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Overcoming barriers to effective immunotherapy: MDSCs, TAMs, and Tregs as mediators of the immunosuppressive microenvironment in head and neck cancer

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Abstract

A significant subset of head and neck cancers display a T-cell inflamed phenotype, suggesting that patients with these tumors should respond to therapeutic approaches aimed at strengthening anti-tumor immune responses. A major barrier to the development of an effective anti-tumor immune response, at baseline or in response to immunotherapy, is the development of an immunosuppressive tumor microenvironment. Several well described mechanisms of effector immune cell suppression in the head and neck cancer microenvironment are discussed here, along with updates on current trials designed to translate what we have learned from pre-clinical and correlative clinical studies into improved responses in patients with head and neck cancer following immune activating therapies.

Introduction

The Role of Immunity in Head and Neck Cancers

Advances in understanding the role of the immune system in preventing the development and progression of clinically significant cancers have encouraged interest in therapeutic strategies targeting immunity. Head and neck squamous cell carcinoma (HNSCC), with its poor prognosis despite refinements in surgical and chemoradiation protocols, is no exception. The Cancer Genome Atlas (TCGA) has clarified the common genetic alterations contributing to development and progression of HNSCC, and the rational design of agents targeting specific oncogenic pathways in HNSCC remains a valuable approach that can be effective in subsets of patients [1]. However, the heterogeneity of human tumors, ability of plastic cancer cells to adaptively resist single agent small molecule inhibitors, and toxicity

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commonly observed with combination small molecule inhibitors represent significant challenges [2–4]. Immune-based therapies are less likely to depend on common genetic alterations and carry the potential to generate systemic, durable anti-tumor responses. Compared to many other malignancies, HNSCC has a high genetic mutation rate and generates immunogenic tumors with robust immune cell infiltration [5–8]. Accordingly, a significant subset of patients HNSCC patients objectively respond to immunotherapies such as antibody-based checkpoint inhibition [9]. For these reasons and others, immune-based therapeutic strategies dominate the landscape of new clinical trials in cancer treatment [10].

Key mediators of effective anti-tumor immune responses include tumor-infiltrating lymphocytes (TILs) and natural killer (NK) cells. Activated TILs are cytotoxic to target cells in an MHC-restricted, antigen-specific fashion through well-defined mechanisms of cytotoxicity [11,12]. Alternatively, NK cells, broadly considered to be part of the innate immune response, recognize tumor cells through non-specific mechanisms including detection of low cell surface MHC [13]. Increased TILs and tumor-infiltrating NK cells correlate with better response to standard therapies and, accordingly, prognosis and survival [14,15].

Cancer development and progression is predicated upon evasion of immune recognition and destruction. Mechanisms of immune escape can be divided into lack of initial activation of an anti-tumor immune response and/or suppression of an activated response. Although both mechanisms are likely crucial to tumor development and progression, the majority of HNSCCs demonstrate measurable infiltration of effector immune cells suggesting the presence of immune suppression within the tumor microenvironment (TME) [6,8,16]. Perhaps even more critically, these same mechanisms of immunosuppression that allow an upper aerodigestive tract malignancy to develop and progress are likely the same that confer resistance to immune activating treatments. Understanding how to modulate the TME will be critical as we aim to enhance patient responses to such treatments. This review will focus on known mechanisms of immunosuppression in the HNSCC microenvironment, with particular attention to myeloid-derived suppressor cells (MDSCs), polarized macrophages, regulatory T cells (Tregs), and work being done to translate pre-clinical knowledge into clinical advances.

Discussion

Mechanisms of Immunosuppression in the Tumor Microenvironment

Many different cell types and functional states contribute to the overall immunosuppressive or immunopermissive status of the TME, and this can vary between patients and even regionally within one tumor [17]. The ability to characterize the overall immune status of large cohorts of tumors has been aided by genomic sequencing and array profiling techniques [1,6], but is still handicapped by tumor sampling and difficulties analyzing representative tissue from a heterogeneous source. In its most basic form, immunosuppression within the TME is mediated directly by HNSCC tumor cells or indirectly by the stroma or through recruitment and polarization of host cells [10,16,18]. All of these factors are important, and ultimately the immunogenicity of an individual HNSCC tumor (and subsequent response to immune-activating therapies) is determined by the

balance between recruitment and activation of innate and antigen-specific effector immune cells, and immunosuppressive forces levied by tumor, stromal and other infiltrating immune cells.

Direct Tumor Effects—The physical characteristics of tumors themselves represent a barrier to the infiltration of immune cells. Rapid cellular growth during tumor development leads to formation of abnormal vasculature and lymphatics, which are inherently leaky and cause elevated interstitial pressure within the tumor that physically blocks immune cell infiltration [19]. This abnormal vasculature also contributes to a hypoxic TME, which induces secretion of T-cell inhibiting compounds galectin-1 and adenosine [19,20].

While immune cells overcome these barriers and infiltrate into tumor cell nests in many HNSCC tumors, unfortunately HNSCC cells utilize many direct and indirect mechanisms of immune suppression and tumor cells with this capacity are likely selected for during tumorigenesis [21]. HNSCC tumor cells can directly express immunosuppressive cytokines TGF- β , IL-6 and IL-10 that suppress T-cell proliferation and cytolytic function [22,23]. In addition to direct release of immunosuppressive cytokines, tumor cell metabolism can deplete nutrients critical to effector immune cell function [24,25]. Overexpression of enzymes within cancer cells such as indoleamine 2,3-dioxygenase (IDO1) deplete local tryptophan pools, profoundly inhibiting T-lymphocyte function [26].

Cancer cells also express surface ligands that bind and inhibit effector T-cells [27]. Known as checkpoints, T-cells express positive and negative co-stimulatory receptors on their surface that normally function to regulate immune responses and prevent autoimmunity. Well known checkpoints expressed on activated T-cells include programmed death 1 (PD1) and cytotoxic T-lymphocyte protein 4 (CTLA4) [28]. Checkpoint ligands, including PD-Ligand 1 (PD-L1) and CD80, are often expressed on HNSCC cells either constitutively downstream of oncogenic signaling pathways or in response to interferon (IFN) present within the TME [29,30]. Induced expression of PD-L1 in response to IFN from activated local T-cells and NK cells is termed *adaptive resistance* and represents a major barrier to effective immune cell function in an otherwise T-cell inflamed TME [28,31].

Role of Host Cells—In addition to direct mechanisms of immunosuppression detailed above, HNSCC cells also secrete chemokines that recruit hematopoietic cells to the TME [32]. These include cytotoxic immune cells required for an effective immune response, but also include many types of immunosuppressive immune cells. Immature or naïve immune cells that are highly plastic can be polarized into an immunosuppressive phenotype by the cytokine milieu within the TME after their recruitment [10,16]. Well-characterized cell subsets are discussed here.

Myeloid derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells that were first characterized in humans as a population of CD34+ suppressor cells in patients with HNSCC [33]. Around the same time, a mouse immunosuppressive myeloid cell equivalent was characterized in syngeneic models of HNSCC [34,35]. In healthy humans these immature cells rapidly differentiate into mature macrophages, granulocytes, or

dendritic cells and only make up ~0.5% of peripheral blood mononuclear cells (PBMCs) [36]. However, in cancer patients this differentiation is partially arrested, resulting in expansion of the immature population [32]. The presence of expanded MDSCs peripherally and within the TME is associated with metastasis and recurrence after definitive treatment in HNSCC patients [33,37]. Manipulation of MDSCs within the TME alters effector T-cell function and responses to anti-cancer therapies, making MDSCs a major focus of research [33,38,39].

Mechanisms of MDSC Recruitment—MDSCs are characterized as CD11b⁺CD14⁺CD33⁺HLA-DR⁻ in humans [32]. In mice, two MDSC subtypes have been identified: CD11b⁺Ly6G⁺Ly6C^{int} granulocytic MDSCs (gMDSCs) and CD11b⁺Ly6G^{low}Ly6C^{hi} monocytic MDSCs (mMDSCs). Similar subsets of MDSCs have been characterized in humans. Both subtypes suppress T-cell activity, and the relative role of gMDSCs and mMDSCs appears to be model dependent.

MDSCs are recruited into the HNSCC TME by a chemotactic gradient involving GM-CSF, MCP-1, CXCL1, IL-8, and CSF1 released from the TME (**Figure 1**) [38,40–42]. Murine mMDSCs often express the chemokine receptor CCR2, and melanoma studies in transgenic mice have demonstrated recruitment of this population into the TME with subsequent T-cell suppression in response to tumor produced GM-CSF [41]. gMDSCs express chemokine receptors CXCR2 and CSFR1, for which CXCL1, CXCL8 (IL-8 in humans) and CSF1 are the primary ligands. Indeed, murine and human HNSCC often significantly express CXCL1 and IL-8 [43]. Studies using CXCR2-deficient mice or CXCR2 blocking antibodies have demonstrated decreased gMDSC accumulation with subsequent decreased tumorigenesis, decreased primary tumor growth rates and enhanced responses to therapy [38,40,44], strongly pointing to MDSCs as a major barrier to effective immunotherapy and immune clearance of tumor cells.

While peripheral MDSCs express chemokine receptors and can be recruited into the HNSCC TME, some evidence exists that PBMCs can be recruited and converted into MDSCs by the local TME cytokine milieu. G-CSF, M-CSF, IL-10, prostaglandins, TNF- α and VEGF have all been shown to induce MDSCs from PBMCs [32,45,46]. Cytokines expressed by HNSCC cell lines can directly contribute to the generation of MDSCs from PBMCs *in vitro*, with IL-6 and GM-CSF playing dominant roles [46]. These inflammatory cytokines and chemokines are constitutively expressed by many HNSCC cell lines and tumors secondary to upstream genetic alterations, forming a link between dysregulated oncogenic signaling and development of an immunosuppressive TME [35,47].

Mechanisms of MDSC immunosuppression—Classic mechanisms of T-cell suppression by MDSCs that have been demonstrated in many tumor models include production of arginase 1 (Arg-1), nitric oxide synthase (iNOS), and reactive oxygen species (ROS) (**Figure 2**) [32]. L-arginine is a common substrate for Arg-1 and iNOS. Upregulation of these enzymes depletes L-arginine from the TME, which directly inhibits T-cells. Expression of Arg-1 depends on COX2-mediated production of prostaglandin E2 [48]. iNOS converts L-arginine into nitric oxide, which is subsequently converted into peroxynitrite.

This reactive nitrogen species potently suppresses T-cell function and correlates with a poor prognosis in a variety of cancers [32,45].

Recently, additional metabolic mechanisms of MDSC-mediated immunosuppression have been identified. Similar to tumor cells themselves, altered tryptophan metabolism through IDO expression can deplete local tryptophan and suppress T-cell function [49]. Further, IDO catalyzes conversion of tryptophan into immunosuppressive kynurenine metabolites. MDSCs also deplete environmental cysteine, which T-cells are unable to synthesize for themselves [50]. The MDSC-mediated depletion of metabolites required for effective T-cell function represents a formidable barrier to anti-tumor immunity in a hostile TME characterized by intense competition for metabolic substrates.

Signal transducer and activator of transcription (STAT) family members are key transcription factors responsible for development of an MDSC phenotype in myeloid populations [32]. Increased STAT3 activation has been observed in MDSCs of tumor-bearing compared to naïve mice, and this activation has been associated with increased expression of pro-growth/survival genes Myc, Bcl-xL, and cyclin D1 [32]. Upregulation of MDSC Arg-1 is largely dependent upon STAT1, STAT3, and STAT6, whereas IDO has been linked to STAT3 and iNOS to STAT1 [32,49]. STAT activation within MDSCs is influenced by TME cytokines including IFN γ , IL-4, -6 and -13 as well as TGF β [45]. In HNSCC patients, MDSCs isolated from the blood, lymph nodes, and HNSCC tumors have been shown to suppress T-cell proliferation at least through STAT3-dependent Arg-1 activity [51].

The hypoxic TME influences MDSC function through the transcription factor hypoxia-inducible factor-1 α (HIF-1 α) [52]. HIF-1 α induces PD-L1 expression on MDSCs and other immune cells within the TME. Interestingly, antibody blockade of PD-L1 function following HIF-1 α induced overexpression appears to reduce MDSC production of the inflammatory and immunosuppressive cytokines IL-6 and 10, suggesting a link between hypoxia and multiple immunosuppressive mechanisms of MDSCs. Peripheral MDSCs from HNSCC patients also suppress T-cell function through surface expression of PD-L1 and the release of TGF β [53], suggesting a role for PD-L1 in MDSC-mediated T-cell suppression outside of the hypoxic tumor microenvironment as well.

MDSCs also appear to be capable of functionally suppressing effector T-cells in an antigen-specific fashion. MDSC presentation of peptide to antigen-specific T-cells results in nitration of T-cell surface molecules, leading to antigen-specific T-cell receptor (TCR) dysfunction and subsequent unresponsiveness to the same antigen presented by professional antigen presenting cells (APCs) [54]. This suggests that the presence of MDSCs in the TME of an otherwise antigenic tumor may result in silencing of antigen-specific T-cells. Another significant immunosuppressive mechanism of MDSCs is the activation of CD4 regulatory T cells (Tregs). Tregs are a FoxP3+CD25+ subset of CD4 T-cells that normally maintain self-tolerance, but can be dysregulated in the TME to cause local immunosuppression [55].

The link between MDSCs and Tregs has been established in many different animal models [56], and has been confirmed in MDSCs derived from cancer patients [57]. In various cancer models, MDSC-mediated induction and activation of Tregs depends upon IL-10, IFN γ ,

CD40, and arginase expression and may occur following uptake of tumor antigen and activation of antigen-specific Tregs [56,58,59]. Through Treg activation and induced TCR dysfunction, MDSCs suppress antigen-specific adaptive immune responses. Clearly, the presence of MDSCs in the TME potently suppresses anti-tumor immunity and alters the inflammatory balance in favor of immune escape and tumor progression.

Targeting MDSCs in HNSCC—Given the above evidence, suppression of either MDSC function or recruitment has become a major research focus for many cancer types including HNSCC. A variety of approaches including forced differentiation, small molecule inhibitors and chemotaxis blockade are under investigation in various stages of development (**Figure 3**).

Vitamin D3 Analogs: As MDSCs represent an immature myeloid cell phenotype, initial efforts focused on inducing differentiation of these immature cells into mature, less immunosuppressive phenotypes. 1,25-dihydroxyvitamin D3 induces differentiation of peripheral blood CD34+ MDSCs isolated from HNSCC patients into antigen-presenting dendritic cells [60]. Although no objective responses were observed, an initial pilot of 1,25-hydroxyvitamin D3 in patients with advanced HNSCC demonstrated reduction in peripheral MDSCs and increased plasma concentrations of IL-12 and IFN- γ [61]. Further study revealed decreased MDSC accumulation, increased mature dendritic cell, CD4 and CD8 T-cell and enhanced T-cell CD69 expression in the TME of HNSCC patients treated with neoadjuvant 1,25-dihydroxyvitamin D3 before surgical extirpation [62]. Given these promising immune correlates and low toxicity profile, a clinical trial of 1,25-hydroxyvitamin D3 in combination with the COX2 inhibitor celecoxib in surgically resectable oral cavity HNSCC has recently been completed, with results pending publication (NCT00953849).

Small Molecule Inhibitors: As two well-known mediators of MDSC immunosuppression that act through L-arginine depletion, Arg-1 and iNOS presented promising targets. Although inhibition of Arg-1 with nor-NOHA and iNOS with L-NMMA mechanistically reduced the suppressive effect of murine and human tumor-derived MDSCs, these inhibitors are limited by their toxicity in humans [63].

Alternatively, identification of IDO as a mechanism of MDSC immunosuppression has led to development of IDO inhibitors capable of inhibiting MDSC function in vitro [49]. A number of phase I/II clinical trials using IDO inhibitors alone (NCT02048709) or in combination with other immune-based therapies (NCT02327078, NCT02178722, NCT02559492) are currently underway in patients with advanced solid tumors. See table 1 for a list of active immunotherapy-based clinical trials discussed in this manuscript. For an exhaustive discussion of immunotherapy trials in currently enrolling HNSCC patients, please refer to a recent review by Allen, et al. [10].

As a transcriptional regulator of Arg-1 and IDO expression in MDSCs, STAT3 represents another logical target. A number of STAT3 inhibitors are in development and have successfully reduced MDSC function through Arg-1 and IDO inhibition in preclinical experiments [49,51]. Success in early phase trials of the STAT3 inhibitors OPB-51602 and OPB-31121 has been limited by toxicities, including early termination of one trial

(NCT02058017) due to intolerable metabolic acidosis [64]. Recent development of the antisense oligonucleotide STAT3 inhibitor ISIS 481464 (AZD9150) has shown promise in initial clinical trials in lymphoma [65]. Currently, a phase I/II trial of PD-L1 blocking antibody in combination with AZD9150 is recruiting patients with recurrent/metastatic HNSCC (NCT02499328).

Targeted Antibodies: Initial pre-clinical work utilized anti-Ly6G or anti-Ly6C antibodies or depleted MDSCs systematically in mice, and this has led to promising anti-tumor and immune enhancing effects in multiple cancer models [66,67]. Translationally, most MDSCs express the chemokine receptor CXCR2. CXCR2 knockout mice demonstrate substantially reduced myeloid recruitment into the TME, and the majority of HNSCCs express significant levels of the CXCR2 ligand CXCL1. Thus, targeted inhibition of CXCR2 represents a rational therapeutic strategy. Monoclonal antibody blockade of CXCR2 results in reduced recruitment of MDSCs into the TME and primary tumor growth suppression [38,44]. Mechanistically, this may be due to increased TIL activation[38], removal of MDSC-liberated proinflammatory cytokines and growth factors[44], or both. More recent work has focused on the combination of CXCR2 blocking antibodies with immune activating therapies, such as checkpoint inhibitors. Indeed, eliminating CXCR2+ MDSCs from the TME enhances responses observed with PD1 targeting immunotherapy in different animal models [38]. A number of small molecule inhibitors of CXCR2 are also in development [68]. A clinical trial of an anti-PD-L1 antibody combined with a STAT3 inhibitor or anti-CXCR2 antibody for patients with metastatic HNSCC (NCT02499328) is underway.

Phosphodiesterase 5 Inhibitors: Phosphodiesterase 5 (PDE5) inhibitors such as sildenafil and tadalafil are used to treat erectile dysfunction and pulmonary hypertension. In addition to mediating these vasodilatory effects, PDE5 inhibition suppresses production of Arg-1 and iNOS, leading to functional inhibition of MDSCs [39]. In several syngeneic murine cancer models, sildenafil treatment slowed tumor growth, increased tumor infiltration with CD8+ T-cells, upregulated T-cell activation markers CD69 and CD25, and decreased T_{reg} proliferation [37,39,58]. MDSCs from sildenafil-treated mice demonstrated decreased ability to suppress T-cell proliferation [39]. In addition, peripheral T-cells from HNSCC patients demonstrated recovery of proliferative capacity in response to ex vivo treatment with sildenafil.

Based on this promising pre-clinical data, tadalafil has been tested in two randomized placebo-controlled phase II trials in HNSCC [37,69]. In both studies, neoadjuvant tadalafil prior to definitive therapy was well tolerated and enhanced objective markers of anti-tumor immunity. Weed et al. observed a significant reduction in the numbers of MDSCs and Tregs in peripheral blood and the TME of treated patients compared to placebo [37]. The proliferative capacity of circulating CD8+ T-cells also improved following tadalafil treatment.

Califano et al conducted a parallel study comparing high-dose tadalafil to placebo, in which a reduction in peripheral MDSC numbers and an increase in ex vivo T-cell proliferation were also observed [69]. Consistent with previous results, MDSC expression of Arg-1 and iNOS were also reduced. This MDSC suppression correlated with enhanced anti-tumor immunity.

Interestingly, evidence of enhanced CD4 and CD8 T-cell activation in response to tumor antigen was observed. Currently, tadalafil is in two phase II trials in HNSCC patients as an adjuvant to standard of care and immune activating treatments (NCT01697800 & NCT02544880).

Tumor Associated Macrophages

Mature macrophages, once recruited into the TME, exhibit high plasticity and appear to be heavily influenced by the TME cytokine milieu. Tumor-associated macrophages (TAMs) develop into one of two phenotypes: tumor limiting M1 or tumor enhancing M2. M2 TAMs support tumor growth through secretion of growth promoting cytokines and local immunosuppression, whereas M1 macrophages possess tumoricidal characteristics and can directly limit primary tumor growth [70,71]. Although the M1/M2 paradigm represents extreme phenotypes of a large spectrum of macrophage functions, in general the macrophage infiltrate in the HNSCC TME is primarily M2 in composition [72]. High levels of M2 TAM infiltrate correlate with metastasis and poor prognosis in HNSCC patients [8,72,73].

TAMs can be recruited to the tumor from the bone marrow, or arise from mMDSCs that differentiate into macrophages within the TME (**Figure 1**) [74]. Macrophage recruitment to the tumor is largely dependent upon colony stimulating factor 1 (CSF1) and monocyte chemotactic protein 1 (MCP-1) signaling with contributions from GM-CSF, VEGF and other chemokines [75]. Hypoxia appears to be a major driver of the induction of signals that recruit macrophages.

Mechanisms of TAM Immunosuppression—TAMs functionally contribute to the immunosuppressive TME through secretion of enzymes, soluble factors, and expression of surface molecules (**Figure 4**). Like MDSCs, M2 TAMs also express Arg-1 and iNOS, leading to depletion of L-arginine and metabolic suppression of T-cell function. Also like MDSCs, TAMs express surface PD-L1 and tend to be polarized toward an immunosuppressive M2 phenotype under hypoxic conditions [52,75]. In a recent analysis of pools of PD-L1 in the TME of syngeneic murine oral cavity HNSCC, macrophages represented the population of cells within the tumor with the highest intensity of PD-L1 staining [76]. TAMs also express the checkpoint ligands CD80/86 (B7-1/2) that inhibit TILs through binding CTLA4 [77]. In some cancer models, surface expression of specific MHC class I proteins can activate or suppress effector immune cells, with macrophage expression of less common HLA-G and E demonstrating ability to suppress T and NK cell function [78]. TAMs also secrete immunosuppressive cytokines IL-1 β , IL-6, IL-10, and TGF β [74]. IL-10 inhibits effector T-cell function and activates Tregs [79]. Further establishing the relationship between TAMs and other regulatory immune cells, TAMs (along with tumor cells, themselves) recruit Tregs to the TME through chemokines CCL22, CCL20, and CCL5 [80–82].

Targeting TAMs in HNSCC

Chemotaxis blockade: A main approach to targeting TAMs in cancer therapy has focused on inhibiting their recruitment into the TME. mMDSCs share a dependence on CSF1/

CSF1R for recruitment, so CSF1R inhibition may target both mMDSCs and TAMs. Studies of anti-CSF1R antibodies and small molecule inhibitors of CSF1R in a variety of cancer models have demonstrated depletion of TAMs from the TME and reprogramming of the remaining macrophages from the M2 pro-tumor to the M1 anti-tumor phenotype [42,83,84]. Although blockade of CSF1/CSF1R signaling resulted in only modest growth suppression in a murine cancer model, combination with anti-PD1 and anti-CTLA4 therapy resulted in regression of established tumors [42]. Anti-CSF1R antibodies have been used to deplete TAMs in patients with a variety of cancers, including one with advanced HNSCC [85]. Two trials combining anti-CSF1R antibody with PD-1 checkpoint blockade are currently recruiting patients with advanced solid tumors (NCT02452424 and NCT02526017). Antibody or tyrosine kinase inhibitor-mediated blockade of other chemokine systems including VEGF receptor and CXCL12 can also reduce accumulation of TAMs [75,83,86].

Reversing TAM polarization: Another attractive approach to targeting TAMs is inducing polarization away from an immunosuppressive M2 toward a cytolytic M1 phenotype. Not only does this approach decrease tumor promoting M2 TAMs, but increases anti-tumor M1 TAM activity. Successful approaches in animal models have included toll-like receptor 3 ligation with Poly(I:C) [87], nanoparticle-mediated delivery of hydrazinocurcumin [88], zoledronic acid treatment [89], and deposition of histidine-rich glycoproteins within the TME [90]. Recent evidence suggests that type I IFN signaling through STING agonists can also re-educate M2 TAMs into an M1 phenotype [91]. In mouse models, STING agonists improve responses to radiation therapy by enhancing the adaptive immune response against the tumor antigens released by radiation [92]. These approaches are in various stages of development and have great capacity to serve as adjuvants to standard and immune therapies moving forward.

Regulatory T-cells

Tregs represent the third major cell type that contributes to the immunosuppressive TME in HNSCC. These cells are a subset of CD4 T-cells that express the transcription factor FoxP3 and commonly express CD25, CTLA4, glucocorticoid-induced TNF receptor (GITR) and OX40 (CD134) on their cell surface [55]. Studies have shown mixed results with regards to the prognostic value of tumor-infiltrating Tregs in HNSCC. In some studies, increased peripheral and tumor-infiltrating Tregs were associated with a negative prognosis in HNSCC patients [37,93]. However, Badoual, et. al. observed the opposite, with Treg infiltration correlating with favorable locoregional control [94]. These authors hypothesized that this unexpected result may be due to the ability of Tregs to inhibit tumor-promoting inflammation as well as anti-tumor immunity. Interestingly, greater numbers of circulating Tregs were observed in HNSCC patients with complete response to treatment compared to untreated patients with active disease, indicating that anti-cancer therapies may increase Treg expansion and provide a rationale for therapeutically targeting them to prevent recurrence [95].

Mechanisms of Treg recruitment—Tregs can be broadly subclassified as either natural or inducible Tregs. Natural Tregs develop as CD4⁺CD25⁺ Tregs in the thymus and are subsequently recruited into the TME [82,96]. Inducible Tregs, on the other hand, are

undifferentiated CD4⁺ T-cells that are polarized into CD4⁺CD25⁺ Tregs by the HNSCC cytokine profile [97]. Tumor-infiltrating T_{regs} demonstrate more potent immunosuppression than those in the periphery, suggesting that the HNSCC TME influences the suppressive activity of these cells [98]. Evidence supports recruitment of Tregs to the TME through tumor-derived CCL22 binding the chemokine receptor CCR4 on the surface of Tregs [55,82]. In melanoma patients, tumor-infiltrating FOXP3⁺ T-cells were found to primarily express CCR4, whereas peripheral Tregs lacked CCR4, suggesting a critical role for this receptor in Treg trafficking [99]. Additional signals implicated in Treg recruitment to the TME include CCL28-CCL28R and CXCL10-CXCR3 interactions [100,101].

FOXP3-T-cells can be converted into FOXP3⁺ Tregs within the TME in the presence of IL-10, TGF- β and retinoic acid [56,102,103]. CD80 binding of CTLA4 on the surface of effector T-cells also induces differentiation into TGF- β ⁺ Tregs in an IL-10-dependent manner [104]. On the transcription factor level, STAT3 appears to be necessary for FoxP3 expression and an immunosuppressive phenotype [105]. Whether they are recruited to the TME or induced from undifferentiated CD4 T-cells already present in the TME, Tregs in the TME expand following recognition of tumor-associated self antigens in the presence of IL-2 [55,106]. Further, mounting evidence suggests that Tregs mediate their immunosuppressive effects in an antigen-specific manner [107,108]. These concepts have critical implications for rational design of immunotherapy approaches involving Treg manipulation.

Mechanisms of Treg immunosuppression—Tregs derived from HNSCC patients suppress effector immune cell function, at least in part, via the production of IL-10 and TGF- β [109,110]. Studies have also demonstrated the ability of tumor-infiltrating Tregs to kill effector NK and CD8⁺ T-cells in a granzyme B- and perforin-dependent manner [111]. In addition, tumor-infiltrating Tregs in HNSCC patients express the cell surface molecules CTLA4 and CD39 as well as surface bound TGF- β 1 [98]. Conditional knockout of CTLA4 in Tregs impaired their suppressive function and protected from tumor development [112]. CTLA4 downregulates CD80/CD86 expression by APCs and thereby limits CD28 binding and costimulation of naïve T-cells. CD39 (ENTPD1) on the surface of Tregs catalyzes degradation of ATP and ADP into AMP, which is subsequently degraded into immunosuppressive adenosine by CD73 [113].

Targeting Tregs in HNSCC—Strategies aimed at targeting Tregs in the TME have focused on surface markers including CD25, CCR4, CTLA4, OX40 and GITR (**Figure 5**).

Anti-CD25 Monoclonal Antibodies: CD25 is the α chain of the IL-2 receptor required for effector function. It is constitutively expressed on the surface of many Tregs, making it a target for monoclonal antibody development. This strategy has shown promise in preclinical studies in a variety of cancer types [114–116], but clinical studies have shown mixed results. In a trial of metastatic melanoma patients treated with anti-CD25 mAb in combination with a dendritic cell vaccine, activated effector T-cells expressing CD25 were depleted in addition to the targeted Tregs, potentially contributing to a lack of observed clinical responses [114]. Conversely, a recent clinical trial of metastatic breast cancer patients treated with anti-CD25 mAb in combination with peptide vaccination demonstrated depletion of Tregs and

improved peptide-specific CD4/CD8 T-cell responses [115]. Clearly more work is needed to understand the impact of therapeutic antibodies targeting CD25 in HNSCC patients.

Anti-CCR4 Monoclonal Antibodies: The preferential expression of CCR4 on the surface of activated Tregs makes it a promising target [99]. Ex vivo depletion of CCR4+ T-cells from PBMCs of melanoma patients resulted in significant induction of antigen-specific effector T-cells [99]. Treatment of adult T-cell leukemia-lymphoma patients with anti-CCR4 mAb revealed depletion of Tregs and induction of antigen-specific T-cell response [99]. A phase I study of the same anti-CCR4 mAb KW-0761 (Mogamulizumab) in lung and esophageal cancer patients demonstrated effective Treg depletion from PBMCs following treatment [117]. Four phase I/II clinical trials evaluating anti-CCR4 antibody alone (NCT02281409) or in combination with other immune-activating therapies (NCT02301130, NCT02705105, NCT02444793) are currently recruiting patients with advanced solid cancers including HNSCC.

Anti-CTLA4 Monoclonal Antibodies: The anti-CTLA4 mAb Iplimumab is FDA-approved for the treatment of metastatic melanoma alone or in combination with PD checkpoint inhibition [118]. This strategy was originally thought to mediate its therapeutic effects through blockade of the inhibitory CTLA4 signal on the surface of effector T-cells. However, recent studies have demonstrated that anti-CTLA4-mAb may primarily mediate its anti-tumor effects through Treg depletion [119]. CTLA4+ Tregs have also been implicated in resistance of HNSCC patients to the anti-EGFR mAb cetuximab, suggesting a possible role for Iplimumab to improve responses to EGFR targeting and other therapies [120].

Anti-OX40 and GITR Agonist Antibodies: Tumor-derived Tregs commonly express two costimulatory molecules of the TNF receptor family; GITR and OX40 [121,122]. Although these receptors are considered positive costimulatory signals when found on the surface of effector T-cells and APCs, in the context of tumor-infiltrating Tregs they appear to play an inhibitory role leading to reduced Treg function and subsequent immune activation in the TME [122]. In HNSCC patients, tumor-infiltrating Tregs express a number of T-cell activation markers, including OX40 [123]. Ligation of OX40 on newly activated CD4 T-cells plays a role in their differentiation into effector T-cells or Tregs, depending upon the TME cytokine milieu [124]. Agonism of the OX40 receptor by either OX40L or an agonistic antibody improved survival and tumor-specific immunity in several mouse models – an effect which was initially only attributed to proliferation of effector and memory T-cells [122]. When anti-OX40 agonist antibody was studied in murine cancer models, the majority of tumors were rejected; this rejection was dependent upon OX40 agonism on both Tregs and effector T-cells [125]. In further preclinical studies, the combination of anti-OX40 agonist and the alkylating agent cyclophosphamide resulted in regression of established tumors and apoptosis of Tregs [126].

Two humanized anti-OX40 antibodies (MEDI6469, PF-04518600) are currently under study in phase I clinical trials. Studies of MEDI6469 in combination with radiation are underway in select tumor types (NCT01303705, NCT01862900, NCT02559024). A phase I trial of PF-04518600 monotherapy is currently recruiting patients with advanced/metastatic solid tumors (NCT02315066). In addition, a murine anti-OX40 antibody (MEDI6469) is currently

in a phase I clinical trial as monotherapy prior to resection for locally advanced HNSCC (NCT02274155).

GITR is a co-stimulatory receptor found on the surface of APCs and CD4 T-cells [127]. Ligation of GITR with an agonist antibody directly inhibits naïve Treg-mediated immunosuppression *in vitro*, eradicates established tumors, and protects from melanoma cell line challenge *in vivo* [121,127,128]. However, it was initially unclear whether these anti-tumor effects were mediated by stimulation of effector T-cells, inhibition of Tregs, or both [128]. Recent work has confirmed that *in vivo* ligation of GITR leads to down-regulation of FoxP3 expression and reduced production of IL-10 by Tregs [129]. In addition, anti-GITR agonist antibody therapy synergizes with anti-CTLA4 blocking antibody in several solid tumor models [127,130]. Recent development of two humanized anti-GITR antibodies (TRX518, MEDI1873) has facilitated translation into phase I trials recruiting patients with advanced malignant melanoma or advanced solid tumors (NCT01239134, NCT02628574, NCT02583165).

Conclusions and Future Directions

With advances in our understanding of the intricacies of anti-tumor immunity, and the development and clinical application of checkpoint inhibitors and adoptive T-cell therapies, immunotherapy has taken center stage for many cancer types, including HNSCC. Advances in the clinical application of sequencing technology and MHC binding prediction algorithms will likely allow clinicians to identify immunotherapy-responsive HNSCC tumors in the treatment planning phase. Immunosuppression within the TME, mediated largely by tumor-infiltrating MDSCs, TAMs and Tregs, limits the efficacy of any type of immune activating therapy. While durable response rates of 15-30% with current immunotherapeutic strategies are significant, there is good evidence to support the concept that reversing local immunosuppression within the HNSCC TME could increase responses. Whether checkpoint inhibition or other immune activating approaches are utilized, future clinical trials will need to address the problem of local immunosuppression as a major limitation to the development of effective anti-tumor immunity. Challenges lie in how to best shift the balance of inflammation within the HNSCC TME toward effector immune cell activation and tumor rejection without damaging the anti-tumor immune response, itself, or inducing irreversible immune-related adverse events.

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Highlights

1. The majority of head and neck cancers display high effector immune cell infiltration
2. Head and neck cancer cells utilize many defined mechanisms to escape immune elimination
3. An immunosuppressive immune infiltrate contributes heavily to local immune suppression
4. Modulation of immune escape mechanisms may enhance responses to standard and immune therapies

Figure 1. Myeloid Cell Differentiation and Recruitment

Myeloid-derived suppressor cells (MDSCs) accumulate under the influence of factors including GM-CSF, IL-6, IL-10, COX2, IL-1β, IDO, and VEGF, and are recruited to the tumor microenvironment by secreted CXCL1, IL-8, GM-CSF and CSF1. gMDSCs are terminally differentiated, while mMDSCs can further differentiate into macrophages and dendritic cells. Macrophages can also be recruited to the tumor microenvironment by CSF1.

Figure 2. Immunosuppressive Mechanisms of MDSCs

Under the influence of transcription factors STAT1, STAT3, STAT6 and HIF1 α , MDSCs suppress TILs through a variety of mechanisms. Arg-1 inhibits TILs through depletion of arginine and activates Tregs. iNOS inhibits TILs through production of reactive oxygen and nitrogen species in addition to depletion of environmental arginine. MDSCs also secrete immunosuppressive cytokines IL-6 and IL-10, and express the immune checkpoint ligand PD-L1. Intracellular IDO expression has also been proposed as a mechanism of TIL inhibition through depletion of tryptophan and production of kynurenine metabolites.

Figure 3. Therapeutic Targeting of MDSCs

CXCR2 and CSFR1 antibodies inhibit chemotaxis of MDSCs to the tumor microenvironment. Vitamin D3 reduces MDSC numbers by promoting differentiation into macrophages and dendritic cells. PDE5 inhibitors increase intracellular cGMP, thereby inhibiting the immunosuppressive enzymes iNOS and Arg-1. Small molecule inhibitors of IDO, iNOS, Arg-1, and STAT3 have also been developed. Depleting antibodies targeted against Gr1 and Ly6G surface markers reduce MDSC numbers.

Figure 4. Tumor-Associated Macrophages

M2 macrophages inhibit T-cells through surface expression of HLA-G, CD80/86, and PDL1/PDL2. Like MDSCs macrophages secrete Arg1, which inhibits TILs through depletion of arginine. Macrophages also secrete immunosuppressive cytokines IL-1 β , IL-6, IL-10 and TGF β which inhibit TILs and activate Tregs. Surface expression of HLA-E also may inhibit NK cells in some cancer models. The central approach to targeting TAMs has been through the development of CSFR1 antagonists and antibodies.

Figure 5. Therapeutic Targeting of Regulatory T-Cells (Tregs)

In addition to secreting immunosuppressive cytokines IL-10 and TGF β , Tregs inhibit TILs through surface expression of CTLA4 and CD39. Antibodies against surface molecules CTLA4, CCR4, and CD25 have all been shown to deplete Tregs in various cancer models. Agonist antibodies against GITR and OX40 stimulate effector T-cells in addition to inhibiting Treg function.

Table 1

Current Immunotherapy Trials in HNSCC Targeting the Immunosuppressive Tumor Microenvironment

Targets	Treatments	Phase	Clinical Trial ID	Patient Eligibility
IDO1	GDC-0919	I	NCT02048709	Recurrent/refractory solid tumors
IDO1 PD-1	Epacadostat + Nivolumab	I/II	NCT02327078	Recurrent/metastatic solid tumors including HNSCC
IDO1 PD-1	INCB024360 (Epacadostat) + MK-3475 (Pembrolizumab)	I/II	NCT02178722	Recurrent/metastatic solid tumors including HNSCC
IDO1 JAK1	INCB024360 (Epacadostat) + INCB039110	Ib	NCT02559492	Advanced/metastatic solid tumors including HNSCC
PD-L1 STAT3 CXCR2	MEDI4736 + AZD9150 or AZD5069	Ib/II	NCT02499328	Recurrent/metastatic HNSCC
PDE5	Tadalafil	II	NCT01697800	Untreated nonmetastatic HNSCC
PDE5 Tumor Mucin 1 (MUC1)	Tadalafil Anti-MUC1 Vaccine	I/II	NCT02544880	Resectable HNSCC
CSF1R PD-1	PLX3397 + MK-3475 (Pembrolizumab)	I/IIa	NCT02452424	Recurrent/metastatic solid tumors including HNSCC
CSF1R PD-1	FPA008 +/- Nivolumab	Ia/Ib	NCT02526017	Advanced solid tumors including HNSCC
CCR4 PD-L1 CTLA-4	KW-0761 (Mogamulizumab) + MEDI4736 or Tremelimumab	I	NCT02301130	Advanced solid tumors including HNSCC
CCR4 PD-1	KW-0761 (Mogamulizumab) + Nivolumab	I	NCT02705105	Advanced/metastatic solid tumors
CCR4 4-1BB	KW-0761 (Mogamulizumab) + PF-05082566	Ib	NCT02444793	Advanced/metastatic solid tumors
CCR4	KW-0761 (Mogamulizumab)	I/II	NCT02281409	Advanced/metastatic solid tumors
OX40 4-1BB	PF-04518600 +/- PF-05082566	I	NCT02315066	Advanced/metastatic solid tumors including HNSCC
OX40	MEDI6469	Ib	NCT02274155	Resectable HNSCC
GITR	TRX518	I	NCT01239134	Advanced/metastatic melanoma or solid tumors
GITR	TRX518	I	NCT02628574	Advanced solid tumors
GITR	MEDI1873	I	NCT02583165	Advanced solid tumors