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Improving head and neck cancer therapies by immunomodulation of the tumour microenvironment

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Abstract

Targeted immunotherapy has improved patient survival in head and neck squamous cell carcinoma (HNSCC), but less than 20% of patients produce a durable response to these treatments. Thus, new immunotherapies that consider all key players of the complex HNSCC tumour microenvironment (TME) are necessary to further enhance tumour-specific T cell responses in patients. HNSCC is an ideal tumour type in which to evaluate immune and non-immune cell differences because of two distinct TME aetiologies (human papillomavirus (HPV)-positive and HPV-negative disease), multiple anatomic sites for tumour growth, and clear distinctions between patients with locally advanced disease and those with recurrent and/or metastatic disease. Recent technological and

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Author contributions

All authors researched data for the article and wrote the article. R.L.F, T.B. and D.Z. contributed substantially to discussion of the content and reviewed and/or edited the manuscript before submission.

Competing interests

L.V. is a co-inventor of a methodology licensed to INmune Bio, Inc., where soluble TNF sequestration using DN-TNF can be used to prevent or treat malignancies. D.P.Z. declares competing interests with Blueprint Medicines (advisory board), Macrogenics (consulting), Prelude Therapeutics (advisory board), and Merck (advisory board) and research support (institutional) from Merck, BMS, AstraZeneca, GlaxoSmithKline, Aduro, Astellas, Macrogenics, Lilly, Bicara, Checkmate Pharma, and Novasenta. R.L.F. declares competing interests with Aduro Biotech, Inc. (consulting), AstraZeneca/MedImmune (clinical trial, research funding), Bristol-Myers Squibb (advisory board, clinical trial, research funding), EMD Serono (advisory board), MacroGenics Inc. (advisory board), Merck (advisory board, clinical trial), Novasenta (consulting, stock, research funding), Numab Therapeutics AG (advisory board), Pfizer (advisory board), Sanofi (consultant), Tesaro (research funding) and Zymeworks Inc. (consultant). T.C.B. declares competing interests with Walking Fish Therapeutics (Scientific Advisory Board). The other authors declare no competing interests.

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scientific advancements have provided a more complete picture of all cellular constituents within this complex TME and have evaluated the interplay of both immune and non-immune cells within HNSCC. Here, we include a comprehensive analysis of the complete ecosystem of the HNSCC TME, performed utilizing data-rich resources such as The Cancer Genome Atlas, and cutting-edge techniques, such as single-cell RNA sequencing, high-dimensional flow cytometry and spatial multispectral imaging, to generate improved treatment strategies for this diverse disease.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide (890,000 new cases and 450,000 deaths annually[1,2]) and occurs in the mucosal surfaces of four major anatomical sites: oral cavity, sinonasal cavity, pharynx and larynx[3]. A modest improvement in survival of patients with HNSCC has been demonstrated over the past decade, partially attributed to the decreased use of tobacco in the elderly population globally[4]. However, the incidence of HNSCC continues to rise, with a 30% anticipated increase by 2030 (refs.[1,3,5]), mostly attributed to the increase in human papillomavirus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC) in younger individuals who is caused primarily by HPV16 and, to a lesser extent, HPV18. Indeed, the fraction of HPV-positive OPSCC in the United States increased from 16.3% in the 1980s to more than 72.7% in the 2000s[3,6]. As the effectiveness of prophylactic HPV vaccination is less well defined for OPSCC than for indices such as cervical cancer, a decreased incidence in HPV-related disease might not be evident before 2060. The prognosis of patients with OPSCC that is HPV-positive is more favourable than that of patients with HNSCC that is HPV-negative as they have improved responses to chemotherapy and radiotherapy owing to fewer co-existing conditions than patients with HPV-negative HNSCC, who are often physiologically compromised by chronic tobacco and alcohol use[7]. Further, HPV-positive OPSCCs display an increased immune infiltrate, and the overall microenvironment and cellular ecosystems of these tumours can be quite different to those from patients with HPV-negative disease[3]. In addition to HPV status, consideration of locally advanced versus recurrent and/or metastatic (R/M) disease is important owing to the potential impact of prior therapies and tumour progression in the tumour microenvironment (TME)[8]. Indeed, although the treatment of both locally advanced and R/M HNSCC has benefited a cohort of patients, there is room for improvement. HNSCC of the oral cavity is generally treated with surgical resection, followed by adjuvant chemotherapy plus radiotherapy[3]. Molecularly targeted agents, such as the epidermal growth factor receptor (EGFR) inhibitor cetuximab, have shown modest success in locally advanced disease[9]. Finally, two immune-checkpoint inhibitors (ICIs) targeting PD1 (nivolumab and pembrolizumab) are approved for the treatment of R/M HNSCC. Unfortunately, only 15–20% of patients with HNSCC achieve a durable response to these agents despite a twofold to threefold higher expression of PD1 and PDL1 within the tumour[10]. In addition to the clear genomic differences between HPV-positive and HPV-negative HNSCC (Table 1), which should be considered for next-generation immunotherapies, we can begin to assess how various microenvironmental drivers affect the immune and stromal landscape of these disease aetiologies. With the incidence of HNSCC increasing, a clear need exists for improved therapeutics for both locally advanced and R/M disease. Furthermore, locally advanced disease provides a

window of opportunity to study the dynamic infrastructure of the HNSCC TME, knowledge of which is critical for improving therapies for patients that progress to R/M disease. This comprehensive Review of the cellular ecosystems of the two unique HNSCC TMEs (HPV positive and HPV negative) will discuss current treatment modalities and provide new combinatorial therapeutic avenues aimed at improving patient survival.

Interrogating cellular ecosystems of HNSCC

Important microenvironment differences between patients with HNSCC have been uncovered utilizing The Cancer Genome Atlas (TCGA). Specifically, the HNSCC cohort in TCGA (97 patients with HPV-positive disease and 423 patients with HPV-negative disease) contains bulk RNA sequencing data collected from tumour specimens resected from the different sites of HNSCC[11]. This rich resource has improved our understanding of the immunogenomic landscape in these two TMEs and how these landscapes are shaped by clinical features, including HPV status, tumour stage, mutational burden, and tobacco and alcohol use. Several reports using gene set enrichment analysis and unsupervised clustering to deconvolute and identify cell types stratified patients with HNSCC by immune signatures: cold (no immune infiltrate), lymphocyte (enrichment for $CD4^+$ T cells, $CD8^+$ T cells, B cells and plasma cells) and myeloid dendritic cell (DC) (enrichment of neutrophils, macrophages, monocytes, DCs, T regulatory (T_{reg}) cells and eosinophils)[12–15]. Classifying patient tumours based on immune infiltrate alone is becoming more limited as assessing cellular neighbourhoods (that is, tertiary lymphoid structures; TLS) within these patients will offer key insights into the dynamic interplay of multiple cell types in patients with HNSCC (regardless of HPV status). Adding this further metric to immune high and immune low HNSCC tumours could be important in predicting response to immunotherapy as shown in adult sarcomas[16]. Further, TLS are important considerations in HNSCC as the presence of a mature TLS (that is, one with a germinal centre; GC) is associated with increased survival regardless of HPV status although survival is enhanced further in patients with HPV-positive tumours[17]. In terms of total abundance of each individual cell type, patients with HPV-positive HNSCC have a significant increase in B cells, plasma cells, T helper type 1 (T_H1) $CD4^+$ T cells, T_H2 $CD4^+$ T cells, natural killer (NK) cells and $CD8^+$ T cells compared with patients with HPV-negative HNSCC. Monocytes, macrophages, NK T cells and neutrophils are higher in patients with HPV-negative HNSCC[12,13,15]. Reports regarding T_{reg} cell abundance in HNSCC have been conflicting. One study reported via bulk RNA sequencing that the abundance of T_{reg} cells was higher in patients with HPV-positive HNSCC than in patients with HPV-negative HNSCC while another reported no significant difference[12,13]. In general, lymphocyte gene signatures correlated with a longer overall survival than the cold and myeloid DC signatures, especially in patients with HPV-positive disease. Immune cold and myeloid DC gene signatures correlated with later-stage disease and shorter overall survival[12,13,15]. The molecular smoking signature, defined by the proportion of mutational processes in each tumour attributable to tobacco smoking, correlated inversely with immune infiltration in both HPV-positive and HPV-negative tumours and correlated positively with high total non-synonymous mutational burden (the total number of non-silent missense mutations per tumour)[13].

Comparing individual differentially expressed immune-related genes (IRGs) in the TCGA-HNSCC cohort revealed a total of 65 IRGs that were associated with prognosis, 56 of which were significantly associated with overall survival rates in patients with HPV-negative HNSCC[11,14]. In this IRG prognosis model, *SEMA3G*, *GNRH1*, *TNFRSF4* and *ZAP70* were positively correlated with overall survival[11] and *PLAU*, *SH2D1A*, *CCL26*, *DKK1*, *GAST*, *PDGFA* and *STC1* were negatively correlated with overall survival. Further, the 5-year survival rate for patients whose tumours expressed more of the negatively correlated genes was 31.2% and that for patients whose tumours expressed more of the positively correlated genes was 66.8%[11]. This study focused on HPV-negative HNSCC tumours, which have low infiltration. Indeed, TCGA analyses that have focused on both TMEs have uncovered significant differences in several other immune pathways. For example, $CD8^+$ T cells show significant upregulation of PD1, T cell immunoglobulin and mucin domain 3 (TIM3), lymphocyte-activation protein 3 (LAG3), and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) in HPV-positive tumours compared with HPV-negative tumours[18–22]. In addition, $CD8^+$ T cells in HPV-positive tumours also showed increased expression of the T cell activation marker CD137 and ectonucleotidase CD39 compared with HPV-negative tumours[23]. KIR inhibitory receptor genes, which inhibit NK cell function, are increased in HPV-positive tumours and B cell-associated genes, such as *CD200*, *VCAM1*, *BCL2* and *ICOSLG*, are also increased HPV-positive tumours[13,18].

Bulk RNA sequencing datasets require deconvolution to identify cell types within samples and thus might under-represent the composition and distribution of cell types within a given sample[24–27]. Thus, such analyses have limited ability to detect intratumoural heterogeneity and cell-to-cell variability in gene expression. Further, differences in transcript quantification, read depth and sequencing platforms can compromise the reproducibility of TCGA analyses[25,27]. The development of single-cell RNA (scRNA) sequencing has revolutionized transcriptomic analysis of all cellular populations in the HNSCC TME (Fig. 1). Specifically, scRNA sequencing can uncover rare cell populations and novel protein–protein interactions, and track differentiation of cell types within a specific cell lineage[24,27]. Indeed, scRNA sequencing analyses paired with high-dimensional flow cytometry and spatial multispectral imaging (Fig. 1) have revealed immense differences among innate and adaptive immune cells within HPV-positive and HPV-negative HNSCC tumours that were not captured by initial bulk TCGA analyses[28–30]. Furthermore, non-immune cells, such as fibroblasts and mesenchymal stem cells, are also diverse in these TMEs and have now been included in scRNA sequencing landscape analyses[28,31]. In this Review, we discuss new technologies and analyses that more completely and discretely identify the differences in immune and non-immune compartments in patients with HPV-positive and HPV-negative HNSCC.

Maximizing cellular and humoral immunity

Functional $CD8^+$ T cells are key to a robust immune response in HPV-positive and HPV-negative disease

$CD8^+$ T cells can recognize antigens presented by tumour cells and directly kill them by releasing pro-inflammatory cytokines and cytolytic granules (Fig. 2). Although the

overall abundance of $CD8^+$ T cells decreases considerably in patients with HPV-negative HNSCC, those with comparable proportions of $CD8^+$ T cells to proportions in those with HPV-positive tumours tend to have similarly favourable outcomes[32–34]. For example, patients with HPV-negative OPSCC who had comparable numbers of tissue-resident $CD8^+$ T cells ($CD103^+$) to patients with HPV-positive OPSCC demonstrated a similar outcome, suggesting a critical role for tissue-resident $CD8^+$ T cells in HNSCC antitumour immunity irrespective of HPV status[23,35].

One of the hallmarks of the $CD8^+$ T cell response in cancer and chronic viral infections is T cell dysfunction, sometimes termed exhaustion, a cell state characterized by hierarchal loss of effector function (production of IL-2, TNF and IFNγ) and co-expression of the immune inhibitory receptors PD1, TIM3, LAG3, CTLA4 and TIGIT[36–39]. Although flow cytometric studies revealed increased frequencies of $PD1^+CD8^+$ tumour-infiltrating lymphocytes (TILs) in HPV-positive tumours, the frequencies of $CD8^+PD1^{high}$ TILs (which exhibit impaired IFNγ production) are higher in HPV-negative tumours[40]. Furthermore, $PD1^{high}$ expression on $CD8^+$ TILs predicts response to anti-PD1/PDL1 therapies by patients with HPV-positive disease but has limited prognostic value in those with HPV-negative disease[40]. These data underscore the importance of further interrogating the state of $CD8^+$ TILs in HPV-positive and HPV-negative HNSCC. scRNA sequencing that compared global changes in transcriptomic profiles of $CD8^+$ TILs has revealed five broad cell states in HPV-positive and HPV-negative disease, including cycling, cytotoxic, pre-dysfunctional, early activated, and terminally exhausted T cells[28,29]. Diffusion pseudotime analysis revealed that $CD8^+$ T cells follow a shared differentiation trajectory in HPV-positive and HPV-negative HNSCC, moving from early activated $CD8^+$ T cells towards terminally differentiated $CD8^+$ T cells marked by co-expression of inhibitory receptors[29]. Shared transcriptional profiles of exhausted $CD8^+$ T cells have also been reported in paired lymph node metastases of patients who are treatment naive with locally advanced HPV-negative HNSCC[30].

High-dimensional immune profiling studies using scRNA sequencing have revealed that $CD8^+$ TILs are a phenotypically and functionally diverse population within the antigen-specific pool. scRNA sequencing analysis of HPV-specific T cells sorted from HPV-positive tumours and paired metastatic lymph nodes revealed three distinct subsets: stem-like ($PD1^+TIM3^-CD39^-TCF7^+$), transitory ($PD1^+IFN\gamma^{high}$) and terminally differentiated ($PD1^+TIM3^+CD39^+$)[41]. T cell receptor (TCR) clonotypes directed at HPV proteins E2, E5 and E6 were shared between the three subsets, which suggests that terminally differentiated $CD8^+$ T cells derive from stem-like $CD8^+$ T cells[41]. HPV-specific stem-like $CD8^+$ T cells effectively proliferate upon in vitro HPV peptide stimulation and immune-checkpoint inhibition[41]. This finding might imply that strategic reinvigoration of HPV-specific stem-like $CD8^+$ T cells by ICIs will benefit patients with HPV-positive HNSCC. Stem-like $CD8^+$ T cells are also present in HPV-negative HNSCC tumours and produce abundant levels of TNF, although their antigen specificity was not determined[42]. More work is needed to determine how and if non-viral neoantigen-specific $CD8^+$ T cells differentially contribute to response to therapy in both TMEs.

Leveraging conventional CD4+ T cells for improved help within the HNSCC TME

CD4+ T cells are also essential for eliciting antitumour immune responses through differentiation into distinct effector subtypes upon antigen stimulation and secretion of different cytokines[43,44]. T_H1 CD4+ T cells produce pro-inflammatory cytokines, particularly IFNγ and TNF, which induce major histocompatibility complex (MHC) class I and II expression in the TME, enhancing tumour antigen recognition by other immune cells, leading to increased killing of tumour cells[43,44]. T_H17 cells produce IL-17, IL-2, IL-8, and TNF and have an important role in pathogen protection[45]. Both pro-tumour and antitumour roles have been described for T_H17 in both mouse and human cancer but studies are limited in human tumours[46]. Higher numbers of T_H1 CD4+ T cells are observed in HPV-positive than in HPV-negative HNSCC. RNA sequencing analyses demonstrated that CD4+ TILs have increased RNA expression of the T_H1 marker *TBX21* (which encodes transcription factor T-bet and regulates IFNγ production) as well as increased expression of the T_H17 markers *RORA* and *RORC* in HPV-positive HNSCC compared with HPV-negative HNSCC[47]. Further, HPV16-specific CD4+ T cells predominantly express IFNγ and IL-17, suggesting that T_H1 and T_H17 cells are enriched in the HPV-positive TME[48,49]. In addition, HPV16-specific CD4+ T cells release IFNγ and TNF and synergize with cisplatin-based therapy to control tumour cell growth, highlighting their importance inpatient response to cancer therapies[50]. T_H17 cells also accumulate in the peripheral blood and tumour-draining lymph nodes of patients with HNSCC, and primary HNSCC tumours express the T_H17-inducing cytokines IL-6, IL-23 and IL-1β[51,52]. Functionally, T_H17 cells were able to suppress HNSCC tumour growth and decrease production of angiogenesis proteins by HNSCC tumour cells in vitro[51,52]. Future studies should further evaluate the antigen specificity and function of T_H17 cells in both HPV-positive and HPV-negative HNSCC TMEs to determine their impact on patient outcomes. CD4+ T follicular helper (T_{FH}) cells, which express CXCR5 and PD1, produce the chemokine CXCL13, which is important for recruiting B cells and CD8+ T cells into the tumour[53–55]. T_{FH} cells are important for the activation and maturation of B cells through IL-21 production and expression of co-stimulatory ligands such as inducible T cell co-stimulator (ICOS) and the CD40 ligand[56,57]. Recent scRNA sequencing analysis demonstrated that a T_{FH} gene signature is enriched in HPV-positive HNSCC and correlates with improved progression-free survival[17,29]. In addition, multispectral imaging revealed co-localization of T_{FH} cells with B cells within TLS in HPV-positive HNSCC tumours[17,29]. More work is needed to evaluate the role of CD4+ T cell subsets in HNSCC and their potential for immunotherapeutic targeting. However, promoting the appropriate local intratumoural CD4+ T cell differentiation should be considered in the next generation of immunotherapies to augment CD8+ T cell function.

Refining T_{reg} targeting to decrease immunosuppression in the TME

T_{reg} cells are a subset of CD4+ T cells defined by the expression of transcription factor Foxp3 (forkhead box protein 3) and CD25, and they suppress immune effector cells and promote tumour progression[58]. T_{reg} cells exert immunosuppressive functions in tumours through the secretion of inhibitory cytokines such as IL-10, IL-35 and TGFβ, upregulation of inhibitory receptors, disruption of metabolism via CD39 and CD73, and depriving the local TME of IL-2 via high CD25 expression[58–60]. T_{reg} cells are present in the peripheral blood and tumours of patients with HNSCC[60–62]. Patients with HPV-positive HNSCC

have higher frequencies of intratumoural T_{reg} cells than those with HPV-negative HNSCC but recent scRNA sequencing analysis demonstrated that T_{reg} cells share transcriptomic profiles in HPV-positive and HPV-negative HNSCC[29]. Interestingly, T_{reg} cells exhibit unique transcriptional cell states distinguished by enrichment of interferon-response genes or TNF receptor (TNFR) family genes[29]. Interferon-response genes correlated with early activation of T_{reg} cells while TNFR genes, such as *GITR* (also known as *TNFRSF18*), *4–1BB* (also known as *TNFRSF9*) and *OX40* (also known as *TNFRSF4*), were expressed later in differentiation, suggesting reciprocal control of T_{reg} cell effector function[29]. Key inhibitory receptors, such as CTLA4, have also been described on T_{reg} cells in HNSCC[63]. CTLA4 expression is high on intratumoural T_{reg} cells in patients with HNSCC and is further upregulated after cetuximab treatment[63]. Further, CTLA4$^+$ T_{reg} cells inhibit NK cell function during cetuximab treatment, possibly owing to TGFβ production, and treatment with ipilimumab restores NK cell function by depleting T_{reg} cells[63,64]. Other inhibitory receptors have now been more thoroughly examined in patients with HNSCC[65,66]. Expression of neuropilin 1 (NRP1) on intratumoural T_{reg} cells was shown to correlate with GITR and 4–1BB expression on T_{reg} cells[61]. NRP1$^+$ T_{reg} cells have enhanced suppressive function and correlate with overall poor prognosis in patients with HNSCC[61]. NRP1 expression on T_{reg} cells is driven by T cell activation signals and can be reduced by inhibiting molecules within the TCR signalling pathway[61]. Intratumoural TIM3$^+$ T_{reg} cells also limit naive CD8$^+$ T cell proliferation, thus inhibiting effector cell function in HNSCC[21,22]. Lastly, intratumoural T_{reg} cells have high expression of CD39 and CD73 in HNSCC, which allows them to convert ATP to adenosine for T cell suppression via adenosine receptor 2A[60,67]. Taken together, these data suggest that T_{reg} cells have diverse mechanisms to construct an immunosuppressive TME in HNSCC and that specifically inhibiting T_{reg} cells in the HNSCC TME might enhance immuno-therapeutic response.

B cells within tertiary lymphoid structures increase antitumour humoral immunity

Several distinct subsets of B cells exist, and these subsets have diverse functional capacities that extend beyond antibody production and include antigen presentation, initiation of TLS formation, cytokine production and direct cell lysis[68–74]. Furthermore, studies have revealed the prognostic impact of B cells and TLS as predictors of patient survival and response to ICI therapy[16,75–77]. Thus, their role as potential immunotherapeutic targets warrants further investigation.

Infiltration by intratumoural B cells was significantly increased in samples from patients with HPV-positive HNSCC compared with samples from patients with HPV-negative disease, while infiltration of the invasive tumour margin was not significantly different between the two groups[78–80]. Overall, high densities of CD20$^+$ B cells were associated with favourable outcomes in HNSCC[79,81]. B cells are predominantly found within TLS rather than as immune aggregates that have not formed a TLS in both HPV-positive and HPV-negative disease[17]. HPV-positive HNSCC tumours that occurred in the tonsil or tongue had more TLS than HPV-negative tumours that occurred in the same sites[17]. Multispectral imaging revealed that B cells co-localize with both CD8$^+$ T and T_{FH} cells in TLS in patients with HNSCC[17,76,81,82]. Strikingly, scRNA sequencing analysis revealed that, compared with other immune cells, B cells showed larger transcriptional differences between HPV-positive

and HPV-negative disease[17,29]. Specifically, B cells within HPV-positive tumours were enriched for GC and naive B cell gene signatures whereas B cells in HPV-negative HNSCC were enriched for plasma cells and class-switched memory B cells (MBCs)[29]. Several other studies have quantified subsets of B cells within HNSCC tumours. B regulatory cell subsets, such as $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD25^{hi}$ cells, were increased in HNSCC tumours compared with non-cancerous mucosa and healthy peripheral blood mononuclear cells[78,83]. Oropharyngeal sites of HNSCC (tonsil, base of tongue and oropharynx) have increased total levels of MBCs ($CD38^{-}IgD^{-}$), GC B cells ($CD38^{+}IgD^{-}$) and plasma cells ($CD38^{hi}IgD^{-}$) when frequencies of tumour-infiltrating B cells are high in the TME[84]. MHC class I, MHC class II, CD70, CD86 and CD40 expression were also significantly increased on B cells in these patients[78,84]. Intratumoural GC B cells that express canonical GC markers, such as BCL6 and semaphorin 4A (SEMA4A), are increased in HPV-positive HNSCC[17]. Intratumoural GC B cell subsets, including dark zone, light zone and a novel transitional GC population, were present in HPV-positive tumours with unique patterns of surface marker expression, suggesting that GC formation in HNSCC is not impaired[17,76,82]. An increased number of GC B cells correlated with superior overall survival in both HPV-positive and HPV-negative HNSCC[17] (Fig. 3).

The presence of active GCs in HNSCC tumours suggests that B cells undergo selection and could potentially have high-affinity antigen receptors directed at tumour-associated antigens. Indeed, IgG^{+} antibody-secreting cells (ASCs) specific for HPV E6 and E7 antigens were observed in both metastatic lymph node and primary tumours of patients with HPV-positive HNSCC[82] (Fig. 3). Interestingly, IgG^{+} ASCs were also specific for HPV E2 and more ASCs produced antibodies specific for E2 than for E6 or E7 (ref.[82]). Intratumoural HPV-specific IgG^{+} ASC responses correlated positively with titres of HPV-specific IgG in patients. However, very few HPV-specific ASCs were found in the peripheral blood, suggesting that the generation of HPV-specific ASCs occurs locally in the TME[82]. HPV-specific IgG^{+} MBCs were also present in peripheral blood of patients. Circulating HPV-specific antibodies in patients have been shown to be predominantly of the IgG1 isotype, suggesting that HPV-specific antibodies can participate in activation of antibody-mediated killing of tumours via antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis[85,86]. HPV-specific human monoclonal antibodies (mAbs) were generated from intratumoural B cells and ASCs were shown to have a high degree of somatic hypermutation, which suggest that B cells in these tumours experience chronic antigen exposure[82]. Patients with HPV-negative disease have higher levels of circulating antibodies directed towards tumour-associated antigens, such as p53, melanoma antigen gene (MAGE) proteins and cyclin-dependent kinase inhibitor 2A (CDKN2A), compared with patients with HPV-positive disease[78]. The quality of tumour-associated antigen responses in HNSCC is yet to be evaluated. Taken together, these data indicate that distinct B cell-focused targeting strategies might be needed in HPV-positive and HPV-negative HNSCC but should focus on inducing TLS and driving antigen-specific intratumoural maturation of B cells.

Innate immune cells in the HNSCC TME

NK cells could improve tumour cell killing in patients with HNSCC

NK cells are highly malleable effector cells of the innate immune system that are regulated by a sophisticated interplay of activating and inhibitory surface receptors that scrutinize MHC class I expression and stress-induced ligands on potential target cells[87].

NK cells are generally segregated into two major subsets: $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$. $CD56^{bright}CD16^{-}$ cells are primarily known for their cytokine-producing capacity, while $CD56^{dim}CD16^{+}$ cells are best known for their cytotoxic capacity[13,88]. Increasing evidence indicates that $CD56^{dim}CD16^{-}$ NK cells might be the third major subset[89,90]. $CD56^{dim}CD16^{-}$ NK cells are primarily known for efficient IFNγ production in response to tumour challenge and display the most diverse chemokine receptor profile of the three subsets ($CCR2^{+}CCR3^{+}CCR4^{+}CCR5^{+}CCR7^{+}CXCR1^{+}CXCR3^{+}$), making them highly capable of chemo-attraction[89,91,92]. Available studies suggest that the $CD56^{dim}CD16^{-}$ subset might be the most frequent tumour-infiltrating NK cell subset in HNSCC, but little is known about how disease aetiology affects NK cell subset distribution[93]. This observation is highly important as cetuximab, a humanized IgG1 mAb used to treat EGFR-expressing tumours, is believed to mediate tumour elimination in part by promoting $CD16/Fc\gamma RIII^{+}$ NK cell-mediated ADCC against $EGFR^{+}$ tumours[94–96]. A decrease in $CD16^{+}$ NK cell frequencies within the TME would suggest that ADCC might be a mechanism preventing R/M HNSCC but not of debulking the primary tumour. Increased understanding of NK cell subset functions within HNSCC is needed to optimize future immunotherapies.

Comparative transcriptomic analyses indicate that HNSCC have the highest median $CD56^{dim}$ NK cell infiltration amongst the most common tumour types[13]. When comparing HPV-positive and HPV-negative HNSCC aetiologies, NK cell infiltration is more prominent in HPV-positive tumour beds and surrounding stroma than in HPV-negative tumours and surrounding stroma[89,92]. An elevated NK cell infiltrate is associated with increased progression-free and overall survival in patients with HPV-negative and HPV-positive HNSCC (Fig. 4). The importance of NK cells in containing HPV-driven development of dysplastic lesions was highlighted in a case report of a patient with a dysfunctional NK cell compartment who displayed multiple recurrent HPV-associated skin and mucosal dysplasias[93]. The patient achieved long-term remission of all lesions following allogeneic haematopoietic cell transplantation that led to restoration of NK cell cytotoxicity[93]. Given the likely potency of NK cells in the TME, efforts should be made to increase their influx into the HNSCC TME.

Conventional DCs bridge innate and adaptive immunity in the HNSCC TME

Known as professional antigen-presenting cells, conventional DCs are critical for the initiation and polarization of tumour antigen-specific immunity. By presenting tumour antigen-derived peptides in the context of MHC class I and II molecules, they can effectively prime and polarize naive $CD4^{+}$ and $CD8^{+}$ T cells, respectively[97]. Upon maturation, DCs can also effectively recruit NK cells (via CXCL8 (also known as IL-8), CXCL10 (also known as IP-10) and CX3CL1 (also known as fractalkine) release) and activate them (via IL-12p70

secretion and cell–cell contact via transmembrane TNF and trans-presented IL-15), which leads to reciprocal crosstalk and activation between these two cell types[89,98] (Fig. 4).

In HNSCC, DC infiltration is directly associated with increased T cell and NK cell infiltration, decreased rates of tumour dissemination, and improved patient survival[99,100]. The impact of HPV status on DC infiltration and function remains ambiguous. Available studies suggest that HPV-positive tumours might have higher frequencies of tumour-infiltrating DCs than HPV-negative tumours; however, increased DC infiltrates are positively correlated with improved outcome only in patients with HPV-negative disease[101]. DC infiltration directly correlated with MIP3α/CCL20 expression[102]. The potential role of MIP3α/CCL20 in DC recruitment has also been implicated in HPV-driven cutaneous malignancies, where the HPV16 oncogenes E6 and E7 were shown to inhibit MIP3α transcription in human keratinocytes[103]. Two potential ways to improve the prognostic potential of DC-based immune scoring methods is to account for spatial relations of DC with other cell types found within the TME as well as their activation state and/or functional polarization. In the context of cetuximab therapy, ADCC-activated $CD56^{dim}CD16^{+}$ NK cells secrete IFNγ, which leads to increased DC maturation, type 1 skewing (characterized by increased production of IL-12p70, transmembrane TNF and trans-presented IL-15 as well as of NK cell-recruiting chemokines CXCL8, CXCL10 CX3CL1) and elevated expression of the NKG2D ligand MICA, which in turn further enhances NK cell activation[98,104,105].

TAMs in patients with HNSCC have plasticity associated with HPV status

Tumour-associated macrophages (TAMs) are one of the major immune populations that infiltrate cancers[106–108]. In HNSCC, macrophage infiltration was elevated in tumours in comparison to normal mucosa and correlated with R/M disease and poor patient outcomes[109]. Increased TAM infiltration coincided with elevated MCP1/CCL2 and MIP3α/CCL20 expression in HNSCC[109]. Using scRNA sequencing, flow cytometry and multispectral imaging, TAMs were identified as the primary contributors of PDL1 within the HNSCC TME, regardless of disease aetiology[28]. Not only do TAMs express the highest levels of PDL1 on their cell membranes but $PDL1^{+}$ macrophages spatially associate with $CD8^{+}$ T cells within the HNSCC TME, which is imperative for cell–cell interactions[28].

Macrophages are generally categorized into two functionally distinct/divergent subtypes of the activation–polarization continuum: M1 and M2 macrophages[110]. Tumour-ablating M1 macrophages are generated from monocytes polarized by IFNγ and granulocyte–macrophage colony-stimulating factor (GM-CSF)[110]. Pro-tumoural M2 macrophages can differentiate from monocytes or monocytic myeloid-derived suppressor cells (M-MDSCs)[111]. Monocyte-derived M2 macrophages are differentiated in the presence of macrophage colony-stimulating factor (M-CSF), IL-4, IL-10 and IL-13, while M-MDSC-derived M2 macrophages are generated in response to GM-CSF and endogenous S100A9 expression[108,111–113]. While M1 TAMs produce IL-12 for T_H1-skewing and attack and eliminate tumour cells through reactive oxygen species (ROS) and nitric oxide (NO) release or by ADCC, M2 TAMs exhibit a variety of tumorigenic functions, including immune suppression via arginase 1, IL-10, PGE2 and TGFβ production, tumour vascularization, and promotion of tissue remodelling that facilitates tumour dissemination and radiotherapy

resistance[114–117]. Thus, a high number of $CD163^+$ M2 TAMs is associated with poor prognosis in HNSCC[107,118,119] (Fig. 4). High infiltration of M1 TAMs and low infiltration of M2 TAMs were shown to correlate with superior prognosis in both HNSCC aetiologies, with HPV-positive tumours displaying a higher M1 to M2 TAM ratio[15,47,115]. Owing to the plasticity of TAMs, reprogramming tolerogenic M2 TAMs into antitumour M1 TAMs is a very promising tumour treatment strategy[112].

Reducing immunosuppression in the HNSCC TME by targeting MDSCs

MDSCs are a heterogeneous group of highly immunosuppressive innate immune cells that expand owing to skewed haematopoiesis under pathological conditions such as cancer[120,121]. In nasopharyngeal carcinoma, a recent scRNA sequencing analysis demonstrated that MDSCs are developmentally similar to TAMs but are transcriptionally distinct cells[122]. They interact with T cells, DCs, macrophages, and NK cells and suppress their functions through a variety of mechanisms, including chronic production of ROS, NO, anti-inflammatory cytokines, arginase 1 and prostaglandin E2 (PGE2)[120]. Two key MDSC subsets have been identified: M-MDSCs and polymorphonuclear MDSCs (PMN-MDSCs)[121]. M-MDSCs and PMN-MDSCs are defined as $CD14^+CD11b^+HLA\text{-}DR^{lo/-}CD15^-$ and $CD15^+CD11b^+CD14^-$, respectively. M-MDSCs mediate immunosuppression primarily through arginase 1, NO, PGE2, TGFβ and IL-10, while PMN-MDSCs inhibit immune responses via ROS, TGFβ and IL-10 (ref.[121]). Tumour infiltration of these MDSC subsets is mediated by specific chemokines: MCP1/CCL2 and RANTES/CCL5 for M-MDSCs, and CXCL8 for PMN-MDSCs[121].

Although there is no evidence to suggest that MDSC infiltration in HNSCC is driven by disease aetiology, elevated MDSC infiltration is observed in HNSCC in general, and correlates with high clinical stage and pathological grade[123]. In patients with melanoma, high numbers of circulating and tumour-infiltrating MDSCs are associated with poor patient prognosis and lack of responsiveness to immune-checkpoint blockade[124,125]. Immune-checkpoint blockade resistance can also be acquired as the number of intratumoural MDSCs increases following immune-checkpoint blockade[124]. To this end, a 2021 study reported that $CD155^+PDL1^+$ MDSCs are enriched in patients with HNSCC and that blockade of the TIGIT–CD155 axis effectively enhanced the response rate of patients with HNSCC to PDL1 blockade[125].

Multiple strategies have been developed that target MDSC accumulation and differentiation (for example, all-trans-retinoic acid and sunitinib), tumour infiltration and function (for example, CXCR2-blocking and CSFR1-blocking antibodies, PDE5 inhibitors, triterpenoids, non-steroid anti-inflammatory drugs), or viability (for example, gemcitabine, 5-fluorouracil and DR5 antibodies)[126–129]. In vivo studies have shown that resistance to immune-checkpoint blockade can be partially alleviated by targeting MDSC accumulation (using, for example, anti-IL-18 antibodies, phenformin or selective inhibitors of soluble TNF), function (using PI3Kδ/γ and CSF1R inhibitors) and trafficking (using CXCR2)[126,130,131]. Clinical translation of these drugs has been hampered by their off-target toxicities, selective impact on specific MDSC subsets and limited antagonism of suppressive functions.

Stromal tissue regulates the HNSCC TME

When designing new therapeutics, consideration of the contribution of non-immune stromal cells is important as these cells can influence the phenotype and function of immune cells and control the nutritional and structural support for the TME (Fig. 5). Here, we focus on tumour stromal cells, including fibroblasts and mesenchymal stromal cells, and their interactions with malignant cells and immune cells in the HNSCC TME.

CAFs have vast functional differences in the HNSCC TME

scRNA sequencing performed on patients with treatment-naive HNSCC with primary tumours and lymph node metastasis revealed heterogeneity among tumour cells in patients with HPV-negative HNSCC tumours[30]. Seven expression patterns related to cell cycle, hypoxia, and epithelial genes and partial epithelial to mesenchymal transition (pEMT) were observed. Notably, pEMT-high cells were spatially localized to the leading edge of primary tumours near cancer-associated fibroblasts (CAFs) and expressed ligands that correspond to receptors expressed by malignant cells, particularly of interactions important for pEMT transition[30,132]. In late-stage HNSCC tumours, fibroblasts constitute up to 80% of the cellular composition and have multiple functions, including secreting collagen and fibrous macromolecules that build the extracellular matrix (ECM), maintenance of tissue homeostasis, recruitment of immune cells via secretion of cytokines, chemokines and growth factors, regulation of cell mobility within tissue through degradation of the ECM via matrix metalloproteinases (MMPs), and metabolic reprogramming. CAFs have been reported to be quite variable in phenotype in human solid tumours and thus to differ in function[106,133–136].

The composition of CAFs in HNSCC was recently characterized in two independent scRNA sequencing studies[28,31]. In the first study, eight CAF subpopulations were uncovered using gene set enrichment analysis[31]. Seven of these subpopulations were significantly enriched in HNSCC tumour tissue compared to normal tissues. Subpopulations of myofibroblasts, EMT-positive CAFs, and MHC class II plus CAFs were associated with poor overall survival. Pathway enrichment analysis revealed that these three subpopulations had distinct biological functions related to the cyclic guanosine monophosphate-dependent protein kinase (cGMP–PKG) signalling pathway and the oxytocin signalling pathway, to oxidative phosphorylation and ECM receptor interactions, and antigen processing and presentation, respectively[31]. Distinctions between HPV-positive and HPV-negative HNSCC were not made in these analyses. In another scRNA sequencing study, nine subpopulations of fibroblasts were identified that could be grouped into three major subgroups: classical CAFs, normal activated fibroblasts and elastic fibroblasts[28]. Classical CAFs were enriched for genes encoding proteins such as fibroblast activation protein (FAP), platelet-derived growth factor receptor (PDGFRA), lysyl oxidase (LOX) and MMPs. Normal activated fibroblasts showed low expression of CAF markers. Elastic fibroblasts were enriched for tropoelastin (ELN), fibrillin 1 (FBLN1) and microfibril-associated protein 4 (MFAP4). The CAF gene signature correlated with poor overall survival in HPV-positive but not in HPV-negative HNSCC. Patients with HPV-positive HNSCC with both a low frequency of elastic fibroblasts and a classical CAF signature had the best overall survival[28].

Functional analysis of CAFs derived from patients with HNSCC revealed that CAFs secrete hepatocyte growth factor (HGF), which promotes glycolysis in HNSCC tumour cells[137,138]. HNSCC tumour cells in turn secrete basic fibroblast growth factor (bFGF), which induces oxidative phosphorylation, proliferation and migration of CAFs by binding to the fibroblast growth factor receptor (FGFR). HGF regulates glycolysis in HNSCC tumour cells via the c-Met tyrosine kinase receptor expressed on HNSCC tumour cells. Inhibiting both c-Met and FGFR with small-molecule inhibitors attenuated CAF-associated HNSCC tumour growth. Interestingly, in 2D and 3D cultures, HPV-negative HNSCC cell line-conditioned media could activate normal oral fibroblasts to secrete HGF, IL-6, and CXCL8 and induce cell migration by fibroblasts while HPV-positive HNSCC cell lines could not[134,139]. Future studies should explore the secretory profile, proliferation and migration potential of CAFs in ex vivo HPV-positive and HPV-negative HNSCC tumours. The metabolic relationship between CAFs and tumour cells in HNSCC could be a potential targetable axis and enhance current ICI therapies. However, the metabolic relationship between CAFs and immune cells has not been fully elucidated. Notably, the heterogeneity in CAF phenotype and function can be patient specific and might provide a barrier to the therapeutic targeting of CAFs[28,132,135].

MSCs can regulate immune responses in the HNSCC TME

Mesenchymal stromal cells (MSCs) are also referred to as mesenchymal stem cells because of their potential to differentiate into osteoblasts, chondrocytes, adipocytes or myocytes depending on environmental conditions[136,140]. This capability makes MSCs extremely important for tissue repair and regeneration. MSCs can also ameliorate tissue-damaging inflammation and immune responses through the inhibition of both innate and adaptive immune cells and can induce immune tolerance[140]. This function is mediated by the secretion of immunosuppressive soluble factors, such as PGE2, TGFβ, indoleamine 2,3-dioxygenase (IDO) and sHLA-G, and through the expression of inhibitory and apoptotic ligands such as PDL1, galectin 1 and 9, and FasL[140,141]. In malignant stroma, MSCs promote tumour progression and metastasis[142].

MSCs are increased in HNSCC tumours compared to normal tissue, and when MSCs were isolated from patients with HNSCC, they inhibited in vitro cell proliferation and cytokine production by polyclonally stimulated $CD4^+$ and $CD8^+$ T cells[143]. HNSCC tumour-derived MSCs were also shown to inhibit T cell proliferation via IDO production and have a similar phenotype to normal bone marrow-derived MSCs[143]. Direct comparisons of MSCs in HPV-positive and HPV-negative tumours were not performed in this study most likely owing to the small number of patients included. However, of the 13 tumours analysed, 11 occurred in traditional HPV-negative sites. HNSCC-derived MSCs have also been reported to support survival of tissue-resident memory T cells through secretion of IL-7 and IL-15 (ref.[144]). Ex vivo, tissue residence markers (CD69 and CD103) were increased on $CD4^+$ and $CD8^+$ T cells in HNSCC and normal tissues compared to peripheral blood[144]. Co-culture of HNSCC-derived MSCs with healthy donor T cells increased CD69 and CD103 expression and migration of T cells. Vascular cell adhesion protein 1 (VCAM1) was shown to mediate interactions with HNSCC-derived MSCs and T cells, and blocking VCAM1 inhibited the upregulation of resident memory T cell markers[144]. Again, distinctions between HPV-positive and HPV-negative MSCs were not made; however, these studies suggest that

HNSCC MSCs might have both pro-tumour and antitumour roles and their function in the TME warrants further investigation.

Current successes in targeting the TME

As highlighted in the Introduction, HNSCC can be divided into different diseases both biologically and clinically. The most common primary sites (oral cavity, larynx, hypopharynx, oropharynx) for HNSCC are divided by HPV status. Specifically, OPSCC is routinely divided into HPV-positive and HPV-negative with all other sites being predominantly HPV-negative (larynx, oral cavity, hypopharynx). In the setting of curative intent, locally advanced HPV-positive OPSCC can further be subdivided based on clinical staging and smoking status into low-risk disease with the best prognosis and intermediate-risk disease. For example, based on smoking status (≤10 pack-years or >10 pack-years) and nodal stage, 3-year overall survival of locally advanced low-risk HPV-positive, intermediate-risk HPV-positive and HPV-negative OPSCC was 90%, 70% and 30%, respectively[145,146]. For low-risk HPV-positive OPSCC, the field is evaluating de-escalation strategies through clinical trials, with the goal of maintaining the excellent outcomes in these patients but lowering the toxicity from treatment, especially long-term toxicity, which can negatively impact quality of life. Clinical trials continue to evaluate de-escalation strategies, such as reducing radiation dose and/or field, and/or replacing or omitting cisplatin chemotherapy with immunotherapy, either after minimally invasive surgery or in lieu of surgery. In the HPV-negative group, outcomes have not improved for decades in the locally advanced setting; ongoing trials therefore look to escalate therapy beyond our current therapeutics, including the addition of immunotherapy or novel radiosensitizers to standardize chemoradiation therapy (CRT). Patients with intermediate-risk HPV-positive OPSCC are often included in escalation strategies.

Paramount to the integration of immunotherapy in the locally advanced setting is an improved understanding of its interaction with standard CRT, with both standard modalities having stimulatory and suppressive immune effects. The first reported phase III escalation trial (JAVELIN 100) evaluating chemoradiation with or without the anti-PDL1 mAb avelumab given during and after CRT did not meet its primary objective of prolonging progression-free survival[147]. The lack of benefit with the addition of immunotherapy in this trial points to a need for understanding the best sequence of anti-PD1/PDL1 blockade and standard CRT. To this end, an ongoing randomized phase II trial specifically evaluating the timing of anti-PD1 (concurrent versus after CRT) has completed accrual with results currently pending (NCT02777385). Interestingly, in exploratory analysis of JAVELIN, the only subgroup in whom benefit of avelumab added to CRT was observed was that of patients who had high PDL1 (>25%) expression at baseline[147]. This finding suggests that perhaps only an immune-enriched subset would benefit from the addition of anti-PD1/PDL1 in the locally advanced setting. Additional phase III trials evaluating pembrolizumab concurrent with and after CRT (KEYNOTE412; NCT03040999) and atezolizumab starting after CRT (IMvoke 010; NCT03452137) are ongoing as is a phase III trial using anti-PD1 mAb therapy in the adjuvant setting post resection (RTOG 1216; NCT01810913).

Only 10% of patients with HNSCC present with distant metastatic disease but a large proportion, especially of those who are HPV negative, will either recur after curative intent therapy within the head and neck and/or develop metastasis to distant organs. These patients face tremendous morbidity and mortality and there is a great need for improvement in outcomes with systemic therapy. While patients with HPV-positive OPSCC have better outcomes than those with HPV-negative disease in the R/M setting, the difference is less than that seen in the locally advanced setting and systemic therapy alone is palliative[148]. However, great gains have been seen in the setting of R/M OPSCC over the past 5 years, first with the approval of nivolumab and pembrolizumab in 2016 in the platinum failure setting, and more recently with pembrolizumab with or without chemotherapy in the frontline setting in 2019 (refs.[149–151]). With this progress have come new questions, including how to improve outcomes in the anti-PD1-naive setting with combination therapy, an increased need for predictive biomarkers, and where immunotherapy fits after anti-PD1/PDL1 failure.

Efforts to improve outcomes in the frontline setting include immunotherapy combinations and immunotherapy in combination with targeted therapy. Unfortunately, two phase III trials evaluating anti-PD1/PDL1 plus anti-CTLA4 mAbs, Checkmate 651 and Kestrel, evaluating nivolumab plus ipilimumab and durvalumab plus tremelimumab, respectively, both failed to improve overall survival compared to chemotherapy for the primary trial populations evaluated, although the nivolumab plus ipilimumab regimen did appear to be superior to the EXTREME chemotherapy triplet[152,153]. A phase II/III trial with anti-PD1 plus an ICOS agonist did not achieve the efficacy needed to transition to the phase III portion[154]. Other potentially promising combinations for the frontline anti-PD1-naive setting include anti-PD1 plus anti-B7H3 mAb or cetuximab. Cetuximab in combination with pembrolizumab showed a response rate of 45% in patients who are anti-PD1 naive with R/M HNSCC, most of whom had failed platinum chemotherapy[150]. This result is nearly three times the response rate expected with pembrolizumab alone. Immunotherapeutics that can be given via intratumoural injection are also promising. Based on activity in melanoma, a frontline trial with the TLR agonist CMP-001 plus pembrolizumab is under way (NCT046332789). Although only a subset of patients with R/M HNSCC will have a lesion amenable to intratumoural injection, a reliable response at least in the accessible lesion could have the potential to reduce morbidity. Many novel combination immunotherapy strategies are under way in the anti-PD1/PDL1 failure setting. The majority are phase I studies with expansion cohorts that include HNSCC but two randomized phase III trials are ongoing. One trial is evaluating the multi-targeted tyrosine kinase inhibitor lenvatinib plus pembrolizumab (NCT04428151). The other is evaluating the natural killer group 2A (NKG2A) inhibitor monalizumab in combination with cetuximab compared with cetuximab alone in patients who have failed both platinum chemotherapy and anti-PD1 mAb therapy based on a response rate of 20% with the combination, with stable disease in an additional 38% of patients, from a single-arm trial of patients who had failed multiple lines of therapy, including immunotherapy[155].

Most trials include all patients and stratify by HPV status but some trials are focusing solely on R/M HPV-positive OPSCC, primarily those investigating vaccines and adoptive T cell therapy. Treatment with ISA 101, a synthetic long-peptide HPV16 vaccine, in

combination with nivolumab had an impressive response rate of 33% in a single-arm trial and a randomized phase II trial is ongoing, including frontline patients and those with platinum failure[156]. Data are limited on adoptive T cell therapy targeting HPV. Two small trials with autologous genetically engineered T cells expressing a TCR against either HPV16 E6 or E7 have been reported. A trial including 12 patients targeting E6 had 1 patient with OPSCC who achieved only stable disease, and another study with T cells targeting E7 included 4 patients with HNSCC, 2 of whom had a partial response and 2 of whom achieved stable disease. In both studies, the benefit was acute, only lasting 3–4 months[157,158].

Although blockade of the anti-PD1/PDL1 pathway has revolutionized the field of oncology (including in R/M HNSCC), only a minority of patients benefit from these agents. The successes have been limited because efforts to target the complete HNSCC TME have not been made. Indeed, most immunotherapeutic regimens have been T cell centric (ICIs such as anti-CTLA4 and anti-PD1, HPV-specific vaccines, and adoptive T cell therapies). We have seen some new examples of targeting the diversity of the HNSCC TME (for example, cetuximab for T_{reg} cell and NK cell targeting and intratumoural injection for increased myeloid cell function), but unique therapeutics are still limited. While efforts focusing on HPV are still ongoing, it is likely that we will have to take an even more personalized approach based on the characteristics of each individual patient's TME for the field to take the next big leap with immunotherapy. Important to this goal are neoadjuvant window trials to better define the mechanism of action of current and novel agents and prospective biomarker-driven selection of combinations that also considers the known differences in HPV-positive and HPV-negative disease.

Lessons from anti-PD1 and novel treatments

With more therapeutic choices, including those in clinical trials, predictive biomarkers of efficacy are even more important. As biomarkers are being evaluated, we can learn a lot about the future of immunotherapeutics from anti-PD1 in patients with HNSCC. In anti-PD1-treated R/M HNSCC, increased PDL1, particular IFNγ-related immune gene expression profiles (GEP) and an increased tumour mutational burden (TMB) have all been associated with greater efficacy with anti-PD1 mAb monotherapy[149,151,159]. Multiple studies have shown that PDL1 shows increased predictive value when its expression on tumours and immune cells is combined versus its expression on tumour cells alone[160,161]. The predictive value of these biomarkers in combination has been evaluated in R/M HNSCC. PDL1, TMB and GEP were independently associated with response to anti-PD1 mAb therapy, with the highest efficacy in patients with high TMB and high GEP or high TMB and high PDL1, with correlation only found between PDL1 and GEP[159]. Remarkably, the response rate was still only 34% in patients with a favourable combination of these biomarkers[159]. Intratumoural hypoxia has also been shown to be predictive in R/M HNSCC and, importantly, is a biomarker that has the potential to be therapeutically targeted[162]. Data are conflicting as to whether HPV positivity is associated with a higher chance of response, with a more consistent increased response observed in HPV-positive than in HPV-negative disease when both groups expressed PDL1 (refs.[159,162]). What is clear and important is that both patients with HPV-positive and patients with HPV-negative HNSCC benefit from anti-PD1 mAb therapy over standard-of-care chemotherapy and that the magnitude of benefit is similar in

both groups[163]. While data are consistent in that the TME is more immunogenic in patients with HPV-positive disease, the great majority of these data are from patients at the time of their initial presentation with earlier-stage disease. An analysis of paired primary and recurrent HNSCC tumours demonstrated a significant decrease in $CD8^+$ T cells, NK cells and B cells in recurrent tumours, with chemoradiation as part of their initial therapy at diagnosis being associated with a more immune suppressed TME at recurrence[164]. Although more data are needed, these findings suggest that the immune infiltration and activation status of HPV-positive and HPV-negative tumours might be more similar at recurrence than it is at initial presentation, potentially accounting for comparable efficacy of anti-PD1 mAb therapy in HPV-positive and HPV-negative HSNCC tumours.

Conclusions

With these lessons in mind, the complete HNSCC TME needs to be considered when designing next-generation immunotherapies (Fig. 6). Specific to $CD8^+$ T cells, additional work on how non-viral neoantigen-specific $CD8^+$ T cells could differentially contribute to therapeutic responses in patients with both HPV-positive and HPV-negative tumours will be important. Furthermore, how to augment $CD8^+$ T cells with local intratumoural $CD4^+$ T cell differentiation should be considered in the next generation of immunotherapies while specific therapeutics to dampen tumour T_{reg} cell activity should be employed to release the brake on $CD8^+$ T cell function. Cell types other than T cells might also greatly enhance antitumour activity in patients with HNSCC. Distinct B cell targeting strategies might be needed in patients with HPV-positive and HPV-negative HNSCC tumours but, overall, these strategies should focus on inducing TLS formation and driving antigen-specific intratumoural B cell maturation. Furthermore, an increased understanding of NK cell subset functions in patients with HNSCC is necessary for improved immunity and, specifically, to increase the influx of NK cells into the TME and improve the spatial interplay with myeloid cells such as DCs and macrophages. In addition, reducing M2 polarization and decreasing MDSC-related immunosuppression could alleviate pro-tumour immunity in both HPV-positive and HPV-negative TMEs. Finally, future studies should explore the effect of CAFs and MSCs in the TME and in the regulation of immunity before and after immunotherapy. New technologies, such as scRNA sequencing, multiplexed spatial imaging and spatial transcriptomics, will also reveal new targets of the TME to consider as next-generation therapies combined with current immunotherapies such as anti-PD1; however, other technologies, such as immunoPET, and preclinical models will be necessary to validate and implement these new targets. ImmunoPET is a new technology that combines the specificity of mAbs with highly sensitive PET to enable a 3D probing of the TME, which will be particularly useful in imaging the complex cellular neighbourhoods in the TME, in particular, TLS formation and maturation. Furthermore, while current preclinical markers for HNSCC enable a basic evaluation of the TME, it will be important to develop, in the longer term, more physiological murine models that reflect the differences outlined in this Review for target testing and validation. In this Review, we have summarized several new targets to further interrogate, which go far beyond the T cell. It is not just one immune player that must be targeted but, rather, an entire orchestra of immune and non-immune players that must synchronize for robust improvements in therapeutic strategies for patients

with HPV-positive and HPV-negative HNSCC tumours. These new technologies and robust analyses of the TME can help with biomarker identification and, more importantly, could lead to personalized therapeutics in patients with HNSCC.

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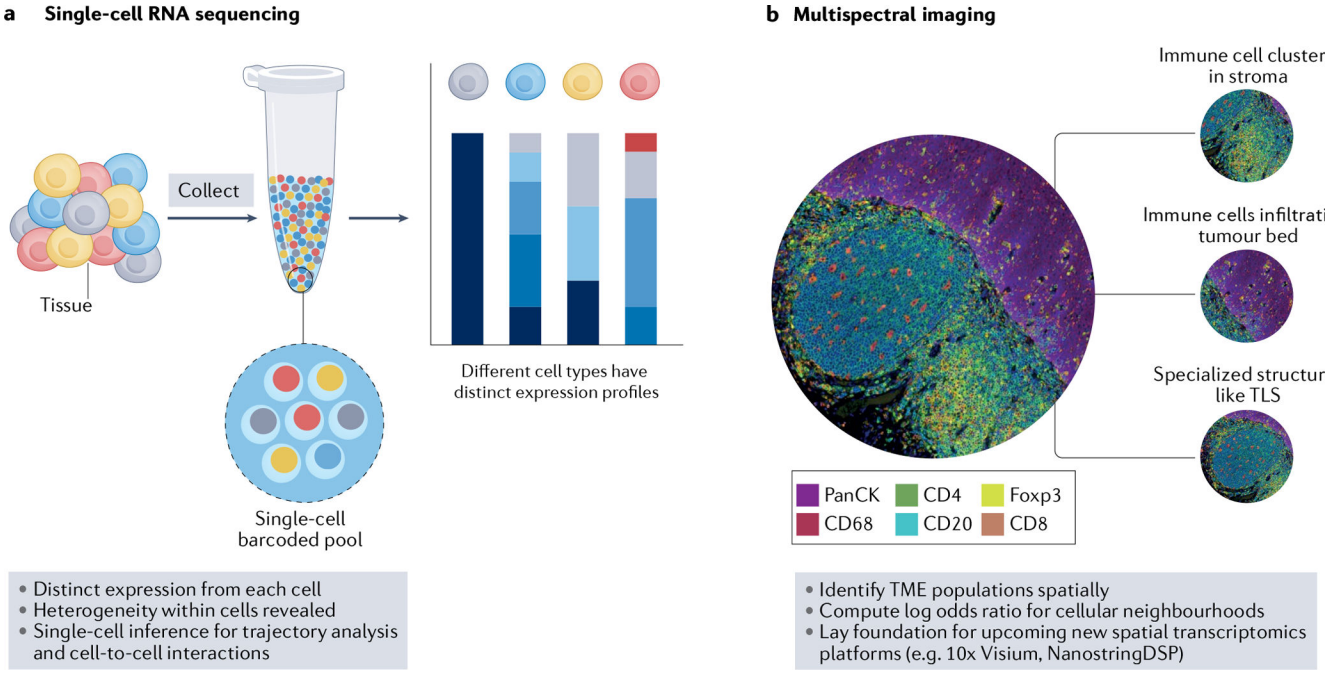

Fig. 1 |. Novel technologies have begun to elucidate the clear complexity of the HNSCC TME.

a, Single-cell RNA sequencing enables distinct expression from each cell and reveals heterogeneity within immune and non-immune cell subsets. Furthermore, bioinformatic analyses can provide single-cell inference for trajectory analysis and cell-to-cell interactions. **b**, Multispectral imaging is paramount to identifying tumour microenvironment (TME) populations spatially and analysing cellular interactions within and between tumour and stroma. Bioinformatic analyses can also determine cellular neighbourhoods within the head and neck squamous cell carcinoma (HNSCC) TME, and these imaging technologies have laid the foundation for new and upcoming spatial transcriptomic platforms that will increase our spatial knowledge of the HNSCC TME. TLS, tertiary lymphoid structure.

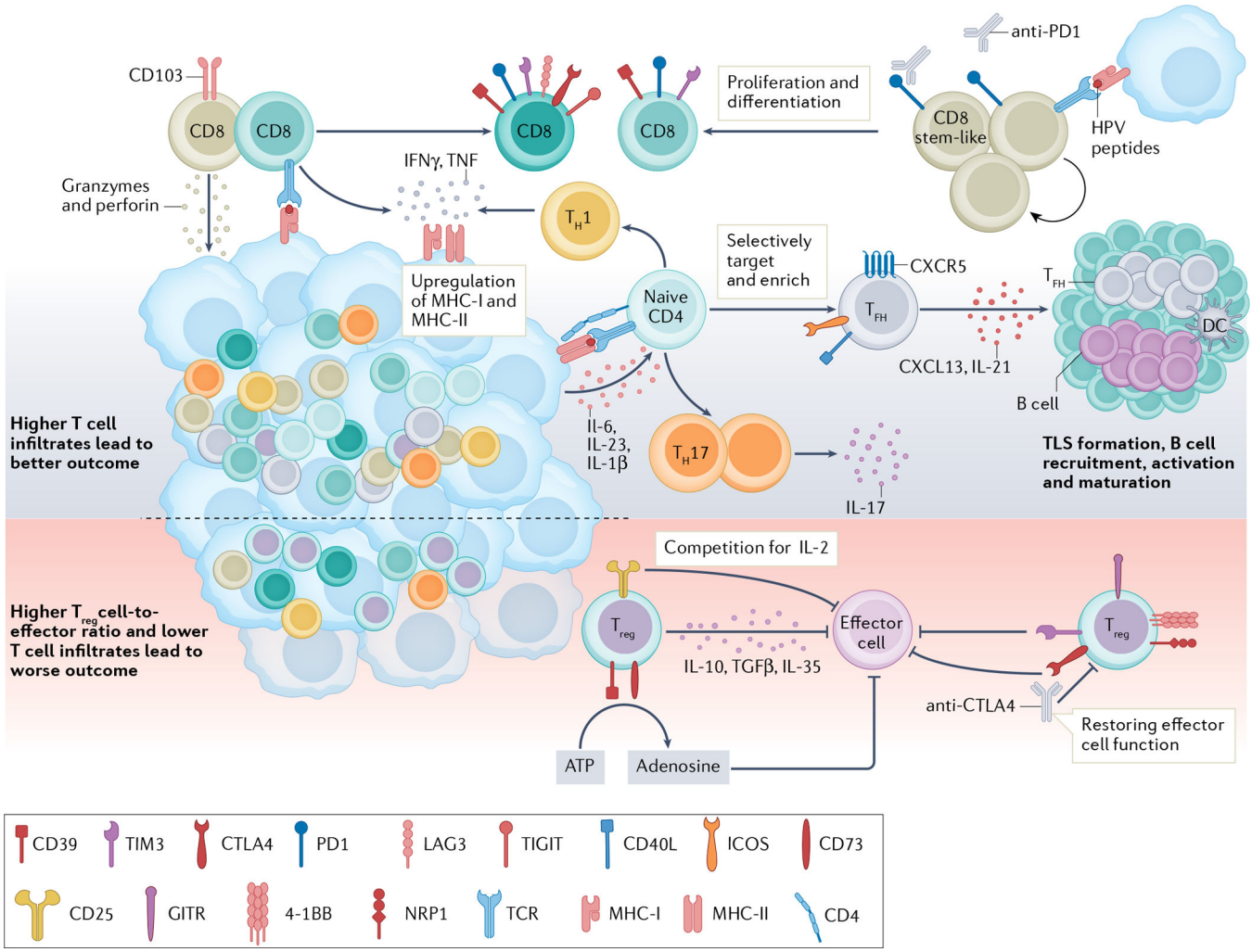

Fig. 2 |. New T cell targets in the HNSCC TME.

T cells are important in antitumour immunity, and human papillomavirus (HPV)-positive tumours generally show higher T cell infiltration and are associated with better outcomes than HPV-negative tumours[32–34]. $CD8^+$ T cells can directly lyse target cells by releasing granzymes and perforin in an antigen-directed manner. Chronic engagement with tumour antigens leads to a dysfunctional state characterized by high expression of immune-checkpoint receptors (PD1, T cell immunoglobulin and mucin domain 3 (TIM3), lymphocyte-activation protein 3 (LAG3) and CTLA4)[36–39]. An HPV16-specific $CD8^+PD1^+$ T cell population in HPV-positive tumours contains a stem-like $CD8^+$ T cell subset. These cells are capable of proliferating and differentiating into effector cells upon HPV peptide stimulation and represent a population of future therapeutic targets[28]. $CD4^+$ T lymphocytes recognize major histocompatibility complex (MHC) class II antigens and differentiate into different subtypes upon antigen stimulation. A higher number of T helper 1 (T_H1) and T_H17 cells are found in HPV-positive tumours than in HPV-negative tumours[48,49]. T_H1 cells release IFNγ and TNF and promote MHC class I and II upregulation on cancer cells, facilitating tumour elimination[43,44]. IL-17-releasing T_H17 cells are induced by IL-6, IL-23 and IL-1β produced by primary tumour cells[51,52]. $CXCR5^+PD1^+ICOS^+CD40L^+$ T

follicular helper (T_{FH}) cells produce CXCL13 and IL-21, recruiting B cells into the tumour microenvironment (TME)[56,57]. T_{FH} cells are essential for B cell activation, maturation and tertiary lymphoid structure (TLS) formation in tumours, and their presence is associated with improved outcomes[29]. T regulatory (T_{reg}) cells have an immunosuppressive role in the TME. T_{reg} cells can suppress effector cells through the release of IL-10, TGFβ and IL-35. High levels of CD25 on T_{reg} cells compete with effector cells for local IL-2, so that effector cells might not have enough IL-2 to survive and function[58–60]. T_{reg} cells also express CD39 and CD73, which convert extracellular ATP to adenosine and impair effector T cell function[58,67]. Co-expression of 4–1BB, GITR and neuropilin 1 (NRP1) on intratumoural T_{reg} cells demonstrated an enhanced suppression function[29,61,63]. Immune-checkpoint receptors, such as TIM3 and CTLA4, are reportedly expressed by T_{reg} cells in head and neck squamous cell carcinoma (HNSCC). Both are highly expressed by intratumoural T_{reg} cells and able to suppress effector cell functions[21,22,63,64]. Therapeutically targeting T_{reg} cells might help rejuvenate effector cell function. DC, dendritic cell; ICOS, inducible T cell co-stimulator; TCR, T cell receptor; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains.

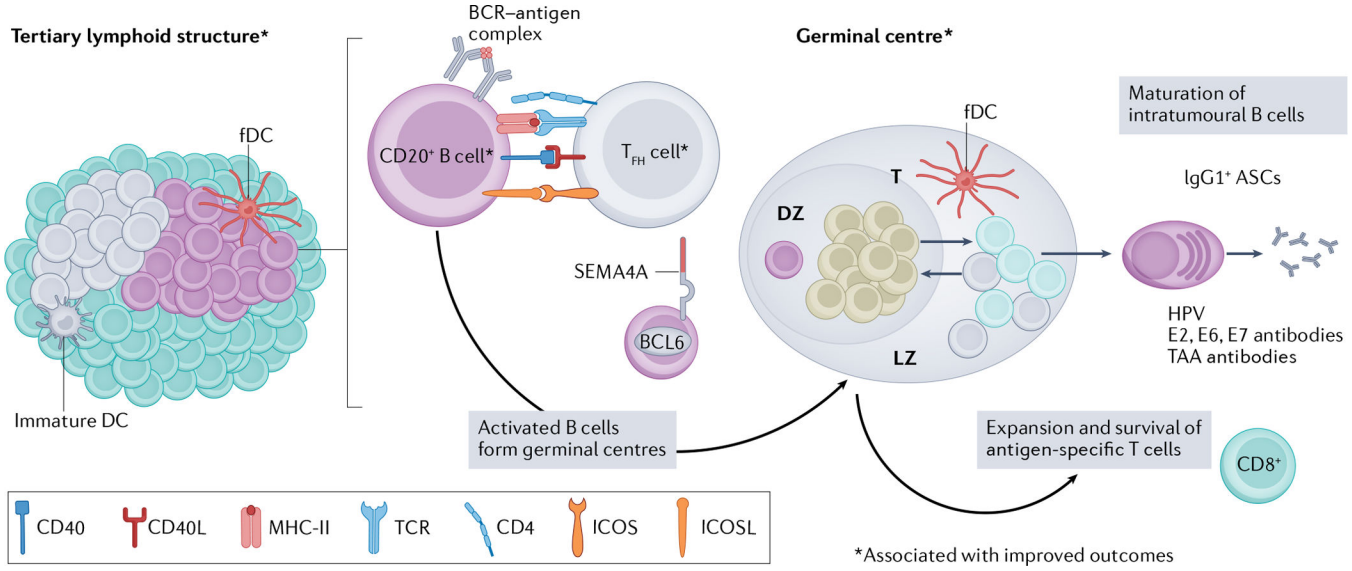

Fig. 3 |. Increasing cellular interactions with tertiary lymphoid structures in the HNSCC TME for maximal humoral and cellular immunity.

Tertiary lymphoid structures (TLS) form within the tumour or surrounding the tumour stroma in patients with head and neck squamous cell carcinoma (HNSCC)[17,78,84]. TLS vary in size and cell composition in patients, but an increased presence of TLS correlates with improved survival and reduced risk of recurrence in both human papillomavirus (HPV)-positive and HPV-negative HNSCC[17]. Within TLS, B cells co-localize with T follicular helper (T_{FH}) cells and receive activation signals via CD40, inducible T cell co-stimulator ligand (ICOSL) and major histocompatibility complex class II (MHC-II) engagement[17,29,84]. Activation of B cells via T_{FH} cells leads to germinal centre formation, which is regulated by transcription factor B cell lymphoma 6 (BCL6) and marked by surface expression of semaphorin 4A (SEMA4A) on germinal centre B cells[17,165]. Intratumoural germinal centre B cells in HPV-positive HNSCC have three distinct cell states: dark zone (DZ), light zone (LZ) and transitional (T), marked by unique patterns of gene expression that are important for the selection and maturation of B cells[17]. Formation of germinal centres within TLS is an independent predictor of superior progression-free survival in HNSCC[17]. As a result of successful germinal centre formation in HPV-positive HNSCC, germinal centres produce terminally differentiated somatic hypermutated antibody-secreting cells (ASCs) that have undergone immunoglobulin class-switching to an IgG1 isotype[82]. $IgG1^+$ ASCs in patients with HPV-positive disease recognize HPV viral proteins E6, E7 and E2 (ref.[82]). Antibodies directed at tumour-associated antigens (TAAs) have also been detected in the serum of patients with HPV-positive and HPV-negative HNSCC[78]. Activated B cells might also provide co-stimulatory signals to $CD8^+$ T cells, resulting in their expansion and survival in HNSCC tumours[81,84]. Co-localization of B cells with $CD8^+$ T cells is associated with favourable outcomes in HNSCC[81,84]. Together, these features provide a new targetable axis for future immunotherapies, which should be directed at enhancing TLS formation, maturation of B cells and increasing B–T cell interactions within the TME of HNSCC. BCR, B cell receptor; fDC, follicular dendritic cell; ICOS, inducible T cell co-stimulator; TCR, T cell receptor.

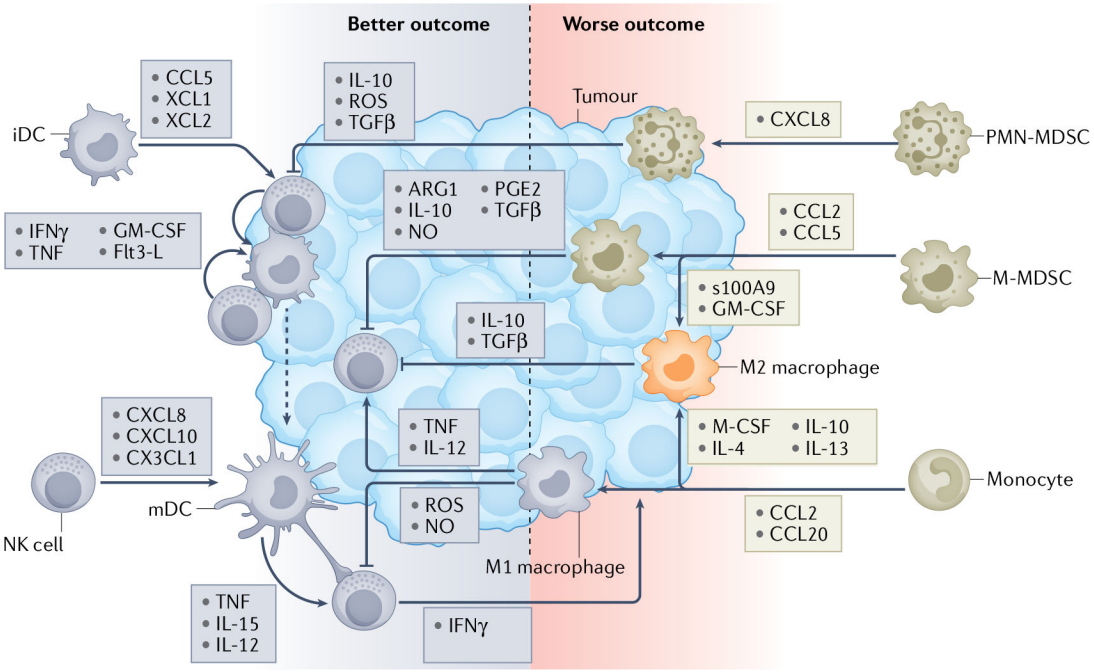

Fig. 4 |. Innate cell interactions generate inflammatory signals that drive outcomes in patients with HNSCC.

Innate immunity, an evolutionary defence against pathogens or tissue damage, plays a central role in antitumour defence as it dictates the robustness and function of tumour immune infiltrates. Human papillomavirus (HPV)-positive lesions generally have a greater level of innate immune infiltrates than HPV-negative lesions, which generally correlates with a better clinical outcome. Natural killer (NK) cell–dendritic cell (DC) crosstalk plays a pivotal role in antitumour immunity[89,92]. Enhanced NK cell and DC infiltrates correlate with better patient survival[99,100]. Tumour-infiltrating NK cells can recruit immature DC (iDCs) into the tumour microenvironment (TME) by releasing CCL5, XCL1 and XCL2 (ref.[166]). Once inside the tumour, iDCs can take up cell debris from killed tumour cells, which is the first step required for effective antigen presentation[105]. As antigen-loaded DCs start to mature, they can engage and crosstalk with NK cells with their long dendrites. NK cells enhance DC maturation and polarization through release of IFNγ, TNF, granulocyte–macrophage colony-stimulating factor (GM-CSF) and Flt3-L[89,98]. Maturing DCs (mDCs) in turn enhance NK cell activation through IL-12 secretion as well as cell-to-cell contact mediated by transmembrane TNF and *trans*-presented IL-15 (ref.[104]). Furthermore, they release a number of chemokines, including CXCL8, CXCL10 and CX3CL1, that enhance the number of NK cells infiltrating the tumour[89,167]. This cascade of events initiates and potentiates tumour-specific adaptive T cell responses that are required for effective tumour elimination[105]. The myeloid compartment is highly diverse and consists of various cell subsets that exhibit a broad range of immune functions. Myeloid cells commonly infiltrate the tumours and associated stroma and their presence influences outcomes in patients with head and neck squamous cell carcinoma (HNSCC). Patients with worse clinical outcome generally display higher numbers of tumour-infiltrating macrophages and myeloid-derived suppressor cells (MDSCs)[107,118,119,123,125]. Monocytes, monocytic MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) are recruited into a tumour by specific

chemokines[109,121]. Once in the tumour, monocytes can differentiate into antitumour M1 macrophages and immunosuppressive M2 macrophages depending on the inflammatory signals they receive within the TME[110]. M-MDSCs can maintain their immature myeloid profile within the TME or can further differentiate into M2 macrophages under the influence of endogenous S100A9 and exogenous GM-CSF[108,111–113]. M2 macrophages, M-MDSCs and PMN-MDSCs all have a variety of mechanisms by which they can suppress NK cell and DC activation and skew their polarization towards an immunoregulatory phenotype. M1 macrophages, which can activate NK cells by IL-12 and TNF production, can also inhibit NK cell function by reactive oxygen species (ROS) and nitric oxide (NO) release[116,117].

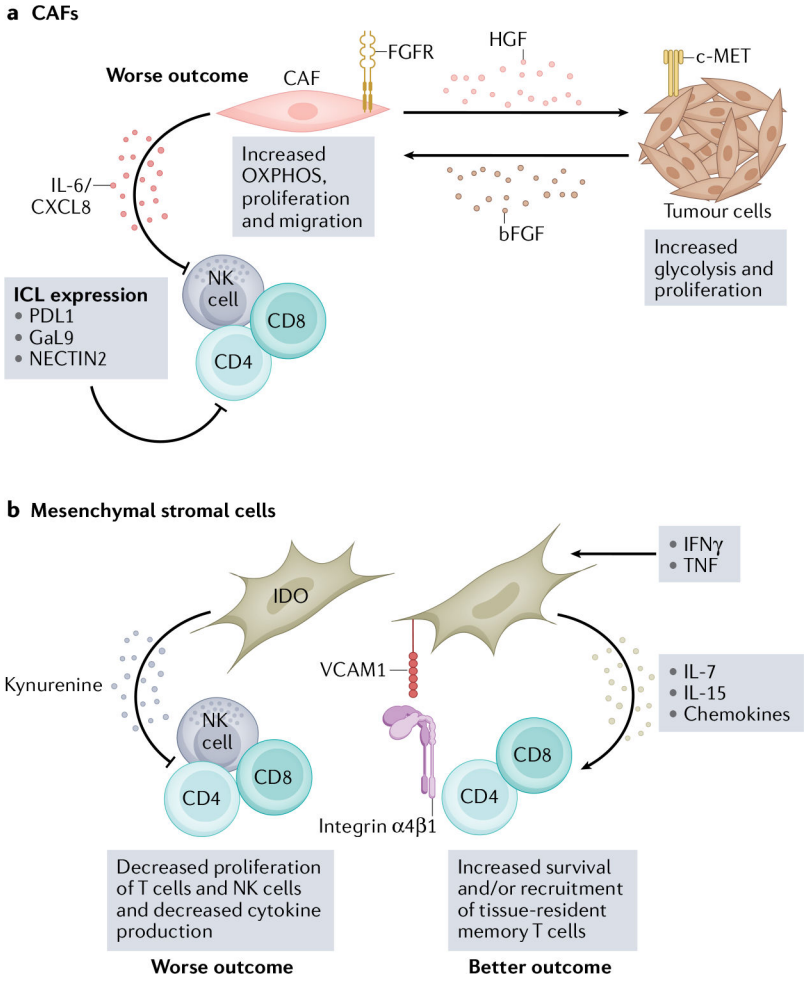

Fig. 5 |. The stromal microenvironment is functionally important for the HNSCC TME.
The head and neck squamous cell carcinoma (HNSCC) tumour microenvironment (TME) consists of a diverse stromal cell compartment — which includes cancer-associated fibroblasts (CAFs) and mesenchymal stromal cells (MSCs) — that interacts with neighbouring tumour cells and infiltrating immune cells[106,143]. These interactions primarily promote tumour growth, progression and metastases but, under some conditions, can provide a supportive environment for immune cells and recruit immune cells to the HNSCC TME[132,141]. Combining immune-checkpoint therapy with therapies that target the stromal compartment might improve patient outcomes in HNSCC. **a**, CAFs and tumour cells have a metabolic relationship mediated by hepatocyte growth factor (HGF) and basic fibroblast growth factor basic (bFGF), which increases oxidative phosphorylation (OXPHOS) in CAFs and glycolysis in tumour cells[137]. Increased oxidative phosphorylation in CAFs leads to IL-6 and CXCL8 production, which suppress immune cell function[134,137]. CAFs also express several immune-checkpoint ligands (ICL), including PDL1, galectin 9 (GaL9) and nectin cell adhesion molecule 2 (NECTIN2), which interact with corresponding inhibitory receptors on natural killer (NK) cells and T cells[28]. **b**, MSCs can also suppress immune cell function in vitro via indoleamine-pyrrole 2,3-dioxygenase (IDO), which metabolizes tryptophan into kynurenine and is cytotoxic to T cells and NK cells[140,142,143]. However,

some in vitro studies suggest that MSCs can support tissue-resident memory T cells via IL-7 and IL-15 production in the presence of IFNγ and TNF[144]. VCAM1, vascular cell adhesion protein 1.

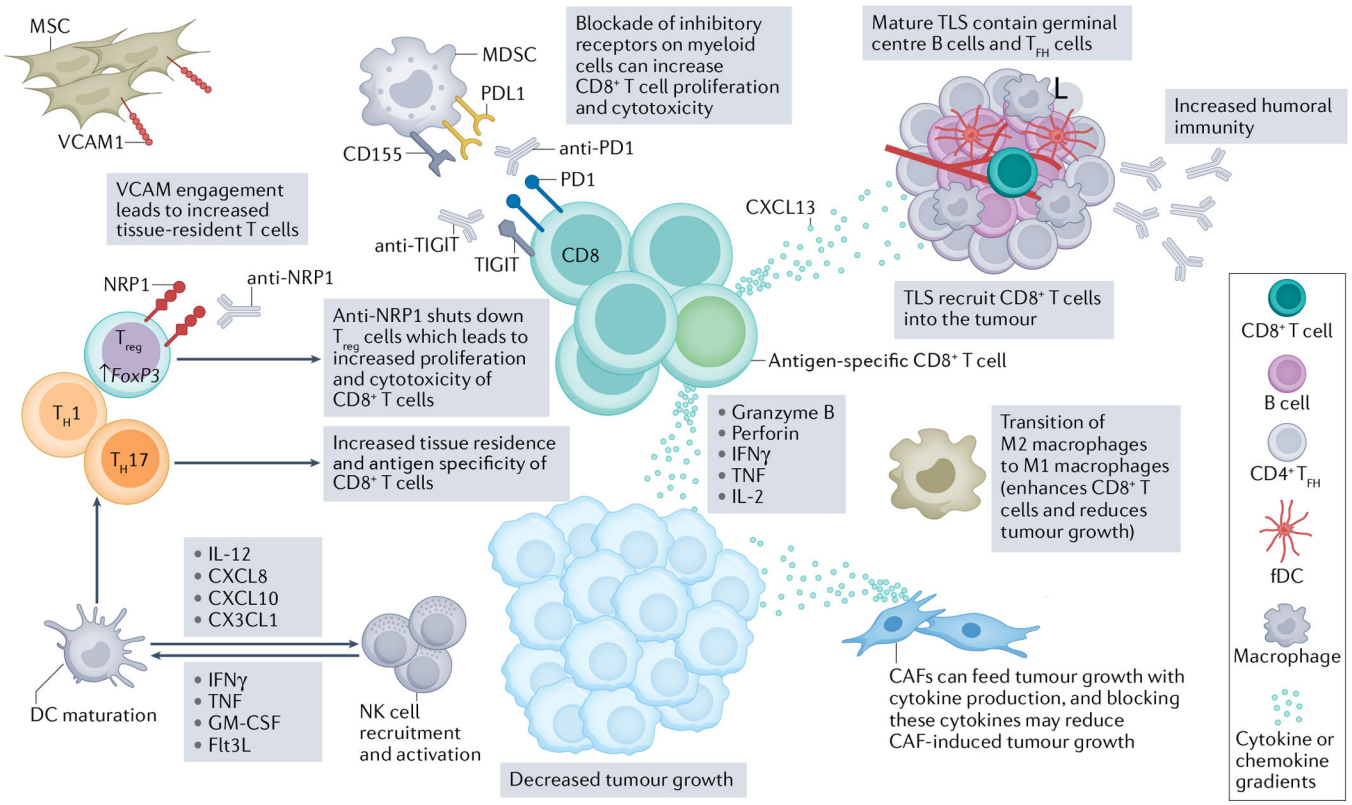

Fig. 6 |. Immune and non-immune therapeutic targets in the HNSCC TME.

The head and neck squamous cell carcinoma (HNSCC) tumour microenvironment (TME) hosts a complex interplay of immune and non-immune cells that can amplify tumour killing. Augmenting aspects of antitumour immunity while suppressing pro-tumour immunity will be key in next-generation therapeutics. Although the $CD8^+$ T cell has been the centre of immunotherapeutic strategies for several years, several other axes can be targeted for improved antitumour $CD8^+$ T cell responses. First, blockade of the PD1–PDL1 pathway on myeloid cells, in particular myeloid-derived suppressor cells (MDSCs), has been an important consideration for improved intratumoural $CD8^+$ T cell function. Additionally, other inhibitory receptors, like T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), can be blocked on myeloid cells to improve responses to anti-PD1 immunotherapy[125]. Further, reinvigoration of these antigen-specific $CD8^+$ T cells can result in increased production of key chemokines, such as CXCL13, that can trigger tertiary lymphoid structure (TLS) development in the TME[168]. Amplifying mature, germinal centre-containing TLS will lead to increased recruitment of $CD8^+$ T cells and increased antitumour humoral immunity. Further, directly enhancing germinal centre B cells and T follicular helper (T_{FH}) cells could be paramount for increased antitumour immunity as they correlate with increased prognosis[17,29]. T_{FH} cells also secrete CXCL13, which helps with further recruitment of B cells and $CD8^+$ T cells into the TME and TLS[53–55]. Evidence exists to indicate that the transition of M2 macrophages to M1 macrophages will further enhance antitumour immunity[112]. Furthermore, dendritic cell (DC) maturation can be fuelled by natural killer (NK) cells via secretion of IFN**γ**, TNF, granulocyte–macrophage colony-stimulating factor (GM-CSF) and Flt3L[89,98], which in turn will also help to activate

and recruit more NK cells via IL-12, CXCL8, CXCL10 and CX3CL1 (refs.[89,104,167]). DC maturation is also instrumental in educating productive T helper 1 (T_H1) and T_H17 responses, which ultimately lead to increased tissue residence and antigen specificity of $CD8^+$ T cells. While T regulatory (T_{reg}) cells have dampened $CD8^+$ T cells in the TME, new therapeutics, such as neuropilin 1 (NRP1) blockade, can specifically reduce immunosuppression without any effects on homeostatic balance in patients[61,65]. Finally, non-immune cells, such as cancer associated fibroblasts (CAFs) and mesenchymal stromal cells (MSCs), have a dichotomy of function in the TME. CAFs can feed tumour growth via cytokine production, and the metabolic crosstalk between CAFs and tumour cells could be a targetable axis to suppress tumour growth. MSCs can be advantageous in recruiting tissue-resident T cells to the TME in a vascular cell adhesion protein 1 (VCAM1)-dependent manner[144]. Ultimately, next-generation immunotherapies for HNSCC will be multi-faceted to improve patient response and treatment durability. fDC, follicular dendritic cell.

Table 1 |

HNSCC: one disease, two distinct tumour microenvironments

HNSCC type	Carcinogen	Anatomical site	Incidence	Tumour microenvironment
HPV-negative HNSCC	Tobacco, alcohol	Oral cavity, pharynx, larynx	Decreased since 2000s	Decreased immune infiltration
HPV-positive HNSCC	HPV	Oropharyngeal	Increased since 2000s	Increased immune infiltration

HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus.