Advances in radioimmunotherapy 1

Radioimmunotherapy for the treatment of head and neck cancer

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Evidence to date shows that immune checkpoint inhibitors have little benefit in most patients with head and neck squamous cell carcinoma (HNSCC). Intense interest is focused on identifying and developing rational combinations of immune checkpoint inhibitors and different therapeutic interventions to enhance response rates and overcome immune checkpoint inhibitor resistance. Combining radiotherapy, a primary HNSCC treatment modality, with immunotherapy has been shown to induce potent antitumour immune responses in many cancers including HNSCC. In addition to its direct cytotoxic effect on the cancer cell, radiotherapy can shape the tumour microenvironment to affect the abundance and composition of tumour-infiltrating immune cells and therefore change responses to immune checkpoint inhibitor therapy. In this Series paper, we examine how radiotherapy can be used to its maximum therapeutic potential in the setting of immunotherapy treatment for HNSCC by focusing on published clinical and preclinical data. We rely on preclinical evidence for this disease to discuss how radiotherapy can help create and maintain an immunologically permissive environment. Our hope is that such mechanistic insights will provide a foundation for maximising the use of radioimmunotherapy in disease control, designing future trials, interpreting emerging immunotherapy data, and accelerating discovery within radioimmunotherapy interventions for HNSCC.

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This is the first in a **Series** of five papers about advances in radioimmunotherapy

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Introduction

Radiotherapy is standard of care for most patients with head and neck cancer treated definitively or adjuvantly. Aside from the effects of radiotherapy through direct DNA damage and indirect damage from free radical formation, radiotherapy can also induce antitumour immune responses that contribute to indirect tumour cell killing.[1,2] These effects include interferon (IFN)-dependent expression of MHC class I (MHC-I), enhanced expression of innate immune ligands, expanded diversity of T-cell receptor repertoires within tumours, and promotion of cGAS-dependent sensing of cytoplasmic DNA and subsequent hSTING (downstream adaptor stimulator of interferon genes)-dependent production of type I IFNs.[1] However, the antitumour immune effects of radiotherapy can be blunted by mechanisms of immune evasion and immune suppression leading to upregulation of PD-L1 on tumour cells, and infiltration of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages.[1] These mechanisms have been thoroughly discussed in multiple review articles.[1,3–11] However, in this Series paper, we draw on available clinical evidence to explain how such mechanisms contribute to therapeutic response to radioimmunotherapy in patients with head and neck squamous cell carcinoma (HNSCC). HNSCCs represent biologically different diseases depending on human papillomavirus (HPV) positivity, smoking history, the site of origin of primary disease within the head and neck, the burden of disease locally, regionally, and distantly, and the extent of previous treatment and the variations of treatment modality a patient has received. As such, the variable goals of radioimmunotherapy include enhancing local control outcomes, controlling distant metastatic disease, inducing abscopal effect, and facilitating treatment de-escalation in low-risk patients. Given the scarcity of relevant literature, however, and the fact that many radioimmunotherapy trials are still accruing, we only summarise relevant literature that is mostly focused on HNSCCs and review some of the salient observations and difficulties facing the field. A discussion of newly emerging biological and radiographical biomarkers from clinical trials and retrospective and prospective studies is included. Finally, we review the controversies and potential effect of radiotherapy dosing, fractionation, and sequencing of radioimmunotherapy.

Biomarkers of response

Much published research on biomarkers of response to immunotherapy has focused on the identification of diverse alterations in immune cell populations and regulation of immune checkpoints, or defects in the antigen presentation machinery that could act as predictors of immune evasion. An immunohistochemical evaluation of PD-L1, tumour-infiltrating lymphocytes (TILs), or both has been proposed[12–16] as these potential biomarkers are of promising prognostic value for response to immunotherapy in HNSCCs. For patients treated with surgery followed by chemoradiotherapy, high PD-L1 expression and CD8 TILs in the tumour compartment, as analysed by immunohistochemistry, positively correlate with survival outcomes independent of other clinicopathological factors such as resection status, grade, extracapsular extension, or N stage.[17] Similarly, in the definitive chemoradiotherapy or radiotherapy setting, infiltration of CD3 lymphocytes in

the tumour compartment, not the stromal compartment,[18] or PD-L1 expression on tumour cells or immune cells,[12] showed prognostic correlation with survival outcomes or response to radiotherapy.[12–14] Additionally, immune-cell expression of PD-L1 combined with high CD8 TILs according to immunohistochemical analysis correlates with improved prognosis in patients with HNSCCs receiving definitive chemoradiotherapy or radiotherapy.[15]

Some of the variability in the findings of immune response studies associated with chemoradiotherapy or radiotherapy, particularly in the definitive treatment setting, can be attributed to challenges with immunohistochemical analysis. Tissue variability, both in terms of intertumour and intratumour heterogeneity, is a major confounder. In this disease, tissue sampling varies from small diagnostic biopsies or resection material from primary tumours to resected lymph nodes or other metastases. PD-L1 scores as assessed on biopsy specimens tend to underestimate PD-L1 expression as evaluated on primary tumour specimens.[16] Furthermore, primary tumour and nodal metastasis had poor to fair agreement when comparing biopsy material versus resection tissue.[16] Given that many patients with HNSCC are treated with definitive chemoradiotherapy and tissue availability is low, a quest for more sensitive and predictive biomarkers of response to immunotherapy is crucial for this disease. The composite positive score, [16,19–21] developed by Kulangara and colleagues in 2017[22] has the potential to overcome these challenges, but awaits further validation. In multiple studies, retrospective analyses and meta-analyses have shown that the baseline circulatory neutrophil-to-lymphocyte ratio is a strong predictor of survival outcomes for chemoradiotherapy or radiotherapy treatment for patients with HNSCC.[23–27]

A pertinent question that emerges in many of these studies is the prognostic relevance of HPV status in terms of PD-L1 expression and TIL expression levels. Despite the substantial clinical, genomic, and immunological differences between HPV-positive and HPV-negative HNSCC, tumour HPV status has not yet predicted response to immunotherapy in clinical trials of metastatic disease[28,29] after controlling for PD-L1 status, TIL status, or analysis of interactions.[17] Some studies have observed that high intratumour expression of PD-1 on T cells predicts better prognosis in HPV-positive cancers.[17,30] Since PD-1 is a marker of T-cell exhaustion, Kansy and colleagues[31] sought to solve the problem of how dysfunctional T cells could be associated with a subset of patients known to have a better prognosis. They showed that the higher expression of PD-1 on CD8 T cells, not the mere presence of PD-1 expression, was associated with negative disease outcomes. Although high PD-1 expression on CD8 T cells was more prevalent in HPV-negative patients, subsets of HPV-positive patients who had high PD-1 expression on CD8 T cells also had worse outcomes. In an orthotopic mouse model of HPV-positive tonsillar cancer treated with radiotherapy, which is known to induce high PD-L1 expression on tumour cells and PD-1 expression on T cells,[31] treatment with PD-1 inhibitors restored the functionality of high PD-1 expression on CD8 T cells, enhanced proliferation of the PD-1-low T cells, or both, thus enabling antitumour response. These data suggest relative levels of expression of PD-1 and the subsequent effects on T effector cells' functionality might predict response to anti-PD-1 immunotherapy.[31]

A 2018 analysis identified two key variables that, if validated prospectively, might predict T effector cell functionality, and therefore also predict the relative activity of immune checkpoint inhibitors.[32] In a large retrospective pan-tumour genomic study, which included patients with HSNCCs, tumour mutational burden (TMB) and inflammatory biomarkers (T-cell inflamed gene expression profile [GEP] and PD-L1 expression) can jointly stratify human cancers into responders and non-responders (table).[32] The objective response rates were highest in those tumours that were high for both T-cell-inflamed GEP and TMB compared with those that were low for either one or both, and this outcome remained significant on multivariate analysis.[32] Although the specific effect of HPV status was not evaluated, a different analysis in HPV-positive HNSCC showed no statistically significant pretherapy association between TMB and response from inhibition of the PD-1 pathway.[33] Data from the melanoma literature shows that the dynamics of TMB (change in TMB from early therapy to 4 weeks after immune checkpoint inhibitor treatments) are more closely associated with response to therapy.[34] This parameter remains challenging to implement in clinical practice, however, because of the difficulties of doing a biopsy while on therapy. New technologies aimed at non-invasively assessing circulating cell-free DNA should better enable the implementation of this method in the future.

The requirement of both high TMB and T-cell inflamed GEP for eliciting maximum responsiveness to immune checkpoint inhibitors is consistent with the results of a comparative analysis between patients with lung cancer that were heavy smokers and patients with head and neck cancer that were heavy smokers.[35] Heavy smokers with lung cancer are known to have a higher response rate to immune checkpoint inhibitor therapy, and better overall survival, than individuals with HNSCC. Desrichard and colleagues[35] identified the tumour immune microenvironment to be a major difference between the responses of the two cohorts. Despite both populations having a high TMB (a smoking signature), smokers with lung cancer had a highly inflamed tumour microenvironment compared with smokers with HNSCC.[35] In the lung cancer context, the effects of increased neoantigen load are counterbalanced by the high proinflammatory tumour microenvironment compared with HNSCC where the increased TMB exists in an immunosuppressive microenvironment. This idea might partly explain the discrepant results between the two

	Controversies	Conclusions	Bottom line
Tregs	Definition of Treg varies; method of analysis varies; tissue analysis varies; heterogeneous pool of studies; studies did not control for HPV status and other prognosticators on multivariate analysis	Treg measurement in isolation does not represent net extent of immune activation or suppression; Treg numbers vary with broader patterns of immune infiltrates	Tregs are suppressive and negative prognosticators; Treg to CD8 ratio or Treg to T effector cell ratio is more indicative of response; unified definition of Tregs as CD4-positive, CD25-positive, and FoxP3-positive cells proposed; Tregs are relatively unresponsive to radiotherapy and contribute to resistance; effect of radiotherapy fractionation is unknown
PD-L1 composite positive score	Studies have been done in the setting of recurrent metastatic head and neck cancer, but have not included a control for radiotherapy as a variable; tissue variability, both in terms of intertumour and intratumour heterogeneity; technical variability using immunohistoschemical analysis	PD-L1 composite positive score is defined as number of PD-L1-staining cells (tumour cells, lymphocytes, or macrophages) divided by total number of tumour cells, multiplied by 100; composite positive score ≥1% leads to better response rates to anti-PD-1	Prognostic relevance in setting of radiotherapy unknown; ignores TMB and other gene expression profiles within tumour microenvironment
PD-L1 expression levels	Same as PD-L1 composite positive score	Baseline expression of tumoural PD-L1 are prognostic of therapeutic response to immunotherapy in patients with recurrent or metastatic disease undergoing first-line or second-line treatment; exact cutoff remains controversial	Prognostic relevance of baseline pretreatment expression level is uncertain in the setting of radiotherapy especially since radiotherapy itself increases PD-L1 expression levels
TILs	Often defined as number of infiltrating CD8 T cells; subject to technical and tissue variability, as with composite positive score; effect of stromal *vs* tumour compartment unclear	CD3 lymphocytes are probably important in both compartments	High baseline CD8 TILs in combination with high anti-PD-L1 expression predictive of response to radiotherapy and chemoradiotherapy; whether TILs predict response to radioimmunotherapy awaits validation in a clinical trial
HPV	No clear data justify a selection of HPV-positive patients; responses in some trials are higher than in HPV-negative patients many of these HPV-negative tumours are highly immunogenic at baseline	No definitive conclusion can be drawn; response could be dependent on the type of immunotherapy used (anti-PD-1 *vs* anti-PD-L1)	After controlling for PD-L1 level and TILs, HPV status does not appear prognostic; trials not done in radiotherapy context; analysis did not control for radiotherapy variables
TMB or GEP	TMB currently assessed by NGS, but the exact cutoff for immunotherapy response prediction is unknown; reporting methods are not standardised	Each independently predictive and the two together are more predictive; effect of treatment before with chemotherapy reportedly prognostic but controversial	Retrospective tissue analysis suggests a strong correlation with response to immunotherapy; prospective data are not available; effect of radiotherapy is unknown, especially in non-pretreated patients

Treg=regulatory T cell. HPV=human papillomavirus. TMB=tumour mutational burden. TIL=tumour-infiltrating lymphocyte. HPV=human papillomavirus. GEP=gene-expression profile. NGS=next-generation sequencing.

Table: **Controversies and conclusions of biomarkers of response to head and neck squamous cell carcinoma radioimmunotherapy treatment**

trials[36,37] that combined hypofractionated radiotherapy (stereotactic body radiotherapy [SBRT]) with anti-PD-1 antibody. Although in the advanced non-small-cell lung cancer cohort, combination pembrolizumab preceded by SBRT doubled the objective response rate (ORR) from 19% in the control group to 41% in the experimental group at 12 weeks,[36] adding SBRT to nivolumab in M1 HNSCC did not improve ORR.[37] Although the studies differed in their choice of PD-1 antibody and the inclusion of virally driven HNSCC, the superiority in synergy between SBRT and anti-PD-1 was evident for the non-small-cell lung cancer cohort when compared with the HNSCC cohort. Whether this superiority is due to a tumour microenvironment with a low T-cell-inflamed GEP or a low TMB remains to be established. Until quantitative yields of misrepair after SBRT become available, it will not be possible to establish whether the problem is an issue with achieving a high enough TMB or whether radiotherapy is simply acting as an immunological adjuvant that can create a highly inflamed tumour microenvironment on existing tumour antigens via necrotic or immunogenic cell death.[38] These data indicate that the intrinsic capacity of intratumoural T cells to recognise adjacent tumour tissue can be rare and variable, and suggest that clinical efforts to reactivate intratumoural T cells will benefit from approaches that simultaneously increase the quality of the intratumoural T-cell receptor repertoire.

Whether radiotherapy in patients with HNSCC can create a proinflammatory tumour microenvironment and increase the T-cell inflamed GEP should be confirmed with results from future clinical trials combining radiotherapy with immunotherapy. In preclinical animal models of HNSCCs, radiotherapy converts tumour

unresponsiveness to checkpoint inhibitors into response.[39] This response correlates with tumour microenvironment inflammation.[39] Radiotherapy initiates cell death and the production and release of cytokines and chemokines, such as CXCL9 and CXCL10, into the tumour microenvironment, leading to infiltration of dendritic cells, macrophages, promotion of antigen-specific T-cell priming and cytotoxic T-cell infiltration and function.[3,40] Radiotherapy, however, can also modulate infiltration of the immunosuppressive cell population in a dose-dependent manner. Successful clinical translation of using radiotherapy to invigorate immune response will depend heavily on promoting cytotoxic cell kill while overcoming immunosuppressive effects. Suggested mechanisms have been discussed elsewhere.[3,40]

In tumours with low TMB, speculation has arisen that radiotherapy might improve the efficacy of immune checkpoint inhibitor therapy by causing radiation-induced TMB. However, many have questioned whether conventionally fractioned radiotherapy, such as that used in HNSCC treatment, can sufficiently raise TMB to induce antigenicity.[38] For this event to occur, radiotherapy would have to induce DNA modifications to the point that error-free DNA damage repair becomes increasingly saturated and the rate of misrepaired DNA alterations is considerably amplified.[41] Some have argued that these circumstances can be overcome with the delivery of single high doses of radiotherapy or large doses per fraction of hypofractionated radiotherapy (eg, in SBRT).[38] Clinical studies on immune checkpoint inhibitor therapy combined with SBRT in other non-HNSCC tumour models have shown impressive responses and improved overall survival.[38] Combining SBRT with nivolumab in patients with metastatic HNSCC, however, has yielded disappointing results in eliciting systemic responses.[37] Collectively, these data show the discordance between TMB and immunogenicity in HNSCCs. Even if radiotherapy can increase the number of DNA alterations, these alterations will probably be subclonal mutations that do not drive an efficient antitumour immune response rather than neoantigens derived from clonal mutations,[42,43] although further data are needed in HNSCCs to confirm such a hypothesis.

HPV-positive HNSCCs: role of viral oncoproteins in the immune response

HPV-positive HNSCCs are biologically different diseases to HPV-negative HNSCCs and might be particularly dependent on aberrant immune checkpoint inhibitors that create an immune-privileged site for HPV infection and function as an adaptive resistance mechanism of the tumour against the host.[44] HPV proteins E6 and E7, the first viral oncoproteins expressed following infection, are considered good targets to activate the immune response to recognise the cancer since they are foreign entities for the host.[45] In response to E6 and E7 HPV-derived oncoproteins, polyfunctional and active antigen-specific T cells have been shown to undergo expansion in most HPV-positive disease, and correlated with improved survival outcomes after chemoradiotherapy in a clinical trial.[46]

To exploit HPV viral neoantigens as therapeutic targets, a phase 1b/2 clinical trial evaluated immune responses in HPV-positive patients with HNSCC receiving antigenic stimulation using DNA-based immunotherapy with synthetic plasmids targeting HPV-16 and HPV-18 E6 or E7 antigens (VGX-310, Inovio Pharmaceuticals, Plymouth Meeting, PA, USA).[47] This treatment elicited antigen-specific, long-lasting, humoral and functional cellular immune responses. A cellular immune response against HPV-16 and HPV-18 was even detected in circulating peripheral blood mononuclear cells, with an increase in IFNγ, T-cell activation, and CD8 to FoxP3 ratio. Evidence for HPV-specific T cells was corroborated in patients that progressed on the trial, who when later treated with anti-PD1 therapy, had complete response and expansion of antigen specific PD-1-positive CD8 cells with cytolytic potential.[47] This result suggests that DNA-based HPV-specific immunotherapy induced HPV-specific cytotoxic T cells, but that coexpression of PD-1 inhibited their antitumour effects. This finding will enable future trials aimed at combining such antigen-specific DNA immunotherapy for immune priming with checkpoint inhibitors in the treatment of HPV-positive HNSCCs.

Use of these vaccines in HPV-positive HNSCCs in the setting of concurrent radiotherapy warrants further study. Radiotherapy might drive anticancer immunity towards tumour-related antigens and act as an adjuvant to boost antigen presentation. In a preclinical HPV-positive immunocompetent mouse model of lip cancer, Mondini and colleagues[48] tested the efficacy of local radiotherapy in boosting the response to a Shiga Toxin vaccine (STxB-E7). In response to radiotherapy, enhanced infiltration of HPV protein E7-specific CD8 T cells was observed, with memory response sufficient to inhibit local and distant recurrence at re-challenge. This response was evident without intranasal (mucosal) immunisation with the vaccine, supporting the conclusion that radiotherapy itself is sufficient to induce above-threshold antigen-specific CD8 T cells in combination with the vaccine to overcome tumour growth and result in tumour eradication.

Treg and myeloid populations of an immunosuppressive environment

The prognostic potential of Tregs in HNSCCs has been a controversial matter that deserves special consideration. Higher numbers of Tregs have been reported to associate with better prognosis in a small retrospective series and in a meta-analysis pooling these heterogenous studies together.[49–51] These studies were not only limited by the variability of the patient population, treatment characteristics, and methods of tissue analysis, but also did not adjust for other immune cells present within the tumour. In an analysis of The Cancer Genome Atlas by

Mandal and colleagues,[52] the prognostic significance of Tregs was lost in a multivariate analysis after adjusting for the presence of other immune cells. These results indicate that the trend in Treg numbers in HNSCC changes with broader patterns among T-cell populations, and that Treg numbers in isolation might not represent the overall net level of immune activation or suppression in the tumour microenvironment (table). These findings agree with a hypothesis in which Tregs are recruited to the tumour microenvironment (or converted to Tregs from conventional CD4 T cells) in response to immune signals to maintain immune homoeostasis. Supporting this hypothesis are results from a cross-sectional study showing that in the setting of postoperative chemoradiotherapy, Tregs increased in frequency and persisted afterwards, especially in those with active disease, and could be responsible for suppression of antitumour immune responses and recurrence in HNSCC (table).[53] These results were also supported by a multiparameter flow cytology analysis[54] of patients with recurrent or metastatic HNSCCs showing that patients with high Tregs had a median survival of only 13 months compared with a median survival of 84 months in those with lower Treg populations. These data collectively suggest that high Tregs within the HNSCC tumour microenvironment are exerting an immunosuppressive effect within tumours (table).

In the context of radiotherapy, Tregs have been shown to be so-called radioresistant, as they persist or reaccumulate and, in some cases, increase after radiotherapy (figure 1, table).[47,55,56] Preclinical orthotopic models of HNSCC are highly enriched with Tregs, and this has been shown to contribute to resistance to radiotherapy in combination with anti-PD-L1, an effect that was not overcome by hypofractionated radiotherapy[57] or by adding other immune checkpoint inhibitors such as anti-Tim-3 or anti-CTLA-4.[58] Only when Tregs were depleted were tumours rendered sensitive to radiotherapy, with an associated increase in T effector cell responses, and in which tumour eradication was achieved.[58] Strikingly, only when Treg depletion is done in combination with radiotherapy did eradication become possible. Treg depletion alone was not sufficient to induce an antitumour response in these so-called cold tumours.[57] A local insult to the tumour appears necessary to allow T effector cells to infiltrate and orchestrate an antitumour immune response. Thus, combining Treg depletion strategies with immunotherapy in immunogenic-poor tumours will probably not be effective, as the absence of Tregs is not sufficient to expose tumour neoantigens and activate signals that attract cytotoxic immune cells. Radiotherapy is well suited for transforming immunogenic-poor tumours, as it has been shown to expose tumour neoantigens, induce MHC-I, increase secretion of effector T-cell chemokines (including CXCL9 and CXCL10, leading to increased T effector cell infiltration), and activate the hSTING pathway.[1] Such a potent synergistic effect when radiotherapy is combined with Treg depletion highlights that the two modalities can act on distinct aspects of antitumour immunity that are not sufficient on their own to provide tumour control.

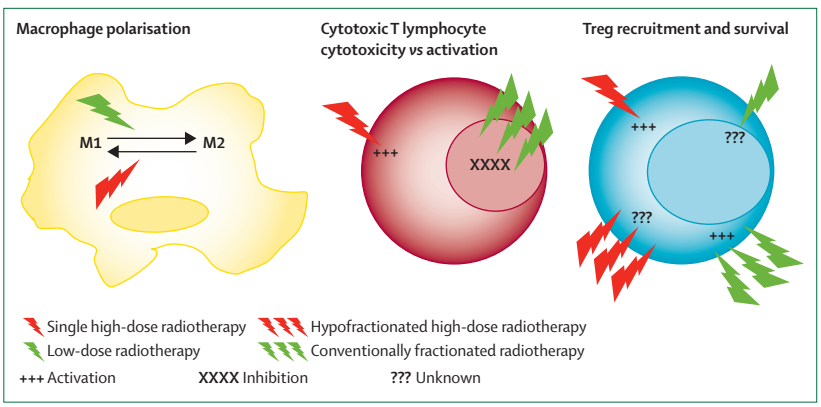

Figure 1: **Radiotherapy effects on the immune microenvironment dependent on dose and fractionation**
Much remains unknown in the head and neck tumour microenvironment. Low-dose radiation tends to favour macrophage polarisation in favour of a tumoricidal M1 phenotype, whereas a single high-dose fraction favours the tumour-promoting M2 phenotype. Both phenotypes are likely to upregulate PD-1 expression on cytotoxic T lymphocytes. A high-dose single fraction activates cytotoxic T lymphocytes whereas conventionally fractionated radiotherapy is cytotoxic. According to preclinical data, single-dose hypofractionation and conventionally fractionated radiation are both likely to activate and recruit Tregs, but it is unknown how low-dose radiotherapy or high-dose hypofractionation will affect Treg populations. The effect will vary depending on the magnitude of the dose per fraction. The effect will also vary as a function of time, which is not depicted here. Treg=regulatory T cell.

MDSCs are a major cellular component of the tumour microenvironment that promote immune evasion and tumour growth by preventing T-cell infiltration and activation. Local radiotherapy results in substantial intratumoural infiltration by MDSCs, accompanied by upregulation of their activation products and production of chemotactic factors.[57,59] In preclinical HPV-positive models of HNSCCs, inhibition of the TGFβ pathway in combination with radiotherapy has been shown to decrease differentiation of MDSCs into functional myeloid cells that have reduced both immunosuppressive activity and antitumour effects.[60] In HPV-negative preclinical models of HNSCCs, MDSCs were shown to be key drivers of resistance in the radiotherapy setting, especially in models resistant to Treg depletion.[57] Strategies aimed at targeting MDSC recruitment into the tumour microenvironment, such as via CXCL2 or by targeting their differentiation, will be vital to overcoming immunosuppression in this setting. Similar to MDSCs, myeloid precursor cells can differentiate to M1 (tumouricidal) or M2 (tumour-promoting) macrophages,[61] and the M1 to M2 ratio has been shown to be prognostic in the HNSCC patient population.[62,63] For HNSCCs, radiotherapy polarises macrophages into an M2 phenotype (figure 1),[64] and their depletion before irradiation has a more robust antitumour response.[65] Preclinically, Treg depletion with radiotherapy also transforms myeloid populations, decreasing M2 macrophages and MDSCs and increasing M1 macrophages.[57]

Radiation dose effects

The effects of radiation dose and fractionation on immune cells is discussed in figure 1. The optimal radiotherapy dose and fractionation to invigorate the immune response remains unknown. Clinically, in the definitive or adjuvant setting of HNSCCs, radiotherapy is delivered in multiple daily fractions of 1·8–2·0 Gy. Although it is uncertain whether this fractionation regimen stimulates an immune response, data from the clinical literature show that such fractionation can result in severe lymphopenia[66,67] due to the repeated exposure of radiosensitive T cells to radiotherapy (figure 1),[68] negating the therapeutic benefit of this fractionation regimen. However, these data are based on an analysis of peripheral T cells and do not reflect T-cell functionality or diversity. Within the irradiated tumour, conventionally fractionated daily radiotherapy has been shown to increase the local expansion of pre-existing T-cell clones and diversification of the T-cell receptor repertoire,[69] so additional data are needed to fully appreciate the immunogenic effects of varying radiotherapy fractionation.

Hypofractionated radiotherapy, which involves the delivery of large doses of radiotherapy in one to five fractions, offers enough time between doses for repopulation of T cells, and therefore provides an effective response to PD-L1 blockade. Studies making use of different models have shown that hypofractionation or single high-dose radiotherapy can induce T-cell infiltration for cancer.[70,71] Compared with conventionally fractionated radiotherapy in an HNSCC model, Morisada and colleagues[72] similarly showed that hypofractionated radiotherapy (figure 1) using 8 Gy in two fractions maintained peripheral and tumour-infiltrating CD8 T-cell number and activity, and decreased peripheral MDSC accumulation,[72] despite similar control of primary tumour growth between the two study groups. Additionally, in the tumour-draining lymph nodes, conventionally fractionated radiotherapy at 2 Gy in ten fractions (but not 8 Gy in two fractions) suppressed tumour-specific T-cell responses. This finding might explain why only the hypofractionated radiotherapy regimen (8 Gy in two fractions) synergised with anti-PD-1. These data were gathered from highly immunogenic MOC1 tumours subcutaneously transplanted as flank xenografts. In orthotopic models using less immunogenic models, Oweida and colleagues[57] revealed that a single-dose of 10 Gy radiotherapy yielded a statistically significant increase in effector CD8 and CD4 T cells, but no response was observed with 10 Gy in five fractions.

Whether hypofractionated radiotherapy delivered in multiple fractions is superior to a single high-dose fraction in stimulating immune response remains a controversial subject. In mammary and colon tumour flank xenograft models, high single-dose radiotherapy of 20 Gy blunts cGAS-dependent and hSTING-dependent type I IFN responses compared with 24 Gy in three fractions of hypofractionated radiotherapy.[73] However, these data have yet to be verified in HNSCCs. In orthotopic HNSCC models that resemble HPV-negative disease in their behaviour and immunophenotyping, combining either single high-dose radiotherapy of 10 Gy versus hypofractionated radiotherapy of 8 Gy in three fractions in combination with anti-PD-L1 yielded similar immune profiles and treatment response.[58] Finally, when radiotherapy is given in a single large fraction, the optimal threshold required to trigger a potent response appears to be in the range of 7·5 Gy in preclinical models of HNSCCs aimed at examining the synergy between Shigela toxins and radiotherapy.[48] The applicability of these results to other preclinical models of HNSCCs or to human cancers remains to be tested.

Within the tumour microenvironment, the response of macrophages differs depending on radiotherapy dose, time, and fractionation.[1,74] Tumour-associated macrophages have been associated with poor prognosis in relapsed HNSCCs.[75] In preclinical animal models, a single dose of 12 Gy radiotherapy was shown to polarise macrophages towards an M2 phenotype,[76] whereas conventional radiotherapy dosing of 2 Gy fractions promotes the M1 phenotype, although the latter has been shown in other models.[74,77] Consistent with these data, a phase 2 trial showed that low-dose fractionated radiotherapy of 50 cGy per fraction (two each on days 1, 2, 8, and 15) potentiated the effects of induction chemotherapy.[78] It would be of great interest to examine the effect of such tumour-associated macrophages, or even circulating monocytes, from patients treated with this regimen and compare it with historical controls treated with daily 2 Gy radiotherapy.[79] Finally, in a novel application of radioimmunotherapy, the increase in tumour-associated macrophages in response to high-dose radiotherapy (delivered as a single 5 Gy fraction) has been exploited to enhance delivery of therapeutic nanoparticles.[79]

Assessing radiographic response to radioimmunotherapy

Assessment of response to combination immunotherapy and radiotherapy remains a challenge, as the radiographic response patterns to immune checkpoint inhibitor therapies can differ substantially, especially compared with cytotoxic chemotherapy. New guidelines have been implemented to track tumour response in patients undergoing immunotherapy,[80] but outside of tumour burden, little HNSCC literature exists that identifies patterns of radiological response data to predict which patients will benefit from combination immune checkpoint inhibitor therapy and radiotherapy.[81] Similarly, optimal imaging criteria in the setting of radiotherapy remains to be established, with emerging data showing higher sensitivity of the Hopkins criteria (78%) using PET scans alone when compared with conventional Response Evaluation Criteria In Solid Tumors (62%).[82]

These data show the challenges in using conventional imaging parameters when combining chemoradiotherapy with immunotherapy in curative patients with HNSCC. Multiple groups are working on developing imaging biomarkers where the radiological features can more closely associate with underlying gene expression levels in response to radioimmunotherapy. One method is immuno-PET/CT imaging using Zr-89-labelled anti-mouse PD-L1 monoclonal antibody, reported to differentially detect anti-PD-1 response to radiotherapy in preclinical murine models of HNSCCs or melanoma.[83] Interestingly, melanoma tumours more readily upregulated PD-L1 expression by immuno-PET and flow cytometry following a low dose of conventionally fractionated radiotherapy (2 Gy in four fractions) compared with HPV-positive HNSCC tumour models, which required a higher dose of conventionally fractionated radiotherapy (2 Gy in ten fractions).[83] If translated into a clinical setting, this imaging modality could serve as a non-invasive, predictive biomarker that can quantify PD-L1 increases after radiotherapy, and thus show who is likely to benefit from supplementing chemoradiotherapy with immunotherapy. New modalities aimed at imaging Tregs or MDSCs within the tumour microenvironment can potentially predict resistance to therapy and guide clinical practice.

Timing and sequencing of radiotherapy and immunotherapy

Understanding the temporal framework for the progression of innate and adaptive immune cell dynamics in response to radiotherapy in HNSCCs is crucial for optimal sequencing of radiotherapy and immunotherapy. Although no HNSCC-specific clinical data exist to guide clinical practice or design, much can be learned from understanding the biology of immune responses to radiotherapy in preclinical models of HNSCC. Radiotherapy will initiate a wave of cell death, recruiting antigen-presenting cells such as dendritic cells to sample the bolus of tumour debris released in the microenvironment and traffic it to lymph nodes to present antigens and prime T cells. This process is not instantaneous, and might take hours or days to align with circadian fluxes of lymphocytes through the lymph nodes to reach a maximal level of antigen-presenting cell to T-cell interaction. This event is also accompanied by an increase in antigen presentation, increased cytotoxic T-cell infiltration, and a robust increase of PD-L1 expression on tumours in response to T-cell-derived IFNγ as a mechanism of adaptive resistance.[84] This mechanism provides a rationale for concurrent administration of PD-L1 or PD-1 antibodies with radiotherapy to elicit antitumour immunity and immunological memory. Dovedi and colleagues[85] showed that this event occurred only when radiotherapy was given concurrently or immediately following radiotherapy, but delayed administration of anti-PD-L1 therapy by 7 days after completion of radiotherapy, thus abrogating any therapeutic benefit.[85]

The choice of immunotherapeutic agent and its targets is important in establishing the sequencing of immunotherapy in relation to radiotherapy. Unlike T effector cells, Tregs in HNSCC tumour models have a very small and short-lived reduction in numbers after radiotherapy.[39] This reduction is swiftly overcome by a substantial increase in Tregs, MDSCs, and M2 to M1 ratio, and an accompanying reduction in cytotoxic effector T-cell numbers and function. In the radiotherapy setting in preclinical HNSCC models, only when the tumour microenvironment was preconditioned with Treg targeted therapy was tumour eradication possible.[57,64] These findings are similar to radiotherapy responses in Treg-enriched colon cancer models with response enhancement occurring when tumours are subjected to Treg depletion before initiation of radiotherapy.[86,87] In other HNSCC tumours, such as MOC2 tumours, resistance to radioimmunotherapy, even when combined with Treg-depletion strategies, is driven by recruitment of MDSCs into the tumour microenvironment that ensues following radiotherapy initiation.[57]

For clinical translation, achieving optimal therapeutic efficacy might depend on preconditioning the tumour microenvironment with therapeutics aimed at reversing the immunosuppressive effects of radiotherapy before the delivery of radioimmunotherapy. Equally important will be the choice of agent to target immunosuppressive populations and better tools to precisely predict the response to temporal sequencing of new immunotherapeutics with respect to radiotherapy. The role of maintenance immune checkpoint inhibitor therapy delivered either monthly or by in-pulse dosing remains a subject of debate.

Abscopal effects: T-cell priming and the cytotoxic T lymphocyte permissive tumour microenvironment

The rate of inducing abscopal effects (ie, regression of a tumour mass outside the radiation field) by radiotherapy alone is quite low, with 46 total cases reported in the literature.[88] To elicit a systemic response, sustained T-cell priming that allows for cytotoxic T-cell activation in both the irradiated and occult metastatic sites has to occur.[89,90] The likelihood that such priming will occur without immunotherapy is low as self-sustained, continuous generation of new effector T cells is necessary to support the activity of a tumour-specific T-cell response.[89,90] Preclinical models of different cancers, as well as in multiple human trials, show that combination immunotherapy and high biologically effective dose radiotherapy, such as SBRT, is more likely to elicit abscopal effects.[73,91–93] Even in HNSCCs, data from animal models have supported the concept that combination hypofractionated SBRT-type dosing of 8 Gy in two fractions (but not conventional dosing of 2 Gy in ten fractions) induced an

abscopal effect.[72] However, results from a 2018 clinical trial in patients with metastatic HNSCC did not show any abscopal responses when hypofractionated SBRT was combined with nivolumab.[37] These findings are difficult to explain, especially when this combination yielded abscopal effects in other cancers combining SBRT and immunotherapy.[91]

In HNSCCs, the tumour microenvironment probably poses a major challenge for the induction of abscopal effect. In principle, radiotherapy induces tumour cell destruction and release of antigens and danger signals.[1] These antigens are presented to naive T cells by dendritic cells, which also provide the appropriate costimulatory and cytokine signals needed to induce T-cell clonal expansion and effector differentiation. Effective antitumour immunity and abscopal effects, though, require both priming of tumour-specific T cells and a cytotoxic-T-lymphocyte-permissive tumour microenvironment. Even though radiotherapy and anti-PD-1 therapy raises T-cell immune response, immune suppression within the tumour microenvironment soon ensues because of negative feedback control on T-cell effector function. As previously discussed, these mechanisms include Treg activity and the suppressive activity of myeloid cells, stromal cells, and even the tumour cells themselves.[1] For abscopal effect to work in poorly immunogenic HNSCCs, one might therefore speculate that radiotherapy has to be combined rationally with immunotherapy that functionally creates sustained, tumour-specific T-cell priming (radiotherapy and anti-PD-1), and a cytotoxic-T-lymphocyte-permissive tumour microenvironment that can overcome the immunosuppressive signals (eg, anti-Treg-directed therapy).

Even in the context of immune checkpoint inhibitor therapy and a permissive tumour microenvironment, the likelihood of eliciting an abscopal effect with single-site irradiation has been challenged.[94] Given the low clinically documented response rates in this context,[88] Brooks and Chang[94] have argued that single-lesion irradiation in the setting of diffuse metastasis is unlikely to generate enough tumour-specific antigens, T-cell priming, or intratumoural infiltration. To that effect, they propose that these events, and therefore abscopal responses, are more likely to occur with multisite irradiation of as much of the tumour burden as safely possible than restricted irradiation of a single site. Although this idea could explain the negative results of a clinical trial combining SBRT and nivolumab in the setting of metastatic disease,[37] this hypothesis awaits validation in future trials.

The role of regional lymph node irradiation

The contribution of elective nodal irradiation to an improved response rate for radioimmunotherapy, either in terms of local control or abscopal effect, is highly relevant for a disease like HNSCC. Patients with HNSCCs are at high risk for regional lymph node metastasis and typically receive radiotherapy targeted to their primary tumour site as well as elective nodal irradiation even when the cancer does not involve lymph nodes. The cytotoxic effects of radiotherapy elicit the release of danger signals that prompt the maturation of dendritic cells and other antigen-presenting cells and enable T-cell activation, leading to clonal expansion and activation of antitumour T cells in the draining lymph nodes.[1,2] The tumour-draining lymph nodes are essential for the generation of tumour-specific effector T cells, given that they are where dendritic cells prime antigen-specific CD8 T cells.

The fundamental clinical question of how lymph node radiation in HNSCC affects tumour immune response remains understudied in a disease where nodal irradiation is currently a standard component of therapy. The only study that has examined this question was done in flank preclinical cell line models of colorectal cancer and melanoma,[95] which showed that adding elective nodal irradiation of uninvolved nodes to immune checkpoint blockade reduced antigen-specific immune infiltrates favouring increased Treg to CD8 ratio, altered the chemokine milieu with a decreased cytotoxic T-lymphocyte chemoattractant profile, and shortened overall survival.[95] In another preclinical breast cancer model, pharmacological blockade of T-cell egression from the draining lymph nodes resulted in substantial trapping of newly primed CD8 T cells, hindering their ability to infiltrate the tumour and obliterating the response to radioimmmunotherapy.[96] Although these data are provocative, they are merely hypothesis-generating. Care must be exercised in extrapolating these data to HNSCCs, given their high propensity for micrometastatic lymph node involvement, especially compared with melanoma models where clinical practice does not include elective nodal irradiation. HNSCC-specific data in either preclinical or clinical models remain an unmet need, and such studies should help inform future directions.

Novel approaches

A growing list of pharmaceuticals exists with immune-oncologic effects that, on the basis of their mechanisms of action and an understanding of how radiotherapy affects the tumour microenvironment, can represent a novel way to synergise with radiotherapy and balance its immunotherapeutic ratio in HNSCCs (figure 2). TGFβ1, which is upregulated in the setting of radiotherapy, prevents T effector cell proliferation and activation, drives development of inducible Tregs, polarises macrophages towards the tumour-promoting M2 phenotype, and enhances myeloid-cell differentiation toward MDSCs.[2] Inhibition of TGFβ1 in combination with radiotherapy represents an ideal target. A bifunctional protein targeting both TGFβ1 and PD-L1 (M7824; EMD Serono, Darmstadt, Germany) has established a safety profile in phase 1 trials,[97] and future trials incorporating M7824 with radiotherapy in HSNCC are being developed.

The discovery that radiotherapy can activate transcriptional pathways that mediate T-cell exhaustion and

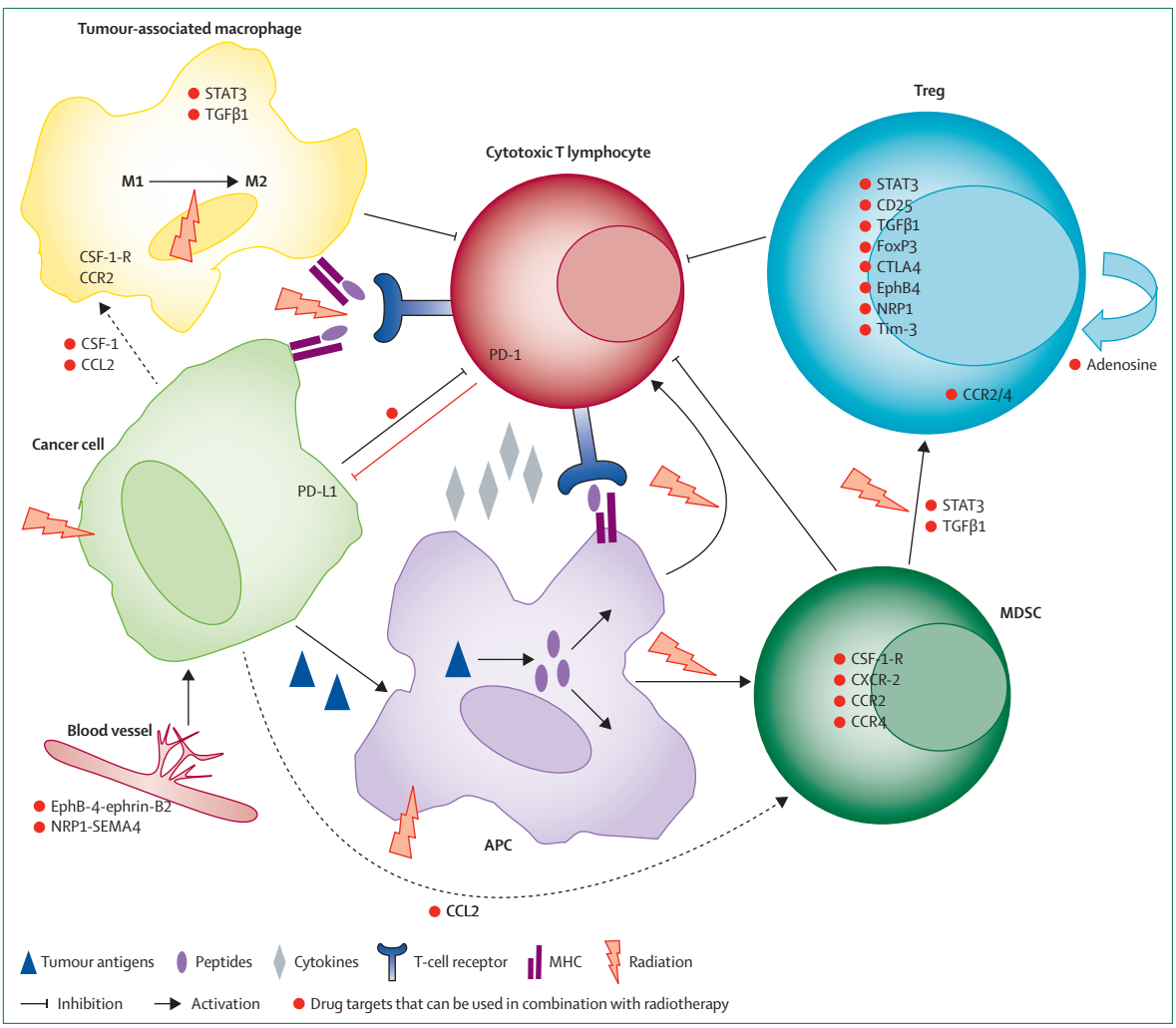

Figure 2: **Schematic for radioimmunotherapy combinations in head and neck cancer**
Radiotherapy has activating and suppressing effects on immune cells and can enhance antigen presentation and activation of cytotoxic T lymphocytes but can also enhance the recruitment and activation of suppressor cells. Leveraging the benefits of radiotherapy will depend on combining it with targets based on mechanistic insights of radiotherapy's modulation of the immune microenvironment. The examples shown here are illustrative and not comprehensive. APC=antigen-presenting cell. MDSC=myeloid-derived suppressor cell. Treg=regulatory T cell.

contribute to an immunosuppressive tumour microenvironment has led to new attractive approaches to combine with radiotherapy in targeting the immune microenvironment. One such target is anti-STAT3, for which phase 1 testing has been completed in clinical trials.[98] Conversion of CD4 T cells to Tregs involves activation of STAT3 via interleukin (IL)-2-induced FoxP3 expression.[99–101] Similarly, the production of TGFβ1 and IL-10 by CD4 T cells has also been shown to be dependent on STAT3 signalling.[102] Genetic ablation of *Stat3* in the haematopoietic system was found to reduce tumour infiltration of Tregs and increase proliferation of CD8 T cells, leading to a robust CD8 T-cell-dependent anti-tumour immune response.[100] In 2019, radiotherapy was shown to induce phosphorylation of STAT3 on CD4 T cells, resulting in an increase in Treg numbers in a dose-dependent manner in vitro,[57] an effect that can be reversed with STAT3 inhibition. In preclinical HNSCC models, STAT3 inhibition in combination with radiotherapy leads to significant delay in tumour growth, downregulation of immunosuppressive cytokines, decreased Tregs, MDSCs, and M2 macrophages, and enhanced effector T cells and M1 macrophages.[57] Clinical trials making use of anti-STAT3 antisense oligonucleotide AZD9150 (AstraZeneca, Boston, MA, USA) in different disease sites, including in HNSCC, (eg, NCT03421353, NCT02983578, and NCT01839604) are ongoing.

Disruption of chemokine signalling to reduce intra-tumoural infiltration of immunosuppressive cells represents a highly attractive target. In murine HNSCC models, both Treg and myeloid chemoattractants CCL20

Search strategy and selection criteria

We did a PubMed search using the terms "radiation", "immunotherapy", and "head and neck cancer" for papers published between Sept 1, 2009 and March 1, 2019. 444 papers matched these key terms. Those discussed in this Series paper were manually chosen at the discretion of the authors. Only papers published in English were considered.

(C-C motif chemokine 20) and CCL2 were highly expressed along with their cognate receptors CCR4 or CCR6, and CCR2 or CXCR-2.[103,104] Numerous other chemokines have been shown to be upregulated in the HNSCC tumour microenvironment,[103] and a comprehensive review of the topic has been previously published.[104] Of note, the CCR4-CCL2 and the CCR2-CCL2 axes are highly expressed in patients with HNSCC Tregs and tumour-associated macrophages, and their blockade in murine HNSCC models resulted in reduced recruitment of Tregs and tumour-associated macrophages, tumour growth, and enhanced survival.[103,105] The anti-CCR4 antibody, mogamulizumab, which has been approved for treatment in refractory adult T-cell leukaemia and lymphoma, was tested in a phase 1 dose-escalation trial with anti-PD-L1 or anti-CTLA-4 in patients with solid tumours including those with HNSCCs.[106] Given that Tregs have been described to reaccumulate with radiotherapy treatment, and given the preclinical data showing that radiotherapy and Treg depletion can eradicate tumours, a combination trial of this drug with radiotherapy might have a useful purpose. CCL2-CCR2 recruitment of monocytes and Tregs was shown in the radiotherapy setting to be a mechanism for resisting Treg depletion, highlighting its potential therapeutic relevance in HNSCCs.[105] The anti-CCR2 antibody PF-04136309 (Pfizer, New York, NY, USA) has been tested in a phase 2b trial with FOLFIRINOX in patients with locally advanced pancreatic cancer, which elicited a robust treatment response.[107] This site might be a potential target to test in trials in combination with radioimmunotherapy or radiotherapy alone in patients with HNSCC. Finally, pharmacological inhibitors targeting radiation-induced recruitment of tumour-associated macrophages can potentially be combined with radiotherapy in patients with HNSCC. In preclinical models of HNSCCs, tumour-associated macrophage recruitment and survival has been shown to be regulated by CSF-1-R.[108] Potentially relevant therapeutics for radioimmunotherapy include the anti-CSF-1-R monoclonal antibody AMG 82 (Amgen, Thousand Oaks, CA, USA) that was tested in a phase 1 safety trial in solid tumours[109] or the small molecule PLX3397 (Roche Innovation Center, Munich, Germany) that was tested in phase 1 and 2 trials.[110]

Since most HNSCCs have EGFR overexpression, understanding the mechanisms by which cetuximab affects the immune tumour microenvironment will be crucial to optimising its efficacy. Although cetuximab can induce tumour antigen-specific cytotoxic T cells, it also appears to increase CTLA-4-expressing Tregs.[111] Dual blockade with anti-CTLA-4 and cetuximab was tested in a phase 1 clinical trial;[112] however, skin-related toxic effects developed at the higher dose of ipilimumab. Although this trial did not examine efficacy, the negative results from an anti-CTLA-4 trial in recurrent metastatic HNSCC (NCT02369874) and the reported toxicity of currently available anti-CTLA-4 inhibitors[113] have prompted hesitation about adding current anti-CTLA-4 inhibitors to standard-of-care therapies. However, given the crucial role Tregs have in mediating radiotherapy resistance in HNSCCs,[57] the search is renewed for safer and more effective Treg-directed therapies such as Fc-optimised anti-CD25,[114] EphB4 (ephrin type-B receptor 4)-ephrin-B2 inhibitors,[64] molecules targeting NRP1 (neuropilin-1)-SEMA4A (semaphorin-4A),[115] anti-TIM3,[58] adenosine,[116] and new generation anti-FoxP3 or anti-CTLA-4 inhibitors.

Other novel approaches being investigated include additional therapeutics targeting the vascular and fibrotic stroma as a way to modulate immune response. Several features of the HNSCC tumour microenvironment, including tumour cell glycolysis or hypoxia, the vascular network, and a fibroblast-rich stroma, are associated with so-called immune-cold head and neck cancer.[117] These features will probably represent evasion mechanisms to immunotherapeutic strategies. In fact, to identify mechanisms implicated in immune checkpoint blockade resistance, Cristescu and colleagues[32] examined genetic differences between patient tissue samples with high TMB and high GEP, where the survival rates were superior, and compared them with patients with mediocre survival rates when one or both parameters were low.[32] No single gene or qualitative difference in T-cell inflammation markers explained the phenotypic differences between the groups or their response to immunotherapy. Instead, gene-set enrichment analysis identified key differences in proliferative, endothelial, myeloid, and stromal biology and tumour type-specific dysregulation of tumour cell-intrinsic signalling pathways. Given radiotherapy's role in modulating fibrosis, and vascular remodelling,[40] further examination of the roles of these pathways in the radiotherapy setting will be vital to optimising responses to radioimmunotherapy for HNSCCs.

Conclusion

The therapeutic potential of radioimmunotherapy in HNSCCs is enormous and the field awaits results from ongoing clinical trials. Borrowing from patient data in the recurrent or metastatic setting, and data in other tumour models, the HNSCC immune tumour microenvironment is evidently diverse and heterogeneous. Immunological and genomic analyses from radioimmunotherapy trials will be essential not only in providing prognostic information about tumour behaviour but also in guiding strategies to modulate the immune system for therapeutic benefit. Caution is advised in extrapolating outcomes in

the metastatic HNSCC setting with immunotherapy alone to a setting where radiotherapy is incorporated, as radiotherapy can substantially alter the tumour microenvironment to enhance the effects of immunotherapy. Preclinical HNSCC studies have shown that radiotherapy has benefits and downsides, with both immune stimulatory and inhibitory effects, and that using immunotherapy will depend on balancing the two strategies through rational combinations. As a final note, given that cancers of the head and neck are of mucosal origin and will have a substantially different tumour microenvironment to other cancers, and thus different responses to immunotherapy depending on the site of tumour implantation,[118] the efficacy of immunotherapy combinations with radiotherapy must be preclinically tested in orthotopic models that recapitulate human disease.

Declaration of interests
SDK reports receiving research grants from AstraZeneca, outside the submitted work. DR serves as a consultant with AstraZeneca and Suvica and is on the advisory boards and honorariums for AstraZeneca, Merck, Genentech, Nanobiotix, and EMD Serono.

Contributors
SDK did the literature search, figures, study design, data collection, data analysis, data interpretation, writing, preparation, and organisation of the manuscript. DR contributed to data analysis, data interpretation, and writing.

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