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Chapter 9

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Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cellular Cytotoxicity
APC	Antigen Presenting Cells
CAR	Chimeric Antigen Receptor
CDC	Complement-Dependent Cytotoxicity
CEA	CarcinoEmbryonic Antigen
CpG	Cytosine-phosphate-Guanine motif
CR	Complete Response
CRT	Calreticulin
CRu	Unconfirmed Complete Response
DAMP	Damage-associated molecular pattern molecules
DC	Dendritic Cells
Flt3-L	Fms-related tyrosine kinase 3 - Ligand
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
Gy	Gray
HMGB1	High Mobility Group Box 1
ICAM-1	InterCellular Adhesion Molecule 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IU	International Unit
LFA-3	Lymphocyte Function- Associated Antigen 3
mAb	monoclonal Antibody
MHC	Major Histocompatibility Complex
MIP1α	Macrophage Inflammatory Protein 1α
MM	Multiple Myeloma
MTD	Maximum Tolerated Dose
NHL	Non-Hodgkin B-cell Lymphoma
NK	Natural Killer
NKG2D	Natural Killer Group 2D receptor
ORR	Overall Response Rate
PBMCs	Peripheral Blood Mononuclear Cells
PR	Partial Response
RAIT	Radioimmunotherapy
SD	Stable Disease
TAA	Tumor Associated Antigens
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor α
VCAM-1	Vascular Cell Adhesion Molecule-1

Abstract

Radiation therapy has long been considered as immunosuppressive, therefore its impact on the immune system and other aspects which could be involved in raising efficient anti-tumor immune responses have been neglected. However the recent demonstration of the immunogenic properties of ionizing radiation is rapidely modifying the radiation oncology field and it also opens new and promising perspectives for the development and improvement of radioimmunotherapy. In this chapter, we first review the immunogenic properties of irradiation before discussing available evidence of the benefits of radiation therapy and immunotherapy combinations in the context of lymphoma.

Introduction

The use of ionizing radiation in cancer treatment armamentarium has become predominant as over half of the patients developing a tumor are now treated with irradiation during the course of their treatment [1,2]. Radioimmunotherapy (RAIT) remains a small fraction of such therapy despite the demonstration of its efficacy and safety in non-Hodgkin B-cell lymphoma (NHL) [3] and the promising results obtained in specific clinical settings of solid tumors [4,5]. For decades, research into improving RAIT have focused almost entirely on the approach itself, and significant progress have been made in humanization of monoclonal antibodies, development of new vectors, new radionuclides, more stable chelates, new delivery systems, better dosimetric models and definition of new target antigens. In parallel, radiobiological studies have addressed the direct and indirect (bystander) effects of ionizing radiation on the tumor cells to some extent, but for long, the complex interactions between the tumor, its microenvironment, inflammation and the immune system have been ignored by the field.

Among the established hallmarks of cancer are resistance to cell death, evading immune system, and creation of a tumor microenvironment [6]. Multiple immunosuppressive mechanisms are implemented by tumors to escape immune recognition and destruction which involve the tumor itself and its microenvironment [7]. For long, ionizing radiation and RAIT, often used in combination with chemotherapy, were also considered as immunosuppressive treatments. As a result, studies largely failed to appreciate the effects of ionizing radiation on immunity despite the fact that clinical cases of "abscopal effect" after radiotherapy were reported and that some patients achieved long term CR after a single dose of RAIT. The elucidation of the mechanisms underlying the off-target effects after irradiation and the demonstration that immunogenic tumor cell death is inducible by ionizing radiation have changed the perception of radiation therapy. And at a time where numerous and promising new immunotherapies are emerging, it also opens a new era of combination therapy options where the immunogenic effects of radionuclides could be a key factor for the success of treatment.

Ionizing Radiation and anti-tumor immunity

Abscopal effect: an aftermath of ionizing radiation involving the immune system

The abscopal effect, originally described by Dr. RH. Mole in 1953 [8], comes from the latin "ab" meaning "far" and "scopos" which means « target ». The abscopal effect refers to effects outside the irradiation field of the target, which can result in anti-tumor responses and the elimination of non-irradiated tumor cells. More generally, abscopal effect stands for any systemic effect that is observed after a local treatment. A growing set of preclinical and clinical data point out that the therapeutic potential of ionizing radiation does not reflect only the antiproliferative and cytotoxic activities of X or γ radiation but also implies bystander and systemic (distant) effects [9-11].

This effect is rarely observed in the clinic, however, it has been documented in patients with hematological malignancies like lymphoma [12,13], leukemia [14,15], and also in patients with a wide variety of solid tumors [16-21]. Investigations of the possible mechanisms underlying the abscopal effect in animal models have demonstrated that it might be possible to favor the development of such event by modulating the immune system. Chakravarty et al. have shown in a syngeneic and immunocompetent metastatic lung cancer model that combining radiotherapy and injection of Flt3-Ligand (Flt3-L), a growth factor for immune cells and especially for dendritic cells (DC) [22], reduced lung metastases, significantly improved survival and resulted in 56% of disease free animals. Notably, the abscopal effect was abolished in nude mice lacking T-lymphocytes which demonstrates that this systemic anti-tumor effect is mediated by the adaptive immune system [23]. In a comparable study using a syngeneic immunocompetent breast carcinoma mouse model, Demaria et al. demonstrated that irradiation of a tumor implanted on the right flank combined with systemic injection of Flt3-L induced regression of a second tumor engrafted on the left flank of the animals. The combined treatment was ineffective if the second tumor was from another cell type than the irradiated breast carcinoma one or if the mice were deficient in T-cells [24]. So, in those studies, the abscopal effect was promoted by a tumor specific

response relying upon T-lymphocytes. Another study, combining radiotherapy and injection of ECI301, a recombinant MIP1α chemokine showed the involvement of $CD4^+$ and $CD8^+$ T-cells or natural killer cells (NK) depending on the tumor type [25]. In addition, in this study, irradiation of healthy tissues did not promote any abscopal effect, which suggests that radiation-induced tumor cell death or damages are mandatory for the development of such anti-tumor response.

All these data indicate that ionizing radiation can initiate immune responses involving DC, NK and T-cells with systemic effects on tumor growth, highlighting the importance of the interactions between ionizing radiation and the immune system to foster an efficient anti-tumor response. It is therefore crucial to understand how irradiation acts on the tumor, its microenvironment and on the immune system.

Immunological effects of ionizing radiation

Cancer development is strongly influenced by inflammation, innate and adaptive immunity and the very complex interrelationships and modulations between those different components can either lead to tumor growth or to tumor regression. Although ionizing radiation has been mainly used to treat cancer through its direct cytotoxicity, there is now evidence that irradiation also modulates inflammation and the immune system at multiple levels including production of reactive oxydative species, generation of danger signals, release of cytokines and other soluble factors, activation of immune cells and induction of various type of cell death. Depending on the low or high dose of irradiation, the generation of acute or chronic inflammation, these underlying mechanisms can have immunosuppressive or immunostimulatory effects [26-30]. It is therefore important to understand the links between ionizing radiation and the immune response to cancer to try and develop treatments that could limit the immunosuppressive effects of radiation while boosting anti-tumor immunity. In the present chapter, we will focus on the studies that have shown the various mechanisms by which radiotherapy and chelated radionuclides might boost the immune system.

Modulation of tumor cell immunogenicity

Although radiation therapy has been used traditionally to destroy tumor cells, the dose received by a number of cells within a given tumor mass, is too low to cause their destruction (event which is further emphasized in hypoxic areas). Several preclinical studies have shown however that such low radiation doses are capable of inducing phenotypic changes in neoplastic cells, which help their recognition and their destruction by the immune system. The molecules described to be up-regulated at the surface of tumor cells by such ionizing radiation doses are Tumor-associated antigen (TAA), MHC-Class I molecules, the death receptor Fas (CD95), NKG2D ligands, the costimulatory molecule B7-1 (CD80) and adhesion molecules including LFA-3 (CD58 or lymphocyte function-associated antigen 3) and ICAM-1 (Intercellular adhesion molecule 1) [31-37], Fas, MHC-Class I molecules, ICAM-1 and TAA such as CEA (Carcinoembryonic antigen) and the mucin glycosylated phosphoprotein Muc-1 have also been shown to be upregulated on tumor cells after irradiation with β-particle emitters ^{153}Sm [38] and ^{90}Y [39]. Interestingly, the B7-1 costimulatory molecule is also up-regulated in B-cell lymphoma following irradiation [40]. All these molecules are known to play a role in tumor destruction by cytotoxic $CD8^+$ T-cells and the development of an anti-tumor immune response.

One of the major consequences induced by tumor destruction after irradiation is the exposure of a large amount of TAA to the immune system. The delivery of tumor antigens is done because of tumor cell necrosis, apoptosis or the release cell fragments [41,42]. The increased availability of those TAA allows circulating DC to capture, present and then induce a specific T-cell response against the tumor. One study demonstrated that irradiated tumors expressing low levels of antigen, as MHC-peptide complex, provide a sufficient amount of TAA to allow the destruction of tumor cells by cytotoxic $CD8^+$ T cells [43].

Additionally, cell death induced by irradiation may allow the release of new TAA that will be captured by the DC in the tumor microenvironment and lymph nodes. Reits *et al.* have demonstrated that radiotherapy increases, within tumor cells, the repertoire of peptides available for MHC-Class I molecule presentation to cytotoxic $CD8^+$ T-cells. This broader repertoire does not only result from an increased degradation of the existing proteins, but

also in activation of the mTOR pathway, which leads to increased protein translation and thus the creation of a new peptide repertoire [36].

Immunogenic cell death

All cell deaths do not promote an immune response. The immune system is able to distinguish between an immunogenic death and a non-immunogenic death which results either in the activation of adaptive immunity or in the persistence of tolerance. Tumor cell death induced by ionizing radiation can be quite immunogenic and potentiates the presentation of TAA by DC to activate T-cells and the development an immune response [44,45]. Several molecular danger signals, DAMP (Danger associated molecular pattern) have been identified among the main features of an immunogenic cell death.

1) The translocation of calreticulin (CRT), an endoplasmic reticulum chaperone protein, to the outer face of the plasma membrane of the cells undergoing apoptosis is an important "eat-me" signal for the professional antigen-presenting cells (APC) such as DC [46-49].

2) The release of HMGB1 (high mobility group box 1, a non-histone protein associated to chromatin and in the cell nucleus) by dying cells will transmit proinflammatory signals after binding to TLR4 (Toll-like receptor 4) [50-52]. Those DAMPs, CRT and/or HMGB1 are induced following exposure of tumors cells to external irradiation [46,53] but also to α-particle emitter like ^{213}Bi [54].

3) The third signal is the release of ATP from the cells undergoing apoptosis. ATP functions both as a "find-me" signal to professional APC and as a potent pro-inflammatory signal through binding to the P2X7 purinergic receptor thereby triggering inflammasome activation [55-60]. Its release has not been demonstrated following irradiation yet. But, since autophagy is necessary for the release of ATP [61], and that ionizing radiation promotes autophagy [62-64], the third signal may be generated by ionizing radiation when autophagy precedes cell death.

4) Heat-shock proteins, especially HSP70, are expressed at the cell surface but also released during tumor cell stress or cell death after exposure to ionizing radiation like X-rays [65,66] or α-particles [54], and stimulates innate and adaptive immune responses mediated by NK, DC cells and T-cells through antigen cross-presentation [67].

All these experimental data support that tumor cell stress and death resulting from ionizing radiation, are sensed by the immune system as "danger" signals which in turn can stimulate an immune response.

Secretion of cytokines

Radiotherapy also modifies tumor microenvironment by generating a proinflammatory environment [29,68]. For example, CXCL9, CXCL10 and CXCL16 chemokines promote the recruitment of $CD8^+$ effector T-cells, and Th1 helper $CD4^+$ T-cells and are induced following ionizing radiation in various types of tumors [69-71]. Irradiation also promotes the production of proinflammatory cytokines such as IL-1β, type I and type II IFN (IFN-α, -β, -ω and IFN-γ) and TNFα, involved in the cytotoxic and cytostatic effects on cancer cells after irradiation, including tumor regression, inhibition of proliferation, tumor cell death and immune cell recruitment [68,69,72-75]. Such an inflammatory context after radiotherapy may facilitate the initiation and amplification of an anti-tumor immune response.

Blood vessels

After antigen activation, T-lymphocytes must reach and infiltrate tumors. Ionizing radiation can promote this process in many ways. For example, the radiation-induced remodeling of abnormal tumor vessels results in an effective tumor infiltration by anti-tumor T-cells following adoptive transfer in a transgenic mouse model of insulinoma [76]. In an experimental model of melanoma, increased expression of VCAM-1 adhesion molecule (Vascular cell adhesion molecule-1) induced by ionizing radiation boosts T-cell infiltration of the tumor [77].

Overall, these data demonstrate that ionizing radiation can drive an immune response to cancer in a number of ways, it is therefore important to consider these beneficial effects while designing cancer treatments. This also constitutes a strong rationale for combining radiation therapy with immunotherapy in order to improve current therapies.

Combining Ionizing Radiation and Immunotherapy

Preclinical evidence in hematopoietic cancer models

Radiotherapy and immunotherapy

By taking advantage of the immunogenic properties of ionizing radiation described in the previous sections, numerous preclinical studies have successfully combined radiotherapy with immunotherapies in solid tumors to obtain impressive responses (reviewed in [68],[78]). Here, we will focus on the available data for radiotherapy and immunotherapy combinations in preclinical models of lymphoma.

In 1997, the group of Batterman in Utrecht assessed the efficacy of supplementing local radiotherapy with locoregional low-dose injection of interleukin-2 (IL-2) in a subcutaneous model using the spontaneously arisen SL2 T-lymphoma [79,80]. IL-2 is a potent T cell activator, which has proved its efficacy and safety in the SL2 preclinical model [81,82] as well as in human patients with Hodgkin's and non-Hodgkin's lymphoma [83-85]. In their SL2 model, they demonstrated that the combination of local radiation therapy (20Gy) followed by 2 cycles of 4-day injection of IL-2 (7000 IU/day) peri-tumorally led to 93% of long-term disease-free survival compared to 17% with radiation alone ($p<0.0001$). Additionally, in a setting where they inoculated mice with 2 subcutaneous tumors (one on each thigh), they showed that treatment of one tumor with irradiation and IL-2 led to anti-tumor effects in the second, untreated tumor in 80% of mice and local response was increased to 100%. When the second, non-irradiated tumor was also treated with peritumoral IL-2, both local and distant responses increased to 100% and disease free survival reached 70%. Interestingly, in an attempt to reproduce more closely the radiotherapy scheduled applied in clinic, they reproduced the experiments with a fractionated regimen of radiotherapy (2.5Gy/day for 10 days). Fractionated therapy was far less efficient than single dose regimen and led to only 12% local response and no disease-free survival. However, even in these settings, combination with IL-2 therapy improved treatment outcome up to 90% local response and 10% disease-free survival. The authors postulated that the selected

fractionation schedule was not optimal for the SL2 model, possibly because it is a highly aggressive tumor that metastasizes quickly and therefore needs a rapid rather than prolonged treatment. They did not observe any toxicity related to IL-2 and therefore showed that IL-2 therapy was both safe and efficient in improving both local and systemic response to radiotherapy. This study was the first to demonstrate the potential of radiotherapeutic association with immunotherapy in a lymphoma model.

In 2003, the group of Illidge in Manchester tested the combination of total body irradiation with an agonistic anti-CD40 antibody on the murine A31 and BCL_1 B-lymphoma models [86]. CD40 is a co-stimulatory protein expressed on APC such as DC, B-cells, monocytes and macrophages, and participates to their activation. Interestingly, CD40 is also expressed on various tumors, in particular B-cell lymphomas. Therapeutic treatment of lymphoma using agonistic antibodies targeted to CD40 can have multiple complementary anti-tumor effects. Indeed, activated APC are able to generate antigen-specific T-cell responses while the targeting of $CD40^+$ tumor cells can have a direct tumoricidal effect by inducing apoptosis [87]. Interestingly, it has also been shown that anti-CD40 agonists can sensitize multiple myeloma (MM) and B-lymphoma cell lines to γ-radiation *in vitro* [88]. Anti-CD40 antibodies are the object of several ongoing clinical trials in leukemia, MM and NHL [89]. Recently, a phase II trial using an agonistic anti-CD40 as a monotherapy on patients with relapsed diffuse large B-cell lymphoma demonstrated low toxicity but only modest efficacy [90], suggesting that these therapies need combination with other cancer treatment modalities to reach their full potential. In this study, Honeychurch *et al.* showed that radiotherapy (5Gy) with anti-CD40 (1mg) led to an impressive long-term disease free survival of 100% of treated mice as opposed to 0% for single-agent treatments in both orthotropic models of lymphoma (A31 and BCL_1). Mice treated with a single treatment survived slightly longer than untreated controls but eventually all succumbed to their lymphoma. The effect of the combination treatment was not due to combined cytotoxicities as the anti-CD40 Ab did not induce cell death of lymphoma cell lines, nor did it sensitize the tumor cells to radiation. Instead the effect was mediated by a strong specific $CD8^+$ T-cell response which was long-lasting, as demonstrated by protection against later tumor challenge (therefore suggesting the onset of immune memory), and transferable to naive recipients. Interestingly, they observed that the combined therapy was less efficient on smaller tumor loads, indicating that radiation

cytotoxicity needs to liberate a critical amount of TAA to allow anti-CD40 activated APCs to mount an efficient immune response. This study therefore brought to light mechanisms by which radiation therapy can synergize with immunotherapy by simultaneously reducing tumor load and providing antigens for an optimal immune response against lymphoma.

More recently, the same group published a study testing the association of local radiotherapy with the TLR7 agonist R848 in subcutaneous B-cell (A20) and T-cell (EL4, EG7) lymphoma models [91]. TLR7 is a pattern recognition receptor that is expressed on the endosomal membranes of DC (mainly plasmacytoid DC) and B-cells [92]. It has been shown to induce DC, B-cell and T-cell activation *in vivo* and lead to an effective anti-tumor cytotoxic T-cell response when combined with doxorubicin in a murine T-lymphoma model [93]. Studies also demonstrated that *ex vivo* stimulation of cutaneous T-cell lymphoma patients' PBMCs with TLR7 agonists induced the secretion of IFN-α, IFN-γ and led to NK cell and T-cell activation *in vitro* [94,95]. A phase II clinical trial also showed that treatment with the TLR7 agonist 825A was well tolerated in patients with refractory hematological malignancies and associated with evidence of immune activation [96]. Finally, treatment with TLR7 agonists has also been shown to potentiate the efficacy of radiotherapy in preclinical models of solid tumors [97,98]. In their study, Dovedi *et al.* first demonstrated that systemic injection of R848 appeared well tolerated and led to increased levels of IL-6, IFN-γ, TNF-α and IL-5 and activation of B and T-lymphocytes in EG7 tumor-bearing mice. They then showed that the combination of local radiotherapy (10Gy) and intravenous injection of R848 (3mg/kg) could lead up to 75% of long term survival as compared to only 25% with either monotherapy. This improved outcome was not due to combined cytotoxicities as R848 did not sensitize EG7 tumor cells to radiation. Instead the effect was mediated by a specific $CD8^+$ T-cell response and led to the generation of long-lived specific memory T-cells. Depletion of B-cells with anti-CD20 Ab also showed that the efficacy of treatment combination in the T-lymphoma model was independent on B-lymphocytes. Interestingly, they showed that although radiotherapy alone induced the release of HMGB1 by tumor cells and led to their phagocytosis by DC, it was not sufficient to trigger DC activation. Addition of R848 was required to induce up regulation of CD80 and CD86 after irradiation of tumor cells, suggesting that both radiation and TLR7 stimulation were required to mount an efficient T-cell response. Finally they showed that using fractionated radiation regimen (5x2Gy) led to

better responses in both EL4 T-lymphoma and A20 B-lymphoma models, leading to 100% of long-term survival. Interestingly, weekly injections of R848 for 5 weeks was more efficient than a single-dose, suggesting that repeated irradiation and immune stimulation could act as immune boosters for anti-tumor immunity and prevent the re-establishment of a suppressive tumor environment.

Although one could question the use of immune adjuvants in diseases originating from immune cells and hypothesize that stimulation with IL-2 in a T-cell malignancy could sustain tumor growth, or that CD40 and TLR agonists could promote B-lymphoma cell survival, those 3 studies demonstrate no such effect. On the contrary, the immunostimulants tested in these hematological malignancies all improve survival outcome with no apparent induced toxicity. These studies also clearly demonstrate that combining the immunogenic properties of ionizing radiation associated to tumor antigen release with immune stimulation can lead to efficient anti-tumor immunity. This immunity seems mainly driven by DC activation of specific cytotoxic $CD8^+$ response, leading to the establishment of long-lasting immune memory. B cells do not seem to participate in the observed anti-tumor responses but other immune populations could potentially be involved and further investigations should address this possibility. Altogether, those findings warrant further trials of various immune-modulatory molecules and radiotherapy schedules in order to find the best combinations for the treatment of lymphomas.

Radioimmunotherapy and immunotherapy

To date, very few studies have investigated combination therapy of RAIT and immunotherapy in preclinical tumor models. To our knowledge, Chakraborty *et al.* have been the first to report such combination, in a study where RAIT was combined with cancer vaccination to treat human CEA expressing murine carcinoma in CEA transgenic mice [39]. This group had previously demonstrated that tumor cells were more susceptible to T-cell killing after exposure to non-lytic doses of external radiation therapy [34]. They thus hypothesized that delivering RAIT to a tumor mass might have the same effect. To this end, they used an ^{90}Y-labeled anti-CEA mAb and a recombinant vaccine containing the CEA, B7-1, ICAM-1 and LFA-3 genes. They observed that survival of tumor engrafted mice was

significantly increased after a single dose of RAIT in combination with vaccine compared to vaccine or radiolabeled mAb alone. Analysis of the immune response showed that in mice receiving the combination therapy, the amount of CEA-specific $CD8^+$ T-cells infiltrating the tumor was significantly increased over vaccine alone. Interestingly, the animals cured after treatment with the combination therapy demonstrated a broadening in the anti-tumor immune response, since in addition to $CD4^+$ and $CD8^+$ T-cell responses against CEA which was encoded by the vaccine, they also observed T-cell responses against other TAA [39]. More recently, our group investigated the possibilities to promote an efficient and long-lasting anti-tumor response by combining α-RAIT and adoptive transfer of tumor specific T-lymphocytes in a multiple myeloma murine model expressing the TAA CD138 and ovalbumine (OVA) [99]. The therapeutic efficacy was evaluated by treatment with a ^{213}Bi-labeled anti-CD138, followed by an adoptive transfer of OT-I cells, which are OVA-specific $CD8^+$ T-cells. We observed a significant tumor growth control and an improved survival in the animals treated with the combined treatment over radiolabeled mAb or OT-I cell transfer alone. Both studies demonstrate that not only radiotherapy by also RAIT in combination with immunotherapy promotes effective antitumor response, which may have implications in the design of future clinical trials.

Clinical Evidence

Radiotherapy and immunotherapy

The occurrence of abscopal effects after radiotherapy without concurrent immune stimulation is a rare event in the clinic. Although this may be due in part to underreporting, it is likely a consequence of tumor-derived immunosuppression and suggests that the threshold for anti-tumor immune activation is high in clinical settings. Notably, most of the reported cases of abscopal effect occurred in renal cell carcinoma, melanoma and lymphoma [100], indicating that these cancers are the most likely to benefit from combination with immunotherapy. Clinical trials have assessed the efficacy of various immunotherapies in combination with radiotherapy [101] and many trials are still ongoing [102] but most of the work has been performed on patients with solid non hematopoietic tumors, in particular melanoma [103].

The Stanford group is currently investigating the potency of combined treatments in patients with lymphoma in three trials testing the efficacy of radiotherapy associated with the TLR9 agonist SD-101 (NCT02266147, NCT01745354) and the anti-CTLA4 mAb Ipilimumab (NCT02254772). They also already published one study on the subject [104]. In this study, 15 patients with recurrent stage III or IV low grade B-cell lymphoma were treated with local radiotherapy combined with intra-tumoral TLR9 agonist (CpG PF-3512676) injection at one site only while distant tumor sites were evaluated for response. There was no treatment limiting adverse event and all patients completed the full course of therapy. They obtained 27% of overall objective response rate at the distant untreated sites with 1 CR lasting 61 weeks, 3 PR lasting 20, 64 and over 111 weeks and 8 SD. Tumor reactive $CD8^+$ T-cells were detected in peripheral blood of several responding patients but no significant correlation between T-cell immunity and clinical response was found. Interestingly, some patients' tumor were able to induce a T-reg phenotype in autologous $CD4^+$ T-cells *in vitro* and those patients had significantly shorter progression free survival. This suggests that tumor-derived immunosuppression may be the main obstacle to treatment efficacy. These preliminary results warrant confirmation, nevertheless, this is the first study to demonstrate that association of radiotherapy with intratumoral injection of an immunostimulant can be safe and trigger efficient systemic responses in patients with lymphoma.

Radioimmunotherapy and immunotherapy

Only one trial tested the combination of RAIT with an immune stimulant so far [105] and the same team also recently completed a trial in which they tested the association of ^{90}Y-ibritumomab tiuxetan with rituximab, G-CSF and IL-11 (NCT00012298) but the results have not been published at the time this manuscript was produced. In the former study, 30 patients with relapsed or refractory $CD20^+$ B-cell NHL have been treated with ^{90}Y-ibritumomab tiuxetan (0.4 mCi/kg) in association with rituximab (250 mg/kg) and CpG 7909, a TLR9 agonist. Four doses of CpG 7909 have been tested (0.08, 0.16, 0.32 and 0.48 mg/kg) without reaching the MTD, demonstrating the safety of treatment. They obtained an impressive ORR of 93%, with 63% CR/CRu and 30% PR, and responses were durable with a median time to progression of 42.7 months. T-cell responses have not been evaluated in this

study but analysis of serum cytokines showed a statistically significant decrease in IL-10 and TNFα and increase in IL-1ß, consistent with the development of an immune response. It is important to note that IgG themselves can trigger immune responses. In particular, chimeric IgG such as rituximab have been shown to trigger CDC and ADCC *in vitro*. However the extent to which these phenomena participate in rituximab efficacy *in vivo* is still unclear [106]. In this trial, measurement of ADCC induced by rituximab was very variable between subjects and did not show any statistically significant difference between groups. Although they warrant confirmation, these phase I results are extremely encouraging. Nevertheless, further studies should assess the mechanisms and importance of the immune response in the efficacy of this treatment combination.

Based in part on the observations that Ab treatments could induce anti-tumor responses through the induction of CDC, ADCC but also through Ab-targeted tumor antigen cross-presentation [107], it has been postulated that RAIT combined with maintenance anti-CD20 Ab treatment may trigger protective T-cell responses in lymphoma patients [108]. There has been several studies testing the efficacy of ^{90}Y-ibritumomab tiuxetan after treatment with rituximab and chemotherapy [109-111] and all obtained very good response rate. However, only Jacobs *et al.* used rituximab as a maintenance treatment after RAIT and none of these trials assessed the presence of an anti-tumor immune response. Besides, chemotherapies used in those studies, such as fludarabine, cyclophosphamide and prednisone, can induce important immunosuppression and lymphopenia and may therefore limit the induction of an effective immune response against lymphoma.

Overall, results obtained from combination of RAIT and immune-related treatments in patients with lymphoma are very encouraging. However, there are still very little data available on the implication of the immune system in patient responses to these treatments. Notably, it will be of prime importance in the future to assess the effect of vectors on the immune response to tumors in RAIT.

Conclusions and perspectives

Within the past two decades, important advances have been done in our knowledge of the complex interplay between ionizing radiation, inflammation and the immune system. The immunogenic properties of irradiation are now clearly demonstrated and, even though most of the data comes from external radation therapy, the few reports using radionuclides and RAIT strongly support that α- and β-particle emitters can also drive an anti-tumor immune response. More importantly, these immunogenic aspects have opened a new era of research in radiation oncology by the initiation of clinical trials combining ionizing radiation and immune-based therapy. Notably, preliminary results in patients with lymphoma are very encouraging. Combination therapies appeared safe and, to date, neither limiting adverse effects nor cumulative or overlapping toxicity were observed in any of the trials. These trials are initial investigations and there are still a lot of parameters to optimize in order to overcome tolerance and maximize the synergy of combined therapies towards tumor cell destruction. In that aspect RAIT may be of great interest in the treatment of disseminated and poor prognostic metastatic solid cancer as this approach will generate locally but at hundreds tumor sites: high dose to the tumor and cell death, production of ROS, release of TAA, acute inflammation and other immunogenic effects which should represent an ideal springboard for the combined immune-based therapy. In order to develop a systemic anti-tumor response, and to ultimately achieve an immune memory and long term protection, future directions will have to address which radionuclide, treatment schedule (single vs fractionation) and dose to use for different pathologies and different patients. Some clinical studies have already been completed but several more are about to start exploring radiation therapy in combination with immunotherapies using growth factors like Flt3-ligand or GM-CSF [112] or checkpoint inhibitors such as anti-CTLA4 (Ipilumumab), anti-PD-1 (Nivolumab) [102] or anti-PD-L1 (Atezolizumab) mAbs. On july 2016, searching the clinicaltrials.gov website for checkpoint inhibitor mAb + radiation gives 41 results: 2 trials for Ipilumumab, 35 trials for Nivolumab , 4 trials for Atezolizumab. Among these 41 clinical trials, all but one use external radiation therapy, the remaining one will use ^{90}Y glass microspheres in hepatocellular carcinoma (NCT02837029). Despite the limited use of radionuclides so far, RAIT in combination with cytokines or immune checkpoints blocker do represent an exciting

option. Several other attractive combination opportunities come from the development adoptive T-cell therapies and Chimeric Antigen Receptors (CAR) (for review [113,114]) and other class of small molecules designed for immuno-oncology treatment (for review [115]). This multitude of options implies to define biomarkers to identify patients who are the most likely to benefit from such combined treatment and especially from the immune-based therapy.

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