Chapter 3

Immunosuppressive Microenvironment in Head and Neck Cancer

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1. Introduction

Typically, the immune system detects and eliminates tumor cells through adaptive immunity, but that is not always the case in the local tumor microenvironment. In cancer patients, local tumor cells progress persistently—even though the bloodstream is full of tumor-specific cytotoxic T lymphocytes (CTLs), which are primed with tumor antigens in the lymph nodes and are able to kill tumor cells. In about 70% of patients with human papillomavirus (HPV)-associated head and neck squamous cell carcinoma (HNSCC), CTLs enter into the tumor tissue and become tumor-infiltrating lymphocytes (TILs). But those TILs fail to kill tumor cells in the local tumor microenvironment [1-3]. Instead, they become anergic, exhausted, or apoptotic when they are surrounded by tumor cells, especially when the tumor cells are positive for a cell surface marker called programmed death ligand 1 (PD-L1). PD-L1 is also known as B7-H1 or CD274 [4-8].

In general, about 20% of cancer patients have tumor cells positive for PD-L1. Moreover, about 20% of patients with HNSCC [9], as well as 80% to 90% of patients with orolaryngeal cancer [2, 3], are infected by HPV. In patients with HPV-associated HNSCC, PD-L1 positivity is as high as 70% [1]. PD-L1 expression in tumor cells has been shown to make them aggressive, with a poor clinical outcome. Patients with renal cell carcinoma with high PD-L1 expression are 4 to 5 times more likely to die of their disease [7]. However, the clinical outcome may be better for patients with HPV-associated HNSCC; in such patients, whether PD-L1 positivity indicates a poor or a better clinical outcome depends on tumor types [10-12].

TILs express CD3, CD8, and programmed death 1 (PD-1) surface marker proteins and are surrounded by tumor cells. In that local tumor microenvironment, PD-1 on the TIL side is

inevitably contacted by PD-L1 on the tumor cell surface and activated by PD-L1. The activation of PD-1 on TILs inactivates their function. Thus, TILs are incapable of killing tumor cells in the tumor tissue. Immune negative regulatory pathways, exemplified by the PD-L1/PD-1 pathway, probably act in concert to counteract effective immune responses of TILs in the local tumor microenvironment [6].In such a context, the function of TILs is reshaped and manipulated by the tumor microenvironment, so that they fail to kill tumor cells.

Tumor cells employ several strategies to evade an immune response. An immunosuppressive network is known to exist, involving multiple immunosuppressive pathways plus regulatory cell populations, all of which can act as "checkpoints" to successfully restrict immune cell activation. The PD-L1/PD-1 pathway is the representative pathway for suppressing immune responses in tumors [13]. In certain cancer patients, this pathway deeply affects and shapes cell-based immunity and thus can serve as an immunotherapeutic target [14]. In addition, regulatory T cells (Treg), natural killer (NK) T cells, and myeloid-derived suppressor cells (MDSCs) are involved in manipulating immune responses, thereby promoting the development and growth of tumor cells. Naturally occurring immunosurveillance occurs when T-cell receptors (TCRs) interact with the peptide-major histocompatibility complex (MHC) complex; yet immunotolerance may occur when PD-L1 activates PD-1 on TILs.

PD-L1 is frequently induced by chronic infections of oncogenic viruses, such as HPV and hepatitis B virus (HBV) [15].Chronic inflammation can promote the tumorigenesis of HNSCC in all phases of malignant stages, including susceptibility, initiation, progression, dissemination, morbidity, and mortality of tumor cells [16, 17]. Conversely, the normal microenvironment has antitumorigenic forces that must be overcome by tumor cells. The typical molecule that can overcome the normal microenvironment's antitumorigenic forces is PD-L1. Accumulating evidence demonstrates that the adverse tumor microenvironment facilitates immune escape of tumor cells through active manipulation of PD-L1. The interaction between PD-L1 and PD-1 inactivates T cells, making them powerless [18].

2. Inflammatory mediators and cytokines

Generally, HNSCC is rich in inflammatory cytokines and mediators in the tumor tissue [19, 20], especially when associated with HPV infection [21]. HNSCC is characterized by profound chronic inflammation [20, 22, 23]. It is highly regulated by interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and relevant regulatory transcription factors. Those factors include the inhibitor of differentiation family members, ID1 and ID3 [24], as well as immune and inflammation relevant transcription factors such as nuclear factor kappa B (NF-κB) [19, 25, 26].

ID1 is an oncoprotein that dictates the proliferation of squamous cells. An immediate-early gene, it responds to mitogenic signals, infectious agents, and bone morphorgenetic proteins (BMPs) [27]. It is an important transcription factor critical for the production of IFN-γ and TNF-α [28].

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ID3 is a critical transcription factor involved in the proliferation of B cells and in antibody isotype switching [29]. Both ID1 and ID3 are involved in the adaptive immune response in HNSCC via the regulation of IFN-γ and TNF-α and via the proliferation of B cells and antibody isotype switching.

NF-κB is a well-known transcription factor activated by infection, either bacterial or viral. Clinical studies indicate that NF-κB plays an important role in the carcinogenesis of HNSCC, in conjunction with ID1 [24, 28, 30]. As shown in our recent studies, ID1 and NF-κB subunit p65 together transduce immortalized keratinocytes into malignant keratinocytes, both in vitro and in vivo [31]. In HNSCC specimens, the major inflammatory cytokines in tumor tissues include interleukin-8 (IL-8), TNF-α, and IFN-γ [19, 24]. HPV-associated HNSCC generally induces a robust immune response and has emerged as a distinct entity, with a different profile of risk factors and with a more favorable response to therapy than HPV-negative HNSCC. More than 50% of patients with HPV-associated HNSCC are infected by HPV type 16 [10-12]. The percentage is similar in patients with HPV-associated cervical carcinoma [32, 33].

Chronic inflammation is involved in tumor development and growth, in both nonimmune and immune ways, via the following mechanisms: (1) the production of reactive oxygen species, such as peroxynitrites, which promote DNA mutation; (2) the production of proangiogenic factors, such as vascular endothelial growth factor (VEGF), which promotes tumor neovascularization; (3) the production of matrix metalloproteases (MMPs), such as MMP9, which facilitate invasion and metastasis; and (4) the perturbation of myelopoiesis and hemopoiesis, thereby causing a deficiency in antigen-presenting cells, as well as dysfunctional cell-based antitumor immunity [34].

One result is the unleashing of MDSCs to dispose of dendritic cells. MDSCs are immature myeloid cells that inhibit both innate and adaptive immunity, thus subverting immunosurveillance. They are thought to be a disturbed differentiation of dendritic cells by inflammatory mediators, such as VEGF, IL-6, cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2) [20]. MDSCs are present in patients with HNSCC [35, 36]—especially those with oropharyngeal cancer, [37] 80% to 90% of whom are infected by HPV. Inflammatory cytokines and mediators (e.g., IL-6, VEGF, COX-2, PGE_2,) induce the formation of MDSCs and cause immunosuppression in most cancer patients; MDSCs are an impediment to all immunotherapies that require an active immune response by the host. Blockade of PD-L1 improves T-cell activation mediated by myeloid dendritic cells, in turn downregulating T-cell IL-10 and upregulating IL-2 and IFN-γ [38].

Thus, blockade of PD-L1 is indirectly relevant to counteracting the inhibition of Treg cells. A subset of CD4+ regulatory T lymphocytes, Treg cells inhibit effector T lymphocytes. Tumor cells and microenvironmental macrophages produce chemokines to attract Treg cells into the tumor tissue [39]. Even though Treg cells inhibit immune responses, they have no value, according to many studies, in predicting the prognosis of patients with HNSCC. Nonetheless, the $CD8^+/FoxP3^+$ (effector:regulatory) ratio, the $CD8^+/CD4^+$ (effector:helper) ratio, and the Treg cell subset ($CD45RA^-FoxP3^+$) may be useful in predicting the prognosis [40-42]. PD-L1 costimulates the secretion of IL-10, which promotes the function of Treg cells [4].

2.1. ID1-IFNγ/TNFα-PD-L1 signaling

BMPs induce the expression of ID1 [43, 44]. ID1 regulates the expression of INF-γ and TNF-α in T cells [28]. IFN-γ and TNF-α are mainly produced by activated T cells and NK cells [45] responsible for macrophage activation and differentiation [46]. IFN-γ induces transcription of several proinflammatory genes, such as inducible NO synthase (iNOS), COX-2 [47], and IL-1β, as well as MHC proteins [46]. In mouse macrophages, IFN-γ regulates TNF-α expression [45]. ID1 is critical for recruiting γδ T cells into the skin tissue via CXC chemokine receptor type 4 (CXCR4), a receptor specific for stromal derived factor 1 (SDF-1). Involved in the "settling down" of tumor cells in a new organ or tissue, SDF-1 is thus implicated in the metastasis of tumor cells. *ID1*$^{-/-}$ mice are *more* susceptible to skin tumorigenesis (as compared with their wild-type counterparts) because they lack CXCR4 in their blood vessels [48] and have far fewer γδ T cells entering into their skin tissue via CXCR4.

Moreover, ID1 and ID3 proteins are required for tumor angiogenesis [49] via VEGF [50, 51]. The activation of the TGF-β1 signaling pathway downregulates ID1 expression [52], whereas the activation of the BMP pathway upregulates it [43, 44]—although both pathways act through the SMAD proteins. The effect of inflammation on PD-L1 in the tumor cells is summarized in Fig. 1.

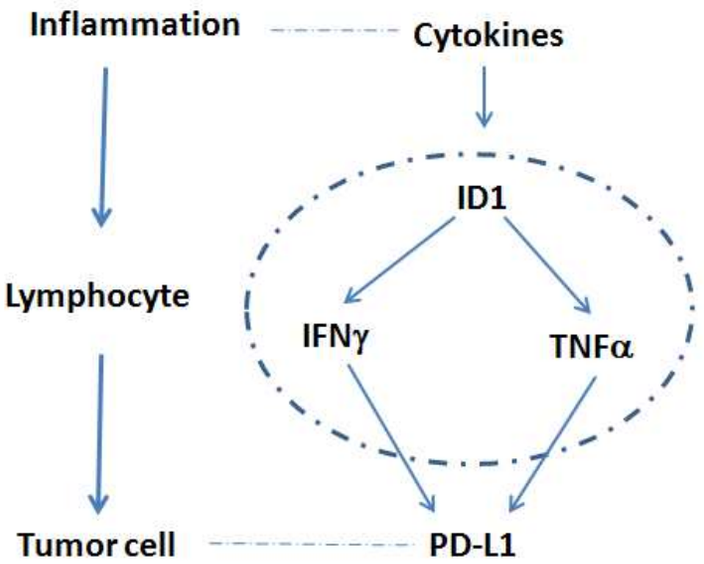

Figure 1. ID1 drives the expression of PD-L1 on tumor cells. Chronic inflammation induces cytokine expression, which, in turn, triggers the early responding gene expression of ID1. ID1 then induces IFNγ and TNFα expression in lymphocytes (T cells and NK cells), eventually inducing PD-L1 expression in the tumor cells.

3. Immunosuppressive microenvironment

At the molecular level, many inhibitory factors are involved in the tumor microenvironment. In HNSCC, PD-L1 and PD-1 interact with TILs and tumor cells [1, 13], constituting a negative

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signaling pathway for immune responses. The TGF-β signaling pathway inhibits the production of IL-2 and thus suppresses the proliferation of T and B cells, forming another signaling pathway for immunosuppression in HNSCC. The enzyme CD73 metabolizes adenosine triphosphate (ATP) into adenosine monophosphate (AMP), which inhibits immune responses in HNSCC. In addition, galectin-1, indoleamine 2,3-dioxygenase, and arginase might play specific roles in the carcinogenesis of HNSCC by suppressing local immunity.

Stratified by the presence or absence of PD-L1 expression and of TILs, patients with HNSCC can be divided into these 4 subsets:

1. $TILs^+/PD\text{-}L1^+$
2. $TILs^+/PD\text{-}L1^-$
3. $TILs^-/PD\text{-}L1^+$
4. $TILs^-/PD\text{-}L1^-$

About 70% of patients with HPV-associated HNSCC are in subset (1), with about 30% in subset (2). But those percentages are reversed in patients with HPV-negative HNSCC: about 29% are in subset (1), with about 71% in subset (2) [1]. In 110 human primary and metastatic melanoma studies, the distribution of patients was as follows: 38% in subset (1), 1% in subset (2), 20% in subset (3), and 41% in subset (4) [53-55]. Clearly, immunotolerance is more common in HPV-associated HNSCC than in HPV-negative HNSCC and in other subtypes.

At the cellular level, Treg cells and MDSCs comprise the inhibitory cellular populations [41]. In patients with HNSCC, as well as with numerous other solid tumors, $CD4^+FoxP3^+/CCR4^+$Treg cells have been shown to be upregulated [41]. In patients with HNSCC, such upregulation may potently suppress effector T-cell responses, in both an antigen-specific and an antigen-independent fashion, constituting a negative force for innate and adaptive immune responses. Treg cells make TGF-β; active TGF-β is important in order for Treg cells to mediate immunosuppression and to help maintain peripheral tolerance [56].

4. Tumor-Infiltrating Lymphocytes (TILs)

About 70% of patients with HPV-associated HNSCC have TILs in their tumors [1]. TILs provide insight into the immunologic activity against tumor cells. The presence of TILs in the tumor tissue marks an antigen-based immune response in the host. Theoretically, this action would eliminate tumor cells in the body. But in reality, many coinhibitory signaling pathways provide feedback to TILs from the tumor side and inhibit their function. For example, the PD-L1/PD-1 pathway frequently determines and controls the function of TILs or CTLs [18, 57-59]. In most cancer patients, freshly isolated TILs are usually inactive against autologous tumor cells, although they can be activated after incubation in vitro with IL-2 [60, 61]. A high level of $CD8^+$ TILs is associated with a favorable outcome [40], yet a low level of Treg cells has no such influence on the prognosis.

TILs predispose patients with HNSCC to a favorable response to chemoradiotherapy [42]. TILs are specific to tumor cells and are able to recognize them through the transmembrane coreceptor CD8, which binds to peptide-loaded MHC class I molecules expressed on the surface of tumor cells; thus, tumor cells are killed via the cytotoxic action of TILs [62, 63]. However, MHC class I molecules on the tumor cell surface are frequently downregulated, so the activation of T-cell cytotoxicity is impaired in the tumor microenvironment. CD3, consisting of 4 chains (ε, γ, δ, and ζ), is a pan-T cell marker and represents a coreceptor of the TCR (Fig. 2), which delivers activation signals down to subsequent pathways and eventually activates T cells. At the same time, PD-1 is upregulated when T cells are differentiated and become activated $CD3^+CD8^+PD\text{-}1^+$ T cells; thus, PD-1 is also a typical cell marker for activated TILs.

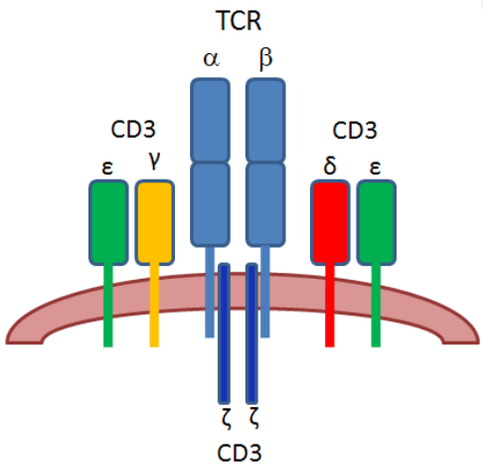

Figure 2. TCR and its coreceptor CD3 are expressed on the T-cell surface. CD3 consists of 4 chains (ε, γ, δ, and ζ), with ζ in the cytosol and ε, γ, and δ on the surface. TCR consists of α and β chains in which signals are converted into the cytosol via the CD3 ζ chain, thereby activating T cells.

Problematically, PD-1 is a surface receptor for PD-L1, which is highly expressed in patients with HNSCC, especially those infected by HPV. In that local tumor microenvironment, PD-1 receptors on the surface of TILs are inevitably surrounded by numerous PD-L1 molecules on the tumor cells and thus are activated by PD-L1. Given the inflammatory nature of HPV-associated HNSCC, a robust infiltration by $CD3^+CD8^+PD\text{-}1^+$ cytotoxic T cells in the tumor tissue is quite common. In the literature, this phenomenon is observed in different tumor types, including HNSCC as well as colorectal, breast, esophageal, renal, lung, ovarian, and anal carcinoma [40].

5. Immunotherapy

Enhancing the specific antitumor immune response is the primary goal of immunotherapy. That goal can occasionally be achieved by the nonspecific stimulation of innate immunity, for example, by activating cytokine-induced killer cells [AS MEANT?] or reinfusing TILs treated by IL-2 outside of the body. But such nonspecific measures are not effective enough to kill tumor cells in the body. In the clinical setting, a more plausible option might be to block the

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PD-L1/PD-1 pathway, along with other possible signaling pathways (such as the CTLA-4 pathway), and to retarget T cells [14, 64, 65]. Increasing the number of tumor-specific cytolytic $CD8^+$T cells available to infiltrate into the tumor is widely believed to be a key component of effective immunotherapy; therefore, numerous approaches for enhancing the tumor antigen-specific immune responses are being actively investigated., Combined therapy with 2 antibodies (anti-PD-1 and anti-CTLA-4 monoclonal antibodies) is highly effective in patients with advanced melanoma, as compared with anti-PD-L1 or anti-PD-1 monoclonal antibody alone [66, 67]; in fact, in a recent study, combined therapy reduced tumor size in about 80% of patients [68]. Similar strategies based on that same principle might apply to the treatment of patients with HNSCC.

Because ID1 regulates both IFN-γ and TNF-α expression in lymphocytes (including NK cells, T cells, and B cells), controlling the ID1 expression levels in tumor cells and TILs is important. Activated NK cells are characterized by both IFN-γ and TNF-α expression. We and others have demonstrated, in mutant mice, that CD137(4-1BB) is a target for treatment of cancer and that application of monoclonal antibodies against the 4-1BB molecule eradicates established tumors by increasing T-cell activity [69-71]. Those earlier findings suggest that the local microenvironment of HNSCC features inhibitory forces that block the activity of tumor-specific T cells. At the 2014 American Society of Clinical Oncology (ASCO) annual meeting, results of an early-phase clinical trial of anti-PD-1 treatment (pembrolizumab, MK-3475) were presented: the best overall response rate in patients with HNSCC positive for HNSCC was only 20%.

6. Future directions

An effective strategy for reversing specific immunosuppressive mechanisms prominent in the HNSCC microenvironment is to target immune checkpoints' that modulate T-cell activity. In light of our current understanding, PD-1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) would be good choices for this purpose [72]. CTLA-4 is a negative costimulatory molecule for T cells, which are usually immature; it is the target of ipilimumab (Yervoy), an immunotherapeutic monoclonal antibody for blocking the immune proteins on the surface of TILs. Ipilimumab has been approved for the treatment of cutaneous melanoma [73]. Polymorphisms in CTLA-4 have been shown to influence the prognosis of patients with HNSCC; the implication is that CTLA-4 may be a rational target in order to block the negative regulation of TILs in HNSCC, just as in cutaneous melanoma [74, 75].

PD-1 (the negative receptor for CTLs) and its ligand, PD-L1, are both overexpressed in patients with HPV-positive HNSCC [1]. In a mouse model of HNSCC, blocking antibody to PD-1 or to PD-L1 has been shown to confer cell-based immunity [76, 77]. The safety, and evidence of antitumor efficacy, of anti-PD-1 have been demonstrated in a phase 1 clinical trial of refractory solid tumors, such as melanoma, non-small-cell lung cancer (NSCLC), and renal carcinoma [76, 77]. However, that approach has not yet been applied to HNSCC. Given the data gained from melanoma and NSCLC, a combination of both anti-CTLA-4 and anti-PD-1 monoclonal antibodies would be ideal for treating HNSCC in the future: CTLs are either inhibited by

CTLA-4 or suppressed to death by PD-1 (Fig. 3). Particularly in the subset of patients with HPV-associated HNSCC, in whom TILs are abundant, treatment with anti-PD-1 monoclonal antibody might enhance the efficacy of chemoradiotherapy.

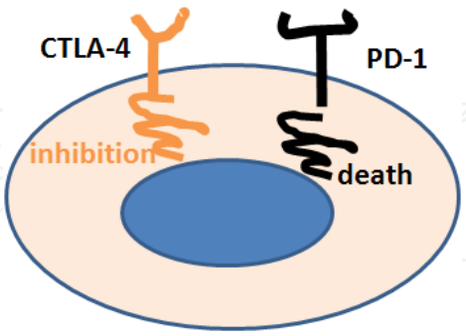

Figure 3. TILs are inhibited by CTLA-4 or suppressed to death by PD-1 in the tumor microenvironment. Blockade of both the CTLA-4 and the PD-1 signaling pathways by anti-CTLA-4 or anti-PD-1 monoclonal antibodies would be ideal for optimal activation of CTLs in the tumor tissue.

7. Conclusion

The majority of HNSCC is full of TILs in the tumor tissue. On one hand, these TILs are primed with tumor antigens and ready to fight off cancer. On the other hand, these TILs are suppressed in their functions due to the expression of PD-L1 on the surface of tumor cells. Immunotherapy is generally effective in those patients when both TILs and PD-L1 are positive. More clinical studies are needed to improve the efficacy of immunotherapy with anti-PD-1 or anti-PD-L1 monoclonal antibody.

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