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The potential of radiotherapy to enhance the efficacy of renal cell carcinoma therapy

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Keywords: antitumor immunity, immunotherapy, radiotherapy, renal cell carcinoma, targeted therapy, treatment combination

Abbreviations: APCs, antigen presenting cells; APM, antigen processing machinery; ASMase, acid sphingomyelinase; ATP, adenosine triphosphate; ccRCC, clear cell renal cell carcinoma; CRT, calreticulin; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte associated protein 4; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; ER, endoplasmic reticulum; HFRT, hypofractionated radiotherapy; HIF-1α, hypoxia-inducible factor α; HMGB1, high-mobility group box 1; HSP70, heat shock protein 70; ICAM-1, intercellular adhesion molecule 1; ICD, immunogenic cell death; IDO, immune regulating enzyme indoleamine-2,3-dioxygenase; IFNγ, interferon γ; IL-2, interleukin 2; IL-6, Interleukin 6; IL-10, interleukin 10; IL-12, Interleukin 12; M1 macrophages, pro-inflammatory macrophages; M2 macrophages, anti-inflammatory macrophages; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MICA, MHC class I-related chain A; mTOR, mammalian target of rapamycin; NK cells, natural killer cells; PDGFR, platelet-derived growth factor receptor; PD-L1, programmed death ligand 1; RCC, renal cell carcinoma; ROS, reactive oxygen species; SBRT, stereotactic body radiotherapy; STAT3, signal transducer and activator of transcription 3; TCR, T cell receptor; TGF-β, transforming growth factor β; Th1 cells, T helper 1 cells; Th 2 cells, T helper 2 cells; TILs, tumor infiltrating lymphocytes; TIM-3, T cell immunoglobulin and mucin domain 3; TKIs, tyrosine kinase inhibitors; TNFα, tumor necrosis factor α; Tregs, regulatory T cells; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

Renal cell carcinoma (RCC) is an immunogenic tumor, but uses several immune-suppressive mechanisms to shift the balance from tumor immune response toward tumor growth. Although RCC has traditionally been considered to be radiation resistant, recent evidence suggests that hypofractionated radiotherapy contributes to systemic antitumor immunity. Because the efficacy of antitumor immune responses depends on the complex balance between diverse immune cells and progressing tumor cells, radiotherapy alone is unlikely to induce persistent antitumor immunity. Therefore, the combination of radiotherapy with drugs having synergistic immunomodulatory properties holds great promise with the optimal timing and sequence of modalities depending on the agent used. We highlight the immunomodulatory properties of targeted therapies, such as tyrosine kinase inhibitors, mammalian target of rapamycin (mTOR) inhibitors and vascular endothelial growth factor (VEGF) neutralizing antibodies, and will suggest a combination schedule with radiotherapy based on the available literature. We also address the combination of radiotherapy with innovative treatments in the field of immunotherapy.

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Introduction

RCC presents with metastatic disease in about 30% of patients, while another third of patients with localized advanced disease will ultimately develop metastases.[1,2] Molecular therapies that block the VEGF or mTOR pathways are currently considered the mainstay treatment[3] for metastatic RCC. Nevertheless, a durable response to targeted therapy is rare and most patients eventually develop progressive disease.[4,5] We therefore have to look at new therapeutic options to improve the outcome of these patients. Since RCC is considered an immunogenic tumor,[6-8] we might find the answer in the field of immunotherapy. There are some clinical cases in RCC describing responses outside the irradiated regions, following high-dose stereotactic body radiotherapy (SBRT) to metastases.[9,10] These responses are termed "abscopal effects." Both pre-clinical and clinical data[11–13] suggest that these effects are immune mediated.[14,15] Despite these observations, both the tumor and its microenvironment seem to be able to evade the immune system in the majority of cases. Radiotherapy alone is probably unlikely to induce persistent antitumor immunity and a combination with synergistic immunomodulatory agents might be necessary to induce long-term clinical results, as suggested by promising preclinical and clinical data.[12,16-20]

The current review offers insights in the specific immune escape mechanisms present in RCC with a specific focus on the potential role of radiotherapy in combination with systemic treatment to improve clinical responses by enhancing antitumor immunity.

Immune Modulation in RCC

Although the immune system tries to control the proliferation of RCC, the tumor is able to progress. By evasion of the antitumor immune response, RCC is able to shift the balance from tumor immune response toward tumor growth (**Fig. 1**). In the next paragraphs, these evasion mechanisms of RCC influencing both the innate[21] and adaptive immune system are highlighted.[22]

RCC is able to escape cytotoxic T lymphocyte (CTL)-mediated killing through different mechanisms (**Fig. 2**). T cells are initially stimulated to recognize cancer cells through cross-priming by dendritic cells (DCs). However, RCC interferes with DC activation by secreting immunosuppressive factors. Consequently, only a minority of the DCs show signs of activation[23] and are able to prime naïve T cells. Moreover, deficiencies in both the proteasome and transporter associated with antigen processing, reduction of other antigen processing machinery (APM)-components, and altered expression of major histocompatibility complex (MHC)-I molecules, allows RCC to escape recognition by CTLs.[24]

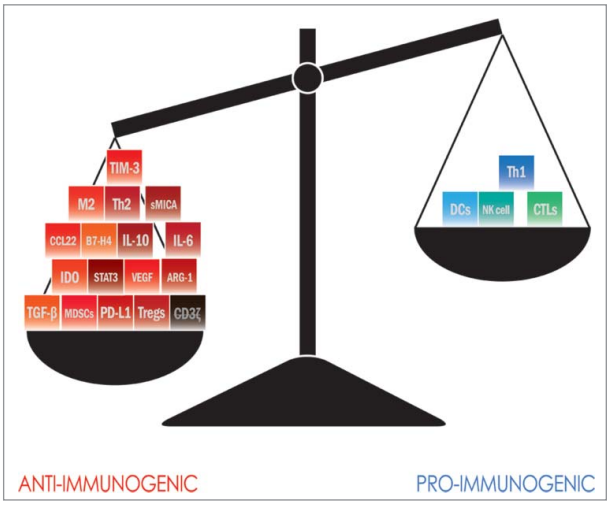

Figure 1. The balance between pro-immunogenic and immunosuppressive factors in the tumor microenvironment of RCC. The immune system plays a protective role in tumor control. Dendritic cells (DCs) take up apoptotic and necrotic tumor fragments and present processed tumor-derived peptides to T-helper (Th) lymphocytes as well as cross-present to cytotoxic T lymphocytes (CTLs). Tumor-activated NK cells kill tumor cells by releasing their cytotoxic granules onto the surface. On the other hand, RCC is able to evade antitumor immune responses. RCC stimulates the secretion of immunosuppressive soluble factors such as IL-10, IL-6, vascular endothelial growth factor (VEGF), arginase-I (ARG-1) and indoleamine-2,3-dioxygenase (IDO). RCC also activates transforming growth factor β (TGF-β), signal transducer and activator of transcription 3 (STAT3), promotes the accumulation of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and pro-tumorigenic M2 macrophages. RCC also impairs T cell function by the decreased expression of the CD3ζ chain and the increased expression of the co-inhibitory molecules PD-L1, B7-H4 and T cell immunoglobulin and mucin domain 3 (TIM-3). Finally, RCC impairs NK cell activity by shedding soluble MHC class I-related chain A (MICA) into the circulation.

Most RCCs are highly vascularized because of mutations of the von Hippel–Lindau (VHL) tumor suppressor gene. pVHL is needed for the degradation of hypoxia-inducible factor α (HIF-1α). Deficient pVHL leads to accumulation of HIF-1α and stimulation of angiogenesis through HIF-induced VEGF production.[25] In addition to stimulating tumor angiogenesis, VEGF also arrests the differentiation of myeloid cells, resulting in accumulation of immature myeloid cells. These immature myeloid cells are myeloid-derived suppressor cells (MDSCs) and block T cell responses by producing IL-10, transforming growth factor (TGF)-β, prostaglandin E2,[26] reactive oxygen species (ROS)[27] and arginase I.[26,28] Compared to healthy controls, higher levels of MDSCs are found in the peripheral blood of RCC patients,[24] associated with a 6–10-fold increase in arginase activity.[26] Arginase production by MDSCs results in a decreased expression of the CD3ζ chain on tumor-infiltrating lymphocytes (TILs) of RCC.[29] The CD3ζ chain is part of the T cell receptor (TCR) complex and normally plays a critical role in the proximal signaling events leading to T cell activation. Its reduced expression leads to impaired TCR signaling, causing a disturbed lytic function of the TILs.[22] VEGF, along with IL-6 and IL-10, also induces signal transducer and activator of transcription 3 (STAT3) activation.[30] STAT3 activation is thought to be involved in the accumulation of immunosuppressive cells, such as MDSCs and regulatory T cells (Tregs),[31,32] and in the absence of functional DCs.[31] In addition, STAT3 activation might be responsible for the reduced CTL reactivity in RCC, since STAT3 is required for the expression of HIF-1α,[33] constitutively activated in the majority of RCC,[34] and gene silencing of HIF-1α was seen to restore the susceptibility of tumor cells to CTL-mediated killing.[30] These mechanisms might explain why both VEGF expression in tumor tissue and serum levels of VEGF are associated with poor prognosis in RCC patients.[35,36]

T cells are only activated when the balance between co-stimulatory and co-inhibitory signals crosses the threshold for T cell activation.[37] Therefore, the expression, by both primary and metastatic RCC tumor cells, of the co-inhibitory molecule programmed death ligand 1 (PD-L1) might shift the balance toward T cell inhibition.[38] The expression of the co-inhibitory molecule PD-L1 in RCC is associated with aggressive tumor behavior and poor outcome.[39,40] RCC tumor cells also often express the negative co-stimulatory molecule B7-H4. Its expression is associated with adverse clinical features.[38,41] Recently, a new co-inhibitory molecule, T cell immunoglobulin and mucin domain 3 (TIM-3), was described. The molecule is expressed by Th1 cells and CTLs and induces cell death by binding its ligand, galactin-9. Furthermore, the upregulated expression of TIM-3 on tumor-specific and tumor-infiltrating $CD8^+$ T cells from patients with clear cell (cc)RCC was associated with poor prognosis.[42,43]

Since Th1 cells are considered to be effector cells with antitumor activity, achieving a Th1-dominated immune response

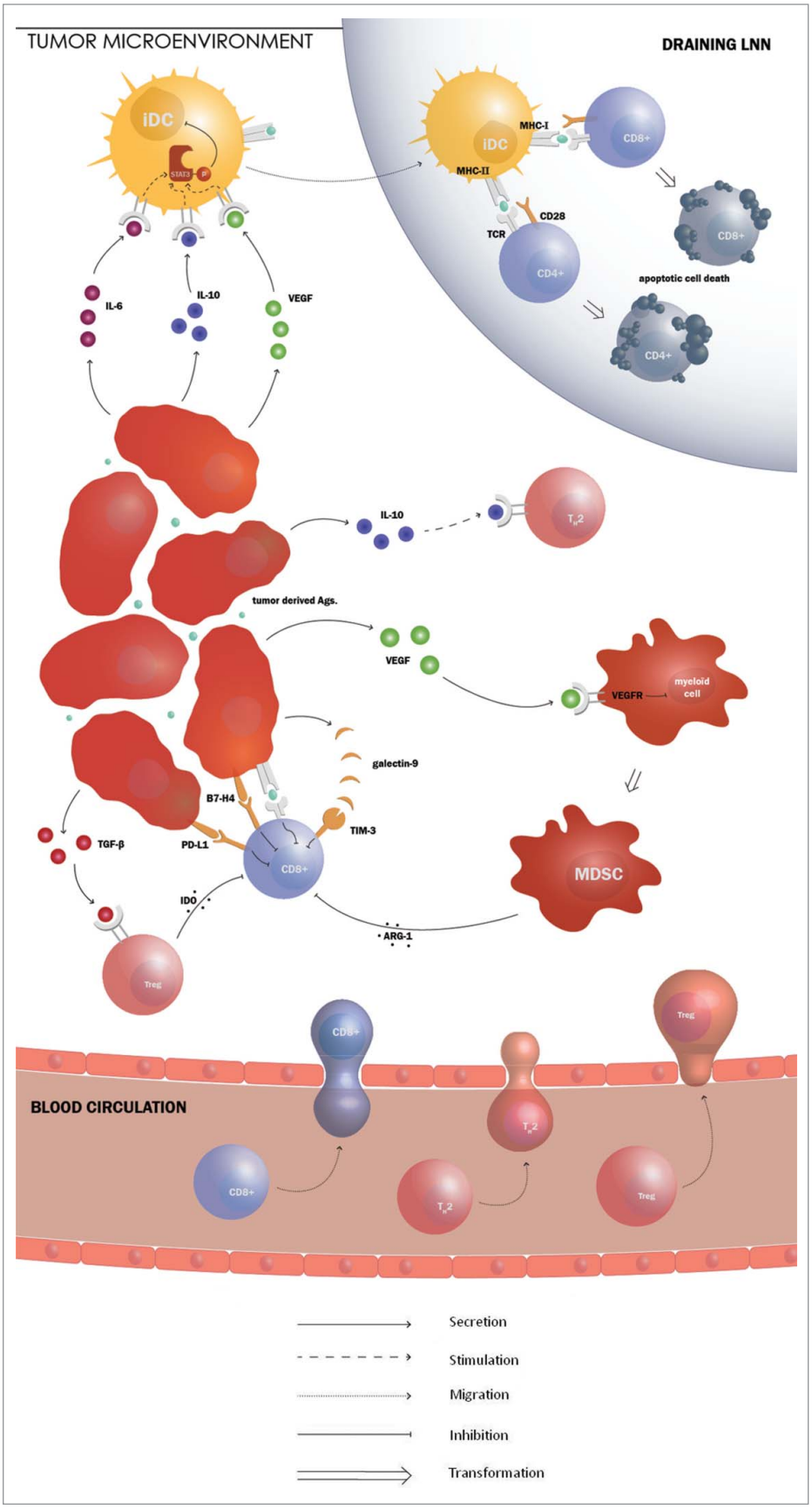

Figure 2. The immune evasion mechanisms of RCC hinder the adaptive immune response. Production of vascular endothelial growth factor (VEGF) by renal cell carcinoma (RCC) arrests the differentiation of myeloid cells, resulting in the accumulation of immature myeloid cells. These immature myeloid cells are called myeloid derived suppressor cells (MDSCs) and block T cell responses by producing immunosuppressive agents such as arginase I. Arginase production by MDSCs results in impaired T cell receptor (TCR) signaling, causing a disturbed lytic function of the CD8+ T cells. VEGF, along with IL-6 and IL-10, also induces the activation of signal transducer and activator of transcription 3 (STAT3). STAT3 activation is thought to be involved in the absence of functional DCs. The expression the co-inhibitory molecule programmed death ligand 1 (PD-L1) by RCC might stimulate T cell inhibition. RCC cancer cells also often express the negative co-stimulatory molecule B7-H4. Recently, a new co-inhibitory molecule, T cell immunoglobulin and mucin domain 3 (TIM-3), was described. The molecule induces T cell death by binding its ligand, galactin-9. RCC is able to counteract Th1 cell differentiation. Production of IL-10 by the tumor cells causes Th1 cell loss and Th2 cell prevalence. RCCs also produce transforming growth factor (TGF)-β, which is known to stimulate the recruitment and activation of regulatory T cells (Tregs). They downregulate the function of immune effector cells through secretion of immunosuppressive factors such as indoleamine-2,3-dioxygenase (IDO).

against RCC cancer cells would be desirable. However, RCC is able to counteract Th1 cell differentiation. Production of IL-10 by the tumor cells causes Th1 cell loss and Th2 cell prevalence.[24] Additionally, RCCs do not produce the necessary cytokines, such as IL-2 and IL-12, to foster an optimal development of tumor-specific T cells. On the contrary, they produce TGF-β, which is known to stimulate the recruitment and activation of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs.[22,23] Under the influence of the chemokine CCL22, Tregs accumulate at the tumor site.[44] They downregulate the function of immune effector cells through secretion of IL-10, TGF-β[27,45] and the immune-regulating enzyme indoleamine-2,3-dioxygenase (IDO).[37,46] Tregs are detectable in the peripheral circulation. Frequencies of Tregs in the peripheral circulation of patients with RCC were

elevated 3-fold compared to healthy controls[24] and increased frequencies were associated with a shorter overall survival.[40,47,48]

RCC also influences the innate immune system (**Fig. 1** Supplementary data). In patients with RCC a high frequency of natural killer (NK) cells in the lymphocytic infiltrate of the primary tumor seems to predict a better prognosis.[49,50] However, in advanced RCC, NK cell frequency and activity are often decreased, correlating with poor survival.[24] One possible mechanism for the impaired NK cell activity is the shedding of MHC class I-related chain A (MICA), a soluble NKG2D ligand, from the tumor cell surface into the circulation.[24] This causes a down-modulation of the NK cell-activating receptor, NKG2D, resulting in decreased cytotoxicity.[51] In addition, by secreting IL-10, cancer cells induce the polarization of tumor-associated macrophages from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype.[24,52] It is STAT3 signaling that plays an important role in this conversion.[31] M1 macrophages are hypothesized to bear antitumor activities because they produce high levels of inflammatory cytokines, such as IL-12 and tumor necrosis factor α (TNFα). On the contrary, M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and IL-6.[47,53] In RCC, M2 macrophages are associated with a more advance tumor stage, while the opposite is held true for M1 macrophages.[24,47,52]

Immunogenic Potential of Radiotherapy in RCC

The role of radiotherapy in metastatic RCC is used to palliate symptomatic metastases[3] as RCC has been traditionally considered a radiation-resistant tumor. Although RCC might be resistant to conventional fractionated radiation (daily fractions of 1.8–3.0 Gy), a recent review suggested the opposite for hypofractionated radiotherapy (HFRT), typically delivering $\geq$ 5 Gy per fraction, in a single or a few fractions. HFRT, results in a different tumor radiobiology compared to conventional fractionated radiotherapy. One of the effects involves increased endothelial cell apoptosis, triggered by acid sphingomyelinase (ASMase)-induced ceramide release. Others have suggested that HFRT activates de novo synthesis of ceramide. Ceramide is able to initiate an apoptotic cell death through the release of mitochondrial cytochrome c.[54] Therefore, HFRT, in contrast to conventional radiotherapy, efficiently destroys tumor microvasculature and is expected to have better results in tumors that are highly dependent on angiogenesis, such as RCC. This is supported by the excellent local tumor control of HFRT.[55] HFRT has already been proven to be very safe in the treatment of oligometastatic disease. A systematic review of Kothari et al. reported one year local control rates of 88% and 86% for intra- and extracranial metastases, respectively. Grade 3–4 toxicity ranged between 0 and 6%.[56] A prospective phase II trial for patients with brain metastases from so-called radio-resistant primary tumors, including RCC, showed median survival rates with stereotactic radiosurgery (SRS), which were comparable to surgical series.[57] A prospective phase II trial using extracranial HFRT in mRCC or inoperable primary RCC showed local control in 98% of treated lesions,[58] making it an excellent alternative to metastasectomy for treatment of extracranial metastases that are technically inoperable. Future randomized trials are required to confirm the additional benefit of HFRT above conventional radiotherapy.

The encouraging results of HFRT might also be explained by the effect radiotherapy has on the immune system.[59] In the next paragraphs, we provide evidence for the potential of radiotherapy in shifting the balance back toward tumor control (**Fig. 3**). To date, little is known about which dose/fractionation regimens optimally enhance the antitumor immune response (25), but the majority of preclinical studies has investigated the effect of HFRT (22, 25) (**Table 1**).

Radiotherapy is able to hinder RCC in escaping CTL-mediated killing on different levels (**Fig. 4**). Firstly, irradiated dying cells provide a source of multiple tumor antigens[60,61] for cross-presentation by circulating DCs.[62-64] Radiotherapy stimulates DC activation by inducing immunogenic cell death (ICD), a cell death modality that is part of a ROS-dependent endoplasmic reticulum (ER) stress response.[59,62] ICD stimulates an immune response against dead-cell associated antigens[65] and is characterized by exposure of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT)[60] and heat shock protein (HSP)70 and release of high-mobility group box 1 (HMGB1)[66] and adenosine triphosphate (ATP).[60,67] These DAMPs are able to stimulate DC maturation,[68] diversifying the TCR repertoire of tumor-specific T cells.[69] Therefore, irradiated tumor cells might serve as an in situ autologous tumor vaccine.[63] Radiation also induces interferon (IFN)γ production within the tumor microenvironment,[70,71] which has been shown to enhance the level of APM-components and to increase the expression of MHC-I molecules on the surface of the tumor cells.[14,60,62] Activation of the ceramide pathway in response to HFRT, triggers vascular endothelial cell apoptosis via the ASMase pathway. Such damage also stimulates expression of MHC molecules.[72-74]

Secondly, the upregulation of IFNγ following radiotherapy also plays a role in the trafficking of $CD8^+$ T cells[68,75,76] leading to the accumulation of $CD8^+$ T cells in the tumor. The efficacy of high-dose radiotherapy has been proven to depend on the presence of these $CD8^+$ T cells, since antibody-mediated depletion of $CD8^+$ T cells completely abolished the therapeutic effect.[59,71] The accumulation of $CD8^+$ T cells is the result of different IFNγ-induced mechanisms, such as the expression of the adhesion molecules vascular cell adhesion molecule (VCAM)-1[70] and intercellular adhesion molecule (ICAM)-1[62,74] on tumor vasculature, facilitating T cell adhesion before transmigration and the secretion of CXCL9 and CXCL10, important T cell chemo-attractants with an anti-angiogenic effect.[70] Besides T cells, they also attract monocytes who replenish the amount of DC.[77] The expression of the co-stimulatory molecule CD80 on DCs in the tumor microenvironment has also been found to be increased by radiation[78] and could therefore shift the balance toward T cell activation.

Thirdly, radiotherapy is able to restore the limited recruitment of Th1-polarized lymphocytes in the tumor microenvironment of RCC[14] by shifting the balance from a tumor

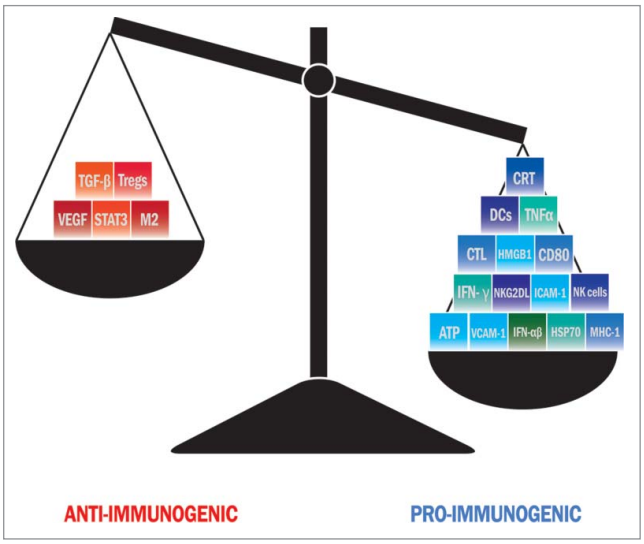

Figure 3. The balance between pro-immunogenic and immunosuppressive effects of radiotherapy and tumor rejection. Radiation promotes the antitumor immune response. Key molecular signals that promote priming of antitumor cytotoxic T cells (CTLs) by dendritic cells (DCs) loaded with tumor antigens include exposure of calreticulin (CRT) and heat shock protein (HSP) 70 and release of ATP and high-mobility group box 1 (HMGB1). These signals are released by the tumor cells undergoing a radiation-induced immunogenic cell death. Tumor infiltration by T cells that produce interferon γ (IFNγ) and tumor necrosis factor α (TNF-α) is facilitated by upregulation of vascular cellular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on tumor endothelium. Radiation-induced upregulation of major histocompatibility complex class 1 (MHC-1), NKG2D ligands (NKG2DL) and the co-stimulatory molecule CD80 on surviving tumor cells improves their recognition and killing by T cells. On the other hand, radiation activates immunosuppressive transforming growth factor β (TGF-β) and Signal transducer and activator of transcription 3 (STAT3), stimulates the secretion of vascular endothelial growth factor (VEGF), and promotes accumulation of regulatory T cells (Tregs) and pro-tumorigenic M2 macrophages.

microenvironment dominated by TGF-β toward a tumor microenvironment enriched with IFNγ, which is responsible for the differentiation of $CD4^+$ T cells into Th1 cells. This is important, because a Th2-dominated response was consistently observed as a poor prognostic factor for patients with RCC.[79] Consequently, radiotherapy is able to induce tumor-specific Th1 cells in the non-irradiated draining lymph nodes of the irradiated tumor and favor the trafficking of effector cells into tumors.

However, radiotherapy also induces immunosuppressive mechanisms by activating TGF-β,[80,81] stimulating Tregs and inducing the activation of STAT3 and VEGF.[82] STAT3 and TGF-β, might hinder the response to ICD.[14]

Total dose, fractionation, dose distribution and timing of radiotherapy are key variables in determining the effects of radiotherapy on the immune system.[83] In a murine melanoma model, a hypofractionated regimen with two fractions of 7, 5 Gy gave the best tumor control and tumor immunity while maintaining low Treg numbers.[84] However, the optimal radiation regimen may not necessarily be the same for all tumor types or settings.

Radiotherapy influences components of the innate immune response as well (**Fig. 1** Supplementary data). Radiation increases the surface expression of NKG2D ligand,[85] which binds the NK cell-activating receptor NKG2D, increasing the susceptibility of NK cells. On the other hand, radiation might also decrease the expression of the NK cell-activating NKG2D receptor,[86] through the release of TGF-β.[80] In addition, radiotherapy induces the expression of MHC-I molecules. Since NK cells destroy cells that have downregulated expression of MHC class I molecules, induction of MHC-I expression might decrease recognition by NK cells.[87] Therefore, it is difficult to predict the net effect of radiation therapy on NK cells. In addition, after radiotherapy a misdirected tissue repair response can promote tumor recurrence and progression. This wound healing response is orchestrated by M2 macrophages who stimulate angiogenesis and contribute to the suppression of antitumor immunity by secreting cytokines such as IL-10.[73,88] In contrast, other studies show that HFRT results in the priming of MHC-I molecules and augments cytolytic activity.[89,90] Radiotherapy is also able to prime macrophages for pro-inflammatory signaling in a dose-dependent manner, as shown by enhanced IFNγ-mediated NO production and increased TLR-mediated TNF-α secretion.[91,92] Furthermore, conventional fractionated radiotherapy was observed to skew macrophage function to an antitumor mode in different murine carcinoma models[93] and both conventional and HFRT caused a significant increase of tumor-infiltrating M1 macrophages.[94] Importantly, radiotherapy was able to enhance M2 activity in C57BL/6 mice, while increasing M1 activity in CBA/CaJ mice.[95] Thus, not only depending on the modulation of cytokine production, but also on the experimental model, radiotherapy has been reported to have different effect on tumor-infiltrating macrophages, therefore the net results in clinical practice is still unclear.

Repurposing of Molecular Targeted Therapies

Because the efficacy of antitumor immune responses depends on the complex balance between diverse immune cells and progressing tumor cells, radiotherapy alone is unlikely to induce persistent antitumor immunity in all treated patients. Therefore, a new role for radiotherapy in combination with synergistic immunomodulatory agents is emerging.[62] Significant progress in the understanding of RCC biology has led to the development of targeted therapies such as tyrosine kinase inhibitors (TKIs), mTOR inhibitors and VEGF neutralizing antibodies. Since the pro-oncogenic pathways targeted by these therapies also drive many of the immune-evasion mechanisms of RCC, target therapies have the capacity to optimize antitumor immune responses.[96] In the next paragraphs, we highlight the immunomodulatory properties of these agents and will suggest a combination schedule with radiotherapy based on the available literature. The agents and their effects are summarized in **Table 2**.

Table 1. Immunogenic potential of radiotherapy

A. Pro-immunogenic effects			
Effect on the TME	**Conventional RT**	**HFRT**	**References**
Increases the surface expression of NKG2D ligand	Unknown	Yes	81
Provide tumor antigens	Unknown	Yes	57,58
DC activation	Yes	Yes	56,61
CRT exposure	Unknown	Yes	57
ATP secretion	Unknown	Yes	57
Release of HMGB1	Unknown	Yes	57
Increase of MHC-I expression	Yes	Yes	57,70
Increase of ICAM-1 expression	Unknown	Yes	57,70
Induction of IFNγ production	Unknown	Yes	66
Induction of type 1 IFN	Unknown	Yes	72
Stimulation of $CD8^+$ effector T cells	Unknown	Yes	72
B. Immunosuppressive effects			
Effect on the TME	**Conventional RT**	**HFRT**	**References**
Induction of TGF-β	Yes	Yes	77
Secretion of VEGF	Yes	Yes	78
Induction of M2 macrophages	Unknown	Yes	69,84
Activation of STAT3	Unknown	Yes	14,84

(A) Pro-immunogenic effects: Hypofractionated radiotherapy (HFRT) (fraction sizes more than 5 Gy) is known to promote the antitumor immune response by upregulation of NKG2D ligands (NKG2DL), major histocompatibility complex class I (MHC-I) and intercellular adhesion molecule 1 (ICAM-1). HFRT activates dendritic cells (DCs) through exposure of calreticulin (CRT) and release of ATP and high-mobility group protein B1 (HMGB1). Activated DCs migrate to local lymphoid organs and stimulate $CD8^+$ effector T cells. $CD8^+$ effector T cells will infiltrate the tumor and produce interferon γ (IFNγ).
Conventional radiotherapy (daily fractions of 1.8–3.0 Gy) is known to promote the antitumor immune response by upregulation of MHC-I and activation of DCs.
(B) Immunosuppressive effects: HFRT is also known to activate signal transducer and activator of transcription 3 (STAT3), promote the secretion of vascular endothelial growth factor (VEGF) and the accumulation of pro-tumorigenic M2 macrophages. Conventional radiotherapy has been observed to activate the immunosuppressive transforming growth factor β (TGF-β) and VEGF.

In RCC, TKIs not only inhibit angiogenesis and tumor growth, but also have the potential to interact with the immune system.[97] TKIs approved for treatment of advanced RCC currently include sunitinib, sorafenib, pazopanib and axitinib. They all target VEGFR, PDGFR and c-kit tyrosine kinases, be it with a different affinity. The most-studied TKI in the treatment of RCC, sunitinib, has important immunostimulatory capacities. It causes downregulation of immunosuppressive Tregs and MDSCs.[97,98] It reduces the level of MDSCs through three different mechanisms: the inhibition of STAT3,[99] the inhibition of c-kit[100] and the inhibition of VEGF receptors.[96] Additionally, sunitinib stimulates T cell priming by DCs. It also reduces the expression of co-inhibitory molecules, such as PD-1 and CTL-associated protein 4 (CTLA-4).[97] Importantly, sunitinib has already been observed to safely potentiate the radiation-induced antitumor response.[17] To optimize the therapeutic effects of this combination, we suggest that the administration of sunitinib should be started prior to radiotherapy, since it affects T cell priming and increases radiation sensitivity by normalizing tumor vasculature.[101,102] Because sunitinib also antagonizes the immunosuppressive tumor microenvironment by reducing the levels of MDSCs, Tregs and co-inhibitory molecules, it should be continued after radiotherapy as treatment consolidation.[96] Unlike sunitinib, sorafenib has some immune suppressive effects on DC and $CD8^+$ T cell function.[103] Sorafenib lowers cytokine secretion by DCs, prevents upregulation of co-stimulatory molecules and reduces the capacity of APCs to stimulate T cell proliferation.[104] Even though sorafenib also has some pro-immunogenic activity,[105–107] we believe it might not be an ideal candidate to use before radiotherapy, since it might hinder tumor-specific T cell priming.[96]

Pazopanib and axitinib are more novel TKIs that also inhibit VEGFR and c-kit kinases.[108,109] Not much is known about their immunomodulatory capacities. They might have similar effect as sunitinib on the level of MDSCs. Treatment with axitinib in combination with DC-based vaccination was observed to stimulate antitumor immune responses, by reducing the number of intratumoral MDSCs and Tregs and activating tumor-specific $CD8^+$ T cells.[110] We suggest that pazopanib and axitinib treatment should be combined with radiotherapy with the same sequence as proposed for sunitinib.[96] The safety of the combination of pazopanib with conventional radiotherapy has already been investigated.[111]

mTOR inhibitors, temsirolimus and everolimus, are known to promote Tregs.[112] Combining radiotherapy with a mTOR inhibitor might further boost the stimulation of immunosuppressive Tregs. Even though mTOR inhibition could also increase the quantity of memory T cells,[113] it is difficult to predict to which side the balance would be shifted when radiotherapy is added to treatment with mTOR inhibitors.

Bevacizumab is a monoclonal antibody neutralizing VEGFA. Treatment with bevacizumab in combination with IFN-α is also a first-line treatment in metastatic RCC. VEGFA blockade blocks

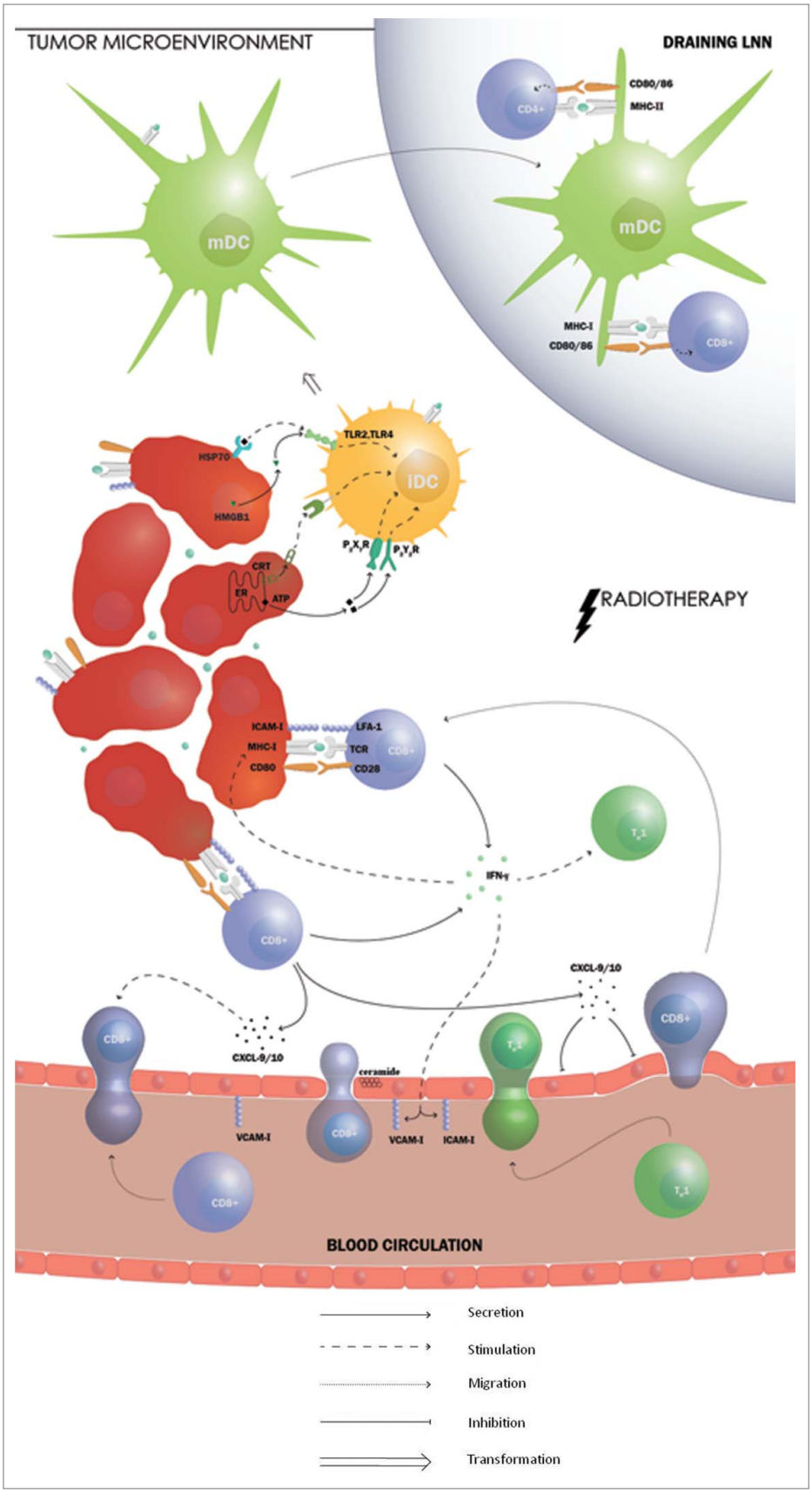

Figure 4. Radiotherapy stimulates the adaptive immune response in RCC. Radiotherapy is able to hinder renal cell carcinoma (RCC) in escaping cytotoxic T lymphocytes (CTL)-mediated killing on different levels. Irradiated dying cells provide a source of multiple tumor antigens for cross-presentation by circulating dendritic cells (DCs) and increases the expression of major histocompatibility complex class I (MHC-I) molecules on the surface of the tumor cells. Furthermore, radiotherapy stimulates DC activation by inducing immunogenic cell death (ICD), a cell death modality that is characterized by exposure of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT) and heat shock protein (HSP)70 and release of high-mobility group box 1 (HMGB1) and adenosine triphosphate (ATP). These DAMPs are able to stimulate DC maturation. Radiation induces interferon (IFN)γ production which plays a role in the accumulation of $CD8^+$ T cells and restores the limited recruitment of Th1-polarized lymphocytes. IFNγ also induces the expression of the adhesion molecules vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on tumor vasculature, facilitating T cell adhesion before transmigration. Radiotherapy stimulates the secretion of CXCL9 and CXCL10, which are known to be important T cell chemo-attractants with an antiangiogenic effect. Furthermore, activation of the ceramide pathway in response to hypofractionated radiotherapy triggers vascular endothelial cell apoptosis. Finally, the expression of the co-stimulatory molecule CD80 on DCs has also been found to be increased by radiation and could therefore shift the balance toward T cell activation.

STAT3 signaling and stimulates the antigen presenting capacity of DCs which results in increased T cell proliferation.[114] Therefore, combination of radiotherapy with bevacizumab might promote the formation of a radiation-induced antitumor immune response in patients with RCC. Since bevacizumab stimulates DC maturation and T cell priming and increases radiation sensitivity, we suggest that bevacizumab should be administrated prior to radiotherapy.[96]

Immunotherapy

The combination of radiotherapy with immunotherapies that possess

Table 2. General working mechanism of approved targeted therapies and their effect on immune cells

Drug	General working mechanism	Effect on the immune system	Refs.
Sunitinib	Blocks multiple tumor-associated tyrosine kinases, including VEGFR and PDGFR and c-kit tyrosine kinases	Immunostimulatory: Blocks STAT3 Decreases numbers and effectiveness of MDSCs and Treg cells Stimulates T cell priming by DCs Blocks VEGF signaling Reduces the expression of co-inhibitory molecules PD-1 and CTLA-4	85–89,92
Sorafenib	Blocks multiple tumor-associated tyrosine kinases, including VEGFR and PDGFR and c-kit tyrosine kinases	Immunostimulatory: reduces Tregs, decreases NK cell inhibition, stimulates pro-inflammatory activity of macrophages Immunosuppressive: prevents upregulation of co-stimulatory molecules, reduces T cell proliferation, lowers cytokine secretion by DCs	92,94–96
Pazopanib	Blocks multiple tumor-associated tyrosine kinases, including VEGFR and PDGFR and c-kit tyrosine kinases	Unknown	97
Axitinib	Blocks multiple tumor-associated tyrosine kinases, including VEGFR and PDGFR and c-kit tyrosine kinases	Immunostimulatory: Reduces Tregs Reduces MDSCs	98,99
Temsirolimus and Everolimus = mTOR inhibitors	Blocks mTOR pathway	Immunostimulatory: enhances $CD8^+$ T cell activation, enhance IFNγ production, enhance $CD8^+$ T cell differentiation into memory T cells and decreases IDO expression Immunosuppressive: augments the responsiveness of Tregs to antigen	85,102
Bevacizumab	Blocks angiogenesis	Immunostimulatory: Blocks STAT3 Increases DC maturation Shifts DC differentiation toward mature DCs instead of MDSCs Increases DC priming of T cells	85

Summary of the most important immunomodulatory properties of approved targeted agents in the treatment of renal cell carcinoma. The immunomodulatory properties of not all the targeted agents have been thoroughly studied already.
Abbreviations: VEGFR: vascular endothelial growth factor receptor, PDGFR platelet derived growth factor receptor, STAT3: signal transducer and activator of transcription 3, MDSCs: myeloid-derived suppressor cells, Tregs: regulatory T cells, DC: dendritic cell, VEGF: vascular endothelial growth factor, PD-1: programmed cell death protein-1, CTLA-4: cytotoxic T lymphocyte associated protein 4, NK cell: natural killer cell, mTOR: mammalian target of rapamycin, *IFN*γ: interferon γ, IDO: indoleamine-2,3-dioxygenase.

synergistic immunomodulatory properties might also be promising.

Since IL-2 is known to stimulate Th1 responses and treatment with high-dose IL-2 occasionally has been observed to induce complete responses in patients with RCC,[6] combining it with radiotherapy may improve clinical effects. A phase 1 study evaluating the combination of SBRT and IL-2, could not detect any dose-limiting adverse effects related to SBRT. Furthermore, response to the combination therapy was correlated to an increased frequency of proliferating early effector $CD4^+$ memory T cells in the peripheral blood.[13]

Preclinical and clinical evidence suggest that inhibition of CTLA-4, a known inhibitory competitor for the co-stimulatory molecules CD80 and CD86,[115] might increase the stimulation of antitumor T effector cells. Ipilimumab, an anti-CTLA-4 antibody, was able to induce tumor regression in 10% of patients with metastatic RCC in a phase II study.[116] Since anti-CTLA-4 antibodies decrease co-inhibitory signaling, they might also be able to increase the strength of radiotherapy-induced T cell stimulation. In a murine carcinoma model, the combination of anti-CTLA-4 treatment and radiotherapy was observed to inhibit tumor growth through the formation of a stable interaction between TILs and tumor cells. This stable interaction was largely due to the improved formation of a NKG2D-mediated immunological synapse, complementing weak stimulatory signals from the tumor cells.[117] There are already clinical cases and a phase I/II clinical trial[69] describing an immune-mediated abscopal effect in melanoma patients receiving a combination of high-dose radiotherapy and ipilimumab.[11,12] We suggest that anti-CTLA-4 antibodies should be administered before radiotherapy since they stimulate the removal of Tregs and continued following radiotherapy to prolong the proliferation of antitumor T effector cells.[96]

Since resistance to the combination of HFRT and ipilimumab in metastatic melanoma patients was correlated to an upregulation of PD-L1, addition of PD-L1 blockade might reverse T cell exhaustion and prevent resistance to the combination therapy. Importantly, preclinical evidence suggests that the combination with radiotherapy is mandatory as dual checkpoint blockade alone proved to be inferior.[69] As previously described, the co-inhibitory molecule PD-L1 suppresses T cell responses in RCC, by binding PD-1, and could shift the balance toward tumor progression.[29] Furthermore, the expression of PD-L1 in RCC is associated with aggressive tumor behavior and poor outcome.[39,40] Blocking PD-1 pathways, therefore, has the

potential to increase antitumor immunity in RCC patients. Blockade of PD-1 induced responses in 27% of patients with RCC in a phase 1b study[118] and response rates were higher in patients with greater percentages of TILs and PD-L1 expression. Preclinical data confirm that HFRT in combination with anti-PD-1 and anti-PD-L1 treatment synergistically promote antitumor immunity.[19,20] In RCC tumors with low inflammation, HFRT might create a more permissive tumor microenvironment, thereby increasing response rates to anti-PD-1/PD-L1 treatment in otherwise non-responding patients. As blockade of PD-1 pathways prevents T cell exhaustion, we suggest it should be administrated directly following radiotherapy. Finally, combinations of radiotherapy with inhibitors of B7-H4 and TIM-3 have not yet been investigated, but are interesting options considering their importance in the antitumor immune response as described above.

Conclusion

RCC is considered an immunogenic tumor, but uses several immune suppressive mechanisms to shift the balance from tumor immune response toward tumor growth. Radiotherapy tries to shift the balance back. However, radiotherapy alone is unlikely to induce persistent antitumor immunity. Therefore, the combination of radiotherapy with drugs having synergistic immunomodulatory properties holds great promise in preventing the immune escape in RCC and might result in superior therapeutic responses. Consequently, prospective trials examining these combinations hold great potential. It should be considered that HFRT might increase the risk of inflammatory reactions. Therefore, phase I trials, assessing the safety of these novel combinations, are essential. In addition, preclinical evidence suggests that high-dose radiation, such as typically delivered by HFRT, results in increased antitumor immunity. Preclinical data also indicate that fractionated radiotherapy might be preferable to single dose radiation. However, these findings need to be confirmed in clinical studies. Besides the optimal HFRT pattern, it is just as important to determine the optimal timing of each treatment combination. Since the optimal treatment sequence, leading to maximum immunologic and clinical benefit while maintaining tolerable toxicities, may vary depending on the specific type of agent used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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