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ORIGINAL ARTICLE

Allogeneic gene-modified tumor cells (RCC-26/IL-7/CD80) as a vaccine in patients with metastatic renal cell cancer: a clinical phase-I study

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Despite novel targeted agents, prognosis of metastatic renal cell cancer (RCC) remains poor, and experimental therapeutic strategies are warranted. Transfection of tumor cells with co-stimulatory molecules and/or cytokines is able to increase immunogenicity. Therefore, in our clinical study, 10 human leukocyte antigen (HLA)-A*0201$^+$ patients with histologically-confirmed progressive metastatic clear cell RCC were immunized repetitively over 22 weeks with 2.5–40$\times 10^6$ interleukin (IL)-7/CD80 cotransfected allogeneic HLA-A*0201$^+$ tumor cells (RCC26/IL-7/CD80). Endpoints of the study were feasibility, safety, immunological and clinical responses. Vaccination was feasible and safe. In all, 50% of the patients showed stable disease throughout the study; the median time to progression was 18 weeks. However, vaccination with allogeneic RCC26/IL-7/CD80 tumor cells was not able to induce TH1-polarized immune responses. A TH2 cytokine profile with increasing amounts of antigen-specific IL-10 secretion was observed in most of the responding patients. Interferon-γ secretion by patient lymphocytes upon antigen-specific and non-specific stimulation was substantially impaired, both before and during vaccination, as compared with healthy controls. This is possibly due to profound tumor-induced immunosuppression, which may prevent induction of antitumor immune responses by the gene-modified vaccine. Vaccination in minimal residual disease with concurrent depletion of regulatory cells might be one strategy to overcome this limitation.
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INTRODUCTION

Despite implementation of several molecularly targeted agents in systemic therapy of metastatic renal cell cancer (RCC), long-term outcome of most patients remains poor. Furthermore, more than 50% of patients who have undergone nephrectomy for locally advanced disease, subsequently relapse with local or distant metastases. Therefore, new treatment modalities are highly warranted, even in the era of targeted therapies with kinase inhibitors and other anti-angiogenic drugs.

During the past two decades, clinical results with therapeutic vaccines in advanced cancer have been largely disappointing.[1–3] However, tumor vaccination may be appropriate in a more favourable setting, such as minimal residual disease after surgery or successful systemic therapy.[4] Therefore, clinical development of vaccines remains a potentially useful experimental approach. In clinical trials, the most widely used vaccines were antigenic peptides/proteins plus adjuvant or cellular vaccines, such as antigen-loaded dendritic cells or modified tumor cells.[1,5] Tumor cell vaccines based on autologous tumor cells must be modified *in vitro* in order to render them more immunogenic.[1,5] Gene transfer using different cytokines or co-stimulatory molecules, such as CD80 (B7.1), was an approach pursued by several groups, both in animal models and in humans.[6–8] Granulocyte-macrophage colony stimulating factor and interleukin (IL)-2 belong to the most widely used cytokines for the engineering of cytokine-secreting tumor cells (reviewed in Mocellin *et al.*[1], webtables I and II). Granulocyte-macrophage colony stimulating factor is a powerful chemoattractant of antigen-presenting cells, inducing their maturation into dendritic cells, thereby enhancing T-cell responses.[9–11] IL-2 is a cytokine, which stimulates the proliferation of cytotoxic T cells (CTLs), helper T cells and natural killer cells, all of which may contribute to a cellular antitumor immune response.[12] IL-7 is a pleiotropic cytokine inducing CD8$^+$ and CD4$^+$ T-cell proliferation, and particularly the activation of CTLs.[13] A synergistic effect of IL-7 and CD80 on T-cell stimulation has been demonstrated. Furthermore, antitumor immune responses induced by IL-7 in animal models were completely T-cell dependent.[13]

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We have previously shown in a Balb/c mouse model with syngeneic mammary adenocarcinoma cells (TS/A) that tumor cells cotransfected with IL-7 and CD80 induced protective T-cell-dependent immunity against a subsequent tumor challenge with wild type tumor cells. IL-7/CD80 cotransfected TS/A cells were superior to single-gene transfected tumor cells and to the adjuvant corynebacterium parvum.[13] These data were the rationale for the clinical study reported here. We developed a genetically-modified allogeneic RCC vaccine based on a well-characterized tumor cell line (RCC26) which was transfected with genes coding for IL-7 and CD80.[14,15] Transfection with CD80 and cytokine genes led to increased immunogenicity of RCC26 cells.[16] The use of allogeneic tumor cells offers several advantages: the approach (1) circumvents the laborious and cost-intensive Good Manufacturing Practice (GMP) production of a patient-individualized vaccine, with variable efficiency of gene-modification and subsequent immunogenicity; (2) allows for the large-scale production of highly standardized and antigenically well-defined tumor cells, which are ready for clinical use in a large number of patients and (3) improves comparisons within a study through the use of cells from a single batch. The allovaccine can be used in partially human leukocyte antigen (HLA)-matched patients, particularly when the HLA-restriction of immunogenic antigenic peptides is known, but it can also be used across HLA-barriers as cross-priming through autologous antigen-presenting cells is likely to occur.[17]

We selected patients carrying the HLA-A*0201 allele for inclusion in this trial as the vaccine cells expressed this HLA allotype and extensive preclinical studies revealed that surrogate peptides presented by HLA-A2 molecules on RCC26 cells could be used for immune monitoring.

RESULTS

Vaccine cells

Genetically-modified RCC26 tumor cells were successfully transfected with IL-7 and CD80 (B7.1). After thawing, 1×10^6 RCC26/IL-7/CD80 vaccine cells were able to produce $\sim4.5\times10^3$ pg IL-7. IL-7 secretion peaked on day 2 after thawing and irradiation. Compared with nonirradiated vaccine cells, irradiation did not impair IL-7 production at least for up to 3–4 days after thawing, compared with nonirradiated vaccine cells (Figure 1a). In six independent experiments, IL-7 secretion of 1×10^6 thawed irradiated cells was 760–4830 pg after 24 h. and 1365–4030 pg after 48 h. IL-7 production in the cell batch used for clinical vaccination was 1205 pg per 10^6 cells after 24 h and 2320 pg per 10^6 cells 48 h after thawing. CD80 expression on vaccine cells after thawing was in the range of 80–90% and dropped to $\sim$50% during the next 70 h in the case of irradiated cells. Within 20 h after thawing CD80 expression on the vaccine cells did not substantially differ between irradiated and nonirradiated cells. During the following 50 h, a difference of 20–30% in favour of the nonirradiated cells became apparent (Figure 1b). However, the percentage of CD80-positive vaccine cells after thawing was always $\geqslant$50% during the first 3 days. In six independent experiments, CD80 expression of irradiated cells was in the range of 85–94% 4 h after thawing, 56–84% after 24 h and 52–81% 48 h after thawing. CD80 expression in the cell batch used for clinical application was 90% 4 h after thawing, 67% after 24 h and 69% after 48 h.

Clinical results

All patients included in the study protocol had been previously nephrectomized; nine patients belonged to the intermediate risk group and one patient belonged to the poor risk group, according to the Memorial Sloan Kettering Cancer Center criteria[18] (Table 1). Almost all patients had multiple metastatic sites, mostly involving lungs, lymph nodes and bones. Three patients were typed as

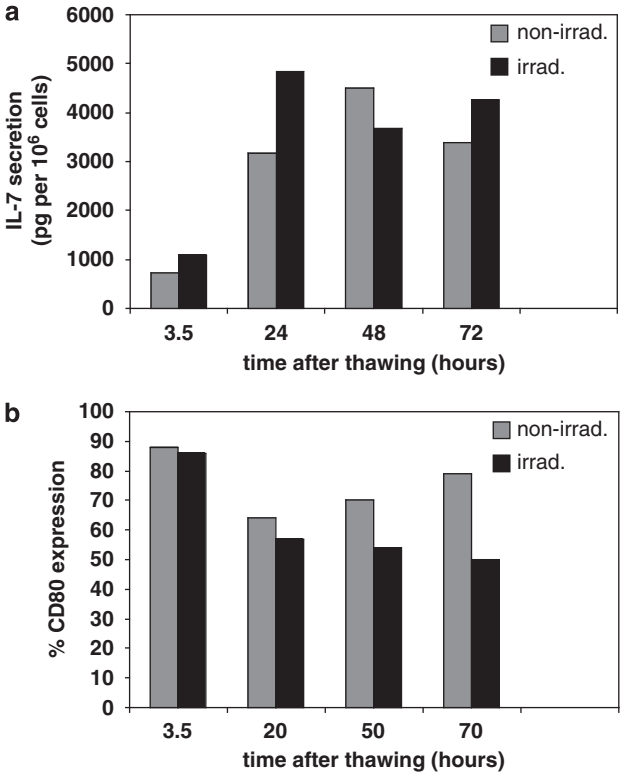

Figure 1 IL-7 production and CD80 expression of RCC26/IL-7/CD80 vaccine cells. (**a**) RCC26/IL-7/CD80 vaccine cells were thawed and cultured for 3.5–70 h. IL-7 secretion per 10^6 cells was determined by enzyme-linked immuno sorbent assay over 70 h. Irradiated cells were compared with nonirradiated cells after thawing. In six independent experiments, IL-7 secretion by 1×10^6 thawed irradiated cells was 760–4830 pg after 24 h and 1365–4030 pg after 48 h. (**b**) RCC26/IL-7/CD80 vaccine cells were thawed and cultured for 3.5–70 h. CD80 expression was determined by flow cytometry. Irradiated cells were compared with nonirradiated cells after thawing. In six independent experiments, CD80 expression of thawed irradiated cells was 85–94% after 4 h, 56–84% after 24 h and 52–81% after 48 h.

HLA-A*0201 homozygous, whereas seven patients were heterozygous (data not shown). Patients characteristics are shown in Table 1. All patients were evaluable for response. Six of the ten patients (nos. 1, 3, 4, 5, 8 and 9) received all scheduled vaccinations through week 22, four patients (nos. 2, 6, 7 and 10) were taken off study owing to disease progression at weeks 8, 8, 4 or 18, according to the protocol. Vaccination was clinically feasible and safe, with no grade 3/4 toxicities being observed. Mild local skin reactions not fulfilling criteria for a positive delayed-type hypersensitivity (DTH) reaction were observed at the injection site in most patients. Grade 1 transient transaminase elevation was observed in three patients (nos. 2, 3 and 4); in one patient (no. 5) grade 1 transient and clinically-asymptomatic lipase elevation was detected during weeks 8 and 18; one patient (no. 8) reported grade 1–2 nausea and constipation; one patient (no. 8) reported grade 2 arthralgias during week 4 of the treatment. Another patient (no. 1) developed grade 2 dyspnea in week 9 of treatment, caused by pleural effusions of unknown origin. As there was no clinical or radiological signs of disease progression and cytological evaluation of the effusion showed marked eosinophilia, but no tumor cells, the clinical picture was interpreted as a hypersensitivity reaction, possibly related to vaccination. Treatment was continued under careful clinical monitoring and the pleural effusions disappeared by week 14.

Table 1 Patient characteristics

		N[a]
Patient no.		10
Patients evaluable for response		10
Age (median/range)	63/37–74	
Sex	Female	4
	Male	6
Metastatic sites	1	1
	2	5
	⩾3	4
	Only lung	0
	Lungs	7
	Lymph nodes	5
	Liver	2
	Bone	4
	Soft tissue	1
	Kidney	2
	Other	2
Tumor nephrectomy		10
Time interval between primary diagnosis and diagnosis of metastases	⩽12 months	3
	⩾24 months	4
	Synchronous	3
	Unknown	0
Risk group[b]	Good	0
	Intermediate	9
	Poor	1

[a]Number of patients.
[b]Risk group according to the Memorial Sloan Kettering Cancer Center.

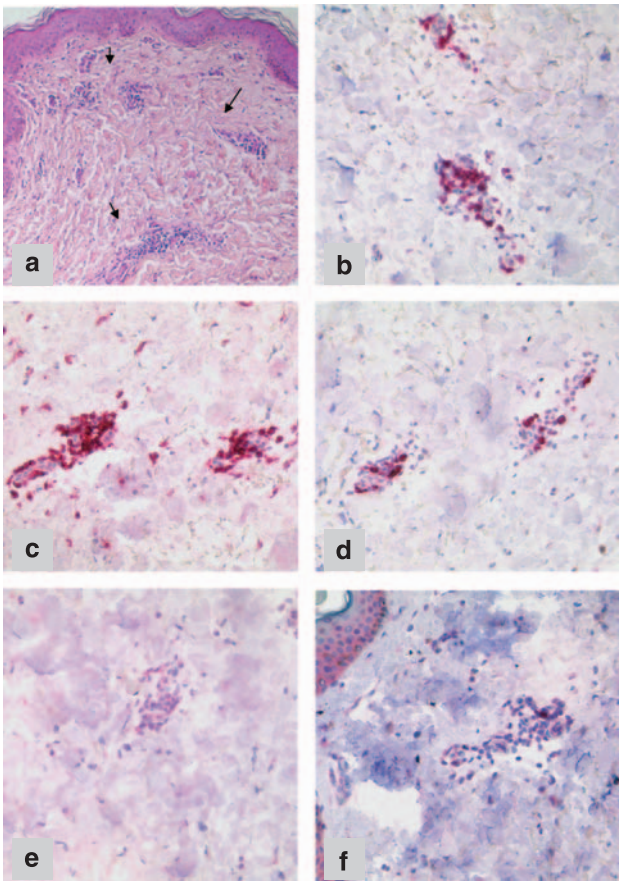

Figure 2 Immunohistology of skin biopsies taken from DTH challenge sites. (**a**) hematoxylin-eosin staining: a perivascular infiltrate is observed in the dermis (arrows). The infiltrate consists mainly of lymphocytes and some plasma cells. Immunohistological stainings: most of the lymphocytes are CD3-positive T cells (**b**). 2/3 of the T cells belong to the CD4 subpopulation (**c**) whereas 1/3 are CD8-positive T cells (**d**). No CD56-positive cells are detectable (**e**). Only a few B cells are present (**f**). The figure shows the biopsies of patient no. 9.

No objective responses could be observed. In all, 50% of the patients showed stable disease throughout the study and the median time to progression (TTP) was 18 weeks (range 4–69), with the longest TTP being 69 weeks in patient no. 3 (Table 4). The median overall survival was 40 months (range 13–56), with three patients (nos. 5, 9 and 10) still alive in December 2009, at a median follow-up of 41.5 months.

DTH reactions of the skin remained negative in six patients (nos. 1, 2, 3, 4, 6 and 7), while mild to moderate DTH reactions were observed in four patients (nos. 5, 8, 9 and 10) (data not shown). Skin biopsies at the injection sites 48 h after the last vaccination revealed scarce cellular lymphoid infiltrates in seven patients (nos. 1, 3, 4, 5, 8, 9 and 10). These infiltrates consisted predominantly of T cells with a $CD3^+CD4^+$ phenotype (Figure 2); in five patients (nos. 1, 3, 5, 8 and 9) eosinophilic infiltration was observed. A clear correlation could not be established between the composition of the cellular infiltrate and the number of vaccinations or TTP (data not shown).

Immunological results

Peptide-specific T-cell responses. Antigen-specific T-cell responses against several HLA class-I-restricted peptides that were shown to be over-expressed in the RCC26 cell line (data not shown)[19] were determined before and during vaccination by interferon-γ enzyme-linked immunospot (IFN-γ ELISpot) and by cytometric bead array (CBA), which assessed different TH1 (IFN-γ, tumor necrosis factor-α, IL-2) and TH2 (IL-10, IL-4, IL-5) cytokines. No significant peptide-specific T-cell responses were detected in most patients before treatment. Antigen-specific T cells were present at very low frequencies in only two patients (nos. 5 and 7). Following vaccination, T cells in patient no. 5 recognizing thymidylate synthase (TYMS), vimentin and G250 were detected by IFN-γ ELISpot (Figure 3). These T cells had a predominant TH2-cytokine profile as measured by CBA (Figures 4 and 5). Patient no. 6 and no. 9 also showed some reactivity to TYMS and/or G250 in the IFN-γ ELISpot (Figure 3). Overall their T-cell responses were predominantly TH2 (Figures 4 and 5), as detected by CBA. In patient no. 7, T cells against TYMS, vimentin, adipophilin and G250 were detected by CBA; the immune response of this patient initially also had a TH1 component for vimentin and G250, which then switched to TH2 (Figure 4). Vaccination with RCC26/IL7/CD80 tumor cells was not able to induce a significant TH1-polarized T-cell response against RCC26-associated antigens in most of the patients. An amplification of antigen-specific responses against some of the tumor-associated peptides (survivin, adipophilin, vimentin, G250, TYMS, Insulin-like growth factor (ILGF), c-Met) was observed in a few patients, but these immune responses showed a predominant TH2-cytokine pattern in nearly all patients (Figure 4). There was an induction of peptide-specific IL-10 secretion in four patients (nos. 5, 6, 7 and 9) against the target antigens survivin, TYMS, vimentin, G250, adipophilin and ILGF (Figure 5). The lack of

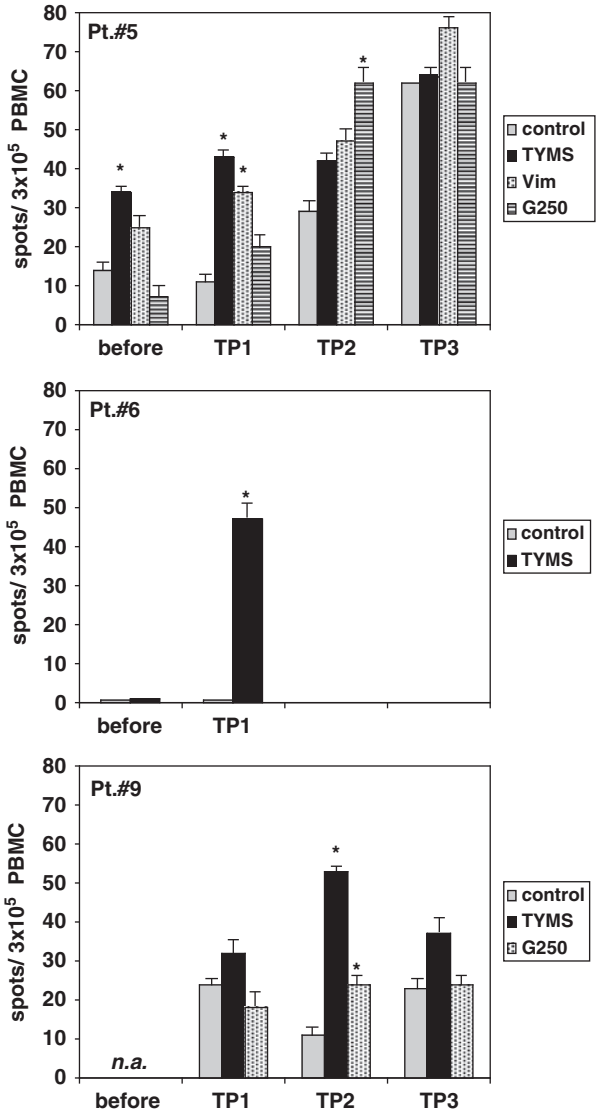

Figure 3 Peptide-specific T-cell responses in IFN-γ ELISpot. PBMC from patients before vaccination and at different time points after vaccination were stimulated *in vitro* with different tumor-associated peptides from survivin (surv), vimentin (vim), adipophilin (ADFP), TYMS, cyclin D1, c-Met, ILGF and G250. Peptide-specific T-cell responses were measured by IFN-γ ELISpot, an HLA-A2-restricted peptide from the CML-specific fusion protein bcr/abl was used as control peptide. Results are expressed as spots per 3×10^5 cells. TP, time point (TP1=week 6, TP2=week 14, TP3=week 22); Asterisk indicates peptide-specific response according to predefined criteria (see patients and methods section on ELISpot).

IFN-γ secretion upon antigenic stimulation, as assessed by ELISpot, seemed not to be restricted to tumor antigens as IFN-γ T-cell responses against HLA-A2-restricted control peptides of cytomegalo-, EBV- and influenza (flu)-virus were also severely impaired in most of the patients (Table 2). Interestingly, the diminished capacity of the T cells to secrete IFN-γ upon stimulation was even confirmed after non-specific stimulation with phorbol myristate acetate in most of the patients, and there was a strong correlation between impaired IFN-γ secretion after phorbol myristate acetate stimulation and a lack of IFN-γ response to HLA-A2-restricted cytomegalo-, EBV- and influenza (flu)-virus peptides (Table 2).

Functional responsiveness of peripheral blood T cells. In order to exclude general unresponsiveness of the patients' T cells, proliferation assays were performed after stimulation with phytohemagglutinin (PHA) and IL-2 or allogeneic peripheral blood mononuclear cell (PBMC). Responses were compared with control cells of healthy donors. After stimulation with PHA/IL-2, the proliferative capacity of patients' PBMC did not change during vaccination and was not significantly different from healthy donors (Figure 6a). Furthermore, stimulation with a mixture of allogeneic PBMC revealed proliferative responses of patients' cells that were comparable with PBMC of healthy donors (Figure 6b), demonstrating that patients' PBMC had a normal proliferative responsiveness to non-specific and allogeneic stimulation.

Induction of allo- and auto-antibodies. Patients were tested repeatedly for the presence of HLA antibodies. Eight of ten patients remained HLA-antibody negative throughout the study. In contrast, two patients (nos. 5 and 9) developed HLA class-I antibodies on days 112 and 202. HLA antibodies in patient no. 5 were specific for HLA-A29 and -A32, whereas the antibodies in patient no. 9 detected HLA-A25, -A32, -A33, -A34, -B51, -B52, -B53 and -B49. Patient no. 9 showed DTH reactivity with lymphoid infiltrate, IFN-γ responses in ELISpot and a longer TTP, in addition to the broad allo-antibody response, encompassing two HLA antigens expressed by RCC-26 (HLA-A33 and -B51). The specificity of most HLA class-I antibodies that were detected (except B49) could be explained by broad cross-reactivity among different HLA allotypes. Anti-HLA class-II antibodies were not detected. In two patients (nos. 2 and 5), anti-nuclear antibodies were transiently detected at week 8 and disappeared thereafter; the antibody titer was 1:80 in patient no. 2 and 1:320 in patient no. 5.

Analysis of peripheral blood effector cells. Flow cytometric analysis of lymphocyte subpopulations ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD56^+$, $CD3^+DR^+$, $CD19^+$) before and during vaccination did not show any significant changes (data not shown). In order to detect clonal expansion of T cells, even the T cells with unknown specificity, peripheral blood samples of nine patients were analysed by standardized T-cell receptor (TCR)γ- and TCRβ-PCR before, and at different time points during vaccination. However, no vaccination-associated T-cell clones were found (data not shown). Finally, the presence of stable FoxP3 expressing regulatory T cells (Tregs) per $CD4^+$ T cells in PBMC of the patients before and during vaccination was monitored by PCR-based detection of a demethylated FoxP3 gene locus (TSDR): before vaccination the percentage of $TSDR^+$ CD4-T cells did not differ substantially from healthy controls (4.9 ± 3.4 vs 6.4 ± 1.6% in healthy controls). During vaccination there was a decline of TSDR+ CD4-T cells in three patients (nos. 3, 6 and 10). In three patients (nos. 2, 5 and 7) TSDR+ CD4-T cells increased during vaccination, whereas in patients nos. 1, 8 and 9 there was no significant change (Treg after last vaccination as compared with Treg before first vaccination) (Table 3).

DISCUSSION

The past decade has shown that active immunization mostly fails to induce clinically relevant tumor responses in metastatic cancer. Nevertheless, there is increasing evidence that vaccination may have a role in preventing relapse from minimal residual disease. In this context, gene-modified allogeneic tumor cells remain an attractive clinical approach as they can present a broad spectrum of tumor-associated antigens and they help to circumvent a time-consuming and cost-intensive patient-individualized GMP production. Partial

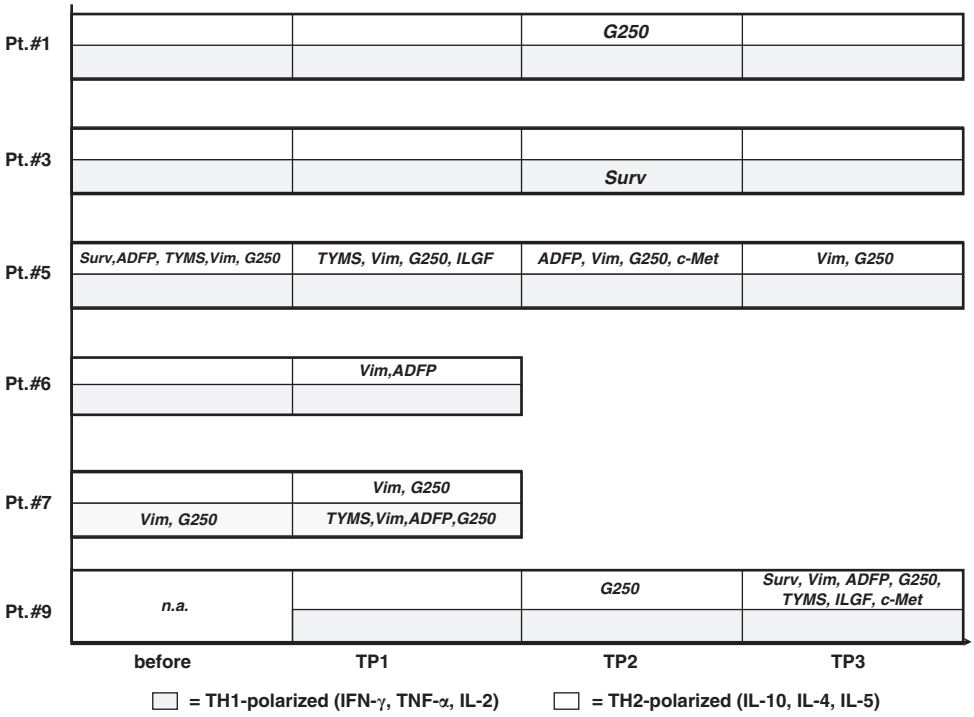

Figure 4 Cytokine bead array detecting the cytokines IFN-γ, IL-2, tumor necrosis factor-α, IL-4, IL-5 and IL-10. PBMC from patients before vaccination and at different time points during vaccination were stimulated with different tumor-associated peptides (survivin (surv), vimentin (vim), adipophilin (ADFP), TYMS, cyclin D1, c-Met, ILGF and G250). Peptide-specific cytokine secretion was measured by flow cytometry. The TH-polarization (TH1 vs TH2) of the cytokine profile is indicated for each time point and for each antigen. Grey field=TH1 profile, white field=TH2 profile. For the definition of peptide-specific cytokine secretion see patients and methods section. TP, time point (TP1=week 6, TP2=week 14, TP3=week 22).

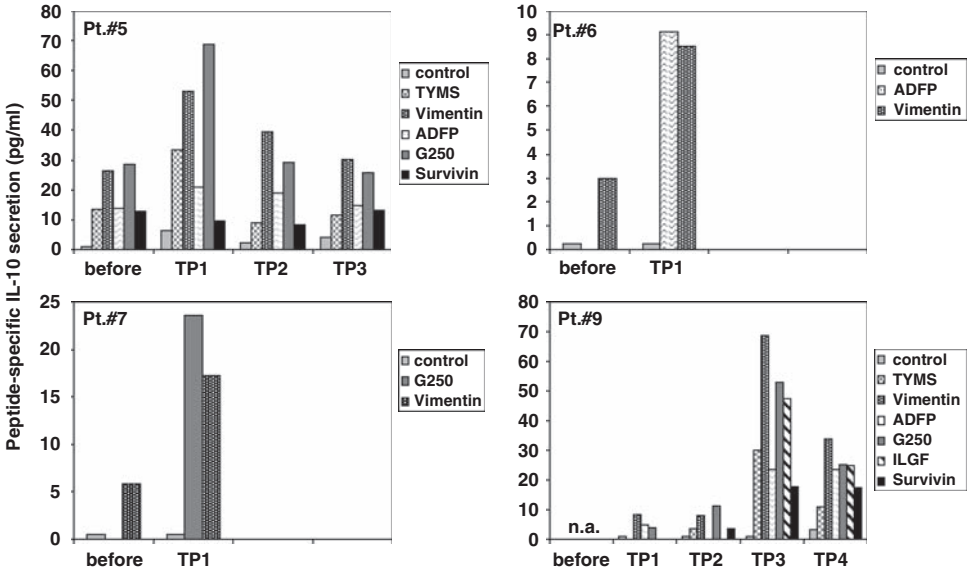

Figure 5 Induction of peptide-specific IL-10 secretion by the vaccine. The peptide-specific response of patients' PBMC was determined by CBA. Cytokine-secretion upon stimulation with RCC-associated peptides was measured before vaccination and at different time points thereafter (TP1-4). Results show significant IL-10 induction by the vaccine in 4/10 patients (patient nos. 5, 6, 7 and 9). IL-10 was determined in pooled samples from duplicates. In test series with repetitive measurements, the s.d. of the assay was 3.0–7.8% for IL-10. TP, time point (TP1=week 6, TP2=week 14, TP3=week 22, TP4=week 36).

HLA-mismatch of patients with vaccine cells is likely to increase activation of the immune system through induction of an allo-response.[20] IL-7/CD80-transfected RCC26 cells were previously shown to be immunogenic[14,16,21] and the vaccine was tested for the expression of several RCC-associated tumor antigens. According to this antigen-expression profile, a number of peptides were selected for the use as surrogate markers for immune monitoring. It was assumed that possible tumor-specific responses induced by the vaccine would

Table 2 IFN-γ secretion of PBMC from patients and HD after stimulation with PMA or HLA-A2-restricted viral peptides (CEF) as measured by IFN-γ ELISpot (in spots per 3×10^5 PBMC)

Patient no.	*PMA (HD)*	*PMA (patients)*[a]	*CEF (patients)*[a]
1	>1000	8/7	NA/NA
2	>1000	12/75	1/3
3	>1000	12/55	1/1
4	>1000	16/203	0/1
5	>1000	>600/>1000	541/728
6	>1000	17/7	NA/NA
7	>1000	>1000/>1000	475/405
8	>1000	1216/252	4/0
9	>1000	NA/>1000	61/138
10	>1000	15/59	10/2

Abbreviations: CEF, cytomegalo-, EBV- and influenza (flu)-virus; HD, healthy donor; IFN-γ ELISpot, interferon-γ enzyme-linked immunospot; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; NA, not available.
[a]Data represent values pre/post vaccination with the post vaccination sample taken after the last immunization. PBMC from HD were used as positive control in each assay.

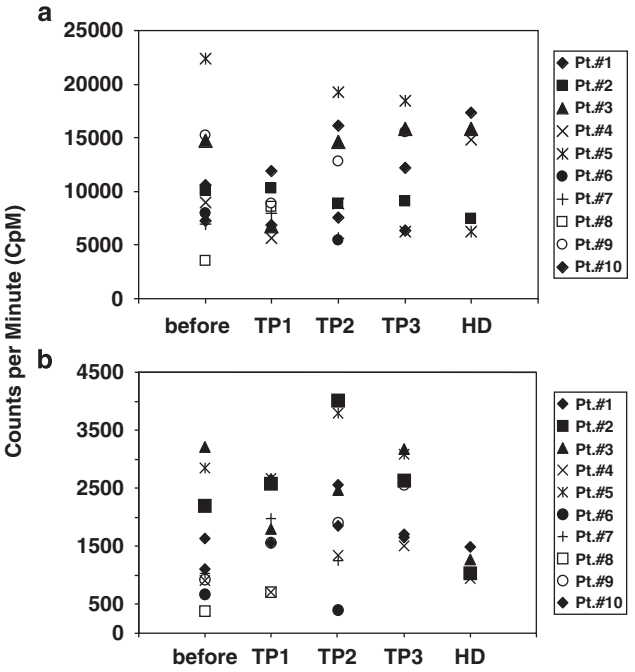

Figure 6 Proliferative responses of PBMC. (**a**) Proliferative responses of PBMC from patients before vaccination and at different time points after vaccination in response to PHA/IL-2 as measured by a ^{3}H-thymidine assay. Results were compared with PBMC from healthy donors (n=5) showing no significant differences in proliferative capacity. (**b**) Proliferative responses of PBMC from patients before vaccination and at different time points after vaccination in response to irradiated allogeneic PBMC from healthy donors. Proliferative capacity was measured in a ^{3}H-thymidine assay. Results were compared with PBMC from healthy donors (n=5) showing that the patients' PBMC did not have impaired alloresponses. Without stimulation, proliferation of responder cells from patients before vaccination was 452±457 CpM, proliferation of responder cells from healthy donors was 308±74 CpM. Background values for irradiated stimulator cells were <250 CpM. CpM, counts per minute; TP, time point (TP1=week 6, TP2=week 14, TP3=week 22); HD=healthy donor.

be based on tumor antigens shared by the vaccine and other RCC.[19] Indeed, such responses were detected by ELISpot studies of patients evaluated in another study using an RCC26-based vaccine.[19]

Clinical application of increasing doses of genetically modified and irradiated allogeneic RCC26/IL-7/CD80 cells was feasible and safe. Pleural effusions, which transiently occured in one patient, might reflect a systemic hypersensitivity reaction and were possibly related to vaccination as there were no signs of disease progression or congestive heart failure. This event, however, was relatively mild and resolved spontaneously without any need for intervention.

Disease stabilization for up to 69 weeks was observed in the patients in this trial and the median overall survival of 40 months compares favourably with the median overall survival of 10 months observed in large cohorts of patients with advanced RCC,[22] particularly in consideration that progressive disease was one of the inclusion requirements. However, these results have to be interpreted with caution as most patients received further treatment after progression and selection bias is likely to influence overall survival in small phase-I/II vaccination studies. Disappointingly, immune monitoring in our patients revealed that the *in vivo* immunogenicity of the RCC26/IL-7/CD80 vaccine was rather low: four patients (nos. 5, 8, 9 and 10) showed a DTH reaction and two of these patients also developed T-cell responses against RCC-associated antigens. However, six patients did not develop any DTH reactions of the skin, indicating insufficient immune activation by the vaccine. In two patients with positive DTH reactions (nos. 5 and 9) peptide-specific IFNγ release was detected upon stimulation with tumor-associated peptides, indicating a TH1-polarized component of the emerging immune responses (Figure 3). In most of the patients, the vaccine was not able to induce a TH1-polarized T-cell response against RCC-associated peptides. This does not exclude an immune response against yet unknown antigens, however, even in this case the clinical benefit would not have been satisfactory. A peptide-specific response could be detected in six patients, encompassing the target antigens survivin, ADPF, vimentin, TYMS, c-Met, ILGF and/or G250. However, the cytokine secretion profile indicated a more predominant TH2 type of immune response with increasing amounts of IL-10 in many cases. Some scant TH1-polarized T-cell responses were detectable; however, these responses were often weak and short-lived, indicating insufficient T-cell activation by the vaccine. A clear correlation between clinical response, TTP and the type of immune response could not be established in our small cohort of patients. The most important clinical and immunological results are summarized in Table 4 for each patient.

The lack of appropriate peptide-specific immune responses was not due to general unresponsiveness of the patients' PBMC, as the viability of the thawed cells and functional evaluation of T-cell proliferation showed normal reactivity in response to general stimulation with PHA/IL-2 or allogeneic PBMC. On the contrary, the mixed lymphocyte reaction experiments showed that there was a tendency towards a higher allo-responsiveness in the RCC patients compared with normal controls. This difference is most likely due to allo-immunization induced by blood transfusions in the RCC patients.

Interestingly, the lack of IFN-γ release in cells of the patients in response to stimulation was not restricted to tumor-associated peptides and cytomegalo-, EBV- and influenza (flu)-virus peptides. A severely impaired IFN-γ secretion was also observed after stimulation with non-specific stimuli such as phorbol myristate acetate (Table 2). As the lack of IFN-γ secretion upon stimulation could not be explained by reduced viability or generally impaired T-cell responsiveness, we conclude that the observed difference reflects a deficit in IFN-γ secretion in this cohort of RCC patients. This could be explained by preexisting tumor-induced immunosuppression, as has

Table 3 Percent TSDR$^+$ CD4-T cells in the peripheral blood of patients before and during vaccination

Patient no.	*Before*	*TP1*	*TP2*	*TP3*	*TP4*
1	3.4	2.9	2.0	1.4	3.3
2	3.8	7.5			
3	5.5	6.6	4.1	3.5	2.0
4	4.7	6.4	6.8	7.0	7.4
5	0.2	2.9	4.5	5.8	
6	13.7	8.4	8.4		
7	4.3	7.9			
8	3.1	0.1	6.7	4.5	3.6
9	2.9	6.4	2.5	2.8	3.1
10	7.3	2.2	5.9	4.9	

Abbreviation: TP, time point (TP1=week 6, TP2=week 14, TP3=week 22, TP4=week 36).
Mean % TSDR+CD4-T cells (patients before vaccination): 4.9 ± 3.4%.
Mean % TSDR+CD4-T cells (healthy controls): 6.4 ± 1.6%.

Table 4 Clinical and immunological results: overview

Patient no.	*Clinical response*	*TTP (weeks)*	*Immune response*			
			DTH	*Peptide specific*	*Cytokine profile*[a]	*IL-10 release*[b]
1	SD	25	–	(+)	TH2	–
2	PD	8	–	–		
3	SD	69	–	(+)	TH1	–
4	PD	18	–	–		
5	SD	26	+	+	TH2>TH1	+
6	PD	9	–	+	TH2>TH1	+
7	PD	4	–	+	TH1→TH2[c]	+
8	SD	26	+	–		
9	SD	58	+	+	TH2>TH1	+
10	PD	18	+	–		

Abbreviations: CBA, cytometric bead array; DTH, delayed-type hypersensitivity reaction of the skin; PD, progressive disease; SD, stable disease; TTP, time to progression.
[a]Peptide-specific cytokine release on CBA/predominant cytokine pattern (TH1 vs TH2).
[b]Induction of peptide-specific IL-10 release by the vaccine as measured by CBA.
[c]Shift from TH1 to TH2 during vaccination.

been previously described in RCC.[23] Immunosuppression might be caused by high tumor burden and may explain the inability of the vaccine to induce efficient anti-tumor immunity. Alternatively, the insufficient anti-tumor immune response could be attributable to the vaccine itself, such that a lack of immunogenicity is inherent to RCC26/IL-7/CD80 cells. Low immunogenicity of the vaccine is also suggested by the limited number of patients developing a DTH reaction of the skin. Our data do not allow us to distinguish whether this was mainly due to patient characterictics (that is, profound systemic tumor-induced immunosuppression), an insufficient immunological activity of the vaccine, or both. Immunological data from another clinical trial performed with the same RCC26 cell line cotransfected with IL-2 and CD80[19] suggest that RCC26/IL-2/CD80 cells are more immunogenic than RCC26/IL-7/CD80. Whether this is due to quantitative (amount of IL-7 production too low for T-cell activation and/or co-stimulatory signal too weak) or qualitative (IL-2 better than IL-7) differences remains an open question. It has been demonstrated that exposure of anergic T cells to IL-2 is able to restore antigen responsiveness.[24] Further *in vitro* studies are necessary in order to dissect the differences between the two gene-modified tumor cell vaccines and their impact on immune responses *in vivo*.

Genetically-modified tumor cells have previously been used as vaccines in clinical trials in both autologous and allogeneic settings.[1,2,6,7] Allogeneic whole cell tumor vaccines have been clinically studied in melanoma, neuroblastoma, breast cancer, colorectal cancer, pancreatic cancer and prostate cancer. These studies showed that the approach is clinically feasible and safe. Vaccine-induced immune responses were a common observation in many of these trials, whereas clinical responses were reported only occasionally. Mocellin *et al.*[1] published a comprehensive review of these studies in 2004 (webtables I and II), additional clinical studies have been recently published.[2,25–29] In RCC, autologous tumor cells were retrovirally modified to secrete granulocyte-macrophage colony stimulating factor. Use of granulocyte-macrophage colony stimulating factor-secreting autologous tumor cells was feasible and safe; after vaccination of RCC patients with advanced disease strong DTH reactions of the skin were observed, together with cellular infiltrates at the injection site as well as occasional immunological and clinical responses.[30,31] Two other clinical studies used CD80-transfected autologous tumor cells in RCC patients.[32,33] Both studies confirmed the feasibility of the approach and clinical responses were reported in a few patients; however, assessment of clinical efficacy was hampered by coadministration of s.c. IL-2 in these trials. All of these trials with autologous tumor cells, as well as ours with an allogeneic vaccine, show that vaccination is not able to induce clinically relevant responses in most patients with advanced disease. Our data support the hypothesis that this is very likely due to profound immunosuppression in these patients. Tumor-induced immunosuppression can be demonstrated both in animal models and in humans, and seems to be mediated by different mechanisms.[34–36] Tregs, which can be induced by TGF-β and by inadequate (non-immunogenic) antigen presentation, obviously have an important role in maintaining a state of tolerance, thereby preventing induction of anti-tumor responses. In our patients, baseline levels of stable FoxP3 expressing Tregs (TSDR$^+$ CD4-T cells) did not differ significantly from normal controls. However, an increase in the percentage of TSDR$^+$ CD4-T cells during vaccination (pre- vs post-vaccination) was observed in four patients, whereas the percentage of TSDR$^+$ CD4-T cells did not change in three patients. Interestingly, in the remaining three patients who demonstrated a decrease in the percentage of TSDR$^+$ CD4-T cells (nos. 3, 6 and 10) throughout vaccination, two showed immune responses with a TH1 component. In RCC and melanoma, a correlation between decreasing numbers of Tregs and response to IL-2 therapy was described previously.[37] In our trial, induction of antigen-specific IL-10 secretion in patients may not only be explained by a TH2-polarization of the immune response. Alternatively, IL-10 release could also be the consequence of immunosuppressive networks induced by the vaccine, in particular Tregs.[38] Therefore, in future vaccination studies concomitant inhibition of immunosuppressive mechanisms, such as Tregs depletion, may help to augment the clinical efficacy of the vaccine, particularly in minimal residual disease. Recent reports on successful phase-III vaccination trials in follicular lymphoma,[39] prostate cancer[40] and melanoma[41] suggest that there will be a role for vaccines in the future management of malignant diseases in particular clinical situations, and encourages us to further investigate the role of allogeneic vaccines in renal cancer.

PATIENTS AND METHODS

Allogeneic tumor cell line and vaccination schedule

RCC26 tumor cells, derived from a HLA-A*0201 renal cancer tumor cell line, were used for vaccine production according to GMP guidelines. The HLA type of the tumor cells was HLA-A2, A33, B41, 51, Bw4, 6, Cw15, 17, DRB1 15, 4, DRB4 53, DRB5 51, DQB1 06, 03. Transfection of RCC26 tumor cells with human IL-7 and human CD80 (B7.1) was performed by electroporation using

the plasmid vector pKEx-IL-7-IR-B7, which contained an internal ribosomal entry site and a hygromycin resistance gene as described previously.[16] GMP production of the genetically-modified vaccine (RCC26/IL7/CD80) was performed in our GMP facility. External quality control of the master cell bank for viral contamination was performed by BioReliance (Stirling, Glasgow, Scotland). For vaccine production, transfected cells were cultivated in Nunc cell factories (Nunc, Naperville, IL, USA) using RPMI 1640 (BioWhittaker, Verviers, Belgium) in the presence of 1 ml per 100 ml non-essential amino acids (BioWhittaker), 1 ml per 100 ml sodium pyruvate (BioWhittaker), 1 ml per 100 ml ITS-X (insulin-transferrin-selen) (InVitrogen, Leek, The Netherlands), 1.2 ml per 100 ml hygromycine (InVitrogen) and 20 ml per 100 ml fetal calf serum (Summit Technology, Collins, CO, USA). Cells were seeded at a density of $3\times10^3\,cm^{-2}$ and were harvested at a density of $6\times10^4\,cm^{-2}$ using trypsin/ethylenediaminetetraacetic acid (PAA Laboratories, Linz, Austria). After washing with Hank's Buffered Salt Solution (HBSS) (BioWhittaker) aliquots containing 2.5–40×10^6 RCC26/IL7/CD80 cells were frozen in cryovials (Nunc) in the presence of HBSS, 7.5% dimethyl sulfoxide (Sigma, St. Louis, MO, USA) and 20% human serum albumin (Octapharma, Langenfeld, Germany). Aliquots were stored in the vapour phase of liquid nitrogen until clinical use. For safety reasons, all cryopreserved aliquots of the vaccine were irradiated (120 Gy) according to a standardized protocol which completely prevented long-term survival of tumor cells *in vitro*. IL-7 production of the vaccine cells was measured *in vitro* by enzyme-linked immunosorbent assay (Biozol, Eching, Germany), CD80 surface expression was determined by flow cytometry using phycoerythrin-labelled anti-CD80 antibody (Becton-Dickinson, Heidelberg, Germany). IL-7 production and CD80 expression by the vaccine cells were determined in accordance with local regulatory authorities: IL-7 transfection (as measured by IL-7-secretion into the cell culture supernatant) was analyzed in our GMP facility and confirmed by an external institution (Bender MedSystems, Vienna, Austria). The clinical protocol did not contain a prespecified level of IL-7 production as a release criterion for the vaccine cells, as no data on a possible dose–response correlation suggesting critical IL-7 levels were available at that time. Therefore, release criterion, as requested by regulatory agencies, was detectable IL-7 production at different time points after thawing (4, 24 and 48 h). CD80 expression of irradiated vaccine cells for clinical application had to be >60% after thawing. The phase-I vaccination protocol was performed according to the following schedule: 2.5×10^6 cells s.c. at weeks 1, 2, 4 and 6; 10×10^6 cells at weeks 8, 10, 12 and 14; and 40×10^6 cells at weeks 18 and 22.

Patients

A total of 10 HLA-A*0201$^+$ patients with American Joint Committee on Cancer stage IV RCC were enrolled in this phase-I monocentric protocol. Inclusion criteria were age 18–74 years, histologically proven clear cell RCC with at least one measurable marker lesion, Karnofsky performance score $\geqslant$70, interval between last systemic therapy, and enrolment in the study of at least 3 months and informed written consent. Exclusion criteria were severe impairment of organ function, clinically active autoimmune disease, immunodeficiency or constant treatment with immunosuppressive drugs, organ allografts, history of severe allergy, pregnancy or nursing and evidence of another malignant disease during the past 5 years. Patients characteristics are given in Table 1. Primary objectives of the study were feasibility and safety as well as the induction of a measurable T-cell response against RCC-associated antigens. Secondary objectives were clinical response, and the assessment of a correlation between clinical and immunological response. The protocol was approved by the local ethics committee and by the national ethics committee for somatic gene therapy of the German Medical Association.

Clinical monitoring

Clinical examination and routine blood checks were performed at every visit (weeks 1, 2, 4, 6, 8, 10, 12, 14, 18, 22, 24). Patients were assessed for signs of autoimmune disease by measurement of thyroid hormones and pancreatic enzymes, screening for anti-nuclear antibodies as well as monitoring of rheumatoid factor and complement factors. Blood samples for immune monitoring were taken before vaccination and at weeks 6, 14, 22 (and 36 in some patients). In the case of progressive disease, the last blood sample was taken when the patient was set 'off study'. Restaging with Computed Tomography (CT) was performed at weeks 8, 18, 24 and 36. Patients were withdrawn from the study upon evidence of tumor progression, according to the response evaluation criteria in solid tumors.[42]

Immune monitoring

DTH testing. The patients were assessed for a DTH reaction of the skin after subcutaneous injections of irradiated vaccine after 4, 8, and 10 vaccinations at weeks 6, 14, and 22, respectively. Furthermore, skin tests were performed by intradermal injection of 2.5×10^6 cells at a site distant from the vaccination sites in the inguinal region. DTH reactions were evaluated 48 h later and were judged as being positive if an induration or erythema of at least 10 mm diameter was observed. Skin biopsies were also taken 48 h after intradermal injection.

Fluorescence-activated cell sorting analysis of peripheral blood

Ethylenediaminetetraacetic acid-blood was analysed by two colour immunofluorescence staining using Pharm Lyse solution (Becton-Dickinson, Heidelberg, Germany) according to the manufacturer's instructions. Direct immunofluorescence staining was performed in whole blood using the following fluoroisothiocyanat- and phycoerythrin-conjugated monoclonal antibodies: CD3, CD4, CD8, CD19, CD56, CD25, HLA-DR, and isotype control (Becton-Dickinson). 1×10^4 cells were analyzed on a fluorescence-activated cell sorting FACS Calibur (Becton-Dickinson). Analysis was made on gated PBMC.

ELISpot assays

PBMC were prepared from heparinized whole blood by density gradient centrifugation on Lymphoprep (Progen, Heidelberg, Germany). Cells were harvested and washed four times at low speed to remove platelets. PBMC were then stored in the vapour phase of liquid nitrogen until assays could be performed at the end of the study. For IFN-γ ELISpot assays, PBMC were thawed, washed with CTL WashTM supplement medium (Cellular Technology Ltd., Cleveland, OH, USA) plus 1% L-glutamine and Benzonase nuclease (50 Units per ml; Novagen Merck Biosciences, Darmstadt, Germany), rested 2–5 h in serum-free culture medium (CTL Test medium) plus 1% L-glutamine and seeded at 1.0×10^5 cells in triplicates on PVDF plates which had been coated with antibody (Mabtech AB, Nacka, Sweden). PBMC were stimulated directly with selected peptides ($10\,\mu g\,ml^{-1}$) (see below) in serum-free culture medium (CTL Test medium), supplemented with 1 µg per ml anti-CD28 antibody (BD Biosciences, San Jose, CA, USA) and 2 U per ml recombinant IL-2 (Proleukin Chiron, Emeryville, CA, USA). Incubation was performed overnight, ELISpot plates were developed according to the manufacturer's instructions. Spots were counted using the AID reader system with the software version 3.1 (AID Autoimmun Diagnostika GmbH, Strassberg, Germany) and controlled by human audit. Results were expressed as spots per 3×10^5 PBMC. Peptide-specific responses were defined as having (1) a ratio of specific peptide: irrelevant control peptide >2 and (2) an absolute number of spots >10.

Peptides for immune monitoring were selected from sequences of TAA shown to be overexpressed in RCC26 cells[15] (and unpublished observations) using HLA-A*0201 motif-based epitope predictions available on the web (http://www.syfpeithi.de), or as published in the literature. Peptides specific for the following antigens were used:

survivin (ELTLGEFLKL$_{95-104}$, TLPPAWQPFL$_{5-14}$,)[43]; heteroclitic survivin-related peptide (LMLGEFLKL$_{69-104}$,[44] and EKVRRAIEQL$_{129-138}$); cyclin D1 (LLGATCMFV$_{101-109}$, RLTRFLSRV$_{228-236}$)[45]; adipophilin (SVASTI TGV$_{129-137}$, TLLSNIQGV$_{327-335}$)[46]; c-Met proto-oncogene (YVDPVI TSI$_{654-662}$, VLAPGILVL$_{6-14}$, GLIAGVVSI$_{31-39}$)[46]; TYMS (VLEELLWFI, RILRKVEKI, YMIAHITGL); ILGF (AALTLLVLL, LLDGRGLCV) vimentin (DLERKVESL$_{218-226}$, ILLAELEQL) and G250 (HLSTAFARV$_{217-225}$; GLLFAVTSV). Peptide synthesis was performed as described previously.[47] An irrelevant HLA-A2-restricted peptide from the CML fusion protein bcr3/abl2 (SSKALQRPV$_{926-934}$)[48] was used as a negative control and the HLA-A2 cytomegalo-, EBV- and influenza (flu)-virus peptide pool (CMV pp65 NLVPMVATV$_{495-503}$, EBV-BMLF1 GLCTLVAML$_{280-288}$, EBV-LMP-2 CLGGLLTMV$_{426-434}$, influenza M1 protein GILGFVFTL$_{58-66}$ and influenza RNA polymerase PA FMYSDFHFI$_{46-54}$) with 0.2 µg of each peptide per well as a positive control (PANATecs GmbH, Tübingen, Germany).

Phorbol myristate acetate (500 ng ml^{-1}) was used as a positive control for IFN-γ release in samples of both patients and healthy donors.

CBA

CBA in assays permit simultaneous quantification of multiple cytokines in solution by capturing these to spectrally distinct beads. The cytokine pattern measured by the CBA used in this study (CBA, Becton-Dickinson) consists of IFN-γ, IL-2, tumor necrosis factor-α, IL-4, IL-10 and IL-5. 1.0×10^5 cells were stimulated directly with selected peptides (10 μg per ml of each peptide) in serum-free culture medium (CTL Test medium), supplemented with 1 μg per ml anti CD28-antibody (BD Biosciences, San Jose, CA, USA) and 2 U per ml recombinant IL-2 (Proleukin Chiron, Emeryville, CA, USA). Incubation was performed in duplicates overnight in a 96-well tissue culture plate (Falcon, Becton-Dickinson, San Jose, CA, USA). Culture supernatant was frozen at −80 °C until cytokine analysis was performed according to the manufacturer's instructions. Duplicates were pooled for cytokine analysis. A peptide-specific response was defined as having a ratio 'cytokine secretion of specific peptide: cytokine secretion of irrelevant control peptide' >2 and mean fluorescence intensity of specific peptide : mean fluorescence intensity of irrelevant control peptide >2.

Mixed lymphocyte reaction and proliferation assays

For proliferation assays, triplicates of 10^5 PBMC per well were used as responder cells in RPMI 1640 medium supplemented with 4 mM L-glutamine, 25 mM HEPES, 1 mM Na-pyruvate (Lonza, Verviers, Belgium) and 50 μg per ml gentamycin (Ratiopharm, Ulm, Germany), and 10% heat-inactivated fetal calf serum (HyClone, Thermo Fisher Scientific, Logan, UT, USA), further referred to as complete medium. PBMC from healthy donors were used as a positive control. Viability of thawed PBMC was >70% in nearly all samples, as measured by trypan blue or propidium iodide staining. In all, 10^5 irradiated (30 Gy) allogeneic PBMC (mixture from four different healthy donors) were used as stimulator cells. Proliferative capacity of the responder cells was measured in a standard 5 day ^{3}H-thymidine proliferation assay. During the last 16 h of the 5-day culture, 1 mCi per ml ^{3}H-thymidine (Amersham Life Science, Buckingham, UK) was added. Cells were harvested and radioactivity was measured in a scintillation counter. Maximum proliferation rate was determined by cultivation of 10^5 PBMC in the presence of PHA and IL-2. Minimum proliferation rate was determined by cultivation of PBMC alone. Median values were determined from the triplicates.

HLA typing

HLA typing was performed initially at a low-resolution level by the PCR using sequence-specific oligonucleotide probes (Dynal RELI_ SSO HLA Typing Kits from Dynal, Dynal Biotech A.S.A, Oslo, Norway). HLA-A2 high resolution subtyping was subsequently performed by PCR, using sequence-specific primers (Olerup SSP- kits, Genovision/Qiagen, Vienna, Austria).

HLA antibody screening

Screening for HLA class I and class II antibodies was performed by means of an enzyme-linked immunosorbent assay-based HLA screening assay (Lambda Antigen Tray LATM, OneLambda, Canoga Park, CA, USA), and determination of the HLA antibody specificity was accomplished by the HLA specification assay LAT1288 (One Lambda).

Immunohistological analysis

Four-micrometer-thick sections from paraffin-embedded cutaneous biopsies were deparaffinized and subjected to an antigen-retrieval pretreatment using pronase or high-pressure cooking with citrate buffer. After pretreatment, sections were incubated with primary antibody. The following antibodies were used in this study: CD3 (clone LN10, Leica Biosystems, Newcastle, UK, concentration 1:100), CD4 (clone 4B12, Leica Biosystems, concentration 1:25), CD8 (clone C8/144B, Dako, Glostrup, Denmark, concentration 1:100), CD56 (clone 123C3, Monosan, Sanbio Germany, concentration 1:50), TCR ßF1 (clone 8A3, Endogen USA, concentration 1:10), CD68 (clone PGM1, Dako, concentration 1:50) and CD20 (clone L26, Dako, concentration 1:50). Bound antibodies were visualized using the alkaline phosphatase-anti alkaline phosphatase method, employing FastRed as chromogen (reagents obtained also from Dako).

TCR analysis by PCR

PBMC were prepared from 10 ml of citrate blood by density gradient centrifugation through Ficoll-HyPaque (Pharmacia, Freiburg, Germany). Genomic DNA was prepared from $\sim1\times10^6$ cells by a standard procedure using proteinase K digestion. Subsequently, the DNA of the blood samples was amplified by TCR-PCR as previously described.[39,40] All primers were purchased from BioTez (Berlin, Germany). Primer synthesis included a standard purification by gel filtration. Each reaction was screened by electrophoresis on a 1.5% agarose gel, followed by GelRed staining before fluorescence fragment analysis (FFA, see below). Each TCR-PCR showing a clonal pattern in the FFA was repeated in order to confirm the corresponding profile of the FFA. Following PCR and screening on an agarose gel, products were subjected to FFA on the ABI 310 PRISM capillary sequencing instrument using the Gene Mapper 3.7 software (Applied Biosystems, Weiterstadt, Germany). A successful PCR from DNA of polyclonal T cells displayed approximate Gaussian profiles fitting the relevant size ranges. Details of the FFA, including evaluation of the profiles by calculation and classification of height ratios for predominant ('clonal') peaks, have been described elsewhere.[49] Values of peak height ratios were applied for the assessment of the FFA from all TCR-PCR. The detection threshold of the PCR varied between 1 and 3% (TCRγ) or 1 and 10% (TCRβ) of clonally rearranged T cells.[50]

FOXP3 TSDR quantification

Genomic DNA was isolated from PBMC samples using the QIAamp^R DNA Blood Mini kit (Qiagen, Hilden, Germany). In all, 500 ng eluted genomic DNA was used in a subsequent bisulfite treatment (EpiTect^R, Qiagen). A minimum of 60 ng bisulfite-treated genomic DNA was used in a Realtime-PCR to quantify the *FOXP3* TSDR as previously described.[51] Realtime-PCR was performed in a final reaction volume of 20 μl containing 10 μl FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany), 50 ng per μl Lamda DNA (New England Biolabs, Frankfurt, Germany), 5 pmol per μl methylation or non-methylation specific probe, 30 pmol per μl methylation- or non-methylation-specific primers and 60 ng bisulfite-treated DNA or respective amounts of plasmid standard. Primers and standards were provided by Epiontis GmbH (Berlin, Germany). The samples were analyzed in triplicates on an ABI 7500 cycler, using the following cycling conditions: 1 cycle of 10 min 95 °C and 45 cycles of 15 s 95 °C followed by 1 min 61 °C. The % *FOXP3* TSDR content was then calculated by dividing the non-methylated copy number by the total genomic *FOXP3* copy number. As PBMC samples were used, the obtained results were adjusted to the individual percentage of CD4$^+$ T cells determined by flow cytometry.[51] The classification of changes in the percentage of TSDR+ CD4-T cells (increase vs decrease vs unchanged) is based on a comparison of the respective values pre- and post-vaccination as these parameters reflect the outcome of the whole vaccination period.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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