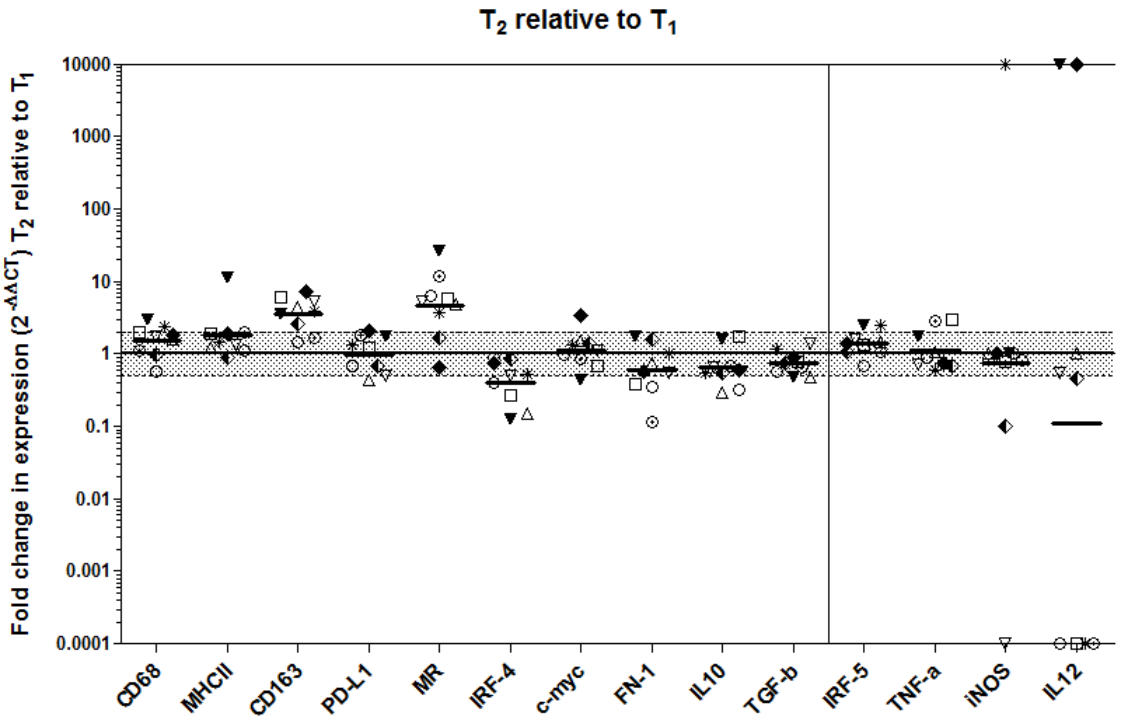
Supplementary Figure S3: ΔCT levels of original cDNA correlate with ΔCT levels of preamplified cDNA

cDNA of paraffin-embedded tumor material was preamplified to enable the evaluation of additional genes. The ΔCt levels of the original cDNA were plotted against the ΔCT levels of the preamplified cDNA for CD68 and CD163. ΔCt was calculated by normalizing Ct values to the Ct values of CD45. Each symbol represents an individual sample. Result of Spearman Rho correlation test is displayed in the graph.

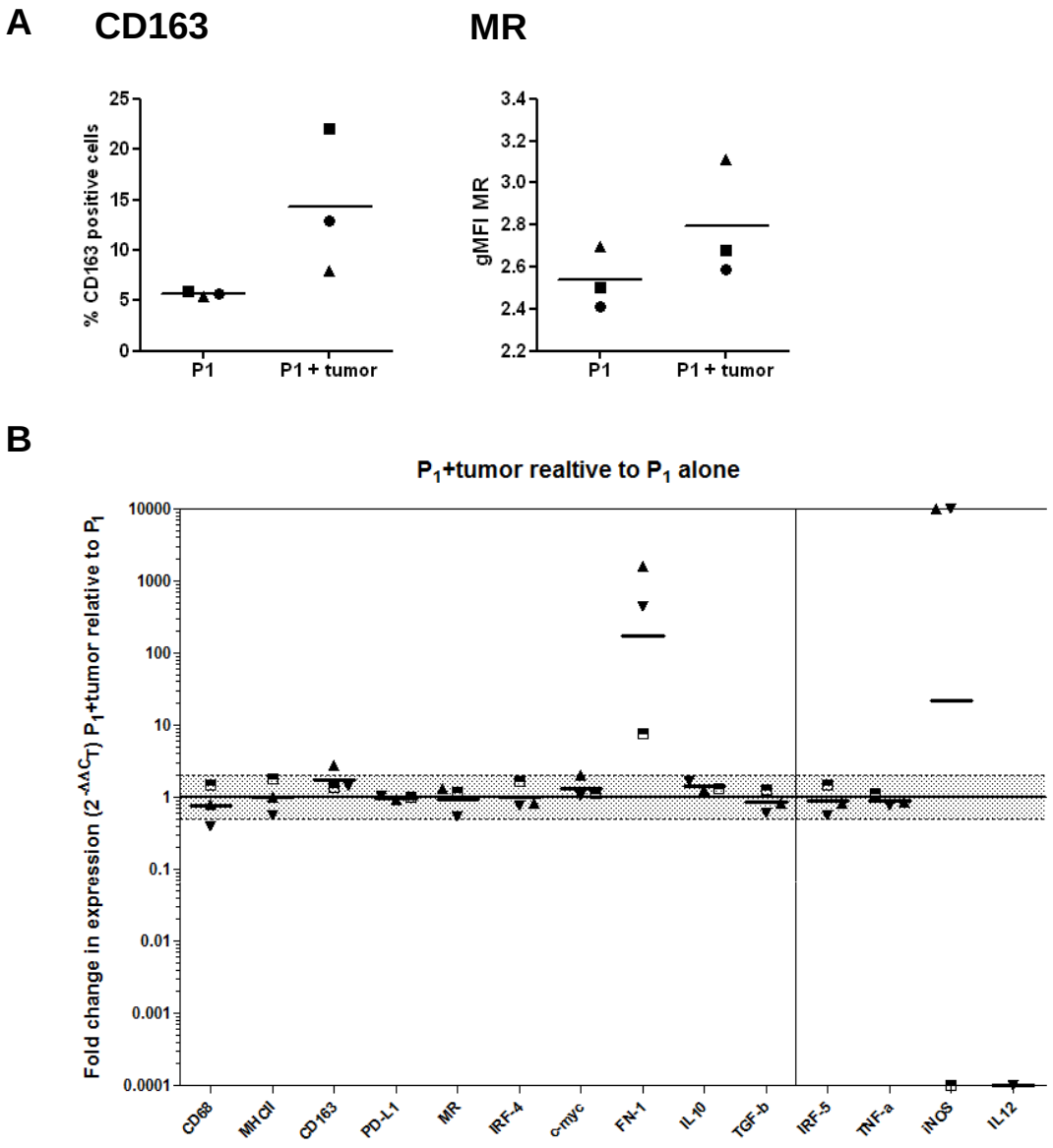


Supplementary Fig. S4: Representative staining for regulatory molecules on T_1, T_2 and P_1 myeloid populations

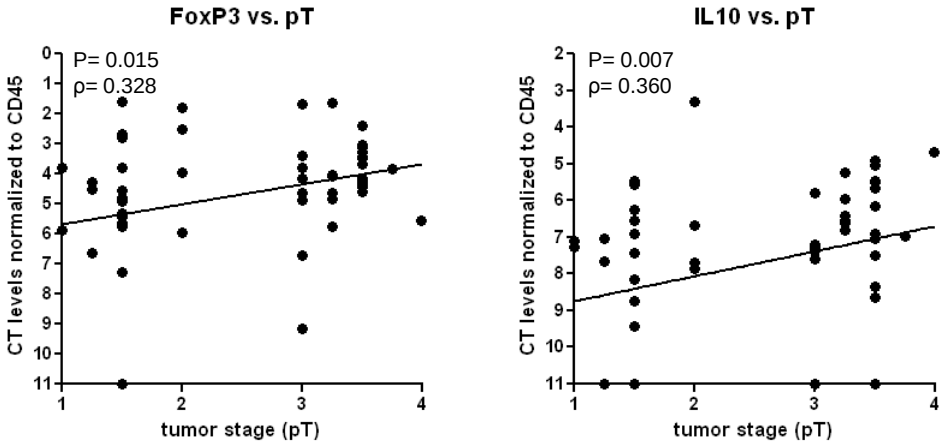
PBMCs and paired TILs were stained and T_1, T_2 and P_1 subpopulations were determined after gating on live $CD45^+CD3^-CD19^-$ cells. Representative dot plots of PBMCs (left) and TILs (right) are shown and histograms of the expression of CD68 (intra-cellular), CD163, PD-L1, MR and MHC class-II after gating on T_1, T_2 and P_1.



Supplementary Figure S5: Comparison of T_2 and T_1 fraction via qRT-PCR analysis
qRT-PCR analysis of FACS-sorted T_2 and T_1 fractions. Genes displayed on the left side of the vertical line are related to M2 TAMs, on the right side to M1 TAMs. ΔCt levels were calculated by normalizing the Ct values of the target genes to the Ct values of PPIA. Results are presented as fold change in expression level of T_2 relative to T_1. Fold differences in expression within the shaded area are considered as not significant. Symbols at the 10000 or 0.0001 line on the y-axis represent samples of which the fold change could not be calculated, since expression was only detected in one of the fractions. Each symbol represents an individual patient.



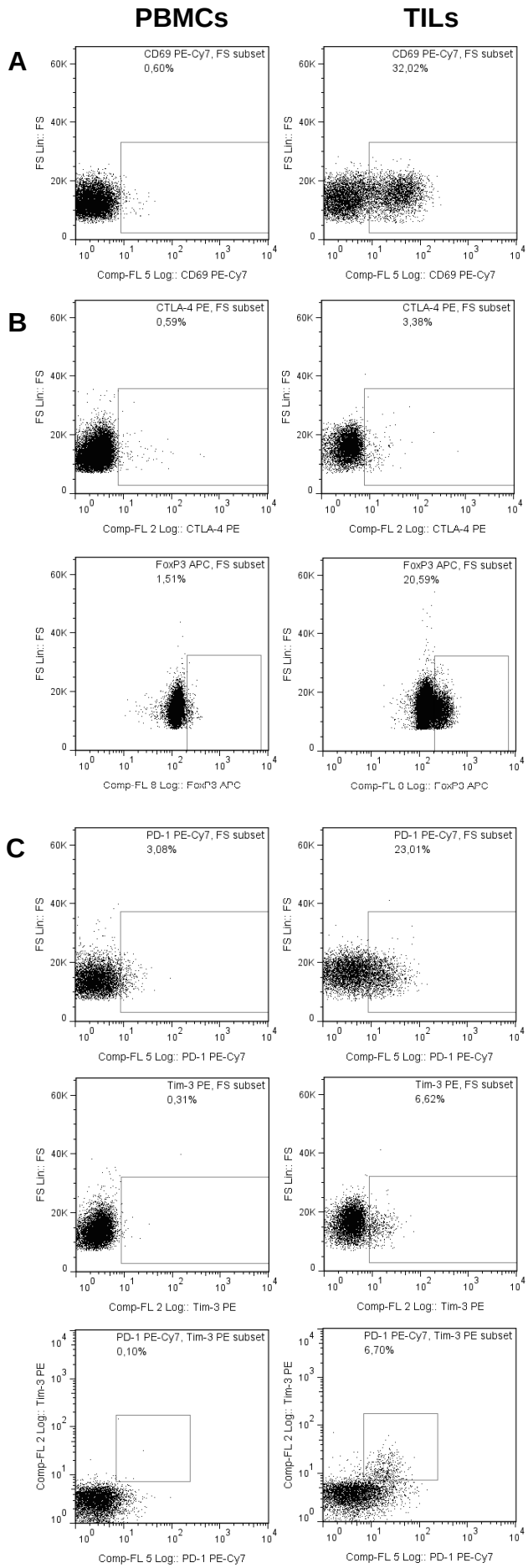
Supplementary Figure S6: Tumor cells promote transition of an M1 to an M2 phenotype
FACS-sorted blood-derived CD11b$^+$ cells were cultured with and without sorted autologous CD45$^-$ tumor cells for 48 h at a 1:3 ratio. (A) Results of FACS staining for different macrophage-associated markers after gating on CD45$^+$ CD11b$^+$ cells. (B) qRT-PCR for different M1- and M2-associated genes after preamplification of the cDNA. Genes displayed on the left side of the vertical line are related to M2 TAMs, on the right side to M1 TAMs. ΔCt levels were calculated by normalizing the Ct values of the target genes to the Ct values of CD45. Results are presented as fold change in expression level of CD11b$^+$ cells cocultured with CD45$^-$ tumor cells (P_1+tumor) relative to CD11b$^+$ cells cultured without (P_1 alone). Fold differences in expression within the shaded area are considered as not significant. Symbols at the 10000 or 0.0001 line on the y-axis represent samples of which the fold change could not be calculated, since expression was only detected in one of the fractions. Each symbol represents an individual patient. Geometric means of each group are depicted.



Supplementary Figure S7: Positive correlation of FoxP3 and IL-10 with tumor progression
qRT-PCR was performed on cDNA of FFPE material from ccRCC tumors and gene expression was correlated with tumor stage (pT). Results of Spearman Rho correlation analysis are shown for FoxP3 and IL-10. Each symbol represents an individual patient. ΔCt levels were calculated by normalizing the Ct values of the target genes to the Ct values of CD45. pT was defined as 1= pT1, 1.25= pT1a, 1.5=pT1b, 2=pT2, 3=pT3, 3.25=pT3a, 3.5=pT3b, 3.75=pT3c and 4=pT4.

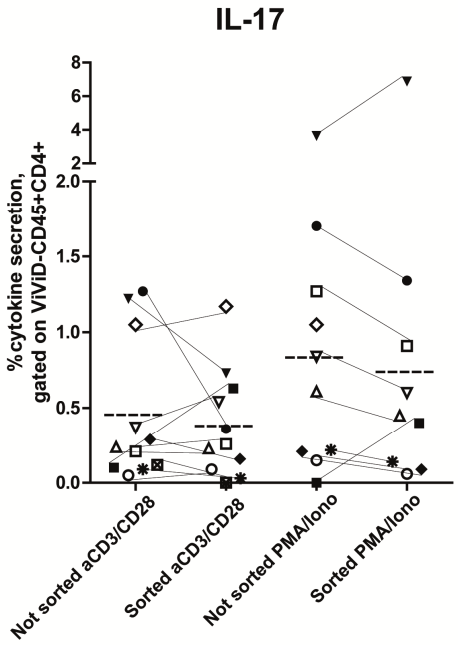
Supplementary Table S3: Patients' characteristics of fresh tumor material

Histology	clear cell renal cell carcinoma	
Patient number	n=20	
Age (years)	37-84 (60.5 ± 11)	
Pathological Stage	pT1	0
	pT1a	9
	pT1b	3
	pT2	3
	pT3	0
	pT3a	3
	pT3b	2
	pT3c	0
	pT4	0



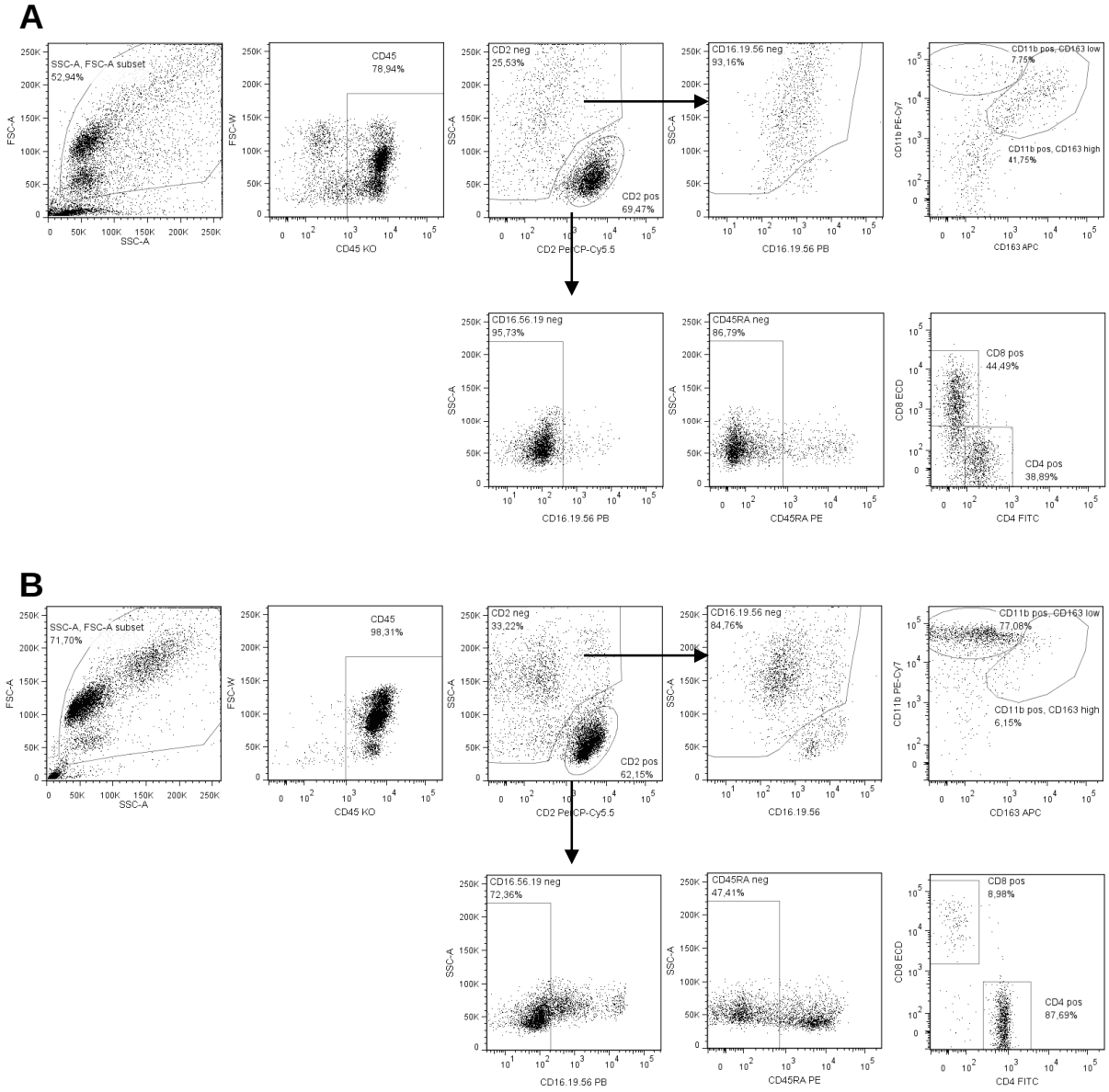
Supplementary Figure S8:
Representative staining of co-inhibitory molecules on blood- and tumor-derived T cells

PBMCs and autologous TILs were stained with fluorochrome-labeled antibodies for specific surface markers and their expression is depicted after gating on (A) live $CD45^+$ $CD3^+$ cells, on (B) live $CD45^+$ $CD4^+$ cells or on (C) live $CD45^+$ $CD8^+$ cells.



Supplementary Figure S9: The tumor microenvironment does not impact on the capacity of CD4⁺ T cells to produce IL-17

Intracellular staining for IL-17 after 6h *ex vivo* stimulation with anti-CD3/CD28 beads or with PMA + ionomycin in the presence of brefeldin A and monensin. Cells were stimulated within the tumor digest or after sorting of CD45⁺ CD4⁺ T cells and were analyzed after gating on live CD45⁺ CD4⁺ T cells. Each symbol represents an individual patient; results of unsorted and sorted T cells from the same patient are connected by a thin line, the mean of each group is depicted.



Supplementary Figure S10: Sorting strategy for myeloid and T cells from fresh ccRCC biopsies and blood

Sorting strategy for the simultaneous isolation of myeloid cells and T cells from (A) digested ccRCC tumors and (B) autologous PBMCs. After exclusion of cell debris, gates were set on $CD45^+$ cells. For the sorting of T cells gates were set on $CD2^+$ cells, $CD16^-$ $CD19^-$ and $CD56^-$ cells, followed by gating on $CD45RA^-$ cells and finally on either the $CD4^+$ or $CD8^+$ population. For the isolation of myeloid cells gates were set on $CD2^-$ cells, then on $CD16^-$ $CD19^-$ and $CD56^-$ cells, and finally on the $CD11b^+$ $CD163^{low}$ population (P_1 and T_1 fraction) or on the CD11b+$CD163^{high}$ population (T_2 fraction).

SUPPLEMENTARY MATERIALS AND METHODS

Flow Cytometry for phenotypical analysis

Cells were stained with combinations of following fluorochrome-conjugated antibodies CD4 FITC, Tim-3 PE, CTLA-4 PE, CD8 ECD, CD3 PerCP, PD-1 PE-Cy7, CD69 PE-Cy7, CD45RA PacificBlue, CD45 KromeOrange, FoxP3 APC, CD45RA APC, CD25 APC-Cy7, CD68 FITC, PD-L1 PE, CD14 ECD, MR PerCPCy5.5, CD11b PE-Cy7, CD3 PacificBlue, CD19 PacificBlue, CD45 KromeOrange, CD163 APC or PD-L1 APC, HLA-DR APC-Cy7.

FACS sorting of T cells and TAMs from TILs and PBMCs

(i) Sorting of T cells only: processed tumors or PBMC

Surface staining mix: CD45 PerCP-Cy5.5, CD8 ECD and CD4 KromeOrange.

(ii) Sorting of T cells plus TAMs: processed tumors or PBMC

Surface staining mix: CD45 KromeOrange, CD19, CD16, CD56 PacificBlue, CD8 ECD, CD4 FITC, CD11b PE-Cy7, CD163 APC, CD2 PerCP-Cy5.5, and CD45RA PE or PacificBlue.

Polyclonal stimulation and intracellular cytokine staining (ICS)

(i) Stimulation of T cells in presence or absence of tumor microenvironment

Surface staining mix: CD45 PerCP-Cy5.5, CD8 ECD, CD4 KromeOrange, CD14, CD16, CD19 PacificBlue; intracellular staining mix: IFN-γ FITC, IL-17 PE, IL-2 PE-Cy7, IL10 APC.

(ii) Stimulation of T cells in presence or absence of TAMs

Surface staining mix: CD45KromeOrange, CD4 FITC, CD11b PacificBlue with (A) or without TGF-β1 PE (B); intracellular staining mix: (A) IL-4 PE-Cy7, IL-2 PerCP-Cy5.5, IL-10 APC, (B) IL17 PE, IFN-γ PE-Cy7, IL-13 APC, TNF-α PerCPCy5.5.

RNA isolation from paraffin material

Deparaffinization of the paraffin punches was performed in 300 µL elution buffer (1 M Tris pH 8, 0.5 M EDTA pH 8, 20% SDS (all Ambion), ultrapure water (Sigma)) for 10 min at 95°C while shaking. Samples were subsequently centrifuged (Eppendorf centrifuge 5417R, Omnilab) for 10 min at 14'000 rpm and 4°C

and digested with 3 µL of Proteinase K (18 +/- 4 mg/ml, Roche) for 72 hours at 55°C. Thereafter samples were centrifuged for 2 min at 14'000 rpm and 4°C and 250 µL of the obtained supernatants were transferred into new tubes. 750 µL TRIzol LS Reagent (Invitrogen) was added to each sample and tubes were mixed by vortexing. Samples were homogenized by centrifugation for 2 min at 14'000 rpm and 4°C using QIA-shredder columns. RNA purification was performed by phenol and chloroform extractions: 200 µL of chloroform was added to each flow-through, then samples were mixed by inverting the tubes, incubated at room temperature for 5 min until two phases were visible and centrifuged for 15 min at 14'000 rpm and 4°C. The upper aqueous phase containing the RNA was transferred into a new tube, 20 µg glycogen (Invitrogen) was added and the RNA precipitated by adding 0.5 mL isopropanol (99.9% V/V, Kantonsapotheke Zürich). Samples were subsequently incubated for 15 min at room temperature and centrifuged for 20 min at 14'000rpm and 4°C. After removing the supernatant the pellet was washed with 1 mL 75% ethanol (absolute for analysis, Merck), air-dried, dissolved in RNase free water (Sigma) and digested with 80 U/ml DNAse I (New England Biolabs) for 15 min at room temperature followed by an inactivation by 2 mM EDTA (Ambion) for 10 min at 65°C.