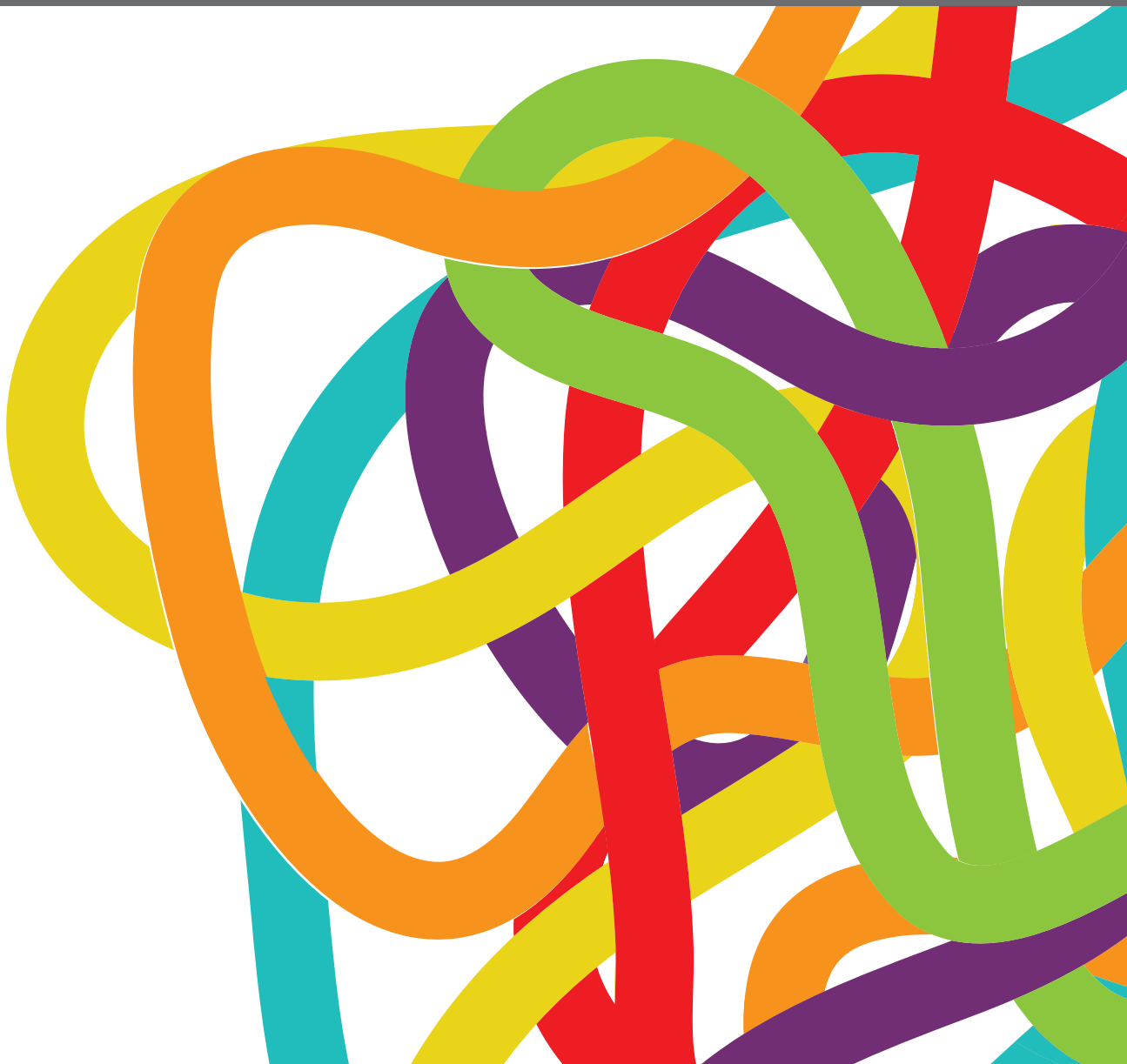# ADVANCES IN HEAD AND NECK CANCER IMMUNOLOGY AND IMMUNOTHERAPY

EDITED BY: Rasha Abu-Eid and John E. Janik
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ADVANCES IN HEAD AND NECK CANCER IMMUNOLOGY AND IMMUNOTHERAPY

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Understanding the immunology of different cancers has led to great advances in developing cancer immunotherapies which are successfully used in generating effective anti-tumour immune responses. Head and neck cancers are no exception and various immunotherapies are now under study for the treatment of this diverse group of diseases.

The articles in this eBook provide a range of topics that highlight some of the latest advances in head and neck cancer immunology and immunotherapy. The authors of these articles provide their unique insight and expertise and suggest future directions for translational clinical research.

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Editorial: Advances in Head and Neck Cancer Immunology and Immunotherapy

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Editorial on the Research Topic

Advances in Head and Neck Cancer Immunology and Immunotherapy

Head and neck cancers are a group of malignancies that affect the head and neck region. These include cancers of the oral cavity, oropharynx, larynx, lips, sinuses, and nasal cavity. The incidence of head and neck cancer is on the rise which is mainly attributed to changes in risk factors including increased alcohol and tobacco consumption and betel chewing habits in certain parts of the world. Additionally, human papilloma virus (HPV) infections have contributed to the rise of head and neck cancers, in particular oropharyngeal cancers, especially in younger patients.

Despite advances in cancer diagnosis and treatment, the mortality rate of head and neck cancer remains unchanged. The high mortality rate is mainly attributed to late diagnosis, and the lack of effective treatments for late stage cancers.

Cancer immunotherapy is among the greatest advances in cancer therapy. Various treatment modalities that target different components of the immune system have proven successful in controlling disease progression and even producing long lasting cures in some types of cancer. Cancer immunotherapy's biggest successes were reported in melanoma and lung cancer. However, many other cancer types are benefiting from the advances in cancer immunotherapy including head and neck cancer. In fact, several immunotherapies have been approved for the treatment of head and neck cancer, including immune checkpoint inhibitors for the management of recurrent or metastatic cancers.

Although the success of cancer immunotherapy cannot be disputed, many patient responses are transient and short lived. This can be explained by different immune escape mechanisms deployed by cancer cells including dampening the immune response through modulating immune checkpoints, in addition to the recruitment and *de novo* differentiation of suppressive immune cells such as regulatory CD4 T cells.

In this research topic, two review articles (Forster and Devlin; Ward et al.) discussed the use of immune checkpoint inhibitors in the treatment of head and neck cancer.

Forster and Devlin provided a review of different co-inhibitory and co-stimulatory checkpoints that could be targeted by immune checkpoint inhibitors in the context of immunotherapy. Their article included a review of the role of the PD-1/PDL-1 axis and GITR in cancer immunology and immunotherapy. The review article by Forster and Devlin presented a comprehensive and exciting review of different therapeutic combinations with checkpoint inhibitors, including different immune modulators, viral therapies, and chemoradiotherapy. The review then summarized the adverse effects associated with immune checkpoint inhibitor therapy and highlighted the importance of biomarkers for the prediction of disease progression and response to therapy.

In their manuscript, Ward et al. provided an overview of the timeline for FDA approvals of different immune checkpoint inhibitors in the treatment of different cancers. The manuscript detailed the history, function, and application of anti-CTLA-4, anti-PD-1, and anti-PDL-1 antibodies in cancer immunotherapy before providing an in depth review of the use of immune checkpoint inhibitors in head and neck cancer. The manuscript by Ward et al. provided an interesting review of possible modifications of existing checkpoint inhibitors including antibodies that target a soluble isoform of CTLA-4 (sCTLA-4).

The presence of suppressive immune cells in the tumor microenvironment, and in particular regulatory CD4 T cells, has been shown to adversely affect the patient prognosis and the anti-tumor immune response. Several strategies are under study to selectively target these suppressive cells as part of cancer immunotherapy. However, there is some conflicting evidence in the literature regarding the role of regulatory CD4 T cells in head and neck cancer. The systematic review by O'Higgins et al. investigated this controversy and systematically analyzed the available evidence. Their findings revealed a deficiency in fully characterizing regulatory T cell phenotypes in the studied head and neck tumors, especially with regard to HPV status, which could contribute to the discrepancy in describing the role of regulatory CD4 T cells in tumor progression. Furthermore, the findings of the systematic review by O'Higgins et al. uncovered a real need for developing robust markers for phenotyping T cells and for detecting regulatory CD4 T cells systemically and within the tumor microenvironment.

HPV positive head and neck cancers represent a particularly appealing target for cancer immunotherapy because of their intrinsic immunogenicity. In addition to the immune response mounted in response to the virus itself, tumors positive for HPV over-express E6 and E7 which can be recognized by the immune system as non-self antigens and can therefore be ideal targets for vaccine based immunotherapies. In their review article, Wang et al. discussed the use of cancer vaccines in the prevention and the treatment of head and neck cancer. They discussed the differences between HPV positive and HPV negative tumors and provided a comprehensive review into various target antigens (viral antigens, neoepitopes, and tumor associated antigens) and different vaccine platforms (DNA, mRNA, peptide, viral, bacterial vector, and cellular vaccines).

Measures for predicting patient survival in head and neck cancer are still lacking. Wichmann et al.'s original research article reported the potential use of a Human Leucocyte Antigen (HLA) score to predict progression free survival in head and neck cancer patients. In their study, Wichmann et al. used HLA traits known to be predictors of progression free survival to build a scoring system using genetic information from HLA typing for predicting prognosis. The findings of their study have significant clinical potential for predicting relapse and for the stratification of patients for clinical trials and informing personalized treatment.

Given the complexity of the immune response to cancer, no single therapeutic agent is capable of enhancing the effector arms of the immune response while simultaneously targeting the suppressive arm. The collection of articles in this research topic suggests a role for combination therapies in the treatment and management of head and neck cancer. Many clinical trials are ongoing that are testing various combinations of modulators of the immune system for the management of head and neck cancer.

The combination of diverse immunotherapies that target different arms of the immune response is gaining acceptance in the clinical setting and could potentially provide a solution for sustaining short lived anti-tumor immune responses.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Targeting Head and Neck Cancer by Vaccination

Chuan Wang, James Dickie, Ruhcha V. Sutavani, Catherine Pointer, Gareth J. Thomas and Natalia Savelyeva*

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Head and neck cancer (HNC) is a heterogeneous group of squamous cell cancers that affect the oral cavity, pharynx, and larynx. Worldwide, it is the sixth most common cancer but in parts of Southern and South-East Asia, HNC is one of the most common cancers. A significant proportion of HNC is driven by human papillomavirus (HPV) infection, whereas HPV-independent HNC is associated with alcohol, smoking, and smokeless tobacco consumption. Here, we review the past and present experience of targeting HNC with vaccination focusing on HPV-derived antigens as well as non-viral antigens for HPV-negative HNC. Novel therapeutic approaches for HNC will focus not only on effective vaccine platforms but will also target the stroma-rich immunosuppressive microenvironment found in those tumours.

Keywords: head and neck cancer, human papillomavirus, human papillomavirus independent, cancer antigens, cancer vaccines

INTRODUCTION

Head and neck cancer (HNC) is a heterogeneous group of squamous cell cancers that affect the oral cavity, pharynx, and larynx. Overall, it is the sixth most common cancer worldwide with an annual estimated incidence of 550,000 cases and around 300,000 deaths (1–3). In parts of Southern and South-East Asia, HNC is one of the most common cancers, and the actual incidence in these developing countries is probably underestimated (1, 4, 5). While the aetiology of HNC is usually associated with smoking and alcohol, a significant subset of oropharyngeal cancers is driven by human papillomavirus (HPV) infection, and these cancers account, at least in part, for the significant increase in HNC in recent years (3, 6, 7).

Combinations of surgery, chemotherapy, and radiotherapy form the standard current first-line treatment regimens for HNC. But despite improvements, these are associated with significant morbidity and a relatively static 5-year survival rate of around 40–50% (1). HPV-positive HNCs have a better prognosis than HPV-negative HNC. Recent clinical trials have demonstrated a clear survival advantage in advanced head and neck squamous cell carcinoma patients treated with immune checkpoint blockade [for review, see Ref. (8)]. In a recent KEYNOTE 012 trial, treating HNC patients with anti-PD1 produced an overall response rate of 24.8% (9). Most patients (around 80%), however, do not respond to checkpoint inhibitor monotherapy aimed to boost pre-existing anti-tumour immune responses. The focus is now on induction of anti-tumour immune responses using cancer vaccines.

HPV-POSITIVE VERSUS HPV-NEGATIVE HNC

The incidence of HNC has risen dramatically since the later 1970s and this has been linked to HPV infection. Around 25–50% of HNCs are HPV-driven with a higher percentage in developed countries

(10–14). This percentage is expected to increase in coming years due to many patient cohorts being infected before prophylactic vaccination against HPV started. HPV-positive HNC predominantly tend to be restricted to the oropharynx, conversely most oropharyngeal cancers are reportedly HPV-positive (11).

Human papillomavirus is an asymptomatic, sexually transmitted DNA virus that infects squamous epithelium *via* micro abrasions which expose the deeper basal epithelial cells (15). Most of HPV-positive HNC (90%) are driven by high-risk HPV16 in contrast to 70% of cervical cancers that are linked to either HPV16 or 18 (11, 13, 16, 17). Other high-risk types involved include HPV 18, 31, and 33. The HPV genome encodes eight genes which are expressed early (E1, E2, E4, E5, E6, and E7) or late (L1 and L2) in the virus life cycle. E6 and E7 are the first viral proteins expressed following infection (15). They inhibit tumour suppressors p53 and pRb, respectively, resulting in uncontrolled host DNA synthesis and cell division; the first step towards malignant transformation (16). E2 protein differentially regulates E6/E7 expression through control of their transcription (18, 19). E5 is known to play an anti-apoptotic role and is thought to contribute to the early stages of oncogenesis (20, 21) by cooperating with E6 and E7 to immortalize cells (22). E5 is not necessary for the maintenance of the transformed phenotype and is often lost. L1 and L2 are structural proteins and form the viral capsid required for infectious viral particles (23).

For HPV-negative HNC incidence, habitual and cultural factors play a major role. In high-income countries, smoking and alcohol [70 and 30%, respectively, or 80% combined (24)] contribute, while in developing countries of Southern and South-Central Asia, HNC and in particular oral squamous cell carcinoma (OSCC), are primarily linked to smokeless tobacco and paan (1). Chewing of paan or betel quid has been strongly attributed to both OSCC and oral premalignancy (25). Besides tobacco, areca nut included in betel quid is also a known carcinogen and the mixture of tobacco, areca nut, and slaked lime forms a potent carcinogenic combination.

HPV-negative HNC also differs from HPV-positive genetically, and common genetic alterations which lead to inactivation of cell-cycle suppressors p53 and p16 and amplification of CCND1 (cyclin D) have been found in the HPV-negative HNC subset. Further alterations in the genes associated with smoking such as those involved in oxidative stress CUL3, KEAP1, and NFE2L2 are also associated with the HPV-negative subset (26–28).

PROPHYLACTIC VACCINATION AGAINST HPV

Several prophylactic vaccines including Cervarix, Gardasil® and more recently Gardasil®9 have been approved by the FDA to protect from HPV infection as well as HPV-associated diseases such as genital warts and cancer (**Figure 1A**) (29–32). The prophylactic effect specifically on HNC is assumed without relevant epidemiological studies available at present.

These vaccines are based on virus-like particles (VLPs) consisting of different HPV capsid proteins L1 (33). For example, Gardasil consists of VLP derived from genital warts-inducing

HPV6 and 11, and oncogenic strains of HPV16 and 18. One VLP is made of one type L1 molecule. When L1 is expressed using recombinant protein expression systems it self-assembles into VLPs *in vitro* (34–36). Superior properties of VLPs in induction of antibody are largely accounted for by their multimeric structure, and their ability to stimulate naive B cells has been demonstrated (37). Prophylactic HPV vaccines target the viral infection itself by inducing neutralising antibody and are effective in preventing HPV-induced malignancies but are not effective in treating them (38). The vaccine target L1 is not expressed during the oncogenic process. Hence, antigens expressed in the tumour have to be targeted by therapeutic vaccination.

THERAPEUTIC CANCER VACCINES

Target Antigens

For HPV-positive cancers, the expressed viral antigens are available. For HPV-negative cancers, other antigens have to be considered. Cancer antigens can be broadly classed into two categories: tumour-specific antigens (TSAs) and tumour-associated antigens (TAAs). Here, we describe TSAs as proteins only expressed in cancer cells and mutated self-proteins (neoepitopes), and TAAs as unmutated self-proteins such as glycosylated proteins MUC1 and CEA or cancer testis antigens (CTAs) (39). TSAs often generate strong immune responses, but are comparatively less available than TAAs. TAAs are generally well conserved in populations, but tend to generate a weaker immune response (40, 41).

To cover antigens which have been targeted to date, current clinical trials for HNC were queried at the NIH ClinicalTrials.gov database utilising "Head and Neck Cancer" or "Oropharyngeal Cancer" or "Oral Cancer" as the disease, and "Vaccine" as the search string, yielding 55 studies. Terminated, withdrawn, suspended trials as well as trials with unknown recruitment statuses were excluded and the results are summarised in **Table 1**. The WHO International Clinical Trials Registry Platform was also queried with the same search strings yielding two additional trials not included on the NIH ClinicalTrials.gov database, but registered on the Japanese UMIN-Clinical Trials Registry (UMIN000008379, UMIN000000976).

Viral Antigens

Viral proteins are considered to be good targets since they are foreign, and hence, the available T-cell repertoire has not been subjected to central tolerance. Most vaccination strategies for HNC target the HPV-positive subset where HPV antigens can be used. HPV E6 and E7 play a critical role in carcinogenesis of HNCs, similar to ano-genital cancers. During malignant transformation when HPV frequently integrates into the host genome, E6 and E7 are thought to be the only proteins expressed and hence have been targeted by many types of vaccines. Vaccines against these antigens have demonstrated efficacy in HPV-induced cervical dysplasia (42–46) and are currently in clinical trials for both cervical cancer (e.g., NCT02128126) and HNC (**Table 1**).

Other potential targets are E2 and E5. E2 has been successfully targeted in ano-genital intraepithelial lesions (47). In

FIGURE 1 | Approaches for prophylactic **(A)** and therapeutic **(B)** vaccination for head and neck cancer. Abbreviations: VLP, virus-like particle; iTME, immunosuppressive tumour microenvironment; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; CAF, cancer-associated fibroblast.
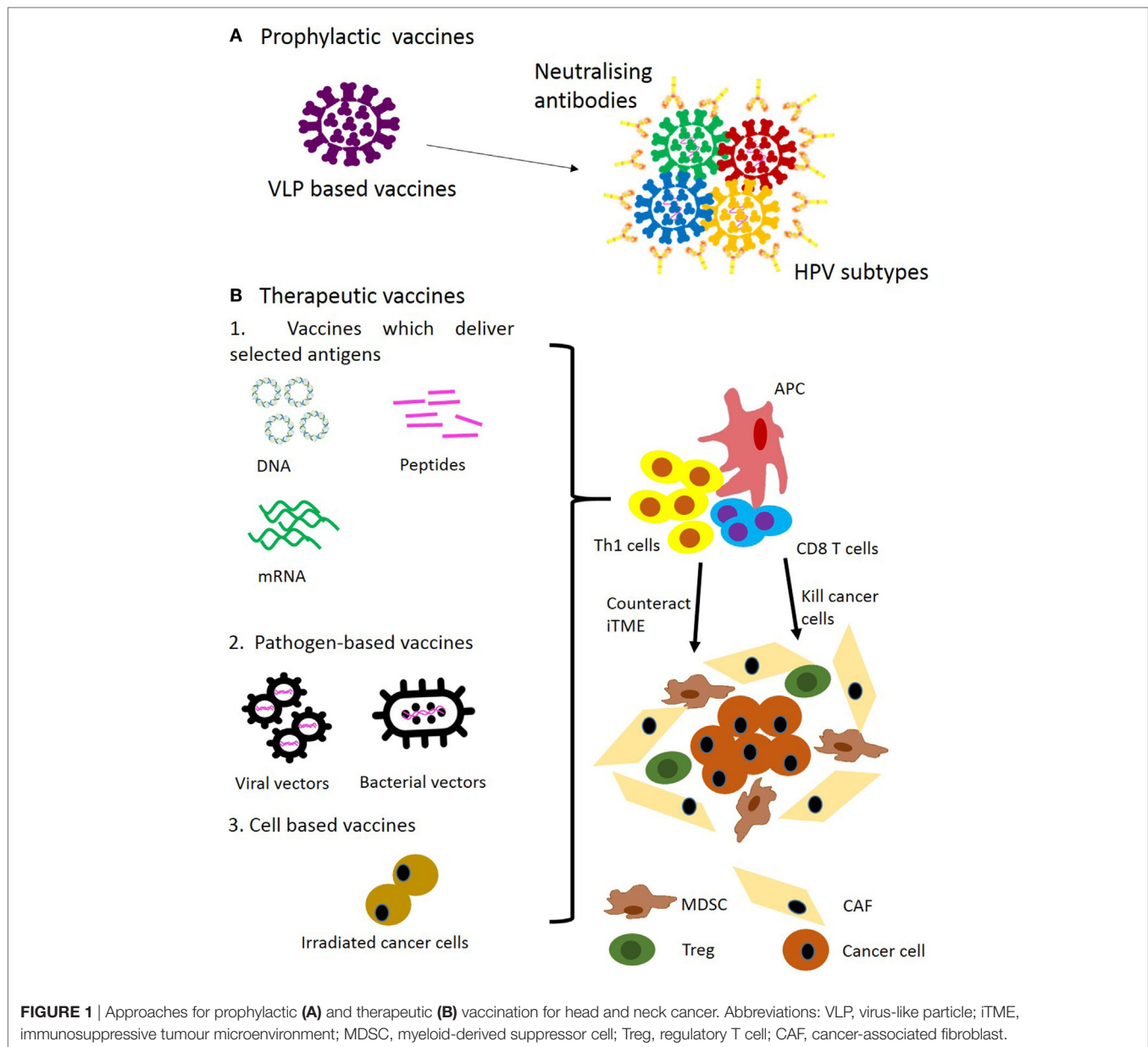
HNC, E2 is not always lost, and can be retained in episomal HPV DNA (48) (and unpublished data from our lab). A number of vaccines targeting E5 are in preclinical development (49–52). No clinical data on targeting E2 and E5 in HNC are currently available.

HPV-negative HNC lacks the immunogenic HPV viral proteins of HPV-associated HNC and appears less responsive to current treatments (53). A viral target which may be present in HPV-negative cases is EBV, which is strongly associated with nasopharyngeal cancer (54), though only around 6–21% of HNC cases express EBV RNA (55, 56). Vaccines targeting EBV are currently in phase I clinical trials (**Table 1**; NCT01147991), and appear safe and well tolerated, inducing only grade I/II adverse events, while reporting increased circulating CD4 cells and antigen-specific T cell responses (57, 58).

Neoepitopes

Despite earlier reports that HPV-negative HNC had a greater mutation rate than HPV-positive (59), more recent reports have found no significant difference to the mutation rate as a result of HPV status (26, 27). However, these reports do find significant differences in the mutational spectrum based on HPV status, which influence vaccine-targetable mutations. Targeting p53 and RAS is more likely to benefit HPV-negative cases, as these proteins are mutated in HPV-negative cases, but degraded in HPV-positive cases (60). Early trials targeting mutated p53 or RAS have been completed (61). The RAS phase II trial was completed in 2007, but no results have been reported to date (**Table 1**; NCT00019331).

Conventional mutation targeting in cancer therapy focuses upon driver mutations, but in the last decade has arisen a view that other mutations may be relevant and make for potential

TABLE 1 | Target cancer antigens in head and neck cancer.

Vaccine	Target antigens	Type	Phase	Identifier	Relevant references
Pre-active/active clinical trials					
ADXS11-001	Human papillomavirus (HPV)16-E6/E7	Viral Ag	Phase II	NCT02002182	(136)
			Phase I/II	NCT02291055	N/A
DPX-E7	HPV16-E7	Viral Ag	Phase I/II	NCT02865135	N/A
MEDI-0457 (INO-3112)	HPV16/18-E6/E7	Viral Ag	Phase I/II	NCT03162224	N/A
TG4001	HPV16-E6/E7	Viral Ag	Phase I/II	NCT03260023	N/A
ISA101/101b	HPV16-E6/E7	Viral Ag	Phase II	NCT02426892	N/A
			Phase II	NCT03258008	N/A
ISA201 (Hespecta)	HPV16-E6/E7	Viral Ag	Phase I	NCT02821494	(121)
HARE-40	HPV16-E6/E7	Viral Ag	Phase I/II	NCT03418480	N/A
Trojan	MAGE-A3 and HPV16-E7	Viral Ag and tumour-associated antigen (TAA)	Phase I	NCT00257738	(81)
MUC1 vaccine	MUC1	TAA	Phase I/II	NCT02544880	N/A
NANT	MUC1/CEA/HER2/Brachyury/Ras	TAA	Phase I/II	NCT03169764	N/A
MVX-ONCO-1	Allogeneic tumour-irradiated	Cellular	Phase II	NCT02999646	N/A
AlloVax	Allogeneic tumour-chaperone-rich cell lysate	Cellular	Phase I/II	NCT01998542	N/A
			Phase II	NCT02624999	N/A
Completed clinical trials					
Peptide pulsed dendritic cell	P53	TAA	Phase I	NCT00404339	(69)
Ras vaccine	Ras	TAA	Phase II	NCT00019331	N/A
MEDI-0457 (INO-3112)	HPV16/18-E6/E7	Viral Ag	Phase I/II	NCT02163057	(111)
P16 vaccine	P16	TAA	Phase I/II	NCT01462838	(120)
			Phase I	NCT02526316	N/A
GI-6207	CEA	TAA	Phase I	NCT00924092	N/A
EBV vaccine	EBV	Viral Ag	Phase I	NCT01147991	(58)
TRICOM-CEA(6D)	CEA	TAA	Phase I	NCT00027534	N/A
Peptide with IFA	CDCA1, LY6K, and IMP3	TAA	Phase I/II	UMIN000008379	(87)
Survivin-2B vaccine	Survivin-2B	TAA	Phase I	UMIN000000976	(70)

vaccine targets (62, 63). Advances in immunotherapeutics and bioinformatics in recent years have increased the practicality of targeting these neoantigens *via* vaccination. However, as each case exhibits its own unique mutanome (64), each vaccine must be created for the specific individual, making it expensive and time consuming. Recent studies reported around 100 days are required for production and analytical testing (NCT02035956, NCT01970358) (65, 66). Despite the current great expense of both time and money, early results suggest great efficacy from targeting neoantigens (65, 66).

Tumour-Associated Antigens

Well-characterised antigens including MUC1 and CEA have demonstrated immunogenicity in patients and their potential is being translated into clinical efficacy (67, 68). In HNC, phase I/II trials targeting MUC1 are on-going (**Table 1**; NCT02544880), while trials targeting CEA (**Table 1**; NCT00924092, NCT00027534) have been completed but have yet to report results. The p53 phase I trial targeting wt p53 T-cell epitopes completed with modest improvement to 2-year disease-free survival (DFS) [**Table 1**; NCT00404339 (69)], but has yet to progress to a phase II trial. A phase I trial targeting Survivin-2B has been performed in

oral cancer (**Table 1**; UMIN000000976), but demonstrated low efficacy (70). The NANT vaccine (**Table 1**; NCT03169764) is a novel combination immunotherapy combining metronomic chemo-radiotherapy with vaccines targeting well-established and molecularly confirmed TAAs, and off-the-shelf NK cell therapy. This experimental therapy is part of the Cancer Breakthroughs 2020 global initiative.

Cancer testis antigens are a class of TAAs that make for promising vaccine targets. While there is evidence of central tolerance for CTAs (71), CTA expression in the periphery is normally restricted to healthy male germ cells, immune privileged cells lacking MHC I (72). Thus, CTAs are only presented to the immune system in the periphery by cancer cells (73), and thus frequently demonstrate immunogenicity (74–76).

Using either SEREX or TIL-derived T cells, many CTAs have been described over the past decades, most prominently the MAGE family, BAGE family, SSX family, PRAME, and NY-ESO1 (77–79). A recent analysis of HNC selected several potential CTA targets for further preclinical study, although they did not differentiate between HPV-associated and HPV-negative cases (80). A phase I clinical trial targeting MAGE-A3 and HPV antigens in HNC is on-going (**Table 1**; NCT00257738) (81).

Novel CTAs including LY6K, CDCA1, and IMP3 have been identified through genome wide microarray analysis of various cancer tissues (82–84). A multivalent vaccine targeting HLA-A24 restricted LY6K, CDCA1, and IMP3-derived peptides recently was tested in phase I and phase II clinical trials in oesophageal cancer (85, 86), and significantly improved DFS in HLA-A24 cases was reported. This success encouraged targeting of these antigens in HNC (**Table 1**; UMIN000008379) (87), which increased overall survival (OS) when administered to HLA-A24 patients with advanced refractory HNC (HPV-negative with the exception of one patient) and correlated with peptide specific CTL responses.

Immune Mechanisms for Cancer Attack

Most target antigens in HNC including HPV antigens, neoepitopes as well as the majority of TAA are intracellular antigens. Intracellular antigens are generally presented as 8-11-mer peptides bound to MHC I on cancer cells and these are targeted by CD8 cytotoxic T cells (CTLs). CTLs are powerful effector cells which directly kill target cells *via* a variety of mechanisms including perforin/granzyme and Fas-mediated attack. For induction of long-lasting CTLs, CD4 T helper (Th)1 cells must also be co-induced (88). These provide T-cell help *via* dendritic cells (DC)-licensing by binding to 12-15mer or longer peptides presented in the context of MHC II on DCs which leads to DC activation (89, 90). These Th1 subsets do not have to be specific for the target cancer antigen. Approaches for recruiting Th1 cells specific for foreign antigens have been extensively explored in the development of cancer vaccines (91–94). The advantage of this approach is that these Th cells escape tolerogenic mechanisms and are therefore available to provide help to CTLs specific for cancer-derived antigenic peptides [for review, see Ref. (95)]. Tetanus-derived Dom helper sequence has been used to recruit CD4 T cell for induction of CD8+ CTLs using DNA vaccine (96). A recent phase II clinical data demonstrated this approach led to induction of CD8+ T-cells response to CEA detected post-vaccine with indication of clinical benefits (67).

For effective cancer attack, Th1 cells that play not only a helper role to CTLs but a more direct role in anti-tumour immunity are important (97–99). Those specific for the target antigen Th1 cell subsets may be involved in recruitment of tumouricidal macrophages or reprogramming of the tumour microenvironment (100, 101). In HPV-associated cancer, targeting co-induction of broad CD4 and CD8 T-cell responses correlated with vaccine efficacy (44). This is in keeping with the data obtained in other solid tumours (65, 66).

VACCINE PLATFORMS

Delivering antigenic epitopes in an immunogenic context which leads to the induction of a durable T-cell response is the goal. The available vaccine platforms are illustrated in **Figure 1B**. DNA and RNA vaccines encoding selected tumour antigens or synthetic long peptides (SLPs) vaccines co-delivering CD4 and CD8 epitopes have recently been highlighted as optimal cancer vaccine modalities (97). These focus on delivery of selected target antigens without co-delivering the backbone-encoded antigens as in the case of pathogen-derived bacterial or viral vectors.

The latter often generate strong pathogen-derived CD8 epitopes and can focus the immune response on the vector itself (96). Nevertheless, several viral and bacterial vaccines have demonstrated induction of CD8 responses against dysplastic disease and cancer together with clinical efficacy (47, 68).

DNA Vaccines

DNA vaccines represent a simple approach of directly injecting a plasmid DNA encoding one or more antigens driven by a eukaryotic promoter. Not only is the antigen made directly in the body but the DNA backbone also acts as an immunological adjuvant (102). Multiple innate sensors for plasmid DNA have been identified including endosomal toll-like receptor (TLR) 9 as well as several cytosolic sensors DAI, AIM2, cGAS-STING, and others (103). Flexibility, simplicity of preparation, stability, and safety are the advantages. However, low immunogenicity in patients has been highlighted in early clinical trials (104). The situation improved upon combination of DNA vaccine injection and *in vivo* electroporation [EP, for review, see Ref. (105)]. *In vivo* EP increased cellular DNA uptake leading to generation of more antigen available for immunisation and potentially made DNA more visible to the cytosolic innate sensors. This led to significant increase in immunogenicity. Combination of DNA and EP induced durable antibody and T-cell responses in cancer patients (42, 67, 104, 106). Other methods of DNA delivery including liposomes, tattooing and cationic polymers have also been also investigated (107, 108).

For targeting of HPV, oncogenes E6 and E7 by DNA vaccines modifications have been made to their sequences to prevent E6 and E7 binding to p53 and pRb, respectively (42, 109, 110). Most HPV-targeting DNA vaccines so far have been trialled in the setting of cervical intraepithelial neoplasia (CIN). In the recent phase IIb clinical trial, VGX-3100 DNA vaccine encoding E6 and E7 in combination with DNA vaccine encoding IL-2 administered i.m. with EP has shown promising clinical results in women with HPV16- and 18-associated CIN2/3. Robust T-cell responses were induced and regression of premalignant lesions was demonstrated in 50% of vaccinated women (42). VGX-3100 was subsequently moved to the HNC setting where it was also delivered with EP plus DNA vaccine encoding IL-12 (the combined treatment defined as INO-3112; **Table 1**; NCT02163057). Initial results from the study were very promising. HPV E6/E7-specific antibody was successfully generated in four of the five HNC patients analyzed. Increased HPV-specific cellular responses were observed in nine out of 10 evaluable patients by ELISPOT. Seven of eight evaluable patients had HPV-specific granzyme/perforin positive CD8 T cells by flow cytometry (111). A phase I/II trial to assess the vaccine (now called MEDI-0457, MedImmune) safety and anti-tumour efficacy in combination with PD-L1-blocking mAb Durvalumab is now recruiting HPV-positive HNC patients (**Table 1**; NCT03162224).

DNA vaccine targeting HPV16 E7, pNGVL4a-CRT/E7 (detox), based on E7-calreticulin (CRT) fusion demonstrated the ability to enhance MHC I presentation and exhibited an anti-angiogenic effect (112). In a preclinical study, E7-specific antibody and T-cell responses were generated with protection from the TC-1 tumour challenge (113). A pilot clinical study using pNGVL4a-CRT/E7

(detox) for the treatment of patients with HPV16-associated CIN2/3 was recently conducted (114). EP was not used in this study but one arm investigated the particle-mediated epidermal delivery using a needle free ND10 delivery system. The results demonstrated mild but manageable toxicity predominantly localised to the injection site but only a small increase in systemic T-cell responses was observed with no increase in regression above the control. A phase I trial assessing safety and feasibility of this DNA vaccine in combination with cyclophosphamide in HPV16-associated HNC patients has been terminated (**Table 1**).

Immunogenicity and efficacy of a novel linear closed end DNA vaccine, doggybone (db) DNA (dbDNA™, Touchlight Genetics), have recently been demonstrated in the HPV E6 and E7 tumour model (115). dbDNA™ vaccine was developed using a bacteria-free manufacturing platform which relies on bacteriophage Phi29 polymerase for amplification. Minimal purification is required and safety is improved because of exclusion of antibiotic-resistant genes irrelevant for this platform. dbDNA™ vaccine operated through STING-mediating pathways but was independent of TLR9 recognition. Importantly, HPV16 E6 and E7 dbDNA™ vaccine and conventional plasmid DNA delivered with EP generated similar levels of CD4 and CD8 T cells as well as antibody. dbDNA™ was also able to suppress established TC-1 tumours similar to plasmid DNA. This novel DNA vaccine represents a promising alternative to a plasmid DNA vaccine for targeting of HPV E6 and E7 antigens.

mRNA Vaccines

mRNA vaccines are becoming increasingly attractive in recent years. They can accumulate at high concentration in the cytoplasm ensuring high antigen expression. mRNA is a natural ligand for TLR3, TLR7/8, and several cytosolic sensors (i.e., RIG-I, MDA5), which induce innate immune response to enhance vaccine efficacy [for review, see Ref. (116)]. For *in vivo* delivery, mRNA is complexed with a lipid carrier that protects from degradation as well as targets DCs (117). The efficacy of a first-in-human personalised mRNA vaccine targeting patients' mutanome in melanoma patients has been reported recently (65). T-cell responses against multiple mutanome-derived neoepitopes were induced in all patients. Four out of five patients with progressing metastasis at the start of vaccination demonstrated either a partial or a complete clinical response after vaccination. Interestingly, one patient benefited from vaccination followed by anti-PD1 mAb. A phase I/II clinical trial using an mRNA vaccine targeting HPV16 E6 and E7 has recently started recruiting at our institution (University of Southampton, led by Prof C. Ottensmeier and Dr E. King; NCT03418480). The vaccine will be given to patients with HPV-positive HNC intradermally either alone or in combination with anti-CD40 costimulatory antibody.

Peptide Vaccines

Peptide-based vaccines are safe and easy to produce, but they are also expensive and poorly immunogenic by themselves. They are often CD8+ epitopes predicted for a particular HLA allele or include long single or overlapping peptides which often contain both CD8 and CD4 epitopes. The latter approach circumvents HLA restriction issues. Unlike DNA and RNA vaccines peptides do not carry "inbuilt" adjuvants. The efforts to enhance their immunogenicity have focused on combining with appropriate adjuvants. MF59®, emulsion of squalene oil approved in both Europe and USA, has been used in earlier trials (97). A number of clinical trials have used SLPs targeting HPV antigens combined with oil-in-water adjuvants for treatment of CIN and vulvar neoplasia which resulted in regression of premalignant lesions (43–46). However, this approach has not been successful when tested in recurrent cervical cancer with low T-cell responses and no survival benefit (118). This failure highlighted the need to use more potent adjuvants and combinational therapeutic approaches to overcome the tumour microenvironment (119).

Several peptide-based vaccines against HPV-associated HNCs are now in clinical trials. A phase I/II trial to assess safety and efficacy of a short peptide-based vaccine targeting HPV16 E7 (11–19), in combination with low dose of cyclophosphamide intending to deplete regulatory T cells, has started recently in HLA-A2 patients with incurable HPV16-associated oropharyngeal, cervical, and anal cancer (**Table 1**; NCT02865135). In another clinical trial, patients with advanced HPV-associated cancers were vaccinated weekly with a SLP derived from p16 (27, 37–62), the tumour suppressor induced as a result of HPV-linked transformation, after the completion of a standard treatment. The vaccine containing both CD8 and CD4 epitopes was emulsified with Montanide™ ISA-51 VG (oil-in-water adjuvant, SEPPIC). Both cellular and humoral responses to the peptide were induced with no unexpected serious adverse reactions. Out of 14 evaluated patients, nine had stable disease as their best overall response and five patients developed progressive disease [**Table 1**; NCT01462838 (120)]. A subsequent on-going trial using the same vaccine has been evaluating different routes of vaccination, i.e., subcutaneous and intradermal (**Table 1**; NCT02526316). Two phase II clinical trials using 13 HPV16 E6/E7 overlapping SLPs in combination with anti-PD-1 antibody (Nivolumab) or in combination with anti-CD137 immuno-stimulatory antibody (Utomilumab) have been initiated to treat patients with HPV16-associated HNC as well as other HPV-associated malignancies (ISA101/ISA101b; **Table 1**; NCT02426892 and NCT03258008) (119). A phase I trial using two HPV16 E6 SLPs (ISA201) together with TLR1/2 agonist adjuvant Amplivant® (ISA pharmaceutical), for HPV16-positive tumours and premalignant lesions has also been initiated [**Table 1**; NCT02821494 (121)].

A multivalent vaccine targeting HLA-A24 restricted short peptides from three CTAs (LY6K, CDCA1, and IMP3) in combination with IFA injected subcutaneously has recently cleared phase II clinical trials in HNC patients in Japan [**Table 1**; UMIN000008379 (87)]. The vaccine increased OS when administered to HLA-A24 patients, which was correlated to peptide specific CTL responses. Interestingly, those patients that demonstrated response to all three peptides had extended OS versus those who responded to one or two peptides only.

Viral and Bacterial Vector-Based Vaccines

Viral vector-based vaccines employ attenuated viruses that deliver antigen of interest in the infected cells. Alphaviruses, adenoviruses, and vaccinia viruses are the examples that have been explored to deliver HPV-associated antigens.

Replication-deficient alphaviruses including Semliki Forest virus (SFV) and Venezuelan equine encephalitis virus (VEE) have been demonstrated to be safe [for review, see Ref. (122)]. These RNA viruses preferentially infect APCs and are able to efficiently activate the adaptive immune system [for review, see Ref. (123)]. SFV- and VEE-based vaccines against HPV16 E6/E7 have demonstrated the ability to induce specific CTLs that can kill HPV16 E6/E7-expressing tumour cells *in vitro* and clear tumours in mouse models (124–126), including in the HLA-A*0201 transgenic mice (127, 128).

The first clinical trial of a recombinant vaccinia virus targeting HPV was conducted more than 20 years ago. The TA-HPV vaccine was based on a live vaccinia virus and was engineered to express E6 and E7 proteins from HPV16 and 18. Two patients with advanced cervical cancer remained tumour free 15 and 21 months after vaccination, in one of them an HPV-specific T-cell response was also induced (129). Two more clinical trials using the same vaccine to treat HPV-associated advanced cervical cancer and vulval/vaginal intraepithelial neoplasia had been reported with partly successful results, but also some side effects manifesting as erythema and swelling followed by ulceration with scab formation at the site of vaccination (130, 131). Safety concerns related to the use of live vaccinia virus prompted the development of vaccines based on the attenuated virus, i.e., modified vaccinia virus Ankara (MVA) (132, 133). MVA also preferentially infects APCs (134) and through recognition of its viral DNA by TLR9 and cytosolic DNA sensors is able to activate APCs leading to effective activation of T-cell immunity. The safety as a result of restricted replication has been demonstrated in many clinical trials (132).

Efficacy of an MVA-based vaccine encoding modified HPV16 E6 and E7 together with IL-2 TG4001 (Transgene) was demonstrated in patients with HPV16-related CIN2/3. In 7 out of 10 patients who were evaluated as clinical responders, cytological and colposcopic regression together with HPV16 mRNA clearance was observed (135). A phase I/II clinical trial assessing TG4001 in HPV16-associated HNC patients has recently started recruiting (**Table 1**; NCT03260023).

A well-characterized attenuated facultative intracellular bacterium *Listeria monocytogenes* (Lm) incorporating a non-toxic version of the listerionlysin O (LLO) as adjuvant has been utilised to target HPV16 E7 oncoprotein (Lm-LLO-E7 also known as AXAL, ADXS11-001) [(136); for review, see Ref. (137)]. Several clinical trials using this vaccine in cervical and anal cancers are on-going, including one which progressed to phase III last year in women with high-risk locally advanced cervical cancer (NCT02853604). With regard to HNC, one phase I clinical trial in oropharyngeal cancer was terminated in 2016 as one patient suffered a dose limitation toxicity (NCT01598792). Two more phase II trials using the vaccine in combination with anti-PD-L1 mAb (**Table 1**; NCT02291055) or surgery (**Table 1**; NCT02002182) are still active and recruiting. The latter trial has recently reported specific T-cell responses in the blood and increased T-cell infiltration in the tumour in five out of eight and four out of eight patients, respectively (138).

Cellular Vaccines

Using autologous tumour cells as vaccines ensures that patients are vaccinated with cells containing the same tumour antigens that their tumour expresses saving time and effort needed to identify TSAs. Irradiated cells are used but undesired immune responses are still a potential safety concern. To enhance immunogenicity cells genetically modified to express costimulatory molecules, TLR ligands or cytokines have been utilised (139). Phase I/II trials are underway for personalised HNC vaccines Allovax and MVX-ONCO-1, utilising allogeneic tumour cells as a source of antigen (**Table 1**; NCT02999646, NCT01998542, NCT02624999). MVX-ONCO-1 contains irradiated autologous tumours cells expressing GM-CSF combined with encapsulated cellular technology that allows continuous supply of GM-CSF (140). It has demonstrated reasonable safety in a phase I trial for solid tumours, with no systemic serious adverse events (NCT02193503) (141). AlloVax™ is Charperone Rich cell lysate combined with AlloStim™ cells which are allogeneic Th1 effector cells [Allostim, Immunovative Therapies Ltd. (142)].

THE ROLE OF THE IMMUNOSUPPRESSIVE TUMOUR MICROENVIRONMENT: CANCER-ASSOCIATED FIBROBLASTS (CAFs)

Several immunosuppressive immune subsets have been found in HNC including tumour-associated macrophages, myeloid-derived suppressor cells, and regulatory B and T cells. The role of these have been reviewed elsewhere (143, 144), but it is clear that vaccination must induce the correct immune milieu for therapy to be effective. Similarly, we have identified several features of the tumour microenvironment, including tumour cell glycolysis/hypoxia and a CAF-rich stroma that are associated with "immune cold" HNC (145, 146). It has been suggested that CAFs suppress T-cell infiltration into cancers, and also through secretion and activation of TGF-β, modulate multiple types of immune cells towards a more suppressive phenotype, including tolerization of CD4 T cells and promotion of a regulatory T cell phenotype. Tumours with this type of stromal response may not be effectively targeted by vaccination; it is possible, however, that such evasion mechanisms could be targeted as part of a vaccination strategy; for example, we have shown recently that CAFs can be specifically targeted by inhibiting the NADPH oxidase, NOX4 (146).

CONCLUDING REMARKS

Incidence of both HPV-positive and -negative HNC is on the increase. The trend is unlikely to change at least in the near future, particular for HPV-independent HNC, especially OSCC, where the habitual and cultural causes are unlikely to disappear.

Human papillomavirus targets E6 and E7 are considered to be less challenging because of their foreign nature. Their targeting by DNA, peptides, and other vaccines has already demonstrated clinical efficacy in HPV-driven dysplasia. These vaccines are now in clinical trials for HPV-driven cancers including HNC. On the contrary, HPV-independent HNC has received less attention largely because such targets have not been available. A number

of interesting antigenic targets has started coming through; these include personalised mutanome-derived neoepitopes but also novel TAAs (87). The mutanome-based approach has started demonstrating clinical efficacy but it is unlikely to have an impact on the disease in the developing parts of the world. Therefore, the focus is on TAAs.

More affordable vaccine modalities such as DNA vaccines combined with a simple *in vivo* delivery are promising. However, these will still need to be developed within a strategy that overcomes a suppressive tumour microenvironment and further work is required to develop these therapeutic approaches.

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Check for updates

Immune Checkpoint Inhibition in Head and Neck Cancer

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Head and Neck Squamous Cell Carcinoma (HNSCC) is the 6th most common cancer globally and commonly presents with locally advanced disease, which has a recurrence rate of around 50% despite aggressive multi-modality treatment involving surgery, radiotherapy and chemotherapy or EGFR inhibition where appropriate. As understanding of the underlying cancer biology and the complex interactions within the tumor microenvironment improves, there is gathering interest in and evidence for the role of immunomodulating agents in the management of HNSCC. Immune checkpoint inhibitors, which aim to hinder the inhibitory interaction between programmed cell death protein 1 (PD-1) and its ligand PD-L1, have demonstrated durable improvements in patient outcomes in advanced / metastatic HNSCC, with both pembrolizumab and nivolumab being granted FDA approval in 2016. There are numerous ongoing clinical trials exploring the role of checkpoint inhibitors both as single agents and in combination, administered with established treatment modalities such as chemotherapy and radiotherapy, as well as alongside other novel immune modulators. These trials are not limited to advanced / metastatic HNSCC, but also to the neo-adjuvant or adjuvant settings. As studies complete and more results become available, the role immunotherapy agents will have within the treatment strategies for HNSCC may change, with increasing biomarker selection resulting in personalized therapy aiming to further improve patient outcomes.

Keywords: immune checkpoint inhibitors, head and neck cancer, head and neck squamous cell carcinoma, cancer immunology, cancer immunotherapy

Head and neck cancer encompasses malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx. In Europe, there are around 139,000 new cases of head and neck cancer per year, 90% of which are squamous cell carcinoma (HNSCC) (1). While both smoking and alcohol consumption have long been established as risk factors for the development of HNSCC, human papillomavirus (HPV) has emerged as a driver for a significant proportion of oropharyngeal squamous cell carcinomas and increasingly is being recognized as its own distinct clinical entity with a more favorable prognosis than non-HPV associated HNSCC (2, 3).

The 2016 FDA approval of the Programmed death-1 (PD-1) monoclonal antibodies pembrolizumab and nivolumab for the treatment of HNSCC heralded the dawn of a new era of treatment for a patient population that historically has a 50% recurrence rate despite aggressive multi-modality treatment involving surgery, radiotherapy, chemotherapy and, where appropriate, EGFR inhibition (4).

Intact immune surveillance is critical to control carcinogenesis and in order to propagate locally and metastasise cancer cells must develop mechanisms that allow them to evade elimination by the host immune system. Immunotherapy works on the premise that the host immune system can be activated to overcome these acquired mechanisms, allowing the recognition of cancer as non-self and eliminate it.

In order for this to happen, T-lymphocytes must be able to infiltrate the tumor and mount appropriate responses (5) and higher numbers of CD3+, CD8+ and FOXP3+ tumor infiltrating lymphocytes (TILs) are associated with a favorable outcome in in several malignancies, including HNSCC (6). In particular, the presence of CD8+ "effector" T-cells and the ratio between CD8+ and FOXP3+ regulatory T-cells (Tregs) correlates with improved prognosis. Tregs are known to actively suppress immune responses (7) and as such their presence within the tumor microenvironment may assist immune evasion in head and neck cancers. However, further understanding is required as although their presence is linked to a decreased survival in several tumor types, a positive correlation has been reported in other cancers such epithelial ovarian cancer, colorectal cancer and lymphoma (8–10).

Some cancers evade T-cell directed immune effects by developing ways of excluding the T-cells from the tumor microenvironment. However, HNSCC has been found to be one of the most immune-infiltrated cancer types (11) suggesting other mechanisms are involved and T-cell homing, infiltration and activity are under a number of influences generated by the tumor. In HNSCC several suppressive mechanisms have been identified that include:
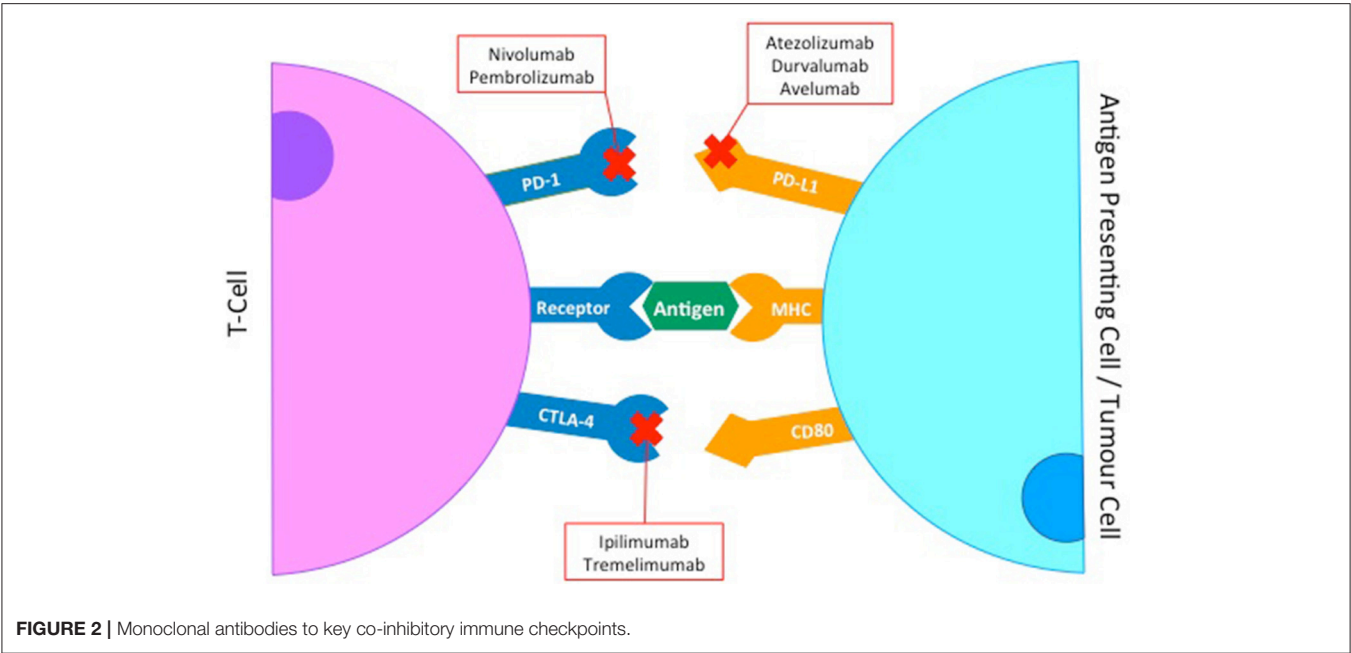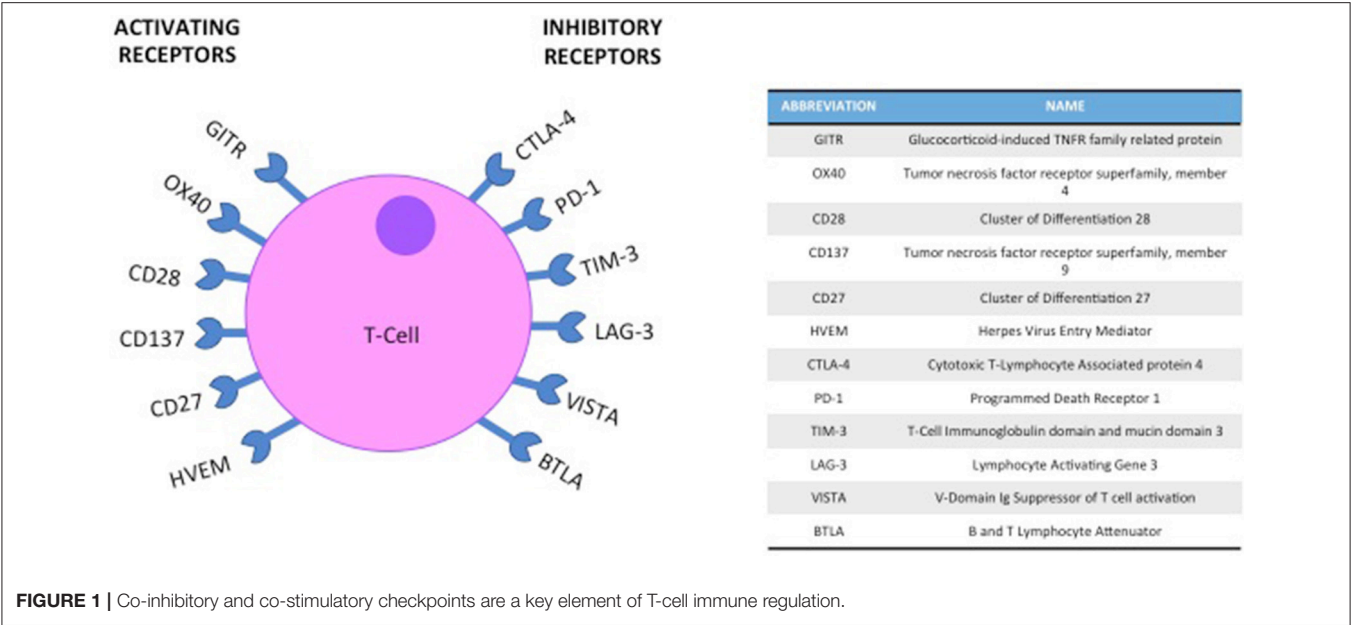
1. Deficiencies or alterations of tumor human leukocyte antigen (HLA) class I molecules expression (12, 13) along with overexpression of antigens causing T-cell tolerance (14)
2. Increases in immunosuppressive cytokines such Il-10, (15) IL-6 (16), and TGF-β (17)
3. Aberrant activation of the transcription factors Signal Transducers and Activators of Transcription 3 (STAT3) (18) and NF-kB, (17) which are notably linked to IL-6 and TGF-β signaling respectively.

To avoid autoimmunity a series of checkpoints exist on the surface of immune cells, with the activation of a T-cell response being a careful balance of co-inhibitory and co-stimulatory molecules and their ligands (19) **(Figure 1)**. In HNSCC immunomodulatory agents exist that block interaction between co-inhibitory receptors such as Cytotoxic T-Lymphocyte-associated antigen 4 (CTLA-4), Programmed death-1 (PD-1) or Lymphocyte activation gene-3 (LAG-3) and their ligands. Conversely, biologics that mimic ligand activated signaling in co-stimulatory molecules such as Glucocorticoid-induced tumor necrosis factor receptor (GITR) have also been evaluated. Both these approaches aim to achieve the same outcome; an enhanced activation of an immune response to tumor cells **(Figure 2)**.

PD-1/PD-L1 AXIS

PD-1 is a member of the CD28 receptor family which is expressed on activated T- and B-cells, monocytes and a subset of thymocytes. The expression of PD-1 on activated T cells enhances the suppressive function of Tregs and this effect is mediated by the interaction with its ligands, PD-L1 and PD-L2, which are expressed on antigen presenting cells, endothelial and epithelial cells as well as on activated lymphocytes. The interaction between PD-1 and PD-L1 negatively regulates immune responses by decreasing cytokine production and inducing T lymphocyte anergy and apoptosis. Upregulation of PD-L1 can occur in tumor cells and allows cancer cells to escape from host immune systems by functionally inactivating T-cell immune surveillance. The inhibition of this interaction can enhance T-cell response and mediate clinical anti-tumor activity (20, 21). Expression of PD-L1 is often high in HNSCC tumors, with positivity being quoted between 46 and 100% across several studies, this wide range likely owing to differences in staining technique, sample preservation and possibly sampling error (22).

Nivolumab is an IgG4 PD-1 monoclonal antibody designed to block co-inhibitory signaling through the PD-1/PD-L1 axis. Following early phase studies demonstrating promising data, a phase III trial, CheckMate 141, compared nivolumab with the physician's choice of second line single agents (docetaxel, methotrexate or cetuximab) in a 2:1 randomisation in patients with platinum resistant recurrent / metastatic HNSCC. It recruited 361 patients and response rates (RR) were low in all arms, but higher in the nivolumab cohort (13.3%) with 6 complete responses (CR) and 26 partial responses (PR). In the chemotherapy arm, RR was 5.8%, including 1 CR and 6 PR. The median overall survival (OS) was 7.5 months with nivolumab, vs. 5.1 months in the control arm, and patients who received nivolumab had a statistically significant 30% lower risk of death (HR 0.70; 95% CI 0.51 to 0.96). There was also an improvement in the estimated progression free survival (PFS) at 6 months; 19.7% with nivolumab and 9.9% for those who received physician's choice. This increased benefit at later time-points gives promise to durable benefit for responding patients, as seen with nivolumab in other tumor types where clinical data are more mature (23). In addition to the higher efficacy seen, treatment with nivolumab was also more tolerable than the standard therapies, with a reduction in grade 3 or 4 adverse events; 13.1 vs. 35.1% and a relative improvement in patient reported outcomes (PROMS) and other Quality of Life parameters (24). Outcomes of patients randomized to nivolumab who were Treated Beyond Progression (TBP) were presented at the European Society of Medical Oncology 2017 Annual Congress (ESMO 2017). Of those patients who had progressed, 62 patients (42%) received at least one further dose of nivolumab and of these 15 (24%) had a subsequent reduction in target lesion size with 3 patients achieving >30% reduction, with no increase in grade 3 or 4 toxicities (25). These findings of unusual patterns of response to immune checkpoint inhibitors have also been described in other patient groups (26, 27) and offers reassurance that continuing therapy beyond progression is safe and may have a role to play in patients maintaining clinical benefit.

FIGURE 1 | Co-inhibitory and co-stimulatory checkpoints are a key element of T-cell immune regulation.

FIGURE 2 | Monoclonal antibodies to key co-inhibitory immune checkpoints.

Pembrolizumab, another IgG4 PD-1 antibody, has also been examined as a treatment for patients with recurrent or metastatic HNSCC. KEYNOTE-012 was a phase Ib study which included an expansion cohort of 132 HNSCC patients. A response rate of 18% at 9 months was reported (including 4 CR and 20 PR), with a median OS of 8 months and a 6-month PFS of 23%. Treatment was well tolerated with grade 3 or 4 toxicity reported in 9% of patients (28) These data led to the accelerated approval of pembrolizumab by the FDA. Outcomes from the confirmatory phase III trial comparing pembrolizumab with standard therapies (KEYNOTE-040) in patients with platinum-resistant relapsed /

metastatic head and neck squamous cell cancers were also presented at ESMO 2017 (29), although the final published manuscript is awaited. This study was similar in design to the CheckMate 141 study, although with a complex hierarchical statistical analysis plan. It demonstrated a median OS of 8.4 months with pembrolizumab compared to 7.1 months in the control arm, with a hazard ratio of 0.81 (95% confidence interval 0.66–0.99), which did not reach statistical significance ($p = 0.020$) within the multiplicity statistical model. The overall survival data may have been confounded by some patients (12%) within the control arm going on to receive immune checkpoint

inhibitors post progression (cross-over effect). The evaluation of other inhibitors of the PD-1/PD-L1 axis in HNSCC, such as durvalumab, atezolizumab and avelumab, are currently ongoing.

GITR

GITR is a member of the Tumor Necrosis Factor superfamily, expressed on the surface of CD25+ CD4+ Tregs. GITR activation by its ligand (GITRL) reduces Treg recruitment and abrogates their suppressive function (30, 31).

AMG228 is a IgG1 antibody that binds to GITR and recently completed phase I clinical trial. Of the 30 patients treated, 10 had HNSCC. Of the 29 evaluable patients, none had an objective response. Treatment-emergent toxicity was experienced in 90% of patients; most common being hypophosphatemia, fatigue, anemia, nausea and pyrexia. There was no evidence of altered T cell activity observed despite complete target coverage in both tumor and peripheral blood (32).

The promise of PD-1 inhibitors has been realized for a small proportion of patients with advanced HNSCC. Further work is ongoing, exploring mechanisms of primary and secondary resistance to these agents within HNSCC, aiming to find better predictive biomarkers and methods to increase likelihood for response.

COMBINATION CHECKPOINT INHIBITION

Emerging evidence has implicated the upregulation of alternative immune checkpoints in resistance to PD-1/PD-L1 axis interruption (33). The use of combination checkpoint inhibitors (CPI) has been successful in improving response rates and survival in other tumor types, with an increasing number of immune checkpoint inhibitors targeting many co-stimulatory and co-inhibitory interactions transitioning from pre-clinical to clinical evaluation in HNSCC.

CTLA-4 is commonly expressed on the surface of activated T cells, where it binds to B7, preventing interaction with co-stimulatory CD28, leading to negative regulation of T cell proliferation and IL-2 production. Blockade of CTLA-4 correlates with an increase in T-Cell activation and maintenance of high-frequency T-cell receptor clonotypes (34, 35). Dual blockade of PD-L1 and CTLA-4 has been shown to improve response rate and anti-tumor activity when compared to monotherapy alone in metastatic melanoma (36) and the combination is being explored in other solid cancers. In HNSCC, this combination approach is being evaluated in four separate trials (**Table 1**).

LAG-3 is expressed on the surface of activated CD4+ and CD8+ T-cells and certain subtypes of natural killer and dendritic cells. It is thought to negatively regulate T cell activation and proliferation and is also expressed by Treg cells and required for their optimal suppressive function (37–41). CA224-020 is a phase I/IIa dose escalation and expansion study, exploring BMS-986016 (an anti-LAG-3 antibody) alone and in combination with Nivolumab in advanced solid tumors, including a HNSCC

cohort (NCT01968109). Data from the head and neck cohorts are eagerly awaited.

CHECKPOINT INHIBITION IN COMBINATION WITH OTHER IMMUNE MODULATORS

The immune ecosystem is a complex network of interconnected cells, cytokines and signaling pathways and as such augmenting the antitumor effect of checkpoint inhibitors is not limited to CPI combinations alone. As understanding of this complex biology improves, a number of other agents with the potential to modulate the tumor microenvironment are currently being investigated for the treatment of HNSCC.

SCORES is a phase Ib/II trial of Durvalumab (a PD-L1 inhibitor) in combination with either AZD9150 or AZD5069 in advanced solid tumors including recurrent / metastatic HNSCC (NCT02499328). AZD9150 inhibits STAT3, with pre-clinical evidence of activity in lymphoma and lung cancer (42). Pre-clinical studies have also shown that STAT3 inhibition can increase chemo and radiotherapy sensitization in HNSCC, particularly Nasopharyngeal Cancer (NPC) (43, 44). AZD5069 is a novel selective antagonist of CXC Chemokine receptor 2 (CXCR2), a G protein-coupled receptor for a number of cytokines. It is overexpressed in HNSCC and is implicated in disease proliferation via IL-8 signaling (45, 46). Initial results of patients with HNSCC treated in the AZD9150 and Durvalumab arm were announced at the ESMO Congress 2017. Of 35 patients, 15 had prior PD-L1 treatment and 20 were CPI-naïve. In the CPI-naïve arm, a 25% objective RR was reported (4 confirmed PR and 1 unconfirmed PR) with a 45% DCR at 12 weeks and 30% patients remaining on treatment at 25 weeks. Overall the combination was felt to be tolerable, with G3/4 thrombocytopenia and increase in liver enzymes reported for 3.4% of those dosed, and two treatment related discontinuations (unspecified). These early data are promising and mature results are awaited. In the PD-L1 pretreated arm, 1 complete response and 1 unconfirmed response were reported, with a 20% DCR at 12 weeks (47).

Indoleamine 2,3-dioxygenase 1 (IDO1) is a catabolizing enzyme that induces immune tolerance by suppressing T-cells and has been found to be associated with poor outcome in laryngeal squamous cell carcinoma (48). IDO1 is being heavily investigated as a novel target for immune therapies, with several inhibitors in clinical development. KEYNOTE-037 is a phase I/II study evaluating pembrolizumab given concurrently with epacadostat, an oral inhibitor of IDO1. Patients with recurrent / metastatic HNSCC were eligible for this study if they had received at least one line of platinum based chemotherapy and were CPI-naïve. An interim update of this study was presented at ASCO 2017, when data from 38 patients were presented, 36 of whom were efficacy-evaluable at this early cut off. An ORR of 31% was reported, with a disease control rate of 58%, regardless of the number of previous lines of treatment. The most common treatment related SAEs were fatigue (24%), nausea (11%) and decreased weight (11%). These data suggesting

TABLE 1 | Ongoing trials evaluating combination PD-L1 and CTLA-4 blockade in HNSCC.

Trial	Phase	Cohort	Drugs	Trial code
CheckMate 356	I/II	EBV positive nasopharyngeal cancers, HPV positive HNSCC	Ipilimumab and Nivolumab	U111-1166-0687
CheckMate 714	II	Recurrent or metastatic HNSCC	Ipilimumab and Nivolumab vs. Nivolumab and Placebo	NCT02823574
CheckMate 651	III	First line treatment for HNSCC	Ipilumumab and Nivolumab vs. Cetuximab with platinum and flurouracil	NCT02741570
KESTERAL	III	Recurrent or metastatic HNSCC	Durvalumab and Tremelimumab vs. Durvalumab monotherapy	NCT02551159

Durvalumab, IgG1 antibody to PD-L1; EBV, Epstein-Barr Virus; Ipilimumab, IgG1 antibody to CTLA-4; Tremelimumab, IgG2 antibody to CTLA-4.

promising anti-tumor activity with good tolerability have led to plans for a phase III study (49).

CHECKPOINT INHIBITION IN COMBINATION WITH VIRAL THERAPY

Oncolytic viruses have been found to reduce tumor burden and prime an anti-tumor immunity in a number of preclinical studies (50, 51) and when used in combination with PD-1 inhibition may overcome CPI resistance by broadening neoantigenome-directed T-cell responses (52). Several oncolytic viruses are currently being evaluated in HNSCC. KEYNOTE-137 is an ongoing phase Ib/III randomized study exploring the combination of Talimogene Laherparepvec (T-VEC) with pembrolizumab in recurrent metastatic HNSCC (NCT02626000). T-VEC is a modified, live, attenuated herpes simplex virus type 1 that is designed to promote an antitumor response through selective replication in tumor cells and production granulocyte macrophage colony-stimulating factor (GM-CSF) to stimulate systemic antitumor immunity. It has already been licensed as a single agent for the treatment of unresectable metastatic melanoma after demonstrating an improved durable response rate (DRR) and mOS relative to GM-CSF (53, 54).

CHECKPOINT INHIBITION IN COMBINATION WITH CHEMORADIOTHERAPY

In addition to interest in combining immune checkpoint inhibitors to other novel agents, there is also a rationale to combine with both chemotherapy and radiotherapy, which are both known to modulate the tumor microenvironment as well as inducing immunogenic tumor cell death. There is particular interest in exploring CPI in combination with chemo-radiation in locally advanced head and neck cancers. Upregulation of PD-L1 by tumor cells following administration of chemo-radiotherapy (CRT) has been demonstrated in a number of preclinical models (55–57) with an improvement in RR, PFS and median time to death being achieved with the addition of adjuvant durvalumab to CRT in locally advanced, unresectable Non-Small-Cell Lung Cancer as demonstrated in the large phase III PACIFIC study (58).

Higher numbers of CD3+ and CD8+ TILs have been shown to positively correlate with clinical outcome to definitive CRT in HNSCC (59). In addition, in a pilot study of 20 patients, CRT was shown to alter the immune landscape in HNSCC, with an increase in the number of CD8+ T effector cells, CD4+ regulatory cells and T cells expressing PD1, TIM3 and LAG3. It is important to note that in this study, most patients were male (90%) with locally advanced human papillomavirus (HPV) associated disease, 80% of which originated from the oropharynx and thus results may not be representative of all HNSCC entities (60).

A safety study demonstrating the tolerability of pembrolizumab in addition to cisplatin-based CRT for locally advanced HNSCC was presented at ASCO 2017. This 27-patient study delivered a fixed dose of pembrolizumab 4–7 days prior to CRT, 3 weekly for the duration of CRT and a further five doses following completion. Patients predominantly had HPV positive oropharyngeal tumors (74%) and all received their planned RT dose, with 85% achieving target cisplatin dosing and 78% completing the planned doses of pembrolizumab. The addition of checkpoint blockade was not felt to significantly increase the toxicity experienced by patients, however three patients did have treatment discontinued due to immune related adverse events (G2 peripheral motor neuropathy, G3 AST elevation and G1 Lhermitte-like syndrome). The study has now progressed into expansion cohorts of both HPV positive and negative tumors to confirm tolerance and gain preliminary evidence of efficacy (61).

Cetuximab is also used with RT for the radical treatment of locally advanced HNSCC. It is an influencer of natural killer cell response and consequently dendritic cell maturation (62, 63) and is thought to increase the expression of inhibitory checkpoints PD-1, TIM-3 and CTLA-4 on TILs (64, 65). There are a number of current trials attempting to determine the benefit of checkpoint inhibition alongside varying combinations of cetuximab and RT in locally advanced HNSCC, with the combination with avelumab as part of REACH (NCT02999087) being of particular interest due to the propensity of both avelumab and cetuximab to activate the antibody-dependent cellular cytotoxicity (ADCC) pathway (63, 66).

IMMUNE-RELATED ADVERSE EVENTS

Treatment with CPIs can have inflammatory side effects which are termed immune related adverse events (irAEs). Although the

exact mechanism is unknown, it is likely due to the role that immune checkpoints have in maintaining immune homeostasis and by inhibiting their action, T cells are able to react with self-antigens, with different checkpoint inhibitors having distinct immune toxicity profiles (67). These autoimmune manifestations are more common in patients with pre-existing autoimmune disorders however they may still be safely administered to this population if used with caution and appropriate patient selection (68).

A pooled retrospective analysis of the safety profile of nivolumab in 576 patients with advanced melanoma found the 49% of patients had an irAE; most commonly skin, gastrointestinal, endocrine and hepatic and were classed as grade 3–4 in 4% of patients. The time of onset varied depending on the organ system involved, with skin irAEs manifesting at 5 weeks whereas renal toxicity had had median time of onset of 15 weeks. Approximately 24% required systemic immunosuppressive treatment with the majority of cases resolving (69). In CheckMate 141, the side effect profile of nivolumab in a HNSCC population showed lower rates of both gastrointestinal and hepatic toxicities when compared to the standard of care treatments but did demonstrated an increase in skin toxicity (15.7%), endocrinopathies (7.6%) and pneumonitis (2.1%) (24).

The results of a 114-case series of patients with metastatic HNSCC treated with anti-PD-1 therapy was presented at ASCO 2018, demonstrating that patients who manifested an irAE had improved outcomes compared to those that did not. In total, 59 irAEs were recorded in 49 patients with ORR being higher in irAE positive group (30.6 vs. 12.3% $p = 0.02$) and an improvement in both PFS (6.9 vs. 2.1 months; $p = 0.0004$) and mOS (12.5 vs. 6.8 months; $p = 0.007$) were reported, which remained significant on multivariate analysis (70). A similar observation has been noted in patients with metastatic melanoma and persisted regardless if they required treatment with a systemic immunosuppressant for treatment of their irAE (69).

The development of irAE's can be serious and in some cases fatal, and as such careful consideration must be taken before initiating their use, particularly in the adjuvant or neo-adjuvant setting. As our understanding of the mechanism that drive these systemic manifestations improve, we may develop biomarkers that help identify those who are more likely to develop them which will assist in informed decision making and toxicity monitoring.

BIOMARKERS

As improvements in the understanding of the interaction between cancer and the host immunity are complimented by increasing numbers of immunomodulatory drugs, there has been a drive to develop potential biomarkers to select patients most likely to benefit from treatment and assist in monitoring response.

Both the nivolumab and pembrolizumab studies outlined above explored the impact of HPV on outcome. In Checkmate-141, OS appeared to be longer with nivolumab regardless of p16

status, however the increase was more pronounced in patients with p16 positive tumors (mOS of 9.1 months with nivolumab vs. 4.4 months with standard therapy) than in p16 negative tumors (mOS 7.5 vs. 5.8 months respectively) (24). KEYNOTE-012 also reported better outcomes in the patients with HPV-positive tumors relative to HPV negative ones (RR 32% vs. 14%, 6 month PFS 37% vs. 20% and 6-month OS 70% vs. 56%) (28).

These trials also examined tumor cell PD-L1 expression as a potential biomarker, with data suggesting increased benefit in PD-L1 positive disease. In the overall survival analysis of Checkmate-141, patients with PD-L1 expression >1% treated with nivolumab had a hazard ratio for death of 0.55 (95% CI 0.36–0.83) when compared to standard therapy. Where PD-L1 expression was <1%, this HR was 0.89 (95% CI 0.54–1.45) (24). Similarly, KEYNOTE-012 reported tumor PD-L1 expression using immunohistochemistry, with positivity being defined as >1%. Patients with PD-L1 expression >1% who received pembrolizumab had improved RR of 22 vs. 4% in those with PD-L1,1%, with median OS 303 vs. 151 days respectively (28). KEYNOTE-040 described an OS HR of 0.54 (95% CI 0.35–0.82) with pembrolizumab in patients with tumors with PD-L1 expression >50% but also evaluated PD-L1 expression on both tumor and associated immune cells (CPS score) describing OS HR of 0.75 for PD-L1 CPS >1% with pembrolizumab compared to control patients.

Of the 61 PD-L1 positive HNSCC in KEYNOTE-012, 43 had RNA expression profiling and survival data were evaluated with multi-gene expression signatures that had previously been derived in melanoma patients. Of these signatures, the 6-gene INF-γ was the top-performing, with significant associations to OR ($p = 0.005$) and PFS (<0.001). On evaluation of the individual signature genes, INF-γ inducible MHC-II expression was felt to be the biological link. Using an optimal cutoff for INF-γ, positive predictive value for response was 40% with a negative predictive value of 95%; AUC = 0.8 (95%CI 0.61–0.95) within this patients population, which may assist in identifying clinical benefit from anti-PD-1 therapy in patients who are PD-L1 positive (71).

The somatic mutational load (ML) and INF-γ gene expression profile were found to be independently predictive of response to pembrolizumab in the 73 patients within KEYNOTE-012 who had HPV and Epstein-Barr Virus (EBV) negative HNSCC, with ML and INF-γ gene expression profile being significantly associated with OR ($p = 0.064$ and $p = 0.001$; AUROC 0.82 and 0.74 respectively). The INF-γ gene expression profile also remained a significant predictor in HPV and EBV positive patients showing promise as a predictor of response regardless of viral status (72).

Combinations of these biomarkers may give additive value to patient selection for CPI therapy. Other potential biomarkers that have shown promise include epigenetic modification of genes associated with homologous recombination, such as RAD51 and XRCC3, which are thought to alter checkpoint expression (73)

and the identification of different HNSCC subtypes, each with a distinct tumor microenvironment (74) but how these influence survival or response to immunotherapy has yet to be addressed.

CONCLUSION

Immunotherapy looks set to revolutionize the treatment of HNSCC, with the approval of nivolumab and pembrolizumab (FDA) already offering new therapeutic options in recurrent / metastatic disease. As our knowledge of the biological processes driving HNSCC improves, along with greater understanding of the important features of the tumor microenvironment, so too does the rationale for combination strategies and the parallel development of predictive biomarkers. These approaches should

support an era where a personalized approach to immunotherapy treatment translates into improved outcomes for patients with this disease.

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On the Road to Immunotherapy—Prospects for Treating Head and Neck Cancers With Checkpoint Inhibitor Antibodies

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Head and neck cancers (HNC) represent a heterogeneous cluster of aggressive malignancies that account for 3% of all cancer cases in the UK. HNC is increasing in frequency particularly in the developing world, which is related to changes in risk factors. Unfortunately, the mortality rate is high, which is chiefly attributed to late diagnosis at stages where traditional treatments fail. Cancer immunotherapy has achieved great successes in anti-tumor therapy. Checkpoint inhibitor (CI) antibodies enhance anti-tumor activity by blocking inhibitory receptors to drive tumor-specific T and NK cell effector responses. Since their introduction in 2011, CI antibodies have been approved for many cancer types including HNC. Here, we examine the development of CI therapies and look forward to future developments for treatment of HNC with CI therapies.

Keywords: head and neck squamous cell carcinoma, checkpoint inhibitor, immunotherapy, T cells, PD-1

INTRODUCTION

The notion of boosting anti-tumor immunity as a means of treating cancer has been escalated by the recent unveiling of exciting new immunotherapies including the checkpoint inhibitor (CI) antibodies (1). CI antibodies selectively activate adaptive immunity to locate and obliterate tumors anywhere in the body and can also generate an enduring disease remission (2). The recent approval of six CI antibody therapies for treating a range of cancers (3–8) heralds a golden age of immunotherapy, with the promise of further novel, better immune-boosting technologies and combination treatment strategies to come (9). But there are also caveats such as poor patient response frequency, the potential for serious immune related side effects and generally a lack of biomarkers which can guide the use of these therapies (10). In this review, we reflect on the developing prospects for CI therapy to treat head and neck cancers (HNC).

HEAD AND NECK CANCERS

Head and neck cancers, of which the majority are squamous cell carcinomas (HNSCC), represent a collection of neoplasms that are difficult to treat and whose incidence in the UK and worldwide has increased by around 30% since 1990. In 2015, 12,000 individuals were diagnosed with HNC in the UK [CRUK, oral cancer statistics, 2018], representing 3% of all cancers. Annually, there is an estimated 600,000 cases worldwide, which affect the oral cavity, oropharyngeal, hypopharyngeal,

and laryngeal tissues (11, 12). Increased incidence is associated with known risk factors including high use of both tobacco and alcohol. In certain parts of the world, in particular South East Asia, the incidence of HNC is much higher and is reported as high as 30% of all cancers in India, with the major risk factor being betel quid (pan) chewing in all its forms, which almost invariably includes tobacco (smokeless tobacco) (11, 12). Many cases of HNC are also associated with infection by human papillomavirus (HPV) strains 16 and 18, well established high-risk viral types in other malignancies, most notably cervical cancer (13). Patients with HPV$^+$ tumors, however, have a better outcome in terms of both survival and reduced risk of recurrence compared with HNC in which no virus can be detected (13). This latter observation may reflect a greater intrinsic immunogenicity associated with HPV infection and this perception is supported by immune profiling studies that find increased effector T cell infiltrates in HPV$^+$ compared with HPV$^-$ tumors (14).

The current treatment standard of care for HNSCC is to treat recurring or metastatic tumors with cetuximab, an anti-epidermal growth factor receptor antibody, together with platinum based cis- or carboplatin chemotherapy plus 5-flurouracil and methotrexate, which is further supported where appropriate by surgery and radiotherapy (15), and in some instances augmented by the taxanes, docetaxel and paclitaxel.

In 2016, two anti-PD-1 checkpoint inhibitor monoclonal antibodies (mAbs), pembrolizumab and nivolumab provided new options for cisplatin resistant recurring or metastatic HNSCC following accelerated FDA approval based on encouraging clinical trial data (16, 17) and precedence of response efficacy in large phase III clinical trials of melanoma and non-small cell lung cancer in which both antibodies had already demonstrated significant improvements in patient outcomes compared to current standard of care therapy (4, 5, 18, 19). This has led to further clinical trials for HNSCC with larger patient cohorts primarily with the aim of comparing anti-PD-1 antibodies alone or together with current platinum-based therapies and cetuximab. Currently, there are more than 90 clinical trials involving established CI inhibitor therapies and HNSCC.

IMMUNE CHECKPOINTS

The immune system is a decision-making entity which, when not required remains quiescent but vigilant for the emergence of a new pathogenic challenge. Once that challenge arrives, the immune system ramps up immune processes shaped to deal specifically with each new pathogenic threat, powerful enough to clear the pathogen, but which may also carry some risk of bystander damage to host cells and tissues. After pathogen clearance, the immune system also needs to return to its former quiescent state to avoid any further damage. Immune checkpoints, primarily receptors on immune cells, regulate both immune response intensity to prevent host tissue damage, and also resolve the immune response after pathogen clearance (20). Interactions between checkpoint receptors and their ligands can be restricted to immune cell subsets but can also take place

between immune and non-immune cells (21). Environmental cues within an inflammatory lesion up-regulate expression of receptors on non-hematopoietic and non-lymphoidal cells such as epithelia that then engage with immune effector cells to suppress and eventually quell their activity (22, 23).

There are several immune checkpoint receptors, all of which have individual expression patterns on a variety of immune cells and, therefore, contribute to immunoregulation at different levels. Perhaps the best known and most fundamental checkpoint receptor is CTLA-4 (CD152) (24), which plays a role both in the priming of naïve T cells and also control of effector T cell response intensity (25–27). Other checkpoints include PD-1 (CD279) (28, 29), ICOS (CD278) (30), 4-1BB (CD137) (31), OX40 (CD134) (32), LAG-3 (CD223) (33), TIM-3 (34), TIGIT (35), VISTA (36), BTLA (CD272) (37), and GITR (38), which display a hierarchy of expression on different cell types and therefore exert a more selective control over interactions both between immune cell subsets and between immune and non-immune cells (39). Analyses of immunogenic HNSCC, suggest that in many individuals the tumors appear "primed" to make potent anti-tumor effector T cell responses and can therefore be considered suitable for CI therapy. There is an urgent need, therefore, to develop genetic and histological response biomarkers that efficiently identify and stratify responsive patient cohorts to one or more of these CI therapies, together with other therapies designed to increase tumor immunogenicity (40, 41).

CHECKPOINT INHIBITOR ANTIBODIES

Checkpoint inhibitor (CI) antibodies target immune cell checkpoint receptors to selectively activate antigen-specific anti-tumor T cell responses. In 2013, CI therapies together with CAR-T cell immunotherapy were considered to be the most important scientific breakthrough of the year by *Science* (42). The efficacy of CI therapies has been ground-breaking in the treatment of melanoma, non-small cell lung cancer (NSCLC) and other cancers, including HNSCC, offering clear advances over other established chemo- and immunotherapies in terms of patient response frequency, as well as efficacy and durability of response. In general, tumor immunogenicity is considered to be the most important factor in determining whether or not a particular type of cancer will respond to CI therapy (43), but this has not prevented CI therapies from being tested in most types of cancer in ongoing clinical trials and it will be some time before it becomes apparent which cancers are most responsive to this type of CI immunotherapy.

The six CI antibodies with FDA approval so far have specificities for CTLA-4 (ipilimumab), PD-1 (pembrolizumab, nivolumab) and PD-L1 (atezolizumab, durvalumab, and avelumab) checkpoint receptors. Collectively, they have been approved for advanced melanoma, non-small cell lung cancer, renal cell carcinoma, urothelial and bladder cancer, HNSCC, metastatic Merkel cell carcinoma, refractory classical Hodgkin lymphoma and gastric cancer (**Figures 1–3**). Another CTLA-4 antibody, tremelimumab, is in advanced stages of clinical trials, while cemiplimab an anti-PD-1 IgG$_4$ antibody is likely to be

FIGURE 1 | Time line of FDA approvals for ipilimumab (July 2018).

FIGURE 2 | Time line of FDA approvals for the anti-PD-1 antibodies pembrolizumab and nivolumab (July 2018).
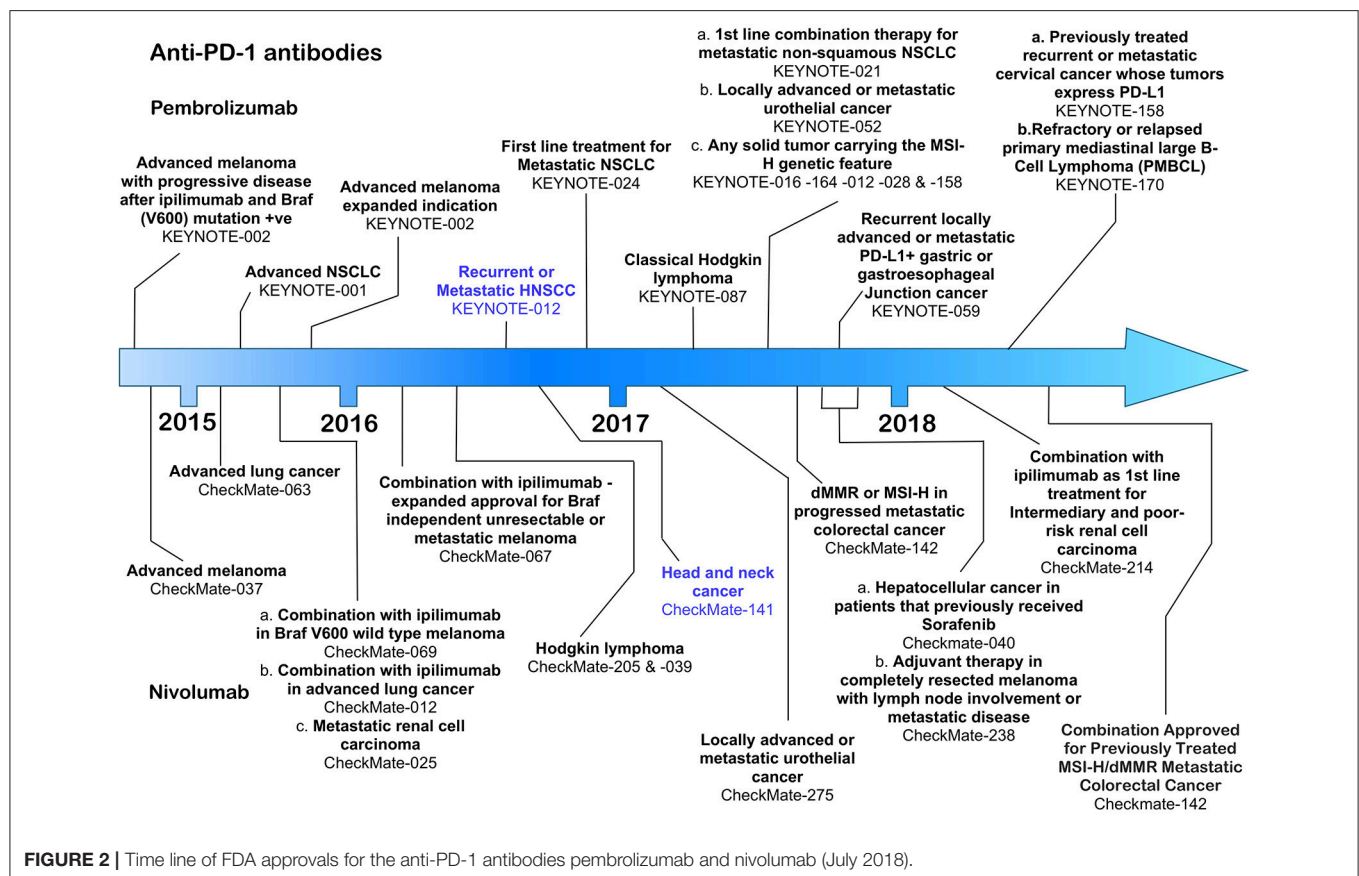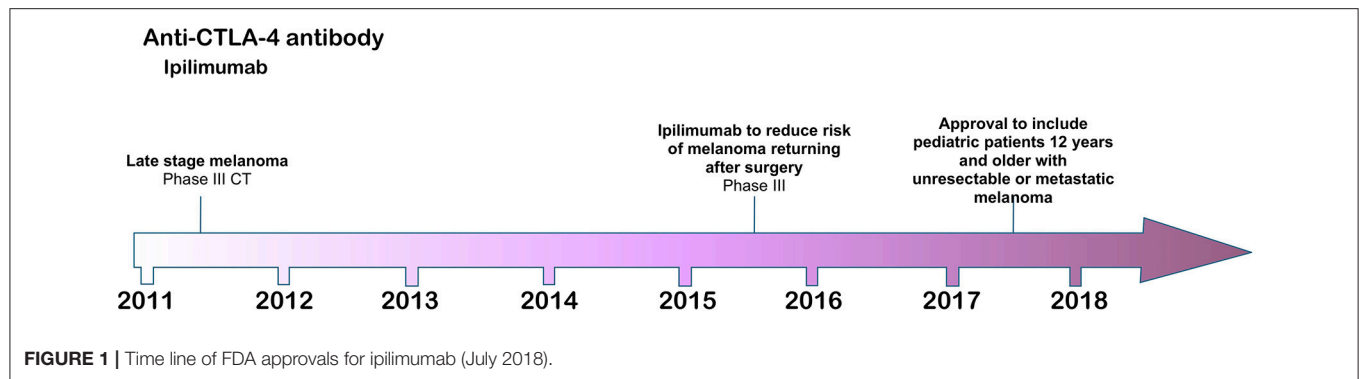
FDA approved soon for the treatment of advanced cutaneous squamous cell carcinoma. Many more CI therapies are under development (44), and by 2025, the checkpoint inhibitor market is expected to exceed $40 billion worldwide.

CTLA-4

The first checkpoint inhibitor antibody, ipilimumab, was approved by the FDA in 2011 for the treatment of metastatic melanoma (3) based on a phase III clinical trial in which the antibody significantly extended melanoma patient survival compared with standard of care therapy. The CTLA-4 (CD152)

target of ipilimumab is an inhibitory regulator of T cell costimulation (45, 46), modulating the priming and activation of naïve T cells, as well as the intensity and potency of both $CD4^+$ T helper and $CD8^+$ cytotoxic effector T cell responses. CTLA-4 is an inhibitory counterpart to the stimulatory CD28 receptor on T cells, which is an essential component of antigen-specific naïve T cell costimulation during initial priming by dendritic cells (DC). Like CD28, CTLA-4 on T cells interacts with the B7.1/B7.2 (CD80/CD86) ligands on DC, but with higher affinity to out-compete its costimulatory partner and thus to inhibit T cell activation (47). As a CI therapy, therefore, antibody blockade of CTLA-4 functions at a fundamental level to restore effective DC

FIGURE 3 | Time line of FDA approvals for anti-PD-L1 antibodies Atezolizumab, Avelumab, and Durvalumab (July 2018).
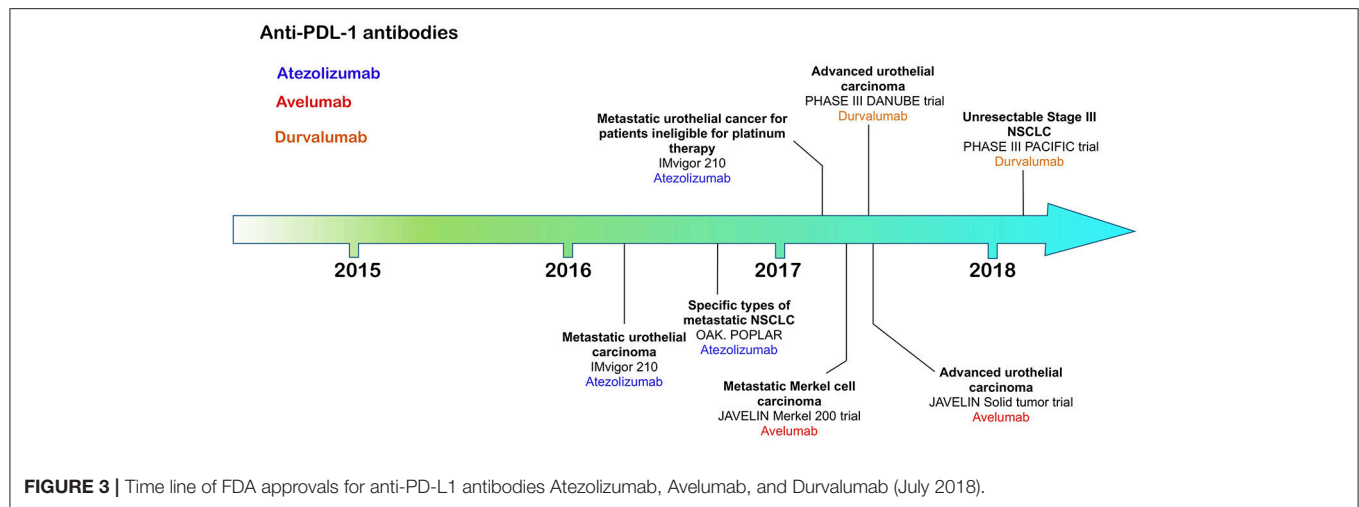
priming of naïve T cells, inducing full activation and expansion of nascent effector T cell populations against tumor neoantigens (48).

The non-redundant function of CTLA-4 in regulating immune cell homeostasis was demonstrated by CTLA-4 knockout mice, which die soon after birth from massive lymphocytic infiltration of tissues and organs (49, 50). Healthy homeostasis in these mice could be restored, however, by infusion of recombinant soluble CTLA4-Ig (51) or by generating chimeric mice in which CTLA-4$^+$ T cells are able to regulate CTLA-4$^-$ T cells to prevent their unregulated expansion (52, 53). In humans, analyses of heterozygous CTLA-4 gene haploinsufficiencies, or reduced expression of CTLA-4 caused by mutation in the LRBA gene (encoding the lipopolysaccharide-responsive and beige-like anchor protein), which is thought to regulate CTLA-4 protein turnover (54), have also revealed complex pathological phenotypes that correspond to unregulated T cell responses, including Treg dysfunction, effector T cell hyper-proliferation, non-lymphoid organ infiltration and autoantibody production (55, 56). Patients with these pathologies also respond well to abatacept, the human recombinant soluble CTLA4-Ig. These observations demonstrate that CTLA-4 can be used by immune cells to extrinsically regulate effector T cell populations. CTLA-4 is constitutively expressed in higher amounts on regulatory T cells (Treg) and is essential for their immunoregulatory function (57), although how these cells utilize CTLA-4 to control effector T cell populations still needs to be fully elucidated (58).

The therapeutic potential of CTLA-4 antibody blockade was first demonstrated in murine cancer models of melanoma, mammary and prostate cancer (59–61). In the B16-BL6 and B16-F10 murine models of solid and metastatic melanoma, in particular, anti-CTLA-4 antibody blockade of T cells alone was not effective in eliminating tumors, and their potential for driving anti-tumor immunity was revealed only after treatment of the mice with a granulocyte/macrophage colony-stimulating factor (GM-CSF)-expressing tumor cell vaccine, which enhanced dendritic cell activity and also enhanced the generation of B16 melanoma-specific T cells (60). This increase in immunogenicity

provided the antigen required by tumor specific effector T cells, allowing complete dissolution of the tumors. These experiments in mice shaped the future therapeutic strategy for CI blockade in humans and it also highlighted the requirement for tumor immunogenicity as an essential requirement for successful treatment. These early experiments in CI therapy highlighted the importance of the mechanisms and environmental cues that drive the release and display of tumor associated and tumor specific neoantigens (62).

Ipilimumab was approved by the FDA in 2011 for use in metastatic melanoma refractory to conventional treatments, in which patients receiving ipilimumab with or without the melanoma-derived gp100 tumor-associated antigenic peptide co-vaccine demonstrated increased overall survival of 10 months compared with 6.4 months in patients receiving the vaccine alone (3). An interesting and important feature of ipilimumab CI therapy is that it can induce an enduring remission from melanoma disease in approximately 22% of metastatic melanoma patients receiving the therapy (2). Despite this success, ipilimumab has little beneficial effect for most patients receiving it and it can also provoke very severe immune related adverse events, including dermatitis, colitis, hypophysitis, and other inflammatory events (3). Further, there is currently no reliable biomarker with which to stratify patients by identifying those responsive to the therapy. Ipilimumab is generally administered four times over a period of approximately 3 months at a dose of 3 or 10 mg/kg body weight.

Despite the ground-breaking success of ipilimumab as the first CI therapy, there is still some controversy of how it is able to "release the brakes" of the immune system to drive anti-tumor immunity. Although the simple hypothesis is that CTLA-4 blockade generally inhibits CTLA-4 engagement with its B7 ligands, thereby allowing CD28 costimulation and full T cell activation to take place, there is some evidence that other factors may also be at play. Currently, the best accepted hypothesis is that anti-CTLA-4 antibodies mediate at least some of their immune boosting effects by engaging with activating Fc gamma receptors (FcγR) by binding to CTLA-4 on Treg to induce macrophage

mediated depletion of the Treg through antibody-dependent cell-mediated cytotoxicity (ADCC) within the tumor environment or at the infusion site (63). This depletion would therefore allow reactivation of tumor infiltrating lymphocytes (TIL) to drive productive anti-tumor immunity. Other groups have provided evidence that anti-CTLA-4 antibodies can directly engage with CTLA-4$^+$ tumor cells to drive ADCC (64, 65), but this is unlikely to fully explain the profound anti-tumor effects of CTLA-4 based CI therapy.

No CTLA-4 based CI therapy has been approved for the treatment of HNSCC but there are several clinical trials underway involving either ipilimumab or tremelimumab. Almost all of these are clinical trials involving combinations of anti-CTLA-4 mAbs either with other CI therapies, or current standard of care HNSCC therapies such as cisplatin and cetuximab.

PD-1/PD-L1

Programmed death-1 (PD-1) is a checkpoint receptor primarily expressed by T cells that plays a crucial role in regulating and resolving adaptive effector T cell immune responses (28). PD-1 binds two ligands PD-L1 (CD274, B7-H1) (66) and PD-L2 (CD273, B7-DC) (67). PD-L1, has a very broad distribution on normal tissues and PD-1: PD-L1 interactions between immune and non-immune cells within inflammatory milieus are thought to maintain peripheral tolerance by suppressing effector T cell responses (68, 69). Induction of signaling through the PD-1 receptor suppresses IL-2 production in T cells and renders them less antigen-responsive (70). This anergic "exhaustion" phenotype is reversible by selective blockade of either PD-1 or PD-L1 (71). PD-L2 expression is less abundant and restricted mainly to professional antigen presenting cells but induces similar effects in T cells (72).

The therapeutic benefits of CTLA-4 antibody blockade were identified in murine tumor models, but the potential for PD-1 or PD-L1 blockade as a novel CI therapy arose from observational studies in humans. Soon after initial identification of PD-L1, analysis of renal cell carcinoma (RCC) patient survival outcomes following nephrectomy identified that patients with high expression levels of PD-L1 on either RCC tumor cells, RCC tumor infiltrating lymphocytes, or both, were at significantly increased risk of death from aggressive tumor progression (73). This important observation, together with extensive analyses of PD-L1 on tumor cell lines (67) and tumors including HNSCC (74), raised the notion of a novel immune evasion mechanism through which cancer cells nullify anti-tumor effector T cell responses by engaging PD-1 and inducing the exhaustion phenotype (75). These latter observations in HNSCC were further qualified by more recent studies in which analysis by PCR and immunohistochemistry of 41 esophagectomy tumors identified elevated levels of PD-L1 to be associated with a poor prognosis particularly in advanced tumors (76), while another study, however, did not find a clear correlation between tumor cell expression of PD-L1 and poor prognosis, but did identify elevated expression of PD-L1 on infiltrating immune cells, including T cells, macrophages and

dendritic cells, to correlate significantly with increased overall survival (77). In addition, increased abundance of CD3$^+$ and CD8$^+$ T cell infiltrates also associated with prolonged survival outcomes (77).

With regard to HPV$^+$ tumors, the PD-1:PD-L1 nexus may be especially relevant given that the effector T cell exhaustion phenotype, induced by engagement of PD-1 with PD-L1, is often associated with viral infection (70), and is likely a critical element in the induction of an artificial immune privileged microenvironment (78). HPV$^+$ oropharyngeal tumors are associated with increased levels of T cell infiltrates and following conventional therapy overall survival and reoccurrence are both improved compared with HPV$^-$ tumors (77, 79) suggesting that they are in effect primed for an anti-tumor response because of the anti-viral response. However, although HPV positive status signals a better outcome for HNSCC, recent studies investigating HPV integration into the host genome suggest that HPV integration into key gene sites including the PD-L1 gene may be a critical marker for patient outcome with reduced survival in patients with integration positive HPV tumors (13).

These studies have led to the rapid development of both anti-PD-1 and anti-PD-L1 checkpoint inhibitor therapies to block the PD-1: PD-L1 axis, which have since demonstrated significant improvements in patient outcomes in clinical trials for a range of cancers including HNC over the last five years.

Antibodies specific for the PD-1 receptor were the first CI therapies to be introduced after ipilimumab, initially for the treatment of melanoma, and have had much greater success than ipilimumab clinically and commercially. In 2017, sales of Keytruda^{TM} (pembrolizumab) and Opdivo^{TM} (nivolumab) were reported as $3.8 billion and $4.95 billion respectively with worldwide growth in sales from 2016 to 2017 of 171 and 31% respectively. Because CI therapies, including the PD-1 antibodies, target the immune system rather than the tumor, CI antibodies can theoretically be used to treat many forms of cancer and this notion has been successfully translated into the clinic with regard to anti-PD-1.

Unlike the view of anti-CTLA-4 antibodies in which binding FcγR may indirectly contribute to their therapeutic effects, anti-PD-1 mAbs are mechanistically straightforward and function simply by blocking engagement of PD-1 with its ligands PD-L1 and PD-L2. Indeed, engagement of FcγR was detrimental to their therapeutic potency (80) and thus most anti-PD-1 antibodies are of the IgG$_4$ antibody subclass, which has weak binding associations with FcγR.

So far, the anti-PD-1 antibodies have been FDA approved for the treatment of advanced melanoma, non-small cell lung cancer, renal cell carcinoma, classical Hodgkin's lymphoma, urothelial cancer, gastric cancer and head and neck cancer (**Figure 2**). Throughout clinical trials, anti-PD-1 antibodies demonstrated an increase in response frequency in patients compared with current standard of care and were accompanied by lower risk and frequency of serious immune related adverse events compared with ipilimumab (81). Both pembrolizumab and nivolumab were first approved for use by the FDA after being granted an accelerated approval protocol in 2014 for the treatment of

unresectable metastatic melanoma in patients carrying the V600 BRAF mutation (5, 82).

ANTI-PD-1 THERAPY IN HNSCC

Pembrolizumab, a humanized IgG_4 antibody was approved on August 5th, 2016 under the FDA's accelerated approval programme, based on data from the KEYNOTE-012 phase 1b clinical trial, which assessed the therapeutic effects of pembrolizumab in patients with HNSCC, triple negative breast cancer, gastric cancer and urothelial cancer (16). HNSCC patients whose disease had progressed following platinum-based therapy received pembrolizumab at either 10 mg/kg body weight every 2 weeks ($n = 53$) or a fixed dose of 200 mg every 3 weeks ($n = 121$) until disease progression or the development of intolerable toxicity (16). Patients received treatment for a maximum of 24 months. The overall response rate in the combined HNSCC patient cohorts was 16 with 5% of those achieving a complete response. The duration of response in 82% of the responsive patients lasted more than 6 months. Pembrolizumab has also shown clinically significant activity in patients with both HPV$^+$ or HPV$^-$ tumors (83). Among the immune related adverse events associated with therapy were pneumonitis, colitis, hepatitis, adrenal insufficiency, diabetes mellitus, and skin toxicities (16). Despite these promising results, however, pembrolizumab failed to meet its pre-specified primary endpoint of overall survival in the larger phase III KEYNOTE-040 clinical trial, which compared treatment with pembrolizumab at a fixed dose of 200 mg every 3 weeks with cetuximab, methotrexate (ESMO 2017 Press Release). Another phase III KEYNOTE trial (KEYNOTE-048, NCT02358031) is currently underway in which pembrolizumab alone (fixed 200 mg dose in 3 weekly cycles for up to 24 months), or pembrolizumab (fixed 200 mg dose) together with a platinum-based therapy plus 5-fluorouracil and compared with cetuximab combined with a platinum-based therapy and 5-flurouracil. The primary completion date for this trial is 31st December 2018. In addition, more than 50 clinical trials involving pembrolizumab in HNSCC are underway, most of which are focussed on therapies that combine radiotherapy or platinum-based therapies with pembrolizumab.

Nivolumab, a fully human IgG_4 was also FDA approved in 2016 following completion of the CheckMate-141 open-label, phase III clinical trial in which 361 HNSCC patients with recurrent squamous-cell carcinoma of the head and neck whose disease had progressed within 6 months after platinum-based chemotherapy, were treated with nivolumab or standard therapy alone (17). Patients received nivolumab at a dose of 3 mg/kg every 2 weeks with the end point of overall survival as the critical marker of improvement over the standard of care therapy. Patients receiving nivolumab were compared at a 2:1 ratio with patients receiving post-platinum standard of care therapies including methotrexate, docetaxel and cetuximab (17). Patients receiving nivolumab had an overall survival median of 7.5 months [95% confidence interval [CI], 5.5 to 9.1] in the nivolumab group compared with 5.1 months (95% CI, 4.0 to 6.0) in the standard-therapy

group (17). PD-L1 expression levels were examined in 72% of the 361 patients in the clinical study and 57.1% of those had PD-L1 expression levels of $\geq$ 1%. Individuals with PD-L1 levels $\geq$ 1% responded better to nivolumab compared with the patient cohort in which PD-L1 was less than 1% (17). Patients specifically with oropharyngeal HNSCC also responded better to nivolumab independent of their HPV status. This study also revealed that quality of life measures in patients receiving nivolumab remained stable or improved slightly, whereas patients receiving standard therapy suffered significant deterioration at 15 weeks after commencement of therapy (17). Toxicities included pneumonitis, dermatitis, and endocrine dysfunction, although serious adverse events were significantly lower in the nivolumab compared with standard care study arm (17). As for pembrolizumab, nivolumab is currently the subject of several further clinical trials, primarily in which it is paired with other treatment options including anti-CTLA-4 ipilimumab (84).

ANTI-PD-L1 ANTIBODIES

The most recently FDA approved CI antibodies are three anti-PD-L1 antibodies—atezolizumab, avelumab and durvalumab; approved for urothelial/bladder cancer (6–8), non-small cell lung cancer (atezolizumab, durvalumab) and Merkel cell carcinoma (avelumab). All of these therapies rely on expression of PD-L1 on the target tumor, allowing both patient stratification through increased response frequency based on tumor expression levels of PD-L1.

Atezolizumab, a humanized IgG_1 antibody engineered to reduce any potential for ADCC or CDC, was the first anti-PD-L1 antibody to be approved for use in advanced urothelial carcinoma patients whose disease had worsened after a platinum-based therapy (6). Bladder tumors have relatively high expression levels of PD-L1 compared with other tumors identifying this type of cancer as a suitable target for anti-PD-L1 antibodies (85). Patients in this phase II clinical trial were segregated according to tumor-infiltrating immune cell and tumor levels of PD-L1 expression by immunohistochemistry using the Ventana SP142 assay (6). Immune cell (IC) PD-L1 status was grouped into IC0 ($<$1%), IC1 ($\geq$1% but $<$5%) and IC2/3 ($\geq$5%). Over 26% of patients with PD-L1 positive tumor TIL experienced an anti-tumor response compared with 9.5% negative for PD-L1 supporting need for PD-L1 screening.

Avelumab, a fully human IgG_1 antibody with *retained* potential to induce ADCC, was first approved for the treatment of Merkel cell carcinoma (MCC) (7), an aggressive cancer associated with polyomavirus infection with poor prognosis, ineffective chemotherapeutic options and low survival compared with other skin cancers. Avelumab therapy increased significantly overall survival, progression-free survival and durability of response compared with chemotherapy (7). The efficacy of avelumab was independent of PD-L1 tumor expression or polyomavirus infection (7).

Durvalumab, a fully human IgG1 engineered to reduce ADCC or CDC, was first approved for the treatment of urothelial

carcinoma followed by approval for stage III unresectable NSCLC (8, 86).

For HNSCC, all three anti-PD-L1 antibodies are currently in clinical trials and in nearly all cases they are being combined with other experimental or established therapies.

COMBINATION THERAPY

Although all of the current six CI therapies can be used as single agent therapeutics, the emphasis now is on identifying combinations of CI therapy or CI therapy with other traditional therapies that will increase both anti-tumor efficacy and patient response frequency. Since 2015, many combination therapies have increased patient responses compared with single CI therapies alone. In metastatic melanoma, combination of nivolumab and ipilimumab (87) were significantly more effective in generating productive shrinkage of tumors in a higher frequency of patients than either of the CI therapeutics alone. For HNSCC, there are >100 clinical trials registered and most of those are combination therapies (**Figures 4**, **5**). The predominant partner therapy for anti-PD-1 and PD-L1 antibody therapies is radiotherapy in either stereotactic body or intensity modulated forms, which is often further supported by established chemotherapy. Radiotherapy seems a particularly good partner for CI therapies, because it can expose tumor-associated neoantigens that in turn can induce the nascent effector T cell responses that develop under cover of checkpoint blockade (88, 89). With regard to surgery followed by radiotherapy and standard chemotherapy, there is an interesting dichotomy of how checkpoint inhibitors have been combined in current clinical trials. While one strategy is to administer either anti-PD-1 (pembrolizumab) or PD-L1 (durvalumab) antibodies in the weeks prior to resection, another strategy is to administer these checkpoint inhibitors just after surgery. Presumably, the strategy of administration prior to surgery is based on the notion that pre-treatment will prime an anti-tumor immunity to enable the immune system to clear any residual tumor cells missed during the surgical procedure. Anti-PD-L1 antibody, durvalumab, is notable for its entry into several HNSCC clinical trials together with tremelimumab, the anti-CTLA-4 mAb (see **Figure 5**).

Anti-PD-1 and PD-L1 antibodies are also in clinical trials in combination with a wide range of experimental treatments that can be broadly divided into therapies that either target and activate host immunity or target and impair tumor survival. Inhibitors that target the enzyme indoleamine 2,3 dioxygenase (IDO) (90, 91), which is used by regulatory immune cells to deplete tryptophan availability to effector T cells, are particularly notable in these experimental combination therapies. In a similar vein, Toll-like receptor agonists, receptive to nucleic acids are also well-represented. Recent evidence suggests the endoplasmic reticulum associated DNA sensor stimulator of interferon genes (STING) to be a key player in a pathway to sense cytosolic nucleic acids (92, 93) and reverse tumor immunosuppression (94). DC activation through the STING pathway can promote tumor rejection after conventional cancer therapies such as
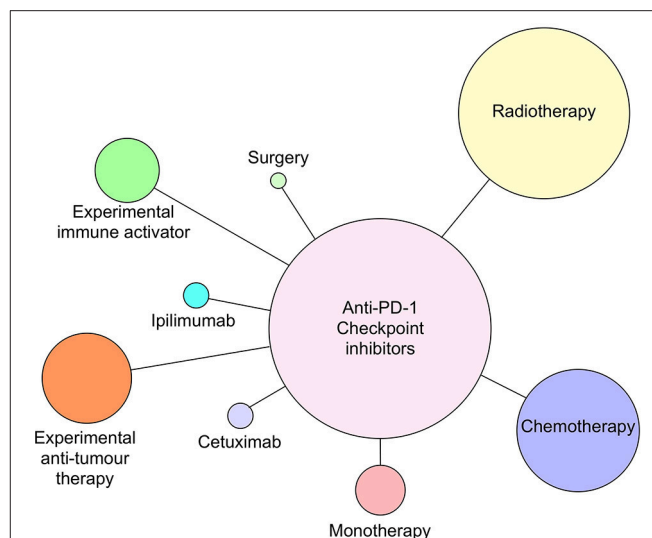
FIGURE 4 | Anti-PD-1 antibodies pembrolizumab and nivolumab currently in clinical trials as monotherapy or combined with radiotherapy, chemotherapy, cetuximab, ipilimumab (anti-CTLA-4), surgery, or novel immune activating or tumor suppressive therapies as of July 2018. Circle size reflects relative number of times each type of therapy has been combined with anti-PD-1 therapy.
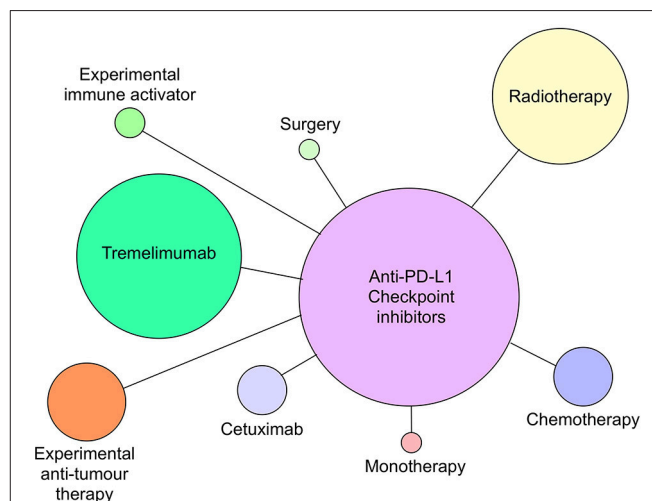
FIGURE 5 | Anti-PD-L1 antibodies atezolizumab, avelumab and durvalumab currently in clinical trials as monotherapy or combined with radiotherapy, chemotherapy, cetuximab, tremelimumab (anti-CTLA-4), surgery, or novel immune activating or tumor suppressive therapies as of July 2018. Circle size reflects relative number of times each type of therapy has been combined with anti-PD-L1 therapy.

radiation therapy (95). In preclinical studies, STING agonists have been shown to be effective alone or in combination with PD-1/PD-L1 blockade, particularly with established tumors that are refractory to checkpoint blockade alone (96, 97). Signalling cascade inhibitors, e.g., PI3K inhibitors (98, 99) or novel tumor associated peptides are examples of therapies that target tumor cells (100).

CI therapies in combination with other immune activating therapies offer the potential to increase response rates for a range of cancers including HNSCC, but there will also be intense focus on immune related adverse events, especially with regard to both bystander damage of otherwise healthy tissues and to local peritumoral tissues. This will be particularly important for HNSCC given the delicacy of many of the structures within and surrounding the oral cavity and oropharynx. Analysis of safety in patients with renal cell carcinoma that received different combination doses of nivolumab and ipilimumab indicated that the frequency of treatment related adverse effects were ubiquitous but manageable (101). Very few of the adverse events, however, were specific to the tumor site and were typically general, e.g., diarrhea and pyrexia (101).

OTHER CI THERAPIES

CTLA-4 and PD-1 are not the only immunoregulatory receptors associated with anti-tumor T cell immunity. There are several more checkpoint proteins under investigation, which may have direct therapeutic use or might be used to improve patient stratification and prognosis. These can be divided into two categories—immunosuppressive and immunostimulatory receptors with examples of the former being Lag-3, Tim3, TIGIT, BTLA and VISTA, and of the latter 4-1BB (CD137), OX40 (CD134), ICOS, and CD40. The expression of these receptors on tumor cells, myeloid, and lymphoid immune cells is variable and tumor dependent, and it is likely that some of them will find future therapeutic value as CI therapies (9).

MODIFYING CURRENT CI THERAPIES

The development of novel CI therapies over the next few years will continue to be an important focus and a critical aspect for future improvement is to fully elucidate how checkpoint inhibitor therapies are functioning at a molecular level. As a corollary to improving CI therapies, identifying mechanisms that will also condition T cells to respond consistently and effectively following CI therapy is also paramount. Much of this work involves identifying next generation vaccines or mechanisms to shape effector T cell phenotypes with potent anti-tumor activity. Both of these strategies will lead to higher patient response frequencies, better safety and hopefully an enduring immunity in most patients.

A generally less studied aspect of CI receptors is that of the functional effects that their soluble counterparts may have on therapeutic outcomes. These alternate receptor isoforms are either actively secreted by the cell or in some cases cleaved off the cell surface to exert their effects (102–105). Both CTLA-4 and PD-1 have soluble counterparts that are produced by alternative splicing of each gene during translation and are therefore under transcriptional control of the cell that expresses them. Soluble CTLA-4 (sCTLA-4) is produced from the omission of exon 3, encoding the transmembrane domain during alternative splicing of the CTLA-4 gene (46). In addition, a frame shift during splicing of exon 2 to 3 gives rise to a unique C terminal amino

acid sequence that replaces the cytoplasmic domain of the CTLA-4 receptor. The soluble isoform of PD-1 (sPD-1) is also produced by omission of exon 3 during alternative splicing. These soluble isoforms may be useful as response biomarkers for patients receiving CI therapy but may also impinge upon the therapy itself.

Soluble CTLA-4 is produced by Treg, but also resting T cells, monocytes, B cells and is capable of binding B7.1, B7.2 and B7-H2 (ICOS-L) on APC (106). This secretable isoform can also be produced by some non-immune cells such as pituitary cells (107). Analysis of several autoimmune diseases originally identified high serum levels of sCTLA-4 compared with healthy donors raising the notion that this isoform actively contributes in some way to immune regulation. Indeed, selective antibody blockade of sCTLA-4 enhanced antigen-specific T cell responses *in vitro* significantly increasing cell proliferation and effector cytokine production compared with isotype or anti-CTLA-4 antibodies. Further, in the diffuse B16F10 murine model of metastatic melanoma, selective blockade had reduced the number of tumor lesions comparably with conventional anti-CTLA-4 antibody treatment (106).

The CTLA-4 receptor exists on cell surfaces as a dimer but the dimerizing cysteine residue at position 122 of the receptor isoform is lost during alternative splicing, which has led to the assumption that sCTLA-4 is secreted as a monomer and therefore has less potency that its dimeric cell-bound counterpart. However, another cysteine is present in the C terminal unique amino acid sequence of sCTLA-4 raising the possibility that sCTLA-4 may be as functionally relevant as the receptor isoform in terms of immune regulation.

Does sCTLA-4, therefore, have any effect on current anti-CTLA-4 based CI therapy? A recent retrospective study of melanoma patient responses to ipilimumab CI therapy demonstrated that patients with relatively high serum levels of sCTLA-4 were more likely to respond to ipilimumab treatment compared to individuals with low or absent serum levels (108). Indeed, selective antibody blockade of sCTLA-4 enhanced antigen-specific T cell responses *in vitro* significantly increasing cell proliferation and effector cytokine production compared with isotype or anti-CTLA-4 antibodies (109). These studies suggest that measuring sCTLA-4 serum levels may be a useful biomarker to stratify patients most likely to respond to therapy and even hint that sCTLA-4 may form a target for therapy. Indeed, analyses of CTLA-4 and sCTLA-4 in cancer cells lines suggest that some tumors may use either or both isoforms as part of a previously overlooked immune evasion strategy (110). Several cancer cell lines and tumor sections have been identified to express CTLA-4 with some evidence that some may also be able to produce sCTLA-4 to suppress effector T cell responses (64). In a seminal analysis of primary melanoma cell lines, Laurent et al. identified some cell lines to express and secrete sCTLA-4 (110). Relatively high levels of sCTLA-4 could also be detected in the sera and pleural effusions of mesothelioma patients, suggesting that it may be contributing to some aspect of immune regulation (111). One hypothesis is that tumor cells or induced Treg

secrete sCTLA-4 within the local tumor milieu to suppress effector anti-tumor T cell responses. Even if sCTLA-4 has no functional activity at all, anti-CTLA-4 antibodies will bind sCTLA-4, which over time could reduce the amount of antibody available to target the receptor isoform. Although there is evidence of exosome production of PD-L1 as an immunosuppressive mechanism in HNSCC (112), the role that soluble isoforms of CI receptors play in HNSCC is still largely unexplored.

CONCLUSIONS

Checkpoint inhibitor antibodies for the treatment of HNSCC have demonstrated clear benefits in terms of patients' survival and durability of response but can also induce serious immune related adverse events coupled with an inability to consistently and accurately identify patients likely to respond to this type of therapy. The focus now must be on understanding the genetic signatures most likely to be associated with a productive response to CI therapy, while augmenting current therapies to improve their reliability. Soluble isoforms of CI receptors must also be factored to account for any immunoregulatory role or impact on current therapy that they might have.

AUTHOR CONTRIBUTIONS

All authors contributed areas of their expertise to this review. FW: CTLA-4 and sCTLA-4; LD: soluble isoforms in disease; RA-E: pathology and insights into anti-tumor immunity in HNSCC.

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Development of a Human Leukocyte Antigen Score to Predict Progression-Free Survival in Head and Neck Squamous Cell Carcinoma Patients

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Background: In personalized medicine and treatment stratification of head and neck squamous cell carcinoma (HNSCC), the heterogeneous genetic background of patients is not considered. Human leukocyte antigen (HLA) alleles and HLA haplotypes (HLA traits) are linked to development of HNSCC and affect progression-free survival (PFS) of HNSCC patients but most head and neck oncologists are not familiar with HLA typing. Hence, we developed an HLA-score abstracting from complexity of HLA-typing results to facilitate potential use of HLA-associated hazard ratios (HR) for prognostic stratification.

Methods: The HR for PFS of 8 HLA traits shown to be independent predictors (*Pi*) of PFS in a test cohort (TC) of 90 HNSCC patients were used to build the HLA-score based on the natural logarithm (ln) of the *Pi*-associated HR. Crude ln-transformed HR of the eight *Pi*, alleles B*13 (2), B*35 (1), B*51 (2), DQB1*06 (1), homozygous Cw (1), homozygous DRB4 (2), and haplotypes A*01/B*08 (−6) and B*08/C*07 (4), were summed up to yield the individual patient's HLA-score. Receiver operating characteristic (ROC) and Kaplan–Meier curves were used to proof the suitability of the HLA-score as prognostic marker for PFS. An independent validation cohort (iVC) of 32 patients treated in the larynx-organ preservation trial DeLOS-II was utilized for validation.

Results: The individual HLA-scores (range −2 to 6) in TC classified HNSCC patients regarding PFS. ROC analysis (area under the curve = 0.750, 95% CI 0.665–0.836; *P* = 0.0000034) demonstrated an optimum cutoff for the HLA-score at 0.5 (97.9% sensitivity, 34.7% specificity), and 70/90 patients in TC with HLA-score > 0 had significant reduced PFS (*P* = 0.001). Applying the same classifier (HLA-score > 0) confirmed these findings in the iVC revealing reduced PFS of 25/32 patients (*P* = 0.040).

Conclusion: HLA traits constitute critical *Pi*. Considering the HLA-score may potentially facilitate the use of genetic information from HLA typing for prognostic stratification, e.g., within clinical trials.

Keywords: head and neck squamous cell carcinoma, head and neck cancer, human leukocyte antigen, human leukocyte antigen haplotype, progression-free survival, larynx-organ preservation trial, biomarker score, independent predictor

INTRODUCTION

The genetic background of head and neck squamous cell carcinoma (HNSCC) patients and a potential genetic predisposition for development of HNSCC and relapse after successfully applied curative treatment are almost completely ignored. By contrast, somatic mutations, epigenetic changes and divergent gene-expression patterns in HNSCC and tumor-infiltrating lymphocytes present in the tumor are assessed as prognostic and predictive biomarkers (1–8). Main risk factors for carcinogenesis of HNSCC are tobacco and/or alcohol consumption, but the risk is modified by genetic polymorphisms (8–13). In addition, infection with oncogenic subtypes of the human papillomavirus (HPV), especially HPV16 and other high-risk subtypes, is etiologically involved in development of HNSCC of the oropharynx (14). HPV-related HNSCC are characterized by distinct molecular features (2–6, 8, 15). High level tobacco smoking, daily alcohol drinking as well as HPV-related carcinogenesis and especially their simultaneous presence are accompanied by immunotoxicity, genotoxicity, mutagenesis plus increased expression of cytokines and growth factors and exhausted immune response resulting in loss of proliferation control (16). Therefore, and because of the multitude of studies demonstrating the association of these risk factors with HNSCC and outcome, the reasons for developing HNSCC and also relapse after initially curative treatment appear to be obvious. Consequently, and in contrast to many other cancer entities, both occurrence and relapse of HNSCC are mostly seen as attributable to the patient's lifestyle. However, tobacco smoking and alcohol together explain only 73% of upper aerodigestive tract cancer incidence totally ranging from below 50% in HNSCC-affected women to about 85% in laryngeal and hypopharyngeal HNSCC in men (12). The risk for development of HNSCC is increased by genetic variants in genes encoding enzymes involved in DNA repair or metabolism of alcohol (9–11, 13); Fanconi anemia patients have a 700-fold increased risk for HNSCC and high rate of relapses (17, 18). Moreover, research implicated that polymorphisms in cytokine genes and members of the immunoglobulin supergene family including human leukocyte antigens (HLAs) are involved in either improved or impaired ability to control somatic mutations by adequate immune responses and maintenance of immune surveillance: we recently demonstrated in a German cohort of white Caucasian genetic descent that polymorphisms in HLA, in particular HLA-B antigens and homozygosity in HLA-Cw and DRB4, are associated with increased risk for HNSCC (19). Moreover, the highly frequent disruption of functionally coupled HLA antigens (haplotypes) and presence of uncommon haplotypes in a significant proportion of patients are linked not only to development of HNSCC but even more to reduced progression-free survival (PFS) independent of lifestyle-associated risk factors (19). By contrast, compared with healthy blood donors, some HLA traits are detected in HNSCC in lower frequency. The fewer carriers of such haplotypes have improved PFS, whereas those that are over-represented did not. Hence, genetic heterogeneity seems to account for altered risk of developing HNSCC but also PFS (19). The hazard ratios (HR) for PFS of B*13, B*35, B*51, HLA-DQB1*06, homozygous Cw and DRB4, and the haplotypes

A*01/B*08 and B*08/C*07 were stably significant independent predictors (Pi) in multivariate analyses (19). They also may be considered as prognostic factors in comparative analyses, e.g., in clinical trials.

However, the use of raw low-resolution tissue-typing results to assess the risk for PFS according to presence of a particular HLA trait and an individual risk attributed to any risk factor including Pi appears to be not very useful to estimate the individual patient's risk for relapse in clinical routine. For the latter purpose, an abstraction from individual polymorphisms could be helpful. Very desirable but not at hand is an easy-to-use way to assess the individual patient's risk attributable to his/her HLA type. As the aggregation of independent risk factors in a score offers a way to abstract from individual risk factors by summarizing only their (potential) impact as Pi (20), we had the hypothesis that HR for PFS may be useful to construct an HLA-score. Therefore, we newly defined an HLA-score based on published HR from our recent findings in a test cohort (TC) (19). Here, we aim to verify a potential impact of the scored HLA traits on PFS of HNSCC and to particularly elucidate, if this HLA-score reliably predicts outcome differences in the context of clinical trials. Hence, low-resolution HLA typing of leukocytes from an independent validation cohort (iVC), 32 laryngeal/hypopharyngeal HNSCC patients treated in the DeLOS-II larynx-organ preservation trial (20, 21), was performed. Related to their HLA-scoring the PFS in the iVC was analyzed and confirmed the prognostic value of the HLA-score.

MATERIALS AND METHODS

HNSCC Patients and Study Population

This study was carried out in accordance with the recommendations of the guidelines of the ethics committee of the Medical Faculty of the University Leipzig. The protocol was approved by the ethics committee of the Medical Faculty of the University Leipzig (vote no. 201-10-12072010 and no. 202-10-12072010). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Test Cohort

Blood samples were from histopathologic confirmed HNSCC patients (**Table 1**) of white Caucasian genetic descent diagnosed and treated between 08/2010 and 05/2011 at the ENT Department of the University Hospital Leipzig. 12 of the 90 patients in the TC were treated in the larynx-organ preservation trial DeLOS-II (21) (NCT00508664; advanced HNSCC of the hypopharynx or larynx receiving induction chemotherapy followed by radiotherapy ± cetuximab; n = 12). For HLA typing, genomic DNA was isolated using the salting out procedure (22) from leukocytes of blood samples. Low-resolution DNA-typing was performed using PCR-SSP for HLA-A, B, Cw, HLA-DRB1, DRB3/4/5, and DQB1 as described elsewhere (18).

Independent Validation Cohort

DNA samples from peripheral blood of additional 32 of the 52 LHSCC patients treated in the DeLOS-II trial in Leipzig

TABLE 1 | Main characteristics of the head and neck squamous cell carcinoma patients of the test cohort (TC; $N = 90$) and independent validation cohort (iVC; $N = 32$) investigated.

Covariate	Category	TC n	TC (%)	iVC n	iVC (%)	P value
Sex	Female	12	(13.3)	5	(15.6)	0.748
	Male	78	(86.7)	27	(84.4)	
Localization	Oropharynx	28	(31.1)	0	(0.0)	<0.001
	Other	62	(68.9)	32	(100.0)	
	Oropharynx	28	(31.1)	0	(0.0)	<0.001
	Hypopharynx	20	(22.2)	19	(59.4)	
	Larynx	24	(26.7)	13	(40.6)	
	Other	18	(20.0)	0	(0.0)	
T category	Tx	1	(1.1)	0	(0.0)	0.025
	T1	13	(14.4)	0	(0.0)	
	T2	21	(23.3)	3	(9.4)	
	T3	21	(23.3)	15	(46.9)	
	T4a	32	(35.6)	14	(43.8)	
	T4b	2	(2.2)	0	(0.0)	
N category	N0	32	(35.6)	3	(9.4)	0.010
	N1	7	(7.8)	1	(3.1)	
	N2a	5	(5.6)	0	(0.0)	
	N2b	22	(24.4)	13	(40.6)	
	N2c	9	(10.0)	14	(43.8)	
	N3	5	(5.6)	1	(3.1)	
N category	N0	32	(35.6)	3	(9.4)	0.005
	N+	58	(64.4)	29	(90.6)	
M category	M0	87	(96.7)	32	(100.0)	
	M1	3	(3.3)	0	(0.0)	
Stage	UICC I	8	(8.9)	0	(0.0)	0.055
	UICC II	11	(12.2)	0	(0.0)	
	UICC III	9	(10.0)	4	(12.5)	
	UICC IVA	53	(58.9)	27	(84.4)	
	UICC IVB	6	(6.7)	1	(3.1)	
	UICC IVC	3	(3.3)	0	(0.0)	
Stage	Early	19	(21.1)	0	(0.0)	0.006
	Advanced	71	(78.9)	32	(100.0)	
Human papillomavirus (HPV) status	High-risk HPV-DNA+	17	(18.9)	0	(0.0)	0.008
	High-risk HPV-DNA−	73		32	(100.0)	
	HPV16-DNA+	13	(14.4)	0	(0.0)	0.023
	HPV16-DNA−	77	(85.6)	32	(100.0)	
	HPV16-DNA + RNA+	8	(8.9)	0	(0.0)	0.081
	HPV16 RNA−	82	(91.1)	32	(100.0)	
Tobacco smoking behavior	Non-smoker	19	(21.1)	0	(0.0)	0.004
	Smoker	70	(77.8)	32	(100.0)	
	Missing	1	(1.1)	0	(0.0)	
	Non-smoker	19	(21.1)	0	(0.0)	0.046
	<10 pack years	2	(2.2)	2	(6.3)	
	10 < 20 pack years	5	(5.6)	5	(15.6)	
	20 < 30 pack years	8	(8.9)	4	(12.5)	
	30 < 40 pack years	26	(28.9)	9	(28.1)	
	≥40 pack years	29	(32.2)	12	(37.5)	
	Missing	1	(1.1)	0	(0.0)	
Alcohol consumption	No	9	(10.0)	1	(3.4)	0.218
	Yes	80	(88.9)	31	(96.6)	
	Missing	1	(1.1)	0	(0.0)	
Alcohol consumption category	0	10	(11.1)	1	(3.4)	0.455
	>0 < 30 g/day	31	(34.4)	11	(34.4)	
	30 < 60 g/day	24	(26.7)	8	(25.0)	
	≥60 g/day	24	(26.7)	12	(37.5)	
	Missing	1	(1.1)	0	(0.0)	

(Continued)

TABLE 1 | Continued

Covariate	Category	TC n	TC (%)	iVC n	iVC (%)	P value
Therapy	Surgery (Op)	19	(21.1)	0	(0.0)	<0.001
	Radiotherapy (RT)	6	(6.7)	0	(0.0)	
	Op + PORT	19	(21.1)	0	(0.0)	
	Op + PORChT	16	(17.8)	0	(0.0)	
	Primary concurrent RChT	3	(3.3)	0	(0.0)	
	DeLOS-II (IC + RT)	12	(13.3)	32	(100.0)	
	IC + Op + PORT	13	(14.4)	0	(0.0)	
	Best supportive care (BSC)	2	(2.2)	0	(0.0)	
Therapies applied	Monomodal	25	(27.8)	0	(0.0)	0.002
	Multimodal	63	(70.0)	32	(100.0)	
	BSC	2	(2.2)	0	(0.0)	
	Received Op	68	(75.6)	5	(15.6)	<0.001
	No	22	(24.4)	27	(84.4)	
	Received RT	69	(76.7)	32	(100.0)	0.003
	No	21	(23.3)	0	(0.0)	
	Received chemotherapy (ChT)	44	(48.9)	32	(100.0)	<0.001
	No	46	(51.1)	0	(0.0)	
	Received Op + RT	49	(54.4)	5	(15.6)	<0.001
	No	41	(45.6)	27	(84.4)	
	Received ChT + RT	44	(48.9)	32	(100.0)	<0.001
	No	46	(51.1)	0	(0.0)	
	Received Op + ChT + RT	30	(33.3)	5	(15.6)	0.057
	No	60	(66.7)	27	(84.4)	

(20, 21) underwent low-resolution HLA typing utilizing BMT OneLambda SSO-typing kits for HLA-A, B, Cw, DQ, and DR according to the manufacturer's instructions. Blood samples of eight DeLOS-II patients were not available as three patients were already deceased, two patients were reluctant to participate in the study and donate blood for genotyping, and three patients were lost to follow-up. Avoiding overlap with the 12 DeLOS-II patients in TC, the iVC consists of $N = 32$ independent samples. Presence of the eight HLA traits constituting Pi of PFS in the TC was assessed as described (19).

Development of the HLA-Score

The recently published HR of HLA traits detected in the TC that consistently remained significant Pi for PFS after bootstrapping with 1,000 iterations (19) were used to build the HLA-score. This data set from the TC (19) fulfils all prerequisites to classify the patients based on a score combining up to eight independent predictors ($n = 90 > 64 = 8^2$). The HR for each of the eight HLA traits was transformed into its natural logarithm, ln HR (**Table 2**). Absence of a genetic predictor scored 0, while its presence scored with the crude ln HR denoted in brackets: HLA-B*13 (2), HLA-B*35 (1), HLA-B*51 (2), HLA-DQB1*06 (1), homozygous HLA-Cw (1), homozygous HLA-DRB4 (2), haplotype A*01/B*08 (−6), haplotype B*08/C*07 (4). These scores were summed up to build the HLA-score of the individual patient.

Statistical Analysis

Pearson's chi-square (χ^2) test for contingency tables was used to analyze differences between groups. PFS was chosen as end point in survival analyses and measured from registration date until

TABLE 2 | Significant independent predictors for progression-free survival of head and neck squamous cell carcinoma patients according to published data from the multivariate Cox regression model (18) used to define the human leukocyte antigen (HLA) score.

Covariates	TC					Validation cohort			
	HR	(95% CI)	P value (2-sided)	P value (2-sided; in bootstrapping)[a]	n (%)	n (%)	P value (2-sided)[b]	ln HR	HLA-score
HLA-B*13	7.460	(2.212–25.16)	0.0012	0.025	11 (12.2)	4 (12.5)	0.967	2.010	**2**
HLA-B*35	2.630	(1.543–4.485)	0.0004	0.017	15 (16.7)	5 (15.6)	0.891	0.967	**1**
HLA-B*51	9.278	(2.270–37.92)	0.0019	0.015	8 (8.9)	4 (12.5)	0.556	2.228	**2**
HLA-DQB1*06	1.890	(1.152–3.101)	0.0117	0.037	40 (44.4)	10 (31.3)	0.192	0.637	**1**
Homozygous HLA-Cw	4.292	(1.864–9.888)	0.0006	0.001	27 (30.0)	10 (31.3)	0.895	1.457	**1**
Homozygous HLA-DRB4	9.513	(2.787–32.47)	0.0003	0.007	10 (11.1)	7 (21.9)	0.131	2.253	**2**
Haplotype A*01/B*08	0.003	(0.000–0.054)	0.000056	0.003	11 (12.2)	3 (9.4)	0.664	−5.809	**−6**
Haplotype B*08/C*07	74.856	(12.58–445.4)	0.000002	0.001	16 (17.8)	5 (15.6)	0.782	4.316	**4**

Hazard ratio (HR plus 95% CI in brackets) and the associated 2-sided P value, plus P values from bootstrapping applying 1,000 iterations are shown accompanied by their frequencies in the test cohort (TC) (N = 90) and independent validation cohort (N = 32).
The HLA-scores (highlighted bold) for the individual respective HLA trait are derived from the natural logarithm (ln)-transformed HR.
[a]Bootstrapping using 1,000 iterations.
[b]Comparison of frequencies in TC versus iVC using Pearson's χ² test.

date of either cancer-related death or relapse, censoring patients without any malignancy at last follow-up, or deaths not related to cancer. As relapse were considered local recurrence, lymph node or distant metastasis, or second primaries. PFS was analyzed using Kaplan–Meier curves (SPSS version 20, IBM Corporation, Armonk, NY, USA) applying log-rank tests (Mantel–Cox). Receiver operating characteristics (ROC) for PFS versus HLA-score were assessed using SPSS. P values < 0.05 from two-sided tests were considered significant.

RESULTS

Table 1 shows the characteristics of both cohorts. Some significant inequalities in risk-factor distributions were detected. According to the study protocol of the DeLOS-II trial all 32 LHSCC patients of the iVC were of advanced stage (UICC III, IV) with higher T and N categories. They were exclusively smokers and not HPV-related (P < 0.01), and based on the per-protocol treatment in DeLOS-II (20, 21) their treatment differed significantly from the TC (**Table 1**). Despite the lower case number in iVC, a comparable distribution and frequency of the HLA traits included in the HLA-score was observed and found being without any significant difference (**Table 2**).

The individual patient's HLA-score as defined by the sum of the crude ln-transformed HR of the eight HLA traits resulted in a comparable distribution of HLA-scores (P = 0.683). These scores were found in cohorts TC/iVC: −2 (n = 3/2; 3.3/6.3%), −1 (n = 5/1; 5.6/3.1%), 0 (n = 12/4; 13.3/12.5%), 1 (n = 29/6; 32.2/18.8%), 2 (n = 22/11; 24.4/34.4%), 3 (n = 9/4; 10/12.5%), 4 (n = 4/0; 4.4/0%), 5 (n = 3/2; 3.3/6.3%), and 6 (n = 3/2; 3.3/6.3%).

Figure 1A shows Kaplan–Meier analyses of PFS in the TC according to HLA-score quartiles. The PFS of HNSCC patients is inversely correlated with HLA-score quartiles. Applying the log-rank test, a significant different PFS of patients of the TC was observed (P < 0.00001).

Receiver operating characteristic analyses revealed a significant area under the curve (AUC) for PFS event versus HLA-score (AUC = 0.750, 95% CI 0.665–0.836; P = 0.0000034) with
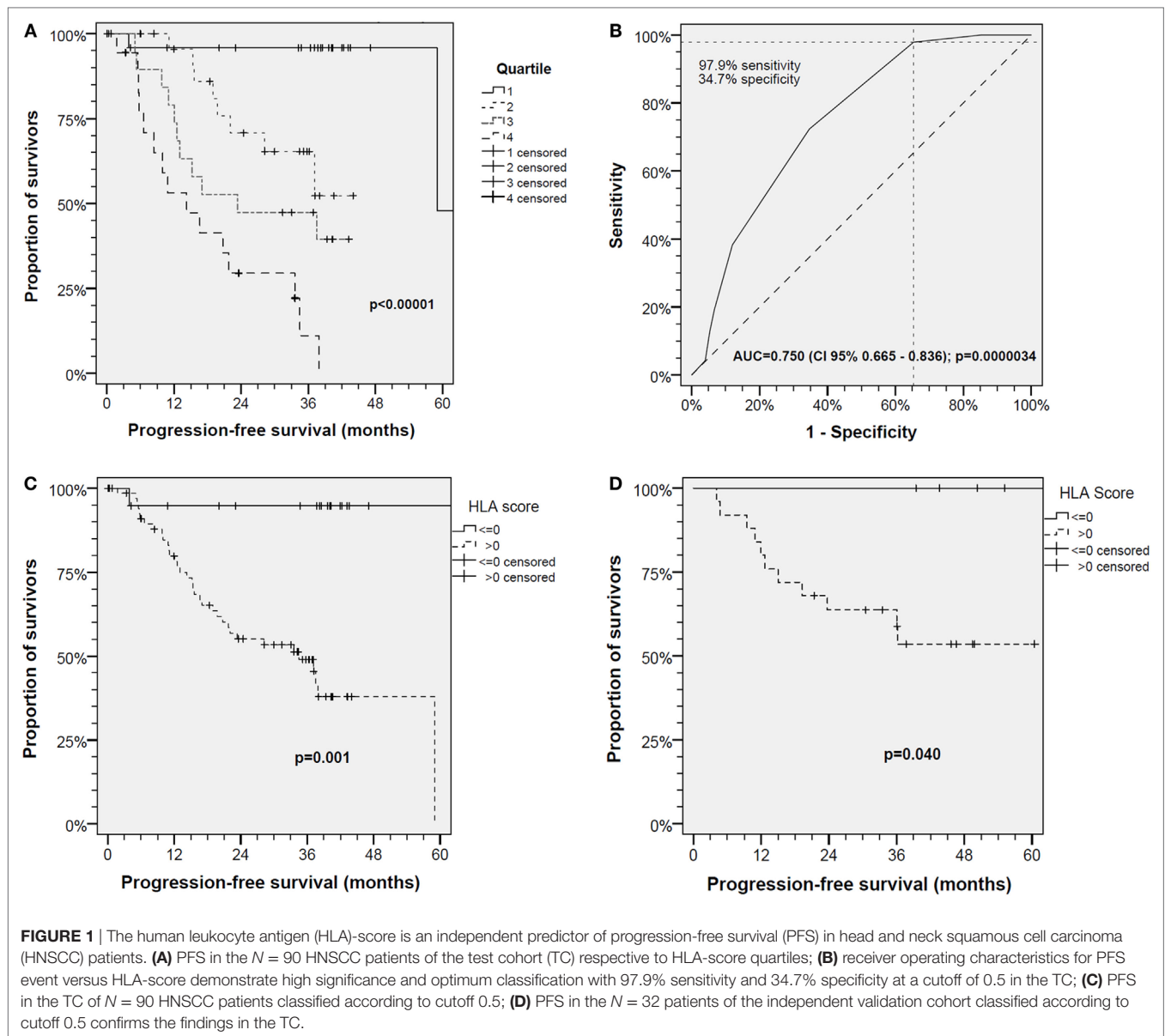
HLA-score 0.5 being the optimum cutoff for discrimination of HNSCC patients with good *versus* impaired PFS in the TC (**Figure 1B**). Binary classification of TC patients applying this cutoff offers 34.7% specificity and 97.9% sensitivity corresponding to a negative predictive value (NPV) of 94.7% of the HLA-score ≤ 0 for relapse or cancer-related death.

Kaplan–Meier analyses confirmed the optimal binary classification into groups of patients without HLA-attributable risk (HLA-score ≤ 0; n = 20) versus those at risk (HLA-score > 0; n = 70) and achieved in the TC the most significant discrimination between groups with deviating PFS (P < 0.001; **Figure 1C**).

The impact of the HLA-score on PFS was further analyzed in the iVC. The HLA-score ≤ 0.5 had in this cohort an NPV of 100%. In full agreement, and despite the smaller sample size of N = 32, the HLA-score ≤ 0 and >0 exactly predicted in Kaplan–Meier curves either prolonged or shortened PFS, respectively, of the iVC patients (P = 0.040; **Figure 1D**).

DISCUSSION

The TC of 90 HNSCC patients demonstrated altered frequencies of HLA antigens and two-locus haplotypes, as well as high frequent homozygosis in Cw and DRB4. The eight HLA traits identified as stable *Pi* respective to a significant impact on PFS of HNSCC patients can be combined to build an HLA-score. As shown here for the first time, the HLA-score of an HNSCC patient, which is the sum of crude ln-transformed HR of the eight HLA traits, is inversely correlated with the PFS and is a *Pi*. Multivariate analysis in the TC revealed significant altered PFS in carriers of homozygous Cw and DRB4, four HLA-B alleles, and two haplotypes (19). The in Wichmann et al., 2017 (19) applied Cox model (due to inclusion of HLA traits) no longer included N category N0 versus N+ (P = 0.520), alcohol consumption (P = 0.541), sex (P = 0.118), and age at diagnosis (P = 0.253), as these covariates lowered its overall significance (19). The here newly established HLA-score defines groups of HNSCC patients with significant different PFS independent from these "classical" risk factors for HNSCC (19). This may be seen also in the context

FIGURE 1 | The human leukocyte antigen (HLA)-score is an independent predictor of progression-free survival (PFS) in head and neck squamous cell carcinoma (HNSCC) patients. **(A)** PFS in the $N = 90$ HNSCC patients of the test cohort (TC) respective to HLA-score quartiles; **(B)** receiver operating characteristics for PFS event versus HLA-score demonstrate high significance and optimum classification with 97.9% sensitivity and 34.7% specificity at a cutoff of 0.5 in the TC; **(C)** PFS in the TC of $N = 90$ HNSCC patients classified according to cutoff 0.5; **(D)** PFS in the $N = 32$ patients of the independent validation cohort classified according to cutoff 0.5 confirms the findings in the TC.

of unexplained risk for development of HNSCC outside the main risk factors tobacco and alcohol (12).

The cutoff 0.5 allowed for discrimination of patient groups with different PFS according to their HLA-score also in the iVC. Patients with HLA-score > 0 had a significant higher risk for relapse ($P = 0.040$) compared with patients with HLA-score ≤ 0 who were without event and confirmed the findings in the TC (**Figure 1D**). This suggests that the HLA-score is potentially able to summarize the HR of HLA traits in a single measure that inversely correlates with the PFS and may be useful as stratification factor for clinical trials, observational studies or in personalized medicine.

Taken together, the results obtained by applying the HLA-score demonstrate the possibility that HLA traits are able to explain at least partially the high level of variance in outcome within clinical trials as demonstrated by the 32 patients of the iVC (**Figure 1D**)

treated in the DeLOS-II larynx-organ preservation trial (20, 21). The distribution of HLA traits between study arms therefore may affect the outcome in clinical trials. Frequencies of antigens and haplotypes shown to be *Pi* in the TC range between a few percent (4.4 up to 23.9%), and homozygosity in Cw and DRB4 was detected in 30.0 and 11.1%, respectively (19). As effective randomization regarding multiple risk factors each of them individually present in low frequency requires prohibitive high case numbers, unevenly distributed HLA traits could explain failure or irreproducibility of clinical trials even if higher case numbers are compared. This will occur as long as the multitude of low frequent *Pi* is not considered in stratification before randomization. The HLA-score may allow for overcoming this issue by assessment of the risk associated with particular HLA traits which are *Pi*.

What are the reasons behind the effect of HLA traits and the HLA-score on PFS of HNSCC? In HNSCC oncology, the

exposure to tobacco smoke and alcohol are the dominant and most accepted risk factors for development and relapse of HNSCC as they are observed in high frequency and shown to be causative for mutations, e.g., in oncogenes or inactivation of tumor-suppressor genes but also resistance to immune surveillance (23). Research showed a broad spectrum of mutated genes and affected signaling pathways in HNSCC (2–5, 8). However, most sporadic somatic mutations or viral infections potentially causing neoplastic transformation are controlled by the immune system. Consequently, altered peptides derived from mutated or viral proteins are in majority efficiently presented to CTL (CD8+ cytotoxic T lymphocytes) via HLA-A, B, and Cw enabling antigen-specific CTL to bind and delete cancerous cells expressing aberrant or viral proteins. Obviously, these mechanisms work well in most people but not so well in most HNSCC patients. This may be caused either by inadequate binding of T cell receptors to MHC:peptide complexes or incapability of the HLA-proteins to process tumor-associated antigens (TAA) by proteolytic cleavage and to bind particular TTA-derived peptides. Besides the often observed MHC class I loss in HNSCC (24, 25) allowing immune escape, there are indeed huge differences between certain HLA antigens to bind TAA-derived peptides (26). HLA antigens combined in particular haplotypes may have gaps in the capability to bind and present particular TAA-derived peptides. Consequently, the HLA antigens and haplotypes differ in the probability to appropriately activate T cells and get rid of cancer cells expressing peculiar proteins (27). Such impaired competence to maintain immune surveillance is suggested to be related to particular HLA alleles (and haplotype combinations) to efficiently bind and present altered peptides (26) and trigger deletion of the mutated cell by CTL (27). This may cause varying numbers of tumor-infiltrating CD8+ T cells within HNSCC as shown recently (5, 8, 28).

The HLA-score reliably predicts PFS of HNSCC patients. Even without any clinical information, the stratification using the HLA-score distinguished HNSCC with significant deviating PFS in TC and iVC (**Figure 1**). We detected significant superior outcome in patients with HLA-score ≤ 0 with strongly improved relative risk and odds ratios by optimum classification identified in ROC analyses (**Figure 1B**). It might be important that the HLA-score is able to indicate significant outcome differences even within the small sample of 32 patients in the iVC which moreover had a huge heterogeneity in many response-associated parameters (20). This is even more important as the case number of 32 is much too low to demonstrate significant outcome differences between LHSCC patients related to the treatment, e.g., arm A versus B (20).

Within our study, we noticed that sole presence of one of the eight *Pi* constituting the HLA-score not necessarily predicts the outcome regarding reduced PFS. This is in full agreement with the general explanations by Powers (29) who stated that the directions of implications are not in general dependent: if *Pi* is one of several independent possible causes of the condition *R* (i.e., PFS), *Pi* → *R* is strong, but *R* → *Pi* is in general weak for any specific *Pi*. If *Pi* is one of several contributing factors to the condition *R*, *Pi* → *R* is weak for any single *Pi*, but *R* → *Pi* is strong (29). Regarding our HLA-score, this means that each of the eight included independent predictors *Pi* for the condition *R* (event regarding PFS) alone is weak in explaining the outcome

(PFS), and presence of a particular *Pi* (either alleles HLA-B*13, B*35, B*51, DQB1*06, haplotypes B*08/Cw*07 and A*01/B*08, homozygous DRB4 or Cw) stands not against good outcome in general. *Vice versa*, the HLA-score ≤ 0 summarizing the eight *Pi* strongly predicts superior outcome, and the outcome explains the high predictive value of the HLA-score (**Figure 1**).

Our study has some limitations. The impossibility of familial HLA typing due to unavailability of DNA from parents of our patients allowed only for evaluation of estimated haplotypes (phenotypic combinations). However, this is the appropriate and most-often used method for analysis of HLA haplotypes and disease associations (30–32). The small sample size in the iVC might have caused reduced power to replicate the findings in the TC regarding particular HLA traits which are present in frequencies below 10%, e.g., HLA-B alleles and estimated haplotypes, on PFS. However, the abovementioned causality according to *Pi* → *R* applies (29).

Nevertheless, the recently detected HLA-trait dependence of PFS and the possibility to use the newly developed HLA-score requests further investigations to provide proof of reproducibility within cohorts of different genetic background. The possibility exists that the HLA-score can apply only to patients of white Caucasian genetic descent as particular alleles/antigens may behave in a different way when present in a different genetic environment. Some similarities may exist (e.g., the role of homozygosity in particular of Cw respective to an impaired prognosis), but ethnicity-dependent differences in distribution of particular HLA antigens/alleles and the association of varying haplotypes, e.g., with autoimmune diseases suggest the existence of such inequalities. They might have been at least partially responsible for unresolved issues of varying outcome especially observed in multinational clinical trials. For instance, in the SPECTRUM trial a huge difference was seen outcome of patients treated in Europe and in the U.S. of America compared with patients from the Asian-Pacific region (33) which we expected being at least partially related to genetic heterogeneity in HLA traits of the ethnicities. Therefore, it would be very welcome if HLA typing at least at the low-resolution level is performed within clinical trials including HNSCC patients of other ethnicities to elucidate HLA traits with the potential to differently affect outcome. This should clarify if patient stratification according to HLA traits is possible in multinational trials and if the here presented HLA-score is able to improve reproducibility in future clinical trials.

The identification of a subgroup of patients based on the HLA-score in both cohorts with uniquely superior PFS argues for consideration of HLA traits as stratification factors in head and neck oncology.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study are available on request from GW, gunnar.wichmann@medizin.uni-leipzig.de.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the guidelines of the ethics committee of the Medical

Faculty of the University Leipzig. The protocol was approved by the ethics committee of the Medical Faculty of the University Leipzig (vote no. 201-10-12072010 and no. 202-10-12072010). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

GW designed and coordinated the study. CH, CL, SW, MH, AD, and GW sampled biological specimen from HNSCC patients. Clinical data were provided by SW, MH, AD, and GW. CH, CL, and GW performed HLA typing. CH and GW assessed antigen and haplotype distribution. GW performed statistical analyses and developed the HLA-score. MK and GW discussed statistical models and interpreted the data. GW wrote the first version of the paper. All the authors approved the report.

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Deciphering the Role of Regulatory CD4 T Cells in Oral and Oropharyngeal Cancer: A Systematic Review

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Background: Recruiting regulatory CD4 T cells (Tregs) into the tumor microenvironment is an important tumor escape mechanism. Diminishing these suppressive cells is therefore one of the targets of cancer immunotherapy. Selective depletion of Tregs has proven successful in enhancing anti-tumor immunity and therapeutic efficacy in multiple tumor types. However, the role of Tregs in oral/oropharyngeal cancers is unclear with conflicting evidence regarding the effect of these suppressive cells on tumor prognosis. In this study, we sought to review the role of Tregs in oral/oropharyngeal cancer with the aim of deciphering the controversy regarding their effect on tumor progression and prognosis.

Methods: A systematic review of the literature pertaining to the role of Tregs in oral/oropharyngeal cancer was performed using Scopus, Embase, and PubMed. Forty-five records were deemed eligible and data describing methodology of Treg detection, tumor type, and association with prognosis were extracted.

Results: Of the 45 eligible manuscripts accepted for this systematic review, thirty-nine studies reported data from human subjects while the remaining studies focused on animal models. Sixteen studies were carried out using peripheral blood samples, while samples from the tumor site were analyzed in 18 studies and 11 studies assessed both blood and tumor samples. The transcriptional factor, Foxp3, was the most commonly used marker for Treg identification (38/45). The findings of 25 studies suggested that an increase in Tregs in the tumor microenvironment and/or peripheral blood was associated with poorer prognosis. These conclusions were attributed to the suppression of immune responses and the consequent tumor progression. Conversely, nine studies showed an increase in Tregs in peripheral blood and/or tumor microenvironment was related to a favorable prognosis, particularly in the presence of human papilloma virus (HPV), the status of which was only assessed in 11 studies.

Conclusions: This review underlines the importance of host immunity in the behavior of oral/oropharyngeal cancer. Furthermore, we report an apparent lack of clarity regarding the true role Tregs play in oral/oropharyngeal cancer progression which could be attributed to inconsistent detection techniques of Tregs. Our results therefore highlight the need for clearer methodologies and more robust phenotyping when defining Tregs.

Keywords: regulatory T cells, oral cancer, oropharyngeal cancer, patient outcome, tumor microenvironment

INTRODUCTION

Head and neck cancer is the sixth most common malignancy with an estimated 686,000 new cases and 375,000 deaths reported annually (combined worldwide laryngeal, oral, and pharyngeal cancer incidence) (1). The majority of head and neck cancers are squamous cell carcinoma (SCC). Along with alcohol consumption, smoking and various forms of betel quid chewing [which have long been associated with the development of oral and oropharyngeal squamous cell carcinoma (OPSCC)], it is now recognized that human papilloma virus (HPV) infection plays an important role in the onset of HPV positive OPSCC (2).

Despite advances in diagnosis and treatment, OPSCC mortality rate has improved little over the years, with 5 year survival rates as low as 53% reported in England for cancers of the oral cavity (3). This is mainly attributed to late diagnosis and the absence of predictors of disease progression in oral premalignant lesions.

Recently a growing emphasis is being placed on the role of the immune system and its association with the occurrence and progression of cancer. Indeed, cancer immunotherapy is among the most important developments in cancer treatment. It was therefore not surprising that cancer immunotherapy was named the scientific breakthrough of the year in 2013 (4). Despite the impressive successes in cancer immunotherapy, the response in patients is sometimes short lived. This is due to factors that hamper the immune response against cancer such as the presence of the suppressive regulatory CD4 T cells (Tregs) in the tumor microenvironment (5).

Tregs are a subpopulation of CD4$^+$ T lymphocytes which are capable of discerning self-antigens from non-self-antigens and suppressing the expansion of effector cells directed against self. The major subpopulations of Tregs include thymus-derived Tregs (tTregs), Tregs which have been induced peripherally by different cytokines (pTregs), and induced Tregs which are induced *in vitro* in the lab, (iTregs). All Treg types maintain regulatory functions, and their development and function are thought to be dependent on the expression of the transcription factor Forkhead box P3 (FoxP3), known as the "master regulator" of Treg regulatory functions (6, 7).

Within the tumor microenvironment, Tregs have an opposing action to cytotoxic CD8 T cells (8), and reducing the number of Tregs was found to reinvigorate anti-tumor immunity and promote tumor regression in different types of cancer (9–14).

The role of Tregs in oral/oropharyngeal cancer is not fully understood and different studies have reported conflicting evidence regarding the role of Tregs in oral/oropharyngeal

cancer progression and prognosis. Some studies emphasized the suppressive role of Tregs within the tumor microenvironment or the periphery, thus negatively impacting the patient clinical outcome (15–20), others reported a positive clinical outcome associated with an increase in circulating or tumor infiltrating Treg (21–29).

It is therefore important to fully comprehend the causes of these contradictions to enable full understanding of the role that Tregs play in oral/oropharyngeal cancer. This will enable designing novel immune-therapeutics that optimize the anti-tumor immune response and ultimately clinical outcome.

In this study, we sought to review the role of Tregs in oral/oropharyngeal cancer with the aim of deciphering the controversy regarding their effect on tumor progression and patient prognosis.

METHODS

We conducted and reported this systematic review following the PRISMA statement (30).

Search Strategy

A systematic search of PubMed, Embase, and Scopus (from their commencements to May 2017 when the search was performed), for studies in the English language with no species restrictions and for studies related to the role of Tregs in oral and oropharyngeal cancer. The following keywords were used in searching: ("head and neck cancer" or "head and neck malignancy" or "oropharyngeal") and ("epithelial dysplasia" or "oropharyngeal premalignancy") and ("tumor microenvironment" or "cancer immunology" or "tumor infiltrating lymphocytes" or "TILs" or "circulating immune cells" or "peripheral immune cells") and ("regulatory T cells" or "regulatory T lymphocyte" or "regulatory CD4 T lymphocyte" or "regulatory CD4 T cells" or "Treg" or "Tregs" or "Foxp3+ or CD4+Foxp3+" or "CD25+" or "CD4+CD25+" or "suppressive immune cells" or "suppressive lymphocytes").

We scrutinized the reference lists of the identified reports, reviews, meta-analyses, and other relevant publications to find additional pertinent studies. The "related articles" function was also used to broaden the search.

Our inclusion criteria were:

1- Studies must have been published as original articles
2- Studies must have been published in English
3- Studies assessing the role of Tregs in oral and oropharyngeal cancer.

Our exclusion criteria were:

1- Letters to the editor, conference abstracts, review, and systemic review articles
2- Studies that focused on thyroid, laryngeal, esophageal, and salivary gland tumors.

Data Extraction

The studies which met the inclusion criteria were summarized and data extraction was performed using a pre-defined form by one of the authors (CO) and accuracy checks were performed on over 75% of the manuscripts by (RA). Data extracted included: author, journal, year of publication, sample size, tumor type, tumor site, species, whether blood or tumor sample were used, method of sample analysis, markers used to detect Tregs, role of Tregs in tumor progression/prognosis, HPV status, correlations between HPV status and Tregs, tTregs vs. pTregs, and any data related to oral epithelial dysplasia.

Due to the huge variation in the study designs, the number of samples, the tumor site, and the method for detecting Tregs within tumor or blood samples, meta analyses of the results were not possible.

RESULTS

Manuscripts Included in the Systematic Review

Of 715 identified citations, we identified 54 articles which met the inclusion criteria. Following full text screening, 45 articles were deemed to be eligible for inclusion in this study. Reasons for exclusion included irrelevant manuscripts which did not tackle the role of Tregs in oral and oropharyngeal

cancer ($n = 478$), manuscripts that focused on tumors other than oral or oropharyngeal; laryngeal/esophageal ($n = 82$), salivary gland ($n = 44$), thyroid gland ($n = 32$) or gastric tumors ($n = 18$), review articles ($n = 13$), one study looked at the role of Tregs in periodontal disease and two articles were excluded because they assessed the expression of Foxp3 in tumor cells rather than assessing Tregs. **Figure 1** shows the flow diagram of the studies retrieved for this systematic review.

Data Summary

The full characteristics of the study populations are displayed in **Table 1**.

Tumors

The majority of the studies [$n = 39$ (86.7%)] assessed human samples (15–18, 20–29, 31, 33, 36–40, 42–51, 54–60), one study assessed both human and murine samples (32), two studies looked at murine samples (19, 34), one at rat (41), and two at canine samples (52, 53). With the exception of two studies that looked into multiple myeloma in canines (52, 53), all studies focused on oral and/or oropharyngeal SCC [$n = 43$ (95.5%)]. Within the 45 studies, the site of the tumor varied and included tumors of the oral cavity (tongue, floor of the mouth, base of the tongue, gingiva), oropharynx, hypopharynx, lower lip, tonsil, epipharynx, and lymph node metastasis. Three studies used head and neck cancer cell lines (19, 40, 58).

Table 2 details the methodologies used to detect Tregs, the markers used and the changes in Tregs observed with treatment and with disease progression. **Table 2** also summarizes the suggested role of Tregs in oral and oropharyngeal cancer for each included manuscript.
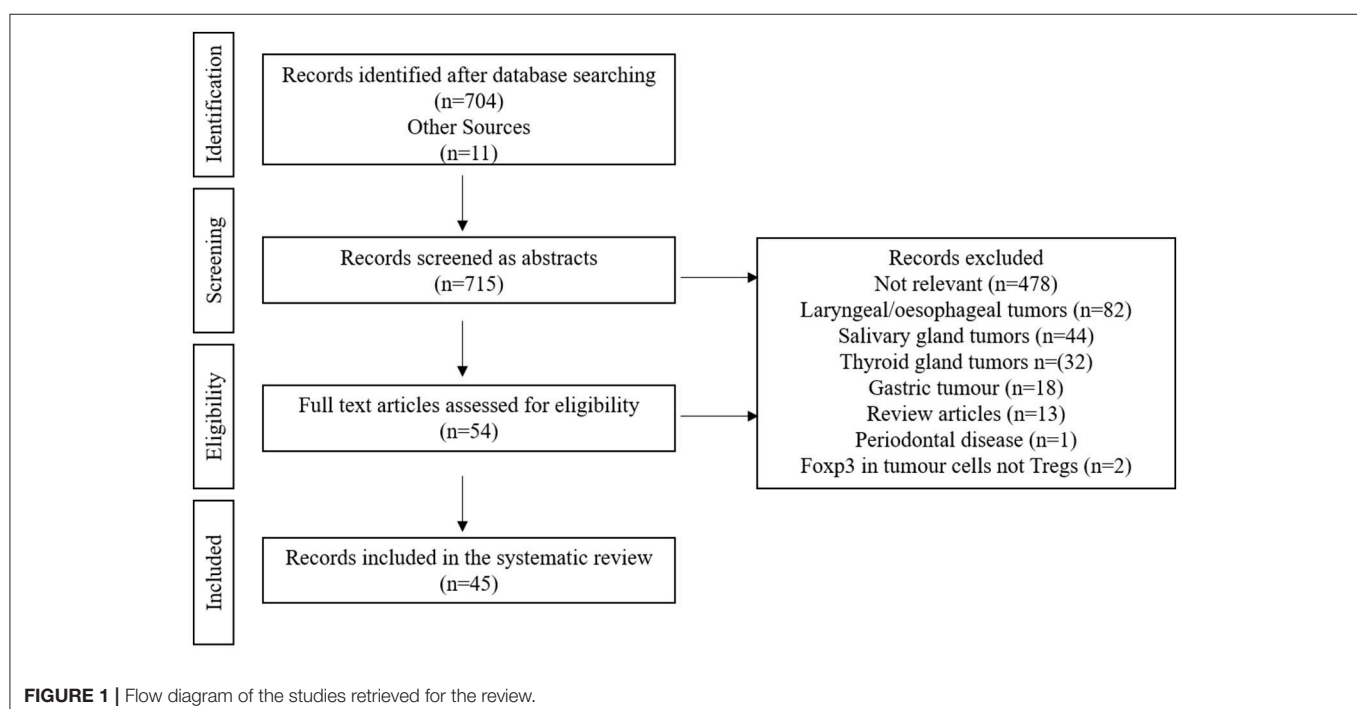

FIGURE 1 | Flow diagram of the studies retrieved for the review.

TABLE 1 | Basic information about the 45 studies that met the inclusion criteria for this systematic review.

References	Tumor type	Tumor site	Number of samples	Sample	Species
Stasikowska-Kanicka et al. (20)	SCC	Oral cavity (floor of the mouth)	78 Patients (41 poor prognosis, 37 better prognosis) 18 Controls (normal mucosa)	Tumor	Human
Hussaini et al. (31)	SCC	Oral cavity	25 Patients 12 Controls (inflammatory hyperplastic tissue)	Tumor	Human
Ihara et al. (17)	SCC	Oral cavity, oropharynx, nasopharynx, larynx, maxillary sinus	46 SCC 23 Controls	Blood	Human
Ma et al. (32)	SCC	HNC	**Human samples:** 43 Normal 48 Dysplastic 165 Primary HNSCC 12 Recurrent HNSCC 17 HNSCC with induction chemotherapy **Murine samples:** 6 WT normal tongue 6 WT tumor bearing mice 6 KO tumor bearing mice	Tumor	Human/Murine
Zhou et al. (33)	SCC	Tongue	46 SCC 46 Paired tumor adjacent non-neoplastic tongue epithelium 20 Metastasis lymph nodes 20 Paired normal cervical lymph nodes	Tumor	Human
Nguyen et al. (25)	SCC	Larynx, oral cavity, oropharynx, hypopharynx	278 SCC	Tumor	Human
Miki et al. (34)	SCC	Tongue	20 Controls 20 4NQO 40 4NQO treated with COX-2 inhibitor	Tumor	Murine
da Cunha Filho et al. (35)	SCC	Low lip	50 Patients, 10 microscopic fields per patient	Tumor	Human
Montler et al. (36)	SCC	Base of the tongue, tonsil, oropharynx, nasal, oral tongue, mandibular gingiva, maxillary sinus, larynx, floor of the mouth	29 Patients	Tumor blood	Human
Takahashi et al. (37)	SCC	Oral cavity, oropharynx, hypopharynx, larynx, paranasal cavity	20 Healthy controls 44 Patients treated with surgery/radio/radio-chemotherapy 16 Chemotherapy	Blood	Human
Jie et al. (18)	SCC	Oral cavity, oropharynx, larynx, hypopharynx	22 Patients treated with cetuximab plus cisplatin/paclitaxel/radiotherapy followed by 6 months of maintenance single agent cetuximab 18 Patients received single-agent cetuximab	Tumor blood	Human
Partlova et al. (38)	SCC	Tongue, tonsil, larynx, verbal base, hypopharynx, Gl. submandibularis, floor of mouth	54 Patients	Tumor blood	Human
Wolf et al. (29)	SCC	Oral cavity: tongue, upper alveolus, floor of mouth, hard palate, buccal mucosa, and retromolar	39 Patients	Blood	Human
Sun et al. (39)	SCC	Oral cavity, hypopharynx, nasopharynx, oropharynx, larynx	112 Patients 31 Healthy donors	Blood	Human
Schipmann et al. (40)	SCC	Oral cavity and skin	**FOXP3 mRNA expression:** 13 Cutaneous cSCC 8 Oral SCC 14 SCC metastases **Immunohistochemistry:** 10 Cutaneous SCC 8 Oral SCC 4 SCC metastases 10 Normal skin control **Cell lines:** Primary human adult skin fibroblasts Human squamous cell carcinoma cell line	Tumor	Human

(Continued)

TABLE 1 | Continued

References	Tumor type	Tumor site	Number of samples	Sample	Species
Lim et al. (23)	SCC	Oral cavity	39 Patients 24 Controls	Blood	Human
Hanakawa et al. (16)	SCC	Tongue	34 Patients	Tumor	Human
Ward et al. (28)	SCC	Oropharynx	149 HPV+ 121 HPV–	Tumor	Human
Lukesova et al. (24)	SCC	Oral cavity, oropharynx	60 Patients	Blood	Human
Zhao et al. (41)	SCC	Tongue	16 Controls 32 4NQO	Tumor blood	Rat
Jie et al. (42)	SCC	Oral cavity, oropharynx	27 Patients	Tumor blood	Human
Park et al. (26)	SCC	Tonsil	79 Patients	Tumor	Human
Weed et al. (43)	SCC	Tongue	49 Patients	Tumor	Human
Drennan et al. (44)	SCC	Oropharynx, larynx	14 Controls 39 Patients	Blood	Human
Bron et al. (22)	SCC	Oral cavity, oropharynx, hypopharynx, larynx	35 Patients	Tumor blood	Human
Judd et al. (19)	SCC	Oral cancer cell line injected in flank	Not disclosed in manuscript	Tumor	Murine
Gaur et al. (45)	SCC	Oral cavity	45 Patients 40 Controls	Blood	Human
Wansom et al. (27)	SCC	Oropharynx	46 Patients	Tumor blood	Human
Wild et al. (46)	SCC	Oral cavity, pharynx, larynx	35 Patients 17 Controls	Tumor blood	Human
Näsman et al. (47)	SCC	Tonsil	31 HPV+ with a good clinical outcome 21 HPV+ with a poor clinical outcome 11 HPV– with a good clinical outcome 20 HPV– with a poor clinical outcome	Tumor	Human
Lee et al. (48)	SCC	Oral cavity	38 Patients 5 Controls	Tumor blood	Human
Schuler et al. (49)	SSC	Oral cavity, pharynx, larynx	9 Patient samples for dendritic cell culture 13 Patient samples for Treg frequency	Blood	Human
Alhamarneh et al. (50)	SCC	Larynx, oropharynx, oral cavity, hypopharynx, nasal cavity, lymph node metastasis, unknown primary site	107 Patients pretreatment 43 4–6 weeks posttreatment 40 Controls	Blood	Human
Al-Qahtani et al. (51)	SCC	Oral cavity	34 Patients	Tumor	Human
Tominaga et al. (52)	MM	Oral cavity	7 Patients 10 Controls	Tumor Blood	Canine
Horiuchi et al. (53)	MM	Oral cavity	15 Patients 10 Controls	Blood	Canine
Schott et al. (54)	SCC	Epipharynx, oropharynx, hypopharynx, larynx, oral cavity	16 Patients with active disease 16 Patients with no evidence of disease 21 Controls	Blood	Human
Gasparoto et al. (55)	SCC	Oral cavity, lip	9 Patients 10 Controls	Tumor Blood	Human
Boucek et al. (15)	SCC	Oral cavity, hypopharynx, larynx	112 Patients 20 Controls	Blood	Human
Distel et al. (56)	SCC	Oral cavity, hypopharynx, oropharynx	62 Low-risk group patients with early disease 53 High-risk group inoperable patients with advanced disease	Tumor	Human
Schwarz et al. (57)	SCC	Oral cavity	15 Patients 15 Controls	Tumor	Human

(Continued)

TABLE 1 | Continued

References	Tumor type	Tumor site	Number of samples	Sample	Species
Bergmann et al. (58)	SCC	HNSCC cell lines from primary tumors	Cell culture of irradiated HNSCC cell lines from primary tumors with blood samples from 10 healthy donors	Blood	Human
Chikamatsu et al. (59)	SCC	Oral cavity, oropharynx, hypopharynx, larynx, paranasal sinuses	43 Patients 24 Controls	Blood	Human
Badoual et al. (21)	SCC	Oral cavity, oropharynx, hypopharynx	84 Patients	Tumor	Human
Schaefer et al. (60)	SCC	Larynx, oral cavity, pharynx, hypopharynx	24 Patients 17 Controls	Blood	Human

SCC, Squamous Cell Carcinoma; MM, Multiple Myeloma; HNC, Head and Neck Cancer; HNSCC, Head and Neck Squamous Cell Carcinoma.

Samples and Treg Analyses

Eighteen studies assessed tumor samples (16, 19–21, 25, 26, 28, 31–35, 40, 43, 47, 51, 56, 57), 16 assessed blood samples (15, 17, 23, 24, 29, 37, 39, 44, 45, 49, 50, 53, 54, 58–60), and 11 studies assessed both tumor and blood samples (18, 22, 27, 36, 38, 41, 42, 46, 48, 52, 55).

With regards to the methodologies used to detect and assess Tregs, immunohistochemistry was used in 21 studies (16, 20, 22, 24–29, 31–36, 40, 47, 48, 51, 56, 57), flow cytometry in 25 studies (15, 17–19, 23, 24, 32, 36–39, 41, 42, 44–46, 48–50, 52–55, 58–60), Immunofluorescence in six studies (21, 31, 32, 43, 46, 52), PCR in six studies (24, 34, 38, 40, 46, 48), ELISA in four studies (23, 46, 50, 58), Histopathology and morphology in three studies (20, 34, 35), and Western blots in two studies (32, 58). The majority of the studies (30 out of 45 studies) used only a single method for detecting Tregs (15–19, 21, 22, 25–29, 33, 37, 39, 41–45, 47, 49, 51, 53–57, 59, 60). The most common single method was flow cytometry (16 out of 30) (15, 17–19, 37, 39, 41, 42, 44, 45, 49, 53–55, 59, 60), followed by immunohistochemistry (12 out of 30) (16, 22, 25–29, 33, 47, 51, 56, 57) and immunofluorescence was used as a single method for Treg detection in two studies (21, 43). Eight out of the 45 studies used two methods of Treg assessment (20, 23, 31, 35, 36, 40, 50, 52) while seven studies used three or more methodologies (24, 32, 34, 38, 46, 48, 58).

As for the markers used to detect Tregs and assess their function, Foxp3 was the most commonly used marker, as it was used in 38 out of the 45 studies (16–22, 25–29, 31–36, 39–43, 45–58, 60). Foxp3 was the sole marker for Treg detection in 13 studies (16, 20, 22, 25, 27–29, 34, 35, 40, 47, 51, 56). Foxp3 in combination with T cell markers CD3, CD4, and/or CD25, was used as a marker in 14 studies (15, 19, 24, 26, 33, 41, 43, 45, 49, 52, 53, 57, 59). CD25 was used as a marker (on its own or with other markers) in 24 studies (15, 18, 21, 23, 24, 26, 36–39, 41, 42, 44–46, 48–50, 54, 55, 58–60). Seven studies identified Tregs using a combination of CD4+CD25+CD127^{low} with or without other markers (17, 23, 37, 38, 44, 46, 54). CTLA-4 was used as a marker of Treg phenotype or suppressive function in seven studies (18, 36, 42, 50, 54, 55, 58), GITR was assessed in four studies and as a marker of Treg function (50, 54, 55, 60), TGF-β was assessed in four studies (18, 42, 48, 55), and IL-10 was used in two studies (55, 58).

HPV Status

Only 11 manuscripts looked at the HPV status of the tumors (24–29, 32, 36, 38, 43, 47), and out of those, only 10 included HPV positive cases in their studies (24–29, 32, 36, 38, 47). Half these studies reported no difference in Treg levels between HPV positive and negative tumors (24, 27, 29, 32, 36). Two manuscripts reported a decrease in Treg proportion in HPV positive (28, 38) [one associated with an increase in TIL (28)], and three reported an increase in Treg associated with an overall increase in TIL (24, 25, 47). Four studies associated HPV positive tumors with better survival compared to HPV negative (24, 27, 28, 47). One study found no correlation between HPV status and survival (29). Three studies correlated an increase in Tregs in HPV positive tumors with better prognosis (24, 26, 28), however, one of the studies suggested that it was associated with the overall increase in TIL (28).

Correlation of Tregs With Clinical Outcome

Twenty-four studies reported a clear increase in Tregs (whether intratumoral or circulating) in cancer patients in comparison to healthy controls and/or in more advanced disease (15, 20, 23, 31–34, 37, 39–41, 44, 45, 48–55, 57, 59, 60). Only three studies reported a decrease in Tregs with more advanced disease (22, 29, 35).

Out of the 45 papers included in this study, 25 studies (55.6%) found a correlation between Tregs and poor clinical outcome and disease progression (15–20, 32, 33, 37, 39–42, 44–46, 48, 49, 51–55, 58, 59), nine manuscripts (20%) correlated Tregs to good clinical outcome (21–29), and 11 (24.4%) did not reach a conclusion regarding the role of Tregs in tumor progression (31, 34–36, 38, 43, 47, 50, 56, 57, 60).

No apparent correlation was found between the site of the tumor and the outcome. Only one study reported higher numbers of Tregs in Oral SCC lesions in comparison to oropharyngeal tumors (24).

With regards to the type of samples assessed for Tregs, interestingly, the majority of the studies that could not conclude the role of Tregs [7 out of 11 studies (63.6%)] looked only at tumor samples (31, 34, 35, 43, 47, 56, 57). Four of the studies that only assessed tumor samples showed an association between Tregs and good prognosis (21, 25, 26, 28), while seven showed association with poor prognosis and clinical

TABLE 2 | Method and markers used to detect Tregs, and reported role of Tregs in HNC.

References	Method for detecting Treg	Treg markers	Changes in Tregs observed with treatment	Changes in Tregs observed with disease progression	Suggested role of Tregs
Stasikowska-Kanicka et al. (20)	IHC, morphometry	Foxp3+	N/A	The mean number of Foxp3+ cells was significantly increased in poor prognosis group in comparison to the better prognosis and control groups	Higher mean numbers of Tregs associated with poorer prognosis
Hussaini et al. (31)	IHC, IF	FoxP3+, TLR2	N/A	Significantly more single-stained FoxP3+ cells and double-stained FoxP3+TLR2+ cells in the OSCC than in the control group	FoxP3+TLR2+ cells may represent dendritic cell dependent pathway of inhibiting Treg suppression. Exact role in disease progression not disclosed
Ihara et al. (17)	FC	CD4, CCR4, CD127^{low}, CD45RA-, Foxp3^{high}	Low frequency of CD45RA-Foxp3 High Tregs before treatment showed a better clinical outcome, even in patients with advanced stage tumors		High frequency of CD45RA-Foxp3^{high} Tregs correlated with a poor prognosis and recurrence
Ma et al. (32)	FC, IHC, IF, WB	CD4, Foxp3, A2AR	A2AR blockade reduces CD4+ Foxp3+ Tregs in HNSCC mouse model. A2AR blockade enhances the anti-tumor response of CD8+ T cells in HNSCC mouse model	A2AR was correlated with higher pathological grade and significantly correlated with Foxp3	A2AR blockade reduces CD4+ Foxp3+ Tregs in HNSCC mouse model and enhances the anti-tumor response of CD8+ T cells
Zhou et al. (33)	IHC	CD4, FoxP3	N/A	Increased number of Tregs in SCC and metastatic lymph nodes Tissue in comparison to adjacent tissues	Expression of Tregs in SCC lesions was inversely associated with overall survival and associated with worse prognosis
Nguyen et al. (25)	IHC	FoxP3	N/A	N/A	Higher levels of FoxP3 infiltrates were associated with improved overall survival but not for relapse free or disease specific outcomes
Miki et al. (34)	Histopath, RT-PCR, IHC	Foxp3	No difference in the number of Foxp3+ cells between the control group and the groups treated with the COX-2 inhibitor regardless of the dose of COX-2 inhibitor	Foxp3 expression in the tongues of mice treated with 4NQO was significantly higher than normal control group (weeks 15 and 20), but significantly decreased with tumor progression	The authors could not conclude the exact role of Tregs in SCC
da Cunha Filho et al. (35)	Histopath, IHC	FoxP3+	N/A	Decrease in FoxP3+ T Cells with more advanced lesions and lymph node metastasis	Tregs are probably involved in early stages of lip carcinogenesis. Exact role not concluded
Montier et al. (36)	IHC, FC	CD25, Foxp3, OX40, PD-1, CTLA-4	N/A	N/A	High expression of OX40, as well as CTLA-4 and PD-1 in the TIL Tregs. Role in SCC not concluded

(Continued)

TABLE 2 | Continued

References	Method for detecting Treg	Treg markers	Changes in Tregs observed with treatment	Changes in Tregs observed with disease progression	Suggested role of Tregs
Takahashi et al. (37)	FC	CD3, CD4, CD25, CD127^{low}	The proportion of Tregs decreased significantly at day 6 following treatment, but the activation marker increased at day 21	The proportion of Tregs was significantly higher in SCC patients compared to healthy donors	Chemotherapy can trigger a transient reduction of Tregs associated with an activation of CD8 T cells suggesting a tumor progressive role of Tregs in HNC
Jie et al. (18)	FC	CD4+, CD25^{hi}, Foxp3+, CTLA-4, TGF-β, CD39	Cetuximab significantly increased the frequency of intratumoral Treg expressing CTLA-4, CD39, and TGF-β. significant increase was only observed in circulating Treg expressing CTLA-4		The frequency of CTLA-4+ Treg were significantly increased among the non-responder patients
Partlova et al. (38)	FC, RT-PCR	CD4+, CD25+, CD127^{low}	N/A	N/A	No statistically significant differences were observed in the numbers and proportions of Tregs were observed between HPV+ and HPV– tumors. The role of Treg could not be concluded
Wolf et al. (29)	IHC	Foxp3	N/A	Levels of Tregs were higher in early stage cancers. Mean TIL levels for CD4, CD8, and FoxP3 cells were significantly correlated with each other and were higher in surviving patients	The findings suggest that Tregs are associated with better survival
Sun et al. (39)	FC	CD3, CD4, CD45RA-, Foxp3, CD25	N/A	Tregs increase in the peripheral circulation of HNSCC patients, and correlate with tumor stage and nodal status	The findings suggest a role for Treg in tumor progression
Schipmann et al. (40)	IHC, RT-PCR	Foxp3	N/A	Foxp3 expression much higher in SCC compared to normal controls	Oral and skin SCC recruit Tregs into the tumor microenvironment to suppress immunosurveillance
Lim et al. (23)	FC, ELISA	CD4+CD25^{hi}CD127^{low}	N/A	Tregs increased in SCC compared to normal controls	High levels of CD4+CD25^{high}CD127^{low} Tregs is associated with better survival
Hanakawa et al. (16)	IHC	Foxp3	N/A	N/A	High intraepithelial and stromal infiltration of Tregs correlated with significantly worse 5-year disease-free survival
Ward et al. (28)	IHC	Foxp3	N/A	The proportion of Foxp3+ cells was reduced in HPV+ compared with HPV– tumors	Tregs were associated with improved survival, but might be a reflection of the overall increase in TIL
Lukesova et al. (24)	FC, PCR, IHC	CD4, CD3, CD25	N/A	Higher numbers of Tregs in oral tumors than oropharyngeal tumors	High level of Tregs in blood is associated with better survival
Zhao et al. (41)	FC	CD4+ CD25+ FoxP3+	N/A	Tregs were significantly higher in OSCC than controls and increased with the progression of 4NQO-induced rat tongue carcinogenesis	The results of this study suggest a role for Tregs in tumor progression

(Continued)

TABLE 2 | Continued

References	Method for detecting Treg	Treg markers	Changes in Tregs observed with treatment	Changes in Tregs observed with disease progression	Suggested role of Tregs
Jie et al. (42)	FC	CD4+, CD25+, Foxp3+ TGF-β CD39 CTLA-4	N/A	Intratumoral Treg exhibited more suppressive activity than peripheral blood Treg	The findings of this study suggest a suppressive function associated with disease progression
Park et al. (26)	IHC	Foxp3+ CD25+	N/A	Foxp3 expression is associated positively with p16 expression, and is a favorable prognostic factor for overall survival	Tregs are up-regulated in HPV+ SCC and Foxp3 is related to a favorable prognosis
Weed et al. (43)	IF	CD4+, Foxp3+	N/A	Cytoplasmic Foxp3 is associated with a lower possibility of recurrence, while nuclear Foxp3 is associated with a higher possibility of recurrence	The overall expression of FoxP3 does not Correlate with Clinical Outcome. However, elevated number of TILs expressing Foxp3 in the cytoplasm are indicative of a favorable prognosis while TILs expressing nuclear Foxp3 are associated with recurrence
Drennan et al. (44)	FC	CD4+ CD25^{high} CD127^{low}	N/A	Level of peripheral Tregs increased with advanced tumor stage and lymph node involvement	The findings of this study suggest a role for Tregs in tumor progression
Bron et al. (22)	IHC	Foxp3	N/A	Treg more frequent in patients without lymph node involvement	High numbers of total FOXP3+ Tregs within the TIL were significantly associated with prolonged overall survival
Judd et al. (19)	FC	CD4+, Foxp3	Depletion of Tregs using anti-CD25 antibody resulted in a decrease in growth rate	N/A	Tregs contribute to the aggressive tumor growth in the studied model
Gaur et al. (45)	FC	CD4+ CD25+ Foxp3+	N/A	Increase in Th17/Tregs ratio in early stages and a decrease in this ratio in later stages due to a higher frequency of Tregs in later stages and in lymph node metastasis	The findings of this study suggest that Tregs are associated with more advanced disease and promote metastasis
Wansom et al. (27)	IHC	FOXP3	N/A	N/A	Higher levels of Tregs (Foxp3+) in TIL was associated with better disease specific and overall survival
Wild et al. (46)	FC, IF, RT-PCR, ELISA	CD4+, CD25+, FoxP3+, CD127^{low}, TLR4	N/A	HMGB1 promotes suppressive function of Treg in HNSCC patients	The findings of this study suggest a role for Tregs in immune escape and tumor progression
Näsman et al. (47)	IHC	Foxp3	N/A	Higher number of Foxp3+ TILs HPV+ compared to HPV- SCC. No difference in Treg levels between poor and good prognosis	Although a High CD8+/Foxp3+ Ratio is Linked to a Good Clinical Outcome, no diff in Treg levels was observed related to clinical outcomes, indicating that the better prognosis is attributed to the elevated CD8 Levels

(Continued)

TABLE 2 | Continued

References	Method for detecting Treg	Treg markers	Changes in Tregs observed with treatment	Changes in Tregs observed with disease progression	Suggested role of Tregs
Lee et al. (48)	FC, IHC, RT-PCR	Foxp3, CD4, CD25, ICOS, TGF-β, CCR6	N/A	Within the TILs, the percentages of Th17 and Treg cells were inversely correlated. The prevalence of IL-17-producing FOXP3+ CD4+ in TIL is increased in SCC and possess suppressive function similar to Tregs	The findings of this study suggest a tumor promoting role for Tregs (regardless of their IL-17 production ability)
Schuler et al. (49)	FC	CD4+, CD25high, Foxp3+	RCT had diverse effects on Treg frequency	The mean frequency of Tregs was significantly increased SCC prior to Rct compared to healthy controls	Although this study reported unpredictable effect of RCT on Tregs, it reported an increase in Tregs in SCC patients suggesting an active mechanism of immune escape and tumor promotion
Alhamarneh et al. (50)	ELISA, FC	CD4+CD25high, GITR, CTLA-4, Foxp3	Post-treatment Treg levels were significantly higher than pre-treatment levels	Patients had significantly higher percentages of circulating Tregs compared with normal controls	The levels of Treg cells were elevated significantly SCC, however, they failed to correlate with disease progression or tumor burden
Al-Qahtani et al. (51)	IHC	Foxp3	N/A	Treg levels were higher in poorly differentiated SCC.	A linear positive correlation was established between tumor grade and number of Tregs suggesting a role in tumor promotion
Tominaga et al. (52)	IF, FC	CD4+, FoxP3+	N/A	Dogs with MM had increased numbers of circulating Tregs and TILs compared to healthy control dogs	The findings suggest a tumor promoting effect of Tregs
Horiuchi et al. (53)	FC	CD4+, Foxp3+	N/A	The percentage of circulating Treg increased with the tumor stage in dogs with oral MM	The findings of this study suggest Tregs possess a suppressive role for anti-tumor immunity, thus promoting tumor progression
Schott et al. (54)	FC	CD4+, CD25high, GITR, CTLA-4, CD122, CD127low, CCR7, Foxp3, CCL22	Increased Treg levels were found even in patients with no active disease several years after tumor resection	Increased ratio of Tregs within total CD4+ population in SCC patients. Increased level of GITR and CCR4 expression in Tregs from SCC patients	Increased Tregs in SCC patients might correspond to reduced anti-tumor immunity and therefore contribute to tumor progression or recurrence
Gasparoto et al. (55)	FC	CD4, CD25, FoxP3, GITR, CD45RO, CD69, TGF-β, CTLA-4, CCR4, IL-10	N/A	High frequency of Tregs in SCC patient blood with stronger suppressive ability than Tregs from healthy donors	Tregs suppress immune responses both systemically and in the tumor microenvironment, thus promoting tumor progression
Boucek et al. (15)	FC	CD3+, CD4+, CD25	N/A	Treg counts were higher in SCC patients compared to controls and were higher in recurrent disease	The levels of Treg in the peripheral blood correlate with a higher probability of early recurrence of SCC

(Continued)

TABLE 2 | Continued

References	Method for detecting Treg	Treg markers	Changes in Tregs observed with treatment	Changes in Tregs observed with disease progression	Suggested role of Tregs
Distel et al. (56)	IHC	Foxp3	N/A	In the low risk group, CD3+/Foxp3+ ratio had a clear impact on NED-survival with a low ratio being associated with a better prognosis. This was not observed in high risk patients	The results of this study suggest that intratumoral Treg infiltration on its own does not have an impact on tumor control or survival rates. CD3+/Foxp3+ ratio impacted NED-survival in the low risk group
Schwarz et al. (57)	IHC	Foxp3, CD25	N/A	Tregs were significantly elevated in SCC compared to control tissues	The authors could not conclude the role that Tregs play in tumor progression
Bergmann et al. (58)	FC, ELISA, WB	CD3, CD4,CD25+, Foxp3, IL-10, CTLA-4	N/A	overexpression of COX-2 and secretion of PGE2 by tumor cells induce the highly suppressive type 1 Treg (Tr1) subset of suppressor cells	The induction of Tr1 suppressor cells by SCC contribute to carcinogenesis by creating a suppressive microenvironment that promotes tumor growth
Chikamatsu et al. (59)	FC	CD4+, CD25+	N/A	Circulating Tregs are increased in patients with SCC compared to controls	Although there were no associations between Treg and tumor stage or histological differentiation, Treg percentage inversely correlated with that of total CD8+ T cells in cancer patients and was associated with inhibition of cytokine expression in CTLs suggesting a possible role in the downregulation of antitumor immune response
Badoual et al. (21)	IF	CD3, CD4, CD25, Foxp3, CD69	N/A	Overall, high levels of CD4+CD69+, CD4+CD25+ or CD4+Foxp3+ are associated with better survival and locoregional control	The findings of this study suggest that tumor infiltrating Tregs are associated with a better prognosis
Schaefer et al. (60)	FC	CD3, CD4+, CD25+, Foxp3, GITR, CCR7	N/A	Patients had significantly higher percentages of circulating Tregs than controls	Although the effect of Treg on downregulating the immune functions of other T cells subsets was shown, the exact role of Treg on disease progression could not be confirmed in this study

FC, Flow Cytometry; IHC, Immunohistochemistry; IF, Immunofluorescence; WB, Western Blot.

progression (16, 19, 20, 32, 33, 40, 51). Regarding the studies that assessed blood samples only, the majority [11 out of 16 (68.7%)] reported an association between Tregs and poor outcome (15, 17, 37, 39, 44, 45, 49, 53, 54, 58, 59), while three studies reported a good outcome (23, 24, 29) and two studies could not conclude a definite role (50, 60). When both tumor and blood samples were assessed, only two studies (out of 11) could not define a role for Tregs in disease progression (36, 38), two studies found a positive correlation with good outcome (22, 27) while the remaining seven studies reported a correlation with poor outcome and disease progression (18, 41, 42, 46, 48, 52, 55).

Regarding the method used to detect Tregs, interestingly, flow cytometry was only used in two of the nine studies that concluded a good prognosis (23, 24), and four out of the studies that made no conclusion (36, 38, 50, 60). On the other hand, immunohistochemistry was used in seven out of the nine studies that found a positive connection between Treg and a better clinical outcome (22, 24–29), and seven out of the studies that made no conclusion (31, 34–36, 47, 56, 57).

As for the markers used, remarkably, Foxp3 was the only marker used to identify Tregs in five out of the nine studies that suggested a positive clinical outcome with an increase of Tregs (22, 25, 27–29). Five out of the seven studies that used CTLA-4 as a marker of Treg correlated the presence of Treg with a poor clinical outcome (18, 42, 54, 55, 58). The remaining two did not conclude a definitive role for Tregs (36, 50). On the other hand, out of the four studies that assessed GITR, two associated Tregs with poor clinical outcome and disease progression (54, 55), while the other two did not conclude a role (50, 60). All four studies that assessed TGF-β as a marker of Treg function found a negative clinical outcome associated with Treg (18, 42, 48, 55).

DISCUSSION

Cancer immunotherapy to reactivate anti-tumor immunity is one of the most important recent developments in cancer treatment. For some patients, targeting the immune system to boost its anti-tumor activity can generate enduring disease remission, but despite the impressive successes in cancer immunotherapy, the response in patients is sometimes transient. This is attributed to multiple factors including the exhaustion of tumor-specific CD8 T cells in addition to induced suppression of the immune response against cancer. One of the major immune escape mechanisms in cancer patients is the conversion and dominance of suppressive immune cells within the tumor microenvironment that hamper the function of anti-tumor effector T cells. Regulatory CD4 T cells (Tregs) are among the most studied suppressor cells in the tumor microenvironment and their role in mediating tumor progression has been reported in many types of cancer. Indeed, reducing the number of Tregs has been reported to enhance anti-tumor immunity and promote tumor regression (9–14).

However, in head and neck cancer and particularly in OPSCC, the role of Tregs in mediating tumor progression and affecting

the overall clinical outcome is not clear. In fact, there are conflicting reports in the literature; while a considerable number of studies reported a similar role for Tregs in mediating tumor escape mechanisms and facilitating tumor progression (15–20), other studies reported an opposite role and associated Tregs with a positive clinical outcome (21–29).

In this systematic review, we attempted to assess the body of knowledge available about the role that Tregs play in head and neck cancer with the aim of understanding the reasons for this contradiction in describing the role that Tregs play in disease progression and the clinical outcome.

Our findings emphasized the controversy in the literature. An elevated level of Tregs in patients was observed in some studies (15, 20, 23, 31–34, 37, 39–41, 44, 45, 48–55, 57, 59, 60), while no significant differences were reported between patients and healthy controls in others and a decrease in Tregs with more advanced disease was observed in three studies (22, 29, 35). While more than half of the reviewed studies reported a poor prognosis associated with increased levels of Tregs (15–20, 32, 33, 37, 39–42, 44–46, 48, 49, 51–55, 58, 59), many studies reported a better prognosis (21–29). A considerable number of studies did not conclude a role for Tregs in tumor progression or clinical outcome (31, 34–36, 38, 43, 47, 50, 56, 57, 60).

One of the potential reasons for the controversy in the literature, is different reports from different species. We therefore included all the manuscripts from all species to assess whether the species under study affected the reported outcome. The only study that assessed both human and murine samples reported a role for Tregs in promoting tumor progression (32). One of the two studies that assessed murine sample did not reach a conclusion about the role of Tregs (34), while the second murine study reported a role for Tregs in enhancing tumor progression (19). Similar results about the role of Tregs in promoting disease progression were reported in the only study that assessed rat samples (41) and the two canine samples (52, 53). These findings ruled out any role for inter-species variability in the controversy in the literature.

In recent years, the incidence of HPV positive oropharyngeal cancers has increased and is on the rise. Surprisingly, we report that HPV status was assessed in only 11 studies out of the 45 included in this systematic review. HPV-associated tumors are a distinct subtype with different intra-tumoral immune cell infiltration and better prognosis (24, 27, 28, 47). Therefore, phenotyping tumors according to their HPV positivity is essential when assessing the role of different immune cells in anti-tumor immunity.

Despite the advances in head and neck cancer diagnosis and treatment, the mortality rate is still high. This is mainly attributed to late diagnosis and the lack of predictors of disease progression. Premalignant lesions are altered tissues that carry a higher risk of developing into malignancy, but unfortunately markers to predict malignant transformation into malignancy in these lesions are lacking. Surprisingly, among all the reviewed articles in this study, only three studies assessed premalignant lesions in animal models (32, 34, 41), and only one of these studies assessed samples from human patients (32). All three studies reported an increase in suppressive Tregs with disease progression from

normal through dysplastic to neoplastic lesions (32, 34, 41). In their study, Ma et al. reported a correlation between disease stage and Tregs and in particular in A2AR expression. They reported that blocking A2AR reduced Tregs in the tumor bearing mice and enhanced anti-tumor immune response (32). Understanding the immune response within premalignant lesions is crucial to predict their progression into malignancy and to design treatments to modulate the immune response to eliminate these lesions before transforming into cancerous lesions.

Interestingly, distinction between thymic vs. peripheral Treg within the tumor microenvironment was not made nor assessed in any of the identified manuscripts in this systematic review. This is not a surprise given the lack of markers that accurately determine the origin of Tregs, but certainly measuring intratumoral numbers of converted Treg, defined by markers such as CD103 or S1PR may yield more precision for the role that Treg play within the tumor microenvironment.

In this systematic review, we found that Foxp3 is the most commonly used marker for Treg identification. In fact, it was the only marker used in 13 studies (16, 20, 22, 25, 27–29, 34, 35, 40, 47, 51, 56). Many of the studies used immunohistochemistry and a single stain to detect Foxp3. This causes a potential problem as Foxp3 is highly expressed in other activated T cell subsets including effector T cells. In fact, it has been proposed that under certain inflammatory condition, Foxp3+ Tregs might become unstable adopting a phenotype that is more characteristic of effector CD4+ T cells (61). Foxp3 might be a marker of activation rather than a marker of regulation and a key identifier of Tregs. Therefore, using dual staining and co-localization of markers such as CD4 and Foxp3 could clarify the role of Treg TIL subset in the tumor microenvironment more accurately.

Furthermore, Foxp3 is expressed in tumor cells. In fact, it has been reported that tongue SCC tumor cells express Foxp3 and its expression significantly associated with disease progression and poor patient outcome (62). The same group found that Foxp3 expressed in tumor cells has distinct biological functions compared with that in Tregs (63). On the other hand, the expression of Foxp3 in tumor cells is associated with an increase in the secretion of sCTLA-4, which was recently reported to be a favorable predictor of clinical outcome in advanced cancers (64). This could explain the reported association between Tregs, as identified by Foxp3 expression, and a favorable clinical outcome.

This adds to the controversy regarding the role of Tregs and emphasizes the need to use more than one method and different markers to detect Tregs within the tumor microenvironment. Remarkably, immunohistochemistry was the only used method to assess Tregs in 21 studies (16, 20, 22, 24–29, 31–36, 40, 47, 48, 51, 56, 57). A study further suggests that the overall expression of Foxp3 in Tregs by itself is not an important predictor of clinical outcome, but rather the localization of Foxp3 is the important predictor of outcome. Weed et al. reported that in oral SCC, nuclear Foxp3 is associated with a higher probability of early disease recurrence in comparison to cytoplasmic Foxp3, which is associated with a lower probability of recurrence (43). Immune profiling and the pattern of TIL

within the tumors is of great importance as reported by Feng et al. after we conducted our search. They reported that the distance between the suppressive Foxp3+ Tregs and the effector CD8+ T cells are predictive of patient overall survival (65). Surprisingly, despite the great advances in different assays that added to the confidence in defining Treg markers, our study did not find any major changes in the assays or markers used to detect Tregs over the 12 year period (2005-2017) that the manuscripts included in this review covered. These reports emphasize the importance of using different markers, assays and analyses in the study of immune cells in cancer patients.

Checkpoint inhibitor antibodies represent a novel type of cancer immunotherapy that has seen notable success in the treatment of different cancers (66). One of the major targets of checkpoint inhibitors is CTLA-4, which is highly expressed on Tregs. Only seven studies out of the 45 studies that we assessed looked at the expression of CTLA-4 in Treg or used it as an identifier for these suppressor cells (18, 36, 42, 50, 54, 55, 58), five of which correlated the presence of Treg with a poor clinical outcome (18, 42, 54, 55, 58). One of these studies reported a higher frequency of CTLA-4+ Tregs (identified as CD4+CD25^{hi}Foxp3+) in non-responder patients to Cetuximab (18), suggesting a potential use of this immune checkpoint as a biomarker for response to therapy.

GITR, another immune checkpoint was assessed in four studies as a marker of Treg function (50, 54, 55, 60), two of which associated Tregs with poor clinical outcome and disease progression (54, 55). TGF-β was assessed in four studies as by the membrane bound form (LAP), or using RT-PCR (18, 42, 48, 55), all of which correlated Tregs to poor clinical outcome.

CONCLUSIONS

In conclusion, our systematic review emphasized the existing controversy regarding the role of Tregs in head and neck cancer, and in particular in OPSCC. We conclude that similar to most cancer types, Tregs contribute to tumor escape mechanisms and are therefore associated with poor clinical outcome. The inconsistent results reported in the literature could be due to the use of different markers to identify Tregs, variation in patient recruitment criteria or a heterogeneous cancer population. Indeed, we observed major differences in the reported outcomes between studies that assessed tumor samples, and those that assessed blood samples, suggesting the need to assess both to reach a more definitive understanding of the role of different immune cells in disease progression. HPV status, an important prognostic marker in OPSCC, was not assessed in majority of the studies, which could explain some of the discrepancy in the findings.

Our findings therefore suggest the need to define a better and more robust method to detect Tregs in the tumor microenvironment and in the periphery using a combination of methodologies, markers and analyses. We suggest using a combination of markers to define Tregs in the periphery and within TIL, including CD4, CD3, CD25, CD127lo, FoxP3,

and CTLA-4. We also propose incorporating the regulatory properties of tumor cells as well as TIL for a complete picture of the tumor microenvironment.

AUTHOR CONTRIBUTIONS

CO performed the search, screening and data extraction. FW contributed to results interpretation and writing the manuscript.

RA designed the study, checked the accuracy of the data extraction, and contributed to results interpretation and writing the manuscript.

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