REVIEW

Update on vaccine development for renal cell cancer

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Abstract: Renal cell carcinoma (RCC) remains a significant health concern that frequently presents as metastatic disease at the time of initial diagnosis. Current first-line therapeutics for the advanced-stage RCC include antiangiogenic drugs that have yielded high rates of objective clinical response; however, these tend to be transient in nature, with many patients becoming refractory to chronic treatment with these agents. Adjuvant immunotherapies remain viable candidates to sustain disease-free and overall patient survival. In particular, vaccines designed to optimize the activation, maintenance, and recruitment of specific immunity within or into the tumor site continue to evolve. Based on the integration of increasingly refined immunomonitoring systems in both translational models and clinical trials, allowing for the improved understanding of treatment mechanism(s) of action, further refined (combinational) vaccine protocols are currently being developed and evaluated. This review provides a brief history of RCC vaccine development, discusses the successes and limitations in such approaches, and provides a rationale for developing combinational vaccine approaches that may provide improved clinical benefits to patients with RCC.

Keywords: renal cell carcinoma, vaccines, immunotherapy, combinational therapy, cellular immunity

Introduction: immunotherapy for renal cell carcinoma

Renal cell carcinoma (RCC) accounts for approximately 3% of all cancers in adults, with metastases identified in 20%–30% of patients at the time of diagnosis. Metastatic RCC, if left untreated, has a 5-year disease-free survival rate of 2%–11%.[1] Following nephrectomy, conventional treatments with standard chemotherapeutic agents, hormones, and radiotherapy have shown minimal success. This has prompted extensive evaluation of alternate treatment strategies, including immunotherapies, in the adjuvant and advanced disease settings.

Optimism for the use of biological response modifiers and vaccines has been buoyed by past findings, which suggest that, like melanoma, RCC progression and regression may be regulated by immunologic mechanisms.[2–5] Patients with RCC exhibited a low but significant incidence of spontaneous regression,[6,7] and patients under chronic immunosuppression regimens to retain kidney allografts displayed an increased risk of developing RCC.[8] The degree of tumor infiltration by lymphocytes has been used as a prognostic indicator for patient survival.[9,10] In particular, T cells of the type-1 polarization profile (ie, capable of producing interferon [IFN]-γ and mediating the cytotoxic death of RCC tumor cells) and proliferative potential[11,12] have proven

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to represent primary immunologic mediators of objective clinical responses (OCRs).

However, patients with RCC are frequently characterized with a state of "immune dysfunction",[13–16] where type-1 responses directed against tumor-associated antigens are muted in comparison with type-2 (normally associated with antibody production and allergic reactivity) and/or T-regulatory (Treg) responses, which are immunosuppressive in nature.[17–20] Furthermore, when the responses can be identified, type-1 anti-RCC T cells may be proapoptotic under a chronic state of stimulation with specific tumor antigens in patients with cancer.[21,22]

Rationale for RCC vaccines

As depicted in Figure 1, therapeutic normalization of type-1, antitumor T-cell-mediated immunity in patients with RCC requires one or more of the following processes to occur: (1) existing tumor antigen-experienced T cells exhibiting anergy or nontype-1 functional polarization need to be reactivated or retrained to become type-1 polarized; (2) the survival and functionality of existing type-1 T cells

must be extended; (3) new type-1 effector cells must be "primed" from the naive cohort of T cells (a process that may require the "breaking" of operational tolerance); (4) effective trafficking of renal cell carcinoma – associated antigen (RCCAA)-specific T cells to the tumor microenvironment (TME); and/or (5) blunting of regulatory T cells (Treg) that suppress effector T-cell activation, function, and durability. Each of these immunologic end points may be theoretically achieved via the implementation of tumor-specific vaccines that contain and/or condition antigen-presenting cells (APCs) in situ to assume type-1 function (typically associated with the ability of APCs to differentially secrete interleukin [IL]-12 vs IL-10).[23–25] Reports have shown that type-2 "memory" T-cell responses (ie, characterized by strong IL-4 and IL-5 production) may be repolarized toward type-1 immunity *in vitro* by (re)stimulation with antigen-pulsed dendritic cells (DCs) that were preconditioned with proinflammatory cytokines, toll-receptor ligands, and other costimulatory adjuvants.[23,26,27] In humans, type-1 effector T cells have exhibited extended survival, function, and conversion into the memory cells when provided signals
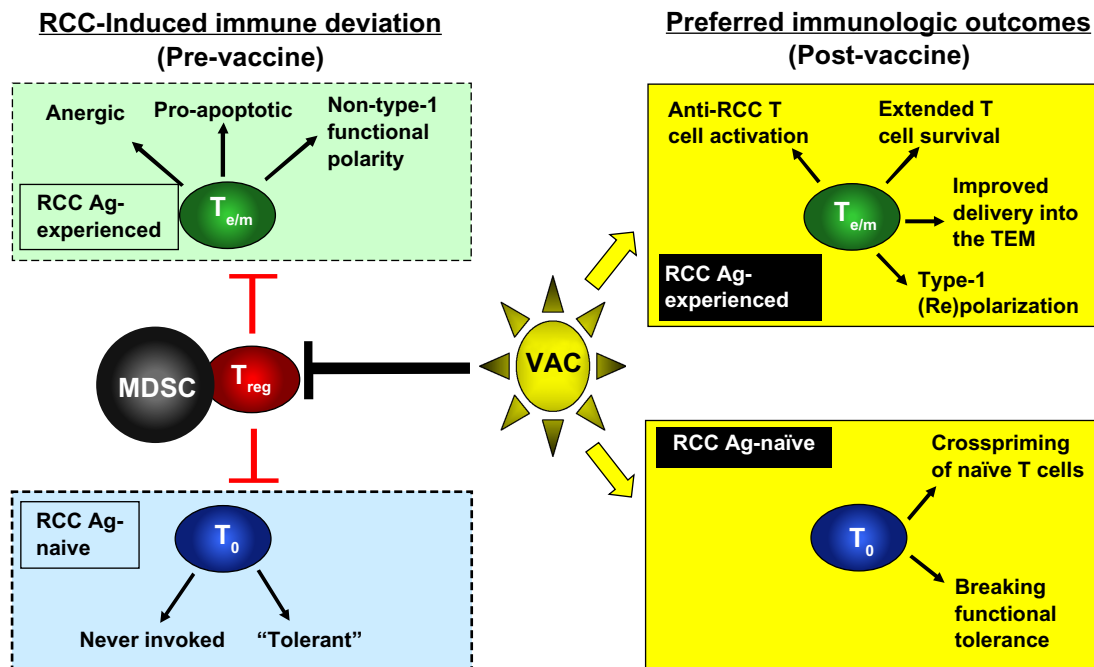

Figure 1 Paradigm for effective renal cell carcinoma (RCC) vaccines. Antitumor T cells in patients with RCC are frequently anergic, hyporesponsive, or they may mediate functions that are nonprotective. T effector (T_e) and memory (T_m) cells (cumulatively indicated as $T_{e/m}$) may also be prone to apoptotic death based on conditioning by tumor cells or their elaborated products *in vivo*. Naive (T_0) antitumor T cells may be rendered nonresponsive or exhibit specificities against "subdominant" RCC-associated antigens (RCCAAs) or epitopes that have failed to become activated productively. Furthermore, the vitality and function of antitumor T cells may be inhibited by regulatory T cells and myeloid-derived suppressor cells (MDSCs), particularly in the tumor microenvironment (TME). Effective vaccine formulations would at least partially correct such defects by (re)activating $T_{e/m}$ and promoting their extended survival and delivery into the TME. Importantly, given some plasticity in functional T-cell polarization, effective RCC vaccines may promote a conversion of nontype-1 T-cell responses towards type-1 immunity, which has been commonly associated with improved clinical prognosis. Such vaccine-induced repolarization in T-cell function may foster the breaking of operational tolerance against additional RCCAAs and the cross-priming of a broadly reactive antitumor T-cell repertoire. If sustained (through booster vaccination), this vaccine-initiated T-cell response may extend time to disease recurrence or progression and overall patient survival.

from CD16^{+} monocyte-derived DCs.[28] Furthermore, type-1 polarized or conditioned DCs appear superior to alternate APC types in their capacity to activate and drive naive T-cell differentiation into type-1 CD4^{+} and CD8^{+} T effector cells *in vitro* and *in vivo*.[23,29,30] Although much of these data have been developed translationally in the context of cell (ie, DC-based) therapeutics, it would also be predicted that cell-free vaccine formulations including the appropriate tumor antigens and conditioning adjuvants would activate APC in situ with similar type-1-polarizing potential.[31–33]

RCCAA and vaccine construction

Vaccines designed to promote specific adaptive immunity against RCC have been traditionally grouped into 4 general categories. One type of tumor vaccine is RCC cells themselves (either autologous or allogenic cells that express unique and shared tumor-associated antigenic proteins). More than 20 years ago, Miller et al[34] trialed autologous RCC tumor cells using *Cryptosporidium parvum* as an adjuvant. Later, Tani et al[35] and others modified the autologous tumor cell vaccine by using granulocyte macrophage-colony-stimulating factor (GM-CSF) or other inflammatory cytokines as adjuvant. Thereafter, others used genetically modified patient tumor cells that expressed inflammatory cytokines, including GM-CSF, IFN-γ, and IL-2.[36] Another tumor vaccine formulation is represented by RCC-APC fusion hybrids, which generate APCs that are capable of expressing RCC gene products and presenting their derivative peptide epitopes to T cells. Avigan et al[37] were one of the few groups that used this strategy to treat patients with RCC. They fused autologous tumor cells to DCs from normal donors using serial electrical pulses. Another approach involves RCC-derived total mRNA or cDNA (encoding the complete repertoire of RCCAA). Although most published work using these vaccines has been limited to preclinical models,[38,39] Su et al[40] used autologous DCs transfected with total RCC RNA. More recently, several laboratories have been moving toward a more specified vaccine formulation using peptides, protein, mRNA, or cDNA derived from or encoding one or more molecularly defined RCCAAs (Table 1). Wierecky et al[41] and Bleumer et al[42] have vaccinated RCC patients with mucin (MUC1) and carbonic anhydrase (CA-IX) peptides, respectively, loaded on to autologous DCs. The clinical outcomes associated with these various vaccine formulations will be discussed later in this review.

A myriad of genetic aberrations can potentially develop within the evolving heterogeneous RCC lesion over many months to years under immune selective pressure. The first

3 categories of vaccines cited earlier theoretically provide the greatest variety of RCCAAs, which promote the broadest antitumor T-cell repertoire, when applied in the context of a vaccine. In vaccines based on whole tumor cells, tumor-APC hybrids, and/or tumor-derived mRNA or cDNA, RCCAAs derived from mutant proteins with alternate open reading frames (ORFs), antisense transcripts, or unique protein-splicing events (Table 1) will be incorporated without knowing the identity of the RCCAA. However, these approaches have limitations from an immunologic perspective. Complex mixtures of unknown RCCAA may merely reinforce an existing, yet failing, immune repertoire given the immune dominance of certain RCCAAs over others. Competition by hundreds or thousands of peptide epitopes for loading into major histocompatibility complex (MHC) molecules expressed by (cross-presenting) APCs *in vivo* could prevent attaining immunogenic quantities of RCCAA peptides. These types of vaccines would also introduce an array of immunsuppressive genes and gene products (ie, IL-10, transforming growth factor [TGF]-β, B7-H1, indoleamine 2,3-dioxygenase [IDO], etc) into the vaccine site that may negate the immunostimulatory potential of the treatment.

A less-dynamic, but better-controlled, vaccine approach involves the use of molecularly defined RCCAAs identified by tumor cell or tumor "genome-" or "proteome-based" approaches. Such a formulation reduces the effects of confounding immunosuppressive signals or competing ligands for MHC presentation. Among the many RCCAAs identified and defined as targets for T-cell recognition over the past 10–15 years, most of these gene products represent proteins that are 1) nonmutated, 2) frequently overexpressed by tumor vs normal kidney tissue, and 3) upregulated as a consequence of the hypoxic or hypomethylating conditions prevalent in the TME (Table 1). The conditional and/or preferential (over)expression allows for the differential expression of antigenic peptides in MHC complexes expressed by tumor cells vs normal cells. This property of RCCAAs encourages the development of therapeutic vaccines capable of eliciting antigen-specific T effector cells that may strategically eradicate tumor cells without manifesting pathologic autoimmune correlates.

Several well-defined RCCAAs have been (or could be) implemented in phase I/II vaccine trials for patients with RCC. A partial list of more than 30 such candidates is provided in Table 1. Nearly 2 dozens of these gene products were reported to be (over) expressed in the majority of RCC specimens evaluated, making them tenable candidates for inclusion in a "general" vaccine for treating patients with

Table 1 RCC-associated antigens (RCCAAs) recognized by T cells

Antigen	Antigen category	Frequency of expression among RCC tumors (%)	CD8+ T-cell recognition: patients with HLA class I allele(s)	CD4+ T-cell recognition: patients with HLA class II allele(s)	References
Survivin[a]	ML	100	Multiple	Multiple	114
OFA-iLR	OF	100	A2	NR	115, 116
IGFBP3[a,b]	ML	97	NR	Multiple	117, 118
EphA2[a]	ML	>90	A2	DR4	17, 44, 119
RU2AS	Antisense transcript	>90	B7	NR	120
G250 (CA-IX)[a,b]	RCC	90	A2, A24	Multiple	47, 51
EGFR[a,b]	ML	85	A2	NR	121, 122
HIFPH3[a]	ML	85	A24	NR	123
c-Met[a]	ML	>80	A2	NR	124
WT-1[a]	ML	80	A2, A24	NR	125–128
MUC1[a,b]	ML	76	A2	DR3	46, 129, 130
5T4	ML	75	A2, Cw7	DR4	54, 131–133
iCE	aORF	75	B7	NR	134
MMP7[a]	ML	75	A3	Multiple	117, 135, 136
Cyclin D1[a]	ML	75	A2	Multiple	117, 137, 138
HAGE[b]	CT	75	A2	DR4	139
hTERT[a,b]	ML	>70	Multiple	Multiple	140–142
FGF-5	Protein splice variant	>60	A3	NR	143
mutVHL[a,b]	ML	>60	NR	NR	144
MAGE-A3[b]	CT	60	Multiple	Multiple	145
SART-3	ML	57	Mulitple	NR	146–149
SART-2	ML	56	A24	NR	150
PRAME[b]	CT	40	Multiple	NR	151–154
p53[a,b]	Mutant/WT ML	32	Multiple	Multiple	155, 156
MAGE-A9[b]	CT	>30	A2	NR	157
MAGE-A6[b]	CT	30	Multiple	DR4	18, 158
MAGE-D4[b]	CT	30	A25	NR	159
Her2/neu[a]	ML	10–30	Multiple	Multiple	45, 160–164
SART-1[a]	ML	25	Multiple	NR	165–167
RAGE-1	CT (ORF2/5)	21	Multiple	Multiple	151, 157, 168, 169
TRP-1/gp75	ML	11	A31	DR4	151, 170–172

Note: A summary is provided for RCCAAs that have been defined at the molecular level. RCCAAs are characterized with regard to their antigen category, their prevalence of (over) expression among total RCC specimens evaluated, whether RCCAA expression is modulated by hypoxia or tumor DNA methylation status, and which HLA class I and class II alleles have been reported to serve as presenting molecules for T-cell recognition of peptides derived from a given RCCAA. [a]Hypoxia-induced. [b]Hypomethylation-induced.
Abbreviations: CT, cancer-testis antigens; ML, multilineage antigens; NR, not reported; OF, oncofetal antigen; aORF, altered open reading frame; ORF, open reading frame; RCC, renal cell carcinoma; WT, wild type.

RCC. Notably, those RCCAAs that may function as general RCCAAs for use in vaccines include survivin, an inhibitor of apoptosis whose expression is correlated with poorly differentiated, advanced-stage RCC;[43] the receptor tyrosine kinases (RTKs) EphA2, epidermal growth factor receptor (EGFR), c-Met, and Her2/neu;[44,45] MUC1;[46] CA-IX, also known as G250;[47] and the oncofetal antigen 5T4.[48]

Of the aforementioned RCCAAs, CA-IX and 5T4 are 2 of the more frequently overexpressed RCC markers. CA-IX overexpression in RCC is associated with a defect in the Von Hippel-Lindau (VHL) tumor suppressor gene via activation of hypoxia-inducible factor-α (HIF-α).[49] In conjunction with

regulation by VHL-HIF, CA-IX expression is also driven by the methylation status of the C9 gene, as the C9 promoter has been reported to be hypomethylated in all CA-IX+ RCC cell lines and to be hypermethylated in all CA-IX− RCC cell lines.[50] CA-IX is beginning to be exploited as a therapeutic target for RCC only now.[51] Only within the last few years has an appropriate animal model been established to study CA-IX-expressing tumors, using human CA-IX-transduced murine RCC cells.[52] Recently Bauer et al[53] published promising results on a G250 – tumor necrosis factor fusion antibody administered with IFN-γ to RCC xenograft-bearing nude mice; however, no CA-IX-based therapies have yet made it to

clinic. The heavily glycosylated transmembrane protein 5T4 is overexpressed by RCC tumor cells.[54] In a phase II trial for RCC, a recombinant fusion protein composed of a modified form of staphylococcal enterotoxin A (SEA) linked to mouse Fab specific for 5T4 has exhibited antitumor efficacy, albeit in the context of moderate toxicity.[55] Therapeutic viral vectors have also been used as a vaccination strategy, one being TroVax, a highly attenuated vaccinia virus, modified vaccinia ankara (MVA), which encodes the human 5T4 antigen. In a phase II trial, TroVax with IL-2 appeared to confer some clinical advantage, with some patients showing disease stabilization and an improved ratio of CD8+ effectors to Treg cells.[56,57] Patients cotreated with TroVax and IFN-α, however, did not show significant clinical improvement, despite having 5T4-specific antibody and/or cellular responses.[58,59]

In most cases, RCCAA-derived peptide epitopes recognized by CD8+ and/or CD4+ T cells have been identified. Such peptides may be used in vaccines for treating patients with relevant human leukocyte antigen (HLA) class I or II tissue types, which are required for presenting a given peptide epitope to the immune system. Recently, Rahma et al[33] published results from a pilot study testing a mutant VHL peptide vaccine customized to each patient's specific mutation and HLA haplotypes, with 80% of the treated patients exhibiting specific immune responses. In Table 1, we list the HLA allelic molecules to which known RCCAA peptides bind and are recognized by CD4+ and CD8+ T cells. These peptides are also applicable in immune monitoring of specific *in vitro* T-cell responses of patients being treated with any form of vaccine or immunotherapy. Vaccination against a single peptide can potentially promote "epitope spreading" in the evolving antitumor CD8+ T-cell repertoire based on cyclic and longitudinal cross-priming events within (tumor-draining) lymph nodes.[41,60,61] Additionally, several peptides (from one or more RCCAAs) can be constructed into a single vaccine formulation using mixtures of peptides or an extended "bead-on-a-string" protein construct in which multiple epitopes are expressed in tandem.[62–64] Such formulations focus the T cell on a more limited, but potentially more effective, cohort of RCCAA epitopes that may be immunologically subdominant and/or ignored under standard conditions, thereby unveiling a previously undertapped population of antitumor effector cells.

RCC vaccines in the clinic

Most of the clinical vaccine trials in patients with RCC performed till date have involved the use of whole tumor cells, tumor lysates, or the fusion of RCC cells with DCs.[65,66]

Vaccine formulations based on specific RCCAAs (protein, cDNA) or their derivative (MHC-presented) peptides have lagged behind due to the comparatively recent molecular identification of the applied RCCAA. Rather than providing a traditional tabulated summary of the data resulting from such trials, we provide a schematic diagram of trial outcomes based on the clinical (primary) and immunologic (secondary) end points defined in these protocols (Figure 2). We have further delineated vaccines based on the type of RCCAA utilized in each trial.

Despite recent discussions stating that immunotherapies should not be evaluated based on the "acute" response criteria (Response Evaluation Criteria in Solid Tumor [RECIST]) defined for chemotherapeutic agents because immunotherapies may depend on the gradual build up of adaptive immunity over a protracted period of time,[67,68] virtually all reported RCC vaccine trials have still done so. Hence, in Figure 2, we have depicted OCR frequencies based on partial responses (PRs), complete responses (CRs), or stabilization of disease (SD) per RECIST criteria as reported by the primary investigators. The consensus of such information suggests that current RCC vaccines are generally safe and well tolerated,[69] but are curative in only a very minor subset of treated patients. Although PRs increase in frequency somewhat after vaccine treatment, the major benefit of these cancer vaccines is reflected in many patients who exhibit stable disease, leading to increased progression-free and overall survival when compared with control groups.[69–72] Notably, each of the various vaccine formulation categories listed yielded similar clinical impact based on RECIST criteria (Figure 2), with roughly 50% of treated patients exhibiting stable disease, 20% showing partial response, and <20% developing CRs.

Since these represent immunotherapies rather than chemo- or radiotherapies, immunologic end point analyses are critical for determining the biological efficacy of these approaches and how such strategies may be improved based on our current understanding of RCC immunobiology. In this regard, the diverse array of RCC vaccine trials performed over the past 15 years has implemented a number of immune assessment assays to determine specific immune response to active vaccination, including analyses of patient tumor-specific T-cell responses *in vitro* (TRIV) and delayed-type hypersensitivity (DTH) responses to vaccine components *in vivo*. Assays for TRIV have dramatically evolved over the past decade, with established proliferation (ie, ^{3}H-thymidine incorporation) and cytokine (ie, enzyme-linked immunosorbent assay [ELISA]) assays now being supplanted by methods capable of discerning the frequency and/or functionality of
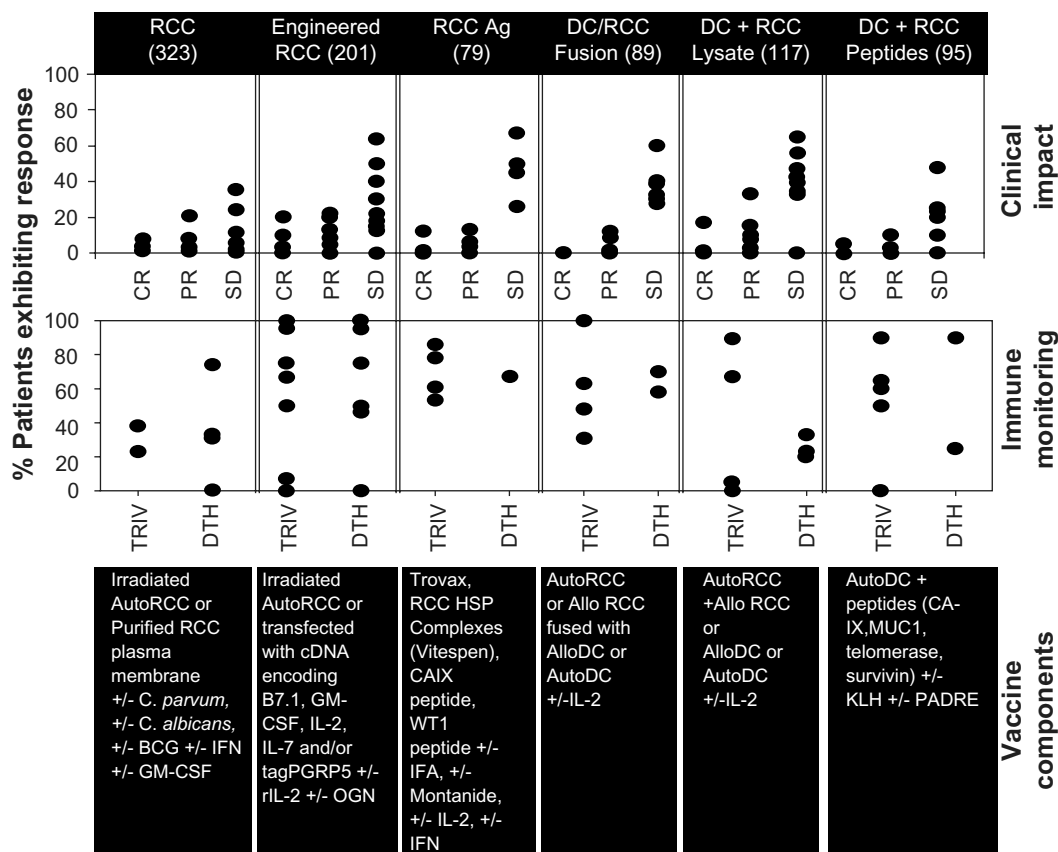
Figure 2 Renal cell carcinoma (RCC) vaccine trials: summary of clinical and immunologic monitoring results. Phase I/II clinical trials were performed in patients with RCC using one of the six indicated types of RCC-associated antigens (RCCAAs), with the cumulative number of patients treated with a given modality indicated in parentheses. RECIST criteria were applied to define patient response to therapy: CR, complete response; PR, partial response; SD, stable disease. The bottom panels show the components used in individual clinical trials. Data are only summarized from published trials in which coordinate immune monitoring for specific T-cell reactivity post- vs prevaccination was performed. Immune monitoring was performed by assessing T-cell responses *in vitro* (TRIV) using proliferation or cytotoxicity assays, or via the analysis of IFN-γ production by T cells using intracellular staining (in concert with flow cytometry), ELISA or ELISPOT assays. Alternatively, type-1 T-cell responses were deduced based on cutaneous delayed type hypersensitivity (DTH) responses to vaccines *in vivo*. We report the percentage of treated patients exhibiting specific clinical impact outcomes and increases in specific T-cell responses based on the immune monitoring criteria established for a given protocol. Each filled circle represents the data reported for an individual clinical trial.

clonal T-cell responses (ie, cytokine [IFN-γ] enzyme-linked immunosorbent spot [ELISPOT] assays, intracellular staining of T cells for cytokine [predominantly IFN-γ] production and reactivity of T cells with fluorescently-labeled, recombinant MHC-tumor peptide multimers). The merits and perceived weaknesses of these various methods have been well discussed in the past.[73–75]

Here, we provide a sample of the reported clinical trial data, which strongly support the capacity of RCC vaccines (implementing each of the 6 major formulations shown in Figure 2) to promote an increase in RCC-specific T-cell responsiveness. Although the frequency of immunologically responsive patients was highly variable within a given treatment type, the majority of treated patients in many cases exhibited detectable increases in TRIV at some point after vaccination. Similarly, DTH analyses suggest that RCC vaccines have been generally competent to promote tissue inflammation at sites of vaccination (mediated by

type-1 T cells, as shown in Figure 2). However, detectable TRIV and DTH as determined by current methods, even at high percentages, do not seem to directly correlate with the clinical outcome.

The general consensus regarding the immunologic monitoring of cancer patients receiving immunotherapy is that more than one assay system should be applied to monitor the changes in a patient's T-cell immune response to specific antigens. However, there are currently no acknowledged immunologic surrogate markers associated with OCR in treated cancer patients. Nevertheless, a number of vaccine trials have reported that 1) patients exhibiting OCR do typically fall within the cohort of patients exhibiting increased specific T-cell responses after vaccination;[41,76] 2) clinical benefit may be associated with an increased ratio of CD8+ T effector cells vs Treg cells;[56,57] 3) patients exhibiting OCR may have an expansion in their functional – cell repertoire against RCCAA specificities that were not included in the

vaccine formulation (ie, "epitope spreading"); and (4) patient pretreatment levels of type-1 chemokines, such as IP-10, in serum may portend to better clinical outcome.[72] Data collected from animal tumor models have shown that the effectiveness of a given vaccine or immunotherapy was linked to the recruitment of RCCAA-specific T effector cells into the TME via CXCR3 ligands, such as IP-10/CXCL10. Interestingly, *in vitro* studies have shown that IP-10 may promote apoptosis of CXCR3B^{+} vascular endothelial cells (VECs) in the TME, implicating the role of at least this particular type-1 chemokine in regulating angiogenesis.[77]

Combinational RCC vaccines: rationale and past efforts

Thus, existing RCC vaccine platforms increase the frequency of circulating anti-RCC T cells (and antibodies) based on immunological readouts established as secondary end points in clinical trials performed to date. However, few things remain completely unclear: (1) what threshold of such antitumor T cells must be reached to be biologically active against tumor? (2) what (poly)functionality and operational avidity should these T effectors exhibit in the TME (or elsewhere) to promote optimal antitumor impact? (3) do optimized vaccines include a risk of autoimmune pathology by inducing high-avidity T effector cells capable of recognizing normal tissues expressing low levels of "self" RCCAA? or (4) would even the most immunostimulatory approaches be dampened via normal compensatory mechanisms as bursts in Treg numbers have been reported in certain RCC vaccine trials?[72,78] If "epitope spreading" in the T-cell repertoire is indeed a requisite to achieve and maintain objective clinical benefits, it will also be critical to normalize DC function within the TME. Specifically, it will be important to foster the ability of these APCs to effectively and reiteratively cross-prime antitumor T cells, some of which have to be retained for extended periods as memory cells.

Table 2 provides a partial list of additional immunomodulatory agents that could reinforce existing RCC vaccines by impacting the adaptive response at various levels suggested in Figure 1, namely, by (1) facilitating the process of cross-priming by normalizing APC function in the TME; (2) expanding the pool of treatment-induced antitumor T cells and improving their survival; (3) enhancing the function of these cells, including conversion into memory cells; (4) enhancing the recruitment of such T cells into the TME; and (5) reducing the suppression of T effector cells mediated by myeloid-derived suppressor cells (MDSCs) and Treg cells. Although we have indicated in a qualitative manner whether

a given agent is likely to benefit or detract from a given biological parameter based on findings in the literature, it is important to acknowledge that no agent is perfectly suited to address all aspects in optimizing the therapeutic paradigm. Combinations of multiple agents may be necessary to yield complementary benefits. Realistically, such multicomponent vaccines may also have significant off-target toxicities, including the aforementioned autoimmune sequela that will require careful clinical monitoring. For instance, two of three RCC patients treated with autologous T cells engineered to express a high-avidity T cell receptor (TCR) reactive against the CA-IX (G250) RCCAA developed severe liver toxicity due to T-cell targeting of CA-IX expressed by bile duct epithelial cells.[79]

To a certain degree, the process of developing combinational RCC vaccines has been initiated in phase I/II trials, based on the use of long-standing cytokines, such as IL-2, IFN-α, IFN-γ, and GM-CSF, as "adjuvants" to support the function of T cells and DCs. Other cotherapeutics such as ONTAK® (Ligand Pharmaceuticals, LaJolla, CA, USA) (anti-CD25), CTLA-4Ig, or all-trans retinoic acid (ATRA) have been used to alleviate the inhibitory action of Treg cells and/or MDSCs. However, only a limited number of prospective randomized trials have been conducted till date.

Fenton et al[80] reported minimal impact of treating RCC patients with an irradiated, autologous tumor cell vaccine in concert with low- or high-dose of recombinant human interleukin (rhIL-2) vs the tumor vaccine alone in a phase I trial. This lack of benefit may relate, at least in part, to the well-known ability of rhIL-2 to support Treg expansion in treated patients.[78,81] In a phase II study evaluating the efficacy of a MVA vaccine encoding the RCCAA 5T4 (TroVax) plus recombinant interferon α (rIFN-α), no benefit was observed in the cohort of patients receiving IFN-α cotherapy.[59] Indeed, patients treated with vaccine + IFN-α tended to be less likely to exhibit antigen-specific TRIV and displayed shorter median progression-free survival and median overall survival when compared with patients treated with the vaccine alone. No rationale for this regulatory effect was suggested, but it is conceivable that the potent antiviral activity of IFN-α promotes more rapid viral clearance by activating neutralizing antibodies or antiviral T cells and actually limits the efficacy of booster vaccinations in patients receiving the TroVax vaccine.

In contrast to the aforementioned studies, patients with RCC receiving vaccines containing Newcastle virus-infected, irradiated, autologous RCC cells in combination with low dose of rhIL-2 and recombinant human interferon α2a

Table 2 Potential vaccine coimmunotherapeutics.

Cotherapeutic agent	Expected impact on Teff vs suppressor cells					
	Teff priming	**Teff function**	**Teff survival**	**Teff (TME)**	**Treg/MDSC**	**References**
Cytokines						
IL-2	↑	↑	+/−	↑	↑ (Treg)	173–175
IL-7	↑	↑	↑	↑	↑ (Treg)	176–178
IL-12	↑	↑	↑	↑	− (Treg), ↓ (MDSC)	179–181
IL-15	↑	↑	↑	↑	↑ (Treg)*	182, 183
IL-18	↑	↑	↑	?	↓ (Treg)	184–186
IL-21	↑	↑	↑	↑	+/− (Treg)	187–190
IFN-α	↑	↑	↑	↑	+/− (Treg)	175, 191–194
IFN-γ	↑	↑	−?	↑	↓ (Treg)	195–197
GM-CSF	↑	↑	↑	↑	↓ (Treg)	198–202
Coinhibitory antagonist						
CTLA-4	↑	↑	?	↑	↓ (Treg)	203, 204
PD1/PD1L	↑	↑	↑	↑	↓ (Treg)	205–207
Costimulatory agonist						
CD40/CD40L	↑	↑	↑	↑	↑ (Treg); ↑ (MDSC)	208–211
GITR/GITRL	↑	↑	↑	↑	↓ (Treg); ↓ (MDSC)	212, 213
OX40/OX86	↑	↑	↑	↑	↑↓ (Treg); ↓ (MDSC)	214–219
4-1BB/4-1BBL	↑	↑	↑	↑	↑ (Treg)	220–224
TLR agonists						
Imiquimod (TLR7)	↑	↑	↑	↑	?	225–227
Resiquimod (TLR8)	↑	↑	↑	?	?	228, 229
CpG (TLR9)	↑	↑	↑	↑	↓ (Treg)	230–232
Antiangiogenic						
VEGF-trap	−	−	?	?	−	233
Sunitinib	↑	↑	?	↑	↓ (Treg/MDSC)	98, 100, 234
Sorafenib	↓	↓	↓	?	↓ (MDSC)	235
Bevacizumab	↑	↑	?	?	↓ (MDSC)	236, 237
Gefitinib (IRESSA)	?	?	?	?	?	238, 239
Cetuximab	?	↑	?	?	?	240
mTOR inhibitors						
Temsirolimus/everolimus	↓	↓	↓	?	↓ (Treg)	241
Treg/MDSC inhibitors						
Iplimumab (CTLA-4)	↑	↑	?	↑	↓ (Treg)	242, 243
ONTAK (CD25)	+/−	+/−	?	?	↓ (Treg)	244
Anti-TGFβ/TGFβR	↑	↑	↑	↑	↓ (Treg)	245–247
Anti-IL10/IL10R	↑	↑	↑	+/−	↓ (Treg)	248, 249
Anti-IL35/IL35R	↑?	↑?	↑?	↑?	↓ (Treg)	250
1-Methyl trytophan	↑	↑	?	?	↓ (MDSC)	251
ATRA	↑	↑	?	?	↑ (Treg), ↓ (MDSC)	90–93

Note: Agents that are currently or soon-to-be used in clinical trials are summarized with regard to their anticipated impact(s) on type-1 antitumor T cell (T_e) activation, function, survival, and recruitment into the TME. Additional anticipated effects of drugs on suppressor cells (Treg and MDSCs) are also summarized.
Key: ↑, agent is expected to increase parameter; ↓, agent is expected to inhibit parameter; +/−, minimal increase or decrease is expected in parameter as a consequence of treatment with agent; ?, unknown effect of agent on parameter.
Abbreviations: ATRA, all-trans retinoic acid; CTLA-4, cytotoxic T lymphocyte antigen 4; GITR(L), glucocorticoid-induced TNF receptor (ligand); GM-CSF, granulocyte macrophage-colony-stimulating factor; IFN, interferon; IL, interleukin; MDSC, myeloid-derived suppressor cell; PD1/PD1L, programmed cell death 1 (ligand); TGF-β(R), tumor necrosis factor-β (receptor); TLR, Toll-like receptor; TME, tumor microenvironment; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

(rhIFN-α2a) appeared to display improved relapse-free and overall survival when compared with the cohort of patients receiving only the vaccine.[82] Furthermore, combinational therapy benefit was also suggested in a study by Simons et al[83] who performed a randomized, double-blinded phase II

study comparing the efficacy of vaccines composed of control or GM-CSF cDNA-engineered autologous, irradiated RCC cells. In this trial, the GM-CSF-engineered vaccine was well tolerated with no dose-limiting toxicities or autoimmunity reported. It appeared to be capable of promoting superior

type-1 T-cell response to RCCAA (based on patient DTH readouts) when compared with vaccines composed of control tumor cells alone. Dannull et al have shown that partial depletion of Treg cells using ONTAK, an anti-CD25 antibody coupled to diphtheria toxin, conditions the patients with RCC for improved type-1 T-cell responses against autologous DCs transfected with mRNA isolated from autologous tumor cells.[84] However, its impact on the clinical course was not reported. A recent phase I/II study by Holtl et al used allogenic DCs pulsed with autologous tumor lysates in conjunction with cyclophosphamide (CY) pretreatment to deplete Treg cells and provide "space" for homeostatic T-cell expansion. Results showed only a slight improvement in patients treated with CY, with a median overall survival of 23.2 months vs 20.3 months without CY.[85]

Combinational RCC vaccines: moving forward

A plethora of agents exist for consideration in the design of combinational vaccines (Table 2). Going forward, it will be crucial that all vaccine trials develop as randomized, prospective protocols that integrate secondary immunologic end points. These immunological end points must determine the actual impact of the vaccine on RCC-specific T-cell responses over the duration of the study. This will be important not only to validate whether the perceived immunologic benefits of the combined approach are indeed met, but also to see how well they are met, so that future trials may be designed using agents that further augment or complement mechanisms currently believed to underlie optimal immunotherapeutic benefit.

In 2007, the NCI Immunotherapy Agent Workshop established a list of the top 20 (from among 124) agents that were considered by participants to have a high likelihood for efficacy in cancer therapy.[86] Thirteen of these top 20 are cited in Table 2, including IL-15; anti-PD-1 (or anti-B7-H1); IL-12; anti-CD40 and/or anti-CD40L; IL-7, CpG, 1-MT, anti-4-1BB; anti-TGF-β; anti-IL10 or anti-IL10R; anti-GITR; anti-OX40; and resiquimod. As the merits of these agents are well delineated with regard to shaping, sustaining, and directing antitumor T-cell responses in the NCI Workshop report, we will focus our attention on a small cohort of these agents in the following paragraphs.

A major barrier to effective vaccine therapy in RCC is the immune regulatory component in cancer patients. As such, eradication or inhibition of Treg cells and MDSCs would promote vaccine efficacy. One mechanism that some groups have been targeting is CTLA-4 signaling of Treg cells.[84] CTLA-4 blockade has been applied as a monotherapy for RCC, where it has been reported to yield partial clinical responses (PRs) in approximately 25% of treated patients with RCC for up to 18 months.[87] However, autoimmune hypophysitis and colitis have been reported in patients with RCC receiving CTLA-4-blocking agents.[88,89] Such toxicities might be exacerbated by vaccine combinations and will require careful monitoring in prospective clinical trials.

It has recently been reported by multiple groups that ATRA can ablate the number and/or suppressive function of MDSCs, while augmenting the function of DCs isolated from patients with RCC resulting in improved type-1 TRIV.[90,91] Furugaki et al[92] have also shown that ATRA serves as an effective adjuvant when combined with genetic immunization, promoting long-term survival in a murine promyelocytic leukemia model. However, ATRA has also been reported to regulate the balance between developing Treg vs Th17 cell responses in the CD4+ T-cell compartment, favoring the Treg outcome.[93] This seems to be based on signals mediated via nuclear retinoic acid receptor-α (RARα), making ATRA (or other RARα antagonists) a potential double-edged sword when considering the design of combinational RCC vaccines.

Intriguing candidates for integration into combination RCC vaccine design include small molecules, such as tyrosine kinase inhibitors (TKIs). Several clinical trials have utilized TKIs to inhibit various RTKs, such as vascular endothelial growth factor receptor (VEGFR), as an antiangiogenic agent to treat RCC. One such TKI is sunitinib, which has exhibited pronounced (albeit transient) efficacy in phase I/II clinical trials as a first-line treatment for patients with RCC.[94–96] Even though this drug was initially developed as an antiangiogenic agent, a series of very recent reports suggest that patients treated with sunitinib exhibit reductions in peripheral blood levels of MDSCs and Treg cell populations and normalized type-1 TRIV after mitogenic stimulation.[97–100] Murine tumor modeling suggests that sunitinib suppresses STAT3 activation[101] and bolsters the efficacy of immunogenetherapy by promoting RCCAA-specific T effector cells and concomitantly suppressing MDSCs and Treg cells in vivo.[98,99,102] Alternative TKIs, such as sorafenib (which currently serves as a second-line therapy for RCC), may not be as preferred as a vaccine or cotherapeutic agent, given its reported inhibitory effects on DCs and T cells.[103] An additional immunotherapeutic benefit of sunitinib and other antiangiogenic drugs, is predicated on their ability to "normalize" the tumor vasculature, leading to a decrease in interstitial fluid pressure and improved delivery of

chemotherapeutic drugs and effector T cells into the TME.[104] However, enhanced T-cell infiltration into the TME is not a passive event. Our group has recently shown that sunitinib activates tumor VEC expression of VCAM-1 and Mig (CXCL9), which serve to recruit (CXCR3+) type-1 tumor infiltrating lymphocyte (TIL) (unpublished data). In theory, sunitinib cotherapy may condition the TME for improved recruitment and sustained function of RCC vaccine-induced T effector cells,[105] thereby prolonging the dramatic early OCR characteristic of sunitinib.

Summary and additional considerations

Early clinical results published over a decade ago showed that IFN-α_{2a}– and IL-2-based immunotherapy provided survival advantage to patients compared with control groups.[106,107] However, a recent study has called these earlier findings into question by demonstrating that there may be little survival benefit from either of these cytokines when applied alone or in combination, and that they may induce a significant risk of toxicity.[108] IL-2 is known to promote Treg cell responses *in vitro* and *in vivo*.[109] As such, its less-than-ideal activity might have been predicted, given its ability to potentiate aspects of the dysfunctional or inappropriate polarization by the adaptive immune system in the majority of patients with tumors.[78,110] Given the information, we have collated and proposed that a successful combinational therapeutic platform must include the integration of appropriate immunogeneic tumor antigens in order to focus immunity toward carcinoma cells with adjuvants that activate and "license" APCs to preferentially prime or activate type-1 antitumor T cells plus additional stimuli that may 1) support vaccine-activated T-cell survival, resistance to tumor-induced immune deviation and conversion to memory status; 2) prevent or remove the opposing influence of existing immune suppression (tumor, MDSC, Treg); 3) normalize the TME, thereby allowing for improved delivery of vaccine-induced T effector cells (and type-1 APCs) into the TME; and 4) promote reiterative rounds of T-cell cross-priming in the tumor draining lymph nodes leading to an expansion in the functional antitumor T-cell repertoire via a process akin to "epitope spreading" (a classical autoimmune paradigm). In such optimized protocols, one would expect a minor cohort of patients to exhibit a complete response (based on RECIST criteria), but perhaps of equal importance, a sizeable cohort of patients develop stabilization of their disease or tumor dormancy. If vaccines are successful in attaining even a transient state of dominance for type-1 antitumor immunity, one would predict

the consequential development of compensatory regulatory immunity to limit this "autoimmunity", allowing for the resumed growth of micro- or macroscopic metastases. Given such concerns, and despite their potential to unveil untoward autoimmune pathology, maintenance booster vaccines will likely be mandated to significantly prolong median time to progression and overall survival.

Notably, it has recently also become possible to consider the immune targeting of the tumor vasculature based on vaccines formulated using antigens that are differentially expressed by either VECs (ie, EphA2)[111] or pericytes (ie, PDGFRβ[112] or NG2[113]). In such cases, specific T cells may destroy or dysregulate tumor angiogenesis in a prolonged fashion (based on T-cell memory) and in a manner that is independent of other therapeutics, such as TKIs, which may put selective pressure on tumor cells. Furthermore, focusing type-1 T-cell responses on tumor-associated vascular cells may foster corollary recruitment (based on CXCR3 ligand chemokine production and endothelial cell expression of VCAM-1) of type-1 T cells that have been cross-primed against additional RCCAAs.

We have come far in the past decade in developing a better understanding of how the immune system recognizes RCC and how the optimal function of protective anti-RCC T-cell-mediated immunity may be altered in chronic disease states. Only now, we are becoming skilled at applying agents in the appropriate combinations, quantities, and schedules to allow for the normalization and maintenance of protective immunity. By adopting a stepwise progression through randomized prospective trials integrating sensitive and appropriate immunomonitoring methodologies, we will soon develop a consensus regarding optimal (combinational) vaccine-based immunotherapy approaches for the treatment of RCC. Upon consideration of the frustration in treating metastatic RCC and the distress of such patients, combinational protocols that integrate anti-RCCAA vaccines and angiogenic inhibitors promoting tumor apoptosis should be prioritized in the immediate future, as such strategies may greatly improve the outcome of patients with advanced-stage disease.

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Disclosure

The authors report no conflicts of interest in this work.

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