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Spontaneous Peripheral T-cell Responses toward the Tumor-Associated Antigen Cyclin D1 in Patients with Clear Cell Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is a heterogeneous group of kidney cancers with clear cell RCC (ccRCC) as the major subgroup. To expand the number of clinically relevant tumor-associated antigens (TAA) that can be targeted by immunotherapy, we analyzed samples from 23 patients with primary ccRCC for the expression and immunogenicity of various TAAs. We found high-frequency expression of *MAGE-A9* and *NY-ESO-1* in 36% and 55% of samples, respectively, and overexpression of *PRAME*, *RAGE-1*, *CA-IX*, *Cyclin D1*, *ADFP*, *C-MET*, and *RGS-5* in many of the tumor samples. We analyzed the blood of patients with HLA-A2⁺ ccRCC for the presence of CD8⁺ T cells specific for TAA-derived HLA-A2-restricted peptides and found spontaneous responses to cyclin D1 in 5 of 6 patients with *Cyclin D1*-positive tumors. Cyclin D1-specific CD8⁺ T cells secreted TNF- α , IFN- γ , and interleukin-2 (IL-2), and degranulated, indicating the presence of polyfunctional tumor-specific CD8⁺ T cells in the blood of these patients with ccRCC. The high frequency (43%) of *Cyclin D1* overexpression and the presence of functional cyclin D1-specific T cells in 83% of these patients with ccRCC suggest that cyclin D1 may be a target for immunotherapeutic strategies. *Cancer Immunol Res*; 1(5); 1–8. ©2013 AACR.

Introduction

The immune system recognizes and controls tumors through a process called cancer immune surveillance (1). Studies showed a correlation between tumor infiltration by T cells and patient survival in various cancers (2). Furthermore, spontaneous immunity against tumor-associated antigens (TAA) can be detected in patients with cancer (3). TAAs include cancer-testis (CT) antigens, differentiation antigens, mutated proteins, overexpressed proteins, and viral antigens (4). Boosting spontaneous TAA-specific immunity is a low-toxicity strategy that resulted in objective clinical responses in some patients with cancer (5).

Renal cell carcinoma (RCC) is a heterogeneous cancer that encompasses about 90% of all human kidney tumors, and clear cell RCC (ccRCC) is the major histologic subgroup (6). About one third of RCC has already metastasized at the time of diagnosis, and 20% to 50% of resected patients develop meta-

stasis (7). Treatment of advanced and metastatic RCC is challenging due to its relative resistance to chemo- and radiotherapy (8).

Evidence supporting RCC as an immunogenic cancer includes cases of spontaneous regressions, increased incidence in immunosuppressed patients, and the high density of tumor-infiltrating leukocytes (TIL; ref. 9). Since the 1980s, immunostimulatory compounds such as interleukin-2 (IL-2) and IFN- α are being used to treat RCC. Despite modest therapeutic efficacy in some patients, the concomitant severe systemic toxicity remains a problem (8). Furthermore, data showing TAA-specific immunity in patients with RCC are scarce.

We conducted quantitative reverse transcription PCR (qRT-PCR) on samples from 23 patients with primary ccRCC for the expression of genes encoding cancer-testis antigens MAGE-A1 (CT1.1), -A3 (CT1.3), -A4 (CT1.4), -A9 (CT1.9), -A10 (CT1.10), synovial sarcoma X breakpoint 2 (SSX2/CT5.2), New York esophageal I (NY-ESO-1/CT6.1), L antigen 1 (LAGE-1/CT6.2) and MAGE-C1 (CT7), carbonic anhydrase IX (CA-IX/G250), renal antigen 1 (RAGE-1), preferentially expressed antigen of melanoma (PRAME), adipose differentiation-related protein (adipophilin/ADFP), C-MET proto-oncogene, cyclin D1 (CCND1), and regulator of G-protein signaling 5 (RGS-5). We also determined whether spontaneous T-cell responses were elicited against any of these antigens.

Materials and Methods

Patients

Patients with ccRCC underwent full or partial nephrectomy as part of their standard treatment at the Department of

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Table 1. Patient information

Histology	ccRCC	
Patient number	<i>n</i> = 23	
Age, y	37–84 (64.1 ± 11)	
Pathologic stage	pT1	1
	pT1a	8
	pT1b	4
	pT2	3
	pT3	0
	pT3a	3
	pT3b	4
	pT3c	0
	pT4	0

Urology, University Hospital Zurich (Zurich, Switzerland), between 2008 and 2011. Tumor specimens and peripheral whole blood were obtained following informed consent in accordance with the Declaration of Helsinki. The local ethics committee approved the study (EK-1017 and EK-1634). Detailed patients' characteristics are listed in Table 1. All patients were HLA-A2–typed using fluorescein isothiocyanate (FITC)–conjugated HLA-A2–specific or isotype control antibodies (BioLegend), on a CyAn ADP 9 flow cytometer (Beckman Coulter), and data were analyzed using FlowJo software (TreeStar).

Processing of blood and tumor samples

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll (Ficoll-Paque PLUS; GE Health-

care) density centrifugation. Immediately after surgery, a small piece of tumor tissue was snap-frozen in liquid nitrogen for RNA isolation. The remaining tumor was digested with 6 U/mL DNase I type IV (Sigma), 1 mg/mL collagenase IV (Sigma) in Dulbecco's modified Eagle medium (DMEM; Gibco), 50 U/mL penicillin, and 50 U/mL streptomycin (Gibco) for 1 to 2 hours at 37°C. The resulting single-cell suspensions and the PBMCs were cryopreserved at –80°C until analysis.

RNA isolation

Total RNA was extracted from snap-frozen ccRCC tumor samples using the RNeasy Mini Kit (Qiagen) followed by digestion with DNase I (New England BioLabs) according to the manufacturer's instructions.

Quantitative real-time PCR

The concentration and purity of RNA were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), 500 ng of RNA was reverse transcribed according to the manufacturer's instructions; cDNA was stored at –20°C until qRT-PCR analysis was conducted on a Rotor-Gene Q real-time PCR cycler (Qiagen) using the commercially available TaqMan reagents with optimized primer and probe concentrations (TaqMan gene expression assays; Applied Biosystems; Table 2). After an initial hold for 2 minutes at 50°C and 10 minutes at 95°C, the probes were cycled 45 times at 95°C for 15 seconds and at 60°C for 60 seconds. All PCR reactions were carried out in triplicates. Threshold cycle (C_t) values were determined with the Rotor-Gene Q Series software 1.7. ΔC_t values were calculated by normalizing the

Table 2. TaqMan assays of TAAs and derived HLA-A2–restricted peptides

	TaqMan assay ID	Position	Peptide sequence	Peptide source
MAGE-A1	Hs00607097_m1	278–286	KVLEYVIKV	Not used
MAGE-A3	Hs00366532_m1	271–279	FLWGPRALV ^d	Not used
MAGE-A4	Hs00365979_m1	230–239	GVYDGREHTV	Not used
MAGE-A9	Hs00893224_m1	223–231	ALSVMGVYV	Thermo Scientific
MAGE-A10	Hs01560792_m1	254–262	GLYDGMHL	Not used
SSX2	Hs00817683_m1	41–49	KASEKIFYV	Not used
NY-ESO-1	Hs00265824_m1	157–165	SLLMWITQC	Bio Synthesis
LAGE-1	Hs00535628_m1	41214	MLMAQEALAF	Not used
		157–165	SLLMWITQV	Not used
MAGE-C1	Hs00193821_m1	959–968	ILFGISLREV	Not used
		1083–1091	KVVEFLAML	Not used
PRAME	Hs00196132_m1	425–433	SLLQHLIGL	Thermo Scientific
		100–108	VLDGLDVLL	Thermo Scientific
RAGE-1	Hs00179504_m1	32–40	PLPPARNGGL	Thermo Scientific
		352–360	LKLSGVVRL	Thermo Scientific
CA-IX	Hs00154208_m1	254–262	HLSTAFARV	Thermo Scientific
CCND1	Hs00765553_m1	101–109	LLGATCMFV	S. Stevanovic, Tuebingen
		228–236	RLTRFLSRV	S. Stevanovic, Tuebingen
ADFP	Hs00765634_m1	129–137	SVASTITGV	S. Stevanovic, Tuebingen
C-MET	Hs00179845_m1	654–662	YVDPVITSI	S. Stevanovic, Tuebingen
RGS-5	Hs00186212_m1	5–13	LAALPHSCL	S. Stevanovic, Tuebingen

target mRNA levels to the endogenous control 18s-rRNA (Hs03928990_g1; Applied Biosystems). Testis cDNA generated from human testes total RNA (Invitrogen) served as positive control for expression analysis of cancer-testis antigens. As qRT-PCR for *MAGE-A9* suggested low expression in the healthy kidney control (CT 35, ΔC_t 30), expression in ccRCC specimens was considered positive at a $CT < 35$ and a $\Delta C_t < 30$. For overexpressed antigens, ΔC_t levels of tumors were compared with ΔC_t levels of healthy kidney control (human kidney total RNA; Invitrogen) using the $2^{-\Delta\Delta C_t}$ formula. Only changes 2-fold or more were considered as overexpression.

Genomic DNA extraction

Punch biopsies from paraffin-embedded ccRCC samples were incubated (10 minutes at 95°C) with 300 μ L of buffer containing 20 mmol/L Tris pH 8.0, 20 mmol/L EDTA and 1% SDS. After cooling down, 3 μ L of Proteinase K (18 \pm 4 mg/mL; Roche) was added to each sample and incubated at 55°C for 72 hours. Digested samples were centrifuged and 4 volumes of RLT buffer (AllPrep DNA/RNA Mini Kit; Qiagen), were added to the supernatant. Genomic DNA was extracted following the manufacturer's protocol.

Cyclin D1 sequencing

Purified genomic DNA was amplified using the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) with the following PCR conditions: 10 minutes at 98°C, followed by 34 cycles of 30 seconds at 98°C, 30 seconds at 68°C, 20 seconds at 72°C with a final elongation of 5 minutes at 72°C. Sequences encoding the HLA-A2-restricted CTL epitope corresponding to cyclin D1 amino acids 101–109 or 228–236 were amplified by using the forward primer 5'-TGCGAGGAACAGAAAGTGC-GA-3'/reverse primer 5'-TCCAGTGGTTACCAGCAGCTC-3' or forward primer 5'-TGCTCACAGCCTCCTTCCCT-3'/reverse primer 5'-TCGGCATTTCCGTGGCACTA-3' respectively. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) and sequenced using the same amplification primers.

MACS sorting and *in vitro* stimulation

T-cell stimulation was conducted as described previously (10). Briefly, CD8⁺ and CD4⁺ T cells were isolated sequentially from PBMCs by positive selection using MACS (Miltenyi Biotech) according to the manufacturer's instructions. The remaining CD8⁺CD4⁺ fraction was used as antigen-presenting cells (APC), which were loaded with 10⁻⁵ mol/L of each peptide (Table 2). For *in vitro* stimulation, 5 \times 10⁵ CD8⁺ T cells were incubated with 5 \times 10⁵ loaded and irradiated (30 Gy) APCs in 96-well flat-bottomed plates. Cells were cultured for 9 days in 200 μ L of TC-RPMI [i.e., RPMI (Gibco), supplemented with NaHCO₃ (2 g/L; Sigma), L-glutamine (2 mmol/L, Sigma), penicillin and streptomycin (50 U/mL; Gibco), minimum essential medium nonessential amino acids (1 \times ; Gibco), sodiumpyruvate (1 mmol/L; Gibco), 10⁻⁴ mol/L β -mercaptoethanol (Sigma), and 10% pooled human serum plus DNase I (6 U/mL; Sigma)]. T-APCs were generated from purified autologous CD4⁺ T cells stimulated with 1 μ g/mL phytohemagglutinin (PHA; Sigma) and 100 U/mL recombinant IL-2 (R&D Systems) during the

9-day culture. T-APCs were used as APCs during the short *in vitro* stimulation before intracellular cytokine staining (ICS). Medium was exchanged every second day with TC-RPMI containing 25 U/mL of IL-2.

Intracellular cytokine staining

At day 9, the presence of TAA-specific T cells was tested by short-term restimulation with relevant peptides, followed by ICS. Briefly, T cells were stimulated with peptide-loaded autologous T-APCs at a 1:2 ratio for 5 hours in the presence of brefeldin A (10 μ g/mL; Sigma), monensin (10 μ g/mL; Sigma), 1 μ g/mL anti-CD28/49d (BD), and appropriately diluted phycoerythrin (PE)-labeled anti-CD107a antibody. Before addition to the assay, T-APCs were labeled with 2 μ mol/L carboxy-fluoresceinsuccinimidyl ester (CFSE; Sigma), which allowed their exclusion before analysis. Surface staining for CD45-PerCP, CD3-Pacific Orange (Invitrogen), CD8-ECD (Beckman Coulter), CD14-FITC, CD16-FITC, CD19-FITC, and live-dead staining (LIVE/DEAD Fixable Green Dead Cell Stain Kit; Invitrogen) was conducted in PBS [NaCl (136 mmol/L; Fluka), Na₂HPO₄ (8 mmol/L; Roth), KH₂PO₄ (1.5 mmol/L; Roth), pH 7] for 20 minutes at room temperature. Subsequently, cells were fixed with 4% formalin (Kantonsapotheke Zurich) and incubated for 5 minutes at room temperature with permeabilization buffer [PBS supplemented with 2 mmol/L EDTA (Sigma), 2% fetal calf serum (FCS; PAA Laboratories), 0.05% NaN₃ (Sigma), and 0.1% Saponin (Sigma)]. Intracellular staining was conducted using IFN- γ -APC, TNF- α -PECy7 (ebiosciences), and IL-2-Pacific Blue antibodies in permeabilization buffer for 20 minutes at room temperature in the dark. Cells were washed once with permeabilization buffer and resuspended in PBS containing 1% formalin. Samples were evaluated by flow cytometry and data were analyzed with FlowJo software as described earlier. Unless stated differently, all antibodies were purchased from BioLegend.

Tetramer staining

PBMCs or digested tumor samples were stained with tetramers for the presence of TAA-specific CD8⁺ T cells. Briefly, cells were resuspended in PBS and incubated for 10 minutes at 37°C with PE-labeled tetramers consisting of HLA-A2 plus cyclin D1_{101–109}, cyclin D1_{228–236}, CMV_{495–503} or FLU-MATRIX_{58–66}, followed by incubation with anti-CD45 APC, anti-CD8 Pacific Blue (both BioLegend), and live-dead stain (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen) for 15 minutes at 4°C. Cells were washed once with PBS and resuspended in PBS containing 1% formalin. Samples were evaluated by flow cytometry and data were analyzed with FlowJo software. The percentage of tetramer-positive cells was determined after gating on live, CD45⁺ CD8⁺ cells.

Western blotting

The presence of cyclin D1-specific immunoglobulin G (IgG) in patient sera was tested by Western blotting as described previously (11) using recombinant human cyclin D1 (Abnova cat. no. H00000595-PO1). Sera from 22 patients with ccRCC were used at a 1:250 dilution. MaxPab mouse polyclonal anti-

human CCND1 (Abnova cat. no. H00000595-BO1P) was used as positive control at 1:1,000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Immuno-Research; 115-035-008) was used as positive control and HRP-conjugated goat anti-human IgG (Jackson Immuno-Research 109-035-006) for serum samples at a dilution of 1:25,000. Blots were developed with Western Lightning Plus enhanced chemiluminescence (ECL) reagents (PerkinElmer cat. no. NEL104001EA) and signals were measured using a Fusion FX7 machine (VILBER LOURMAT).

Results

Frequent expression of TAAs in primary ccRCC tumor samples

The expression of nine cancer-testis and seven other anti- gens in 23 primary ccRCC specimens was analyzed by qRT-PCR. We found expression of *MAGE-A9* and *NY-ESO-1* in 36%

and 55% of samples, respectively, and did not find expression of the other seven cancer-testis antigens in any samples (Fig. 1A). Although *MAGE-A9* and *NY-ESO-1* were frequently expressed, their expression levels were very low (Fig. 1B). Overexpression of *RAGE-1* in 13%, *C-MET* in 30%, *PRAME* in 39%, *Cyclin D1* in 43%, *ADFP* in 65%, *RGS-5* in 83%, and *CA-IX* in 96% of ccRCCs specimens was also observed (Fig. 1A and B). More than 50% of the ccRCC specimens coexpressed four to six different TAAs (Fig. 1C). We found no evidence for preferred coexpression of particular TAAs (Fig. 1D).

Spontaneous cyclin D1-specific CD8⁺ T-cell responses in patients with primary ccRCC

Because of limited specimen availability, we selected HLA-A2⁺ patients and used previously described HLA-A2-restricted peptides for stimulation of PBMCs (<http://www.cancerimmunity.org/CTdatabase>; refs. 12–14; summarized in Table 2).

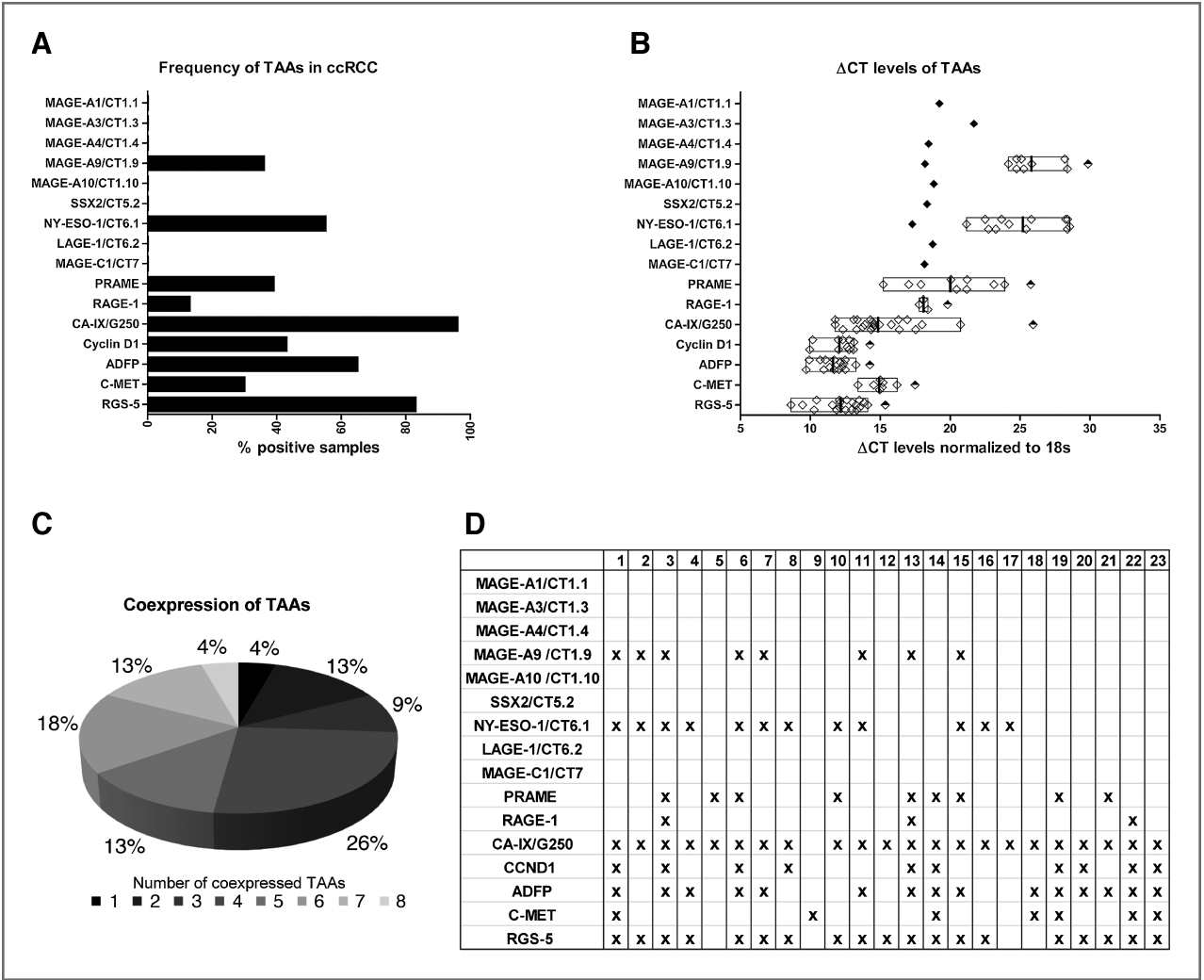
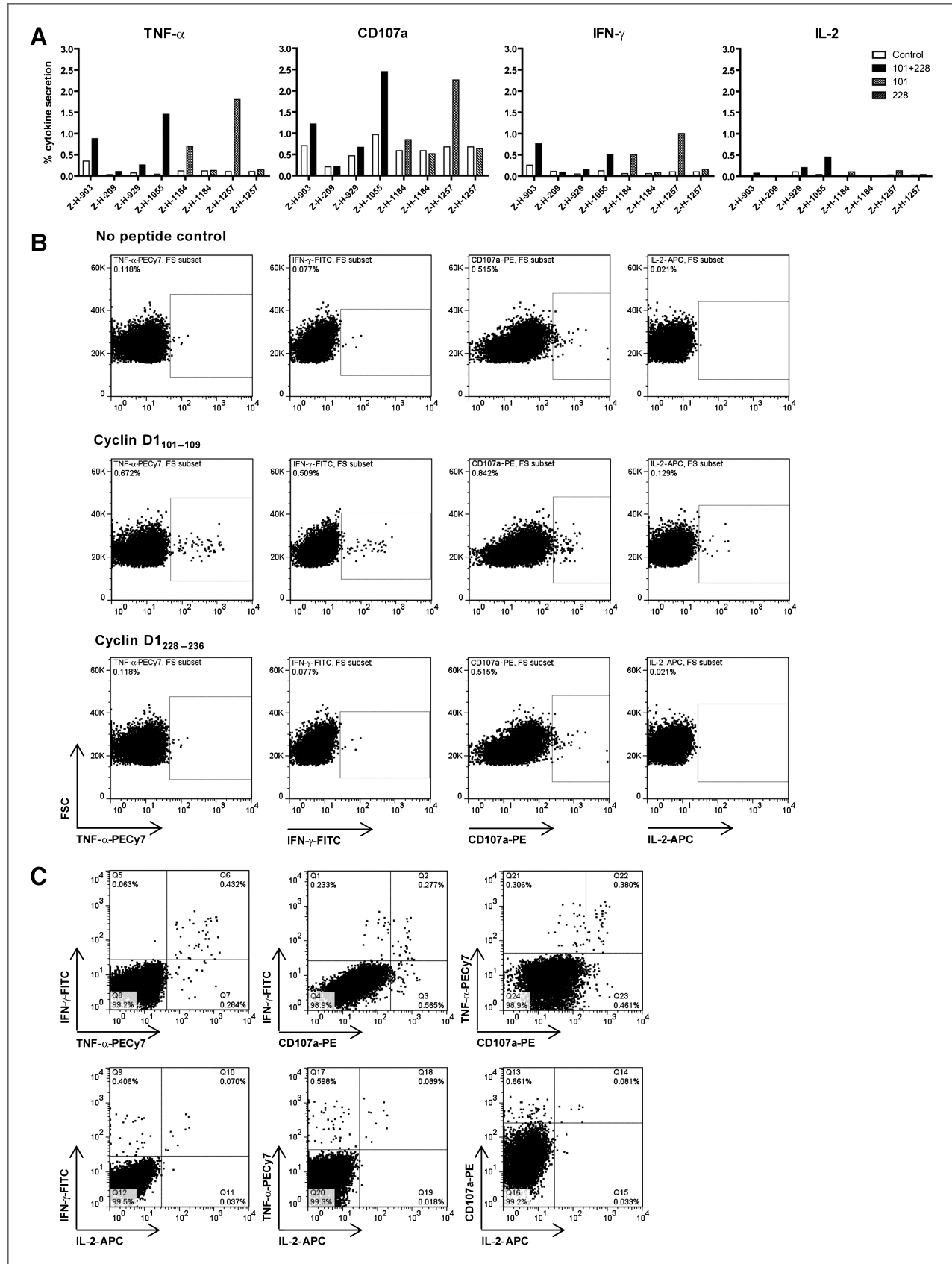


Figure 1. Expression of TAA transcripts in ccRCC specimens. RNA was isolated from 23 primary ccRCC samples and reverse transcribed, and cDNA was used as a template for qRT-PCR analysis. A, frequency of expressed TAAs in primary ccRCC samples. B, detected Δ CT levels for the different TAA transcripts of the individual tumor samples after normalization to the endogenous control (18S rRNA). Results from tumor samples are represented as open, from testis as filled, and from healthy kidney as half-filled symbols. C and D, coexpression of TAAs in primary ccRCC samples.



After *in vitro* culture for 9 days with the relevant peptides, we conducted a 5-hour restimulation with the same peptides in the presence of brefeldin A and monensin, followed by ICS for effector cytokines TNF- α , IFN- γ , and IL-2, and staining for degranulation (surface CD107a). We found CD8⁺ T cells specific for cyclin D1–derived peptides in 5 of 6 HLA-A2⁺ patients, whose tumors overexpressed *Cyclin D1* (Fig. 2A), but not for any of the other peptides tested (data not shown). Because of limited material, the readout for patient samples Z-H-903, Z-H-209, Z-H-929, and Z-H-1055 was conducted with both cyclin D1–derived peptides (cyclin D1_{101–109} and cyclin D1_{228–236}) together. Because there were enough specimens from patients Z-H-1184 and Z-H-1257, we analyzed the two cyclin D1–derived epitopes separately and found that responses in both patients were directed against cyclin D1_{101–109} as illustrated by representative staining of specimens from patient Z-H-1184 (Fig. 2B). Furthermore, the cyclin D1–specific CD8⁺ T cells in all 5 patients were polyfunctional although only a few cells secreted IL-2 (Fig. 2C). To exclude the possibility that the cyclin D1–specific CD8⁺ T-cell responses were due to *in vitro* priming rather than *in vivo* induction by *Cyclin D1*–overexpressing tumors, we carried out a similar experiment with PBMCs from 3 healthy, HLA-A2⁺ donors. CMV_{495–503} was used as positive control because these donors were selected by the presence of CMV tetramer-specific CD8⁺ T cells. We did not find cyclin D1–specific CD8⁺ T-cell responses in these healthy donors, whereas CMV-specific CD8⁺ responses were readily detectable by ICS (data not shown).

To confirm the presence of cyclin D1–specific T cells, we carried out *ex vivo* tetramer staining on PBMCs (Supplementary Fig. S1A) and TILs (Supplementary Fig. S1B) from patients with ccRCC when sufficient material was available. In PBMCs from patient Z-H-209, who did not show a cyclin D1–specific response after *in vitro* stimulation, we also did not detect cyclin D1 tetramer-positive T cells. However, in PBMCs from patients Z-H-903, Z-H-1055, and Z-H-1184, who responded to *in vitro* cyclin D1–specific peptide stimulation, we detected cyclin D1–specific T cells by tetramer staining (Supplementary Fig. S1A). Although we did not find cyclin D1 tetramer-specific T cells in TILs of patient Z-H-929, whose PBMCs responded weakly to cyclin D1–specific peptide stimulation *in vitro*, we detected cyclin D1 tetramer-specific T cells in TILs of patients Z-H-1184 and Z-H-1257, whose PBMCs secreted cytokines upon peptide stimulation *in vitro* (Supplementary Fig. S1B).

To investigate whether cyclin D1 induced humoral immune response in patients with ccRCC, particularly those who overexpress *Cyclin D1*, we tested for cyclin D1–specific antibodies by Western blotting but did not find cyclin D1–specific antibodies in the sera of the 22 patients tested (Supplementary Fig. S2).

Discussion

To expand the number of clinically relevant TAAs that can be targeted by immunotherapy in patients with RCC, we investigated the expression of nine cancer-testis antigens and seven other antigens (15) in samples from 23 patients diagnosed with primary ccRCC. Most tumors expressed more than one TAA, which has been described for other tumor entities (16–18), and which enables therapeutic-targeting of multiple antigens at the same time.

Except for *MAGE-A9* and *NY-ESO-1*, we did not detect expression of the other seven cancer-testis antigens in our patient cohort. Although two studies showed expression for *MAGE-A3* and *-A4* in RCC (19, 20), our results are in line with those described in a previous review on cancer-testis antigens (21). The expression frequency of *MAGE-A9* in RCC is similar to that shown by Oehlrich and colleagues (12); however, we found only very low levels of *MAGE-A9* transcripts. As Oehlrich and colleagues have reported (12), we also detected a signal for *MAGE-A9* in the healthy kidney tissue control. The frequency of *NY-ESO-1* expression in CCR was unexpected on the basis of immunohistochemistry data published over a decade ago (22–24). The discrepancy may be explained by the highly sensitive qRT-PCR method we used that allowed the detection of very low levels of *NY-ESO-1* transcripts. Although *RAGE-1* was the first antigen recognized by autologous T cells from a human RCC cell line, it was only detected in 1 of 57 RCC samples (25). Our results (13%) are in agreement with studies that identified higher expression frequencies, which presumably is also due to the more sensitive methods used (12, 22). The frequency of *PRAME*-expression (39%) in ccRCC is in accordance with previously published reports (20, 22).

The von Hippel-Lindau (VHL) protein is mutated in most ccRCC samples, leading to reduced proteolytic degradation of hypoxia-inducible factor- α (HIF- α), resulting in upregulated HIF- α –mediated transcriptional programs (26). CA-IX is an HIF- α target gene that is frequently expressed in RCC but very rarely detected in normal kidney tissue (27). We observed an overexpression of CA-IX in all but one ccRCC samples. CA-IX has been identified as a therapeutic target for RCC (13, 28, 29), and evaluated in clinical studies (30–32), but it has not yet induced strong antitumor immune responses. In addition to CA-IX, *ADFP*, *Cyclin D1*, *C-MET*, and *RGS-5* are also overexpressed in ccRCC (14). In contrast to published reports (33, 34), we found that *ADFP* expressed more frequently (65%) than *C-MET* (30%). Cyclin D1 is a cell-cycle regulator crucial for the G₁–S transition (35) and is overexpressed in many cancers including colorectal and breast carcinoma (36, 37). We found that cyclin D1 was overexpressed in 43% of the primary ccRCC samples, a finding that was similar to the previous published data (38–40).

Many factors affect the success of immunotherapy (22). Despite the high frequency of expression (55%) of *NY-ESO-1*

Figure 2. Cyclin D1–specific CD8⁺ T-cell response in PBMCs of patients with ccRCC. CD8⁺ T cells from PBMCs of HLA-A2⁺ patients with cyclin D1–overexpressing tumors were stimulated for 9 days with cyclin D1–derived peptides in the presence of IL-2. Restimulation was carried out using both cyclin D1–derived epitopes (cyclin D1_{101–109} and cyclin D1_{228–236}) together, separately, or no peptide as highlighted in the figure, followed by surface staining for CD107a and intracellular staining for TNF- α , IFN- γ , and IL-2. Cytokine production of CD8⁺ T cells was measured after gating on live CD45⁺, CD3⁺, CD8⁺, CD14⁺, CD16⁺, and CD19⁺ cells. A, summary of results from 6 individual patients. B, representative staining (patient Z-H-1184) of single cytokine secretion and (C) polyfunctional cytokine response.

in ccRCC samples, which is one of the most immunogenic cancer-testis antigens (24, 41), we did not find NY-ESO-1-specific CD8⁺ T-cell responses in any of the patients with HLA-A2⁺ ccRCC, which may be explained by the low level of *NY-ESO-1* expression. ADFP, C-MET, and RSG-5 are not immunogenic in patients with ccRCC, as we did not detect T-cell responses specific for these antigens, despite their frequent overexpression. This finding is in agreement with previously published data on rare T-cell responses in patients with ccRCC (15), even though ADFP- and C-MET-specific T cells could be expanded from the blood of healthy donors (33, 34). *RGS-5* overexpression did not result in detectable RGS-5-specific T-cell responses in our cohort, although such responses were reported in the blood of healthy donors and patients with acute myeloid leukemia (42). In contrast, we detected cyclin D1-specific CD8⁺ T cells in the blood of 5 of 6 patients with HLA-A2⁺ ccRCC, whose tumors overexpressed *Cyclin D1*. Unlike cyclin-dependent kinase 4 (CDK4) in melanoma (43), we did not find a mutation in the two *Cyclin D1* epitopes in ccRCC (data not shown).

Ex vivo tetramer staining confirmed the presence of cyclin D1-specific T cells in patients whose CD8⁺ PBMCs secreted cytokines upon *in vitro* cyclin D1-specific peptide stimulation. Although we detected a higher frequency of T cells specific for cyclin D1_{228–236} by tetramer in blood and TILs, we found a higher frequency of T cells specific for Cyclin D1_{101–109} after *in vitro* restimulation. Because cyclin D1_{228–236} is the immunodominant peptide, it may induce more extensive *in vivo* proliferation of specific T cells and thus compromise their ability to further expand *in vitro*. Alternatively, tetramer-positive T cells may not be functional, suggesting a preferential functional exhaustion of T cells specific for cyclin D1_{228–236}.

There is no association identified between *Cyclin D1* expression and ccRCC prognosis (40), although *Cyclin D1* overexpression is associated with shorter patient survival in other cancers and thus represents an interesting therapeutic target (44). However, cyclin D1 is difficult to target as it is expressed in the cytosol and lacks intrinsic enzymatic activity. One approach is to block its activity indirectly by inhibiting associated kinases with kinase inhibitors (45); however, this strategy does not interfere with its kinase-independent tumor-promoting effects.

Cyclin D1 induces T-cell responses in mantle cell lymphoma and colon cancer, and antibodies in prostate cancer (46–49), and was identified as a target for immunotherapy in mantle cell lymphoma (50).

To our knowledge this is the first study that describes naturally occurring cyclin D1-specific CD8⁺ T-cell responses in patients with cancer. Importantly, these responses have polyfunctional effector characteristics. We therefore propose cyclin D1 as a target for immunotherapy in patients with ccRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.R. Dannenmann, T. Hermanns, A. Knuth, M. van den Broek

Development of methodology: S.R. Dannenmann, A. Bransi, H. Moch, A. Knuth, M. van den Broek

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.R. Dannenmann, T. Hermanns, A. Bransi, C. Matter, L. von Boehmer, H. Moch, A. Knuth

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.R. Dannenmann, A. Bransi, A. Knuth, M. van den Broek

Writing, review, and/or revision of the manuscript: S.R. Dannenmann, A. Bransi, S. Stevanovic, P. Schraml, H. Moch, A. Knuth, M. van den Broek

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Hermanns, A. Bransi, C. Matter, L. von Boehmer, S. Stevanovic, P. Schraml, H. Moch, A. Knuth, M. van den Broek

Study supervision: H. Moch, A. Knuth, M. van den Broek

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