Immunotherapy of Kidney Cancer

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Abstract: Renal cell carcinoma (RCC) accounts for 4% of all new cancer cases in males and 3% in females in the US. Compared to other solid tumors, it does not respond to traditional management modalities, such as chemotherapy and radiation therapy. However, it appears to be an immune-responsive tumor and several immunotherapeutic strategies have been investigated in the management of RCC with variable degrees of success. Active immunotherapy refers mainly to the use of vaccines, while adoptive (passive) immunotherapy includes the use of autologous immune cells, allogeneic immune cells (stem cell transplantation, donor lymphocyte infusion), as well as antibody delivery. Cytokine delivery with IL-2 has resulted in long-term disease-free survival in a small proportion of patients with metastatic disease. The continuous understanding of the mechanisms that underlie the immune complex networks has led to the identification of key molecules that play a major role in the immune response process. A panel of immuno-modulatory compounds that target such molecules has been tested in the preclinical and clinical setting. At the post-genomic era, the development of novel biomarkers can contribute to more accurate patient selection, resulting in higher responses and less toxicity of immunotherapeutic approaches.

Keywords: Immunotherapy, renal cell carcinoma, kidney cancer, interleukin-2, cytokine, stem cell transplantation, vaccines, immune system.

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 4% of all new cancer cases in males and 3% in females in the US [1]. It is estimated to account for 58,240 new cases and 13,040 deaths in the US in 2010. Approximately one third of patients initially present with metastatic disease. Another third develops recurrence after initial primary surgery with curative intent [2]. RCC encompasses a group of distinct histologic subtypes, with clear cell being the most common. It has been traditionally resistant to cytotoxic and hormonal therapy, as well as irradiation [3, 4]. However, immunotherapy has been used in the management of RCC with variable degrees of success. Cytokine immunotherapy has resulted in long-term disease-free survival in a small proportion of patients with metastatic disease [5]. This comprehensive review aims at describing the different immunotherapy modalities in RCC, focusing on their significance and current role. It presents important previous preclinical and clinical data, salient developments and landmark studies, discussing at the same time present and future perspectives in the field.

IMMUNOLOGY PRINCIPLES IN CANCER

The immune system is a complex network, comprising of cellular components, antibodies and soluble biological factors, such as cytokines, that are responsible for the intercellular communication of the immune system. The cellular component comprises of a variety of different elements. Antigen-presenting cells (APCs), such as monocytes, macrophages, and dendritic cells, "present" antigenic epitopes to immune effector T cells, resulting in the activation of the latter and initiation of an immune response. Tumor-associated antigens can be presented *via* MHC class I or II molecules, which are expressed in the membrane of APCs. Recognition of this membrane complex *via* T-cell receptor by CD8+ MHC I restricted T cells can activate these cells that have the potential to attack and lyse target (tumor) cells expressing the antigenic epitope [6]. These cells are called cytotoxic T lymphocytes (CTLs) and their activation requires the expression of specific co-stimulatory molecules in the membrane of APC and the interaction of those molecules with specific receptors on the T cells [6]. Dendritic cells are considered the most potent APCs, and they can stimulate naïve T cells [7]. They highly express co-stimulatory molecules, such as CD80 and CD86, which are required for T cell activation. [7]. CTLs receive cytokine signals from helper T cells (CD4+ MHC class II restricted), such as interleukin 2 (IL-2) and interferon γ (IFN-γ) in response to antigen binding. Interleukin-2 is a potent growth factor for T cells, and it can also stimulate and activate another subpopulation of immune cells, natural killers (NK) cells [8]. NK cells differ from the classic T lymphocytes, as they recognize target cells *via* a non-MHC-restricted mechanism, and their ontogenesis is not thymic-dependent. They express CD56 and are considered to be involved in immune surveillance, targeting cells that have lost MHC expression [9]. Another cellular component of the immune system is the B lymphocyte. These cells mediate the production of antibodies against antigens expressed by target cells. B cells are derived from the bone marrow, where they acquire the ability to proliferate clonally and produce antibodies which can bind and neutralize target antigens. The components of the immune system are continuously in a

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dynamic "cross-talk", *via* the presence of multiple communication signals, which co-ordinate their complex interaction, resulting in an integrated immune response against a specific target.

In addition to the cells and signals involved in immune activation there is another aspect of the immune response that is present to offset or suppress immune activation. This immune suppressive or regulatory system is also composed of cellular components and immunologically active molecules. Specific immune regulatory cells identified include immune-regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC). Immunosuppressive molecules include cytokines, such as IL-10 and TGF-beta, as well as specific receptors, such as CTLA-4 and PD-1 [10, 11]. A representative depiction of the immune system components is illustrated in Fig. (**1**).

IMMUNOTHERAPY STRATEGIES

Selective immunotherapy strategies are listed in Table **1**. Active immunotherapy is characterized by immunization of patients with agents that increase the immune response towards a tumor antigen. The development of tumor vaccines has been the best example of such an approach. One form of tumor vaccines encompasses the use of tumor antigenic molecules that can be isolated, purified and administered to the host, in order to elicit immune response against the tumor itself. Another approach can incorporate *in vitro* transfection of cytokine-producing genes into a vector, which is subsequently administered to the host to "boost" the immune response against a tumor. Moreover, intra-lesional injections of genetic elements, which can enhance MHC expression or trigger cytokine secretion, can also be implemented within the "concept" of tumor vaccine development.

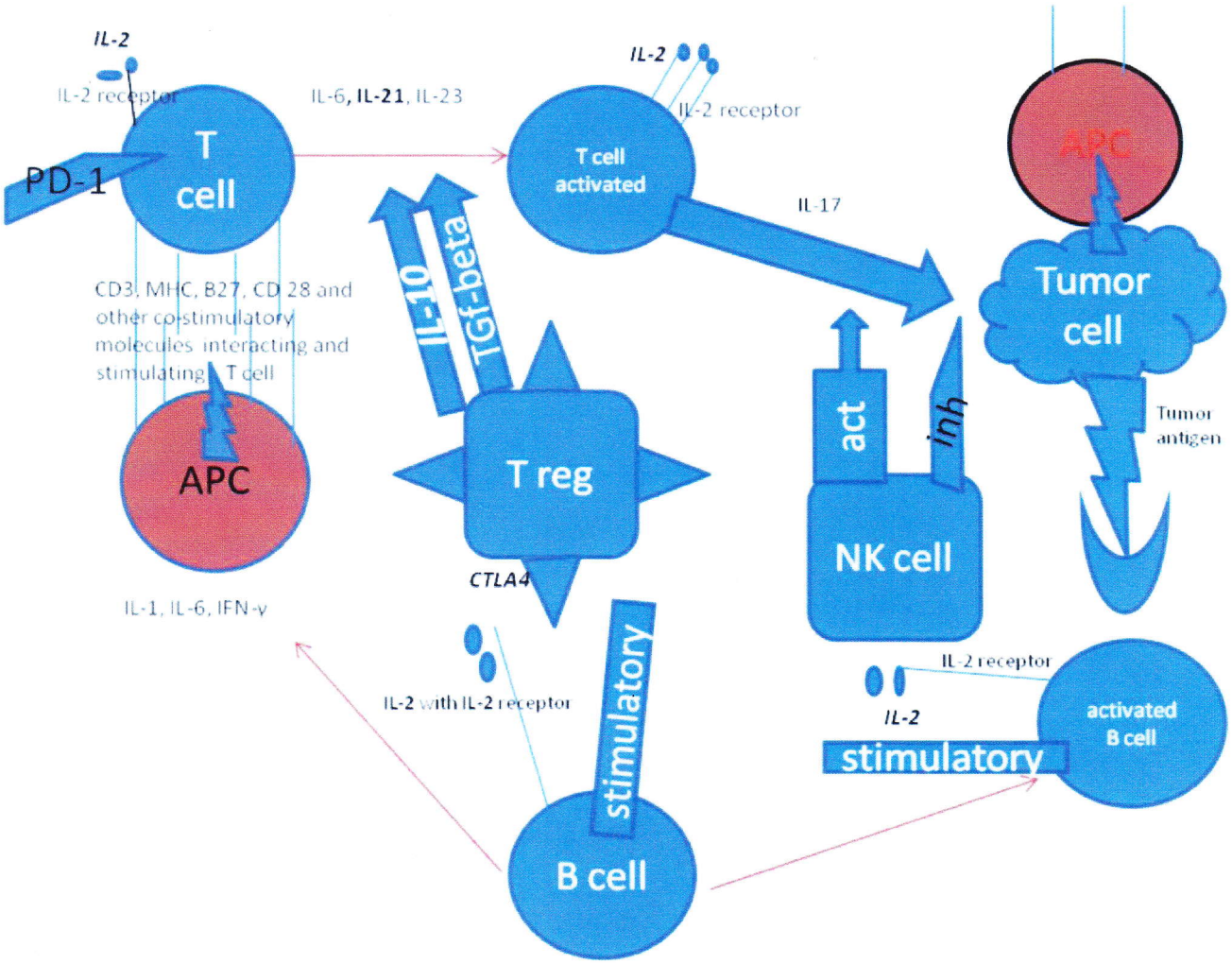

Fig. (1). Tumor-associated antigens can be presented to T cells by APCs. Recognition of such an antigen *via* T-cell receptor by CD8+ MHC I restricted T cells can trigger cell lysis. Naïve T cells can be stimulated *via* highly expressed co-stimulatory molecules expressed by APCs. Cytotoxic T and NK cells receive cytokine signals from CD4+ helper T cells in response to antigen binding. NK cells can recognize target cells *via* a non-MHC-restricted mechanism and express both activating (act) and inhibitory (inh) receptors; therefore their activity depends on the presence of specific signals and activation of corresponding pathways. B lymphocytes produce antibodies against target cell antigens. Immune system components are in a dynamic interplay *via* secreted communication signals and ligand-receptor interactions, resulting in an integrated immune response. The immune suppressive (regulatory) system is composed of cellular components and immune-modulatory molecules. Immune regulatory cells include T cells (Treg) and myeloid derived suppressor cells (MDSC). Immunosuppressive molecules include cytokines (IL-10, TGF-beta), and receptors (CTLA-4, PD-1).

Table 1. Immunotherapy Strategies in RCC

a. Active immunotherapy
- Tumor vaccines
b. Adoptive (passive) immunotherapy
- autologous immune cells
- allogeneic immune cells (stem cell transplantation, donor lymphocyte infusion)
- antibody delivery
c. Cytokine delivery
d. Immuno-modulatory molecules

Adoptive (passive immunotherapy) entails the transfer of immune cells or antibodies that can exert direct anti-tumor activity. Immune cells can be derived from self (autologous), and can often be "trained" *in vitro* to attack specific tumor cells, and therefore mediate tumor cell lysis *in vivo*. Immune effector cells can also be derived from non-self (allogeneic), and be "transplanted" into the host, to recognize target tumor cells as foreign. A characteristic paradigm of the latter approach is the allogeneic stem cell transplantation (ASCT), which has been successfully implemented in the management of hematologic malignancies.

Cytokine delivery has been evaluated as monotherapy and as "adjunctive" therapy to the administration of tumor vaccines and adoptive cell infusions. Parenteral administration of IL-2 has been used in the management of metastatic RCC with modest response rates and is the only FDA-approved immunotherapy for advanced RCC.

Novel immune-modulatory molecules have been tested for the treatment of RCC and have shown different results in both the preclinical and clinical setting. The continuous understanding of the underlying mechanisms that describe the immune complex network has shed light on the identification of key molecules that play a major role in the immune response process. Targeting these molecules *via* the use of specific compounds can affect the activity of immune mediators and improve tumor immune response. A number of these compounds are currently under clinical investigation, and may contribute to the anti-tumor armamentarium.

ADOPTIVE IMMUNOTHERAPY

Autologous T and NK Cell-Based Immunotherapy

The first attempt to apply an adoptive strategy in RCC implemented non specific lymphokine activated killer (LAK) cells, isolated from patient's peripheral blood, incubated *in vitro* with IL-2 and subsequently re-administered to the patient. An early phase II trial with IL-2 and LAK cells in metastatic RCC revealed an overall objective response rate of 16%; 2 patients had complete and 3 patients had partial responses [12]. However, three separate phase III trials failed to show improved outcome when this approach was compared to standard high dose IL-2 in metastatic RCC [13-15].

Pilot trials, employing autologous cytokine-induced NK (CIK) cells in the management of RCC, have been launched [16, 17]. Preliminary data shows this approach is safe and potentially efficacious, resulting in increased numbers of "effector" cells, with significant anti-tumor immune function. In a trial of 16 patients with metastatic RCC, CIK immunotherapy produced 3 complete responses, 1 partial response, while 6 patients had stable disease [17]. The paradigm is now shifting to the implementation of allogeneic NK cells, with a recent phase I clinical trial of 11 RCC patients reporting encouraging results [18]. At the same time, autologous and/or allogeneic dendritic cells can be co-cultured with NK cells, producing potent anti-tumor "mediating" cells. A recent study reported that such techniques can result in high numbers of cytotoxic cells with enhanced anti-tumor activity [19]. Larger scale trials need to verify these promising results.

Encouraging data on the *in vitro* "large scale" expansion of NK cells have been published [20]. NK cells expanded with irradiated with Epstein-Barr virus-transformed lymphoblastoid cells *in vitro* were found to exhibit up-regulated expression of activating and death receptor cytotoxic ligands, as well as enhanced cytokine secretion. Expanded NK cells can be used in conjunction with the proteasome inhibitor bortezomib, which is shown to sensitize human renal cell carcinomas to Tumor necrosis factor–Related Apoptosis-Inducing Ligand (TRAIL) - mediated apoptosis [21]. At the same time, bortezomib was found to result in T regulatory cell depletion, thus augmenting NK-mediated immune response [22]. Moreover, a recent study reported the characterization and *in vitro* expansion of cytotoxic NK cells from cord blood mononuclear cells, which may provide an additional option of adoptive immunotherapy in patients treated with cord blood ASCT, as shown below [23].

Another strategy incorporates the combination of IL-2 with tumor infiltrating lymphocytes (TIL). These cells infiltrate the tumor, can be isolated after biopsy, cultured and expanded *in vitro* with various immune regulatory molecules, resulting in cell growth, cytotoxicity and multi-cytokine synthesis [24]. Reactive cells can be selected by their IFN-γ secretion when cultured with autologous or allogeneic MHC-matched cancer cell lines. These cells exhibit immunologic memory to tumor cells, and mediate anti-tumor cytotoxicity. They constitute a heterogeneous cell population that can exert cell toxicity *via* MHC-restricted and MHC-non restricted mechanisms, and seem to possess 50-100 times more potent anti-tumor activity compared to LAK cells [25]. A large national trial investigating the benefit of cellular adoptive therapy in combination with IL-2 in metastatic RCC has been completed and results are pending (identifier NCT00093574). The main drawback of adoptive TIL immunotherapy is the labor intensive and time consuming process of reactive T cell isolation and expansion *in vitro*. Moreover, poor definition of tumor antigens, poor localization of T cells in the tumor microenvironment, loss of T cell cytotoxic function upon adoptive transfer, and tumor resistance to TIL-mediated apoptosis can be additional explanations of the suboptimal benefit from this strategy [26-28].

"Programming" of the genetic material of T lymphocytes has been pursed as a mechanism to increase their selective

reactivity against tumor cells [29]. This process attempts to produce increased numbers of tumor specific T cells in a short time by the introduction of tumor antigen-specific T cell receptor (TCR) or a chimeric immune receptor (CIR) [30]. Another trial demonstrated that T cell receptor gene transfer appeared to confer high tumor antigen-specific cellular avidity and reactivity in nonreactive peripheral blood mononuclear cells (PBMC) or TIL [31]. The successful *in vitro* introduction of cloned RCC-antigen-reactive TCR into human T cells corresponded with the recent initiation of a phase I/II trial using T cells transduced with a receptor that recognizes TRAIL bound to DR4 receptor, as a therapeutic maneuver in patients with metastatic RCC (identifier NCT 00923390) [29].

Alternatively to the use of full length T cell receptor, "shorter" receptor versions have also been examined. Chimeric immune receptors consist of single-chain antibody fragment recognizing tumor protein fused to a transmembrane region with intracellular signaling activity [32]. A clinical trial investigated the use of a single-chain antibody-type receptor based on the murine monoclonal antibody (mAb) G250 [33]. This trial reported clear *in vivo* reactivity of autologous T-cells, but was terminated early because of liver toxicity, which was probably an on-target effect. Strategies need to be developed to attenuate the activity of re-targeted T-cells against normal tissues, which express the target antigen. An *in vivo* limitation of CIR-using strategies is the inadequate activation of T cells, leading to suboptimal antigen-dependent IL-2 secretion, thus lower T cell proliferation and growth [34]. Second generation CIR T cells (e.g. incorporating CD28 domain in the CIR) have been developed to address this concern [35]. Such cells can proliferate after stimulation with tumor antigens, even without IL-2 administration, and exhibit increased cytokine secretion and resistance to apoptosis [36].

This novel technique of gene-modified T cells has limitations, such as lack of *in vivo* persistence of reactive T cells, possibly related to stimulation of immune reaction against *in vitro*-genetically engineered T cells. There is theoretical concern of inducing tumorigenesis in the host cells, in case viral vectors with potential random insertion of genetic material in the genome are used [37]. Despite the sophisticated development of next-generation modified T cells with more promising activity, caution should be exerted considering the recently reported death temporarily associated with such an approach [38].

A recent study attempted to identify T-cells with shared tumor-specific recognition specificities to develop TCR-engineered T cells for adoptive immunotherapy in RCC [39]. The recent description of HLA I and II epitopes identified for RCC-associated antigens can potentially improve the *in vitro* yield of identification and selection of potent and specific immune-mediating cells [40, 41]. Moreover, "young" and non-selective TIL can shorten the required *in vitro* cell expansion time [42]. Immune cell selection and expansion protocols need to be improved. Expression analysis of specific biomarkers, such as CD137, the use of genetically modified APCs and cytokines, such as INF-γ, can all contribute to the development of high-quality protocols [43-46]. Recent data suggest that IL-12 administration fully expanded short-term cultured CIK cells *in vivo*, resulting in significant anti-tumor efficacy in a mouse breast cancer model [47]. IL-15 has also been shown to play a major role in the development, function and growth of immune cells, and specifically of RCC-reactive T cells [48, 49]. IL-7 is also essential for early T cell development, resulting in the expansion of naïve T cells subpopulations and relative decrease in the number of regulatory T cells [50, 51].

ALLOGENEIC STEM CELL TRANSPLANTATION

Graft *vs* tumor (GVT) effect against a solid tumor was initially described in a murine model of lymphosarcoma and was extensively studied in other animal models [52, 53]. Initial evidence of GVT against a solid tumor of epithelial origin in a human was suggested by the incidental regression of a metastatic breast adenocarcinoma, with ASCT, performed for the treatment of a patient with AML [54]. Because of the significant morbidity and mortality noted with fully myeloablative conditioning regimens, sub-myeloablative or reduced intensity conditioning (RIC) approaches were investigated in the management of RCC.

A pilot trial of RIC ASCT in 17 patients with refractory malignancies (7 patients with RCC) and HLA-matched siblings evaluated the feasibility, toxicity and engraftment of such an approach and attempted to document evidence of GVT effect [55]. Grade II/III acute GVHD occurred in 5 patients. Among patients with a follow-up more than 100 days, 2 complete and 3 transient partial responses were observed. The first clinical trial investigating the potential benefit of RIC ASCT in patients with cytokine-refractory RCC was conducted at the NIH [56]. Clear cell histologic type, development of GVHD, and metastatic deposits in the lung were associated with response. Transplant-related mortality was not existent in patients younger than 40 years, but it was 20% in patients 50-59 years. An update from the same group in 45 patients with solid tumors treated with RIC ASCT included 20 patients with significant tumor regression including 3 with long-term complete regressions [57].

The European ASCT experience with 124 patients from 21 centers reported 4 complete and 24 partial responses, achieved in a median of 150 days post-transplant [58]. These responses were associated with the time from diagnosis to ASCT, mismatched donor and acute GVHD II-IV. Patients with less than 3 metastatic sites and a Karnofsky performance status score >70% had better outcome. Post-transplant DLI and limited chronic GVHD improved survival. A phase II trial assessed the safety and efficacy of RIC ASCT in 10 related and 6 unrelated donor transplants [59]. All patients achieved donor chimerism; 7 patients developed acute grade II/III GVHD, 6 patients developed chronic GVHD, and the transplant-related mortality was 12%. In the related donor cohort, there was 1 complete response, 3 partial responses, while 3 patients had stable disease. In the unrelated donor cohort, there was 1 complete response and 1 patient had stable disease. The first experience from RIC ASCT derived from cord blood is derived from a 69 year-old male with cytokine-refractory metastatic RCC, who achieved durable donor engraftment with regression of metastatic disease, along with grade II skin and gut GVHD [60]. Disease progression was documented during treatment with tacrolimus

for GVHD. However, disease stabilization was achieved after discontinuation of the immunosuppresion. Cord blood contains a greater proportion of highly proliferative hematopoietic progenitor cells and provides immediately available cells without risks for the donor [61]. However, prolonged graft recovery, susceptibility of rejection and unavailability of DLI are considered drawbacks. Nevertheless, a recent study reported that characterization and *in vitro* expansion of cytotoxic NK cells from cord blood mononuclear cells is feasible and can be used post-transplant [62].

Long-term follow-up data of 25 patients with metastatic RCC who underwent RIC ASCT from an HLA-identical sibling donor were published [63]. At a median follow up period of 65 months, 5 patients were alive, 1 with complete response, 1 with partial response and 3 with stable disease. Using multivariate analysis, C-reactive protein level before transplant, the number of CD34 + infused cells and disease status at day +90 were associated with survival. Regression of metastatic RCC following RIC ASCT consistent with GVT effect was also reported [64]. RCC-reactive donor-derived CD8+ T cells were detected in the blood of ASCT recipients. Using c-DNA expression cloning, a 10-mer peptide (CT-RCC-1) was identified as a target antigen of these T cells. The genes encoding this antigen were derived from human endogenous retrovirus (HERV) type E, were expressed in RCC cell lines and fresh RCC tissue, but not in normal kidney or other tissues. This was the first solid tumor antigen identified using allogeneic T cells from a patient undergoing ASCT, providing evidence that HERV-E is activated in RCC and it encodes an over-expressed immunogenic antigen, providing a potential target for cellular immunotherapy.

ANTIBODY DELIVERY

Monoclonal antibodies that target selective tumor antigens and mediate humoral anti-tumor immune response have also been employed in the management of RCC. G250 is an IgG1 kappa light-chain chimeric monoclonal antibody that binds to carbonic anhydrase IX (G250/MN), which is present in more than 95% of RCC of the clear cell type. The suggested mechanism of action of G250 is antibody-dependent cell-mediated cytotoxicity (ADCC). A multicenter phase II trial evaluated the safety and efficacy of repeated doses of cG250 in 36 patients with metastatic RCC after nephrectomy [65]. In the follow-up, 1 patient demonstrated a complete response in week 38 and another patient with stable disease showed a significant tumor size reduction in week 44; 6 patients with progressive disease at study entry remained stable for more than 6 months. Another phase I trial evaluated the safety, pharmacokinetics and biodistribution of repeated doses of G250 in 13 patients with unresectable/metastatic clear cell RCC [66]. One patient achieved a complete response, 9 had stable and 3 had progressive disease, 1 patient received 11 six-week cycles of treatment with no toxicity or evidence of immunogenicity.

The number of activated immune "effector" cells could be increased by a cytokine pulsing schedule, so the antibody administration can be combined with cytokine delivery. A pilot study evaluated the addition of daily low-dose SC IL-2 to G250 for the treatment of 9 patients with unresectable/metastatic or locally advanced clear cell RCC [67]. A trend for higher percentage of circulating CD3-/CD16+CD56+ NK cells was observed. A few patients had enhanced ADCC or LAK activity; however no anti-tumor responses were reported. A multicenter study investigated the role of G250 combined with low dose-IL-2 in 35 patients with progressive RCC [68]. Partial response was noted in 3 patients with stable disease for 24 weeks in additional 5 patients. A phase I/II trial evaluated the efficacy and safety of G250 combined with low-dose IFN-α in 31 patients with progressive metastatic clear cell RCC, after nephretctomy [69]. The antibody was well tolerated. Two patients achieved partial remission and 14 had stable disease. While the above studies are of interest it is unfortunate that to date no long-term clinical benefit has been achieved with antibody-based therapy.

CYTOKINE DELIVERY

Cytokines are critical soluble factors in the generation and efficacy of an immune response. They can act as lymphocyte growth factors and communication signals integrating immune system functions. IL-2 and INF-α have been the two main cytokines that have traditionally been used in the management of RCC. A very detailed database literature review provided a thorough synopsis of the data with the use of IL-2 and INF-α, derived from 53 clinical trials involving a total of 6117 patients [70]. Combined data for a variety of cytokine based immunotherapy regimens revealed an overall remission rate of 12.9% in 99 study arms, compared to 2.5% in 10 non-immunotherapy control arms, and 4.3% in two placebo arms. IL-2, unlike INF- α, has no direct anti-tumor activity, but is a potent stimulator of T, and NK cells. There is evidence that local or generalized "effector" cell dysfunction, which is commonly observed in patients with advanced malignancies, can be reversed with IL-2 administration [71].

Recombinant IL-2 has well documented efficacy in metastatic RCC and has been FDA-approved for this indication since 1992. When administered as a high dose regimen in patients with intact organ function and good performance status treatment results in durable complete responses in about 5-7% of patients and partial responses in an additional 10%-15% [5]. In 7 phase II trials, recombinant IL-2 (600,000-720,000 IU/Kg) was administered as a 15-minute IV infusion every 8 hours over 5 consecutive days, up to 14 consecutive doses. A course of therapy consisted of such two 5-day treatment cycles, beginning on days 1 and 15. Patients who respond and those who had stable disease were retreated approximately every 3 months for a maximum of 3 courses [72]. The toxicities of IL-2 are mediated by cytokines and other small molecules secreted by IL-2 receptor-expressing cells. The common mechanism for IL-2-induced multiorgan dysfunction seems to be a capillary leak syndrome directly related to the production of pro-inflammatory molecules. A complete list of the most common toxicities, derived from high dose IL-2 is presented in Table **2**. The vast majority of these toxicities are reversible and manageable with appropriate supportive care and medications, and the treatment-related mortality is less than 1% [73].

In an early clinical trial, 283 patients with metastatic melanoma or RCC, who had failed standard treatment, received high-dose IL-2 IV every 8 hours for a maximum of

Table 2. High Dose IL-2 Toxicity

• Fevers, chills, rigors, fatigue, weakness, arthralgias, myalgias
• Nausea, vomiting, diarrhea, anorexia
• Hypotension (requiring IV pressors)
• Shortness of breath, tachypnea
• Peripheral edema, fluid retention
• Renal failure, oliguria
• Neurotoxicity (confusion, disorientation, seizures)
• Skin rash/pruritus
• Cardiac arrythmias (supraventricular tachycardia, atrial fibrillation, ventricular tachycardia), and ischemia
• Metabolic acidosis
• Hepatotoxicity (elevated transaminases)

15 doses per cycle [74]. Ten patients (7%) with metastatic RCC experienced complete and 20 (13%) partial regression. Of the total of 19 patients (melanoma and RCC) with complete regression, 15 maintained it from 7 to 91 months. Three treatment-related deaths (1.1%) occurred early, but as experience with high-dose IL-2 accumulated, no treatment-related deaths were observed during the last 5 years. The same group published data on a cohort of 409 patients with either metastatic melanoma or RCC treated with this IL-2 scheme [75]. Complete response was seen in 9.3% of patients with metastatic RCC. The absence of prior treatment with immunotherapy, total dose of IL-2, and maximal rebound lymphocytosis after cessation of IL-2 were associated with complete response. The same group evaluated the role of successive courses of high-dose IL-2 in 350 patients with metastatic melanoma or RCC [76]. Of the 201 patients with RCC, 18 achieved complete and 20 partial responses, with an overall response rate of 19%. Based on this retrospective analysis, patients who have objective response can receive additional courses until either complete response or intolerance. Patients who do not achieve objective response after 2 courses should not receive further treatment.

Because of the toxicity and cost associated with high-dose IL-2, it has been limited to specialized centers and a highly selected patient population. In an attempt to decrease the toxicity seen with the high dose regimen lower dose regimens have been evaluated. A phase II trial investigated a lower SC IL-2 dose in 27 unselected patients, with advanced RCC [77]. After 6 weeks, 26 patients were evaluable for response; 2 patients had a complete remission, 4 had a partial remission, and 13 had stable disease. The median survival for all patients was 13 months. One patient died from myocardial infarction and brain stem ischemia. Systemic toxicity in other patients was tolerated, and included transient inflammation and local induration at the injection sites, fever, chills, and nausea. Another trial evaluated the efficacy and safety of low-dose SC IL-2 in 41 patients with metastatic RCC and reported no complete response, a 17.1% partial response rate with 46.3% of patients achieving stable disease [78]. The median time to progression was 6 months. The 1-year survival rate was 71.2%, with a median overall survival of 22.5 months. A large NCI clinical trial randomized 125 patients with metastatic RCC to receive IV bolus IL-2 every 8 hours at either 720,000 IU/kg or 72,000 IU/kg to the maximum tolerated number of doses, with a maximum of 15 doses [79]. There were no treatment-related deaths. There was a greater incidence of grade III/ IV thrombocytopenia, malaise, and hypotension with high-dose compared to this "moderate" dose regimen. Only 3% of courses with moderate dose required vasopressor support, compared to 52% with high-dose. The moderate dose resulted in a 7% complete and an 8% partial response rate, while high-dose led to a 3% and a 17%, respectively. This moderate dose IL-2 regimen was effective, with preliminary results from this trial comparable to high-dose IL-2, resulting in significantly fewer complications, lower rate of vasopressor support, and fewer admissions to the intensive care unit. However this moderate dose regimen still required inpatient care.

A more recent randomized trial from the same group, which compared the efficacy of high *vs* low-dose IL-2, reported superior efficacy of the high-dose [80]. Toxicities, especially hypotension, were less frequent with low dose IV IL-2, but there were no treatment-related deaths in any arm. There was a higher response rate with high dose compared to low-dose IV IL-2 (21% *vs* 13%; p =.048), but no overall survival difference was noted, due to the relatively low response rates. The response duration and survival in complete responders were superior with high-dose compared to the low-dose IV regimen (p =.04). A large randomized phase III clinical trial with192 patients with metastatic RCC from the Cytokine Working Group (CWG) showed that low-dose IL-2 combined with IFN-α2b produced inferior response rates when compared to standard high-dose [81]. Response rate (23.2% *vs* 9.9%; p =.018), 3-year progression-free rate (10 *vs* 3 patients; p =.082), median response duration (24 *vs* 15 months; p =.18) and median survival (17.5 *vs* 13 months; p= .24) showed a trend that favored the high-dose arm.

Updated follow-up data from seven phase II trials of high-dose IL-2 was reported [82]. The median response duration of the original cohort of 255 patients with metastatic RCC treated with high-dose IL-2 was 54 months. The median duration for complete responses was not reached, but was at least 80 months. The median survival for all patients was 16.3 months, with a 10–20% overall long-term (5-10 years) survival. The NCI Surgery Branch published their 20-year experience with high-dose IL-2 in the management of 259 patients with metastatic RCC [83]. Predictive factors for response and survival were investigated; response to IL-2 was the major determinant for long-term survival. Twenty-three patients experienced a complete and 30 had a partial response, with an overall objective response rate of 20%. All partial but only 4 complete responders had developed relapse at the time of last follow-up. Despite the observed toxicity, there were only 2 treatment-related deaths. A series of 212 RCC patients treated with high-dose IL-2 recently reported a 20% overall response rate, with an 8% durable responses, corresponding with a median survival of over 10years [84]. Based on a systematic database review, no randomized trial of high-dose IL-2 compared to a non-immunotherapy control

or to INF-α evaluating survival has been reported [70]. Low-dose IL-2 provided equivalent survival to high-dose with less toxicity, but with inferior and less durable response rates in the large randomized phase III trials. High-dose IL-2 remains the standard regimen for the treatment of highly selected patients with clear cell carcinoma, good performance status and no major co-morbidities, resulting in durable responses in a minority. A summary of the clinical trials with IL-2 in metastatic RCC is presented in Table **3**.

Despite the modest benefit and the approved indication in the metastatic setting, high-dose IL-2 failed to provide benefit when tested in the adjuvant setting. A pilot study was conducted to investigate the toxicity and tolerance of low-dose SC IL-2 in 41 patients with resected RCC at high risk for recurrence [85]. Grade III/IV toxicity was seen in 20% of patients and the 3-year survival rate was 70%. There was no significant difference between the treatment arms. A large, prospective, randomized phase III trial evaluated the role of 1 course of high dose IL-2 (600,000IU/kg) every 8h on days 1-5 and days 15-19 (maximum of 28 doses) after radical nephrectomy in 69 high risk patients with locally advanced or metastatic disease, resected to no evidence of disease [86]. The study closed early when an interim analysis showed that the 30% targeted improvement in 2-year disease-free survival could not be achieved despite full accrual. The toxicity was as anticipated without therapy-related mortality.

PREDICTORS OF IL-2 RESPONSE

The limited number of responders and the significant toxicity associated with the use of IL-2 has resulted in attempts to identify selective patient subsets which are more likely to benefit from IL-2 therapy. The predictors of response to high-dose IL-2 can be divided into clinical, histological and molecular. CWG showed that ECOG PS 0 (p=.07), presence of bone or liver metastases (p=.001), presence of primary tumor (p=.040) predicted for response to IL-2 [81]. A retrospective analysis proposed a predictive model of response to IL-2 in metastatic RCC, after nephrectomy, known as Survival After Nephrectomy and Immunotherapy (SANI) scoring system [87]. According to this model, both response rates and survival were worse in patients with lymph node involvement, constitutional symptoms, metastatic sites other than bone or lung or multiple metastatic sites, thyroid-stimulating hormone (TSH) >2mIU/ml and sarcomatoid histologic type (Table **4**). In this report, 71% of patients died from their disease with a mean follow-up of 3.2 years. The mean and median time from the radical nephrectomy to death was 19 and 13 months. The estimated survival rates at 1, 3 and 5 years for the entire cohort were 66% 33%, and 22%, respectively. Based on the five prognostic markers, patient were stratified into 3 risk categories; low (no markers), intermediate (1-3 markers) and high-risk (>3 markers). The risk groups exhibited differential response and survival after IL-2 therapy.

Table 4. SANI Scoring System

• Lymph node involvement
• Constitutional symptoms
• Metastatic sites other than bone or lung or multiple metastatic sites
• TSH >2mIU/ml
• Sarcomatoid histologic type

Data on the role of pathological predictive markers has been presented. In a retrospective study, pathology examination was performed in primary and metastatic RCC tissue specimens from patients who had participated in CWG trials,

Table 3. Clinical Trials with IL-2 in Metastatic RCC

Patients Number	Disease	Dose	Endpoint	Reference
283	melanoma/RCC	high 720,000IU/kg	7% CR, 13% PR	Rosenberg [74]
409	melanoma/RCC	high 720,000 IU/kg	9.3% CR	Rosenberg [75]
201	RCC	high 720,000IU/kg	9% CR, 10% PR	Lindsey [76]
125	RCC	high 720,000 IU/kg *vs* low IV 72,000 IU/kg	3% *vs* 7% CR, 17% *vs* 8% PR	Yang [79]
306	RCC	high 720,000 IU/kg *vs* low IV 72,000 IU/kg	7% *vs* 4% CR, 14% *vs* 9% PR	Yang [80]
192	RCC	high 600,000 IU/kg *vs* low SC $5x10^6$IU/m^2 +IFN-α	23.2 *vs* 9.9% RR	McDermott [81]
255	RCC	high 600,000 -720,000IU/kg	7% CR, 8% PR	Fisher [82]
259	RCC	high dose 720,000 IU/kg	8.9% CR, 11.6% PR	Klapper [83]
212	RCC	high	8% CR, 12% PR	Belldegrun [84]
157	RCC	IL-2 + IFN-α	9% CR, 21% PR	Belldegrun [84]
26	RCC	low SC 9-18$x10^6$ IU	8% CR, 15% PR	Sleijfer [77]
41	RCC	low SC 9$x10^6$ IU	0% CR, 17.1% PR	Sheng [78]

CR: complete response, PR: partial response, RR: response rate.

with the attempt to identify pathological predictors of response to IL-2 [88]. The presence of clear cell histologic type, presence of more than 50% alveolar pattern, absence of granular morphology and the presence of renal vein involvement in primary tumors were associated with response. In a different study, higher levels (>85%) of carbonic anhydrase IX (CAIX) expression in the tissue was associated with higher response rate and longer survival [89]. This enzyme is highly expressed in clear cell type. Similar results were reported from another study, showing that higher (>85%) CAIX expression was more common in responders and was associated with longer survival [90].

With the advent and support of significant technological advances, the characterization of molecular biomarkers of response has become the focus of current research. In a gene expression and tissue microarray analysis, a set of 73 genes, including CAIX, P-TEN, CXCR-4 appeared to predict response to IL-2 [91]. More recent data, derived from array-based comparative genomic hybridization revealed that loss of genetic material in the chromosome 9p, a region which contains CAIX, pS6, and B7H1genes, predict fewer responses to IL-2 [92]. Additionally, data derived from RNA-based Afymmetrix gene chips followed by bioinformatics analysis described a set of 6 genes, which comprised the "IL-2 response signature" [93]. This gene set was able to separate responsive from non-responsive tumors in a training set of 22 samples with 100% accuracy. Further evaluation revealed that HLA DQalpha1 is highly expressed in responsive tumors. Proteomics analysis, using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, identified a proteomic profile that predicts response to IL-2 with 86% accuracy [94]. Results were validated in an independent data set with 72% accuracy. On multivariate analysis, the proteomic profile was significantly associated with IL-2 response when corrected for lymph node status ($p<0.04$). Immunostaining analysis was performed to evaluate the expression levels and predictive value of 10 molecular markers (Aurora-A, Bcl-2, clusterin, heat shock protein 27, heat shock protein 90, Ki-67, matrix metalloproteinase-2, matrix metalloproteinase-9, p53 and vascular endothelial growth factor) in radical nephrectomy specimens from 40 patients with metastatic RCC who received combined immunotherapy with IFN- α and low-dose IL-2 [95]. One patient achieved a complete response and 10 patients had partial responses, while 15 patients had stable disease. Higher expression levels of Bcl-2 and Ki-67 were associated with fewer responses and decreased cancer-specific survival. On univariate analysis, performance status, presence of metastases at diagnosis, metastatic organ site and C-reactive protein also correlated with cancer-specific survival. Ki-67 expression levels, and metastases at diagnosis, were independent predictors of cancer-specific survival on multivariate analysis. Additional validation is necessary before these tools are incorporated into the clinical practice. A phase II trial was recently launched to prospectively validate predictive models of response to high-dose IL-2 [96].

INTERFERON-α

IFN-α is a family of molecules encoded by closely related genes on chromosome 9, which encode proteins variably glycosylated. These proteins consist of almost 150 amino acids, and can bind to specific receptors on the surface of immune cells. This family of proteins exerts profound and diverse effects on gene expression. IFN-α exhibits immunomodulatory, anti-viral, anti-proliferative, and anti-angiogenic properties. Common toxicities associated with IFN-α include constitutional symptoms, such as fatigue, weakness, fever, chills, and myalgias, depression, elevated transaminases and autoimmunity [97].

The activity of IFN-α in metastatic RCC has been extensively evaluated in several large, well-designed clinical trials [98-102]. Using a variety of preparations, doses and schedules, the overall response rate was up to 15%; the median time to response was approximately 4 months and most responses were partial and did not last more than a year. Two large randomized trials evaluated the role of initial nephrectomy prior to IFN-α in highly selected patients with metastatic disease at diagnosis and minimal symptoms. Despite minimal improvement in the remission rate, both studies showed that the addition of up-front nephrectomy improved the median survival by 4.8 months [103, 104]. Results from 4 studies with 644 patients indicated that INF-α was superior to inactive control arms, with the average median survival benefit of 3.8 months. Doses 5-10X10^{6}U correlated with the most clinical benefit; however there was no clear dose-response association [70]. More recently a large randomized phase III trial with 750 patients demonstrated superiority of sunitinib over IFN-α as first-line treatment for metastatic RCC in terms of response rate, progression-free and overall survival, resulting in FDA-approval of sunitinib [105]. IFN-α monotherapy is not FDA-approved and currently has no role as a single agent for the treatment of RCC.

INTERLEUKIN-21

IL-21, a recently described common γ-chain cytokine, can induce the maturation and cytotoxicity of NK and CD8+ T cells, as well as the proliferation of CD40-stimulated B cells [106]. Exogenous administration of IL-21 exerts anti-tumor effects in murine models through immunological mechanisms. The combined stimulation of target-cells with IFN-α and IL-21 triggers an increased activation of signal transduction and activator of transcription-3 (STAT3), stimulating a selective increase in MHC I expression and NK and CD8+ T cell-mediated cytotoxicity [107].

A phase I trial of 43 patients with metastatic melanoma and RCC evaluated the safety, maximum tolerated dose, pharmacokinetics, pharmacodynamics, and preliminary anti-tumor activity of recombinant human (rIL-21) [108]. The maximum tolerated dose was found to be 30μg/kg. The most common adverse events included flu-like symptoms, pruritus and rash. One patient developed reversible grade IV hepatotoxicity with additional cycles of therapy. Serum concentrations of rIL-21 increased in a dose-proportional manner, while dose-dependent increase in soluble CD25 level reflected lymphocyte activation. Anti-tumor activity was observed in patients with RCC; 4 obtained partial response, and 13 had stable disease. Another phase I dose-escalation trial investigated different dose levels [109]. A therapy-related effect on soluble CD25 level was observed at all dose levels and was dose-dependent. Higher doses of IL-21induced IFN-γ, perforin, and granzyme B mRNA expression in peripheral

blood, and granzyme B protein expression in both CD8+ T cells and NK cells, corresponding with the activation of cytotoxic lymphocytes. Two patients with RCC achieved a partial response. A comprehensive review regarding IL-21 pharmacologic properties, safety, current development status and clinical efficacy was just published [110]. Overall, it appears to have an acceptable safety profile and encouraging single agent anti-tumor activity.

VACCINES

The aim of active immunization is to stimulate the host immune system to elicit an immune response against tumor antigens. The tumor antigen component of a vaccine can be generated by whole tumor cells (live, necrotic or apoptotic), tumor-derived cellular lysates, or tumor genetic material [111]. There are 3 types of cell-based vaccines, which have been investigated in RCC; autologous tumor cell-, gene-modified (engineered) tumor cell- and dendritic cell-based vaccines [112]. The first type involves autologous tumor lysates expressing antigens, which can be recognized and targeted by cytotoxic T cells. In the second paradigm, genetic engineering of either autologous or allogeneic tumor cells is employed, in order to "boost" local immune response when administered by stimulating local cytokine secretion, thus activating immune cells in the tumor microenvironment. In the third case, autologous dendritic cells "pulsed" with tumor antigens are used to act as antigen-presenting cells, and thus stimulate specific T cells as well as NK cells and even humoral responses against tumor cells [113]. This strategy has a lower risk of inducing autoimmune phenomena; however, the biologic and immunologic complexity of the host as well as the quality of manufacturing process should be considered as possible limitations [114]. The genes incorporated in such vaccines code for co-stimulatory molecules, such as B7; growth factors, such as GM-CSF; as well as cytokines, such as IL-2 and IL-6 [115-118]. Moreover, peptide-based vaccines, using tumor peptides, such as G250 membrane protein (carbonic anhydrase IX), telomerase and survivin, can also be used [111]. An extensive, systematic review on the clinical data with all vaccine types was presented [119]. The authors commented on the relative safety of vaccines over current therapies, such as cytokine delivery, encouraging the continuation of vaccine clinical research in RCC.

There have been randomized phase III clinical trials investigating the role of therapeutic vaccines in the adjuvant treatment of RCC. The first phase III trial was performed with 558 patients with stage pT2-3b pN0-3, non-metastatic RCC, scheduled to undergo radical nephrectomy [120]. All patients were randomized before surgery to receive either autologous RCC vaccine or no treatment. The updated report confirmed the progression-free survival benefit from the vaccine, but there was no difference in the overall survival in the intention-to-treat analysis. A 10-year survival analysis of patients with non-metastatic RCC treated with the same vaccine in the adjuvant setting was reported [121]. In the overall population, the 5- and 10-year overall survival rates were similar. Another phase III trial investigated the use of an autologous, tumor-derived heat-shock protein (glycoprotein 96)-peptide complex (HSPPC-96; vitespen) as an adjuvant modality in patients at high risk of relapse after resection of locally advanced RCC [122]. In this open-label trial, 818 patients were randomly assigned 1:1 to receive either vitespen or observation, after nephrectomy. Patients were stratified by performance status, lymph node status, and tumor grade. The recurrence and death rate were similar; however, overall survival data were not mature. In predefined exploratory analyses by tumor stage, the recurrence rate in patients with stage I/II disease favored the vitespen group. The most common adverse events in the vaccine group were injection-site erythema and induration. Only 1 severe adverse event (grade II autoimmune thyroiditis) was reported in the vitespen group; no treatment-related grade III/IV adverse events were reported.

OTHER IMMUNO-MODULATORY MOLECULES

Continued investigations of the mechanisms that underlie immune system processes have revealed molecules that participate actively in the regulation of anti-tumor immune responses. Programmed death-1 (PD1) is a T cell inhibitory receptor, which when bound with its ligand, suppresses anti-tumor immunity. Inhibition of this receptor or its ligand PD-L1 (B7-H1) could potentially result in the development of immune response against tumor cells. MDX-1106 is a fully human IgG4 PD1 blocking antibody, which has shown anti-tumor activity and limited toxicity, with an intermittent dosing scheme. A multicenter clinical trial evaluated the safety, anti-tumor activity, pharmacokinetics, and immunological correlates of escalating doses of this antibody. The study included patients with treatment-refractory metastatic solid tumors, including RCC, and with no history of autoimmune disease [123]. Grade I/II drug-related adverse events included fatigue (56.3%), nausea (25%), and diarrhea, xerostomia, and pruritus (18.8% each). One patient with RCC had complete response and another had partial response, which were ongoing at the time of the report. Expansion cohort enrollment was nearly complete at the time of the report and grade III/IV adverse events were uncommon.

CD137 (4-1BB) is a leukocyte differentiation antigen expressed specifically on the surface of activated T cells, NK cells, and dendritic cells. Agonist antibodies against CD137 receptor on the surface of antigen-primed T lymphocytes act as artificial stimulatory ligands and enhance tumor immunity with curative potential against transplantable murine tumor models. A fully human IgG4 anti-CD137 antibody is under development with signs of clinical activity, but with reports of severe liver toxicity that appear to be on-target and dose-dependent effects. A review of the clinical experience with anti-CD137 and anti-PD1 therapeutic antibodies was just published [124].

The inhibitory receptor cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) has a critical role in peripheral tolerance of T cells for both normal and tumor-associated antigens. Experiments in murine models suggested that blockade of this receptor might result in anti-tumor activity, while clinical experience with the anti-CTLA4 antibody ipilimumab in patients with metastatic melanoma showed durable tumor response in a number of patients. A phase II trial of ipilimumab was conducted in patients with metastatic RCC to assess response [10]. Major adverse events were enteritis and endocrine deficiencies, possibly autoimmune.

Five of 40 patients at the higher dose had partial responses; responses were observed in IL-2-refractory patients. One third of patients had grade III/IV immune-mediated toxicity. There was a highly significant association between autoimmune events and tumor response. A phase I trial evaluated the combination of tremelimumab (CP-675206; anti-CTLA4 antibody) combined with sunitinib in 28 patients with metastatic RCC who had received up to 1 previous systemic treatment. Overall, acute renal failure was the most common dose-limiting adverse event [125]. From 21 evaluable patients, 9 experienced partial response and 4 of those were ongoing at the time of the report. A thorough review on the new therapeutic paradigm, exploiting the role of PD1 and CTLA4 signaling inhibition was just published [11].

Sunitinib is a FDA-approved receptor tyrosine kinase inhibitor with significant impact in metastatic RCC. Sunitinib appears to have immuno-modulatory properties. Sunitinib has been reported to reverse type-1 immune suppression and decrease circulating T regulatory cells [126]. A decrease of regulatory T cells correlated with overall survival in metastatic RCC after sunitinib-based therapy [127]. Sunitinib may potently reverse MDSC accumulation and T cell inhibition, even in patients without tumor response [128]. Both monocytic and neutrophilic splenic MDSC were highly repressible by sunitinib. However, MDSC within the tumor microenvironment were highly resistant to sunitinib, and ambient T cell function remained suppressed. Proteomic analyses comparing tumor to peripheral compartments showed that granulocyte macrophage colony-stimulating factor (GM-CSF) predicted resistance to sunitinib and recombinant GM-CSF conferred sunitinib resistance to myeloid-derived suppressor cell *in vivo* and *in vitro* [129]. MDSC conditioning with GM-CSF uniquely inhibited STAT3 and promoted STAT5 activation. However, STAT5 abrogation in these cells rendered them sensitive to sunitinib in the presence of GM-CSF *in vitro*. Compartment-dependent GM-CSF exposure in resistant tumors may explain this effect of sunitinib upon host MDSC modulation and ancillary strategies attempting to decrease such escape might enhance the potency of sunitinib as immunomodulator [129]. Moreover, sunitinib-induced STAT3 inhibition was found to induce RCC apoptosis and reduce immunosuppressive cells [130]. The anti-tumor activity and related mechanisms of a novel STAT3 inhibitor (WP1066) was investigated *in vitro* in RCC lines and *in vivo* in murine xenografts, providing encouraging results [131].

CONCLUSION

RCC appears to be an immune-responsive tumor. Various immunotherapeutic strategies have been investigated with diverse results. Cytokine delivery with IL-2 has been the only FDA-approved immune-related modality in metastatic disease. Clinical trials implementing ASCT have been conducted, showing a correlation between occasional responses and development of GVHD, implying the role of GVT. Adoptive immunotherapy with T and NK cells is still under investigation, and results from both the preclinical and clinical setting have been encouraging; however, definitive positive results from large randomized clinical trials are lacking. The use of vaccines is another emerging strategy, with one large randomized phase III clinical trial, demonstrating a trend towards benefit in the adjuvant setting in selected patients. A number of immune modulating molecules are under development and their role in immune response is being characterized. In the post-genomic era, the development of novel biomarkers may contribute to more accurate patient selection, resulting in higher response rates and less toxicity of immunotherapeutic strategies. Rational, biology-driven combinatorial approaches can be tested *in vitro* and *in vivo* models that might lead to improved outcome in the immunotherapy of RCC.

CONFLICT OF INTEREST

None declared.

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