Assessing the interactions between radiotherapy and antitumour immunity

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Abstract | Immunotherapy, specifically the introduction of immune checkpoint inhibitors, has transformed the treatment of cancer, enabling long-term tumour control even in individuals with advanced-stage disease. Unfortunately, only a small subset of patients show a response to currently available immunotherapies. Despite a growing consensus that combining immune checkpoint inhibitors with radiotherapy can increase response rates, this approach might be limited by the development of persistent radiation-induced immunosuppression. The ultimate goal of combining immunotherapy with radiotherapy is to induce a shift from an ineffective, pre-existing immune response to a long-lasting, therapy-induced immune response at all sites of disease. To achieve this goal and enable the adaptation and monitoring of individualized treatment approaches, assessment of the dynamic changes in the immune system at the patient level is essential. In this Review, we summarize the available clinical data, including forthcoming methods to assess the immune response to radiotherapy at the patient level, ranging from serum biomarkers to imaging techniques that enable investigation of immune cell dynamics in patients. Furthermore, we discuss modelling approaches that have been developed to predict the interaction of immunotherapy with radiotherapy, and highlight how they could be combined with biomarkers of antitumour immunity to optimize radiotherapy regimens and maximize their synergy with immunotherapy.

Since the approval of the immune checkpoint inhibitor (ICI) ipilimumab, an anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4) antibody, for the treatment of metastatic melanoma in 2011, immunotherapy has revolutionized the management of patients with cancer, with response rates and outcomes that have previously not been achieved with traditional cytotoxic therapies[1,2]. Nonetheless, although some patients have durable responses to immunotherapy, many will ultimately develop progressive disease[3]. Accordingly, interest has been growing in combining immunotherapy with other agents, both systemic and local, to maximize response rates and, in turn, improve patient outcomes. One such modality receiving major research interest is radiotherapy, a widely available and effective treatment that can be precisely tailored to a given tumour target, with extensive safety data being available. Importantly, as radiotherapy has both local and systemic effects on the immune system, the combination of immunotherapy with radiotherapy (immunotherapy–radiotherapy combinations) has the potential to maximize the antitumour immune response and induce durable disease control[4].

Optimism regarding the potential synergy between radiotherapy and immunotherapy has led to a large increase in the number of clinical trials evaluating immunotherapy–radiotherapy combinations; as of May 2019, 361 clinical trials evaluating immunotherapy–radiotherapy combinations with a total enrolment of >28,000 patients have been registered on ClinicalTrials.gov. Moreover, a 2018 review of the landscape of trials evaluating anti-programmed cell death protein 1 (PD-1) and anti-programmed cell death 1 ligand 1 (PD-L1) ICIs found 114 trials involving combinations with radiotherapy that are currently recruiting patients[5].

Many of these immunotherapy–radiotherapy trials are designed to use radiation-induced in situ tumour vaccination to stimulate systemic immune-mediated antitumour effects, even though only a single site (or, in some patients, a few sites) are irradiated[4,6,7]. The rationale for in situ tumour vaccination is that irradiation of the tumour induces immunogenic cell death and, consequently, local release of tumour-derived antigens that promote crosspresentation by dendritic cells to T cells, leading not only to a systemic tumour-directed immune response at all sites (also termed the abscopal effect — irradiation of one site leading to responses outside the irradiated field) but also to long-term immunological memory[8,9]. This priming of the immune system by

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Key points

- Peripheral absolute lymphocyte count is a high-level marker of systemic immune status and is correlated with survival after radiotherapy in multiple tumour types.
- Investigation continues into additional markers of immune status, including circulating lymphocyte subsets, humoral markers, cytokines, and tumour-infiltrating lymphocytes; these markers tend to be highly indication-specific and context-specific.
- A variety of imaging methods based on MRI, single-photon emission CT (SPECT), and PET enable imaging of both the current distributions and dynamics of immune cell populations.
- Mathematical models of the tumour–immune interaction and the influence of radiotherapy on this system can be broadly classified as either systemic or regional-interacting models.
- Both circulating markers and advanced imaging methods will be needed to refine these models based on clinical patient data.

radiotherapy can, in turn, synergize with immunotherapy to enhance the immune response.

The designs of the regimens used in immunotherapy–radiotherapy combination trials are based on results from animal studies, which have confirmed the existence of synergy between immunotherapy and radiotherapy using various combination regimens[10–14]. Crucially, these experiments have also revealed that this synergy depends on the total dose, sequencing, timing, and fractionation of radiation used[14–17]. However, contradictory results have challenged the interpretation of these studies, and the translational relevance from animals to humans is unclear. Experiments involving the combination of anti-CTLA-4 ICIs with radiotherapy have shown major systemic responses to the combination when the drug is given before radiotherapy, as demonstrated using the syngeneic CT26 colorectal tumour-bearing mouse model[16]. By contrast, in a similar study using a syngeneic 4T1 mammary carcinoma mouse model, anti-CTLA-4 antibodies administered after radiotherapy generated the best response[13]. In terms of optimal dosing and timing, such models can be influenced by differences in the intrinsic characteristics of the selected cell line and/or animal model[13,16]; thus, such data cannot be directly translated into clinical trials[18].

Initial interest in immunotherapy–radiotherapy combinations stemmed, in part, from case reports describing the abscopal effect in patients receiving treatment with immunotherapy who also received radiotherapy for palliative purposes[19–22]. Subsequently, prospective phase I and II trials, as well as retrospective series, have reported encouraging results regarding potential synergy between radiotherapy and immunotherapy, with some radiographic and durable responses seen even in heavily pretreated patients[7,23–28]. However, several trials have not demonstrated a substantial improvement in patient survival with immunotherapy–radiotherapy combinations[26]. Accordingly, the question remains as to what underpins this clinical heterogeneity and whether improved biomarkers of antitumour immunity can help us understand these differences in outcomes and, in turn, develop better and more rational combinations. An additional, elusive question is whether computational models based on patient data might enable exploration of the vast number of possible combinations of immunotherapy and radiotherapy (including variations in dose, sequencing, timing, and fractionation) that are impractical to explore in animal experiments, and even less so in clinical trials.

Full realization of the potential of immunotherapy–radiotherapy regimens will require an improved understanding of two important factors — the risk of toxicities, which will enable the safe treatment of patients, and the individual patient's immune response to radiotherapy, which will improve outcomes. The former aspect has been reviewed elsewhere[29]. In this Review, we address the latter aspect and discuss the current landscape of potential biomarkers, both circulating and radiographic, that might indicate a patient's immune response to radiotherapy. We also highlight how tumour modelling can be employed to not only simulate the tumour microenvironment (TME) but also to make the best use of all the information provided by biomarkers to improve the prediction of the interaction between radiotherapy and the immune system at the patient level.

Biomarkers of immune status

Radiotherapy has both immunostimulatory[12] and immunosuppressive[30] effects, which complicates the interpretation of the immune landscape in patients receiving radiotherapy. Recent advances in immunotherapy, particularly the development of ICIs, and a growing interest in the potential synergy between radiotherapy and immunotherapy have increased the importance of identifying reliable biomarkers of immune status and treatment response in patients undergoing radiotherapy with or without concurrent immunotherapy. Potential biomarkers of the immune response among patients undergoing radiotherapy, both circulating (for example, circulating cytokines and other proteins associated with inflammatory and immune responses) and cellular (for example, circulating and tumour-infiltrating lymphocytes), have been identified[31–34]. Characteristics of the ideal biomarker have been described in detail elsewhere[35]. Briefly, the ideal biomarker is: noninvasively accessible (for example, using blood and/or urine sampling or imaging); stable in vivo and after collection; minimally variable by sex, age, ethnic origin, and other variable population-based characteristics; operator-independent; cost-effective; and based on a known biological mechanism. Biomarkers can be prognostic or predictive, correlating with outcomes in a particular disease state irrespective of treatment or with response to a particular therapy, respectively. To date, a number of candidate biomarkers of immune response after and during radiotherapy have been reported, with various advantages and disadvantages related to biomarker characteristics (BOX 1).

Increasing evidence suggests that responses to immunotherapy, radiotherapy, or immunotherapy–radiotherapy combinations cannot be adequately predicted using a single gene or protein, such as those that are commonly used in patient selection for targeted therapies (for example, *KRAS* mutational status to identify patients with colorectal cancer who are unlikely to respond to anti-EGFR therapy). For example, a 2018 meta-analysis of eight randomized controlled trials (including studies in

Box 1 | **Candidate immune-based biomarkers of response to radiotherapy**

Clinical blood count measurements
Examples
- Absolute lymphocyte count (ALC)
- Absolute neutrophil count (ANC)
- ANC:ALC ratio (NLR)
- Platelet:ALC and platelet:ANC ratio

Advantages
- Easily accessible and often available
- Clinically consistent between laboratories
- Robust prognostic data (especially for ALC)

Disadvantages
- Nonspecific to antitumour immunity
- Might be confounded by coexisting infection, frailty, or other comorbidities

Circulating immune cell subpopulations
Examples
- T cell subtypes
- Myeloid-derived suppressor cells
- Tumour-antigen-specific lymphocytes

Advantages
- Easily accessible
- Specific to antitumour immunity
- Might be useful for monitoring the evolving antitumour response

Disadvantages
- Operator-dependent
- Lack of clinical validation

Cell surface markers
Examples
- Programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1)
- FAS ligand
- Tumour-antigen-specific cytotoxic T lymphocytes

Advantages
- Might predict response to immune checkpoint inhibitors

Disadvantages
- Lack of clinical validation
- Possibly confounded in the setting of active anti-PD-1 or anti-PD-L1 therapy

Functional assays of lymphocyte activity
Examples
- In vitro antigen stimulation
- Response to vaccine challenge
- Natural killer cell activity assay

Advantages
- Reflects actual capacity of surviving immune cells
- Can test response to tumour antigens

Disadvantages
- Expensive
- Operator-dependent
- Lack of clinical validation

Tumour-infiltrating lymphocytes
Examples
- $CD8^+$ tumour-infiltrating lymphocytes (TILs)
- $CD8^+PD\text{-}1^+$ TILS
- Tumour-antigen-specific TILs

Advantages
- Highly specific to antitumour immunity

Disadvantages
- Limited sample availability (requires invasive sampling access)

Circulating inflammatory markers
Examples
- C-reactive protein (CRP)
- Lactate dehydrogenase
- Albumin
- CRP:albumin ratio (modified Glasgow Prognostic Score)

Advantages
- Widely available in the clinical setting
- Generally consistent between laboratories
- Extensive prognostic data available

Disadvantages
- Nonspecific for antitumour immunity
- Confounded by comorbidities (for example, infection and malnutrition)

Circulating cytokines
Examples
- Interleukins (IL-2, IL-6, IL-7, IL-8, IL-10 and IL-15)
- TGFβ1

Advantages
- Provides a snapshot of the physiological regulation of inflammatory and immune status

Disadvantages
- Highly contextual
- Operator-dependent
- Lack of clinical validation

Humoral markers
Examples
- NY-ESO-1 antibodies
- MART-1 antibodies
- gp100 antibodies

Advantages
- Highly specific to functional antitumour immunity

Disadvantages
- Considerable variability in antitumour epitopes (both interindividual and intraindividual)
- Might not be able to detect immune escape

non-small-cell lung cancer (NSCLC), renal cell carcinoma (RCC), melanoma, head and neck cancer, and urothelial carcinoma) demonstrated that anti-PD-1 and anti-PD-L1 ICIs are associated with improved survival outcomes both in patients with PD-L1-positive and PD-L1-negative disease, although the apparent magnitude of the survival benefit was greater in patients with PD-L1-positive disease[31]. Site-specific randomized trials of ICIs, including in

Study	Indication	*n*	OR (95% CI)
Grossman et al., 2011[61]	High-grade glioma	96	1.8 (1.05–2.6)
Mendez et al., 2016[58]	High-grade glioma	76	2.8 (1.3–5.9)
Rudra et al., 2018[60]	High-grade glioma	210	1.8 (1.2–2.8)
Liu et al., 2017[59]	Nasopharyngeal cancer	413	1.8 (1.1–2.8)
Davuluri et al., 2017[53]	Oesophageal cancer	504	1.6 (1.05–2.4)
Campian et al., 2013[54]	NSCLC	47	1.7 (0.8–3.6)
Tang et al., 2014[50]	NSCLC	711	1.7 (1.05–2.7)
Cho et al., 2016[52]	SCLC	73	2.6 (1.2–5.7)
Balmanoukian et al., 2012[56]	Pancreatic (resectable)	53	2.9 (1.5–5.4)
Wild et al., 2013[57]	Pancreatic (unresectable)	101	2.2 (1.2–4.1)
Chadha et al., 2017[55]	Pancreatic (unresectable)	177	1.7 (1.1–2.5)

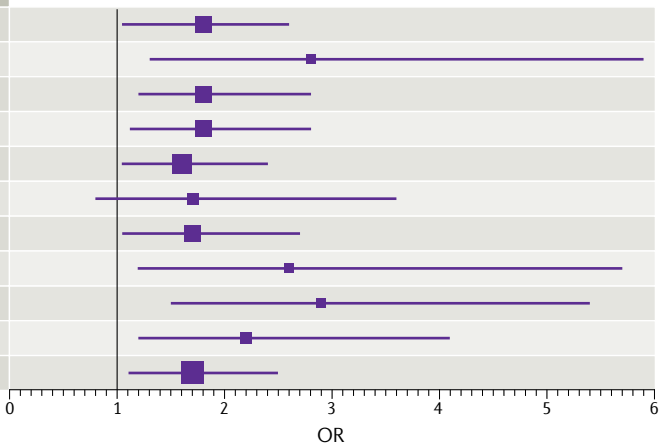

Fig. 1 | **Correlation of radiation-induced lymphopenia with survival.** Forest plot of the odds ratio (OR) with 95% confidence interval (CI) for death in patients with radiation-induced lymphopenia from published cohort studies investigating the effect of lymphopenia on survival. An OR of 1 signifies no effect on survival outcomes, whereas an OR >1 indicates that patients with lower lymphocyte counts have inferior survival. NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

NSCLC[32] and RCC[36], have also demonstrated the benefit of ICI therapy in PD-L1-negative patients, even when patients were stratified for PD-L1 status[37], suggesting that additional data beyond PD-L1 status alone is needed to optimize patient selection for treatment with ICIs. The response to ICIs in PD-L1-negative patients might be partly explained by sampling error due to heterogeneous intratumoural PD-L1 expression[38] or shifting patterns of PD-L1 expression over time[39]. The effects of radiotherapy on the immune system are far more diverse and complex than those of targeted monoclonal antibodies, which block the action of a single known protein on immune cells. Even in the setting of treatment with targeted immunotherapy drugs such as ICIs, biomarker interpretation is not straightforward; thus, a single biomarker will probably not be able to serve as the sole marker of a radiotherapy-induced immune response, and a combination of biomarkers will likely be required to obtain the most accurate evaluation of the immune response to radiotherapy.

Circulating immune cells

Circulating immune cells are a promising class of immune biomarkers in patients undergoing radiotherapy (BOX 1). These cells are accessible in the peripheral blood and, therefore, can be sampled frequently at low cost and with low risk to the patient.

Radiation-induced lymphopenia. A low pretreatment absolute lymphocyte count[40] (ALC), as well as increased pretreatment neutrophil:lymphocyte[41] and platelet:lymphocyte[42] ratios, have been identified as adverse prognostic factors in patients with solid tumours. However, the clinical syndrome of radiation-induced lymphopenia (RIL), which develops in up to 70% of patients undergoing conventionally fractionated external-beam radiotherapy, with[32] or without[43–45] concurrent chemotherapy, is distinct from baseline lymphopenia and from other markers of inflammation status in patients with solid tumours. RIL is characterized by normal lymphocyte counts at baseline, the rapid increase in lymphocytes within the first 2–3 weeks of radiotherapy initiation, and the preferential depletion of $CD4^+$ T lymphocytes and B lymphocytes[46,47]. Abnormalities in circulating lymphocyte subpopulations can persist for decades following radiation exposure, as demonstrated both by data obtained from individuals suffering from accidental radiation exposure[38] and in patients undergoing radiotherapy for clinical indications[48,49]. Importantly, RIL is an independent risk factor for inferior overall survival (OS) outcomes in patients with solid tumours (FIG. 1), as shown in a pooled analysis of the effects of RIL on survival outcomes in patients with malignant glioma, lung cancer, and pancreatic cancer[32]. The majority of studies included in the pooled analysis used an ALC cut-off value of <500 cells/μL at 2 months after the start of radiotherapy[32], but other investigators have identified a continuous relationship between ALC nadir and decreased OS[50]. A range of ALC nadir values (200–390 cells/μL) have been reported to be associated with poor OS[51–60]. Thus, further investigation is needed to identify the optimal time point for ALC measurement after radiotherapy and to determine whether there is a threshold ALC value above which no association with survival is observed.

Although ALC is the best-studied predictive biomarker in patients with cancer undergoing radiotherapy, decreases in other lymphocyte subsets after radiotherapy, particularly $CD4^+$ T cells, also seem to be correlated with survival outcomes[61,62]. However, these changes are highly contextual and can vary with primary tumour histology or treated site, radiation technique (photons versus protons or heavy ion therapy), dose schedule (hypofractionated versus conventionally fractionated), and the time point at which blood is sampled (before, during, or after radiotherapy). For example, an increase in the $CD4^+$:$CD8^+$ T cell ratio has been shown to be correlated with an improved response rate in patients with prostate cancer after treatment with carbon ion radiotherapy[63]. By contrast,

increased $CD8^+$ T cell counts, a decreased $CD4^+$:$CD8^+$ T cell ratio, and an increased proportion of activated $CD8^+$ T cells and PD-1^+CD8^+ T cells relative to $CD4^+$ T cells were all associated with clinical benefit (defined in the study as a partial response, complete response, or stable disease lasting at least 6 months for lesions outside of the radiation field) in a phase I study in which patients with lung or liver metastases from any type of solid tumour received stereotactic ablative body radiotherapy plus ipilimumab[25]. Several explanations might account for these seemingly contradictory observations. Lymphocyte subtypes are not uniformly distributed within the human body (for example, B cells tend to accumulate in the spleen, and antigen-naive lymphocytes accumulate in the lymph nodes)[64] and, therefore, preferential depletion of certain lymphocyte subsets might depend on the radiosensitivity of specific subsets as well as the irradiated site, although data from systematic investigations of these effects are not currently available. Currently available data suggest that B cells, as well as $CD4^+$ T lymphocytes and perhaps naive T cells, are more radiosensitive than $CD8^+$ T lymphocytes, memory T cells, and regulatory T cells[46,47,65,66]. Even among patients treated in the same anatomical region, differences in circulating lymphocyte distributions have been reported following radiotherapy for tumours of different histologies. Indeed, Grassberger et al.[67] reported differential effects of radiotherapy on circulating lymphocyte subsets between patients with hepatocellular carcinoma (HCC) and those with intrahepatic cholangiocarcinoma treated with the same hypofractionated proton beam radiotherapy regimen. Furthermore, different lymphocyte subtypes were found to be prognostic in each tumour type; in intrahepatic cholangiocarcinoma, only high baseline levels of $CD4^+CD25^+$ and $CD4^+CD127^+$ T cells were associated with improved OS whereas, in HCC, an increase in activated cytotoxic T cells ($CD8^+CD25^+$) during treatment was associated with increased OS.

MDSCs and eosinophils. Several studies have also identified cells of myeloid lineage, including myeloid-derived suppressor cells (MDSCs) and eosinophils, as mediators of the immune response to cancer[58–60]. These observations suggest that assays of whole blood, as opposed to isolated peripheral blood mononuclear cells, might assist in the identification of novel biomarkers of antitumour immunity in patients undergoing radiotherapy. MDSCs are associated with systemic immunosuppression, and decreased circulating levels of MDSCs have been reported to be associated with tumour response in patients with HCC receiving radiotherapy[68] and in patients with oligometastatic solid tumours treated with concurrent stereotactic body radiotherapy and sunitinib[49]. Conversely, eosinophils might enhance the antitumour immune response by stimulating the recruitment of T cells into tumours and by inducing changes in the TME that enhance cytotoxic antitumour immune responses[69].

Lymphocyte clonal diversity. Despite the promise of evaluating lymphocyte subpopulations, this approach yields limited insight into T cell clonal diversity and its importance to response. Over the past few years, T cell receptor sequencing[70] has been used to evaluate T cell diversity and clonal dynamics in various indications[71–73]. In a 2018 study, Formenti et al.[18] used T cell receptor sequencing to evaluate the dynamic changes in clonal T cell subpopulations isolated from patients with NSCLC after treatment with the anti-CTLA-4 antibody ipilimumab and radiotherapy. The investigators not only observed a markedly larger number of expanded T cell clones in responders than in nonresponders, but also a substantially larger number of contracted clones among responders. In addition, a rapid expansion of $CD8^+$ T cells recognizing a specific neoantigen encoded by a gene upregulated by radiation was observed in a single patient with a complete response to radiotherapy plus CTLA-4 blockade. Both observations confirm previous findings from preclinical models[74,75] and show the importance of quality versus quantity in a patient's T cell repertoire. Several authors[75,76] have simplified the observed T cell diversity into 'richness', which quantifies the number of unique elements in the population, and 'evenness', which relates to their frequency distribution, based on the Shannon entropy metric[77].

Diminished functional immunity. Reports of diminished functional activity of circulating lymphocytes following conventionally fractionated radiotherapy date back to the mid-20th century. For example, in 1976, Fuks et al.[78] described the decreased responsiveness to nonself antigenic stimulation of both T and B lymphocytes isolated from patients with Hodgkin lymphoma who had undergone radiotherapy compared with those from untreated patients and individuals without Hodgkin lymphoma. In 2014, Parikh et al.[79] performed serial lymphocyte subtyping and assessed anti-human papillomavirus (HPV) immune responses in 18 patients undergoing chemoradiotherapy for HPV^+ head and neck squamous cell carcinoma (HNSCC). Of the 13 patients who initially had an anti-HPV T cell response, 10 had lost this response by the end of chemoradiotherapy. Whether this loss of response reflects active immunosuppression, which would be detrimental to tumour control, or decreased antigen levels owing to the diminished tumour load or trafficking to the tumour, which could be beneficial, is unclear. PD-1 expression on lymphocytes also increased substantially during chemoradiotherapy. Unfortunately, this study had a very small sample size, and no data on tumour control or survival are available. A 2018 longitudinal study employing a cell-free assay of plasma autoantibody levels showed the decreased responsiveness of patients with breast cancer who received radiotherapy as a component of multimodality therapy to stimulation by a preselected 32-antigen panel of candidate breast tumour antigens, again suggesting that radiotherapy might be associated with systemic immunosuppression in this group of patients[80]. Further research is needed to determine whether radiation-induced inhibition of functional immunity leads to inferior tumour control.

Biomarkers for ICIs

The development of ICIs, which interrupt T cell inhibitory signalling pathways to promote effector T cell activation and enhance the antitumour immune response, have ushered in a new era for cancer immunotherapy[31–35,80].

These drugs have three primary targets — the CTLA-4 (ipilimumab and tremelimumab) and PD-1 receptors (pembrolizumab and nivolumab), which are both expressed on activated lymphocytes, and the PD-1 receptor ligand PD-L1 (atezolizumab, avelumab and durvalumab). Biomarkers, including PD-1 and PD-L1 expression levels on tumour and immune cells, can function as prognostic biomarkers, assist in patient selection for treatment with ICIs, and serve as a way to track changes in immune status with radiotherapy (BOX 1). Of note, our understanding of the utility of these markers in the setting of patients undergoing radiotherapy, with or without associated ICI therapy, is rapidly evolving, and definitive recommendations cannot be made regarding the applicability of PD-1 and/or PD-L1 expression levels for clinical decision making in the radiation oncology clinic.

PD-L1 expression. PD-L1 expression is an attractive candidate biomarker for the response to anti-PD-1 or anti-PD-L1 antibodies, as it is generally only expressed by macrophages in the absence of a malignancy but is commonly expressed by tumour cells[81]. PD-L1 expression in tumour cells is predictive of prolonged response to anti-PD-1 or anti-PD-L1 antibodies, although patients whose tumour cells do not express PD-L1 might also benefit from treatment with anti-PD-1 or anti-PD-L1 antibodies, suggesting that PD-L1 status cannot be used as a stand-alone predictive biomarker for the selection of patients for anti-PD-1 or anti-PD-L1 therapy[31,82].

Data on tumour cell PD-L1 expression as a prognostic or predictive biomarker specifically in patients treated with radiotherapy are limited to a few small retrospective studies. Pretreatment expression of PD-L1 in tumour cells has been identified as a potential prognostic biomarker in patients with squamous cell carcinoma (SCC) of the anus and HNSCC treated with chemoradiotherapy[33,83]. In both cancer types, tumour cell PD-L1 expression was also correlated with HPV status, although the mechanism underlying the apparent increased immunogenicity of HPV-related cancers remains unclear. Conversely, PD-L1 upregulation during preoperative chemoradiotherapy has been associated with poor survival outcomes in small retrospective cohorts of patients with oesophageal SCC[84] and NSCLC[85]. Similarly, in patients with Merkel cell carcinoma (MCC), Merkel cell polyomavirus infection is associated with tumour cell PD-L1 expression[86]. Furthermore, serial sampling of circulating immune cells during treatment of patients with MCC showed that MCC-targeting T cells isolated from the blood had higher levels of PD-1 expression than T cells specific to other human viral antigens, and that populations of MCC-specific T cells increased with increasing MCC tumour burden and decreased in response to MCC treatment[34]. These findings suggest that the PD-1–PD-L1 inhibitory axis is a biomarker of disease load and a promising therapeutic target in MCC. Furthermore, in patients with gastric cancer, Epstein–Barr-virus-driven tumours have a high level of PD-L1 expression (that is not seen in Epstein–Barr-virus-negative tumours)[87], which is generally correlated with a poor prognosis[88].

Tumour-infiltrating lymphocytes. Some investigators have suggested that the presence of $CD8^+PD\text{-}1^+$ tumour-infiltrating lymphocytes (TILs) in the context of PD-L1-positive tumours reflects an adaptive antitumour response and portends a favourable response to ICIs[89]. However, data from detailed immunophenotyping of tumours before and after radiotherapy are limited, and available studies have reported conflicting results regarding the role of $CD8^+PD\text{-}1^+$ TILs in the context of radiotherapy. A type I tumour phenotype, characterized by high numbers of $CD8^+$ TILs and high tumour cell PD-L1 expression, has been correlated with improved outcomes in the setting of treatment with definitive radiotherapy, predominantly in patients with HPV-driven cancers such as anal SCC[83] and oropharyngeal SCC[90]. However, in patients undergoing radiotherapy, the degree of $CD8^+$ TIL infiltration is not always associated with a favourable prognosis. For example, in patients with extrahepatic cholangiocarcinoma treated with adjuvant radiotherapy, $CD8^+PD\text{-}1^+$ TILs were strongly associated with inferior OS, although tumour cell PD-L1 expression was not[91].

Of note, most investigations have only focused on the tumour immunophenotype at baseline, and data on changes in immune cell infiltrates and on tumour cell PD-L1 expression after radiotherapy are extremely limited. A particular lack of data exists regarding the interesting question as to whether alterations in TIL populations correlate with changes in total peripheral blood lymphocyte counts and/or circulating lymphocyte subpopulations during and after radiotherapy. Such studies might provide additional prognostic and predictive information. For example, Fujimoto et al.[85] reported that a decrease in tumour cell PD-L1 expression after neoadjuvant chemoradiotherapy is associated with improved relapse-free survival and OS in patients with NSCLC; $CD8^+$ TILs were not identified as a prognostic factor in this study. Similarly, in a matched-pair analysis of 123 patients with rectal adenocarcinoma, both tumour cell PD-L1 expression and $CD8^+$ TIL levels increased following neoadjuvant chemoradiotherapy[92]. In this study, high levels of PD-L1 expression at baseline and after chemoradiotherapy were independently associated with decreased OS and a shorter disease-free survival, whereas low $CD8^+$ TIL density was associated with high PD-L1 expression levels but was not an independent risk factor for death.

Circulating immune biomarkers

Circulating biomarkers of the immune response include both nonspecific indicators of inflammation and immunosuppression, and biomarkers that are more specific to the antitumour immune response, including circulating levels of cytokines and antitumour autoantibodies (BOX 1).

Nonspecific immune indicators. A considerable amount of retrospective research has attempted to correlate survival outcomes with levels of circulating inflammatory markers in patients with cancer; an exhaustive summary of these data is beyond the scope of this Review.

An updated systematic review and meta-analysis of the prognostic value of systemic inflammatory markers in patients with cancer was published in 2017 by Dolan et al.[42]. The most commonly studied biomarkers in this class include serum lactate dehydrogenase[93], C-reactive protein (CRP)[42], and albumin[94], as well as prognostic scores such as the Glasgow Prognostic Score and the modified Glasgow Prognostic Score[42], which integrate CRP and albumin levels as a measure of systemic inflammation; high CRP and low albumin levels reflect increased inflammation and are associated with a worse prognosis[95]. Although these biomarkers might be useful for pretreatment prognostic classifications, they are not highly specific to antitumour immunity, and can be confounded by common comorbidities such as infection, cachexia, and/or autoimmune disease.

Circulating cytokine levels. Cytokines are small proteins that have an important role in intercellular immune signalling. Radiotherapy stimulates cytokine production in a variety of immune and nonimmune cells, including production of interferons, IL-4, and IL-5 by lymphocytes[96], transforming growth factor-β (TGFβ) production by platelets[97], and production of IL-1β, IL-6 and tumour necrosis factor by intestinal epithelial cells[98]. Circulating levels of certain interleukins, such as IL-6 (REF.[99]) and IL-8 (REF.[100]), have been correlated with survival in patients with solid tumours undergoing radiotherapy. However, the use of cytokines as biomarkers of the immune response following radiotherapy is complicated by multiple factors. Substantial variability has been reported in cytokine expression between individuals in the setting of radiotherapy-induced chronic inflammation, even in patients whose tumours are matched for site, radiotherapy dose, and stage[100]. In addition, tumours themselves can produce certain active cytokines, such as TGFβ1 (REF.[101]). Most importantly, cytokine levels are highly variable and contextual, and the signalling response of individual immune cell types to a particular cytokine is often idiosyncratic; for example, TGFβ1 inhibits IL-7-induced proliferation in $CD4^+$ memory T cells, but not in naive $CD4^+$ T cells[102]. Finally, owing to problems with multiple hypothesis testing resulting from evaluating the associations between large numbers of candidate cytokine-based biomarkers and patient outcomes, care must be taken in validating such biomarkers to avoid spurious conclusions.

Box 2 | **Imaging modalities and labelling approaches**

Imaging modalities

MRI

- Excellent temporal and spatial resolution
- Exquisite soft-tissue contrast
- No ionizing radiation

PET and single-photon emission CT

- Large library of contrast agents offers more specificity than MRI
- Excellent quantification and sensitivity of signal

Labelling methods

In vivo

- Simple imaging procedures
- Lower regulatory hurdles than ex vivo methods
- Provides whole-body assay of target distribution and density

Ex vivo

- Superb specificity and signal-to-noise ratios
- Provides assay of rates of infiltration and trafficking of cells labelled ex vivo

Circulating antitumour autoantibodies. A final subcategory of circulating immune biomarkers includes circulating autoantibodies against tumour-associated antigens, which are markers of humoral immunity. The abscopal effect has been hypothesized to be mediated by the radiation-induced generation of circulating antitumour autoantibodies, which have the potential to be highly specific markers of antitumour immunity[12]. However, variations in tumour antigen expression between patients limit the generalizability of such biomarkers. Furthermore, as tumours evolve and because cells within a tumour and at metastatic sites are often genetically heterogeneous[103], the presence of autoantibodies against a single tumour epitope might not always correlate with a clinical response. In addition, decreases in antitumour autoantibodies might not reflect reduced tumour burden, given that, as the tumour progresses, antibody levels might decrease as immune-tolerant clones preferentially proliferate[104]. Nonetheless, proof-of-principle evidence of the potential of circulating antitumour autoantibodies for monitoring the response to concurrent radiotherapy and immunotherapy has been provided by several case reports on circulating immune biomarkers of the abscopal effect. For example, in a patient with melanoma treated with ipilimumab and hypofractionated radiotherapy, the appearance of antibodies against cancer–testis antigen 1 (NY-ESO-1) in the serum following hypofractionated radiotherapy coincided with tumour regression[19]. Additional case reports have described humoral responses involving antibodies against melanoma-associated antigen 3 (MAGEA3)[104], melan-A (MART-1)[91], and glycoprotein 100 (gp100)[105] in patients with melanoma.

In summary, any one biomarker described in this section will probably not fulfil all the criteria for an 'ideal' biomarker of immune response to radiotherapy[35]. Continued research is needed to validate existing candidate biomarkers and discover novel immune-based predictors of response to radiotherapy to improve our understanding of the complex effects of radiotherapy on the immune system and to optimize the potentially synergistic combination of radiotherapy with immunotherapy.

Imaging the immune response

Inflammation is a characteristic of multiple major disease processes, including infection, autoimmune disease, and cancer, and major progress has already been made in developing noninvasive and clinically useful imaging techniques to evaluate the immune response. These advanced imaging techniques can be applied to patients undergoing radiotherapy and/or immunotherapy as an effective method of visualizing and quantifying immune cell-mediated targeting of tumours following treatment.

Here, we focus on imaging approaches for monitoring the immune response that are already in clinical use or nearing clinical application, but are not necessarily exclusive to oncology (BOX 2). Fluorescent or near-infrared imaging methods for cell tracking, although potentially useful in the preclinical setting, are limited

in their clinical utility by their low level of resolution and limited tissue penetration and, therefore, will not be discussed in this Review. However, reviews of these methods are available in the literature[106]. Furthermore, we highlight imaging techniques that enable a mechanistic interpretation of the immune response in the patient, either by enabling specific cell populations or expression of proteins relevant to the immune response to be imaged. Emerging efforts involving radiomics or similar machine-learning approaches based on the use of imaging data to predict response to immunotherapeutic agents[107] are beyond the scope of this Review and will not be discussed.

MRI-based immune tracking

With its exquisite spatial resolution and lack of ionizing radiation, MRI is a powerful tool for clinical imaging. Several magnetic contrast agents can assist in visualization of the immune response. Nanoparticles containing paramagnetic metal ions such as gadolinium (Gd^{3+}) and manganese (Mn^{2+}) have been used to detect inflammatory responses in atherosclerotic plaques, in which these large contrast agents are endocytosed by macrophages, enabling contrast in macrophage-rich plaques[108]. However, gadolinium-based contrast agents are associated with rare, but severe, adverse events, particularly in patients with pre-existing kidney dysfunction[109]. A large body of work has been performed in another family of contrast agents — superparamagnetic iron oxide nanoparticles (SPIONs). These contrast agents are phagocytosed by monocytes and macrophages, enabling image contrast under inflammatory conditions such as infection[110], atherosclerotic plaques[111], inflammatory bowel disease (IBD)[112], and multiple sclerosis[113]. In addition, SPIONs (in conjunction with cell-penetrating agents or electroporation) have been used for the ex vivo labelling of isolated (both allogenic and autologous) immune cells, which can be reinjected into an individual to visualize the localization and trafficking of specific white blood cell (WBC) populations, such as dendritic cells[114] or other leukocytes[115,116]. This technique has been demonstrated with the labelling of tumour-associated macrophages with the SPION ferumoxytol in a breast cancer mouse model, which enabled the MRI-based visualization of tumour-associated macrophage dynamics[117].

MRI-based techniques have the advantage of excellent spatial resolution, although they also have two major shortcomings. First, MRI has a low level of sensitivity compared with imaging techniques such as PET or single-photon emission CT (SPECT), and cannot detect areas of inflammation that are not densely populated by the cells of interest[118]. The use of high doses of contrast agents might enhance sensitivity but also increases the risk of complications, such as iron overload[119]. Second, the direct quantification of the signal (for example, the molar concentration of contrast agents) can be difficult using MRI. This limitation is due to a broad range of factors, including differing relaxivities of contrast agents based on their local agglomeration states, discrimination of cell uptake and haemorrhage, and magnetic susceptibility artefacts. These limitations reduce the clinical utility of this approach for precise disease staging or treatment monitoring[118,120].

SPECT-based methods

The most common SPECT-based methods for imaging the immune response use leukocytes labelled with either ^{111}In-oxyquinoline (^{111}In-oxine) or ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO). With these methods, autologous leukocytes are purified from blood samples taken from the patient, incubated with either ^{111}In or ^{99m}Tc, and then reinjected into the patient's blood[121,122]. These methods are currently used in the clinic to detect IBD[123], infected prostheses and osteomyelitis[124], and endocarditis[125]. Limitations of these techniques include the fact that the entire WBC population is labelled, thereby reducing the specificity of the results. Furthermore, cell labelling is not always stable, with ^{99m}Tc-HMPAO particles eluting from reinjected cells at a rate up to 7% per hour, which increases the background signal over time[126].

In the context of immunotherapy, genetic engineering of reporter genes can also be used to enable systemic administration of contrast agents targeting the product of those genes. In murine models, chimeric antigen receptor (CAR) T cells have been modified to express reporter genes that enable imaging with ^{99m}Tc pertechnetate ($^{99m}TcO_4^-$)[113]. This approach enables the correlation of CAR T cell location and number with therapeutic efficacy, and allows for repeated and persistent imaging of the population of interest.

Many investigations involving the in vivo labelling of immune cells with SPECT tracers, which circumvents the need for ex vivo cell labelling and enables in situ imaging of specific cell populations, are currently ongoing. Indeed, ^{99m}Tc-labelled antigranulocyte antibodies (Leukoscan, Immunomedics) have been used in a range of applications for imaging inflammation[123,127]. A large body of work has also been completed regarding the use of ^{99m}Tc-labelled antitumour necrosis factor antibodies for imaging of macrophages in patients with rheumatoid arthritis or other inflammatory diseases[128]. These radiolabelled antibody methods, although still in the early stages, are a growing area of noninvasive whole-body assays of the distributions of specific immune responses. However, the development of these compounds, although promising, might be limited by additional complications regarding clinical approval and large-scale synthesis compared with other small-molecule contrast agents.

The lymphatic mapping agent ^{99m}Tc-labelled tilmanocept (Lymphoseek, Norgine) is comprised of multiple mannose units and has high affinity for the CD206 mannose receptor, which is found at high concentrations on macrophages and dendritic cells, enabling the SPECT-based imaging of these WBC subpopulations[129]. Lymphoseek is used for lymphatic mapping and guiding sentinel lymph node biopsies in solid tumours. This compound has also been used to visualize macrophage accumulation in patients with arthritis[130] and in atherosclerotic plaques from patients infected with HIV[131]; compared with healthy control individuals, much higher levels of Lymphoseek uptake were seen in the aortic arch and

descending aorta of HIV-infected patients. Additionally, the levels of Lymphoseek uptake were closely correlated ($r = 0.87$) to noncalcified plaque volume, as determined by CT angiography imaging. Compared with MRI, SPECT-based WBC imaging methods are more sensitive and better suited to quantitative analyses. However, SPECT studies are restricted by limited spatial resolution (12–15 mm in conventional clinical systems[132]) and involve exposure of patients to ionizing radiation. Despite these limitations, the cost of SPECT instrumentation and scans remains much lower than that of PET and MRI, thus enabling a wider range of clinics to conduct these studies and thereby increasing the clinical utility of contrast agents developed for this modality.

PET-based imaging

With its high level of sensitivity, the ability to provide quantitative data, and its improved spatial resolution (5–7 mm in clinical systems) compared with that of SPECT, PET is a very promising platform for imaging the immune response. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is by far the most common PET tracer in this setting. By exploiting the increased metabolic activity of immune cells at the site of inflammation, ^{18}F-FDG–PET imaging has been used to detect the immune response in osteomyelitis and joint infections[124], IBD[133], infections of cardiac devices[134,135], and other infections[136]. In addition, ^{18}F-FDG–PET imaging has been widely investigated as a PET tracer for imaging atherosclerotic plaques[137–139]. Despite its wide clinical utilization and availability, ^{18}F-FDG–PET has several major drawbacks for use in immune imaging. Most importantly, ^{18}F-FDG is not specific to any particular leukocyte population, or to leukocytes in general, leading to problems with signal-to-background ratios, especially near areas of high glucose utilization (such as the brain, heart, or FDG-avid tumours) or hypoxia[139]. In oncology, this issue is further complicated by the phenomenon of pseudoprogression, where the increased immune activity in response to successful therapy is difficult to distinguish from increased tumour metabolism in response to therapeutic failure[140]. These confounding factors can make the quantification of inflammation difficult, and in vivo imaging of aortic injury in rabbits has shown that ^{18}F-FDG–PET results do not necessarily correlate with the degree of inflammation in atherosclerosis[141].

These limitations have led to the development of a family of PET tracers targeting biomarkers of the inflammatory response that are more specific than ^{18}F-FDG uptake. Somatostatin receptor type 2, a G-protein coupled receptor that is upregulated on the surface of activated macrophages, has been used as a target for PET tracers such as ^{68}Ga-DOTATATE in investigations of atherosclerotic inflammation[142–144]. Similarly, the translocator protein, which is expressed broadly in immune cells involved in neuroinflammatory processes, has been used as a target for PET tracers, the most common of which is ^{11}C-PBR28 (REFS[145–147]). CXC-motif chemokine receptor 4 (CXCR4), which is expressed on a wide range of immune cell types, has also been used as an imaging target for PET tracers such as ^{64}Cu-AMD3100 (REF.[148]). However, given the broad expression of the cellular protein targets across immune cell types, these tracers still lack a high degree of specificity.

A growing amount of preclinical data suggest that radiolabelled antibodies and antibody fragments can be used to image specific WBC populations, thereby overcoming some of the limitations of the aforementioned PET tracers. Detailed reviews describing these families of tracers have been published elsewhere[106,149]. For example, preclinical studies have reported the successful imaging of T and B cell populations using radiotracer-labelled antibodies or antibody fragments against T cells and B cells[150–153]; these compounds are highly specific, with little off-target uptake outside the organs of clearance (liver and kidney). Radiolabelled SPIONs have also been used in combination with PET imaging in the preclinical research setting to image phagocytic cells (monocytes and/or macrophages)[154] and general inflammation[155]. These studies have shown that trafficking of phagocytic cells can be monitored by microPET and CT imaging for up to 7 days after tracer injection in healthy mouse models, and that inflamed tissues are clearly visualized by microPET and CT imaging with highly asymmetric lymph node uptake favouring nodes draining the site of injury in murine peripheral limb injury models. Similar to MRI, these radiolabelled nanoparticles can be modified to enable the ex vivo labelling of specific cell populations[156], and such modifications have been used to label and track B cells in vivo in mouse models[157]. Similar to SPECT imaging, PET imaging exposes patients to radiation; however, PET imaging has superior spatial resolution to that of SPECT, but is still inferior to MRI in terms of resolution.

Investigators and clinicians must take care in the application of the aforementioned imaging contrast agents to ensure that compounds are synthesized using Good Manufacturing Practices (GMP)[158,159] and abide by the specific regulations imposed by the relevant agencies, such as the FDA and the European Medicines Agency (EMA)[160,161].

Modelling the immune response

Traditional mathematical models of tumours focused on tumour growth[162,163], the effects of therapeutic approaches such as radiotherapy and chemotherapy on tumours[164–166], and the evolutionary path of cancer (that is, how nontransformed cells acquire additional mutations that confer malignant potential and how tumours themselves evolve)[167,168]. However, realization of the importance of the TME[169] in tumour development and progression has highlighted the role of other noncancerous cell populations (such as immune cells, fibroblasts, endothelial cells, and others) and their interactions. To model the interactions between the individual components of the TME and tumour cells, mathematical models have been used to replicate the essential behaviour of the system and to study the fundamental mechanisms that explain our observations in patients and animal models[170–172].

With the increase in the clinical application of immunotherapeutic approaches over the past decade, interest has grown in the mathematical modelling of the interactions between the tumour and the immune

system. Based on fundamental work from the 1970s[173], the vast majority of these models assume a predator–prey-like interaction between immune cells and tumour[174–176]. However, given the multitude of immunotherapy approaches, ranging from cytokine therapy to CAR T cells and ICIs, a large number of variants of this classical mathematical model have been developed to simulate the interaction between the tumour and the immune system[175,177,178]. Most of these models focus on the interaction between tumour cells and WBCs, and usually describe the antitumour activity of $CD8^+$ T cells, natural killer cells, and macrophages (often explicitly including IL-2 dynamics)[179], and focus on personalizing immunotherapy for specific patients[180,181].

In this section, we focus on models that were specifically developed to study the interaction between radiotherapy and the immune system and that can, therefore, be used to predict synergy between immunotherapy and radiotherapy. The models discussed here, including systemic and regional-interacting models (BOX 3), are distinct from unstructured machine-learning approaches owing to their limited number of parameters and their underlying mechanistic 'structure', meaning that the equations describing their behaviour are derived from our mechanistic understanding of the tumour–immune interaction. In addition, the lower dimensionality and the mechanistic underpinnings of these models make them particularly useful in the immunotherapy–radiotherapy combination setting, in which data are usually limited and where intrapolation, or even extrapolation, between radiation time points or fraction sizes is desirable.

Systemic models

Systemic models are defined here as those describing the entire tumour as a single compartment and that do not distinguish between various possible metastatic sites. This approach differentiates systemic models from the regional-interacting models and has important consequences for the clinical questions to which they can be applied. Systemic models are often phenomenological in nature, meaning they synthesize the empirical high-level relationships between the tumour and the immune system, and do not describe the entirety of the detailed cell–cell interactions and processes observed in laboratory experiments.

Sotolongo-Grau et al.[182] described a simple model of the tumour–immune system interaction and the effects of radiotherapy on each aspect. The model was based on a previously introduced tumour–immune model[183] and was modified to include a population of nonclonogenic tumour cells. It introduces an immune system efficiency metric that quantifies the strength of a patient's immune response and that is predictive of the success of an immunotherapy–radiotherapy combination regimen. Serre et al.[184] designed a specialized model explicitly aimed at simulating the combination of radiotherapy with anti-CTLA-4 and anti-PD-1 ICIs. In a subsequent publication, the authors proposed a simplified approach aimed at describing the immunomodulatory effects of different fractionation regimens[185]. To this end, they introduced the immunologically effective dose, which is conceptually related to the biologically effective dose metric used in clinical radiotherapy[164], and which quantifies the abscopal response induced by a given radiation schedule. Chakwizira et al.[186] modified the Serre model for applicability in experiments using the syngeneic RG2 glioma rat model treated with radiation combined with an indoleamine 2,3-dioxygenase–tryptophan 2,3-dioxygenase inhibitor.

Owing to advances in understanding of the emergence of a tumour-directed immune response, groups have started developing mechanistic models that describe the tumour–immune interaction in much greater detail than the aforementioned systemic models. Kosinsky et al.[187] presented a very detailed mechanistic model that was based on individual tumour size trajectories from mouse experiments. Their model incorporates a detailed description of the immune response to radiotherapy, starting from radiation-induced dendritic cell maturation to the recruitment, proliferation, and

Box 3 | **Mathematical models of the interactions between immunotherapy and radiotherapy**

Systemic models

Explicitly modelled compartments

- Always includes tumour cells and at least one immune cell population
- Depending on the approach: nonclonogenic tumour cells, tumour antigens, endogenous IL-2, IFNα, dendritic cells and others

Characteristics

- Can be phenomenological or mechanistic
- Often focused on one specific drug or immunotherapeutic modality, with compartments and their interactions tailored to a specific question

Possible applications

- Together with systemic inputs, these models are best suited to questions concerning radiotherapy scheduling, dosing and fractionation
- In early and locally advanced stages of disease, modelling of one tumour compartment might suffice

Regional-interacting models

Explicitly modelled compartments

- Tumour cells and lymphocytes at each metastatic site
- Requires modelling of the interaction between immune response at different sites, for example via blood flow compartments

Characteristics

- Does not include multiple immune cell populations
- Interaction modelling can require large number of parameters
- Focused on modelling trafficking between metastatic sites

Possible applications

- Main application is selection of target sites when irradiating metastatic disease
- Ideal input could be derived from dynamic imaging modalities that can quantify trafficking of cell populations

differentiation of T cells and the resulting upregulation of PD-L1 expression in tumour cells, as well as describing the pharmacokinetics and pharmacodynamics of anti-PD-1 or anti-PD-L1 antibodies.

Regional-interacting models

The term 'regional-interacting' originates from the fact that these models explicitly simulate the primary tumour and various metastatic sites, each with a possibly different immune environment, as well as the interactions between them.

Poleszczuk et al.[188] developed a model that incorporates physiological information on T cell trafficking via the circulatory system, including separate compartments such as the lungs, gastrointestinal tract and spleen, liver, and other organs. This model simulates the probability of a T cell activated at one metastatic lesion trafficking from this lesion and extravasating at another metastatic lesion. Thus, an immunogenicity index can be defined for each metastatic lesion (that is, how effectively activated T cells from one lesion can reach other metastatic sites to induce an abscopal effect). In a follow-up study, the investigators demonstrated how their model could be used to select irradiation sites in specific patients[189]. Walker et al.[190] further extended this model to simulate the cytotoxic and immunogenic consequences of surgery or radiotherapy. In an extension of their research, Poleszczuk and Enderling[191] adapted their model to integrate experimental results from a typical syngeneic TSA mouse model of breast carcinoma using delayed left–right flank tumour initiation. They combined various fractionation regimens with anti-CTLA-4 antibodies to calibrate their model to measure tumour volumes and to extract the necessary parameters, and further concluded that the optimal per-fraction doses required to elicit an antitumour immune response are in the range of 10–13 Gy.

The parameters used in the systemic and regional-interacting models are either derived from animal experiments or estimated to produce sensible results. The fact that none of these models are based on parameters tailored to data on human tumour–immune system interactions is the most important drawback, and is an area of active research[192–194].

Remaining questions

Immunotherapy has revolutionized the management of patients with advanced-stage cancers, and the combination of radiotherapy and immunotherapy could be a powerful tool to help maximize response rates and potentially induce durable disease control. Initial data on radiotherapy in conjunction with immunotherapy have been encouraging and have, in turn, led to the development of numerous prospective trials in both the localized and metastatic settings. However, multiple questions remain as to how different immunotherapeutic modalities should be combined with radiotherapy, and how the choice of modality affects the optimal timing, dose, and target site for radiotherapy to maximize the immune response. Crucially, ongoing trials exploring these questions should ideally incorporate biomarkers of an antitumour immune response into their design, as assessment of clinical response data without predictive biomarkers of immune response could limit the future clinical application of these combinations.

Systemic and local immune biomarkers

Numerous biomarkers of immune response have been explored, including the aforementioned peripheral or circulating biomarkers such as cytokines and circulating immune cells, tumour-specific biomarkers such as PD-L1 expression, and radiographic biomarkers such as PET and SPECT tracer imaging. Circulating biomarkers are certainly the most easily accessible and cost-effective biomarkers available at this time. Moreover, blood draws are less invasive and less prone to complications than tumour biopsy sampling and can be easily obtained at multiple time points, enabling serial assessment of the immune response over time. However, circulating biomarkers are partly limited by providing only a view of the immune response at the systemic level. This snapshot might be sufficient for patients with limited sites of disease, although it does not provide enough granularity to distinguish clinically significant heterogeneity in terms of response among metastatic sites. The ability to predict and monitor immune responses at a local level will be key to predicting and optimizing patient outcomes.

The fact that circulating biomarkers provide a systemic-level view of the immune response has a number of simultaneous advantages and disadvantages (BOX 3). The systemic-level view might provide sufficient information to assess response and aid in clinical decision making regarding treatment (particularly in patients with localized disease) and investigate questions regarding sequencing of radiotherapy and immunotherapy. However, for questions pertaining to intratumoural heterogeneity and for investigations of differential responses among metastatic sites in the same patient, a high level of granularity will be needed to distinguish between local and systemic immune responses. For these situations, approaches that enable investigators to monitor the immune response at a local level are required to predict response and optimize patient outcomes.

To this end, the aforementioned advanced imaging techniques, such as MRI, PET, and SPECT, can potentially differentiate between the immune response at different metastatic sites. Although these techniques are more expensive than the majority of circulating biomarkers, they also provide a more refined overview of the immune response to radiotherapy. This information is particularly valuable in trials of immunotherapy–radiotherapy combinations in patients with metastatic cancers, in which selection of the target site for radiotherapy is currently often guided by ease of delivery rather than the potential for maximal stimulation of the immune system[178]. Indeed, the optimal target lesion for radiotherapy is probably not only histology-specific, but also patient-specific.

Application of mathematical models

The serum-based and imaging-based biomarker classes intuitively relate to the aforementioned systemic and regional-interacting mathematical modelling approaches, respectively (FIG. 2). Systemic models

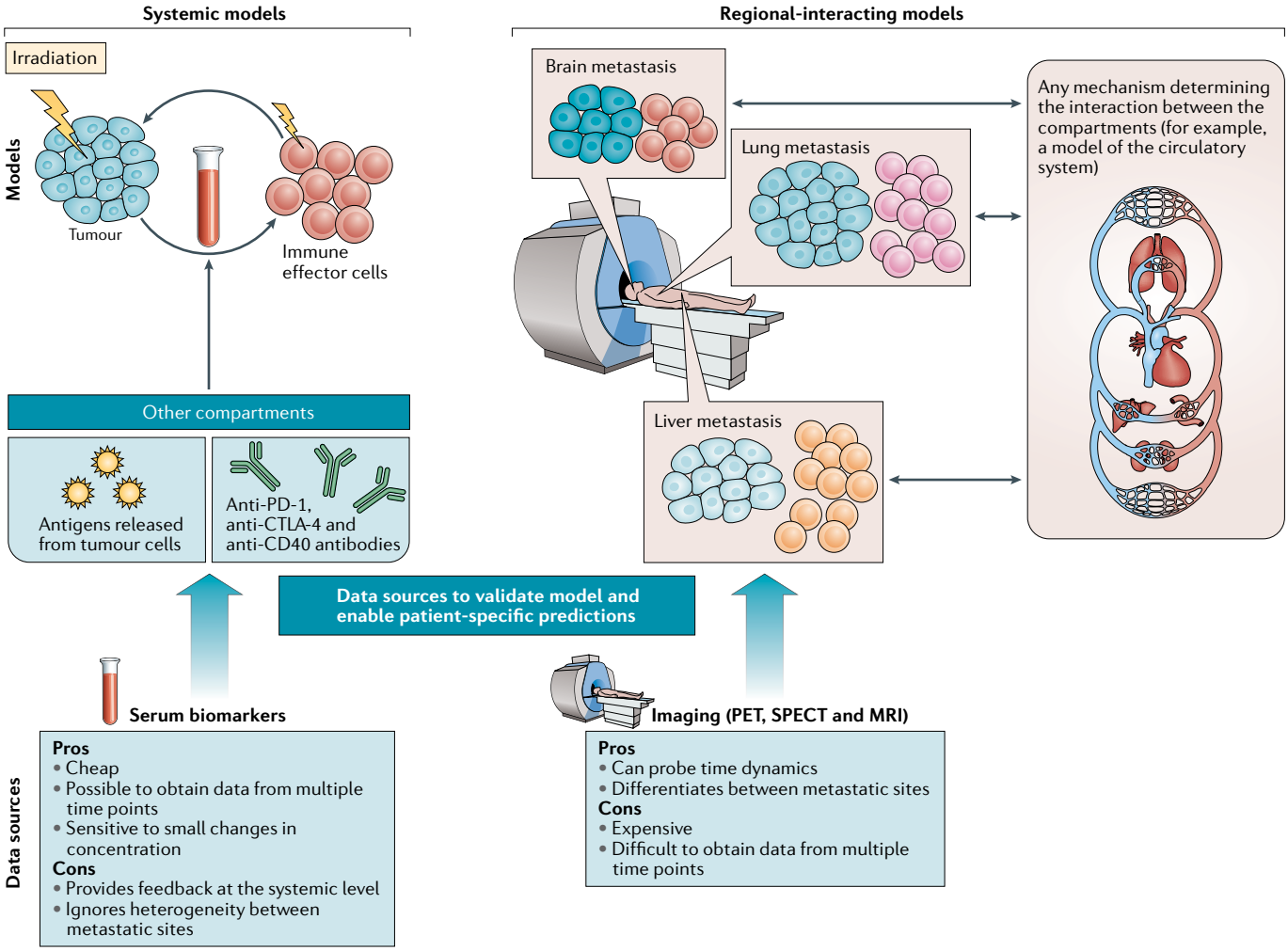

Fig. 2 | **Integration of biomarker data with mathematical models.** The figure illustrates the connection between available patient data, including serum-based and imaging-based biomarkers, and parameters needed to validate mathematical models of the immune response to radiotherapy. Systemic models, shown on the left, only feature one tumour compartment, interacting with immune effector cells and various other compartments depending on the question being investigated. Regional-interacting models, shown on the right, explicitly model separate compartments for each disease site, each with their own regional immune environment. These immune compartments can interact, for example via modelling the blood flow between them. The lower part of the figure describes the types of patient-specific data that can be used to inform the above models, together with their advantages and drawbacks. CTLA-4, cytotoxic T lymphocyte antigen 4; PD-1, programmed cell death protein 1; SPECT, single-photon emission CT.

can be optimally informed by serum biomarkers, as they both relate to the interaction between the tumour and the immune system at the systemic level. However, PET, SPECT, and MRI imaging techniques can examine the tumour response in a site-specific context and only the regional-interacting models can incorporate this localized information into patient-specific predictions. Consequently, these two classes of mathematical models are ideal for investigating distinct sets of clinical questions.

Systemic models can be applied to the general questions of radiation sequencing, dosing, and fractionation, and are well suited to study the interactions between immunotherapy and radiotherapy in locally advanced stages of disease, or earlier (BOX 3), given the absence of gross disease outside of the radiation field at these disease stages. An example of the potential application of systemic models is provided by the 2018 FDA approval of the anti-PD-L1 antibody durvalumab after concurrent chemoradiotherapy in patients with unresectable stage III NSCLC, based on findings from the phase III PACIFIC trial[195]. Systemic models of tumour–immune interactions, supported by serum biomarkers during the radiotherapy stage of the treatment, could guide the adaptation of the total radiation dose and fractionation to maximize synergy with durvalumab in individual patients. Circulating lymphocyte counts could have a central role as immune biomarkers in this scenario, as they have been shown to correlate with outcomes after definitive chemoradiotherapy in patients with NSCLC[50], as well as for patients with metastatic cancer on treatment with ICIs receiving radiotherapy[24]. This approach could enable the personalization of the relative proportion of the radiotherapy

and immunotherapy components of the overall treatment strategy to account for differences in the initial state of a patient's immune system and its response to radiotherapy.

Both the sequencing and timing of therapies are expected to have a key role in the design of such novel combination regimens. In the PACIFIC trial, initiation of durvalumab within 14 days of completion of chemoradiotherapy was associated with a marked improvement in OS on multivariable analysis compared with initiation of durvalumab 15–42 days after chemoradiotherapy completion[195]. Although this result might have been influenced by the performance status of patients, given that patients with excellent performance status could, in theory, initiate durvalumab sooner after completion of chemoradiotherapy, it also highlights a potential synergy between radiotherapy and immunotherapy in this setting. Multiple preclinical studies have also demonstrated the importance of sequencing for achieving synergy between immunotherapy and radiotherapy[16,196].

Conversely, regional-interacting models are ideal for studying emerging questions related to target selection in irradiation of disease that has already metastasized. These models could be particularly useful for adoptive cell immunotherapies, in which a cell population can be tagged with imaging tracers before injection into the patient[121,122]. Although the choice of radiotherapy target might be made directly based on imaging findings, models of the interaction between the disease sites could yield important information about precisely when to intervene with radiotherapy.

The aforementioned allocation of the systemic and regional-interacting models to specific sources of patient data (in order to inform and/or validate said models) should not be considered a strict classification. In the same sense that systemic and regional-interacting models are complementary, the combined use of blood-based assays and imaging together will improve our understanding of the entire spectrum of the effects of immunotherapy–radiotherapy combinations, and could be particularly useful for investigating possible correlations between peripheral lymphocyte counts and intratumoural dynamics.

The importance of lymph nodes

Over the past decade, the role of elective nodal irradiation (ENI) has been re-evaluated in a number of disease sites, including NSCLC[197,198], small-cell lung cancer, oesophageal cancer, and early-stage breast cancer, leading in some instances (for example, in NSCLC) to the elimination of ENI from standard clinical recommendations[199–201]. Although randomized trials of ENI in breast cancer demonstrated an improvement in progression-free survival without a statistically significant improvement in OS, mixed results have been reported in other disease sites, with several studies, particularly in NSCLC and small-cell lung cancer, failing to demonstrate an improvement in survival outcomes with ENI. However, even when lymph nodes are excluded from radiotherapy target volumes, they are also not considered to be organs at risk in current treatment paradigms. Eliminating ENI would be a paradigm shift in many disease sites, including HNSCC, anorectal, gynaecological, and many breast cancers, and must be undertaken with caution. However, given the strong correlation between field size and both the risk and severity of radiation-induced lymphopenia[30,50], reconsidering the clinical benefits of prophylactically irradiating large draining nodal fields might be worthwhile. Furthermore, emerging preclinical data suggest that nodal irradiation can attenuate the efficacy of immunotherapy–radiotherapy combination regimens, perhaps by interfering with lymphocyte–antigen interactions within the TME or in nearby lymph nodes[202]. The advanced imaging biomarkers described in this Review could enable the identification of lymph nodes that either should be targeted or that can be excluded from the radiotherapy field, thereby informing rational field design. The various imaging modalities would offer different advantages, depending on the clinical issue under investigation.

The wide array of imaging methods that can be used to image the inflammatory response can be divided into two broad groups. The first group comprises tracers that can be administered systemically and provide images of the current distribution and extent of the immune response, either generally (using tracers with broad specificity, such as ^{18}F-FDG) or specifically (using tracers such as anti-CD20 antibodies). The second group includes imaging methods that involve the ex vivo labelling of immune cells and their subsequent readministration to the patient (such as ^{99m}Tc-HMPAO with SPECT imaging, or SPIONs with MRI). Despite the technical and logistical difficulties with the second group of methods (such as maintaining sterility), they offer a distinct advantage over systemic administration. Specifically, these ex vivo labelling methods enable imaging of the trafficking of specific cell populations and their infiltration into tissues or accumulation in lymph nodes, either at steady state (that is, under normal conditions) or in response to immunotherapies.

Of note, imaging-based evidence of inflammation in irradiated areas might not necessarily reflect an enhanced immune response. Chronic inflammation, in addition to promoting tumour growth, might be associated with immunosuppressive effects, both within the TME and systemically[203]. For example, cytokines associated with the inflammatory response, including TGFβ and IL-10, can trigger T cell exhaustion via chronic stimulation of inhibitory receptors such as PD-L1, CTLA-4, and others[204]. Other immunosuppressive effects of chronic inflammation include the proliferation of cancer-associated fibroblasts and immunosuppressive plasma cells[205].

Imaging alone might offer sufficient information to inform which lymph nodes should be spared, but mathematical models are required to optimize the timing of irradiation (that is, at what time point irradiation of the lymph nodes is expected to be least detrimental to the development of a robust immune response). This is possible given that patients with metastatic disease receiving immunotherapy can be treated with radiotherapy at different time points, unlike those with

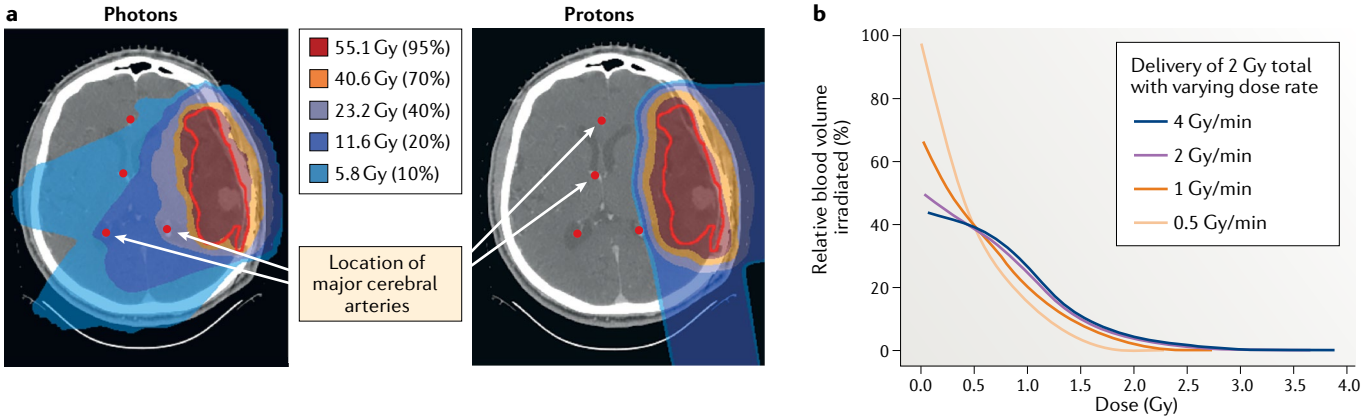

Fig. 3 | **Effect of radiotherapy technique on immune response.** Radiotherapy technique, including the integral dose and the dose rate, can have an important role on lymphocyte depletion during radiotherapy and, therefore, has implications for immunotherapy–radiotherapy combination regimens. **a** | Depicting the dose distributions for photon (left) and proton (right) therapy treatment plans for a patient with an intracranial tumour (red contour). Note the large difference in dose bath extending to the contralateral side of the brain. The locations of the major arteries supplying blood to the supratentorial brain are indicated with red dots. **b** | The blood dose–volume histogram shows the relative blood volume irradiated for delivery of 2 Gy using photon-based radiation with different dose rates. Data from part **a** courtesy of H. Shih and J. Daartz, Massachusetts General Hospital, USA. Data from part **b** courtesy of D. Craft, Massachusetts General Hospital, USA.

locally advanced disease, who require radiotherapy at the time of diagnosis. Furthermore, animal experiments have demonstrated that the improved therapeutic efficacy of combination regimens is sensitive to the timing of administration and is further dependent on the mechanism of action of the immunotherapy modality used[16].

Radiotherapy technique

In the context of lymphocyte depletion during radiotherapy, the radiation delivery technique used could also have important implications. Indeed, large differences can exist between the dose distributions for photon and proton therapy in a given patient with an intracranial tumour, caused by the low-dose bath, which is the large volume irradiated to relative low doses that extends to the contralateral side of the brain with photon therapy (FIG. 3a). Although the effect of this low-dose bath is heavily debated, is dependent on the organ at risk and end point, and does not necessarily influence clinically relevant toxicities, emerging data indicate that this low-dose bath might affect the degree of radiation-induced lymphopenia in patients[53,60].

A similar argument exists for the speed with which radiation is delivered. A faster rate of irradiation enables a higher dose to be delivered to a smaller fraction of circulating lymphocytes while sparing the majority (FIG. 3b). Thus, in the context of immunotherapy–radiotherapy combination regimens, conformal modalities (such as proton therapy[206]) and the dose rate achievable with a given technique could possibly have clinical implications.

Conclusions

Preclinical models are key for the development of not only effective systemic therapies but also for selection of the optimal radiotherapy regimen and its integration with immunotherapy. Studies have provided intriguing data on the variability in the immune response to different doses and fractionation schedules of radiotherapy[4,8,14,16], which are not necessarily related to the amount of direct tumour cell killing by radiation. However, although these data are intriguing and hypothesis-generating, they must be interpreted with caution given the immunological differences between animals, particularly mouse models, and humans. Moreover, these animal models are limited in their ability to track variations in the immune response over time. Ultimately, models that do not rely on data from animal experiments but are based directly on observations in patients, such as those presented in this Review, will be key to fully understanding the immune response to radiotherapy.

Thus, given the lack of validated prospective data on the 'optimal' biomarker for predicting the immune response to radiotherapy, we favour a broad approach to biomarker inclusion in clinical trial design. Inclusion of circulating biomarkers, although limited in their potential to provide a nuanced assessment of site-specific responses, will, at the very least, enable tracking of key immune cell populations over time. Importantly, circulating biomarkers will probably be the most cost-effective of all the potential biomarkers, which will be important for their widespread application. We also strongly encourage supplementing circulating biomarkers with advanced imaging techniques to facilitate assessments of the variability in immune response between different sites. When combined with clinical response data, these comprehensive datasets will provide the greatest potential for fully understanding and optimizing the immune response to radiotherapy.

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