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Immune-relevant aspects of murine models of head and neck cancer

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Abstract

Head and neck cancers (HNC) cause significant mortality and morbidity. There have been few advances in therapeutic management of HNC in the past 4 to 5 decades, which supports the need for studies focusing on HNC biology. In recent years, increased recognition of the relevance of the host response in cancer progression has led to novel therapeutic strategies and putative biomarkers of tumor aggressiveness. However, tumor-immune interactions are highly complex and vary with cancer type. Pre-clinical, in vivo models represent an important and necessary step in understanding biological processes involved in development, progression and treatment of HNC. Rodents (mice, rats, hamsters) are the most frequently used animal models in HNC research. The relevance and utility of information generated by studies in murine models is unquestionable, but it is also limited in application to tumor-immune interactions. In this review, we present information regarding the immune-specific characteristics of the murine models most commonly used in HNC research, including immunocompromised and immunocompetent animals. The particular characteristics of xenograft, chemically-induced, syngeneic, transgenic, and humanized models are discussed in order to provide context and insight for researchers interested in the in vivo study of tumor-immune interactions in HNC.

Keywords

experimental models; immunology

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Competing interests

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References are cited in the manuscript (text and table) followed by an asterisk (*) can be found in the appendix

Introduction

Head and neck cancer (HNC) is the sixth most common cancer worldwide (1). Most HNCs (>90%) are squamous cell carcinomas, which are aggressive tumors that are associated with high treatment morbidity. Although survival rates for most types of cancer have improved from 2005 to 2011, the prognosis of HNC remains poor; approximately 40% of patients die within 5 years of diagnosis (2). Loco-regional spread causes significant morbidity due to functional and esthetic impairment (3). Approximately 40% of HNC metastasize to cervical lymph nodes (4–6), which is a significant negative prognostic factor; 60–75% of patients with metastases die within 5 years (7–9). Lymphatic, hematogenous and perineural spread frequently occur together as these structures are anatomically and functionally 'bundled' together, although distinct mechanisms may be involved for each (9, 10).

The poor prognosis for HNC highlights the relevance for improved understanding of mechanisms of tumor progression. Oncogenic phenotypes, including proliferation, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, survival, angiogenesis, invasion and metastasis (11), are all influenced by the immune system. In support of the relevance of the immune system, chronic inflammation is associated with various cancers (12); *Helicobacter pylori* infection and gastric cancers (13), inflammatory bowel disease and colon cancer (14), prostatic inflammation and prostate cancer (15), and smoking and obesity and lung cancer (16). In fact, cancer-related inflammation has been proposed as the seventh hallmark of cancer (11), a recognition of the importance of the immune response in the development, prognosis, progression and treatment of cancer.

Recent increased interest in HNC immunology is illustrated by the results of an online search on the NCBI/PubMed database in March 2018 using the string "Head and neck cancer AND immune response", which returned an average of 125 papers in 2016 and 2017 compared to 36 in 2006 and 2007. Studies on tumor immunology have provided breakthroughs in understanding how immunoevasion and immunosubversion allow and promote, respectively, tumor growth and progression. These breakthroughs have facilitated the development of therapies to rescue immune competence in detecting and eliminating cancer (17).

In the past 30 years, studies on etiopathogenesis and treatment of HNC have focused on neoplastic cells exploring genetic and epigenetic changes that potentially regulate prognosis, invasion, and therapeutic targets. These studies used diverse in vivo models, typically xenografts of human neoplastic cells or tissues in immunocompromised animals. Information derived from studies using immunodeficient models has limitations in applicability to human HNC, which may be one reason why anti-tumor effects of drugs that are observed in pre-clinical studies are not always duplicated in humans.

It is important to consider specific characteristics of different in vivo models when selecting these models for investigations of tumor-immune interactions (Tables 1 and 4). Although excellent reviews of in vivo models of HNC have been published in the past 12 years (18–24), these have not specifically addressed the immune-related characteristics and suitability for investigations of tumor-immune interactions. The purpose of this review is to summarize

the immune-related aspects (Figure 1, Tables 2 and 3) and to discuss the suitability of common murine models of HNC for investigations of tumor-immune interactions (Table 4).

Immunocompromised models

The majority of studies of HNC are performed in immunocompromised mice. A search of NCBI/PubMed database using the string "Head and Neck Squamous Cell Carcinoma" and the filter for 'Other Animals' considering only publications in English from 2017 till April 2018, revealed that the athymic nude mouse and the severe combined immunodeficient (SCID) mouse are the most frequently used animal models, with 133 and 29 published studies, respectively. These investigations have limited relevance to tumor-immune interactions since the animals are immunocompromised. Immunocompetent models include chemically-induced models in mice and rats (15 studies) and hamster cheek-pouch (10 studies); syngeneic mouse models (8 studies), genetically-modified animals (5 studies) and genetically-modified humanized mice (2 studies).

Athymic nude mice have an autosomal recessive mutation in the transcription factor, *Foxn1* (forkheadbox n1) gene, and lack of thymus development. The phenotypic characteristics are defective hair growth and shorter lifespan compared to euthymic mice (25, 26). Athymic mice lack mature T-cells, which is the basis for their utility in cancer research, since these animals are unable to reject allografts and xenografts (27, 28). However, these mice are not completely immunodeficient, since innate immunity and T cell-independent immune functions are preserved and even heightened compared to wild-type animals (29, 30).

The athymic nude rat has also been proposed as a model for HNC mainly because of its relative robustness (reduced severity of wasting allowing for longer periods of study) in comparison to mice, and due to advantages for imaging studies including lower doses of whole-body radiation (31). However, the larger size requires more housing space and may increase the cost of therapeutic studies.

There is a high rate of cell engraftment in nude mice and rats, but tumors tend to grow encapsulated and without metastases to liver or lungs (32, 33). These growth characteristics are observed both in heterotopic and orthotopic models, and may be associated with the increased numbers of NK, γT, and B cells. Heterotopic (e.g. subcutaneous at the dorsum of flank) or orthotopic (e.g., tongue, floor or mouth) location of the xenograft is of relevance for the assessment of progression, invasion and metastasis, as these outcomes are highly influenced by the anatomy and histology of the microenvironment. It is possible that tumor-immune interactions with xenografts is influenced by variations in the density of microvasculature, nerves, lymphatic vessels and proximity to regional lymph nodes; moreover in orthotopic models, the oral microbiome adds an additional immune-modulating aspect. Nevertheless, nude mice and rats have no defect in the non-MHC-restricted response and the cytotoxic activity of NK cells is significantly higher in comparison with euthymic animals, which may be a compensatory mechanism (34). Over 90% of circulating lymphocytes in nude mice and rats are B cells. Innate immune cells (dendritic cells, neutrophils, and monocytes/macrophages) are preserved in number and function, and innate immune responses are more pronounced and efficient than in euthymic animals (29, 30, 35).

In engineered antibody therapeutics, monocytes/macrophages, dendritic cells and NK cells mediate cytotoxic activity of anti-tumor monoclonal antibodies in nude mice through their Fcγ receptors (36, 37). Some studies have used nude animals to explore the mechanisms associated with co-opting macrophages (38–40) and neutrophils (41–43) to enhance solid tumor progression.

A random mutation causing SCID syndrome was initially observed in mice (44) and was later traced to changes in genes encoding DNA-dependent protein kinase catalytic subunits (DNA-PKcs) (45). These mutations disrupt non-homologous end joining recombination (NHEJ), which is required for genetic rearrangement of variable (V), diversity (D) and joining (J) genes. Development of all lymphocytes is abrogated, so these animals do not have B or T cells (46). Due to severe immunodeficiency, the successful take rate of xenografts in SCID mice is higher than in nude animals. In contrast to nude animals, NK cell function in SCID mice varies from normal (C.B-17) to defective (NOD/SCID, C3H/SCID), in different strains (47, 48).

Xenograft survival in SCID animals is still limited as the remaining innate immune components are involved in rejection of human cell xenografts, independently of functional B or T cells (49, 50). Importantly, both nude and SCID animals may present a 'leaky phenotype', with development of some functional B and T cells arising from residual and low frequency NHEJ (51). This 'leakiness' varies with the background strain of the animals, housing conditions, and age (47). An alternative to the SCID mutation in DNA-PKcs is the disruption of *Rag2* gene, which is also involved in NHEJ and is required for maturation of B and T cells (52). In contrast to SCID animals, deletion of *Rag2* results in lack of CD3+, TCRαβ, TCRγδ, CD4+/CD8+ and CD4-/CD8- cells; however a minute population of single positive (CD4+ or CD8+) cells is still detectable. *Rag2*-deleted animals do not have a leaky phenotype and their immune phenotype is similar to that of SCID animals (53). Similarly, *Rag1*-knockout mice have no mature B or T lymphocytes (54), indicating that both Rag1 and Rag2 are required for V(D)J recombination in vivo.

With advancement and improvement of rodent genetic manipulation techniques a number of other mutations causing various levels of immunosuppression have been described and many are available through commercial suppliers. More recently, X-SCID mice (55) and X-SCID rats (56), were generated with mutation in the gene of the common gamma chain (γc) of the IL-2 receptor (*Il2rg*) in the X-chromosome (56), which is homologous to the X-linked form of severe combined immunodeficiency syndrome in humans (57). Lack of *Il2rg* renders the cells unresponsive to various cytokines, including IL-2, IL-4, IL-7, IL-15 and IL-21. The T cells express TCR, CD3, CD28 and are responsive to γc-independent stimuli (e.g., PMA +ionomycin, CD3/CD28 antibodies); however cytokine production is markedly reduced and there is no evidence of MHC-I activation and cytolytic ability. B cells are also markedly reduced in number and maturation in X-SCID mice, with a corresponding decrease in all immunoglobulin classes, except for IgM (55). Both monocyte/macrophage and neutrophil populations are increased in X-SCID mice (58). Successful take of human ovarian cancer cell xenografts that were otherwise rejected in immunocompetent wild-type rats of the same strain supported their utility in cancer and transplant research (56).

In contrast to nude animals, peripheral lymph nodes and lymphatic structures such as Peyer's patches are either absent/undetectable or severely underdeveloped in SCID (59), X-SCID and *IL2rg* KO (55, 58), *Rag2* KO (60), *Rag1* KO (54) and NOD/SCID *IL2rg* KO (NOG) mice (61). The lack or underdevelopment of these secondary lymphoid structures influences antigen presentation and T-cell priming in the adaptive immune response, which is another important peculiarity of these mice that should be considered when studying tumor-immune interactions.

Models using patient-derived xenografts (PDX) of HNC have been introduced more recently (62) taking advantage of these severely immunocompromised mouse strains to enhance survival of xenografts and to study tumor-immune interactions when associated with reconstitution with patient-derived immune cells or hematopoietic stem cells (see the syngeneic and humanized models section for comment). In immunocompromised animals, PDX models allow investigations of growth and invasion mechanisms and response to treatment with a phenotype corresponding to the individual patient (63, 64), as opposed to the use of established human cell lines that have more homogenous genetic and phenotypic traits. It is important to consider the difficulties associated with the establishment of heterotopic PDX in immunocompromised mice, which involve optimization of animal conditioning (pre-grafting irradiation, antibiotic treatment) and preparation of patient-derived samples (size of the fragments, enzymatic digestion, depletion of infiltrating T cells) (62). The reported 17% efficacy of successful engraftment of PDX HNC tissues in immunocompromised mice is low and may also be affected by the phenotype of the tumors, as successful take of the tumors is greater for more aggressive, poorly-differentiated tumors associated with lymph node involvement (64). Although these PDX models in non-humanized (i.e., non immune-reconstituted) mice are appealing from the perspective of personalized medicine, the assessment of tumor-immune interactions is subject to the same limitations already discussed for immunocompromised animals (e.g., lack of a fully competent immune system, and the microenvironment comprising mouse-derived extracellular matrix, mesenchymal cells and remaining immune cells) in addition to the possible influences related with heterotopic or orthotopic location of the xenografts (Table 4).

In summary, immunodeficient rodent models facilitate investigations of interactions between innate immune and HNC cells, but their defect in cellular and humoral immunity must be considered for implications to tumor immunology. Although this drawback appears obvious, immunodeficient animals have been used in the study of tumor-immune interactions in vivo, such as the correlation between expression of class I and II MHC antigens by neoplastic epithelial cells and their tumorigenicity (65), or co-opting innate immune cells to favor solid tumor progression (40, 41).

Immunocompetent – chemically-induced models

These models use chemicals to induce DNA damage leading to random mutations and oncogenic transformation that more closely resemble the genetic heterogeneity of human disease. In contrast to models directly grafting tumor cells or tissues, there is no 'abrupt' and 'overwhelming' challenge to the immune system. The chemical agents are titrated and either

topically applied or dissolved in drinking water. Also, in contrast with xenograft models, tumor development is preceded by epithelial dysplasia and chronic inflammation in the connective tissue (66, 67), similar to human HNC (68).

The first immunocompetent murine model was the hamster cheek-pouch (69), primarily used for chemopreventive studies (70–73). The buccal pouch mucosa is exposed to 9,10 dimethyl-1,2 benzathracene (DMBA), a carcinogen. Importantly, although the cheek pouch is a normal anatomic structure in the hamster oral cavity, humans have no similar structure. While histologically similar to human oral mucosa, the cheek pouch is characterized by reduced or absent lymphatic drainage, which limits its utility to study tumor-immune interactions (74, 75). Some studies assessed inflammation in the cheek pouch (76–78) mainly due to convenient access and easy documentation attributable to translucence of the thin tissue. However, the dense connective tissue underlying the epithelium, coupled with the lack of lymphocytic infiltrations and lymphatic vessels (79, 80) and lack of rejection of xenograft transplants at this site indicate its immunoprivileged status (81, 82). A variation to this model is topical application of a carcinogen on the tongue, bypassing issues with lymphatic drainage. This model is usually associated with induction of mechanical trauma to the dorsum of the tongue to increase exposure of the tissues to the chemical. However, most tumors developed as papillomas with a wide range of variability in histopathological features. Moreover, there are no distant or regional lymph node metastases in this model (83).

Mice and rats have also been used in chemically-induced models primarily by exposure to the carcinogen 4-nitroquinoline 1-oxide (4-NQO). Since the initial description of its topical application to the palatal mucosa of rats (84), there have been variations, including topical application to the tongue and addition to drinking water. Tumor development occurs between 8 and 36 weeks, depending on mode of administration, concentration of 4-NQO, and the use of inbred or outbred animals (67). In mice, 16-week treatment with 4-NQO in drinking water (100ug/mL) caused papillomas and invasive squamous cell carcinoma (SCC) in 100% of animals assessed at 28 weeks; in contrast, only 20% of the animals developed papillomas and 5% developed invasive SCC with topical application (5mg/mL) to the dorsum of the tongue (85). An initial inflammatory response concurrent with the development of pre-malignancy (epithelial dysplasia) precedes the appearance of SCC (86).

Most cell lines derived from 4-NQO-induced tumors in inbred rats were tumorigenic in syngeneic and immunocompetent hosts, but lesions regressed spontaneously and were associated with a mild to moderate inflammatory infiltrate. When grafted in immunodeficient nude mice, the same cell lines showed a higher rate of tumor development, progressive growth and a sparse to mild inflammatory infiltrate (67).

The 4-NQO model showed a progressive increase in macrophages and lymphocytes, proportional to the duration of exposure (66). Macrophage-like cells predominated in tumors, followed by T cells; CD8+ T cells or NK cells were rare (66). There was no reduction in expression of class I MHC antigens in pre-neoplastic or neoplastic epithelium (67) whereas in human primary HNC complete loss of class I MHC was reported in up to 50% of cases (87). In fact in bone marrow metastases of HNC, loss of class I MHC is

inversely correlated with the degree of cell differentiation and prognosis in HNC (88). Topical or systemic administration of 4-NQO also causes a rapid increase in inflammatory cytokines, chemokines and other biological mediators of inflammation, which is associated with activation of NF-kB (89).

There are similarities between lesions in the 4-NQO model and primary HNC, including changes in expression patterns of keratins 1 and 14, reduced cell cycle inhibitor p16, increased proliferation, and changes in expression of EGFR (89), p53 (90), E- and P-cadherin (91), apoptosis-regulating Bcl-2 and Bax (92), and point mutations in H-ras (93). Despite similarities, chemically-induced tumors seldom metastasize to lymph nodes and are not usually as aggressive as primary HNC (18). However, increasing the concentration of 4-NQO in drinking water and extending the treatment period to 20 weeks and the observation period to 40 weeks allows for the development of lymph node metastases (94).

Chemically-induced models present histopathologic heterogeneity ranging from flat pre-cancer to papillomas, non-invasive (in situ) and invasive HNC (85). Other drawbacks are risks for research personnel who handle carcinogens, duration to develop lesions, and appearance of ectopic tumorigenesis in the lips, paws (associated with grooming behavior) and esophagus, which is not usually observed with human HNC. Chemically-induced models are primarily useful to study early events, as they induce pre-neoplastic lesions that are similar to humans (66). These models may be particularly useful to assess the role of inflammation in progression of pre-cancer to cancer (Table 4).

Immunocompetent – syngeneic and humanized models

Grafting of neoplastic cell lines established from experimental mice into genetically identical (syngeneic) counterparts allows investigations of tumor-immune interactions in immunocompetent animals. Initially described with heterotopic VII/SF, a SCC cell line derived from C3H/HeJ mice (95), orthotopic models were developed using this same model (96). It is important to be aware that this cell line was derived from spontaneous tumors that developed in the abdominal wall of these mice, and not from the head and neck region (97). Moreover, C3H/HeJ mice have a genetic mutation in the TLR4 gene that affects its function (98). TLR4 is responsive to LPS and endogenous signals (DAMPs), and may be important in immune activation during cancer progression and for immunotherapeutic strategies. In fact, less severe cachexia may be associated with tumor burden in orthotopic models in TLR4-deficient C3H/HeJ mice than the immunocompetent C3H/HeN strain due to inflammation-associated wasting; however tumor progression was accelerated in C3H/HeJ mice (99). No pulmonary metastasis or lymph node invasion were detected in either strain, albeit the tumors were described as unencapsulated with a wide invasive front that compromised connective tissue, muscle, and eroded bone (99).

Another syngeneic model is the AT-84 cell line, derived from spontaneously arising oral SCC in C3H mice (100). An orthotopic modification of this model with injection of cells at the base of the tongue showed aggressive behavior, with lung metastases (101*); but no lymph node involvement (101*, 102*). More recently, a murine syngeneic model particularly suited to the study of tumor-immune interaction in HNC has been introduced

(103*). In this model, two cell lines (MOC1, MOC2) established in C57BL/6 mice generate phenotypically distinct tumors associated with different immunological features: less aggressive tumors, with increased CD8 T cell infiltration, increased expression of IFN-gamma, MHC class I and PD-L1 expression (MOC1 cell line); or more aggressive tumors associated with lower immunoreactivity, characterized by reduced expression of MHC class I and increased prevalence of CD4+ Tregs (MOC2 cell line) (104*). This model has been used in the assessment of immune-based therapy (105*) and on the effectiveness of biochemical and small molecule inhibitors of signaling pathways in the context of immunogenic and poorly immunogenic tumors (106*).

Introduction of neoplastic cell lines, generated by exposure of normal primary murine oral keratinocytes to 4-NQO, into syngeneic Balb/c mice generated tumors but no metastases (107*). This heterotopic model was used to show that expression of T cell-activating co-receptor CD80 by neoplastic cells is associated with a less aggressive phenotype (107*) and that peri-tumoral administration of IL-12 alone or with IL-2 effectively regresses CD80+ but not CD80- tumors (108*). DBA/2 neoplastic cell line in DBA/2 mice is another syngeneic in vivo model that was used to assess immunotherapy using peritumoral injection of IL-2 and activated lymphocytes associated with radiotherapy (109*).

Immunodeficient nude (110*, 111*) and SCID (112*, 113*) mice have been used for immune reconstitution using human hematopoietic tissues (bone marrow, fetal liver, thymus, lymph nodes) and cells. Engraftment methods for cells included intra-hepatic, intra-peritoneal (usually in newborn animals) and intravenous (usually in adult animals) (110*-113*). Successful engraftment was limited because of graft-versus-host disease and due to remaining innate immunity (114*). Additional genetic modifications in SCID mice, such as backcrossing the SCID mutation into strains presenting other types of immunodeficiency or disrupting other immune-relevant genes increased the success of immune reconstitution with human cells (115*). Even in these profoundly immunosuppressed mice, additional procedures (such as marrow cell ablation by irradiation) are used to enhance successful cell engraftment (114*), indicating a role for remaining innate immunity and a possible source of bias in the assessment of tumor-immune interactions (116*). Various strains of these immunocompromised animals are commercially available, including NOD-SCID (Non Obese Diabetic mice presenting defects on complement, macrophage and NK cell function backcrossed with SCID mice presenting spontaneous mutation of DNA-*Pkc* gene), NOG or NSG (NOD-SCID with deletion of *Il2rg* gene), BRG (Balb/c strain with deletion of both *Il2rg* and *Rag1* or *Rag2*) or NRG (NOD-SCID with deletion of both *Il2rg* and *Rag1* or *Rag2*) mice. More recently, NSG mice were compounded with a homozygous mutation in c-Kit designated NSGW mice, which further suppresses endogenous hematopoiesis and allows for successful engraftment of human CD34+ hematopoietic stem cells without the need for irradiation to ablate the bone marrow (117*).

Engraftment of human hematopoietic CD34+ stem cells in these animal strains results in successful development of human CD45+ cells that may represent up to 30% of leukocytes in the peripheral blood after 5 months (118*). These hematopoietic human cells undergo multi-lineage differentiation resulting in B (CD19+), macrophage/monocytes (CD33+),

plasmacytoid DCs (BDCA-2+), NK (CD3-CD56+) and even, surprisingly, T cells (both TCRαβ and TCRγδ), T helper (CD3+CD4+) and T cytotoxic (CD3+CD8+) cells. These human immune cells are functional. Furthermore, human T cells proliferate in response to mitogens and display human leukocyte antigen (HLA)-dependent cytotoxic activity (118*). The efficiency of human cell engraftment and the frequency of immune cell subsets effectively reconstituted vary according to the source and method of preparation of human hematopoietic CD34+ cells and also with the strain of mice (119*).

Other humanized mouse models incorporate primary tumor cells/tissues derived from patients. These PDX models could be searched on a database for gene expression profiles, mutations, treatment history/outcome and response to treatment and may be used to study tumor-immune interactions and chemo- and immune-therapeutic strategies (120*). However, limitations such as costs and labor-intensive protocols need to be considered. In addition, unless xenograft tissues/cells and the hematopoietic cells used to generate humanized mice originate from the same individual, the HLA antigens will most likely be different. Tumor growth in these conditions differ from the clinical situation possibly due to reduced expression of class I MHC antigens by neoplastic cells, which may be associated with increased expression of negative immunoregulatory checkpoint molecules such as PD-L1 (120*). Moreover, one has to consider that in contrast to the clinical situation, healthy human immune cells reconstituted in the experimental animal are suddenly faced with a severe neoplastic challenge; which may introduce a bias in the tumor-immune interaction.

In fact, immune reconstitution of mice using hematopoietic stem cells associated with engraftment of PDX is a powerful pre-clinical model to study tumor-immune interactions from the perspective of personalized medicine, as it is the closest replication of a human autologous tumor microenvironment. These models allow investigations of the relationship between tumor cells with different components/ cells of the immune system, preserving genetic traits and phenotype of an individual patient. One model generates humanized mice using grafts of human hematopoietic stem cells expanded in vitro into immunocompromised mice and then uses the successfully reconstituted animals (called XactMice) for PDX cell engraftment (121*). Interestingly, growth of heterotopic xenograft tumors was similar in immune-reconstituted XactMice, NOD/SCID/IL2rg$^{-/-}$ immunocompromised mice, and athymic nude mice. However, human immune cells and myeloid-derived stromal cells were observed in the tumor microenvironment of reconstituted XactMice, but not in NOD/SCID/IL2rg$^{-/-}$ or athymic nude mice. Moreover, xenograft tumors grown in XactMice show greater preservation of the original phenotype observed in patient-derived tumor samples in terms of expression of extracellular matrix-, EMT- and immune-related genes in comparison with xenografts grown in immunosuppressed animals (121*). Notably, these results were not dependent on gender- or HLA-matching of the donors of tumor and hematopoietic stem cells. Whereas this model allows for the study of tumor-immune interactions using human cells, there are limitations associated with the presence of mouse-derived stromal and residual innate immune cells, as well as issues associated with the interaction of human cells and cytokines with mouse stroma, lack of HLA-matching, and heterotopic location of the xenografts.

Using human hematopoietic stem cells from the bone marrow and HNC cells isolated from the primary tumor of the same patient, Fu et al. (122*) demonstrated that suppressing STAT3 signaling in neoplastic cells reduced tumor growth and increased infiltration with myeloid and lymphoid cells, which was associated with a decrease in the expression of arginase-1 (M2 macrophage marker). Importantly, these authors (122*) demonstrate that in addition to allowing the study of tumor-immune interactions, this novel model can be used to assess the effectiveness of immunomodulatory treatment approaches. Of note, the full autologous reconstitution of patient-derived immune and neoplastic cells required initial establishment of the immune reconstituted (humanized) animals (a process that takes 6 weeks); whereas the primary human neoplastic cells were expanded subcutaneously in immunocompromised mice and then used for implantation in the immune-reconstituted animals, which may cause some initial genetic drift in the neoplastic cells due to the interaction with mouse-derived stroma. The use of non HLA-matched immune and neoplastic cell lines (such as the XactMice model) is an alternative; however this loses the major advantage of matching individual genetic and phenotypic characteristics that influence the immune response and neoplastic cells in a given patient.

Human T cells in lymphoid organs, peripheral tissues and blood of animals may result from a thymus-independent pathway by expansion of mature CD3+ T cells contaminating the CD34+ hematopoietic stem cell preparation (123*), such as in the case of the engraftment of mature PBMCs (124*). However, mature T cells may also result from a thymus-dependent pathway in grafted mice, particularly with additional treatment of the animals with IL-7 (115*). Atrophy or underdevelopment of the thymus is a serious hindrance to human T cell development in mice used to generate human immune cell chimeras, including the NSG and NSGW mice. The absolute and relative numbers of human T cells are usually low and are selected in mouse epithelial cells expressing mouse MHC molecules; which make these cells inadequate for human studies. Human thymopoiesis and human T-cell development were reported with transplantation of human fetal liver and thymus tissues under the kidney capsule of NOD-SCID mice (112*); however these cells were not able to mediate effective immune responses in vivo. A modification that included grafting of CD34+ human hematopoietic stem cells associated with human fetal liver and thymus transplants (BLT or Bone marrow, Liver and Thymus mice) resulted in repopulation of multi-lineage human immune cells, including T and B cells, CD11c+ dendritic and CD14+ monocytic cells. Strikingly, 18–25 weeks after transplantation, the mesenteric, axillary, brachial, renal, inguinal and cervical lymph nodes of these mice were populated by over 90% of multi-lineage human CD45+ cells, including B and T lymphocytes and dendritic cells. B cells produced human IgM and IgG and the mice effectively rejected a pig skin xenograft, indicating a competent and functional adaptive immune response in vivo (125*). This same approach has been used in different strains of mice, such as C57BL/6 (126*).

Interestingly, mesenteric lymph nodes, the only lymph nodes found in reconstituted mice, present both CD3+ T-cell and CD19+ B-cell compartments (127*). Repopulation of secondary lymphoid organs is required for antigen presentation and T-cell priming in adaptive immune response, thus the lack of regional lymph nodes reconstituted with human immune cells limits the study of tumor-immune system interactions in these humanized mice.

Thus, although the humanized mouse is an FDA-recommended, powerful pre-clinical model to study tumor-immune cell interactions, there are many variables and a significant cost to these labor-intensive models. Success of human immune cell engraftment and distribution of immune cell subsets in lymphoid tissue may vary with genetic background, strain and age of animals, source of human cells, and method of engraftment (118*, 127*, 128*). Humanized mice tend to present a B-cell bias (129*, 130*) and have limitations in reconstitution of NK cells, (127*, 131*), which may be related to species-specific incompatibility between mouse cytokines required for development of these cells and human cell receptors (132*). This limitation has been addressed by administration of human cytokines and by creation of transgenic mice expressing human cytokines (133*–135*). Recently a novel humanized mouse expressing human IL-6 (NOG-hIL6 Tg, a NOD-SCID with deletion of Il2rg and constitutive transgene expression of hIL6) was engrafted with human CD34+ hematopoietic cells and 12–14 weeks later with a human HNC cell line that constitutively expresses IL-6, M-CSF, VEGF and IL-8. In these mice, ectopic tumors were infiltrated with CD68+ human macrophages that presented a M2, CD163+ HLA-DRlo/- regulatory/immunosuppressive phenotype, similar to tumor-associated macrophages (TAMs) in humans. However, in control NOG mice (without transgenic expression of human IL-6), there was a reduced infiltrate of macrophages, indicating that the tumor microenvironment associated with HSC4 cells-derived tumor was insufficient to induce M2 immunoregulatory-suppressive TAMs (136*).

Immunocompetent – genetically engineered models

Advances in mouse genetics have allowed development of genetically modified mouse models with spatially and temporally regulated expression of oncogenes or tumor suppressor genes. An example is a strain of mice with mutant K-*ra*s under the control of keratin 5 or 14 promoters, which target expression of the mutated transgene to the basal cells of the oral mucosa, allowing for spatial control of transgene expression. Transgene expression may be induced by tetracycline or by induction of Cre recombinase-mediated excision of a LoxP flanked stop codon (137*), allowing for temporal control. The first model developed for HNC combined transgene expression of cyclin D1 in oral epithelium and global deletion of the p53 tumor suppressor gene, which resulted in invasive oral-esophageal cancer (138*). Other models involve mutation, overexpression or deletion of cell cycle genes (139*), oncogenes or tumor suppressor genes (140*), or signaling intermediates (141*), mimicking changes described in primary cancers (for excellent recent reviews of genetically-engineered mouse models, please see (24).

In conditional *Pten/Tgfbr1* double knockout mice, deletion of the floxed target genes in oral epithelium is mediated by K14 promoter-driven expression of Cre recombinase (142*). Conditional expression of Cre-recombinase depends on topical application of tamoxifen to induce nuclear translocation of the fusion protein between the tamoxifen responsive hormone-binding domain of the estrogen receptor and Cre recombinase to catalyze excision of the target gene (143*). Loss of PTEN promotes epithelial proliferation, whereas loss of TGFBR1 results in increased inflammation in the subjacent connective tissue, with increased expression of cytokines, chemokines, and infiltration of granulocytes and macrophages. Mice with conditional deletion of only *Pten* developed epithelial dysplasia, but only 21%

developed SCC in 12 months. In contrast, all *Pten*/*Tgfbr1* conditional double knockout mice developed well-differentiated SCC in 10 weeks (142*), indicating the critical role of inflammation in oral tumorigenesis in this model.

Various models combine genetic manipulation with an exogenous trigger, such as sustained exposure to a chemical carcinogen (144*, 145*), suggesting that some genetic manipulations are insufficient to initiate tumorigenesis. Less than 4% of mice with tamoxifen-initiated Cre-mediated deletion of *Tgfbr1* in oral epithelium developed tumors spontaneously; however when these mice were subjected to topical DMBA, there was an increased infiltrate of inflammatory cells, as well as increased Tgfb1 expression in the connective tissue stroma (146*). Thus, loss of Tgfbr1 expression in epithelium may result in enhanced effects of Tgfb1 in stromal cells (147*), which may have immunosuppressive effects. This is supported by the finding of reduced CD4+ and CD8+ T-cells and increased CD4+/CD25+/Foxp3+ T regulatory cells in cervical lymph nodes of these mice (146*). This highlights that an epithelial-specific genetic change can influence the phenotype of immune cells in the subjacent stroma thereby altering the tumor microenvironment. Other studies also report increased inflammation in the stroma, such as in mice with conditional and epithelium-specific deletion of *Smad4*. The decreased production of TGFb1 by epithelial cells in these mice triggers a compensatory increase in production of TGFb1 by stromal cells and activation of Smad1/5/8. These changes are associated with increased expression of chemokines and chemokine receptors and increased inflammation in the connective tissue, characterized by enhanced migration of granulocytes, macrophages and Th17/CD4+ T cells (141*).

It is important to note that the immune phenotype in these transgenic animals with genetic changes in tumor suppressors or in oncogenes is seldom characterized. Moreover, the proper backcrossing of these animals is not always described in published studies, which may result in animals with a mixed genetic background. There is evidence indicating that the genetic background of different strains of mice may affect the immune phenotype and thus tumor-immune interactions (148*–150*).

Skewed activation of the immune response is relevant for oncogenesis and tumor progression, for example by selective suppression of anti-tumoral cytotoxic T CD8+ and NK cells by cAMP induced activation of the adenosine A2 receptor (A2AR). Blockade of A2AR significantly reduced the tumor burden in *Pten*/*Tgfbr1* 2cKO mice by decreasing suppressive CD3+/CD25+/Foxp3+ Tregs and increasing the cytotoxic activity of CD8+ T cells (151*).

Furthermore, constitutive activation of Akt results in pre-cancerous lesions in the oral cavity but few aggressive SCCs, which indicates a role for Akt in tumorigenesis. Combination of constitutive Akt activation and stratified epithelial-specific ablation of p53 tumor suppressor gene resulted in SCC in the oral cavity with lymph node and lung metastases and correlated with increased activation of NF-kB and STAT3, which are activated by cytokines and also participate in cytokine expression (152*). *Pten*/*Tgfbr1* 2cKO mice have been used as a model system (151*, 153*–155*), to demonstrate a pro-tumoral role for the NLRP3 inflammasome by regulating the self-renewal activity of cancer stem cells; and to show that

the NLRP3 inflammasome is activated by bacterial LPS and ATP and has a role in HNC progression in vivo (153*).

Due to the value of in vivo immunocompetent models, more consideration should be given to the immune response in studies using these genetically modified models for HNC. The specificity of the transgene activation/inactivation is usually assumed to preserve the integrity of the immune response; however this may not always be the case. For example, macrophages from *p53* KO mice produce more IL-1, IL-6 and IL-12 (156*). Polarization of Th17 cells from *p53* KO mice is enhanced, whereas differentiation of CD3+/CD25+/Foxp3+ T regulatory cells is compromised (157*). These effects are related with increased activation of NF-kB and STAT3 (158*), and may correlate with chronic inflammation and tumorigenesis. Mutation or deletion of other oncogenes may not result in an immune phenotype, for example *H-ras* KO mice (159*); whereas deletion of the *N-ras* isoform in mice reduces the anti-viral immune response and impairs CD8+ T cell development, activation and function (160*), which may have important implications in tumor immunity. An activating mutation of *K-ras* induces lung tumors with increased prevalence of IL-22+ cells; tumor development and burden were significantly reduced in IL22 KO mice (161*). Interestingly, IL-22 is expressed by immune cells (e.g., CD4+ Th1, Th17, Th22 cells, natural killer T cells, γδT cells and CD8+ T cells) but exerts its biological effects almost exclusively on cells of non-hematopoietic origin, which express the cognate receptor IL22R1 (162*, 163*). Mice with global deletion of the pro-inflammatory cytokine macrophage migration inhibitory factor (*Mif*) are less susceptible to 4-NQO induced oral cancer and present a reduced inflammatory cell infiltrate in the surrounding stroma and decreased expression of various pro-inflammatory mediators, including Il-1, Tnf, Cxcl1, Cxcl6, Ccl3, Mmp1 and Ptgs2 (164*), which support a pro-tumoral role for the immune response. Surprisingly, there are relatively few studies in HNC using transgenic animal models of cytokine/immune relevant genes (e.g., interleukins, interferons, chemokines); whereas multiple studies have been conducted using transgenic mice for immune-relevant genes in models of breast (165*, 166*), prostate (167*, 168*), liver (169*, 170*), pancreatic (171*), colon (172*), cervical (173*), ovarian (174*) and lung (175*) cancer.

Thus, the impact of global changes in gene expression on the immune response should be considered in interpreting the effects of oncogenes or tumor suppressor genes on tumor development. Tumors investigated in genetically modified mice are of autochthonous, *in situ* origin, which is more representative of human tumors than xenografts. However, human tumors usually are heterogenous and display important variation in genetic alterations, whereas tumors generated in transgenic models present a limited number of genetic alterations. Moreover, these genetic changes are expressed in non-physiological levels by all cells of a specific tissue under the control of heterologous promoters, in contrast to the few genetically-altered cells giving rise to primary human tumors.

Concluding remarks

Murine models are the most frequently used experimental models for pre-clinical research in HNC. However, every model has limitations, which should be taken into consideration in experimental planning and data interpretation (Table 4). Thus, the different models discussed

in this review may be selected on the basis of their suitability for investigations of a specific aspect of tumor-immune interactions in HNC. With the widespread recognition of the fundamental role of the immune system in tumorigenesis, tumor progression, and as a treatment component in HNC, it is important to consider the influence of immune cells on outcomes assessed in immunocompromised models, such as nude mice and SCID mice or rats. Continuous advances in the development of novel genetically modified animals, including transgenic animals used to generate human immune chimeras (humanized mice), are certainly of great importance for the study of tumor-immune interactions. However, even these sophisticated models have limitations as the complexity and heterogeneity of HNC tumorigenesis cannot be completely recapitulated in any single experimental model. Rather, the quality and relevance of the information on tumor-immune interactions in HNC is complemented by studies in different in vivo models.

Supplementary Material

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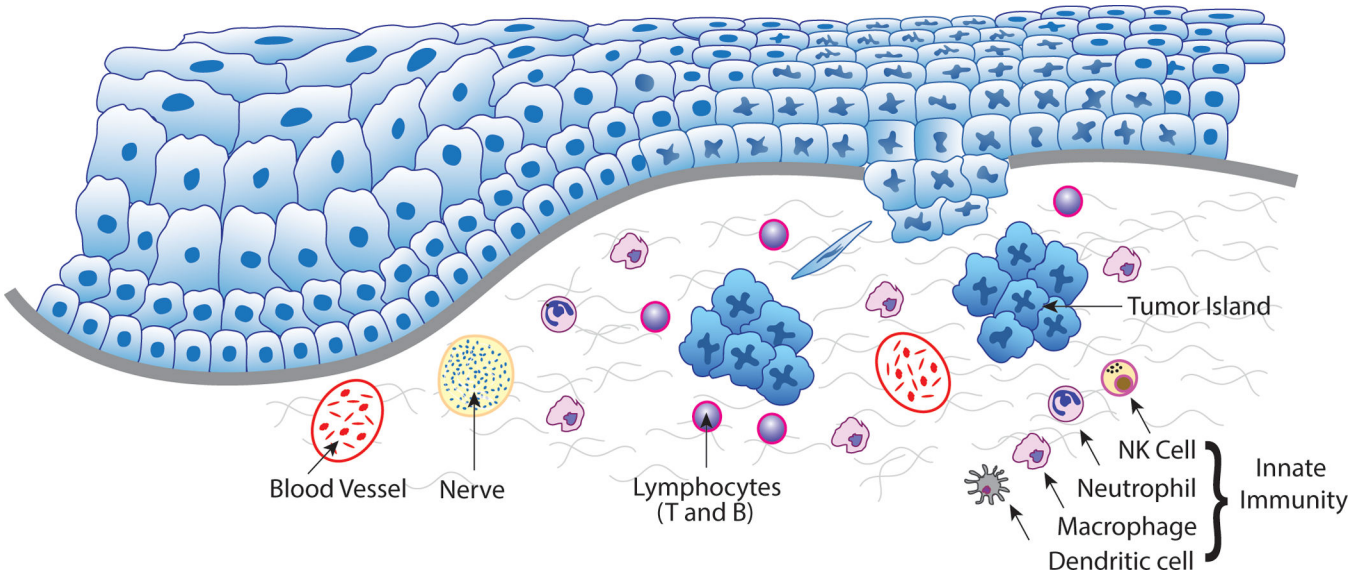

Figure 1 –.

Schematic representation of the tumor microenvironment in HNC showing main cells of the adaptive (T- and B-lymphocytes) and innate immune response.

Table 1:

Summary of immune characteristics/phenotypes of major murine models of HNC. Information derived from specific studies cited in the table.

Immunity	Murine model	Immune aspects	References
Compromised	Nude / athymic	High NK cell activity, no T cells. B cells, PMNs, macrophage /monocytes, dendritic cells are functional; may have increased activity. Altered lymph nodes and secondary lymphatic structures.	(Holub et al., 1984, Vetvicka et al., 1984, Budzynski & Radzikowski, 1994, Watts et al., 2009)
	SCID	NK cell activity varies with strain, no T or B cells, PMNs, macrophage /monocytes, dendritic cells present. Development of some functional T and B cells ('leaky phenotype') possible. No/underdeveloped peripheral lymph nodes.	(Blunt et al., 1996, Nonoyama et al., 1993, Lin et al., 2005, Kotloff et al., 1993)
	Rag1/*Rag2* mutant	Similar to SCID, but no leakiness.	(Shinkai et al., 1992, Mombaerts et al., 1992)
	X-SCID / *Il2rg* mutant	Reduced numbers of T and B cells with limited functionality. Monocytes/macrophages and neutrophils may be increased. No/underveloped peripheral lymph nodes.	(Cao et al., 1995, Ohbo et al., 1996)
	NOD-SCID	Combines characteristics of SCID mutation with defects in complement activation, macrophage and NK cell function. Used in humanized models.	See references listed under 'humanized models' below.
	NOG / NSG *	Combines characteristics of NOD-SCID animals with inactivation of Il2rg gene. Used in humanized models.	
	BRG / NRG	Balb/c mice (BRG) with deletion of both *Il2rg* and *Rag1* or *Rag2*. NRG combines NOD-SCID characteristics with deletion of both *Il2rg* and *Rag1* or *Rag2*. Used in humanized models.	
	NSGW	NSG mice with additional mutation of c-Kit to further suppress endogenous hematopoiesis. Used in humanized models.	
Competent	Hamster / chemically-induced	Immune-privileged site without lymphatic drainage and no counterpart in human anatomy	(Aromando et al., 2008, Ghiabi et al., 1992)
	Other murine / chemically-induced	Immune response normal within the characteristics of the specific genetic background.	(Matthews et al., 1986, Thomas et al., 1995)
	Syngeneic	Immune response normal according to the genetic background.	(Vahle et al., 2012)
	Transgenic	Immune response may be directly or indirectly affected by specific genetic modification or by genetic background	
	Humanized	Human immune cell reconstitution in immunodeficient animals. May be used with grafts of human SCC cell lines or characterized human primary tumor tissues or cells (PDX models). Variety of immunosuppressed transgenic mice may be used in this model, see 'Immune compromised' section of this table.	(Ishikawa et al., 2005, Shultz et al., 2007, Shultz et al., 2005, Wang et al., 2018, Dykstra et al., 2016, Rongvaux et al., 2014, Hanazawa et al., 2018, Skelton et al., 2018, McIntosh et al., 2015)

* NOG/NSG mice with transgene expression of human cytokines (e.g., IL-6, IL-3, GM-CSF, human stem cell factor) have been recently used to enhance engraftment, to support development of specific human immune cells or to recapitulate characteristics of the human tumor microenvironment (e.g., increased prevalence of tumor-infiltrating M2 macrophages in hIL-6 expressing animals).

Table 2:

Major innate and adaptive immune cell types, function and presence in murine models of HNC. Information derived from (Krijgsman et al., 2018*, Affara et al., 2014*, Hansen & Andersen, 2017*, Moses & Brandau, 2016*, Rei et al., 2015*, Haeryfar et al., 2018*, Albini et al., 2018, Kotas & Locksley, 2018, Mittrucker et al., 2014*, Burger & Wiestner, 2018*, Simoni et al., 2017*) and from studies cited in the table. References with an asterisk (*) are included in the appendix.

Immune response	Cell type	Function	Murine models
Innate	Neutrophil (PMN)	Phagocytosis, oxidative metabolism, neutrophil extracellular trap (NET) that may promote tumor metastasis, matrix-degrading, immunosuppressive and angiogenic bioactive molecules. May polarize into different phenotypes: N1 – anti-tumorigenic, antimicrobial, pro-inflammatory; and N2 – regulatory, pro-angiogenic, pro-tumorigenic	Nude/athymic[1], SCID, transgenic, immunocompetent [1]defective maturation(Wysoczynski et al., 2017*), increased activity (Holub et al., 1984)
	Natural killer (NK) / Natural killer T cells* (NKT)	Major NK cell phenotypes (CD3-, also classified as ILC1 cells) include $CD56^{dim}/CD16^{+}$ with strong cytotoxic activity and $CD56^{bright}/CD16^{-/low}$, which produce IFNγ and are more immunoregulatory. NKT cells also express TCR (CD3+) and may be further subdivided into type I and type II cells based on the repertoire of TCR chains expressed. Both type I and II NKTs recognize antigens presented by APCs or on the surface of stromal cells via CD1d molecules (i.e., non MHC-restricted). NKT cells may assume different phenotypes analogous to those of T-helper cells: Th1-like (IFNγ and TNF-secreting cells); Th2-like (IL4 and IL13 producing cells) and Th17-like (IL17 secreting cells) and Treg-like (FOXP3+, IL10 secreting cells).	Nude/athymic[1], SCID[2], transgenic[2], immunocompetent [1]increased cytotoxic activity in comparison to wild-type mice [2]not present in transgenic animals with *Il2rg*-deletion/inactivation
	Monocyte / macrophage	Phagocytosis, oxidative metabolism, antigen presentation to T (helper and cytotoxic) and B cells, may assume pro-inflammatory/ anti-tumorigenic (M1) or repair/pro-tumorigenic (M2) phenotypes.	Nude/athymic[1], SCID[2], transgenic, immunocompetent [1]reduced numbers, defective maturation and enhanced function/activity (Wysoczynski et al., 2017*, Vetvicka et al., 1984, Holub et al., 1984) [2]impaired function in some strains of SCID mice(Shultz et al., 1995*)
	Dendritic cells	Phagocytosis, antigen presentation to T (helper and cytotoxic) and B cells. Cytokine and chemokine expression regulates other immune cell types. May activate the immune response or induce tolerance and	

Immune response	Cell type	Function	Murine models
		assume anti- or pro-tumorigenic roles.	
	γδT cells	These cells have both innate-like properties, (rapid secretion of immune mediators in response to danger signals) and adaptive-like properties (may undergo clonal expansion and develop antigen-specific memory). Most are infiltrated in the tissues and are divided into two basic types according to TCR chains: type I/Vδ2 and type II/Vδ1. Both may have cytotoxic effects on tumor cells, but Vδ1 cells are more common in solid tumors. Phenotypical subsets may include Th1-, Th2-, Th17-like and regulatory/ immunosuppressive γδT cells. These cells may also polarize CD4+ αβT cells to a Th1 or Th2 phenotype.	Nude/athymic[1], immunocompetent, transgenic[2] [1]present these cells as intra-epithelial lymphocytes [2]there are TCRδ-deficient mice that lack γδT cells
	Immature myeloid cells (MDSC and DC)	May be recruited into the tumor microenvironment by soluble factors. Characterized by an immature phenotype and production of pro-angiogenic and immunosuppressive molecules.	Nude/athymic, SCID, immunocompetent, transgenic
	Innate lymphoid cells (ILC)	Phenotype dictates their biological role. Largely analogous to T-helper cell subsets (see below): ILC1/Th1 anti-tumorigenic, ILC2/Th2 pro-tumorigenic, ILC3/Th17 pro-tumorigenic	Nude, SCID, transgenic[1], immunocompetent [1]absent in animals with inactivation or deletion of the IL2rg chain gene
	Mucosal-associated invariant T cells (MAIT)	Present in various mucosal surfaces, inflamed sites and in the peripheral blood. Develop in the thymus and express a semi-invariant form of TCR. Respond to MHC-related protein 1 (MR1)-restricted antigens by producing immunoregulatory cytokines (may assume a Th1-like or Th17-like phenotype). These cells also present membrane and cytosolic effector molecules that mediate direct cytotoxicity.	Low in most mouse strains, except for CAST/EiJ mice. Absent in nude/athymic and SCID mice.
Adaptive	αβT-helper cells (Th, CD4+)	Regulation of the activity of other innate and adaptive immune cells, indirect cytotoxicity. May assume distinct phenotypes (see table 2) with varying biological activities including pro- and anti-tumorigenic effects	Transgenic*, immunocompetent (*except for animals with genetic disruption of functional IL2rg, TCRβ or CD4- / CD8-specific knockouts)
	αβT-cytotoxic cells (CTL, CD8+)	MHC class I-restricted activation. Cells may assume different phenotypes analogous to CD4 T-helper cells (see table 2), including: Tc1 (classic CTL/cytotoxic,	

Immune response	Cell type	Function	Murine models
		direct cytotoxicity and expression of IFNγ/TNF), Tc2 (expression of IL5 and IL13, lower cytotoxic activity), Tc9 (express IL9 and IL10, lower cytotoxic activity but a greater anti-tumor activity than Tc1 cells), Tc17 (produce IL17 and IL21, may have pro- or anti-tumorigenic activity) and CD8+ Tregs (these cells are restricted by non-classical MHC class Ib antigens and may suppress immune response by direct cytotoxicity of activated CD4+ T cells, as well as via IL10 in a non-cytolytic manner)	
	αβT-regulatory cells (Treg)	Regulation of activity of other immune cells (see table 2)	
	B cells	Antigen presentation to T-helper cells, antibody production. Role in solid tumors is less understood; may have pro-tumorigenic effects in pancreatic ductal adenocarcinoma and squamous cell carcinoma mediated by direct paracrine effects of secreted products (e.g., IL35) on cancer cells or by instructing other immune cells in tumor microenvironment.	Nude/athymic, transgenic, immunocompetent
	Intra-epithelial lymphocytes (IEL)	Naturally occurring lymphocytes (IEL) take residence in epithelium early in life; are γδT cells that have cytotoxic profile (and related with both innate and adaptive immunity, see above). Both αβT-CD4+ and CD8+ conventional lymphocytes may be adaptively induced and recruited into the epithelium by local antigens. These cells take residency and undergo phenotypic changes that turn them into stable-resident memory cells without the capacity to recirculate. IEL usually noted in intestinal epithelium, also detected in oral epithelium.	Nude/athymic[1], SCID[2], transgenic, immunocompetent [1]only γδT cells as IEL [2]intestinal mucosa present CD3-CD8+ granulated IEL

Table 3:

CD4+ T-helper lymphocyte subsets specified by clues from the microenvironment, the major cytokines characteristic of each subset and their possible role in HNC. Information derived from (Kim & Cantor, 2014*, DuPage & Bluestone, 2016*, Caza & Landas, 2015*, Geginat et al., 2013*). References with an asterisk (*) are included in the appendix.

T-helper subsets	Cytokines	General function and role in HNC
Th_1	IFNγ, lymphotoxin, TNFα	Protection against intracellular pathogens, cell-mediated immunity, antibody class switch to IgG. Reduced in HNC. Increased prevalence correlated with better prognosis.
Th_2	IL4, IL5, IL10, IL13	Help B cells in humoral immunity, participate in allergic reactions and defense against extracellular parasites. Increased in HNC, associated with worse prognosis.
Th_9	IL9, IL10	Mucosal immunity, protection from helminth infections in the intestines, activation of Th17 cells. Limited pre-clinical evidence suggests anti-tumoral role in HNC.
Th_{17}	IL17, IL17F, IL22, IL6, IL10, TNFα	Defense against bacteria and fungi in mucosal surfaces, protection against microorganisms not covered by Th1/Th2 cells. Reduced in HNC. Increased prevalence is associated with better prognosis.
Th_{22}	IL13, IL22, FGF, CCL15, CCL17, TNFα	Wound repair, protection against bacterial, viral, fungal infections in epithelial surfaces. No known role in HNC.
Th_{25}	IL25, IL4, IL5, IL13	Stimulation of non-lymphoid cells to produce effector cytokines in response to extracellular pathogens. No known role in HNC.
T_{fh}	IL21	B cell class-switching and chemotaxis to germinal centers in the lymph nodes. No known role in HNC.
T_{reg} *	IL10, TGF-β	Suppression of immune response, immune tolerance and protection against autoimmunity. Increased in HNC and associated with worse prognosis.
Th_1/Th_{17}	IFNγ, GM-CSF, IL17	Associated with autoimmune conditions (type I diabetes, juvenile rheumatoid arthritis, Crohn's disease). No known role in HNC.

*This subset include natural T_{reg} derived in the thymus or induced/adaptive-T_{reg}s differentiated in periphery in the presence of high concentrations of TGF-β and in the absence of proinflammatory cytokines. Induced T_{reg}s include cells involved in oral tolerance (Th3) and T regulatory type 1 (Tr1) cells induced by IFNα secreted by dendritic cells. Natural CD4+CD25+Foxp3+ T_{reg} cells are also heterogeneous and may present an active suppressive ($CD45RA^-$, $CD25^{hi}$, $FOXP3^{hi}$) phenotype, a resting/non-suppressive ($CD45RA^+$, $CD25^{moderate}$, $FOXP3^{lo}$) phenotype and a non-suppressive phenotype ($CD45RA^-$, $CD25^{moderate}$, $FOXP3^{lo}$) associated with production of IL-2 and IFNγ.

Table 4 –

Summarized immune-relevant characteristics the murine models most commonly used in HNC research. Note that the characteristics and limitations of each model may be explored to address specific questions.

Model	Uses/ Characteristics	Limitations
Immunocompromised Nude/athymic	Xenograft cells, orthotopic/heterotopic models, overactive innate immune cells, increased B cells	No T cell response, murine stroma, induced tumors of limited genetic heterogeneity, leaky phenotype
Immunocompromised SCID	Xenograft cells and tissues (PDX), orthotopic/heterotopic models, NK cell activity	No T or B cells, murine stroma, induced tumors
Immunocompromised Genetically-engineered	Improved survival of human xenografts of cells and tissues (PDX), can be used for immune resconstitution, some models preserve NK cell response	No lymphocytes, induced tumors, murine stroma, normal/reduced innate immunity
Immunocompetent Chemically-induced	Orthotopic, high genetic heterogeneity, genetic mutations resemble human disease, induced tumors that recapitulate the oncogenic process of human disease (dysplasia, carcinoma-in-situ, carcinoma), histologically similar to human mucosa	Reduced control over location and number of lesions, mixed lesions include dysplasia and papillomas, murine tumors and stroma, length of time to develop tumors, reduced metastases and node invasion, immune-privileged site in hamster cheek-pouch, possibility of concomitant lesions in the esophagus and skin
Immunocompetent Syngeneic	Induced tumors, orthotopic /heterotopic	Murine neoplastic cells and stroma, reduced genetic variability, immune response may vary with the specific strain/genetic background
Immunocompetent Genetically engineered	Induced/spontaneous tumors, immune phenotype not always characterized	Murine tumors of reduced genetic heterogeneity, murine stroma
Immunocompetent Reconstituted (humanized)	Xenografts of human cells and PDX, orthotopic/heterotopic, human immune response to human neoplastic cells or tissues, assess immunotherapeutic approaches, personalized medicine approach if using neoplastic and hematopoietic cells from the same individual	Labor intensive, cost, procure and prepare human hematopoietic stem cells, HLA-matching, possible residual murine innate immune cells, murine stroma and myeloid-derived suppressor cells, possible genetic drift if initially expanding neoplastic cells/tissues in immunocompromised mice.